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Individual variation in aquatic toxicology: Not only unwanted noise

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ABSTRACT

The mean value of any parameter and its changes are usually discussed, when ecotoxicological studies are carried out. However, also the variation of any parameter and its changes can be important components of the responses to environmental contamination. Although the homogeneity of variances is commonly tested, testing is done for the use of correct statistical methods, not because of exploring the possibility that variability and its changes could be important components of environmental responses. We evaluated recent aquatic toxicological literature and found that in the majority of articles indicating that homogeneity of variances was tested and giving the result of testing, the assumption of homogeneity was not fulfilled. Further, it was observed that in some studies experimental treatment clearly affected the variability. In this commentary we discuss the reasons for variability: measurement errors, experimental design, genetic heterogeneity and phenotypic plasticity, and conclude that even after accounting for experimental design and genetic makeup significant variability remains. This plasticity may change in environmental responses as suggested by a hypothetical example, and as confirmed by experimental data. As a consequence, the changes of variability can be significant, even when the means do not differ. Because of this, variability and its changes should always be analysed and reported. This will be easy, since the datasets are exactly the same for comparing the variances and means, and as normally variances are tested for homogeneity. It is likely that much new information about the responses of organisms to environmental contamination will be obtained. However, the present journal practises tend to discourage one from concentrating on anything but the mean. In contrast, we think it is imperative that variability is always included as an endpoint in data analysis in the future.

1. The importance of individual variation: is it taken into account in publications?

The capacity of organisms to respond to environmental contamination depends on how much variation the affected population has, but also on how plastic the individual phenotypes of the population are and on the possibility of changes in phenotypic plasticity within the population. The more variation (including phenotypic plasticity) there is in the population, the greater environmental changes the population can tolerate (i.e. at least some members of the population are able to survive and reproduce). Further, natural selection depends on variability. These undisputable statements indicate that it is not only mean value but individual variation that should be taken into account when exploring how organisms are influenced by environmental contamination. Already more than 30 years ago Bennett (Bennett, 1987) pointed out that individual variability is an underutilized resource in environmental biology, arguing that many phenomena could be better explained by analysing variability together with the mean than by analysing the mean alone. He actually went as far as saying that we are suffering from the tyranny of the golden mean. He took as an example articles from comparative physiology journals in 1985, pointing out that out of the more than 250 articles he analysed, only one analytically examined the observed variation. In accordance with this view is the statement that variation in phenotypic plasticity is a character in its own right, separate from the mean value of a character over all environments (Via et al., 1995). One can argue that work in aquatic toxicology requires similar understanding of variability as comparative and ecological physiology. We have examined more than 100 articles dealing with aquatic toxicology published in Aquatic Toxicology, Ecotoxicology and Environmental Safety, and Science of the Total Environment after June 2018. Our major question was: is variation taken into account in analysing the results? The results are given in Table 1. Surprisingly, only 52% of the articles published indicated that the data were checked for homogeneity of variances. Of the articles mentioning that the variance was tested, 39% did not indicate if the results showed that the variances were either homo- or heterogeneous. When this was done, only 6 (i.e. 18% of the articles giving the result of testing) reported homogeneous variances, leaving the vast majority of articles

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Table 1
Evaluation on how 103 aquatic toxicological articles from the Science of the Total Environment, Ecotoxicology and Environmental Safety and Aquatic Toxicology from June to August 2018 have taken individual variation into account.

The way variation was taken into account	Number of articles	Number of articles
Testing of the homogeneity of variance was not stated	49	
Homogeneity of variance was tested	54	
The equality/inequality of variances was not indicated		*21*
The variances were homogenous		*6*
The variances were not homogeneous		*22*
It could be estimated that treatment affected variance		*5*

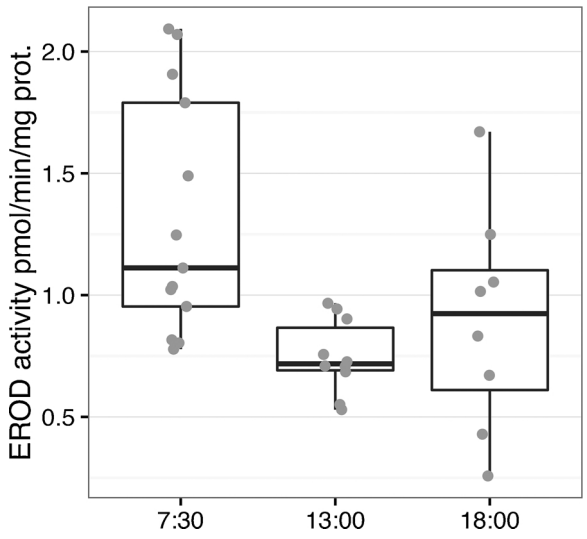

Fig. 1. The activity of CYP1A as measured by ethoxyresorufin O-deethylase (EROD) assay in the liver of three-spined stickleback under normoxic conditions. Samples were collected at three time points; light period lasted from 07:00 h to 19:00 h. It can be concluded that the EROD activity at 07:30 h is significantly higher than that at 13:00 h (P = 0.006, MCMCglmm; Prokkola et al., 2015). Data from Prokkola et al. (2015). It further appears that the variation of EROD activity at 13:00 is smaller than at either 7:30 or 18:00. The figure is reproduced from Prokkola & Nikinmaa, Journal of Experimental Biology 221, jeb179267 (2018) by permission. Data are given as a boxplot, where the dots indicate individual data points, the line across the box indicates median, the ends of the box the upper and the lower quartile and the ends of the lines the upper and the lower extreme.

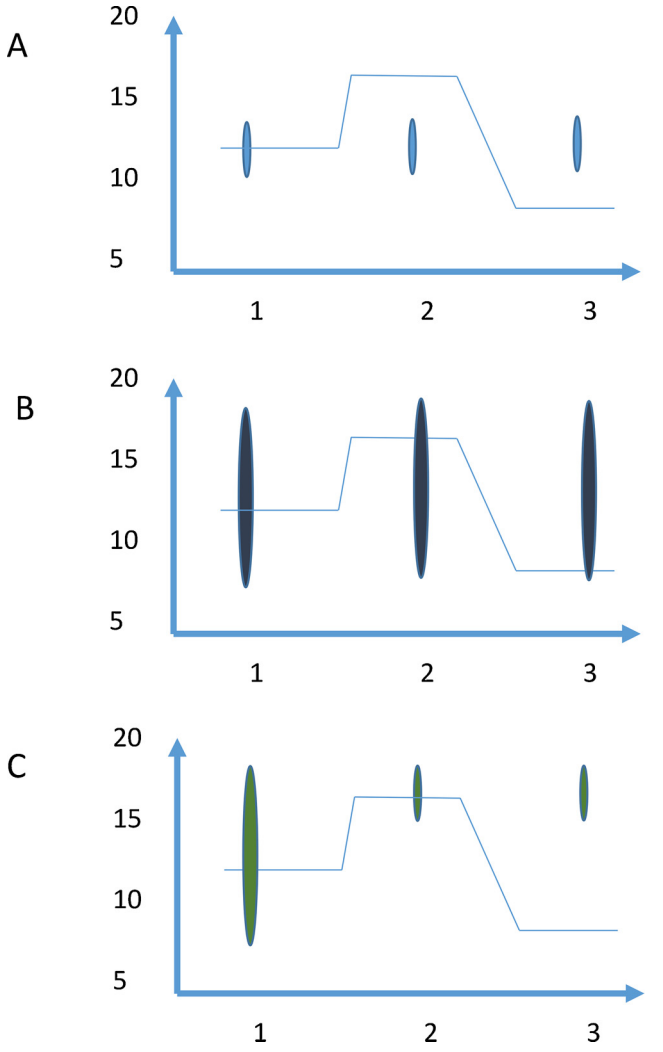

Fig. 2. A hypothetical example of how increased variation between individuals helps some of the organisms to reproduce successfully in conditions, where population with smaller variation would become extinct. The positive effect of large variation is greatest, if it is not diminished by reproduction in new conditions. In the hypothetical example, temperature is taken as the environmental condition. A. The individual variation is too small to enable any specimen of the population to reproduce successfully at the high or low temperature. (The line indicates ambient temperature; when the symbol crosses the line, some specimens are able to reproduce) B. The large variability enables some specimens to reproduce at high and low temperature. If the plasticity is unaltered (remaining large with the same tolerated temperature range as originally) in the offspring of animals surviving at the high temperature, some specimens will survive and are able to reproduce, when the temperature decreases again. C. The variation in the population depends on genetic variability, whereby some specimens have the genetic properties enabling them to tolerate high temperature. However, the phenotypic plasticity of the different genetically distinct specimens is much smaller than the overall variation of the genetically heterogeneous population. Consequently, the animals, which have the genetic makeup enabling them to tolerate high temperatures, cannot reproduce successfully, when temperature is reduced. Y-axis gives temperature (°C), x-axis gives generation.

where homogeneity or heterogeneity of variance was given (this is 21% of all articles) to show that the variances measured in the study were not similar. As is advised by basic statistics textbooks, the studies used, e.g., log transformation to make it acceptable to use parametric statistical testing (ANOVA etc.). When even this did not make the variances similar, the researchers resorted to the use of non-parametric testing. The outset of transformations is that if there had been an infinite number of measurements, the variability in the different treatments/groups had been similar, i.e. the whole idea behind is derived from thinking that variability is not a factor that responds to environmental disturbances. Since the number of biological replications was usually extremely low, often only 2–3, in most cases it was not possible to evaluate the correctness of this assumption. However, out of the articles, which showed heterogeneous variances, in 19% the experimental treatment appeared to result in a change in variance. To us this is enough to prove that one would need to evaluate if the environmental change affected variation in the population. This is actually done, whenever the equality of variances is tested, e.g., by Levene's test: the researchers would only need to notice the importance of the result as a finding and not treat it merely as a reason to do data transformation. Similarly, Norin et al. (Norin et al., 2016) observed that the individual variation in integrative measurements related to metabolic rate changed as a result of environmental change. Below, we discuss the possible sources of individual variation.

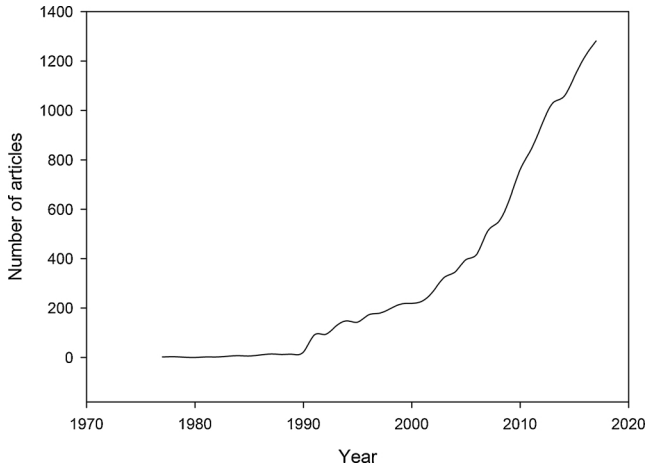

Fig. 3. The number of articles per year found in the Web of Science with the key phrase "phenotypic plasticity" in 1977-2017. The term was introduced in 1977; before that studies investigating the same thing used, e.g., non-genetic variability or inter-individual variability. In the initial year two articles used the term. By 2017 the number had increased to approximately 1300.

2. Sources of individual variation

2.1. Measurement errors and experimental design

The simplest reason for variation in obtained data is measurement errors. Because of this in laboratory studies, technical replications are commonly used, i.e. measuring the sample several, usually at least three, times. Because the value of a parameter should be the same in all the measurements, any variation shows measurement errors. Thus, this variability is very different from biological variation. Although the source of variation because of technical points should not be confused with biological variability, and although ecological literature always makes a point of adequate number of biological samples, it is surprisingly common that far-reaching conclusions are based on only 2–3 biological replicates in published toxicological articles, especially the ones utilizing high-end molecular methods. An important reason for the small number of biological replicates is the cost of analysis. While it is difficult to abolish this problem, the researchers should acknowledge it, and, when possible, resolve it. Even when the costs are not insurmountable, the scientists are following the ways that are commonly used in biomedical studies with mice and laboratory rats. The point about addressing biological variation in molecular biomedical studies has been emphasized, e.g. by Vaux et al. (Vaux et al., 2012): they pointed out that if a researcher took cells from one mouse, and did a treatment to the cells of that mouse 10 times, measuring the response in every treatment, the responses of a single mouse were still evaluated. Two to three is slightly better, but certainly not adequate to indicate the response of a population of organisms. What if the 2–3 organisms represent marginal values? They can, for example, be animals, which were most easily caught.

Another source of variation, which is solely caused by inadequate experimental design, stems from not taking circadian (or seasonal) variation into account when designing studies (Prokkola and Nikinmaa, 2018). This is a significant problem, since many environmental responses and values of measured parameters depend on the time of day (Fig. 1) and the mean daily variations can further depend on the ambient conditions (Prokkola et al., 2018, 2015; Zhao and Fent, 2016; Zhao et al., 2016). While in laboratory experiments usually control and treatment samples are taken from organisms that differ only in the experimental treatment, this is not possible in field studies, where it is, e.g., impossible to guarantee that the experimental subjects would have got similar amounts of food. The only way to address this source of variation is to increase the number of organisms sampled.

2.2. Genetic variation in the studied population

There is a wealth of information indicating that there are genetic differences between populations of a species inhabiting different environments, and that there is a clear genetic basis of adaptations to environmental changes (Powers et al., 1991). Further, it has been shown that rapid genetic adaptation has taken place to enable animals to tolerate highly polluted environments (Whitehead et al., 2012, 2010; Williams and Oleksiak, 2008). Although this as such does not indicate a genetic component in individual variation, it shows how genetic variability is utilized in responses to environmental disturbances. A huge problem is that there is a large distance from a genetic difference to whole-animal performance (Dalziel et al., 2009); the same whole-animal response can involve very different changes in gene expression of components of a pathway leading to a final response (Nikinmaa and Waser, 2007). Taking this into account, one of the reasons for individual variations is the genetic heterogeneity of most natural populations, where differences in gene function can be found at different steps of the final response. Further, the sequence difference in the genome can be found in protein-coding areas, in areas that transcribe non-coding RNAs or occur in the regulatory regions of protein-coding genes (Dalziel et al., 2009). Thus, from sequence differences it is very difficult to draw other conclusions of individual variation except saying that since populations are usually genetically heterogeneous, the possibility for individual variation is increased. Notably, as compared to laboratory rodents, even zebrafish strains are genetically more variable (Guryev et al., 2006), and this is usually associated with larger individual variations in zebrafish studies than in mouse studies.

Apart from genetic heterogeneity as such, individual variation is

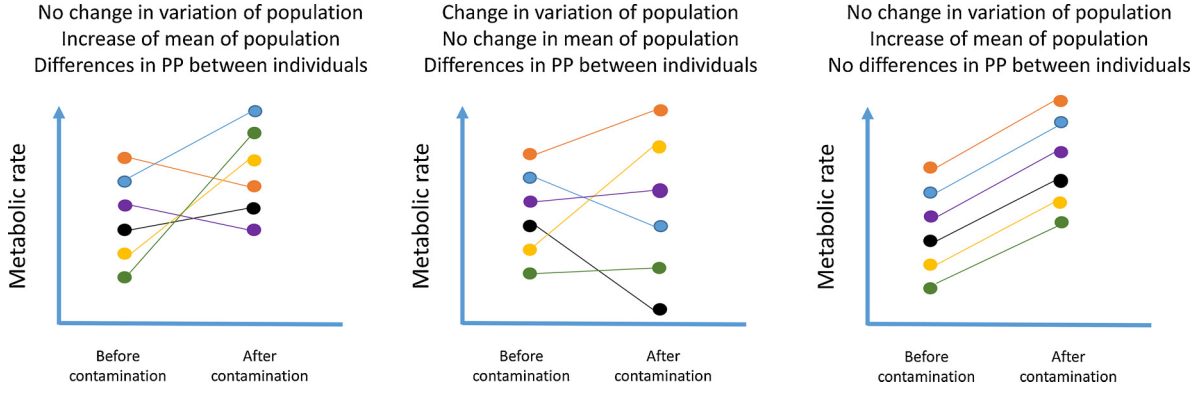

Fig. 4. Three hypothetical examples on how environmental contamination can influence phenotypic plasticity (PP, a change in physiological parameter, e.g., metabolic rate), and how population variability and mean are consequently affected.

affected by the fact that all organisms appear to show phenotypic plasticity, i.e. one genotype has several phenotypes. The phenotypic plasticity can be considered genetically adaptive or non-adaptive (Ghalambor et al., 2007), if it is thought that there is a most suitable environment for the measured character. In simple terms, if an optimal environment for the measured character is encountered, a decrease in plasticity is usually beneficial, as then all animals are close to the optimal phenotype. However, if continuous variations in environmental conditions are a rule, genetic adaptations should favour large phenotypic plasticity in order to enable the species to survive in every condition (Fig. 2). If phenotypic plasticity is a character in its own right, separate from the mean value of a character over all environments, then it is expected to have some genetic control. Scheiner's extensive work has investigated the degree to which genetic components may underlie phenotypic plasticity (Scheiner, 1993, 1998, 2002, 2013, 2014; Scheiner et al., 2012; Scheiner and Berrigan, 1998; Scheiner and Callahan, 1999; Scheiner et al., 1991, 2015; Scheiner and Holt, 2012; Scheiner and Lyman, 1989, 1991; Scheiner and Yampolsky, 1998). In addition to Scheiner's studies, other workers have also shown that plasticity is partly genetically controlled (Dayan et al., 2015; Healy and Schulte, 2012; Pigliucci, 2005). Overall, it has become clear that phenotypic plasticity plays a role in environmental adaptation (Ghalambor et al., 2015, 2007). Notably, studies of genetic components behind phenotypic plasticity have been much helped by recent methodological advances: with high-throughput methodology (microarrays, RNA sequencing) researchers are able to determine the genomic make-up of plastic traits (Aubin-Horth and Renn, 2009). The studies of Dayan et al (Dayan et al., 2015) suggest that the suites of genes behind direct genetic adaptation and those behind phenotypic plasticity are different. This result is in direct disagreement with the notion that plasticity is not independent of trait means (genetic adaptation) (Via, 1993), and actually supports the notion that plasticity should be considered independently from mean.

2.3. Phenotypic plasticity independent of genotype

In addition to genetic background, individual variations may be affected by the conditions during development, previous exposure of the organism, or by the conditions that the parents have experienced. Despite this, the non-genetic phenotypic plasticity and its possible regulation have received little attention in animal biology. For example in physiology, one has usually been interested in responses themselves, and not in how the environment influences the responses of different individuals with consequent effects on the variation in the population (Nikinmaa and Waser, 2007). Although the role of physiology in evolutionary biology has nowadays started to achieve increasing attention (Noble, 2013), the plasticity of functions is a major component of evolutionary responses which deserves more thorough studies. Such studies are needed to evaluate what role variation has in the physiological mechanisms influencing the fitness of populations. Earlier, one has seen variation as an unwanted phenomenon in physiology, and virtually always considered the mean as the only important measure thinking that any variation is just unwanted noise. Terms related to variation like "error bar", "standard error of mean" and "confidence interval" indicate that variation is thought to be an error in measurement, mean being the important value – the smaller the errors bars the higher the quality of data that the researchers have gathered. Recently, however, there has been increased interest on investigating direct environmental effects on individual variation and even on individual variation in phenotypic plasticity in animals, and on how plasticity develops (Serobyan and Sommer, 2017). It has, e.g., been shown that individual differences in integrative functions such as some measures of behaviour occur in clonal fish reared in identical conditions (Bierbach et al., 2017), indicating that unknown effects can be behind phenotypic plasticity. The independency of plasticity and genetic variation has been noted in a study investigating the ambient oxygen variation and genetic differentiation of an African cichlid (Crispo and Chapman, 2008). In the case of physiological responses, the repeatability of the response can be studied by doing the same measurement after an appropriate rest period. However, in the case of toxicological studies the first toxicant exposure may have caused irreversible damage to the organism regardless of the "wash-out" period between the first and second exposure.

The possible molecular mechanisms of phenotypic plasticity and their significance in environmental responses and the tolerance of adverse conditions have been reviewed by Kelly et al. (Kelly et al., 2012). Experimentally, environmental effects on non-genetic phenotypic plasticity can be teased apart from genetic influences on the phenotype by minimizing genetic variability of the studied organisms (Serobyan and Sommer, 2017). The following alternatives can all cause non-genetic phenotypic plasticity as a response to environmental variations: (a) Developmental plasticity, (b) Maternal effects and (c) Epigenetic changes (we define the maternal effects to mean that changes in mother-derived molecules such as hormones, yolk etc. affect the plasticity of the offspring; in our view epigenetic changes mean that the plasticity is affected by changes in transcription, mRNA metabolism and translation, which are independent of the physiological influences exerted by the mother). There are several examples of studies investigating the plasticity associated with each of these: developmental plasticity, e.g., (Scott and Johnston, 2012; Wiens et al., 2014), maternal effects, e.g., (Evans et al., 2010; Laurila et al., 2002), epigenetic changes, e.g., (Metzger and Schulte, 2016, 2017; Zambonino-Infante et al., 2017). However, it is impossible to address the role of variation behind environmental non-genetic phenotypic plasticity as such, before conclusive studies, measuring the variability as an endpoint, have been done. Up to the present, even when very valuable information about the possible involvement of plasticity in environmental adaptation and the role of development in it has been obtained (Gibbin et al., 2017), the work has concentrated on the changes of mean as a result of plasticity. The possibility that differences in variation (Fig. 2) could, as such, be important, has not been explored. Usually phenotypic plasticity is considered as a component of genetic assimilation, where it first enables some organisms to tolerate a new, unfavourable environment, whereafter the property that supports tolerance is genetically enriched (Schlichting and Wund, 2014). However, since the number of studies on phenotypic plasticity has increased from 0 to 1300 per year (Fig. 3) in 42 (the term was introduced in 1977; before that studies investigating the same thing used e.g. non-genetic variability or inter-individual variability) years, we expect that studies investigating variation as such will increase in near future.

3. Conclusion: analysing individual variation can add significantly to observations within aquatic toxicology

Present publishing practises discourage from using a measure of individual variation as an endpoint in ecotoxicological studies. However, the fact that statistically significant changes in variability can commonly be detected in aquatic toxicological experiments strongly suggests that individual variation should be evaluated as well as the mean. Hypothetically, environmental contamination can affect the phenotypic plasticity as described in Fig. 4. It is of note that the changes of variability can be significant even when the means do not differ not only in hypothetical cases, but when experimental data are evaluated (see time points at 13:00 and 18:00 in Fig. 1). This being the case, variability and its changes should be analysed in all studies, especially since all the measurements to do the analysis are already available. It is possible that much new information will be obtained.

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