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Résumé d'auteur : Concentration of uranium (U), a naturally encountered radioactive element in earth's crust, can be enhanced in freshwater ecosystems ($\mu\text{g.L}^{-1}$ – 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-denaturing 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.

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

4

5 **Abstract**

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

21

22 **Keywords**

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

24

25 **1. Introduction**

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

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

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

50 conditions ($20 \mu\text{g.L}^{-1}$, 20 days, pH=6.5). For this work, the combination of non-denaturing
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 2nd to 5th 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.*, $790\text{-}3500 \text{ ng.g}^{-1}$ dry
72 weight (dw) for wild roaches exposed *in situ* vs. c.a. $960 \pm 3.5 \text{ ng.g}^{-1}$ dw zebrafish (mean values)
73 (Eb-Levadoux et al., 2017; Mounicou et al., 2019) after a similar waterborne exposure.

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

82 **2. Material and methods**

83 **2.1. Wild and caged roach samples**

84 Roach fish used (20.8 ± 4.6 g, 12.7 ± 1.3 cm, $n=12$) in this study were sampled from the
85 Jaladys pond, an abandoned open pit U mine in the South West region of France. The U
86 concentrations (total and dissolved) and main abiotic parameters ($T^{\circ}\text{C}$, pH, O_2) have been
87 characterised in a water column (Mounicou et al., 2019). Six female roaches (numbered 62,
88 63, 64, 65, 66, 68) were sampled on the 12th of June 2014 ([U]: $15 \mu\text{g.L}^{-1}$ (0-2 m depth); [U]: 62
89 $\mu\text{g.L}^{-1}$ (15 m depth)) and another set of 6 females (90, 91, 93, 94, 95, 97) in 17th July 2014 ([U]:
90 $10 \mu\text{g.L}^{-1}$ (0-2 m depth); [U]: $32 \mu\text{g.L}^{-1}$ (15 m depth)). The concentrations given here are the
91 total ones in which 80-90% of U were dissolved (Mounicou et al., 2019). It appeared that June
92 and July roaches cannot be considered as different from many perspectives, *i.e.*, GSI,
93 reproduction status, U accumulation in gonads, protein and multi-elemental distribution
94 patterns. Therefore, they have all been considered as reproduced roaches (Geraudie et al.,
95 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 ($[U] < 5 \mu\text{g}\cdot\text{L}^{-1}$)
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 $\text{mL}\cdot\text{min}^{-1}$), where proteins at 280 nm and ^{238}U , ^{31}P , ^{56}Fe , ^{64}Zn , ^{63}Cu were successively detected

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

122 **3. Results**

123 **3.1. Uranium and metal biomolecule distribution in roach gonads – Description** 124 **and relationship with ovary bioaccumulation**

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

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

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

142 29.5 min, <3 kDa). In this fraction, some elements monitored co-eluted as a substantial peak
143 and with an intense UV peak.

144 Among the five P chromatographic peaks (**Fig 1.B**), the major one coeluting with U was
145 found in the void volume of the column (Fraction 1) and to a decreasing extent in Fractions 2,
146 and 3. The second dominant P fraction was found associated with a small peak of U (Fraction
147 4, biomolecules < 3 kDa). Fe chromatogram (**Fig 1.C**) displayed five peak apexes, while three
148 of them are shared with four U fraction apexes. The major ones are Peaks 2 and 3, then Peak
149 1~Peak 4, the least intense one is Peak 5. Among the five Zn peak apexes (**Fig 1.D**), only the
150 first one eluted with U in Fraction 1. In the Cu chromatogram (**Fig 1.E**) the two most intense
151 Cu peaks in the 40-3 kDa region didn't match the apexes of U, whereas in some extracts the
152 two poorly intense Cu peaks in Fractions 1 and 2 did.

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

155 To compare the uranium-protein complex distribution in the gonad of all females, the
156 relative area (ratio of the area of the fraction to the total area of the chromatogram from 10
157 to 40 min) was calculated for each fraction for the 12 female roach samples (**Figure 2a**).

158 First, the U distribution within cytosolic biomolecules follows the same trend for all individuals
159 with four main fractions detected (as presented in Fig1.a) and regardless of the sampling date
160 of the roaches. For most wild individuals sampled in July and June, about 56 to 67% (average
161 values) of U is mainly associated to 3-40 kDa proteins in Fraction 3 for roaches sampled in July
162 and June, respectively. The uranium proportion in Fraction 2 remains constant at around 8 -
163 9% for the two groups, whereas it ranges from 13% to 21% in Fraction 1, which is likely to
164 compensate for fluctuations in Fraction 3. The remaining U (~13%) consisted of a low signal

165 (near baseline level) outside the defined plots. However, some important discrepancies can
166 be observed between individuals regardless of their sample periods and their U content. For
167 example, the two roaches labelled 64 and 65 had about 90% of the U in Fraction 3, in contrast
168 with some individuals (*i.e.*, 90 and 93) showing a fairly equal distribution between Fractions 1
169 and 3. Additionally, this U peak intensity of individuals numbered 64 and 65 was about 50-fold
170 higher than the average peak intensity for fish of the other group sampled in July (Fig. S1).
171 For upstream caged roaches, distribution is slightly different with a main Fraction 1 having
172 45% of U, Fractions 2 and 3 with around 20% of U, and finally Fraction 4 with 15%.

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

183 **3.2. Metal-protein complexes cytosolic distribution: roach vs. zebrafish**

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

188 intensity (left y-scale for zebrafish, right one for roaches) but also in terms of U distribution
189 along cytosolic biomolecules. Indeed, a c.a. 50-fold signal difference and a clear elution time
190 shift could be noticed between the major U peak of each chromatogram at 22.8 min
191 (equivalent to 21 kDa at peak apex, fraction MW range: 54-8 kDa) and 24.6 min (equivalent to
192 11 kDa at peak apex, fraction MW range: 21-4 kDa) for the zebrafish and roach cytosols,
193 respectively. It is also important to note the distinct U relative proportion between the two
194 fish, with a high U proportion (around 90%) at 22.8 min peak apex and consistent for all
195 zebrafish females analysed (Eb-Levadoux et al., 2017), contrary to the more variable but
196 homogeneous U distribution among F1 and F3 proteins of female roaches.

197 Other endogenous elements (**Fig3. B-E**), P, Fe, Cu, Zn exhibited a similar biomolecular
198 distribution in zebrafish and roach cytosols, except that the abundance of each element
199 differed according to the fish species investigated. However, few noticeable differences are
200 observed. Indeed, the P pattern is slightly different in the 4-89kDa region where two peaks (at
201 18 and 24.6 min) are eluted in roaches, compared to only one, more intense, (at 22.8 min) in
202 reproduced zebrafish (**Fig3.B**). The other noticeable difference is the appearance of a Zn peak
203 at 22.8 min, with an increase in the Fe level at the same retention time for the roach cytosol
204 (**Fig3.D**). This Zn-containing protein fraction was absent in the zebrafish cytosol
205 chromatogram, where 90% of U is found. Finally, UV-protein elution profiles (**Fig3. F**) were
206 consistent between the two fish species according to the 280 nm UV detection, but the
207 relative proportion of the protein fraction was different between species. It is noteworthy
208 that, to the same extent for the two fish species, the 280 nm UV signal is close to the baseline
209 level in Fraction 3 where U is predominant.

210 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 $\mu\text{g}\cdot\text{L}^{-1}$) 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

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

262 In addition, the investigation of uranium and endogenous element distributions among
263 cytosolic biomolecules in roach gonads has shown the co-elution of uranium with some
264 endogenous elements-containing proteins, mainly phosphorus, confirming once more the
265 known affinity of uranyl ions with phosphorylated proteins, as already reported (Basset et al.,
266 2008; Bucher et al., 2014b; Eb-Levadoux et al., 2017; Huynh et al., 2016; Safi et al., 2013).
267 Therefore, P-containing biomolecules seem to play an important role in U trafficking in gonad
268 cytosol. However, uranyl appears to be somehow specific to some phosphorus-containing
269 proteins, as it doesn't coelute homogeneously with P but preferentially in Fraction 3, where
270 the P signal is the least intense. Interestingly, the same molecular weight range for the main
271 fraction and the linearity of the P and U percentage relationship in this main fraction were
272 also found after zebrafish exposure (Eb-levadoux et al 2017). According to the P content and
273 to the MW elution range of this fraction (40-3 kDa), U could be expected to bind
274 phosphorylated fragments of vitellogenin (Vtg). In addition, the relationship between U and P
275 in Fraction 2 (670-92 kDa) suggested the presence in roach ovaries of uranium binding with a
276 high molecular weight protein complex between 320 and 610 kDa that could be the entire Vtg
277 (Hara et al., 2016; Wallace and Selman, 1985).

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

280 undergo enzymatic degradations, *i.e.*, leading to lipovitellin and phosvitin, in order to be used
281 as vitellin reserve by the future progeny (Gerbron et al., 2014; Örn et al., 2003). Thus, the
282 presence of the entire Vtg in ovaries is controversial. Often described as immediately
283 processed after its entry into the ovary (Amano et al., 2008), it has already been detected in
284 ovaries of the fish *Tanichthys albonube* (Zhong et al., 2014). According to the species, Vtg
285 (MW, enzymatic products) and its enzymatic degradation can be different (Yilmaz et al., 2016).
286 Vtgs of zebrafish have three main domains, the heavy chain lipovitellin (120 kDa), the light
287 lipovitellin (30-35 kDa) and phosvitin (6 kDa) chains, leading to several combinations for the
288 by-products after enzymatic degradation. For instance, these include lipovitellin-phosvitin
289 complexes (Byrne et al., 1989). The hypothesis of U complexation by Vtg (more particularly by
290 a highly phosphorylated phosphvitin complex) in ovaries has already been made after
291 zebrafish exposure (Eb-levadoux et al 2017), especially as zebrafish exhibit a decrease in the
292 U accumulation in ovaries after spawning and an U accumulation in eggs. Unfortunately, the
293 phosvitin domain, which is more or less phosphorylated depending on the species, cannot be
294 identified by mass spectrometry due to its chemical composition (Samaraweera et al., 2014).
295 In our study, the molecular weight (MW) of the biomolecule fraction binding U in roaches (~11
296 kDa) and the P content signal in this medium MW range are different from those in zebrafish
297 (~21 kDa and high P content). In contrast, no MW difference was noticed for the other
298 endogenous elements in this region, except for Zn.

299 A search on the UniProtKB engine revealed the identification in roaches (*R. rutilus*), of a 237-
300 residue vitellogenin fragment (c.a. 27.7 kDa, 3% phosphorylated serines,
301 <https://www.uniprot.org/uniprot/C6ZNM7>) and a 103-residue vitellogenin fragment (11.2
302 kDa, 2.9% phosphorylated serine, <https://www.uniprot.org/uniprot/A0A221LCK1>) against
303 20% and up to 40% of the phosphorylated serine content in the phosvitin domain of the most

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

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

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

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

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

333 **4.2. U containing protein: a possible marker of biological effects on reproduction?**

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

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

351 **5. Conclusions**

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

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370 **7. Figure captions**

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

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

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

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

390

391 **8. References**

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