Heritable and environmental influences on innate immunity across three populations of threespine stickleback (*Gasterosteus aculeatus*)

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6 **Keywords: threespine stickleback, innate immunity, ecoimmunology, microbial environment,**

7 **16S DNA, granulocytes, ROS.**

8 **Abstract**

9 Environmental variation plays a key role in immune development and function; factors such as

10 pathogen exposure history, seasonality, and resource availability all affect an individual's immune

11 phenotype. However, the relative contributions of heritable and non-heritable factors remain unclear

12 for most immune phenotypes. We used populations of threespine stickleback (*Gasterosteus*

13 *aculeatus*) with heritable differences in immune function to investigate the relationship between

14 immunity, genetic divergence, and the environment. Fish were raised in tanks with different flow

15 rates (continuous or intermittent) that harbored differing microbial communities. After long-term

16 acclimation to one tank environment, a subset of adult fish were moved to the alternate flow regime

17 and allowed to acclimate for eighteen weeks. We then measured the effects of starting environment,

18 transfer between environments, and final environment across several immune parameters. Both

19 population and treatment significantly affected immune function. Sticklebacks from a population

previously found to display the highest heritable levels of innate immunity displayed the highest oxidative burst capacity (ROS) regardless of tank environment. However, all fish in intermittent flow tanks (both resident and transfers) tended to have higher ROS production, more granulocytes, and greater spleen mass than in continuous flow. Variation in liver mass was mainly driven by population effects. Together, this work demonstrates that a simple change in water flow dynamics can shift aquarium microbial conditions, creating opportunities to explore immune flexibility. Perhaps more importantly, we also highlight the need for further research examining how naturally evolved genetic differences and environmental factors individually and jointly influence the magnitude and direction of immune responses.

Introduction

Differential immune responses facilitate an organism's ability to maintain high fitness in the face of changing pathogen risk. While some elements of immunity are highly innate, others are influenced primarily by either environment or by genotype-by-environment interactions. Ecoimmunology has traditionally focused on environmental factors such as seasonality, resource availability, and exposure to pathogens (1). This is largely due to difficulty in identifying heritable differences in wild organisms, which is further complicated when environmental influences vary across the life of an organism. For example, many species have a critical window where immune system development is sensitive to external inputs. In these cases, the adult immune function is contingent on early life exposures (2,3). In contrast, other aspects of immunity display plastic shifts depending on current environmental conditions. This includes the large body of work examining how exposure to toxins impacts immunity and disease e.g., (4,5). However, a large research gap exists when considering whether environmentally induced immune shifts are common to all individuals of a species or parceled into genotype-specific effects where only some individuals respond to cues (i.e., genotype-environment interaction; reviewed in Bolnick et al. (6).

44 The microbial environment surrounding an organism has strong impacts on host immunity. In
45 mammals, the gut microbiome plays an important role in immune development and function,
46 including maintenance of homeostasis and disease resistance (7). Much of what we know in this area
47 comes from laboratory work on captive, inbred lines of house mice (*Mus musculus*). In addition to
48 their limited genetic diversity, housing mice in sterile lab environments may limit our power to
49 establish links between microbes and host immune phenotypes. Indeed, lab mice lack certain immune
50 cell populations that are found in pet store mice (8) and have much lower levels of immune activation
51 than wild animals due to limited pathogen exposure (9). Wild immune phenotypes can also be
52 recapitulated through "rewilding" experiments that acclimate lab mice to natural outdoor enclosures.
53 For example, wild-acclimated mice greatly increase granulocyte production and immune activation
54 relative to laboratory animals (10,11). Similarly, inoculating lab mice with wild gut microbiomes
55 lowered levels of inflammation and improved resistance to viral infection (12). Although most
56 immunology research has focused on rodent models, teleost fish represent an emerging model for
57 connecting environmental and microbial variation with immune development and function.

58 Fish models offer several useful avenues for studying immune variation. One clear benefit of
59 these systems is that their mucosal organs are in constant contact with the aquatic environment and
60 can quickly respond to environmental changes (13). Mucosal tissues such as respiratory surfaces
61 harbor important microbial communities and act in the first line of immune defense (14). Numerous
62 studies have examined how environmental factors such as salinity, pH, temperature, and pollutants
63 affect fish mucosal immunity (reviewed in Morshed and Lee, (15)). Another large body of work
64 addresses connections between pathogens and innate immunity, mostly focusing on organ specific
65 immune responses (reviewed in Dezfuli et al, (16)). Apart from tissue or response specific studies,
66 the ability to easily breed and rear fish across a spectrum of environments also offers research
67 opportunities that are often not available with rodent models. For example, housing similar strains in

68 lab, controlled outdoor (i.e., aquaculture), and wild settings, offers great opportunities for
69 disentangling genetic and environmental effects on immunity. Moreover, in instances where genetics
70 differences drive immune variation, several fish systems offer the opportunity to understand how and
71 why these phenotypic differences evolved (17,18)

72 Threespine stickleback fish (*Gasterosteus aculeatus*) are well known as models for studying
73 the process of adaptation via natural selection. This ancestrally marine species repeatedly colonized
74 and adapted to freshwater ecosystems across the northern hemisphere as glaciers receded 11,000
75 years ago (19,20,21). Recent work suggests that population-specific immune divergence is an
76 important factor in the adaptive radiation of stickleback into freshwater environments. Differences in
77 innate immune function have been documented between numerous ecotypes, including marine-
78 freshwater (22), sympatric benthic-limnetic (23), parapatric lake-stream ecotypes (24), and variation
79 over larger, continental scales (25,26). While some of these studies include more modern measures of
80 immune gene expression (18,23,27), many rely on two classic flow cytometric assays of immune
81 cells collected from the head kidney (i.e., the primary hematopoietic organ). In one case, forward and
82 side scatter are used to categorize populations and relative ratios of granulocytes and lymphocytes in
83 the head kidney, with greater proportions of granulocytes suggesting greater investment in innate
84 immunity (26). The second assay measures the oxidative burst response of granulocytes, where
85 fluorescent dyes allow the quantification of reactive oxygen species (ROS) production after cells are
86 exposed to an immune stimulant. (24,26). In general, populations with high natural macroparasite
87 exposure tend to evolve higher proportions of granulocytes and larger oxidative burst responses
88 (23,24). In addition to flow cytometric assays, the splenosomatic index (spleen mass/body mass, SSI)
89 is frequently used to evaluate immune activity in fish, with an increased SSI corresponding to higher
90 pathogen related immune response (28,29), potentially resulting from a greater need to filter
91 circulating leukocytes. Another simple measure, the hepatosomatic index (liver mass/body mass,

92 HSI) provides a proxy for individual condition because the liver is used to store fatty acids in fish
93 (30).

94 We took advantage of threespine stickleback populations with recently evolved heritable
95 differences in immune function to examine the effects of environment, most specifically microbial
96 community, on host immunity. By rearing fish in a highly controlled laboratory setting, we were able
97 to focus on two experimental variables: the effect of ancestry (i.e., host population) and the effect of
98 water flow into tanks (i.e., constant versus intermittent flow). We used three different stickleback
99 populations for this experiment: a 'resistant' population with a high oxidative burst response, a
100 'tolerant' population with a dramatically reduced oxidative burst capacity, and an ancestral 'marine'
101 population which had not had its immune phenotype previously categorized. Based on previous
102 results (18,26), we expected the cestode-resistant population to exhibit the highest immune response.
103 We also hypothesized that variation in water flow rate would affect the composition of microbes in
104 our fish tanks; specifically, we predicted that intermittent flow tanks would have more diverse
105 microbial communities due to higher environmental stability. Finally, we predicted that housing adult
106 fish in different water flow environments would reveal flexibility in adult innate immunity, likely
107 because of different microbial communities. Specifically, if the intermittent flow tanks drive higher
108 microbial diversity, fish housed in those tanks should also exhibit the largest immune responses. As
109 most of our fish measurements focused on cellular immunity, to understand more generalized effects
110 of water flow we also compared plasticity in two organs. We predicted that increased immune
111 investment would correlate with increases in spleen size, and that decreased liver size would indicate
112 increased energetic expenditure, potentially connected to immune activation.

113 **Materials and Methods**

114 *Animal Care & Husbandry*

115 Wild fish (F0-generation) were obtained from two sites on Vancouver Island, British Columbia:
116 Roberts Lake ("resistant" population, 50.222, -125.541); Gosling Lake ("tolerant", 50.061, -
117 125.504); and from the Kenai River Estuary in southeast Alaska ("marine", 60.542, -151.218).
118 Offspring were generated via in-vitro fertilization (F1-generation) in the field in summer 2019 and
119 fertilized eggs were shipped to the University of Wisconsin-Madison (ADFG license P-19-005;
120 Canada: NA19-457335). F1 fish were bred in the lab after reaching maturity in 2021, and those
121 offspring (F2) were used in this experiment. This breeding design should minimize maternal effects
122 on measured phenotypes, as all parents shared the same laboratory environment. Fish were housed in
123 18L tanks in full-sibling groups (n/tank = 2 – 15) with a water temperature of 17°C. The room was
124 kept on a 16:8 light cycle. Fish were fed daily with a mixture of freeze-dried bloodworms and mysis
125 shrimp, and each tank had a section of PVC pipe and two artificial plants for environmental
126 enrichment.

127 *Acclimation Design*

128 We used two water flow regimes to create different tank environments. Continuous-flow tanks had a
129 volume of 18L and input flow rate of 1L/minute. The intermittent flow tanks had a volume of 23L,
130 and water changes involved 1L/minute for 5 minutes every 12 hours. The entire volume of water was
131 replaced every 30 minutes in continuous flow tanks, as opposed to every 48 hours in intermittent
132 flow tanks. The intermittent flow tanks were also provided standing bubbling filters to maintain
133 water quality and promote microbial growth on the filter sponge. All tanks in the experiment shared a
134 common water system, and water was run through a UV filter before recirculation to limit microbe
135 movement between tanks. Due to a limited number of intermittent flow tanks, fry and young fish
136 were all initially reared in constant flow tanks. All fish used for this experiment had been in their
137 current tank for at least 11 months.

138 Fish ranged in age from 14 – 24 months at the start of the experiment and were randomly
139 assigned to one of four treatments: continuous flow only ('c'), intermittent flow only ('i'), move from
140 continuous to intermittent flow ('ci'), and move from intermittent to continuous flow ('ic'). After the
141 latter two treatments were initiated, fish were maintained in the new tank for approximately eighteen
142 weeks. After acclimation, we fasted fish for 24 hours and then euthanized via a combination of MS-
143 222 overdose (500 mg/L) and pithing. Prior to dissection fish were weighed and standard length
144 recorded. The liver and spleen were removed and weighed with an analytical balance (±0.0001), and
145 the head kidneys (HKs) were removed for flow cytometric assays.

146 *Flow Cytometry Assay*

147 Maximum ROS production in HK cells was quantified via oxidative burst assays. Organs were
148 placed in ice cold media (0.9× Roswell Park Memorial Institute medium containing 10% fetal bovine
149 serum, 100 μM nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, and 55 μM
150 β-mercaptoethanol) immediately after removal. Single cell suspensions were generated by manually
151 disrupting and filtering the tissue on a cell strainer (35 μm) with a pipette. We washed the cell
152 suspension once in 4 mL of cold media (300g, 4°C, 10 min), poured off most of the supernatant, and
153 then resuspended the cell pellet in the remaining media. Live HK cells were counted using a
154 hemocytometer (Hausser Scientific 3520) via Trypan blue exclusion (Corning 25-900-CI). Each
155 individual had 2×10^5 HK cells distributed to three separate wells on a 96-well plate. Each well
156 received either (1) 200 μL of HK medium or (2 & 3) HK medium with DHR-123 (ROS indicator
157 fluorochrome, 2 μg/mL, Sigma D1054). Samples were incubated at 18°C, 3% CO_2 for 10 minutes.
158 Next, PMA (Phorbol 12-myristate-13-acetat, 130 ng/mL; Sigma P8139) was added to well 3 to
159 stimulate ROS production while wells 1 and 2 received equivalent volumes of HK media. Cells were
160 incubated for a final 20 min at 18°C, 3% CO_2, placed in a sealed bag on ice for transport, and run on
161 a ThermoFisher Attune NxT V6 flow cytometer.

162 *Data Analysis*

163 Flow cytometry data was analyzed in FlowJo (TreeStar). The magnitude of ROS production during
164 oxidative burst assays was determined by comparing median fluorescence intensity of PMA-
165 stimulated and unstimulated cells. The proportion of granulocytes and lymphocytes was determined
166 by flow cytometry of the unstained, unstimulated HK cells and forward and side scatter gating
167 described in Weber et al. (18).

168 All other data analysis was done in R v4.2. Because the amount of ROS produced by head
169 kidney cells depended on time since exposure to PMA, we included flow cytometry sample order as
170 a covariate. Specifically, we made separate linear models for PMA-stimulated and unstimulated cells
171 with sample order as a random effect with R package *lme4* v35.5 (31) and used the model residuals
172 as the corrected median fluorescence intensity. We then built a series of linear models for
173 associations between four traits of interest (ROS production, percent granulocytes, liver and spleen
174 mass) and a suite of potential predictor variables: population, sex, family, tank environment
175 (continuous/intermittent), flow treatment (c/ci/ic/i), movement between tanks (ic and ci groups were
176 moved, c and i were not), body mass (only used for spleen and liver), and all relevant combinations
177 of predictor variables and interaction terms. We used the R package *performance* v12.4 (32) to
178 compare models and selected the model with the lowest AIC score for each response variable. The R
179 package *emmeans* v1.11 (33) was used to estimate group averages and test for significant differences
180 between multiple factors.

181 *Tank 16S Sequencing & Analysis*

182 We selected a subset of tanks for 16S sequencing to characterize microbial community differences
183 between flow regimes. Five continuous and five intermittent flow tanks were sampled using a
184 microbiome testing kit from Aquabiomics LLC (Junction City, OR). Two samples were taken from

185 each tank. First, 60 mL of tank water was slowly pushed through a 22-micron mixed cellulose ester
186 filter, followed by 2 mL of 90% ethanol; the filter was allowed to dry then sealed in a small plastic
187 container. Second, each tank's water intake tube was swabbed vigorously with a cotton swab, the
188 swab was dunked in a 1.5 mL cryotube filled with 90% ethanol, allowed to dry, then sealed in a
189 plastic tube.

190 Sequence data were curated in R v4.2 with the *DADA2* pipeline (v1.16, 34). Reads were
191 filtered and trimmed (command: 'filterAndTrim') with parameters maxN=0, truncQ=2,
192 rm.phix=TRUE and maxEE=c(2,5). Paired reads were then merged (command: 'mergePairs') and
193 chimera sequences were removed (command: 'removeBimeraDenovo'). R package *DECIPHER*
194 (v.2.26, 35) was used to construct an amplicon sequence variants (ASVs) table (command: 'IdTaxa')
195 after training on a reference dataset (SILVA 138 SSU; 36). Analyses of species richness and
196 differences between communities were completed with the R package *phyloseq* (v1.42, 37).

197 **Results**

198 *Tank Microbe Communities*

199 Despite collecting and submitting 20 microbial samples for 16S library preparation and sequencing,
200 we only recovered usable data from four tanks: three continuous flow and one intermittent. We were
201 unable to acquire additional tank microbial samples because the experiment had concluded by the
202 time that we learned of the sequencing error (i.e., we no longer had access to the relevant fish
203 populations in each tank environment). Although the small number of tanks limits our confidence in
204 measurements of microbial differences between water flow environments, we nonetheless identified
205 several patterns. 2D ordination (Figure 1) separated the filter and swab samples along the first NMDS
206 axis, and the intermittent flow tank separated from the three continuous flow tanks along the second
207 axis. The intermittent flow tank also had the highest Shannon diversity index score (Figure S1),

208 though it did not diverge greatly from the continuous flow tanks. We generated a list of the top ten
209 ASVs that had the largest difference in read counts between the tank environments. ASVs in the
210 genera *Sediminibacterium*, *Polynucleobacter*, and *Undibacterium* were overrepresented in both filter
211 and swab samples in the intermittent tank, whereas an ASV in the genus *Sphingopyxis* was
212 underrepresented in the intermittent samples compared to continuous flow. Together, this suggests
213 that the water flow regime likely altered the microbial communities where fish were housed.

214 *Reactive Oxygen Species (ROS) Generation*

215 The best fit regression model for ROS production included fixed effects for population and flow
216 treatment, and family as a random effect. The resistant stickleback population produced significantly
217 higher ROS than tolerant (mean of 0.55 median fluorescence intensity vs -0.48 mfi, Cohen's d =-
218 3.56, t-test $p<0.00001$, Table 1, Figure 2) or marine populations across all treatments (d =-1.72,
219 $p<0.00001$). The tolerant population also produced less ROS than marine fish (d=-1.84, $p<0.00001$).
220 These data support a strong role of population ancestry and evolutionary divergence in shaping
221 oxidative burst capacity. Across all populations the c treatment had the lowest ROS production,
222 which was significantly different from the i (d=-0.27, p = 0.004) and ci treatments (d=0.505, p =
223 0.0001) and marginally different from the ic treatment (d=0.37, p = 0.051). Fish in intermittent flow
224 tanks tended to have greater ROS production than those in continuous flow (Figure S6, d=-0.11, p =
225 0.002). There was no effect of sex on ROS generation (p = 0.74).

226 *Granulocyte:Lymphocyte Ratio*

227 The best supported model for percent granulocytes included fixed effects for population, sex, and
228 flow regime, and family as a random effect. Intermittent flow tanks averaged significantly higher
229 proportions of granulocytes in the head kidney than continuous flow tanks (38.1% vs 34%; t-test p =
230 0.003, Cohen's d =-0.03, Figure 3). The resistant population also had higher proportions of

granulocytes than the tolerant (41.2% vs 34.4%, $p = 0.02$, $d=-0.83$) or marine (32.6%, $p = 0.001$, $d=-1.05$) populations. The latter two populations were not significantly different ($p = 0.47$).

Liver and Spleen Mass

Variation in spleen and liver mass was driven by fish mass, population, sex, and tank environment. Fish from the tolerant population had significantly smaller livers than resistant fish after controlling for mass (-0.44 vs 0.27 mass-scaled liver size, Cohen's $d = -2.1$, t-test $p<0.00001$, Table 1, Figure 4). The marine population was intermediate in liver size and not significantly different from either tolerant or resistant populations. Fish in the c treatment had significantly larger livers than the ci (0.16 vs -0.12, $d=0.81$, $p=0.03$) and ic treatments (0.16 vs -0.13, $d=0.83$, $p=0.02$) and were marginally larger than the ii treatment (0.16 vs -0.08, $d=0.67$, $p=0.06$). Males averaged smaller livers than females, but this difference was not significant in multiple comparisons (-0.04 vs 0.04, $d = 0.23$, $p=0.16$). The best-supported model for liver mass included population, sex, and flow treatment as fixed effects and family as a random effect.

The marine population averaged larger spleens than both resistant (0.16 vs -0.08 mass corrected spleen size, $d = 0.49$, $p = 0.01$, Figure 5) and tolerant populations (0.16 vs -0.08, $d = 0.55$, $p = 0.006$). Male fish had larger spleens than females (0.11 vs -0.11, $d=0.45$, $p=0.01$), and fish in intermittent flow had marginally larger spleens than continuous flow (0.06 vs -0.07, $d = -0.28$, $p=0.05$). The best-supported model for spleen mass included population, sex, and flow regime. Mass-corrected spleen size was not significantly correlated with either ROS production or granulocyte:lymphocyte ratio (Pearson's correlation coefficient, $p>0.1$). Mass-corrected liver size was positively correlated with ROS production ($r=0.31$, $p=0.005$), but not granulocyte:lymphocyte ratio ($p=0.18$).

Discussion

254 We leveraged evolved differences in immunity across stickleback populations to investigate how
255 variation in the laboratory environment affects immune function. We found that simply transferring
256 fish between tanks with constant or intermittent water flow, a relatively modest environmental
257 change, led to significant changes in the inflammatory responses and composition of head kidney
258 cells. Intermittent water flow not only drove significantly higher levels of ROS production but also
259 greater proportion of granulocytes–a category that includes neutrophils likely to drive strong
260 oxidative burst responses. After eighteen weeks of exposure, the magnitude of both immune
261 measures approximated levels observed in fish that had been housed in each environment for a long
262 period. This demonstrates that environmentally induced flexibility in stickleback innate immune
263 responses is a relatively rapid process.

264 Apart from classic measures of innate immunity, we also tested the impacts of water flow
265 regime on liver and spleen size. Fish that ended the experiment housed in intermittent flow tanks had
266 significantly higher SSI, consistent with a plastic shift to greater innate immune allocation. In
267 contrast, there was no simple relationship between HSI and water flow treatment. Stable (non-
268 transplant) rearing under constant water flow yielded significantly higher HSI than the other
269 treatment groups. We had too few tanks available to perform sham transfers (i.e., c-c or i-i), so we
270 cannot rule out a handling effect in the ci or ic treatments. However, this seems unlikely given that
271 fish were allowed to acclimate to their new tanks for approximately eighteen weeks. The tolerant
272 population had the lowest HSI overall. This was likely due to this population's fast growth rate,
273 which is also associated with liver size in fish (38). Marine and resistant fish did not differ in relative
274 liver size even though marine fish are significantly larger.

275 All populations showed a similar magnitude of flexibility for ROS production, granulocyte
276 proportion, and SSI, and the heritable differences between populations were by far the strongest
277 predictor of immune response (Table 2). Population accounted for 89% of the variance in ROS

278 production, 42% of the variance in granulocyte proportion, and 43% of the variance in SSI. Several
279 recent studies have highlighted the utility of population level comparisons of immune variation (e.g.,
280 17, Robertson et al 2016, Weber et al 2022), but this remains a relatively neglected component of
281 most experimental designs (Sasser et al 2023).

282 Variation in ROS production in these populations evolved recently in response to parasitism
283 by the tapeworm *Schistocephalus solidus* which is not present in marine environments (18). We
284 found a similar magnitude of difference between resistant and tolerant populations compared to
285 previous work, although overall our measurements were lower than those reported in Weber et al
286 (18). Our measures of granulocyte proportion were comparable to our previous results (18) but
287 significantly higher than those reported in wild stickleback from Germany (24). This study was the
288 first to include measures of ROS production in ancestral marine stickleback and demonstrated that
289 the marine population is intermediate between the cestode resistant and tolerant freshwater
290 populations.

291 Because our experiment was performed in a laboratory with highly controlled environments,
292 we hypothesized that reducing the frequency of tank water changes would primarily lead to increased
293 microbial diversity. Our results, with the caveat that they represent only a few tanks, support this
294 prediction. The intermittent flow tank had the highest level of microbial diversity in swabs taken
295 from the tank outflow pipes, and the second highest diversity from filtered water samples (Figure
296 S1). Perhaps more striking was the divergence in microbe communities when accounting for ASV
297 abundance. Whether considering microbes filtered directly from the water or from a swab of the tank
298 outflow, 16S divergence along the first NMDS axis was strongly captured by flow regime (Fig S1).
299 While the present study was a preliminary examination of whether variation in water flow could
300 separately influence tank microbial environment and fish immunity, our positive results beg several
301 questions: Do changes in tank microbial environment alter the fish microbiome and, if so, are the

302 effects general or specific to areas such as the skin or gut? And are shifts in host immunity caused by
303 microbial exposure, alterations in microbiome, or both events? More directed experiments are needed
304 to address these questions, but our results do at least support a microbe-immune connection. The
305 higher ROS and granulocyte levels of head kidney cells from stickleback in intermittent flow tanks
306 suggests an increased systemic immune stress (40). This is consistent with pathogen response,
307 potentially mediating via migration of antigen presenting cells from the periphery to head kidney
308 (41).

309

310 **Ethics Statement**

311 This experiment was reviewed and approved by the University of Wisconsin-Madison IACUC under
312 protocol L006460-A01.

313 **Conflict of Interest**

314 The authors declare that the research was conducted in the absence of any commercial or financial
315 relationships that could be construed as a potential conflict of interest.

316 **1. Author Contributions**

317 Conceived and designed the experiments: JW and CW. Performed the experiments, analyzed the
318 data, and prepared the figures: CW. Wrote the first draft: CW and PC. Provided funding: JW. All
319 authors contributed to the article and approved the submitted version.

320 **2. Funding**

321 This work was funded by a startup award from the University of Wisconsin-Madison Department of
322 Integrative Biology (JNW) and the National Institute of General Medical Sciences (5R35GM142891;
323 JNW)

324 3. Acknowledgments

325 We thank Dr J Benavente Paredes and M Chotlos for assistance with data collection; J Kosmopoulos
326 and Dr K Anantharaman for advice on 16S data analysis; and E Kerns, K Yu, J Abels, and L Rippin
327 for helpful feedback on this manuscript.

328 References

329 1. Schoenle, Laura A., Cynthia J. Downs, and Lynn B. Martin. "An introduction to
330 ecoimmunology." Advances in comparative immunology (2018): 901-932.

331 2. Stinson LF. Establishment of the early-life microbiome: a DOHaD perspective. J Dev Orig
332 Health Dis. 2020 Jun;11(3):201–10.

333 3. MacGillivray DM, Kollmann TR. The Role of Environmental Factors in Modulating Immune
334 Responses in Early Life. Front Immunol [Internet]. 2014 Sep 12

335 4. Campolim CM, Schimenes BC, Veras MM, Kim YB, Prada PO. Air pollution accelerates the
336 development of obesity and Alzheimer's disease: the role of leptin and inflammation - a mini-review.
337 Front Immunol. 2024 Jun 12;15:1401800.

338 5. Kreitinger JM, Beamer CA, Shepherd DM. Environmental Immunology: Lessons Learned
339 from Exposure to a Select Panel of Immunotoxicants. The Journal of Immunology. 2016 Apr
340 15;196(8):3217–25.

341 6. Bolnick DI, Fuess LE, Graham AL, Khan I, Steinel NC, Vaziri GJ. Evolutionary
342 Immunology. Annual Review of Ecology, Evolution, and Systematics [Internet]. 2025 May 6

343 7. Zheng D, Liwinski T, Elinav E. Interaction between microbiota and immunity in health and
344 disease. Cell Res. 2020 Jun;30(6):492–506.

345 8. Beura LK, Hamilton SE, Bi K, Schenkel JM, Odumade OA, Casey KA, et al. Normalizing the
346 environment recapitulates adult human immune traits in laboratory mice. Nature. 2016
347 Apr;532(7600):512–6.

348 9. Abolins S, King EC, Lazarou L, Weldon L, Hughes L, Drescher P, et al. The comparative
349 immunology of wild and laboratory mice, Mus musculus domesticus. Nat Commun. 2017 May
350 3;8(1):14811.

351 10. Lin JD, Devlin JC, Yeung F, McCauley C, Leung JM, Chen YH, et al. Rewilding Nod2 and
352 Atg16l1 Mutant Mice Uncovers Genetic and Environmental Contributions to Microbial Responses
353 and Immune Cell Composition. Cell Host & Microbe. 2020 May;27(5):830-840.e4.

354 11. Yeung F, Chen YH, Lin JD, Leung JM, McCauley C, Devlin JC, et al. Altered Immunity of
355 Laboratory Mice in the Natural Environment Is Associated with Fungal Colonization. Cell Host &
356 Microbe. 2020 May;27(5):809-822.e6.

357 12. Rosshart SP, Vassallo BG, Angeletti D, Hutchinson DS, Morgan AP, Takeda K, et al. Wild
358 Mouse Gut Microbiota Promotes Host Fitness and Improves Disease Resistance. Cell. 2017
359 Nov;171(5):1015-1028.e13.

360 13. Cabillon NAR, Lazado CC. Mucosal Barrier Functions of Fish under Changing
361 Environmental Conditions. Fishes. 2019 Jan 10;4(1):2.

362 14. Nochi T, Jansen CA, Toyomizu M, Eden WV. The Well-Developed Mucosal Immune
363 Systems of Birds and Mammals Allow for Similar Approaches of Mucosal Vaccination in Both
364 Types of Animals. Front Nutr. 2018 Jul 12;5:60.

365 15. Morshed SM, Lee TH. The role of the microbiome on fish mucosal immunity under changing
366 environments. Fish & Shellfish Immunology. 2023 Aug;139:108877.

367 16. Sayyaf Dezfuli B, Lorenzoni M, Carosi A, Giari L, Bosi G. Teleost innate immunity, an
368 intricate game between immune cells and parasites of fish organs: who wins, who loses. Front
369 Immunol. 2023 Oct 16;14:1250835.

370 17. Peuß R, Box AC, Chen S, Wang Y, Tsuchiya D, Persons JL, et al. Adaptation to low parasite
371 abundance affects immune investment and immunopathological responses of cavefish. Nat Ecol
372 Evol. 2020 Jul 20;4(10):1416–30.

373 18. Weber JN, Steinel NC, Peng F, Shim KC, Lohman BK, Fuess LE, et al. Evolutionary gain
374 and loss of a pathological immune response to parasitism. Science. 2022 Sep 9;377(6611):1206–11.

375 19. Bell, M. A., and S. A. Foster, eds. 1994. The evolutionary biology of the threespine
376 stickleback. Oxford Univ. Press, Oxford, U.K

377 20. McKinnon JS, Rundle HD. Speciation in nature: the threespine stickleback model systems.
378 Trends in Ecology & Evolution. 2002 Oct;17(10):480–8.

379 21. Reid K, Bell MA, Veeramah KR. Threespine Stickleback: A Model System For Evolutionary
380 Genomics. Annu Rev Genom Hum Genet. 2021 Aug 31;22(1):357–83.

381 22. Whiting JR, Magalhaes IS, Singkam AR, Robertson S, D'Agostino D, Bradley JE, et al. A
382 genetics-based approach confirms immune associations with life history across multiple populations
383 of an aquatic vertebrate (Gasterosteus aculeatus). Molecular Ecology. 2018 Aug;27(15):3174–91.

384 23. Stutz WE, Lau OL, Bolnick DI. Contrasting Patterns of Phenotype-Dependent Parasitism
385 within and among Populations of Threespine Stickleback. The American Naturalist. 2014
386 Jun;183(6):810–25.

387 24. Scharsack JP, Kalbe M, Harrod C, Rauch G. Habitat-specific adaptation of immune responses
388 of stickleback (Gasterosteus aculeatus) lake and river ecotypes. Proc R Soc B. 2007 Jun
389 22;274(1617):1523–32.

390 25. Hamley M, Franke F, Kurtz J, Scharsack JP. An experimental approach to the immuno-
391 modulatory basis of host-parasite local adaptation in tapeworm-infected sticklebacks. Experimental
392 Parasitology. 2017 Sep;180:119–32.

393 26. Weber JN, Steinel NC, Shim KC, Bolnick DI. Recent evolution of extreme cestode growth
394 suppression by a vertebrate host. Proc Natl Acad Sci USA. 2017 Jun 20;114(25):6575–80.

395 27. Robertson S, Bradley JE, MacColl ADC. Measuring the immune system of the three-spined
396 stickleback – investigating natural variation by quantifying immune expression in the laboratory and
397 the wild. Molecular Ecology Resources. 2016 May;16(3):701–13.

398 28. Kaufmann J, Lenz TL, Kalbe M, Milinski M, Eizaguirre C. A field reciprocal transplant
399 experiment reveals asymmetric costs of migration between lake and river ecotypes of three-spined
400 sticklebacks (Gasterosteus aculeatus). J of Evolutionary Biology. 2017 May;30(5):938–50.

401 29. Rajkov J, El Taher A, Böhne A, Salzburger W, Egger B. Gene expression remodelling and
402 immune response during adaptive divergence in an African cichlid fish. Molecular Ecology. 2021
403 Jan;30(1):274–96.

404 30. Chellappa S, Huntingford FA, Strang RHC, Thomson RY. Condition factor and
405 hepatosomatic index as estimates of energy status in male three-spined stickleback. Journal of Fish
406 Biology. 1995 Nov;47(5):775–87.

407 31. Bates D, Mächler M, Bolker B, Walker S. Fitting Linear Mixed-Effects Models Using lme4. J
408 Stat Soft. 2015 67(1). Available from: http://www.jstatsoft.org/v67/i01/

409 32. Lüdecke D, Ben-Shachar M, Patil I, Waggoner P, Makowski D. performance: An R Package
410 for Assessment, Comparison and Testing of Statistical Models. JOSS. 2021 Apr 21;6(60):3139.

411 33. Lenth RV. emmeans: Estimated Marginal Means, aka Least-Squares Means. 2025.

412 34. Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. DADA2: High-
413 resolution sample inference from Illumina amplicon data. Nat Methods. 2016 Jul;13(7):581–3.

414 35. Murali A, Bhargava A, Wright ES. IDTAXA: a novel approach for accurate taxonomic
415 classification of microbiome sequences. Microbiome. 2018 Dec;6(1):140.

416 36. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal
417 RNA gene database project: improved data processing and web-based tools. Nucleic Acids Research.
418 2012 Nov 27;41(D1):D590–6.

419 37. McMurdie PJ, Holmes S. phyloseq: An R Package for Reproducible Interactive Analysis and
420 Graphics of Microbiome Census Data. Watson M, editor. PLoS ONE. 2013 Apr 22;8(4):e61217.

421 38. Pelletier D, Dutil JD, Blier P, Guderley H. Relation between growth rate and metabolic
422 organization of white muscle, liver and digestive tract in cod, Gadus morhua. J Comp Physiol B.
423 1994 Jul;164(3):179–90.

424 39. Sasser KT, Weber JN. A call for more ecologically and evolutionarily relevant studies of
425 immune costs. Evol Ecol. 2023 Feb;37(1):203–14.

426 40. Speirs ZC, Loynes CA, Mathiessen H, Elks PM, Renshaw SA, Jørgensen LVG. What can we
427 learn about fish neutrophil and macrophage response to immune challenge from studies in zebrafish.
428 Fish & Shellfish Immunology. 2024 May;148:109490.

429 41. Iliev DB, Thim H, Lagos L, Olsen R, Jørgensen JB. Homing of Antigen-Presenting Cells in
430 Head Kidney and Spleen – Salmon Head Kidney Hosts Diverse APC Types. Front Immunol. 2013
431 June;137:1-13

432

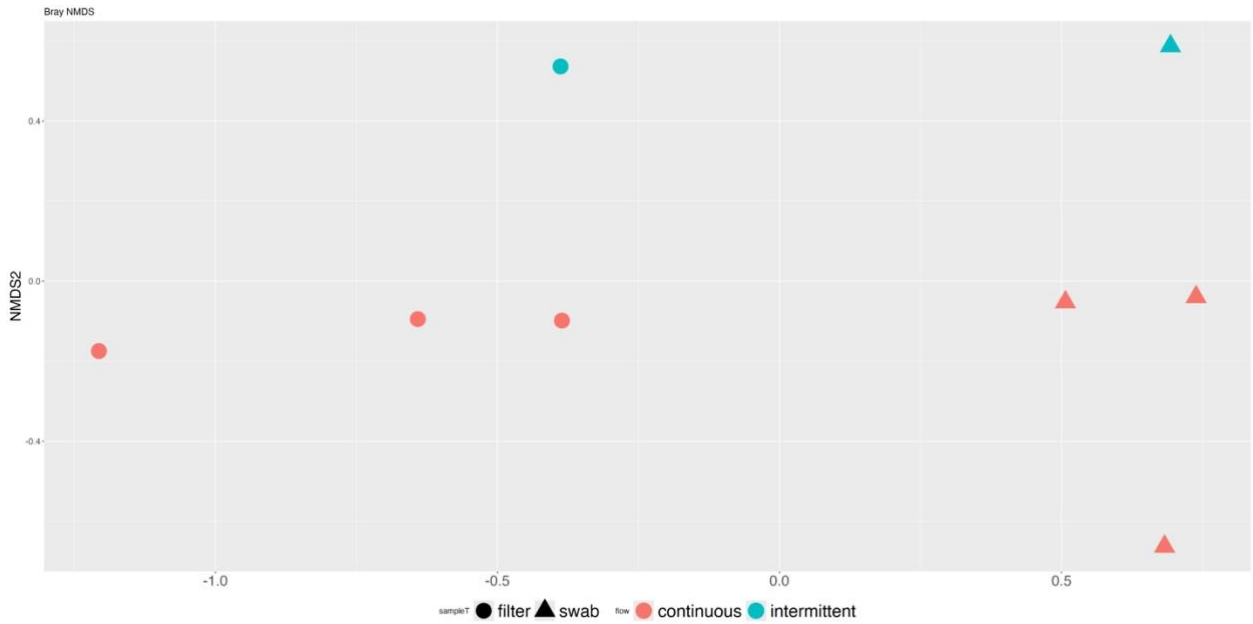


Figure 1: Bray-Curtis non-metric multidimensional ordination scaling plot of tank microbial communities based on 16S data. Color indicates flow regime (red – continuous flow, blue – intermittent flow) and shape indicates sampling location (square – tank outlet swab, triangle –tank water sample).

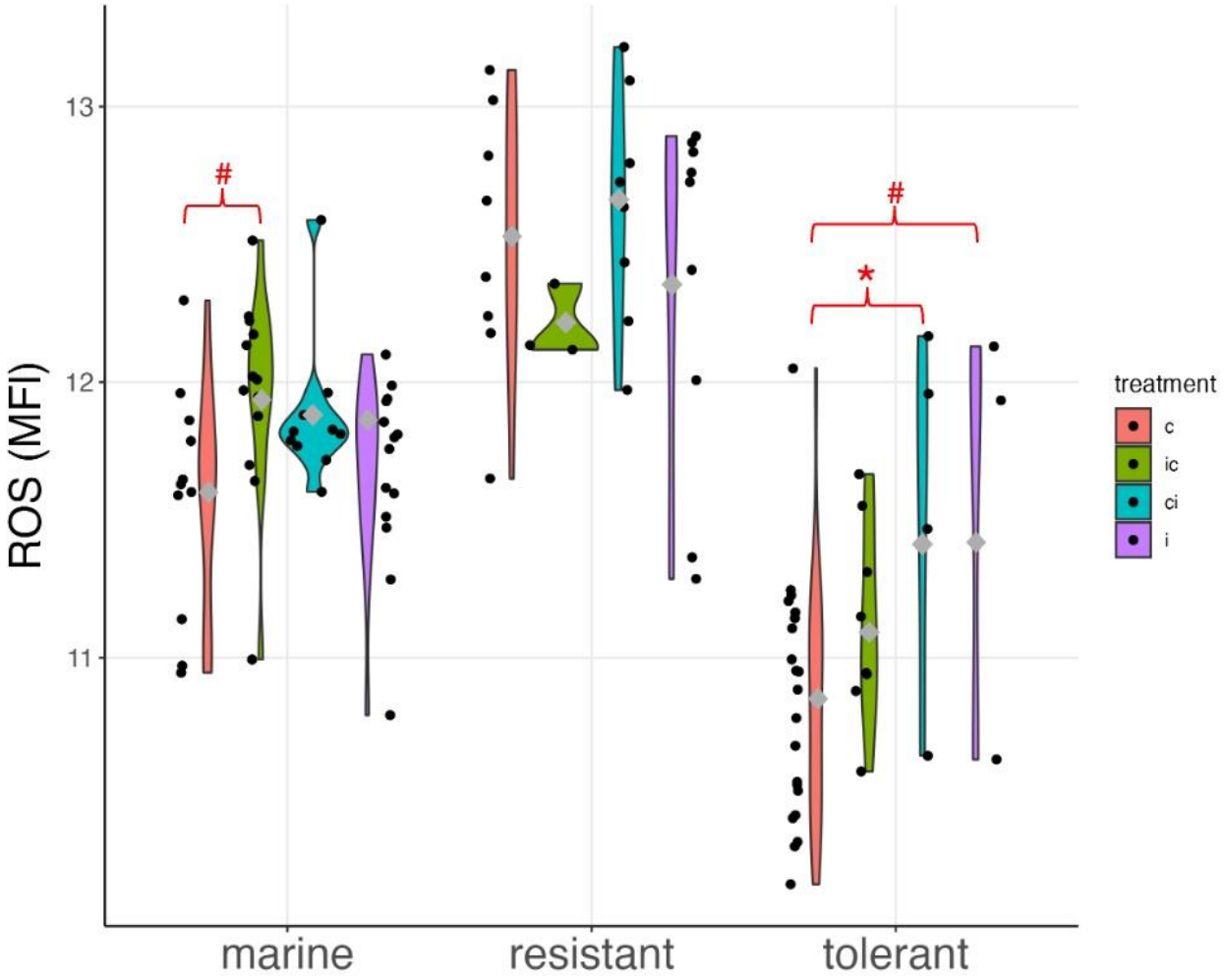


Figure 2: ROS production during oxidative burst assay in head kidney cells by population. Color indicates treatment (red – continuous only, green – intermittent to continuous, blue – continuous to intermittent, purple - intermittent only. Symbols indicate significant differences (# - $p<0.05$, * - $p<0.01$)

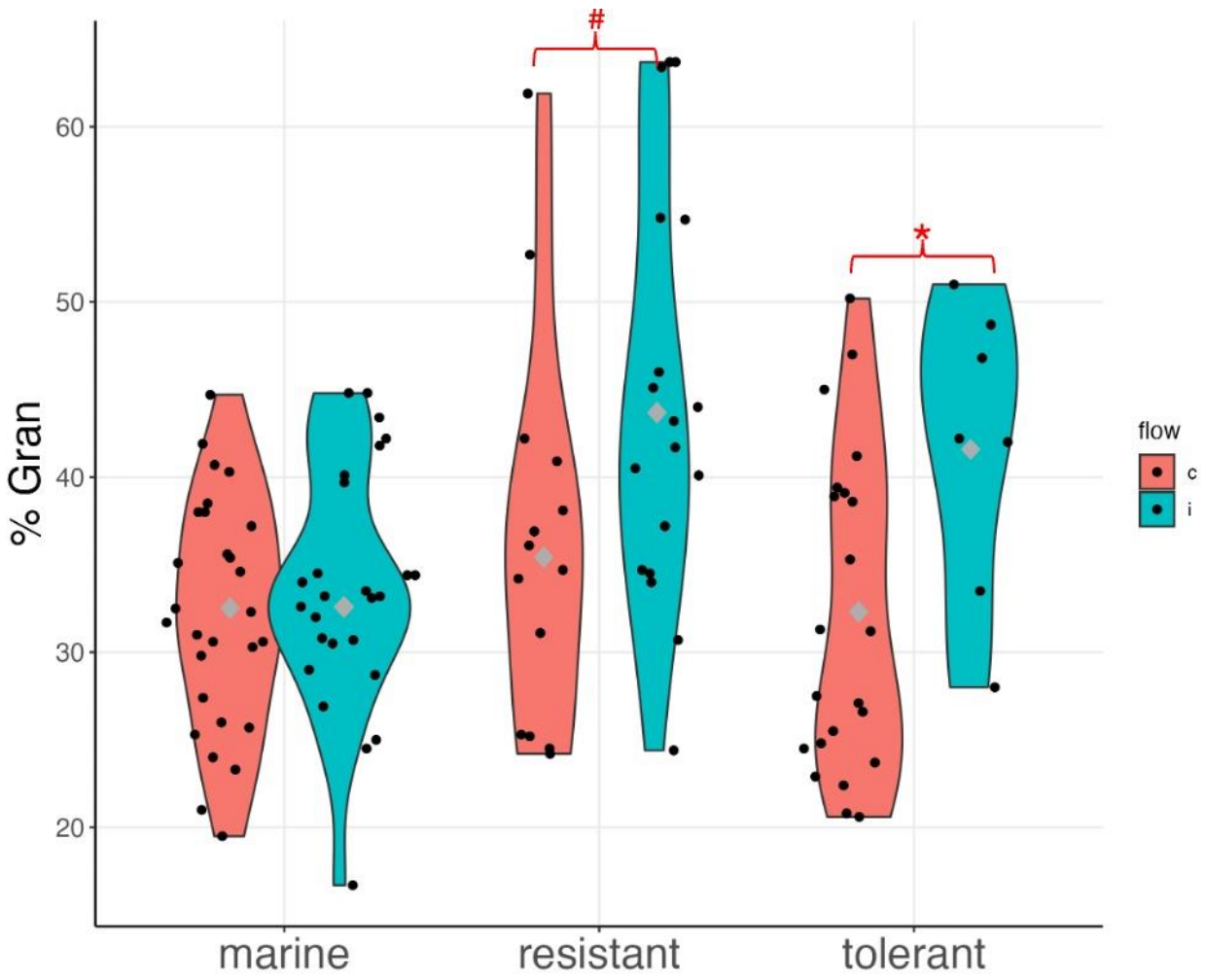


Figure 3: percent granulocyte cells in head kidney by population. Color indicates flow regime (red – continuous flow, blue – intermittent flow. Symbols indicate significant differences (# - $p<0.05$, * - $p<0.01$)

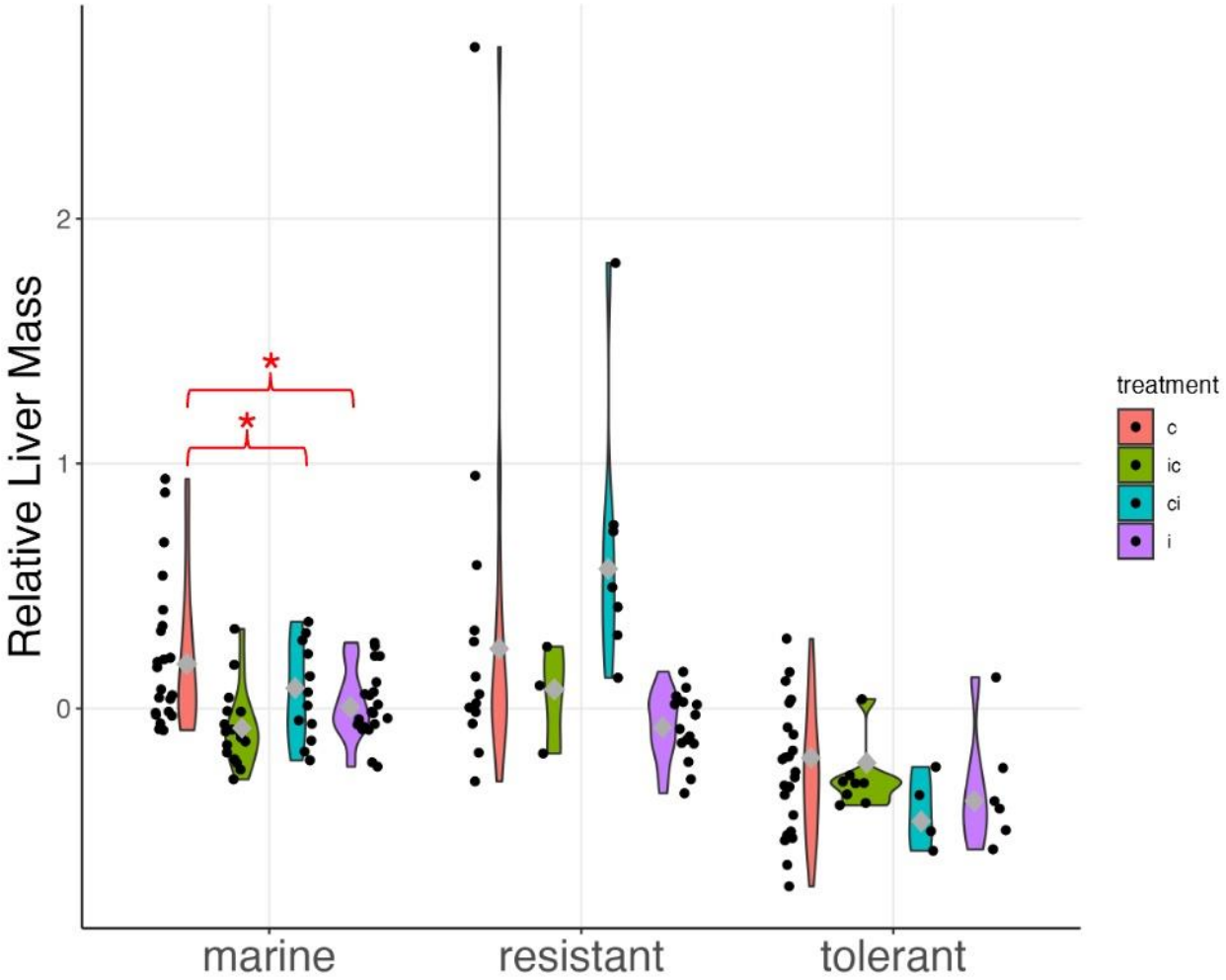


Figure 4: relative liver mass (corrected for body mass) by population. Color indicates treatment (red – continuous only, green – intermittent to continuous, blue – continuous to intermittent, purple - intermittent only. Symbols indicate significant differences (# - $p<0.05$, * - $p<0.01$)

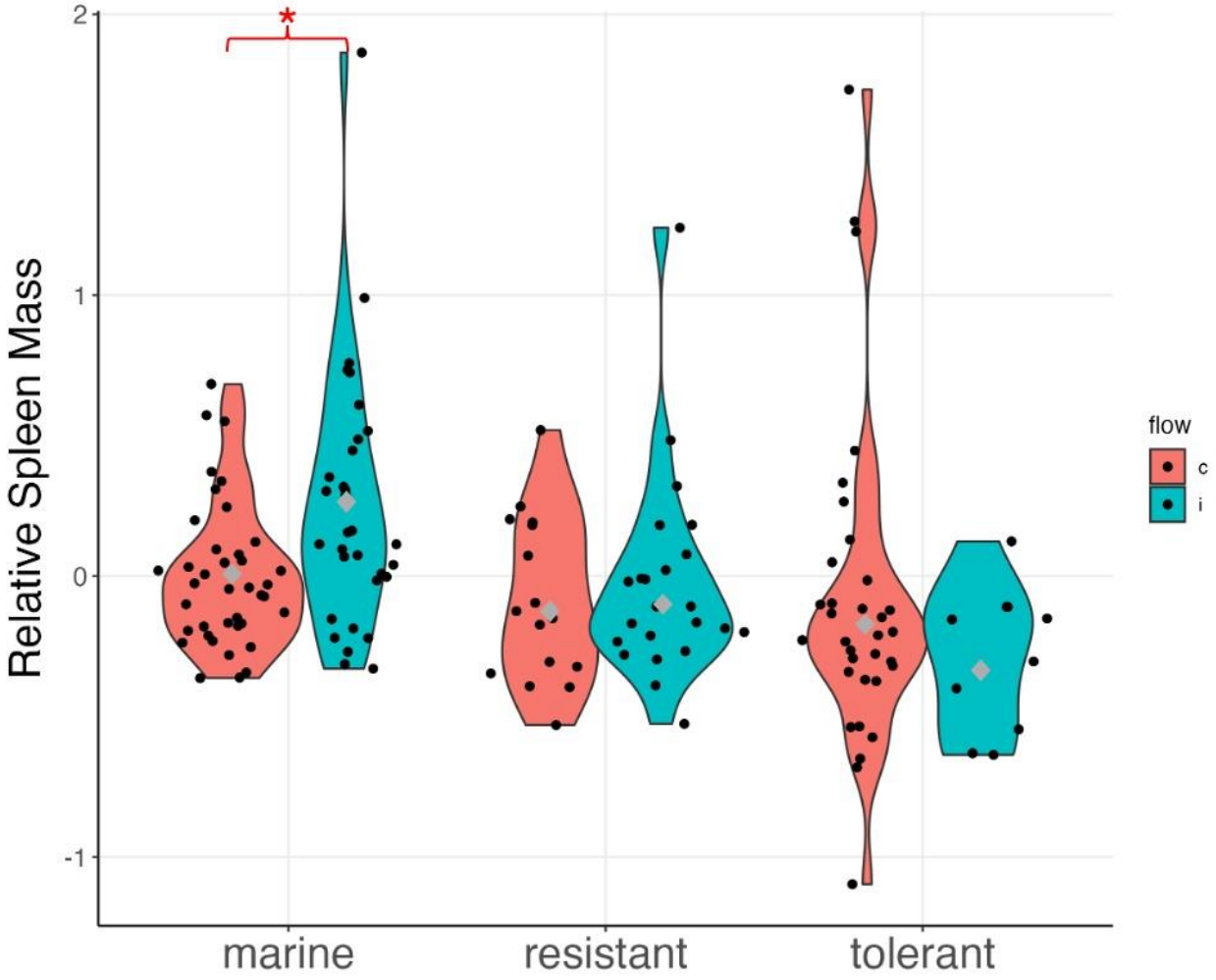
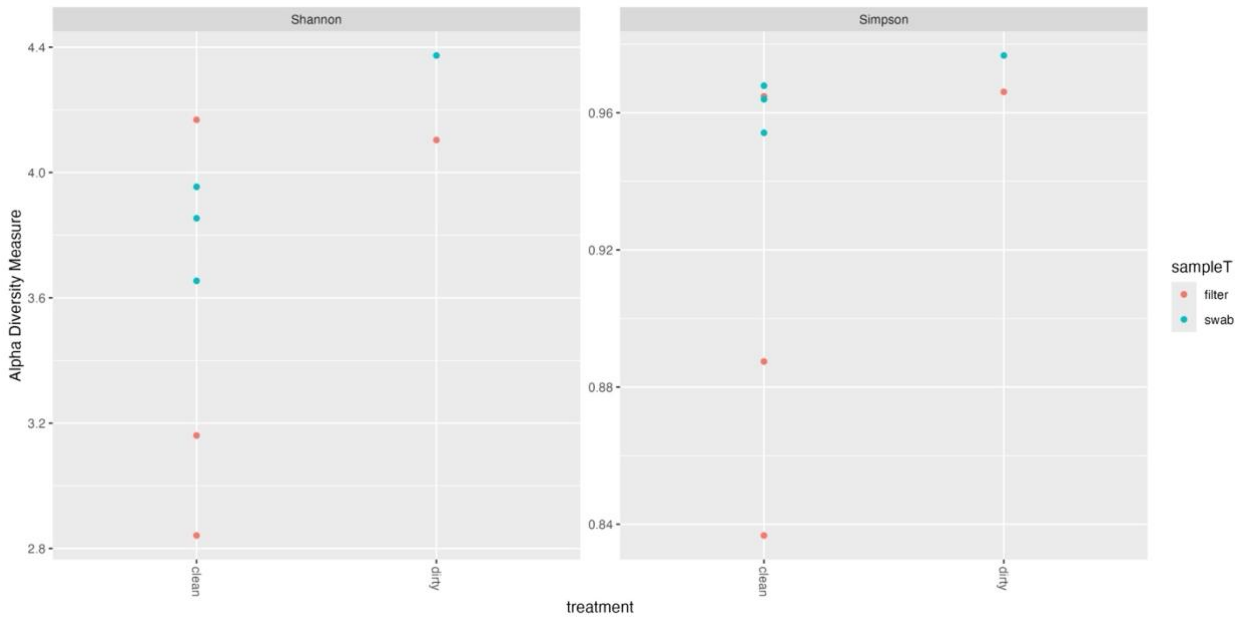
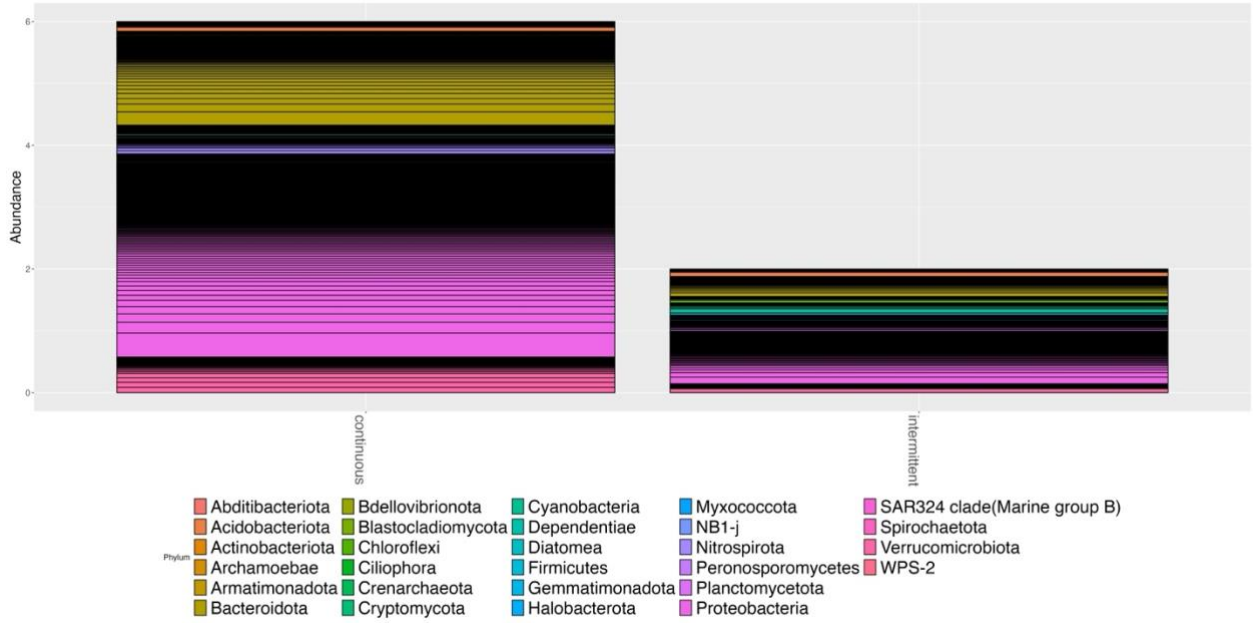


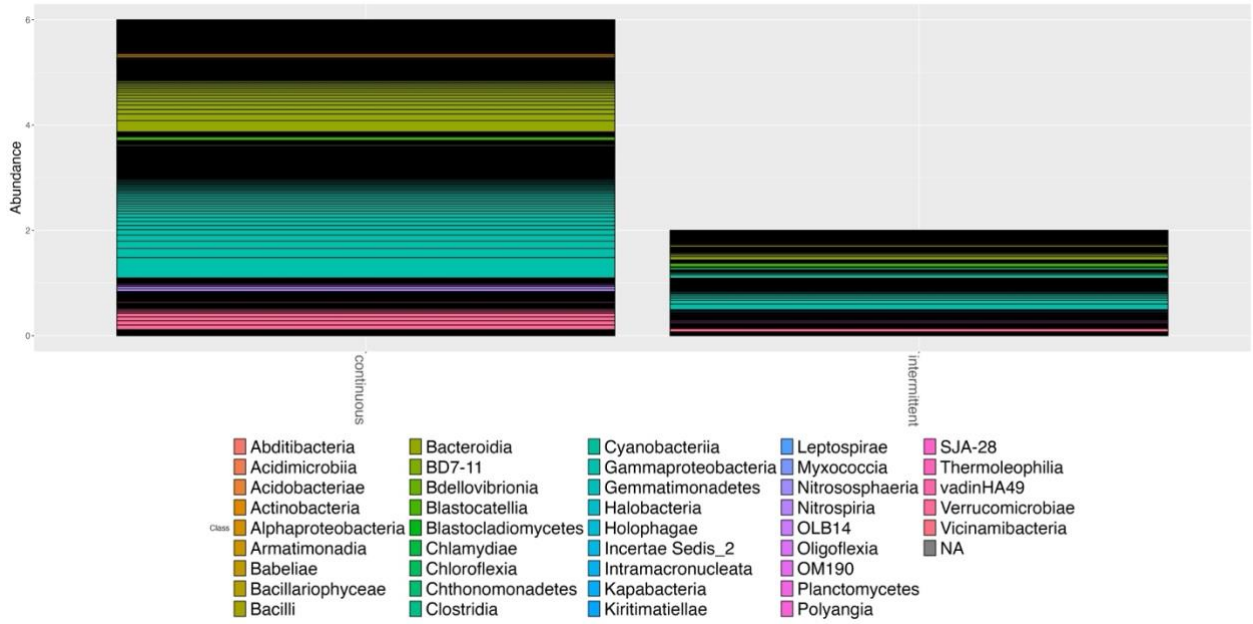
Figure 5: relative spleen mass (corrected for body mass) by population. Color indicates flow regime (red – continuous flow, blue – intermittent flow. Symbols indicate significant differences (# - $p<0.05$, * - $p<0.01$)



Supplemental Figure 1: diversity indices of tank microbial communities based on 16S data by flow regime. Color indicates sampling location (blue – tank outlet swab, red –tank water sample).



Supplemental Figure 2: abundance of bacterial phyla by flow regime.



Supplemental Figure 3: abundance of bacterial classes by flow regime.