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Application in a biomonitoring context of three-spined stickleback immunomarker reference ranges

Adrien Marchand^{a,1}, Cleo Tebby^{b,1}, Audrey Catteau^a, Cyril Turiès^a, Jean-Marc Porcher^a, Anne Bado-Nilles^{a,*}

^a Institut National de l'Environnement Industriel et des Risques (INERIS), UMR-I 02 SEBIO, Parc Technologique Alata, BP 2, 60550 Verneuil-en-Halatte, France

^b INERIS, Unit of Models for Ecotoxicology and Toxicology (METO), Parc Technologique Alata, BP 2, 60550 Verneuil-en-Halatte, France

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ABSTRACT

The relevance of a biomarker for biomonitoring programs was influenced both by the knowledge on biomarker natural inter-individual and site variabilities and by the sensitivity of the biomarker towards environmental perturbations. To minimize data misinterpretation, robustness reference values for biomarkers were important in biomonitoring programs. Specific three-spined stickleback, *Gasterosteus aculeatus*, immune reference ranges for field studies had been determined based on laboratory data and one reference station (Contentieuse river at Houdancourt). In this study, data obtained in one uncontaminated and three contaminated sites were compared to these reference ranges as a validation step before considering them for larger scale biomonitoring programs. When the field reference range were compared to data from the uncontaminated station (Béronelle), only few deviations were shown. In this way, data coming from uncontaminated station (Béronelle) was integrated in the field reference ranges to improve the evaluation of site variability. The new field reference ranges provided better discrimination of sites and spanned a larger range of fish lengths than the initial reference ranges. Furthermore, the results suggest lysosomal presence during several months and phagocytosis capacity in autumn may be the most relevant immunomarkers towards identifying contaminated sites. In the future, combining this reference value approach with active biomonitoring could facilitate the obtention of data in multiple stream conditions.

1. Introduction

High variability of biological responses, though beneficial for populations in reacting to stress, hinders quantification of adverse effects in ecotoxicological studies (Devin et al., 2014). In this way, it is important to characterize the natural variability of the selected biomarkers to better understand biological impact of contaminants during biomonitoring studies. As previously recommended, determination of reference values may help to consider natural variations in environmental diagnostics and improve data interpretation (Burgeot et al., 2010; Coulaud et al., 2011; Marchand et al., 2019, 2020). Currently, two methods are used to better determine effects of confounding factors on biomarker responses. The first method, which consists in collecting organisms on the same reference site during several years to integrate effects of many confounding factors (Burgeot et al., 2010; Barrick et al., 2016, 2018), provides results only representative of the water quality of the studied site and suffers from confounding factors, the effects of

which cannot be clearly quantified. On the other hand, the second method consists of separate evaluations of each confounding factor which are then integrated into a larger model of biomarker responses (Maltby et al., 2002; Coulaud et al., 2011; Hanson, 2011; Krell et al., 2011; Marchand et al., 2019).

Among the set of biomarker reference ranges, recent studies have demonstrated benefits of immunomarker reference ranges in biomonitoring (Marchand et al., 2019, 2020). In fact, immunological parameters reflect both environmental contamination and fish health status (Bols et al., 2001) which improve the identification of risks associated to an ecosystem contamination (Bado-Nilles et al., 2014). Among all the measurable parameters that represent innate and acquired components of the immune system, biomarkers related to innate immune functions were highly relevant due to their response being non-dependent of previous exposure to foreign antigens (Monserrat et al., 2007). Furthermore, phagocytosis capacity was at the interface between innate and adaptive response (Biller and Takahashi, 2018)

* Corresponding author.

E-mail address: Anne.Bado-Nilles@ineris.fr (A. Bado-Nilles).

¹ These authors contributed equally to the publication.

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which explains their central role in cellular immune processes in fish (Bols et al., 2001; Neumann et al., 2001; Biller and Takahashi, 2018). In ecotoxicology, from phagocyte recruitment to pathogen destruction, each step of the phagocytosis mechanism can be used as a biomarker of field pollution in fish (Betoulle et al., 2000; Bols et al., 2001; Reynaud and Deschaux, 2006). For example, phagocytic efficiency and capacity were repressed in rainbow trout (*Oncorhynchus mykiss*) after one week of exposure to Montreal municipal sewage effluent (Salo et al., 2007) or in an industrial waste incineration site (Benchalgo et al., 2014). In the same way, respiratory burst (Bado-Nilles et al., 2014, 2015b) and lysosome modifications (Köhler, 1991; Köhler et al., 2002) were strongly affected in field by many pollutants. Moreover, these two immunomarkers were currently associated to both necrotic and apoptotic cell death (Holtzman, 1989; Risso-de Faverney et al., 2001; Guicciardi et al., 2004; Kurz et al., 2008).

The present works want to test the field reference ranges, previously defined by Marchand et al. (2020), on multiple uncontaminated and contaminated sites from a French sampling campaign before being used for large scale biomonitoring programs. Since the field reference ranges (R1) were developed in Marchand et al. (2020) using one single uncontaminated reference site (*Contentieuse* at Houdancourt), in the first step we compared this data to one other uncontaminated site (*Béronelle* at Breuil-le-Sec) before developing a new field reference range (R2) which account for natural variations between sites. Then, in a second step, the R1 and R2 were matched by comparison of data collected from a highly contaminated station due to urban runoff (*Réveillon* at Villecresnes), from a station highly polluted by agricultural practices (*Fossé Traxin* at Pontpoint) and from a site exposed to domestic runoff (*Rivière de Tancarville* at Tancarville).

2. Materials and methods

2.1. Sampling station and fish sampling

Five distinct stations (Fig. 1, Table 1) were investigated. The *Contentieuse* (Houdancourt, Oise, France, from October 2016 to April 2018) was an uncontaminated site located immediately upstream of a water-cress exploitation (Marchand et al., 2020). The *Béronelle* station (Breuil-le-Sec, Oise, France, from August 2017 to April 2018) was a small stream which was considered as uncontaminated due to absence of chemical pollution around the sampling site. The three others were considered as contaminated. The *Réveillon* river (Villecresnes, Val de Marne, France, from October 2016 to April 2018) was highly disturbed due to high urban contamination and hydromorphological alterations. The *Rivière de Tancarville* (Tancarville, Seine Maritime, France, October 2016) was poorly urbanised and contaminated. The *Fossé Traxin* station (Pontpoint, Oise, France, from August 2017 to April 2018) was located on a small canal highly impacted by agricultural practices surrounding this site. Along the manuscript, each station was named as: reference station (*Contentieuse*), uncontaminated station (*Béronelle*), highly urban contaminated station (*Réveillon*), moderately urban contaminated station (*Rivière de Tancarville*) and agricultural contaminated station (*Fossé Traxin*).

In each station, 20 adult fish by date were caught by electrofishing. After capture, on field, all fish were anaesthetized (tricaine methanesulfonate, 100 mg/L, Sigma), sacrificed, measured, weighed, and the spleen was removed to measure, in the laboratory, immune responses. Furthermore, at each sampling date and for each station, temperature, dissolved oxygen, pH and conductivity were monitored.

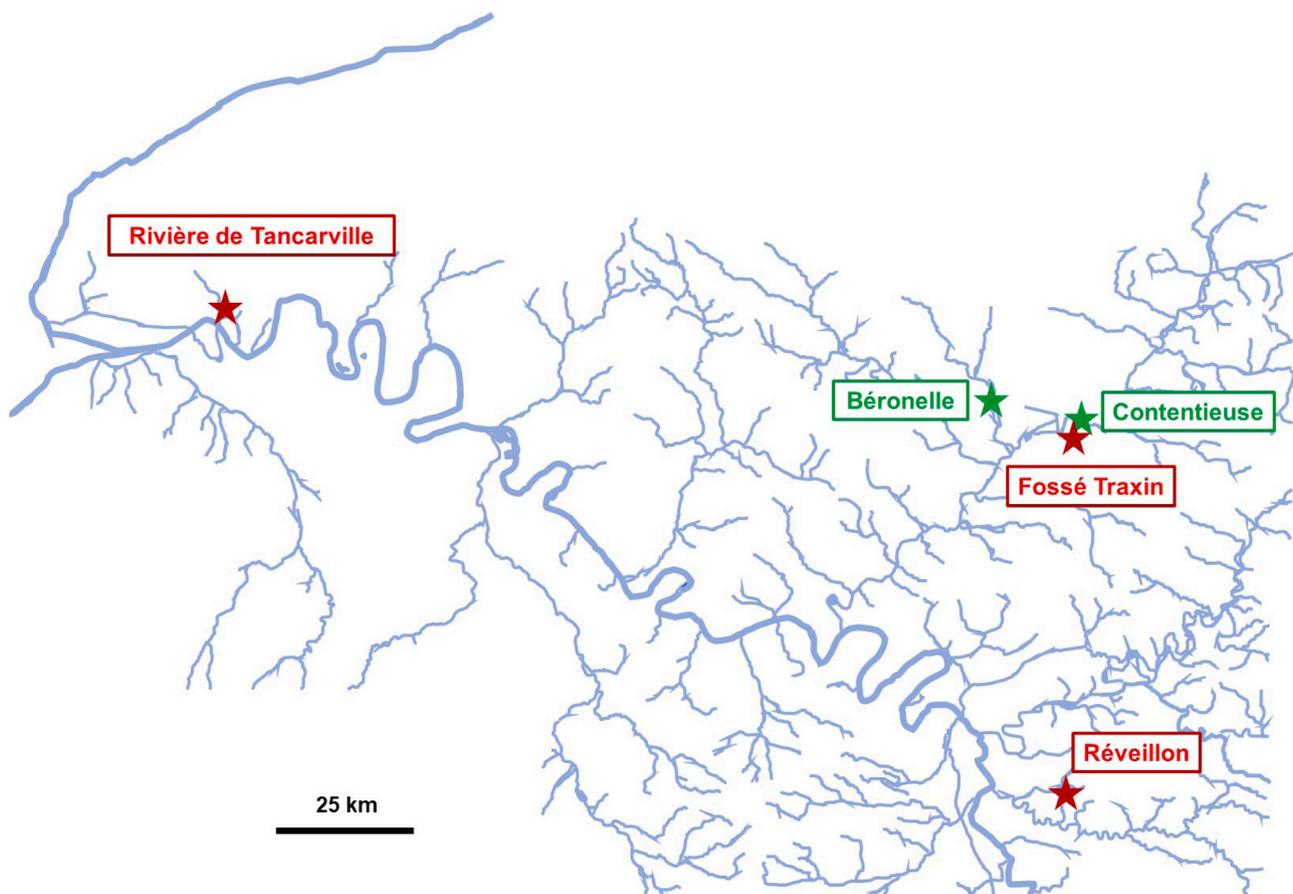


Fig. 1. Localization of studied sites in the north of France corresponding to reference station (*Contentieuse*), uncontaminated station (*Béronelle*), highly urban contaminated station (*Réveillon*), moderately urban contaminated station (*Rivière de Tancarville*) and agricultural contaminated station (*Fossé Traxin*).

Table 1
Fish characteristics and water physical parameters of each site at each sampling period, except for the reference station (Contentieuse) (Houdancourt, Oise, France) which were previously described (Marchand et al., 2020).

	Uncontaminated						Highly urban contaminated						Agricultural contaminated						Moderately urban contaminated					
	Béronelle at Breuil-le-Sec						Réveillon at Villecresnes						Fosé Traxin at Pontpoint						Rivière de Tancarville at Tancarville					
	Oise (60)						Val-de-Marne (94)						Oise (60)						Seine-Maritime (76)					
	49.372513; 2.445940						48.7204737; 2.543006						49.305696; 2.636935						49.486074; 0.466481					
	Physical parameters						Physical parameters						Physical parameters						Physical parameters					
	Size (mm)	T° (°C)	pH	Conductivity (µS.cm ⁻²)	O ₂ (%)	n (M; F)	Size (mm)	T° (°C)	pH	Conductivity (µS.cm ⁻²)	O ₂ (%)	n (M; F)	Size (mm)	T° (°C)	pH	Conductivity (µS.cm ⁻²)	O ₂ (%)	n (M; F)	Size (mm)	T° (°C)	pH	Conductivity (µS.cm ⁻²)	O ₂ (%)	
October 2016	-	-	-	-	-	20 (8; 12)	46-56	12.3	8.06	615	85.5	-	-	-	-	-	-	-	17 (7; 10)	30-48	12.8	7.48	398	87.0
August 2017	-	-	-	-	-	16 (8; 8)	43-53	a	a	a	a	-	-	-	-	-	-	-	-	-	-	-	-	-
December 2017	20 (11; 8)	35-52	9.3	7.66	737	62.8	20 (11; 9)	39-53	10.2	8.14	600	90.6	20 (8; 12)	35-44	8.4	7.62	844	79.2	-	-	-	-	-	-
February 2018	20 (7; 13)	42-58	8.6	7.64	524	85.6	20 (11; 9)	38-50	10.1	8.04	642	95.7	20 (11; 9)	35-49	6.4	7.58	701	94.1	-	-	-	-	-	-
April 2018	12 (7; 5)	36-48	11.7	7.72	473	92.6	20 (8; 12)	37-46	12.1	8.09	621	101.7	20 (9; 11)	38-51	11.1	7.82	609	111.7	-	-	-	-	-	-
TOTAL	52 (25; 27)	35-58	-	-	-	96 (46; 54)	37-56	-	-	-	-	60 (28; 32)	35-51	-	-	-	-	17 (7; 10)	30-48	-	-	-	-	

Oise, France) which were previously described (Marchand et al., 2020).

^a Water parameters was not monitored at this period due to technical issues.

2.2. Leucocyte isolation and innate immune biomarker analysis

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

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

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

The measurement of leucocyte respiratory burst was performed using a modification of the Chilmoneczyk and Monge (1999) technique, adapted for stickleback. Determination of reactive oxygen species (ROS) production in unstimulated cells depends upon the cell incorporating 2', 7'-dichlorodihydrofluorescein diacetate acetyl ester ($\text{H}_2\text{DCF-DA}$, Invitrogen, final concentration: 29.30 mg/L), a stable non-fluorescent molecule which was hydrolysed to dichloro-dihydro-fluorescein diacetate (DCFH) by cytosolic enzymes. When leucocytes were stimulated with phorbol 12-myristate 13-acetate (PMA, Invitrogen, final concentration: 9.25 mg/L), the most specific inductor of respiratory burst (Ambrozova et al., 2011; Chadzinska et al., 2012), $\text{H}_2\text{DCF-DA}$ was hydrolysed by H_2O_2 . Finally, the DCFH obtained was oxidized to the fluorescent dichlorofluorescein (DCF) to enable quantification, after 30 min of incubation at room temperature, by flow cytometry of unstimulated and stimulated cells in FL1. The respiratory burst index was determined as the ratio of fluorescence of PMA stimulated cells ($\text{H}_2\text{DCF-DA}$ plus PMA) to that of unstimulated cells ($\text{H}_2\text{DCF-DA}$).

Intracellular lysosomal presence was determined by incubation of spleen leucocyte suspension with 0.3 mg/L of acridine orange (AO, Sigma), a lysosomotropic weak base, for 20 min in the dark at room temperature. Then, fluorescence was measured in FL3 (Bado-Nilles et al., 2013).

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

2.3. Development of improved field reference ranges

As previously described by Marchand et al. (2020), the initial field reference ranges (hereafter referred to as R1) made use of data from laboratory, outdoor mesocosms and one French single reference station

(Contentieuse) – data provenance (laboratory, mesocosm, field) was taken into account in the models. This R1 data was compared to uncontaminated station (Béronelle) data using a *t*-test ($p \leq 0.05$) or a Wilcoxon test ($p \leq 0.05$), depending on whether the normality and homoscedasticity assumptions were fulfilled. Since the data were comparable, a new field reference range (hereafter referred to as R2) was developed by adding data from uncontaminated station (Béronelle) station to R1. Statistical significance of the number of uncontaminated station (Béronelle) data points outside R1 was evaluated using a unilateral binomial test compared to the expected 5%.

Finally, since the R2 could be safely used, type II analyses of covariance (ANCOVA) (Shaw and Mitchell-Olds, 1993) (“Anova” function of R package “car”, $p \leq 0.05$) were used to develop the R2 reference ranges based on a linear model with their binary and ternary interactions (biomarker ~ period + origin + sex + size + period:sex + period:origin + period:size + size:origin + size:sex + origin:sex) selected stepwise and downward.

2.4. Comparison of new field data to R1 and R2 field reference ranges

The predictability of new field data was evaluated for R1 and R2 by using highly urban contaminated station (Réveillon), moderately urban contaminated station (Rivière de Tancarville) and agricultural contaminated station (Fossé Traxin) data. Firstly, the set of data were compared to the two reference ranges (R1/R2) using either an ANCOVA followed by a Dunnett test ($p \leq 0.05$) for parametric data. Then, the coefficient of determination (R^2) and the percentage of data outside the reference ranges (noted error percentage) were calculated for each period of each site and with each reference range (R1 and R2) (Marchand et al., 2019).

2.5. Statistical analysis

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

3. Results

A summary of the innate immune responses of the highly urban contaminated station (Réveillon), the moderately urban contaminated station (Rivière de Tancarville) and the agricultural contaminated station (Fossé Traxin) was proposed in the Table S1.

3.1. Agreement between uncontaminated station (Béronelle) data and previously determined reference ranges

The data obtained from the uncontaminated station (Béronelle) was mostly within the R1 reference ranges: among the 27 reference ranges compared (seven biomarkers at three periods with differences between sex for two biomarkers) only 12 showed discrepancies and only 6 showed significant numbers of datapoints from uncontaminated station (Béronelle) out of the reference ranges (Fig. 2, Table 2). In fact, in February, a reduced lysosomal presence was shown in uncontaminated station (Béronelle) (2.26 ± 0.33 MFI) compared to reference station (Contentieuse) (2.64 ± 0.42 MFI) (Dunnett test *p*-value = 0.003) confirmed by a significant percentage of data outside the R1 reference range (25%, binomial test *p*-value = 0.003). In the same way, whatever the sex, necrotic percentages detected in April were lower in uncontaminated station (Béronelle) ($1.35 \pm 1.07\%$ for male; 1.62 ± 1.02 for female) than reference station (Contentieuse) ($2.24 \pm 1.17\%$ for male; 2.59 ± 1.00 for female) (Dunnett test *p*-value = 0.010) with also 25% of data outside the R1 reference range (binomial test *p*-value = 0.020). Although the respiratory burst was not significantly different between sites in April (Dunnett test *p*-value = 0.446), a significant percentage of

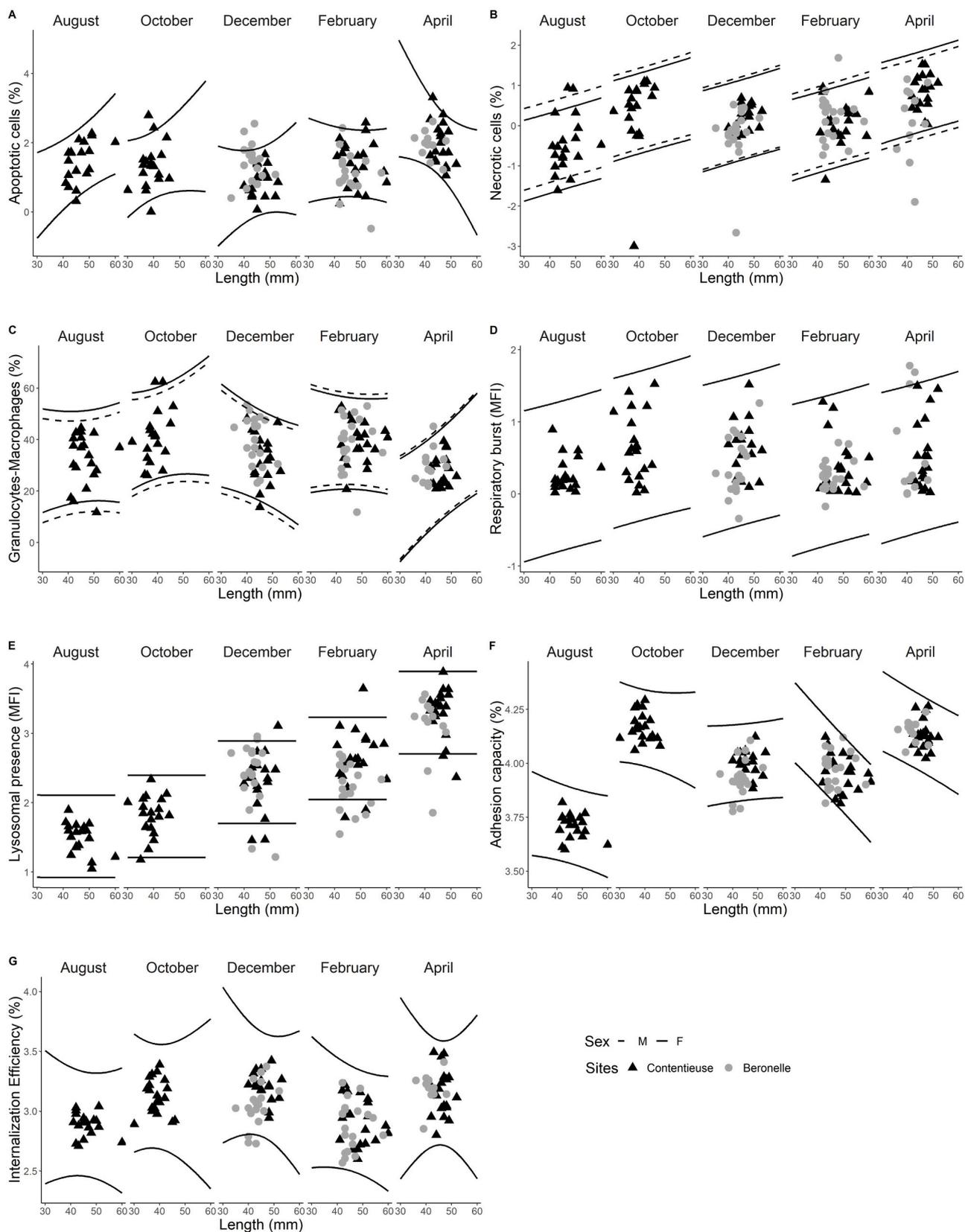


Fig. 2. Projection of uncontaminated station (Béronelle) data on the R1 field reference ranges, corresponding to reference station (Contentieuse) data. For apoptosis (A), respiratory burst index (D), lysosomal presence (E), adhesion capacity (F) and internalization efficiency (G), the lines represent the values predicted by the model and the prediction interval at 95%. For necrotic cells (B) and granulocyte-macrophage percentages (C), the full lines represent prediction intervals for female and the dotted lines represent prediction intervals for male. With MFI = Mean Fluorescent Intensity and e.u = experimental units.

Table 2

Summary of the statistical differences obtain between reference station (Contentieuse) and uncontaminated station (Béronelle) for innate immune immunomarkers in three-spined stickleback, *Gasterosteus aculeatus*. The *t*-test (parametric data, $p \leq 0.05$) or the Wilcoxon test (non-parametric data, $p \leq 0.05$) were used to test statistical differences between males and females and between station. The error % correspond to percentage of data outside the Contentieuse-based reference ranges (R1) for uncontaminated station (Béronelle) tested using a “greater than 5%” unilateral binomial test ($p < 0.05$). With $n = 12-20$ (mean \pm standard deviation); *, ** and *** indicates significant difference at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ levels respectively; M = male; F = female.

		December			February			April		
		Reference	Uncontaminated	Error %	Reference	Uncontaminated	Error %	Reference	Uncontaminated	Error %
Apoptosis (%)	M/	2.65	4.74**	20*	4.94	3.63	15	8.58	4.96	0
	F	± 1.15	± 2.83		± 3.16	± 2.33		± 5.95	± 2.73	
Necrosis (%)	M	1.30	0.98*	5	1.13	1.98	5	2.24	1.35**	25*
	F	± 0.51	± 0.37		± 0.60	± 1.59		± 1.17	± 1.07	
Granulocytes-macrophages (%)	M	29.27	38.44*	0	41.20	38.40	5	28.86	33.35	8
	F	± 14.90	± 9.11		± 7.46	± 12.81		± 6.96	± 8.70	
Respiratory burst index (Experimental units)	M/	1.99	1.57	0	1.57	1.32	0	1.90	2.32	25*
	F	± 0.85	± 0.69		± 0.74	± 0.30		± 1.09	± 1.86	
Lysosomal presence (Mean fluorescence intensity)	M/	2.30	2.39	20*	2.64	2.26**	25*	3.30	3.10	16.7
	F	± 0.42	± 0.47		± 0.42	± 0.33		± 0.36	± 0.48	
Adhesion capacity (%)	M/	55.07	51.17**	15	52.40	53.15	5	62.36	63.39	0
	F	± 3.18	± 4.40		± 4.56	± 4.36		± 4.33	± 3.22	
Internalization efficiency (%)	M/	25.17	21.58**	15	18.74	18.16	0	23.86	24.70	0
	F	± 3.06	± 3.75		± 3.62	± 3.66		± 4.66	± 3.05	

data outside the R1 reference ranges (25%, binomial test p -value = 0.020) was shown. December have more disparity between the two reference sites: increased values were observed for uncontaminated station (Béronelle) compared to reference station (Contentieuse) for apoptotic percentage ($4.74 \pm 2.83\%$, $2.65 \pm 1.15\%$, respectively, Dunnett test p -value = 0.003) with 20% of error (binomial test p -value = 0.016) and for granulocyte-macrophage percentages ($38.44 \pm 9.11\%$ and $29.27 \pm 14.90\%$ for male; $41.70 \pm 9.54\%$ and $33.81 \pm 8.11\%$ for female, respectively, Dunnett test p -value = 0.026) without data outside R1 reference ranges (binomial test p -value = 0.623). Significant differences between uncontaminated station (Béronelle) and reference station (Contentieuse) were observed for necrotic percentage ($0.98 \pm 0.37\%$ and $1.30 \pm 0.51\%$ for male; $0.92 \pm 0.44\%$ and $1.24 \pm 0.38\%$ for female, respectively, Dunnett test p -value = 0.015), for adhesion capacity ($51.17 \pm 4.40\%$, $55.07 \pm 3.18\%$, respectively, Dunnett test p -value =

0.003) and for internalization efficiency ($21.58 \pm 3.75\%$, $25.17 \pm 3.06\%$, respectively, Dunnett test p -value = 0.003), without any significant number of data points outside the reference ranges (binomial test p -value = 1.000, p -value = 0.076, p -value = 0.076, respectively). Even if the lysosomal presence was not significantly different between sites in December (Dunnett test p -value = 0.143), a significant percentage of data outside the R1 reference ranges (20%) was shown (binomial test p -value = 0.016).

3.2. Changes in reference ranges caused by adding data from the Béronelle uncontaminated station site

Pooling data obtained from the uncontaminated station (Béronelle) site with the field data previously used to build the R1 reference ranges resulted in few changes in reference ranges (Table 3). The month

Table 3

Effects of sampling period, origin (laboratory, mesocosm or field data), sex and fish size (and interactions) on the non-specific immune parameters of the three-spined stickleback, *Gasterosteus aculeatus*. Results were obtained by performing a type II ANCOVA ($p \leq 0.05$) on data including one reference station (Contentieuse) (see Marchand et al., 2020, “R1” field reference range) and on data including reference station (Contentieuse) and uncontaminated station (Béronelle) (“R2” field reference range). With *, ** and *** indicates significant effects of factor (F test) at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ levels respectively. R^2 were calculated on the model including only the significant explicative variables and interactions. With MFI = Mean Fluorescence Intensity and e.u = experimental units.

“R1” field reference range (Marchand et al., 2020)											
	Explicative variables										R^2
	Month	Origin	Sex	Size	Month:origin	Month:sex	Origin:sex	Month: size	Origin:size	Sex:size	
Log (Apoptosis, %)	***	*			***			***	*		0.33
Log (Necrosis, %)	***	***		**	***	**					0.43
Granulocytes-macrophages (%)	***	***		**	***	*		***	*		0.52
Log (Respiratory burst index, e.u)	***	*		*	***						0.29
Lysosomal presence (MFI)	***	***			***						0.53
Log (Adhesion capacity, %)	***	***		***	***			***			0.63
Log (Internalisation efficiency, %)	***	***		***	***			***	*		0.80
“R2” field reference range											
	Explicative variables										R^2
	Month	Origin	Sex	Size	Month:origin	Month:sex	Origin:sex	Month: size	Origin:size	Sex:size	
Log (Apoptosis, %)	***	***		*	***			**			0.32
Log (Necrosis, %)	***	**		*		***					0.28
Granulocytes-macrophages (%)	***	***		***	***	*		***			0.29
Log (Respiratory burst index, e.u)	***	***		**	***						0.12
Lysosomal presence (MFI)	***	***		***	***		*				0.71
Log (Adhesion capacity, %)	***	***		***	***			***	**		0.81
Log (Internalisation efficiency, %)	***	***		***	***			***	*		0.44

remained the most important factor on all biomarkers ($p \leq 0.001$). As previously, there was no main effect of sex throughout the year, but the significant interaction between sex and month suggests that sex has an effect in certain months on necrosis percentage ($p \leq 0.01$ for R1; $p \leq 0.001$ for R2) and on granulocyte-macrophage percentage ($p \leq 0.05$ for R1 and R2). In both reference ranges, the effects of origin (laboratory, mesocosm, or field) and of the interaction between month and origin have a strong impact on all immunomarkers. However, the interaction between origin and sex was only statistically significant for lysosomal presence ($p \leq 0.05$) in R2 reference ranges. The effect of size was not significant for the same immunomarkers in R1 and R2 reference ranges. In fact, even if size effect was detected for the two reference ranges for granulocyte-macrophage percentages ($p \leq 0.01$ for R1 and $p \leq 0.001$ for R2), respiratory burst ($p \leq 0.05$ for R1 and $p \leq 0.01$ for R2) and adhesion capacity ($p \leq 0.001$ for R1 and R2), the effect of size was significant on necrosis percentage ($p \leq 0.01$) and internalization efficiency ($p \leq 0.001$) in the models for R1 reference ranges and on apoptosis percentage ($p \leq 0.05$), lysosomal presence ($p \leq 0.001$) and adhesion capacity ($p \leq 0.001$) in the models for R2 reference ranges. For each biomarker, the interaction between month and size was either significant in both R1 and R2, or not significant in either R1 or R2. Interactions between month and size are significant for apoptosis percentage ($p \leq 0.001$ for R1; $p \leq 0.01$ for R2), granulocyte-macrophage percentage ($p \leq 0.001$ for R1 and R2), adhesion capacity ($p \leq 0.001$ for R1 and R2) and internalization efficiency ($p \leq 0.001$ for R1 and R2).

As a consequence from pooling the data obtained from uncontaminated station (Béronelle) and reference station (Contentieuse), the R^2 for field data was decreased for necrosis percentages, granulocyte-macrophage percentages, respiratory burst and internalization efficiency were lower in R2 reference ranges (0.28, 0.29, 0.12 and 0.44, respectively) compared to R1 reference ranges (0.43, 0.52, 0.29 and 0.80, respectively) (Table 3). On the other hand, lysosomal presence and adhesion capacity were higher represented by the R2 reference ranges

(0.71 and 0.81, respectively) compared to the R1 reference ranges (0.53 and 0.63, respectively).

Including the data from uncontaminated station (Béronelle) in the construction of the R2 reference ranges widened the reference ranges but actually only resulted in a slight decrease in the number of data points from presumably contaminated sites that were outside the R2 reference ranges compared to R1 (Table 4, Fig. S1). Nevertheless, some unusual discrepancy could be shown. Major dissimilarities concerned a more important percentage of error with R1 reference range than R2 (apoptosis in August for highly urban contaminated station (Réveillon), necrosis in April for highly urban contaminated station (Réveillon) and agricultural contaminated station (Fossé traxin), respiratory burst in October for moderately urban contaminated station (Rivière de Tancarville), lysosomal presence in February for highly urban contaminated station (Réveillon) and in December for agricultural contaminated station (Fossé traxin) and adhesion capacity in October for moderately urban contaminated station (Rivière de Tancarville). Only one percentage of error were more important by using R2 reference range than R1 (adhesion capacity in February for agricultural contaminated station (Fossé traxin)).

3.3. Comparison of field data to R2 reference ranges

Deviations between data from presumably contaminated field sites and R2 reference ranges (Table 4, Fig. 3) were observed for highly urban contaminated station (Réveillon) in October for four of the biomarkers: granulocyte-macrophage percentages (50% out of range, binomial test p -value = 1.180×10^{-8}), respiratory burst index (20% out of range, binomial test p -value = 0.016), adhesion capacity (25% out of range, binomial test p -value = 0.003) and internalization efficiency (55% out of range, binomial test p -value = 5.380×10^{-10}) (Table 4). Apoptosis and lysosomal presence data from highly urban contaminated station (Réveillon) were also out of the reference ranges in December (55% and

Table 4

Percentage of data outside the R1 and R2 reference ranges for each tested site (highly urban contaminated station (Réveillon), agricultural contaminated station (Fossé Traxin) and moderately urban contaminated station (Rivière de Tancarville)). Significant deviations from the 5% assumption error percentage was tested by unilateral binomial test. With *, ** and *** indicates significant difference at $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$ levels respectively.

	Highly urban contaminated station (Réveillon)					Agricultural contaminated station (Fossé Traxin)			Moderately urban contaminated station (Rivière de Tancarville)
	August	October	December	February	April	December	February	April	October
R1 reference ranges									
Apoptosis (%)	50***	15	35***	0	55***	15	0	5	0
Necrosis (%)	12.5	5	0	10	30***	10	25**	35***	47.1***
Granulocytes-macrophages (%)	6.25	80***	10	5	5	5	5	10	17.6*
Respiratory burst index (Experimental units)	0	30***	0	5	0	0	5	0	0
Lysosomal presence (Mean fluorescent intensity)	0	5	35***	20*	75***	20*	55***	80***	5.9
Adhesion capacity (%)	0	75***	5	10	5	10	40***	5	47.1***
Internalization efficiency (%)	0	55***	0	20*	5	0	15	5	94.1***
R2 reference ranges									
Apoptosis (%)	12.5	15	35***	0	25**	15	0	5	0
Necrosis (%)	0	5	0	10	10	5	20*	5	41.2***
Granulocytes-macrophages (%)	6.25	50***	10	5	0	0	5	5	0
Respiratory burst index (Experimental units)	0	20*	0	5	0	0	5	0	0
Lysosomal presence (Mean fluorescent intensity)	0	0	25**	5	70***	10	40***	40***	5.9
Adhesion capacity (%)	0	25**	5	10	10	20*	20*	5	0
Internalization efficiency (%)	0	55***	0	20*	10	0	10	5	64.7***

Concerning the R2 reference ranges, the dark grey cases correspond to statistically significant difference without modification of error percentage between R1 and R2 reference ranges. The grey cases correspond to an increase of significant error percentage (unilateral binomial test, $p < 0.05$) between R1 and R2 reference ranges. The light grey cases correspond to a decrease of significant error percentage (unilateral binomial test, $p < 0.05$) between R1 and R2 reference ranges.

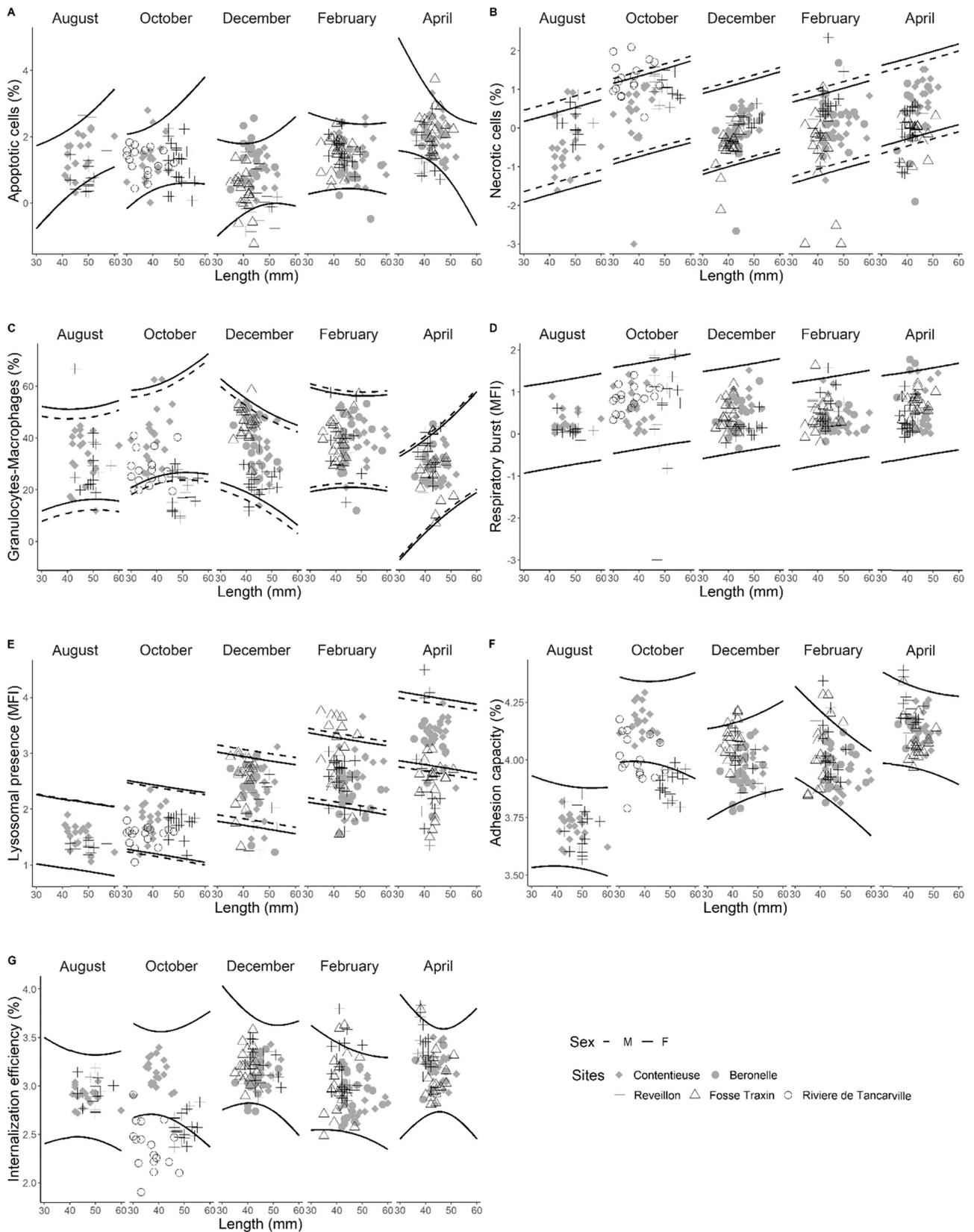


Fig. 3. Projection of contaminated sites data (highly urban contaminated station (Réveillon), moderately urban contaminated station (Rivière de Tancarville) and agricultural contaminated station (Fossé Traxin)) on the R2 field reference ranges (corresponding to reference station (Contentieuse) and uncontaminated station (Béronelle) data). For apoptosis (A), respiratory burst index (D), lysosomal presence (E), adhesion capacity (F) and internalization efficiency (G), the lines represent the values predicted by the model and the prediction interval at 95%. For necrotic cells (B) and granulocyte-macrophage percentages (C), the full lines represent prediction intervals for female and the dotted lines represent prediction intervals for male. With MFI = Mean Fluorescent Intensity and e.u = experimental units.

75% out of range, respectively; binomial test p -value = 3.390×10^{-5} and 0.003, respectively) and April (25% and 70% out of range, respectively; binomial test p -value = 0.003 and 5.590×10^{-15} , respectively). Internalization efficiency data from highly urban contaminated station (*Réveillon*) was also out of the reference range in February (20% out of range, binomial test p -value = 0.016). Data collected in agricultural contaminated station (*Fossé Traxin*) was out of the reference ranges for adhesion capacity in December (20% out of range, binomial test p -value = 0.016) and February (20% out of range, binomial test p -value = 0.016), for lysosomal presence in February (40% out of range, binomial test p -value = 2.860×10^{-6}) and April (40% out of range, binomial test p -value = 2.860×10^{-6}), and necrosis in February (20% out of range, binomial test p -value = 0.016). Data from moderately urban contaminated station (*Rivière de Tancarville*), which was collected in October was clearly out of the reference ranges for necrosis (41.2% out of range, binomial test p -value = 9.730×10^{-6}) and internalization efficiency (64.7% out of range, binomial test p -value = 4.560×10^{-11}).

4. Discussion

The relevance of a biomarker for biomonitoring programs was influenced both by the knowledge of biomarker natural variability and by the capacity of the biomarker to detect environmental perturbations (Flammarion et al., 1998; Van der Oost et al., 2003). Concerning the non-specific immune response of stickleback, the effect of three major confounding factors were modeled by Marchand et al. (2019) and tested in mesocosm and field conditions (Marchand et al., 2020). In the present work, the field reference ranges were tested in various stream conditions.

In a first step, new data from a presumably uncontaminated site was used to complete the previously published reference ranges. The major part of the discrepancy detected between reference station (*Contentieuse*) and uncontaminated station (*Béronelle*) stations was detected in December. The variations of the innate immune response could be due to many factors which could vary between stations, including variable pathogen diversity and quantity (Scharsack and Kalbe, 2014; Stewart et al., 2018), food resource (Waagbø, 1994; Gómez and Balcázar, 2008; Kosiewicz et al., 2014) and dynamics of water physico-chemical properties (Dittmar et al., 2014; Scharsack and Kalbe, 2014). To be representative of natural variations between control stations, the uncontaminated station (*Béronelle*) data were integrated to the previously established reference ranges.

The updated reference ranges differed only slightly regarding the influence of each confounding factor (period, origin, sex and fish size) and the difference between the data from the two stations resulted in decreased predicted variability (R^2) and wider reference ranges (necrosis percentage, granulocyte-macrophage percentage, respiratory burst and internalization efficiency). On the other hand, the R^2 for lysosomal presence and the phagocyte adhesion capacity were increased by the addition of data from uncontaminated station (*Béronelle*) due to good agreement between uncontaminated station (*Béronelle*) and reference station (*Contentieuse*) data in particular regarding the strong effect of the month on those immunomarkers. This could support the usefulness of lysosomal presence and phagocyte adhesion capacity reference ranges for field studies.

Adding data obtained from the uncontaminated station (*Béronelle*) to the data used to build the reference ranges mostly resulted in similar results regarding acceptability of the field data from presumably contaminated sites. Nevertheless, for some sampling dates and immunomarkers, the use of the R2 reference ranges reduced the percentage of data outside reference ranges. For example, in highly urban contaminated station (*Réveillon*) in April, a significant increase of necrotic cell percentage was detected with R1 reference ranges (Table 4) whereas the R2 reference ranges present no significant increase of this immunomarker. The same effect could be shown on different immunomarkers at different periods and whatever the tested site. Some of the

discrepancies between the initial R1 reference ranges and R2 could be explained by the fact that the fish sampled in the uncontaminated station (*Béronelle*) were smaller than those from reference station (*Contentieuse*). The R2 reference ranges may therefore more accurately model the effect of fish body size. So, due to importance of confounding factors on biomarker interpretation (Coulaud et al., 2011; Jolly et al., 2012; Bado-Nilles et al., 2015a), using these R2 new reference ranges could reduce the false positive responses due to impact of confounding factors, such as length.

Comparing data from the three contaminated sites to the R2 reference ranges highlight some differences but which depended on the sampling period. In fact, many seasonal events could induce seasonal immune pattern, such as the seasonal variations of environmental pathogenic load and of fish immune response and the cyclic variations of pollutants released in the environment by human activities (Hedrick et al., 1993; Saha et al., 2002; Duchemin et al., 2007; Buchtková et al., 2011; Bado-Nilles et al., 2015a). In this way, some reflexion must be made concerning the best sampling period for detecting adverse effects on fish immunity. In the present work, autumn and spring seem to be the better period with more important modulation of immune responses in contaminated sites, especially for highly urban contaminated station (*Réveillon*) and moderately urban contaminated station (*Rivière de Tancarville*). Concerning agricultural contaminated station (*Fossé Traxin*), no data had been collected in autumn and the last part of winter was also attractive. Fish appear to be most vulnerable in autumn and spring, due to high pathogenic load and high chemical releases (Hedrick et al., 1993; Saha et al., 2002; Duchemin et al., 2007; Buchtková et al., 2011; Jolly et al., 2012; Bado-Nilles et al., 2015a) which can explain their relevance in biomonitoring studies. Nevertheless, in spring, sexual maturation could induce interfere with immune responses (Ansar Ahmed 2000, Harris and Bird, 2000; Buchtková et al., 2011; Milla et al., 2011) due notably to sexual hormones which interact with hormonal receptors located on leucocytes and lymphopoietic organs (Slater et al., 1995; Ansar Ahmed, 2000). In this way, autumn is the most appreciated period for biomonitoring.

The R2 reference ranges demonstrated many immune effects on contaminated sites corresponding mainly to inhibition of immunological functions. The more impacted station was the highly urban contaminated station (*Réveillon*), a historically reference contaminated site (Sanchez et al., 2008; Bado-Nilles et al., 2013; Santos et al., 2016) with a large variety of chemicals such as heavy metals (e.g. zinc, copper), PAHs, endocrine disruptors (e.g. alkylphenols, nonylphenols, bisphenol A) and pesticides (e.g. endosulfan) (Sanchez et al., 2008; Kinani et al., 2010; Santos et al., 2016). A major part of these substances had immunomodulatory properties (Yin et al., 2007; Tellez-Bañuelos et al., 2009; Yang et al., 2015; Qiu et al., 2016). The agricultural contaminated station (*Fossé Traxin*) and moderately urban contaminated station (*Rivière de Tancarville*), which were less contaminated sites, display lower immunological modulation.

In conclusion, the R2 reference ranges developed in the present work seem to better model the immune response of either uncontaminated or contaminated stations. This statistical model was quite attractive in particular to avoid misdiagnosis on fish immune responses. The R2 reference ranges highlighted two immunomarkers, the lysosomal presence and the phagocytosis adhesion capacity, which seem to be the most relevant to discriminate sites. Furthermore, the present work also suggested the relevance of autumn in discriminating various contaminated stations due to high pathogenic load and high chemical releases. In parallel to this work, to limit inter-individual immunomarker variations, active biomonitoring approaches which use selected organism have been developed with the aim of limiting inter-individual and inter-population immunomarker variations (Le Guernic et al., 2016; Catteau et al., 2021). In this way, future studies must combine comparison of biomarker reference ranges with the caging approach.

CRedit authorship contribution statement

Adrien Marchand: Methodology, Formal analysis, Investigation, Visualization, Project administration, **Cleo Tebby:** Methodology, Validation, Formal analysis, Writing – Original Draft, Visualization, **Audrey Catteau:** Investigation, **Cyril Turiès:** Investigation, Project administration, **Jean-Marc Porcher:** Funding acquisition, Supervision, **Anne Bado-Nilles:** Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft, Visualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.ecoenv.2021.112580](https://doi.org/10.1016/j.ecoenv.2021.112580).

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