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1 **Reliability evaluation of biomarker reference ranges for**
2 **mesocosm and field conditions: cellular innate immunomarkers in**
3 ***Gasterosteus aculeatus*.**

4

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17 Abstract

18 Due to their sensitivity to environmental contamination and their link with fish health status,
19 innate immunomarkers are of great interest for environmental risk assessment studies.
20 Nevertheless, the lack of knowledge about the effect of confounding factors can lead to data
21 misinterpretation and false diagnostics. So, the determination of reference values was of huge
22 interest for the integration of biomarkers in biomonitoring programs. *Laboratory*
23 immunomarker reference ranges (including cellular mortality, leucocyte distribution,
24 phagocytosis activity, respiratory burst and lysosomal presence) that consider three
25 confounding factors (season, sex and body size) were previously developed in three-spined
26 stickleback, *Gasterosteus aculeatus*, from our husbandry. Usefulness of these reference
27 ranges in biomonitoring programs depends on how they can be transposed to various
28 experimental levels, such as mesocosm (outdoor artificial pond) and field conditions.
29 Immunomarkers were therefore measured every two months over one year in one *mesocosm*
30 and in one site assumed to uncontaminated (Houdancourt, *field*). Differences between
31 immunomarker seasonal variations in *mesocosm* and *field* fish on one side and *laboratory* fish
32 on the other side were quantified: in some cases, seasonal trends were not significant or did
33 not differ between mesocosm and laboratory conditions, but overall, models developed based
34 on data obtained in *laboratory* conditions were poorly predictive of data obtained in
35 *mesocosm* or *field* conditions. To propose valuable *field* reference ranges, *mesocosm* and *field*
36 data were integrated in innate immunomarker modelling in order to strengthen the knowledge
37 on the effect of confounding factors. As in laboratory conditions, sex was overall a
38 confounding factor only for necrotic cell percentage and granulocyte-macrophage distribution
39 and size was a confounding factor only for cellular mortality, leucocyte distribution and
40 phagocytosis activity. Confounding factors explained a large proportion of immunomarker
41 variability in particular for phagocytosis activity and lysosomal presence. Further research is

42 needed to test the *field* models in a biomonitoring program to compare the sensitivity of
43 immunomarkers to the confounding factors identified in this study and the sensitivity to
44 various levels of pollution.

45 Keywords: biomarker reference ranges; fish conditions; innate immunity;

46

47 Highlights:

48 1- Similar immunomarker seasonal variations for *laboratory*, *mesocosm* and *field* fish

49 2- Importance of fish conditions on reference ranges

50 3- Better predictability of phagocytosis activity and granulocyte-macrophage percentage

51 4- Development of *field* reference ranges for cellular innate immunomarkers

52

53 **I. Introduction**

54 Biomarkers reflect the physiological state of an organism and can serve to detect exposures to
55 xenobiotics or their early effects. They have therefore been proposed to determine effects of
56 water quality on aquatic wildlife. Nevertheless, to be used currently in biomonitoring
57 campaigns, some criteria, including natural variability (van der Oost et al., 2003), should be
58 considered in data analysis (Flammarion et al., 2000; van der Oost et al., 2003) to reduce data
59 misinterpretation. In fact, many confounding factors, including variations of environmental
60 conditions (temperature, salinity, photoperiod or oxygen levels) and organism's morphology
61 and life history traits (sex, age or sexual maturation) could greatly influence biomarker
62 responses (Bowden, 2008; Bowden et al., 2007; Uribe et al., 2011). For example, Shepherd *et*
63 *al.* (2012) showed differences between sexes in the involvement of the SOCS genes in the
64 yellow perch immune response. Since the immune system interacts in a bidirectional way
65 with the endocrine system, variations in hormone concentrations due to the reproductive cycle
66 of wild fish may be a major confounding factor for the measurement of immune responses. In
67 fact, gonadal sex hormones affect innate immune capabilities by acting directly on hormonal
68 receptors or indirectly through non-hormonal receptor mediated mechanisms (Ansar Ahmed
69 2000). In the same way, sex steroids are known to modulate greatly the immune response of
70 fish (Slater and Schreck 1993, Yamaguchi *et al.* 2001, Cabas *et al.* 2018). Regarding the
71 effect of fish body size on immune response, little information was available. Even so, the
72 attractiveness of fish to parasites was appears to be higher in larger fish notably due to
73 increased contact with water flow. To stop this phenomenon, the host could increase their
74 immune responses (Lo *et al.* 1998). All these sources of variability may disturb biomonitoring
75 results, limit the interpretation of variation between sites, and may lead to incorrect site
76 classification.

77 To limit the risk of false positive and false negative results, many authors suggest comparing
78 biomonitoring results to robust reference values (Barrick et al., 2018, 2016; Burgeot et al.,
79 2010; Kumari et al., 2006; Mohanty et al., 2014; Sanchez et al., 2008). Unfortunately, most of
80 the developed reference values ignore confounding factor effects by providing either only one
81 mean with a confidence interval or a range of means over a sampling period (Kumari et al.,
82 2006; Mohanty et al., 2014; Sanchez et al., 2008). To better determine effects of confounding
83 factors on biomarker responses, two methods were proposed. The first method, suggested by
84 the Convention for the Protection of the Marine Environment of the North-East Atlantic
85 (OSPAR) and by the International Council for the Exploration of the Sea (ICES), consists in
86 collecting organisms on a same reference site during several years to integrate effects of many
87 confounding factors (Barrick et al., 2018, 2016; Burgeot et al., 2010). Nevertheless, this
88 method was only representative of the water quality of the site used and it could be difficult to
89 separate specific effects of each confounding factor. Within the second method, the influence
90 of each confounding factor is evaluated separately before being integrated in a larger model of
91 biomarker responses (Coulaud et al., 2011; Hanson, 2011; Krell et al., 2011; Maltby et al.,
92 2002).

93 Among all developed biomarkers, due to its sensitivity to environmental contaminations and
94 its direct link with individual health status, the immune response is considered as an attractive
95 non-specific marker for environmental biomonitoring (Bols et al., 2001) which could help to
96 better identify risks associated to an ecosystem contamination (Bado-Nilles *et al.* 2014).
97 Among all the measurable parameters composing innate and acquired components of the
98 immune system, biomarkers related to innate immune functions were highly relevant due to
99 their response being non-dependent of previous exposure to foreign antigens (Monserrat et al.,
100 2007). In teleosts, the innate mechanism of phagocytosis appears to be the central cellular
101 immune process. Mainly two leucocyte sub-populations, the granulocytes and the

102 monocytes/macrophages (“granulocyte-macrophage percentage”), are responsible for
103 phagocytosis. This process consists in the adhesion to the leucocyte membrane and the
104 engulfment of the target particle, followed by its destruction by a combined action of aerobic
105 destruction pathway by respiratory burst and anaerobic destruction pathway by lytic enzymes
106 contained in lysosomes (Ellis, 1999; Magnadóttir, 2006). In ecotoxicology, each part of this
107 phagocytosis mechanism has been described as an attractive biomarker of field pollution in
108 fish (Betoulle *et al.* 2000, Bols *et al.* 2001, Reynaud and Deschaux 2006). For example, the
109 phagocytic efficiency was repressed in rainbow trout (*Oncorhynchus mykiss*) after one week
110 of exposure to Montreal municipal sewage effluent (Salo *et al.* 2007). In the same way,
111 phagocytic efficiency and capacity were reduced in rainbow trout coming from industrial
112 waste incineration site (Benchalgo *et al.* 2014). Modifications of respiratory burst index and
113 lysosomal presence were affected by many pollutants (Arnold *et al.* 1995, Roméo *et al.* 2000,
114 Ahmad *et al.* 2004, Santos *et al.* 2006) and were currently associated to cell death (Holtzman
115 1989, Zdolsek *et al.* 1990, Risso-de Faverney *et al.* 2001, Guicciardi *et al.* 2004,
116 Krumschnabel *et al.* 2005, Kurz *et al.* 2008). For example, copper induced both necrotic and
117 apoptotic cell death on rainbow trout hepatocytes by stimulation of ROS production,
118 essentially into mitochondria, which favors destabilization of lysosomal membranes
119 (Krumschnabel *et al.* 2005). In the same way, an increase of ROS production in Nile tilapia
120 (*Oreochromis niloticus*) splenocytes by endosulfan could induce at term lysosomal
121 destabilization and cellular mortality (Tellez-Bañuelos *et al.* 2009). Induction of cellular
122 mortality and decrease of phagocytosis activity could contribute to an increased susceptibility
123 to opportunistic infections in animals (Arkoosh *et al.* 1998, Misumi *et al.* 2005, Bado-Nilles
124 *et al.* 2009, Kreutz *et al.* 2010, Danion *et al.* 2011, Marchand *et al.* 2017). Nevertheless, as
125 previously explained, use of these immunomarkers in biomonitoring campaigns depends on

126 applicability of reference values. Few studies provide information on their natural variability,
127 which partly explains why they are scarcely used by managers in biomonitoring contexts.

128 Reference ranges of some innate immunomarkers were first modelled in function of season,
129 sex, and body size in the three-spined stickleback (*Gasterosteus aculeatus*) (Marchand *et al.*
130 2019). In the present paper, the previously established *laboratory* reference ranges are
131 evaluated at various experimental levels, ranging from the laboratory to the field. In this way,
132 to evaluate the relevance of immunomarker reference ranges previously developed (Marchand
133 *et al.* 2019), data obtained in stickleback in other conditions, (outside artificial pond:
134 *mesocosm*; Houdancourt reference site: *field*) were compared to the *laboratory* reference
135 ranges.

136

137 **II. Material and Method**

138 **a. Experimental level of sticklebacks for each experimental level reference** 139 **range**

140 During this study, all mature three-spined sticklebacks used for laboratory and outside
141 artificial pond experiments were obtained from the INERIS husbandry (Verneuil-en-Halatte,
142 France). The *laboratory* reference ranges used in the present work had been developed based
143 on data obtained in 282 fish in a previous experiment, from December 2015 to August 2016
144 (Marchand *et al.* 2019).

145 To make the *mesocosm* reference ranges, 112 sticklebacks were maintained in one outside
146 artificial pond containing sediments, natural vegetation, phytoplankton, periphyton, and a
147 macroinvertebrate community which were well-suited to stickleback (Jones and Stafford
148 2019). Every two months, from February 2016 to December 2016, 15 - 20 fish were sampled

149 (Table I). Fish were anaesthetized using tricaine methanesulfonate (100 mg/L, Sigma) then
150 sacrificed, measured, weighed, and the spleen was removed to measure immune responses.

151 *Field* reference ranges were developed using sticklebacks sampled at Houdancourt (60, Oise,
152 France – Table I), an uncontaminated site located immediately upstream of a watercress
153 exploitation, from October 2016 to April 2018. At each sampling date, temperature, dissolved
154 oxygen, pH and conductivity were monitored and 20 adult fish were caught by electrofishing,
155 anaesthetized (tricaine methanesulfonate, 100 mg/L, Sigma), sacrificed, measured, weighed,
156 and the spleen was removed to measure immune responses.

157 Mesocosm and laboratory sampling were made strictly at the same time, whereas field
158 sampling was sometimes a few weeks before or after.

159 **b. Leucocyte isolation and innate immune biomarker analysis**

160 The spleen filters plasma, traps blood-borne substances, and enriches blood with new immune
161 cells (Press and Evensen, 1999), and was therefore selected to assess immunological
162 characteristics. A splenic leucocyte suspension was obtained by gently pressing the spleen
163 through sterilized nylon mesh (40 μm , Sigma) with 5 mL Leibovitz 15 (L15) medium (Sigma)
164 containing heparin lithium (100 mg/L, Sigma), penicillin (500 mg/L, Sigma), and
165 streptomycin (500 mg/L, Sigma). To eliminate any bias due to stressful conditions of the fish
166 before sacrifice, samples were stored during 12 hr at 4°C (Bado-Nilles et al., 2014). Then, a
167 Malassez haemocytometer was used to adjust leucocyte concentration in samples to 10^6
168 cell/mL in L15 medium. Analyses were carried out by flow cytometry, on whole leucocytes,
169 using a CyAn™ ADP (Beckman coulter) flow cytometer. A total of 10,000 events per sample
170 were analyzed after cell excitation by a 488 nm-argon laser.

171 Percentage of each leucocyte sub-population (lymphocyte and granulocyte-macrophage) were
172 determined by measuring size (forward scatter, FSC) and complexity (size scatter, SSC)
173 (Bado-Nilles et al., 2014). For ease of reading, only the granulocyte-macrophage percentages
174 among the total leucocyte populations are presented in figures and tables.

175 A double labelling, with Yo-PRO®-1 (Invitrogen, final concentration: 3.14 mg/L) and
176 propidium iodide (Invitrogen, final concentration: 5.01 mg/L) probes, was used to assess the
177 cellular mortality percentages. After 10 minutes of incubation on ice and in the dark, cellular
178 fluorescence parameters were measured. Apoptotic and necrotic cells expressed respectively
179 green (FL1) and red (FL3) fluorescences (Bado-Nilles et al., 2014).

180 The measurement of leucocyte respiratory burst was performed using a modification of the
181 Chilmonczyk and Monge (1999) technique, adapted for stickleback. Determination of reactive
182 oxygen species (ROS) production in unstimulated cells depends upon the cell incorporating
183 2',7'-dichlorodihydrofluorescein diacetate acetyl ester (H₂DCF-DA, Invitrogen, final
184 concentration: 29.30 mg/L), a stable non-fluorescent molecule which was hydrolysed to
185 dichloro-dihydro-fluorescein diacetate (DCFH) by cytosolic enzymes. When leucocytes were
186 stimulated with phorbol 12-myristate 13-acetate (PMA, Invitrogen, final concentration:
187 9.25 mg/L), the most specific inductor of respiratory burst (Ambrozova et al., 2011;
188 Chadzinska et al., 2012), H₂DCF-DA was hydrolysed by H₂O₂. Finally, the DCFH obtained
189 was oxidized to the fluorescent dichlorofluorescein (DCF) to enable quantification, after 30
190 min of incubation at room temperature, by flow cytometry of unstimulated and stimulated
191 cells in FL1. The respiratory burst index was determined as the ratio of fluorescence of PMA
192 stimulated cells (H₂DCF-DA plus PMA) to that of unstimulated cells (H₂DCF-DA).

193 Intracellular lysosomal presence was determined by incubation of spleen leucocyte
194 suspension with 0.3 mg/L of acridine orange (AO, Sigma), a lysosomotropic weak base, for

195 20 min in the dark at room temperature. Then, fluorescence was measured in FL3 (Bado-
196 Nilles et al., 2013).

197 Phagocytosis activity was evaluated after 1 h of incubation in the dark and at room
198 temperature of spleen leucocyte suspension with fluorescent microsphere at a concentration of
199 2.7×10^7 particles/mL (Fluorospheres® carboxylate-modified microsphere, diameter 1 μm ,
200 Invitrogen) (Gagnaire et al., 2004). Phagocytosis activity was characterized by two
201 biomarkers, phagocytic capacity (capacity of leucocyte plasma membrane; fluorescence of at
202 least one bead currently attached to the membrane), and phagocytic efficiency (percentage of
203 leucocytes that had engulfed microspheres; fluorescence of at least three beads).

204 **c. Statistical analysis**

205 Statistical analysis was performed with the software R v.2.14.1 (R Core Team, 2014).
206 Normality was checked using Shapiro's tests and homogeneity of variance was tested using
207 Levene's test ($p \leq 0.05$). When the normality assumption was not fulfilled, data were log-
208 transformed if this improved normality.

209 To evaluate differences in biomarker levels between "experimental levels", three different
210 statistical analyses were made:

- 211 - First, at each sampling period, the differences between *laboratory* and *mesocosm*
212 or *field* data were tested either by an ANCOVA followed by a Dunnett test
213 (parametric data, $p \leq 0.05$) or by a Kruskal-Wallis test followed by a Dunn test
214 with comparison to the control (non-parametric data, $p \leq 0.05$, "dunn.test.control"
215 function of R package "PMCMR").
- 216 - To determine whether *mesocosm* or *field* data fitted within the *laboratory* reference
217 ranges, the percentage of data outside the reference ranges were calculated for each

218 condition. Unilateral binomial tests ($p \leq 0.05$) were performed at each sampling
219 period to detect if there was a significant increase of data out of the ranges between
220 *laboratory* data and *mesocosm* or *field* data.

221 - The predictive power of the previously-built models, which were based on data
222 collected in *laboratory* conditions, towards *mesocosm* and *field* data was also
223 evaluated using the predictive squared correlation coefficient (Q^2) as previously
224 described (Marchand *et al.* 2019).

225 The differences in biomarker levels between the three “experimental levels” highlighted the
226 need for specific *field* reference ranges. These were determined with the following steps:

227 - First, a linear model that included all confounding factors (period, experimental
228 level, sex and size) and their binary and ternary interactions was adjusted for each biomarker.
229 Period, experimental level and sex were included as qualitative variables whereas size was
230 included as a quantitative variable. Though the aim was to build field reference ranges, data
231 obtained in mesocosms were included in order to consolidate confounding effects if
232 appropriate, for example by confirming relationships between size and biomarker levels.

233 - The effects of each confounding factor were evaluated using a type II analysis of
234 covariance (ANCOVA) (Shaw and Mitchell-Olds, 1993) (“Anova” function of R package
235 “car”, $p \leq 0.05$), owing for the fact the data was slightly unbalanced due to postmortem
236 identification of sex. The ANCOVA model for each biomarker was selected using a
237 downwards stepwise procedure based on the AIC and then by removing effects that were not
238 statistically significant according to the F-test, starting from the highest order interactions.
239 Reference ranges were estimated using the 95% prediction interval.

240 - The model was evaluated using the coefficient of determination (R^2), and the
241 predictive squared correlation coefficient determined by Leave-One-Out (Q^2_{LOO}).

242 **III. Results**

243 **a. Normality and heteroscedasticity**

244 The results of normality tests (Shapiro test) and heteroscedasticity tests (Levene's test) made
245 on residuals of the linear models were presented in the Table II. Briefly, after log-
246 transformation of biomarker values, the normality conditions were complete in most of the
247 groups. Nevertheless, some deviations from the normality assumption were found at specific
248 periods, mainly for the respiratory burst index (Table II). Levene's test revealed differences in
249 variance between groups mainly for respiratory burst index, capacity and phagocytosis
250 efficiency (Table II). Observation of the variance in each group showed higher variances in
251 laboratory data regarding respiratory burst index in June, capacity in February, and
252 phagocytosis efficiency in December (data not shown).

253 **b. Comparison between *mesocosm* and *laboratory* data**

254 The results of the comparisons of means between "experimental levels" (Table III) were
255 compared to results based on percentages of *mesocosm* data outside the *laboratory* reference
256 ranges (Figure 1): the two methods provided complementary results for all biomarkers.

257 The results presented hereafter are based on statistical significance levels of 5%. Since the
258 calculating Q^2 were low for granulocyte-macrophage (-1,54) and apoptotic cell (-1,07)
259 percentages, the models predict some discrepancy between *laboratory* and *mesocosm* data in
260 December (40% for apoptotic cell percentage, binomial test: $p = 2.86 \times 10^{-6}$; 20 % for
261 granulocyte-macrophage percentage, binomial test: $p = 0.0159$; Figure 1) and in June (27.8 %
262 for apoptotic cell percentage, binomial test: $p = 0.0155$; Figure 1, Table IV) whereas the

263 comparison of means test does not (Table III). Only the granulocyte-macrophage percentage
264 difference observed in June, with drastically highest values in *mesocosm* condition was
265 observed with both methods (88.8 % of values out of range, binomial test: $p = 1.31 \times 10^{-21}$,
266 Figure 1; Dunn test, $p = 3.2 \times 10^{-8}$, Table III).

267 A better predictivity of the models was shown for phagocytic activity, including efficiency
268 ($Q^2 = -0.40$) and capacity ($Q^2 = -0.36$), and necrotic cell percentage ($Q^2 = -0.22$). The
269 significant high decrease of phagocytic efficiency shown in February (Dunnett test: $p = 1.71 \times$
270 10^{-6} , Table III) and June (Dunnett test: $p = 1.71 \times 10^{-6}$, Table III) were also detected with the
271 predictive model, with 25 % (binomial test: $p = 2.57 \times 10^{-3}$) and 27.8 % (binomial test: $p =$
272 0.00155) of deviation respectively (Figure 1). In the same way, no significant impact was
273 shown with both methods in August and October. On the other hand, in December, the
274 predictive model shown dissimilarity between *laboratory* and *mesocosm* efficiency
275 percentages (60% of deviation, binomial test: $p = 2.11 \times 10^{-11}$; Figure 1) without statistical
276 significance (Table III). On the opposite, in April, statistical test shown a significant light
277 decrease of efficiency percentage in *mesocosm* condition (Dunnett test: $p = 8.5 \times 10^{-18}$, Table
278 III) without increase of error percentage with the model (Figure 1). Concerning the
279 phagocytic capacity, a good projection of *mesocosm* data on *laboratory* reference ranges was
280 observed in February, April, August and October (Figure 1). Nevertheless, the significant
281 light modulation of capacity percentage observed in December (Dunnett test: $p = 1 \times 10^{-6}$,
282 light increase in *mesocosm* condition, Table III) and June (Dunnett test: $p = 1 \times 10^{-6}$, light
283 decrease in *mesocosm* condition, Table III) was not detected by the predictive model (10%,
284 binomial test: $p = 0.264$, and 0%, respectively). The necrotic cell percentage present a quite
285 good projection of *mesocosm* data on *laboratory* reference range compared to statistical test,
286 with significant highest values in *mesocosm* condition detected in June using both methods
287 (77.7 % of deviation, binomial test: $p = 2.12 \times 10^{-19}$; Dunn test, $p = 3.2 \times 10^{-8}$). Nevertheless,

288 in October, the significantly low increase detected with Dunn test ($p = 0.0021$, Table III) was
289 not observed using projection on *laboratory* reference ranges (0 % of deviation, Figure 1).

290 Moreover, positive calculating Q^2 values were shown concerning respiratory burst index ($Q^2 =$
291 0.03) and lysosomal presence ($Q^2 = 0.17$) suggesting better projection deviations for these
292 biomarker models. Lower values of respiratory burst index were found in *mesocosm*
293 compared to *laboratory* experimental level at June period with both methods (27.7 % of
294 deviation, binomial test, $p = 0.0001$; Dunn test, $p = 1.3 \times 10^{-6}$, Table III). Even if the
295 lysosomal presence in *mesocosm* was slightly lower in December (Dunnett test $p = 0.001$) and
296 slightly higher in February (Dunn test, $p = 0.00019$) and in April (Dunn test, $p = 0.034$) in
297 *mesocosm* compared to *laboratory* data (Table III), the projection of *mesocosm* data on
298 *laboratory* reference ranges was good for this biomarker (Figure 1).

299 The more important deviations between *mesocosm* and *laboratory* data were observed in
300 June. On the other hand, in August no significant differences were identified whatever the
301 immunomarker tested and the method used.

302 Overall, for all immunomarkers except lysosomal presence, there were statistically significant
303 differences in seasonal trends between *mesocosm* and *laboratory* data (F-test between nested
304 regression models, $p < 0.05$). When data collected in June was removed from the analysis, no
305 statistically significant differences were identified either regarding necrotic cell percentage.
306 The proportion of total variance explained by period and all related interactions is compared
307 to the proportion of total variance explained by the differences in seasonal trend in Table VI.
308 Seasonal trends were strongest for phagocytosis efficiency and although differences in
309 seasonal trend between *mesocosm* and *laboratory* data were comparatively weak (5.54 % of
310 total variance), they represented significant contributions to the model (F-test, $p = 6.44 \times 10^{-11}$).
311 Differences in seasonal trends were also particularly marked for necrotic cell percentage (due
312 to June, 19.2 % of total inertia, F-test, $p = 5.86 \times 10^{-14}$) and macrophage-granulocyte

313 percentage (18.4 % of total inertia, $p = 6.66 \times 10^{-16}$). Lysosomal presence, for which no
314 differences in seasonal trend were noted between mesocosm and laboratory data, was the
315 immunomarker with the weakest seasonal trend in mesocosm and laboratory data (26.5 % of
316 total inertia).

317 **c. Comparison between *field* and *laboratory* data**

318 Globally, projection of *field* data on *laboratory* reference ranges revealed high discrepancy to
319 the model predictions (Figure 1). Like for *mesocosm* data, when these results were compared
320 to classic statistical analysis (Table III), some dissimilarities were found.

321 With a Q^2 value highly negative, equal to $- 5.26$, the phagocytosis efficiency was the
322 biomarker that fit the less to the *laboratory* model. Indeed, differences between *laboratory*
323 and *field* experimental conditions were found at each period, except in December, with higher
324 values for *field* data in August (Dunnett test: $p = 1 \times 10^{-7}$), but lower values in February
325 (Dunn test: $p = 7.11 \times 10^{-15}$), April (Dunnett test: $p = 1.51 \times 10^{-18}$) and October (Dunn test: p
326 $= 1.51 \times 10^{-18}$) (Table III). These differences were observable by projection of *field* data on
327 *laboratory* reference ranges in February (85 % of deviation, binomial test: $p = 7.52 \times 10^{-20}$)
328 and April (65 % of deviation, binomial test: $p = 6.79 \times 10^{-13}$) but not in August (5 % of
329 deviation, binomial test: $p = 1$ and October (10 % of deviation, binomial test: $p = 2.64$).
330 Moreover, the model predicts a difference in the December period (27.8 % of deviation,
331 binomial test: $p = 0.00155$), not detected by statistical tests (Figure 1).

332 Low predictive power was also found for the granulocyte-macrophage percentage model (Q^2
333 $= - 1.1$), for the respiratory burst index model ($Q^2 = - 0.72$), for the phagocytic capacity model
334 ($Q^2 = - 0.51$), for the necrotic cell percentage ($Q^2 = - 0.44$), and for apoptotic cell percentage
335 ($Q^2 = 0.016$). The significant increase of granulocyte-macrophage percentages shown in
336 December (Dunnett test: $p = 0.01$, Table III), February (Dunn test: $p = 1 \times 10^{-4}$, Table III) and

337 October (Dunn test: $p = 1 \times 10^{-6}$, Table III) were also detected with the predictive model, with
338 33.3 % (binomial test: $p = 1.72 \times 10^{-4}$), 50 % (binomial test: $p = 1.13 \times 10^{-8}$) and 25 %
339 (binomial test: $p = 2.57 \times 10^{-3}$) of deviation, respectively (Figure 1). On the contrary, the
340 significant increase in April (Dunn test: $p = 1 \times 10^{-6}$) was not detected by the statistical model
341 (5 % of deviation, binomial test: $p = 1.00$). Even if the respiratory burst index was higher in
342 February (Dunn test: $p = 2.1 \times 10^{-4}$), April (Dunn test: $p = 0.00015$) and October (Dunn test: p
343 $= 3.8 \times 10^{-5}$), but lower in August (Dunn test: $p = 2.95 \times 10^{-5}$) in *field* compared to *laboratory*
344 data (Table III), the proportion of values out of range was not significantly different from the
345 expected 5%. As it was detected by the statistical model (100 % of values out of range,
346 binomial test: $p = 9.54 \times 10^{-27}$), strong decrease of capacity was observed in August (Dunnnett
347 test: $p = 1 \times 10^{-6}$). On the contrary, the low capacity decreases observed in February (Dunn
348 test: $p = 4 \times 10^{-5}$) and in October (Dunnnett test: $p = 5 \times 10^{-4}$) was not detected by data
349 projection on reference ranges (15 % of deviation and 0 % of deviation respectively; Figure
350 1). For the necrotic cell percentage, the same effects were detected using both methods in
351 February (Dunnnett test: $p = 1.5 \times 10^{-6}$; 40 % of deviation, binomial test: $p = 2.86 \times 10^{-6}$) and
352 August (Dunn test: $p = 3 \times 10^{-4}$) but not in December (Table III). Considering apoptotic cell
353 percentage, significant higher values were found in *field* experimental condition in April
354 (Dunnnett test: $p = 2 \times 10^{-4}$) that matched with the model predictions (35 % of deviation;
355 binomial test: $p = 3.39 \times 10^{-5}$).

356 With a Q^2 equal to 0.23, the model designed for the lysosomal presence was the most
357 predictive for *field* data. Compared to *laboratory* data, lysosomal presence was significantly
358 higher in *field* experimental condition in February (Dunnnett test: $p = 1 \times 10^{-4}$) and April
359 (Dunnnett test: $p = 2.7 \times 10^{-16}$) but lower in August (Dunnnett test: $p = 1 \times 10^{-5}$) and October
360 (Dunnnett test: $p = 0.02$; Table III). These effects were found again using projection on
361 reference ranges in February (25 % of values out of range; binomial test: $p = 0.002$), April 80

362 % of values out of range; binomial test: $p < 2.2 \times 10^{-11}$) and August (50 % of values out of
363 range; binomial test: $p = 1.13 \times 10^{-8}$) but not in October (Figure 1).

364 Statistically significant differences in seasonal trends between field and laboratory data for all
365 immunomarkers (F-test between nested regression models, $p < 0.05$). Seasonal trends were
366 strongest for lysosomal presence, capacity, and phagocytosis efficiency (F-test, $p < 10^{-16}$), for
367 which differences in seasonal trends between laboratory and field data represented 26.6 %,
368 23.8 %, and 18.8 % of total variance respectively. Differences in seasonal trends were less
369 strong for macrophage-granulocyte percentage (3.09 % of total inertia, $p = 2.17 \times 10^{-2}$).

370

371 **d. Evaluation of the impact of the “experimental level” factor on the**
372 **proposed reference ranges**

373 The models designed for respiratory burst index and for apoptotic cells explained only a small
374 proportion of the data variability with R^2 equal to 0.29 and 0.33 respectively. The necrotic
375 cells percentage was slightly better ($R^2 = 0.43$) explained by the confounding factors
376 (“month”, “experimental level”, “sex”, “size”) and their interactions (Table IV). The global
377 type-II ANCOVA revealed that “month”, “experimental level” (for apoptotic cell percentages,
378 respiratory burst and necrotic cell percentages) and “size” (for respiratory burst index,
379 apoptotic cell percentages, and necrotic cell percentages) all had a statistically significant
380 effect on these immunomarkers. In addition to the “month:experimental level” interaction
381 which influenced these three immunomarkers, the apoptotic cell percentages were also
382 impacted by “month:size” interaction and the necrotic cell percentages by “month:sex”
383 interaction (Table IV). The predictivity of the models including all the significant factors and
384 interactions was relatively low for the immunomarkers cited above. With a Q^2 LOO almost
385 equal, the apoptotic cell percentages (Q^2 LOO = 0.23) and the respiratory burst index (Q^2 LOO

386 = 0.24) were the less well predicted biomarkers by the model. Higher, but still low, predictive
387 power was found for the necrotic cell percentages, with a Q²LOO equal to 0.43 (Table VI).

388 Granulocyte-macrophage percentages ($R^2 = 0.52$), lysosomal presence ($R^2 = 0.54$), and
389 capacity ($R^2 = 0.63$) presented better correlation with these factors (Table IV). Indeed, the
390 “month”, the ‘experimental level’ and their interaction have a great influence on these three
391 immunomarkers. With granulocyte-macrophage percentages and capacity the interaction
392 “period:size” was strongly significant. Effects of “size” were more considerable for capacity
393 than for granulocyte-macrophage percentages and lysosomal presence. Granulocyte-
394 macrophage percentages were also influenced by “period:sex” and “experimental level:size”
395 interactions (Table IV). Medium-high level of predictivity was found for these markers, with
396 Q²LOO equal to 0.51 for lysosomal presence, 0.60 for phagocytic capacity and 0.46 for
397 granulocyte-macrophage percentages.

398 The phagocytic efficiency was highly correlated to the factors studied here ($R^2 = 0.80$). The
399 “period” and the “experimental level” factors have a strong influence while the “size” factor
400 influence was less important (Table IV). This marker was also highly influenced by
401 “period:size”, “period:experimental level” and “size:experimental level” interactions. This
402 good correlation with confounding factors was associated with a high predictive power of the
403 model including significant factors and interactions (Q²LOO = 0.77) (Table IV).

404

405 **IV. Discussion**

406 Use of robust reference values for biomarkers is important in biomonitoring programs to
407 minimize data misinterpretation. Nevertheless, due to the influence of environmental factors
408 on biomarkers (Hanson et al., 2010; Jolly et al., 2012; Sanchez and Porcher, 2009), the
409 addition of confounding factors in the statistical model should improve predictions of

410 biomarker reference values in function of site characteristics (Coulaud et al., 2011; Maltby et
411 al., 2002). In this way, many authors developed *laboratory* reference values comprising
412 confounding factor effect for field evaluation (Barrick et al., 2018, 2016; Burgeot et al., 2010;
413 Coulaud et al., 2011; Krell et al., 2011; Maltby et al., 2002). In case of stickleback innate
414 immunomarkers, the first step was performed in a previous study (Marchand et al., 2019) by
415 assessing the effect of sampling period, fish size and sex in laboratory conditions.
416 Nonetheless, before using these reference values on the field, the variations due to natural
417 conditions should be included. Natural modulation of the immune response could be due to
418 variations of environmental conditions, especially temperature and photoperiod (Bly and
419 Clem, 1992; Bowden et al., 2007; Dittmar et al., 2014; Morgan et al., 2008; Porter et al.,
420 2001) and to reproductive status (Hou et al., 1999; Kortet et al., 2003; Slater and Schreck,
421 1998; Zapata et al., 1992). During the one-year sampling, temperature and photoperiod
422 variations were similar in all three experimental conditions and the influence of sexual
423 hormones during breeding period was expected to be similar.

424 To determine if *laboratory* reference values could predict other experimental levels, new data
425 was compared to data obtained in laboratory conditions either using reference ranges obtained
426 with a regression model or by simply comparing means at given periods. The results using
427 both methodologies differed for several biomarkers. The *laboratory* reference ranges
428 developed previously took the effect of fish size into account on most of these
429 immunomarkers, except respiratory burst index and lysosomal presence (Marchand *et al.*
430 2019) for which the models developed in this paper are therefore not expected to be an
431 improvement over comparisons of means at a given period. For the other five
432 immunomarkers, since fish body size did not cover the same range in *mesocosm* and *field* data
433 as in the laboratory data, statistically significant differences in means could actually be due to
434 the confounding effect of fish size. For example, the efficiency possessed a strong “size”

435 effect in December and only the comparison with the *laboratory* reference ranges showed
436 differences between experimental levels. However, this strong effect of size in December was
437 based on laboratory data where two distinct fish sizes were analysed. Thus, this point had to
438 be confirmed with additional data. Sex had also been identified as a confounding factor of
439 necrotic cell percentage and granulocyte-macrophage distribution values.

440 Integration of more data in the regression models of immunomarkers can strengthen the
441 knowledge on the effect of confounding effects of size and sex, even when the new data is not
442 obtained in the same conditions. First, the immunomarkers that had been log-transformed in
443 the first study were again log-transformed in the present study. Secondly, the additional data
444 obtained in mesocosm and field conditions also confirmed that including sex was only
445 relevant for necrotic cell percentage and granulocyte-macrophage distribution. No significant
446 interactions between sex and experimental conditions were observed, which tends to confirm
447 that the effect of sex is independent of the experimental conditions. Thirdly, size had not been
448 included in the regression models based on laboratory data for respiratory burst index and
449 lysosomal presence, but when mesocosm and field data were added, a linear effect of size on
450 respiratory burst index (see Figure 2 in supplementary data) improved model quality,
451 independently of experimental conditions and sampling period. The additional data did not
452 however confirm the strong effect of size on efficiency in December, which appeared to be
453 specific to the set of data obtained in laboratory.

454 Even so, regarding percentages of data outside *laboratory* reference values, Dunn/Dunnett
455 statistical tests, and Q^2 , the *laboratory* reference ranges developed in the precedent work
456 (Marchand et al., 2019) does not fit perfectly with *mesocosm* and even less with *field* data.
457 Seasonal trends varied significantly between experimental levels. Furthermore, although
458 lysosomal presence showed little seasonal variation between mesocosm and laboratory data,
459 large differences were observed in field conditions. Conversely, although the seasonal trends

460 in macrophage-granulocyte percentages were similar in field and laboratory conditions, they
461 were different in mesocosms. All these statistical aspects demonstrated that the “experimental
462 level” factor have a strong effect on all the studied immunomarkers. Furthermore, as
463 demonstrated by the “experimental level:period” interaction, the “experimental level” factor
464 was also dependent of the season for almost all immunomarkers which implies that seasonal
465 variations were not the same in the various experimental conditions. As previously discussed,
466 the immune response could be influenced by many abiotic (Bowden, 2008) and biotic (Kortet
467 et al., 2003; Robertson et al., 2016) factors, which explain this discrepancy between
468 experimental conditions. Since the fish used in the different experimental condition originated
469 from the same regional ecotype and the temperature and photoperiod were quite similar,
470 differences in pathogen diversity and abundance were likely causes. In fact, pathogen
471 presence induces specific adaptations which may lead to differences in immune profiles of
472 populations living in separate environments (Scharsack et al., 2016). Nevertheless, even if this
473 specific adaptation of fish to their pathogenic environment can be responsible for a decrease
474 in immunity variability (Hablützel et al., 2016), the ANCOVA regression model relies on the
475 assumption that residual variability is the same in each condition. The differences in
476 variability observed at specific periods for some immunomarkers imply that at some periods,
477 the reference ranges will be narrower (e.g. respiratory burst index in June) than expected
478 given the variability at that period, and in some cases, they will be slightly larger (e.g.
479 respiratory burst index at periods other than June). Food allocation and intestinal microbiota
480 also impact the fish immune response (Gómez and Balcázar, 2008; Kosiewicz et al., 2014;
481 Waagbo, 1994). In *laboratory* and *mesocosm* conditions, fish were daily fed with bloodworms
482 unlike in *field* conditions where the food may have been more diversified. This parameter may
483 also be an explicative factor in the differences between conditions. Another factor fixed in
484 *laboratory* experimental level but not in *mesocosm* and *field* experimental levels was the fish

485 age. Since this factor was able to modulate fish immunity, in both innate and acquired
486 component, they should be considered (Robertson et al., 2016).

487 As for *laboratory* reference ranges (Marchand *et al.* 2019), the newly created models
488 including the three type of data (*laboratory*, *mesocosm* and *field*) integrated especially the
489 “size” effect. In fact, each immunomarker were differently influenced by the “size”, with
490 more impact on necrotic cell percentages and capacity and no statistically significant effect
491 shown on respiratory burst index and lysosomal presence. Likewise, as shown by the
492 coefficient of determination (R^2) of the models, the part of data variability explained by these
493 confounding factors was also variable between immunomarkers. Thus, whereas the efficiency,
494 the capacity and the granulocyte-macrophage percentages were well-explained by the
495 considered confounding factors, the respiratory burst index and the apoptotic cell percentages
496 were poorly explained. In some cases, high values of the coefficient of determination are due
497 to large effects of experimental conditions. This can be observed for example with lysosomal
498 presence: in our previous paper the R^2 reported was 13 % (Marchand et al., 2019), whereas it
499 is 53 % in the model including all data. The total variability due to experimental level when
500 data from mesocosms or data from field conditions were compared to laboratory conditions is
501 reported in Table VI: when data from field and laboratory are compared, as much as 40.8 %
502 of variability is due to the experimental level (capacity). The importance of experimental level
503 on immunomarkers may hinder extrapolation to other field conditions, since our experiments
504 were designed to limit differences in photoperiod, temperature and fish population
505 characteristics. Further research is need in similar filed conditions with the least possible
506 contaminations. The fact that, for some immunomarkers, confounding factors only have a
507 small effect on an immunomarker, as for respiratory burst index with only 28 % of data
508 variability explained by the four confounding factors, can have several causes and
509 implications. This may indicate either high inter-individual variability, high measurement

510 uncertainty, unidentified inter-individual confounding factors such as reproductive or health
511 status, or a lack of sensitivity to environmental factors. At the present, no studies have
512 established a relationship between sensitivity to environmental confounding factors and
513 sensitivity to various types and levels of pollution: further research is needed to compare
514 values of immunomarkers in similar environmental conditions with different levels of
515 pollution in a biomonitoring context. Depending on the sensitivity of these immunomarkers to
516 various types of contamination, the *field* reference ranges derived in these models could help
517 to define robust reference values useful for large scale biomonitoring programs (Maltby et al.,
518 2002).

519 **V. Conclusions**

520 This study focused on the evaluation of *laboratory*-based reference ranges, created on three-
521 spined stickleback immunomarkers, for *mesocosm* and *field* conditions. The “experimental
522 level” factor influences in a seasonal-dependent pattern all the studied immunomarkers,
523 therefore care must be taken when extrapolating reference values or ranges between different
524 experimental conditions. Finally, this study argued for considering the influence of specific
525 *field* confounding factors. In this way, *field* reference ranges were developed. Nevertheless,
526 before using them for large scale biomonitoring programs, these *field* reference ranges should
527 be tested on multiple uncontaminated and contaminated sites in order to identify the
528 biomarkers which offer the best compromise between sensitivity to contamination, and either
529 reproducibility in similar conditions or well-characterized effects of abiotic confounding
530 factors.

531

532

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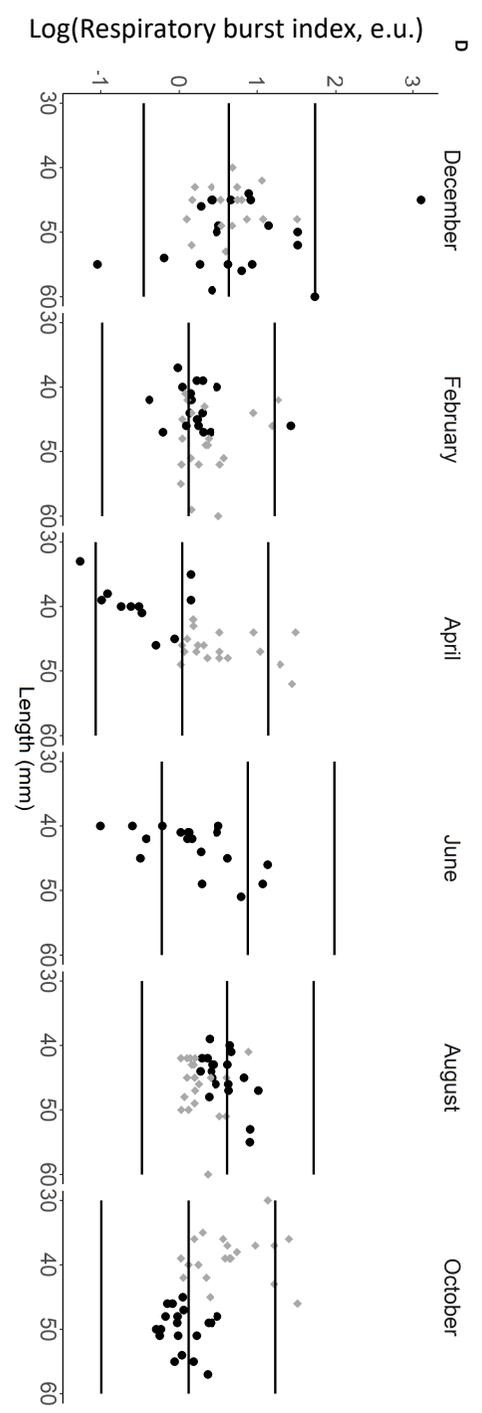
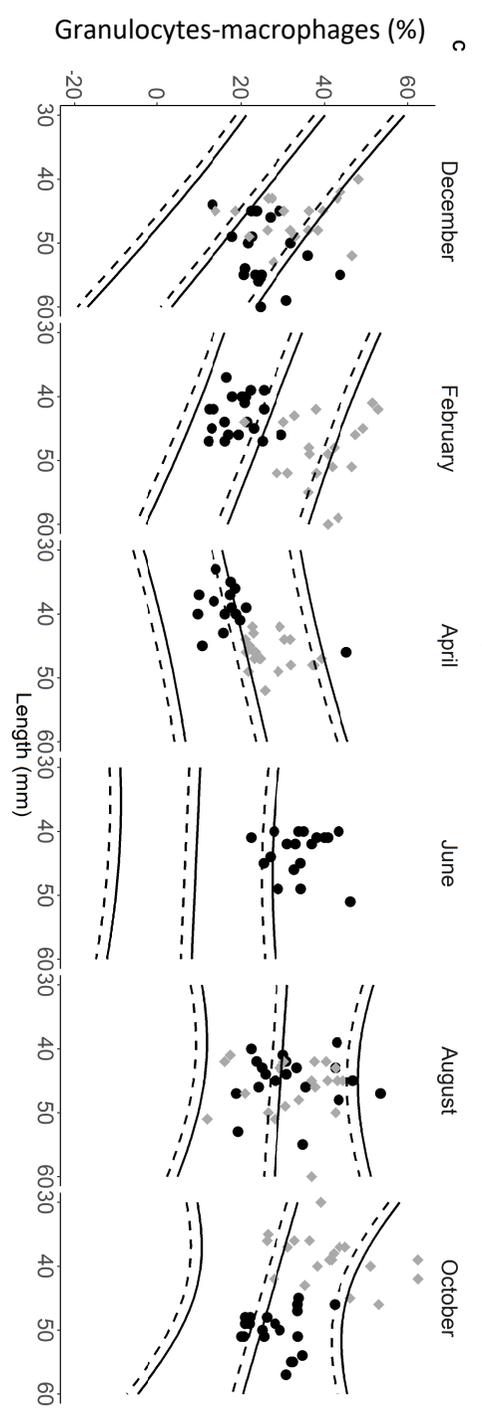
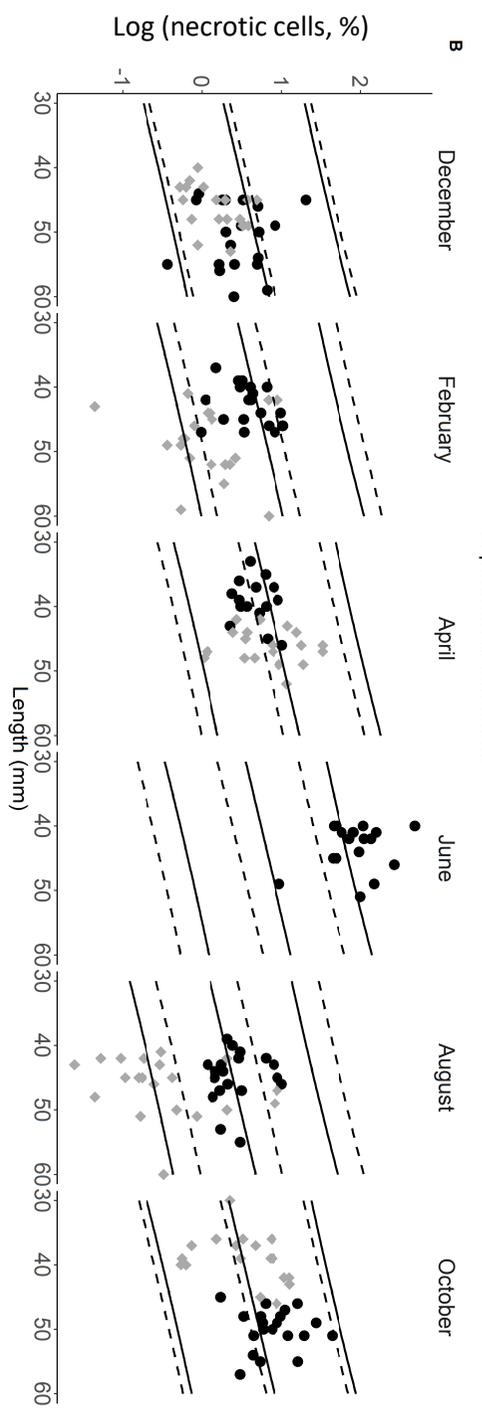
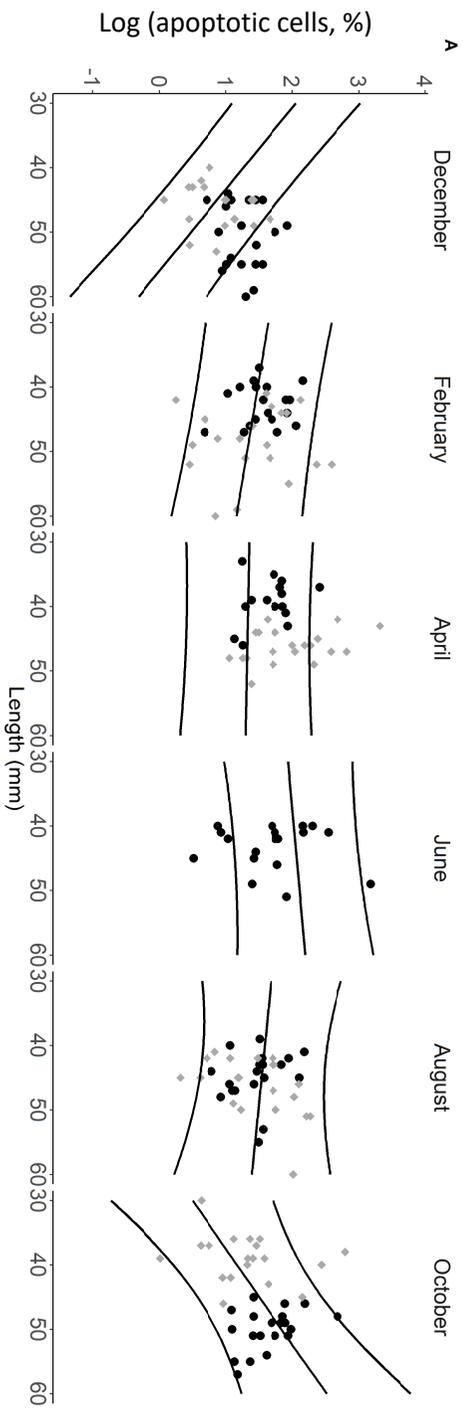
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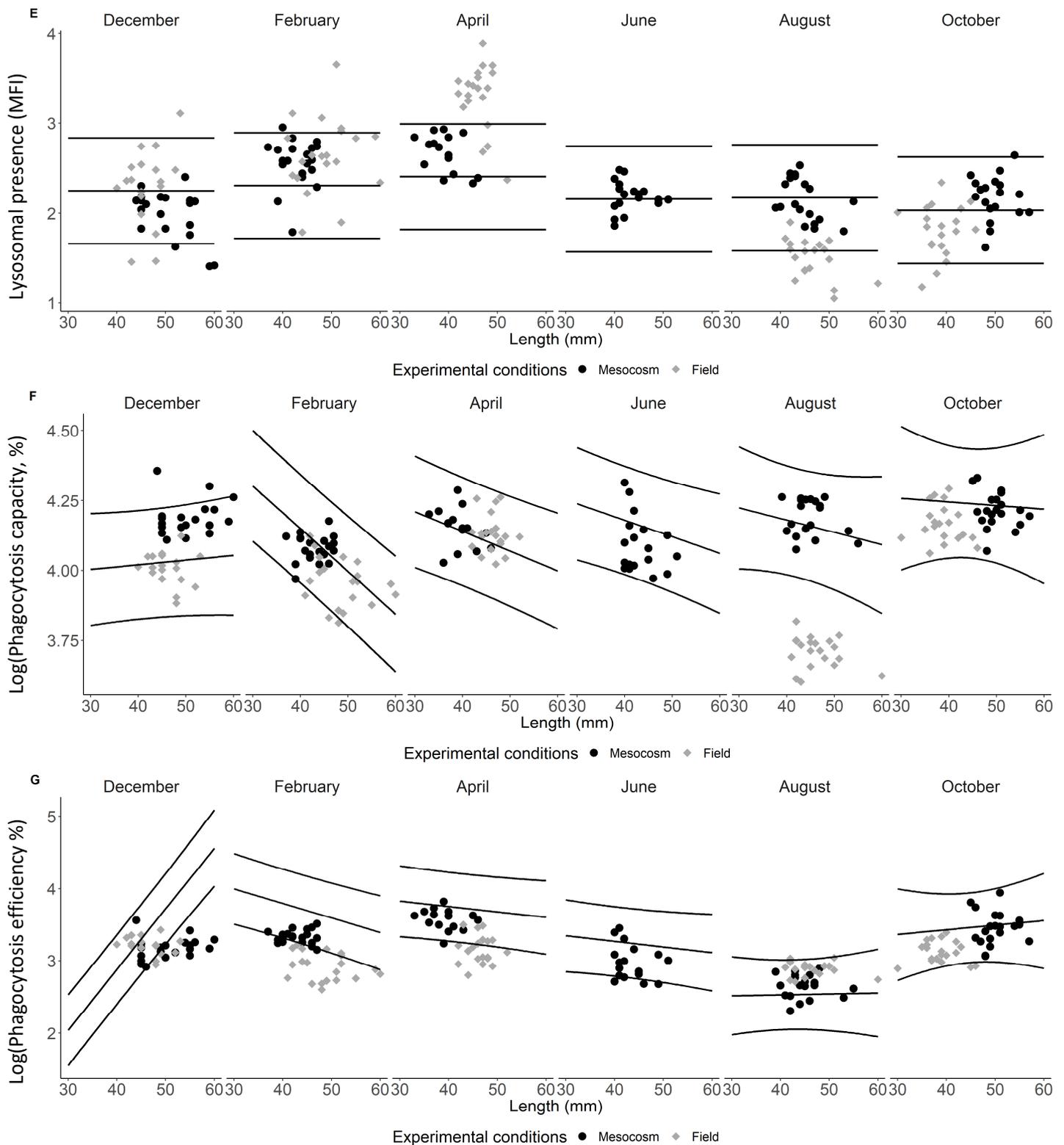


Figure 1: Projection of *mesocosm* (black) and *field* (grey) data on the *laboratory* reference ranges. For apoptotic cells, respiratory burst index, lysosomal presence, phagocytosis capacity and efficiency, the lines represent the values predicted by the model and the prediction interval at 95 %. For necrotic cells percentage and granulocyte-macrophage percentage, the full lines represent prediction intervals for females and the dotted lines represent prediction intervals for males. With MFI = Mean Fluorescent Intensity and e.u = experimental units.

Table I: Description of the two data sets used to perform the comparison to *laboratory* reference ranges in this study. *Laboratory* data set used to create *laboratory* reference ranges was described in Marchand et al. (2019).

MESOCOSM			FIELD						
Sampling period	<i>n</i> (M ; F)	Fish body size (mm)	Sampling period	<i>n</i> (M ; F)	Fish body size (mm)	Water parameters			
						pH	T (°C)	O₂ (%)	Conductivity (µS/cm²)
October 2015	20 (7 ; 13)	45-57	October 2015	20 (8 ; 12)	30 - 46	7.07	12	7.3	544
December 2015	20 (12 ; 8)	44-60	August 2017	20 (6 ; 14)	41 - 60	*	*	*	*
February 2016	20 (9 ; 11)	37-47	December 2017	20 (6 ; 14)	40 - 53	7.14	11.6	68.3	719
April 2016	15 (6 ; 9)	33-46	February 2017	20 (11 ; 9)	41 - 60	7.42	11.3	75.2	706
June 2016	18 (12 ; 6)	40-51	April 2017	20 (10 ; 10)	42 - 52	7.7	13.9	79	799
August 2016	19 (12 ; 7)	39-55							
TOTAL	112 (58 ; 54)	33-60	TOTAL	100 (41 ; 59)	30 - 60				

* Water parameters were not monitored at this period due to technical issues.

Table II: *p*-values from Shapiro tests inside each group and Levene tests for each immunomarker. Analysis were performed on residuals of the full linear model and biomarkers with * were log transformed before performing statistical tests.

LEVENE		SHAPIRO												
		Sex	December		February		April		June		August		October	
			Mesocosme	Field										
Apoptotic cells*	4.54 x 10 ⁻²	Female	0.53	0.39	0.74	0.72	0.33	0.14	0.87	-	0.80	0.26	0.63	0.07
		Male	0.39	0.54	0.27	0.72	0.89	0.58	0.04	-	0.38	0.38	0.77	0.21
Necrotic cells*	2.74x 10 ⁻²	Female	0.55	0.81	0.96	0.86	0.77	0.53	0.19	-	0.59	0.59	0.82	0.30
		Male	0.21	0.93	0.89	0.07	0.01	0.14	0.53	-	0.03	0.35	0.94	0.37
Granulocyte-macrophage	0.16	Female	0.44	0.72	0.30	0.82	0.71	0.06	0.92	-	0.78	0.22	0.25	0.99
		Male	0.20	0.41	0.91	0.41	0.74	0.03	0.86	-	0.64	0.48	0.02	0.28
Respiratory burst index*	6.32 x 10 ⁻⁷	Female	0.09	0.12	0.22	0.66	0.33	0.028	0.26	-	0.28	0.17	0.64	0.31
		Male	0.64	0.82	0.01	0.006	0.009	0.25	0.68	-	0.85	0.16	0.41	0.11
Lysosomal presence	1.84 x 10 ⁻²	Female	0.40	0.08	0.009	0.15	0.32	0.88	0.61	-	0.46	0.73	0.85	0.94
		Male	0.79	0.32	0.84	0.94	0.32	0.40	0.92	-	0.23	0.48	0.81	0.89
Phagocytosis capacity*	3.37 x 10 ⁻⁶	Female	0.55	0.81	0.96	0.86	0.77	0.53	0.19	-	0.59	0.59	0.82	0.30
		Male	0.21	0.93	0.89	0.07	0.01	0.14	0.53	-	0.03	0.35	0.94	0.37
Phagocytosis efficiency*	7.64 x 10 ⁻⁸	Female	0.30	0.38	0.44	0.67	0.55	0.71	0.44	-	0.87	0.94	0.82	0.30
		Male	0.58	0.76	0.73	0.08	0.26	0.64	0.28	-	0.46	0.49	0.06	0.38

Table III: Values correspond to means \pm standard deviations (with $n = 15-20$) for each innate immunomarker tested. Statistical differences between *laboratory* and *mesocosm* or *field* data were tested by a Dunnett test (parametric data, $p \leq 0.05$) or by a Kruskal-Wallis test followed by a Dunn test (non-parametric data, $p \leq 0.05$). With *, ** and *** indicates significant difference at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ levels respectively.

	DECEMBER			FEBRUARY			APRIL			JUNE			AUGUST			OCTOBER		
	Laboratory	Mesocosme	Field	Laboratory	Mesocosme	Field	Laboratory	Mesocosme	Field	Laboratory	Mesocosme	Field	Laboratory	Mesocosme	Field	Laboratory	Mesocosme	Field
Apoptotic cells (%)	3.84 ± 2.21	3.73 ± 1.19	2.65 ± 1.15	4.79 ± 2.37	5.01 ± 1.73	4.94 ± 3.16	4.02 ± 1.39	5.61 ± 2.02	8.58*** ± 5.87	9.15 ± 6.36	6.75 ± 5.15	-	5.27 ± 2.50	4.63 ± 1.84	4.91 ± 2.51	5.22 ± 3.25	5.64 ± 2.66	4.68 ± 3.73
Necrotic cells (%)	1.77 ± 0.54	1.67 ± 0.67	1.25** ± 0.40	2.39 ± 1.81	1.88 ± 0.54	1.20*** ± 0.60	2.36 ± 0.92	1.99 ± 0.42	2.41 ± 1.06	3.68 ± 8.87	7.31*** ± 2.62	-	1.86 ± 1.05	1.60 ± 0.51	0.79*** ± 0.67	2.08 ± 1.90	2.61** ± 0.94	1.75 ± 0.88
Granulocyte-macrophage (%)	25.90 ± 10.32	25.30 ± 6.65	32.80* ± 9.66	27.20 ± 10.00	19.40 ± 4.93	39.30* ± 8.17	18.50 ± 6.21	17.70 ± 8.47	27.20*** ± 5.78	8.19 ± 13.26	34.00*** ± 6.33	-	28.60 ± 9.33	32.20 ± 9.83	32.40 ± 9.82	26.20 ± 7.40	28.40 ± 6.21	41.20*** ± 10.37
Respiratory burst index (experimental unit)	2.11 ± 1.09	3.27 ± 4.67	1.99 ± 0.85	1.27 ± 0.73	1.33 ± 0.71	1.57*** ± 0.74	1.09 ± 0.32	0.66 ± 0.31	1.90*** ± 1.08	3.62 ± 3.71	1.36*** ± 0.77	-	1.98 ± 0.91	1.80 ± 0.43	1.34*** ± 0.36	1.27 ± 0.77	1.07 ± 0.26	2.13*** ± 1.06
Lysosomal presence (Mean Fluorescence Intensity)	2.25 ± 0.27	1.99** ± 0.27	2.30 ± 0.42	2.31 ± 0.28	2.56*** ± 0.26	2.64*** ± 0.42	2.40 ± 0.29	2.67* ± 0.21	3.30*** ± 0.36	2.16 ± 0.26	2.18 ± 0.17	-	2.17 ± 0.34	2.15 ± 0.24	1.52*** ± 0.23	2.03 ± 0.35	2.17 ± 0.24	1.81* ± 0.29
Phagocytosis capacity (%)	55.90 ± 4.09	65.90*** ± 4.21	55.10 ± 3.18	63.40 ± 13.11	59.10 ± 2.84	52.40*** ± 4.56	62.30 ± 6.78	63.60 ± 4.52	62.40 ± 4.27	65.00 ± 5.64	60.10*** ± 6.25	-	64.90 ± 4.00	66.20 ± 4.29	40.90*** ± 2.31	69.50 ± 4.54	67.70 ± 4.28	64.70*** ± 4.37
Phagocytosis efficiency (%)	21.70 ± 11.25	24.10 ± 3.81	25.20 ± 3.06	45.70 ± 16.78	28.00*** ± 2.60	18.70*** ± 3.62	42.40 ± 6.95	35.80* ± 5.08	23.90*** ± 4.60	26.40 ± 5.13	19.90*** ± 5.17	-	12.80 ± 2.44	14.30 ± 2.28	18.10*** ± 1.71	32.30 ± 6.65	32.20 ± 7.67	23.00*** ± 3.41

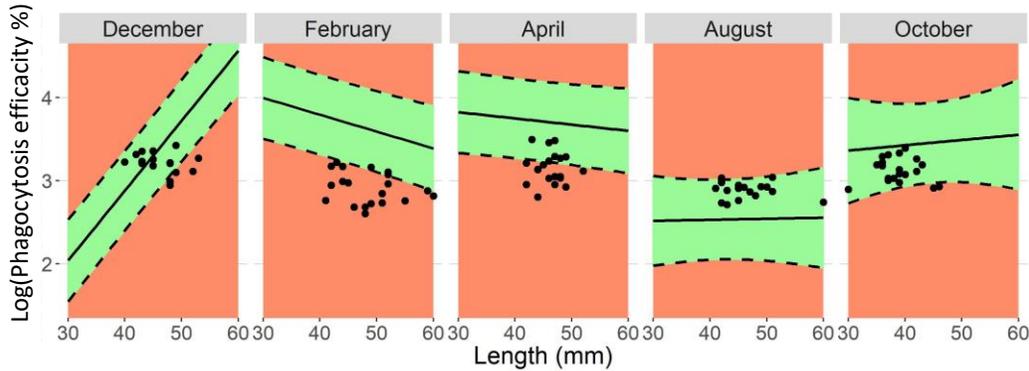
Table IV: Effects of sampling period, experimental level (*laboratory, mesocosm* or *field* data), sex and fish size (and interactions) on innate immune parameters of the three-spined stickleback, *Gasterosteus aculeatus*. Results were obtained by performing a type II ANCOVA ($p \leq 0.05$). With *, ** and *** indicates statistically significant effect at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ levels respectively in F-tests. R² were calculated on the model including only the significant explicative variables and interactions. Q²LOO corresponds to the squared cross-validated correlation coefficient obtained using a leave-on-out procedure. Biomarkers with * were log transformed before performing statistical tests.

	Explicative variables										R ²	Q ² LOO
	Period	Experimental level	Sex	Size	Period:experimental level	Period:sex	Experimental level:sex	Period: size	Experimental level:size	Experimental level:Period:size		
Apoptotic cells*	***				***			***	*	***	0.33	0,24
Necrotic cells*	***	***		**	***	**					0.43	0,43
Granulocyte-macrophage	***	***		**	***	*		***	*		0.52	0,46
Respiratory burst index*	***	***		*	***						0.29	0,24
Lysosomal presence	***	**			***						0.53	0,51
Phagocytosis capacity*	***	***		***	***			***			0.63	0,60
Phagocytosis efficiency*	***	***		***	***			***	*	***	0.80	0,77

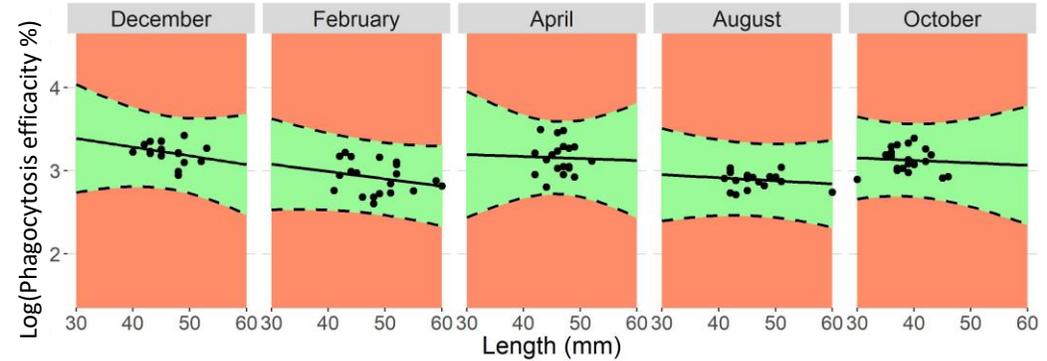
Table V: Proportion of total variability A) explained by period and its interactions (binary and ternary) with other confounding factors B) explained by the period:experimental level interaction and its interactions (ternary) with other confounding factors, C) explained by experimental level and its interactions (binary and ternary) with other confounding factors, for each immunomarker, with the data collected in mesocosms, with and without June, and for the data collected in field, compared to data collected in laboratory conditions. Biomarkers with * were log transformed before performing statistical tests

	Mesocosm			Mesocosm without June			Field		
	<i>Period (%)</i>	<i>Period: experimental level (%)</i>	<i>Experimental level (%)</i>	<i>Period (%)</i>	<i>Period: experimental level (%)</i>	<i>Experimental level (%)</i>	<i>Period (%)</i>	<i>Period: experimental level (%)</i>	<i>Experimental level (%)</i>
Apoptotic cells*	33.4	7.38	7.71	24.0	8.42	9.58	28.3	11.4	13.3
Necrotic cells*	32.1	19.2	22.2	18.4	5.57	6.80	31.0	13.3	26.2
Granulocyte-macrophage	45.1	18.4	21.8	33.9	7.80	11.0	27.1	3.09	19.1
Respiratory burst index*	32.5	6.64	8.20	39.6	4.73	5.19	30.8	11.5	14.8
Lysosomal presence	26.5	5.36	6.89	26.9	5.81	7.64	58.1	26.6	27.9
Phagocytosis capacity*	40.0	15.3	16.3	41.2	13.8	16.2	45.6	23.8	40.8
Phagocytosis efficiency*	79.5	5.54	6.32	81.3	4.89	5.21	77.8	18.8	23.1

Laboratory reference ranges



Development of
new reference ranges



Not adapted to field and mesocosm data

Site-specific field reference ranges