



Phenotypic variation as an indicator of pesticide stress in gudgeon: Accounting for confounding factors in the wild



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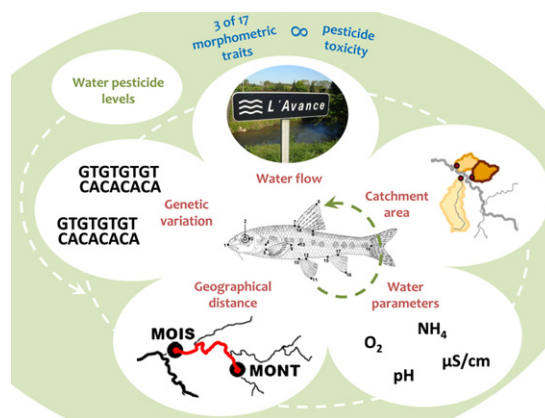
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HIGHLIGHTS

- We tested the link between pesticide levels in rivers and morphology of fish.
- Our model also considered covariables: genetics, geographical distances, etc.
- 1/6 of morphological traits were significantly correlated with pesticide toxicity.
- It is important to consider the many sources of inter-organism phenotypic variability.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:

Received 23 April 2015

Received in revised form 13 August 2015

Accepted 14 August 2015

Available online 29 August 2015

Editor: D. Barcelo

Keywords:

Pesticides

Gudgeon

ABSTRACT

The response of organisms to environmental stress is currently used in the assessment of ecosystem health. Morphological changes integrate the multiple effects of one or several stress factors upon the development of the exposed organisms. In a natural environment, many factors determine the patterns of morphological differentiation between individuals. However, few studies have sought to distinguish and measure the independent effect of these factors (genetic diversity and structure, spatial structuring of populations, physical–chemical conditions, etc.).

Here we investigated the relationship between pesticide levels measured at 11 sites sampled in rivers of the Garonne river basin (SW France) and morphological changes of a freshwater fish species, the gudgeon (*Gobio gobio*). Each individual sampled was genotyped using 8 microsatellite markers and their phenotype characterized via 17 morphological traits. Our analysis detected a link between population genetic structure (revealed by a Bayesian method) and morphometry (linear discriminant analysis) of the studied populations. We then

Abbreviations: Q_{ST} or P_{ST} , genetic differentiation measured with quantitative traits (e.g. body size, growth rate); F_{ST} , genetic differentiation measured with neutral traits, generally measured using neutral loci such as microsatellites; msPAF (multi-substance Predicted Affected Fraction), quantifies the toxic pressure put on an ecosystem due to the presence of a mixture of chemicals, indicating the fraction of all species that is predicted to be exposed above an effect-related benchmark, such as the EC50 (median effect concentration) or the NOEC (no observed effect concentration) for standard toxicity test species; TU (Toxic Units), a risk quotient to reveal whether the measured bioavailable concentrations are higher than the known L(E)C50 (Lethal/Effect) for a certain species.

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Feral fish
Morphometry
Indicator
Confounding effects

developed an original method based on general linear models using distance matrices, an extension of the partial Mantel test beyond 3 matrices. This method was used to test the relationship between contamination (toxicity index) and morphometry (P_{ST} of morphometric traits), taking into account (1) genetic differentiation between populations (F_{ST}), (2) geographical distances between sites, (3) site catchment area, and (4) various physical–chemical parameters for each sampling site.

Upon removal of confounding effects, 3 of the 17 morphological traits studied were significantly correlated with pesticide toxicity, suggesting a response of these traits to the anthropogenic stress. These results underline the importance of taking into account the different sources of phenotypic variability between organisms when identifying the stress factors involved. The separation and quantification of the independent effect of such factors provides an interesting outlook regarding the use of these evaluation metrics as indicators of ecosystem health.

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1. Introduction

At the individual level, phenotype integrates the multiple effects of one or several stress factors upon the development of exposed organisms (Franssen, 2011), and determines part of the fitness of organisms (Orr, 2009). Therefore, via studying phenotypic variation between conspecifics exposed to different environments, we may gain insight from variation in fitness linked to environmental conditions. Phenotypic changes have thus been used as bio-indicators of chronic exposure of various organisms to pollutants as well as when facing habitat degradation (e.g. Klisarić et al., 2014; Monna et al., 2011; Sánchez-Chardi et al., 2013; Smakulska and Górniak, 2004). The developmental stability – reproducible development of a genotype under given environmental conditions – of the individuals of a population can be assessed by studying the degree of morphological variation (direct measurements between body landmarks). While some studies have shown that developmental instability increases with increase of environmental stress, others failed to establish clear relationships.

Because human pressure generally modifies more than one environmental factor at a time, and pressures from several sources often coincide, a multiple-stressor approach is the most adequate when conducting studies in the natural environment (Franssen et al., 2013; Ormerod et al., 2010). In environmental risk assessment, the determination of causality of toxic effects requires specificity of association, i.e., differentiation between stressor independent effects and environmental or genetic variability (Theodorakis, 2003). Indeed, phenotypic variation observed in wild populations can be affected by the presence of contaminants but also by a range of other factors that co-vary spatially with contaminants. Inferring the independent effect of collinear variables is possible through the use of adequate statistical tools (for a technical review see Murray and Conner, 2009). For quantitative traits, the genetic pool of populations can also be a key factor affecting between-population variation, while gene flow among populations can dampen the effect of contaminants on phenotypic variation expected between populations. Although genetic diversity and structure have been regarded as being potentially affected by contaminants (Bouret et al., 2008), they have rarely been considered as potential confounding effects in phenotypically-based ecotoxicological studies. The effect of these potentially confounding factors can be teased apart by comparing, between populations, the genetic differentiation measured with quantitative traits (e.g. body size, growth rate; Q_{ST}) to the genetic differentiation measured with neutral traits (i.e. generally measured using neutral loci such as microsatellites; F_{ST}). Such an approach (the Q_{ST}/F_{ST} approach, or P_{ST}/F_{ST} approach in wild populations) has proven powerful to differentiate the role of natural selection vs. genetic drift on natural populations (Merilä and Crnokrak, 2001; Raeymaekers et al., 2007), but has rarely been applied within an ecotoxicological context.

In this study, the main objective was to develop a method aiming at testing, in the wild, the independent effects of contaminants on the phenotype of individuals, while accounting for confounding environmental

and genetic factors. Such a method was developed on wild populations of a Cyprinid fish (the European gudgeon, *Gobio gobio*) occurring along a river basin gradient (the Garonne watershed, south-western France) and exposed to different levels of water pollution. The method we developed is based on General Linear Models and is rooted on the partial Mantel test framework (Manly, 1991; Legendre, 2000). This method is specifically designed to test the effect of pesticide contamination on the morphometry of gudgeon populations, while simultaneously taking into account the influence of various physical–chemical parameters, the geographic isolation of populations, the genetic structure of these populations, and genetic drift. The applicability of this approach for ecotoxicological assessment of ecosystem health is discussed.

2. Methods

2.1. Site selection and physical–chemical characterization

The 11 selected sampling sites were geographically dispersed throughout different streams of the Adour-Garonne river basin, SW France (Fig. 1), covering a range of varying pesticide levels classified according to a 5-level environmental quality index developed by the Adour-Garonne water agency (MEDD and Agences de l'Eau, 2003). Given the main objective of the study in including a range of different environmental (and genetic) factors in the final phenotype-pesticide contamination assessment, we gathered as much physical and chemical data possible for the selected sampling sites. The following paragraphs explain the structure and specific treatments of each dataset.

In order to characterize each sampling site, the Adour-Garonne water agency pesticide concentration databases between 2006 and 2008 (time period corresponded to the immediate three years prior to field sampling of fish) were used to calculate two toxicity indices: the msPAF (multi-substance Predicted Affected Fraction; Posthuma and De Zwart, 2006) and TU (Toxic Units; Von der Ohe et al., 2008). Each index calculation used the concentrations of 74 pesticides (of 23 different toxic modes of action) measured by the Adour-Garonne water agency in grab-samples taken at each sampling site 3 to 5 times per year.

The msPAF quantifies the toxic pressure put on an ecosystem due to the presence of a mixture of chemicals, indicating the fraction of all species that is predicted to be exposed above an effect-related benchmark, such as the EC50 (median effect concentration) or the NOEC (no observed effect concentration) for standard toxicity test species. As pesticide concentrations varied within and among years, an average msPAF value for each sampling site was calculated according to Posthuma and De Zwart (2006) for each year (2006, 2007 and 2008), and the maximum value out of the three years was retained in our analysis. The calculation of PAF levels is based on chemical-specific species sensitivity distributions (SSDs) that describe the variation in sensitivities for a set of species under acute or chronic exposure to a certain compound. The single substance PAF (ssPAF) can be used as an approximation of the ecological risk of a single substance to the

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