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Metallome evolution in ageing *C. elegans* and a copper stable isotope perspective†

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Ageing is accompanied by important chemical deregulations resulting in bodily metal imbalances. The way and extent to which these deregulations are associated with ageing processes are however poorly understood and their use as potential biomarkers of ageing has not been investigated. In this study, we report whole-body elementary concentrations and copper and zinc isotopic compositions of *Caenorhabditis elegans* in ageing wild type (*i.e.* 'normal'-lived) and mutant (*i.e.* short and long-lived) strains. We show that the strains are characterized by different levels of mutation-related variations such as in phosphorus and magnesium as well as in zinc isotopic composition. During ageing, strains are affected by elemental age-related variations, such as an increase in calcium and iron concentrations and a decrease in the copper isotopic composition and concentration for long-lived mutants. The deregulated metabolism of copper seems to be connected to ageing probably in association with the production of reactive oxygen species. We emphasize that the copper stable isotope composition could serve as a biomarker of normal or accelerated ageing in the future.

Significance to metallomics

Here we report a comprehensive set of metal concentrations and Cu–Zn isotopic compositions of wild type and mutant worms analyzed at different stages of their life. We identified two types of chemical variations: one related to the age, highlighting a critical role of metal deregulations in ageing, and the other specific to mutations. Focusing on age-specific elements, in particular copper, we show that long-lived animals are characterized by distinct early-age variations compared to control worms. Although these results need to be verified by further studies, the present study suggests that copper isotopic compositions could be used in the future to predict lifespan and detect premature ageing conditions.

1. Introduction

By 2050, the number of elderly people over 65 is expected to triple to about 1.5 billion representing 16 percent of the world's population.[1] This rapid increase in life expectancy will encompass dramatic changes such as the development of severe pathological disorders[2] and neurodegenerative diseases[3] making ageing one of the most prominent risk factors for death in the old population. To date, the identification and measurement of biomarkers of ageing have been a major subject of research because biomarkers can shed light on some ageing processes but most importantly, because biomarkers could be potentially useful to detect abnormal and/or premature ageing conditions.[4]

In accordance with the heterogeneity of all the pathways involved in ageing, many biomarkers, being chemical or biological, have already been proposed. These include for example proteostasis impairment,[5] an increase in advanced glycation end products[6] related to glycogen metabolism deregulation (*i.e.* high glucose diet),[7,8] oxidative stress increase[9] and mitochondrial dysfunction,[10] as well as markers linked to DNA processes like DNA methylation,[11,12] telomere shortening[13] or DNA damage accumulation.[14] Predicting biological age is nonetheless a difficult task, and we know that we still do not have a good single index of prediction in humans[15] meaning that we are probably missing a key factor that would work from simple to more complex organisms.

Over the last few decades, the number of studies focusing on age-related changes of the concentrations of major and trace elements has continuously increased supporting the idea that ageing is associated with important elemental deregulations. These elemental deregulations have been observed at the scale of the whole body of the *C. elegans* worm,[16] in various organs in murine models[17–19] and in human blood.[20–23] More recently,

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studies on the worm model have suggested that lifespan could be dependent on metal deregulations. This relies on the observation that high levels of metal exposure including aluminum, iron, copper, zinc, manganese, lead, calcium and cadmium significantly decrease the mean lifespan with a concomitant impairment of several pathological functions such as reproduction, brood size, development and locomotion behavior.[24–26] Conversely, metal binding interventions allowing stopping metal accumulation usually extend the normal lifespan.[16,27,28] All these results are clearly substantial pieces of evidence of the contribution of metallome dyshomeostasis to the ageing process to some extent, although the exact mechanistic links by which they are related to each other remain poorly understood.

Stable isotopic compositions may offer a more comprehensive view and additional means to constrain the metallome evolution during ageing. In two pilot studies, the copper and zinc isotope compositions, δ^{65}Cu and δ^{66}Zn, respectively, were measured for the first time in various organs of sheep raised experimentally with a controlled diet.[29,30] The key observation was that the δ^{66}Zn and δ^{65}Cu values in feces were slightly different from that of the diet, the Zn and Cu isotope compositions in feces being ^{66}Zn-depleted and ^{65}Cu-enriched, respectively. Therefore, mass conservation requires that organisms balance these disequilibria by accumulating a ^{66}Zn-enriched and a ^{65}Cu-depleted component, suggesting that δ^{65}Cu and δ^{66}Zn could be potential biomarkers of ageing. Challenging this hypothesis, Jaouen *et al.*[31] measured the copper and zinc isotope compositions in the blood of volunteers from a remote Yakut population, and found that the δ^{65}Cu and δ^{66}Zn values, respectively, decrease and increase with age, in accordance with previous assumptions. Therefore, although metal concentrations can provide relevant information regarding biological changes over time, additional measurements of isotopic compositions may offer a more comprehensive view of ageing than concentrations alone. Indeed, one advantage of using isotope composition over concentration for a given metal is that the isotopic fractionation, *i.e.* the variation of the isotopic ratio, is independent of the amount of the metal. Thus, isotopic ratios are potentially more reliable biomarkers than concentrations.

In this regard, we report the concentrations of 13 elements as well as the Cu and Zn isotopic compositions during the ageing of wild type and mutant (*i.e. daf-16* short-lived, *daf-2* long-lived and double mutant) *Caenorhabditis elegans* nematodes. We describe the evolution of the metallome of the different strains in terms of mutation-related and age-related variations, discuss the deregulation of the Cu metabolism in relation to the production of reactive oxygen species, and finally propose that the δ^{65}Cu value could be a potential future biomarker of ageing.

2. Materials and methods

2.1. *C. elegans* strains and worms development assay

Four different strains of worms including a wild-type (Bristol N2), a long-lived mutant of the insulin/IGF-1 receptor *daf-2* (*e1370*), a short-lived mutant of the FOXO transcription factor *daf-16*, (*mu86*) and the double mutant for both alleles of CF1588 were used. All the strains were provided by the Caenorhabditis Genetics Center (CGC, University of Minnesota) and were maintained at 20 °C and fed with *E. coli* strain OP50 on nematode growth media (NGM) agar plates as previously described.[32] Bacteria were cultivated within a nutrient-rich microbial growth medium (*i.e.* LB broth-Lennox) containing peptides, amino acids and carbohydrates in a low-salt formulation.

For chemical measurements, synchronized populations of L4 larval stage were placed on 85 mm NGM plates previously seeded with OP50 bacteria and supplemented with 3.95 mg L^{-1} FluoroUracil (FU; Sigma F6627). FU addition was used to prevent egg hatching and young larvae development on plates. Animals were transferred to fresh NGM plates every 5 days to avoid starvation. They were collected before visible age-related declines at three different time points (2 and 10 days for all genotypes as well as 17 days only for the long-lived animals) defined as the period with 100% survival and maximum mobility capacities. This step was carried out under a microbiological safety workbench to ensure sterile conditions and minimize external contaminations. Briefly, worms are collected using a pH-neutral M9 buffer (22 mM KH_2PO_4, 42 mM Na_2HPO_4, 86 mM NaCl, 1 mM $MgSO_4$) followed by 3 rinses with ultra-pure water before being transferred into 2 mL sterilized Eppendorf tubes and frozen at −80 °C for optimized conservation.

2.2. Analyses of concentrations and Cu–Zn isotopic compositions

The Cu and Zn isotopic compositions of worms cannot be determined on a single animal because worms are small (~1 mm). On average, between 10 000 and 30 000 worms are needed per strain and per assay to obtain the minimum amount of Zn and Cu required for precise isotopic measurements. For example, 10 000 worms correspond to about 500 ng of Zn but only 25 ng of Cu. This means that Cu isotopic analyses are highly challenging and require an uncommon and large amount of worms. In addition, since no more than 1000 worms can be deposited per plate, at least 10 plates per condition are necessary. This gives a total of 150 plates for only one assay when including the transfer plates (1 strain collected at 3 timepoints *i.e.* 30 plates + 30 transfer plates and 3 strains collected at 2 different timepoints *i.e.* 60 plates + 30 transfer plates) and 150 additional plates for a single duplicate *i.e.* up to 450 plates for 2 duplicates. Furthermore, keeping the worms alive necessitates a transfer to fresh NGM plates every 5 days, which is time consuming when working with hundreds of plates. Each data point thus represents an important amount of worms and time making the analytical analyses highly difficult and also our capacity to obtain many duplicates as usually carried out in biological studies.

Before each analytical measurement, all samples were first freeze-dried during 48 hours, weighed and then dissolved in a mixture of $HNO_3{\cdot}15N/H_2O_2$ 30% on a hot plate at 130 °C. All the steps of the chemical procedure were carried out in a clean laboratory below laminar flow clean hoods and acids that were doubly distilled to avoid external contaminations. After complete dissolution, major and trace element concentrations as

well as Cu–Zn isotopic compositions were measured at the Ecole normale supérieure (ENS) of Lyon following the procedure described by Maréchal *et al.*[33] and Garçon *et al.*;[34] only brief synopses are provided here.

The concentrations of titanium (Ti), manganese (Mn), iron (Fe), copper (Cu), zinc (Zn), strontium (Sr) and cadmium (Cd) were measured on a quadrupole ICP-MS Thermo iCap-Q and the concentrations of magnesium (Mg), calcium (Ca), potassium (K), sodium (Na), phosphorus (P) and sulfur (S) were measured on an ICP-AES (iCAP 6000 Radial) at the Ecole normale supérieure of Lyon. Indium (In) and scandium (Sc) were used as internal standards on ICP-MS and ICP-AES, respectively, to correct instrumental biases. The validity and reproducibility of major and trace element concentrations are estimated to be better than 10% (2sd) based on the analyses of re-run analysis, complete duplicates and repeated measurements of international (*i.e.* bovine liver, 1577c) and in-house (*i.e.* owe plasma, OEP) reference materials as unknown samples (see Table S1 in the ESI† for the details).

Once elementary concentrations were measured, copper and zinc were isolated using ion chromatography based on the procedure previously detailed by Maréchal *et al.*[33] Briefly, Cu and Zn were purified using quartz columns filled with 1.8 mL of Bio-Rad AG MP-1 (100–200 mesh) anion exchange resin. After removing the matrix phase with 10 mL of 7 N HCl + 0.001% H_2O_2, Cu was first eluted with 20 mL of the same solution, followed by Zn with 10 mL of a 0.5 N HNO_3 solution. Total procedural blanks were less than 4 ng for Zn and lower than 0.4 ng for Cu which represents 1/125 and 1/62 of the amount of element isolated from the samples for Zn and Cu, respectively ($\sim$500 ng for Zn and $\sim$25 ng for Cu).

Isotopic compositions were measured on a Nu Plasma (Nu 500) MC-ICP-MS under wet plasma conditions. On the day of the analyses, Zn and Cu purified solutions were diluted in a Cu (Cu SRM 976, National Institute of Standards and Technology, Gaithersburg, MD, USA) and Zn-doped solution (Zn JMC 3-0749L, Johnson Matthey Royston, UK) respectively to match the concentration of the standard mixture run between the samples (between 75 and 300 μg L^{-1} depending on the sample). The delta values (expressed in ‰) are reported relative to the isotopic solution reference material NIST SRM 976 for Cu and JMC 3-0749L for Zn and are referred to as:

$$\delta^{65}Cu_{sample} = \left[\frac{\left(^{65}Cu/^{63}Cu\right)_{sample}}{\left(^{65}Cu/^{63}Cu\right)_{standard}} - 1\right] \times 1000$$

and

$$\delta^{66}Zn_{sample} = \left[\frac{\left(^{66}Zn/^{64}Zn\right)_{sample}}{\left(^{66}Zn/^{64}Zn\right)_{standard}} - 1\right] \times 1000$$

Instrumental mass fractionation was corrected with an exponential law using an elemental-doping method and instrumental drift over time was controlled with standard sample bracketing as recommended by Maréchal *et al.*[33]

The long-term precision of our results is assessed by repeated analyses of the pure Cu SRM 976 and Zn JMC 3-0749L standard solutions run every two samples and the accuracy is assessed by the analysis of in-house and international reference materials. The reproducibility (2sd) of the Cu SRM 976 and Zn JMC 3-0749L standards was better than 0.1‰ (n = 50) for both $^{65}Cu/^{63}Cu$ and $^{66}Zn/^{64}Zn$ ratios. Our results for the reference standards are 0.16‰ (2sd, n = 9) for OEP and 0.09‰ (2sd, n = 5) for 1577c for $^{65}Cu/^{63}Cu$ ($\delta^{65}Cu_{OEP} = -1.11 \pm 0.16$ (2sd, n = 9), $\delta^{65}Cu_{1577c} = +0.35 \pm 0.09$ (2sd, n = 5)) and better than 0.1‰ for OEP and 1577c for $^{66}Zn/^{64}Zn$ ratios ($\delta^{66}Zn_{OEP} = +0.73 \pm 0.04$ (2sd, n = 3), $\delta^{66}Zn_{1577c} = -0.19 \pm 0.09$ (2sd, n = 6)). All these values are in good agreement with our previous laboratory measurements *i.e.* $\delta^{65}Cu_{OEPref} = -1.15 \pm 0.20$ (2sd, n = 35), $\delta^{65}Cu_{1577cref} = +0.37 \pm 0.14$ (2sd, n = 45) and $\delta^{66}Zn_{OEPref} = +0.73 \pm 0.09$ (2sd, n = 35), $\delta^{66}Zn_{1577cref} = -0.16 \pm 0.15$ (2sd, n = 45) as well as with previous published results ($\delta^{66}Zn_{1577c} = -0.13 \pm 0.02$ (2sd, n = 4)[35]). Given our long-term precision and the accuracy obtained on reference material measurements, the 2sd analytical uncertainty adopted in this study for the Cu and Zn isotopic compositions is ± 0.1‰.

3. Results

Major and trace element concentrations as well as Cu and Zn isotopic compositions ($\delta^{65}Cu$ and $\delta^{66}Zn$, respectively) measured in the four different strains are all reported in Table S1 in the ESI† and shown in Fig. 1. To evaluate the whole pattern of variations, we used a principal component analysis (PCA). Briefly, this statistical technique performs a linear transformation to find a system for the complete dataset with new variables called principal components (PCs). The main result is that all the animals are affected by distinct and specific chemical variations that are either age-related (variations along the x principal component axis; 46.7%) or mutation-related (variations along the y principal component axis; 32.2%).

Along the x principal component axis, the 2 day old animals are less enriched in Cd, Mn, Ca, Sr, Fe, Ti and Cu and have a higher amount of $\delta^{65}Cu$ compared to the 10 day old animals (Fig. 1, 2a and b). An illustration is given in Fig. 2a for Fe showing that concentrations are roughly similar ($\sim$100 ppm) in all the strains at 2 days, and then increase by a factor of 2 at 10 days. This holds for Ca too, which is concentrated by at most a factor of 3 between 2 and 10 days in the N2 strain (Fig. 2b). For all these elements, the major source of variation is age whatever the strain considered is.

Along the y principal component axis, the wild type strains are more concentrated in P, Mg, S, Na and K than the short-lived and the double mutants that are in turn more enriched in these elements than the long-lived worms. An example is given in Fig. 2c for P. The highest P content is observed in the wild type nematodes (23721 ± 3779 (2sd, n = 3) ppm) while the lowest concentrations are measured in the long-lived *daf-2* (e1370) mutants (17573 ± 3214 (2sd, n = 3) ppm). However, for a given strain, subtle variations with age can be observed and, as in the

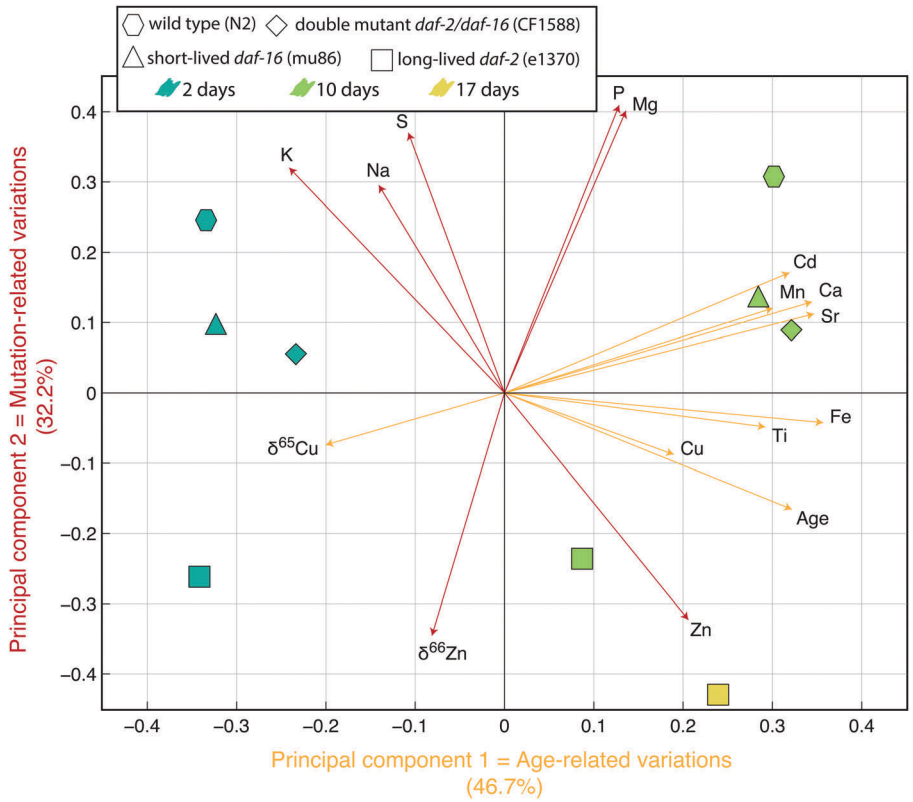

Fig. 1 Principal component analysis (PCA) diagram. PCA is a multivariate method used to highlight strong patterns in a dataset. In this study, this technique was used to classify four different *C. elegans* nematode strains based on several parameters including the chemical concentrations of about 15 elements and copper and zinc isotopic compositions that were normalized to their standard deviation. In more detail, the process of PCA consisted of (1) creating a matrix containing all the raw data (*i.e.* elemental concentrations and isotopic compositions of all the samples). Note that samples for which a measure was missing were excluded. (2) Determining the covariance matrix and (3) calculating the eigen-vectors and the eigen-values of this covariance matrix to determine the principal components (PCs) (PC1 and PC2 correspond to the eigen-vectors with the largest eigenvalues). All these steps were implemented in MATLAB™. PC1 and PC2 explain ~80% of the total variance in chemical composition. Orange and red arrows are graphic representations of loading factors for PC1 and PC2 respectively. Each point is an average of three different complete duplicate analyses (n = 3) except for Cu, Mn and δ^{65}Cu (n = 2). The PCA allows for the classification of chemical variations observed in mutant worms over time into two different groups: (1) the elements related to the age progression (principal component 1, PC1) *i.e.* Cd, Mn, Ca, Sr, Fe, Ti, Cu and δ^{65}Cu and (2) the elements related to the mutation process (principal component 2, PC2) *i.e.* K, Na, S, P, Mg, Zn and δ^{66}Zn.

case of purely age-related variations, trace element concentrations generally increase between 2 and 10 days (Fig. 2c). The type of mutation drives the Zn isotope composition (Fig. 2d), long-lived mutants *daf-2* (e1370) having the highest δ^{66}Zn values ($\delta^{66}Zn_{e1370} = -0.19‰ \pm 0.08$ (2sd, n = 3)), and N2 wild types the lowest δ^{66}Zn values ($\delta^{66}Zn_{N2} = -0.36‰ \pm 0.28$ (2sd, n = 3)). The influence of ageing on the Zn isotope composition is very weak (Fig. 2d).

Focusing on the Cu isotopic composition and concentration, distinct temporal variations are observed between long-lived animals and all the other strains (Fig. 3). Between 2 and 10 days, the δ^{65}Cu value remains constant in the wild type, short-lived and double mutant worms while it decreases in the long-lived mutant from -0.25 ± 0.69 (2sd, n = 2) ‰ to -0.85 ± 0.06 (2sd, n = 2) ‰ reaching -1.08 ± 0.76 (2sd, n = 2) ‰ at 17 days (Fig. 3a). Similarly, the Cu concentration decreases in the long-lived worms from 4.22 ± 1.01 (2sd, n = 2) ppm to 3.00 ± 1.46 (2sd, n = 2) ppm between 2 and 10 days and then tends to stabilize at 17 days (3.15 ± 0.75 (2sd, n = 2) ppm) while it increases in all the other strains from ~2.25 to ~3.75 ppm (Fig. 3b).

Despite the large uncertainties likely resulting from the variability between batches of animals of the same age and the great difficulty to obtain many duplicates as usually carried out in biological studies (see details in Section 2.2.), these preliminary results tend to demonstrate that long-lived animals are chemically different from the other strains. In the future, increasing the number of duplicates would undoubtedly improve the data precision and probably help to strengthen this interpretation.

4. Discussion

4.1. Age *versus* mutation-related chemical variations

The present results show that all the 10 day old worms are enriched in Cd, Mn, Ca, Sr, Fe, Ti and Cu and have lower amounts of δ^{65}Cu compared to the 2 day old worms (Fig. 1, 2a and b). The metal accumulation over time is in agreement with

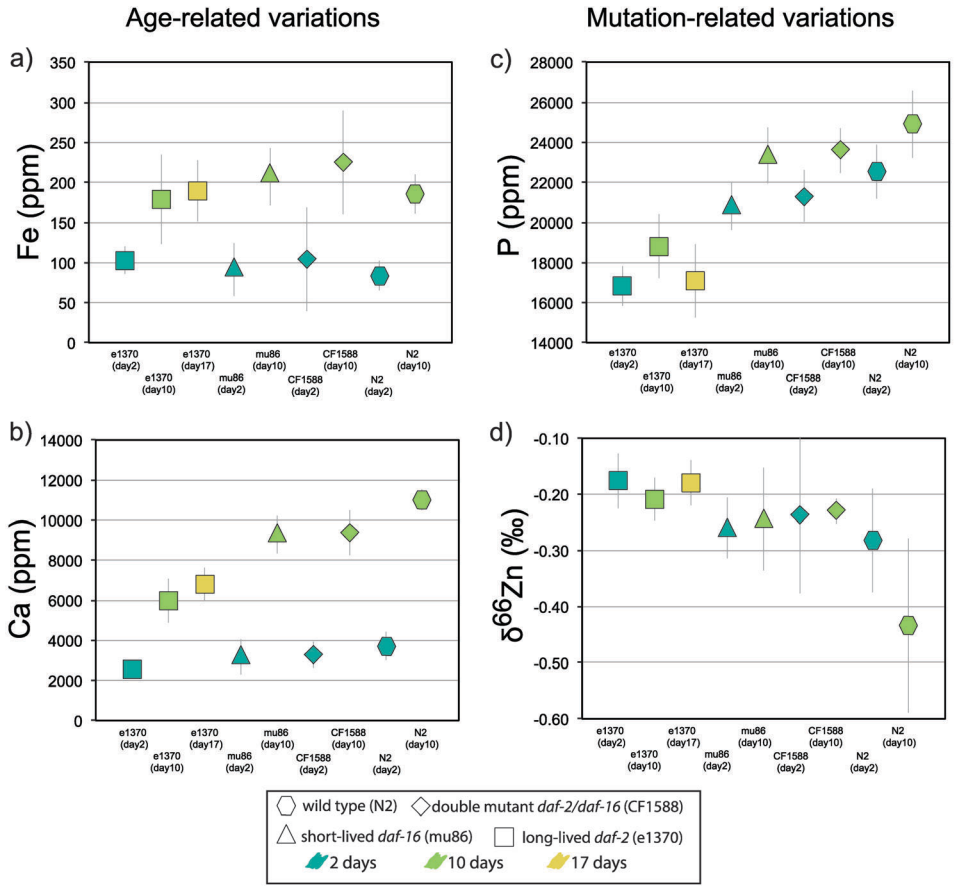

Fig. 2 Distinction between age and mutation-related variations. (a) Iron (Fe) and (b) calcium (Ca) fluctuations over time reflect age-related variations while (c) phosphorus (P) and (d) zinc isotopic compositions (δ^{66}Zn) reflect mutation-related variations. Blue, green and yellow points represent animals collected at 2, 10 and 17 days old respectively. For each value analytical error bars represent 1sd obtained on the average of three different complete duplicate analyses (n = 3).

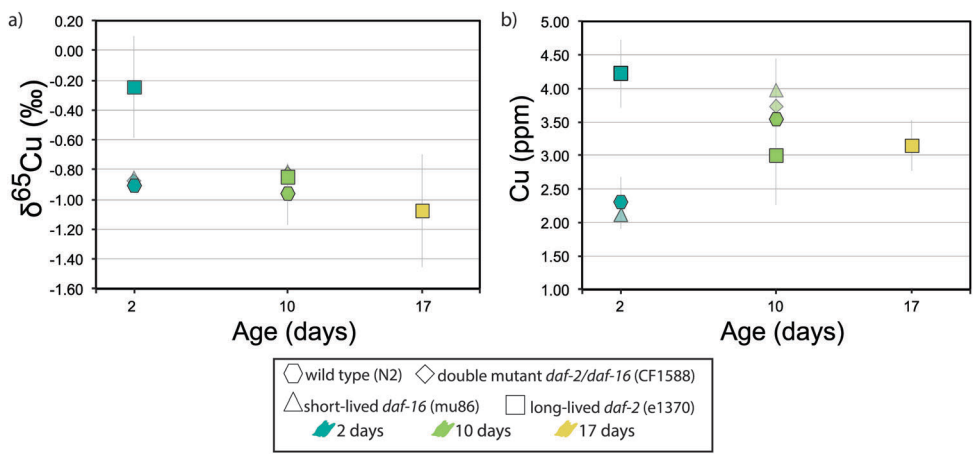

Fig. 3 (a) δ^{65}Cu and (b) Cu fluctuations over time in four *C. elegans* nematode strains including (1) wild-type (N2), (2) short-lived mutants of the DAF-16 FOXO transcription factor: *daf-16*(mu86), (3) long-lived mutants of the insulin/IGF1 receptor: *daf-2*(e1370) and (4) double mutants for both *daf-2* and *daf-16* alleles: CF1588. Reverse early-age chemical fluctuations are observed between wild type (N2) and long-lived (e1370) worms. Each value is an average of two different complete duplicate analyses (n = 2). Analytical error bars are 1sd for long-lived (e1370) and wild type (N2) nematodes. Note that for the δ^{65}Cu diagram, short-lived (mu86) and double mutants (CF1588) are hidden beneath the wild type (N2) at 2 days and the long-lived (e1370) at 10 days. Similarly for the copper concentration at 2 days, the double mutant (CF1588) is hidden beneath the wild-type (N2).

a previous study[16] reporting an increase of Ca, Cu, Fe and Mn levels as a function of age in worms. Similar results have also been reported for mammals (*i.e.* mice and bovines)[17,19,36] and humans[22,37] for which increases of copper and iron have been

observed in organs and plasma, respectively, supporting a critical role of metal accumulation in ageing.

Regarding isotopic ratios, the decrease of the δ^{65}Cu values with lifespan is in line with the results reported by Jaouen *et al.*[31] who measured a decrease of the blood δ^{65}Cu value during ageing in a remote Yakut population. Similarly, the constant δ^{66}Zn value observed over time (Fig. 2d) is consistent with the results of Moynier *et al.*[38,39] who reported a constant δ^{66}Zn value in serum and red blood cells of mice between 9 and 12 months. However, the study of Jaouen *et al.*[31] reports a small increase of the blood δ^{66}Zn value during ageing that is not observed consistently in the present dataset (Fig. 1 and 2d). This is probably because our study includes genetically modified organisms with specific mutations that have a stronger influence on the Zn isotopic composition. As shown in Fig. 2d, the long-lived *daf-2* mutants present a higher isotopic ratio than those of the control mutants, while variations linked to age are rather low.

Genetic mutations affect gene-specific signaling pathways in which trace elements can be involved as co-factors, leading to a potentially important variation of elemental concentrations. The *daf-2* mutation triggers for example inappropriate activation of the downstream DAF-16 transcription factor (*i.e.* a FOXO-family)[40,41] that regulates the expression of several metal detoxification genes including *mtl-1* (metallothionein homologue gene)[42] and *sod-3* (superoxide dismutase gene)[43] *i.e.* two genes encoding the metallothionein and the superoxide dismutase metal-binding proteins, respectively. By chelating, sequestering and then excreting free metal ions out of the cells, these proteins in association with other larger metal-binding proteins prevent metal accumulation and play a major role in metal detoxification; a process referred to as a metal stress response. Consequently, the *daf-2* mutants, which possess an extended lifespan, have a higher metal stress response[42,44,45] that may account for the lower metal content such as K, Na and Mg compared to wild type worms (Fig. 1). While we have not tested these mutants, we can however cite the studies of Davis *et al.*[46] and Roh *et al.*[47] which demonstrated that *cdf-2* and *ttm-1* loss-of-function mutant nematodes displayed reduced Zn content compared to wild type animals. Taken together, these results demonstrate that for a given strain, elemental burden is characteristic of a time-point in the lifespan of *C. elegans*.

4.2. The case of Cu in ageing

The early-age temporal evolution of Cu concentration and isotopic composition is different between long-lived worms and all the other strains (Fig. 3). Between 2 and 10 days, the δ^{65}Cu value tends to remain relatively constant in the wild type, short-lived and double mutant worms while it slightly decreases in the long-lived mutant from -0.25 ± 0.69 (2sd, $n = 2$) ‰ to -0.85 ± 0.06 (2sd, $n = 2$) ‰ reaching -1.08 ± 0.76 (2sd, $n = 2$) ‰ at 17 days (Fig. 3a). Similarly, the Cu concentration tends to decline in the long-lived mutants from 4.22 ± 1.01 (2sd, $n = 2$) ppm to 3.00 ± 1.46 (2sd, $n = 2$) ppm between 2 and 10 days and then tends to stabilize at 17 days (3.15 ± 0.75 (2sd, $n = 2$) ppm) while it increases in the wild type, the double mutant and the short-lived worms from $\sim$2.25 to $\sim$3.75 ppm (Fig. 3b). Despite large uncertainties likely resulting from the variability between the animals of the same age, these results demonstrate for the first time that long-lived mutants slightly differ in their early life from the other strains. Increasing the number of duplicates, which is not straightforward regarding the difficulties represented by precise elemental and isotopic measurements on worm samples, would contribute to improve the data precision and thus help to strengthen this interpretation. The differences observed between the long-lived mutants and all the other strains are not linked to genetic background but rather reflect specific lifespan and probably healthspan. Previous studies indeed showed that long-lived worms have a lifespan but also healthspan which is twice longer than those of wild type, double mutant as well as short-lived worms.[40,48] The long-lived animals can live up to 60 days with approximately 20 days in good health conditions while the maximal lifespan of the three other strains does not exceed 20 to 25 days with less than 8 average days in good health conditions.[48]

Given these preliminary results, although further studies are requested to improve the data precision and strengthen our interpretation, we emphasize that the early-age kinetic of the burden of some metals, especially Cu, may be a reliable marker of ageing and can help to distinguish animals with extended lifespans. This assumption is supported by similar evolution of the concentrations of other metals such as Fe and Ca. These metals accumulate in the body during ageing, but the rate of accumulation is higher in short-lived, double mutants and wild type worms compared with in long-lived mutants (Fig. 2a and b). For instance, while the Ca concentration tends to triple between 2 days and 10 days in the short-lived, double mutant and the wild-type strains, it only doubles in the long-lived mutant between 2 days and 17 days (Fig. 2b).

Interpreting the Cu isotopic data is difficult given that the use of stable isotope for metallomic studies is still in its infancy. We can however propose a mechanistic explanation to account for the observed Cu isotope fractionation during ageing.

In excess and in an environment favorable for oxidation, cuprous (Cu^+) copper ions can be oxidized to cupric (Cu^{2+}) favoring the formation of highly reactive oxygen species (ROS) such as hydroxyl radicals ($^{\bullet}OH$) *via* the Haber–Weiss reaction[49,50] (Fig. 4). Once produced, ROS damage proteins, lipids and DNA[51,52] and consequently accelerate ageing onset and progression. In parallel, reduced Cu^+ compounds are expected to favor light isotopes relative to Cu^{2+}. Therefore, the formation of ROS should preferentially be associated with the oxidation of $^{63}Cu^+$ into $^{63}Cu^{2+}$ contributing to a Cu isotope fractionation favoring light ^{63}Cu over heavy ^{65}Cu; the latter being not involved in ROS production is preferentially excreted leading ultimately to a decrease of the δ^{65}Cu value over time (Fig. 4).

However, this hypothesis does not clarify all the observations made in the present study. For instance, the absence of any decrease in the δ^{65}Cu value is puzzling, in spite of a significant Cu concentration increase, for the short-lived, double mutants and wild type worms. The diminution of the Cu content between 2 and 10 days observed in the long-lived worms (Fig. 3b) probably tends to limit the abnormal generation of ROS resulting in the

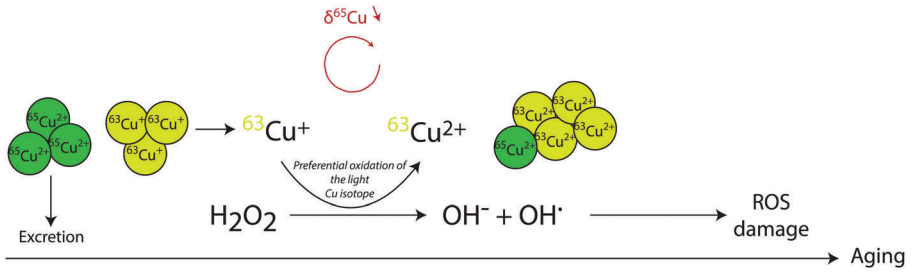

Fig. 4 Conceptual model explaining the temporal diminution of the copper isotopic composition ($\delta^{65}Cu$) with ageing. ROS stands for reactive oxygen species. OH^- (hydroxyl ion) and OH• (hydroxyl radical) are two different types of ROS.

extension of lifespan. If correct, it would suggest that Cu accumulation over time could be a key factor accounting for ROS-induced damages and ageing acceleration. Although these assumptions need to be verified by further experiments including for example the measurement of ROS and lipid peroxidation changes in *C. elegans* exposed to Cu overload, mitigating these chemical processes, notably through chelation, could eventually improve healthy lifespan.

5. Conclusions

Important elemental and isotopic variations were measured in genetically controlled *C. elegans* strains during ageing supporting a critical role for metal deregulations in ageing. Strains are also affected by mutation-related variations suggesting that caution should be taken when comparing the metallome of different genetically modified *C. elegans*. This caution should apply to other genetically engineered organisms, such as the fruit fly and even the rodent models. Focusing on age-related variations, Cu concentration and isotopic composition differences were observed between long-lived strains and their controls. Altogether, these results demonstrate that the simultaneous measurement of elemental concentration and isotopic composition may offer a more comprehensive view on ageing than the elemental concentrations alone and that these chemical parameters can be used to predict *C. elegans* lifespan and detect accelerated ageing.

Conflicts of interest

There are no conflicts to declare.

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