Screening of potential uranium protein targets in fish ovaries after chronic waterborne exposure: differences and similarities between roach and zebrafish.

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Screening of potential uranium protein targets in fish ovaries after chronic waterborne exposure: differences and similarities between roach and zebrafish.

Abstract

Concentration of uranium (U), a naturally encountered radioactive element in earth's crust, can be enhanced in freshwater ecosystems ($\mu g.L^{-1}$ – $mg.L^{-1}$) due to various anthropogenic activities. The consequent aquatic organism exposure to U leads to its accumulation in all organs, particularly in the gonad, and in subcellular fractions (mainly the cytosol); then it is known to affect fish at several biological levels, and more particularly, at a reproduction endpoint, with a decrease in the total number of eggs, spawn events and larvae survival. The understanding of U reprotoxicity requires the fine knowledge of its speciation at molecular level, *i.e.,* its interaction with cytosolic biomolecules. In this study, we focus on the U-protein interactions in gonads. A non-denaturating extraction protocol combined with size exclusion chromatography (SEC) allowed the separation of metal-protein complexes in ovaries of U-contaminated wild roaches before their elemental detection (ICP MS). This enables unprecedented information to be obtained about U distribution in ovaries of autochthonous fish, *Rutilus rutilus*, which is different in some points from that obtained in the model species, *Danio rerio* under controlled laboratory conditions at a similar concentration level. Finally, the ability to transpose results from model to autochthonous fish was briefly discussed.

Keywords

Uranium, speciation, ovary, biomolecules, vitellogenin, zebrafish, roach

1. Introduction

Concentration of uranium (U), a naturally encountered radioactive element in earth's crust, can be enhanced in freshwater ecosystems ($\mu g.L^{-1}$ – $mg.L^{-1}$) due to anthropogenic activities (Betcher et al., 1988; WHO, 2011). The consequent exposure to U of living aquatic organisms has sparked studies on U bioaccumulation and toxicity in diverse organisms, both fish and invertebrates (Goulet et al., 2011). It is known to affect organism growth with DNA and tissues damages and also fish reproduction with a decrease of total number of eggs, spawn events and larvae survival after waterborne chronic and environmentally relevant exposure (Barillet et al., 2011; Barillet et al., 2010; Bourrachot et al., 2014; Simon et al., 2014; Simon et al., 2018; Simon et al., 2019). Reproduction is one of the key biological functions necessary for species sustainability and fitness, and constitutes a key parameter for ecological risk assessment. Comprehensive data on reprotoxicity are therefore necessary to address the U ecotoxic profile. In addition, high bioaccumulation of U was recorded in gonad of zebrafish, without any elucidated mechanism for U transfer to eggs (Simon et al., 2011). One can then wonder if U toxicity in larvae is linked to the initial U concentration in parent ovaries.

The understanding of U toxicity requires a fine knowledge of its speciation at molecular level, *i.e.,* its interaction with cytosolic biomolecules. Indeed, due to its chemical properties, uranium can interfere with essential elements of which homeostasis is regulated. Knowing the weakness of the bond between U and its possible ligand groups (O and N atoms from biomolecules, PO_4^{3-} group) (Van Horn and Huang, 2006) the identification of a U-protein edifice requires tedious non-denaturating extraction and analytical methodologies being set up, and has been discussed in numerous studies (Bucher et al., 2014a; Bucher et al., 2014b; Xu et al., 2014b).

In our previous study, we identified proteins as possible candidates for U complexation in oocyte of the model fish (zebrafish, *Danio rerio*) exposed to U under laboratory controlled

50 conditions (20 µg.L^{-1}, 20 days, pH=6.5). For this work, the combination of non-denaturating
51 separations (Size Exclusion Chromatography (SEC) and Off Gel Electrophoresis (OGE) followed
52 by SEC) of U-protein complexes before elemental (ICP MS) and molecular (ESI FT MS/MS) mass
53 spectrometry detection was performed. Seven relevant proteins have been identified,
54 revealing two main pathways of toxicity mechanisms: one specific to the reproductive organ,
55 with target proteins (vitellogenin (Vtg) fragments and initiation factors) that are involved in
56 oocyte development; and a second generic pathway with proteins involved in oxidative stress
57 (initiation factor protein, glutathion transferase, glyceraldehyde phosphate dehydrogenase
58 and a Cu-Zn superoxide dismutase) and in the oocyte structure (actin and macroglobulin) (Eb-
59 Levadoux et al., 2017).

60 The model organism used, zebrafish (ZF), for which laboratory reproduction is easily
61 achievable and the genome is sequenced, has been at the centre of many genomic and
62 proteomic mechanistic studies. Previous experiments, performed under laboratory chronic
63 exposure conditions with zebrafish, have demonstrated the U tendency to bind proteins.
64 However, the question arises of whether the gained knowledge can be extrapolated to field
65 fish exposed to contamination over several generations. *In situ* experiments were performed
66 with the roach *Rutilus rutilus*, an autochthonous fish from northern Europe, representative of
67 freshwater ecosystems (Mounicou et al., 2019). Unlike the zebrafish, the roach *Rutilus rutilus*
68 has a long reproduction cycle (spawning once every 2 years vs. every 2^{nd} to 5^{th} days for ZF, and
69 has a rather long sexual maturity (2-4 yrs. vs. 3 months for ZF).

70 In our case, both species, which exhibit different reproduction cycles, have been shown
71 to bioaccumulate U in their reproductive organs at similar levels (*i.e.,* 790-3500 ng.g^{-1} dry
72 weight (dw) for wild roaches exposed *in situ* vs. c.a. 960 ± 3.5 ng.g^{-1} dw zebrafish (mean values)
73 (Eb-Levadoux et al., 2017; Mounicou et al., 2019) after a similar waterborne exposure.

Therefore, the objective of this study was (i) to get a screening of U (and other essential metals)-protein complexes in gonads of wild roaches (*Rutilus rutilus*) living in a U contaminated pond, and of caged roaches, and (ii) compare it with those obtained in a model species, the *Danio rerio* under controlled laboratory conditions at similar concentration levels. Non-denaturating extraction protocol combined with size exclusion chromatography (SEC) allowed the separation of metal-protein complexes in ovaries of wild roaches living in a U contaminated pond before their elemental detection (ICP MS). Finally, the ability to transpose results from model to autochthonous fish is briefly discussed.

2. Material and methods

2.1. Wild and caged roach samples

Roach fish used (20.8 ± 4.6 g, 12.7 ± 1.3 cm, n=12) in this study were sampled from the Jaladys pond, an abandoned open pit U mine in the South West region of France. The U concentrations (total and dissolved) and main abiotic parameters (T°C, pH, O_2) have been characterised in a water column (Mounicou et al., 2019). Six female roaches (numbered 62, 63, 64, 65, 66, 68) were sampled on the 12th of June 2014 ([U]: 15 µg.L^{-1} (0-2 m depth); [U]: 62 µg.L^{-1} (15 m depth)) and another set of 6 females (90, 91, 93, 94, 95, 97) in 17th July 2014 ([U]: 10 µg.L^{-1} (0-2 m depth); [U]: 32 µg.L^{-1} (15 m depth)). The concentrations given here are the total ones in which 80-90% of U were dissolved (Mounicou et al., 2019). It appeared that June and July roaches cannot be considered as different from many perspectives, *i.e.*, GSI, reproduction status, U accumulation in gonads, protein and multi-elemental distribution patterns. Therefore, they have all been considered as reproduced roaches (Geraudie et al., 2010) and no distinction was made for data processing.

96 To get a control group, roaches (22.8 ± 2 g, 14.5 ± 0.2 cm, numbered 71, 72, 73, 74, 76)
97 purchased from a fish farm were exposed upstream of the contaminated pond ([U] < 5 µg.L^{-1})
98 for 50 days up until the 17th of July. Roaches were fed manually every day with commercial
99 food.

100 All fish were dissected out on ice; gonads were quickly frozen in liquid nitrogen and
101 were stored at -80°C until further processing.

102 **2.2. Uranium total analysis, speciation and metal-protein extraction from gonads**

103 The procedure set-up for female zebrafish gonads was applied to all female roach
104 gonads (Eb-Levadoux et al., 2017). Basically, for uranium total analysis, organs were digested
105 as previously described. Then, for speciation, metal-protein complexes were extracted from
106 fresh frozen gonad samples (c.a. 65 ± 13 mg) with 1800 µL of 25 mM HEPES, 250mM sucrose,
107 pH 7.4 as buffer and using a Potter-Elvehjem homogeniser for 3 min in ice. Cytosolic metal-
108 protein complexes contained in supernatant were recovered from homogenate by
109 centrifugation (900 g, 20 min, 4°C), and cellular debris (residue) were discarded.

110 **2.3. SEC-ICP SF MS analysis of metal-protein complexes**

111 Immediately after extraction, 80 µL of the supernatant were used for SEC - ICP SF MS,
112 as described in the work by Eb-Levadoux et al., 2017. To sum up the protocol, a Superdex 200
113 10/300 GL column (GE Healthcare, France) was mounted on an Agilent 1200 series liquid
114 chromatographer equipped with a UV detector at the column outlet. The UV detector outlet
115 was connected with PEEK tubing to the nebuliser of the ICP Sector Field Mass Spectrometer
116 (Element XR, Thermo Fisher Scientific, Germany). Metal-protein (and others biomolecule)
117 complexes were eluted from the column with 100 mM ammonium acetate buffer pH 7.4 (0.7
118 mL.min^{-1}), where proteins at 280 nm and ^{238}U, ^{31}P, ^{56}Fe, ^{64}Zn, ^{63}Cu were successively detected

by UV and mass spectrometry in low (for U only) or medium resolution mode, respectively. The SEC column was accordingly calibrated in terms of MW and systematically cleaned after each sample analysis to remove metals bound to the stationary phase of the column.

3. Results

3.1. Uranium and metal biomolecule distribution in roach gonads – Description and relationship with ovary bioaccumulation

The biodistribution of U and essential elements (P, Fe, Zn and Cu) was studied by SEC-ICP SF MS at the molecular level in gonads of three groups of roaches. Accumulation levels in these roach ovaries had been previously measured (Mounicou et al., 2019).

A representative elemental chromatogram in terms of protein biodistribution among wild roaches is presented in **Figure 1;** individual chromatograms are given in **Figure S1 and S2**. The uranium chromatogram (**Fig.1A**) exhibits four main MW fractions. Fraction 1 above the void of the column (>670 kDa) likely corresponds to U binding protein clusters, Fraction 2 to U bound to proteins in the range of 89-670 kDa, and Fraction 3 to U-protein complexes between c.a. 4 to 33 kDa. Fraction 4 is under the total volume of the column, meaning that interactions took place with the stationary phase, and that coeluted proteins are no longer separated in relation to their molecular weight. The apex of U Fractions 1 and 2 matches the apex of the UV peaks at 280 nm (**Fig 1.F**), while a very small UV peak is detected at the apex of U Fraction 3.

The U apex in the void of the column (Fraction 1) was shared with all others elements monitored (*i.e.,* P, Fe, Zn and Cu), but this does not guarantee the binding to the same biomolecule, as no chromatographic separation takes place at that elution time. In contrast, for Fraction 4 only a small U peak could be detected in the low molecular weight region (at

29.5 min, <3 kDa). In this fraction, some elements monitored co-eluted as a substantial peak and with an intense UV peak.

Among the five P chromatographic peaks (**Fig 1.B**), the major one coeluting with U was found in the void volume of the column (Fraction 1) and to a decreasing extent in Fractions 2, and 3. The second dominant P fraction was found associated with a small peak of U (Fraction 4, biomolecules < 3 kDa). Fe chromatogram (**Fig 1.C**) displayed five peak apexes, while three of them are shared with four U fraction apexes. The major ones are Peaks 2 and 3, then Peak 1~Peak 4, the least intense one is Peak 5. Among the five Zn peak apexes (**Fig 1.D**), only the first one eluted with U in Fraction 1. In the Cu chromatogram (**Fig 1.E**) the two most intense Cu peaks in the 40-3 kDa region didn't match the apexes of U, whereas in some extracts the two poorly intense Cu peaks in Fractions 1 and 2 did.

To sum-up, for July autochthonous roaches the dominant U peak mainly coelutes with the P peak, whereas in the June ones, some Fe peaks appear coeluting with P and U (**Fig S1**).

To compare the uranium-protein complex distribution in the gonad of all females, the relative area (ratio of the area of the fraction to the total area of the chromatogram from 10 to 40 min) was calculated for each fraction for the 12 female roach samples (**Figure 2a**). First, the U distribution within cytosolic biomolecules follows the same trend for all individuals with four main fractions detected (as presented in Fig1.a) and regardless of the sampling date of the roaches. For most wild individuals sampled in July and June, about 56 to 67% (average values) of U is mainly associated to 3-40 kDa proteins in Fraction 3 for roaches sampled in July and June, respectively. The uranium proportion in Fraction 2 remains constant at around 8 - 9% for the two groups, whereas it ranges from 13% to 21% in Fraction 1, which is likely to compensate for fluctuations in Fraction 3. The remaining U (~13%) consisted of a low signal

(near baseline level) outside the defined plots. However, some important discrepancies can be observed between individuals regardless of their sample periods and their U content. For example, the two roaches labelled 64 and 65 had about 90% of the U in Fraction 3, in contrast with some individuals (*i.e.,* 90 and 93) showing a fairly equal distribution between Fractions 1 and 3. Additionally, this U peak intensity of individuals numbered 64 and 65 was about 50-fold higher than the average peak intensity for fish of the other group sampled in July (Fig. S1). For upstream caged roaches, distribution is slightly different with a main Fraction 1 having 45% of U, Fractions 2 and 3 with around 20% of U, and finally Fraction 4 with 15%.

To prospect for a link between one of the cytosolic fractions and the bioaccumulation of U in ovaries of both wild and caged roaches, **Figures 2b** and **S3** (for individuals with extreme U burden values, *i.e.,* ten times lower (68, 65, 97) or up to five times higher (90, 95) than the average) show the relation between the U distribution in each fraction and the U concentration in the organ. A positive linear relationship between the distribution in F3 and the U concentration in the organ can be established in Figure 2b (R^2>0.90), in which extreme values of U content were excluded. No linear relationship was observed for individuals with low or high U accumulation levels (**Figure S3**). In addition, a negative linear relationship was also established between the distribution in F1, in F2 and the U concentration in the organ (with R^2>0.72 and R^2>0.95, respectively).

3.2. Metal-protein complexes cytosolic distribution: roach vs. zebrafish

The distributions of uranium and other endogenous elements in cytosol of zebrafish ovaries have already been described in our previous work (Eb-Levadoux et al., 2017). They are compared with those acquired for wild and caged roaches in this study (**Figure 3**). A clear difference can be observed in the U chromatograms (**Fig. 3A**), not only in terms of signal

intensity (left y-scale for zebrafish, right one for roaches) but also in terms of U distribution along cytosolic biomolecules. Indeed, a c.a. 50-fold signal difference and a clear elution time shift could be noticed between the major U peak of each chromatogram at 22.8 min (equivalent to 21 kDa at peak apex, fraction MW range: 54-8 kDa) and 24.6 min (equivalent to 11 kDa at peak apex, fraction MW range: 21-4 kDa) for the zebrafish and roach cytosols, respectively. It is also important to note the distinct U relative proportion between the two fish, with a high U proportion (around 90%) at 22.8 min peak apex and consistent for all zebrafish females analysed (Eb-Levadoux et al., 2017), contrary to the more variable but homogeneous U distribution among F1 and F3 proteins of female roaches.

Other endogenous elements (**Fig3. B-E**), P, Fe, Cu, Zn exhibited a similar biomolecular distribution in zebrafish and roach cytosols, except that the abundance of each element differed according to the fish species investigated. However, few noticeable differences are observed. Indeed, the P pattern is slightly different in the 4-89kDa region where two peaks (at 18 and 24.6 min) are eluted in roaches, compared to only one, more intense, (at 22.8 min) in reproduced zebrafish (**Fig3.B**). The other noticeable difference is the appearance of a Zn peak at 22.8 min, with an increase in the Fe level at the same retention time for the roach cytosol (**Fig3.D**). This Zn-containing protein fraction was absent in the zebrafish cytosol chromatogram, where 90% of U is found. Finally, UV-protein elution profiles (**Fig3. F**) were consistent between the two fish species according to the 280 nm UV detection, but the relative proportion of the protein fraction was different between species. It is noteworthy that, to the same extent for the two fish species, the 280 nm UV signal is close to the baseline level in Fraction 3 where U is predominant.

3.3. Uranium-phosphorus coelution in gonad cytosol: roach vs. zebrafish

211 Biomolecular distribution analyses of uranium and endogenous elements, in roach and
212 zebrafish gonads, showed multiple shared coelutions between U and P. Therefore, the
213 relationship between the P and U distributions in the different fractions was studied in
214 cytosols of female roaches (both wild and caged) and female reproduced zebrafish
215 (reproduced zebrafish were chosen to be homogenous with the "reproduced" status of
216 roaches in our study and to compare oocytes at similar developmental status in both fish).
217 **Figure 4** shows this U/P distribution relationship in zebrafish (left panels) and in roaches (right
218 panels).

219 Regardless of the fish species and the U accumulation levels in the whole organ and
220 the U content in the main F3 fraction, the U percentage in this main fraction is linearly
221 correlated to the P percentage (Figure 4C). The slope is similar, *i.e.*, 2.44 and 2.93 for zebrafish
222 and roaches, respectively, with R^2=0.8 as the minimum value. For upstream caged roaches,
223 the sample size is too low to conclude on the trend. Likewise but with a slope ten times lower,
224 the F4 fraction (Figure 4D), is linearly linked to the P percentage for most roach individuals
225 (both wild and caged ones). In Fraction 2 (Figure 4B), the relationship between U% and P% is
226 not linear whatever the group of fish studied. In Fraction 1 (Figure 4A) a linear relationship
227 with similar slopes is established for wild and caged roaches and not for zebrafish, for which
228 the U proportion in this fraction is 4-fold less than for roaches.

229 ## 4. Discussion

230 The reproduction endpoint is an ecologically relevant parameter that directly
231 influences population dynamics. Understanding the accumulation mechanism of U in ovaries
232 is therefore the first step to determine its observed reprotoxicity profile. Furthermore,
233 elemental speciation is a key parameter to elucidate the toxicity of an element (Sanz-Medel,

234 1998). Our first works focused on *Danio rerio* and led to the identification of candidate
235 proteins for complexation with U. To complete the U profile, this study focuses on uranium
236 speciation in wild fish ovaries in order to evaluate uranium, endogenous elements and protein
237 coelution. The second objective was to compare this U distribution with that found in the
238 zebrafish model, in order to assess the robustness of the extrapolation methodology from
239 model to wild contaminated fish.

240 **4.1. Roach versus zebrafish: evidence for different U speciation**

241 In gonads of roaches, U was distributed among four biomolecule fractions for all
242 individuals investigated. Uranium accumulation levels and U fraction distributions seem to be
243 closely linked, except for Fraction 4. However, some differences were noticed in the relative
244 distribution and the absolute intensity of fractions, without any obvious link with U
245 accumulation levels in the gonads. These results were compared with SEC-ICP SF MS results
246 of U-exposed zebrafish (Eb-levadoux et al 2017). Despite similar accumulation levels in ovaries
247 of both species, the first difference observed is the consistency of the U-biomolecule relative
248 distribution in zebrafish cytosol against the variability of this distribution in the present study.
249 This can partially reflect the different exposure conditions: (i) the zebrafish exposure under
250 well-controlled conditions in the laboratory, ensuring a constant U speciation in water, and (ii)
251 the *in situ* exposure of roaches, where the U concentration (10 to 62 $\mu g.L^{-1}$) and the pH (around
252 6.5 (0-2 m depth); 5.5 (June, 15 m depth); 6.8 (July, 15 m depth) (Mounicou et al., 2019) vary
253 in the water column and throughout the season (variable diet), thus impacting U speciation in
254 water, U bioavailability and U accumulation. This reproducibility observed in zebrafish
255 compared to roaches can also partially arise from the reproduction cycle of these two fish; the
256 roach population is more heterogeneous in age and more likely to be dependent on its

environment for its reproduction (variable reproduction status with variable phosphorylated amino acid content in ovary proteins), whereas zebrafish are more homogeneous in age and synchronous in their reproduction (Gerbron et al., 2014; Lawrence, 2007; Simon et al., 2014). Thus, the distribution pattern in different fish gonads is not linked to the accumulation level only.

In addition, the investigation of uranium and endogenous element distributions among cytosolic biomolecules in roach gonads has shown the co-elution of uranium with some endogenous elements-containing proteins, mainly phosphorus, confirming once more the known affinity of uranyl ions with phosphorylated proteins, as already reported (Basset et al., 2008; Bucher et al., 2014b; Eb-Levadoux et al., 2017; Huynh et al., 2016; Safi et al., 2013). Therefore, P-containing biomolecules seem to play an important role in U trafficking in gonad cytosol. However, uranyl appears to be somehow specific to some phosphorus-containing proteins, as it doesn't coelute homogeneously with P but preferentially in Fraction 3, where the P signal is the least intense. Interestingly, the same molecular weight range for the main fraction and the linearity of the P and U percentage relationship in this main fraction were also found after zebrafish exposure (Eb-levadoux et al 2017). According to the P content and to the MW elution range of this fraction (40-3 kDa), U could be expected to bind phosphorylated fragments of vitellogenin (Vtg). In addition, the relationship between U and P in Fraction 2 (670-92 kDa) suggested the presence in roach ovaries of uranium binding with a high molecular weight protein complex between 320 and 610 kDa that could be the entire Vtg (Hara et al., 2016; Wallace and Selman, 1985).

Vtg is the most abundant glycolipophosphoprotein of oocytes (Garnayak et al., 2013), with a high MW around 400kDa (Hara et al., 2016; Wallace and Selman, 1985). It needs to

undergo enzymatic degradations, *i.e.,* leading to lipovitellin and phosvitin, in order to be used as vitellin reserve by the future progeny (Gerbron et al., 2014; Örn et al., 2003). Thus, the presence of the entire Vtg in ovaries is controversial. Often described as immediately processed after its entry into the ovary (Amano et al., 2008), it has already been detected in ovaries of the fish *Tanichthys albonube* (Zhong et al., 2014). According to the species, Vtg (MW, enzymatic products) and its enzymatic degradation can be different (Yilmaz et al., 2016). Vtgs of zebrafish have three main domains, the heavy chain lipovitellin (120 kDa), the light lipovitellin (30-35 kDa) and phosvitin (6 kDa) chains, leading to several combinations for the by-products after enzymatic degradation. For instance, these include lipovitellin-phosvitin complexes (Byrne et al., 1989). The hypothesis of U complexation by Vtg (more particularly by a highly phosphorylated phosphvitin complex) in ovaries has already been made after zebrafish exposure (Eb-levadoux et al 2017), especially as zebrafish exhibit a decrease in the U accumulation in ovaries after spawning and an U accumulation in eggs. Unfortunately, the phosvitin domain, which is more or less phosphorylated depending on the species, cannot be identified by mass spectrometry due to its chemical composition (Samaraweera et al., 2014). In our study, the molecular weight (MW) of the biomolecule fraction binding U in roaches (~11 kDa) and the P content signal in this medium MW range are different from those in zebrafish (~21 kDa and high P content). In contrast, no MW difference was noticed for the other endogenous elements in this region, except for Zn.

A search on the UniProtKB engine revealed the identification in roaches (*R. rutilus*), of a 237-residue vitellogenin fragment (c.a. 27.7 kDa, 3% phosphorylated serines, https://www.uniprot.org/uniprot/C6ZNM7) and a 103-residue vitellogenin fragment (11.2 kDa, 2.9% phosphorylated serine, https://www.uniprot.org/uniprot/A0A221LCK1) against 20% and up to 40% of the phosphorylated serine content in the phosvitin domain of the most

abundant vitellogenin fragment (Vtg1) of the zebrafish (https://www.uniprot.org/uniprot/A0A2R8Q212)(Hu et al., 2015). Therefore, in zebrafish, the lipovitellin derivatives are likely to contain the phosphorylated phosvitin domain, taking into account the P signal detected at that elution time (c.a. 22.8 min, c.a. 21 kDa). In contrast, the supposed lipovitellin fragment eluting later for the roach gonad cytosol, seemed to be phosvitinless, or at least a fragment containing phosvitin with lower phosphorylated serine residue content because of the low intensity P peak co-eluting.

As phosvitin is also known to chelate cations and particularly Fe, this might explain the small Fe peak tailing coeluting with U and P in the roach lipovitellin MW region. Interestingly, in roach oocyte, Fe, Zn and Cu co-elution (22.8 min, U free and very low P content) perfectly fits the elution time of U- light lipovitellin fragments (Zn free, but Fe, Cu and quite high P content) for zebrafish oocyte. This leads us to assume either the presence in roach oocyte of metalloprotein(s) other than lipovitellin fragments, such as transferrin, Cu-Zn SOD, a subunit of haemoglobin, the latter two already having been identified in zebrafish oocyte (Eb-Levadoux et al., 2017; Xu et al., 2014a, b), or a lipovitellin fragment with a very low absolute phosphorylated serine content so U binding cannot be observed, leaving the binding sites free for complexation with other metals, such as Fe, Zn and Cu.

To conclude, from several points Vtg or Vtg maturation products can be considered as good candidates for U-binding in the ovaries of these two fish. The co-elution with Fe supports this hypothesis, as Vtg is also known to bind Fe and other metals. Unfortunately, the molecular identification could not be done. The roughly homogeneous distribution of uranium in roaches, in contrast to the single main fraction in zebrafish, and its relationship with P content, also support this hypothesis. The reproduction cycle can be the key difference for those

distributions. Indeed, zebrafish continuously produce mature oocytes, with maturation products such as phosphorylated Vtg fragments (~ 90% of the uranium accumulated in the gonad is found in the 21 kDa fraction), compared to once a year for roaches.

Interestingly, high levels of U accumulation (x3-6 compared to ovary) were also observed in the liver of roaches (Mounicou et al 2019), in which Vtg is produced; U speciation in liver should be interesting to perform, in order to explain the origin of U transport in an ovary.

4.2. U containing protein: a possible marker of biological effects on reproduction?

Up to now, the level of accumulation (from a threshold value) in a whole organ could be considered as a predictive marker of deleterious biological effects. The evaluation of toxic effects, through the assessment of speciation and the functional disturbance of U-bound proteins, requires specific approaches, as proposed in this study. This first speciation study on wild roaches shows high variation in protein profiles and essential element levels between individuals, and indicates that U could be linked to Vtg or derivatives. If so, like in zebrafish, U-Vtg will be rapidly digested by future embryos leading to U internalisation in early life stages and finally to toxic effects (Bourrachot et al., 2008). This encourages us to study the reprotoxicity effect of U in the early stages of this wild species. This first study also tends to show that the reproduction status and the protein content in ovaries play a key role in the uranium distribution. Therefore, for fish with reproduction cycle such as roaches, this study underlines the need to consider all peaks as exposure markers in a risk assessment context. So, at this step of knowledge, this is roughly equivalent as considering the total uranium amount in the gonad what is not fully satisfying. Thus, it would be necessary to better characterise uranium complexes from each fraction with target protein identification and

investigate their consequences on reprotoxicity to assess the relevance of each fraction analysis and focus on the best one.

5. Conclusions

Size exclusion chromatography coupled to ICP MS in conjunction with an appropriate non-denaturing sample preparation and separation protocol allowed the monitoring of U-protein complexes in gonads of wild female roaches from a contaminated pond. With regard to P monitoring and other endogenous elements (Fe, Zn and Cu), as well as to previous studies on a sequenced model of fish, hypotheses regarding the protein binding U can be put forward; vitellogenin fragments including its maturation products are expected to bind U. However, compared to the model organism that we investigated earlier, clear differences could be demonstrated in the distribution of U complexes. The reproduction status and different vitellogenin forms can most likely be the origin. Finally, it is noteworthy to underline that any of these hypothesised proteins found in U containing-fractions were not formally identified by mass spectrometry; therefore, further more advanced analysis would be necessary for deeper understanding. This study confirms the contribution of speciation studies in understanding toxic mechanisms and the contribution of fish sequenced models benefiting from efficient molecular tools.

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7. Figure captions

Figure 1. SEC-ICP SF MS distribution of ^{238}U (Panel A), ^{31}P (Panel B), ^{56}Fe (Panel C), ^{64}Zn (Panel D), ^{63}Cu (Panel E), proteins (Panel F) in an ovary cytosol extract of wild *R. rutilus* (G63) sampled from the Jaladys pond.

Figure 2. (A) Relative U distribution (%) in each SEC fraction (F1-F4) of roach cytosolic extracts. For each fraction the dashed line delimitates the different experimental conditions (Wild June, Wild July and Upstream caged); (B) Correlation of U percentage in each SEC fraction and ponderal U (ng.g-1) in roach gonads; data from upstream caged roaches are in the circle. Among the fourteen fish, five individuals with low or high U accumulation levels did not exhibit linear relationship: they were plotted in Figure S3.

Figure 3. Comparison of SEC-ICP SF MS chromatograms of ^{238}U (Panel A), ^{31}P (Panel B), ^{56}Fe (Panel C), ^{64}Zn (Panel D), ^{63}Cu (Panel E) and proteins (Panel F) in an ovary cytosolic extract from female wild roach (G63) sampled from the Jaladys pond, and, of an exposed reproduced female zebrafish (ZF32) under laboratory conditions ([U]: 20µg.L^{-1}, 20 days). F1 to F4 and F1' to F4' are the MW fractions defined for roach and zebrafish samples respectively.

Figure 4. Relashionship between U% and P% in each defined SEC fraction for zebrafish (left panels) and for roach samples (right panels); data coming from autochthonous or caged roaches are identified in each panel. A, B, C, D are the corresponding panels for Fraction F1/F1', F2/F2', F3/F3', F4/F4', respectively. Indicative molecular weight ranges are given for each fraction.

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