

Histochemical characterization of AGD lesions in ballan wrasse
(*Labrus bergylta*)

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Abstract

With the increasing problem of sea lice (*Lepeoptheirus salmonis*) in production of Atlantic salmon (*Salmo salar*), the use of biological and non-drug treatments, such as cleaner fish, like ballan wrasse (*Labrus bergylta*) and lumpfish (*Cyclopterus lumpus*), have become methods of choice in Norwegian aquaculture. Recently, there has been outbreaks of amoebic gill disease (AGD) caused by the amoeba *Neoparamoeba perurans* in facilities producing ballan wrasse. This raised several issues, including that AGD-affected ballan wrasse may act as a vector for the amoebae and transfer it to salmon when they are put into salmon farms for delousing. As ballan wrasse is a new species in aquaculture, it has only recently been found to be susceptible to AGD and that it can transfer the amoebae to salmon. Although there has been a lot of studies on AGD in Atlantic salmon and other salmonids, there is little to nothing known about AGD in ballan wrasse. This study was part of a six-week *in vivo* challenge testing infectivity of UV-irradiated amoebae on ballan wrasse, where for this study, only samples from negative and positive controls were used to look at the cellular and inflammatory response to AGD, and a characterization of the lesions. There was found a significant difference in number of eosinophilic granular cells (EGCs), and apoptotic cells in AGD-associated lesions on filaments in AGD-affected wrasse compared to healthy filaments in AGD-affected fish and non-infected fish. For mucous cells, a significant difference between stains (in AB-PAS) was observed, but no significant difference between the groups. These findings suggest that ballan wrasse, though susceptible to AGD, seems to have a quicker response with more infiltration of EGC and other inflammatory cells, which might correlate with the slower development of the disease than in salmon.

Sammendrag

Lakselus (*Lepeotheirus salmonis*) er det største og stadig økende problemet i norsk akvakultur, og grunnet utvikling av resistens har flere aktører begynt å bruke mer og mer biologiske og ikke-medikamentelle metoder til avlusing. Ved biologisk avlusning brukes i hovedsak berggylt (*Labrus bergylta*) og rognkjeks (*Cyclopterus lumpus*). Nylig har det vært utbrudd av amøbisk gjellesykdom (AGD), forårsaket av amøben *Neoparamoeba perurans*, på landbaserte anlegg som produserer berggylt. Dette kan ha en stor betydning for oppdrett av berggylt, samt muligheten for at berggylten kan være en vektor for amøben og smitte laksen i merdene. Berggylt er en relativt ny art i oppdrettssammenheng, og det er nokså nylig funnet ut at den er mottakelig for amøben, samt at den kan overføre amøber til laks. AGD på laks og annen laksefisk er godt dokumentert og forsket på, men AGD på berggylt er relativt ukjent. Denne studien var del av et større prosjekt, hvor det over seks uker i et *in vivo* forsøk, som testet infektiviteten til UV-bestrålte amøber på berggylt. For denne studien ble det bare brukt prøver fra positiv og negativ kontroll til å se på den cellulære og inflammatoriske responsen hos berggylt, samt en karakterisering av lesjonene. Det ble funnet en signifikant forskjell i andel EGCs (eosinofile granulære celler) og apoptotiske celler i filamenter med lesjoner, sammenlignet med friske filamenter på AGD syk fisk og negativ kontroll. For slimceller ble det ikke funnet signifikant forskjell mellom gruppene, men det ble funnet signifikans mellom fargene, ved AB-PAS farging. Dette indikerer at berggylt, som er mottakelig for AGD, har en raskere respons med mer infiltrasjon av inflammatoriske celler og EGCs. Dette korrelerer med at berggylten utvikler AGD senere enn laks.

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Abbreviations

Abbreviations	Full words
• AB-PAS	Alcian Blue – Periodic Acid Schiff
• AB	Alcian Blue
• AG	Anterior Gradient
• AGD	Amoebic Gill Disease
• ANOVA	Analysis of Variance
• BGD	Bacterial Gill Disease
• CMS	Cardiomyopathy Syndrome
• CO ₂	Carbon dioxide
• CV	Contractile Vacuole
• dH ₂ O	Distilled Water
• ECP	Extracellular Products
• EGC	Eosinophilic Granular Cell
• F	F-statistics
• FIJI	Fiji Is Just ImageJ
• H&E	Hematoxylin and Eosin
• H ₂ O ₂	Hydrogen peroxide
• HSMI	Heart and Skeletal Muscle Inflammation
• IFN	Interferon
• IL	Interleukin
• ILU	Interlamellar Unit
• iNOS	Inducible Nitric Oxide Synthase
• IRF	Interferon Regulatory Factor
• ISH	In-situ Hybridization
• L-ILU	Lesion-associated Interlamellar Unit
• MHC	Major Histocompatibility Complex
• MIC	Molecular Imaging Centre
• mRNA	Messenger Ribonucleic Acid
• NIVA	Norwegian Institute of Water Research
• O ₂	Oxygen
• P	P-value statistics
• PCNA	Proliferating cell nuclear antigen
• PCO ₂	Partial pressure of carbon dioxide
• PAS	Periodic Acid Schiff
• PBS	Phosphate Buffered Saline
• PD	Pancreas Disease
• PCR	Polymerase Chain Reaction
• PGI	Proliferative Gill Inflammation
• PLO	<i>Perkinsella amoeba</i> -like Organism
• TEM	Transmission Electron Microscopy
• TNF	Tumour Necrosis Factor
• TUNEL	Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling
• USD	United States Dollar

1. Introduction

1.1 Norwegian Aquaculture

Norway is the eighth largest when it comes to total aquaculture production, sixth largest producer of farmed fish, and the largest producer of farmed fish in marine and coastal aquaculture (FAO, 2016). In 2015, Norway produced (slaughter weight) 1 234 200 metric tons of Atlantic salmon (*Salmo salar*, Linnaeus 1758), 71 600 metric tons of rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) and about 6000 metric tons of other species farmed for human consumption (Hjeltnes et al., 2016). Norwegian aquaculture production stands for 67% of Norwegian seafood exports and has a net worth of 50,1 billion NOK, about 6,18 billion USD per today's exchange rate. Over the last decade, the seafood export and aquaculture has grown steadily and quantitatively (Anonymous, 2015). The industry has also seen production costs going up due to some costly agents like treatments, mortality, and lost growth. The biggest problems today are salmon lice (*Lepeoptheirus salmonis*, Krøyer), Salmonid alphavirus (PD) sp. and *Neoparamoeba perurans* (AGD) (Hjeltnes et al., 2016).

1.2 Use of cleaner fish in Norwegian aquaculture

Due to the significant problem of sea lice, fish farmers started using wild caught wrasse in the cages as cleaner fish to delouse Atlantic salmon, some companies also started farming cleaner fish, first ballan wrasse (*Labrus bergylta*, Ascanius, 1767), and later some also started farming lumpfish (*Cyclopterus lumpus*, Linnaeus, 1758). In 2015, Norway produced about 10 million lumpfish and about 400 000 – 500 000 ballan wrasse (due to a continuing use of wild caught wrasse) (Hjeltnes et al., 2016). As with the salmon farming, this industry also has its problems with disease, AGD being a significant problem. In 2013, Karlsbakk et al. (2013) found that ballan wrasse are susceptible to AGD, and Dahle (2015) found that ballan wrasse with AGD could potentially transfer amoebae to Atlantic salmon in sea cages. In 2016, lumpfish was also found susceptible to AGD and can transfer amoebae to Atlantic salmon (Haugland et al., 2016).

1.3 *Neoparamoeba perurans*

The causative agent for AGD has been associated with different *Neoparamoeba* spp. (syn. *Paramoeba* spp.), such as *N. permaquidensis* and *N. branchiphila* (Bermingham and Mulcahy, 2007, Dykova et al., 2000, Dykova et al., 2005). In 2007, another *Neoparamoeba* species was described (*Neoparamoeba perurans*) (Young et al., 2007). *N. perurans* was directly associated with AGD lesions when using in-situ hybridization (ISH), and was believed to be the causal agent of AGD. In 2012, *N. perurans* was successfully cultured in-

vitro and through challenge trials, was shown to be the causative agent of AGD and so Koch's postulates were fulfilled (Crosbie et al., 2012).

The separation between the genus *Paramoeba* spp. and *Neoparamoeba* spp. was done mostly because of a morphological presence of organic microscales on the surface of the amoebae found by Fredrick Page in 1987, per Oldham et al. (2016). Recent molecular evidence suggests that *Neoparamoeba* and *Paramoeba* are paraphyletic and can be synonymized (Feehan et al., 2013). Though that would be premature because the SSU rDNA is highly conserved and by itself insufficient to formalize that change (Nassonova et al., 2010, Kudryavtsev et al., 2011). This suggests more scaled amoeba should be sequenced and other genes than SSU rDNA investigated before a change in the nomenclature used today (Young et al., 2014, Oldham et al., 2016). So, throughout this thesis, *Neoparamoeba* will be used for both *Neoparamoeba* and *Paramoeba* species.

N. perurans is a species in the phylum Amoebozoa (Smirnov et al., 2011), the subphylum Lobosoa (Carpenter, 1861 cited in Cavalier-Smith (2009), class Discosea, subclass Flabellina (Smirnov et al., 2005), order Dactylopodia (Smirnov et al., 2005), family Vexilliferidae (Page, 1987), genus *Neoparamoeba* (Page, 1987). *Neoparamoeba* spp. lack cell-surface structures like hexagonal glycostyles of surface scales that occur in other vexilliferids (Dykova et al., 2000, Young et al., 2007). Within the amoeba-cell there are two types of cytoplasm; a hyaline ectoplasm and a granular endoplasm. The ectoplasm can form pseudopodia which extend to various degrees, and produce a range of different shapes. The endoplasm contains the nucleus (5-8 μm), other organelles, vesicles (Dykova et al., 2000, Young et al., 2007) and contractile vacuoles (CVs) that are suggested to be used for osmoregulation (Lima et al., 2016), they are used for osmoregulation in other protozoa (Allen, 2000, Allen and Naitoh, 2002, Fountain et al., 2007). The endoplasm also contains one or more *Perkinsella amoeba*-like organisms (PLOs, syn; parasomes, 3.3-6 μm) adjacent to the nucleus, and is closely related to the flagellate *Ichthyobodo necator* (Dykova et al., 2003, Young et al., 2014). The endosymbiont function is currently not known, although evidence suggest that host cell most likely depends on some biosynthetic capabilities of the endosymbiont, also suggesting a mutualistic obligatory symbiosis, as the PLOs is not known to be able to live separate from their amoeba host (Dykova et al., 2003, Young et al., 2014). The endosymbiont has also been suggested to play a role in inducing disease, although this has not been confirmed (Young et al., 2014). In addition to this, extracellular products (ECP) produced by *N. perurans* have been suggested to be an important virulence factor inducing AGD (Butler and Nowak, 2004, Bridle et al., 2015).

The lifecycle of *N. perurans* is currently not fully known, but the trophozoite is believed to be the only known stage in the cycle. The amoeba has different forms, it can either be free floating or adhered to a surface (locomotive). When locomotive, the hyaline ectoplasm of the amoeba extends to form mamilliform pseudopodia, and the hyaline ectoplasm of free floating amoeba extends to form digitiform pseudopodia. Adherent amoebae are 41-56 µm in diameter, and a distinct plasma membrane can be observed in the outer parts of the ectoplasm (Dykova et al., 2005, Young et al., 2007). Free floating spherical amoebae have been shown to be 22.4-28.5 µm in diameter (Karlsbakk et al., 2013). When exposed to lower salinities, high amoebae density, low abundance of nutrients or other environmental stressors (Wiik-Nielsen et al., 2016), the locomotive amoebae can rapidly change their morphology to “rounded-up” cells by retracting the pseudopodia (Powell and Clark, 2003, Lima et al., 2016). This response is most likely a pseudocystic survival strategy, because most of the affected cells return to normal when the stressor is removed (Lima et al., 2016).

1.4 Host range and transmission of *N. perurans*

As AGD is a disease that occurs all over the world, *N. perurans* has been detected in 25 phylogenetically different finfish species, through experimentally induced or natural infections (Oldham et al., 2016, Adams, 2016, Kim et al., 2017), including Atlantic salmon and other sea-reared salmonid fish such as rainbow trout from Tasmania and chinook salmon (*Oncorhynchus tshawytscha*, Walbaum 1792) from New Zealand (Kent et al., 1988, Munday et al., 1990). *N. perurans* has also been found in marine fish i.e. sharpsnout seabream (*Diplodus puntazzo*, Walbaum) and European seabass (*Dicentrarchus labrax*, Linnaeus) in the Mediterranean, lumpfish and ballan wrasse from Norway, turbot (*Scophthalmus maximus*, Linnaeus) from Spain, purple wrasse (*Notolabrus fuciola*, Richardson, 1840) from Australia and rock bream (*Oplegnathus fasciatus*, Temminck & Schlegel, 1844) (Dykova et al., 1995, Dykova and Novoa, 2001, Santos et al., 2010, Karlsbakk et al., 2013, Karlsbakk et al., 2014, Haugland et al., 2016, Adams, 2016, Kim et al., 2017). This then suggest that *N. perurans* has a low host specificity, though susceptibility of the amoeba does not mean the host will develop AGD, and some species like ballan wrasse might be more resistant to infections of *N. perurans* (Karlsbakk et al., 2013).

The mechanisms responsible for the transmission of *N. perurans* is still unknown, mostly due to a poor knowledge about potential reservoirs. Horizontal transmission between fish has been shown in experimental challenges (Crosbie et al., 2012, Dahle, 2015, Haugland et al., 2016), where two of those studies included cleaner fish (ballan wrasse and lumpfish) as potential

shedders of *N. perurans*. Another form of transmission may be escaped AGD-affected fish, that can be a threat for other fish farms in the area, as escaped Atlantic salmon can travel up to 500 km² within a week (Skilbrei et al., 2010).

Floating stages of the amoebae have been observed with long pseudopodia, which suggests that it may act as a transmission stage of the amoebae (Hjeltnes et al., 2014), and the amoebae may be able to travel vast distances with the currents in this stage.

It has been shown that amoebae from ballan wrasse can infect Atlantic salmon (Dahle, 2015), and as both wild and farmed wrasse are susceptible to *N. perurans*, they will most likely be a source of infection, when introduced to salmon cages (Karlsbakk et al., 2013, Mortensen et al., 2014), the same has also been observed for lumpfish (Haugland et al., 2016).

1.5 Amoebic Gill Disease (AGD)

Amoebic gill disease of Atlantic salmon and rainbow trout was first described by Munday in 1986, cited by Munday et al. (2001) in Tasmania. Later it was also described in coho salmon (*Oncorhynchus kisutch*, Walbaum) by Kent et al. (1988) in Washington and California. The disease has since then been reported in all countries that are considered major producers of Atlantic salmon, except Iceland (Nowak, 2012, Oldham et al., 2016).

In Norway, the first report of AGD was made in 2006 (Nylund et al., 2008, Steinum et al., 2008) at four different sites, costing the industry approximately 12.55 million USD (Shinn et al., 2015). This was mostly due to high mortality rates, where one site reached 80% mortality (Steinum et al., 2008). Other cases of AGD were not reported until 2012, when 5 new sites were diagnosed with AGD. In 2013, 58 sites were diagnosed and another 68 sites in 2014 (Bornø and Lie Linaker, 2015). The disease has since been a regular occurrence in Norwegian aquaculture. In 2013, the first cases of AGD in cleaner fish such as ballan wrasse and corkwing wrasse (*Symphodus melops*, Linnaeus 1758) were diagnosed, showing that AGD had a wider range of hosts and potential to infect other species in Norwegian aquaculture (Karlsbakk et al., 2013, Bornø and Lie Linaker, 2015). In terms of reported outbreaks, the year 2015 was not as severe as the year before (Bornø and Lie Linaker, 2015, Hjeltnes et al., 2016). Perhaps due to improved experience in managing AGD than before, and because of the cooler and wetter summer with most outbreaks occurring later in the autumn, with more fresh water and a lower salinity in coastal areas. In Norway, outbreaks of AGD have occurred along the coast from Vest-Agder in the south, to the northern parts of Nord-Trøndelag, and Helgeland. The amoeba has also been detected in Nordland and Troms using molecular

methods, but there have not been any disease outbreaks of AGD so far (Hjeltnes et al., 2014, Hjeltnes et al., 2016). AGD has also been a major recurring problem on the British Isles since 2011 (Rodger, 2014).

High seawater temperatures (>12 °C) and high salinity seem to be the limiting factors of disease outbreaks (Clark and Nowak, 1999, Douglas-Helders et al., 2001). The most important environmental factor is salinity, and long-term infections with AGD have been associated with high salinities (Clark and Nowak, 1999, Munday et al., 1990). Other factors may also influence disease outbreaks, such as the immune status of the fish, former lesions, water quality, algal blooms, and stocking densities (Clark and Nowak, 1999, Munday et al., 2001, Bermingham and Mulcahy, 2004).

Often other gill infections occur at the same time as AGD, and in Norway particularly the condition termed PGI (proliferative gill inflammation) which also might cause white patches on the gills (Nylund et al., 2008, Steinum et al., 2008). Several pathogens are associated with PGI or gill disease; epitheliocyst-forming bacteria *Candidatus Piscichlamydia salmonis* (Draghi et al., 2004), *Candidatus Clavochlamydia salmonicola* (Karlsen et al., 2008), *Candidatus Sygnamydia salmonis* (Nylund et al., 2015), *Candidatus Branchiomonas cysticola* (Toenshoff et al., 2012), and *Desmozoon lepeophtheirii* (Syn. *Paranucleospora theridion*), *Ichthyobodo salmonis* (Isaksen et al., 2011) and salmonid pox virus (Nylund et al., 2008) are also often present.

1.6 Host response/immune response

The main site of infection for *N. perurans* is the gill epithelium, and the infected gill will often appear pale, and affected regions on the gill can appear as white patches (Adams and Nowak, 2004, Munday et al., 1990). With histological examination, hyperplasia, hypertrophy, complete fusion of lamellae and interlamellar vesicles have been observed in both Atlantic salmon and ballan wrasse (Mitchell and Rodger, 2011, Karlsbakk et al., 2013). This happens because of a strong response in the host with a migration of immunoregulatory cells (leucocytes) to the regions that are affected (Adams and Nowak, 2001, Adams and Nowak, 2003). Amoebae can be found inside interlamellar vesicles or cavitations (“cysts”), enclosed by epithelial cells, that are primarily formed due to the proliferative host response (Kent et al., 1988, Adams and Nowak, 2001, Lovy et al., 2007). Mortality associated with AGD is normally acute and occurs mostly in the late stages of the disease due to respiratory distress, this happens often in correlation with treatment and handling (Powell et al., 2015).

There are significant knowledge gaps when it comes to the host immune response to an AGD infection in Atlantic salmon, even though there have been several studies on gene regulation in rainbow trout and Atlantic salmon affected with AGD (Table 1). The differences in results between the studies seem to be an effect of the sampling in each study, and Pennacchi et al. (2014) demonstrated that the down regulation found in other studies (Morrison et al., 2006, Wynne et al., 2008, Morrison et al., 2012), were likely artefacts of cell type.

Table 1: Up (+) or down (-) regulation of genes in rainbow trout and Atlantic salmon affected with AGD in several studies. 1= upregulation in infected gill tissue, but not in lesions. 2= downregulation of the isoform TNF- α 3. 3= downregulation in lesions, other tissues were not examined.

	(Bridle et al., 2006) (rainbow trout)	(Morrison et al., 2006)	(Morrison et al., 2007)	(Wynne et al., 2008)	(Morrison et al., 2012)	(Pennacchi et al., 2014)	(Benedicenti et al., 2015)
<i>IL-1β</i>	+		+	-	+	+	+
<i>IL-8</i>	?		?		+		
<i>TNF-α</i>	-		-			+	- ²
<i>IFN-γ</i>	-		-				
<i>iNOS</i>	+		-				
<i>MHCI</i>					-	+ ¹	-
<i>MHCII</i>				+	-	+ ¹	
<i>p53</i>		- ³					+
<i>IgM</i>	+		+	+		+	?

Lovy et al. (2007) found eosinophil-resembling cells in gill-lesions of AGD-affected Atlantic salmon that has not been reported in inflammatory responses of other salmonids like chinook salmon and rainbow trout (Lovy et al., 2006). The eosinophil-resembling cells were characteristic in AGD lesions and were the majority of the infiltrating cells. Unlike EGCs, that are often observed in skin, gut, gills and swimbladder of salmonids and other teleostean fish, including pike (*Esox Lucius*, Linnaeus), ballan wrasse, cuckoo wrasse (*Labrus bimaculatus*, Linnaeus) and brown trout (*Salmo trutta*, Linnaeus), these cells were distinctively different (Ferguson, 1989, Reite and Evensen, 2006, Lovy et al., 2007). The cells

described by Lovy et al. (2007) had a granule morphology more like mammalian eosinophils, containing a crystalline inclusion within its centre and were elliptic. This then confirmed that these cells were not EGCs, but more like eosinophils of a lineage different to EGCs. Whereas EGCs are more like mammalian mast cell, having large spherical membrane-bounded granules with a matrix that is homogenous and dense (Ezeasor and Stokoe, 1980, Sire and Vernier, 1995, Reite, 1998, Reite and Evensen, 2006).

1.7 Aim for the study

The aim of this study was to histochemically characterize the host and cellular response to AGD in ballan wrasse, using a range of staining methods. Staining methods used were H&E, Giemsa, AB-PAS, Toluidine Blue and TUNEL, to look at and count different types of cells, including EGCs, mucous cells and apoptotic cells.

Another aim was also to compare the cellular response in ballan wrasse and Atlantic salmon with AGD, as well as the development of the disease.

2. Materials and Methods

2.1 Origin of the *Neoparamoeba perurans* isolates

The amoeba culture used in this study were cultured from a prior *N. perurans* in Atlantic salmon experiment at Solbergstrand Marine Research (Wennberg and Powell, 2015). The culturing and maintenance of the amoeba for this study was performed as described in the prior experiment by Wennberg and Powell (2015).

2.2 Origin and husbandry of the ballan wrasse

The ballan wrasse used in the study were hatched and reared as juveniles at Marine Harvest Labrus AS in Øygarden, then sent to the facility at Stord for ongrowth. The fish were transported by road from Stord to NIVA marine research station at Solbergstrand, where they were put into 8, 700 L tanks, with water taken from 60 m and maintained at 400 L, with approximately 50 fish in each tank and an average of 13.6 °C (± 0.6) and a pH range of 7.98 – 8.02. The fish were fed a commercial diet (Otohime S2 larval fish diet, 1400 μm pellets) consistent with that provided at the hatchery of origin as well as supplement of minced cooked prawns (Powell and Wennberg, 2016).

Table 2: Showing weight and length for ballan wrasse in week 0 (pre-infection) and week 6 (last week of infection), and added is the weight and length for positive (fish infected with amoebae) and negative (non-infected fish) controls.

Week	Weight (g)	Length (cm)
0	18.0 ± 4.6	10.7 ± 0.7
6	19.34 ± 4.8	10.91 ± 0.6
6 Positive controls	20.21 ± 3.8	11.06 ± 0.6
6 Negative controls	19.48 ± 5.8	10.82 ± 0.5

2.3 Study design

Eight tanks were used in the challenge, two tanks with negative controls, two with positive controls, two tanks with amoebae irradiated with low dose UV, and two tanks with amoebae irradiated with high dose UV. Only the fish from the negative and positive controls were used in this study. Before infecting the fish, pre-samples were taken from 16 fish, two from each tank, taking samples for histology, PCR, and blood, and then the volume in each tank was reduced to 200 L (11.02.16) and the amoebae was added to the tanks, flow was set back to normal approximately an hour later.

2.4 Sampling

Sampling was carried out weekly from the start of the infection (week 0, 11.02.16) until week 6 (23.03.16), samples used in this study were taken from week 5 and week 6 post-infection. Prior to the infection (11.02.16), two fish from each tank were taken samples of, and for the rest of the challenge, five fish were taken from each tank. The sampling was done by catching five fish from each of the tanks and giving them an overdose of anaesthesia (MS-222, Metacain 100 mg L⁻¹, Sigma Aldrich, Norway). Weight and length was measured and the fish was examined for external lesions. The gills were then scored for AGD, using the scoring system made for Atlantic salmon described by (Taylor et al., 2009), and then the operculum was removed and all the gill arches were cut out, the second one on the left side was cut in two and both pieces were put into RNAlater for PCR analysis and the rest of the gills were fixed in 10% neutral phosphate buffered formalin (VWR) solution and processed by a standard paraffin wax protocol (Norwegian Veterinary Institute in Bergen), then sectioned to 4 µm using a Leica RM 2155 microtome.

2.5 TUNEL Staining

TUNEL staining was performed to detect apoptotic cells in the gills. TUNEL was performed following the protocol in ApopTag[®] Peroxidase *In Situ* Apoptosis Detection Kit (EMD Millipore Corporation, Temecula, CA, USA). Gill sections were deparaffinized through graded xylene and ethanol baths, pre-treated with freshly made Proteinase K ($20 \mu\text{g mL}^{-1}$) (Sigma Aldrich, Norway) for 15 mins at room temperature and washed twice in water (dH_2O) for 2 mins each. Endogenous peroxidase was quenched with 3% H_2O_2 (Sigma Aldrich, Norway) in PBS for 5 mins at room temperature and then rinsed in water twice for 5 mins each time. Slides were carefully aspirated and immediately applied with equilibration buffer ($75 \mu\text{L } 5\text{cm}^{-2}$) for 10s. Slides were again aspirated and immediately applied with $55 \mu\text{L } 5\text{cm}^{-2}$ working strength TdT enzyme and incubated in a humidity chamber at 37°C for 1 hour. Slides were then dipped in working strength stop/wash buffer, agitated for 15s and incubated for 10 mins at room temperature. Slides were washed 3 times in PBS for 1 min each and then room temperature anti-digoxigenin conjugate was applied ($65 \mu\text{L } 5\text{cm}^{-2}$) on the slides and the slides were incubated for 30 mins at room temperature in a humidified chamber. Slides were then washed with 4 changes of PBS for 2 mins each at room temperature. Peroxidase substrate was then applied ($75 \mu\text{L } 5\text{cm}^{-2}$) to the slides and they were stained for 3-6 mins at room temperature. Slides were washed three times in dH_2O for 1 min each and incubated in dH_2O for 5 mins at room temperature. Slides were counterstained in 0.5 % methyl green (w:v) for 10 mins at room temperature and washed three times in dH_2O . Finally, the slides were washed in 100% N-butanol (Sigma Aldrich, Norway). Negative controls were made by replacing TdT with equilibration buffer. Slides were dehydrated in graded ethanol and xylene and finally mounted using DPX mounting media (Sigma Aldrich, Norway). All reagents were either supplied with the kit or available at the lab. The apoptotic cells were identified with both positive staining and morphological signs of apoptosis.

Apoptotic cell counts were made on digitalized TUNEL sections using image analysis program FIJI (Fiji Is Just ImageJ) to mark out an area (μm^2) on the filament at 20x magnification, scale at $50 \mu\text{m}$, and counting number of positive apoptotic cells in the given area. Ten areas were made per section (50 areas per fish) on selected places on the filaments. Areas were selected based on number of lamellae (average eight per area), for non-lesion filaments, and lesion areas were chosen to include most of a lesion on a filament within the average number of lamellae. The number of apoptotic cells were counted and averaged per section and per fish, and cells/area were also converted to mm^2 . When counting on gills of

AGD-affected fish, separate counts were made for lesion associated areas and non-lesion associated areas (normal).

2.6 Special histology stains

2.6.1 AB-PAS (Alcian Blue- Periodic Acid Schiff)

Sections (4 μm) were deparaffinized in xylene and put through graded ethanol and rehydrated in distilled water. Sections then stained with alcian blue, either at pH 4 or 1.5 (made with alcian blue 8GX (Sigma Aldrich, Norway) and 3% Acetic acid) for 15 min, then washed well in running tap water for 2 min and rinsed in distilled water. Sections were then placed in periodic acid for 5 min and washed in distilled water before staining with Schiff's reagent for 10 min, then washed in running tap water for 5 min (World, 2011). To stain nuclei, sections were placed in haematoxylin (Sigma Aldrich, Norway) for 1 min. Sections were then washed in running tap water for 2 min and then dipped quickly in acid alcohol to differentiate before they were washed in water, dehydrated through ethanol and xylene, and then mounted with DPX mounting media (Sigma Aldrich, Norway). The use pH 4 distinguishes between neutral (PAS+) and acidic mucins (AB+), both sulphated and carboxylated, while the sulphated mucins only stain AB+ with low pH (Jones and Reid, 1978).

Mucous cell counts were made on AB-PAS sections at 40x magnification, following the method described in Roberts and Powell (2003) and adopted from Speare et al. (1997). Five well-orientated filaments (Speare et al., 1997) were selected and 10 interlamellar units (ILUs) from each filament were selected. The number and type of mucous cell (AB+, PAS+ or AB+/PAS+) present on each of the ILUs were counted and results averaged for each section. When counting mucous cells on the gills of AGD-affected fish, separate counts were made for lesion associated ILUs (L-ILUs) and non-lesion associated ILUs (normal).

2.6.2 Giemsa Stain

Giemsa staining was done to detect and observe EGCs in the gill tissue. Gills were sectioned at 4 μm using a Leica RM 2155 microtome, then deparaffinized in xylene and graded ethanol for 3 min each. Slides were then immersed in buffered water with pH 7.4 for 5 min before they were placed into a modified Giemsa solution (1:20 solution) (Sigma Aldrich, Norway) for 15 min. Slides were then rinsed in buffered water, differentiated in acid alcohol, and then dehydrated in graded ethanol and xylene and then mounted and coverslipped (Kiernan, 1990).

Eosinophilic granular cell counts were made on H&E sections at 40x magnification. Five well-oriented filaments were selected and 10 interlamellar units (ILUs) from each filament were selected. The number of EGCs present on each of the ILUs were counted and results

averaged for each section. When counting EGCs on the gills of AGD-affected fish, separate counts were made for lesion associated ILUs (L-ILUs) and non-lesion associated ILUs (normal).

2.6.3 Toluidine Blue stain

Toluidine blue staining was done to observe EGCs in the gill tissue. Gills were sectioned at 4µm using a Leica RM 2155 microtome, then deparaffinized in xylene, graded ethanol, and distilled water for 3 min each. Drops of a 0.1 % solution of toluidine blue were then put on each slide for a few seconds before slides were rinsed under running tap water, and then placed upright to dry before they were mounted, using DPX mounting media (Sigma Aldrich, Norway).

2.7 Transmission Electron Microscopy (TEM)

Gill tissue from ballan wrasse infected with *N. perurans* were fixed 2.5 % glutaraldehyde with 0.1 M sodium cacodylate buffer (Veterinary institute, Oslo, Norway), before being post-fixed in 2 % osmium tetroxide and embedded in EPON. The embedded tissue was then sectioned and ultra-sectioned before being observed using a JEOL JEM-1230 transmission electron microscope at the facilities of MIC (Molecular Imaging Center).

2.8 Statistical analysis

SigmaPlot 10.0, Sigmastat (Systat Software) and Microsoft Excel 2016 were used to plot and determine average values, standard deviations, standard error, and correlation. Two-Way analysis of variance (ANOVA) was used to compare mucous cell stains and treatment groups. One-Way analysis of variance (ANOVA) was used to compare treatment groups in EGC counts and apoptotic cell counts. P values that were less than or equal to 0.05 were considered significant, using all pairwise multiple comparison procedures (Holm-Sidak method). If the data wasn't normal, it was transformed using natural logarithm, and if the data was still not normally distributed or the variance nonhomogeneous after transformation with natural logarithm, it was analysed untransformed, and P values less than or equal to 0.01 were considered significant (Glass et al., 1972). Alternatively, a Kruskal-Wallis one way analysis of variance on ranks was performed. For further analysis, a Pearson product moment correlation was used to correlate the data from EGC and apoptotic cell count.

3. Results

3.1 Mortality

Accumulated mortality was under 8 %, and there was no distinct pattern observed. Mortalities were found to be thin, malnourished, and some also with fin erosions.

3.2 Lesion morphology in ballan wrasse and Atlantic salmon

When observed histologically, there were some differences between lesions in ballan wrasse and Atlantic salmon, whereas the lesions in Atlantic salmon tended to be over several filaments (Fig. 1 and 2), the lesions in wrasse were more localised, with i.e. one filament affected and the rest were unaffected (Fig. 1 and 2). Within the lesions more EGCs were observed in ballan wrasse than in Atlantic salmon, although there were more of other eosinophil-resembling cells in the lesions in Atlantic salmon (Fig. 3 and 4). EGCs observed in wrasse appeared to have large spherical membrane-bounded granules, the eosinophils observed in salmon appear to be more like eosinophils (Fig. 3 and 4), but EGCs were also observed in salmon (Fig. 3). The

granules of the eosinophils in salmon were smaller, although the structure of the granules could not be observed by light microscopy.

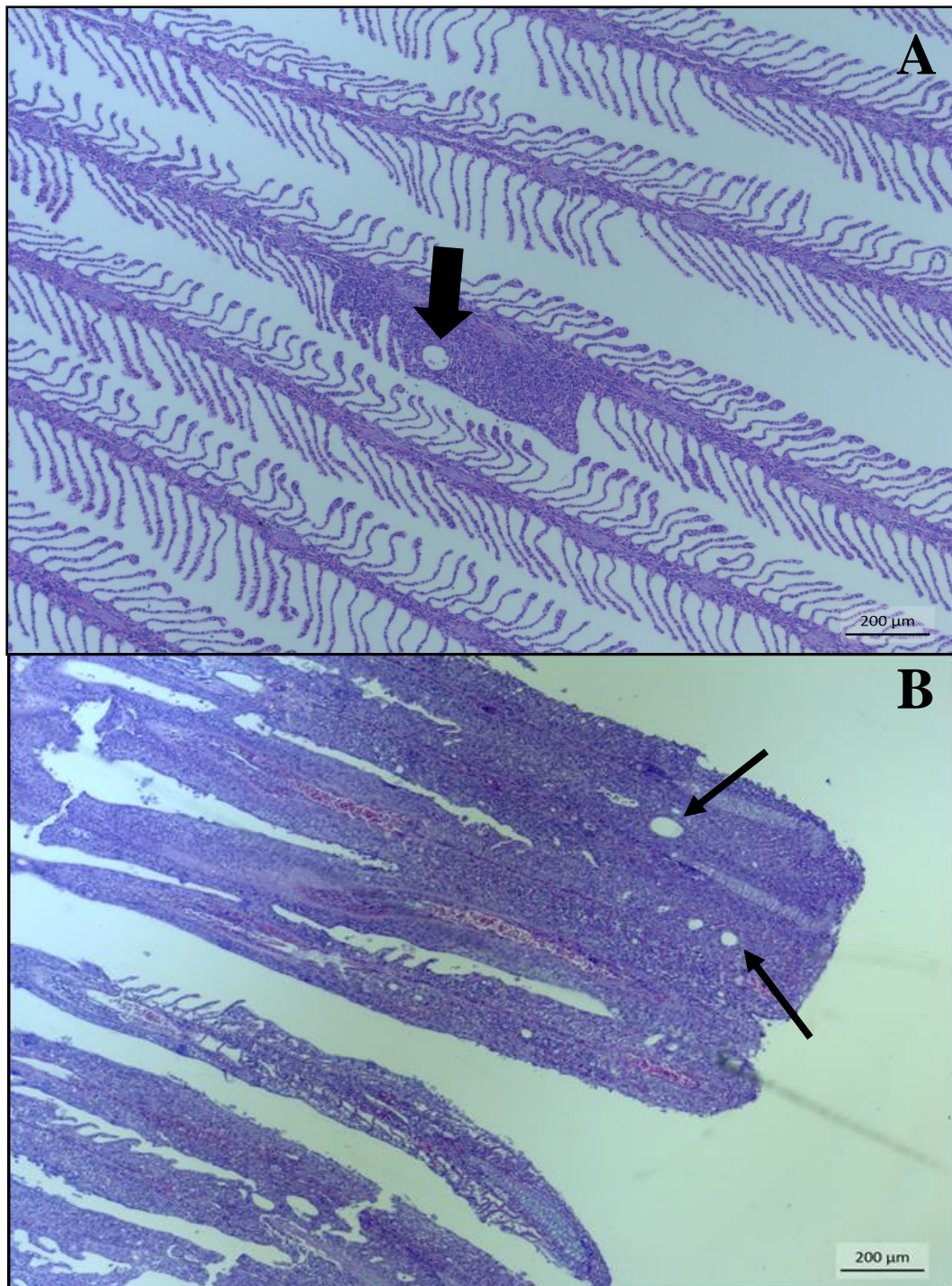


Figure 1: AGD-associated lesions in Atlantic salmon and ballan wrasse, stained with H&E. A: Lesion in early pathogenesis of AGD in Atlantic salmon. An interlamellar vesicle was observed in the lesion (arrow). Scale bar at 200 µm. B: Lesions in filaments of ballan wrasse in late pathogenesis of AGD (5 weeks post-infection). A fusion of filaments was also observed as well as interlamellar vesicles (arrows). Scale bar at 200 µm.

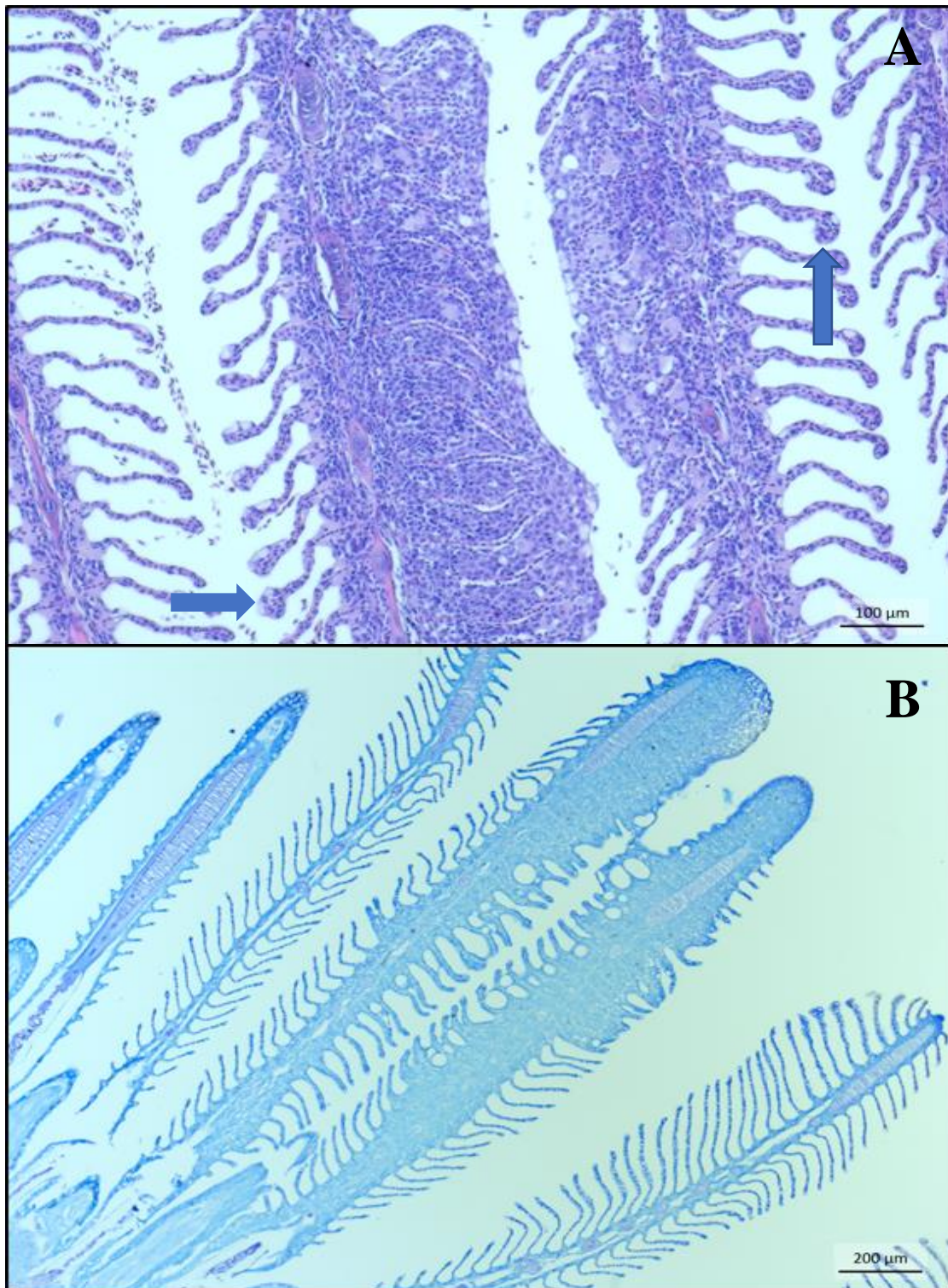


Figure 2: AGD-associated lesions in Atlantic salmon and ballan wrasse. **A:** AGD-associated lesions on filaments of Atlantic salmon, showing multifocal hyperplasia of gill epithelium and fusion of secondary lamellae (early pathogenesis), clubbing of secondary lamellae was also observed (blue arrows). Stained with H&E, scale bar at 100 μm . **B:** AGD-lesioned filaments in ballan wrasse showing hyperplasia of the gill epithelium, fusion of secondary lamellae and interlamellar vesicles. Healthy filaments were observed next to the lesioned filaments. Stained with toluidine blue, scale bar at 200 μm .

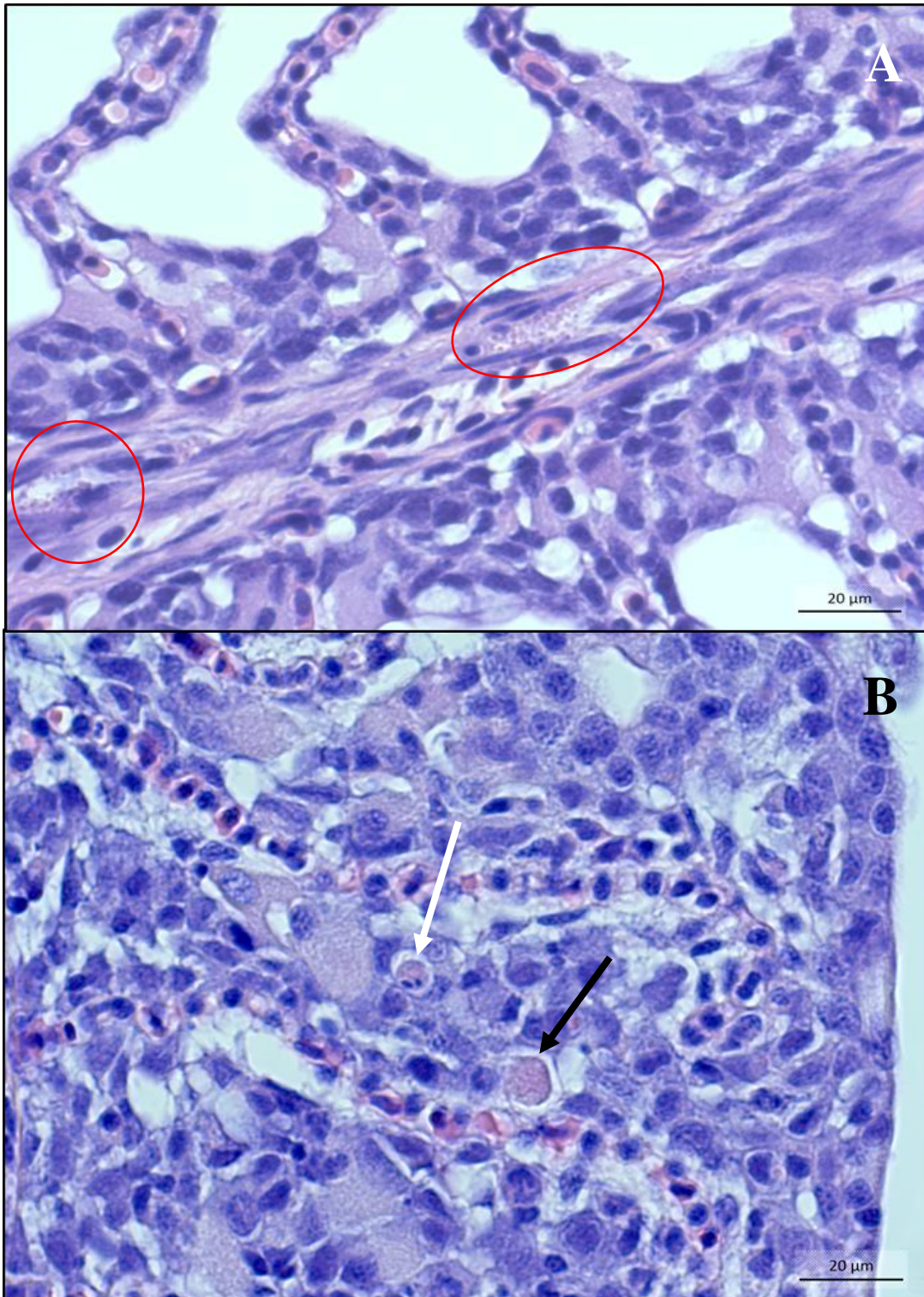


Figure 3: EGCs and eosinophils in Atlantic salmon. A: EGCs in the connective tissue of a filament in Atlantic salmon (circles). Stained with H&E, scale bar at 20 µm. B: Lesion tissue in Atlantic salmon showing an eosinophil (black arrow), and what appeared to be another eosinophil with a poly morphic nucleus (white arrow). Stained with H&E, scale bar at 20 µm.

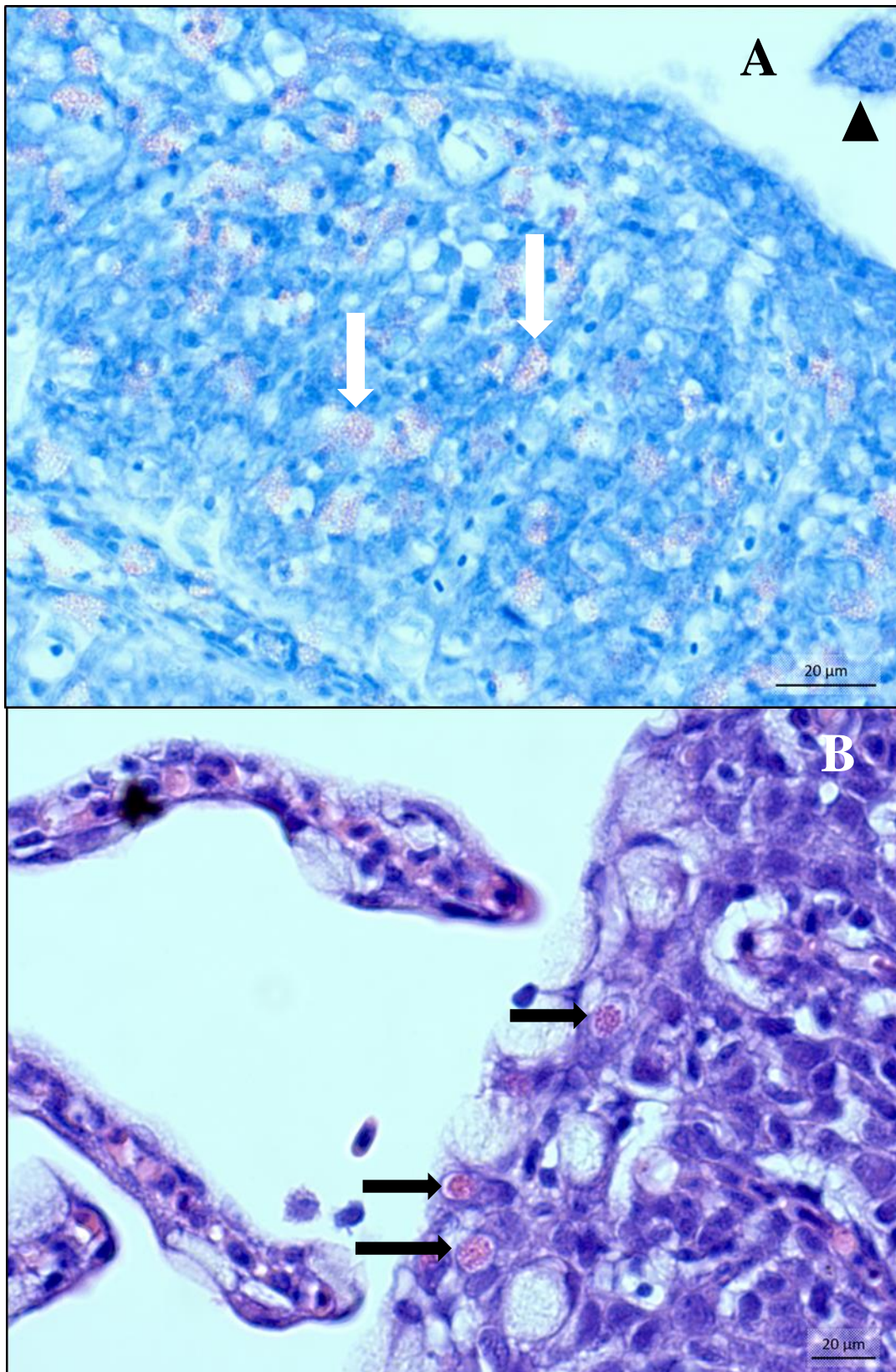


Figure 4: A: EGCs in an AGD-lesion of ballan wrasse (white bold arrows), an amoeba was also observed (black arrowhead). Stained with Giemsa, scale bar at 20 µm. B: Eosinophils (black bold arrows) in lesion tissue of Atlantic salmon. Stained with H&E, scale bar at 20 µm.

3.3 AB-PAS mucous cell density

Mucous cell counts were performed on sections stained with either AB-PAS with alcian blue pH 4 or pH 1.5. Overall on both AB pH 4 and AB pH 1.5, there was a trend with there being more mucous cells per ILU in AGD-affected fish and especially on the lesions (L-ILUs), (Fig. 5 and 6). A Two-Way analysis of variance was used to compare number of mucous cells to treatment (AGD-, AGD+ lesion and AGD+ healthy) and type of stain (AB+, PAS+ and AB+/PAS+). For pH 4, there was no significant difference between treatments ($F_{2, 71} = 1.692$, P value = 0.192), nor between treatment and stain ($F_{4, 71} = 0.0832$, P value = 0.987), but a significant difference was found between stain types ($F_{2, 71} = 5.132$, P value = 0.009) using a P value cut off of 0.01 (Glass et al., 1972). For pH 1.5, there was no significant difference between treatments ($F_{2, 71} = 0.966$, P value = 0.386), nor between treatments and stain ($F_{4, 71} = 0.267$, P value = 0.898). There was a significant difference between stain types ($F_{2, 71} = 6.194$, P value = 0.004). Both mucous cell counts showed high variability, mainly caused by a fish having a large number of mucous cells per ILU. Furthermore the “healthy filaments” on

AGD-affected fish had a lower number of mucous cells than on filaments with lesions, almost at the same cell density as the non-affected fish.

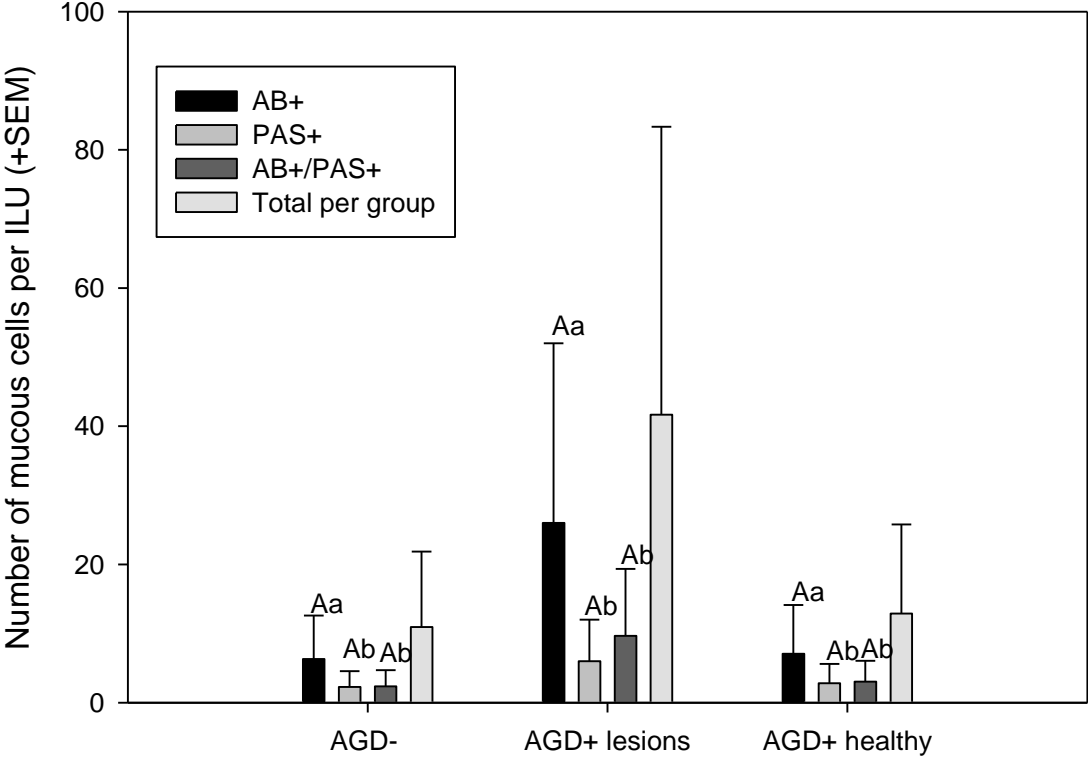


Figure 5: Mean mucous cell density (+ SEM) of alcian blue pH 4 positive mucous cells per ILU (interlamellar unit) on healthy and AGD-affected fish stained with AB-PAS. Different small letters indicate statistical difference between stains independent of treatment, and different capital letters indicate statistical difference between stains within a treatment group.

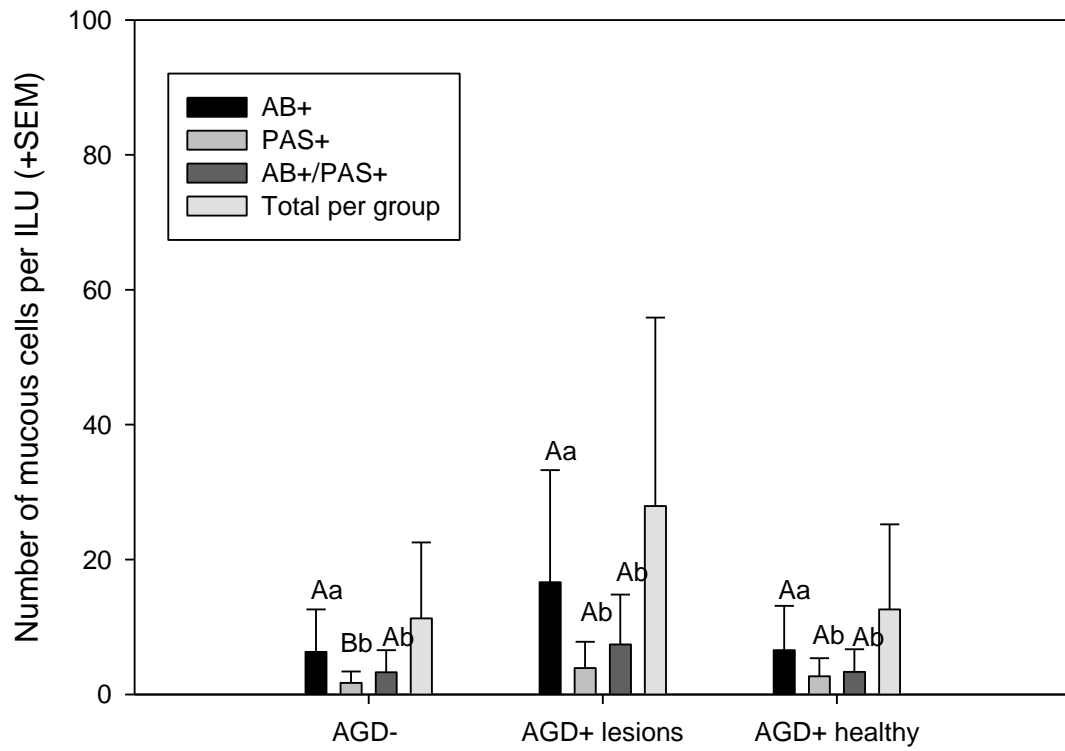


Figure 6: Mean mucous cell density (+SEM) of alcian blue pH 1.5 positive mucous cells per ILU (interlamellar unit) on healthy and AGD-affected fish stained with AB-PAS. Different small letters indicate statistical difference between stains independent of treatment, and different capital letters indicate statistical difference between stains within a treatment group.

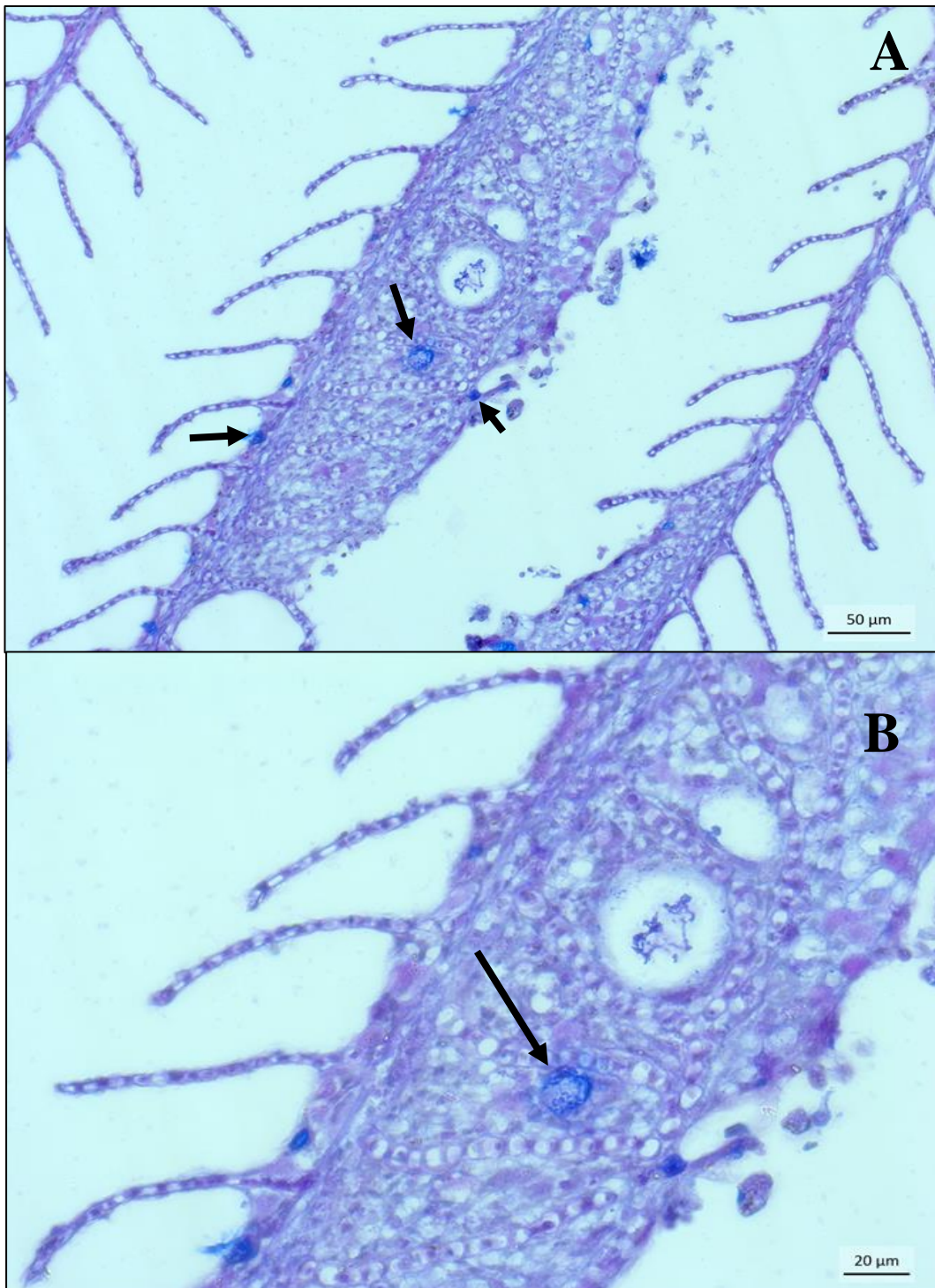


Figure 7: AB-PAS staining of ballan wrasse gills using alcian blue pH 4. A: Filament with focal hyperplasia of gill epithelium and fusion of lamellae with a focal recruitment of mucous cells (AB+) (arrows) on one side of the filament on AGD-affected ballan wrasse. An interlamellar vesicle with cell or amoeba debris was also observed along with sloughing of cells from the epithelium. Scale bar at 50 μm. B: Higher magnification of A, showing the hyperplasia and mucous cell (arrow). Scale bar at 20 μm.

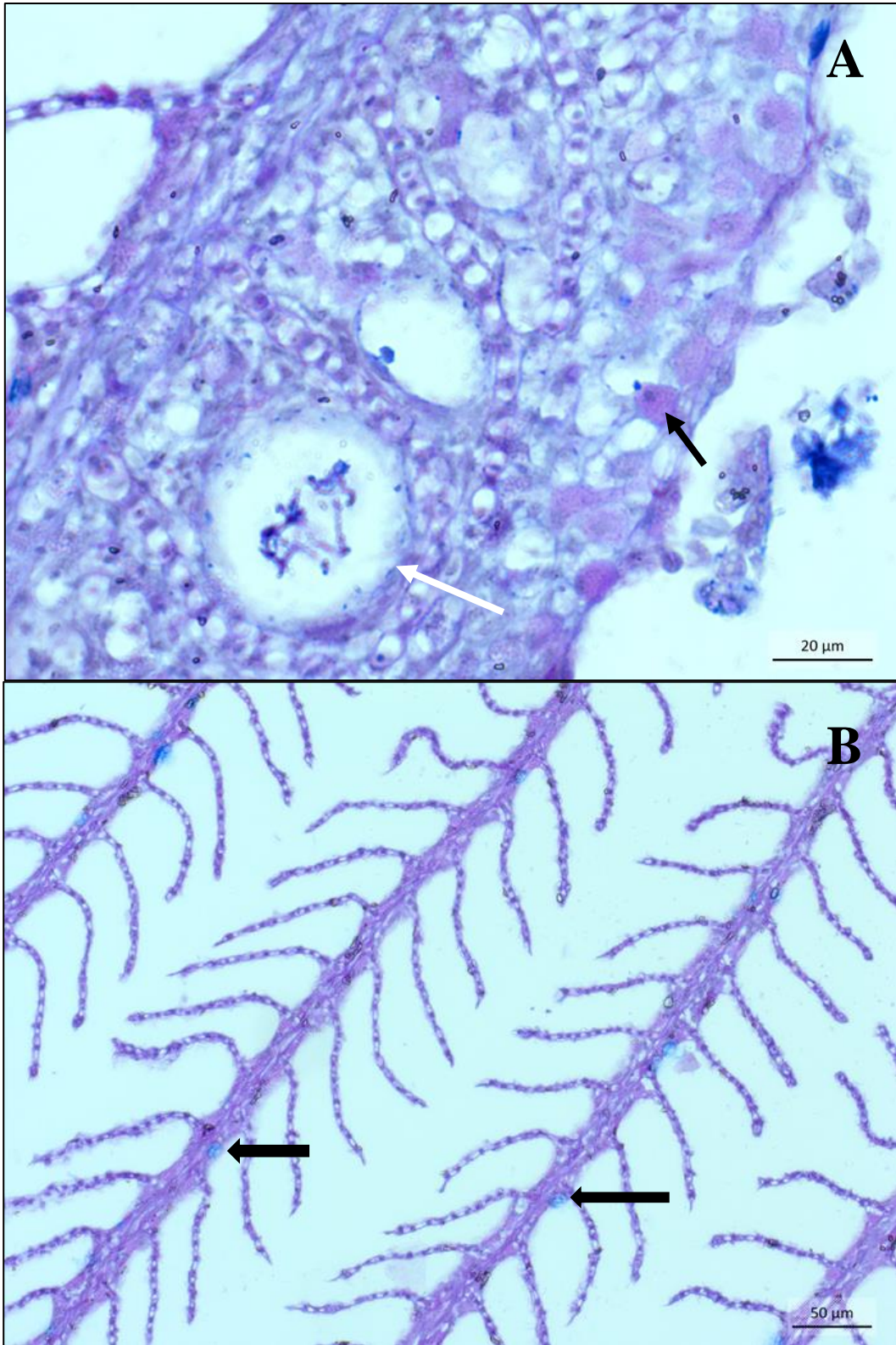


Figure 8: AB-PAS staining of ballan wrasse gills using alcian blue pH 4. **A:** Higher magnification of Fig 7B, showing a cell resembling an EGC (black arrow) as well as the interlamellar vesicle containing cellular debris (white arrow). Scale bar at 20 μm . **B:** Filaments of non-infected ballan wrasse showing some AB+ mucous cells (black bold arrows), mostly located at the base of the lamellae. Scale bar at 50 μm .

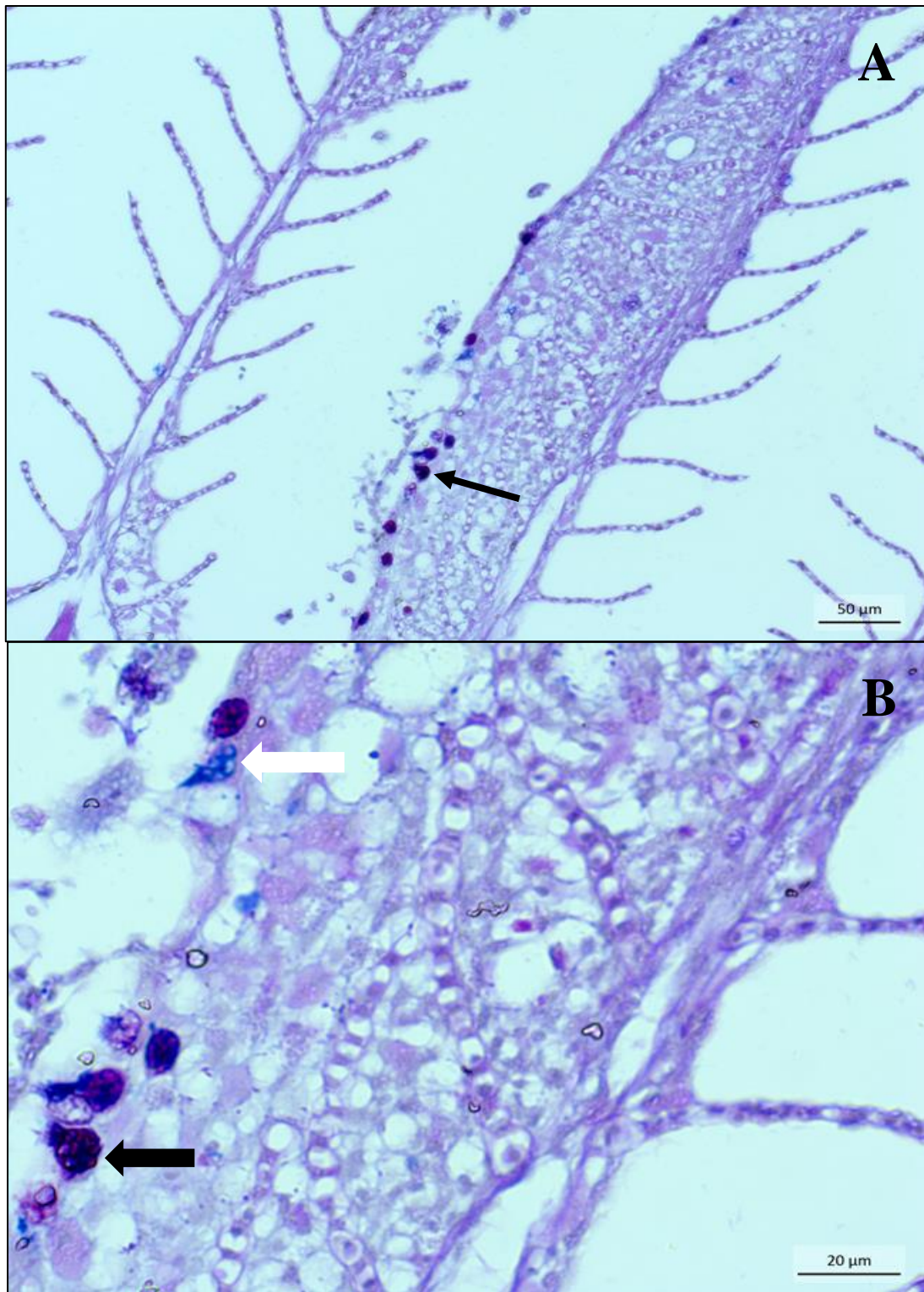


Figure 9: AB-PAS staining of ballan wrasse gills using alcian blue pH 1.5. A: AGD-associated lesion with hyperplasia of gill epithelium and recruitment of mucous cells (arrow). Scale bar at 50 μm. B: Higher magnification of A, showing different mucous cells, AB+ (white bold arrow) and AB+/PAS+ (black bold arrow). Sloughing of cells from the epithelium was also observed. Scale bar at 20 μm.

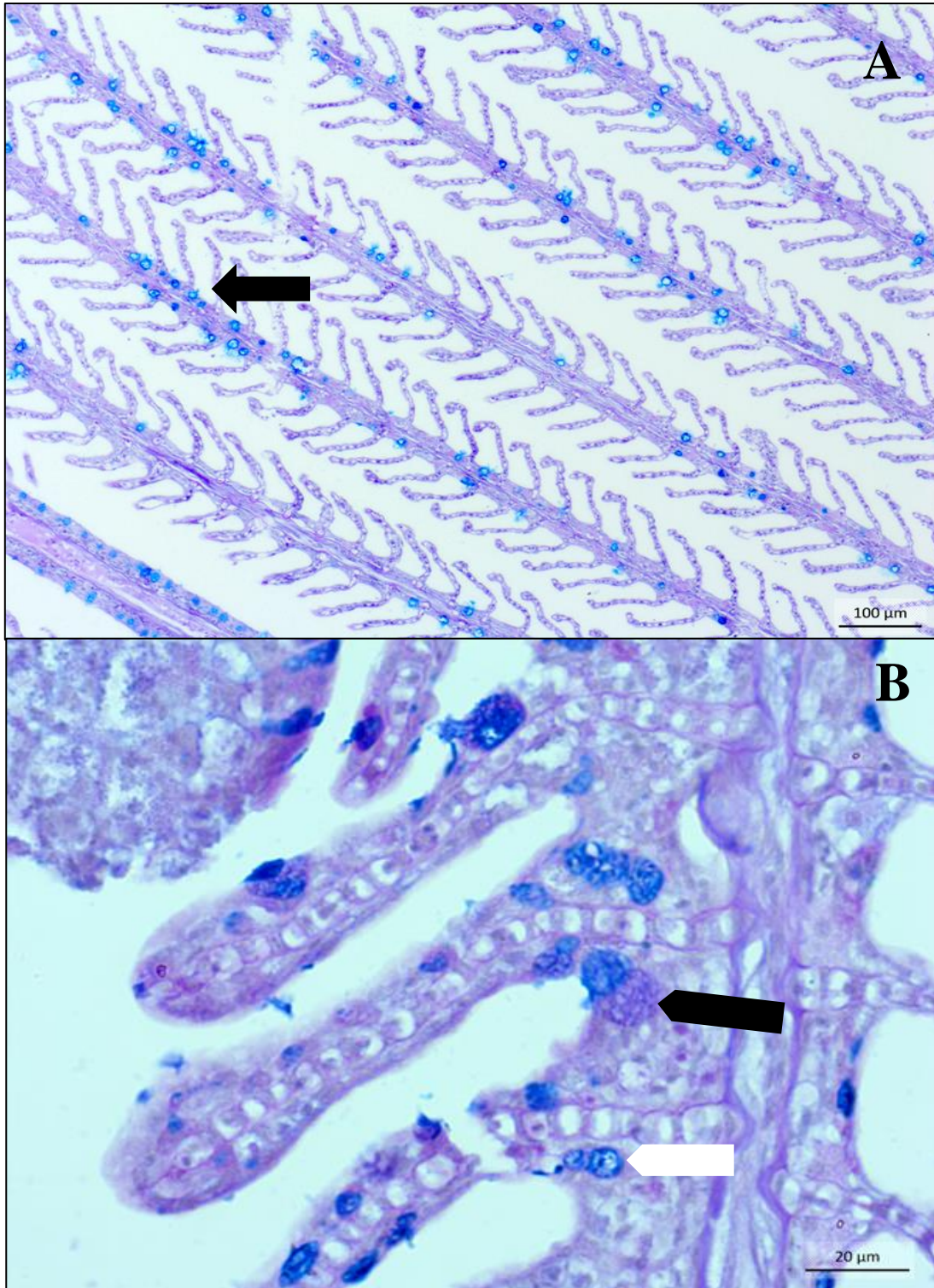


Figure 10: AB-PAS staining of ballan wrasse gills using alcian blue pH 1.5. A: Healthy filaments of an AGD-affected ballan wrasse showing a high abundance of mucous cells (arrow), with most of them AB+. Scale bar at 100 μm . B: High magnification of a lesion filament showing different mucous cells, AB+ (Black arrow) and AB+/PAS+ (white arrow). Scale bar at 20 μm .

3.4 Eosinophilic Granular cell density

Eosinophilic granular cell (EGC) counts were performed on sections stained with H&E and observed on sections stained with AB-PAS, Giemsa, toluidine blue and TUNEL (methyl green). The EGC density showed a trend that in AGD-associated lesions in AGD-affected ballan wrasse there was an infiltration and accumulation of EGCs compared to both healthy filaments on AGD-affected wrasse and filaments on non-infected wrasse ($H_2 = 11.783$, P value = 0.003) (Fig. 15). EGCs observed on sections stained with AB-PAS were shown to be mostly AB and PAS positive (pink/purple) (Fig. 13). EGCs observed with toluidine blue were not metachromatic and were stained light blue/grey or were almost transparent (Fig. 13). The EGCs observed with H&E and Giemsa were stained red (Fig. 11 and 12), and EGCs observed with the TUNEL stain were brightly pink (Fig. 14).

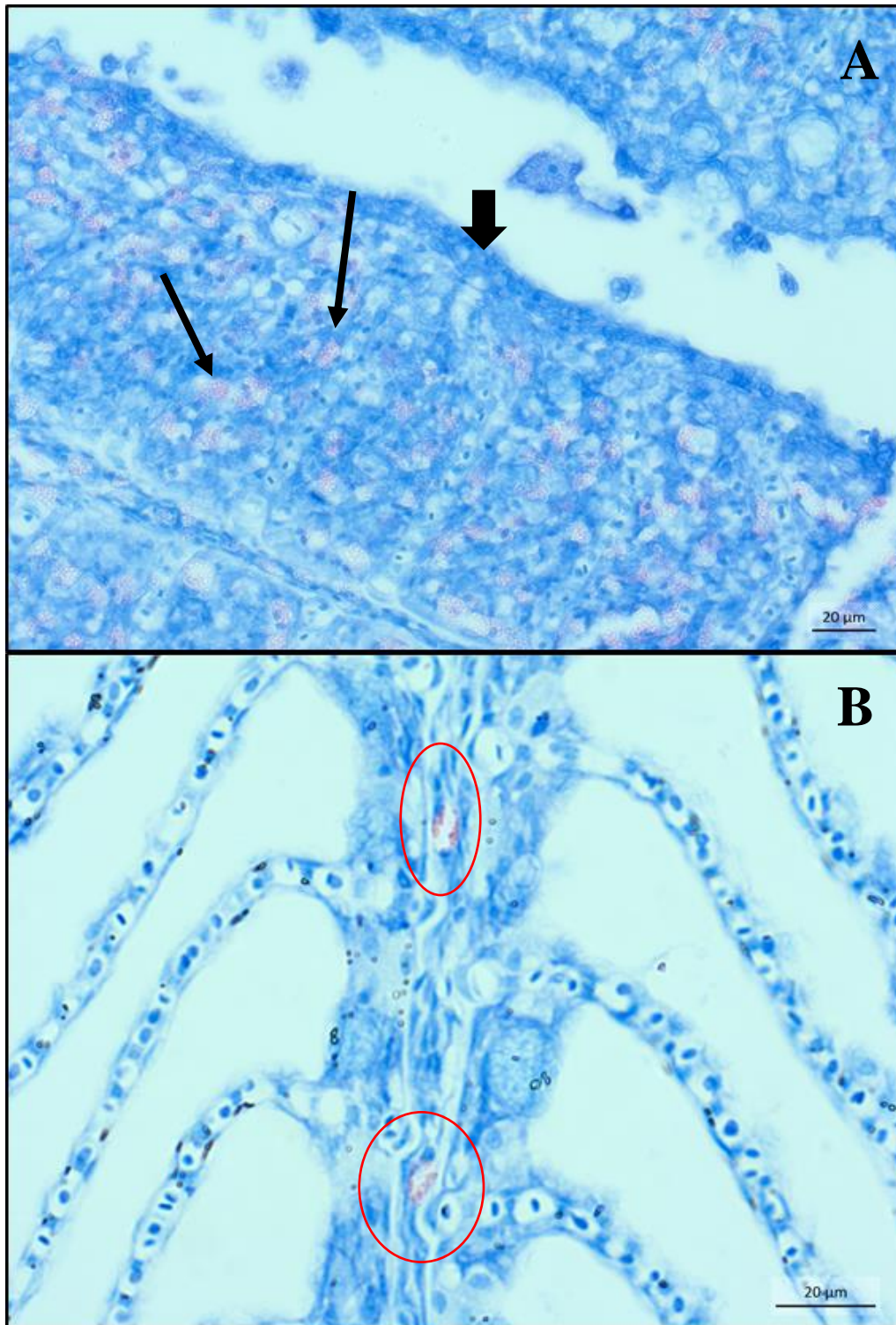


Figure 11: Giemsa staining of ballan wrasse gills. A: Heavy infiltration of EGCs (arrows) in an AGD-associated lesion in ballan wrasse. The squamate epithelial layer on the outside of the lesion was also observed. Scale bar at 20 μm. B: EGCs in the connective tissue in a healthy filament in AGD-affected ballan wrasse (circles). Scale bar at 20 μm.

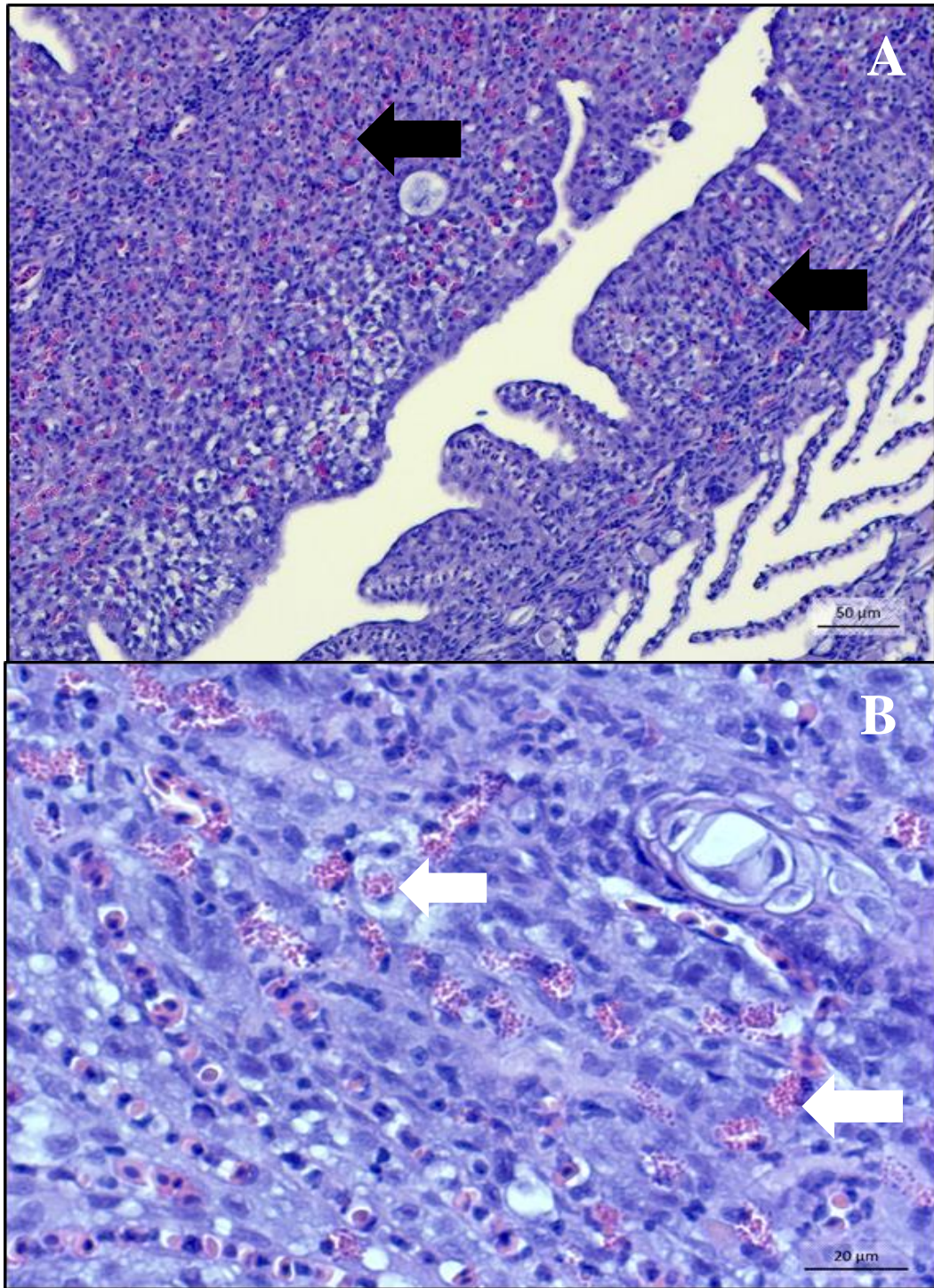


Figure 12: H&E staining of ballan wrasse gills. A: Lower magnification of an AGD-associated lesion with high infiltration of EGCs in the tissue (black bold arrows, red dots), Scale bar at 50 μm . B: High magnification of an AGD-associated lesion with infiltration of EGCs (white bold arrows), the granules were prominent. Scale bar at 20 μm .

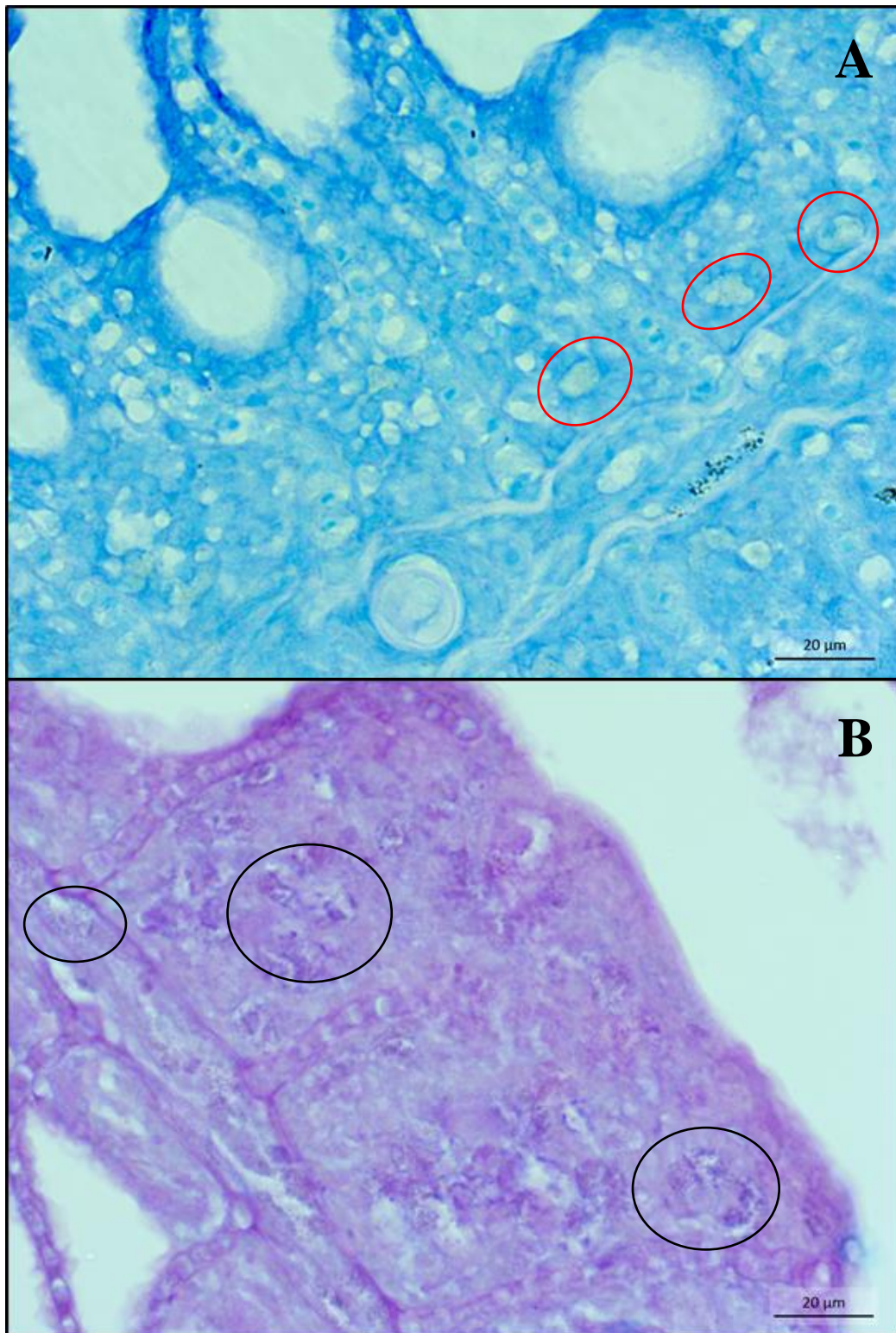


Figure 13: **A:** EGCs staining lightly blue/transparent in an AGD-associated lesion in ballan wrasse stained with toluidine blue (red circles). Scale bar at 20 µm. **B:** EGCs in a hyperplastic lesion in AGD-affected ballan wrasse stained with AB-PAS (black circles), the EGCs stained mostly PAS+ (pink) or AB+/PAS+ (purple), though some were also AB+(blue). Scale bar at 20 µm.

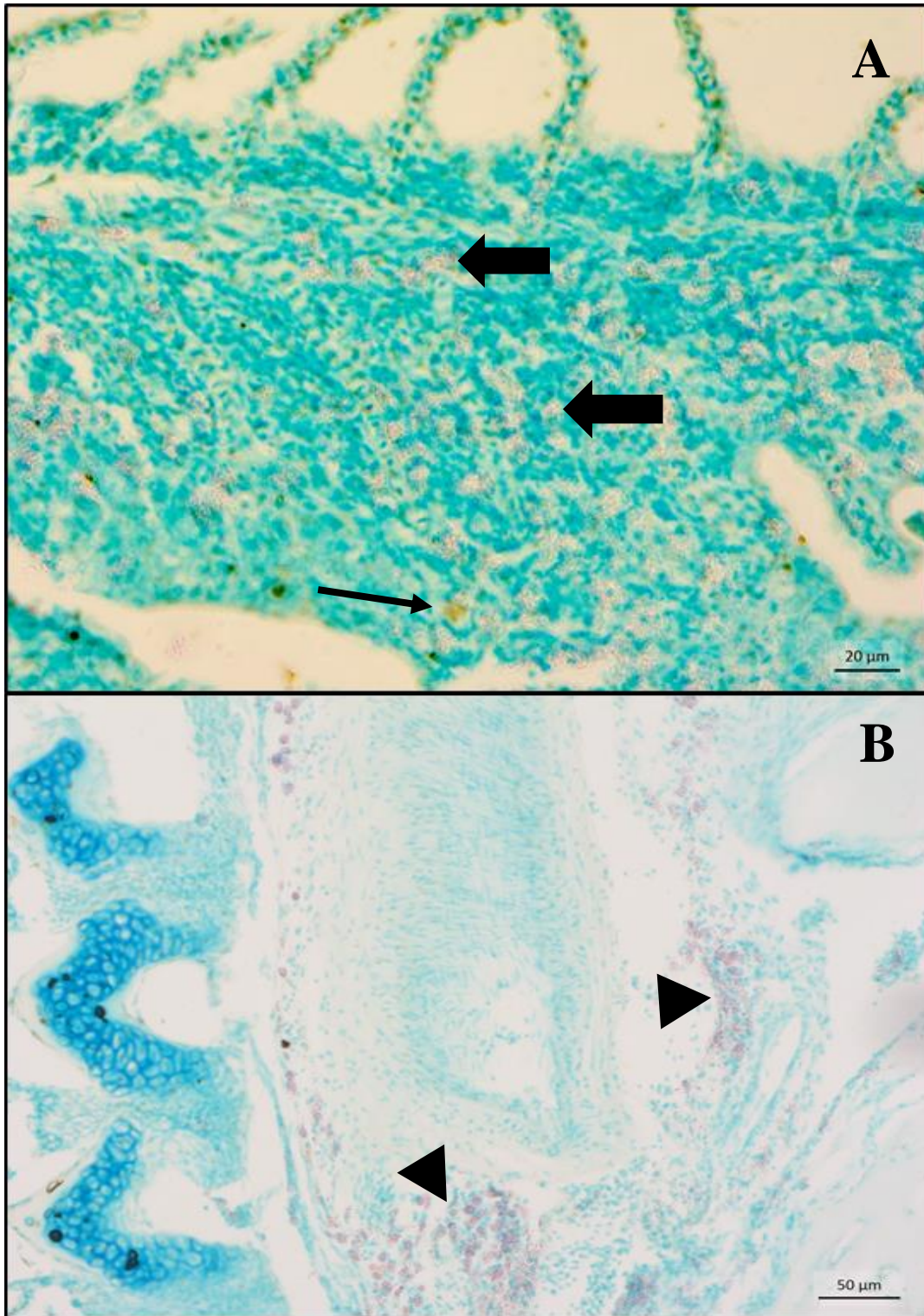


Figure 14: **A:** EGCs in an AGD-associated lesion in ballan wrasse stained using TUNEL (black bold arrows), An apoptotic EGC was also observed (thin arrow). Scale bar at 20 μm . **B:** Heavy infiltration of EGCs in the connective tissue in the base of the filaments in AGD-affected ballan wrasse stained using TUNEL, showing migration of EGCs (black arrowheads). Scale bar at 50 μm .

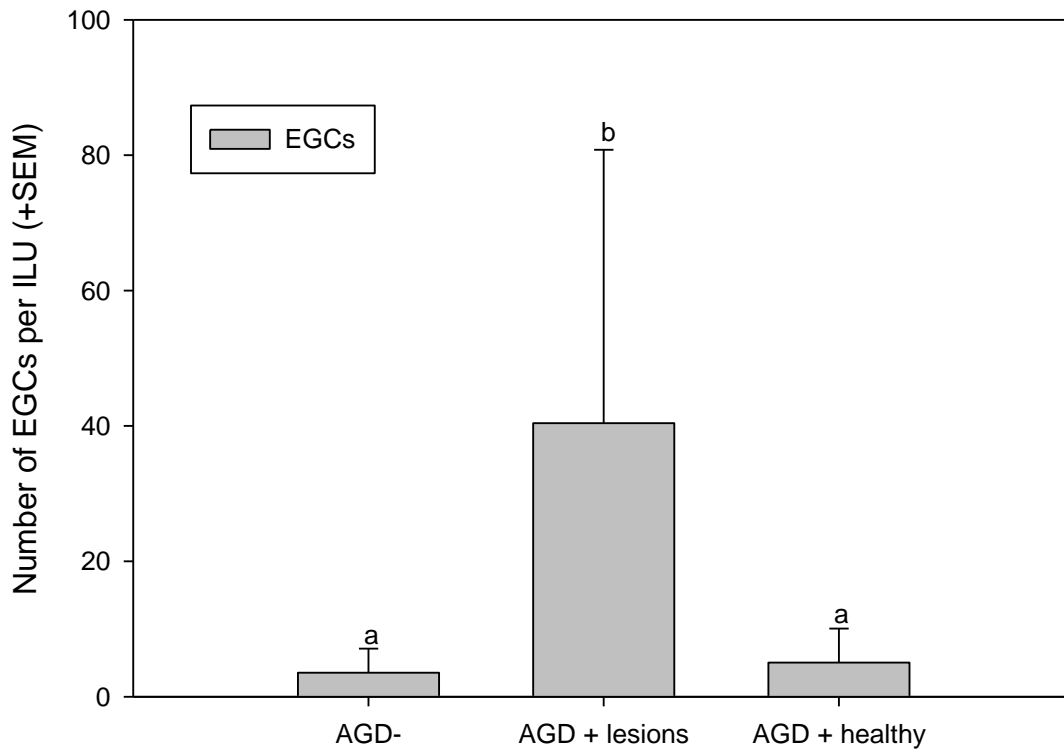


Figure 15: Mean (+SEM) eosinophilic granular cell density on H&E stained sections on healthy and AGD-affected ballan wrasse. Different letters show statistical significant difference.

3.4.1 Transmission Electron Microscopy of EGCs

The observations of EGCs using TEM confirmed that the eosinophil granule-resembling cells observed using other histochemical stains were EGCs (Fig. 16). The granules observed were large and membrane-bounded (Fig. 17). Both degranulated and normal EGCs were observed (Fig. 16). The EGCs observed were in the tissue, either closer to centre of the filament or at the base of the lamellae close to chloride cells (Fig. 16).

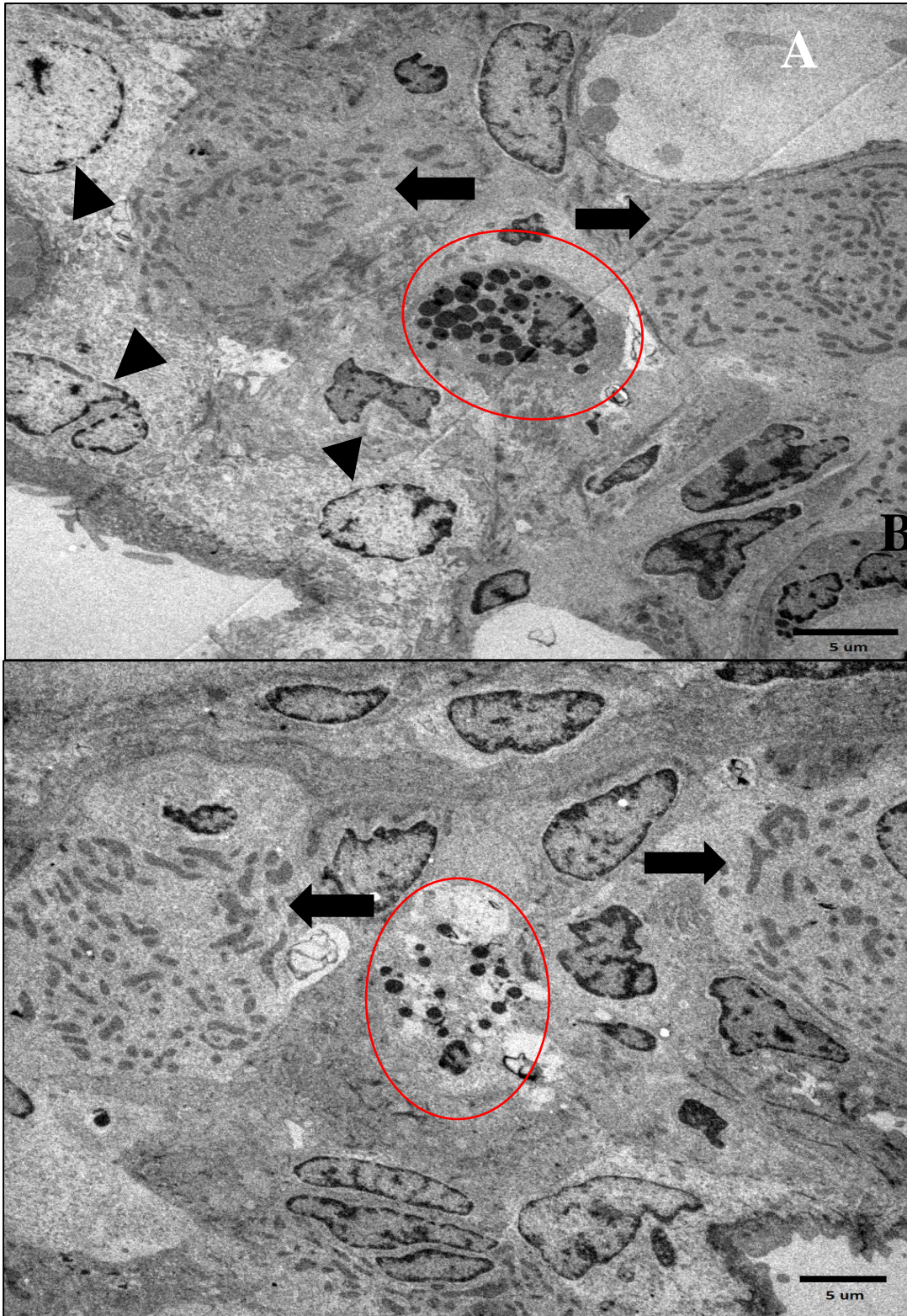


Figure 16: TEM of AGD-affected ballan wrasse. A. An EGC (red circle) in the gill tissue between two chloride cells (black bold arrows). Necrotic tissue and nuclei (arrowheads) were also observed in proximity of the EGC. x5000 and scale bar at 5 μm . B. A degranulating EGC (red circle) in the gill tissue between two chloride cells (black bold arrows). x6000 and scale bar at 5 μm .

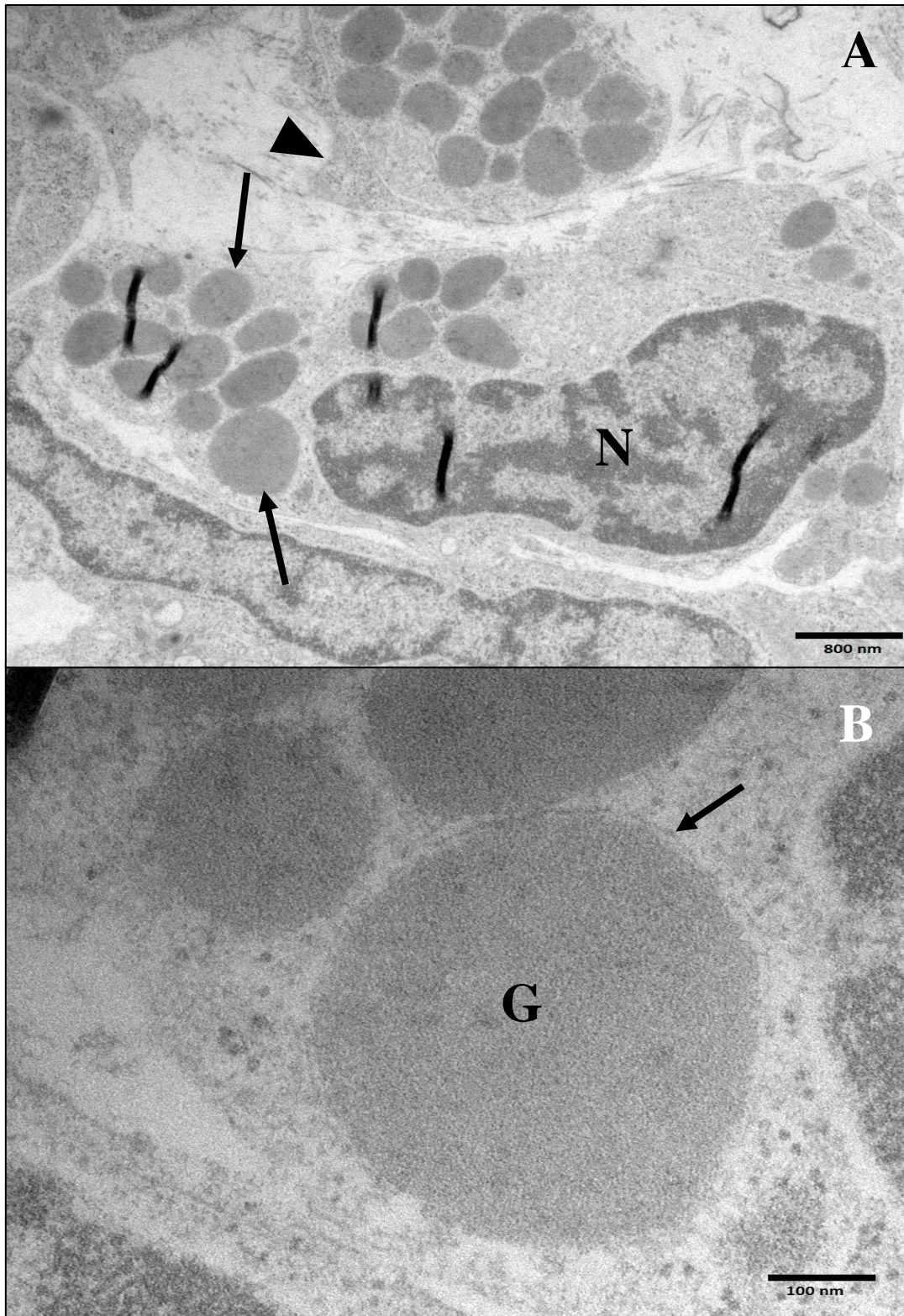


Figure 17: TEM of AGD-affected ballan wrasse. A. An EGC with rounded granules (arrows). A membrane (arrowhead) was observed on the outside of the EGC, possibly an ensheating cell. N = nucleus. x20k and scale bar at 800 nm. B. Higher magnification of a granule, showing the dense homogeneous matrix and the membrane around the granule (arrow). G = granule. x100k and scale bar at 100 nm.

3.5 Apoptotic cell density and observation using TUNEL

There was a tendency for more apoptotic cells and cells/area (mm^2) in AGD-associated lesions, than healthy filaments on AGD-affected fish and filaments on non-infected fish (Fig. 18). There was, however, no correlation between number of EGCs per ILU in the gills and number of apoptotic cells per ILU (Pearson Correlation coefficient = -0.0267, P value = 0.863), nor in AGD+ healthy (Pearson Correlation coefficient = 0.154, P value = 0.143) and AGD- (Pearson Correlation coefficient = -0.0300, P value = 0.716). A One-Way analysis variance was used to compare positive cells/area in the treatments groups, and a significant difference was found ($F_{2, 14} = 15.347$, P value = <0.001). The significantly different groups were AGD+ lesions vs. AGD- and AGD+ lesions vs. AGD+ healthy.

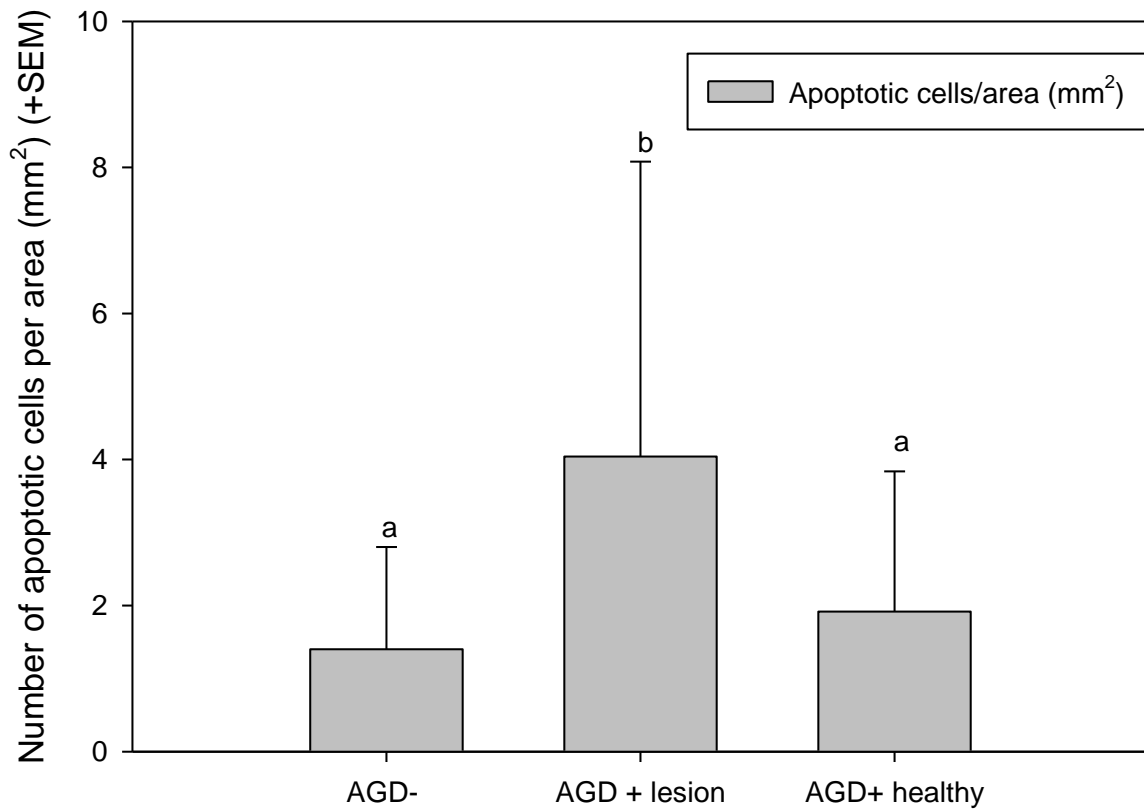


Figure 18: Mean (+SEM) apoptotic cell density on sections stained with TUNEL. Different letters indicate statistical difference.

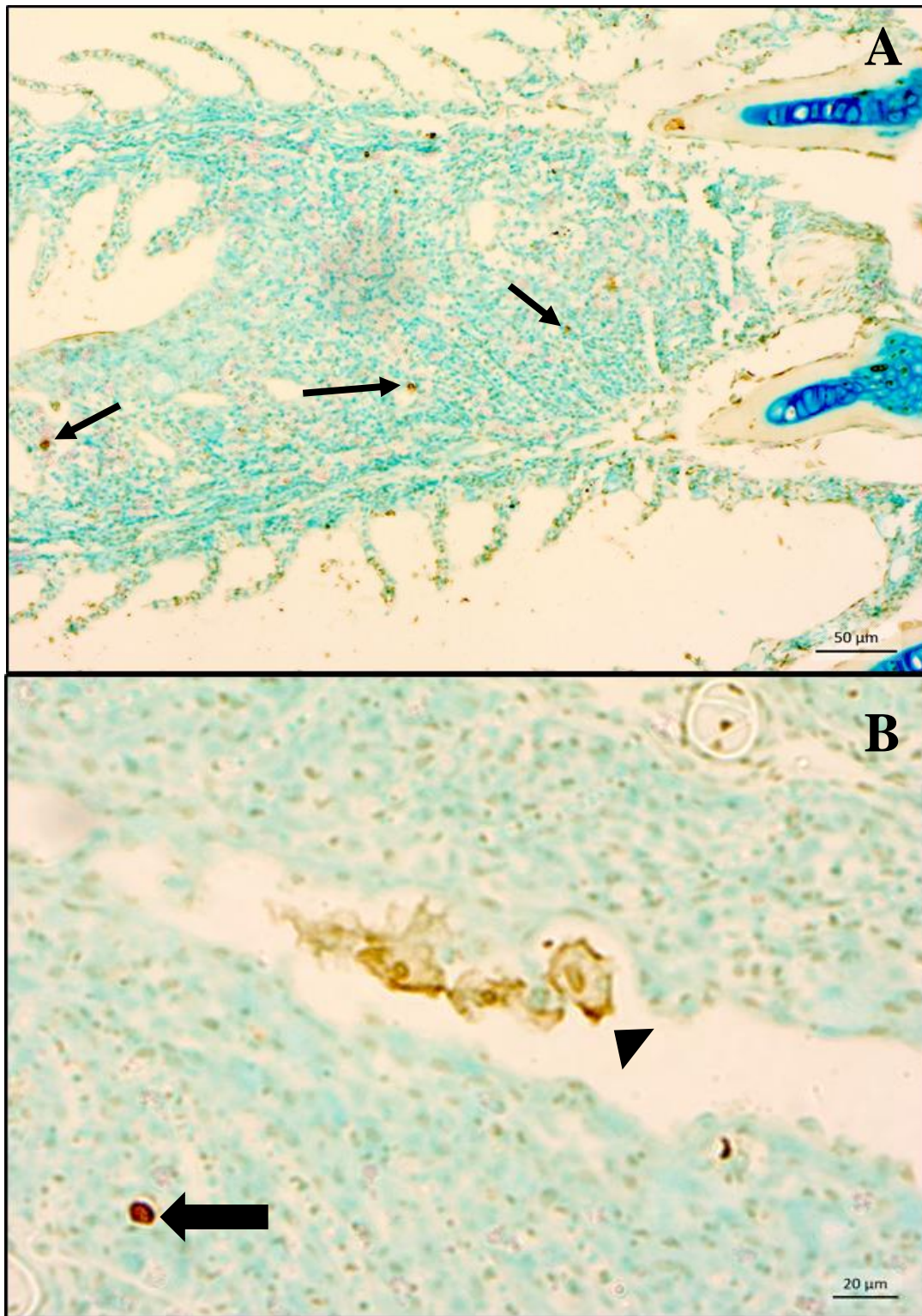


Figure 19: **A:** Apoptotic cells (arrows) in an AGD-associated lesion in ballan wrasse stained using TUNEL, a high infiltration of EGCs was observed. Scale bar at 50 μm. **B:** High magnification of an AGD-associated lesion, stained using TUNEL, with an apoptotic cell (black bold arrow) and amoebae were observed in between the filaments (black arrowhead). Scale bar at 20 μm.

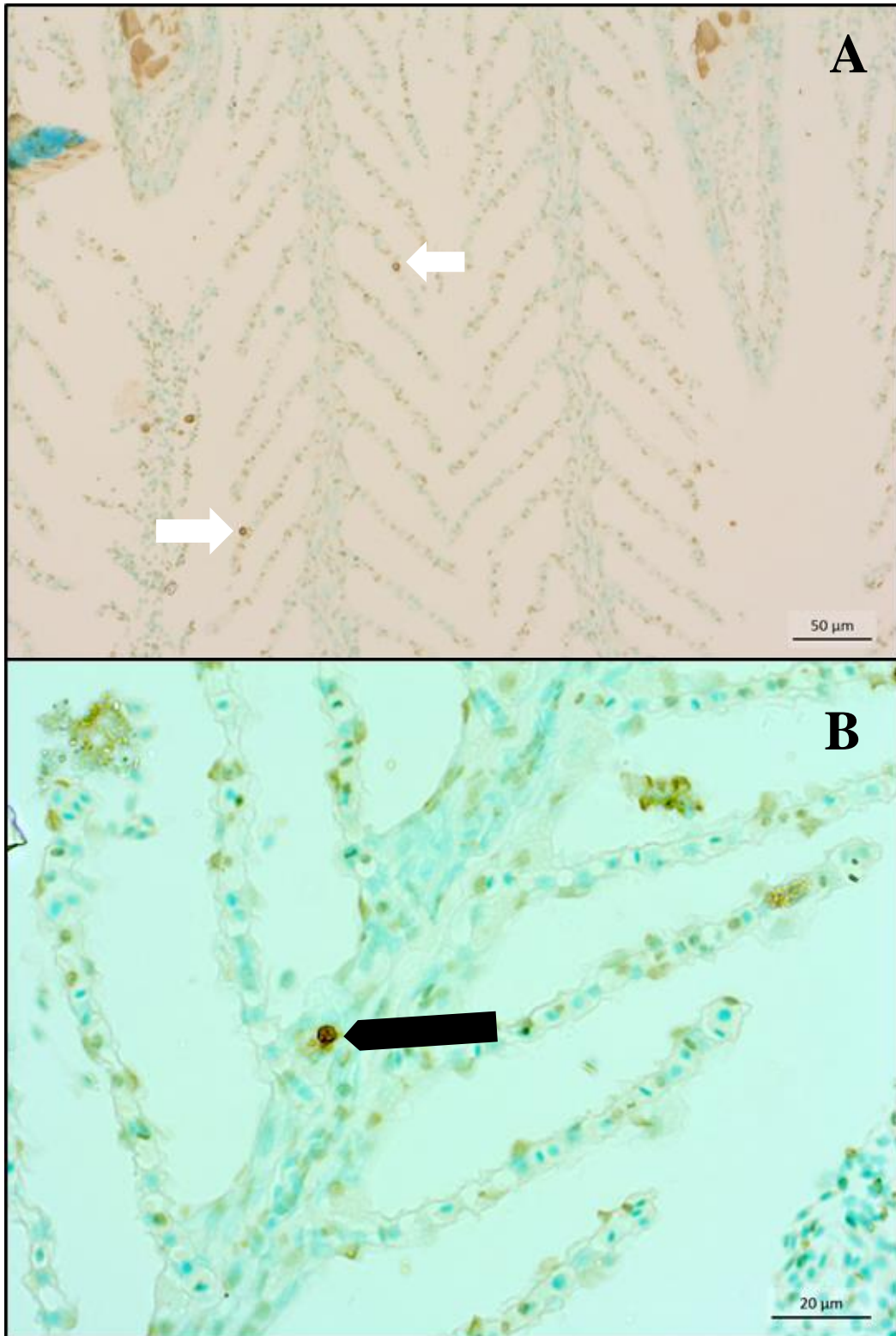


Figure 20: **A:** Filaments on non-infected ballan wrasse stained using TUNEL showing apoptotic cells in the secondary lamellae (white bold arrows). Scale bar at 50 μm . **B:** High magnification of an apoptotic cell (black arrow) on a filament in non-infected ballan wrasse stained using TUNEL. Scale bar at 20 μm .

4. Discussion

4.1 Lesion morphology comparison between ballan wrasse and Atlantic salmon

The pathology of AGD in Atlantic salmon and other salmonids has been thoroughly described to be multifocal hyperplasia of the gill epithelium on the filaments, and a fusion of secondary lamellae and in severe cases fusion of filaments (Fig. 1 and 2) (Munday et al., 1990, Adams and Nowak, 2001, Adams and Nowak, 2003, Adams and Nowak, 2004, Peyghan and Powell, 2006, Powell et al., 2008, Nowak, 2012). Whereas the pathology of AGD in ballan wrasse has only recently been described to be a more focal hyperplasia of the gill epithelium than in Atlantic salmon (Fig. 1, 2, 7 and 9) (Karlsbakk et al., 2013, Dahle, 2015, Lepperød, 2017). The lesions tended to be on single filaments, with no proximity to other affected filaments, in early pathogenesis of the disease.

It has also been observed that ballan wrasse appears to be more resistant to AGD and has a slower development of the disease than Atlantic salmon, and the characteristic white patches on the gill may appear at later pathogenesis of the disease (Dahle, 2015). This may be because of the generally high abundance of immune cells like EGCs in the tissue in and around the filaments and gills of the fish, as well as a quick response and recruitment of immune cells. This has also been observed in lumpfish by Haugland et al. (2016) where at 61 days post-infection only 20 % of the lumpfish had developed gill lesions, and all the Atlantic salmon had severe gill lesions.

4.2 Mucous cells

The mucous cell counts indicated that the abundance of cells increased in the filaments due to AGD, which is similar to what has been found in AGD-affected salmon (Munday et al., 1990, Nowak, 1994, Zilberg and Munday, 2000, Roberts and Powell, 2003, Roberts and Powell, 2005). Roberts and Powell (2005) also discovered a decreased viscosity of the mucus in Atlantic salmon and brown trout, but not in rainbow trout, this is not known for ballan wrasse. An increase in mucous cells on the gills has also been observed in BGD (bacterial gill disease) in rainbow trout (Ferguson et al., 1992), ichthyophthiriasis in goldfish (*Carassius auratus*, Linnaeus) (Tumbol et al., 2001), and infections with dinoflagellates on different teleostean fish (Kim et al., 2000). For the mucous cell histochemistry with AB-PAS, ballan wrasse showed positive for AB+, PAS+ and AB+/PAS+, the opposite of that seen by Roberts and Powell (2005), who did not find AB positive cells in brown trout or rainbow trout. This shows that ballan wrasse, affected with AGD or not, has a higher number of AB positive cells,

which suggest a shift towards more acidic mucin glycoproteins as a response to *N. perurans* (Fig. 5 and 6), contrary to Atlantic salmon, brown trout, and rainbow trout, which was found to have shift towards more neutral mucin glycoproteins (Roberts and Powell, 2003, Roberts and Powell, 2005, Powell et al., 2008).

The reduction in mucus viscosity in Atlantic salmon, has been suggested to be a response to amoebae, with a more effective sloughing of mucus, epithelial cells, and amoebae (Roberts and Powell, 2005), although this might also help spreading the amoebae to new hosts. It has also been showed in rainbow trout that with increased production of mucus and mucous cell hyperplasia, uptake of O₂ is minimally effected, but the excretion of CO₂ will decrease, and could lead to an acid-base disturbance and blood-acidosis (Powell and Perry, 1996, Powell and Perry, 1997, Powell and Perry, 1999, Powell et al., 2008). This has not been fully shown in ballan wrasse, but Lepperød (2017) found an increase in blood pH and a decrease in PCO₂, which was likely associated with hyperventilation, this has also been seen in Atlantic salmon by Leef et al. (2005). This was observed by Lepperød (2017) in the first weeks of the trial, but was unable to collect blood data in the last two weeks, which would probably have indicated an increase in PCO₂ and a decrease in pH, which has been observed in salmon (Powell et al., 2000). Though AGD (represented by gill lesions) was not found to be the clear reason for this, even though a correlation between AGD-lesions and PCO₂ and pH was found (Lepperød, A., pers.comm.). This probably has several reasons, but the increased production of mucus and hyperplasia is a limiting factor for the diffusional window for excretion of CO₂ (Powell and Perry, 1999).

Salinity also influences the number of mucous cells and abundance, Franklin (1990) found an increase in branchial mucous cells after transferring sockeye salmon (*Oncorhynchus nerka*, Walbaum) from freshwater to seawater. The view earlier was that the increase in mucous cells happened with a decrease in salinity (Roberts and Powell, 2003, Shephard, 1994). This was supported by other studies that used seawater adapted fish and transferred them to freshwater (Burden, 1956, Ahuja, 1970, Wendelaar Bonga, 1978). This has also been found to increase when transferring freshwater adapted fish to seawater by Roberts and Powell (2003), but this might also have been because the fish had not acclimated fully to seawater. For ballan wrasse, which is a seawater fish, it would not survive for long in freshwater due to the low salinity. Although brackish water has been used to treat AGD on ballan wrasse (Breck, 2013, Dahle, 2015), no examination of mucous cells in the transfer from seawater to brackish water was undertaken. As brackish water has been the chosen treatment for AGD in ballan wrasse, and it

has a lower salinity than seawater, there may be more mucous cells in the gills of ballan wrasse after a treatment with brackish water, however, this remains to be confirmed.

4.3 Eosinophilic Granular Cells

In non-infected ballan wrasse, the EGCs were observed in the connective tissue of the filament with close proximity to blood vessels, in the tip of the filament and also in either the base or at the tip of the secondary lamellae, this has also been observed in ballan wrasse by Karlsbakk et al. (2013) and in other teleostean fish (Powell et al., 1990, Reite, 1997, Holland and Rowley, 1998, Reite, 1998). In healthy filaments of AGD-affected ballan wrasse, EGCs were observed in the same places on the filaments as in the non-infected fish, although a small increase in number of EGCs was observed, this might be due to the stress of the handling and the disease, as suggested by Holland and Rowley (1998) in rainbow trout. In the lesioned filaments of AGD-affected ballan wrasse, a substantial increase in the number of EGCs was observed, and EGCs were extensively observed in the hyperplastic tissue between secondary lamellae, which supports Karlsbakk et al. (2013) earlier observations (Fig. 11,12, 13 and 14). This has also been observed in Atlantic salmon, rainbow trout and other salmonids (Powell et al., 1990, Reite, 1997, Holland and Rowley, 1998, Lovy et al., 2007).

In Atlantic salmon, eosinophils other than EGCs have been observed, whereas the EGCs observed in this study are thought to be more like mast cells in mammals, the eosinophils described by Lovy et al. (2007) were more akin to mammalian eosinophils, suggesting that they are more like eosinophils than EGCs, and possibly of a different lineage to EGCs and mast cells. These eosinophils, along with EGCs, were also observed on sections with AGD-affected salmon used in this study for comparison (Fig. 3 and 4). Using TEM, it was clearly seen that the granules of the EGCs in ballan wrasse were membrane-bounded with a dense homogeneous matrix (Fig. 17) as described in other studies (Ezeasor and Stokoe, 1980, Reite, 1998, Reite and Evensen, 2006), whereas the eosinophils described by Lovy et al. (2007), had smaller and elliptic granules that contained a crystalline inclusion in the centre of the granules.

EGCs in ballan wrasse were also observed in connective tissue at the base of the filaments, and in blood vessels in the gill arch (Fig. 14) suggesting a migration and recruitment to lesioned-filaments, as suggested in rainbow trout by Powell et al. (1990), the observations of EGCs in and around blood vessels still suggests potential for diapedesis. These observations were made in both non-infected and AGD-affected ballan wrasse, with an increase of EGCs in

AGD-affected ballan wrasse. This suggests that EGCs and other immune cells are possibly circulating around in the fish or are recruited from other areas or tissues (Powell et al., 1990, Matsuyama and Iida, 1999, Sharp et al., 1989).

The EGCs showed some differences to other studies when it comes to staining. In ballan wrasse, EGCs were metachromatic and stained red with Giemsa, whereas Reite (1997) and Holland and Rowley (1998) found them to stain deep blue in other teleostean fish. With AB-PAS staining, EGCs in ballan wrasse were mostly PAS positive (pink/purple), indicating that they are more like EGCs than mast cells, however, a small fraction of EGCs stained blue (AB) positive, which might indicate that some of the EGCs are more like mast cells. These cells were found to be PAS negative in some studies and positive in others (Weinreb and Bilstad, 1955, Ezeasor and Stokoe, 1980, Holland and Rowley, 1998). When stained with toluidine blue, EGCs in ballan wrasse did not show metachromasia, and were stained lightly blue, this was also found by Ezeasor and Stokoe (1980), whom used a 1 % toluidine blue solution, which gives a deeper blue staining, whereas a 0.1 % toluidine blue solution was used in this study. It has also been suggested that fixative can alter the metachromatic properties of EGCs (Weinreb and Bilstad, 1955, Holland and Rowley, 1998). This might be because the occurrence of the metachromatic properties of EGCs in fish are highly dependent on the nature of the used fixative and the subsequent staining method (Reite and Evensen, 1994, Reite, 1996).

4.4 TUNEL and apoptotic cells

The findings in this study suggested that there was a higher turnover and natural cell death in lesioned tissue of AGD-affected ballan wrasse compared to healthy filaments on AGD-affected ballan wrasse and non-infected ballan wrasse. Apoptotic cells have also been found using TUNEL in hearts of rainbow trout with CMS, HSMI and PD (Yousaf et al., 2012, Yousaf et al., 2013), as well as healthy fish (Yousaf et al., 2016). They found a higher number of apoptotic cells in fish with PD or CMS than in fish with HSMI, where PD and CMS have more degenerative changes in the heart, and HSMI shows more inflammatory changes. Apoptosis has also been observed in both normal and diseased tissue in human hearts (Buja and Entman, 1998, Maximilian Buja and Vela, 2008). The high number of apoptotic cells in the lesions on ballan wrasse gills may be because the lesions were “old” and were in regression and had started to go back to a normal state, as the observations and counts were performed on gills taken from week 5 and 6 post-infection (Fig. 19. and 20). If it also had been performed on gills with lesions from earlier weeks, there may have been few apoptotic

cells observed, as the lesions here would be more in a state of growth than regression. This has been observed in gills of crucian carp, where the gills would be covered in hyperplastic tissue in normoxic (aerated) water. When kept in hypoxic water, the hyperplastic tissue would recede, and the secondary lamellae would become more visible and protruding (Sollid et al., 2003). There was no immunohistochemistry done in this study, due to implications with the samples, but to really see if the lesions were in a state of regression, a staining of PCNA (proliferating cell nuclear antigen) to look at the cell proliferation in the lesions should be undertaken. The reason for the regression is unknown and is not known to occur with AGD in salmonids, but it has been observed in some fish affected with AGD (Powell, M. D., pers comm). It may have to do with low temperature and/or salinity, although the temperature and salinity in this study were stable and within the limits of the amoebae. It has also been observed in this study and by Lepperød (2017), that the gross gills score and lesions in ballan wrasse get to a certain score, and stops, this was also observed in lumpfish by Haugland et al. (2016), where there were found fish with score 0 at 93 days post-infection. Whereas in Atlantic salmon the score would continue to increase. Apoptosis detection in other fish species with AGD has not been done, and so a comparison was not possible. Although the same results may be found in other fish that are more resistant to AGD, like lumpfish and other cleanerfish. Whereas in salmon there may be more growth as the gill score does not stop at a certain score as with ballan wrasse and lumpfish.

In the healthy filaments of both AGD-affected and non-infected ballan wrasse, there were few observed apoptotic cells. Generally, more cells were observed in filaments of AGD-affected fish. This suggested that there may be a higher cell turnover and cell death in AGD-affected fish than non-infected fish. Powell et al. (2014) observed apoptotic cells in gills of rainbow trout with and without *Loma salmonae* infection, although they did not find a significant difference between sick and healthy fish. Apoptotic cells have also been widely described in normal and diseased tissues in humans and other mammals, associated with both tissue renewal and tissue death (Kerr et al., 1972, Buja et al., 1993, Majno and Joris, 1995, Buja and Entman, 1998, Maximilian Buja and Vela, 2008).

5. Concluding remarks

Ballan wrasse, though susceptible to AGD, appear to have a slower development of the disease than Atlantic salmon. The lesions in wrasse appeared to be more focal, whereas in salmon, the hyperplastic lesion appeared on different places on different filaments, the lesion seems to appear on a filament, and another filament somewhere else on the gill arch. The pathology of AGD on ballan wrasse appear to be more focal with single filaments being affected at a time rather than several hyperplastic lesions on several filaments at the same time. The finding in this study suggests that there is a higher infiltration of EGCs in ballan wrasse than salmon. There also appear to be more acidophilic mucous cells in wrasse, as a significant difference was found between AGD-associated lesions and healthy filaments on AGD-affected fish and non-infected fish. There were also more apoptotic cells per area in lesions in wrasse with significant difference to healthy filaments of both AGD-affected and non-infected fish, showing that there was a higher turnover and natural cell death in the lesions.

6. Future perspectives

As ballan wrasse is a relatively new species in Norwegian aquaculture, and only recently been found susceptible to AGD, there is more research needed to fully understand the disease in this species. A continuing work on characterizing the cellular and inflammatory response could be undertaken, as well as gene suppression in ballan wrasse with AGD compared to Atlantic salmon and other fish. Further investigation into brackish water treatment and the wrasse's mucosal response and adaptation to low salinities, for a better understanding, should be undertaken.

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Appendix I

1. Solutions used in histological staining

1.1 TUNEL

Solutions used were either supplied with the kit or made at the lab, following the protocol supplied with the TUNEL kit.

100 mL 0.5 % Methyl Green:

1. Prepare 0.1 M sodium acetate, pH 4.0. Dissolve 1.36 g sodium acetate in 80 mL of dH₂O. Adjust pH with acetic acid, and add dH₂O to a final volume of 100 mL.
2. Dissolve 0.5 g of methyl green in 100 mL of 0.1 M sodium acetate pH 4.0.
3. For other volumes, calculations on sodium acetate and methyl green were performed using calculation tools.

1.2 AB-PAS

1 % Alcian blue:

1. 2.5 g alcian blue 8GX dissolved in 250 mL dH₂O.
2. pH adjusted to pH 4 and 1.5 with acetic acid

0.5 % Periodic acid:

1. 1 g periodic acid dissolved in 200 mL dH₂O

Acid alcohol:

1. Coplin jar with absolute ethanol, and add a few drops of acetic acid

Schiff's reagent:

Schiff's reagent was used premade by the histology laboratory at Høyteknologisenteret in Bergen.

Haematoxylin:

Mayer's haematoxylin from Sigma Aldrich, Norway was used.

1.3 Giemsa

Giemsa solution:

1. Dilute Giemsa stain (Sigma Aldrich, Norway) 1:20 with distilled water, colour can be varied by diluting in buffer. For bluer staining water buffered at pH 7.2 may be used.

Acid alcohol:

1. Coplin jar with absolute ethanol, and add a few drops of acetic acid

1.4 Toluidine blue

Toluidine blue:

1 % toluidine blue solution, obtained from the histology lab at Høyteknologisenteret, was diluted to 0.1 % with distilled water

Appendix II

2. Staining protocols

2.1 AB-PAS

1. Deparaffinize through 2 changes of xylene/xylene substitution, 1 change of xylene and absolute ethanol (50/50), 4 changes of graded ethanol (100 % - 25 %) and 2 changes of distilled water for 2-3 min each.
2. Bring sections from distilled water and to a coplin jar with alcian blue and stain for 15 min.
3. Wash well in running tap water for 2 min and rinse in distilled water.
4. Bathe in periodic acid for 5 min.
5. Wash well in distilled water before staining with Schiff's reagent for 10 min.
6. Wash in running tap water for 5 min.
7. Stain nuclei with haematoxylin in a coplin jar for 1 min.
8. Wash in running tap water for 2 min and differentiate by dipping the sections in acid alcohol.

9. Wash in water and dehydrate through distilled water to xylene, clear and mount using mounting media.

2.2 Giemsa

1. Deparaffinize through 2 changes of xylene/xylene substitution, 1 change of xylene and absolute ethanol (50/50), 4 changes of graded ethanol (100 % - 25 %) and 2 changes of distilled water for 2-3 min each.
2. Immerse in buffered water with pH 7.4 for 5 min.
3. Stain with Giemsa (1:20) for 15 min.
4. Rinse in buffered water.
5. Differentiate with acid alcohol, and dehydrate in water, ethanol, and xylene before mounting.

2.3 Toluidine blue

1. Deparaffinize through 2 changes of xylene/xylene substitution, 1 change of xylene and absolute ethanol (50/50), 4 changes of graded ethanol (100 % - 25 %) and 2 changes of distilled water for 2-3 min each.
2. Apply drops of 0.1 % toluidine blue to the slides for a few seconds.
3. Rinse in running tap water and place upright to dry.
4. Mount the sections using mounting media.