Multivariate analyses of evolutionary changes in life history traits of a *Caenorhabditis elegans* population exposed to pollution

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Abstract

Because of their strong intensity of selection, some anthropogenic disturbances force populations to evolve rapidly to resist. However, these environmental changes can also alter the genetic structure of traits, acting on the evolutionary potential of traits. Thus, assessing the effects of pollutants on evolutionary response and genetic structure is important to our understanding of mechanisms entailing specialization or resistance to environmental changes. In this study, we used an experimental evolution approach where we exposed *Caenorhabditis elegans* to uranium, salt and alternating uranium and salt environments during 22 generations. At several generations, we measured individual traits, in particular fertility and growth. Moreover, we evaluated the phenotypic (and potentially genetic) pattern of traits (co)variance through generations in uranium and salt environments. We showed a reduction of the stability of the structure of traits in uranium environment and a higher capacity to response by acclimation, opposed to the results in salt environment. Consequently, the evolutionary responses of traits were generally higher in salt than in uranium. Furthermore, in alternating environment the populations had the strongest evolutionary response for fertility. Thus, the selection of a generalist type did not entail a reduction of resistance for both pollutants. With this multigenerational experiment, we confirmed in *C. elegans* that the effects of pollutants on the evolutionary potential of traits can be extremely fast.

Keywords

experimental evolution; evolutionary response; phenotypic (co)variance; local adaptation; evolution of generalism; *Caenorhabditis elegans*; pollution; uranium

1. Introduction

During the last few decades numerous studies have highlighted the important role of anthropogenic disturbances on the occurrence and speed of contemporary microevolutions in wild populations (reviewed by Hendry & Kinnison, 2001 ; Medina *et al.*, 2007 ; Morgan *et al.*, 2007 ; Hendry *et al.*, 2008). For example, studies have reported cases of increased resistance to heavy metals or pesticides in less than twenty generations in response to strong selection pressures (e.g. Antonovics *et al.*, 1971 ; Klerks & Levinton, 1989 ; Powles *et al.*, 1998 ; Beaudouin *et al.*, 2012).

Speed of evolutionary response is assumed to depend on both the intensity of selection pressures and the level standing genetic variation for the trait under selection (Falconer & Mackay, 1996). However, populations can take different evolutionary trajectories in response to the same novel selection pressures. For instance, in response to a pollutant energy allocation can change to favour detoxification mechanisms (Kraaijeveld & Godfrey, 1997 ; Burdon & Thrall, 2003). Alternatively, the pollutant may affect the evolution of life history characteristics, with an evolution towards faster growth that cause a reduction of the internal concentration of the pollutant (Sibly & Calow, 1989). Furthermore, evolution experiments placing populations in presence of a pollutant over multiple generations have shown that after an early quick drop fitness can increase in as fast as six generations (Xie & Klerks, 2003 ; Ward & Robinson, 2005 ; Salice *et al.*, 2010 ; Jansen *et al.*, 2011b). These results indicate that at least in these conditions populations can show quick evolutionary response to selection in a novel environment.

Selection induced by pollutants is generally directional, continuous, and strong (Posthuma & Van Straalen, 1993 ; Medina *et al.*, 2007). Nonetheless, wild populations affected by pollution generally live in heterogeneous environments where different selection pressures can act on the organism in different directions (Levins, 1968 ; Hedrick, 1974, 1976, 1986). These differential selection pressures have been shown to help maintaining genetic variation in population, from both a theoretical (Haldane & Jayakar, 1963 ; Gillespie & Turelli, 1989 ; Hedrick, 1995) and empirical point of view (Mackay, 1981 ; Hedrick, 1986 ; Gram & Sork, 2001 ; Roff, 2002b). Furthermore, temporally fluctuating environments seem to favour a generalist rather than a specialist way of life (Reboud & Bell, 1997 ; Cooper & Lenski, 2000 ; Turner & Elena, 2000 ; Cooper & Lenski, 2010). In such a case, populations evolving in an alternating presence of stressors in their environment may cope less with each stressor, and their evolutionary response may be slower, than that of populations that have evolved in response to only one of the stressors.

To validate these hypotheses we explore the speed of evolutionary response to selection in populations of *Caenorhabditis elegans* subject to only one type of pollutant or to alternating pollutants in their environment.

An organism can be viewed as an integrated system with functional, developmental, and genetic associations among its different traits (Pigliucci, 2003). Therefore not taking into account the different traits involved in the adaptation of the population facing novel selection pressure can bias the prediction of its evolutionary trajectory (Schluter, 1996 ; Bégin & Roff, 2003). In other words, the evolutionary potential of a group of traits is constrained not only by the additive genetic variation of each trait and the strength of selection pressures acting on them, but also by the magnitude and sign of their genetic associations (Lande & Arnold, 1983 ; Falconer & Mackay, 1996 ; Roff, 2002b). These variances and covariances are included in the **G** matrix of additive genetic variance and covariance (Lande, 1979).

G matrices can be highly stable through time (Arnold *et al.*, 2008 ; Garant *et al.*, 2008), although several studies have shown that its stability can be altered (Maynard Smith, 1989 ; Steppan *et al.*, 2002 ; McGuigan, 2006 ; Doroszuk *et al.*, 2008 ; Berner *et al.*, 2010), and at the extreme following short-term environmental changes (Bégin *et al.*, 2004 ; Sgrò & Blows, 2004). The evolutionary trajectory will depend on diverse aspects of the matrix structure such as its temporal stability and the level of traits (co)variance (Jones *et al.*, 2003 ; Berner *et al.*, 2010). To our knowledge, multivariate analyses of microevolution event in response to a pollutant have not been studied yet, and one of our previous studies has shown that pollutants could highly alter the stability of the **P** and **G** matrices (article II). We proposed here to study experimentally the evolutionary changes occurring in the phenotypic structure of life history traits in *C. elegans* subject to different polluted environments. Although, The phenotypic structure does not always reflect the genetic structure appropriately (Roff, 2002a), in an experimental context where novel environmental conditions are controlled for, we can assume that after four generations in the same environment within- and cross-generation phenotypic plasticity are stable over time (Scheiner, 1993 ; Mousseau & Fox, 1998 ; Räsänen & Kruuk, 2007). Consequently, changes from one generation to another observed at the phenotypic level essentially reflect changes at the genetic level.

In the present study, our goal was to evaluate the evolution of life-history traits in *C. elegans* populations in response to pollution. This nematode is a good model to perform microevolution

experiment because of its short life cycle, its small body length and its great ease of handling (Braendle *et al.*, 2008). Our approach allowed us to provide novel results on: (i) the degree of evolutionary response to selection of life history traits in a genetically diverse population of *C. elegans*, leading to differential increased resistance for two different constants pollutions (i.e. uranium or high salt concentration) and to alternating uranium and salt pollution, and (ii) the evaluation of the changes in the phenotypic/genetic structure over time in these polluted environments.

2. Material and methods

2.1. Population maintenance

We worked with a stock population of *C. elegans* composed of a mixture of 16 wild isolates (Teotónio *et al.*, 2012) to obtain a large genetic diversity. The population was kept in the experimental conditions described in Teotónio *et al.*(2012) for over 140 generations, prior to our study, where recombination-selection equilibrium was mostly achieved without significant loss of genetic diversity. The population was composed of around 30% of males for an androdioecious breeding system (i.e. self-fertilization of hermaphrodites and facultative outcross with males). For our study we changed laboratory conditions: we used 500 individuals in a 9 cm diameter Petri plate (6 replicates) with NGM-modified agar (use of HEPES buffer, see article I). Once NGM was dispensed into Petri plates, plates were left in a laminar flow hood one hour to dry NGM. We also grew *E. coli* OP50 cultures in Lysogeny Broth (LB) rich medium at 37°C overnight. To avoid interaction between LB and uranium in the future U-treatment, we systematically centrifuged bacteria twice, removed the supernatant and re-suspended bacteria with a solution of 85 mM NaCl to obtain a 20:1 mixture of E. coli (OD_{600nm} of 3 in LB). Plates were seeded with 1 ml of this food source and left in a laminar flow hood for one hour to allow the bacterial culture to dry. Then plates were top-exposed to UV doses for 90 s to stop bacterial growth (Bio-Link Crosslinker; λ=254 nm; intensity = 200 $\mu watt.cm^{-2}$). The main aim of this UV treatment was to avoid different bacterial growth in control and polluted plates.

Every 3 days we washed twice the nematodes off the plates with 3 ml of M9-modified solution (use of HEPES buffer) for each replicate and kept a sample per replicate in 15 ml falcon tubes. The number of individuals in a tube was estimated with five sample drops of 5 µl (see

Teotónio *et al.*, 2012), and then the volume corresponding to 500 individuals, from all developmental stages, was placed in a fresh Petri plate. This was done to transfer a representative sample of the age structure of the population at each time and avoid unintentional selection of some specific life history strategies. Nematodes were cultured throughout the experiment at 20°C and 80% of relative humidity.

2.2. Conditions of pollution

After repeating this protocol forty times (i.e. for about 40 generations), the individuals from the six replicates were mixed before transferring, in four different conditions, 500 individuals per plate (six plates per condition). We maintained the novel populations in similar conditions than previously but changed the medium depending on the four conditions of the experiment: (1) a control environment (see above for medium) and three stressful environments, identical to the control, except for the addition in the NGM-modified agar of (2) 1.1 mM U (uranyl nitrate: $UO_2(NO_3)_2$, $6H_2O$; Sigma-Aldrich, France), (3) 308 mM NaCl or (4) alternating uranium and salt at each generation (in the same conditions as for treatment 2 and 3 and salt for odd generations). Thereafter we will refer to different population evolving in these environments as control, uranium, salt and alternating populations. Uranium and salt concentrations were chosen because they entailed a reduction of fertility by 60% at the first generation of exposition corresponding to a strong selection. In all medium we added 51 mM NaCl as in the classical preparation of NGM (Stiernagle, 2006), except in the salt environment where NaCl-concentration was of 308 mM. In the U-treatment, we also added the volume to obtain a concentration of 1.1 mM U (depending on volume of U-medium to prepare at each generation) of a solution of 50 mM U in NGM still liquid after autoclave. This multigenerational experience of selection lasted 66 days and approximately 22 generations (i.e. one generation per three days).

2.3. Traits measurements

During the first four generations we quantified the effects of within-individual and cross-generation phenotypic plasticity by measuring the traits at each generation. We then measured traits once every three generations until generation 22, except for generation 20 during which - for logistical reasons - we did the measurements instead of generation 19. Populations experienced

longer generation time in salt, but to simplify we considered a 3-days generation time for all the treatments thereafter.

At any given generation, after transferring into a novel 6 cm diameter Petri plate the 500 individuals needed for the next generation, we sampled approximately 200 individuals per replicate and placed them into a new Petri plate with a control agar medium. We then estimated more precisely population size by counting individuals that were left on the Petri plates from the given generation after placing them at 4°C for one hour to slow down the movement of individuals.

After cleaning the individuals off the plate, there were still hundreds of eggs adhered to the surface of the plate. We used these eggs for our phenotypic measures. Approximately 100 eggs were taken from the original Petri plate and transferred into a 6 cm diameter Petri plate, contained 10 ml of NGM (same medium than during the three previous generations) and 250 µl of 5:1 UV-killed OP50 (OD_{600nm} of 3), to measure survival and sex-ratio after 48h. We scored as dead eggs that did not hatch and worms that did not move their body or their head in response to three repeated stimulations with a platinum transfer pick (Sutphin & Kaeberlein, 2009). Visual differentiation between males and hermaphrodites starts to be possible at L4 larval stage (Sulston *et al.*, 1980). As the individuals that survived were at least at the L4 stage, we could determine their sex based on morphological criteria. Because of the developmental delay happening in the salt treatment, sex ratio in this treatment was only evaluated at 72h.

We measured phenotypic traits on both hermaphrodites and males. To measure brood size, and index of fertility, three hermaphrodites per replicate were transferred individually in a well of a tissue culture plate (12-well, same medium than in the original Petri plate) containing 2 ml of NGM per well and 75 µl of 5:1 UV-killed OP50. Brood size was measured as the number of hatched progeny produced by a hermaphrodite. An index of early and late (hatched progeny produced before and after 96h of age) fertility was also used.

We measured morphological traits using pictures of individuals taken with a stereomicroscope (Olympus SZX12, 1.6 x 90 magnification) and a computer-connected camera (Nikon D5000). As for fertility, males and hermaphrodites were measured at 96h. Image analyses for males' body length were done by a rapid and automatic procedure, used in batch, developed in Matlab (R2010b, Mathworks ©). First a background subtraction is applied and the body is extracted by a classic thresholding method. A skeletonization algorithm is then used to obtain the

relevant body points, which serve as basis for a spline of interpolation to measure the precise length of each individual (see appendix D for more details on the automatic procedure). We could not measure hermaphrodites with this method because of the presence of bacteria, altering the differentiation of individuals. Consequently, we used ImageJ software (Rasband, 2012) and measured their body length manually. We validated the automatic measures by comparing them with a sample of manual measures in males; and found a very strong correlation between the two methods ($r = 0.97$, $n = 15$). Body length was used as an index of growth between 0 to 96h of age.

We finally measured male body bend frequency at 96 h, as an index of locomotion behaviour. One body bend equals a change in the direction of the anterior part of the worm (including the posterior bulb of the pharynx) along the Y axis using the body of the worm as the X axis (Tsalik & Hobert, 2003). Individuals on a 6 cm Petri plate were washed twice with washing buffer that permitted a rapid sedimentation of individuals in the liquid. The buffer was composed of 5 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 0.5 $g.l^{-1}$ gelatin (Saeki *et al.*, 2001). Then, individuals were placed onto a 6 cm diameter Petri plate containing 10 ml of NGM but no bacteria. After five minutes, we counted the number of body bends over 20 s of three males per replicate. We focused on male body bend: in addition to providing movements necessary for finding the good living conditions in both males and hermaphrodites, locomotion behaviour is also necessary for males to encounter and fertilize hermaphrodites (Pannell, 2002 ; Barrière & Félix, 2005a).

2.4. Effects of pollutants on traits

Changes occurring during the first four generations included both plastic (e.g. individual phenotypic plasticity, maternal, and grand-maternal effects) and evolutionary responses to selection, whereas after generation 4 between-generation phenotypic changes caused by plastic effects were negligible (Scheiner, 1993 ; Mousseau & Fox, 1998 ; Gagliano & McCormick, 2007 ; Räsänen & Kruuk, 2007), leaving essentially evolutionary changes to be responsible for the observed changes across generations. We thus ran models where we estimated evolutionary (i.e. genetic) changes as the changes occurring between generation 4 and 22. Although in this study, we focused on evolutionary changes, we presented analyses for the first four generations in appendix E.

We used a Bayesian model approach in the MCMCglmm package for generalized linear mixed-effects model (Hadfield, 2010) in the R software (R Development Core Team, 2012). We fitted multivariate generalized linear mixed-effects models on hermaphrodite (i.e. quadrivariate models on total, early, and late fertility, and growth) and male traits (i.e. bivariate models on body bend and growth). We also used univariate models to analyse the changes in mean survival, sex ratio (see appendix F for more details on these traits), and population size. For each model we successively added environment, generation (as a continuous variable), and their interactions as fixed effects. For the mixed models, we included replicate populations as a random effect. This allowed us to estimate the (co)variance between replicates and within replicates [or residual (co)variance], and to control for the dependence of data within each replicate across generations. For each selected model we estimated the replicate effects as the sum of (co)variance between replicates divided by the sum of (co)variance within and between replicates. We provide more details on variance between replicates in appendix G. We modelled all the traits with a normal error structure. The multivariate analysis allowed us to estimate a full matrix of posterior distributions of (co)variance for all the traits in the model, and to take into account the fact that associated traits may not evolve independently of each other.

Prior to analyse, we rescaled the traits by subtracting each value by the mean of the sample and dividing it by twice the standard deviation (Gelman, 2008). After having tested different priors (see for example Teplitsky *et al.*, 2011), we retained a proper prior ($nu = k - 1 + 0.002$) with a very low variance parameter ($V = \text{diag}(k) * V_p * 0.05$), where V_p is the phenotypic variance, k the dimension of V (i.e. number of traits). We allowed models to estimate different random and residual variances, and covariances between pairs of traits. After having checked for the convergence of parameters values (i.e. number of iterations, burn-in phase and thinning) and the absence of autocorrelation, we retained 110 000 iterations with a burn-in phase of 10 000, for a total of 1 000 samples for each analysis.

For each trait we previously fitted univariate models with temporal autocorrelation in nlme package (Pinheiro & Bates, 2000). We wanted to check if the significant differences were the same than in our multivariate models without correction for temporal dependency. Although the *p*-values (nlme) were slightly different from *p*-MCMC (MCMCglmm), the significant effects (i.e. $p\text{-MCMC} \approx p\text{-value} < 0.05$) were the same in both models (data not shown). *p*-MCMC is

the proportion of cases where the samples from the MCMC chains is less than the significance level (here 0.05), equivalent to *p*-values (Hadfield, 2010 ; Teplitsky *et al.*, 2011).

We used the posterior distribution of each trait analysed to estimate the parameter value and it confidence intervals (CI). We considered significant differences for a trait between two environments or two different generations, when the 95% interval of highest posterior density (HPDI) for the subtraction between the whole posterior distributions of both estimates did not overlap 0. We used "significant" even if with a Bayesian approach significance reflect more a difference that is considered as non negligible (differ from the significance level commonly used in a frequentist approach). For the study of the evolutionary responses to selection, we used the posterior mode of the distribution of both the intercept (i.e. an estimate of the relative level of the trait for each treatment for a fixed generation) and of the slope of the linear regression of each treatment as a function of generations (i.e. an estimate of the relative change between generations in the value of the trait in one treatment relative to the control). Slopes were considered significant when their 95% HPDI did not overlap 0. We compared deviance information criterion (DIC) of models including different effects. We retained, as best fitted model, the model with the lowest DIC and this DIC differed from the second best fitted model's DIC by more than 5 (Spiegelhalter *et al.*, 2007). When two models had DICs within a range of 5, we retained the most parsimonious one (i.e. with the lowest number of parameters).

2.5. Effects on the phenotypic/genetic structure of traits

We assessed divergence of phenotypic pattern of traits (co)variance trough time and between populations. As we mentioned earlier, we assumed that phenotypic changes occurring between generation 4 and 22 were primarily of genetic origin. Consequently, we interpret these phenotypic changes as changes in the **G** matrix over time. However, we also show the changes observed during generations 1 and 4 as changes in the **P** matrix (without being able to separate genetic from environmental effects).

For these analyses we created matrices for the three constant environments (i.e. control, uranium, and salt) and for traits in hermaphrodites (i.e. total, early, and late fertility, and growth), and in males (i.e. body bend and growth). To increase the robustness of analyses, we assessed the temporal changes in each treatment by pooling data over successive generations (i.e. data from generation 1 to 4, 4 to 10, and 13 to 22). We used these pooled sample to analyse the **P** matrix

($\mathbf{G}_1$: generation 1 to 4), and both the early ($\mathbf{G}_2$: generation 4 to 10) and late ($\mathbf{G}_3$: generation 13 to 22) potential evolutionary changes. For each treatment we ran pairwise comparisons between each successive matrix ($\mathbf{G}_1$ vs. $\mathbf{G}_2$, $\mathbf{G}_2$ vs. $\mathbf{G}_3$). We then compared two matrices (i.e. $\mathbf{G}_1$: generation 1 to 4 and $\mathbf{G}_4$: generation 4 to 22) to analyse the differences between treatments at each time. Results using $\mathbf{G}_2$ and $\mathbf{G}_3$ or only $\mathbf{G}_4$ provided equivalent posterior modes, but larger HPDIs. Below we thus restrict the presentation of the comparisons of $\mathbf{G}_1$ and $\mathbf{G}_4$ only.

We combined Bayesian linear-mixed effect models with a bootstrap (resampling with replacement) procedure to calculate the angle between the first principal components (eigenvectors) of both matrices. Some developments in matrix comparisons have enabled one to compare all the dimensions of **G** matrices (Phillips & Arnold, 1999 ; Roff, 2002a). However, some authors (Berner *et al.*, 2010 ; Johansson *et al.*, 2012) have suggested that differences in the first eigenvector of the matrices may explain most of the variation in the matrix structure, and thus that the method comparing the first eigenvector of these matrices is sufficient to estimate changes of the structure of the matrix. This methods is also the one used to measure of the genetic line of least resistance (Schluter, 1996). For the comparison of the eigenvector of the matrices we ran models not including any fixed effect. However, we included replicate populations as a random effect. After several tests, we decided to keep the same priors and parameters values as in the models on the evolutionary responses. Then after a principal component analysis of the obtained matrices, we measured the cosine of the angle θ, between the first eigenvectors of both matrices by cosine similarity:

$$\cos(\theta) = \frac{\lambda_1 . \lambda_2}{\|\lambda_1\| . \|\lambda_2\|},$$

where λ_i and $\|\lambda_i\|$ are respectively the first eigenvector and the norm of the first eigenvector of the matrix i. We also estimated differences in matrix eccentricity, which is the ratio of the first eigenvalue to the sum of the remaining eigenvalues and in overall matrix size, which is the sum of the eigenvalues. A low matrix eccentricity indicates some instability in the orientation of the primary eigenvector of the matrix and reduction of matrix size suggests a reduction of variance and covariance (Jones *et al.*, 2003). To confirm the estimations of angle, eccentricity or size, we ran a bootstrap procedure with 1 000 iterations for each comparison and calculated posterior

mode of distribution and 95% CI. Moreover, to consider a significant difference between two angles (or another parameter) the 95% CI of subtraction between the distributions of the two angles should not overlap 0. In the bootstrap procedure, we corrected automatically the arbitrary change in the sign of the eigenvectors of any particular axis (axis reflection) and the reordering of axis due to very similar eigenvalues. However, it is easy to check these problems and so rearrange the matrices automatically.

3. Results

3.1. Evolutionary responses

The best fitted model for the temporal changes in hermaphrodite traits included an interaction between treatment and generation and a covariance between traits (Table 6). We found the same result when we limited the analysis to each combination of two traits, except between growth and late fertility (see appendix H for more details on bivariate models). For males the selected model included the interaction between treatment and generation, but including trait covariance did not improve the fit of the model. This indicates that, for both sexes, trait changes across generations differed according to the treatment. The replicate effects explained less than 4% of the (co)variance among traits in these models.

Table 6. Effect of generation and environment (control, uranium, salt, and alternating uranium and salt) on hermaphrodite (i.e. growth, total fertility, early, and late fertility) and on male (i.e. growth and body bend) traits, measured between generation 4 and 22 of the multigenerational experiment.
We used multivariate mixed models with all the traits included as dependent variables, and compared different models using deviance information criterion (DIC). We also ran models only for hermaphrodite growth and total fertility. Left-hand side: characteristics of the fixed effects included in each model (the first DIC value corresponds to a simple model including only replicate as a random effect). Right-hand side: DIC of the model followed by the change (Δ) in DIC value between this model and the previous model that did not include the fixed effect. Except for the models shown at the last line for each sex, covariance between traits was allowed in the priors. In bold: selected models for which $\Delta DIC > 5$, i.e. the model including interaction had a smaller DIC. Replicate effect in these models represents 3.3%, 2.3% and 2.8% of the total variance for hermaphrodite traits – quadrivariate and bivariate models – and for male traits, respectively.

Effects included within the model	DIC	Δ DIC
for hermaphrodite traits		
-	-2506.140	-
environment	-2538.996	-32.856
environment + generation	-2588.716	-49.720
environment x generation	**-2653.526**	**-64.810**
environment x generation (no covariance)	445.699	3098.535
for male traits		
-	701.020	-
environment	683.189	-17.831
environment + generation	631.832	-51.357
environment x generation	605.585	-26.247
environment x generation (without covariance)	**608.146**	**2.561**

Although we show the comparisons between the intercepts (value at generation 4) in the different environments (Table 7), we will focus below on the comparisons between the slopes of each environment. Slopes provide information on the differential rate of evolution of populations in these environments. Traits did not change across generations in the control treatment, except for a slight reduction in late fertility, which did not affect total fertility (Table 7 and Figure 16A). Evolutionary responses between generation 4 and 22 were generally higher in salt (1-5% per generation) than in uranium (1-2% per generation). The strongest evolutionary response for fertility happened in the alternating treatment. The evolutionary response was significant for early fertility in uranium, for late fertility in salt, and for both of these traits in the alternating treatment (Table 7 and Figure 16C, D). Traits related to reproduction produced stronger positive evolutionary response (2-5% per generation) than traits related to growth (1-2% per generation; Table 7, Figure 16B, F and see appendix J Figure J1). Male body bend was affected in the salt and in the alternating treatments, and increased between generation 4 and 22, but did not change significantly in the uranium treatment (Table 7 and Figure 16E).

We started each replicate population with 500 individuals. After three days and at each generation, population size in the control environment reached 20 000 individuals on average (Table 8, Figure 17, and see appendix J Figure J2). In the uranium treatment, population size increased from 10 000 at generation 1 to more than 15 000 at generation 22. Population size varied around 3000 individuals in the salt treatment during the whole experience. In the alternating treatment, population size varied between 3000 and 10000 individuals, without showing any particular temporal trend.

Table 7. Analyses of difference of traits values for hermaphrodites and males in the multi-generational experiment between generation 4 and 22.
Intercept corresponds to the rescaled traits value at the first generation (i.e. generation 4) and slope corresponds to the slope of linear regressions across generations. Values correspond to the estimation given by the posterior mode of the distribution for each parameter (i.e. intercept and slope) in control (first line for each parameter) or for each parameter in each environment relative to the others. Values between brackets correspond to the limit of the 95% highest posterior density interval (HPDI). Values in bold are those for which the 95% HPDI did not overlap 0.

Comparison	Hermaphrodite traits												Male traits					
	Total fertility			Early fertility			Late fertility			Growth			Body bend			Growth		
Intercep																		
Control	**0.727**	**0.626**	**0.841**	**0.643**	**0.549**	**0.741**	**0.380**	**0.204**	**0.583**	**0.497**	**0.370**	**0.631**	**0.363**	**0.206**	**0.521**	**0.422**	**0.297**	**0.546**
Control-Uranium	**-0.763**	**-0.914**	**-0.620**	**-0.688**	**-0.825**	**-0.561**	**-0.351**	**-0.635**	**-0.082**	**-0.389**	**-0.546**	**-0.189**	-0.094	-0.333	0.118	**-0.405**	**-0.573**	**-0.218**
Control-Salt	**-1.277**	**-1.431**	**-1.129**	**-1.172**	**-1.310**	**-1.031**	**-0.549**	**-0.844**	**-0.289**	**-1.140**	**-1.319**	**-0.978**	**-1.048**	**-1.282**	**-0.833**	**-0.925**	**-1.121**	**-0.747**
Control-Alternating	**-1.320**	**-1.465**	**-1.163**	**-1.081**	**-1.217**	**-0.937**	**-0.833**	**-1.114**	**-0.563**	**-0.679**	**-0.845**	**-0.496**	**-0.634**	**-0.857**	**-0.383**	**-0.622**	**-0.793**	**-0.447**
Uranium-Salt	**0.492**	**0.364**	**0.671**	**0.456**	**0.358**	**0.652**	0.195	-0.098	0.473	**0.742**	**0.585**	**0.932**	**0.982**	**0.749**	**1.211**	**0.541**	**0.355**	**0.711**
Uranium-Alternating	**0.566**	**0.398**	**0.705**	**0.437**	**0.246**	**0.538**	**0.525**	**0.231**	**0.786**	**0.312**	**0.113**	**0.461**	**0.498**	**0.328**	**0.773**	**0.215**	**0.032**	**0.389**
Salt-Alternating	0.081	-0.122	0.179	-0.105	-0.251	0.037	0.298	-0.009	0.573	**-0.428**	**-0.622**	**-0.272**	**-0.404**	**-0.629**	**-0.180**	**-0.338**	**-0.474**	**-0.110**
Slope																		
Control	-0.005	-0.012	0.001	0.002	-0.004	0.008	**-0.015**	**-0.028**	**-0.003**	0.004	-0.005	0.011	0.001	-0.010	0.010	0.005	-0.003	0.013
Control-Uranium	**0.012**	**0.002**	**0.020**	**0.012**	**0.003**	**0.020**	0.003	-0.014	0.020	0.005	-0.005	0.015	-0.006	-0.020	0.010	**0.013**	**0.000**	**0.023**
Control-Salt	**0.022**	**0.013**	**0.032**	0.001	-0.007	0.010	**0.048**	**0.030**	**0.067**	**0.015**	**0.005**	**0.025**	**0.031**	**0.016**	**0.045**	0.010	-0.001	0.022
Control-Alternating	**0.035**	**0.025**	**0.044**	**0.024**	**0.016**	**0.032**	**0.031**	**0.014**	**0.048**	0.010	-0.001	0.020	**0.014**	**0.001**	**0.029**	**0.017**	**0.006**	**0.029**
Uranium-Salt	**-0.008**	**-0.020**	**0.000**	**0.011**	**0.002**	**0.019**	**-0.039**	**-0.064**	**-0.027**	-0.009	-0.021	0.000	**-0.039**	**-0.052**	**-0.022**	0.003	-0.009	0.014
Uranium-Alternating	**-0.024**	**-0.032**	**-0.014**	**-0.013**	**-0.021**	**-0.004**	**-0.030**	**-0.046**	**-0.012**	-0.004	-0.015	0.006	**-0.023**	**-0.035**	**-0.006**	-0.005	-0.017	0.007
Salt-Alternating	**-0.011**	**-0.023**	**-0.003**	**-0.021**	**-0.031**	**-0.014**	0.020	-0.001	0.037	0.006	-0.005	0.017	**0.018**	**0.002**	**0.030**	-0.009	-0.019	0.004

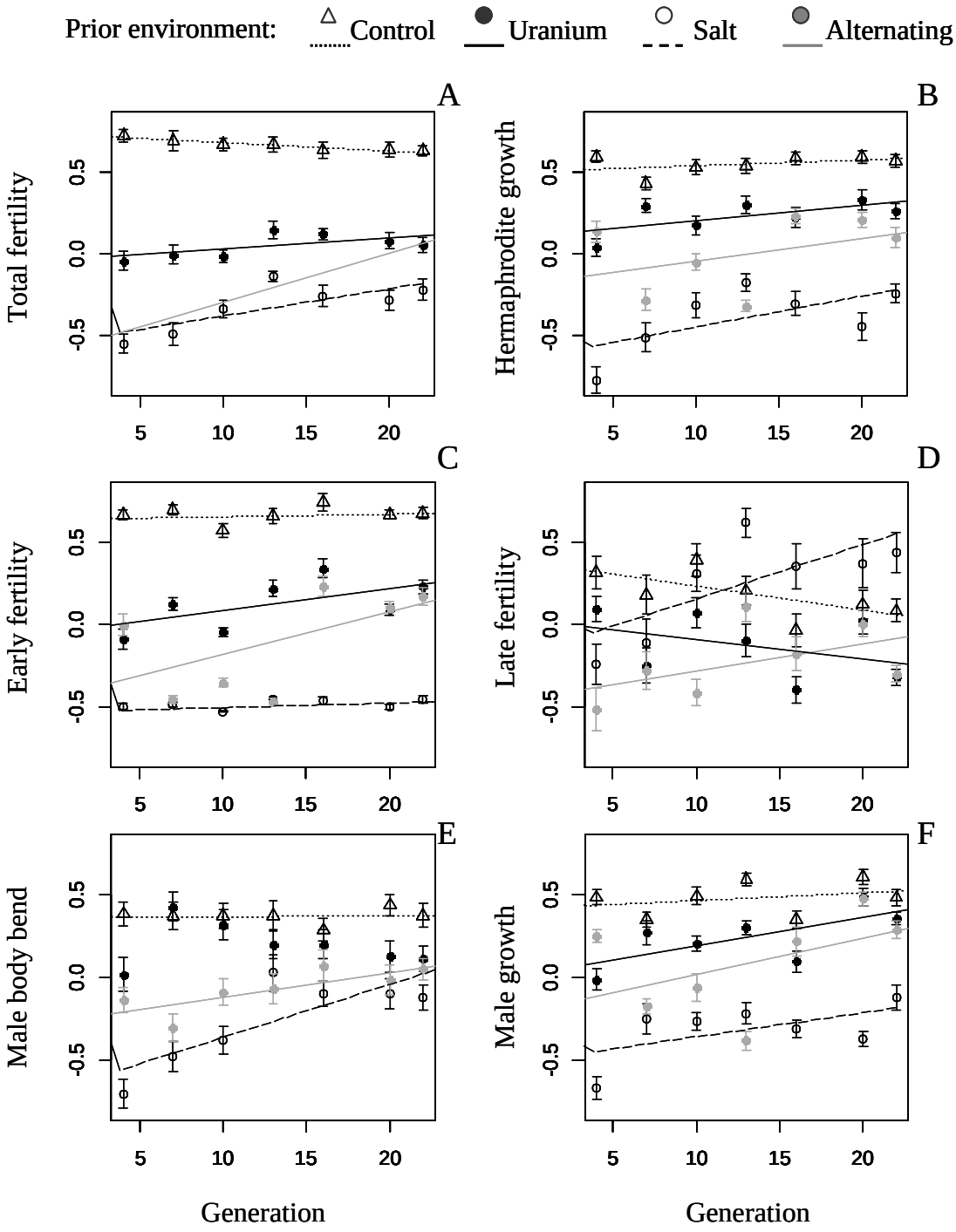

Figure 16. Evolutionary responses of hermaphrodite total fertility (A), growth (B), early (C) and late (D) fertility, and male growth (E) and body bend (F), between generation 4 and 22.
Each symbol corresponds to the mean value of the trait and its standard error (n = 18 individuals/treatment/generation). Traits values were rescaled prior to analysis by subtracting each value by the mean of the sample and dividing it by twice the standard deviation. Control (empty triangle), uranium (filled black dots), salt (empty dots), and alternating uranium and salt (filled grey dots) environments. Regression lines correspond to posterior mode of the distribution for intercept and slope (generation was a continuous fixed effects). Small dashed line: control; black line: uranium; large dashed line: salt; grey line: alternating uranium and salt environments.

Table 8. Effect of generation and environment (control, uranium, salt, and alternating uranium and salt) on population size measured between generation 1 and 22 of the multigenerational experiment.

(A) We used multivariate mixed models with all the traits included as dependent variables, and compared different models using deviance information criterion (DIC). All the models included replicates as a random effect to control for dependence of data across generations within each replicate. The first DIC value corresponds to a simple model including only replicates as a random effect. The next values correspond to the DIC of the next model included a given fixed effect and the associated change (Δ) in DIC between the two model included or not included that fixed effect. In bold, models for which ΔDIC > 5, i.e. the model including interaction had a smaller DIC, for which the replicate effect was 6.2%. (B) Analyses of differences for population size. Intercept corresponds to the population size at the generation 1 and slope corresponds to the slope of linear regressions across generations. Values correspond to the estimation given by the posterior mode of the distribution for each parameter (i.e. intercept and slope) in control (first line) or for each parameter in each environment relative to the others. Values between brackets correspond to the limit of the 95% highest posterior density interval (HPDI). Values in bold are those for which the 95% HPDI did not overlap 0.

A

Effect included within the model	DIC	Δ DIC
-	3661.992	-
environment	3643.314	-18.678
environment + generation	3632.237	-11.077
environment x generation	**3608.861**	**-23.376**

B

Comparison	Intercep					Slope				
Control	20925.9	[	19247.1	;	22489.4	]	-14.9	[	-119.3	;	102.9	]
Control-Uranium	-11971.3	[	-14561.9	;	-9570.2	]	391.7	[	218.5	;	540.9	]
Control-Salt	-18810.6	[	-21262.7	;	-16633.6	]	31.9	[	-130.9	;	173.7	]
Control-Alternating	-15603.9	[	-17895.8	;	-13056.4	]	72.2	[	-78.9	;	233.7	]
Uranium-Salt	**7213.0**	**[**	**4659.8**	**;**	**9304.1**	**]**	**355.4**	**[**	**219.8**	**;**	**510.3**	**]**
Uranium-Alternating	**3799.5**	**[**	**1304.5**	**;**	**6087.3**	**]**	**317.9**	**[**	**172.5**	**;**	**474.8**	**]**
Salt-Alternating	**-3461.8**	**[**	**-5466.2**	**;**	**-817.9**	**]**	-36.0	[	-190.8	;	128.2	]

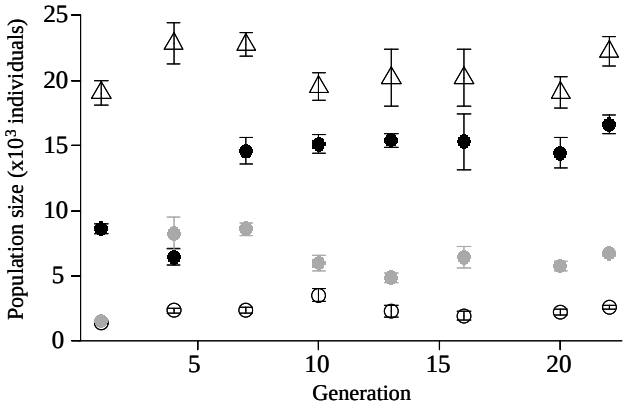

Figure 17. Changes in average population size in the different treatments between generation 1 and 22. Symbols show the mean value and standard error over 6 replicated populations in control (empty triangle), uranium (filled black dots), salt (empty dots), and alternating uranium and salt (filled grey dots) treatments.

3.2. Comparison of (co)variance matrices

We did not find any covariance between growth and body bend of males. Furthermore, tests for traits in males revealed angles of 0 degrees in all environments (data not shown). Below we thus only present matrix comparison for traits of hermaphrodites.

In the uranium treatment we found a strong divergence in phenotypic (co)variance structure between the matrices G_1 and G_2 (>43 degrees and CI for θ were not close to 0, Figure 18 and see appendix K for graphical representation). We found an equivalent divergence between G_1 in uranium and G_1 in control (Figure 19). However, we did not observe any divergence between G_2 and G_3 (Figure 18), or between G_4 in uranium and G_4 in control (Figure 19). In contrast, we found a moderate divergence (>20 degrees and CI for θ were not close to 0) between salt and control populations in both G_1 and G_4 as well as between salt and uranium populations for the same period (Figure 19). The first eigenvector for the matrices G_1 and G_4 accounted for 68% to 80 % of the total variance in all environments, except for G_4 in uranium (57%).

Matrix size for G_4 in salt was significantly higher than for G_4 in control and uranium (Table 9 and 95% HPDI of the subtraction did not overlap 0). Matrix eccentricity for G_4 in control was significantly lower than in salt but higher than in uranium (Table 9 and 95% HPDI of the subtraction did not overlap 0).

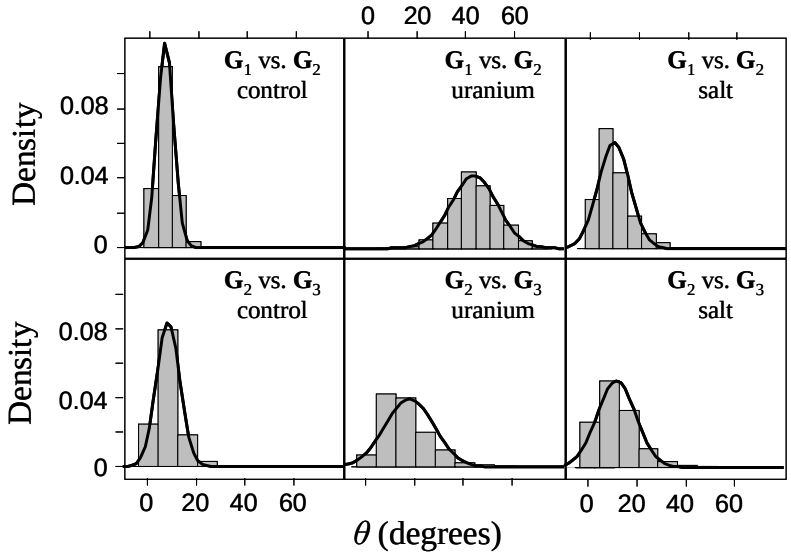

Figure 18. Distribution of density of the angle (θ) between different periods.
θ obtained by a resampling procedure, between the first principal components (eigenvectors) of both matrices of (co)variance for hermaphrodite traits (total fertility, early and late fertility, and growth) between two periods G_1 (generation 1 to 4) vs. G_2 (generation 4 to 10); and G_2 vs. G_3 (generation 13 to 22). We used this procedure for the populations from control, uranium, and salt environments. Error bars denote 95% confidence intervals for θ obtained using a resampling procedure.

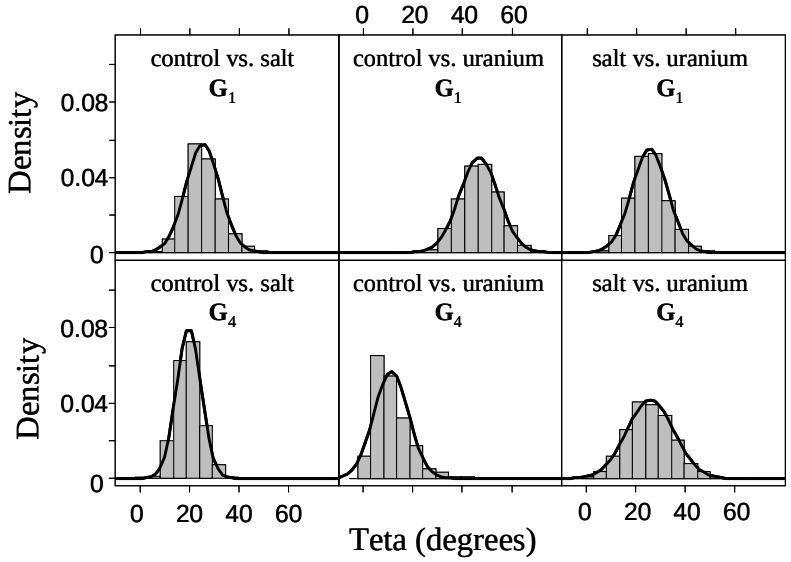

Figure 19. Distribution of density of the angle (θ) between different environments.
θ obtained by a resampling procedure, between the first principal components (eigenvectors) of both matrices of (co)variance for hermaphrodite traits (total fertility, early and late fertility, and growth) between populations from two environments: control vs. salt, control vs. uranium, and salt vs. uranium. We used this procedure for the periods G1 (generation 1 to 4) and G4 (generation 4 to 22). Error bars denote 95% confidence intervals for θ obtained using a resampling procedure.

Table 9. Measures of matrix eccentricity and size and their 95% highest and lowest of confidence intervals (CI), obtained by a resampling procedure, of matrices of (co)variance for hermaphrodite traits (total fertility, early and late fertility, and growth) in the control, the uranium and the salt environments.
We created matrices for the periods G1 (generation 1 to 4), G2 (generation 4 to 10), G3 (generation 13 to 22), and G4 (generation 4 to 22).

Environment	Period	Matrix eccentricity			Matrix size		
		Posterior mode	Lower estimate	Upper estimate	Posterior mode	Lower estimate	Upper estimate
Control	G_1	3.32	1.83	5.26	0.35	0.23	0.47
	G_2	2.91	1.86	4.50	0.26	0.19	0.33
	G_3	1.98	1.15	3.22	0.22	0.16	0.29
	G_4	2.29	1.58	3.17	0.25	0.20	0.31
Salt	G_1	2.44	1.59	3.79	0.38	0.31	0.46
	G_2	2.43	1.44	4.15	0.50	0.38	0.62
	G_3	2.88	1.73	4.85	0.40	0.31	0.51
	G_4	3.09	2.08	4.57	0.47	0.38	0.56
Uranium	G_1	2.20	1.48	3.36	0.49	0.39	0.59
	G_2	1.60	0.95	2.68	0.30	0.22	0.39
	G_3	1.12	0.73	1.64	0.24	0.19	0.30
	G_4	1.35	0.95	1.89	0.28	0.24	0.34

4. Discussion

This laboratory study demonstrated that *C. elegans* populations can increase their resistance to pollutants in only a few generations, and that these changes are very likely evolutionary and adaptive, or correspond to cross-generation genetic changes resulting from novel selection pressures. The intensity of selection pressures in the salt treatment led to a stronger evolutionary response in this medium than in uranium. The evolutionary response of fertility was higher in the alternating than in the other environments. However, unlike for uranium populations, population size in this treatment did not increase significantly across generations. We finally detected changes in the (co)variance structure in uranium and in salt populations compare to control populations, or through time. These results suggest that changes in the genetic structure can depend on the environment in which the populations live, and that selection pressures in a novel environment can quickly alter the genetic structure of a population.

4.1. Microevolution to constant pollution

The presence of microevolutionary change in the different polluted environments go in the same direction as results found by Lopes *et al.* (2008) on the capacity of a genetically diverse population of *C. elegans* to respond to selection by a pesticide. We found previously that traits were heritable in this *C. elegans* population (article II) and now that novel selection pressures were entailed by the pollutants. We thus have all the necessary conditions for populations to quickly adapt to novel stressor in the environment (Xie & Klerks, 2003 ; Ward & Robinson, 2005 ; Salice *et al.*, 2010 ; Jansen *et al.*, 2011b).

Although we cannot rule them out completely, epigenetic effects are highly unlikely to have generated cross-generation changes during the whole study period. They may have strong effects during the first four generations though (e.g. Anway *et al.*, 2005 ; Molinier *et al.*, 2006). We also consider that genetic drift was extremely low since we had low replicate effects in our models (Table 6 and see appendix G for more details on variance between replicates) and we did not see change through time in the control populations. Therefore, the successive phenotypic measures through time allowed us to evaluate the speed of the evolutionary response due to the selection in salt and uranium treatments (Posthuma & Van Straalen, 1993 ; Medina *et al.*, 2007).

Populations responded to both pollutants by increasing their fertility. However we found a stronger evolutionary response in salt than in uranium, with a 3.5% increase per generation for

total fertility in the former (and 4.8% for late fertility). In addition, generation time was longer in the salt than in the uranium or the control treatment (i.e. around four days instead of three; unpublished data and Table 7). Consequently, salt populations were studied for about 17 generations in this environment compared to the other populations, and evolutionary responses were probably underestimated in this environment.

Why would evolutionary response faster in salt than in uranium? We chose the concentration that reduced the fertility of almost 60 % at the first generation for both pollutants. However, despite that precaution, selection pressures could be stronger in salt as suggested by the stronger reduction in survival in this medium (see appendix F for survival analyses). Indeed some genotypes could be removed faster from the salt population. Then the heritability in uranium was lower than in the other treatments (see article II) and this could also constraint the evolutionary potential of traits in that medium (see e.g. Wilson *et al.*, 2006). In uranium, heritability was not even significantly higher than 0 in article II. It could be explained by a lack of power to detect a very low heritability with the method used in this other study since in our current paper we showed a significant evolutionary response in uranium. Our result also show that population reacted quickly to uranium by showing relatively strong acclimation during the first four generations of exposition (see appendix E for analyses of first generations). That quick acclimation may have also reduced the strength of selection on the traits and thus the evolutionary rate in the uranium populations (West-Eberhard, 2003). Moreover, uranium strongly affected the traits at the first generation but its effects were reduced by the third generation. Acclimation in these populations may have thus been related to maternal effects (i.e. response through cross-generation phenotypic plasticity) rather than to within-individual phenotypic plasticity (Mousseau & Fox, 1998). A population's response to a novel environment by acclimation or by adaptive processes can have completely different implications on the future of the population. Although plasticity is also a costly strategy (DeWitt *et al.*, 1998), it does not entail any long-term costs of adaptation, such as a reduction of genetic diversity (Ward & Robinson, 2005 ; Athrey *et al.*, 2007). Consequently, populations that respond to a novel environment by plasticity can cope with a larger range of conditions.

Growth for both sexes in all polluted environments has improved over time compared to control, except for hermaphrodites in uranium. However, in some case, we were not able to clearly conclude that the evolutionary response was significant (i.e. the HPDI slightly overlapped

0). It should be noted that, given the trends observed in our study, the evolutionary response could have become significant if we had run the experiment for a few more generations. Another explanation could be that fitness is more strongly related to fertility than to growth (Maynard Smith, 1989), and thus growth may be subject to weaker selection pressures and not evolve as quickly as fertility.

The locomotory response to uranium treatment was likely plastic since body bend frequency was not significantly affected anymore after generation 4. In contrast there was a positive evolutionary response of salt and alternating populations after a reduction in the first generations (see appendix E for analyses of first generations). Pollutants commonly decrease the frequency of body bends *in C. elegans* (e.g. Wang & Xing, 2008). Since, locomotion behaviour promotes encounter rate between males and hermaphrodites (Pannell, 2002 ; Barrière & Félix, 2005a ; Lopes *et al.*, 2008), outcrossing could be affected in polluted environments. It should be noted that compared to self-fertilization outcrossing permits hermaphrodites double or quadruple their fertility (Hodgkin, 1988 ; LaMunyon & Ward, 1998). However, the ratio of males, also an index of conservation of outcrossing rate (Ward & Carrel, 1979 ; Prahlad *et al.*, 2003 ; Teotónio *et al.*, 2006), was just slightly affected in the uranium and the alternating environments, and not at all in salt (see appendix F for sex ratio analyses). The effects on locomotion, associated to reduction of survival, for salt populations can explain in part the difficulties for population to grow as fast in the uranium and in control treatments.

4.2. Microevolution in response to alternating pollution

Although selection in a heterogeneous environment is assumed to maintain greater levels of genetic variation for fitness (Gram & Sork, 2001 ; Roff, 2002b), we highlighted a stronger evolutionary response for fertility in the alternating treatment. Previous studies have found cases of adaptation to heterogeneous environmental conditions by evolution of a more generalist way of life (Reboud & Bell, 1997 ; Cooper & Lenski, 2000 ; Turner & Elena, 2000). In those cases, the process involved in the evolution of generalism seems to be mutation accumulation (Kawecki, 1994) and not a positive trade-off between the environments. In our study we worked with only 22 generations, and the mutation rate was likely insufficient to be at the origin of the evolutionary changes observed (Mackay *et al.*, 1994 ; Denver *et al.*, 2009). Moreover, it is likely that some temporal fluctuating regimes entailed opposed selection pressures (e.g. alternated light and dark

in *Chlamydomonas* algae; Reboud & Bell, 1997). In contrast, we showed here a positive response on early fertility in uranium and late fertility in salt. We assume that the rapid evolutionary changes observed in the alternating treatment were caused by the same tendencies of directional selection on total fertility for both uranium and salt. Our study may provide the first evidence that the evolution of generalism can happen because of a positive trade-off on life history traits between the environments.

With our alternating treatment we could also have selected individuals having the capacity to face both pollutants. Adaptive plasticity can evolve in environments where phenotypic plasticity is beneficial and its maintenance costs are low (DeWitt *et al.*, 1998). Plasticity may be particularly beneficial and an evolutionary alternative to local adaptation (Kawecki & Ebert, 2004) when populations grow in a spatially heterogeneous environment or when temporal variability is important relative to the life cycle of the organisms (Pigliucci, 1996 ; Ghalambor *et al.*, 2007). For example, a contamination may not be spatially homogeneous in a polluted habitat. The intensity of stress may also be temporally variable and may promote plastic genotypes rather than only resistant, adapted, genotypes (Morgan *et al.*, 2007). Previous theoretical studies came to the same conclusions (e.g. Gomulkiewicz & Kirkpatrick, 1992), but stating that it was necessary that the variability is predictable (Kaplan & Cooper, 1984), as in our alternating treatment. In order to verifying this assumption, constant and alternating populations should be placed in the two constant environments to compare their fitness in both environments.

However, even if survival and fertility increased in alternating treatments across generations the population size did not enhanced throughout the experiment. During adaptation to a stressful environment, effects of genetic variation in population can be restricted to some traits (Agashe *et al.*, 2011). In alternating treatment the genetic variance enhances fitness strongly if the period of oscillation is long (and not sudden as in our study) and the amplitude is large (and not a change at each generation). Otherwise there may be an effect of genetic load (i.e. cost of lost alleles due to selection or mutation) caused by the recurrent change of phenotypic optima (see Lande & Shannon, 1996). Consequently, even if population seems to face this alternating polluted environment, the negative implications on population were severe.

4.3. Strength of structure between traits

Uranium populations only showed divergence in their phenotypic structure (i.e. the first four generations) compared to control populations and also between the first four generation and the period of early potential evolutionary changes. Nonetheless, comparisons of G_4 (i.e. after three generations of exposition) revealed a reduction in matrix size or eccentricity in uranium populations compared to control or salt populations. We thus assume the matrix change in uranium mostly due to environmental effects despite shrinkage of its genetic (co)variance components combined with a reduction of stability in the orientation of its primary eigenvector.

In contrast, the moderate divergence between salt and the other populations was present from the first generations of exposition and stayed equivalent in the subsequent generations. Thus matrix change in salt did not permit us to discriminate potential genetic effects from environmental effects. However, increase of matrix size and higher eccentricity occurred after three generations of exposition. Consequently genetic variance and covariance have probably increased and reinforced the stability of correlation between traits. This implies a stronger association between the traits that may constrain their independent evolution.

Studies on the stability of **G** matrix, in particular on the first eigenvector orientation, already revealed divergence after hundreds or thousands of generation**s** of evolution for natural populations (e.g. Cano *et al.*, 2004 ; Doroszuk *et al.*, 2008 ; Berner *et al.*, 2010 ; Johansson *et al.*, 2012). Moreover, **G** matrix can suffer considerable change**s** over a few generations. For example, Sgrò & Blows (2004) have shown some alteration of the genetic structure in *Drosophila* populations, for populations that had evolved for 30 generations in different heat stress environments. Cross-environment genetic correlation**s** declined as environments became more different, even upon arrival in different stressful environment, the genetic correlation can change of sign (reviewed by Sgrò & Hoffmann, 2004).

Interestingly, the effects of uranium and salt on the structure of traits were opposed. Previously, in isogenic line of the same population of *C. elegans*, we found higher heritability and a positive genetic correlation for fertility and growth in salt than in uranium (article II). This information added to the apparent reduction of stability of structure through generations would suggest that the covariance between growth and fertility traits diminished in uranium. All these differences between environments were probably in part at the origin of the differential evolutionary responses between uranium and salt populations. Moreover, there was a stronger

evolutionary response for fertility in both stressful environments than for the other traits. Thus the positive correlation between hermaphrodite growth and fertility in salt could explain the positive evolutionary response existing for growth in salt and absent in uranium. This method based on phenotypic measures permitted us to highlight alterations of phenotypic structure in polluted environment. These alterations were due to environmental changes but apparently also to genetic changes across generations of exposition, implying induction of changes of life history strategies for populations.

5. Conclusion

We have shown that rapid adaptation to different polluted environments may involve differential and complex evolutionary responses of life history traits. This is due to the action of the strength of selection pressures, capacity to response by acclimation and (co)variance structure between traits. Moreover, in alternating environment the populations had the strongest evolutionary response for fertility. Thus, the selection of a generalist type did not entail a reduction of resistance for both pollutants. Effects of pollutants can be extremely fast on life history traits of populations in few generations. Studies on microevolutionary processes due to pollutants should now focus also on the **G** matrix stability to confirm the feature of rapid divergence of structure between traits and depending on pollutant. This will help to highlight the consequences of pollution on the evolutionary potential of traits.

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