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

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2 in Atlantic salmon, Salmo salar L.

3

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

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16

17 vi. **Abstract**

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

34

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

36

37 vii. Main text

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38 1. Introduction

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

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

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

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

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

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

83 2. Materials and methods

84 Preparation of fixatives

85 Five fixatives were employed for the current study. Three of these were aqueous fixatives: 1)
86 10% neutral buffered formalin (NBF) (i.e. 4 g of Sodium dihydrogen phosphate, 6.5 g of
87 Disodium hydrogen phosphate (anhydrous), 100 ml of Formaldehyde and 900 ml of distilled
88 water); 2) a modified Davidson's solution (i.e. 30 parts 95% ethanol, 20 parts 37-40%
89 formaldehyde, 10 parts glacial acetic acid and 30 parts phosphate buffered saline (PBS)); 3) a
90 modified Davidson's solution (as above) with 2% (w/v) Alcian blue ((Sigma-Aldrich, UK). The
91 two remaining fixatives were non-aqueous solutions: 4) methacarn solution (i.e. methanol-
92 Carnoy's; 60% (v/v) dry methanol , 30% (v/v) chloroform, 10% (v/v) glacial acetic acid; 5)
93 methacarn solution (as above) with 2% (w/v) Alcian blue. All fixatives were freshly prepared
94 immediately before use.

95

96 Fish and sampling

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

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

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

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

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

122

123 Sample processing and staining

124 Comparison of fixatives to preserve mucus on gills

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

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

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142

143 Mucus and mucous cell quantification

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

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

154

155 FIG. 1.

156

157 Lectin histochemistry

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

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

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

174

175 Results

176 Comparison of fixatives to preserve mucus on gills

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

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

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

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

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

201

202 FIG. 2, FIG. 3 and FIG. 4

203

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

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

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

218 FIG. 5, 6

219

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

224

225 FIG. 7

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226

227 Confirmation of mucus preservation using lectin histochemistry

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

233 Regarding the investigation of how the mucus interacts with the amoebae, some hyperplastic
234 lesions could showed lectin labelling (Fig. 10). Also, mucus was well-preserved throughout the
235 gill tissue (Fig. 10 B, C & D). Lacunae formation could be seen again, with (Fig. 10 A) and
236 without (Fig. 10 D) enclosed amoebae. As a double confirmation, we did an additional negative
237 control with the lectin labelling with tissues fixed in 10% NBF solution. As shown in the figure
238 9, mucus is not as present as when methacarn solution is used as fixative. Also the little presence
239 of some mucus remains presents low fluorescence (figure 9, arrows).

240

241 FIG. 8.

242 FIG. 9.

243 FIG.10

244

245 Discussion

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

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

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

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

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

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

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

291 Overall, the present results conclude that both methacarn solution and methacarn solution with
292 2% (w/v) Alcian blue enhanced preservation of mucus. One challenge that was encountered
293 when quantifying the mucus was that it did not present as a uniform layer over the gill
294 epithelium; therefore, the presence of mucus was determined by the enumeration of mucus traces
295 that were still in contact with the originating mucous cells or were fixed in situ across the gill
296 epithelium.

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

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

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

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329 Teleost mucus plays a protective role by inhibiting pathogen binding, but also by acting as a
330 vehicle for mucins and humoral immune factors. As explained earlier, mucus contains high
331 molecular weight glycoproteins that can potentially trap pathogens, acting as a physical barrier
332 (Johansson & Hansson, 2016). Many studies have verified this statement by researching the
333 relationship between pathogens, mucus and mucins. A study by Nagashima et al. (2003)
334 indicated that some pathogenic bacteria could be found attached to the mucus layer and develop=
335 biofilms to protect themselves against the host mucosal immunity. To escape the challenge
336 provided by build-up of biota within the mucus, healthy fish continuously discharge and replace
337 their mucus layer preventing the stable colonization of potential infectious microorganisms as
338 well as invasion of metazoan parasites. Other studies have pointed out that pathogenic
339 microorganisms, such as some Vibrio strains, are capable of utilising mucus as a carbon source,
340 helping the colonisation of these pathogens and eventually supporting the initiation of infection
341 in fish (Bordas et al., 1996). More recently, study of immunological responses within the gill has
342 highlighted the potential role of secreted IgT responses, in part delivered through mucus, as well
343 as gene expression reflecting production of other defensins carried in mucus and acting against
344 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.

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

542

543 Fig. 2: Evaluation of aqueous-based and solvent-based fixatives to preserve mucus layer in
544 Atlantic salmon gills: A) lower magnification and B) higher magnification of gill sample fixed
545 with 10% neutral buffered formalin (10% NBF), stained with Alcian blue and Periodic acid-
546 Schiff's reagent (AB/PAS). Note that there is no evidence of overlying mucus on epithelial
547 layers or associated secretions from mucous cells (black arrow); C) lower magnification and D)
548 higher magnification of gill sample fixed with modified Davidson's solution, stained with Alcian
549 blue and Periodic acid-Schiff reagent (AB/PAS). There is some evidence of patchy preservation
550 of mucus between the secondary lamellae (white arrows) with some mucus secretions from
551 mucous cells (black arrows); E) lower magnification and F) higher magnification of gill sample
552 fixed with Modified Davidson's and 2% Alcian blue solution stained with Periodic acid-Schiff's
553 reagent (PAS). Note increased amount of mucus evident between lamellae (white arrows) and
554 some mucus secretions from mucous cells (black arrows); G) lower magnification and H) higher
555 magnification of gill sample fixed with Methacarn solution stained with Alcian blue and Periodic
556 acid-Schiff's reagent (AB/PAS) showing presence of mucus as a thin attached layer on both
557 interlamellar spaces (white arrows) and on secondary lamellae (black arrows). Note in H)
558 evidence of preservation of mucus being secreted from mucous cells (short arrows). I) Lower
559 magnification and J) higher magnification of gill sample fixed with Methacarn and 2% Alcian
560 blue solution, stained with Periodic acid-Schiff's reagent (PAS). Evidence of mucus as a thin
561 attached layer on interlamellar spaces (white arrows) and also presence of mucous cells (black
562 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
578 express standard error of the mean (s.e.m). (n = 6 control fish; 6 random fields of 20
579 interlamellar spaces; ANOVA test: $p<0.01$).

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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
593 Paramoeba spp. (black arrows) associated with lesion surface showing close interaction with
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

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

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

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

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

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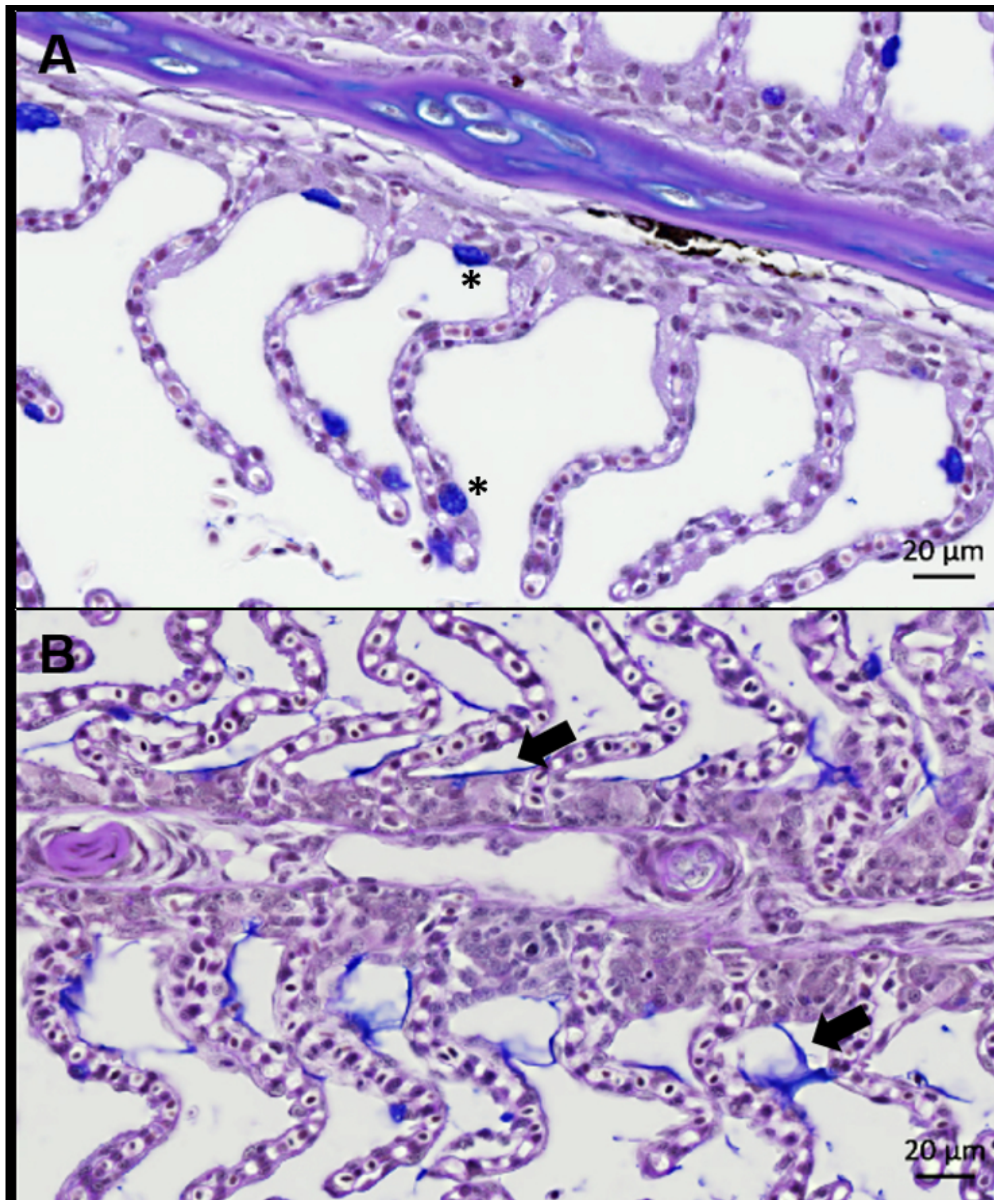

Figure 1: Semi-quantitative analysis method for mucus and mucous cells quantification. Mucus was quantified by counting the absence (A) or presence (B, arrows) of mucus traces (blue) in twenty inter-lamellar spaces from twelve random mid-sections of the primary lamellae. This method was used for all 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).

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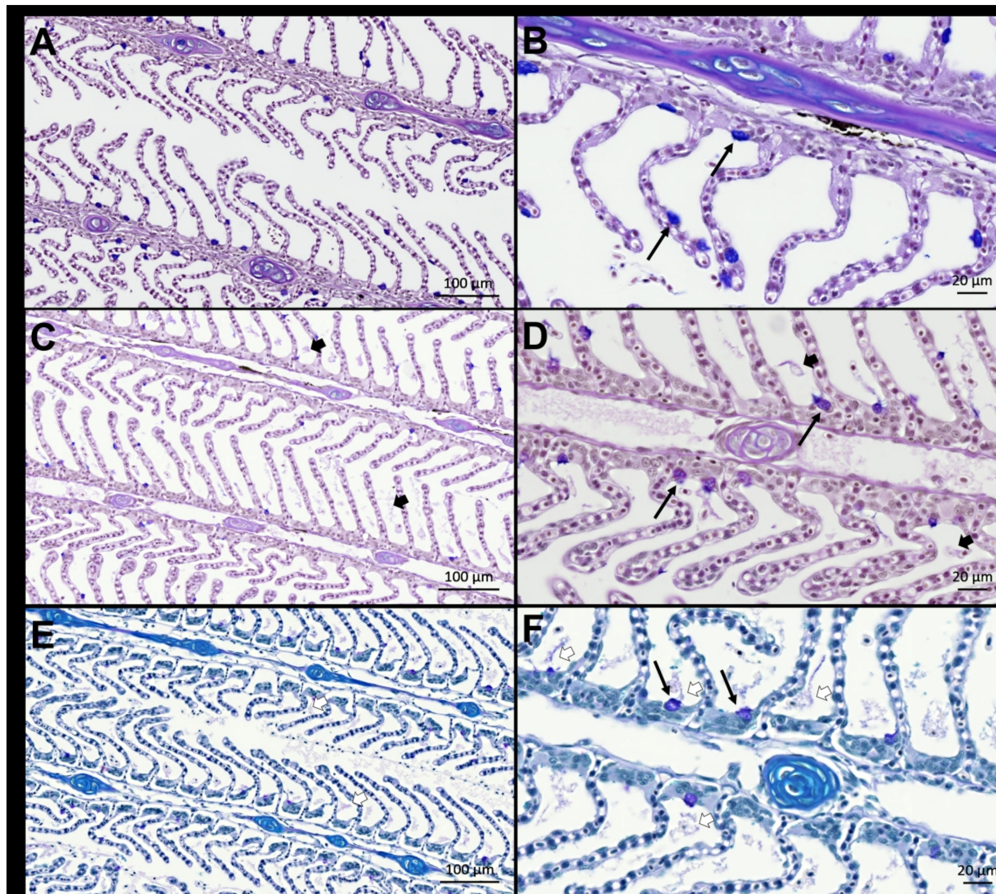

Fig. 2: Evaluation of aqueous-based and solvent-based fixatives to preserve mucus layer in Atlantic salmon gills: A) lower magnification and B) higher magnification of gill sample fixed with 10% neutral buffered formalin (10% NBF), stained with Alcian blue and Periodic acid-Schiff's reagent (AB/PAS). 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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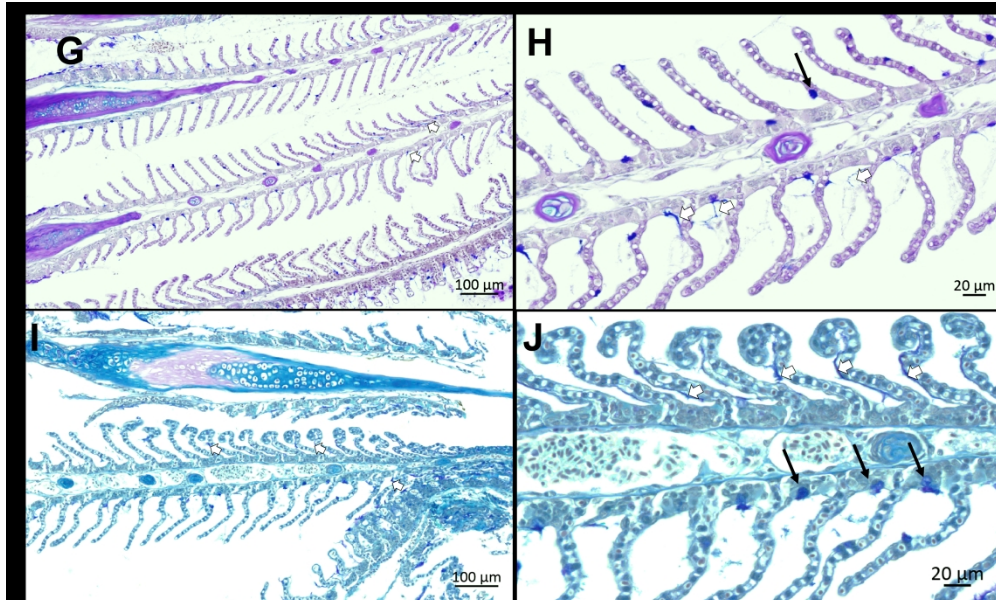

Fig. 2: Evaluation of aqueous-based and solvent-based fixatives to preserve mucus layer in Atlantic salmon gills: A) lower magnification and B) higher magnification of gill sample fixed with 10% neutral buffered formalin (10% NBF), stained with Alcian blue and Periodic acid-Schiff's reagent (AB/PAS). 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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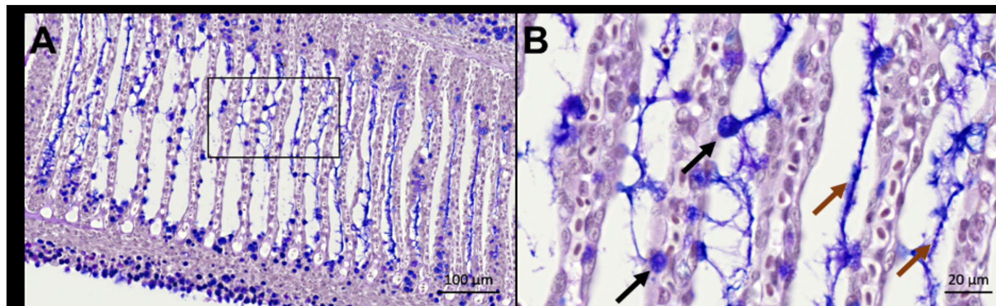

Fig. 3: Transverse sections of methacarn fixed AGD-affected gill tissue stained with Alcian blue and Periodic acid-Schiff reagent (AB/PAS): A) section of gill from Atlantic salmon with gross gill score of 2.5 (showing unaffected area with interlamellar mucus B) higher magnification of boxed area from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

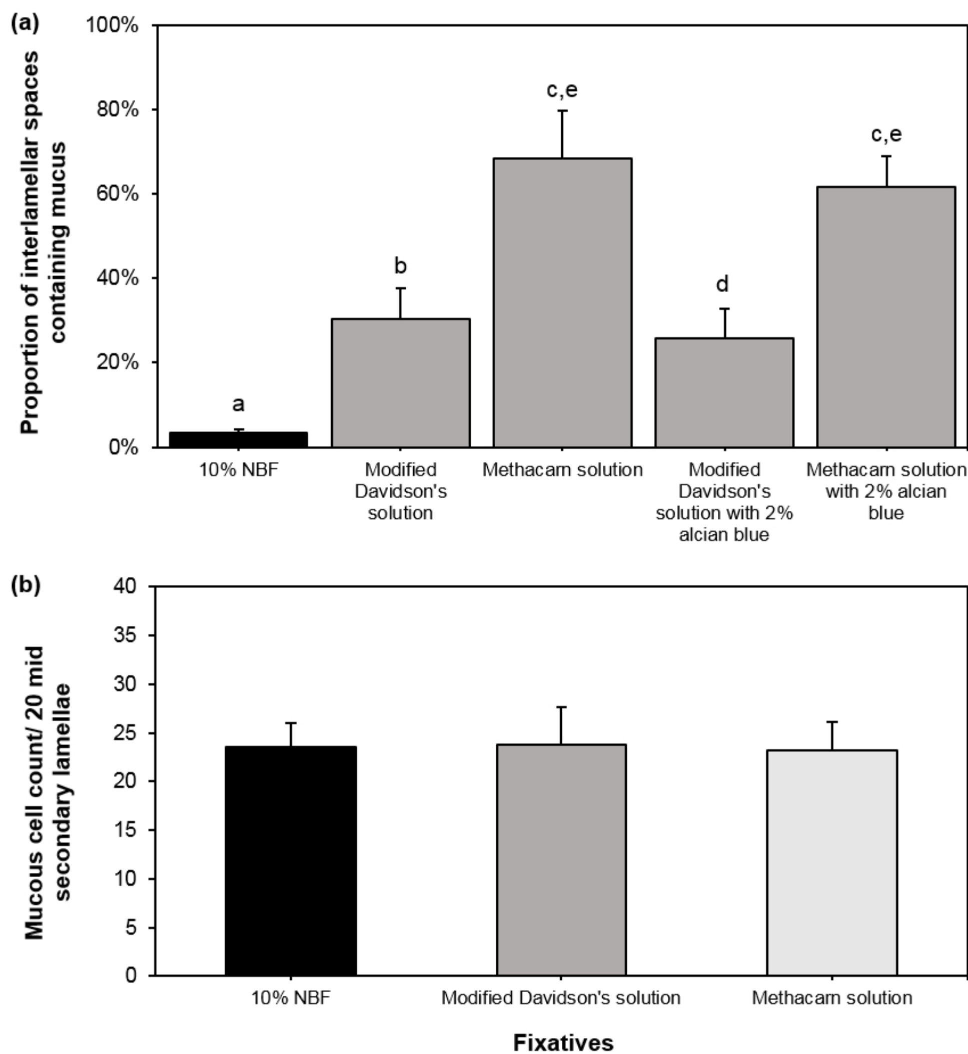

Figure 4. (a) Proportion of examined interlamellar spaces showing the presence of mucus layer for the different fixatives. Methacarn solution and methacarn solution with Alcian blue fixation methods preserve greater amount of mucus. Significant differences between fixatives are denoted with letters (i.e.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

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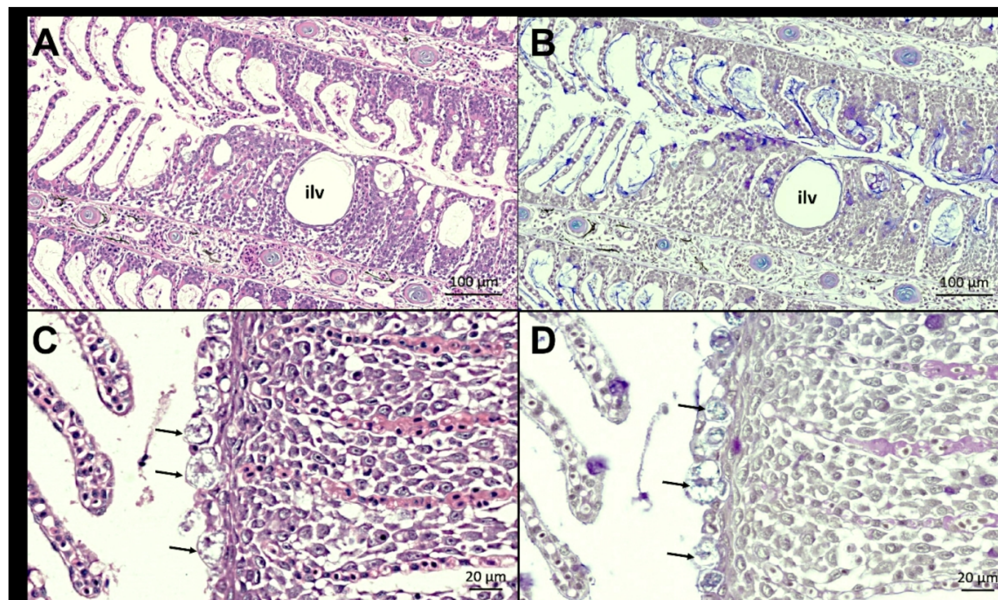

Figure 5: Comparison of histological stains of methacarn fixed Atlantic salmon gill tissue affected by AGD with H&E and AB/PAS staining. A) & B) early hyperplastic lesions with interlamellar vesicles (ilv) from gill tissue of Atlantic salmon with gill score 2.5 stained with A) routine H&E stain and B)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

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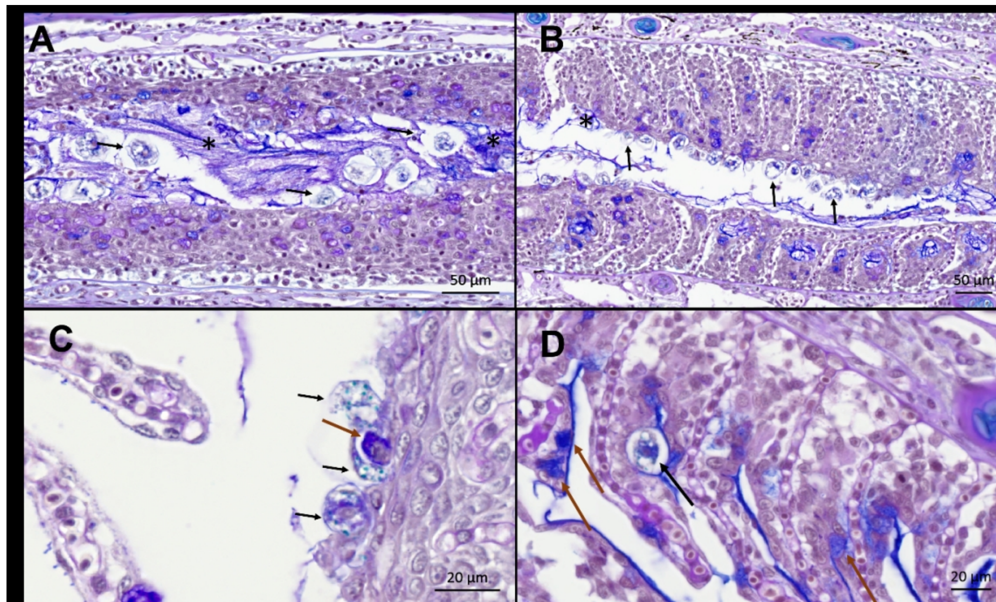

Figure 6: Gill tissue of Atlantic salmon during early stages of infection with AGD fixed in methacarn and stained with AB/PAS. A) & B) Hyperplastic AGD gill tissue with mucous cells and mucus throughout (asterisks), in addition to numerous intralesional trophozoites of Paramoeba spp. (black arrows) associated with lesion surface showing close interaction with overlaying 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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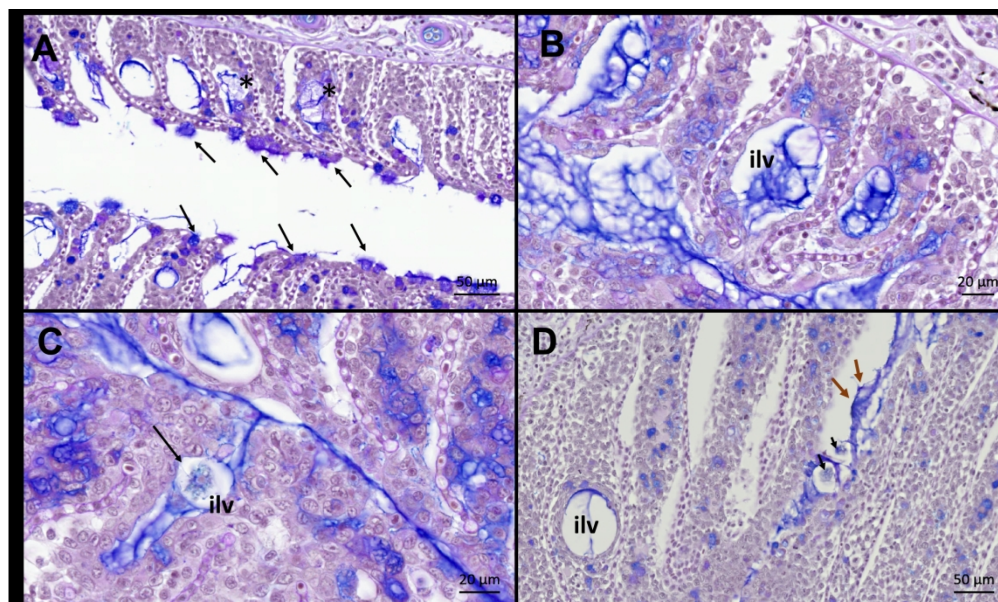

Figure 7: Gill tissue of Atlantic salmon during early stages of infection with AGD fixed in methacarn and stained with AB/PAS. A) Formation of rounded interlamellar vesicle (ilv) across the hyperplastic AGD-affected gill tissue with mucous cells (arrows) and trophozoites of Paramoeba spp. (asterisks) 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).

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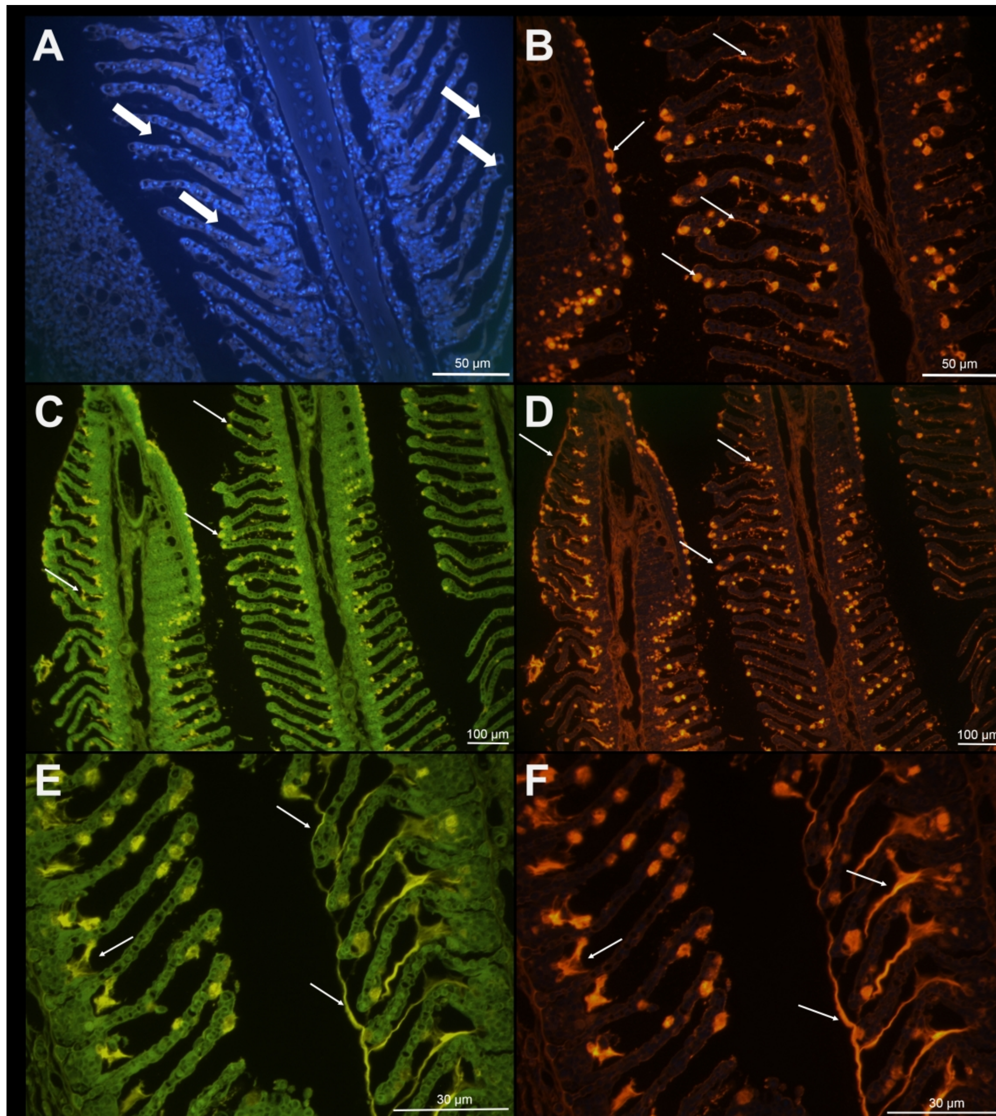

Figure 8: Wheat germ agglutinin (WGA) lectin labelling of carbohydrates of gill tissue fixed with methacarn solution. A) Negative control for the lectin labelling with the lectin buffer. No mucus or mucous cells are presenting any fluorescence (big white arrows). B) D) & F) Gill tissue presenting mucus 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).

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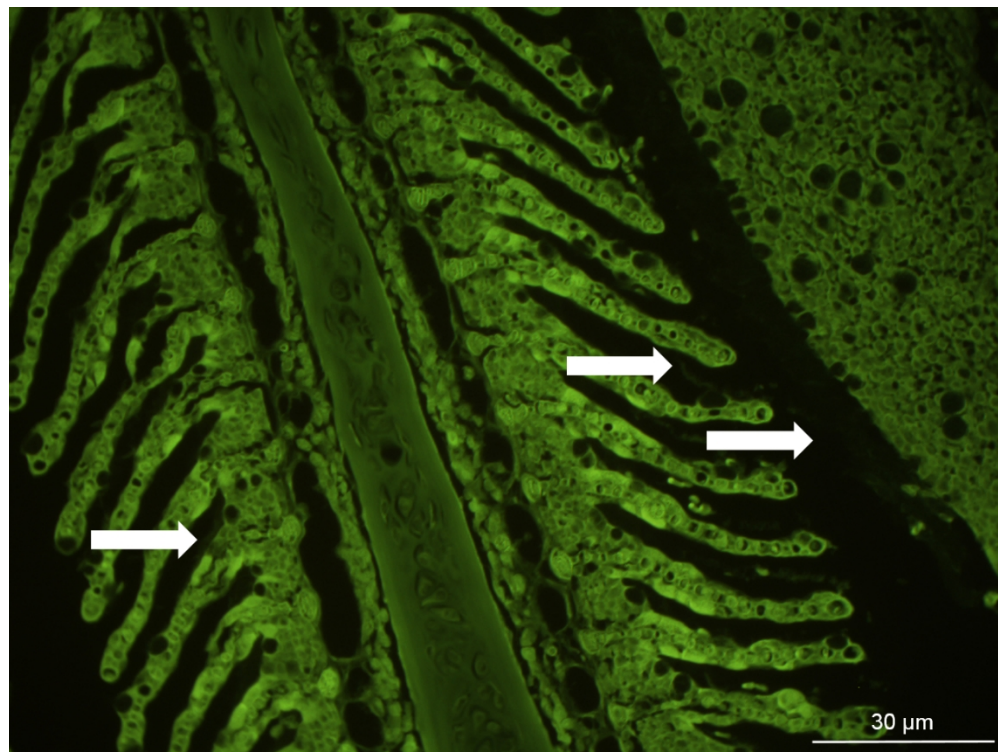

Figure 9: Wheat germ agglutinin (WGA) lectin labelling of carbohydrates of gill tissue fixed with 10% NBF solution. An additional negative control shows how the 10% NBF solution washes off the mucus and shows little fluorescence of the mucus remains (arrows). Images taken with a triple band fluorescence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

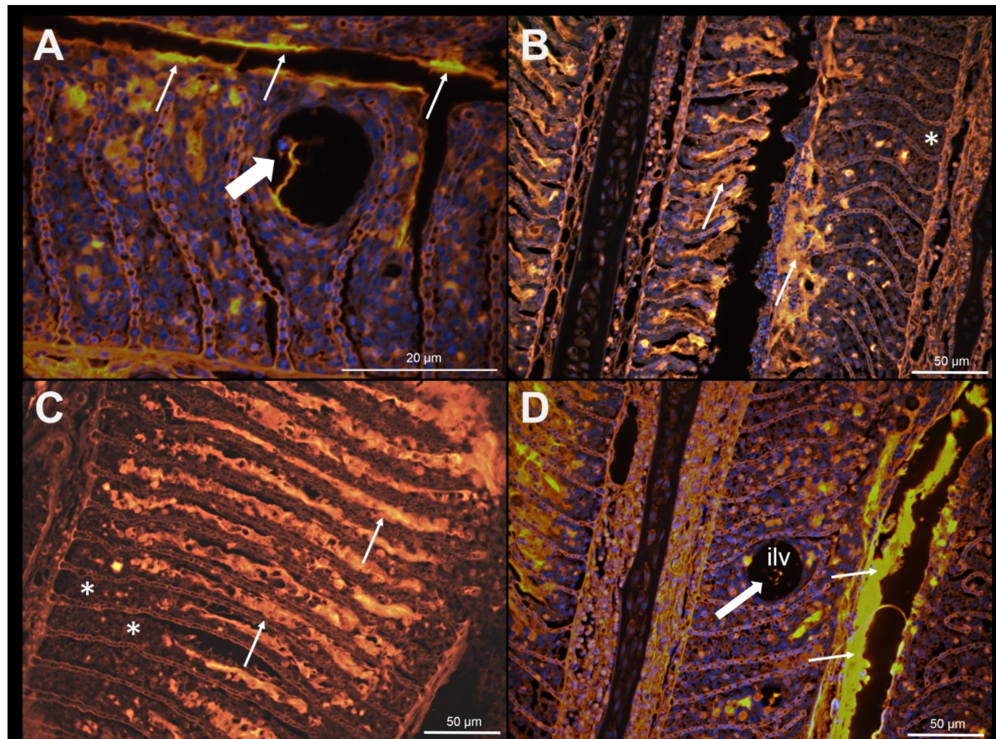

Figure 10: Wheat germ agglutinin (WGA) lectin labelling of carbohydrates of AGD-infected gill tissue fixed with methacarn solution and observation of Paramoeba perurans within. A) Paramoeba spp. trophozoite encapsulated inside interlamellar vesicle with DAPI-stained host and parasite nuclei (big white 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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