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Individual monitoring of immune responses in rainbow trout after cohabitation and intraperitoneal injection challenge with *Yersinia ruckeri*

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1 **Title: Individual monitoring of immune responses in rainbow trout after cohabitation and**
2 **intraperitoneal injection challenge with *Yersinia ruckeri***

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24 **Keywords:** Non-lethal sampling, *Yersinia ruckeri*, intraperitoneal injection, cohabitation,
25 cytokines, antimicrobial peptides.

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26 ## 1. Abstract

27 *Yersinia ruckeri*, the causative agent of enteric red mouth disease (ERM), is a widely studied
28 pathogen in disease models using rainbow trout. This infection model, mostly based on
29 intraperitoneally injection or bath immersion challenges, has an impact on both components
30 (innate and adaptive) of the fish immune system. Although there has been much attention in
31 studying its host-pathogen interactions, there is still a lack of knowledge regarding the impact of a
32 cohabitation challenge. To tackle this we used a newly established non-lethal sampling method
33 (by withdrawing a small amount of blood) in rainbow trout which allowed the individual immune
34 monitoring before (non-infected) and after infection with *Yersinia ruckeri* either by intraperitoneal
35 (i.p.) injection or by cohabitation (cohab). A range of key immune genes were monitored during
36 the infection by real-time PCR, and results were compared between the two infection routes.
37 Results indicated that inflammatory (IL-1β1 and IL-8) cytokines and certain antimicrobial
38 peptides (cathelicidins) revealed a different pattern of expression between the two infected groups
39 (i.p. vs cohab), in comparison to adaptive immune cytokines (IL-22, IFN-γ and IL-4/13A) and β-
40 defensins. This suggests a different involvement of distinct immune markers according to the
41 infection model, and the importance of using a cohabitation challenge as a more natural disease
42 model that likely simulates what would occur naturally in the environment.

43 ## 2. Introduction

44 *Yersinia ruckeri* is the causative agent of enteric red mouth disease (ERM), also known as
45 yersiniosis, affecting mainly salmonids. Infection with this bacterium can cause high mortalities in
46 salmonid aquaculture leading to significant economic losses in the trout farming industry [1-3].
47 The bacteria enters the host initially through the epithelial gill cells [4]. The disease causes general
48 septicaemia in the host as bacteria spread through the body via the blood, resulting in an
49 inflammatory response in most tissues, with haemorrhaging on the surface of the body and oral
50 cavity, the latter giving the name “red mouth” to the disease [2, 3]. Host inflammatory responses

51 in rainbow trout infected with *Y. ruckeri* have been reported through the up-regulation of a range
52 of inflammatory cytokines such as interleukin (IL)-1 family members, IL-6, IL-8, IL-10 and IFN-γ
53 [3, 5, 6]. Another component of immunity has also been investigated, namely cytokines potentially
54 involved in adaptive immune responses, such as IL-22 and IL-17A/F2, that are reported to be
55 induced after infection with the same pathogen [7, 8]. While most studies have focused on
56 analysing responses in lymphoid tissues, knowing that blood plays a crucial role in spreading the
57 bacteria, it is suggested that more attention should be paid to this site of immune cells. In fact,
58 work by Raida et al [9] and Collet et al [10] undertook gene expression analysis in blood samples
59 and confirmed that blood sampling also allows a sensitive detection of immune markers for the
60 purpose of health monitoring. Although many studies have been undertaken in the past years to
61 investigate host-pathogen interactions that occur during ERM infection, most studies focussed on
62 pathogen challenges using an intraperitoneal (i.p.) injection [5, 7, 8] or bath immersion of the
63 bacteria [6, 8, 9]. In the natural environment, transmission of ERM occurs through direct contact
64 between fish and infected carriers, with *Y. ruckeri* able to survive and remain infective in the
65 aquatic environment for long periods of time (more than 4 months), through its presence in faeces
66 of infected animals and microfilms [3, 11]. Cohabitation is a more natural infection model that
67 resembles what occurs in nature and is well established for other bacterial diseases such as
68 *Aeromonas salmonicida*, where it is recommended for use in vaccine efficacy testing [12, 13].
69 However, reported studies undertaking a cohabitation challenge with *Y. ruckeri* are very limited,
70 and are focussed on testing the efficiency of probiotic diets in promoting disease resistance [14].
71 To simulate what would occur naturally in the environment, a cohabitation challenge model was
72 undertaken in this study and the impact on a range of host immune genes determined. A recently
73 reported non-lethal sampling method in Atlantic salmon [10] and rainbow trout [15] was adapted
74 in this study, allowing sequential withdrawal of small amounts of blood from infected fish, to
75 study key aspects of immunity (e.g. innate and adaptive immune components) in the same
76 individual throughout the bacterial challenge. In addition, a comparison of the level of immunity

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77 observed between non-injected (cohabitants) and i.p. injected (shedders) fish at the transcript level
78 was undertaken, providing further insights into the impact of the route of infection on fish immune
79 responses.

80 **3. Methods**

81 *Fish maintenance*

82 Rainbow trout (*Oncorhynchus mykiss*) (average weight 245.6±0.64 g; average total length 275.6±
83 0.19 cm) were purchased from Almond bank (Perthshire) and maintained in 1-m diameter aerated
84 fibreglass tanks supplied with a continuous flow of recirculating freshwater at 14 ± 1 ºC within the
85 aquarium facility in the School of Biological Sciences (University of Aberdeen). Fish were fed
86 twice daily on standard commercial pellets (EWOS, Scotland), and were given a 1-week
87 acclimatisation period prior to treatment. Prior to the bacterial challenge, fish were transferred into
88 3 tanks (20 fish/tank) in the freshwater aquarium pathogen containment facility and fed as above,
89 except on the day of the challenge. After the acclimatisation period, fish were anaesthetised with
90 MS222 (0.08 g/L, Sigma-Aldrich, U.K.) and a Passive Integrated Transponder (PIT)-tag
91 (BioMark, Europe) was inserted into the peritoneal cavity to allow individual identification. Fish
92 were allowed to recover for at least 1 week after this procedure.

93 *Bacteria and challenge experiment*

94 A pathogenic strain (MT3072) of the Gram-negative salmonid pathogen *Yersinia ruckeri* was
95 used. A stock of bacteria stored in phosphate buffered saline (PBS) containing 15% glycerol was
96 prepared and maintained at -80°C as described previously [16]. Briefly, 2 days before the
97 challenge an aliquot of the bacteria was grown in tryptic soy broth medium at 22 ºC in a shaker
98 incubator. After 2-days, the bacterial suspension was serially diluted in sterile PBS until the stock
99 bacteria contained ~10^7 colony forming units (cfu)/mL. For the challenge, thirty rainbow trout
100 were anaesthetised with MS222 (as described above), and injected intraperitoneally (i.p.) with 6.8
101 x 10^6 cfu/mL of *Y. ruckeri* in PBS (0.1 mL/fish). The fish were divided into 3 tanks (10 fish/tank).
102 Ten further fish were added into each tank, which were not injected with bacteria, to act as

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103 cohabitating fish. Past studies using rainbow trout in cohabitation challenges have used a range of
104 ratios of infected to cohabitants, from 1:5 [14] to 3:1 [17] according to the pathogen used and the
105 purpose of the study (e.g. testing vaccine efficacy). In this study, since the purpose was to evaluate
106 and compare the immune response observed in both cohabitant and injected groups, a ratio of 1:1
107 was used. Fish were checked several times a day post-challenge and removed from tanks when at
108 least two signs of the disease were observed to avoid any unnecessary suffering, and were
109 humanely euthanized (Schedule 1 killing method) when appropriate. External symptoms included
110 haemorrhages in the oral cavity, reddening at the base of the fins, abnormal swimming behaviour
111 and dark coloration as reported by Kumar et al [3].

112 *Individual monitoring through non-lethal sampling*

113 Thirty fish from each group (shedders and cohabitants) were anaesthetised with MS222 (0.08 g/L,
114 Sigma-Aldrich, U.K.) and identified with a PIT-tag reader (Biomark, Europe). The sampling
115 method followed a similar procedure to that reported recently [15]. In detail, 150 µL of blood
116 (<1% blood volume) was collected from the caudal vein immediately before the infection with
117 bacteria was undertaken, corresponding to the uninfected day 0 (D0) sampling point. This
118 provided a control for each individual fish that would not be possible with lethal sampling. Blood
119 was also collected 3 days after infection (D3) and at a terminal point (between days 4 and 7), when
120 fish died or were euthanized after showing at least two external signs of disease. After sampling,
121 blood was immediately transferred into heparinised vaccutainers (Midmeds Ltd, U.K.) and kept on
122 ice until RNA extraction was undertaken. Fish were monitored closely after sampling until they
123 were fully recovered from the anaesthesia.

124 *Verification of the cause of death*

125 Fish started to show symptoms of the disease from day 3 after infection, and all fish died or were
126 euthanized within 7 days post-infection. To confirm cause of death/morbidity swabs were taken
127 from the kidney of a proportion of the fish and plated onto tryptic soy agar plates, and incubated
128 for 48h at 22 ºC. Colony PCR was performed using species specific primers for the *Y. ruckeri* 16s

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129 RNA (**Table 1**) and the products visualised after electrophoresis in an agarose gel containing
130 ethidium bromide. DNA was extracted from head kidney samples using a DNA extraction kit
131 (Qiagen, U.K.), performed using the manufacturer's instructions. The head kidney bacterial
132 burden of all samples was assessed by real-time PCR using *Y. ruckeri* 16s RNA specific primers,
133 with the data normalised to the expression of the host MCSF1 gene as described by Harun et al
134 [18] and Gibello et al [19] (**Table 1**). Due to the occurrence of high mortalities observed from day
135 3 and to the inability of obtaining a fresh blood sample from all fish after this time point, only 22
136 fish were successfully bled on three occasions per treatment group and used for real-time PCR
137 analysis.

138 *RNA extraction and cDNA synthesis*

139 RNA from blood samples was extracted using an adapted method from that given in the RNeasy
140 Mini kit (Qiagen, U.K.). Briefly, 30 µL of blood were lysed in RLT buffer (RNeasy kit, Qiagen,
141 U.K.) containing 10 % (v/v) β-mercapto-ethanol (Sigma-Aldrich, U.K.), and homogenised in a
142 Tissue Lyser using a 5-mm bead (Qiagen, U.K.) for 1 min at 25 Hz at room temperature. The
143 remaining steps were undertaken following the manufacturer's instructions, and RNA was eluted
144 in 75 µL of RNAse-free water (Qiagen, U.K.) and stored at -80ºC until further use. cDNA was
145 synthetized using M-MuLV Reverse Transcriptase (New England Biolabs, U.K.) and oligo-d$(T)_{16}$
146 primer (Applied Biosystems, Europe). For this 8 µL of RNA (approximately 0.5 µg) were mixed
147 with 1 µL 10 mM dNTPs (Applied Biosystems, Europe) and 2 µL H_2O, incubated at 65ºC for 5
148 min, and put immediately on ice. The final volume was adjusted to 20 µL by adding Reverse
149 Transcriptase buffer, 10 mM DTT, 0.5 mM each dNTP, 0.5 U RNase inhibitor (Applied
150 Biosystems, Europe) and 200 U M-MuLV Reserve Transcriptase (New England Biolabs, U.K.).
151 The final mix was incubated at 95ºC for 5 min and then at 37ºC for 90 min. The obtained cDNA
152 was stored at -20ºC until further use.

153 *Real-time PCR and data analysis*

154 Real-time PCR was performed using IMMOLASE (Bioline, U.K.) and SYBR Green fluorescent
155 tag (Invitrogen, U.K.) in a LightCycler® 480 System (Roche Applied Science, U.K.). To obtain
156 the expression level of the genes of interest, a standard curve was constructed for each immune
157 gene using serially diluted purified PCR products, amplified from cDNA. The obtained standards
158 were run in duplicate in the same 96-well plate along with the cDNA samples from the challenged
159 fish, and served as reference for quantification. Transcript level was calculated using the
160 integrated software. The relative expression of immune genes was calculated as arbitrary units and
161 normalised against the expression level of rainbow trout elongation factor (EF)-1α, a house
162 keeping gene. All normalised data were multiplied by 1,000 due to the low level of expression
163 found in certain genes. Primers for real-time PCR were designed and pre-tested to ensure they
164 could not amplify genomic DNA, as described by Wang et al [20]. Immune markers selected for
165 real-time PCR analysis included cytokines involved in inflammatory (IL-1β1, IL-6 and IL-8), and
166 adaptive immune responses, with key markers for each putative T_H subset selected (IFN-γ, IL-
167 4/13A, IL-22 and IL-17A/F2). The antimicrobial peptides β-defensin (BD)-3 and BD-4, and
168 cathelicidins (Cath)-1 and Cath-2 were also analysed in this study. The bacterial load in blood was
169 also evaluated by real-time PCR, which has been reported to allow a successful detection of *Y.*
170 *ruckeri* in blood samples [9, 21]. Sequences of primers used are listed in **Table 1**.

171 *Statistical analysis*

172 The expression data was analysed statistically using a general linear model (GLM) for repeated
173 measures test to determine the overall time effect of infection and to evaluate the interaction
174 between delivery routes. Data were Log2 transformed in order to meet the GLM assumptions of
175 homogeneity, sphericity and independency. Statistical significance was taken as a P value of
176 <0.05. Additionally, a paired T-test was used to test for significant differences between the
177 bacterial load in cohabitation and i.p. injection infected groups, where a $P < 0.05$ indicated
178 significant differences between the two groups (n=22). In addition to analysing gene changes
179 during infection, a correlation analysis of expressed genes in infected fish was undertaken to

180 investigate the relationship between gene expression and pathogen load. For this a correlation
181 between bacterial load in blood and gene expression level of immune markers was analysed
182 separately in fish infected by cohabitation or injection, calculating the Pearson correlation
183 coefficient (r) with a $P < 0.05$ (2-tailed) considered statistically significant. A Log2 transformation
184 was also undertaken to improve normality in the data, and the transformed data is presented in the
185 figures. Lastly, a correlation study was applied to compare the pathogen load between terminal
186 head kidney samples and blood. All statistical analysis was performed with SPSS software
187 Version 23 (IBM Corporation, USA).

188 **4. Results**

189 In this study a non-lethal sampling method was used to compare two distinct routes of infection
190 (i.p. injection vs cohabitation) with *Yersinia ruckeri*, the causative agent of enteric red mouth
191 disease (ERM). Thirty rainbow trout (10 per tank) were challenged by i.p. infection with *Y.*
192 *ruckeri* strain MT3072, and were left to cohabitate with 30 non-infected fish (10 per tank). Results
193 revealed that mortalities (fish found dead or killed by a Schedule 1 method) were first observed 3
194 days after infection in the i.p. injected group (**Figure 1**) when 5 mortalities occurred. Mortalities
195 in the cohabitation group were observed 1 day later (7 mortalities). The peak of mortalities
196 occurred at days 4 (10 mortalities) and 5 (17 mortalities) for the injection and cohabitation groups,
197 respectively. The experiment was terminated at 7 days post-infection, since all fish were either
198 found dead or were culled due to the presence of symptoms of the disease, such as haemorrhages
199 in the oral cavity, abnormal swimming behaviour and reddening at the base of the fins by this
200 time. Internally, fish revealed symptoms such as haemorrhaging, enlarged spleen and inflamed
201 tissues. Due to the observed high mortalities only 22 fish per treatment group were successfully
202 sampled on three occasions and used to perform cDNA synthesis and real-time PCR analysis.

203 To confirm the pathogen load in the host, the head kidney was collected at the terminal
204 sampling point and the bacterial load evaluated by real-time PCR. Results indicated that the

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205 expression of *Y. ruckeri* 16s RNA relative to the host MCSF1 expression was significantly higher
206 (121-fold increase) in i.p. injected fish relative to fish infected by cohabitation (**Figure 2A**). Since
207 a non-lethal sampling method was used, it was also possible to detect the presence of the bacteria
208 in the blood during the course of this experiment, by detecting the expression of *Y. ruckeri* 16s
209 RNA relative to the EF-1α expression (**Figure 2B**). For this a small volume of blood was
210 collected from naïve rainbow trout before (corresponding to a true baseline control) and after
211 infection with bacteria. Blood samples from the forty-four rainbow trout where the full range of
212 samples had been collected (i.e. day 0, day 3, and at the terminal point), were analysed.
213 Quantification of the *Y. ruckeri* 16s RNA in blood indicated that the detection of bacterial load
214 increased significantly ($P < 0.01$) at day 3 and the final sampling point post-infection (using a
215 repeated measures statistical test), but no bacteria were detected in either treatment group at day 0
216 (before infection) (**Figure 2B**). A significant interaction between route of infection and pathogen
217 load ($P < 0.01$, F = 13.14) at the terminal time point was also noted, where the injected fish had a
218 higher pathogen load compared to the cohabitation group. Correlation analysis comparing the
219 pathogen load obtained in the head kidney and blood in these rainbow trout at the terminal
220 sampling point (**Figure 3**), showed a significant positive correlation ($r = 0.537$) in the injected fish
221 but not in the cohabitation fish.

222 Expression of selected immune genes was undertaken using real-time PCR in order to
223 enable an overall evaluation of immunity over time in the same individual fish after challenge, and
224 to investigate the effect of the route used for infection (i.p. vs cohabitation). The expression of IL-
225 1β1, a potent pro-inflammatory cytokine, using a repeated measures test indicated that its
226 expression was significantly affected over time ($P < 0.01$, F = 13.1) (**Figure 4A**, **Table 2**). We
227 also observed that there was a significant interaction between time and infection route ($P < 0.01$, F
228 = 11.4), where the i.p. injection group (average relative expression of 49.88, **Supplementary**
229 **Material**) showed a significant up-regulation of IL-1β1 expression in comparison to the
230 cohabitation group (average relative expression of 4.5, **Supplementary Material**). A similar

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231 pattern of expression was observed while monitoring another pro-inflammatory cytokine, IL-8
232 (**Figure 4A, Table 2**). An additional cytokine involved in inflammatory reactions (IL-6) was
233 analysed (**Figure 4A, Table 2**), and although it revealed a similar pattern of expression over time
234 ($P < 0.01$, F = 48.62), there was no significant difference between the injected and cohabitation
235 groups ($P > 0.05$, F = 0.83) (**Table 2**). Cytokines representative of each putative T_H cell subset
236 were also analysed (T_H1, T_H2 and T_H17). Monitoring of IFN-γ, a key T_H1 cytokine, indicated that
237 there was an effect of infection time on its expression ($P < 0.01$, F = 69.5) (**Figure 4B**). However,
238 there was no interaction between time after infection and the route used ($P > 0.05$, F= 2.4), with
239 both cohabitation and injected groups having an upregulation of IFN-γ expression at the terminal
240 time point. A representative of the T_H2 subset, IL-4/13A was also investigated and real-time PCR
241 results revealed that although there was an effect of infection over time, there was no difference
242 between the infected groups (**Figure 4B, Table 2**). When analysing two putative T_H17 cytokines
243 (IL-22 and IL-17A/F2), the results indicated that IL-22 expression was significantly up-regulated
244 ($P < 0.01$, F = 117.1) at the terminal time point, with average relative expressions of 0.84 and 0.59
245 observed for the injected and cohabitation infected groups, respectively (**Figure 4B,**
246 **Supplementary Material**). However, there was no significant difference between the two
247 infection routes ($P > 0.05$, F = 0.65). In the case of IL-17A/F2, only low expression values were
248 obtained in the blood samples (Cp values higher than 35), and so the results were considered
249 unreliable (**Figure 4B, Table 2**). These findings suggest that the route of infection did not affect
250 the expression of these cytokines of adaptive immunity.

251 In addition to the analysed cytokines, several antimicrobial peptides (β-defensins and
252 cathelicidins) were also investigated, due to their important role in mucosal immunity against
253 bacterial pathogens. Regarding the effect of the challenge on the β-defensins (BD-3 and BD-4), a
254 similar pattern was observed whereby there was a significant effect ($P < 0.01$, F = 21.2 and $P <$
255 0.01, F = 40.9, respectively) of the bacterial infection on their expression during the experiment
256 (**Figures 4C**) but no statistical difference was found between the two routes used for infection.

257 Another antimicrobial peptide family, the cathelicidins (Cath-1 and Cath-2), was analysed and
258 both genes studied revealed a similar effect (**Figures 4C**, **Table 2**). However, interestingly, a
259 significant difference ($P < 0.01$) between the two infected groups was found, with a higher
260 significant induction of both Cath-1 and Cath-2 expression in the injected group (average
261 expression of 1.3 and 1.25, respectively) vs the cohabitation group (average expression of 0.06
262 and 0.14, respectively) at the terminal time point (**Figures 4C**, **Supplementary Material**).

263 To evaluate the relationship between immune gene expression and pathogen load a
264 correlation analysis was undertaken using data from all time points. Results indicated that the
265 inflammatory cytokines IL-1β1 and IL-6 showed a positive correlation (statistically significant)
266 with the pathogen load in the i.p. infected group ($r = 0.455$ and $r = 0.543$, respectively) (**Figures**
267 **5A and 5B**). When analysing the cohabitation group, no correlation was observed for IL-6
268 expression whereas IL-1β1 was negatively correlated ($r = -0.403$) (**Figures 6A and 6B**). A similar
269 result was observed when analysing IL-8, where a positive correlation ($r = 0.573$) was only
270 observed in the i.p. group but not in the cohabitant fish (**Figures 5C and 6C**). The adaptive
271 immune cytokines (IFN-γ, IL-4/13A and IL-22) showed a different pattern, where a positive
272 correlation between their expression and the bacterial load was observed in both treatment groups
273 (**Figures 5D-F and 6D-F**) and was highly significant (i.e. $P < 0.01$). A significant correlation
274 pattern was also observed for BD-4 (**Figures 5H and 6H**), with both cohabitant and i.p. groups
275 being positively correlated with pathogen load, whereas BD-3 was only correlated in the i.p. group
276 (**Figures 5G and 6G**). In the case of the cathelicidins Cath-1 and Cath-2, a significant correlation
277 between the i.p. group and pathogen load was also found but again was not observed in the
278 cohabitant group (**Figures 5I, J and 6I, J**).

279 ## 5. Discussion

280 In this study a recently established non-lethal sampling method was used to monitor aspects of the
281 immune response in individual fish before (control) and after bacterial infection. The pathogen

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282 used was *Y. ruckeri*, the causative agent of ERM, an economically important disease in the trout
283 farming industry [1-3]. The only study to date undertaking a similar approach to evaluate immune
284 responses in individual rainbow trout, was a study by Raida et al [9]. However, the design of their
285 experiment was significantly different, as only one non-lethal sampling was undertaken (at day 3
286 post-infection) and fish were subjected to a bath (immersion) challenge. The method used in this
287 study, of withdrawing a small volume of blood (150 μL), has been recently optimised in Atlantic
288 salmon [10] and was found to be very successful to monitor cytokine expression individually in
289 fish experimentally infected with a virus.

290 In the present study mortalities resulting from the disease were first recorded in the
291 intraperitoneally (i.p.) injected group at day 3, and started one day later in the cohabitation group.
292 This is in agreement with previous studies where rainbow trout infected by injection or bath
293 showed the first mortalities from day 3 [4, 9, 18]. That the mortalities in the cohabitation group
294 began from day 4 may be due to the high ratio (infected vs non-infected) used in this study. In
295 terms of bacterial load, it was possible to detect the presence of *Y. ruckeri* 16s RNA in blood 3
296 days post-infection, and at the terminal time point, by real-time PCR. This was also seen by Raida
297 et al [6] who detected the presence of this bacterium (~ 0.25×10^5 cfu/mL) as early as 2 days post-
298 infection by plating 10 μL blood samples onto blood agar plates. The injected group had a higher
299 expression level of *Y. ruckeri* 16s from both blood and head kidney samples collected at the
300 terminal sampling point, when compared to the cohabitation group, as expected. However, this
301 route of delivery is probably not a good model of natural infection compared to bath or
302 cohabitation. For example, injection bypasses natural mucosal defences of the host, such as skin
303 and mucus [22]. Indeed, studies of vaccine efficacy in salmonids have used cohabitation as a
304 model of infection and show that this model, as well as bath challenge, mimics well what occurs in
305 the environment/ fish farms [12, 13, 23]. Findings in this work also suggest that, as reported by
306 others [3, 11], transmission of ERM might occur through direct contact between i.p. infected and
307 non-infected fish, possibly by shedding the bacterium through faeces. However, other

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308 transmission routes, such as the formation of biofilms, are also possible. This highlights the
309 importance of using cohabitation disease models for future studies to provide further clues about
310 the mode of transmission of this bacterium.

311 It has been suggested that the spleen plays a major role against bacterial pathogens, such as
312 *Y. ruckeri*, with its enlargement during infection due to potential recruitment of immune cells
313 activated by inflammatory cytokines [6, 18, 24, 25]. However, a recent study showed that *Y.*
314 *ruckeri* can be detected in the blood and gill epithelial cells within 1 minute post-infection using
315 Optical Projection Tomography and immunohistochemistry [4]. In the same study, severe
316 septicaemia was observed 7 days post-infection, with bacteria found in a wide range of organs
317 such as liver, spleen and heart [4]. This suggests that blood aids the rapid spread of the infection
318 into the internal organs. Indeed up-regulation of inflammatory cytokine transcript levels has been
319 reported in the blood of *Y. ruckeri* bath challenged trout, sampled 3 days post-infection, that
320 subsequently did not survive [9]. Therefore, in this study an optimised non-lethal blood sampling
321 approach was used to evaluate the immune responses between cohabitation and i.p. injection
322 challenged fish, by monitoring blood samples before (control) and after infection (infected group).

323 Real-time PCR analysis of blood samples found that the pro-inflammatory cytokines IL-
324 1β1 and IL-8 were induced over time, during the infection. Other studies [6, 9, 18] also found that
325 both cytokines were highly up-regulated at different time-points upon i.p. injection or bath
326 challenge with *Y. ruckeri* in spleen and blood confirming their involvement in the host response to
327 bacterial infection. Our previous work using a similar sampling method with virus infected
328 Atlantic salmon reported that none of these cytokines were induced during the course of the
329 experiment, with blood sampling points at 0, 4, 8, 12, 16, 21 and 25 days after challenge [10]. This
330 demonstrates that the blood sampling procedure is not inducing cytokine transcription by itself and
331 that very different host responses are seen dependent upon the pathogen encountered. In this study
332 we also show that the route of infection (injection vs cohabitation) used had a differential effect on
333 the transcript level of IL-1β1 and IL-8, with the injected group revealing a high induction over the

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334 non-injected fish. It is known that injection with bacteria induces an influx of neutrophils and
335 macrophages into the peritoneal cavity of rainbow trout, where they mediate phagocytosis [6, 26].
336 This confirms the relevance of the infection method used and its effect on the secretion of
337 inflammatory cytokines, such as IL-1β1 and IL-8, in order to attract phagocytes (e.g. neutrophils)
338 to the local site of infection [27, 28]. The findings were in agreement with the positive correlation
339 observed between the pathogen load and the gene expression of IL-1β1 and IL-8 in the i.p. group,
340 which was not observed in the cohabitants. In fact, a negative correlation was observed for IL-1β
341 in the cohabitant fish.

342 Interestingly, a different result was observed when analysing the adaptive immunity
343 cytokines, IL-22, IFN-γ and IL-4/13A, where such cytokines showed a similar pattern of induction
344 in both injected and cohabitation groups. This was in agreement with the correlation analysis, with
345 the three cytokines showing a positive significant correlation with the bacterial load in both
346 injected and cohabitation groups. This suggests that a common mechanism of adaptive defence is
347 induced independent of the infection route. Previous studies have also reported the involvement of
348 these cytokines upon infection with *Y. ruckeri* by i.p. injection [6, 7, 17] and bath challenge [29],
349 suggesting the response is of a multi-faceted nature during lethal bacterial infections where the
350 host may throw everything in its' armoury at the pathogen in a last ditch attempt to survive.
351 Possibly these cytokines are secreted into the host's circulation to promote both local and systemic
352 immunity via innate lymphoid cell or T_H-driven responses. Overall, findings in this study
353 emphasize the importance of undertaking a comparative study between two commonly used
354 delivery routes and provides an insight for future studies aiming at evaluating the efficacy of
355 vaccines.

356 In addition to the studied cytokines, several antimicrobial peptides, likely involved in
357 promoting innate immune responses against bacterial infections were also studied [30, 31]. The
358 results showed that both β-defensins studied (BD-3 and BD-4) had a similar pattern of expression,
359 being induced in both infection models. Since it is expected that infection by cohabitation will

360 allow the host surface defences to respond to the pathogen, this may explain why molecules
361 potentially involved in mucosal immunity, such as β-defensins [31], as well as IL-22 [7], may be
362 highly induced using this challenge route. Moreover, trout recombinant IL-22 has been shown to
363 be induce the expression of both BD-3 and BD-4 in spleen, confirming the potential relationship
364 between these molecules [7]. To obtain a more general understanding of the roles of AMPs in the
365 responses two further genes from the cathelicidin family (Cath-1 and Cath-2) were analysed and,
366 interestingly, revealed a differential expression pattern dependent upon the route of infection, with
367 injected fish having a significantly higher induction vs the cohabitation fish. This result was
368 confirmed by the correlation analysis, where a significant positive correlation between the
369 bacterial load and gene (Cath-1, Cath-2) expression was observed only in the injected fish. A
370 study by Bridle et al [32] reported that cathelicidins were able to stimulate the expression of IL-8
371 in Atlantic salmon peripheral blood leucocytes, and suggested that both Cath-1 and Cath-2 are
372 involved in promoting local responses by recruiting immune cells through IL-8 activation. The
373 similar patterns of gene expression induction observed here between cathelicidins and IL-8 are in
374 concordance with these findings. Moreover, since injection of pathogens bypasses the natural
375 mucosal defences, it is possible that cathelicidins may have a more important role in promoting
376 immune responses when infections become systemic, whereas β-defensins may be important for
377 both local and systemic defence.

378 In conclusion, this study used a non-lethal sampling method to compare the immune
379 responses elicited in two infection models, i.p. injection and cohabitation. Immune gene transcript
380 levels indicated that adaptive cytokines have a different pattern of expression when compared to
381 pro-inflammatory cytokines, dependent on the infection model. Interestingly, the antimicrobial
382 peptides studied also act differently in these two infection models, indicating different defence
383 mechanisms are activated when mucosal or systemic pathogen detection occurs. Thus, in future
384 studies attention should be drawn to the infection models used, taking into consideration that
385 cohabitation models likely reflect a more natural infection route. However, to provide a more

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386 comprehensive study, future work should also include infection by immersion, which would
387 highlight any potential differences/similarities between two less-invasive infection models.

388 **6. Competing interests**

389 The authors declare that they have no competing interests.

390 **7. Acknowledgements**

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392 Animals in Research (NC3Rs, grant G1100675). The authors are grateful to the aquarium staff at
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395

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492

493 **Figure legends**

494 **Figure 1.** Cumulative mortality of rainbow trout following bacterial challenge with *Yersinia*
495 *ruckeri* (MT3072). Thirty rainbow trout were infected with *Y. ruckeri* by intraperitoneal injection
496 (10^6 cfu/mL) or by cohabitation (cohab).

497 **Figure 2.** Pathogen load in head kidney and blood of fish infected with *Y. ruckeri*. **A.** Total DNA
498 was extracted from head kidney of bacterially infected rainbow trout by cohabitation (cohab)
499 (*n*=22) or intraperitoneal injection (*n*=22) at the terminal sampling point. Real-time PCR analysis
500 was undertaken by detecting the expression of *Y. ruckeri* 16s RNA gene and normalising it to the
501 host gene MCSF1. A paired T-test was performed, with asterisks indicating a significant
502 difference ($P < 0.05$) between cohab and injected groups. **B.** RNA was extracted from rainbow
503 trout blood samples collected before (D0) or after infection with *Y. ruckeri*, at 3 days post-
504 challenge (D3) and at the terminal point (between days 4 and 7) when fish succumbed to the
505 disease (Dterm). Synthesized cDNA was used for real-time PCR, normalising the *Y. ruckeri* 16s
506 RNA gene expression to the house keeping EF-1α gene. Results are averages + standard error
507 (*n*=22 per treatment group). Asterisks indicate significant differences ($P < 0.01$) relative to day 0

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508 (D0). The hash symbol indicates there is a significant ($P < 0.01$) difference between the infection
509 routes, as determined using a general linear model (GLM) for repeated measures.

510 **Figure 3.** Correlation of bacterial load in the head kidney and blood from rainbow trout infected
511 with *Y. ruckeri* by intraperitoneal injection (*n*=22) or cohabitation (cohab) (*n*=22), at the terminal
512 time point. Pearson correlation *r* coefficients are given relative to *Y. ruckeri* load in the blood. A
513 significant correlation ($P < 0.05$) is indicated with an asterisk.

514 **Figure 4.** Expression analysis of selected immune genes in individual trout during *Y. ruckeri*
515 infection by cohabitation (cohab) or intraperitoneal injection. Blood samples were collected before
516 infection (Control, D0), at 3 days post-infection (D3) and at a terminal sampling time (Dterm,
517 between 4 to 7 days post-challenge). RNA was collected for real-time PCR expression analysis, of
518 pro-inflammatory (**A**) (IL-1β1, IL-6 and IL-8) and adaptive immunity (**B**) (IFN-γ, IL-4/13A and
519 IL-22) cytokines, as well as antimicrobial peptides (**C**) (BD-3, BD-4 and Cath-2) genes. Results
520 are presented individually with averages (*n*=22 fish per group) shown as black and grey bars for
521 the injection (i.p.) and cohabitation groups, respectively. Cath-2 was selected to be presented
522 graphically over Cath-1 because it showed a stronger interaction between the routes of infection.
523 Refer to **Table 2** for stats and **Supplementary Material** for relative expression values.

524 **Figure 5.** Correlation of bacterial load and selected immune genes in blood of fish infected with *Y.*
525 *ruckeri* by intraperitoneal injection, across at all time points. Immune markers include IL-1β1 (**A**),
526 IL-6 (**B**), IL-8 (**C**), IFN-γ (**D**), IL-4/13A (**E**), IL-22 (**F**), BD-3 (**G**), BD-4 (**H**), Cath-1 (**I**) and Cath-
527 2 (**J**). Pearson correlation *r* coefficients are given relative to *Y. ruckeri* load in the blood.
528 Significant correlations ($P < 0.05$) are in bold and indicated with an asterisk. *n*=66 samples (22
529 fish per time point).

530 **Figure 6.** Correlation of bacterial load and selected immune genes in blood of fish infected with *Y.*
531 *ruckeri* by cohabitation, across at all time points. Immune markers include IL-1β1 (**A**), IL-6 (**B**),
532 IL-8 (**C**), IFN-γ (**D**), IL-4/13A (**E**), IL-22 (**F**), BD-3 (**G**), BD-4 (**H**), Cath-1 (**I**) and Cath-2 (**J**).

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533 Pearson correlation *r* coefficients are given relative to *Y. ruckeri* load in the blood. Significant
534 correlations ($P < 0.05$) are in bold and indicated with an asterisk. n=66 samples (22 fish per time
535 point).

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Table 1. Oligonucleotide primers used for expression analysis

	Forward primer (5′ TO 3′)	**Reverse primer (3′ TO 5′)**	**Accession No:**
16s RNA (*Y. ruckeri*)	GCGAGGAGGAAGGGTTAAGTG	GAAGGCACCAAGGCATCTCT	X75275
MCSF1	ACCCCGTCTGCCACGAATGA	CAGCTTGGCCCCAGCAACAG	AM901600
EF-1α	CAAGGATATCCGTCGTGGCA	ACAGCGAAACGACCAAGAGG	AF498320
IL-1β1	CCT GGA GCA TCA TGG CGT G	GCTGGAGAGTGCTGTGGAAGAA CATATAG	AJ278242
IL-4/13A	ACCACCACAAAGTGCAAGGAGT TCT	CACCTGGTCTTGGCTCTTCACAA C	FN820501
IL-6	CCTTGCGGAACCAACAGTTTG	CCTCAGCAACCTTCATCTGGTC	DQ866150
IL-8	AGAATGTCAGCCAGCCTTGT	TCTCAGACTCATCCCCTCAGT	AJ310565
IL-17A/F2a	CGTGTCGAAGTACCTGGTTGTGT	GGTTCTCCACTGTAGTGCTTTTCC A	AJ277604
IL-22	ACAGCAGGTGGCTCAACATGCG	CCTTTCCCCTCCTCCATCTCGGA	AM748538
IFN-γ	CAAACTGAAAGTCCACTATAAGA TCTCCA	TCCTGAATTTTCCCCTTGACATAT TT	AJ616215
BD-3	GCTTGTGGAATACAAGAGTCATC TGC	GCATACATTCGGCCATGTACATC C	FM212657
BD-4	TGGTGCTCCTCGCTTTCTTGG	TGGGCGACACAGCATACAAATC	FM212658
Cath-1	ACCAGCTCCAAGTCAAGACTTTG AA	TGTCCGAATCTTCTGCTGCAA	AY594646
Cath-2	ACATGGAGGCAGAAGTTCAGAA GA	GAGCCAAACCCAGGACGAGA	AY542963

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Table 2. Summary of immune gene expression in *Y. ruckeri* infected rainbow trout

Gene	Average Cp Control D0		Overall time effect	Interaction between i.p. and cohab
EF-1α	12.55	F value *P* value	N/A	N/A
IL-1β1	21.14	F value *P* value	**13.08** 0.000	**11.37** 0.000
IL-4/13A	25.79	F value *P* value	**72.79** 0.000	0.907 0.408
IL-6	28.71	F value *P* value	**48.62** 0.000	0.832 0.439
IL-8	25.27	F value *P* value	**26.45** 0.000	**4.98** 0.009
IL-17A/F2a	37.69	F value *P* value	N/A	N/A
IL-22	31.25	F value *P* value	**117.14** 0.000	0.646 0.527
IFN-γ	29.80	F value *P* value	**69.48** 0.000	2.434 0.094
BD-3	23.03	F value *P* value	**21.17** 0.000	0.267 0.767
BD-4	30.29	F value *P* value	**40.88** 0.000	0.021 0.979
Cath-1	25.71	F value *P* value	**14.45** 0.000	**5.02** 0.009
Cath-2	27.89	F value *P* value	**8.13** 0.001	**7.89** 0.001

*Statistically significant differences are highlighted in bold.

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Figure 1.

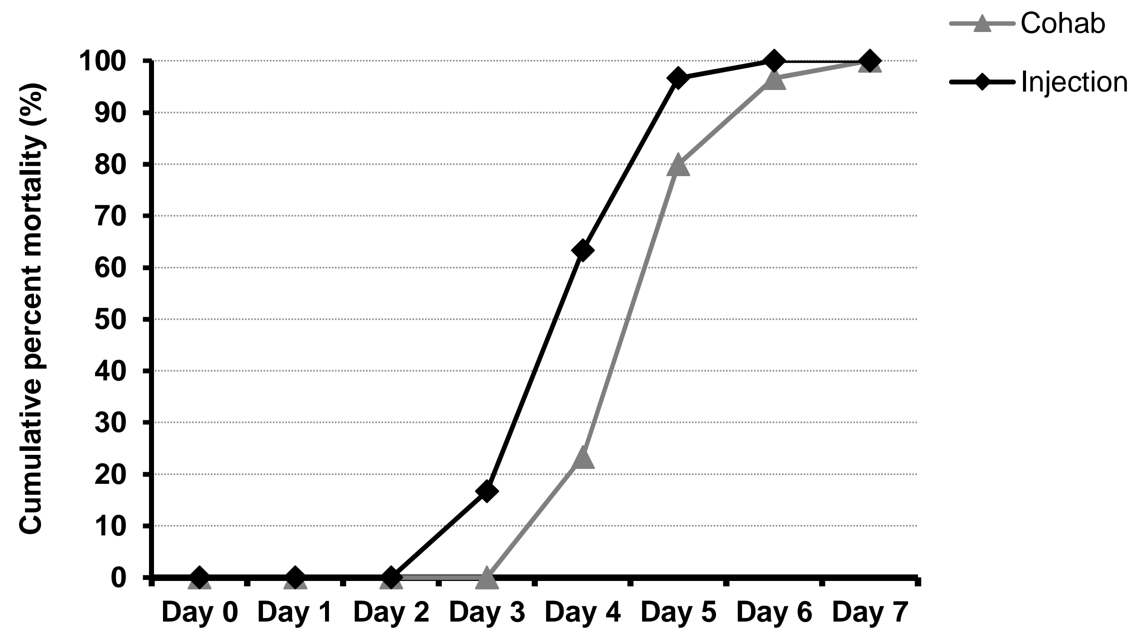


Figure 2.

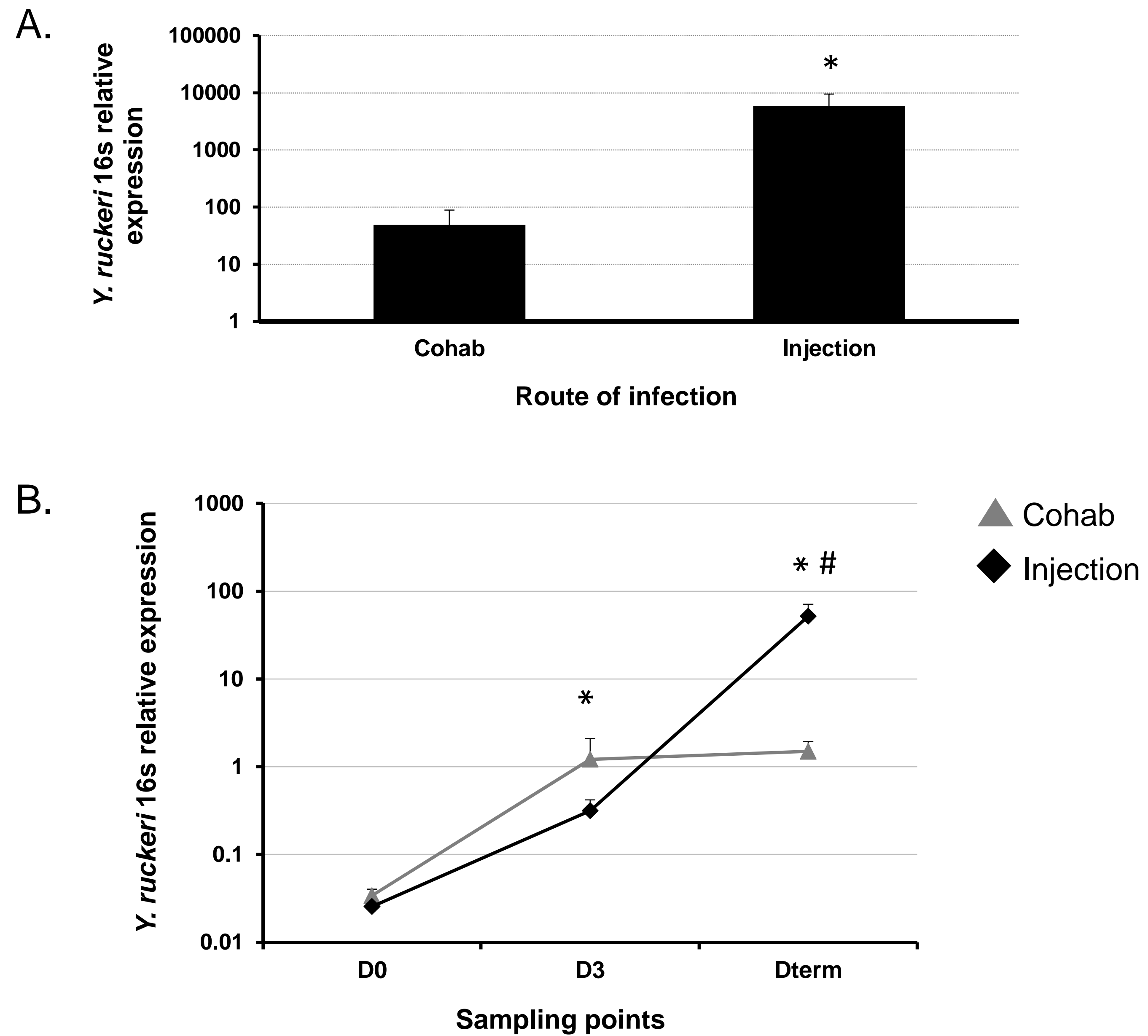
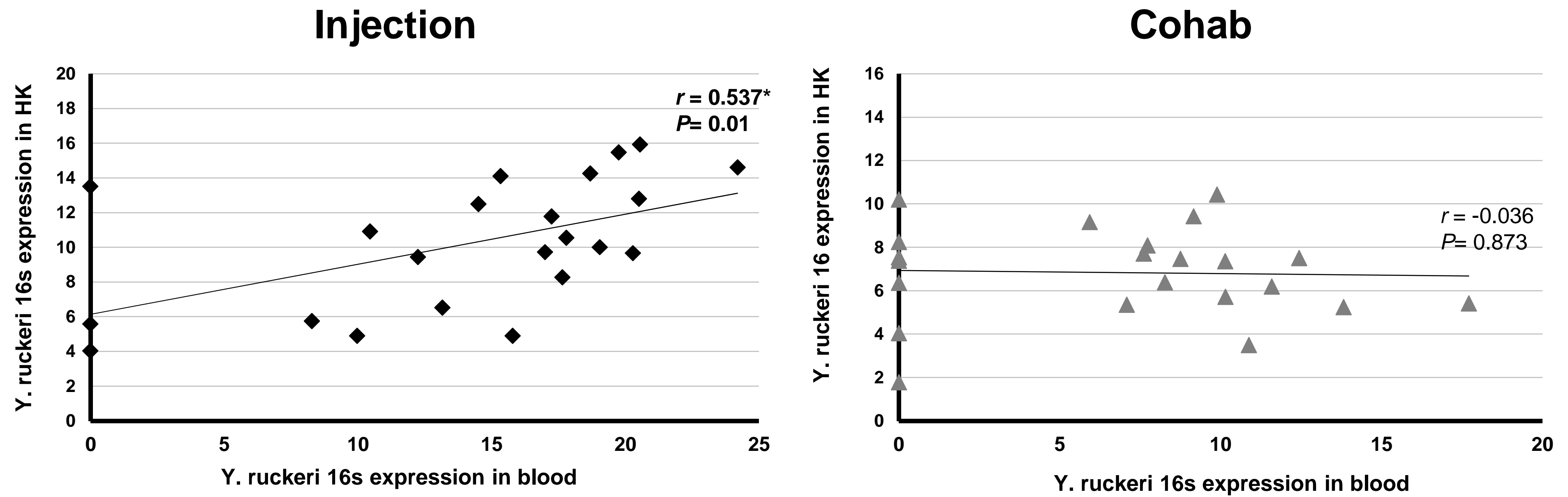


Figure 3.



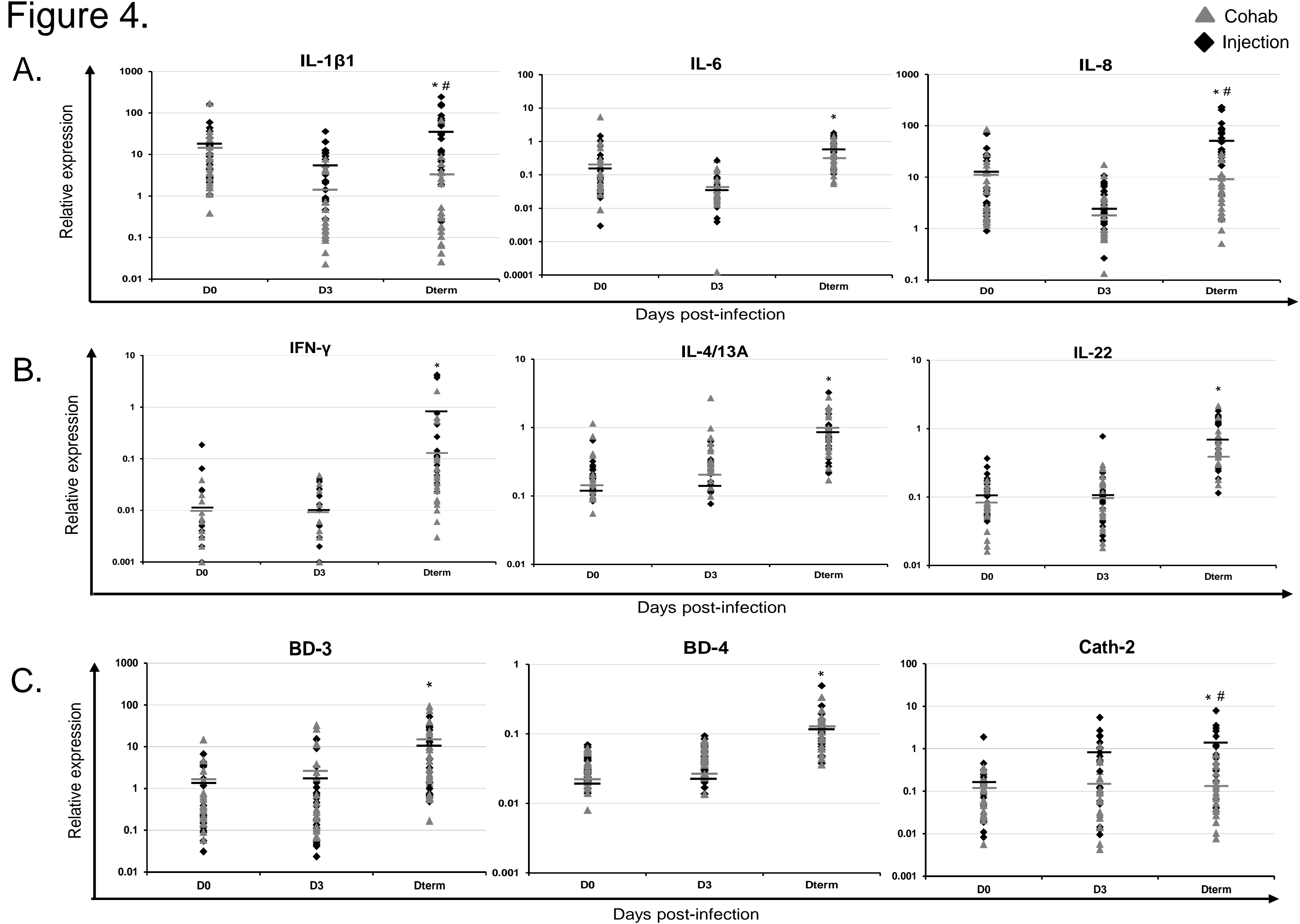


Figure 4.
Cohab
Injection
A.
IL-1β1
IL-6
IL-8
Relative expression
D0
D3
Dterm
Days post-infection
B.
IFN-γ
IL-4/13A
IL-22
C.
BD-3
BD-4
Cath-2

Figure 5.

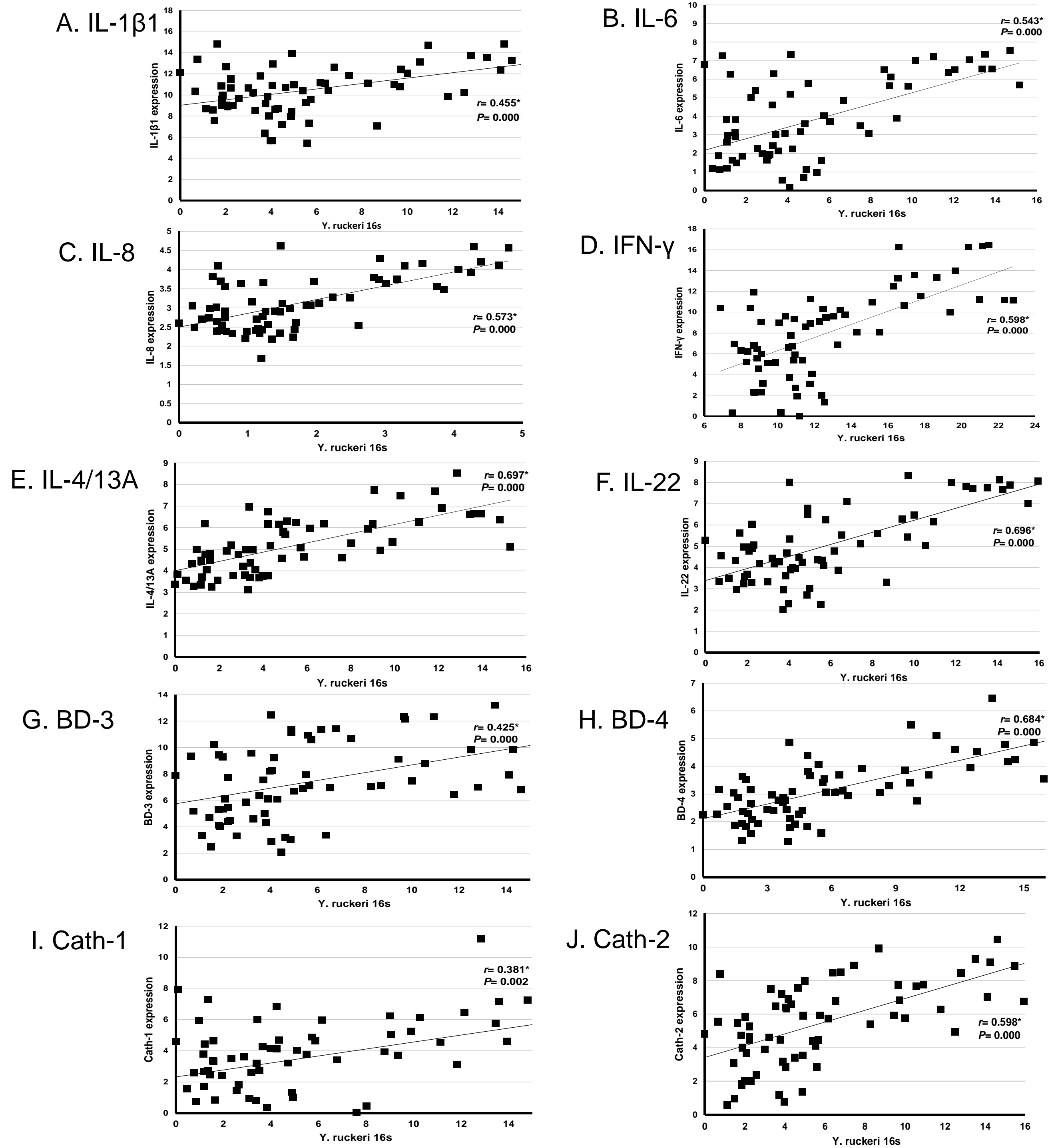


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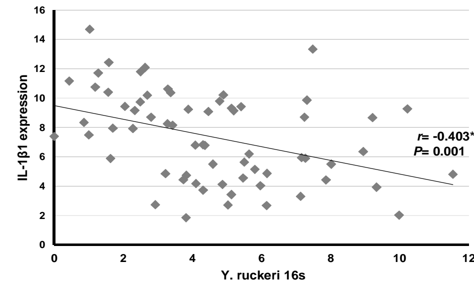
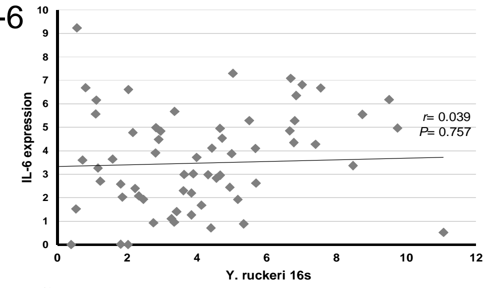
Figure 6.

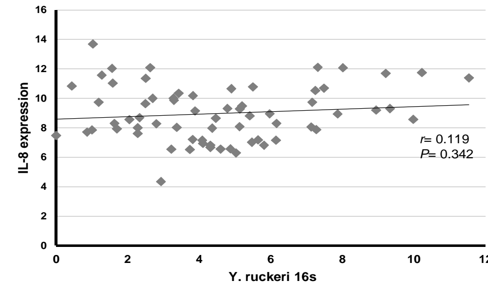
A. IL-1β1



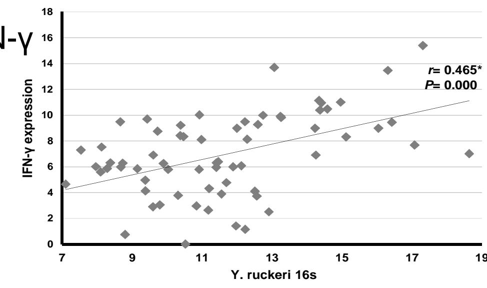
B. IL-6



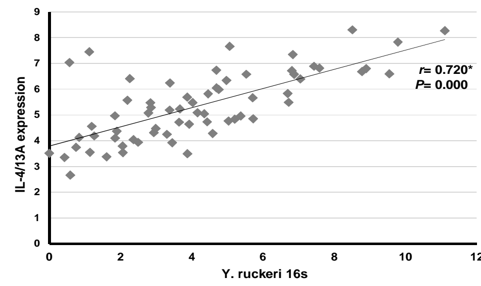
C. IL-8



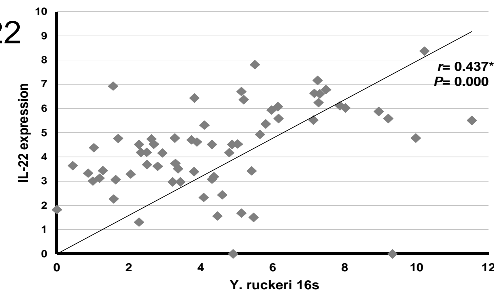
D. IFN-γ



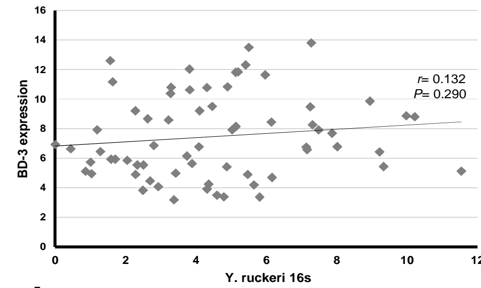
E. IL-4/13A



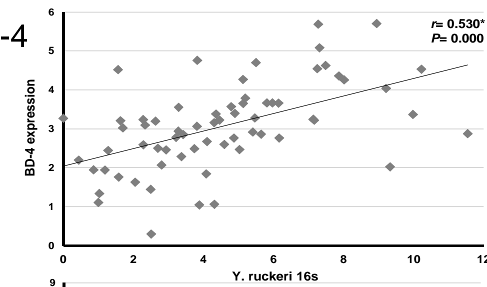
F. IL-22



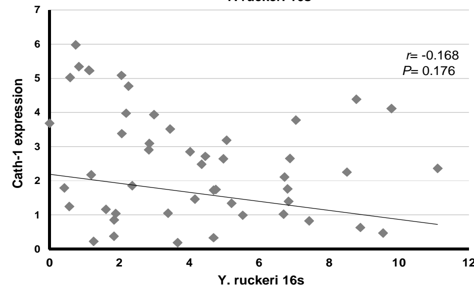
G. BD-3



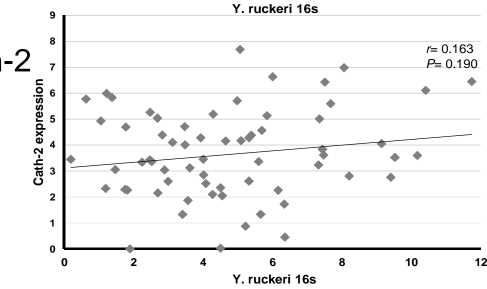
H. BD-4



I. Cath-1



J. Cath-2



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Highlights

- This study reports sequential immune monitoring of individual fish upon infection;
- Two infection models (i.p. injection and cohabitation) were used in this study;
- IL-1β and IL-8 showed a distinct regulation in blood depending on the infection route;
- Adaptive immunity cytokines revealed a similar expression in both infection models;
- Cathelicidins and β-defensins act differently depending on the infection model.

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