

1 **Novel atypical *Aeromonas salmonicida* bath challenge model for juvenile**
2 **ballan wrasse (*Labrus bergylta*, Ascanius)**

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20 *Running title:*

21 Atypical *As* bath challenge model for ballan wrasse

22

23 **Abstract**

24 Atypical *Aeromonas salmonicida* (aAs) is currently one of the most routinely recovered
25 bacterial pathogens isolated during disease outbreaks in farmed cleaner fish, ballan wrasse
26 (*Labrus bergylta*, Ascanius). Vibrionaceae family bacteria have also been isolated from ballan
27 wrasse in Scotland. This study determined the infectivity, pathogenicity and virulence of aAs
28 and Vibrionaceae isolates in juvenile farmed ballan wrasse (n= 50; approx. 2 gr) using a bath
29 challenge and fish were monitored for a period of 16 days. Atypical *As* caused significant
30 mortalities in contrast to Vibrionaceae isolates. Notably, differential virulence was observed
31 between two aAs *vapA* type V strains at similar challenge doses. Diseased fish exhibited a

32 systemic infection where aAs was detected in all analysed tissues (liver, spleen and kidney)
33 by PCR and qPCR. Macroscopically, moribund and survivor fish exhibited hepatomegaly and
34 splenomegaly. In moribund and surviving fish histopathology showed granulomatous hepatitis
35 with eosinophilic granular cells surrounding bacterial colonies and endocarditis along with
36 splenic histiocytosis. This is the first report of a successful aAs bath challenge model for
37 juvenile ballan wrasse which provides an important tool for future studies on vaccine efficacy
38 and immunocompetence.

39 **Keywords:** atypical *Aeromonas salmonicida*, bath challenge, gross pathology, systemic
40 disease, eosinophilic granular cells (EGCs).

41 **Introduction**

42 Among disease challenges faced by the Atlantic salmon (*Salmo salar*, Linnaeus) farming
43 industry, caligid sea lice infections caused by *Lepeophtheirus salmonis* (Krøyer) and *Caligus*
44 *elongatus* (Nordmann) had the most significant impacts since the 1960s (Hastein & Bergsjø,
45 1976; Costello, 2006). Due to large global economic losses to the industry, for example £700
46 million in 2015 alone (Brooker et al., 2018), there has been considerable investment and
47 innovation on developing sea lice control strategies. The most effective control methods have
48 been bath or in-feed medicated treatments but their efficacy is reduced due to resistance
49 development in lice (Treasurer & Feledi, 2014). Alternative mechanical (hydrolicers,
50 thermolicers, and lasers), physical (snorkel cages and lice skirts) and biological (cleaner fish)
51 treatments have been applied in order to control sea lice (Holan et al., 2017). Biological control
52 through the deployment of wild cleaner fish in salmon cages in Norway and Scotland has been
53 effective and gaining popularity as an environmentally friendly pest management strategy
54 (Treasurer, 2012; Skiftesvik et al., 2013). Commercial scale farming of ballan wrasse (*Labrus*
55 *bergylta*, Ascanius) and lumpsucker (*Cyclopterus lumpus*, Linnaeus) is currently being used
56 for this purpose. In Scotland, efforts have recently intensified on the farming of ballan wrasse
57 as opposed to using wild caught animals. However, the bacterial pathogen atypical
58 *Aeromonas salmonicida* (aAs), continues to cause high mortalities during the hatchery and
59 post-deployment stages (Brooker et al., 2018). Depending on the host, typical (salmonids) and
60 atypical (generally non – salmonids) strains of As infect a wide range of fresh and marine
61 water fish species. High mortality events have been recorded in farmed species including
62 Atlantic salmon, rainbow trout (*Oncorhynchus mykiss*, L.), Atlantic cod (*Gadus morhua*, L.),
63 Atlantic halibut (*Hippoglossus hippoglossus*, L.) and turbot (*Scophthalmus maximus*, L.)
64 (Austin & Austin, 2016). Atypical As strains are very heterogeneous and attempts have been

65 made to characterise and categorise the different strain types. To date, four main subspecies
66 have been described: *A. salmonicida* subsp. *achromogenes* (Smith, 1963; Schubert, 1967),
67 *masoucida* (Kimura, 1969a, 1969b), *smithia* (Austin et al., 1989) and *pectinolytica* (Pavan et
68 al., 2000). However, some strains cannot be assigned under any of these groups making
69 vaccine development against aAs difficult. A variety of virulence factors have been
70 characterised for As including the A-layer (Munro et al., 1984). The latter is an outer membrane
71 protein of As (Udey & Fryer, 1978; Kay & Trust, 1991) which plays an important role in the
72 infection of the host as well as providing protection for the bacterium to the host immune
73 responses (Munn et al., 1982; Kay & Trust, 1991; Daly et al., 1996). Gulla et al. (2016a)
74 demonstrated that As strains can be differentiated using the A – layer. Variations can be
75 detected using a partial sequence technique in a region of the virulence array protein gene
76 (*vapA*). In total, 23 A – layer types were differentiated by Gulla et al. (2019) while previously
77 aAs type V (the most predominant in Scotland) and VI (mainly in Norway) were found to be
78 associated with disease in ballan wrasse (Gulla et al., 2016a). Although there are no
79 commercially available registered licensed vaccines to protect ballan wrasse against aAs,
80 autogenous vaccines are currently used as an emergency solution in Scotland (Ramirez
81 Paredes et al., 2020). While efficacy testing is not required for commercialising this type of
82 vaccine, data on immune protection elicited by such vaccines is important for optimising
83 treatment efficacy. Challenge models must therefore be developed for ballan wrasse to
84 undertake vaccine testing and assess protective efficacy.

85 The present study aimed to develop a bacterial bath challenge model for juvenile ballan
86 wrasse using aAs and Vibrionaceae isolates that had been recovered from disease outbreaks
87 in Scotland but without prior knowledge of their virulence. In addition, the infectivity,
88 pathogenicity and virulence of these bacterial isolates was determined by simulating more
89 natural bacterial portals of entry, as opposed to injection challenge, to provide a vaccination
90 testing platform for the future.

91

92

93 **Materials and Methods**

94 **Ethics statement**

95 Bacterial infection and vaccination procedures were performed under UK Government Home
96 Office project licences P8E92D8B3 following approval by the Animal Welfare and Ethical
97 Review Body (AWERB) at the Centre for Environment, Fisheries and Aquaculture Science
98 (Cefas) and University of Stirling. Ballan wrasse were treated in accordance with the Animals
99 (Scientific Procedures) Act 1986 Amended Regulations (SI 2012/3039).

100 **Experimental animals**

101 Juvenile ballan wrasse (n= 1200, 1 ± 0.2 g) were obtained from Otterferry Seafish (Scotland,
102 UK) and transported to Cefas (Weymouth, UK) in January 2018. Fish were kept in four aerated
103 aquaria (50 L) under a 20 h light: 4 h dark photoperiod, with water flows of 1.5 L / min and
104 dissolved oxygen (DO) maintained at 8 ± 0.5 mg / L throughout the experiment. Fish were
105 acclimated to the new environment for three weeks during which temperature was kept
106 constant at 12 ± 1 °C. Temperature was then increased by 0.5 degree / day to reach 15 °C.
107 The fish were fed with Otohime Fish Diet C1 & C2 (Biokyowa, Japan) for 7 h / day using auto
108 feeders during the experiment.

109 **Fish health assessment**

110 The health status of the fish was assessed prior to transportation. Whole larvae (n= 60) were
111 screened for pathogens including common isolated bacteria (aAs and Vibrionaceae) as well
112 as amoebic gill disease (AGD) by histology, bacteriology and molecular techniques
113 (Yamamoto et al., 2000; Young et al., 2008; Klindworth et al., 2013). In addition, the fish health
114 (n= 60) was reassessed upon arrival to Cefas facilities for bacteriology (swabs from head
115 kidney plated onto sea water agar (SWA)), histopathology (fixed in 10% neutral buffered
116 formalin) and molecular methods to provide further assurances of biosecurity in accordance
117 with the methodological approach reported by Ramirez-Paredes et al. (2020). Whole fish were
118 also fixed in transport media (500 mL G-MEM base, 50 mL foetal bovine serum, 5 mL
119 antibiotic antimycotic stabilised solution, 5 mL glutamax 1, 5 mL penicillin – streptomycin
120 solution, 0.8 mL Tris solution, 2.4 mL sterile 7.5% sodium bicarbonate, OIE) for virology to
121 confirm that fish were not carrying any notifiable viral diseases for further biosecurity
122 assurance as listed in the OIE manual of diagnostic tests for aquatic animals (OIE, 2018).
123 Whole fish samples were fixed in 10% neutral buffered formalin to confirm by histology that
124 fish did not have any existing pathological conditions prior to challenge.

125 **Bacteria preparation**

126 The bacterial strains (n= 8) used were previously recovered from diseased ballan wrasse in
127 hatcheries or cage sites in Scotland or Norway (mentioned where applicable) (Biering et al.,
128 2016; Papadopoulou, 2019). The bacteria used were two strains of aAs *vapA* type V (TW 3 /
129 14 and TW 4 / 14) and members of Vibrionaceae family including two strains of *Vibrio*
130 *anguillarum* TW 260 / 16 and 12 – 50 2075 F383 – 1 (Norwegian), *Aliivibrio salmonicida* (TW
131 189 / 16 and TW 322 / 16) and *Photobacterium indicum* (TW 181 / 16 and TW 138 / 16) (Table
132 1). All bacteria were grown in Tryptone Soya Broth (TSB) + 1.5% NaCl at 22 °C for 24 h with
133 continuous shaking at 150 rpm except atypical *Aeromonas salmonicida* isolates which were
134 grown in TSB at 22 °C for 48 h. The bacteria were centrifuged at 2,000 x g for 10 min, bacterial
135 pellets were washed with phosphate buffered saline (PBS) and then re – suspended in sterile

136 sea water to an OD₅₅₀ 1. Colony forming unit (CFU) per mL were counted by the 10 – fold
137 dilution method.

138 **Challenge**

139 Ballan wrasse (1.5 ± 0.3 g) were randomly selected from the holding tanks (n= 4) and split into
140 18 small challenge aquaria (5 L, 50 fish / aquarium). Experimental bacterial infection was
141 performed in duplicate with the exception of an aAs strain (TW 3 / 14) which was found to be
142 less virulent than TW 4 / 14 at similar doses when previously used for an intraperitoneal (i.p.)
143 injection trial in ballan wrasse (Ramirez Paredez et al., 2020). This strain was prepared in two
144 doses (10⁷ and 10⁸ CFU/ mL) to assess changes in virulence. The study consisted of 16
145 challenged (n= 4 bacterial species x 2 strains per species x 2 tanks each) and 2 control groups.
146 The fish were challenged with aAs *vapA* type V and Vibrionaceae isolates by bath in a bacterial
147 suspension of an OD₅₅₀ 1.0 while control groups were exposed to sterile sea water (Table 2).
148 All groups were challenged for 4 h at 15 °C with aeration in static conditions. Fish were
149 monitored at least twice per day, with increasing frequency as clinical signs of infection
150 appeared, up to 16 days post infection (dpi). Individuals displaying more than two clinical signs
151 of disease (generic and pathogen-specific) were considered as moribund and were humanely
152 killed with an overdose of tricaine methanesulfonate MS-222 (Sigma), followed by destruction
153 of the brain to confirm death. Mortalities were removed from the tanks as soon as they were
154 detected.

155 **Sampling**

156 Head kidney swabs were sampled from a representative number of moribund fish (5
157 individuals; 10 %) from each tank and plated onto tryptone soya agar (TSA aAs isolates) or
158 TSA + 1.5 % NaCl (Vibrionaceae) to confirm infectivity and pathogen induced mortality;
159 individuals had only been exposed to one of the four bacteria species. Bacterial colonies were
160 confirmed based on morphology, motility, Gram staining and agglutination test (BIONOR™).
161 Heart, intestine, posterior kidney, liver and spleen samples from moribund fish were preserved
162 in 10 % neutral Buffer Formalin (NBF) for histology. A total of 20 moribund fish (n= 5 x 4 aAs
163 exposed tanks) were sampled during the trial while 5 survivor fish from each tank were
164 sampled at termination and preserved in 100% ethanol for molecular assessment. In addition,
165 all the survivor fish exposed to TW 3 / 14 (dose 10⁷) were sampled (n= 19) at termination as
166 mortalities had not reached a plateau. Samples were screened with molecular assays to
167 determining the presence of aAs as described below. Mid kidneys, liver and spleen tissue
168 were also sampled from control and all the survivor fish challenged with Vibrionaceae isolates
169 and preserved in 100 % ethanol for molecular analysis.

170 **Histology & Gram Twort stain**

171 A representative number of samples (19 moribund, 12 survivor and 6 control) that had
172 previously been fixed in 10 % NBF were dehydrated (Thermo Shandon Citadel 2000),
173 embedded in paraffin wax, sectioned at 5 µm (Leica RM 2135) and stained with Haematoxylin
174 and Eosin (H&E), while slides not previously stained were dewaxed and stained with Gram
175 Twort. Images were captured using Nikon Eclipse Ni microscope (Nikon, UK) and camera with
176 accompanying software.

177 **DNA extraction and quality check**

178 DNA was extracted from liver, spleen, and mid kidney tissue samples (n= 179, 64 fish x 3
179 tissues; 13 spleen samples were not included as they were either too small or not sampled)
180 previously preserved in 100% ethanol (Fisher) using a modified salt precipitation method from
181 (Khanam et al., 2016). The extracted DNA was re-suspended in 5 mM Tris. The concentration
182 and purity of the samples was assessed using a NanoDrop® ND-1000 (Thermo Fisher
183 Scientific) and samples were standardised at 50 ng / µL. The DNA integrity was validated by
184 gel electrophoresis and samples were stored at - 20°C until use.

185 **Conventional PCRs – 16S, *vapA* and aAs type V specific PCR**

186 The presence of bacterial DNA in the fish tissues (liver, spleen and mid kidney) was assessed
187 on the V3-V4 hypervariable region of the 16S rRNA gene – 16S PCR (Herlemann et al., 2011);
188 if samples were negative no further testing was carried out as bacterial load in the samples
189 was considered below the assay detection limit. Positive samples were then screened with a
190 species specific PCR (*vapA*) for the presence of *As* DNA using a previously published PCR
191 protocol targeting the hypervariable region of *vapA* gene – *vapA* PCR (Gulla et al., 2016a).
192 The presence or absence of aAs *vapA* type V was then determined using a previously
193 developed aAs *vapA* type V specific PCR (aAs type V specific PCR) (Papadopoulou, 2019).
194 The primer pair used for the tissue samples screening with conventional PCRs are listed in
195 Table 3. The relative molecular weight of the amplicons was compared against a 100 bp gene
196 ruler (Thermo Fisher Scientific) on 1 % agarose gel and visualised as previously described.

197 **Quantitative PCR (qPCR)**

198 *Aeromonas salmonicida* bacterial loads were assessed on tissue samples that were confirmed
199 positive for *As* with the conventional PCRs using a modified qPCR protocol targeting the *vapA*
200 gene (Gulla et al., 2016b). In brief, a 10 µL reaction was set up and the master mix per sample
201 consisted of 0.03 µM of the forward and reverse (Eurofins) primers (Table 3), 0.02 µM of
202 beacon probe (Eurofins), 5.0 µL of Luminaris colour probe qPCR master mix (Thermo Fisher

203 Scientific), 3.2 μ L of Milli-Q water and 1.0 μ L of sample or control at 100 ng/ μ L. Milli-Q water
204 was used as a negative control and the positive control was aAs type V isolate DNA. The
205 following thermal cycling conditions were run in a LightCycler® 480 Instrument II (Roche
206 Molecular Diagnostics): 1 cycle at 95 °C for 10 min, 45 cycles at 95 °C for 15 sec, 60 °C for
207 30 sec and 72 °C for 30 sec. The analysis was performed with LightCycler® 480 software
208 (Roche Molecular Diagnostics). The *Aeromonas salmonicida* type strain (NCIMB 1102) was
209 used for the development of the standard curve for the qPCR. The bacteria were grown on
210 TSA at 22 °C. After 48 h an inoculum was transferred into TSB and incubated in a shaker
211 incubator at 22 °C, 150 rpm for 48 h and then bacterial DNA was extracted as described
212 previously. The quality and quantity of the DNA was measured with the methods mentioned
213 and 7 serial dilutions (initial concentration of 40 ng / μ L) were conducted using Qubit™
214 (Thermo Fisher Scientific).

215 **Statistical analyses**

216 All statistical analyses were conducted using the statistical software R (R Core Team, 2020) with
217 the packages survival (Therneau, 2020) and survminer (Kassambara et al., 2020). Survival data
218 were analysed using Kaplan–Meier. Differences between aAs challenges were analysed by the
219 log-rank test, using the Bonferroni correction for multiple comparisons.

220 **Results**

221 **Bath challenge**

222 The bacterial infection dosages are shown in Table 2. Control fish did not show any signs of
223 disease and no mortalities were recorded during the trial. No notable mortalities were recorded
224 for the duplicate groups exposed to *V. anguillarum*, *Aliiv. salmonicida* or *Phot. indicum*, with
225 only 2, 1 and 0 mortalities recorded, respectively. In contrast, fish challenged with the two
226 strains of aAs *vapA* type V showed cumulative mortalities of 52 and 60 % (TW 4 / 14 replicates
227 at 2.84×10^7 CFU / mL) and 20 % and 62 % (TW 3 / 14 dose at 1.93×10^7 CFU / mL and 2.04
228 $\times 10^8$ CFU / mL, respectively) (Table 2, Figure 1). Survival was higher for individuals challenged
229 with TW 3 / 14 dose at 1.93×10^7 CFU / mL than with any other aAs challenges. The log-rank
230 shows that the challenges statistically differed significantly at the 5% significance level
231 ($p=0.00011$) and pairwise comparisons indicated that TW 3 / 14 at a dose of 1.93×10^7 CFU
232 / mL is statistically significantly different from the other treatments (Table 2). Fish exhibited
233 clinical signs at 5 and 7 days post infection (dpi), for strains TW 4 / 14 and TW 3 / 14,
234 respectively. The first mortalities were recorded at 7 dpi for both strains at the same dose of
235 10^7 CFU / mL, and 9 dpi for strain TW 3/14 at dose of 10^8 CFU / mL. Mortalities peaked at 13
236 dpi for the TW 3 / 14 isolate (10^7 CFU / mL; one of the replicates) and 14 dpi for strains TW 3

237 / 14 and TW 4 / 14 at 10^7 and 10^8 CFU /mL, respectively (Figure 1). Mortality rate was faster
238 for strain TW 4 / 14 in comparison with fish infected with strain TW 3 / 14 (similar CFU / mL).
239 Pure cultures of aAs isolated from head kidneys of moribund fish grew on TSA within 48 h at
240 22 °C.

241

242 **Gross clinical signs following bath challenge**

243 Moribund fish exhibited external clinical signs of disease including uni or bilateral exophthalmia
244 (Figure 2 A), eroded fins with complete loss of pectoral fins, haemorrhage on dorsal fin and /
245 or tail base (Figure 2 B), skin discoloration, swollen abdomen (ascites), petechiae on the body
246 (Figure 2 B) and haemorrhage between the eyes (Figure 2 A). Exophthalmia and eroded fins
247 were one of the predominant gross pathological changes reported simultaneously with
248 lethargy, loss of buoyancy and imbalance, and loss of appetite. Internally, dark red intestines
249 (Figure 2 C), white nodular lesions in the liver (Figure 2 D), as well as hepatomegaly and
250 splenomegaly were also observed. The liver and spleen were the two main affected organs
251 macroscopically.

252 **Histopathology and Gram Twort staining**

253 In total 6 out of 19 (31.5 %) liver samples from moribund fish analysed exhibited granulomas
254 and increased eosinophilic granular cell activity (Figure 3 A – C). Moribund and survivor fish
255 demonstrated mild scattered single cell necrosis together with mild hepatocellular dissociation,
256 congestion and distended sinusoids (Figure 3 C). In granulomatous livers, small infiltrates of
257 lymphocytes were apparent at the periphery of the granulomas (Figure 3 A - C). The
258 granulomatous formations ranged from large disorganised aggregates of macrophages with
259 eosinophilic granular cells (EGCs) surrounding bacterial colonies (early stage) to well
260 organised, walled off, granulomas (later stage) (Figure 3 A - D). Hepatocellular degeneration
261 was also noted surrounding early lesions (Figure 3 D). Hearts from moribund fish exhibited
262 mild to moderate granulomatous endocarditis and some samples showed endocardial
263 hypertrophy. Intraluminal thrombi containing bacteria were also observed within the atrium
264 (not shown). Over 93.5 % (29 of 31) of the moribund and surviving infected fish that were
265 sampled at the termination of the trial had some degree of peritonitis (Figure 4 C & D),
266 characterised by an increase of EGCs infiltrating the peri-pancreatic fat cells. Control fish had
267 no pancreatic changes (Figure 4 A & B). Spleens of moribund and survivor fish had moderately
268 reactive ellipsoids, mild to moderate histiocytosis and splenic congestion (Figure 5 C & D),
269 which was not evident in control fish spleens (Figure 5 A & B). Spleens from the aAs infected
270 fish also contained a moderate infiltrate of EGCs. Interestingly, no apparent differences in
271 kidneys from both control and infected fish were observed. EGCs were found in the renal

272 interstitium of both groups. The intestines from the fish examined microscopically were also
273 very similar with many goblet cells in both control and infected groups. Control fish did not
274 present any pathology in the livers, hearts or spleens.

275 Gram-Twort stain confirmed the presence of dark pink, Gram-negative rod bacteria forming
276 large colonies within granulomas (Figure 6 C and D) localised in the same area of H&E stained
277 liver sections (Figure 6 A and B).

278 **PCR**

279 **16S PCR**

280 Initially 179 tissue samples (liver, spleen and kidney) from 64 fish were screened with a broad
281 range 16S rRNA PCR for detection of bacterial DNA (Table 4). Of these samples, 103 tissues
282 (from 59 individuals) had bacterial DNA present, specifically, 39/103 (37.9%) in the liver,
283 23/103 (22.3%) in the spleen, and 41/103 (39.8%) in the kidney. The majority of these positive
284 samples were subclinical individuals (33/59) that had survived the challenge trial while the rest
285 were moribund individuals (18/59) and control fish (8/59).

286 ***vapA* PCR**

287 In total, 30 out of 179 (16.8 %) fish tissues (liver, spleen and kidney) screened for *As* species
288 –specific PCR design based on the *vapA* gene of *As* were positive for the bacterium. From
289 the 30 *As* positive organs (from 21 individuals) 13/30 (43.3 %) were detected in the liver, 7/30
290 (23.3 %) in the spleen, and 10/30 (33.3 %) in the kidney of these fish. Ten out of the 21 fish
291 (48.0 %) had survived the challenge trial (subclinical individuals) and 11 (52.0 %) were
292 moribund (Table 4). All control samples tested (30) were negative. There were no differences
293 in aAs detection between the individuals challenged with either of the two aAs *vapA* type V
294 stains. All the individuals challenged with Vibrionaceae isolates were negative for presence of
295 aAs.

296 **aAs type V-specific PCR**

297

298 All control samples tested (30) were negative for aAs type V. In total, 29 out of 64 fish had the
299 expected PCR product for aAs type V (254 base pairs) confirming the presence of this type in
300 the tissues tested (43.5% of liver, 19.6% of spleen and 36.9% of kidney samples). From the
301 29 positive fish, 16 (55.2%) had survived the challenge trial and 13 (44.8 %) were moribund
302 fish (Table 4). There were no discernible differences in the number of positive samples from
303 fish challenged with either of the two aAs *vapA* type V stains. All the individuals challenged
304 with Vibrionaceae isolates were negative.

305 ***As vapA* qPCR**

306 In total, 13 of the 28 fish (or 46.4 %) that were positive by a *As vapA* qPCR were moribund
307 fish and 15 (or 53.6 %) were survivors (subclinical) during the challenge trial. Of the 28 positive
308 fish, 12 (42.8 %) had a high bacterial load with a Crossing point (Cp) value between 18 and
309 24. The liver (16/41; 39.0 %) and kidney (15/41; 36.6 %) samples had higher bacteria loads
310 than the spleen (10/41; 24.4 %). There were no differences between bacteria load for
311 individuals challenged with either of the two a*As vapA* type V strains. All control samples tested
312 (30) were negative.

313 **Discussion**

314 Bacterial disease outbreaks have impeded advancement of the ballan wrasse industry in
315 Scotland. In the present work one of the two a*As vapA* type V strains was more virulent during
316 the bath challenge experiment developed herein. Infected fish displayed clinical signs,
317 however the disease impacts were more remarkable upon post-mortem examination. Internal
318 lesions in challenged fish were severe including multi focal granulomas in the liver,
319 hepatomegaly and splenomegaly. Eosinophilic granular cells and macrophages were the main
320 leukocytes involved in inflammatory response following histological examination. In contrast,
321 bath challenge of ballan wrasse with *V. anguillarum*, *Alliv. salmonicida* and *Phot. indicum*, did
322 not result in notable mortalities, despite challenging under similar conditions as a*As* isolates.

323 The significantly higher mortality rates following bath challenge with a*As* compared to all other
324 bacterial species tested in this study, indicates that juvenile ballan wrasse are more
325 susceptible to this bacterium. Both a*As* strains used in the experimental infection were *vapA*
326 type V, which is a predominant *vapA* type (in addition to VI) to which ballan wrasse have been
327 reported susceptible to (Gulla et al., 2016a; Papadopoulou et al., 2020). Fish were exposed
328 to high doses of bacterial broth cultures (2.84×10^7 CFU / mL for strain TW 4 / 14 and $1.93 \times$
329 10^7 CFU / mL and 2.04×10^8 CFU / mL for strain TW 3 / 14), as required for a successful bath
330 challenge model. The cumulative mortalities were higher for strain TW 4 / 14 than TW 3 / 14
331 at similar doses, which suggested that the former strain was more virulent than the latter even
332 though they belong to the same *vapA* type (V). This is in agreement with our previous findings
333 following an i.p. injection challenge trial (Ramirez Paredes et al., 2020). In addition, in this
334 study, no histological differences, or notable changes in a*As* prevalence, were noted between
335 fish samples collected from fish challenged with the different strains. Furthermore, pure
336 cultures of the bacterium (a*As*) grew on TSA plates from kidney swabs taken from 10 % of
337 moribund fish from each tank and no control fish died throughout the experiment, confirming
338 that a*As* was causing the disease in infected individuals. In a previous study, a*As* (*vapA* type
339 V) induced 75 – 89 % mortalities after i.p. injection in 50 g ballan wrasse and 51 % mortality

340 with a cohabitation infection challenge (Biering et al., 2016). In the same study aAs (*vapA* type
341 VI) was notably less virulent by cohabitation challenge (8 %) while i.p. caused 70 – 85 %
342 mortality. This higher mortality rate is, however, not surprising as i.p. administration of the
343 pathogen bypasses the mucosal tissue barriers (e.g. skin, gill and gut) and results in higher
344 mortalities in fish (Embregts & Forlenza, 2016; Adams, 2019).

345 Gross pathology was similar in fish from all tanks challenged with aAs strains. Exophthalmia
346 and eroded fins were two of the most predominant clinical signs in conjunction with lethargy,
347 loss of equilibrium and inappetence. Externally the response of infected ballan wrasse to aAs
348 following the bath challenge was less severe in comparison with other fish species like
349 salmonids in which large furuncles on the skin of infected fish are often observed (Austin &
350 Austin, 2016). Similarly, the external clinical disease appears less severe than that reported
351 for other non - salmonids species where the clinical picture includes ulceration in cod
352 (Magnadottir et al., 2002), black rock fish (*Sebastes schlegelii*) (Han et al., 2011), sailfin
353 sandfish (*Artoscopus japonicas*) (Wada et al., 2010) and granulomatous dermatitis in turbot
354 (Farto et al., 2011; Coscelli, Bermúdez, Losada, et al., 2014; Coscelli, Bermúdez, Silva, et al.,
355 2014). Internally, however, the pathological impacts of aAs are clear whereby the liver, spleen
356 and hearts of infected ballan wrasse were the most affected organs in this study.
357 Hepatomegaly and splenomegaly was noted macroscopically while internally white nodular
358 lesions were present in these organs.

359 In a recent report of viral haemorrhagic septicaemia (VHS) in the Shetland Islands in Scotland,
360 the presence of EGCs in the pancreas of wrasse species, including ballan wrasse, was
361 described by Munro et al. (2015). Reite and Evensen (2006), in their review on fish
362 inflammatory cells, also reported that the eosinophilic component of leukocytes were dominant
363 in the *Labridae* family. The eosinophilic granular cells observed in the current study were
364 associated with a granulomatous response, surrounding the bacteria in the liver creating the
365 centre of the granulomas. The heart and kidneys were found to be the least affected, despite
366 aAs being present in a considerable proportion of kidney samples tested by PCR/qPCR, thus
367 perhaps indicating rapid clearance of the bacterium. Specifically, no changes were noted in
368 kidneys of control and infected individuals although EGCs were seen in both groups. Even
369 though the role of EGCs is not completely known in ballan wrasse, their function appears to
370 match mast cells in mammals which release chemical mediators and phagocytose foreign
371 particles (Reite, 1998).

372 The granulomatous response was also characterised by macrophage infiltration observed in
373 the liver, pancreas, and spleen of fish infected with aAs. Epithelioid macrophages created the
374 outer layers of the granuloma wall, separating the bacteria from the healthy hepatic cells.

375 Macrophage infiltration was also observed, replacing the normal splenic tissue, which caused
376 congestion and swelling of the organ. The livers of infected ballan wrasse also showed
377 increased vacuolation similar to the aAs infection in walking catfish (*Clarias batrachus*)
378 (Menanteau-Ledouble et al., 2016). Necrosis in the kidney, spleen and gut were previously
379 reported in salmonids during aAs exposure with an increased number of infiltrating
380 macrophages (Menanteau-Ledouble et al., 2016), however such changes were not observed
381 in the kidney or gut in the present study.

382 Bacterial presence in the tissues of infected and control fish was confirmed with a series of
383 molecular assays. Initially a broad range PCR (16S) was used to determine the presence of
384 bacteria DNA in the samples. *Aeromonas salmonicida* are categorised in 23 known A-layer
385 types and for this work *vapA* type V was detected using a *vapA* type V-specific assay
386 (Papadopoulou, 2019). Bacterial loads were also assessed with the *As* species specific qPCR
387 designed on the A-layer of the virulent array protein gene (Gulla et al., 2016b). In total, 59 out
388 of 64 fish (103 out of 179 tissue samples) had bacterial DNA in their tissues including 8 control
389 fish. However, none of the control fish showed a positive result when screened with the aAs
390 specific PCRs (*vapA* and type V) and the absence of mortalities in the control replicates during
391 the trial confirmed the lack of potentially confounding bacterial pathogens. In total 21 fish were
392 positive for the presence of *As* DNA (*vapA* PCR) while 26 were positive for *vapA* type V
393 specific PCR. The higher number of positive fish detected with the *vapA* type V specific PCR
394 may suggest a greater sensitivity of the assay for detecting aAs (type V) DNA in the samples
395 in comparison with *vapA* PCR. Lastly, the aAs loads were measured in the same samples by
396 qPCR. From 64 fish samples 28 were positive, making this assay the most sensitive in
397 comparison with the other two PCRs (*vapA* and *vapA* type V specific). This was expected as
398 the qPCR assay is able to detect as little as 7-8 *As* genomes (Gulla et al., 2016b). The varying
399 bacterial loads seen across different organs and samples most likely indicate different stages
400 of infection following the experimental challenge trial. This concurs with (Gulla et al., 2016b)
401 who also showed that qPCR did not always produce positive results when expected due to
402 variable stages of infection or when the DNA concentration of the bacteria is too low.

403 In this study, the Scottish *V. anguillarum* isolate (TW260/16) caused the second highest
404 cumulative mortality between the different bacteria species tested (4 %). Interestingly, no
405 mortalities were seen from the Norwegian *V. anguillarum* isolate in contrast to a previous trial
406 in Norway where higher mortalities (10 – 15 %) were reported in a bath challenge with two
407 strains of *V. anguillarum* (Biering et al., 2016). Variations in the genome of the bacterium and
408 country of origin of the fish may explain these results (Busschaert et al., 2015; Castillo et al.,
409 2017). The Norwegian isolate included in this study was expected to be more virulent, as
410 bacterial isolates are usually more virulent when coming into contact with hosts from different

411 geographical locations due to the lack of adaptation to the host species (Anderson & Siwicki,
412 1994). *Alliv. salmonicida* and *Phot. indicum* were less virulent to the fish resulting in 1 % and
413 0 % mortalities, respectively. *Alliv. salmonicida* is known to cause cold – water vibriosis in
414 Atlantic salmon and rainbow trout at temperatures between 3 and 10 °C (Egidius et al., 1981)
415 but it does not cause clinical disease at temperatures higher than 14 °C (Colquhoun et al.,
416 2002). This may explain the very low percentage mortality incurred to fish in the present trial
417 despite the fact that these isolates had been isolated from the host at similar ‘higher’
418 temperatures (15 °C). It could also indicate that the isolate in these circumstances acted as
419 an opportunistic pathogen to already immunocompromised hosts. However, it should be noted
420 that the lack of mortalities during the infection with *Phot. indicum* could also be related to low
421 numbers of viable bacteria in the suspension used for challenge (2.66×10^5 and 3.12×10^5
422 CFU / mL) than the target dose 10^8 CFU / mL. This is likely to be due to harvesting of the
423 bacteria towards the end of the exponential phase and perhaps including bacterial cells
424 harvested during the decline (death) phase. We previously noted at least 50 % mortalities in
425 juvenile ballan wrasse when infected by i.p. with *Phot. indicum* (Ramirez Paredez et al., 2020).
426 Growth curve testing and shorter incubation times (< 24 h) may help addressing the problem
427 we encountered in this study to enable reassessment of the pathogenicity of the isolate on
428 juvenile ballan wrasse by bath inoculation.

429 In conclusion, we successfully developed a bath challenge model for aAs *vapA* type V in
430 farmed ballan wrasse for the first time which can now be used as a model for vaccine efficacy
431 testing as it is not invasive and simulates natural bacterial portals of entry in contrast to i.p.
432 injection. The disease caused more severe internal than external pathology in comparison
433 with other species (salmonids and non – salmonids) infected with the same bacterial species.
434 Gross external and internal pathology, alongside histological changes of infected ballan
435 wrasse tissues with the bacterium aAs were described following experimental aAs bath
436 challenge conditions. Microscopically, granulomatous hepatitis with EGCs surrounding
437 bacterial colonies and endocarditis along with splenic histiocytosis in moribund and surviving
438 fish were observed. This bath challenge model is now being applied in immunocompetence
439 and vaccine efficacy studies for juvenile ballan wrasse.

440

441 **Acknowledgements**

442 The authors would like to thank Ms. Debbie Faichney at the Institute of Aquaculture, University
443 of Stirling (Stirling, UK) for her technical advice and support with histology. We would also like
444 to thank Ridgeway Biologicals for providing the bacteria isolates and Dr. Duncan Colquhoun
445 at Norwegian Veterinary Institute for providing the *V. anguillarum* isolate (12 – 50 2075 F383

446 – 1). In addition, we are thankful to MOWI Scotland and Scottish Sea Farms for providing the
447 experimental animals and for supporting the fish transportation. We also like also to thank Dr.
448 Mickael Teixeira Alves at Cefas for the statistical analysis and the aquarists and bacteriology
449 team at Cefas for their technical assistance during the trial.

450 **Funding information**

451 This project was co-funded through the Scottish Aquaculture Innovation Centre (SAIC) and
452 University of Stirling. Scottish Aquaculture Innovation Centre (SAIC) Grant number: SL-2015-
453 01.

454 **Conflict of interest Statement**

455 The authors declare no conflict of interest

456 **Data Availability Statement**

457 The data that support the findings of this study are available from the corresponding author
458 upon reasonable request.

459

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647 Table 1. Bacterial isolates used in this study. All bacterial isolates recovered from diseased
 648 ballan wrasse (*Labrus bergylta*) in Scotland with the exception of a single isolate recovered
 649 from the species in Norway.
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Bacterial species	Isolate ID	Year of isolation
<i>Aeromonas salmonicida</i> (vapA type V)	TW 3 / 14	2014
<i>Aeromonas salmonicida</i> (vapA type V)	TW 4 / 14	2014
<i>Vibrio anguillarum</i>	TW 260 / 16	2016
<i>Vibrio anguillarum</i>	12-50-2075 F383-1*	< 2014
<i>Aliivibrio salmonicida</i>	TW 189 / 16	2016
<i>Aliivibrio salmonicida</i>	TW 322 / 16	2016
<i>Photobacterium indicum</i>	TW 138 / 16	2016
<i>Photobacterium indicum</i>	TW 181 / 16	2016

651 (*) Norwegian isolate

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654 Table 2. Cumulative mortalities of ballan wrasse (*Labrus bergylta*) infected with atypical
 655 *Aeromonas salmonicida*, *Vibrio anguillarum*, *Allivibrio salmonicida* and *Photobacterium*
 656 *indicum*. No mortalities were recorded for control groups.

Bacteria sp.	Bacteria ID	CFU / mL	Cumulative mortalities (%)
<i>Atypical Aeromonas salmonicida vapA</i> type V	TW4/14	2.84E+07	52 ^b
			60 ^b
		1.93E+07*	20 ^a
	TW3/14	2.04E+08*	62 ^b
<i>V. anguillarum</i>	TW260/16	4.81E+07	4
			0
	12-50-2075 F383-1	2.58+08	0
			0
<i>Aliiv. salmonicida</i>	TW189/16	3.85E+07	2
			0
	TW322/16	4.09E+07	0
<i>Phot. indicum</i>	TW138/16	2.66E+05	0
			0
	TW181/16	3.12E+05	0
			0

657 (*) No duplicate tanks

658 (a,b) statistically significant difference at p <0.05.

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667 Table 3. Primers used for amplification of broad bacterial DNA, *Aeromonas salmonicida* and
 668 atypical *Aeromonas salmonicida vapA* type V specific with conventional PCR and primers for
 669 bacterial load with qPCR.

Gene	Oligo sequence	Annealing (°C)	Product size (bp)	Publication
Bacterial rRNA 16S	F341: CCTACGGGNGGCWGCAG R805: GACTACHVGGGTATCTAATCC	54.0	485	(Herlemann et al., 2011)
vapA partial	F2: CTGGACTTCTCCACTGCTCA R3: ACGTTGGTAATCGCGAAATC	53.0	625	(Gulla et al., 2016a)
vapA partial	Vspec– F:CAACGGTTTCTGGAGTAATAACTTT Vspec – R:TGCATCAGCAACAGCGGTAGT	57.0	254	(Papadopoulou et al., 2020)
vapA	F: ACTGTCTGTTACCCTGCCA-3' R: GCTACTTCACCCTGATTGG-3'	60.0		(Gulla et al., 2016b)

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685 Table 4. Ballan wrasse (*Labrus bergylta*) samples (liver, kidney and spleen) from moribund,
 686 survivor and control fish screened with a PCR for bacterial presence (16S), atypical
 687 *Aeromonas salmonicida* (aAs) specific (*vapA* type V) and aAs type specific PCR (*vapA* type
 688 V specific) as well as a qPCR for aAs loads.

689

Tissue / Fish status	Total	16S	<i>vapA</i>	V specific	qPCR
Liver	63	39	13	20	16
Spleen	53	23	7	9	10
Kidney	63	41	10	17	15
Tissues samples	179	103	30	46	41
Moribund fish	20	12	11	13	13
Survivors	34*	35	10	16	15
Control fish	10	8	0	0	0
Total number of fish	64	55	21	29	28

690 (*) includes 19 individuals challenged with TW 3 / 14 (dose 10^7) but mortalities did not reach
 691 a plateau at termination.

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693

694 **Figure 1.** Cumulative mortalities of ballan wrasse (*Labrus bergylta*) bath challenged with
695 atypical *Aeromonas salmonicida vapA* type V strain TW 3 / 14 (interrupted line) and TW 4 / 14
696 (continuous line).

697 **Figure 2.** Gross pathology and Internal gross pathology of infected ballan wrasse (*Labrus*
698 *bergylta*) with atypical *Aeromonas salmonicida* following bath challenge. A) Bilateral
699 exophthalmia and haemorrhage between the eyes, B) eroded pectoral fins and petechiae on
700 the body (arrow head).C) Dark red intestine (circle) D) hepatomegaly and white nodular
701 lesions in the liver (arrow heads).

702

703 **Figure 3.** Ballan wrasse liver infected with atypical *Aeromonas salmonicida*. A) Arrow heads
704 delineating three large granulomas composed of a thick wall of epithelioid macrophages (black
705 arrows) encircling degenerate and non-degenerate eosinophilic granular cells which
706 themselves surround large bacterial colonies B) The region highlighted in a box in A). Detail
707 of wall and centre of granuloma. Degenerate eosinophilic granular cells (E) surrounding
708 atypical *Aeromonas salmonicida* colonies (B), delineated by a dense rim of epithelioid
709 macrophages. Degenerate hepatocytes are present at the periphery (arrow heads) along with
710 small numbers of lymphocytes. C) Multiple poorly organised hepatic granulomas centred on
711 bacterial colonies. Note the moderate distension of hepatic sinusoids throughout the
712 parenchyma. D) Very early site of infection showing bacterial colonies surrounded by a fine
713 rim of karyorrhectic cellular debris (arrow heads), admixed with occasional macrophages,
714 EGCs and lymphocytes. Scale bar 100 µm for A) and C) and 20 µm for B) and D).

715 **Figure 4.** Hematoxylin and Eosin stained slides of ballan wrasse (*Labrus bergylta*) pancreases
716 and peritoneum fixed in 10% neutral buffered formalin from atypical *Aeromonas salmonicida*
717 bath challenge. (A) Healthy pancreas of ballan wrasse (B) Healthy pancreas of ballan wrasse
718 with adjacent fat cells (+), exocrine pancreatic cells, mild infiltrate of eosinophilic granular cells
719 (arrow head). (C) Pancreas of ballan wrasse with peritonitis in a fish infected with atypical

720 *Aeromonas salmonicida*. (D) Pancreas with infiltration of phagocytic macrophages and
721 eosinophilic granular cells replacing the peripancreatic adipose cells (arrow head). Scale bar
722 50 µm for A) and C) and 20 µm for B) and D).

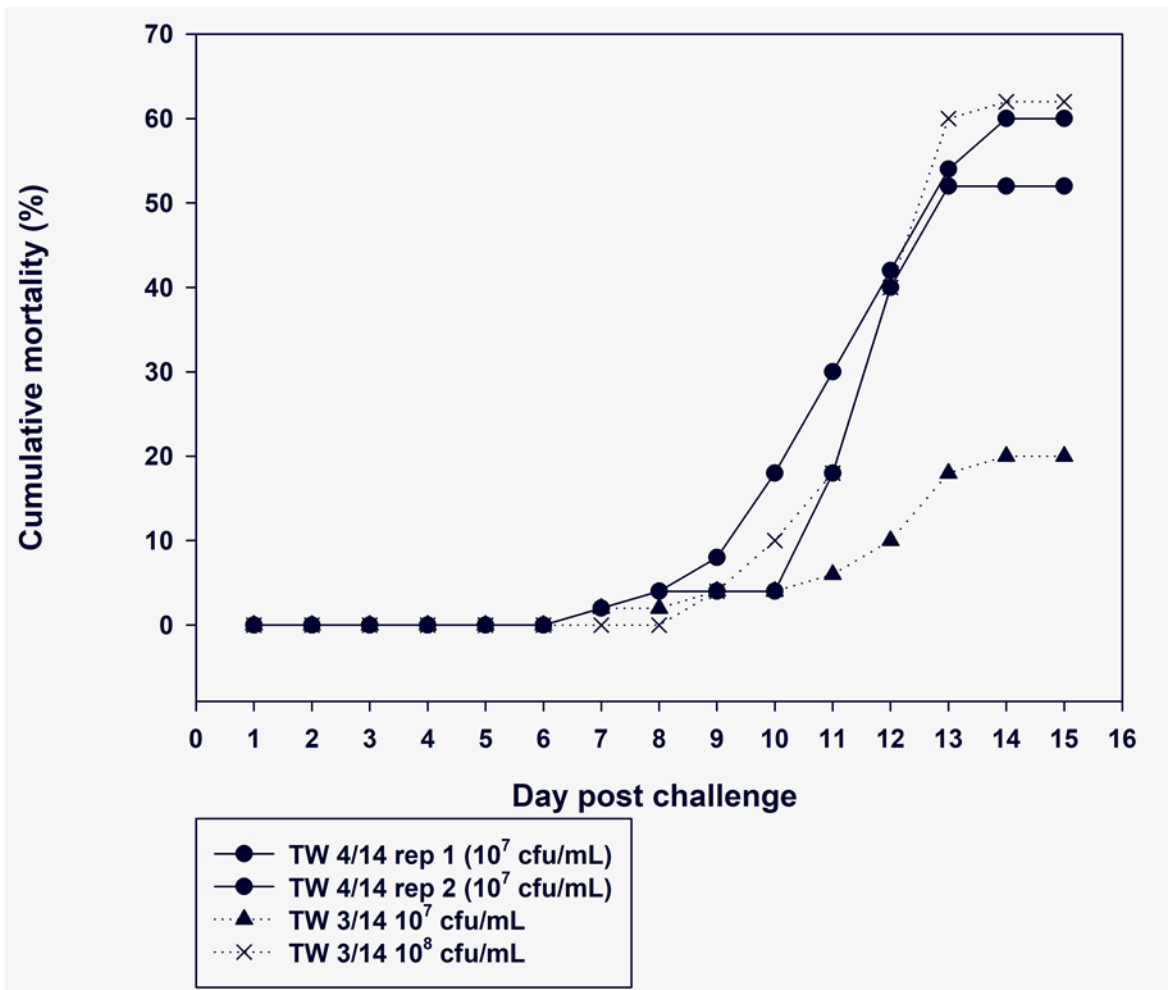
723 **Figure 5.** Ballan wrasse (*Labrus bergylta*) control and infected spleens with atypical
724 *Aeromonas salmonicida* stained with H&E. (A) Healthy spleen of a ballan wrasse with normal
725 splenic haematopoietic tissue and ellipsoids (black arrow). (B) Healthy spleen of a ballan
726 wrasse with visible erythrocytes and haematopoietic tissue. (C) Spleen of a ballan wrasse
727 infected with atypical *Aeromonas salmonicida* with infiltration of macrophages replacing the
728 normal splenic tissue. (D) Spleen of a ballan wrasse infected with atypical *Aeromonas*
729 *salmonicida* with mild congestion and macrophage infiltration (arrow head). Scale bar 20 µm
730 for A) and C) and 10 µm for B) and D).

731 **Figure 6.** Ballan wrasse (*Labrus bergylta*) livers infected with atypical *Aeromonas*
732 *salmonicida*. A and B Gram-negative bacilli-shaped bacteria (arrow head) inside granuloma
733 H&E (scale bar 20 and 10 µm, respectively). B) The region highlighted in a box in A), arrow
734 heads indicate bacilli-shaped bacteria. C and D Gram-negative bacilli-shaped bacteria (arrow
735 head) inside granuloma. D) The region highlighted in a box in C), arrow heads indicate Gram-
736 negative bacilli-shaped bacteria (scale bar 20 and 10 µm, respectively).

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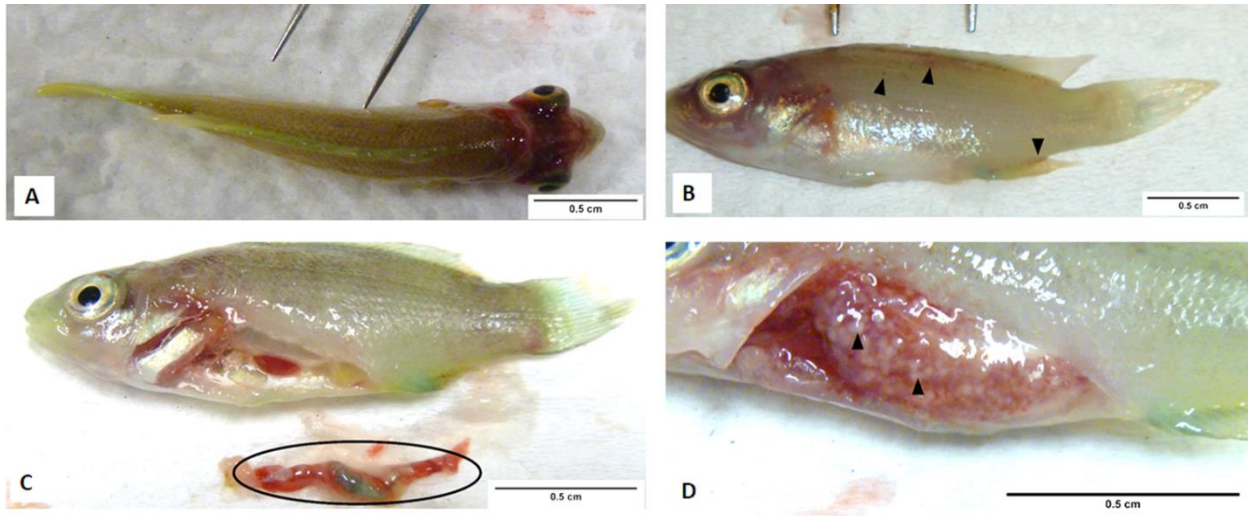
739 Figure 1



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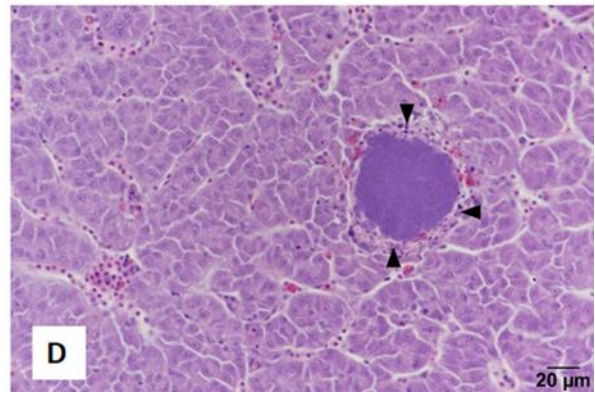
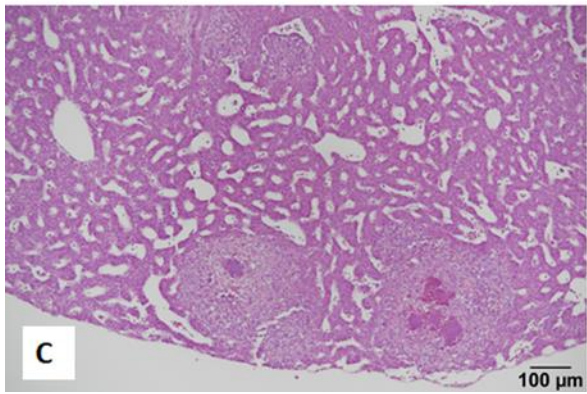
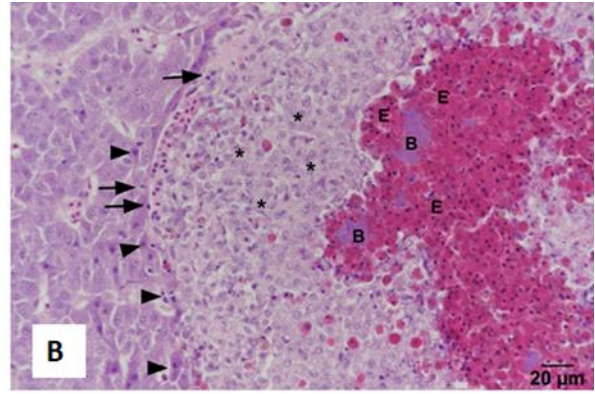
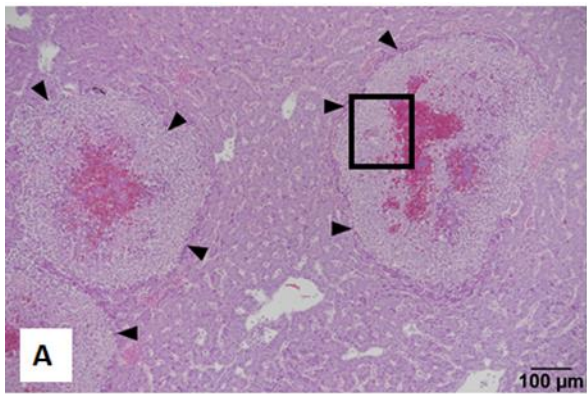
742 Figure 2



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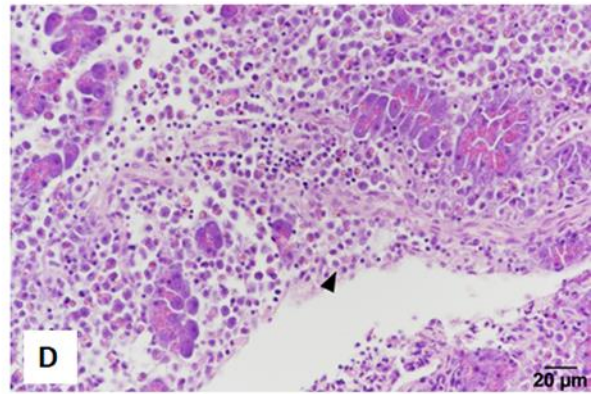
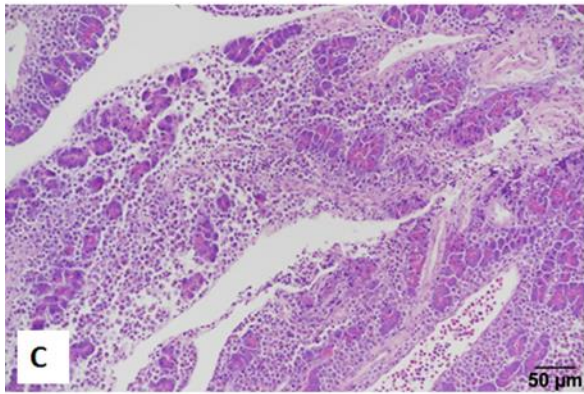
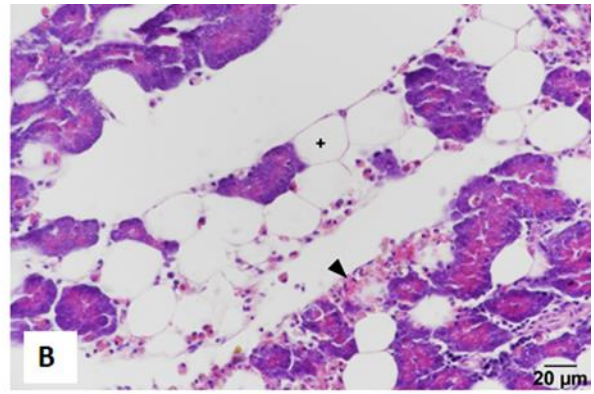
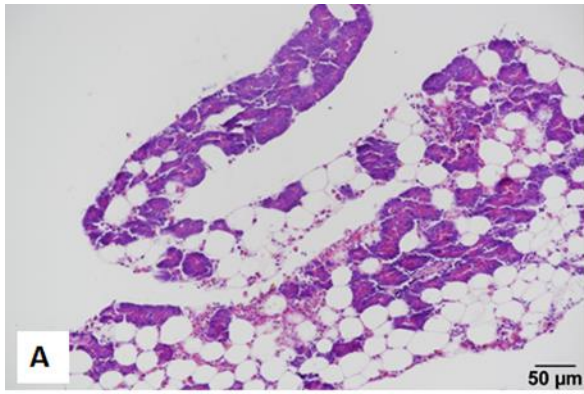
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745 Figure 3

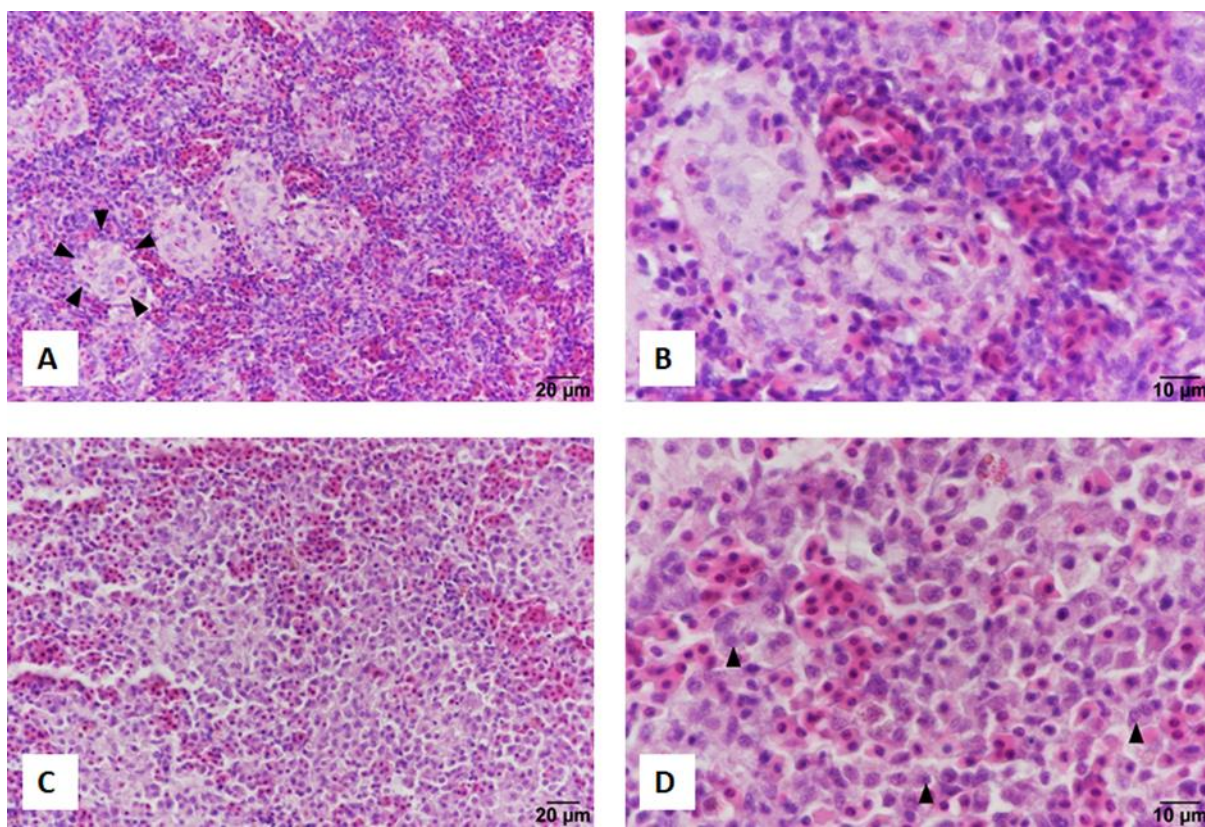


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751 Figure 5



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