Amoebic gill disease susceptibility of farm strain and wild strain Atlantic salmon (*Salmo salar*), and brown trout (*Salmo trutta*) post-smolts challenged with *Paramoeba perurans*

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Abbreviations

AB-PAS	Alcian Blue - Periodic Acid-Schiff
AGD	Amoebic gill disease
BT	Brown trout
CGD	Complex gill disease
DPC	Days post-challenge
DPTF	Days post-transfer
DPTS	Days post-transport
ELA	Etnelaks (Wild strain salmon)
Fig.	Figure
H_2O_2	Hydrogen peroxide
HE	Haematoxylin and Eosin
ILAB	Industrial and aquatic laboratory
ILU	Inter lamellar unit
IMR	Institute of Marine Research
MS222	Metacain; Finquel
NLA	Næringslaks (Farm strain salmon)
PCR	Polymerase chain reaction
PGD	Proliferative gill disease
PGI	Proliferative gill inflammation
qPCR	Real-time PCR
QTL	Quantitative trait locus
RNG	Random number generator
SD	Standard deviation
SE	Standard error
SGPV	Salmonid gill pox virus
WOF	Well oriented filament
$WOF^{1/3}$	WOF with a length of approximately 1/3 of the ventral side of the respective gill arch

Abstract

Gill diseases, including amoebic gill disease (AGD) caused by *Paramoeba perurans*, are regarded as increasing problems in the aquaculture industry in Norway as well as other salmonid farming countries around the world. Worldwide, AGD has been reported from most of the major Atlantic salmon farming countries. AGD has also been observed in a range of different farmed fish, but incidentally, very few wild fish. Despite the potential risk of spreading from AGD-outbreaks in open net-cages, AGD has not been reported in wild Atlantic salmon or wild sea migratory brown trout.

This study was part of a strategic initiative at the Institute of Marine Research. Farm raised post-smolts of a farm strain-, and a wild strain of Atlantic salmon, and brown trout were challenged with low (100 Cells/L), medium (500 Cells/L), and high doses (1000 Cells/L) of *P. perurans* in lab conditions. All three fish types developed gross lesions consistent with AGD. The wild strain Atlantic salmon and the brown trout developed gross lesions later than the farm strain salmon, despite presence of *P. perurans*, confirmed by qPCR.

The results of this study show that farm raised wild strain Atlantic salmon from the Etne river and brown trout from the Matre river develop AGD in lab conditions when challenged with *P. perurans.*

For posterity:

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 theses in a manner which allows them to progress as normally as possible.

1.Introduction

1.1 Atlantic salmon aquaculture

Atlantic salmon (*Salmo salar*) and brown trout (*Salmo trutta*) are both members of the genus *Salmo* in the subfamily Salmoninae of the Salmonidae family in the order Salmoniformes (Helfman, 2009). Wild Atlantic salmon have a long history of both an important food source and a popular fish for recreational fishing in Norway (Liu *et al*., 2011). Similarly, brown trout also have a history of sport fisheries and commercial value (Elliott, 1989). Atlantic salmon have become a significant species in the European, Tasmanian, North-American and Chilean aquaculture industries (FAO, 2020). Whereas, brown trout is less used in aquaculture and more known in relation to recreational fishing (Maynard *et al*., 2016). In 2018, farmed Atlantic salmon (*Salmo salar*) made 4.5% of the world fin fish aquaculture with 24.359 million tonnes (FAO, 2020). Farmed Atlantic salmon from Norwegian aquaculture made 1.282 million tonnes with a value of 64.5 billion NOK (SSB, 2019). A consequence of the large extent of open-net cage salmonid fish farming in Norwegian fjords and coastal waters, is an increased abundance of salmon lice (*Lepeophtheirus salmonis*) (Thorstad *et al*., 2015). The effects of salmon lice from aquaculture facilities on wild salmonid populations in Norway is regarded a considerable threat (Grefsrud *et al*., 2020). Consequently, Produksjonsområdeforskriften (2017) regulates the permitted production capacity in the respective aquaculture production areas in Norway. The estimates of the effects of salmon lice on wild salmonid populations are central in the regulations of permitted production capacity. For now, this is the only infection related factor directly influencing the production capacity of salmonid aquaculture in Norway (Produksjonsområdeforskriften, 2017). However, interpretation of Produksjonsområdeforskriften (2017) indicate that other factors affecting the environment may be implemented in the regulations in the future.

1.2 Gill diseases

Gill diseases are an increasing challenge in marine salmonid aquaculture (Herrero *et al*., 2018, Marcos-López and Rodger, 2020). The gills of fish are, in addition to respiration, central in osmoregulation, pH regulation and excretion of nitrogenous waste (Evans *et al*., 2005). Additionally, the gills are also important in physiological responses to internal- and environmental changes (Evans *et al*., 2005). The gills of fish are continuously exposed to water and make a port of entry for pathogens as a consequence of the short distance from the

surrounding water and the blood circulation (Koppang *et al*., 2015, Herrero *et al*., 2018). The distance from the bloodstream to the surrounding water are 2-3 cell layers in addition to a thin external layer of mucous (Koppang *et al*., 2015). Fish with compromised gill function can show loss of appetite, poor food conversion and increased mortality rates, consequently resulting in economic loss if affecting fish in aquaculture (Herrero *et al*., 2018). Some distinguishable gill diseases are amoebic gill disease (AGD), parasitic gill disease, viral gill disease and bacterial gill disease (Boerlage *et al*., 2020). Additionally, gill diseases can be caused by presence of non-infectious organisms (Herrero *et al*., 2018). Gill diseases in seawater farmed Atlantic salmon are often caused by more than one agent (Gjessing *et al*., 2019, Boerlage *et al*., 2020) and primary agents can often be unclear (Herrero *et al*., 2018, Gjessing *et al*., 2019). Gill diseases with multiple distinguishable agents are often referred to as multifactorial gill diseases (Boerlage *et al*., 2020). Whereas, complex gill disease (CGD), is the current term used for gill disease of variable histopathology and likely multifactorial aetiology (Herrero *et al*., 2018). CGD includes gill diseases previously referred to as proliferative gill inflammation (PGI) and proliferative gill disease (PGD) (Herrero *et al*., 2018). PGI is a diagnosis based on the combination of the following histopathological observations: circulatory disturbances, inflammation, cell death and epithelial cell hyperplasia (Kvellestad *et al*., 2005, Boerlage *et al*., 2020). PGD have been used for similar conditions as PGI, but with less pronounced inflammation and circulatory disturbances (Herrero *et al*., 2018). Confusingly, PGI and PGD have previously also been used interchangeably, but are now, incorporated in the term complex gill disease (CGD) (Herrero *et al*., 2018, Boerlage *et al*., 2020). CGD typically occur from mid-summer to the onset of winter (Herrero *et al*., 2018). Clinical signs of CGD are non-specific and can involve fish swimming near the surface, crowding against the current, increased respiration and reduced appetite (Herrero *et al*., 2018). Gross pathology of CGD can be swollen and shortened gill filaments, petechia and mucus accumulation on the gills (Herrero *et al*., 2018).

Frequently observed agents related to gill disease

Ichthyobodo

Ichthyobodo is a genus of protozoan flagellates, also known as Costia (Robertson, 1985). Two species of the genus are known to infect Atlantic salmon (*Salmo salar*); *Ichthyobodo necator* and *Ichthyobodo salmonis* sp. n. (Isaksen *et al*., 2011). *I. necator* is an established salmonid freshwater parasite (Robertson, 1979), whereas, *I. salmonis* sp. n. can infect Atlantic salmon

both in freshwater and seawater (Isaksen *et al*., 2011). Generally, *Ichthyobodo* spp. are regarded secondary pathogens (Herrero *et al*., 2018, Kvellestad *et al*., 2005), which typically do not cause disease unless the host is already compromised.

Trichodinids

Trichidinids are ectoparacitic peritrichous ciliates, commonly occurring on the skin and gills of fish (Mizuno *et al*., 2016) in freshwater as well as seawater (Mitchell and Rodger, 2011). Trichodinids can cause significant pathology (Mitchell and Rodger, 2011), histologically often epidermal hyperplasia (Mizuno *et al*., 2016). Mortality in Atlantic salmon and brown trout have been reported in freshwater (Mitchell and Rodger, 2011). Mizuno *et al*. (2018) found that both *Ichthyobodo salmonis* and *Trichodina truttae* can be prevented by UV irradiation treatment of inlet water with an irradiation dose of 2.2×10^6 µW s/cm^2.

Epitheliocystis

Epitheliocystis is a term used for membrane bound inclusions in gill and skin-epithelial cells (Mitchell *et al*., 2013). There are several causative agents of epitheliocystis in Atlantic salmon; *Candidatus* Branchiomonas cysticola (Toenshoff *et al*., 2012), *Ca* Pisciclamydia salmonis (Andrew *et al*., 2004) *Candidatus* Clavochlamydia salmonicola (Mitchell *et al*., 2010) and *Candidatus* Syngnamydia salmonis (Nylund *et al*., 2015). *Ca.* Branchiomonas cysticola is widely seen in seawater farmed Atlantic salmon in Norway and Ireland (Mitchell *et al*., 2013) *Ca* Branchiomonas cysticola is an increasing problem in the freshwater phase of salmon farming (Wiik-Nielsen *et al*., 2017) but have an unclear role in CGD (Herrero *et al*., 2018).

Salmonid gill pox virus

Salmonid gill pox virus (SGPV) has been associated with high mortality in Atlantic salmon both in fresh- and seawater (Nylund *et al*., 2008). The target cells of SGPV are lamellar epithelial cells and infected cells are often hypertrophic with degenerated nucleus (Nylund *et al*., 2008, Wiik-Nielsen *et al*., 2017). Changes in chloride cells due to SGPV have been reported and could affect smoltification (Gjessing *et al*., 2017). High mortalities in SGPV-infected fish have been observed before and shortly after seawater transfer (Herrero *et al*., 2018). SGPV is a common finding in seawater reared Norwegian Atlantic salmon (Thoen *et al*., 2020). Garseth *et al*. (2018) found that SGPV is widely distributed in wild populations of Norwegian Atlantic salmon aswell. It is also hypothesized that SGPV infections may facilitate for infections of secondary

pathogens (Gjessing *et al*., 2017). SGPV has also been found in Scotland and the Faroe Islands (Thoen *et al*., 2020).

Desmozoon lepeophtheiri

Desmozoon lepeophtheiri is a microsporidian parasite found in both salmon lice (***Lepeophtheirus salmonis***) and Atlantic salmon (Herrero *et al*., 2018). ***D. lepeophtheiri*** is widely observed in both healthy and CGD-affected Atlantic salmon, however, CGD-affected fish often carry heavier loads of the parasite (Herrero *et al*., 2018). Higher than normal temperature has been associated with histopathological changes related to ***D. lepeophtheiri*** infections (Herrero *et al*., 2018).

Co-infections

Co-infections of two or more pathogens on the gills of seawater farmed Atlantic salmon are common (Gjessing *et al*., 2019, Downes *et al*., 2018, Oldham *et al*., 2016) and have become a highly significant problem in the seawater phase of salmonid aquaculture (Wiik-Nielsen *et al*., 2017, Herrero *et al*., 2018). CGD can lead to compromised gill functions, and consequently poor food conversion, increased mortality and economic losses (Herrero *et al*., 2018). Despite the fact that gill diseases of Atlantic salmon in the marine phase often are caused by several agents (Gjessing *et al*., 2019, Downes *et al*., 2018), they can also be caused by a clear primary pathogen. For instance *Paramoeba perurans* (Herrero *et al*., 2018), that causes amoebic gill disease (AGD) (Crosbie *et al*., 2012). In cases of complex gill disease, AGD have been observed simultaneously (Nylund *et al*., 2008, Gjessing *et al*., 2019). Complex gill pathological situations comprising AGD in addition to other gill pathology have been more commonly observed in recent years and can result in high mortality rates (Herrero *et al*., 2018).

1.3 AGD and *Paramoeba perurans*

AGD was first described in Tasmania in the mid-1980s (Kent *et al*., 1988, Munday *et al*., 1990) and is caused by infection with *Paramoeba perurans* (Young *et al*., 2007, Crosbie *et al*., 2012). AGD has a significant impact on production cost of Atlantic salmon in Tasmania (Munday *et al*., 2001, Oldham *et al*., 2016) and has also become a significant disease in Northern Europe (Wiik-Nielsen *et al*., 2016). Several types of amoebae have been isolated from the gills of AGD-affected Atlantic salmon (English *et al*., 2019). ***Neoparamoeba pemaquidensis*** was for some time the only amoeba isolated from fish with AGD, and regarded the causative agent (Nowak and Archibald, 2018). Later it was shown that the actual causative agent of AGD is ***P. perurans***

(Young *et al*., 2007). *P. perurans* has long been regarded the causative agent of AGD but Koch's postulates were not fulfilled until 2012 by Crosbie *et al*. (2012). In the literature, *Neoparamoeba perurans* is used interchangeably with *Paramoeba perurans* and they are regarded phylogenetically inseparable (Feehan *et al*., 2013). In this text, *Paramoeba perurans* will be used. The first detected AGD-outbreaks in Norway were in the autumn of 2006 (Steinum *et al*., 2008) and has since 2011 been an increasing problem in marine European aquaculture (Boerlage *et al*., 2020). AGD is the main health challenge in seawater reared Atlantic salmon in Tasmania (Oldham *et al*., 2016).

Paramoebae

Paramoebae are marine free living single celled eukaryotes with one or several obligate eukaryotic endosymbiont(s) associated to the nucleus (Young *et al*., 2007). The endosymbiont is surrounded by a single membrane (Dyková *et al*., 2003). *Perkinsiella amoebae*-like organism and "parasome" are other terms that also refer to the endosymbiont (Young *et al*., 2007), which is found to be related to the parasitic flagellate *Ichthyobodo necator* (Dyková *et al*., 2003). The endosymbiont is vertically inherited from the mother cell (Nowak and Archibald, 2018, Sibbald *et al*., 2017). It is not known if the endosymbiont is related to the host amoebae's parasitism of fish (Nowak and Archibald, 2018). In locomotive form, Paramoebae form pseudopodia (Young *et al*., 2007) of varying length and shape (Wiik-Nielsen *et al*., 2016). Paramoebae are variable in both size, and morphology (Nowak and Archibald, 2018). In free form, Paramoebae are approximately 20-30 µm in diameter (Karlsbakk *et al*., 2013) and 41-56 µm in adherent form (Young *et al*., 2007). Despite that Paramoebae feed on bacteria, they also appear to have a more complex symbiotic relationship with bacteria (Nowak and Archibald, 2018). Bacteria have been observed to multiply in the cytoplasm of Paramoebae (Nowak and Archibald, 2018). Paramoebae can be grown in liquid media and on marine agar plates (Nowak and Archibald, 2018).

AGD – Clinical signs and pathology

Clinically, AGD is characterized by whiteish mucoid patches on the gill surfaces (Munday *et al*., 1990, Marcos-López and Rodger, 2020). Clinical signs of AGD can be loss of appetite, altered swimming behavior lethargy and respiratory distress (Boerlage *et al*., 2020). *P. perurans* can attach to healthy gill-epithelium and colonize this shortly after introduction to the gill surface (Zilberg and Munday, 2000). AGD-lesion development is suggested to be initiated by

attachment of *P.* perurans to gill epithelia (Adams and Nowak, 2004) and trigger host-responses resulting in hyperplastic epithelial lesions (Adams and Nowak, 2003b). Pseudopodia of *P. perurans* have been observed to penetrate the lamellar epithelium (Wiik-Nielsen *et al*., 2016, Lovy *et al*., 2007). Three histopathological stages of *P. perurans* infection in Atlantic salmon were described by Adams and Nowak (2003); primary attachment (1st), innate immune response activation and focal epithelial hyperplasia (2nd) and lesion expansion with variable mucous cell recruitment (3rd) (Adams and Nowak, 2003b). Adams *et al*. (2004) suggested that the lesion progression depends on migration and proliferation of amoebae along the filaments. Histopathological observations indicate that amoebae can be sloughed off the gills along with mucous and hyperplastic tissue (Zilberg and Munday, 2000). Hyper secretion of mucous is often observed (Powell *et al*., 2001). The primary spread of the disease is likely to be shedding from infected fish (Zilberg and Munday, 2000). Seawater currents and mobile organisms may be involved in spreading of *P. perurans* between farming locations (Hellebø *et al*., 2017).

Distribution and host register

Amoebic gill disease has been reported from a variety of farmed fish (Nowak *et al*., 2014) and has, among others, been observed in farmed: Atlantic salmon, coho salmon (*Oncorhynchus kisutch*), turbot (*Scophthalmus maximus*), ayu (*Plecoglossus altivelis*), ballan wrasse (*Labrus bergylta*), lumpfish (*Cyclopterus lumpus*) and black seabream (*Acanthopagrus schlegelii*) (Archibald *et al*., 2018). AGD has also been observed in rainbow trout (*Oncorhyncus mykiss*) (Munday *et al*., 1990) and sea water reared brown trout in France (Munday *et al*., 2001). *P. perurans* have been detected on a variety of biofouling organisms in proximity of fish farms with active AGD-outbreaks (Hellebø *et al*., 2017).

Risk factors

Salinity temperature are major risk factors of AGD outbreaks (Adams and Nowak, 2003b, Benedicenti *et al*., 2019). Stocking density may also be a contributary risk factor (Oldham *et al*., 2016). Amoebae can survive and multiply on the gills of dead fish, implying the importance of removal of mortalities (Douglas-Helders *et al*., 2000).

Monitoring and treatment

AGD is commonly monitored by gross gill scores (Downes *et al*., 2017), the extent of visual lesions on the gill surfaces on a scale of increasing severity, ranging from 0-5 (Taylor *et al*.,

2009a). AGD can compromise gas exchange and ion regulation over the gills of affected fish (Hvas *et al*., 2017). If left untreated, AGD will reduce welfare and cause increased mortality rates (Nowak *et al*., 2014). Commercial treatment threshold is when 30% of the fish show industry standard gross gill scores ≥ 2 (Maynard *et al*., 2016). Similarly, in Norway it is recommended to treat the fish in the early stages of AGD-development (Sommerset *et al*., 2020).

There are two treatments against AGD-outbreaks in commercial aquaculture; freshwater treatment and hydrogen peroxide (H_2O_2) treatment (Oldham *et al*., 2016, Powell *et al*., 2015). Freshwater treatment of Atlantic salmon for AGD has shown to reduce the amount of AGD-affected gill filaments, fragment gill lesions and shed hyperplastic tissue associated with lesions (Roberts and Powell, 2003b). Reduction in the number of PCR-positive fish immediately after freshwater treatment has also been reported (Downes *et al*., 2018). Powell *et al*. (2001) observed no significant pathological impact of freshwater bathing on AGD-affected Atlantic salmon. Large-scale freshwater bath treatments for AGD has been used since the mid-1980s (Powell *et al*., 2015), and was for quite some time, regarded the only effective treatment for AGD in salmonids (Munday *et al*., 2001). More recently, hydrogen peroxide (H_2O_2) treatments have been experimentally proven to reduce the amount of amoebae (Adams *et al*., 2012, Thorisdottir *et al*., 2018) and have commonly been used for treatments for AGD and other parasitic infections in salmonid aquaculture (Stratford *et al*., 2020). However, H_2O_2-treatmens have been associated with gill pathology, particularly at temperatures above 13°C (Herrero *et al*., 2018) and are not recommended at gross gill scores of or greater than 3 (reviewed by Oldham *et al*. (2016)).

Functional diets and selective breeding

Functional diets have the potential to become a preventive measure for AGD severity (Mullins *et al*., 2020, Roberts and Powell, 2005a). However, there are interactions between ingredients that complicates elucidation of the effects of each respective ingredient (Mullins *et al*., 2020).

Munday *et al*. (2001) observed that Atlantic salmon appears to be more susceptible to AGD than other salmonids. Bridle *et al*. (2005) observed resistance against severe AGD-infection in a group of Atlantic salmon. The Atlantic salmon showed signs of recovery and lesion repair, indicating capability to constrain the infection (Bridle *et al*., 2005). There has also been observed a correlation of genetic susceptibility or resistance with gill histopathology and

associated gross pathology (Powell *et al*., 2008). Taylor *et al*. (2007) found that there were possibilities for including AGD-resistance in breeding programs and a potential for increasing the AGD-resistance in Tasmanian Atlantic salmon. Gross gill score have shown to be useable as a selection trait for increased AGD-resistance in Atlantic salmon (Taylor *et al*., 2009a). The trait for AGD-resistance is indicated to be suitable for genomic selection and is regarded moderately heritable (Robledo *et al*., 2018). Lillehammer *et al*. (2019) found that there is potential for selective breeding towards AGD-resistance in farmed Norwegian Atlantic salmon as well. In Tasmania, a goal of selective breeding towards increased AGD-resistance is to increase the intervals between freshwater bathing (Taylor *et al*., 2007). Consequently because these treatments are labor-intensive, time-consuming (Harris *et al*., 2005) and have a large economic impact (Taylor *et al*., 2007).

Maynard *et al*. (2016) performed a study of salmonid heterosis for resistance to AGD. They studied Atlantic salmon, brown trout and Atlantic salmon x brown trout hybrids put in research pens in south-eastern Tasmania. All the different fish groups developed AGD, caused by natural infection. Maynard *et al*. (2016) reported that the salmon group reached treatment threshold 50 days post-transfer to seawater. The hybrids and brown trout reached treatment threshold at 78 days post-transfer to seawater, at which time the salmon had reached the treatment threshold for the second time. In total, the salmon needed bath treatment against AGD four times while the hybrids and brown trout only needed one bath treatment during the 177 day long chronic AGD-challenge under field conditions (Maynard *et al*., 2016). This was a field study with natural infection, and the infection dose is subsequently unknown. However, natural infections have been reported with 10-50 amoebae per liter (Douglas-Helders *et al*., 2003). Considering that there are differences in susceptibility to AGD within farm strains of Atlantic salmon (Bridle *et al*., 2005, Taylor *et al*., 2007, Taylor *et al*., 2012), there might also be differences between wild strain salmon and farm strain salmon. However, AGD has not yet been reported in wild Atlantic salmon, despite that there is a risk of spreading from affected farmed fish (Thorisdottir *et al*., 2018).

1.4 Aim of this study

This study was part of a strategic initiative at the Institute of Marine Research with focus on gill and heart. Amoebic gill disease has been observed in a variety of farmed fish, very rarely in wild fish and has not yet been observed in wild Atlantic salmon and wild sea migratory

brown trout. However, AGD is regarded an increasing challenge in the marine phase of Atlantic salmon farming, and it has been suggested that there is a potential risk of spreading from farmed fish to wild populations. This study may enlighten whether wild strain Atlantic salmon and sea migratory brown trout are susceptible to AGD. Genetic differences in AGD-susceptibility have been observed in farm strains of Atlantic salmon, subsequently, there might be differences between farm strains and wild strains as well. The aim of this study is to investigate the susceptibility to AGD in post-smolts of a farm strain of Atlantic salmon, a wild strain of Atlantic salmon from the Etne river, and of brown trout from the Matre river, raised in the same aquaculture facility, and challenged in lab conditions with *Paramoeba perurans*.

Objectives:

1. To assess AGD-severity by gross gill scoring
2. To estimate prevalence of *P. perurans* positive fish based on results from qPCR-screening
3. To examine histological sections with respect to histopathological AGD-like lesions and amoebae
4. To quantify mucous cells in histological sections

2. Material and methods

2.1.1 Project funding

This study was part of a Strategic Initiative at the Institute of Marine Research, project no.: 15555-03.

2.1.2 Ethical considerations

This work was approved by the Norwegian Animal Research Authority under FOTS approval FOTS-ID: 20274.

2.2 Fish

Three fish types were used in this study: farm strain salmon, wild strain salmon and brown trout. They were all farm raised at the facilities of Institute of marine research at Matre, Vestland, Norway.

Table 2.2-1 Overview of the genetic origin, generation in aquaculture and average size of the fish in the different groups used in the study

Fish type	**Origin**	**Generation in aquaculture**	**Average mass (g) ± SD**	**Average length (cm) ± SD**
Farm strain salmon	AquaGen®	12th	201,4 ± 37,5	27,5 ± 2,7
Wild strain salmon	Etne river	1st	101,2 ± 21,2	21,8 ± 1,2
Brown trout	Matre river	1st	70,2 ± 24,5	17,7 ± 2,3

The farm strain salmon used in this study originated from AquaGen® QTL SHIELD and were estimated to be 12th generation in aquaculture. The wild strain salmon originates from the Etne river of Sunnhordland, Vestland, Norway. The wild strain post smolts used in this study were the first generation raised in aquaculture. The brown trout originates from the Matre river in Nordhordland, Vestland, Norway. The smoltified brown trout used in this study are the first generation raised in aquaculture.

2.3 Fish transport, handling and tank setup

2.3.1 Fish Transport

The fish was transported from Matre to Nordnes by a small lorry, a drive of approximately 1 hour and 10 minutes. The fish were put in a 1000 L tank with additional oxygen supply on a the small lorry. The transport tank was filled with brackish water from the Matre facility. Salinity, temperature and oxygen saturation were measured before, during and after fish transfer as well as mid-transport. At mid-transport, the water was hyper-oxygenated (145%)

and the additional oxygen was reduced. At Nordnes, the fish were put into separate 400 L holding tanks with 21‰ salinity, 13.5°C water at a flow rate of approximately 750 L/h.

The brown trout and wild strain salmon were transported together, the farm strain salmon was transported later the same day. A net cage separated the brown trout from the wild strain salmon in the transport tank.

2.3.2 Fin clipping

The farm strain salmon were marked by fin clipping. In batches of 10 ± 1 farm strain salmon were netted out from the holding tank into a bucket of water with MS222 (metacain; Finquel) 100 mg/L. When anesthetized they were handled individually, and the adipose fin was cut by scissors at an angle of approximately 45 degrees posterior towards anterior. After fin clipping the farm strain salmon were put into a recovery bucket (a bucket of clean water like the water of the holding tanks). When every farm strain salmon in the batch had been fin clipped and recovered, they were moved to their respective challenge tank. The wild strain salmon and brown trout were netted out of their holding tanks and into a bucket of water with MS222 (metacain; Finquel) 100 mg/L, 10 ± 1 at a time. They were moved to their allocated tank and netted out of the bucket and into the tank.

2.3.4 Challenge tanks

Eight 250 L tanks were used in this common garden challenge study. Each tank contained 20 farm strain salmon, 20 wild strain salmon and 20 brown trout. (Fig 2.3.4). The waterflow per tank was 700 L/h, salinity of 34‰, average temperature 13,3°C ± 0,2 (±standard deviation) with an average oxygen saturation of 74% ± 4,1 (±standard deviation). The fish were fed to satiation daily. The average biomass during the challenge was estimated by sample time mass and is given in table 2.3.4.

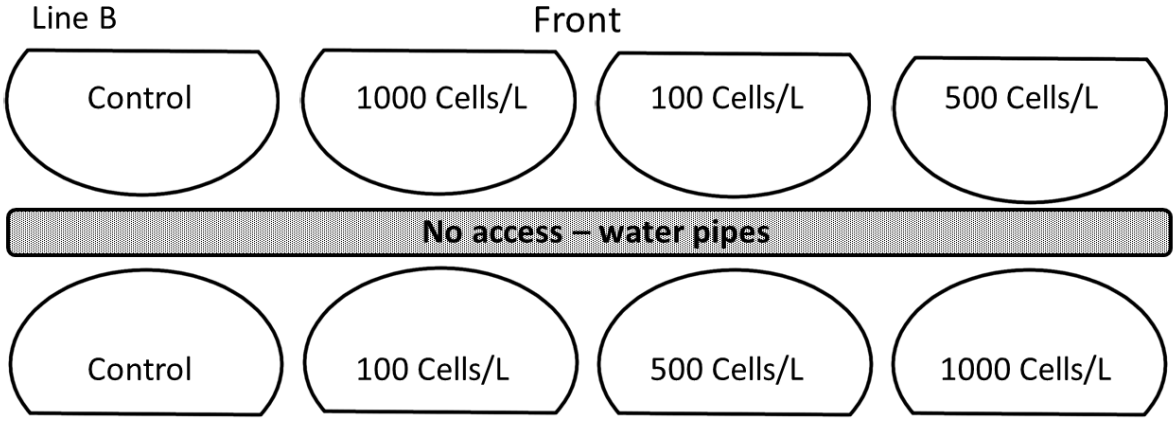

Figure 2.3.4: Schematic illustration of the challenge tank setup, seen from above. Each tank contained 20 farm strain salmon, 20 wild strain salmon and 20 brown trout before challenge.

Table 2.3.4: Average estimated biomass per tank during challenge with *P. perurans*

Weeks post-challenge	Average estimated biomass per tank (kg/m^3) ± Standard deviation
1	28,0 ± 1,2
2	20,8 ± 1,3
3	13,2 ± 1,3
4	6,1 ± 1,0

2.3.5 Challenge

P. perurans culture

The C2 clone of *Paramoeba* perurans (isolated by ILAB, Bergen, Norway) was used in this challenge. It was originally isolated from Sotra, Vestland, Norway in 2013 and has been in continuous culture and was passaged through and subsequently recovered from fish at ILAB Bergen. The cultures were held in flat-bottomed cell culture flasks with medium in an incubator cabinet at 15 ± 1°C. Prior to the challenge, the cultures were subdivided to increase the number of amoebae. Subdividing was performed by carefully pouring the medium of a cell culture flask (containing free amoeba) to a new cell culture flask. Medium was added to both cell culture flasks, as the originate one would contain attached amoebae. The medium of the subdivided flasks was changed after 1-2 days post-subdividing.

Challenge dose calculation and administration of *P. perurans*

The amoeba concentration per mL was estimated by use of a Neubauer counting chamber. The average number of amoebae in 10 large squares on 5 different slides were used in calculation of the estimated amoeba concentration (formula 1).

$$Total\,\frac{cells}{ml} = Total\ cells\ counted\ x\ \frac{dilution\ factor}{\#\ of\ squares}\ x\ 10000\,\frac{Cells}{ml} \quad (1)$$

(Grigoryev, 2013)

The volume of amoebae solution needed to achieve desired challenge concentrations in the 250 L tanks was calculated. The required volume of amoebae solution was measured in a graduated cylinder and poured into the given challenge tank.

2.4 Sampling

Weekly sampling started 7- and 8-days post-challenge. First sampling of line A was 7-days post-challenge (Fig.2.3.4 and Fig.2.3.5). First sampling of line B was 8-days post challenge (Fig.2.3.4 and Fig.2.3.5). For each tank 5 fish of each type were sampled.

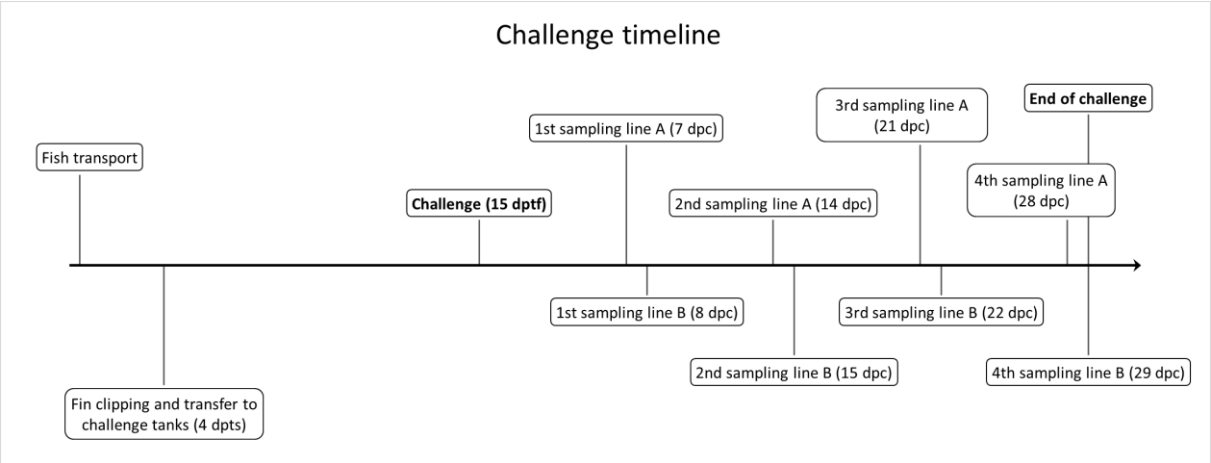

Figure 2.3.4: AGD-challenge timeline, dpts: days post-transport, dptf: days post-transfer, dpc: days post-challenge

2.4.1 Euthanasia

At sampling, the fish were netted out of their tank into a bucket of water and euthanized by an overdose (>100mg/L) of MS222 (metacain; Finquel).

2.4.2 Gill scoring

The operculum was carefully opened by hand and the gill arches and -surfaces were carefully separated without touching the gill filaments. The left operculum was opened by pressing the left-hand thumb towards the ventral part of the operculum. When the operculum started to open, the left-hand thumb slowly slipped onto the inside surface of the operculum, exposing the gills. While exposing the gills as described, the left-hand ring finger or little finger was put through the mouth of the fish, reaching to the gill arches. By carefully pushing one gill arch at a time, the gill surfaces were separated and could be inspected. When gill scoring small fish, the fish was placed on the bench and two pairs of forceps were used, one in each hand. The left-hand forceps were used to lift the operculum by pinching the edge of the operculum before slowly forcing it anterior, exposing the gills. The right-hand forceps were used to carefully separate the gill surfaces by pushing the gill arches apart. All 16 gill surfaces were scored 1-5 according to (Taylor *et al*., 2009b) criteria (Table 2.4.2).

Table 2.4.2: Gill score criteria adapted from Taylor *et al*. (2009)

Gill score	Description
0	No sign of infection and healthy red colour
1	1 white spot, light scarring or undefined necrotic streaking
2	2-3 white spots / small mucus patch
3	Established thickened mucus patch or spot groupings up to 20% of gill area
4	Established lesions covering up to 50% of gill area
5	Extensive lesions covering most of the gill surface

Industry standard gross gill score was determined by the gill surface of highest gill score Average gill score is the average score of all 16 gill surfaces. Lesioned surfaces are the number of gill surfaces with macroscopically visual lesions. Severity is the gill score sum of all 16 surfaces, with a theoretical maximum of 80 (16x5). The data from gross gill scoring was transferred from sampling sheets to an Excel™ spreadsheet. Each cell from F-U contain the gill score for one gill surface. Gross gill score (industry standard) was equal to the gill surface with highest score. The Excel™ function (=STØRST(F:U)) was used to determine this value. Average gill score was calculated by the function (=GJENNOMSNITT(F:U)). Surfaces without visual lesions were found by the function (=ANTALL.HVIS(F:U;0)). The number of lesioned surfaces was calculated by: $16 - (surfaces\ without\ visual\ lesions)$. Each of the abovementioned were assigned columns in the spreadsheet. The average of each within fish type, challenge dose and time were calculated. Standard deviation was calculated by the function (=STDAV()). Standard error (SE) was calculated by $SE = \frac{STDAV}{\sqrt{n}}$.

2.4.3 Measures

Fish mass was measured to the nearest tenth gram. A measure tray was used to determine the fork length of the fish to the nearest millimeter.

2.4.5 Tissue samples for histology

The second left gill arch was sampled for histology. The left side operculum was removed by scissors, cutting from dorsoposterior towards ventral anterior. The gill arch was then placed in a marked histology cassette and put in a bottle of Davidson's Fixative.

2.4.6 Tissue samples for qPCR-screening

The apex of the third left gill arch was sampled for routine qPCR. The third gill arch was exposed due to removal of the operculum and 2nd gill arch. The gill arch was put on the left side of the fish and the apex was cut by scissors and put in a standard PCR sample tube containing RNA-later. In addition to the routine samples, extra samples were taken from lesioned areas of a selection of fish for confirmation of presence of *P. perurans.* The remaining gill tissue was snap frozen in liquid nitrogen. Snap-frozen samples were stored in -80°C freezer and were not used in this study.

2.5 Real time RT-PCR analysis

Real-time RT-PCR screening for *P. perurans* (ParaPer-PA agent) was performed Pharmaq analytiq.

2.5.1 Real time RT-PCR analysis data handling

The number of positives of each fish type within challenge doses at given sample times were calculated by the Excel™ function (=ANTALL.HVIS.SETT). The prevalence (qPCR) of *P. perurans* in each fish type within challenge dose at given sample times were calculated.

2.6 Histology samples

2.6.1 Fixation and processing of tissue samples

Tissue samples for histological investigation were placed in Davidson's seawater fixative (Shaw, 1957) (Howard D.H., 2004), as described under 2.4.5. The samples were fixed in Davidson's solution for 48 hours and then processed or transferred to ethanol 70% before processing. Processing was done in a Leica TP 1020 histokinette. The processing program is given in appendix 2.5. Kunz Instruments Embedding (paraffin) was used for embedding the tissue samples in histowax (paraffin).

2.6.2 Sectioning

The embedded gill arches were sectioned in 3µm sections with a Leica RM 2255 microtome with Accu-Edge low profile microtome blades. All sections were inspected in an Olympus CX31 light microscope to ensure section quality. Two sections were made from every embedded gill arch.

2.6.3 Staining of histological sections

For each embedded gill arch one section was stained with hematoxylin and eosin following standard protocols (Appendix 2.5). The second section was stained combined Alcian blue – Periodic Acid-Schiff (AB-PAS) (H.C,C. 1977) (Appendix 2.5).

2.7 Examination of histological sections

A Leica DMRBE microscope using a Leica MPS52 camera was used to examine histological sections. Software "Spot" was used and plugins in "Image J" was used to add scalebars to the pictures.

A semi-quantitative approach was used to quantify AGD-like lesions, *Ichthyobodo* sp. infection load and amount of epitheliocysts in. For this approach the Speare *et al*. (1997) criteria for well oriented filaments (WOF) were used. In addition to these criteria, the filaments should have a length of approximately 1/3 of the ventral part of the gill arch (Fig.2.8). The filaments that met these criteria are referred to as $WOF^{1/3}$.

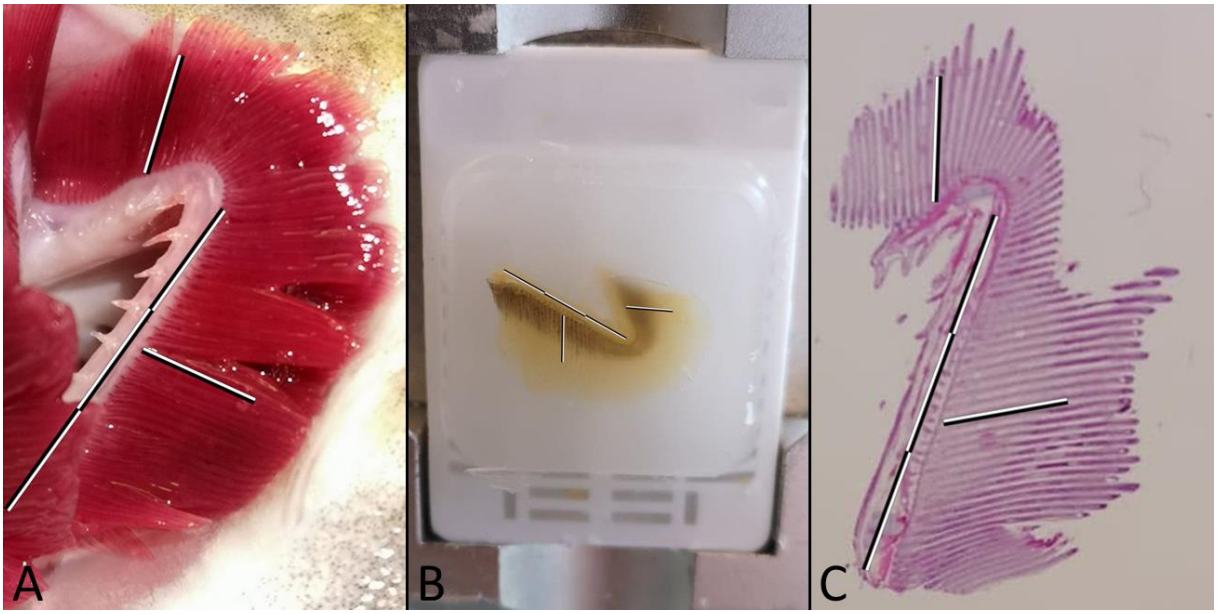

Figure 2.7: Gill arches A: before sampling, B: embedded in paraffin, C: HE stained histological section. Cartography style bars indicate the approximate length of 1/3 of the ventral part of each respective gill arch.

Filaments that met the Speare *et al*. (1997) criteria but were shorter than 1/3 of the ventral side of the gill arch are referred to as WOF. Quantification was performed for up to 10 $WOF^{1/3}$ per section. Epitheliocysts on both sides of $WOF^{1/3}$ were counted. The amount of *Ichthyobodo* sp. in 5 inter lamellar units (ILUs) on the clockwise side of $WOF^{1/3}$ were counted. The procedure

for the examination of histological sections is presented in table 2.7. All sections were scored blindly, by the means that only fish numbers identified the sections.

Table 2.7: Procedure for semi-quantitative examination of histological sections by light microscopy with respect to Ichthyobodo sp., epitheliocystis and AGD-like lesions

Step	Description	Magnification
1	Identify well oriented filaments	25x
2	Assess number of lesioned $WOF^{1/3}$ and lesion type(s)	25x
3	Count *Ichthybobodo* sp. / 5ILUs on the clockwise side of $WOF^{1/3}$	200x
4	Count Epitheliocysts / $WOF^{1/3}$	200x
5	Count AGD-like lesions and size (number of fused lamellae) / $WOF^{1/3}$	200x

2.7.1 Amoebae and AGD-like lesions

The number of $WOF^{1/3}$ with AGD-like lesions were counted at 25x magnification and the number of fused lamellae in the lesions were counted at 200x magnification during examination of histological sections (Fig.2.7.1).

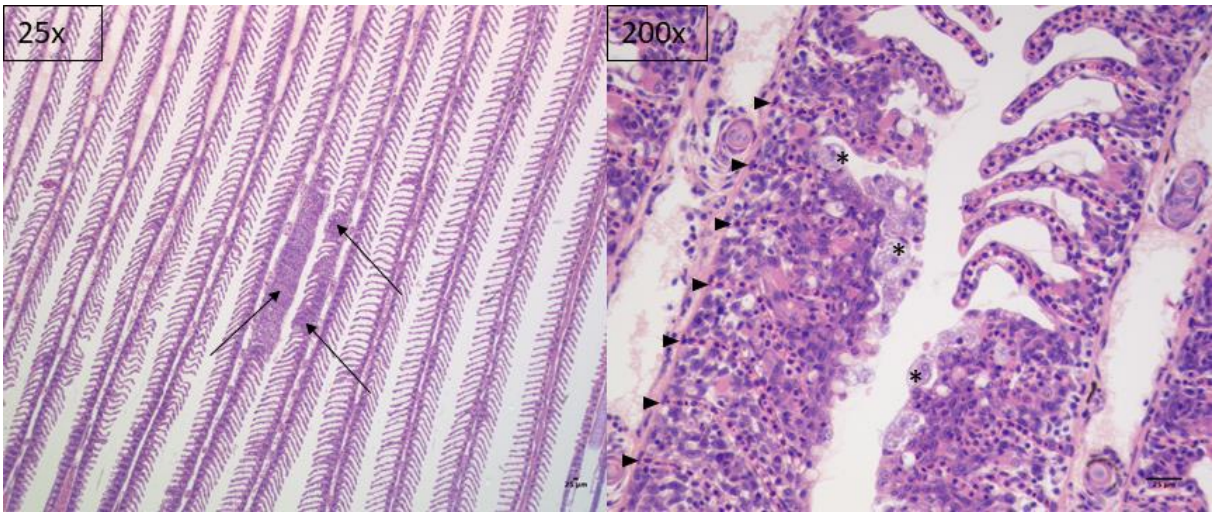

Figure 2.7.1: HE stained histological section of gill tissue with AGD-like lesions. A: 25x magnification, arrows indicate the location of AGD-like lesions, examples of the like that were counted during examination of histological sections. B: AGD-lesion, 200x magnification. Arrowheads indicate the location of some of the lamellae in the AGD-lesion. Some of the amoeba are marked by *.

2.8.2 *Ichthyobodo* sp.

WOF$^{1/3}$ with *Ichthyobodo* sp. lesions were counted at 25x magnification. Examples of filaments with *Ichthyobodo* sp. lesions are given in figure 2.8.2-A. The number of *Ichthyobodo* sp. individuals per 5 inter lamellar units (ILUs) were counted at 200x magnification (Fig.2.7.2-B). The prevalence of *Ichthyobodo* sp. in the different treatment groups within each respective species and the average number of *Ichthyobodo* sp. individuals per 5 ILUs were calculated in Excel™.

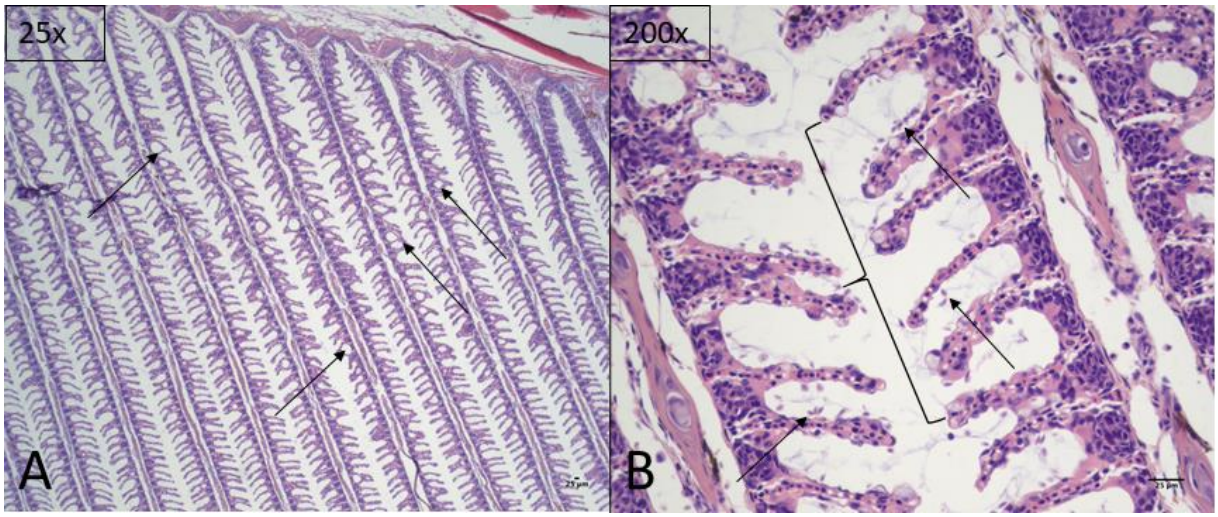

Figure 2.7.2: HE stained section of gill tissue infected with Ichthyobodo sp. A: Ichthyobodo sp. lesions, 25x magnification. Arrows indicate locations of some of the lesioned areas with caverns. B: 200x magnification, arrows indicate the location of some of the Ichthyobodo sp. individuals. The curly bracket indicates 5 inter lamellar units (ILUs), an example of the area size of which the number of Ichthyobodo sp. individuals were counted during examination of histological sections.

2.7.3 Epitheliocystis

The number of epitheliocysts per WOF$^{1/3}$ were counted at 200x magnification (Fig.2.7.3). Prevalence of epitheliocystis and the average number of epitheliocysts per WOF$^{1/3}$ were calculated in Excel™.

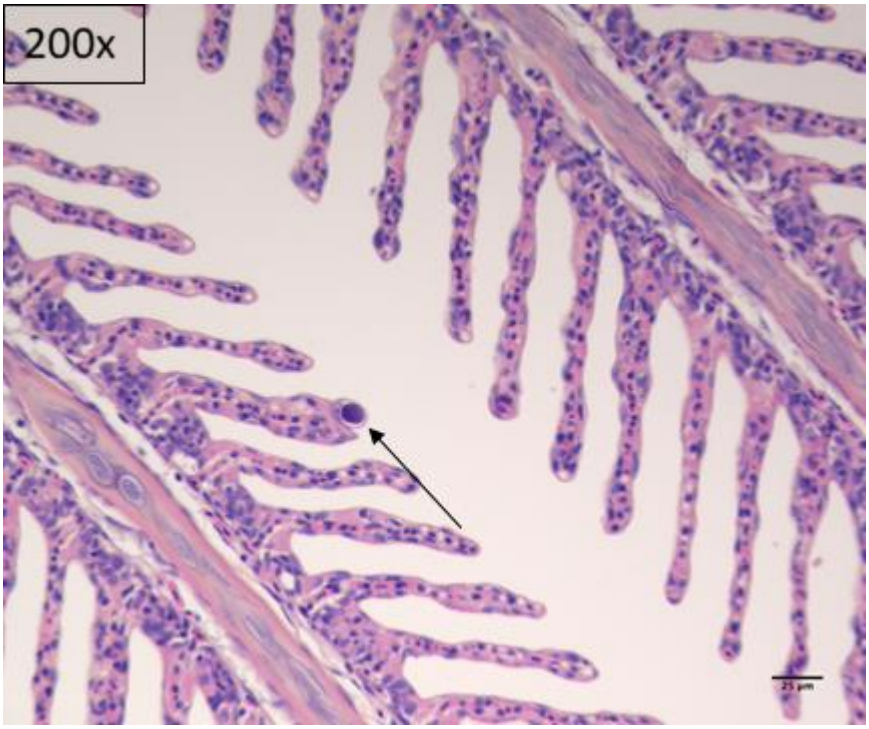

Figure 2.7.3: HE stained histological section of gill tissue, 200x magnification. The arrow indicates the location of an epitheliocyst, an example of the like that were counted during examination of histological sections

2.7.4 Mucous cell counts

For mucous cell counting the Speare *et al*. (1997) criteria for well oriented filaments were used. These filaments are referred to as WFO. Mucous cell counting was performed by use of a Labomed Lx 500 light microscope. Mucous cells of 10 ILUs on the clockwise side of WOFs in the dorsal, middle and ventral regions were counted (Fig.2.7.4-1A and Fig.2.7.4-2).

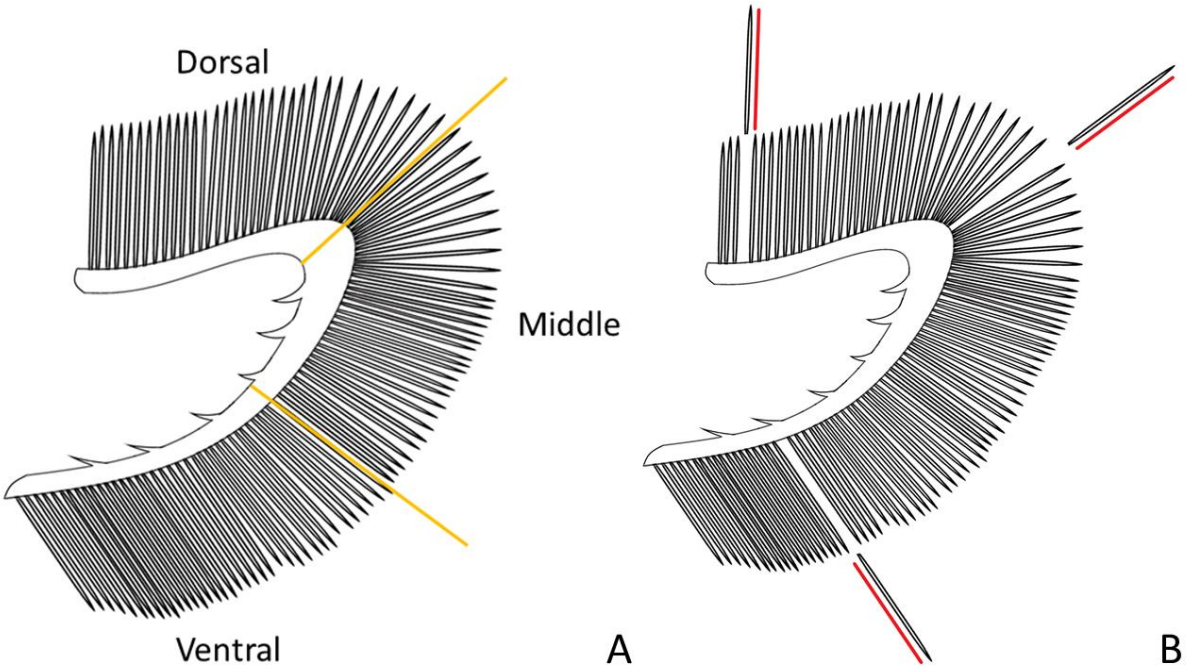

Figure 2.7.4-1: A: Schematic illustration of a gill arch and the divided regions used during mucous cell counts. B: Schematic illustration of a gill arch, the red lines parallel to the detached filaments indicate the clockwise side of the filaments in the different regions given in A

A random number generator, (RNG Plus version 2.4.7, for android), with numbers ranging from 5-50 was used to determine the starting lamella of mucous cell counts. The lamellae were counted from the base of the filament and mucous cell counts were performed from the lamellae number given by the RNG. The average number of mucous cells in the given regions and the overall average were calculated in Excel™. Standard deviation was calculated by (=STDAV) and standard error was calculated by $SE = \frac{STDAV}{\sqrt{n}}$.

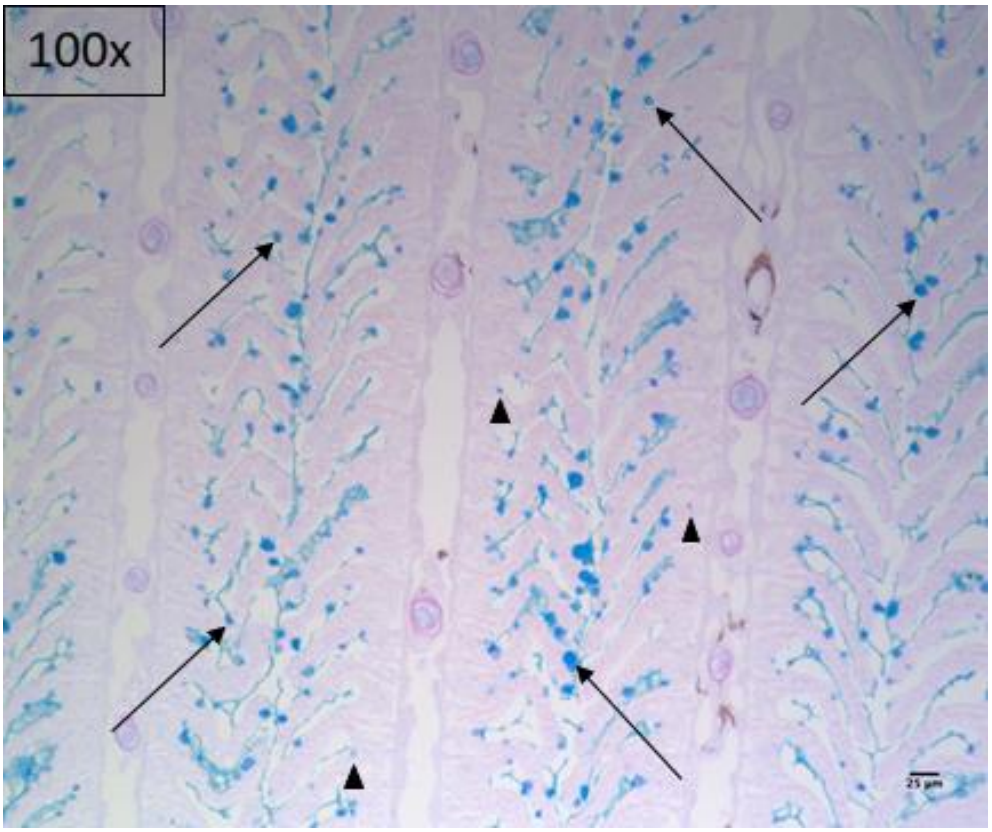

Figure 2.7.4-2: AB-PAS stained section of gill tissue, 100x magnification. Arrows indicate location of different sized mucous cells, both examples of cells that were counted. Arrowheads indicate positions of mucous cells that were not counted.

2.9 Data analysis

Data from gill scoring, semi-quantitative examination of histological sections and mucous cell counts were imported to RStudio, R version 3.5.1 (2018-07-02) for statistical analyses and graph plots. Two-way analyses of variance were performed and the level of significance was adjusted to $p < 0.01$ (Glass *et al*., 1972) for non-normal distributed data. Packages 'emmeans' and 'ggplot2' were used for multiple comparisons post hoc test and graph plots respectively.

3. Results

3.1 Mortality

Moderate scale loss was observed in all three fish types post-transport. At sampling points 1, 2, 3 and 4, during the challenge severe scale loss was observed. No consistent behavior related to skin irritation, e.g. scratching and flashing, were observed during the challenge. Nine days post-transfer to challenge tanks there was an acute mortality in the farm strain salmon (Fig.3.1.1). As this was before the challenge, the mortalities were replaced with fish from the holding tank. No mortality occurred in the farm strain salmon during the AGD-challenge. Mortality was observed in the wild strain salmon from 8-days post-transfer to the challenge tanks and throughout the challenge. The overall mortality of wild strain salmon and brown trout was 15% and 2%, respectively (Fig.3.1.1). As a consequence of the accumulated mortality, there were no wild strain salmon left in the control group after 3-weeks post-challenge. The mortalities during the challenge were not gill scored.

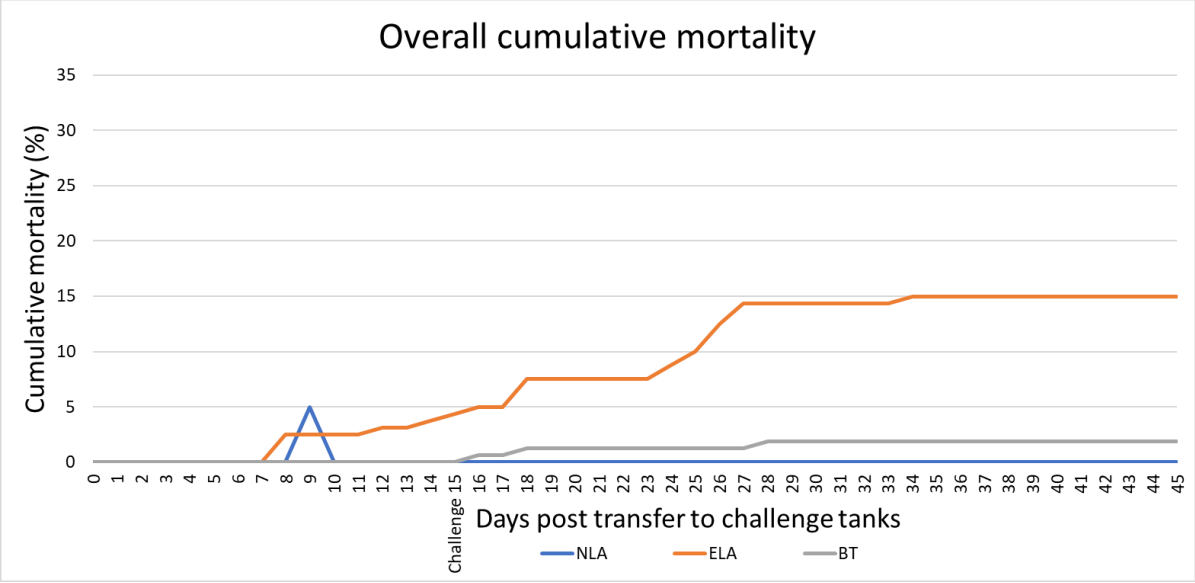

Figure 3.1: Overall cumulative mortality of farm strain salmon (NLA), wild strain salmon (ELA) and brown trout (BT) throughout their stay in the challenge tanks.

3.2 Gross gill scores

3.2.1 Industry standard gross gill scores

None of the fish in the pre-challenge samples had visual patches on their gill surfaces and therefore, gross gill scores 0. There were no significant differences between gill scores among

the controls or the low challenge dose groups within or between species throughout the challenge (Fig.3.2.1-1). Within the farm strain salmon, the high challenge dose group had significantly higher gill scores than the controls 1,2 and 4-weeks post-challenge ($F_{8,81}$ = 4.35, p-value = 0.0023, $F_{8,80}$ = 3.601, p-value = 0.0067 and $F_{8,59}$ = 4.784, p-value = 0.0014 respectively, Fig.3.2.1-1A). Within the wild strain salmon there were no significant differences between gill scores throughout the challenge (Fig.3.2.1-1B). At 4-weeks post-challenge the only significant differences in gill score within the brown trout were observed (Fig.3.2.1-1C). At this timepoint the high challenge dose group had significantly higher gill scores than the controls and the low dose group ($F_{8,59}$ = 4.784, p-values = 0.0010 and 0.0010 respectively). Across species the only significant difference between gill scores was among the high dose challenge groups 1-week post-challenge. At which time the farm strain salmon had significantly higher gross gill scores than both the wild strain salmon and the brown trout ($F_{8,81}$ = 4.35, p-values = 0.0023 and 0.0003 respectively).

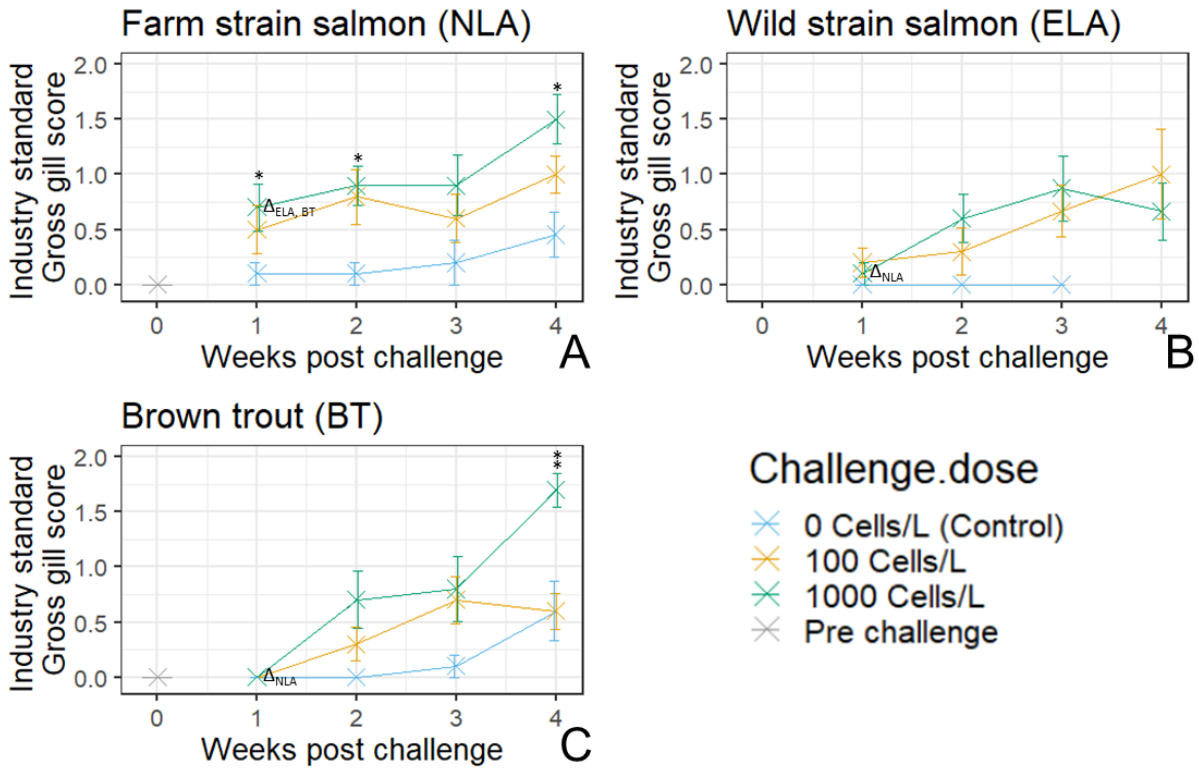

Figure 3.2.1-1: Average Industry standard gross gill scores of A: farm strain salmon (NLA), B: wild strain salmon (ELA) and C: brown trout (BT) during AGD-challenge. Error bars express standard error (SE). *significantly different from controls, **significantly different from controls and low challenge dose group. $\Delta_{species}$ significantly different from named species.

Industry standard gross gill scores = 2 were observed in individuals of all three species, both in the low and high challenge dose groups (Fig.3.2.1-2). However, only one fish in the study had industry standard gross gill score larger than 2. This was a farm strain salmon in the high challenge dose group 4-weeks post-challenge which had a score of 3. There was a trend of increasing percentage of fish with industry standard gross gill scores = 2 over time in the high challenge dose groups of all three species. The high challenge dose groups of farm strain salmon and brown trout both reached commercial treatment threshold 3-weeks post-challenge (Fig.3.2.1-2A and C). However, the wild strain salmon did not reach treatment threshold during the challenge (Fig.3.2.1-2B).

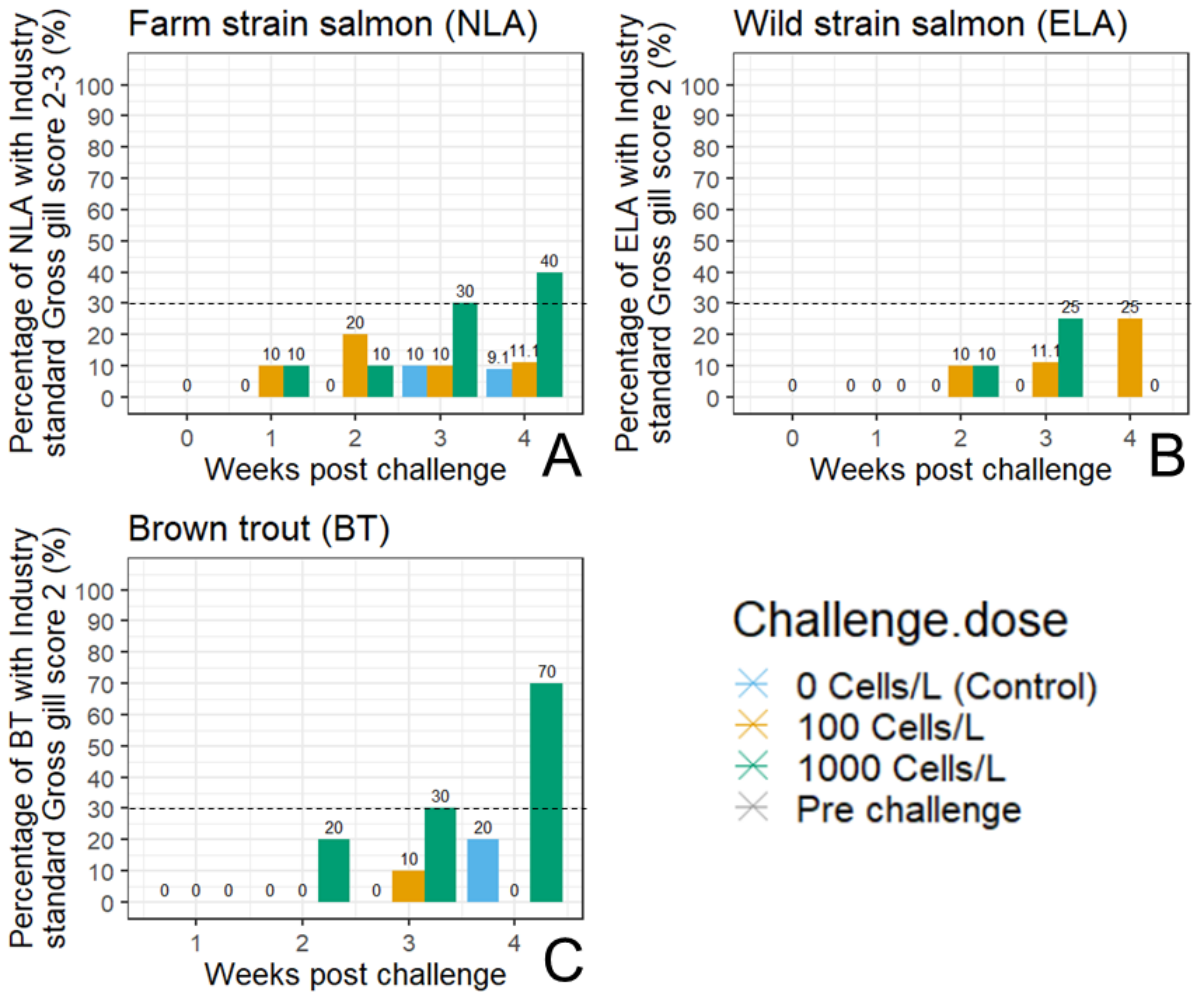

Figure 3.2.1-2: Percentage of A: farm strain salmon (NLA), B: wild strain salmon (ELA) and C: brown trout (BT) with industry standard gross gill scores ≥ 2 during AGD-challenge. The dashed line indicates commercial treatment threshold. Only one fish had industry standard gross gill score above 2. This was a farm strain salmon in the 1000 Cells/L group, 4-weeks post-challenge with an industry standard gross gill score 3.

3.2.2 Average gill score per gill surface

None of the fish in the pre-challenge samples had visual lesions on their gill surfaces and therefore average gill score per gill surface 0. There were no significant differences between average gill score per gill surface among the controls or the low challenge dose groups within or between any of the species throughout the challenge (Fig.3.2.2). Within the farm strain salmon, the high challenge dose group had significantly higher average gill score per gill surface than the controls 1- and 2-weeks post-challenge ($F_{8,81}$ = 4.939, p-value = 0.0003 and $F_{8,80}$ = 3.158, p-value = 0.0031 respectively, Fig.3.2.2-A). At 4-weeks post-challenge this group had significantly higher average gill score per gill surface than both the controls and the low challenge dose group ($F_{7,59}$ = 9.539, p-values < 0.0001 and = 0.0005). Within the wild strain salmon there were no significant differences between average gill score per gill surface throughout the challenge (Fig.3.2.2-B). At 4-weeks post-challenge the high challenge dose group of brown trout had significantly higher average gill score per gill surface than both the controls and the low challenge dose group ($F_{7,59}$ = 9.539, p-values < 0.0001 and = 0.0001 respectively, Fig.3.2.2-C). Among the high challenge dose groups, the farm strain salmon had significantly higher average gill score per gill surface than the wild strain salmon and the brown trout one-week post-challenge ($F_{8,81}$ = 9.939, p-values = 0.0003 and 0.0001 respectively). The high challenge dose group of brown trout had significantly higher average gill score per gill surface than the wild strain salmon 4-weeks post-challenge ($F_{7,59}$ = 9.534, p-value = 0.0061).

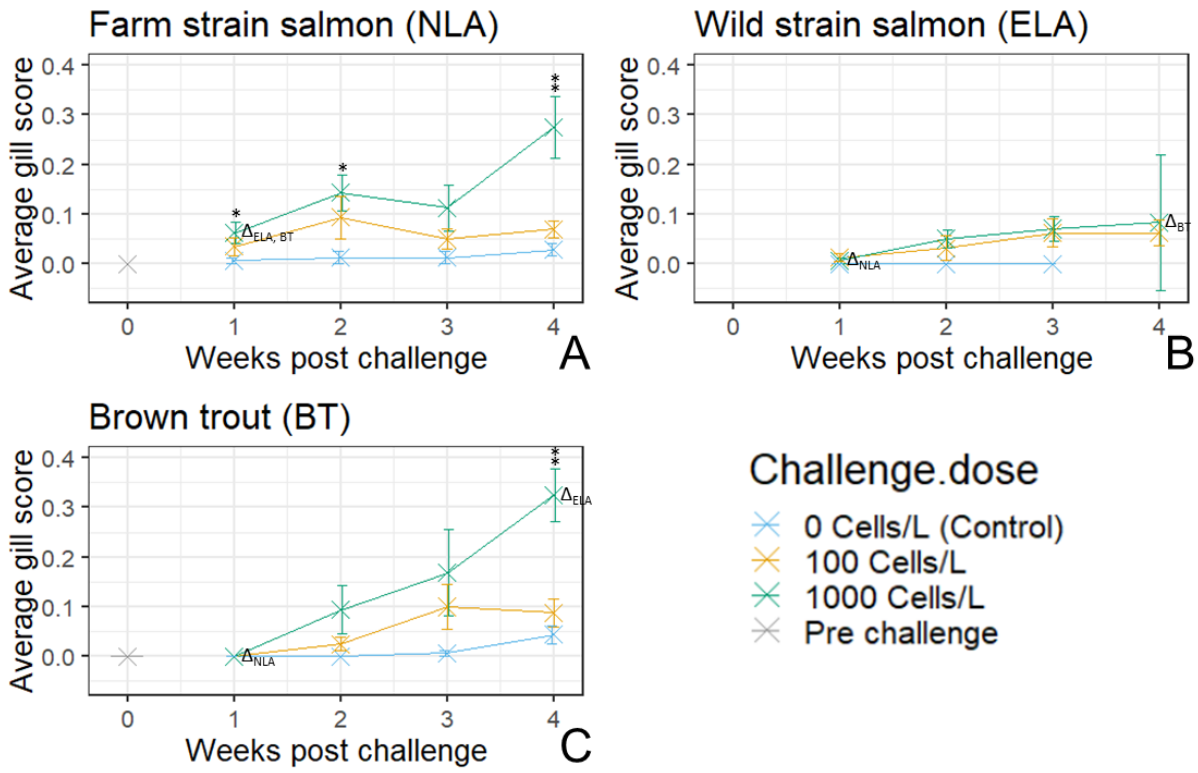

Figure 3.2.2: Average gill scores of A: farm strain salmon (NLA), B: wild strain salmon (ELA) and C: brown trout (BT) during AGD-challenge. Error bars express standard error (SE). *significantly different from controls, **significantly different from controls and low challenge dose group. $\Delta_{species}$ significantly different from named species.

3.2.3 Lesioned gill surfaces

None of the fish in the pre-challenge samples had visual lesions on their gill surfaces and were therefore scored 0 lesioned surfaces. There were no significant differences between number of lesioned surfaces among the controls or the low challenge dose groups, within or between any of the species throughout the challenge (Fig.3.2.3). The high challenge dose group of farm strain salmon had significantly higher numbers of lesioned gill surfaces than the controls 1-,2- and 4-weeks post-challenge ($F_{8,81}$ = 4.58, p-value = 0.005, $F_{8,80}$=3.77, p-value = 0.0008 and $F_{7,59}$ = 10.41, p-value < 0.0001 respectively, Fig.3.2.3-A). At 4-weeks post-challenge this group also had significantly more lesioned surfaces than the low challenge dose group (p-value = 0.0004). Within the wild strain salmon there were no significant differences in number of lesioned gill surfaces between controls or challenge groups throughout the challenge (Fig.3.2.3-B). At 4-weeks post-challenge the brown trout high challenge dose group had significantly higher numbers of lesioned gill surfaces than both the controls and the low challenge dose group ($F_{7,59}$ = 10.41, p-values < 0.0001 and = 0.0001 respectively, Fig.3.2.3-C). Between the high

challenge dose groups, the farm strain salmon had significantly higher numbers of lesioned gill surfaces than the wild strain salmon and the brown trout 1-week post-challenge ($F_{8,81}$ = 4.58, p-values = 0.0005 and 0.0001 respectively). The high dose group of brown trout had significantly higher numbers of lesioned gill surfaces than the wild strain salmon 4-weeks post-challenge ($F_{7,59}$ = 10.41, p-value = 0.0077).

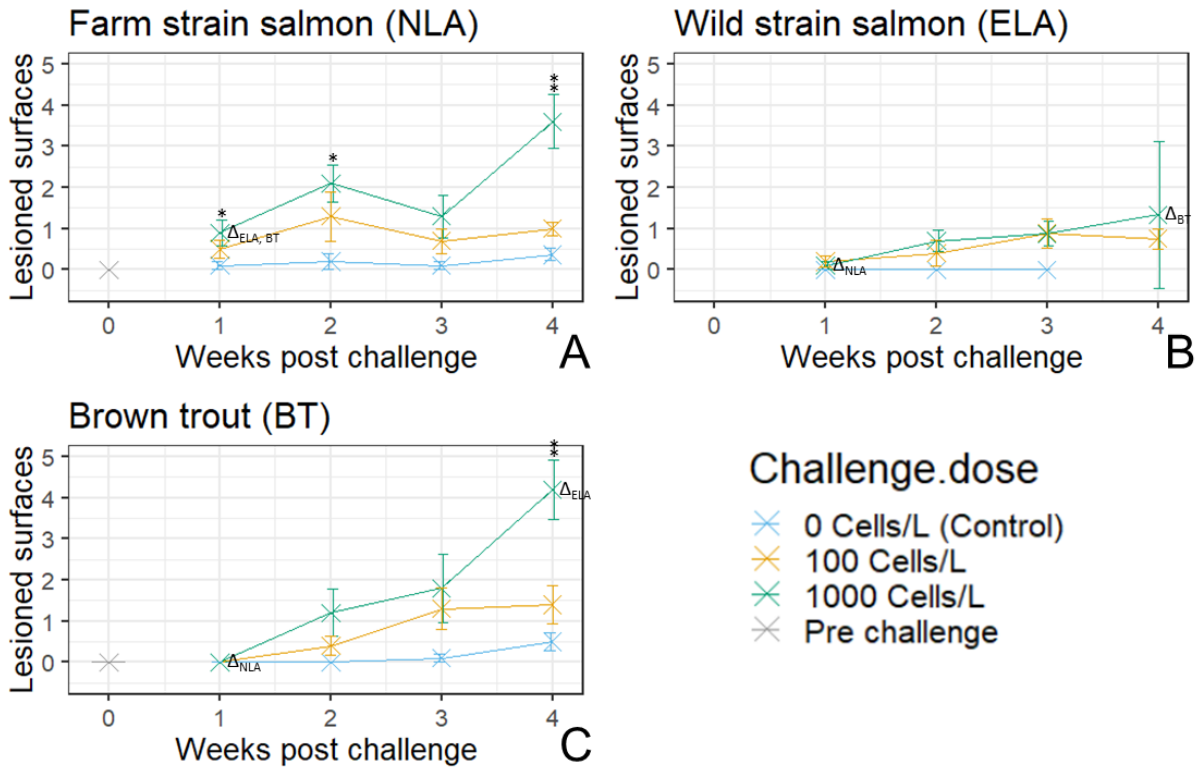

Figure 3.2.3: Lesioned gill surfaces of A: farm strain salmon (NLA), B: wild strain salmon (ELA) and C: brown trout (BT) during AGD-challenge. Error bars express standard error (SE). *significantly different from controls, **significantly different from controls and low challenge dose group. $\Delta_{species}$ significantly different from named species.

3.2.4 Gross gill score severity (the gill score sum of all 16 respective gill surfaces)

None of the fish in the pre-challenge samples had visual lesions on their gill surfaces and therefore gross gill score severity 0. There were no significant differences between gross gill score severity among the controls or the low challenge dose groups within or between any of the species throughout the challenge (Fig.3.2.4). The high challenge dose group of farm strain salmon had significantly higher gill score severity than the controls 1-,2- and 4-weeks post-challenge ($F_{8,81}$ = 4.989, p-value = 0.0003, $F_{8,80}$ = 3.155, p-value = 0.0032 and $F_{7,59}$ = 9.561, p-

value < 0.0001 respectively, Fig.3.2.4-A). At 4-weeks post-challenge the gill score severity of the high challenge dose group was significantly higher than the low challenge dose group as well ($F_{7,59}$ = 9.561, p-value = 0.0006). There were no significant differences in gross gill score severity within the wild strain salmon throughout the challenge (Fig.3.2.4-B). The high challenge dose group of brown trout had significantly higher gill score severity than the controls and the low challenge dose group 4-weeks post-challenge ($F_{7,59}$ = 9.561, p-values < 0.0001 and < 0.0001 respectively, Fig.3.2.4-C). Among the high challenge dose groups, the farm strain salmon had significantly higher gill score severity than the wild strain salmon and brown trout 1-week post-challenge ($F_{8,81}$ = 4.989, p-values = 0.0003 and 0.0001 respectively). Four-weeks post-challenge the high challenge dose group of brown trout had significantly higher gill score severity than the wild strain salmon ($F_{7,59}$ = 9.561, p-value = 0.0054).

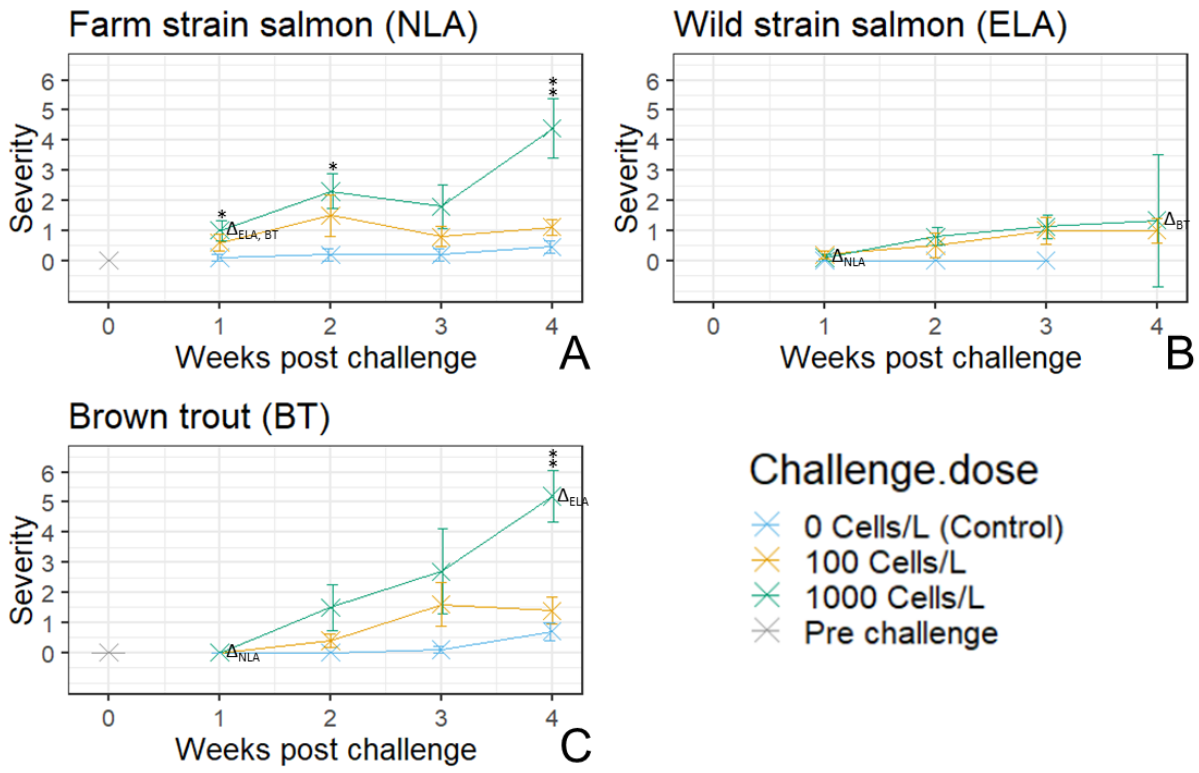

Figure 3.2.4: Gill score severity of A: farm strain salmon (NLA), B: wild strain salmon (ELA) and C: brown trout (BT) during AGD-challenge. Error bars express standard error (SE). *significantly different from controls, **significantly different from controls and low challenge dose group. $\Delta_{species}$ significantly different from named species.

3.3 qPCR-screening for *Paramoeba perurans*

All control fish were qPCR negative for *Paramoeba perurans*. There was a trend of increasing prevalence of *P. perurans* qPCR positive fish in the high challenge dose groups of farm strain and wild strain salmon over time (Fig.3.3.1A and B). This was different to the high challenge dose group of brown trout, which had a relatively stable prevalence of *P. perurans* qPCR positive fish, ranging from 50-60% throughout the challenge (Fig.3.3.1C). Extra qPCR samples from lesioned areas of gill arches were taken for confirmation of presence of *P. perurans*. The qPCR screening of the extra samples revealed *P. perurans* qPCR positive fish not detected by routine samples in the high and low challenge dose groups of farm strain salmon and the low challenge dose group of brown trout (Fig.3.3.2).

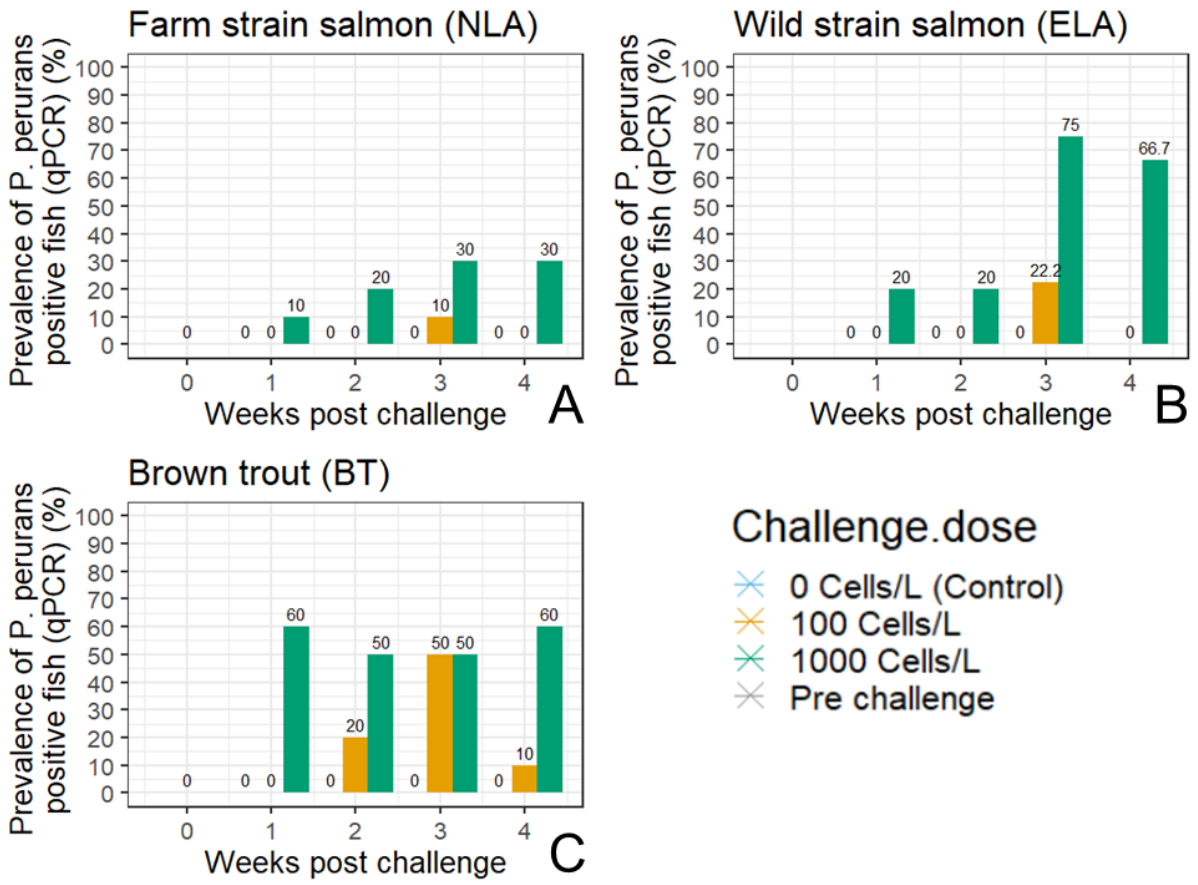

Figure 3.3.1: Prevalence of *Paramoeba perurans* qPCR positive A: farm strain salmon (NLA), B: wild strain salmon (ELA) and C: brown trout (BT) routine samples of gill tissue during AGD-challenge.

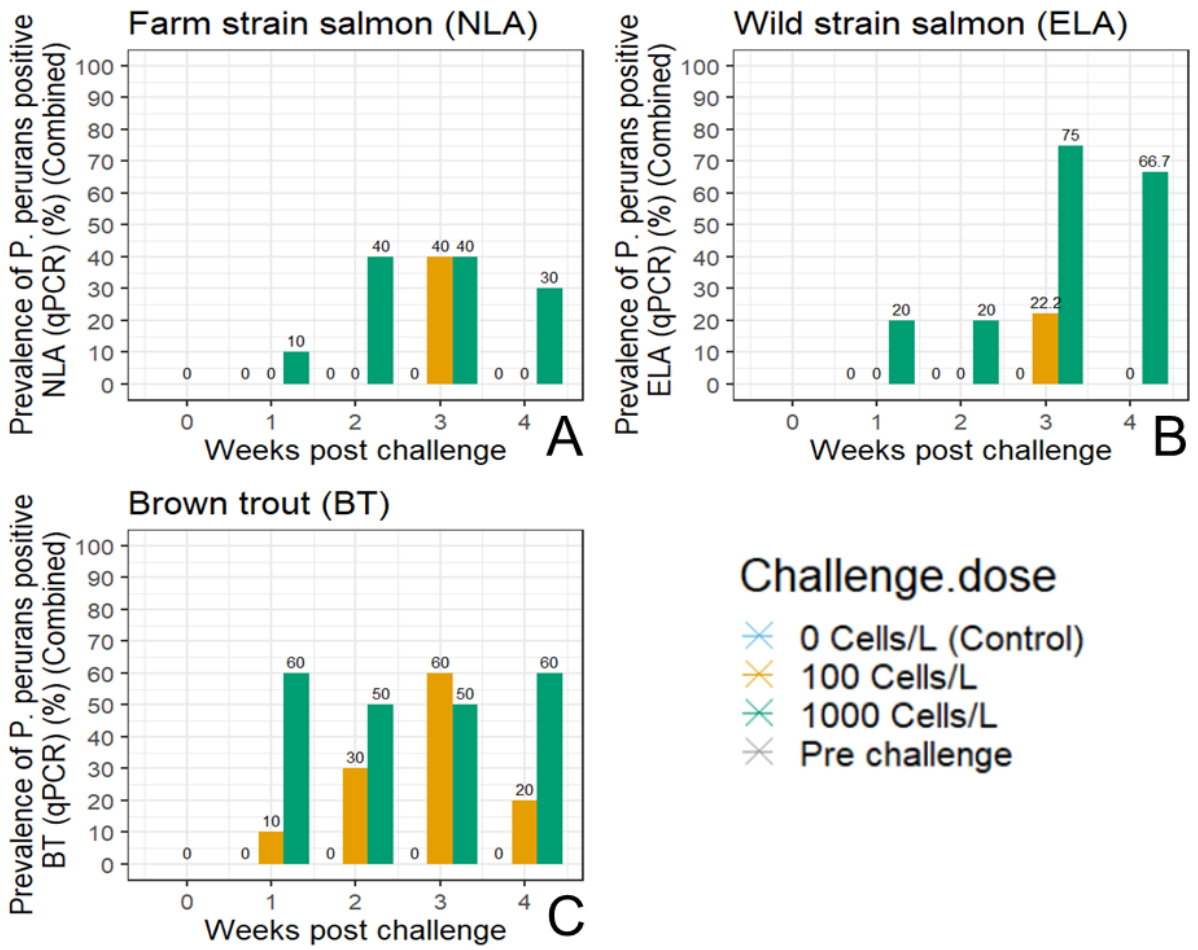

Figure 3.3.2: Prevalence of *Paramoeba perurans* qPCR positive A: farm strain salmon (NLA), B: wild strain salmon (ELA) and C: brown trout (BT) routine samples and extra samples of lesioned areas of gill tissue during AGD-challenge.

3.4 Histology

3.4.1 *Paramoeba perurans* and AGD-like lesions

Histologically, amoebae and AGD-like lesions were seen in few sections (Fig.3.4.1-1). AGD-lesions were only observed in histological sections of farm strain salmon in the high challenge dose group 1-week post-challenge. Histological AGD-like lesions were only observed 1- and 4-weeks post-challenge. One-week post-challenge in sections of farm strain salmon from the high challenge dose group (Fig.3.4.1-2A). AGD-like lesions were also observed in sections of wild strain salmon and brown trout 1-week post-challenge from the low challenge dose groups (Fig.3.4.1-2B and C). Four-weeks post-challenge AGD-like lesions were observed in the low challenge dose group of farm strain salmon.

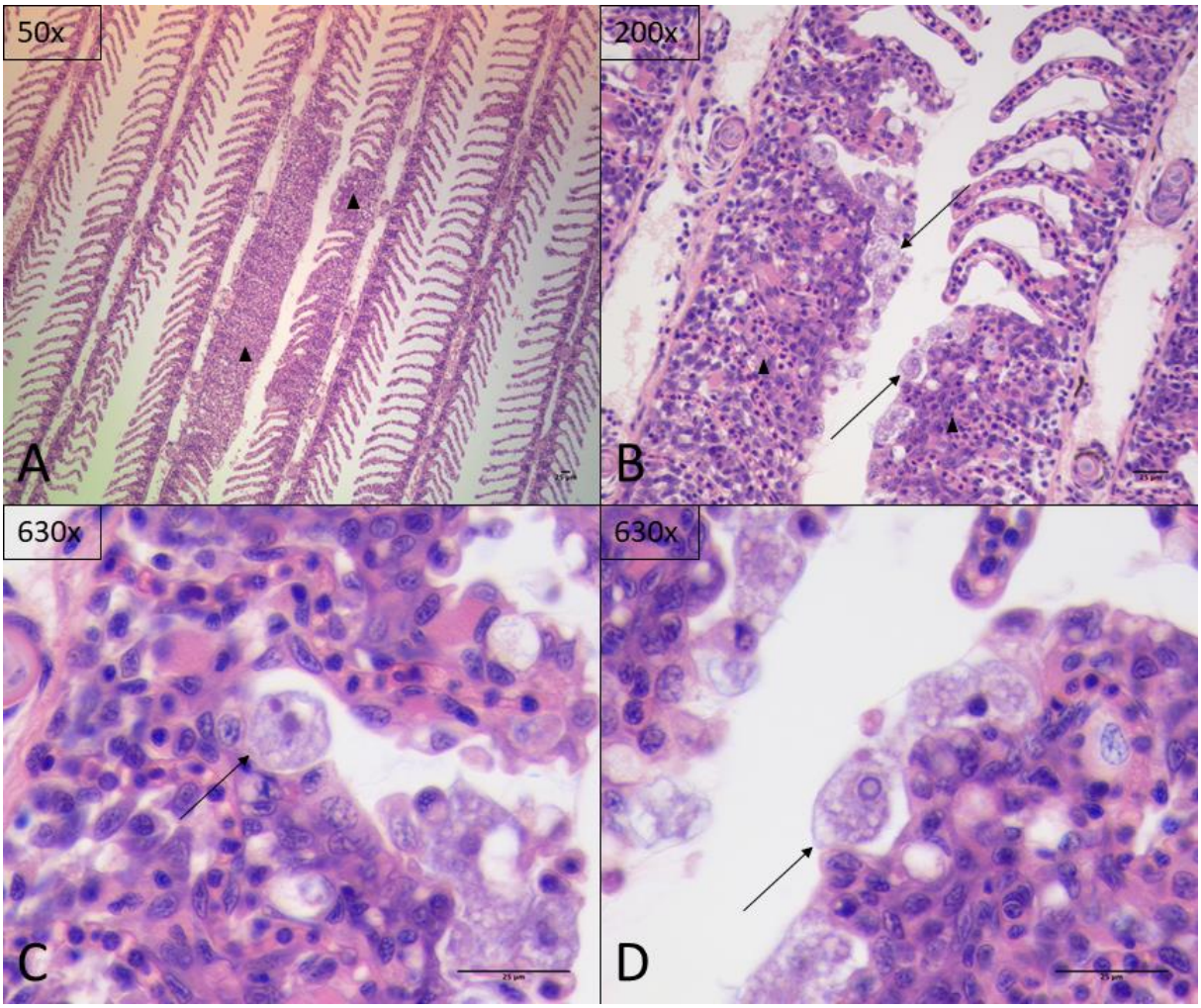

Figure 3.4.1-1: HE stained histological sections of gill tissue with AGD-lesions and amoebae. Magnification is given in the top left corner of each picture, scale bars in bottom right corner of each picture are 25µm. Arrow heads indicate location of AGD-like lesions. Arrows indicate location of amoebae. A: AGD-lesions with hyperplasia of epithelial cells, two lesion areas are indicated by arrow heads, 50x magnification. B: AGD-lesions and amoebae, AGD-lesions are indicated by arrow heads and amoebae by arrows.

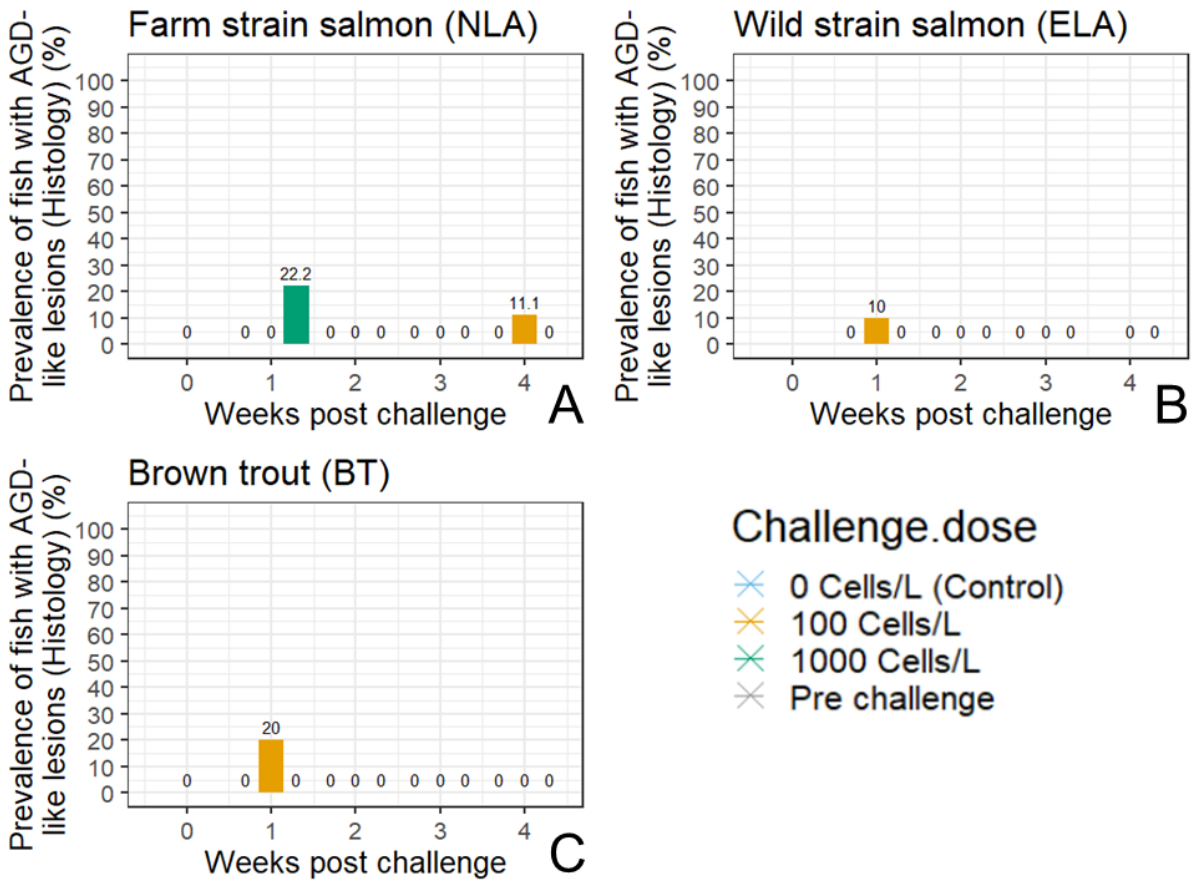

Figure 3.4.1-2: Prevalence of AGD-like lesions in histological sections of A: farm strain salmon (NLA), B: wild strain salmon (ELA) and C: brown trout (BT) during AGD-challenge.

3.4.2 *Ichthyobodo* sp.

Ichthyobodo sp. were observed in histological sections of all three species during the challenge (Fig.3.4.2-1). The first observations of *Ichthyobodo* sp. were made in the pre-challenge samples of farm strain salmon (Fig.3.4.2-2A). However, the wild strain salmon had higher prevalence of *Ichthyobodo* sp. than the farm strain salmon and the brown trout throughout the challenge with *P. perurans* (Fig.3.4.2-2). There was a trend of decreasing prevalence of *Ichthyobodo* sp. positive wild strain salmon over time in the high and low challenge dose groups. A similar trend, but not as profound, was seen among the farm strain salmon groups. *Ichthyobodo* sp. were only observed in brown trout 2-weeks post challenge with *P. perurans* (Fig.3.4.2-C).

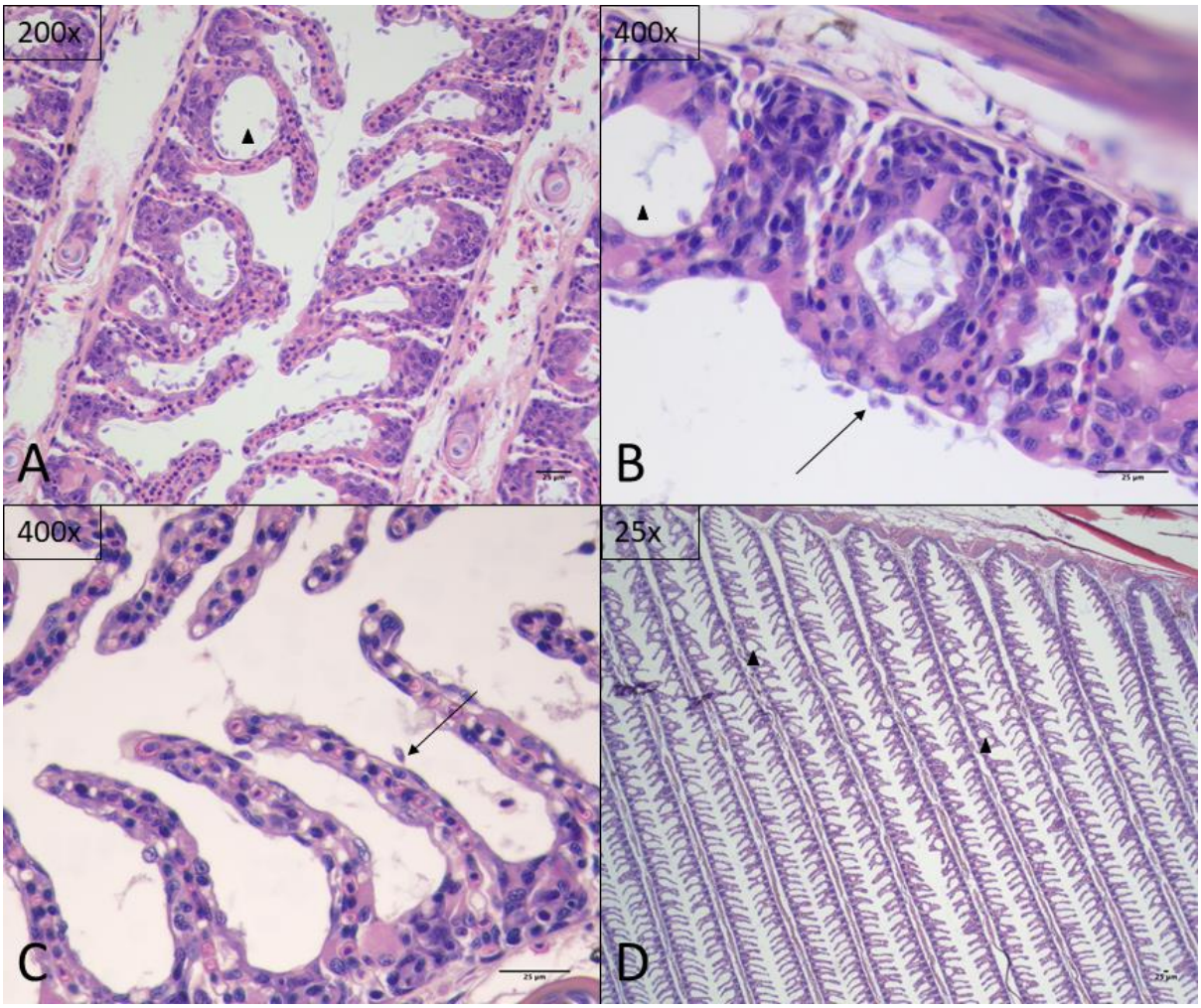

Figure 3.4.2-1: Histological sections of gills with *Ichthyobodo* sp. infections. Magnification is given in the top left corner of each picture, scale bars (bottom right corner) are 25µm. Arrows indicate location of some of the *Ichthyobodo* sp. cells. Arrow heads indicate the location of some interlamellar vesicles. A and B: heavily *Ichthyobodo* sp. infected gill surfaces 200x and 400x magnification respectively. The *Ichthyobodo* sp. cells are seen in caverns as well as on the outward epithelial surfaces. C: lighter *Ichthyobodo* sp. infected gill surfaces with only a few *Ichthyobodo* sp. cells, 400x magnification. D: Typical *Ichthyobodo* sp. lesions, two interlamellar vesicles are indicated by arrow heads, 25x magnification.

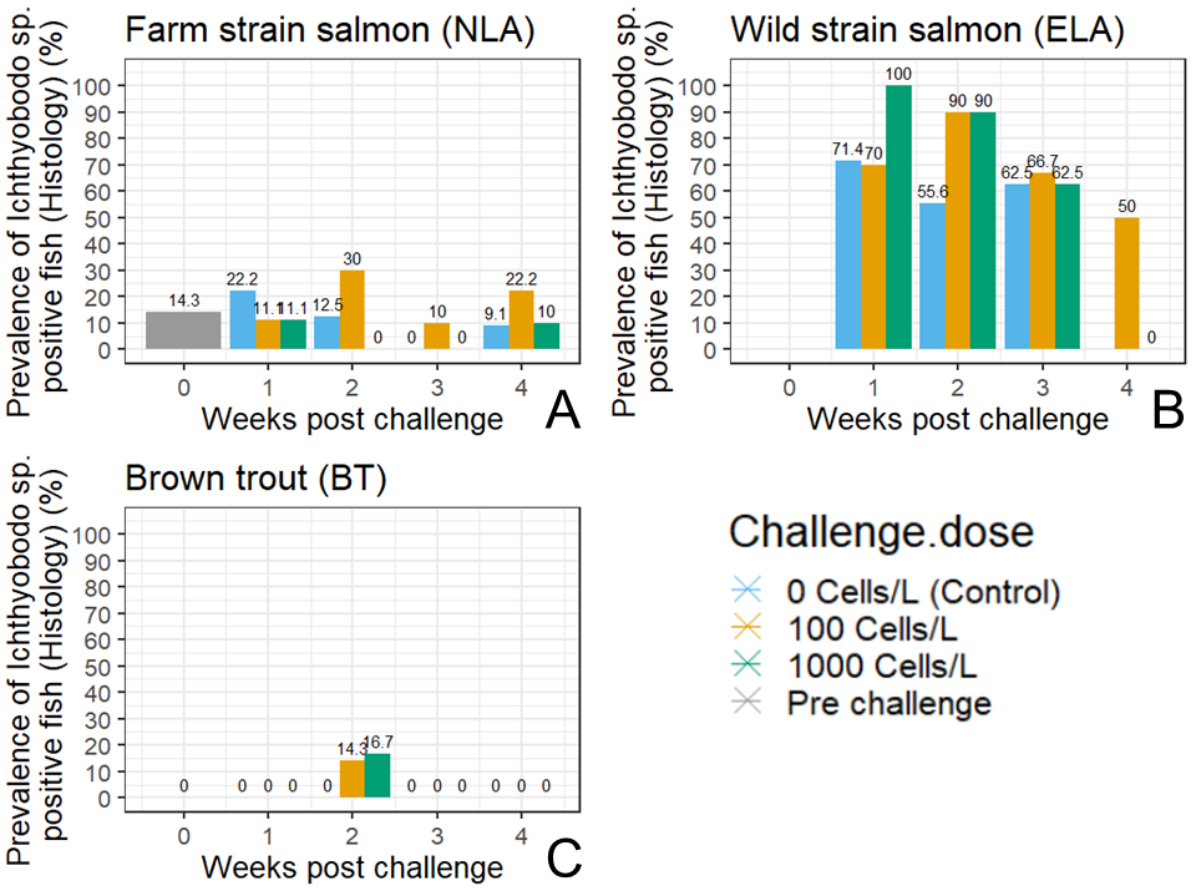

Figure 3.4.2-2: Prevalence of *Ichthyobodo* sp. in histological sections of gill tissue of A: farm strain salmon (NLA), B: wild strain salmon (ELA) and C: brown trout (BT) during AGD-challenge.

There were no significant differences in number of *Ichthyobodo* sp. individuals per 5 ILUs between challenge doses within any of the species throughout the challenge (Fig.3.4.2-3). However, there were significant differences between species. The high challenge dose group of wild strain salmon had significantly higher numbers of *Ichthyobodo* sp. individuals per 5 ILUs than the farm strain salmon 1-week post-challenge ($F_{8,64}$ = 3.158, p-value = 0.0076) and 2-weeks post-challenge ($F_{8,65}$ = 5.767, p-value = 0.0002, Fig.3.2.4-3). Two-weeks post-challenge, the high challenge dose group of wild strain salmon had significantly higher numbers of *Ichthyobodo* sp. individuals per 5 ILUs than the brown trout as well ($F_{8,65}$ = 5.767, p-value = 0.0012, Fig.3.2.4-3C). Among the low challenge dose groups, the wild strain salmon had significantly higher numbers of *Ichthyobodo* sp. individuals per 5 ILUs than the farm strain salmon 2- and 4-weeks post-challenge ($F_{8,65}$ = 5.767, p-value = 0.0013 and $F_{7,48}$ = 4.56, p-value = 0.0001 respectively). The same was seen between the wild strain salmon and the brown trout ($F_{8,65}$ = 5.767, p-value = 0.0021 and $F_{7,48}$ = 4.56, p-value < 0.0001 respectively).

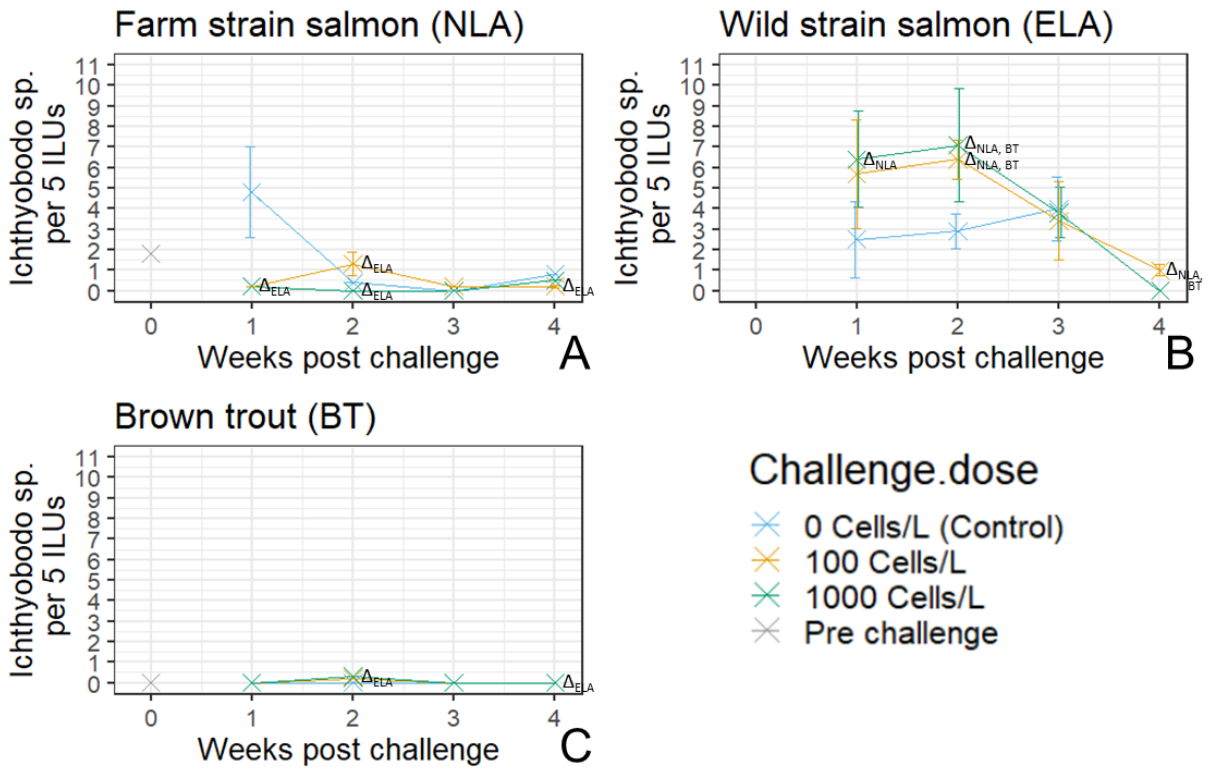

Figure 3.4.2-3: Average amount of *Ichthyobodo* sp. individuals per 5 ILUs in A: farm strain salmon (NLA), B: wild strain salmon (ELA) and C: brown trout (BT) during AGD-challenge. Error bars express standard error (SE). $\Delta_{species}$ significantly different from named species.

3.4.3 Epitheliocystis

Epitheliocysts were observed in histological sections of all three species (Fig.3.4.3-1). In the low challenge dose group of farm strain salmon, there was a trend of increasing prevalence of fish with epitheliocystis over time (Fig.3.4.3-2A). There was a similar trend in the low and high challenge dose groups of wild strain salmon (Fig.3.4.3-2B). In the brown trout there was no clear trend in the prevalence of epitheliocystis (Fig.3.4.3-2C). There were no significant differences in number of epitheliocysts per WOF between challenge doses within or between any of the species throughout the challenge (Fig.3.4.3-3).

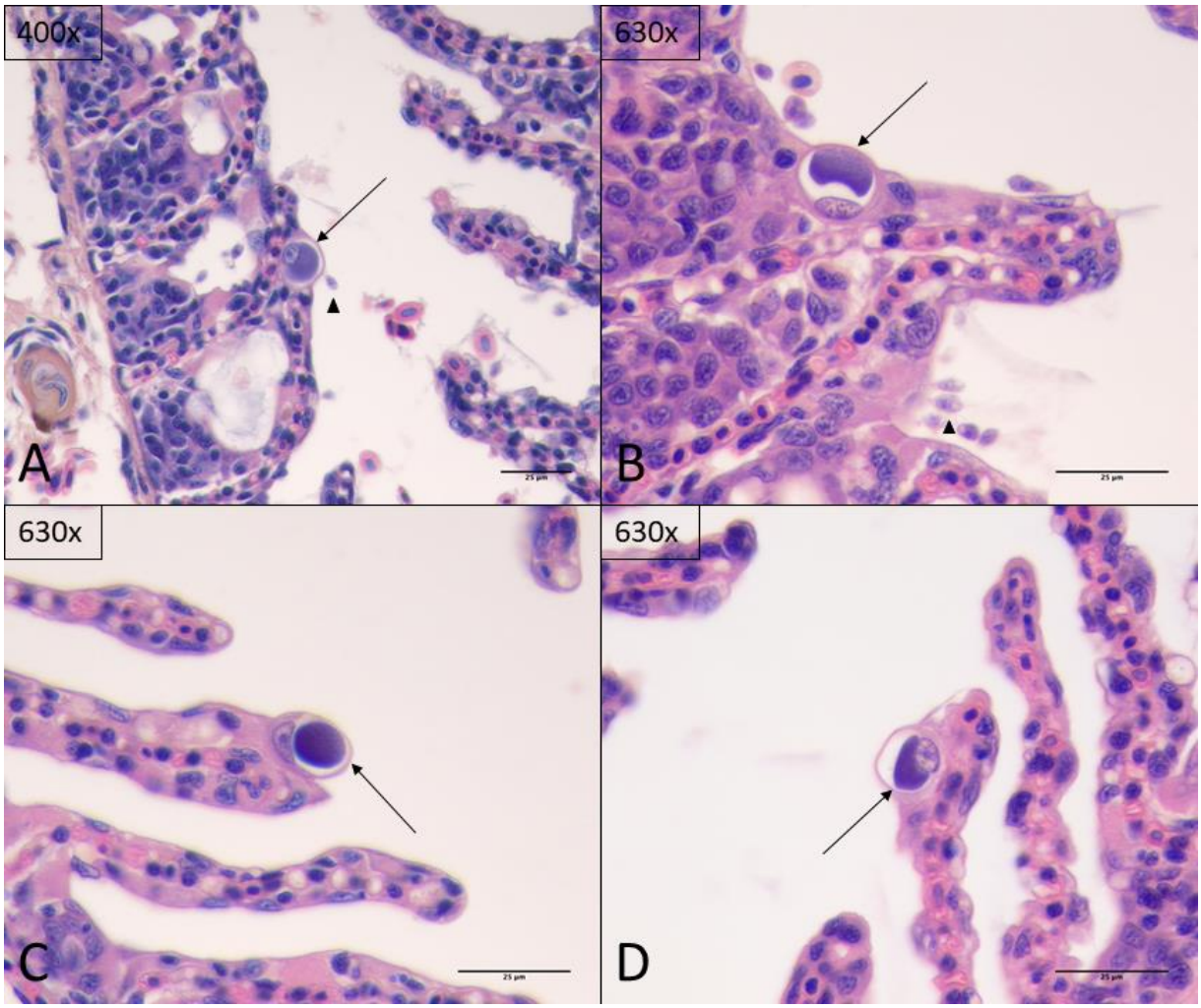

Figure 3.4.3-1: Histological sections of gill tissue with epitheliocysts (A, B, C and D) and *Ichthyobodo* sp. (A and B). Magnification is given in the top left corner of each picture. Scale bars in bottom right corners are 25µm. Arrows indicate location of epitheliocysts. Arrowheads indicate location of some of the *Ichthyobodo* sp. cells.

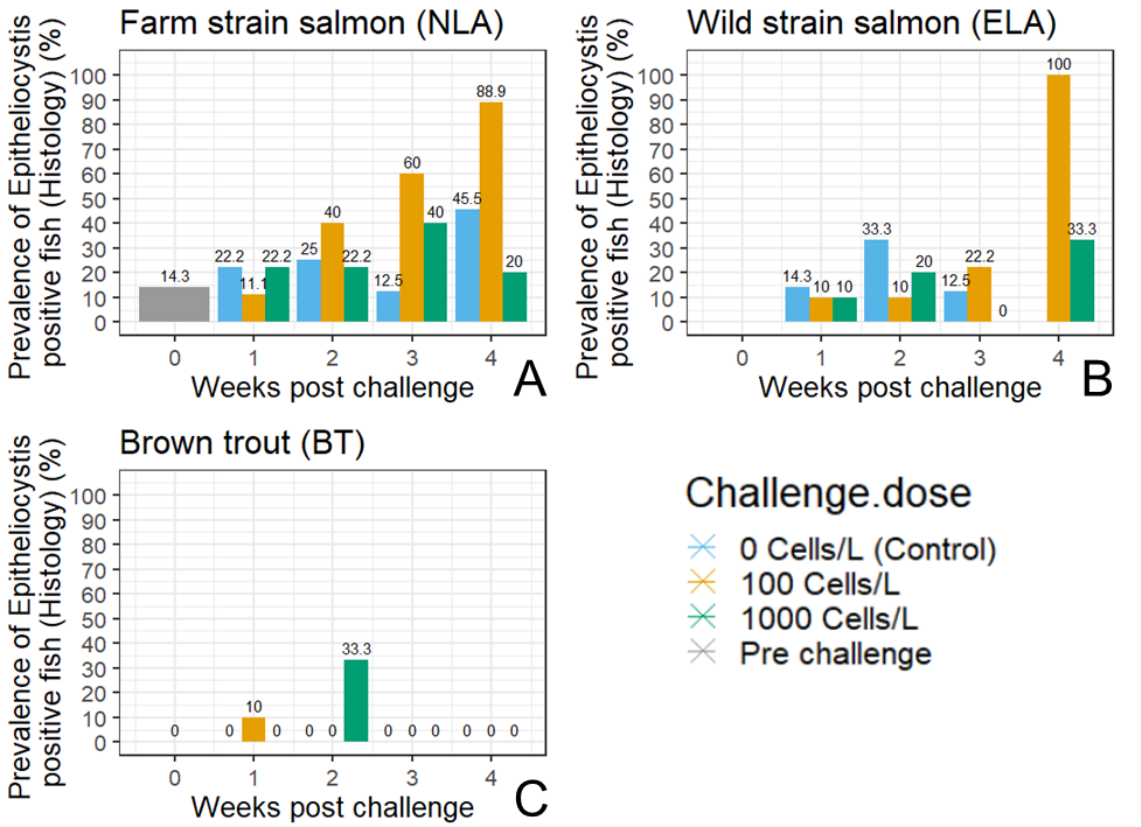

Figure 3.4.3-2: Prevalence of epitheliocystis in histological sections of gill tissue of A: farm strain salmon (NLA), B: wild strain salmon (ELA) and C: brown trout (BT) during AGD-challenge.

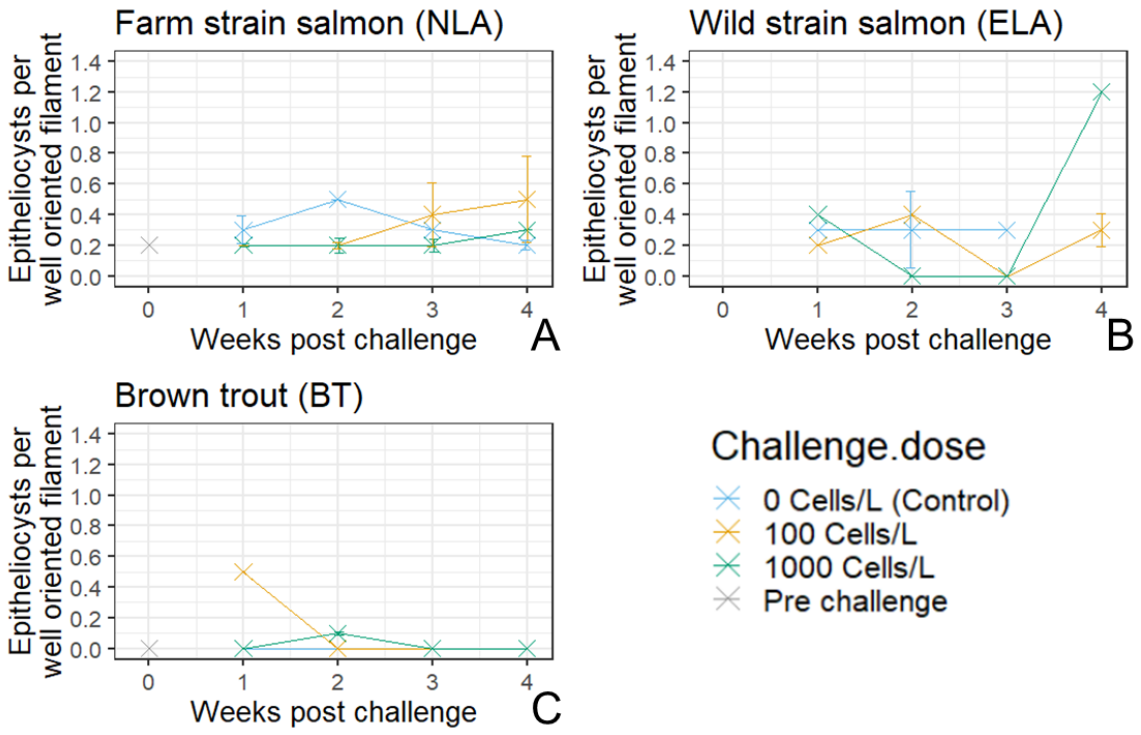

Figure 3.4.3-3: Average amount of epitheliocysts per $WOF^{1/3}$ in A: farm strain salmon (NLA), B: wild strain salmon (ELA) and C: brown trout (BT) during AGD-challenge. Error bars express standard error (SE). $\Delta_{species}$ significantly different from named species.

3.4.4 Other histopathological observations

Trichodinids were observed in three sections, two of wild strain salmon and one of farm strain salmon (Fig.3.4.4-1). Epithelial cells with karyorrhectic nuclei were observed in sections, but not quantified (Fig.3.4.4-2).

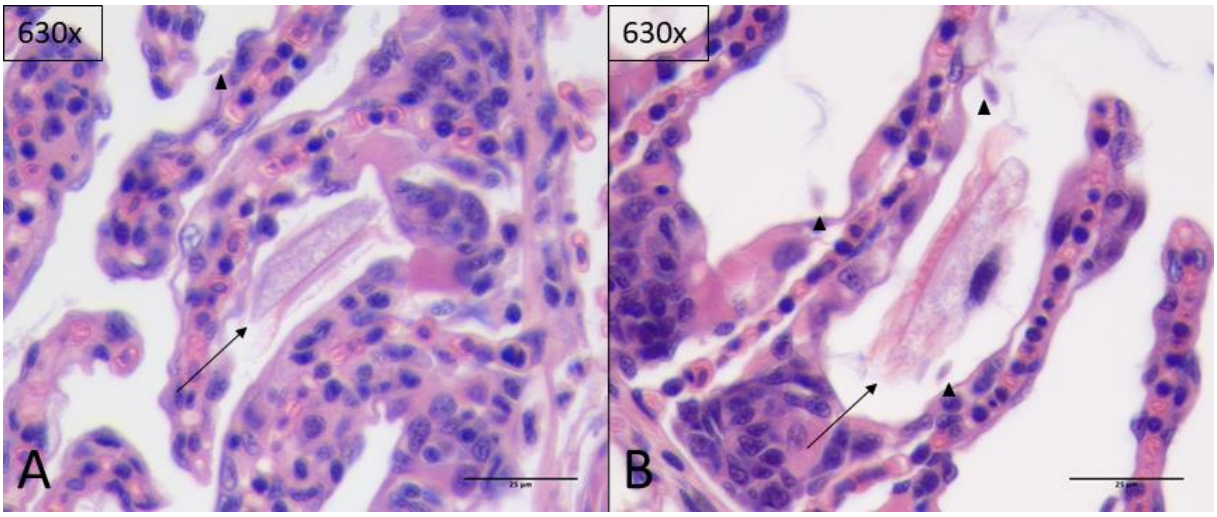

Figure 3.4.4-1: Trichodinids and *Ichthyobodo* sp. in HE stained histological sections of gill tissue of wild strain salmon (ELA). Arrows indicate the position of the trichodinids. Arrowheads indicate the position of some *Ichthyobodo* sp. cells. Magnification is given in the top left corner of each picture. Scale bars are 25µm.

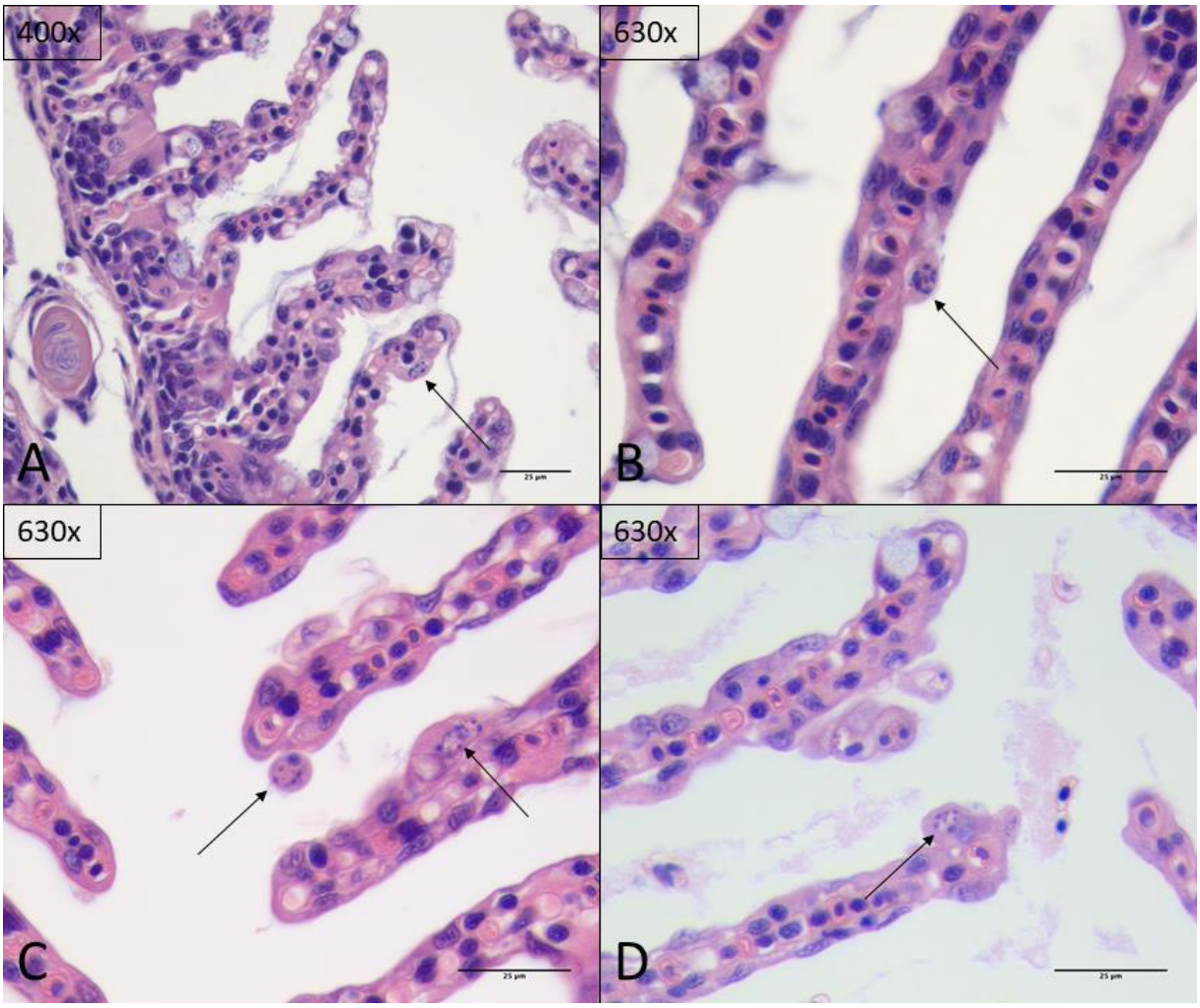

Figure 3.4.4-2: Necrotic epithelial cells (indicated by arrows) with karyorrhectic nuclei in HE stained histological sections of gill tissue of wild strain salmon (A and C) and farm strain salmon (B and D). Magnification is given in the top left corner of each respective picture, scalebars are 25µm.

3.5 Mucous cell counts

Due to failed AB-PAS staining of brown trout sections, the sections were not useable for mucous cell counts (Fig.3.5.1A).

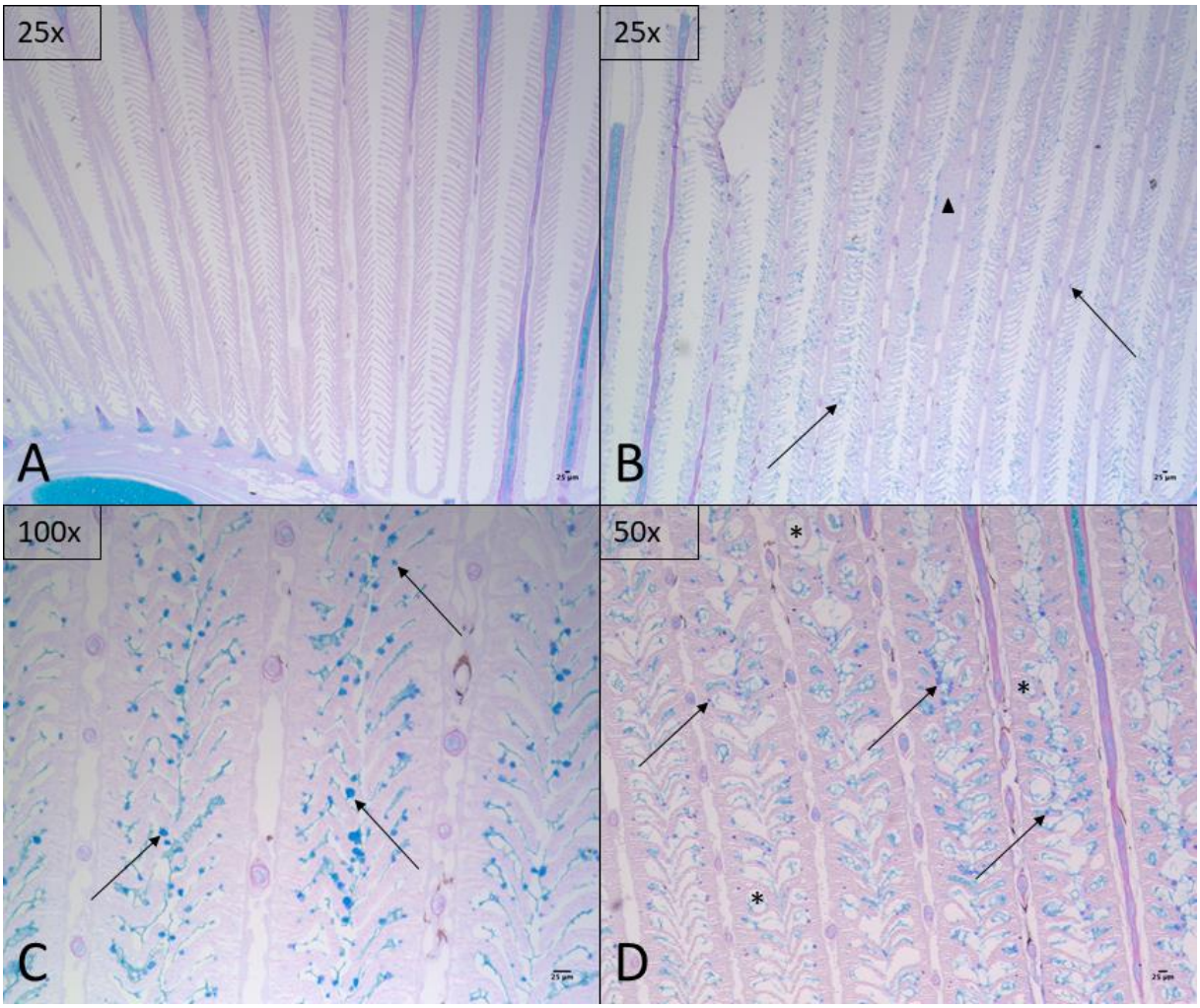

Figure 3.5.1: AB-PAS stained histological sections of gill tissue. Magnification is given in the top left corner of each picture and scale bars in bottom right corners are 25µm. Arrows indicate the location of some of the mucous cells. A: section of brown trout gill arch without visual mucous cells. B: AGD-lesion, indicated by arrowhead. C: Section with 100x magnification, used during mucous cell counting. D: *Ichthyobodo* sp. lesioned filaments, *indicate some of the interlamellar vesicles.

There were no significant differences in overall mucous cell counts within the wild strain salmon throughout the challenge (Fig.3.5.2B). Within the farm strain salmon, the controls had significantly higher numbers of overall mucous cells per 10 ILUs than the low challenge dose group 4-weeks post-challenge ($F_{4,26}$ = 4.523, p-value = 0.0069, Fig.3.5.2A). Between species, both the high and low challenge dose groups of farm strain salmon had significantly higher numbers of overall mucous cells than the wild strain salmon 1-week post-challenge ($F_{5,34}$ = 5.777, p-values = 0.0027 and 0.0010 respectively).

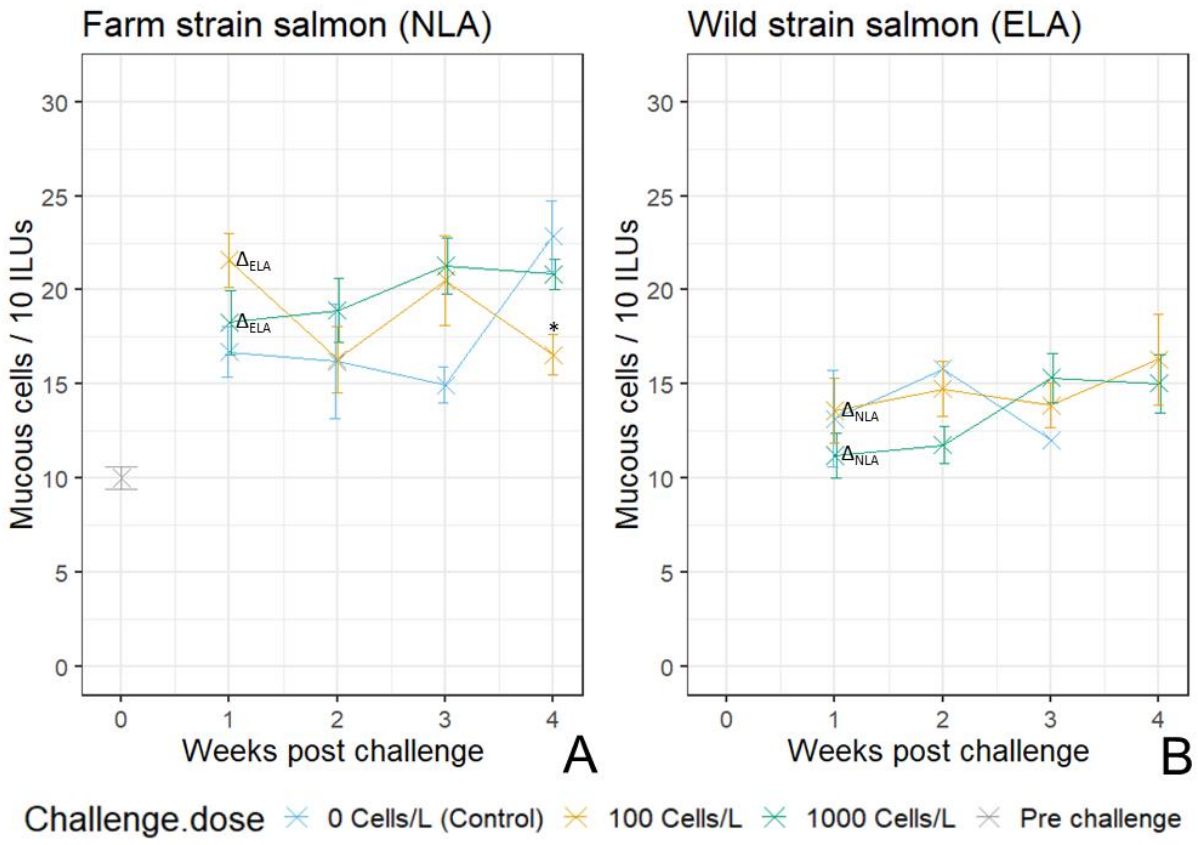

Figure 3.5.2: Average overall mucous cells per 10 ILUs in A: farm strain salmon (NLA) and B: wild strain salmon (ELA) during AGD-challenge. Error bars express standard error (SE.) *significantly different from controls. $\Delta_{species}$ significantly different from named species

Discussion

4.1 AGD-susceptibility: Gross gill scores and *P. perurans* qPCR positive fish

The brown trout and wild strain salmon appeared to have a delayed onset of AGD compared to the farm strain salmon. The brown trout and wild strain salmon developed macroscopically visual lesions later than the farm strain salmon. Even at one-week post challenge, fish from the high challenge dose group of farm strain salmon had developed macroscopically visual lesions, but they were not evident in the wild strain salmon or brown trout (Fig.3.2.1-1). Interestingly, the high challenge dose group of brown trout had a 60% prevalence of *P. perurans* qPCR positive fish 1-week post-challenge, but no macroscopically visual lesions on the gills (Fig.3.3.1). Despite previous studies with brown trout, this has not been previously observed (Maynard *et al*., 2016, Roberts and Powell, 2005b). However, a similar observation has been made with Atlantic salmon, albeit only in one fish (Young *et al*., 2008). The delayed development of lesions in the brown trout is similar to the observations Maynard *et al*. (2016) made during an AGD-field study where brown trout developed gross gill scores later than Atlantic salmon. However, earlier studies only used the identification of visual scores and lesions to confirm AGD and not PCR for confirmation of presence of *P. perurans* on the gill surfaces. The present study lasted for 29-days post-challenge, in contrast to the study performed by Maynard *et al*. (2016) that continued for 177 days post-transfer to seawater. Thus, the fish went through several infection cycles, unlike the fish in the present study which went through one infection cycle. The differences between brown trout and farm strain salmon were only seen 1-week post-challenge in the high challenge dose groups. There were no differences among the low challenge dose groups throughout the study. This may be because of the relatively short challenge length compared to the one performed by Maynard *et al*. (2016). Another possible reason might be that the low challenge dose did not trigger a strong enough response in the respective fish types to observe potential differences between them. Dose-dependent responses to challenge with *P. perurans* and the following development of AGD have previously been described Morrison *et al*. (2004). The brown trout in the Maynard *et al*. (2016) study reached commercial treatment threshold (30% of the fish with industry standard gross gill score ≥ 2) 28 days later than the Atlantic salmon. In the present study, both the farm strain salmon and the brown trout of the high challenge dose group reached commercial treatment threshold at the same time; 3-weeks post challenge.

However, in the low challenge dose group of brown trout, industry standard gross gill scores of 2 were only observed 3-weeks post challenge in 10% of the fish. Unlike the low challenge dose group of farm strain salmon, of which 10% had industry standard gross gill scores of 2 from 1-week post-challenge throughout the study. Similar to the high challenge dose group of brown trout, the wild strain salmon also developed macroscopically visual lesions later than the farm strain salmon. The farm strain salmon had significantly higher gross gill scores than the wild strain salmon one-week post-challenge. Interestingly, the prevalence of *P. perurans* qPCR-positive wild strain salmon was higher than of the farm strain salmon at this sample time. This may suggest that the wild strain salmon are as, or more susceptible to infection with *P. perurans* than the farm strain salmon but develop lesions to a lesser extent or more slowly, consistent with brown trout. Kambestad (2019) screened gill samples from 787 wild fish (Atlantic salmon and brown trout) from fjords and rivers representing western, middle and northern Norway, by qPCR. All samples in the Kambestad (2019) study were qPCR-negative for *P. perurans*. This may depend on the time of year the samples were taken and the presence of AGD around farms near sample cites However, the present study showed that wild-strain Atlantic salmon of the Etne river are susceptible to AGD. Three-weeks post-challenge the prevalence of *P. perurans* qPCR-positive fish in the high challenge dose group of wild strain salmon increased from 22.2% to 75%. Interestingly, there was no marked increase in gross gill scores between 3- and 4-weeks post-challenge. However, the variance in gross gill scores increased. There was observed mortality in the wild strain salmon during the challenge. The mortalities were not gill scored or sampled otherwise. Therefore, it remains unclear whether the mortalities were related to AGD-infection in addition to consequences of severe scale loss. Some of the control fish of farm strain salmon and brown trout had gross gill scores similar to AGD. However, they were all qPCR-negative for *P. perurans* (routine and extra samples from lesioned areas) and AGD-like lesions were not seen histologically. Adams *et al*. (2004) also observed that gill lesions with gross signs similar to AGD are not necessarily AGD-related. Gross AGD-like lesions can e.g. be associated with recovery from focal necrosis or be caused by gill infections with *Trichodina* sp. (Adams *et al*., 2004). Trichodinids were found on histological sections of the wild strain salmon and farm strain salmon. The trichodinids occurred singularly in the sections and were observed in the interlamellar space. Trichodinids were only found in histological sections of gill tissue of three respective fish out of 293 examined. Urawa (1992) observed trichodinids on the skin, and not on the gills of juvenile

chum salmon. However, Khan (1991) found trichodinids both on the skin and the gills of captive Atlantic salmon kelts, thus lower numbers on the gills than on the skin. Mitchell *et al*. (2013) observed trichodinids on the gills of sea water farmed Atlantic salmon that suffered from proliferative gill inflammation (PGI) from 14 Norwegian and three Irish salmon farms. Interestingly, the trichodinids were most frequently observed in samples of fish from the farm with highest mortality rates (Mitchell *et al*., 2013). There is a possibility that other pathogens than *P. perurans* may have caused some of the macroscopically visual lesions in the low and high challenge dose groups as well as in the controls. For example, English *et al*. (2019) found five other amoebae than *P. perurans* on the gills of Atlantic salmon, one of which, *Nolandella* spp., was related to low gross gill scores. Gross gill scoring is not regarded specific enough to solemnly estimate prevalence and severity of AGD in studies (Adams *et al*., 2004). However, gross gill scoring remains the accepted farm-monitoring tool of choice in the aquaculture industry (Adams *et al*., 2004). However, in Europe qPCR and visual confirmation of amoebae in gill scrapes are used when AGD is suspected. The precision of gross gill scoring can be increased by taking all 16 gill surfaces into account. An example is gross gill score severity, which is the sum of the gill scores of all 16 respective gill surfaces. The gill scoring criteria are the same as for the industry standard gross gill scores and the scores of all 16 respective gill surfaces are summed. This gives a theoretical maximum severity of 16x5=80. This method represents the gross gill score situation on all the gill surfaces, rather than just the gill surface with the highest gill score. This can be a useful approach because it may give a measure of the total severity of the gross lesions on all the gill surfaces.

4.2 qPCR-screening for *Paramoeba perurans*

One-week post-challenge the high dose group of brown trout had higher prevalence of *P. perurans* than the high dose groups of farm strain salmon and wild strain salmon. This prevalence is based on qPCR screening results of gill tissue samples from the apex of the 3rd left gill arch. Extra samples were taken to validate suspected lesions on other gill arches than the 3rd left, of which the routine samples were taken. As the extra samples were taken for validation, and not for screening purposes, only a select few from each fish type were taken. In some cases, the extra samples were positive while the routine samples of the respective fish were negative (Fig.3.3.2). The actual prevalence of *P. perurans* positive fish may, therefore, be higher than that observed in the routine sample-based prevalence (Fig.3.3.1 and

3.3.2). The increased chances of detecting *P. perurans* by qPCR at low levels by sampling gill tissue of more than one gill arch is highlighted in an unpublished Master thesis at the University of Bergen (Wiik, 2020). The brown trout typically occupied a different section of the challenge tanks than the salmon. The brown trout mostly stayed near the bottom, in contrast to the salmon that had a more pelagic distribution in the tanks. The water drained from the bottom of the tanks. It could be speculated that this may have caused the fish near the bottom to be exposed to infection longer than the pelagically distributed fish and increased the chance of infection. Particularly, since the amount of amoebae have been correlated to the severity of AGD (Morrison *et al*., 2004, Zilberg *et al*., 2001).

4.3 Semi-quantitative examination of histological sections

AGD-like lesions were observed in few histological sections and there were no clear differences between species. The low detection of AGD-like lesions in the present study, is likely to be consequently related to light infections (Adams and Nowak, 2001). However, there can also be several other additional reasons for the low detection of histological AGD-like lesions. Only the 2nd left gill arch was sampled for histopathological investigation. Lesions would, therefore, have to be present on this gill arch to be detectable. The section would have to be made in the plane of a lesion to be detectable, which can be particularly troublesome for inexperienced histologists (Adams *et al*., 2004). The lesion would have to be on a filament that met the criteria set for well oriented filaments for histopathological examination. Adams *et al* (2004) observed agreement between macroscopically visual gill lesions and histological lesions in moderate and advanced cases of AGD. In light infections with *P. perurans*, lesions are rarely occurring on all gill arches and the chances of histopathological detection are therefore smaller compared to more advanced cases of AGD (Adams and Nowak, 2001). The additional criteria that demanded a certain filament length (WOF$^{1/3}$) contributed to reduced numbers of “approved” filaments, and therefore, reducing the chance of detecting AGD-like lesions. The intent of the length-criterion of WOF$^{1/3}$ was to examine filaments of a certain length relative to the respective gill arch. However, this proved to be challenging in practice. This criterion resulted in a strong reduction of “approved” filaments, and consequently reduced numbers of evaluated filaments. Consequently, the robustness of the quantification of AGD-like lesions, *Ichthyobodo* sp. and epitheliocysts with respect to the gill arch were also compromised in sections with few “approved” filaments. There seemed to be less “approved” filaments in sections of small gill arches compared to larger. The brown trout on average were

smaller than the salmon and, consequently, had more rejected sections than the others. Because of this, the prevalence of AGD-like lesions, *Ichthyobodo* sp. and epitheliocysts, as well as the infection loads of these, could be underestimated. Specific criteria for filament length regarding histopathological examination have been seen in other studies e.g. in Adams and Nowak (2003b) and (Taylor *et al*., 2007), where the central venous sinus should be visible at least 2/3 of the filament. Whereas, for mucous cell quantification, well oriented filaments are typically referred to as filaments that have lamellae of bilaterally equal length to near the tip of the filament (Speare and Ferguson, 1989, Speare *et al*., 1997, Dang *et al*., 2020). This considered, it might be of interest to state criteria for filaments for (semi-) quantitative examination of histopathological changes and/or pathogens with respect to enlighten the extent of the gill area investigated and increase comparability between studies. Mitchell *et al*. (2013) suggested to register histopathological changes on a continuous scale to facilitate identification of statistical differences. This was attempted in the current study by quantifying the number of *Ichthyobodo* sp. cells per 5 ILUs and the number of epitheliocysts per $WOF^{1/3}$, a length relative to the respective gill arch. However, there is room for improvement of the filament length criterion, especially with respect to facilitate for greater use of sections of small gill arches. Gjessing *et al*. (2019) developed a semi-quantitative scoring system for gill disease in seawater farmed Atlantic salmon. Their method was used to score degrees of histopathological lesions related to different infectious agents (scores 0-10). The method was regarded sensitive and robust but time consuming and more applicable to research than diagnostics (Gjessing *et al*., 2019). Mitchell *et al*. (2012) also made a proposition for a histopathological scoring method. Like the Gjessing *et al*. (2019) method, Mitchell *et al*. (2012) also used a semi-quantitative approach to assess the severity of histopathological changes. The observers that performed the histopathological assessment in the Mitchell *et al*. (2012) study, discussed the criteria before histopathological investigation to reduce the inter-observer variations. Similarly, the method by Gjessing *et al*. (2019) was performed by three histopathologists and an inter-observer agreement. However, these approaches were not used in the present study, as they address the severity of histopathological changes rather than quantification of pathogens and/or pathogen-associated lesions. Unlike Gjessing *et al*. (2019) and Mitchell *et al*. (2012), the histopathological examination of sections in the present study focused on a semi-quantitative approach with respect to quantifying the presence of amoebae, *Ichthyobodo* sp. and epitheliocysts in given areas of gill filaments. The degree of

different lesion types and host-responses were not scored. Gjessing *et al*. (2019) rejected sections displaying tissue compression, folded sections and sections with autolyzed tissue. The plane of section was also considered by Gjessing *et al*. (2019). However, it is not clear if there were set criteria for filaments of examination in the method by Gjessing *et al*. (2019). Interpretation of histopathological lesions related to co-infections can be challenging with respect to determining which agents cause the histopathological changes (Gjessing *et al*., 2019, Herrero *et al*., 2018). Amoebae are often seen near the edges of hyperplastic lesions and rarely on the surface of long regions of fully fused lamellae (Adams and Nowak, 2003a, Taylor *et al*., 2007). Accordingly, Adams *et al*. (2004) found more amoebae near the ends of lesions rather than on top of long hyperplastic lesions. Adams *et al*. (2004) suggested serial sectioning to make a more reliable diagnosis for sections with heavy hyperplasia, as these areas seldomly contain amoebae (Adams and Nowak, 2003a). Inflammatory responses in hyperplastic lesions have been documented (Pennacchi *et al*., 2014). Adams and Nowak (2003) suggested that regions with fully fused lamellae make a more exposed surface than the interlamellar spaces. The water velocity may be higher at those regions compared to interlamellar spaces and make attachment more difficult for the amoebae (Adams and Nowak, 2003a). Adams and Nowak (2001) found more lesions in the dorsal region of the gill arches of AGD-affected Atlantic salmon and suggested that the water velocity may be lower in the dorsal region compared to other regions of the gills. One-week post-challenge, both the low and high challenge dose groups of wild strain salmon had significantly lower numbers of mucous cells than the farm strain salmon (Fig.3.5.2). The lower number of mucous cells in the wild strain salmon one-week post-challenge could potentially have been influenced by the infection with *Ichthyobodo* sp. *Ichthyobodo necator*, a relative of *Ichthyobodo* sp., is known to cause reduction in mucous cell numbers in early stages of infection (Bruno *et al*., 2013). AB-PAS stained sections revealed large amounts of mucus in the interlamellar spaces, interlamellar vesicles and between filaments of heavily *Ichthyobodo* sp. infected wild strain salmon. Increased mucous secretion can subsequently result in reduced numbers of mucous cells until the production of mucous cells are increased (Powell *et al*., 2008). Additionally, the apparent increased mucus secretion in heavily *Ichthyobodo* sp. infected wild strain salmon consequently complicated mucous cell quantification. The smallest AB-PAS stained mucous cells were not counted, consequently due to inexperience regarding criteria for mucous cell quantification, and consequently may have led to inaccurate estimation of mucous cell

numbers (Fig.2.8.3-4). However, the presented mucous cell counts indicate the number of mucous cells containing mucus. A lower number of these may, potentially, indicate an increased mucus secretion. Four-weeks post-challenge, the low dose group of farm strain salmon had significantly lower numbers of mucous cells than the controls. At this sample time, the controls had a lower prevalence of *Ichthyobodo* sp. compared to the low challenge dose group. Mucous cell counts were performed at 100x magnification. This relatively low magnification may have influenced the sensitivity of the counts. Zilberg and Munday (2000) counted mucous cells at 400x magnification. However, mucous cell counts have previously been performed at 100x magnification (Roberts and Powell, 2003a). Mucous cell counts could not be performed on AB-PAS stained sections of brown trout gill tissue due to insufficient staining. Previous studies of AGD in brown trout have successfully stained histological sections of brow trout with AB-PAS (Roberts and Powell, 2005b). Zilberg and Munday (2000) found increased numbers of mucous cells in AGD-affected Atlantic salmon 2-weeks post-challenge. Roberts and Powell (2003, 2005), as well as Adams and Nowak (2003), observed increased numbers of mucous cells in AGD-affected fish compared to unaffected fish. Numerous mucous cells are often observed in regions of fully fused lamellae related to AGD-lesions (Adams and Nowak, 2003a, Adams *et al*., 2004, Roberts and Powell, 2003b, Roberts and Powell, 2003a). Adams *et al*. (2004) also observed high numbers of mucous cells near AGD-lesions. Additionally, Adams *et al*. (2004) observed that amoebae rarely occupied mucous cell rich hyperplastic epithelia. Similarly, Adams and Nowak (2003) observed few amoebae in areas with high numbers of mucous cells. *Ichthyobodo* sp. was observed in histological sections of all three species. However, the wild strain salmon generally had a higher prevalence of *Ichthyobodo* sp. and more *Ichthyobodo* sp. individuals per 5 ILUs than the farm strain salmon and brown trout (Fig.3.4.2-2 and 3.4.2-3). Kambestad (2019) identified *Ichthyobodo* spp. in gill samples of wild Atlantic salmon and brown trout from both rivers and fjords in western, middle and northern Norway. The prevalence of *Ichthyobodo* spp. qPCR-positive wild Atlantic salmon ranged from 50-100%. Interestingly, the prevalence of *Ichthyobodo* spp. qPCR-positive brown trout was 97.1-100% (Kambestad, 2019). Thus, it should be noted that the sample sizes of brown trout consistently were smaller than of Atlantic salmon; Atlantic salmon were the main focus of the study (Kambestad, 2019). Parasitic infections often cause increased mucous production (Ferguson, 2006). Considering that mucus plays a central role in defense against infections (Koppang *et al*., 2015) and serves as a first line of defense barrier against infections

(Powell *et al*., 2008), there might be a possibility that the *Ichthyobodo* sp. infection influenced the potential attachment of amoebae. *Ichthyobodo* sp. infections on the gills can cause lamellar fusion (Bruno *et al*., 2013), this was observed in the wild strain salmon groups with large individual variation. The lamellar fusion associated with *Ichthyobodo* sp. infections can be quite distinct from AGD-associated lamellar fusion (Bruno *et al*., 2013). The lamellae typically fuse at the tips and create interlamellar vesicles in the recovery phase of *Ichthyobodo* sp. infections (Bruno *et al*., 2013). This lesion type was seen in heavily *Ichthyobodo* sp. infected wild strain salmon and were often limited to the proximal parts of the filaments. Interlamellar vesicles were still observed in some sections 4-weeks post-challenge, interestingly without visual *Ichthyobodo* sp. cells observed. It could be speculated that the apparent increased mucus secretion, the lamellar fusion and the fact that large numbers of *Ichthyobodo* sp. individuals already occupied large areas of the gill surface in heavily *Ichthyobodo* sp. infected fish could affect attachment of amoebae. There are differences in the properties of mucus between species that may be associated to differences in disease susceptibility (Powell *et al*., 2008). Munday *et al*. (2001) observed that Atlantic salmon appeared to be more susceptible to AGD than rainbow trout. The mucus of rainbow trout is significantly less viscous than the mucus of Atlantic salmon and brown trout (Roberts and Powell, 2005b). AGD has been observed to decrease the mucus viscosity of Atlantic salmon and brown trout, but not the mucus of rainbow trout (Roberts and Powell, 2005b). Atlantic salmon also have more acidic mucous than brown trout and rainbow trout (Roberts and Powell, 2005b). Roberts and Powell (2005) suggested that Atlantic salmon may be more susceptible to AGD than rainbow trout due to its more acidic mucous, as it is found that *P. perurans* requires a negatively charged substrate to be able to adhere (Martin, 1987).

Epitheliocystis was observed in all three species, with a trend of an increasing prevalence in the farm strain salmon and wild strain salmon over time. The increasing prevalence was most prominent in the low challenge dose groups. However, the high prevalence of epitheliocystis in the low challenge dose group of wild strain salmon 4-weeks post-challenge may be an artefact of small sample size. High and increasing prevalence of epitheliocystis has been reported in previous studies (Mitchell *et al*., 2013, Wiik-Nielsen *et al*., 2017, Downes *et al*., 2018). The fish in the present study was not screened for the causative agent(s) of the observed epitheliocystis. Epitheliocystis in Atlantic salmon can be caused by several agents;

Candidatus Branchiomonas cisticola (Toenshoff *et al*., 2012), *Ca* Piscichlamydia salmonis (Andrew *et al*., 2004) *Candidatus* Clavochlamydia salmonicola (Mitchell *et al*., 2010) and *Candidatus* Syngnamydia salmonis (Nylund *et al*., 2015). However, *Candidatus* Branchiomonas cysticola is regarded a common causative agent of epitheliocystis in seawater reared Atlantic salmon (Mitchell *et al*., 2013, Wiik-Nielsen *et al*., 2017).

Karyorrhectic nuclei were observed in epithelial cells in some of the examined sections. However, these findings were not quantified, and the extent of this lesion type is therefore undetermined. Karyorrhexis is a stage of apoptosis (or necrosis) with condensed chromatin and fractioned cytoplasm into membrane bound vesicles (Bruno *et al*., 2013). Apoptosis can be induced as a response to bacterial- and virus infections (Bruno *et al*., 2013). Histopathological observations of gill epithelial cells of Atlantic salmon with karyorrhectic nuclei have been associated with Salmon gill pox virus (SGPV) (Gjessing *et al*., 2015). However, the fish in this study were not screened for SGPV and the potential causative agent(s) for these histopathological changes are not determined. SGPV has previously been observed in co-infection with *Ichthyobodo* spp. and epitheliocystis (Gjessing *et al*., 2017). Co-infections with *P. perurans*, *Ca*. Branchiomonas cysticola and SGPV are common (Gjessing *et al*., 2019). In the present study, *P. perurans,* epitheliocystis and *Ichthyobodo* sp. were observed in histological sections. The causative agent(s) of the epitheliocystis, and potential other pathogens were not identified due to lack of qPCR-screening. However, an apparent multifactorial histopathological situation was observed in the histological sections.

Conclusions

All three fish types in this study; farm strain and wild strain Atlantic salmon and brown trout, developed gross gill lesions consistent with AGD. *P. perurans* was confirmed by qPCR. The wild strain salmon and brown trout appeared to have a delayed onset of AGD compared to the farm strain Atlantic salmon. The brown trout and wild strain Atlantic salmon developed gross gill lesions more slowly than the farm strain salmon, in both the high and low challenge dose groups. This study shows that farm raised wild strain Atlantic salmon from the Etne river and smoltified brown trout from the Matre river are susceptible to AGD in laboratory challenge with *P. perurans*.

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Appendix 2.5: Fixation, processing of tissue samples and staining of histological sections

Table A2.5-1: Components used to make Davidson's fixative

Chemicals	**Volume (ml)**
Formaldehyde 37%	200
Cons. Glycerol	100
Ethanol 96%	300
Filtrated seawater	300
Cons. Acetic acid	100

All the components, except the acetic acid, were added in advance. Before use, the acetic acid was carefully added to the solution a small amount a time.

Table A2.5-2: Histokinette processing program for gill tissue samples

Bath	**Solution**	**Time (hours)**
1	Buffer / 4 % phosphate buffered formalin	1
2	Ethanol 50 %	1
3	Ethanol 70 %	1
4	Ethanol 80 %	1
5	Ethanol 96 %	2
6	Ethanol 96 %	2
7	Ethanol 100 %	2
8	Ethanol 100 %	2
9	Xylene, hist	2
10	Xylene, hist	2
11	Paraffine / Histowax 56 – 58°C	2
12	Paraffine / Histowax 56 – 58°C	2

Table A2.5-3: Protocol for paraffine embedding of tissue samples

Step	Description
1	Place the cassettes with fixated tissues on the heating plate
2	Fill a metal mold with liquid paraffine/histowax (56-58°C)
3	Place the tissue in the mold with the desired cut side facing down
4	Place the metal mold on a cooling plate and carefully move the tissue within the liquid paraffine/histowax towards the bottom of the well with a pair of heated tweezers until the tissue attaches to the bottom.
5	Have the labelled piece of the sampling cassette on top of the metal mold and fill it with paraffine/histowax. Place the metal mold with sampling cassette on the cooling plate.
6	Place the cooling plate with embedded samples in a (-20°C) freezer for 10 - 20 minutes. The paraffine will become solid and loosen from the metal mold.

Table A2.5-4: Protocol for Hematoxylin eosin (HE) staining of 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	Distilled water	1 min
9	Shandon Instant Hematoxylin	3 min
10	Hydrochloric acid 0,1%, aqueous	2 sec
11	Running tap water	3 - 5 min
12	Eosin Y-solution 0,5%, aqueous, working solution	3 min
13	Running tap water	30 sec
14	Ethanol 70%	1 min
15	Ethanol 70%	1 min
16	Ethanol 96%	1 min
17	Ethanol 96%	1 min
18	Ethanol 100%	1 min
19	Ethanol 100%	1 min

20	Xylene	5 min
21	Xylene	5 min

Table A2.5-5: Protocol for AB-PAS staining of histological sections

Step	Description	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	Alcian blue solution	5 min
9	Wash in water, then in distilled water	-
10	1% aqueous periodic acid	2 min
11	Rinse well in distilled water	-
12	Schiff's reagent, 8 minutes	8 min
13	Wash in running tap water	5 - 10 min
14	Rinse in absolute alcohol	-
15	Clear in xylene and mount as desired	-

Table A2.5-6: Procedure for mounting stained histological sections

Step	Description
1	Add a drop of Histokitt (synthetic glue with the same refractive index as glass, contains xylene) to the area of the stained section
2	Place a cover glass on top of the Histokitt drop
3	Gently squeeze the cover glass onto the object glass with a pair of tweezers until there is no longer air between the object glass and cover glass
4	Place the mounted sections in an object folder
5	Have the object folder in a fume hood for 2-3 days