Objectifs et hypothèses

Dans ce chapitre, sont présentés les quatre objectifs spécifiques de ce travail de doctorat, sous forme de questions. Par ailleurs, pour chacune des questions, les hypothèses sont exposées. À la suite, les résultats et les discussions de nos travaux pour chacun de ces objectifs sont présentés sous forme d'articles scientifiques rédigés en anglais.

1. Quels sont les effets à court terme d'une pollution chronique en uranium ?

Avant d'entreprendre toute étude des effets d'un polluant sur l'évolution d'une population (c'est-à-dire étude multigénérationnelle), il est nécessaire de bien connaître à quelles concentrations les effets de ce polluant sont observés sur les traits d'histoires de vie de l'espèce étudiée. Il était en particulier nécessaire de déterminer une gamme de concentrations en uranium allant des concentrations qui ne perturbent pas la reproduction des individus aux concentrations entrainant la mort de 100 % des individus au sein de la population. De plus, à travers cette première expérience, nous voulions mesurer les effets de l'uranium sur les trois premières générations d'exposition pour commencer à appréhender la direction de la réponse évolutive pour chacun des traits étudiés. Nous souhaitions également mesurer la capacité d'acclimatation de la population dans un milieu contaminé à l'uranium. A notre connaissance, il existe extrêmement peu de données concernant les effets de l'uranium sur *C. elegans* (et sur les Métazoaires terrestres plus généralement), et aucunes sur la reproduction ou la croissance (Jiang *et al.*, 2007 ; Jiang *et al.*, 2009).

Hypothèses :

1- L'augmentation de la concentration en uranium dans le milieu affecte les traits d'histoire de vie de C. elegans selon une relation dose-réponse.

2- Par rapport aux effets à la 1ère génération, les effets de l'uranium s'amplifient durant les générations suivantes.

NB : Nous avons également mesuré, sur une seule génération, les effets du sel à différentes concentrations sur *C. elegans* (annexe B).

2. Quels sont les effets à court terme de l'uranium et du sel sur l'expression de la structure génétique des différents traits d'histoire de vie ?

La deuxième étape de ce projet était d'estimer la structure génétique des traits analysés et pour les concentrations en polluants retenues (voir article I et annexe B), car la variabilité génétique est un facteur essentiel à l'évolution d'une population. Les traits que nous avons choisis sont quantitatifs. Cette variation quantitative est supposée être influencée par un grand nombre de gènes aux effets individuels minimes (information génétique transmise dans les œufs pondus) et par l'environnement (milieu de développement). La distribution de ces traits est supposée suivre souvent une distribution normale même si d'autres distributions existent (Falconer & Mackay, 1996).

Afin de faire face à la pollution par la sélection naturelle, la population de *C. elegans* étudiée doit disposer de mécanismes de résistance permettant une résistance, *a minima* chez certains génotypes. Le potentiel évolutif des individus à vivre dans un environnement très stressant dépendra de l'intensité des pressions de sélection, mais aussi de la structure génétique des traits d'histoire de vie. Si l'expression de cette structure peut changer dans un environnement stressant, il est nécessaire de quantifier ces changements afin d'estimer le potentiel évolutif de la population dans ce nouvel environnement. Nous avons donc étudié l'expression de la structure génétique des traits dans trois environnements (contrôle, uranium, sel) chez des lignées isogéniques.

Hypothèses de travail :

1- Il existe une structure génétique des traits d'histoire de vie.

2- La structure génétique est stable malgré les changements d'environnement.

3. Quelle est la vitesse de réponse microévolutive des traits d'une population exposée à une pollution pendant plusieurs générations ?

L'objectif principal de cette partie était de mettre en évidence les modifications des valeurs de traits au travers des générations et de tester si ces modifications étaient associées à des changements génétiques intergénérationnels. En effet, l'habilité d'une population à s'adapter à des stress environnementaux dépend de sa variation génétique pour certains traits d'histoire de vie écologiquement essentiels. Pour ces traits polygéniques, les fréquences des allèles sur de nombreux loci peuvent changer en réponse à la sélection (Falconer & Mackay, 1996). Nous nous sommes intéressés également à la structure phénotypique entre les traits d'histoire de vie pour mettre en évidence tout changement de la structure génétique au cours du temps.

Il est également supposé qu'un environnement fluctuant pourrait exercer des pressions de sélection variables sur une population, ce qui maintiendrait une certaine variance génétique des traits, contrairement à un environnement constant (par exemple Reed *et al.*, 2003). Ainsi, les populations vivant dans un environnement variable devraient révéler une réponse évolutive plus faible. Dans cette expérience, nous avons quantifié les modifications phénotypiques d'une population de *C. elegans* exposée, pendant plusieurs générations, à différentes pollutions de son milieu de vie.

Hypothèses de travail :

1- Les traits répondent à la sélection en quelques générations (microévolution).

2- La vitesse de réponse évolutive dépend du polluant (pressions de sélection différentes).

3- La vitesse de réponse évolutive dépend du type de pollution (constante ou fluctuante).

4- La réponse évolutive est dépendante de l'architecture génétique.

4. La sélection entraine-t-elle des coûts adaptatifs ?

Localement, la diversité génétique des populations naturelles de *C. elegans* augmente fortement par rapport à leur diversité mondiale (Barrière & Félix, 2005a). Or dans le cas où une population s'adapte à certaines concentrations sublétales d'un polluant (voir article III), la force de la sélection peut induire une hyperspécialisation et des coûts sont alors associés à cette adaptation. Toutefois cette population peut être capable de faire plus facilement face à une autre pollution plus sévère, grâce à la sélection de certains individus : les plus résistants ou ceux s'acclimatant le plus vite et le mieux. C'est une sélection de la résistance (par exemple Ward & Robinson, 2005 ; Jansen *et al.*, 2011b). Ainsi, selon le nouveau milieu dans lequel les populations sont placées après une évolution dans un milieu pollué, les répercussions sur les traits d'histoire de vie peuvent être positives ou négatives. Ces risques doivent être identifiés et pris en compte dans l'évaluation des risques écologiques associés à la pollution pour améliorer sa pertinence. Nous avons donc réalisés des expériences en jardins communs et de transplantations réciproques dans différents environnements pour évaluer les coûts adaptifs aux pollutions précédemment étudiées (voir question précédente).

Hypothèses de travail :

1- Une part des modifications phénotypiques est due aux adaptations de la population aux pollutions (différentiation génétique).

2- La valeur sélective des populations adaptées à une pollution est plus faible, dans un environnement sans contamination ou un nouvel environnement stressant, que celle des populations originelles.

3- Les coûts adaptatifs se mettent en place progressivement, en fonction de la réponse évolutive.

4- Les coûts adaptatifs sont plus faibles pour des populations adaptées à une alternance de deux polluants qu'à une pollution constante.

Article I – Rapid phenotypic changes in *Caenorhabditis elegans* under uranium exposure

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Abstract

When exposed to a pollutant affecting fitness, populations can be affected by novel selection pressures related to the exposition, and that depend on the concentration of the pollutant. The main ways to respond to the stress are acclimation (i.e. plastic changes) and adaptation (i.e. genetic changes). Acclimation provides a quick and short response to environmental changes and adaptation can have longer-term implications on the future of the population. One way of studying these responses is to conduct studies on the phenotypic changes occurring across generations in populations experimentally subjected to a selective factor (i.e. multigenerational test). Such never been performed with uranium (U). Here, we explore the phenotypic changes across three generations in experimental *Caenorhabditis elegans* populations exposed to different U concentrations. In the first generation we detected significant negative effects of U on survival, generation time, brood size, body length and body bend. These effects increased with increasing U-concentration. Besides, the strength of selection pressures induced by different U-concentrations changed the direction of the phenotypic response across generations: negative effects were reduced for lower U-concentrations indicating a potential improvement by acclimation, and were amplified for high U-concentrations. Consequently, under high U-concentrations acclimation may not permit the population to avoid extinction and the only way to face pollution seemed to be adaptation to uranium in the subsequent generations. Our results highlight the need to consider changes across generations to enhance environmental risk assessment related to U-pollution.

Keywords

Caenorhabditis elegans, chronic exposure, experimental evolution, phenotypic changes, uranium

1. Introduction

The demographical consequences of pollutants on populations have largely been studied in ecotoxicology. However, most of these studies have focused their attention on within-generation effects, whereas it is well known that pollutants acting on both survival and reproduction could also lead to evolutionary changes occurring across several generations (reviewed by Coutellec & Barata, 2011). Such evolutionary changes can modulate the demographic responses of the population to the pollutant over time (e.g. evolutionary rescue: Bell & Collins, 2008). Therefore, it is important to examine how populations are affected by the pollutant by studying more than one generation.

Populations can show three different types of response to a change in the environment: (i) within-individual phenotypic plasticity (Scheiner, 1993); (ii) cross-generation phenotypic plasticity (i.e. maternal effects; Räsänen & Kruuk, 2007); and (iii) rapid genetic changes through selection of different genotypes (i.e. local adaptation; Hedrick, 2006 ; Hendry & Gonzalez, 2008). The first two types of response can be detected as soon as the first and the second generations that experience the change in environmental conditions, and are generally called acclimation. For example, in the case of pollution, individuals can suffer negative effects on several life history traits during the first generation and its effects can be amplified or attenuated in the next generation (Wang & Wang, 2008a ; Massarin *et al.*, 2010 ; Coutellec *et al.*, 2011). In contrast, selection acts across generation through differential reduction of individuals' fitness depending on their sensitivity to the new environmental conditions or even elimination of the most sensitive individuals (Ward & Robinson, 2005 ; Medina *et al.*, 2007 ; Morgan *et al.*, 2007). When selection pressures are too strong, acclimation may not be sufficient for the population to withstand the stress induced by the new environmental conditions (Morgan *et al.*, 2007). It leads evolutionary response to selection to be the only effect responsible for most of the phenotypic changes observed between generations.

The power of detection of the evolutionary response depends on both the strength of selection pressures and on the heritability of the resistance trait, and increases with the number of generations (Hoffmann & Merilä, 1999). Long-term exposure to a pollutant is assumed to have led to local adaptation and improved resistance in populations exposed to heavy metal pollution (Shirley & Sibly, 1999 ; Reed *et al.*, 2003 ; Hendry *et al.*, 2007 ; Morgan *et al.*, 2007 ; Hendrickx

et al., 2008). Therefore, it is essential to consider multigenerational responses to highlight the evolutionary dynamics of a population to pollution.

In this study, we examined the phenotypic responses of *Caenorhabditis elegans* populations to contamination by different uranium (U) concentrations across three generations. Although the evolutionary changes increase with the number of generations (Lynch & Walsh, 1998), only three generations are necessary to know the direction of that response of the trait to selection. Three generations is the minimum number to be able to evaluate the impact of the three different evolutionary processes that allow a population to respond to changes in their environment. Phenotypic plasticity and selection are combined to modify the affected traits within the first generation subject to the change in the environment (e.g. a novel pollutant). Inter-generational plasticity and selection both can explain the changes in the trait from generation one to generation two. From generation two to generation three, plastic effects are assumed to have the same effect at all the generations and are not responsible for any more changes between generations. So, only selection pressures can explain a further change in the trait (Scheiner, 1993 ; Mousseau & Fox, 1998 ; Gagliano & McCormick, 2007 ; Räsänen & Kruuk, 2007). Information provided by a three-generation experiment can thus improve our knowledge of the risks associated with a range of concentrations of a pollutant, while keeping experiments as short as possible.

The concentration of U, a natural radioactive heavy metal, in sediments or surface soils has increased recently as a result of human activities (e.g. mining, scientific research, military use of depleted uranium). On average, the concentration of natural U is 2 mg U kg^{-1} in soil (~0.008 mM U). However, concentrations in contaminated sites can reach up to 50 to 1000 mg U kg^{-1} (~0.2 to 4.2 mM U; UNSCEAR, 2000). For example, maximum concentrations of 110 mg U kg^{-1} dry wt (~0.5 mM U) have been detected in soils near mining sites in Australia (Lottermoser *et al.*, 2005). Consequently, the organisms in these environments have been found to suffer high U-toxic effects (e.g. terrestrial invertebrates, zooplanktonic invertebrates, epibenthic macro-invertebrates and fish; Sheppard *et al.*, 2005 and references therein). Exposure to natural U, whose main isotope is ^{238}U (99.3%), may induce both chemical and radiological (alpha particle emission) effects. Because of its low specific activity (2.53 x 10^4 $Bq.g^{-1}$ U), U is assumed to have higher chemotoxic than radiotoxic effects (Kuhne *et al.*, 2002). Nonetheless, its radiotoxicity cannot be neglected (Thomas & Liber, 2001).

Free-living forms of the nematode *C. elegans* have been used as a model to assess both (i) the toxicity of heavy metals and other pollutants (Swain *et al.*, 2004 ; Shen *et al.*, 2009; Tominaga *et al.*, 2003 ; Harada *et al.*, 2007 ; Sochová *et al.*, 2007), and (ii) the evolutionary responses to several selective agents (e.g. pesticides: Lopes *et al.*, 2008; starvation: Morran *et al.*, 2009; parasites: Schulte *et al.*, 2010). *C. elegans* is a ubiquitous species, and some populations probably experienced U-contaminated soil. Furthermore, *C. elegans* represents a powerful model to conduct experiments in evolutionary ecotoxicology because of its life cycle, small size and ease of handling and cultivation in various devices (Brenner, 1974). Most of the studies mentioned above have limited their assessment of the selective effects of the pollutant to the first generation, and only a few have reported how exposure to a heavy metal at the first generation affected the population at the second (Wang & Yang, 2007 ; Wang & Wang, 2008a) or the third generation (Wang *et al.*, 2007). Although the observed effects at the first generation are potentially transferable to subsequent generations, their extrapolation is difficult because of the impossibility of disentangling the effects of phenotypic plasticity, parental responses, and of selection occurring during the first two generations. Finally, previous reports have focused on the U concentration-mortality relationship (Jiang *et al.*, 2009) and have neglected potential effects on other life-history characteristics.

In order to understand how U-pollution can act on the evolutionary dynamics of *C. elegans* populations, we monitored the effects of U on changes in life history, morphologic and behaviour traits over three generations in a laboratory population. Studied traits (i.e. survival, generation time, brood size, locomotion and body length) were related to fitness at different degrees and to population dynamics (Mousseau & Roff, 1987 ; Roff, 2002b). We examined if phenotypic responses were affected differently by different U-concentrations (ranging from 0.16 to 2.53 mM) and if the stress-induced evolutionary mechanisms can be highlighted after three generations.

2. Material and Methods

2.1. Strain preparation and metal exposure

We used the wild-type N2 nematode population provided by the *Caenorhabditis* Genetics Center (funded by the NIH National Center for Research Resources). Populations were maintained on nematode growth medium (NGM) plates seeded with *Escherichia coli* strain OP50, at 20°C

(Brenner, 1974 ; Stiernagle, 2006). For the particular needs of our experiments we replaced 25 mM potassium phosphate buffer (pH 6.0) in the NGM with 25 mM HEPES buffer (pH 5.5; Sigma-Aldrich, France) which did not caused any effect on survival, growth, reproduction and locomotion behaviour of worms (Dutilleul, unpublished results). Inorganic phosphate binds with U which can lead to uranyl phosphate formation. This complex formation may decrease the bioavailability and toxicity of U on aquatic plants (e.g. Mkandawire *et al.*, 2007) or *C. elegans* (Dutilleul, unpublished results). U was added to the liquid NGM just before pouring the medium into the plates. For each desired concentration we added 6 ml of a diluted solution of uranyl nitrate ($UO_2 (NO_3)_2$, $6H_2O$; Sigma-Aldrich, France) in 200 ml of NGM. For the purpose of this study we used 10 nominal U-concentrations: 0 (control), 0.1, 0.5, 1, 1.2, 1.4, 1.6, 1.8, 2, 2.5 mM U. NGM was left overnight to solidify. We then collected agar samples (100 µl) from each medium and stored them at 4°C prior to the measurement of U-concentrations. Samples were digested using a combination of 1 ml HNO_3 (65%) and 1 ml H_2O_2 at 90°C, and measurements were obtained using the ICP-AES (Optima 4300 DV, Perkin-Elmer, USA; detection limit 0.04 µM).

We grew *E. coli* OP50 cultures in L Broth rich medium at 37°C overnight. Then 250 µl of saturated culture was seeded on the plates and left overnight to allow the bacterial culture to dry. Plates were then top-exposed to UV doses for 90 sec to kill the bacteria (Bio-Link Crosslinker; λ=254 nm; intensity = 200 µwatt.cm^{-2}). The main aim of this treatment was to avoid different bacterial growth in control and U-contaminated plates.

2.2. Experimental design

Gravid worms were selected from the stock population and placed on a Petri dish at t = 0 h. After 2 h, released fertilized embryos were considered to be age-synchronized. Fifty embryos were placed on U-contaminated plates (4 replicates for each U-concentration). These embryos represented the first generation in our experiment (P0). At t = 96 h, adult worms from P0 were transferred onto new plates with the same U-concentration as in P0, and left for 2 h for embryo collection and preparation of the next generation (F1). The same protocol was repeated to create the third generation (F2). Worms were cultured for these 3 generations at 20°C and 80% relative humidity.

2.3. Life history and behaviour measurements

From t = 48 h, we studied 5 endpoints: (i) survival, (ii) generation time, (iii) brood size, (iv) body length, and (v) body bend frequency. To measure survival we stimulated each worm with a platinum transfer pick. Worms that did not move their body or their head in response to three repeated stimulations were scored as dead (Sutphin & Kaeberlein, 2009).

To assay generation time and brood size we transferred worms into individual wells (12 worms / treatment, 3 / replicate) containing U-contaminated NGM. Each well was visually inspected every 2 h until t = 65 h. Generation time was calculated as the time between the embryo transfer into the well and it first released embryo. Adults were then transferred twice into a new well every 36 h. Hatched progeny were counted the day after each transfer (Swain *et al.*, 2004). Using this method allowed us to fully count brood size.

Body length was measured at t = 72 h. Twenty worms per treatment (including the 12 individuals used to measure generation time and brood size) were photographed using a stereomicroscope with a computer-connected camera (LAS® Leica imaging software). We measured body bend frequency at t = 72 h by picking up worms and placing them onto a new plate without bacteria. We counted the number of body bends over 20 s. 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).

2.4. Statistical analyses

All statistical analyses were performed with the statistical computing software R (R Development Core Team, 2012). We fitted concentration-survival regression for estimation of the LC_{50}. The assumption of normality of the errors was tested with the Shapiro-Wilk test. When this assumption was not satisfied, data were square-root or Box-Cox transformed. Survival at t = 48 h was analysed by ANOVA using Dunnett's comparison with a control test. The other endpoints were tested by linear mixed models, where treatment, generation and treatment * generation were included as fixed effects and replicate was included as a random effect. When the interaction was significant, we ran separate mixed models for each generation and for each treatment, using replicate as a random effect. These analyses were followed by Tukey's all-pair comparison tests (or t-test when only two groups were compared) on treatments in models for each generation and on generations in models for each treatment (Bretz *et al.*, 2010). Differences in brood size among

the different U-concentrations were also tested at each generation using ANCOVA with body length as a covariable.

3. Results

3.1. Contamination of NGM

Initial U-concentrations in the NGM obtained by ICP-AES were 0, 0.16, 0.48, 0.88, 1.14, 1.34, 1.57, 1.75, 1.97 and 2.53 mM, respective to the nominal concentrations (see section *2.1*). These concentrations deviated from the nominal concentrations by 0%, 60%, -4%, -12%, -5%, -4%, -2%, -3%, -2% and 1%, respectively. Actual initial concentrations are used throughout the text and in the figures.

3.2. Trait measurements

After 2 days of exposure, the mean survival at P0 decreased significantly with U-concentration (Figure 12, $F_{9,30} = 51.01$, $P < 0.001$). Mean survival in the control at P0 was 95.5 ± 5.2%. Mean survival varied from 63.3 ± 7.9% at 1.34 mM U to 0% at 2.53 mM U (Dunnett, $P < 0.001$ from 1.34 mM U). The lethal concentration for 50.0% of individuals (LC_{50}) at 48 h was 1.71 mM (95% CI = 1.62-1.80).

For U-concentrations equal to or higher than 1.57 mM, no worms survived after 96 h of exposure. At 1.34 mM U, worms did not release any embryo at all (Figure 13B). As a result, there was no data available after 1.34 mM U for generation time, brood size, body length and body bend frequency. Thus, we could only analyse the effects of treatment for U-concentrations up to 1.34 mM. Moreover, at F1 and F2 survival in the different treatments was not significantly different from the control (Dunnett, $P > 0.05$), except for the higher measurable concentration at F1, 1.14 mM (Dunnett, $P = 0.031$). There was a significant interaction between treatment and generation on the different traits measured (generation time: $F_{7,147} = 37.72$, $P < 0.001$; brood size: $F_{8,140} = 31.52$, $P < 0.001$; body length: $F_{8,296} = 27.27$, $P < 0.001$; body bend: $F_{8,301} = 5.00$, $P < 0.001$).

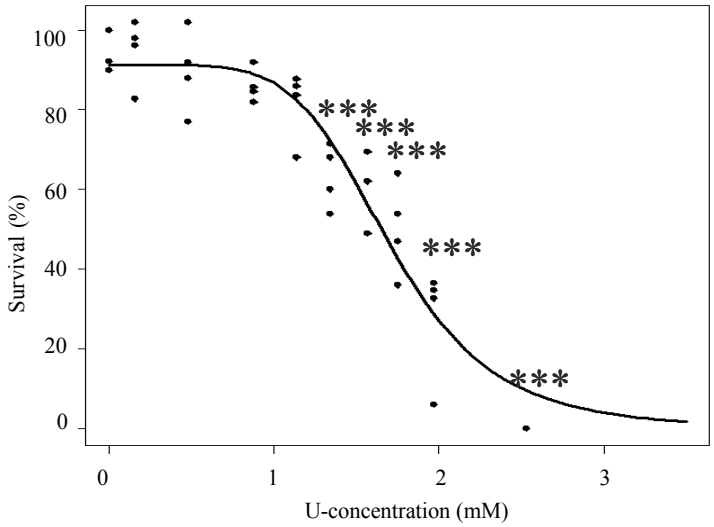

Figure 12. Percentage of *C. elegans* surviving at generation P0 after 48 h of exposure to different U-concentrations. Each concentration was represented by 4 replicates (50 individuals in each replicate). Asterisks indicate significant differences between each U-concentration and the control (***: P < 0.001). The continuous line is the curve concentration-survival: LC50 at 48 h was 1.71 ± 0.09 mM.

When analysed separately from treatment, generation time increased significantly with U-concentration at each generation (P0: $F_{4,47} = 85.07$, $P < 0.001$; F1: $F_{4,53} = 187.19$, $P < 0.001$; F2: $F_{3,44} = 15.37$, $P < 0.001$). The delay in generation time in the U-contaminated media relative to the control started to be significant at 0.48 mM U at both P0 and F1 (Tukey test, $P < 0.001$ in both generations), and at 0.16 mM U at F2 (Tukey test, $P < 0.001$; Figure 13A). The longest delay was observed at 1.14 mM U for F1 (Tukey test, $P < 0.001$). We could not measure generation time for U concentrations higher than 1.14 mM at F2 because of the high variability in the generation time in F1 and F2, embryos hatched inside the adult. A significant delay in generation time was found for F2 worms at 0.88 mM U ($F_{2,28} = 5.33$, $P = 0.01$) and for F1 worms at 1.14 mM U ($t_{21} = 10.05$, $P < 0.001$).

There was an overall significant decrease in brood size with U-concentration at each generation (P0: $F_{4,50} = 27.72$, $P < 0.001$; F1: $F_{4,44} = 117.60$, $P < 0.001$; F2: $F_{4,46} = 238.71$, $P < 0.001$). For example at generation P0, mean brood size in the control was 248 larvae and 145 larvae at 1.14 mM U (Figure 13B; Tukey test, $P < 0.001$). Brood size increased significantly from P0 to F1, at 0.16 and 0.48 mM U ($F_{2,29} = 7.80$, $P < 0.01$; $F_{2,25} = 4.75$, $P = 0.02$), and from P0 to F2 at 0.88 and 1.14 mM U ($F_{2,24} = 7.90$, $P < 0.01$; $F_{2,31} = 404.69$, $P < 0.001$). At 1.14 mM

U, brood size decreased significantly between P0 and F1 (i.e. 48 larvae: Tukey test, $P < 0.001$), and between P0 and F2 (i.e. 5 larvae; Tukey test, $P < 0.001$). The decrease in brood size was not associated with an increase in the number of dead embryos: we observed 98 to 100% of hatchings in all treatments and at all the generations (Dutilleul, unpublished results).

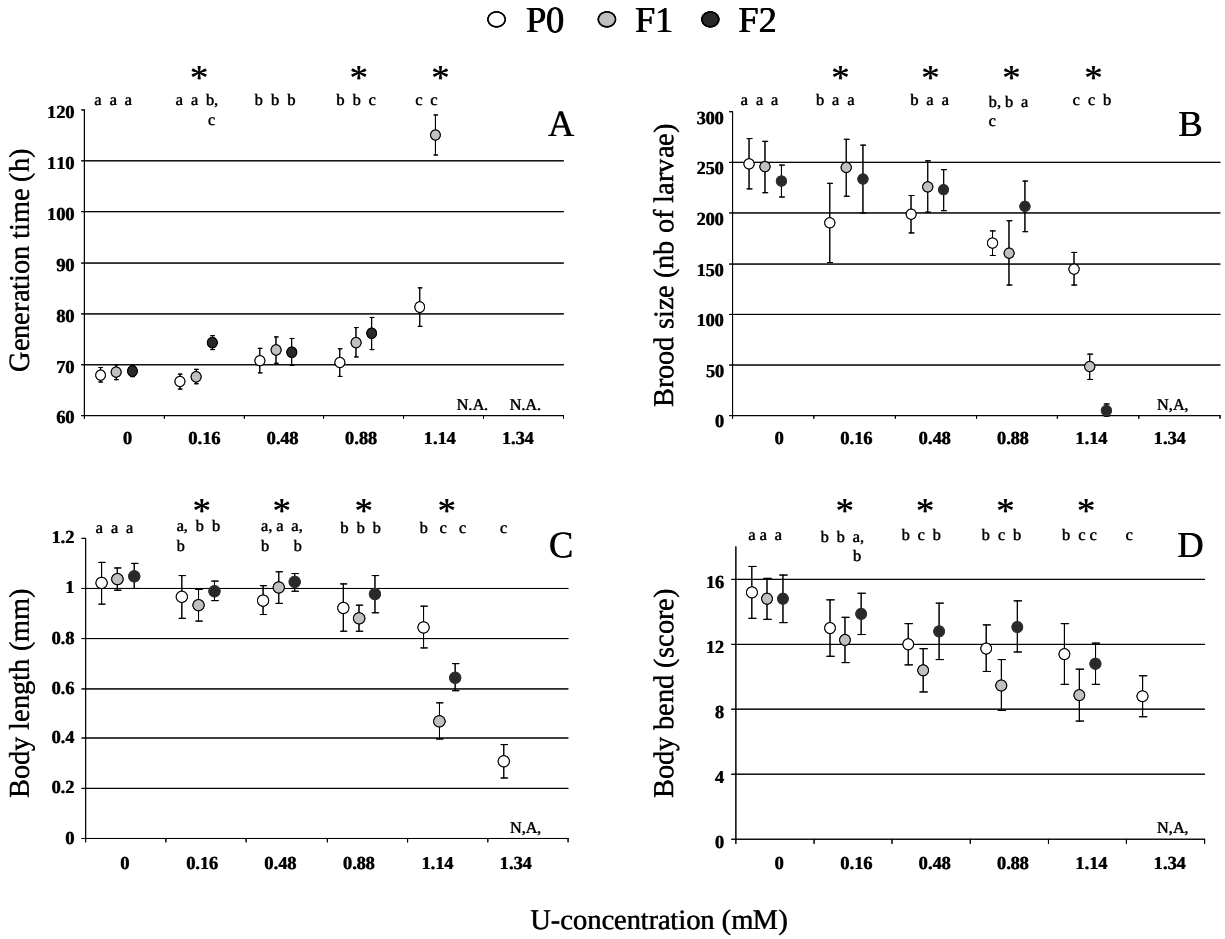

Figure 13. Changes in phenotypic traits (mean ± SD) of *C. elegans* exposed to different U-concentrations and across three generations

P0 (black circle), F1 (grey circle) and F2 (white circle). (A) Generation time; (B) Brood size; (C) Body length; (D) Body bend. Different small capitals and asterisks on top of the histograms indicated significant differences ($P < 0.05$) between two treatments and between generations for the treatment below, respectively. N.A. = non-available data, caused by bagging (eggs hatch inside the adult) or 100% mortality or infertility after a certain U-concentration.

Mean body length was 1.02 mm at 72 h in the control at generation P0 (Figure 13C). It decreased significantly with U-concentration at each generation (P0, $F_{5,95} = 156.41$, $P < 0.001$; F1: $F_{4,91} = 78.68$, $P < 0.001$; F2: $F_{4,104} = 244.6$, $P < 0.001$). From 0.16 to 1.14 mM U, the body length improved at F2, compared to F1, even if the values were below the control and sometimes

below P0. For example at 1.14 mM U, mean body length at each generation was 0.85, 0.47 and 0.64 mm, respectively ($F_{2,52}$ = 122.93, P < 0.001). At P0 generation, mean body length at 1.34 mM U was extremely small: 0.31 mm. However, the worms were not adult and they never reached this stage before dying (data not shown).

Mean body bend frequency over 20 s in the control at generation P0 was 15.2 (Figure 13D). Body bends decreased with U-concentration at each generation (P0: $F_{5,111}$ = 37.04, P < 0.001; F1: $F_{4,95}$ = 51.14, P < 0.001; F2: $F_{4,91}$ = 24.23, P < 0.001). Body bends decreased significantly between 0.48 and 1.14 mM U, respectively, for F1 and F2 (Tukey test, P < 0.001). Moreover, from 0.48 to 1.14 mM U, body bends decreased for F1 but increased again for F2. The values at F2 were at least non-significantly different from the values at P0, or even significantly higher. For example, at 0.88 mM U, mean body bend values at P0 and F2 were 11.8 and 13.1, respectively (Tukey test, P = 0.02).

3.3. Growth-reproduction correlation

We further compared the relationship between brood size and body length at each generation to determine whether reproductive strategy remained the same across the generations. Brood size increased with adult body length for the 3 generations (Figure 14; slopes: t_{135} = 4.26, P < 0.001). The slopes were not significantly nonparallel between P0 and F1 (slopes: t_{135} = 1.51, P = 0.13), therefore, the relationship seemed to be similar. However, the relationship was significantly different between P0 and F2 (slopes: t_{135} = 4.63, P < 0.001). This significance existed due to the highest U concentration. Indeed, if we omit 1.14 mM at F2 generation, the relationship is not significantly different to P0 (slopes: t_{124} = -1.31, P = 0.20).

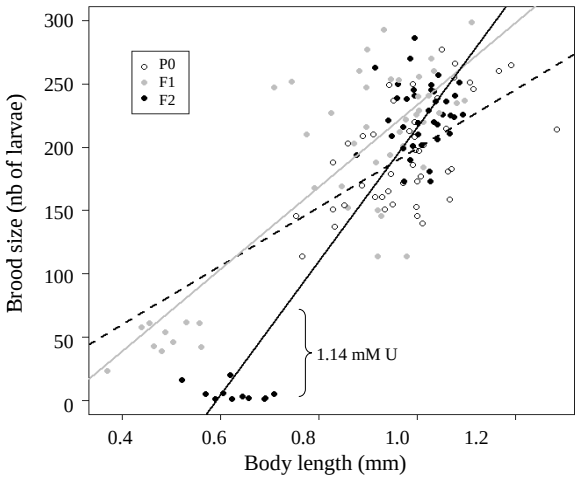

Figure 14. Brood size as a function of adult body length after 72 h of exposure to U-concentrations.
Open circles: generation P0, grey cross: F1, filled circles: F2. Regression lines associated with each generation; dashed line: P0, grey line: F1, black line: F2.

4. Discussion

In this study, we observed different effects of U on several life history traits in *C. elegans*, in only three generations. The effects of U on these traits increased with increasing concentration in the medium. We will first discuss the effects of U on survival and other traits in the first generation. We will then explain how some evolutionary mechanisms can act to produce rapid phenotypic changes in only three generations and their potential consequences on population dynamics.

4.1. Acute toxicity on survival

The range of U-concentrations affecting the survival of the *C. elegans* N2 strain (from 1.34 mM U) differed from other invertebrate models. This range is 10 times higher than for *Chironomus riparius* (Dias , 2008), 1000 times higher than for *Daphnia magna* (Zeman , 2008), but 10 times lower than for *Tubifex tubifex* (Lagauzère *et al.*, 2009). However, time and conditions of exposure differed between each of these experiments, and a comparison of the effect of U on different organisms should be made with caution. To our knowledge, only one study has assessed the effects of U on the survival of *C. elegans* (also strain N2: Jiang *et al.*, 2009). In that study, L1 worms were exposed to U in water medium for 30 min. Survival was assessed 24 h later on NGM plates and the LC_{50} was found to be 66.9 µM ± 30.9, at a concentration 25 times lower than in our bioassay. The

differences between the study by Jiang *et al.*and the present study can be attributed to different environmental conditions. Jiang *et al.* exposed the nematodes to U in water, whereas in our study the nematodes were exposed to U in agar where the effects of U could be reduced, especially because individuals did not develop in a contaminated water column but on a contaminated agar surface. Differences in the mode of exposure can have strong consequences on U bioavailability, which depends on its concentration and also its chemical form according to the conditions of exposure (Zeman *et al.*, 2008 ; Misson *et al.*, 2009).

When compared to the LC_{50} of ten other heavy metals on the same N2 strain (Wah Chu & Chow, 2002), U seems to be one of the heavy metal with the lowest effect on *C. elegans* survival. However, in the study by Wah Chu & Chow(2002), survival was measured in liquid medium over 4 days instead of 48 h in agar medium as in our study. Our results confirm the U-sensitivity of *C. elegans* within the range observed for other heavy metals.

4.2. Chronic toxicity on biological measurements in the P0 generation

C. elegans exposed to U showed a decrease in mean brood size and an increase in generation time. The same trends were found when N2 strain was exposed to nickel or cobalt (Wang *et al.*, 2007 ; Wang & Wang, 2008a). Our results also show an amplification of these two reproductive traits according to increased U concentration, a longer generation time and a smaller brood size. Forbes & Calow(2002) demonstrated that any negative effects on larval production will lead to even more adverse consequences at the population level. Indeed, the progeny production directly affects the population growth rate, an extremely robust endpoint in ecological risk assessment (Forbes & Calow, 1999 ; Billoir *et al.*, 2007).

Growth was negatively affected by exposure to U. At 1.34 mM U, worms had still not reached their adult stage at 3 days and were more than 3 times smaller than control worms. Heavy metals such as cobalt or lead have been shown to cause the same effects on growth (Wang & Yang, 2007 ; Wang *et al.*, 2007). The toxic effects of U on growth have also been found in other invertebrates such as *C. riparius* and *D. magna* (Dias *et al.*, 2008 ; Massarin *et al.*, 2010). The reduction in body size could be explained by the negative effect of U on food assimilation. In *D. magna* and the earthworm *Eisenia fetida*, U severely affected the intestinal epithelium suggesting that this might result in dysfunctions in food assimilation (Giovanetti *et al.*, 2010 ; Massarin *et al.*, 2010).

The lowest concentration of U affected locomotion behaviour and the frequency of body bends decreased with U. It was reported that U produces a central sensorimotor deficit in laboratory rats (Abou-Donia *et al.*, 2002). This could be related to oxidative stress which plays a key role in the mechanism of U neurotoxicity (Lestaevel *et al.*, 2009). In addition, heavy metals commonly decrease the frequency of body bends in the N2 strain (Wang & Xing, 2008).

4.3. Changes after three generations of exposure

After a rapid decline at the first generation, brood size, body length and body bend were enhanced after 3 generations of exposure from 0.16 to 0.88 mM U despite stable environmental conditions during the experiment and for each treatment. These traits are directly or indirectly related to the fitness of individuals, and thus an increase in their values should have positive consequences on the dynamics of the population (Forbes & Calow, 2002). An increase in generation time after 3 generations could also participate in this demographic improvement. Indeed, an increase in generation time may help reduce the negative impact of a pollutant on fitness as the progeny will be in contact with the pollutant as late as possible (e.g. bagging, see below). However, effects on the progeny through the parents should not be ignored and longer generation time directly affects population growth rate (Forbes and Calow, 2002).

There was a general tendency for traits (except generation time) to decrease at F1. As at P0, individual phenotypic values illustrate the direct effect of U on the plasticity of their development, combined with the potential differential survival associated with phenotypic differences (i.e. direct effect of selection within a generation) (Hoffmann & Merilä, 1999). Phenotypic values measured in F1 individuals are subject to both developmental plasticity and direct selection. Heritable traits have been affected by the effect of natural selection occurring in the previous generation (Roff, 1997). In addition, traits can be affected by intergenerational plasticity through the effect of U at P0 on their parents reproductive performance (Gagliano & McCormick, 2007). This hypothesis is supported by results from other studies. For example, similar negative effects on the progeny were observed in *C. elegans* exposed to cobalt, lead and nickel (Wang & Yang, 2007 ; Wang *et al.*, 2007 ; Wang & Wang, 2008a) and on *D. magna* exposed to U (Massarin *et al.*, 2010). Thus, biological defects can be transferred to the progeny even if it does not live in a polluted medium. Phenotypic measures of F2 individuals are still subject to similar developmental plasticity, direct selection, and maternal effects since their parents also

lived in a U-polluted medium. Furthermore, their traits will be affected by the evolutionary response to selection between F1 and F2. Consequently, the improvement in traits (except generation time) at F2, could be due to rapid evolutionary response to U-exposure (Medina *et al.*, 2007). Adaptive processes can occur quickly and for a harsher level of pollution or constraints (Roff, 2002b).

The effect of U on brood size across generations is particularly interesting. At lower U-concentrations, brood size increased significantly between P0 and F1, but only started to increase between F1 and F2 at 0.88 mM. Thus, the possibility for this phenotypic trait to improve is slower for a higher strength of selection pressures. At the highest concentration where it could be measured (i.e. 1.14 mM), brood size showed a decline of 66% between P0 and F1 and of 96% between P0 and F2. When selection pressures of U-exposure were lower, most individuals could probably cope with stress due to acclimation (phenotypic plasticity). For example, individuals could produce enough metallothioneins to protect themselves against U-exposure (Jiang *et al.*, 2009). Under harsher environmental conditions (such as 1.14 mM U), the effects of selection pressures were too strong to overcome it by developmental plasticity or maternal effects, and thus in the generations after F2, the only way to improve fitness will be by evolutionary response. Besides, the strong mortality in a harsh environment reduces both population size and the genetic variance of the traits under selection, which affect the evolutionary potential of those traits (Hoffmann & Merilä, 1999 ; Wilson *et al.*, 2006 ; Bell & Collins, 2008). Under the harshest environmental conditions (i.e. 1.34 mM U), infertility and mortality increased to 100% and annihilated any potential for evolutionary response. It should be noted that we used 50 individuals per replicate in that experiment. Using larger population sizes may have moved the threshold of population extinction to a higher concentration as a few individuals may have had the chance to survive and reproduce. The chance of evolutionary rescue in a population increases with its size (Holt & Gomulkiewicz, 1997 ; Bell & Collins, 2008 ; Willi & Hoffmann, 2009).

The increase in toxic effects on reproduction under high U concentrations is consistent with the toxic effects of U on *D. magna* (Massarin *et al.*, 2010). The delay of first brood for *D. magna* increased and the fecundity at 21 days reduced more and more at the F1 and F2 generation. Tominaga *et al.* (2003) showed more severe effects of phenols on *C. elegans* fecundity at the fourth generation than the first generation. The results of these studies did not show increasing resistance to the pollutant in the first few generations. This does not mean that adaptation is not possible or

more visible a few generations later or with a larger number of individuals. Individuals in populations of *D. magna* and *C. elegans* have the ability to adapt to pollutants (Ward & Robinson, 2005 ; Lopes *et al.*, 2008). Rapid evolutionary changes have been shown to improve the ability of populations to reduce the probability of extinction (Bell & Gonzalez, 2009 ; Willi & Hoffmann, 2009).

At 1.14 mM U, the few L1 individuals that were produced at F2 hatched inside their parents. This explained the different relationship between brood size and adult body length observed between F2 and the first two generations (Figure 14). The phenomenon called bagging (or matricidal hatching) is assumed to represent an example of phenotypic plasticity in *C. elegans* to extreme conditions such as starvation or high salt concentration (Chen & Caswell-Chen, 2003, 2004). In these studies, bagging favoured dauer formation, a dormancy stage that allows the species to survive in stressful conditions (Golden & Riddle, 1984). Studies on heavy metals have never shown the induction of dauer formation. This may be explained by the fact that the dauer cuticle does not confer any resistance to heavy metals (Barsyte *et al.*, 2001 ; Wah Chu *et al.*, 2005). Bagging enhances progeny survival and dispersal (Chen & Caswell-Chen, 2003), however, in our experiment, progeny which hatched in parents never reached sexual maturity or switched to a dauer stage. Therefore, we can say that the attempt to rescue the progeny by bagging failed at higher U-concentrations. Below 1.14 mM U, populations seemed to have the ability to evolve very rapidly for all traits and at 1.14 mM U, individual brood sizes were extremely low at F2 compared to P0 generation (only 4%). Despite a short multigenerational experiment, our results show that it is possible in only three generations to highlight the importance of the strength of the environmental change on the capacity to cope this change by acclimation and if it is not possible, to require adaptation of the population.

5. Conclusion

After 4 days of U-exposure at P0 generation, our results showed that *C. elegans* individuals were on average smaller, slower, less fertile, and developed less fast than the controls. Across generations, phenotypic changes can vary in amplitude and in direction, depending on the concentration of U-exposure. This may be explained by the differential effects of development plasticity, selection pressures, and evolutionary responses of the traits to U-toxicity. Our results

emphasize the need for multigenerational tests for accurate assessment of the environmental risks of U pollution on population dynamics in contaminated environments.

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