



**Use of Methacarn as a fixative to aid interpretation of
amoebic gill disease (AGD) in Atlantic salmon, *Salmo salar*
L.**

Journal:	<i>Journal of Fish Diseases</i>
Manuscript ID	JFD-2018-395
Manuscript Type:	Original Manuscript
Date Submitted by the Author:	20-Dec-2018
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Keywords:	Amoeba, mucous cells, methacarn, gill pathology, AGD

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This is the peer reviewed version of the following article: Fernandez, C, Mascolo, D, Monaghan, SJ, et al. Methacarn preserves mucus integrity and improves visualization of amoebae in gills of Atlantic salmon (*Salmo salar* L.). J Fish Dis. 2019; 42: 883– 894, which has been published in final form at <https://doi.org/10.1111/jfd.12988>. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for self-archiving.

i. **Title:** Use of Methacarn as a fixative to aid interpretation of amoebic gill disease (AGD) in Atlantic salmon, *Salmo salar* L.

ii. **Short running title:** Mucus fixatives for observation of AGD

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v. **Acknowledgements:** The authors would like to thank Ms. Debbie Faichney for her technical advice and support with histology. Many thanks are also owed to Mr. Chessor Matthew, Dr. William Roy and Dr. David Bassett at MERL for provision of tanks and maintenance of fish during the study. This study has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement no. 634429 (ParaFishControl). This output reflects only the author's view and the European Union cannot be held responsible for any use that may be made of the information contained herein.

vi. **Abstract**

The mucous coat of mucosal epithelia is generally lost during routine formalin fixation and paraffin wax embedding procedures. Mucous coat maintenance during processing for histology could potentially help in understanding its relationship with pathogens and pathogenesis; in this case, the aetiological agent of amoebic gill disease (AGD) *Paramoeba perurans*. To examine this question, two aqueous fixation regimes (modified Davidson's solution and modified Davidson's solution with 2% (w/v) Alcian blue) were compared against two non-aqueous fixation regimes (methacarn solution and methacarn solution with 2% (w/v) Alcian blue) in a bid to improve preservation of the mucous coat on AGD-infected Atlantic salmon, *Salmo salar* L., gills. Qualitative and quantitative results revealed a greater preservation of the gill mucus using methacarn solution in comparison to the modified Davidson's solution. The addition of Alcian blue did not enhance preservation of the mucous coat. However, when samples were processed with Alcian blue/Periodic acid–Schiff staining both amoebae and mucous coat were observable. In addition, lectin-labelling was developed to confirm the presence of the mucous coat. The present work demonstrates that the techniques employed for preservation of the mucous coat can indeed avoid the loss of potential mucus-embedded pathogens providing a better understanding of its pathology.

Keywords: amoeba, mucous cells, methacarn, gill pathology, parasite, amoebic gill disease

vii. **Main text**

1. Introduction

Gill mucus is one of the key components of fish mucosal immunity, providing a protective barrier between the organism and the external environment (Shephard, 1994). Components of the gill mucus are similar to the ones found in skin mucus, such as antimicrobial peptides (Cole et al., 2000), enzymes such as lysozyme (Murray & Fletcher, 1976; Costa et al., 2011) and both IgM and IgT (Xu & Klesius, 2013). According to context, surface mucus can provide a barrier preventing host access, a protective matrix or a feeding substrate for a range of obligate, facultative and opportunistic pathogens. It therefore plays a key part in mediating the interaction between pathogen and host and can thus serve an important role in disease development.

Conventional aqueous fixatives provide excellent cytological preservation but fail to deliver the preservation of mucus layers due to them being washed away or dissolved in the fixative more quickly (Mays et al., 1984; Leist et al., 1986; Lee et al., 1995). Results from the use of a number of alternative aqueous and non-aqueous fixatives have proven such approaches to be successful for the preservation of mucus layers in humans (Ota & Katsuyama, 1992), rodents (Nichols et al., 1985; Sims et al., 1991; Geiser et al., 1997), and pigs (Allan-Wojtas et al., 1997). It is suggested that the preservation of mucus in situ can offer similar significant advantages in fish and can aid understanding of host-pathogen interactions and the mechanisms of ports-of-entry and disease development in mucosal tissues.

There has been limited work focusing on the adaptation of these methods in order to observe mucus on fixed mucosal tissues in fish. Most examinations on mucous composition that have been performed on fish involve work on skin. A continuous mucous layer was described to be anchored by the microridges of pavement epithelial cells of various species of teleost and elasmobranch fish (Hughes & Wright, 1970) and, more recently, mucus was found to be preserved on gills of rainbow trout using cryo-scanning electron microscopy (Lumsden & Ferguson, 1994) and Alcian blue staining with electron microscopy (Powell et al., 1992, 1994). However, these techniques were not considered cost-effective in terms of the effort they entailed relative to the gain.

Gill diseases, and diseases associated with gill damage, cause substantial losses in the aquaculture industry, not only through an increased mortality rate among fish but also through impaired growth and costs related to sanitisation and treatment measures. In particular, for farmed Atlantic salmon, *Salmo salar* L., amoebic gill disease (AGD) is recognised as one of the major disease threats. The aetiological agent is the amphizoic protozoan amoeba *Paramoeba perurans* (Young et al., 2007) and its pathology involves extensive epithelial hyperplasia of filaments and lamellae as well as mucous cell proliferation. Investigations into the pathogenesis of AGD, particularly in the early stages of the disease, can be hampered by loss of the mucous coat and its pathogen load during fixation (Zanin et al., 2016). Gross signs of the disease are raised, multifocal white muroid patches on the gills (Adams & Nowak, 2003). These patches are subsequently scored from 0-5 to indicate the fish infection level (Taylor et al., 2009).

In the present work, the technical development of mucus stabilisation through the optimisation of fixation methods has been investigated as a means for examining the relationship between

amoebae, gill mucous layer and the pathogenesis of early and late stages of AGD infection in Atlantic salmon. It is envisaged that such an approach may also be employed more widely for observing surface-associated pathogens in fish and their relationship with the mucous layer in other gill associated conditions where their presence and activities may be obscured due to a loss of mucus coating through use of generic fixation and processing techniques.

2. Materials and methods

Preparation of fixatives

Five fixatives were employed for the current study. Three of these were aqueous fixatives: 1) 10% neutral buffered formalin (NBF) (i.e. 4 g of Sodium dihydrogen phosphate, 6.5 g of Disodium hydrogen phosphate (anhydrous), 100 ml of Formaldehyde and 900 ml of distilled water); 2) a modified Davidson's solution (i.e. 30 parts 95% ethanol, 20 parts 37-40% formaldehyde, 10 parts glacial acetic acid and 30 parts phosphate buffered saline (PBS)); 3) a modified Davidson's solution (as above) with 2% (w/v) Alcian blue ((Sigma-Aldrich, UK). The two remaining fixatives were non-aqueous solutions: 4) methacarn solution (i.e. methanol-Carnoy's; 60% (v/v) dry methanol, 30% (v/v) chloroform, 10% (v/v) glacial acetic acid; 5) methacarn solution (as above) with 2% (w/v) Alcian blue. All fixatives were freshly prepared immediately before use.

Fish and sampling

Gill sampling for the present study was carried out as part of ongoing research into AGD in Atlantic salmon, *Salmo salar* L. at the Marine Environmental Research Laboratory (MERL), Institute of Aquaculture, Machrihanish, Scotland, UK, 55°4'N 5°7'W between May and July 2017. The challenge facility was supplied with flow-through, full strength (35 ‰) fresh seawater filtered at 100 µm. Fish were maintained under ambient temperature (min: 11 °C, max 13 °C) and fed with commercial salmon pellets equivalent to 1% of their body weight per day. Fish were directly observed a minimum of two times per day.

Gill samples were taken from six Atlantic salmon (167.7±21.4 g and 25.6±1.6 cm body weight and fork length, respectively) from amongst a population of stock fish held in a 13000 L tank. For sampling, fish were euthanised by lethal anaesthesia using MS-222 (100 mg/L) (Sigma Aldrich, UK) followed by destruction of the brain, according to Home Office Schedule 1 procedures. Gill pathology was visually assessed and scored for gill lesion severity according to Taylor et al. (2009). Examined fish were found to have a mean gill score of 0.5-1. The third and fourth left gill arches were carefully excised, briefly rinsed in PBS, cut into equal sized parts and fixed in each of the five fixatives described above.

In order to examine the relationship between amoebae and mucus during later stages of AGD infection, a further five fish (324.2±35.6 g and 30.5±12.2 cm body weight and fork length, respectively) were sampled from a 1 m diameter tank (400 L) at the termination of an AGD co-habitation challenge experiment. Fish were euthanised by Schedule 1 methods as described above and gills similarly visually assessed and scored for gill lesion severity. Gills from the

current study were found to have a mean gill score range of 2-3.5. For these fish, the entire second gill arch was removed and fixed in methacarn solution (fixative 4).

Experimental procedures were all approved by the Animal Welfare and Ethical Review Body (AWERB) of the University of Stirling and were conducted under UK Government Home Office project licence 60/4189.

Sample processing and staining

Comparison of fixatives to preserve mucus on gills

After 48 h in their respective fixatives, gill tissues in the aqueous-based fixatives were dehydrated using conventional methods (Thermo Shandon Citadel 2000), embedded in paraffin wax (Histowax, Sweden or Q-Path, France) and sectioned at 5 μ m. Tissues in the solvent-based methacarn solution were processed manually, i.e. washed twice in 100% methanol (30 min), twice in 100% ethanol (20 min) then cleared with two washes in xylene (15 min), impregnated with paraffin wax, and sagittally and transversally sectioned at 5 μ m.

All gill sections where fixatives did not contain Alcian blue were stained using a combined Alcian blue (pH 2.5) and Periodic acid Schiff (PAS) technique according to Mowry (1956), with modifications described in Chalmers et al. (2017). Briefly, sections were de-waxed, rehydrated and immersed in Alcian blue solution (pH 2.5) for 5 min. The residual stain was then removed by washing in water and sections were oxidised in 1% (aq) periodic acid (5 min), washed (5 min) and immersed in Schiff's reagent (20 min). Gill tissue where fixative already contained Alcian blue, i.e. modified Davidson's and methacarn solution with 2% Alcian blue were stained as above, omitting the initial immersion in Alcian blue. Finally, all sections were washed in running tap water (10 min) and counterstained with haematoxylin Z (2 min) before being washed, dehydrated, cleared and mounted. Sections were scanned using an Axio Scan.Z1 slide scanner (ZEISS, Cambridge, UK).

Mucus and mucous cell quantification

Slides generated from all fixed material were assessed to quantify mucus and mucous cells. The mucus was not present as a uniform layer over the epithelium of the lamellae. Therefore, quantification of mucus was achieved through microscopic image acquisition of areas (~1 mm²) of well-preserved gill tissue, counting the number of times mucus traces were not present (Fig. 1-A) or present (Fig. 1-B) or in twelve randomised fields of view of twenty inter-secondary lamellar spaces in the mid-section of the primary lamella (n=6 control fish).

For the quantification of mucous cells the same approach was developed, excluding the fixation solutions with Alcian blue i.e. fixatives 3 & 5. These fixations didn't facilitate the differentiation of mucous cells from other types of cells because all slides presented a generalised blue coloration.

FIG. 1.

Lectin histochemistry

Gill sections from AGD-infected fish were used to characterise glycoproteins and mucins in mucous cells (goblet cells) and epithelial mucus covering by labelling their carbohydrate moieties. To this end, wheat germ agglutinin; *Triticum vulgaris* agglutinin (WGA) was applied to sections for fluorescence microscopic analysis based on its binding affinity to mucopolysaccharides.

Paraffin wax sections (5 µm) were mounted on treated Superfrost® Plus glass slides (Thermo Scientific, UK). Sections were deparaffinised with two changes of xylene (3 min each), hydrated in 100% and 70% ethanol (2 min each), followed by a wash in distilled water (DW) (1 min) and incubated with a rhodamine labelled lectin *Triticum vulgaris* agglutinin (WGA) (Vector laboratory, USA) at 30 mg mL⁻¹ diluted in lectin wash buffer (LWB; 50 mM Tris, 150 mM NaCl, 2 mM MgCl₂), 1 mM CaCl₂). Sections were incubated for 2 h in the dark at room temperature, washed three times with LWB (5 min) and mounted with Vectashield mounting medium with DAPI (Vector, USA). A control was included where lectin solution was replaced with LWB.

Images were captured with Arcturus XT Laser Capture Microdissection System (Applied Biosystems, Life technologies, USA).

Results

Comparison of fixatives to preserve mucus on gills

Overall both the aqueous and the solvent-based fixatives resulted in good maintenance of gill architecture (Fig. 2). The presence of a mucus coating or secretions from mucous cells was not evident in the branchial tissue fixed in neutral buffered formalin (NBF) (Fig. 2 A & B). There was, however, some evidence of patchy/diffuse and weakly stained interlamellar mucus in gills fixed with modified Davidson's solution (Fig. 2 C-F), this being slightly more extensive in tissues fixed with modified Davidson's solution with 2% (w/v) Alcian blue, where some apparent secretions from the mucous cells were preserved (Fig. 2 E & F).

With the non-aqueous based fixatives an improved stabilisation/preservation of mucus was clearly evident; branchial tissue fixed in methacarn solution displayed mucus as a thin attached layer on both interlamellar spaces and on secondary lamellae with mucus extending from mucous cells to form a 'mesh' between the secondary lamellae (Fig. 2 G & H) which can also be seen in transverse sections (Fig. 3 A & B). Fixation in methacarn solution with 2% (w/v) Alcian blue did not improve preservation of mucus, and the mucus layer was patchy and seemed to lift

from the underlying tissue, forming more compact streaks of dark blue stained mucus between the secondary lamellae (Fig. 2 I & J).

Quantitative mucus analysis demonstrated that the preservation of the mucus was significantly different between the different fixatives (Fig. 4). The apparent preservation of mucus was significantly higher when Methacarn solution and Methacarn solution with 2% (w/v) Alcian blue were used ($p=0.0010053$, ANOVA) in comparison to the aqueous fixatives; however, the differences between Methacarn solution and Methacarn solution with 2% (w/v) Alcian blue were not significant ($p=0.899$, Tukey Post-Hoc).

Mucous cell counts remained stable across the different fixation solutions ($p=0.899$, Tukey Post-Hoc), although the Alcian blue fixatives did not enable mucous cells and other types of cells to be distinguished from one another due to the total blue coloration of the tissue.

FIG. 2, FIG. 3 and FIG. 4

Examination of the relationship between amoebae and mucus during early stages of AGD infection

Sections were stained with H&E (Fig. 5 A&C) and AB/PAS (Fig. 5 B&D). Using the AB/PAS stain helped to differentiate between acid and neutral polysaccharides (Fig. 5 B&D), highlighted amoebae with Alcian blue inclusions, and allowed observation of the preserved mucus (Fig. 5 B&D). Also, early hyperplastic lesions were visible with both stains, in addition to the formation of lacunae or interlamellar vesicles (ilv) (Fig 5. A&B). Amoebae sometimes appeared to be enclosed within the gill epithelium although this might be an artefact of slimmed section thickness (Fig. 5 D).

Amoebae is found imbedded within the mucus, suggesting an association between the parasite and mucus, followed by hyperplastic tissue (Fig.6 A&B) caused by the presence of the parasite in the gill epithelium as observed in figure 6 D. The presence of a single amoebae was also showed enclosed in a newly formed vesicle within two lamellae that seemed to be attached together by mucus (Fig. 6 D).

FIG. 5, 6

Hyperplastic lesions associated with amoebic gill disease, were clearly visible with lamellar fusion causing lacunae formation within which are amoebae are embedded within (Fig. 7 B&C). Also a transverse section of the gill shows another lacuna formation and the presence of mucus with amoebae once again embedded into it (Fig. 7 D).

FIG. 7

Confirmation of mucus preservation using lectin histochemistry

Mucus preservation was confirmed by using the wheat germ agglutinin (WGA) lectin labelling on unaffected gill tissues (Fig.8) and AGD-affected gills (Fig. 8). A negative control confirmed that the lectin buffer without containing the lectin did not stain the mucous cells and mucus overlay (Fig. 8 A). Different filters were used for visualising the lectin labelling (Fig. 8 B, C, D, E & F).

Regarding the investigation of how the mucus interacts with the amoebae, some hyperplastic lesions could showed lectin labelling (Fig. 10). Also, mucus was well-preserved throughout the gill tissue (Fig. 10 B, C & D). Lacunae formation could be seen again, with (Fig. 10 A) and without (Fig. 10 D) enclosed amoebae. As a double confirmation, we did an additional negative control with the lectin labelling with tissues fixed in 10% NBF solution. As shown in the figure 9, mucus is not as present as when methacarn solution is used as fixative. Also the little presence of some mucus remains presents low fluorescence (figure 9, arrows).

FIG. 8.

FIG. 9.

FIG.10

Discussion

The present work sought to determine the optimal fixative for preserving both gill structure and aspects of the mucous cells and mucus layer overlying the gill epithelium. Having established an optimum fixative it then sought to examine whether histological/histochemical observation of tissue fixed to retain mucus could provide insights into pathogenesis of AGD.

In the current study, the different aqueous and solvent-based fixatives were all successful in preserving the gill structure. While the aqueous fixatives provided good cytological preservation, the mucus overlying the gill epithelium was lost following fixation. This was presumed to be due to loss of most of the proteoglycan content as reported by Toledo et al. (1996). The solvent-based fixatives, however, demonstrated a significant improvement in the preservation of mucus traces in the studied gill samples. Despite this, no preservation method employed in the current study gave rise to the appearance of a clear and uniform mucus layer as previously observed for rat gut (Sims et al., 1997), pig intestine (Allan-Wojtas et al., 1997), and, more recently, human intestine (Swidsinski et al., 2005). This suggests either that the mucus covering of the gills of Atlantic salmon is less uniformly structured or pronounced than that of mammalian gastric mucosae or that aspects of the sampling and fixation process still need to be optimised.

Several other studies have attempted to optimise mucus stabilisation in teleost skin and gill mucosae, e.g. the oesophageal epithelium in the eel (*Anguilla anguilla* L.) (Humbert et al., 1984)

by implementing freeze-drying of samples; this translated in a improved fixation of mucous coating of the oesophagus. Additional studies, combined this regime of using cryopreservation for freeze-drying of the samples with vapour fixation using osmium tetroxide showing an improved preservation of the mucus coat in skin of rainbow trout (*Oncorhynchus mykiss* Walbaum) (Speare & Mirsalimi, 1992). Another study (Sanchez et al., 1997) also focused on the use of osmium tetroxide, found improved retention of skin's mucus coat using the non-aqueous solvent FC-72 containing 1% osmium tetroxide as compared to aqueous glutaraldehyde in the skin of rainbow trout using transmission electron microscopy in the latter study.

Combined with the previous techniques, some studies implemented the addition of Alcian blue in different fixatives, i.e. aqueous buffered glutaraldehyde (Sims et al., 1997) for the characterisation of the composition and thickness of tracheal mucus in rats. In fish, Alcian blue has also been used as an addition to routine fixatives for both light and electron microscopy in the gills of rainbow trout (Powell et al., 1992). Other non-fish studies, have used Alcian blue as a colorimetric assay for mucous glycoproteins (Hall et al., 1980) or for the characterisation of sialylated, sulphated and mixed mucins (Meyerholz et al., 2009).

Regarding the fixatives used during the present study, the modified Davidson's solution has been previously used for demonstration of *P. perurans* presence in infected gills (Cadoret et al., 2013), as well as for other tissues and species (Black, 1991; Latendresse, 2002). Although the modified Davidson's fixative used in the current study was useful for assessing the number of mucous cells and showing different types of mucous cells (acidic, neutral and basic) in the gill sections, it was found to be less successful in preserving the mucus coating of the epithelium.

Use of the methacarn solutions in the present study proved significantly more successful in stabilising the structure of the mucus layer and retaining it during subsequent processing as it has been proved in previous investigations involving gut and intestinal tissue in mammals (Johansson et al., 2008; Johansson & Hansson, 2012). Particularly, this fixation method has previously given positive results for the immunofluorescent imaging of mucins in pig gut (Earle et al., 2015) showing that there is a greater conservation of the mucus layer structure compared to traditional formaldehyde-based fixatives in which the mucus collapses.

Overall, the present results conclude that both methacarn solution and methacarn solution with 2% (w/v) Alcian blue enhanced preservation of mucus. One challenge that was encountered when quantifying the mucus was that it did not present as a uniform layer over the gill epithelium; therefore, the presence of mucus was determined by the enumeration of mucus traces that were still in contact with the originating mucous cells or were fixed in situ across the gill epithelium.

The lectin-binding study confirmed the fixation results, indicating that the apparent mucus observed using basic histological techniques was indeed mucus or mucin-like glycoproteins. This was achieved by use of WGA (*Triticum vulgaris* (wheat germ)) lectin, which is one of the best studied plant lectins and specifically targets glycoproteins (GlcNAc, its β -(1,4)-oligomers, and N-acetyl neuraminic acid). Its specificity of GlcNAc-carrying ligands for WGA has been investigated through fluorescence methods which were applied to study the interactions of

carbohydrate-binding lectins with glycopolymers, where clustering glycopolymers were shown to induce a much enhanced binding affinity compared to the corresponding mono- and oligosaccharides (Nishimura et al., 1994). Therefore some investigations (Fischer et al., 1984; Madrid et al., 1989; Ferri & Liquori, 1992; Coet-Zee et al., 1995) hypothesised the possibility of this lectin binding to mucopolysaccharides found within the mucus and mucosal cells. They described lectin-binding in goblet cells of both the small and large intestines of animals belonging to at least five different classes of vertebrates studied, i.e. sea bream, frog, tortoise, chicken, rat, hamster, elephant, monkey and human. Regarding fish, the WGA lectin has been used in several studies, including examination of bony fish olfactory epithelium mucus (Wolfe et al., 1998; Ferrando et al., 2006), skin mucus (Guardiola et al., 2014) and N-acetylglucosamine and acetylneuraminic acid residues in the gill epithelium of Argentinean silverside *Odontesthes bonariensis* (Valenciennes, 1835) (Teleostei, Atherinopsidae) (Díaz et al., 2010).

Unsurprisingly, observation of AGD-affected gill tissue in this study demonstrated the presence of amoebae closely associated with the gill epithelium. However, using the mucus-targeted fixation approaches explored and optimised in this study, amoebae were also observed within the retained mucus layers that would normally be lost during standard fixation. Observed pathology was characterized by hyperplasia and hypertrophy, inducing lamellar fusion and the consequent emergence of apparent lacunae or vesicles in the gill lamellae with associated amoebae, as previously observed by other authors (Munday et al., 2001; Adams & Nowak, 2001; Chalmers et al., 2017). Along with these formations, amoebae are found embedded within the mucus which acts as an essential first host barrier against them and prevents to some degree pathogen invasion and subsequent infection. The ability to observe mucus presence and distribution provides considerable scope for improving understanding of the relationship between amoebae, the salmon host and pathogenesis in AGD. Preservation and labelling of mucus in histological sections also allows direct observation / confirmation of levels of mucus production and of adherence of mucus to gills, which may also reflect changes in mucus composition and function.

Teleost mucus plays a protective role by inhibiting pathogen binding, but also by acting as a vehicle for mucins and humoral immune factors. As explained earlier, mucus contains high molecular weight glycoproteins that can potentially trap pathogens, acting as a physical barrier (Johansson & Hansson, 2016). Many studies have verified this statement by researching the relationship between pathogens, mucus and mucins. A study by Nagashima et al. (2003) indicated that some pathogenic bacteria could be found attached to the mucus layer and develop biofilms to protect themselves against the host mucosal immunity. To escape the challenge provided by build-up of biota within the mucus, healthy fish continuously discharge and replace their mucus layer preventing the stable colonization of potential infectious microorganisms as well as invasion of metazoan parasites. Other studies have pointed out that pathogenic microorganisms, such as some *Vibrio* strains, are capable of utilising mucus as a carbon source, helping the colonisation of these pathogens and eventually supporting the initiation of infection in fish (Bordas et al., 1996). More recently, study of immunological responses within the gill has highlighted the potential role of secreted IgT responses, in part delivered through mucus, as well as gene expression reflecting production of other defensins carried in mucus and acting against gill pathogens (Xu et al., 2016; Brinchmann, 2016) and their correlated pathology (Hishida et al.,

1997; Benhamed et al., 2014). Additionally, mucins have been investigated as reliable markers of prognostic and diagnostic value of fish intestinal health (Estensoro et al., 2013).

In conclusion, the current study has explored a number of mucus fixation approaches in the context of studying AGD in Atlantic salmon and has identified an optimal protocol involving methacarn fixation. The study has also demonstrated the utility of taking such deliberate steps to preserve mucus integrity and provides evidence that retention of mucus, particularly in the context of gill diseases, such as AGD or complex gill disease, can provide useful data that would be lost under normal fixation and processing procedures.

viii. References

Adams, M. B., & Nowak, B. F. (2001). Distribution and structure of lesions in the gills of Atlantic salmon, *Salmo salar* L., affected with amoebic gill disease. *Journal of Fish Diseases*, 24(9), 535-542. doi:10.1046/j.1365-2761.2001.00330.x

Adams, M. B., & Nowak, B. F. (2003). Amoebic gill disease: sequential pathology in cultured Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases*, 26(10), 601-614. doi: 10.1046/j.1365-2761.2003.00496.x

Allan-Wojtas, P., Farnworth, E. R., Modler, H. W., & Carbyn, S. (1997). A solvent-based fixative for electron microscopy to improve retention and visualization of the intestinal mucus blanket for probiotics studies. *Microscopy Research and Technique*, 36(5), 390-399. doi:10.1002/(sici)1097-0029(19970301)36:5<390::aid-jemt9>3.0.co;2-p

Benhamed, S., Guardiola, F. A., Mars, M., & Esteban, M. Á. (2014). Pathogen bacteria adhesion to skin mucus of fishes. *Veterinary microbiology*, 171(1-2), 1-12. doi: 10.1016/j.vetmic.2014.03.008

Black, E. A., Whyth, J. N. C., Bagshaw, J. W., & Ginther, N. G. (1991). The effects of *Heterosigma akashiwo* on juvenile *Oncorhynchus tshawytscha* and its implications for fish culture. *Journal of applied ichthyology*, 7(3), 168-175. doi.org/10.1111/j.1439-0426.1991.tb00523.x

Bollard, J. E., Vanderwee, M. A., Smith, G. W., Tasman-Jones, C., Gavin, J. B., & Lee, S. P. (1986). Preservation of mucus in situ in rat colon. *Digestive diseases and sciences*, 31(12), 1338-1344. doi: 10.1007/bf01299812

Bordas, M. A., Balebona, M. C., Zorrilla, I., Borrego, J. J., & Morin, M. A. (1996). Kinetics of Adhesion of Selected Fish-Pathogenic *Vibrio* Strains to Skin Mucus of Gilt-Head Sea Bream (*Sparus aurata* L.). *Applied and Environmental Microbiology* 62(10), 3650-3654.

Brinchmann, M. F. (2016). Immune relevant molecules identified in the skin mucus of fish using-omics technologies. *Molecular BioSystems*, 12(7), 2056-2063. doi: 10.1039/c5mb00890e

- 381 Cadoret, K., Bridle, a. R., Leef, M. J., & Nowak, B. F. (2013). Evaluation of fixation methods
382 for demonstration of *Neoparamoeba perurans* infection in Atlantic salmon, *Salmo salar* L., gills.
383 *Journal of Fish Diseases*, 36(10), 831–839. doi: 10.1111/jfd.12078
- 384 Caceci, T. (1984). Scanning electron microscopy of goldfish, *Carassius auratus*, intestinal
385 mucosa. *Journal of Fish Biology*, 25(1), 1-12.
- 386 Coet-Zee, H. L., Kotze, S. H., & Lourens, N. (1995). Characterization of mucus glycoproteins in
387 the intestinal mucosa of the African elephant (*Loxodonta africana*) following lectin
388 histochemistry. *Onderstepoort Journal of Veterinary Research*, 62, 187-192.
- 389 Cole, A. M., Darouiche, R. O., Legarda, D., Connell, N., & Diamond, G. (2000).
390 Characterization of a fish antimicrobial peptide: gene expression, subcellular localization, and
391 spectrum of activity. *Antimicrob. Agents Chemother.* 44, 2039-2045
- 392 Costa, M. M., Maehr, T., Diaz-Rosales, P., Secombes, C. J., & Wang, T. (2011). Bioactivity
393 studies of rainbow trout (*Oncorhynchus mykiss*) interleukin-6: effects on macrophage growth
394 and antimicrobial peptide gene expression. *Molecular immunology*, 48(15-16), 1903-1916.
395 doi:10.1016/j.molimm.2011.05.027
- 396 Culling, C. F. A. (2013). Handbook of histopathological and histochemical techniques: including
397 museum techniques. Butterworth-Heinemann, 29-30. doi: 10.1016/c2013-0-04011-x.
- 398 Díaz, A. O., García, A. M., & Goldemberg, A. L. (2010). Glycoproteins histochemistry of the
399 gills of *Odontesthes bonariensis* (Teleostei, Atherinopsidae). *Journal of Fish Biology* 77, 1665-
400 1673. doi:10.1111/j.1095-8649.2010.02803.x
- 401 Earle, K. A., Billings, G., Sigal, M., Lichtman, J. S., Hansson, G. C., Elias, J. E., & Sonnenburg,
402 J. L. (2015). Quantitative imaging of gut microbiota spatial organization. *Cell host & microbe*,
403 18(4), 478-488. doi: 10.1016/j.chom.2015.09.002
- 404 Estensoro, I., Caldach-giner, J. A., Pe, J., Kaushik, S., & Sitja, A. (2013). Mucins as Diagnostic
405 and Prognostic Biomarkers in a Fish-Parasite Model . *Transcriptional and Functional Analysis*,
406 8(6), 1–9. doi: 10.1371/journal.pone.0065457
- 407 Ferrando, S., Bottaro, M., Gallus, L., Girosi, L., Vacchi, M., & Tagliafierro, G. (2006).
408 Observations of crypt neuron-like cells in the olfactory epithelium of a cartilaginous fish.
409 *Neuroscience letters*, 403(3), 280-282. doi: 10.1016/j.neulet.2006.04.056
- 410 Ferri, D. & Liquori, G.E. 1992. Characterization of secretory cell glycoconjugates in the
411 alimentary tract of the ruin lizard (*Podarcis sicula*) by means of lectin histochemistry. *Acta*
412 *Histochemica*, 93:341-349. doi: 10.1111/j.1463-6395.2010.00451.x
- 413 Fischer, J., Klein, P.J., Vierbuch EN, M., Skutta, B., Uhlenbruck, G. & Fischer, R. (1984).
414 Characterization of glycoconjugates of human gastrointestinal mucosa by lectins. I.
415 Histochemical distribution of lectin binding sites in normal alimentary tract as well as in benign
416 and malignant gastric neoplasms. *Journal of Histochemistry and Cytochemistry*, 32, 681- 689.
417 doi: 10.1177/32.7.6330198

- 418 Geiser, M., Im Hof, V., Siegenthaler, W., Grunder, R., & Gehr, P. (1997). Ultrastructure of the
419 aqueous lining layer in hamster airways: Is there a two-phase system?. *Microscopy research and*
420 *technique*, 36(5), 428-437.
- 421 Guardiola, F. A., Cuesta, A., Abellán, E., Meseguer, J., & Esteban, M. A. (2014). Comparative
422 analysis of the humoral immunity of skin mucus from several marine teleost fish. *Fish &*
423 *shellfish immunology*, 40(1), 24-31. doi: 10.1016/j.fsi.2014.06.018
- 424 Hall, R. L., Miller, R. J., Peatfield, A. C., Richardson, P. S., Williams, I., & Lampert, I. (1980).
425 A colorimetric assay for mucous glycoproteins using Alcian blue. *Biochemical Society*
426 *Transactions*, 8(1), 72.
- 427 Handy, R. D., & Eddy, F. B. (1990). The interactions between the surface of rainbow trout,
428 *Oncorhynchus mykiss*, and waterborne metal toxicants. *Functional Ecology*, 4 (3), 385-392.
- 429 Hishida, Y., Ishimatsu, A., & Oda, T. (1997). Mucus blockade of lamellar water channels in
430 yellowtail exposed to *Chattonella marina*. *Fisheries science*, 63(2), 315-316. doi:
431 10.2331/fishsci.63.315
- 432 Horne, M. M., & Sims, D. E. (1998). Preliminary ultrastructural studies of the surface mucus of
433 Atlantic salmon. *Bulletin of the Aquaculture Association of Canada*, (2), 85-86.
- 434 Hughes, G. M., & Wright, D. E. (1970). A comparative study of the ultrastructure of the water-
435 blood pathway in the secondary lamellae of teleost and elasmobranch fishes—benthic forms.
436 *Zeitschrift für Zellforschung und mikroskopische Anatomie*, 104(4), 478-493.
- 437 Hulbert, W. C., Forster, B. B., Laird, W., Pihl, C. E., & Walker, D. C. (1982). An improved
438 method for fixation of the respiratory epithelial surface with the mucous and surfactant layers.
439 *Laboratory investigation: a journal of technical methods and pathology*, 47(4), 354-363.
- 440 Humbert, W., Kirsch, R. and Meister, M.F., 1984. Scanning electron microscopic study of the
441 oesophageal mucous layer in the eel, *Anguilla anguilla* L. *Journal of Fish Biology*, 25(1),
442 pp.117-122. doi: 10.1111/j.1095-8649.1984.tb04856.x
- 443 Johansson, M. E. V., Phillipson, M., Petersson, J., Velcich, A., Holm, L., & Hansson, G. C.
444 (2008). The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria.
445 *Proceedings of the National Academy of Sciences of the United States of America*, 105(39),
446 15064–15069. doi: 10.1073/pnas.0803124105
- 447 Johansson, M. E., & Hansson, G. C. (2012). Preservation of mucus in histological sections,
448 immunostaining of mucins in fixed tissue, and localization of bacteria with FISH. *Methods in*
449 *Molecular Biology*, 842, 229-35. doi: 10.1007/978-1-61779-513-8_13
- 450 Latendresse, J. R., Warbritton, A. R., Jonassen, H., & Creasy, D. M. (2002). Fixation of testes
451 and eyes using a modified Davidson's fluid: comparison with Bouin's fluid and conventional
452 Davidson's fluid. *Toxicologic pathology*, 30(4), 524-533. doi: 10.1080/01926230290105721

- 453 Lee, M. M., Schurch, S., Roth, S. H., Jiang, X., Cheng, S., Bjarnason, S., & Green, F. H. Y.
454 (1995). Effects of acid aerosol exposure on the surface properties of airway mucus. *Experimental*
455 *lung research*, 21(6), 835-851. doi: 10.3109/01902149509031766
- 456 Leist, D. P., G. S. Nettleton, and R. C. Feldhoff (1986). Determination of lipid loss during
457 aqueous and phase partition fixation using formalin and glutaraldehyde. *Journal of*
458 *Histochemistry & Cytochemistry*. 34: 437-441. doi: 10.1177/34.4.3081623
- 459 Lumsden, J. S., & Ferguson, H. W. (1994). Isolation and partial characterization of rainbow trout
460 (*Oncorhynchus mykiss*) gill mucin. *Fish physiology and biochemistry*, 12(5), 387-398.
- 461 Madrid, J.F., Ballesta, J., Castells, M.T., Marin, J.A. & Pastor, L.M. 1989. Characterization of
462 glycoconjugates in the intestinal mucosa of vertebrates by means of lectin histochemistry. *Acta*
463 *histochemica et cytochemica*, 22, 1-14. doi: 10.1267/ahc.22.1
- 464 Mays, E. T., R. C. Feldhoff, and G. S Nettleton (1984). Determination of protein loss during
465 aqueous and phase partition fixation using formalin and glutaraldehyde. *Journal of*
466 *Histochemistry & Cytochemistry*. 32: 1107-1127. doi: 10.1152/ajplung.1997.273.5.11036
- 467 Meyerholz, D. K., Rodgers, J., Castilow, E. M., & Varga, S. M. (2009). Alcian Blue and
468 Pyronine Y histochemical stains permit assessment of multiple parameters in pulmonary disease
469 models. *Veterinary Pathology*, 46(2), 325-328. doi: 10.1354/vp.46-2-325
- 470 Mowry, R. W. (1956). Alcian blue techniques for the histochemical study of acidic
471 carbohydrates. *Journal of Histochemistry and Cytochemistry*, 4, 403-407.
- 472
- 473 Munday, B. L., Zilberg, D., & Findlay, V. (2001). Gill disease of marine fish caused by infection
474 with *Neoparamoeba pemaquidensis*. *Journal of Fish Diseases*, 24(9), 497-507. doi:
475 10.1046/j.1365-2761.2001.00329.x
- 476 Murray, C.K. and Fletcher, T.C., (1976). The immunohistochemical localization of lysozyme in
477 plaice (*Pleuronectes platessa* L.) tissues. *Journal of Fish Biology*, 9: 329-334.
- 478 Nagashima, Y., Kikuchi, N., Shimakura, & K., Shiomi, K., (2003). Purification and
479 characterization of an antibacterial factor in the skin secretion of rock fish *Sebastes schlegeli*.
480 *Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology*, 136, 63-71.
481 doi: 10.1016/S1532-0456(03)00174-1
- 482 Nichols, B. A., Chiappino, M. L., & Dawson, C. R. (1985). Demonstration of the mucous layer
483 of the tear film by electron microscopy. *Investigative ophthalmology & visual science*, 26(4),
484 464-473.
- 485 Nishimura, S. I., Furuike, T., Matsuoka, K., Maruyama, K., Nagata, K., Kurita, K., & Tokura, S.
486 (1994). Synthetic Glycoconjugates. 4. Use of omega-(Acrylamido) alkyl Glycosides for the
487 Preparation of Cluster Glycopolymers. *Macromolecules*, 27(18), 4876-4880.

- Ota, H., & Katsuyama, T. (1992). Alternating laminated array of two types of mucin in the human gastric surface mucous layer. *The Histochemical journal*, 24(2), 86-92. doi: 10.1007/bf01082444
- Powell, M. D., Speare, D. J., & Burka, J. F. (1992). Fixation of mucus on rainbow trout (*Oncorhynchus mykiss* Walbaum) gills for light and electron microscopy. *Journal of Fish Biology*, 41, 813–824. doi: 10.1111/j.1095-8649.1992.tb02709.x
- Powell, M. D., Speare, D. J., & Wright, G. M. (1994). Comparative ultrastructural morphology of lamellar epithelial, chloride and mucous cell glycocalyx of the rainbow trout (*Oncorhynchus mykiss*) gill. *Journal of Fish Biology*, 44(4), 725-730. doi: 10.1111/j.1095-8649.1994.tb01248.x
- Sanchez, J. G., Speare, D. J., Sims, D. E., & Johnson, G. J. (1997). Adaptation of a fluorocarbon-based non-aqueous fixation regime for the ultrastructural study of the teleost epithelial mucous coat. *Journal of comparative pathology*, 117(2), 165-170. doi: 10.1016/S0021-9975(97)80033-3
- Shephard K.L. (1994): Functions for fish mucus. *Reviews in Fish Biology Fisheries* 4, 401–429.
- Sims, D. E., Horne, M. M., Edward, P., Island, P. E., Ca, C., David, E., & Heterogeneity, M. M. H. (1997). Heterogeneity of the composition and thickness of tracheal mucus in rats. *American Journal of Physiology*, 273(5 Pt 1), 1036-1041.
- Sims, D. E., Westfall, J. A., Kiorpes, A. L., & Home, M. M. (1991). Preservation of tracheal mucus by nonaqueous fixative. *Biotechnic & histochemistry*, 66(4), 173-180.
- Swidsinski, A., Weber, J., Loening-baucke, V., Hale, L. P., & Lochs, H. (2005). Spatial Organization and Composition of the Mucosal Flora in Patients with Inflammatory Bowel Disease. *Journal of Clinical Microbiology*, 43(7), 3380–3389. doi: 10.1128/JCM.43.7.3380
- Taylor, R. S., Muller, W. J., Cook, M. T., Kube, P. D., & Elliott, N. G. (2009). Gill observations in Atlantic salmon (*Salmo salar*, L.) during repeated amoebic gill disease (AGD) field exposure and survival challenge. *Aquaculture*, 290(1-2), 1-8. doi: 10.1016/j.aquaculture.2009.01.030
- Toledo, O. M. S., Taniwaki, N. N., Saldiva, P. H., & Montes, G. S. (1996). Effect of aqueous and nonaqueous fixatives on the quantitative estimation of collagen-proteoglycan interaction in tissue sections. *Biotechnic & histochemistry*, 71(3), 109-114.
- Wolfe, K. G., Plumb, J. A., & Morrison, E. E. (1998). Lectin binding characteristics of the olfactory mucosa of channel catfish: potential factors in attachment of *Edwardsiella ictaluri*. *Journal of Aquatic Animal Health*, 10(4), 348-360. doi: 10.1577/1548-8667(1998)010<0348:lbcoto>2.0.co;2
- Xu Z., Parra D., Gomez D., Salinas I., Zhang Y.-A., Jørgensen L.v.G., Heinecke R.D., Buchmann K., LaPatra S. & Sunyer J.O. (2013) Teleost skin, an ancient mucosal surface that elicits gut-like immune responses. *Proceedings of the National Academy of Sciences of the United States of America*, 110, 13097–13102.
- Xu, Z., Takizawa, F., Parra, D., Gómez, D., von Gersdorff Jørgensen, L., LaPatra, S. E., & Sunyer, J. O. (2016). Mucosal immunoglobulins at respiratory surfaces mark an ancient

association that predates the emergence of tetrapods. *Nature communications*, 7, 10728. doi: 10.1038/ncomms10728

Zanin, M., Baviskar, P., Webster, R., & Webby, R. (2016). The interaction between respiratory pathogens and mucus. *Cell host & microbe*, 19(2), 159-168.

Figure 1: Semi-quantitative analysis method for mucus and mucous cells quantification. Mucus was quantified by counting the absence (A) or presence (B, arrows) of mucus traces (blue) in twenty inter-lamellar spaces from twelve random mid-sections of the primary lamellae. This method was used for all the fixation and staining techniques (e.g. A. NBF fixation with AB/PAS staining. B. Methacarn fixation with AB/PAS staining). For the mucous cell counts, the same method was performed by counting the presence (asterisk) or absence of mucous cells in twenty inter-lamellar spaces from twelve random mid-sections of the primary lamellae. Images taken by laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

Fig. 2: Evaluation of aqueous-based and solvent-based fixatives to preserve mucus layer in Atlantic salmon gills: A) lower magnification and B) higher magnification of gill sample fixed with 10% neutral buffered formalin (10% NBF), stained with Alcian blue and Periodic acid-Schiff's reagent (AB/PAS). Note that there is no evidence of overlying mucus on epithelial layers or associated secretions from mucous cells (black arrow); C) lower magnification and D) higher magnification of gill sample fixed with modified Davidson's solution, stained with Alcian blue and Periodic acid-Schiff reagent (AB/PAS). There is some evidence of patchy preservation of mucus between the secondary lamellae (white arrows) with some mucus secretions from mucous cells (black arrows); E) lower magnification and F) higher magnification of gill sample fixed with Modified Davidson's and 2% Alcian blue solution stained with Periodic acid-Schiff's reagent (PAS). Note increased amount of mucus evident between lamellae (white arrows) and some mucus secretions from mucous cells (black arrows); G) lower magnification and H) higher magnification of gill sample fixed with Methacarn solution stained with Alcian blue and Periodic acid-Schiff's reagent (AB/PAS) showing presence of mucus as a thin attached layer on both interlamellar spaces (white arrows) and on secondary lamellae (black arrows). Note in H) evidence of preservation of mucus being secreted from mucous cells (short arrows). I) Lower magnification and J) higher magnification of gill sample fixed with Methacarn and 2% Alcian blue solution, stained with Periodic acid-Schiff's reagent (PAS). Evidence of mucus as a thin attached layer on interlamellar spaces (white arrows) and also presence of mucous cells (black arrows). Images taken by laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

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564 Fig. 3: Transverse sections of methacarn fixed AGD-affected gill tissue stained with Alcian blue
565 and Periodic acid-Schiff reagent (AB/PAS): A) section of gill from Atlantic salmon with gross
566 gill score of 2.5 (showing unaffected area with interlamellar mucus B) higher magnification of
567 boxed area from picture A) with mucous cells (black arrows) and mucus layer (brown arrows).
568 Images taken by laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

569

570 Figure 4. (a) Proportion of examined interlamellar spaces showing the presence of mucus layer
571 for the different fixatives. Methacarn solution and methacarn solution with Alcian blue fixation
572 methods preserve greater amount of mucus. Significant differences between fixatives are denoted
573 with letters (i.e. different letters represent statistical differences, whilst same letters express no
574 differences). Bars indicate mean values; error bars express standard error of the mean (s.e.m). (n
575 = 6 control fish; 6 random fields of 20 interlamellar spaces; ANOVA test: $p < 0.01$)(b)
576 Comparison of mucous cell counts across the different fixatives. The number of mucous cells
577 show no variation with the use of the different fixatives. Bars indicate mean values; error bars
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580

581 Figure 5: Comparison of histological stains of methacarn fixed Atlantic salmon gill tissue
582 affected by AGD with H&E and AB/PAS staining. A) & B) early hyperplastic lesions with
583 interlamellar vesicles (ilv) from gill tissue of Atlantic salmon with gill score 2.5 stained with A)
584 routine H&E stain and B) Alcian blue and Periodic acid-Schiff reagent (AB/PAS). C) & D)
585 Advanced hyperplastic lesions with associated *Paramoeba* spp. trophozoites (arrows) stained
586 with C) routine H&E stain and D) Alcian blue and Periodic acid-Schiff reagent (AB/PAS).
587 *Paramoeba* sp. trophozoites (arrows) encapsulated in an interlamellar vesicle (ilv) within
588 hyperplastic lamellae. Images taken by laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

589

590 Figure 6: Gill tissue of Atlantic salmon during early stages of infection with AGD fixed in
591 methacarn and stained with AB/PAS. A) & B) Hyperplastic AGD gill tissue with mucous cells
592 and mucus throughout (asterisks), in addition to numerous intralesional trophozoites of
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594 overlaying mucus (asterisk) C) trophozoites are found attached to the gill epithelium (black
595 arrows) and a mucous cell (brown arrow) D) trophozoite trapped in newly formed interlamellar
596 vesicle (black arrow) surrounded by mucus and mucous cells (brown arrows). Images taken by
597 laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

598

599 Figure 7: Gill tissue of Atlantic salmon during early stages of infection with AGD fixed in
600 methacarn and stained with AB/PAS. A) Formation of rounded interlamellar vesicle (ilv) across

the hyperplastic AGD- affected gill tissue with mucous cells (arrows) and trophozoites of *Paramoeba* spp. (asterisks) B) higher magnification of the formation of interlamellar vesicle (ilv) C) trophozoite trapped in newly formed vesicle in hyperplastic AGD-affected gill tissue D) transverse section of AGD-affected gill with ilv and amoebae attached to the epithelium (arrows) surrounded by mucus layer (brown arrows). Images taken by laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

Figure 8: Wheat germ agglutinin (WGA) lectin labelling of carbohydrates of gill tissue fixed with methacarn solution. A) Negative control for the lectin labelling with the lectin buffer. No mucus or mucous cells are presenting any fluorescence (big white arrows). B) D) & F) Gill tissue presenting mucus as an overlay on the epithelium; mucous cells are also shown in a bright orange colour (thin white arrows). All taken with a triple band fluorescence filter. C) & E) Additional images from the same section showing the mucus and mucous cells (thin white arrows) but with a blue band fluorescence filter. Images taken by laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

Figure 9: Wheat germ agglutinin (WGA) lectin labelling of carbohydrates of gill tissue fixed with 10% NBF solution. An additional negative control shows how the 10% NBF solution washes off the mucus and shows little fluorescence of the mucus remains (arrows). Images taken with a triple band fluorescence filter by laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

Figure 10: Wheat germ agglutinin (WGA) lectin labelling of carbohydrates of AGD-infected gill tissue fixed with methacarn solution and observation of *Paramoeba perurans* within. A) *Paramoeba* spp. trophozoite encapsulated inside interlamellar vesicle with DAPI-stained host and parasite nuclei (big white arrow). Thin arrows show mucus layer on gill epithelium B), C) & D) N-acetylglucosamine / N-acetyllactosamine carbohydrate labelling on transverse sections of gills with presence of mucus layer (thin white arrows) between hyperplastic AGD-affected lamellae (*). Images taken with a triple band fluorescence filter by laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

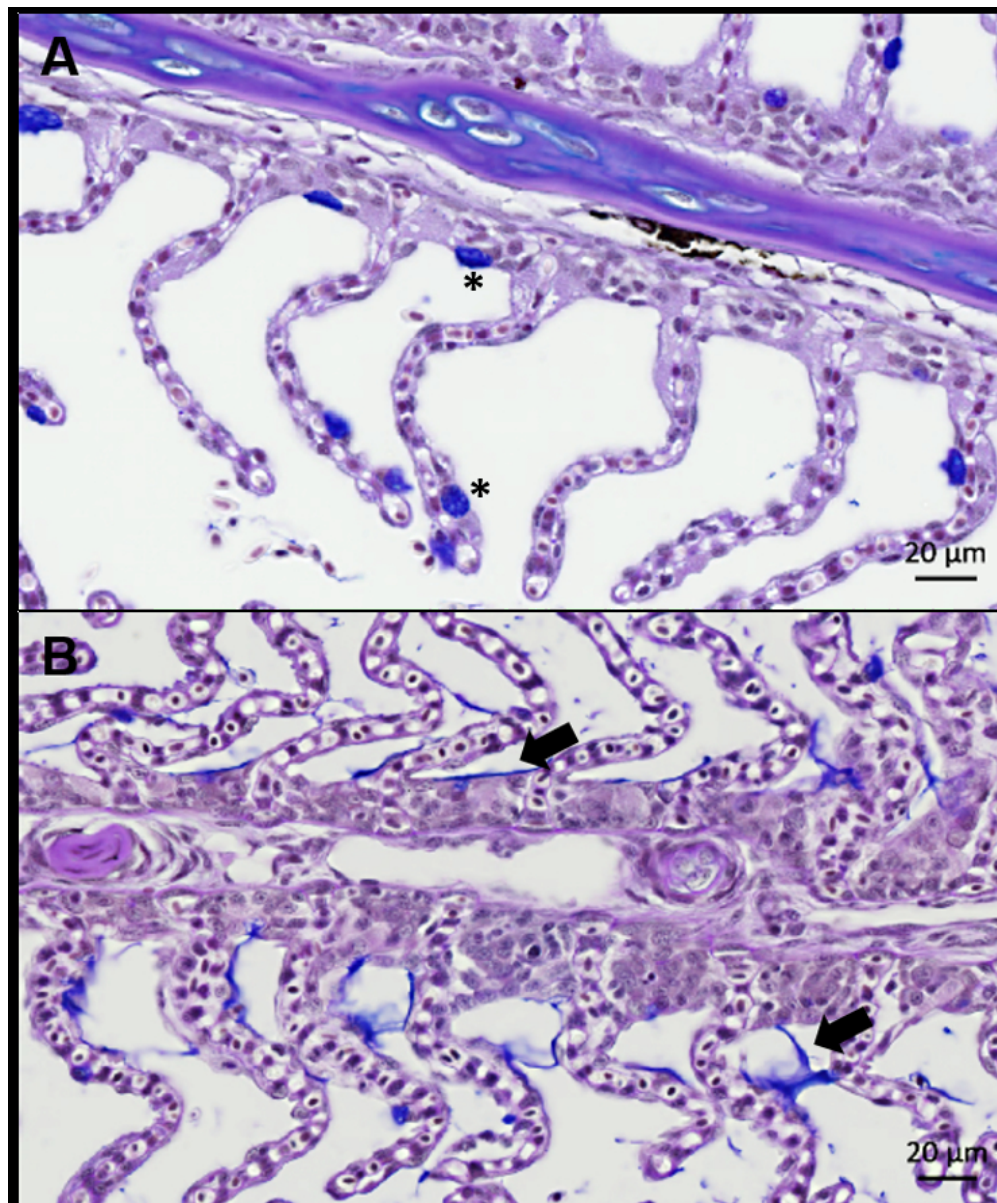


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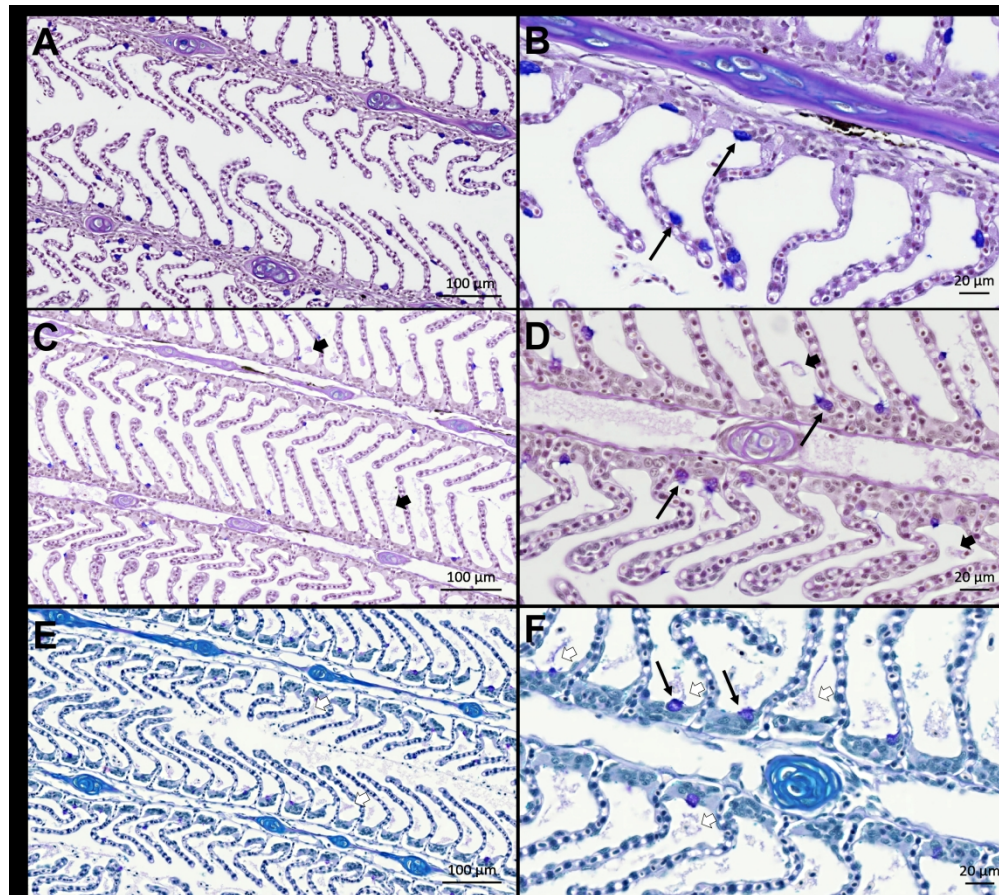


Fig. 2: Evaluation of aqueous-based and solvent-based fixatives to preserve mucus layer in Atlantic salmon gills: A) lower magnification and B) higher magnification of gill sample fixed with 10% neutral buffered formalin (10% NBF), stained with Alcian blue and Periodic acid-Schiff's reagent (AB/PAS). Note that there is no evidence of overlying mucus on epithelial layers or associated secretions from mucous cells (black arrow); C) lower magnification and D) higher magnification of gill sample fixed with modified Davidson's solution, stained with Alcian blue and Periodic acid-Schiff reagent (AB/PAS). There is some evidence of patchy preservation of mucus between the secondary lamellae (white arrows) with some mucus secretions from mucous cells (black arrows); E) lower magnification and F) higher magnification of gill sample fixed with Modified Davidson's and 2% Alcian blue solution stained with Periodic acid-Schiff's reagent (PAS). Note increased amount of mucus evident between lamellae (white arrows) and some mucus secretions from mucous cells (black arrows); G) lower magnification and H) higher magnification of gill sample fixed with Methacarn solution stained with Alcian blue and Periodic acid-Schiff's reagent (AB/PAS) showing presence of mucus as a thin attached layer on both interlamellar spaces (white arrows) and on secondary lamellae (black arrows). Note in H) evidence of preservation of mucus being secreted from mucous cells (short arrows). I) Lower magnification and J) higher magnification of gill sample fixed with Methacarn and 2% Alcian blue solution, stained with Periodic acid-Schiff's reagent (PAS). Evidence of mucus as a thin attached layer on interlamellar spaces (white arrows) and also presence of mucous cells (black arrows). Images taken by laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

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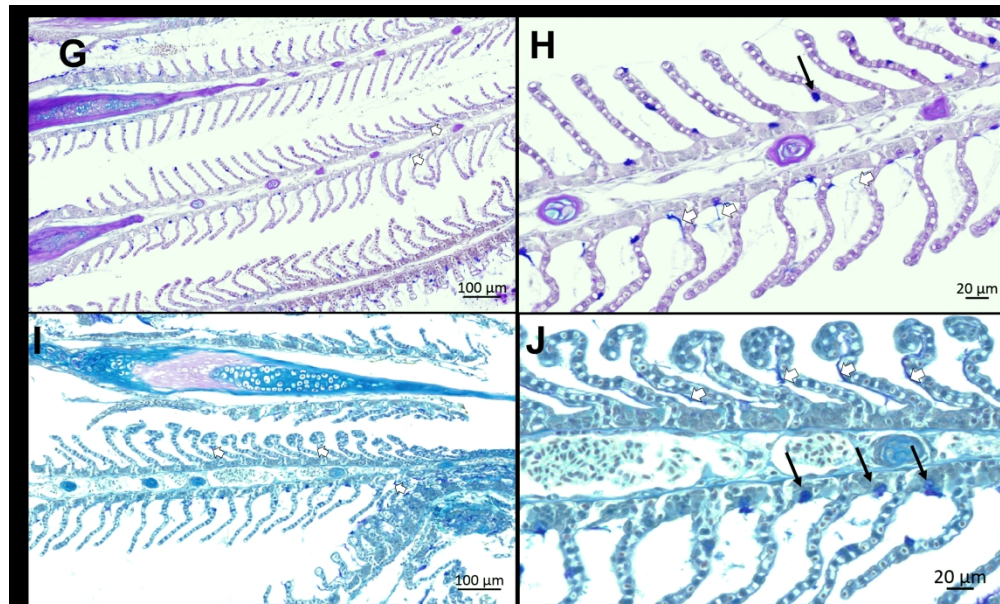


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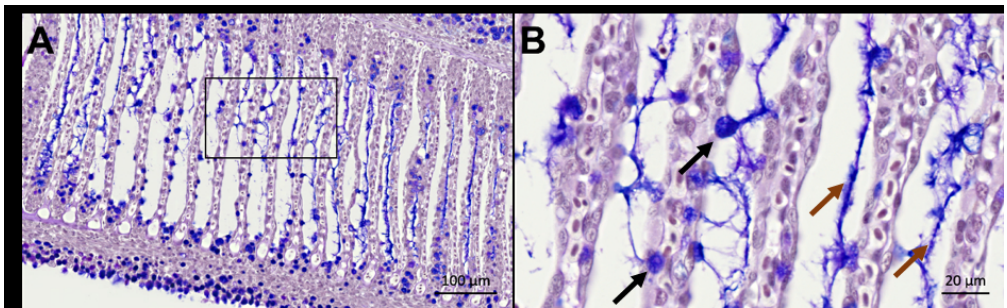


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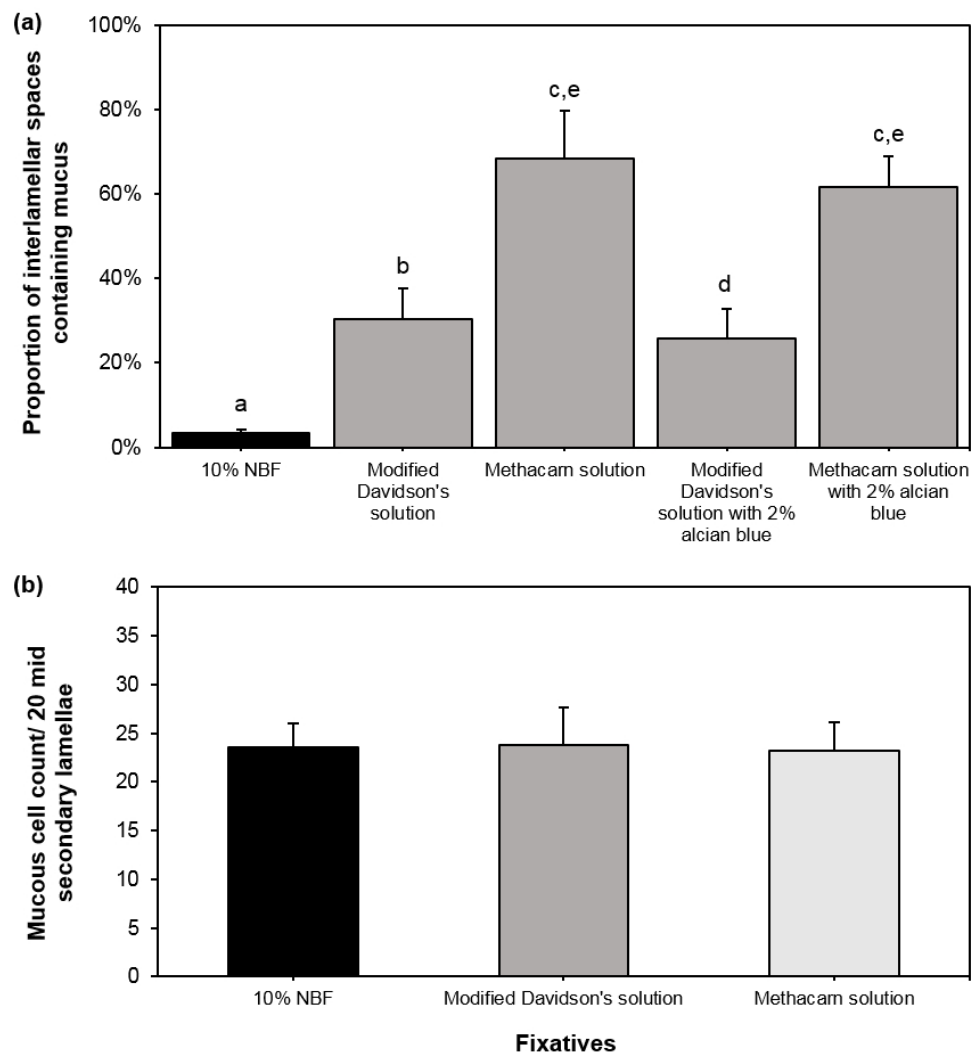


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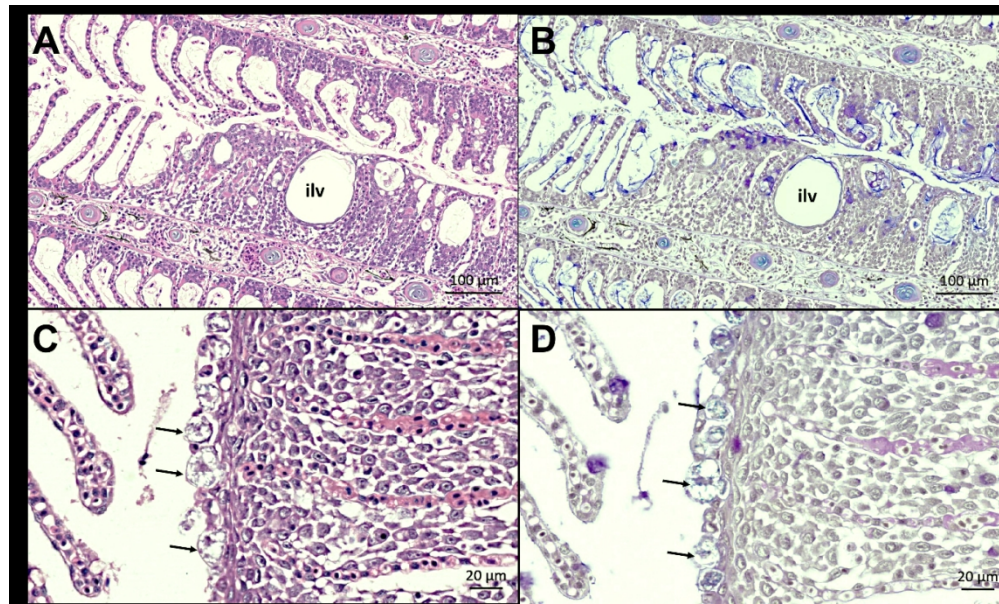


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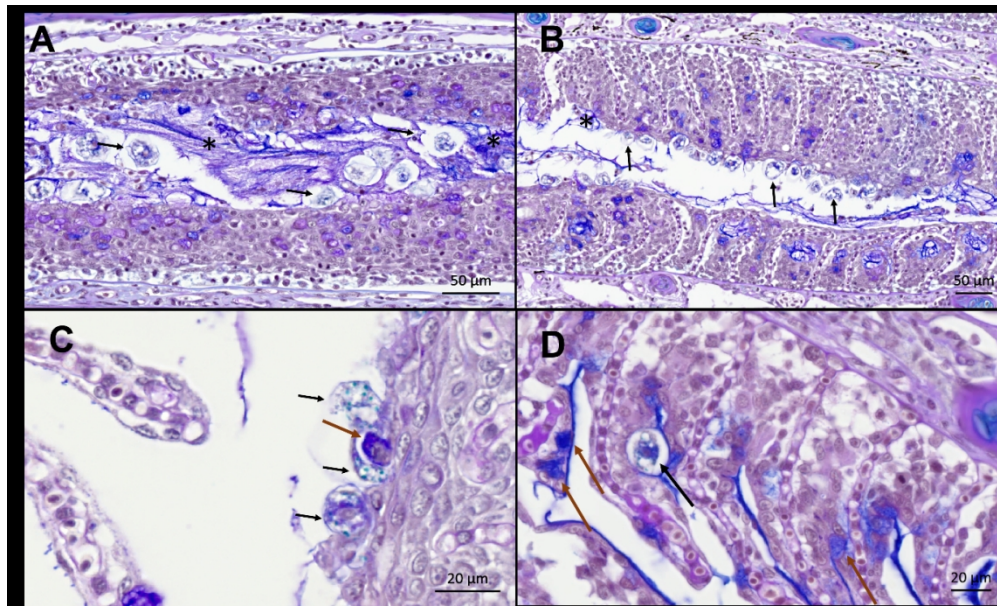


Figure 6: Gill tissue of Atlantic salmon during early stages of infection with AGD fixed in methacarn and stained with AB/PAS. A) & B) Hyperplastic AGD gill tissue with mucous cells and mucus throughout (asterisks), in addition to numerous intralesional trophozoites of *Paramoeba* spp. (black arrows) associated with lesion surface showing close interaction with overlying mucus (asterisk) C) trophozoites are found attached to the gill epithelium (black arrows) and a mucous cell (brown arrow) D) trophozoite trapped in newly formed interlamellar vesicle (black arrow) surrounded by mucus and mucous cells (brown arrows). Images taken by laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

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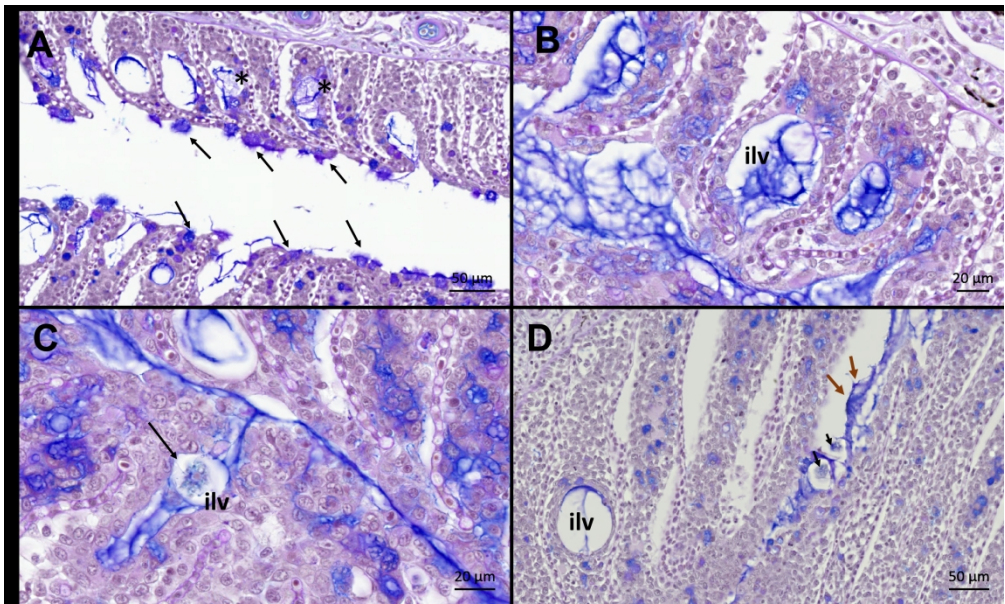


Figure 7: Gill tissue of Atlantic salmon during early stages of infection with AGD fixed in methacarn and stained with AB/PAS. A) Formation of rounded interlamellar vesicle (ilv) across the hyperplastic AGD-affected gill tissue with mucous cells (arrows) and trophozoites of *Paramoeba* spp. (asterisks) B) higher magnification of the formation of interlamellar vesicle (ilv) C) trophozoite trapped in newly formed vesicle in hyperplastic AGD-affected gill tissue D) transverse section of AGD-affected gill with ilv and amoebae attached to the epithelium (arrows) surrounded by mucus layer (brown arrows). Images taken by laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

643x385mm (150 x 150 DPI)

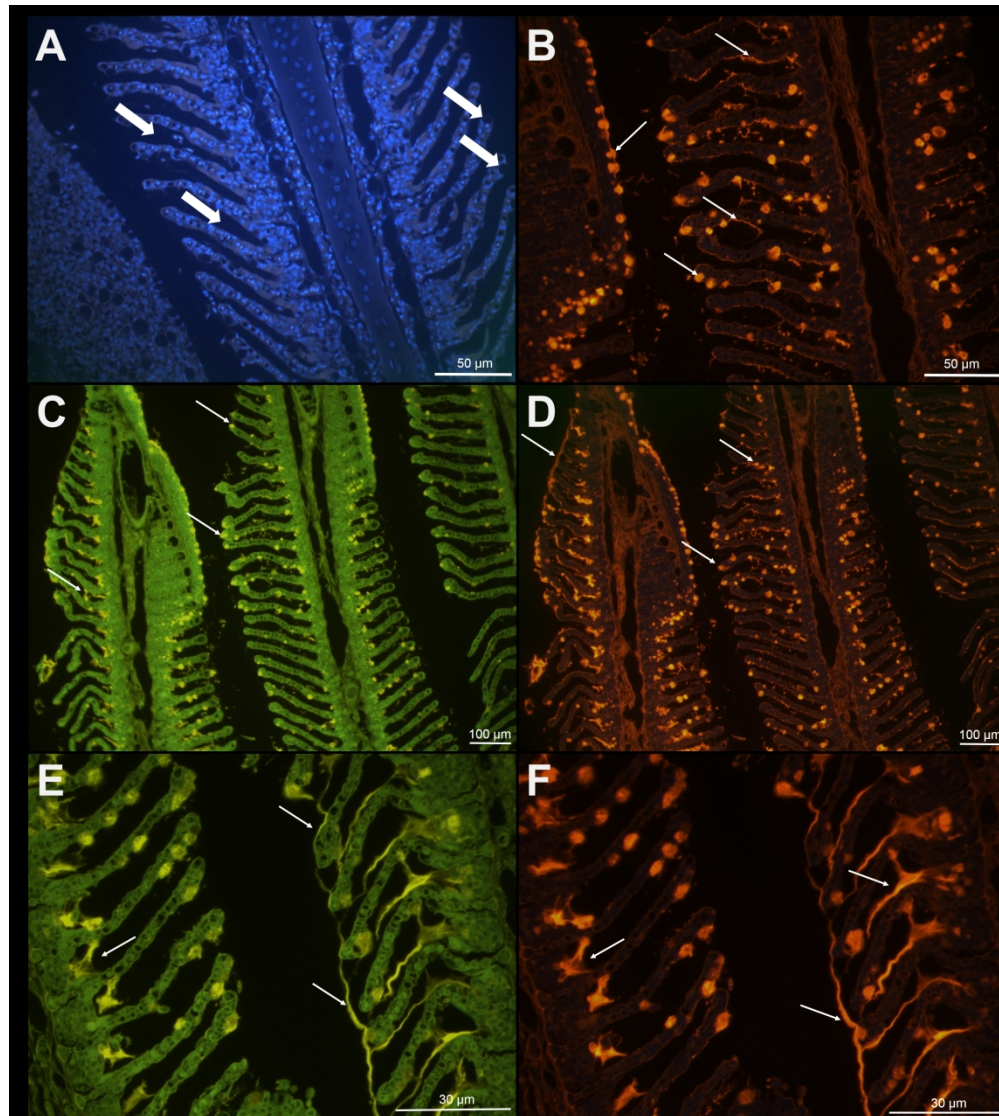


Figure 8: Wheat germ agglutinin (WGA) lectin labelling of carbohydrates of gill tissue fixed with methacarn solution. A) Negative control for the lectin labelling with the lectin buffer. No mucus or mucous cells are presenting any fluorescence (big white arrows). B) D) & F) Gill tissue presenting mucus as an overlay on the epithelium; mucous cells are also shown in a bright orange colour (thin white arrows). All taken with a triple band fluorescence filter. C) & E) Additional images from the same section showing the mucus and mucous cells (thin white arrows) but with a blue band fluorescence filter. Images taken by laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

555x618mm (150 x 150 DPI)

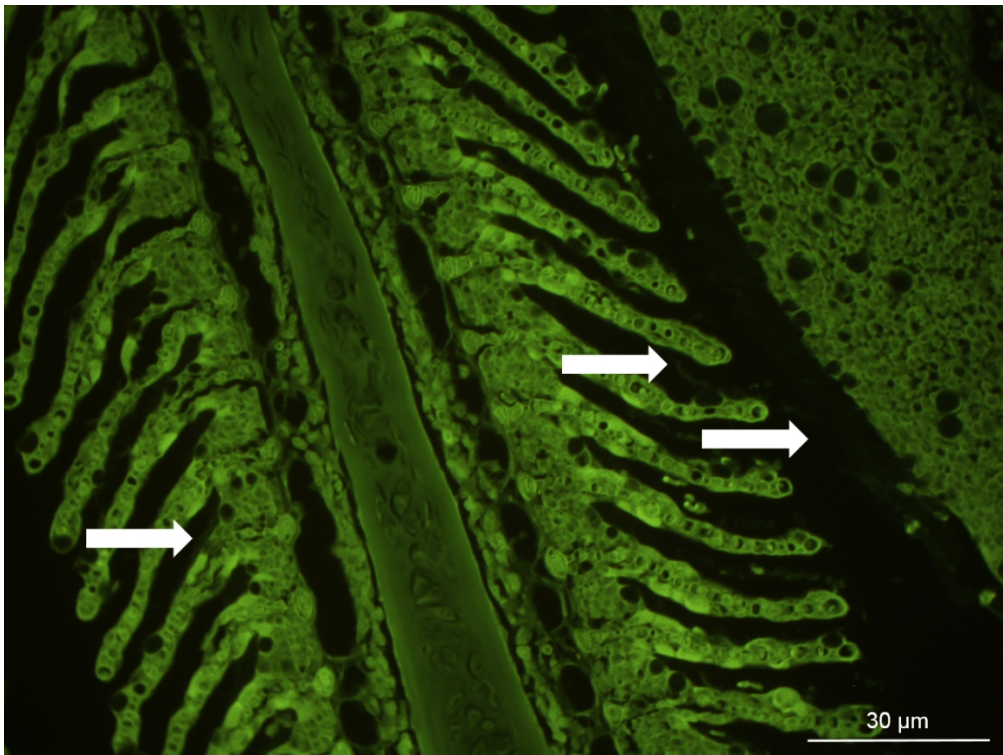


Figure 9: Wheat germ agglutinin (WGA) lectin labelling of carbohydrates of gill tissue fixed with 10% NBF solution. An additional negative control shows how the 10% NBF solution washes off the mucus and shows little fluorescence of the mucus remains (arrows). Images taken with a triple band fluorescence filter by laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

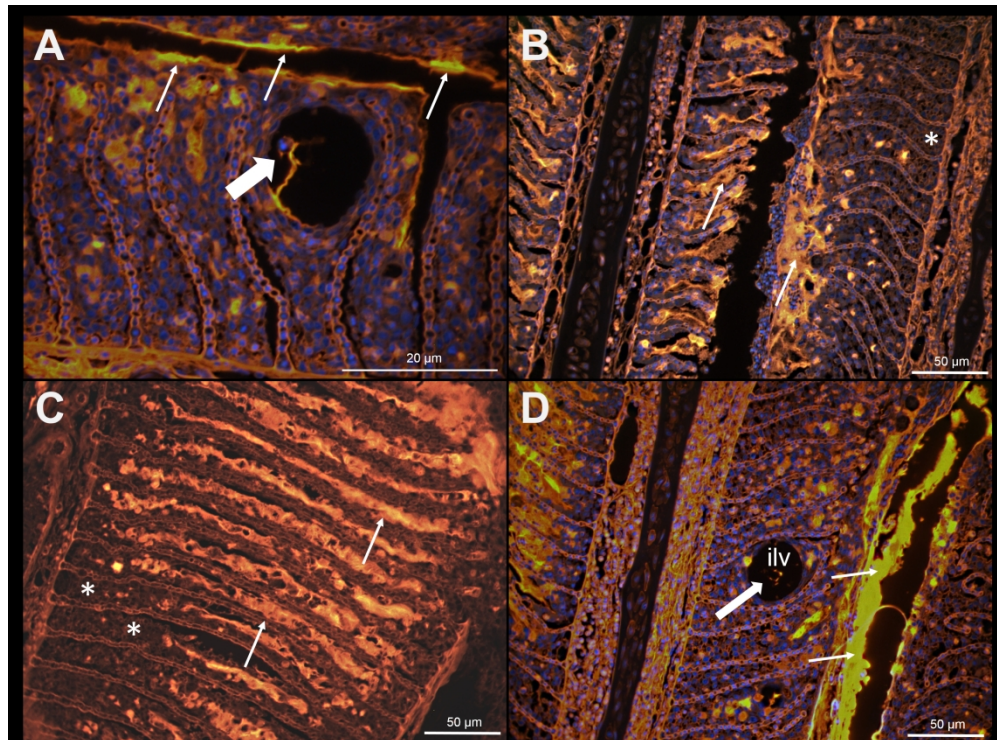


Figure 10: Wheat germ agglutinin (WGA) lectin labelling of carbohydrates of AGD-infected gill tissue fixed with methacarn solution and observation of *Paramoeba perurans* within. A) *Paramoeba* spp. trophozoite encapsulated inside interlamellar vesicle with DAPI-stained host and parasite nuclei (big white arrow). Thin arrows show mucus layer on gill epithelium B), C) & D) N-acetylglucosamine / N-acetyllactosamine carbohydrate labelling on transverse sections of gills with presence of mucus layer (thin white arrows) between hyperplastic AGD-affected lamellae (*). Images taken with a triple band fluorescence filter by laser scanner, Axio Scan.Z1 (ZEISS, Cambridge, UK).

559x413mm (150 x 150 DPI)