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Modelling the effect of season, sex, and body size on the three-spined stickleback, *Gasterosteus aculeatus*, cellular innate immunomarkers: A proposition of laboratory reference ranges

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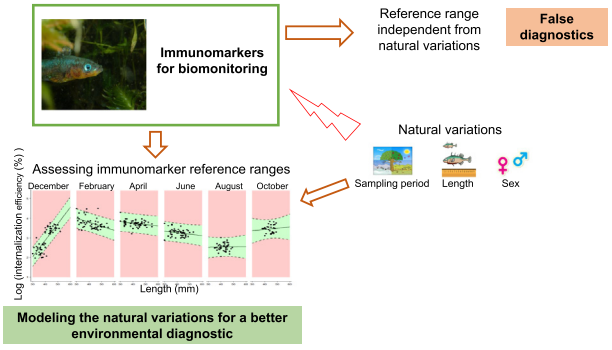
HIGHLIGHTS

- Innate immunomarkers of *G. aculeatus* showed strong seasonal variations.
- Sex and body size were significantly correlated with the immune response in a seasonal dependant way.
- Immunomarker reference ranges were proposed and tested using data from *in vivo* exposures.

GRAPHICAL ABSTRACT



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ABSTRACT

Innate immunomarkers reflect both environmental contamination and fish health status, providing useful information in environmental risk assessment studies. Nevertheless, the lack of knowledge about the effect of confounding factors can lead to data misinterpretation and false diagnoses. The aim of this study was to evaluate the impact of three confounding factors (season, sex and body size) on three-spined stickleback innate immunomarkers in laboratory conditions. Results shown strong seasonal variations in stickleback innate immunomarkers, with higher immune capacities in late winter-early spring and a disturbance during the spawning period in late spring-summer. Sex and body size had a season dependant effect on almost all tested immunomarkers. Reference ranges were established in laboratory-controlled conditions (*i.e.* laboratory reference ranges) and compared with data obtained from *in vivo* chemical expositions. The predictive power of the statistical model depended on the immunomarker, but the control data of the *in vivo* experiments, realized in same laboratory conditions, were globally well include in the laboratory reference ranges. Moreover, some statistical effects of the *in vivo* exposures were correlated with an augmentation of values outside the reference ranges, indicating a possible harmful effect for the organisms. As confounding factors influence is a major limit to integrate immunomarkers in biomonitoring programs, modelling their influence on studied parameter may help to better evaluated environmental contaminations.

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1. Introduction

In environmental monitoring programs, biomarkers are currently proposed to complete the information provided by conventional approaches, such as chemical and ecological data, to monitor regimes defined by the European Water Framework Directive (WFD, Directive 2000/60/EC) (Sanchez and Porcher, 2009). In fact, these integrative tools, currently integrated only in coastal areas monitoring, could help to establish the relationship between biological effects observed at the community level and monitored chemical concentrations. Efforts must now be focused on the integration of biomarkers in the WFD too. In this way, several authors have successfully used biomarkers to discriminate sites regardless of chemical contamination (Bado-Nilles et al., 2014a, 2014b; Burgeot et al., 2010; Sanchez et al., 2008a).

Immune parameters are potentially interesting in biomonitoring studies as they reflect environmental contamination and fish health status (Bols et al., 2001). Hereafter we list cellular innate immune functions and associate them to the biomarkers included in the present study. The induction of cellular mortality, by apoptosis or/and by necrosis, could compromise the immune system by a disturbance of cellular proliferation and modification of leucocyte differential sub-populations ("leucocyte distribution") (Bado-Nilles et al., 2009; Danion et al., 2012). Phagocytes (granulocytes, macrophages or monocytes, and B cells) can be considered to be the central cellular actors of the innate immunity through the mechanism of phagocytosis (Magnadóttir, 2006), described as relevant biomarker in fish (Reynaud and Deschaux, 2006). Pathogens which overcome the physical barriers formed by mucus, skin, and epitheliums are captured by phagocytes ("adhesion capacity"), engulfed ("internalization efficiency"), and then destroyed by a combined action of respiratory burst ("respiratory burst index") which is an aerobic destruction pathway and lytic enzymes contained in lysosomes ("lysosomal presence"), which is an anaerobic destruction pathway (Ellis, 1999; Magnadóttir, 2006). Since phagocytosis plays a major role in defence against pathogens in fish (Ellis, 2001), a decrease in its efficiency could increase susceptibility to opportunistic infections in animals (Kreutz et al., 2010). In this way, fish disturbed by chemical stress might be unable to resist against pathogen invasion (Arkoosh et al., 1998; Kreutz et al., 2010; Shelley et al., 2012a, 2012b).

Nevertheless, fish immunomarkers could be influenced by many environmental parameters like temperature, salinity, photoperiod or oxygen level (Bowden, 2008; Buchtíková et al., 2011; Sanchez et al., 2008b). Moreover, strong seasonal variations, probably related to variations in environmental conditions and to organism physiological cycles (Bowden et al., 2007), were found in several fish species like roach, *Rutilus rutilus* (Kortet et al., 2003), rainbow trout, *Onchorynchus mykiss*, (Morgan et al., 2008), common carp, *Cyprinus carpio*, (Buchtíková et al., 2011), kalbasu, *Labeo calbasu*, (Mohanty et al., 2014), or three-spined stickleback (Brown et al., 2016). Inter-individual variations in parameters like sex, fish age, morphology, or sexual maturation, could also influence immune response and lead to inter-individual immunocompetence variations (Bly and Clem, 1992; Magnadóttir et al., 1999). All these confounding factors limit the interpretation of variation between sites and may disturb the routine environmental risk assessment (Bado-Nilles et al., 2014a, 2014b; Sanchez et al., 2008b). Characterising the remaining natural sources of biomarker variability could allow to discriminate responses induced by pollutant exposure and background noise. In this way, establishment of reference values, that consider the major sources of variation, may be of great interest for environmental monitoring studies.

The three-spined stickleback, *Gasterosteus aculeatus*, is a freshwater fish species commonly used in ecotoxicological studies to assessed pollutant effects *in vivo* (Bado-Nilles et al., 2013; Jolly et al., 2009), and *in situ* (Bado-Nilles et al., 2015; Le Guernic et al., 2016; Sanchez et al., 2008b). Furthermore, this fish has a wide repartition area, in temperate zones of north hemisphere, and can live in highly disturbed streams (Ernst et al., 1991; Svecevičius, 2006). These advantages make it a good sentinel species, interesting for environmental monitoring studies. Seasonal variations of some biomarkers have already been studied in this species, including DNA integrity (Santos, 2013), biotransformation enzymes (Sanchez et al., 2008a), oxidative stress parameters (Sanchez et al., 2008a), neurotoxicity (Sanchez et al., 2008a), and endocrine disruption markers (Sanchez et al., 2008a). Nevertheless, there is a lack of knowledge about the seasonal variations and the effect of inter-individual variations on the stickleback immune system.

Hence, the present study aims at characterising natural variability of non-specific cellular immune response biomarkers to determine a range of variation of reference values for these immunomarkers in laboratory conditions, using statistical tools. Season, sex, and body size were used to predict three-spined stickleback immunomarker values. The obtained reference ranges were then compared with data obtained during laboratory *in vivo* chemical exposures to chlorpyrifos, endosulfan, and 17-β-estradiol.

2. Materials and methods

2.1. Three-spined stickleback and experimental design

2.1.1. Determination of references values

During this study, 282 mature three-spined sticklebacks obtained from one spawning season in INERIS husbandry (Verneuil-en-Halatte, France) were used. Fish were maintained in laboratory conditions and divided in four size class ([30–35 mm], [35–40 mm], [40–45 mm], and [>45 mm]) maintained in four separate 600 L tanks (renewed/recycling water (10 L/min)). The photoperiod and the temperature were artificially changed to mimic natural seasonal variations in western Europe. To study seasonal variations, 15 laboratory fish of each size class were randomly sampled every two months, from December 2015 to August 2016. Two weeks before sampling date, each fish was measured and put to the right size class. Table I summarizes, at each sampling period, the number of sampled fish per size class and per sex. Fish were anaesthetized using tricaine methanesulfonate (70 mg/L, Sigma) then sacrificed, measured, weighed, and the spleen was removed to measure immune response.

2.1.2. Chemical exposures

Data used for the comparison with the reference range values was obtained from three *in vivo* experiments. Fish used during these experiments were obtained from INERIS husbandry, then maintained in laboratory conditions. Briefly, for each experiment, 50 (chlorpyrifos), 60 (endosulfan), and 100 (17-β-estradiol) fish were randomly distributed into 10 L tanks with 10 fish per tank. The fish were then acclimated in each tank for two weeks prior to exposure. Water was renewed daily and fish were fed daily with bloodworm. After the acclimation period, fish were exposed for four days to either chlorpyrifos (1.75 μg/L, 0.88 μg/L, 0.35 μg/L, and 0.18 μg/L, CAS number: 2921-88-2, Sigma), endosulfan (3.5 μg/L and 1.75 μg/L, CAS number: 115-29-7, Sigma), or 17-β-estradiol (4 μg/L, 2 μg/L, 1 μg/L, and 0.5 μg/L, CAS number: 57-63-6, Sigma) previously dissolved using dimethyl sulfoxide (DMSO, Sigma). In the control condition (0 μg/L), fish were exposed to 0.2% of DMSO. Water was renewed daily and the compounds were re-introduced after water change. Fish were fed daily with bloodworm. At the end of the exposure, fish were anaesthetized (tricaine methanesulfonate, 70 mg/L, Sigma), sacrificed by cervical dislocation, measured, weighed, and sampled. For each experiment, the maintaining conditions, fish size class, and the number of fish per treatment (surviving after the four days of exposure) are summarized in Table I. Exposure to chlorpyrifos was performed in April 2015 whereas exposure to endosulfan and 17-β oestradiol was performed in February 2016.