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1 **Subcellular localization of microcystin in the liver and the gonads of medaka fish**
2 **acutely exposed to microcystin-LR**

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16

17 **Abstract**

18 Among the diverse toxic components produced by cyanobacteria, microcystins
19 (MCs) are one of the most toxic and notorious cyanotoxin groups. Besides their potent
20 hepatotoxicity, MCs have been revealed to induce potential reproductive toxicity in
21 various animal studies. However, little is still known regarding the distribution of
22 MCs in the reproductive organ, which could directly affect reproductive cells. In order
23 to respond to this question, an acute study was conducted in adult medaka fish (model
24 animal) gavaged with 10 $\mu\text{g}\cdot\text{g}^{-1}$ body weight of pure MC-LR. The histological and
25 immunohistochemical examinations reveal an intense distribution of MC-LR within
26 hepatocytes along with a severe liver lesion in the toxin-treated female and male fish.
27 Besides being accumulated in the hepatocytes, MC-LR was also found in the
28 connective tissue of the ovary and the testis, as well as in oocytes and degenerative
29 spermatocyte-like structures but not spermatocytes. Both liver and gonad play
30 important roles in the reproductive process of oviparous vertebrates. This observation

31 constitutes the first observation of the presence of MC-LR in reproductive cells
32 (female, oocytes) of a vertebrate model with *in vivo* study. Our results, which provide
33 intracellular localization of MC-LR in the gonad, advance our understanding of the
34 potential reproductive toxicity of MC-LR in fish.

35 **Keywords:**

36 Microcystin; reproductive cell; immunogold electron microscopy; OATP;
37 reproductive toxicity

38

39 **1. Introduction**

40 Recurrent cyanobacterial blooms frequently occur worldwide in eutrophic
41 freshwaters. Various bloom-generating species of cyanobacteria can produce natural
42 toxic components (cyanotoxins), and their blooms threaten human health as well as
43 living organisms of the aquatic environment. Among cyanotoxins, microcystins (MCs)
44 are the most prevalent cyanobacterial hepatotoxins, with 248 structural variants
45 (Spoof and Catherine, 2017), being produced by at least six genera of cyanobacteria
46 (Puddick et al., 2014). Among all these known variants, microcystin-LR (MC-LR) is
47 considered to be the most common and potently toxic (Puddick et al., 2014).

48 MCs have difficulty to penetrate into vertebrate cells via passive transport due to
49 their hydrophilic nature, and organic anion transporting polypeptides (OATPs) are
50 known to be able to transport MCs through cell membranes (Campos and Vasconcelos,
51 2010). Once within the cell, MCs specifically inhibit eukaryotic serine/threonine
52 protein phosphatases 1 and 2A, which causes the disruption of numerous cellular
53 signals and processes (Fischer et al., 2005; MacKintosh et al., 1990). Although there
54 have been 70 members of OATP superfamily identified in the database from humans,
55 rodents and some additional species (Hagenbuch and Stieger, 2013), not all OATPs
56 are capable of transporting MCs through cell membranes. The capability of
57 MC-transport has been demonstrated for OATP1A2 (mainly expressed in the
58 vertebrate brain), liver-specific OATP1BA and OATP1B3 in humans (Fischer et al.,
59 2005). In fish, liver-specific OATPs, such as OATP1d1 in little skate (Meier-Abt et al.,
60 2007) and rtOATP1d1 in rainbow trout (Steiner et al., 2014), have been reported to

61 mediate MC-transport. Different types of OATP distributed in different tissue or
62 organs possess varying levels of affinities and capacities for different MC variants
63 (Fischer et al., 2010, 2005; Steiner et al., 2016). Liver is the organ that presents the
64 highest tropism for MCs (so-called “target”), since it is rich in a few liver-specific
65 OATP members possessing high affinities and capacities to MCs. Several studies have
66 reported the acute hepatotoxicity of MCs characterized by hepatocellular apoptosis
67 (Zhang et al., 2013), necrosis (Mattos et al., 2014), intrahepatic hemorrhaging (Hou et
68 al., 2015), and cytoskeleton disruption (Zhou et al., 2015). Additionally, the
69 distribution of MCs in the liver has been detected using immunohistochemical
70 methods in mice (Guzman and Solter, 2002; Yoshida et al., 1998) and fish (Djediat et
71 al., 2011; Fischer and Dietrich, 2000; Marie et al., 2012).

72 In addition to liver, MCs have been documented to distribute and accumulate in
73 various fish organs including intestine, kidney, gill and gonad (Acuña et al., 2012;
74 Djediat et al., 2010; Mezhoud et al., 2008; Trinchet et al., 2013, 2011). Among these
75 organs, the gonad could be considered as a secondarily most important target of MCs,
76 as several field studies reported the presence of MCs in the gonad of fish. For instance,
77 in Lake Pamvotis (Greece), the gonad of common carp (*Cyprinus caprio*) was
78 reported to contain about 50 ng eq. MC-LR g⁻¹ body weight (bw) using the
79 enzyme-linked immunosorbent assay (ELISA) (Papadimitriou et al., 2012), and in
80 Lake Taihu (China), three variants of MC (MC-LR, -YR and -LR) were determined in
81 the gonad of various fish species using a liquid chromatography-electrospray
82 ionization-mass spectrum system (LC-ESI-MS). Particularly, silver carp
83 (*Hypophthalmichthys molitrix*) and goldfish (*Carassius auratus*) were observed to
84 contain high concentrations of MCs in the gonad (60 and 150 ng MCs g⁻¹ DW,
85 respectively) (Chen et al., 2009). In laboratories, the distribution of MC-LR in the
86 gonad of medaka fish administered with pure toxin was also testified using a
87 radiotracing method (Mezhoud et al., 2008). Although the accumulation of MCs in the
88 gonad has been documented previously through different methods, little is known
89 about the localization of MCs within the gonad tissue, or about the intracellular
90 distribution of MCs. Only one field study reported the presence of MCs in the gonadal

91 somatic tissue, but apparently not in the oocytes in common bream (*Abramis brama*)
92 using immunohistochemical method (Trinchet et al., 2013). To date, it is still hard to
93 know whether MC could enter the reproductive cells or only accumulate in the
94 conjunctive tissue of gonad. Although not the most sensitive detection method, the
95 immunohistochemical examination is a well-established way of providing the precise
96 subcellular localization of MCs in the gonad, which could largely advance our current
97 knowledge of the directly toxic effects of MCs on the gonad.

98 In the present study, in order to investigate subcellular distribution of MCs in fish
99 gonads, an immunohistochemical assay with light microscopy and electron
100 microscopy was conducted in adult medaka fish that were gavaged with acute doses
101 of MC-LR (10 $\mu\text{g}\cdot\text{g}^{-1}$ bw of MC-LR, 1 h exposure). Localization of the toxin in the
102 gonad was shown by MC-LR-specific antibody, MC10E7, which specifically binds to
103 the arginine at position 4 of MC-LR (Zeck et al., 2001). In addition, the toxin
104 distribution and histological change in the liver (the first target of MCs) were also
105 investigated in order to compare the toxic effect in liver and gonad.

106

107 **2. Materials and methods**

108 **2.1 Chemical and reagent**

109 MC-LR, purchased from Novakit® (Nantes, France), was selected as the model
110 MC for the present experiment. 500 hundred μg of MC-LR was dissolved in 500 μL
111 of ethanol and 500 μL of water. The ethanol was evaporated with a Speedvac. The
112 concentration of MC-LR in the obtained solution was quantified using a commercial
113 Adda-specific AD4G2 ELISA test (Abraxis), and then adjusted to be 1 $\mu\text{g}\cdot\mu\text{L}^{-1}$.

114 **2.2 Fish maintenance, exposure and sampling**

115 The experimental procedures were conducted in accordance with the European
116 Communities Council Directive of 24 November 1986 (86/609/EEC) and the
117 supervision of the Cuvier's ethical committee of the Museum National d'Histoire
118 Naturelle (MNHN) concerning the protection of experimental animals.
119 Five-month-old medaka fish (*Oryzias latipes*) of the inbred Cab strain maintained in
120 the lab was used for this experiment. Female and male fish were maintained in 2 glass

121 aquaria, respectively, filled with a mixture of tap water and reverse osmosis filtered
122 water (1:2) in a flow-through system for aeration and filtration, in a temperature
123 controlled room (25 ± 1 °C), with a 12 h:12 h light:dark cycle. Fish were fed three
124 times a day with commercial dry bait for juvenile salmon.

125 Eight females and eight males were randomly selected from the aquaria. The fish
126 were anesthetized with tricaine (150 mg.L^{-1}) before gavage. Five females and five
127 males were gavaged with 5 μL of MC-LR solution ($1 \mu\text{g}.\mu\text{L}^{-1}$) per fish, representing
128 about $10 \mu\text{g.g}^{-1}$ bw of MC-LR (average body weight is 0.52 g, the body weight of
129 individual fish is shown in Supplementary Table). This dose is modified according to
130 a few previous studies in which medaka fish exhibited noticeable tissue damage and
131 toxin presence in liver upon exposure to $5 \mu\text{g.g}^{-1}$ bw of MC-LR (Djediat et al., 2011,
132 2010). Two females and two males were gavaged with 5 μL water as the non-toxin
133 control, and 1 female and 1 male without any treatment were used as the non-gavage
134 control. After 1 h oral exposure, fish were anesthetized with tricaine, sacrificed, and
135 the liver and gonad were collected on ice. One tissue was cut into 2 parts, one part
136 was immediately fixed with formaldehyde fixing solution (Supplemental material 1)
137 according to the protocol provided by the histopathological platform of Ecole
138 Nationale Vétérinaire d'Alfort (ENVA), and the other part was fixed with
139 paraformaldehyde fixing solution (Supplemental material 2) following the instruction
140 of the electron microscopy platform of Muséum National d'Histoire Naturelle
141 (MNHN).

142 **2.3 Histological observation and immunolocalization of microcystins**

143 In the platform of ENVA, the liver and gonad samples fixed in formaldehyde
144 fixing solution at 4 °C for 48 h were dehydrated in successive baths of ethanol (from
145 70 to 100%) and embedded in paraffin. Blocks were cut into 4 μm thick sections.
146 Histology was observed with Hematoxylin-Eosin-Saffron (HES) staining (data not
147 shown). Assessment of hepatic glycogen reserve was evaluated with Periodic
148 Acid-Schiff (PAS) staining. The glycogen reserve analysis performed by ImageJ
149 software (version 1.51d) estimated the hepatic glycogen quantity as a surface
150 percentage of the purple-red pixels on 5 randomly selected photos ($200 \times$

151 magnification, photo size 1388×1040 pixels, as seen in Figure 1) per individual. For
152 immunolocalization of MC, sections were incubated with a monoclonal antibody to
153 MC-LR (MC10E7, Alexis) that recognizes all MCs with Arg in position 4 (dilution
154 1:4000). The immunolabeling was routinely performed with an automated Module
155 Discovery XT (Ventana, Tuscon, USA) using a colorimetric peroxidase-specific
156 staining with diaminobenzidine (DAB), a substrate that produces a highly insoluble
157 brown precipitate. The slides were counterstained with hematoxylin in the end.
158 Negative control slides were prepared by skipping the first antibody MC10E7
159 incubation step. One MC-contaminated medaka liver sample from an acute high dose
160 exposure in the lab previously was included as the technically positive control.

161 In the platform of MNHN, the liver and gonad samples fixed in
162 paraformaldehyde fixing solution at 4 °C overnight were dehydrated in successive
163 baths of ethanol (from 30 to 100%) and embedded into the Unicryl resin. Blocks were
164 cut into 0.5 µm-thick semi-thin sections (stained with toluidine blue staining) and 60
165 nm-thick ultrathin sections for histological observation and immunolocalization of
166 MC with electron microscopy, respectively. The 60 nm-thick ultrathin sections were
167 collected by gold grids. Then, the sections were incubated with the same primary
168 antibody (MC10E7) at 4 °C overnight and rinsed, then incubated with a secondary
169 antibody coupled to gold nanoballs (6 or 10 nm of diameter) at room temperature for
170 1 h. Negative control slides were prepared by skipping the first antibody MC10E7
171 incubation step. After being well rinsed with distilled water, the sections were stained
172 with a saturated solution of uranyl acetate in 50% ethanol and then observed under an
173 H-7700 Hitachi (Tokyo, Japan) transmission electron microscope.

174

175 **3. Results**

176 During the 1 hour of post-gavage, all MC-treated fish exhibit abnormal
177 swimming behaviors, such as difficulty in moving or losing body balance. In the
178 contrary, the fish of control group all swim normally. Histological and
179 immunohistochemical observation results of each individual are summarized in Table
180 1, which shows visible toxic effects and clear toxin distribution in nearly every

181 MC-treated fish, but not in any control fish.

182 (Table 1)

183

184 **3.1 Histological effects with light microscopy**

185 In the liver of all control fish, hepatocytes present compact and characteristic
186 cord-like parenchymal organization and a distinctive central nucleus with a prominent
187 nucleolus (Figure 1 A and B). The cytoplasm of hepatocytes contains mainly
188 glycoprotein and/or glycogen stores that are intensively stained in red-purple with
189 PAS staining (Figure 1 E and F). In contrast, the livers of MC-LR treated fish exhibit
190 noticeable tissue lesions characterized by disintegration of the parenchymal
191 organization and significant cell lysis. The hepatocytes all lose their polyhedral shape
192 and become rounded and swollen. Within the hepatocytes, the reserve vesicles
193 disappear, the cytoplasm presents various small bubble-like structures, and the nuclear
194 chromatin becomes dense. Some hepatocytes exhibit breakdown of the nuclear
195 membrane, mitosis arrest, and dilation of endoplasmic reticulum (Figure 1 C and D).
196 In addition, the hepatic intracellular glycoprotein and/or glycogen quantity seems to
197 decrease, and the mean \pm standard deviation (SD) values of glycogen percentage for
198 female controls, male controls, female toxin-treated fish and male toxin-treated fish
199 are $15\% \pm 9\%$, $11\% \pm 11\%$, $40\% \pm 16\%$ and $29\% \pm 16\%$.

200 For the gonad, there is no apparent cellular difference between the toxin-treated
201 fish and the control ones.

202 (Figure 1)

203 **3.2 Immunolocalization of MC-LR in the liver and the gonad**

204 Through light microscopy, all livers of MC-LR treated fish exhibit a strong
205 positive signal of MC-LR specific antibodies (Figure 2 B) whereas the negative
206 control slide does not display any positive signal (Figure 2 C), being similar to control
207 fish in which no immunolabeling is observed (Figure 2 A). For the toxin treated fish,
208 the brown immunolabelings are evenly distributed in hepatocytes, but not in blood
209 cells which are only stained in blue by hematoxylin. Within hepatocytes, the
210 immunolabeling of MCs is showed in the cytoplasm and more intense labeling is

211 observed in nuclei (Figure 2 B). Immunogold electron microscopy also shows that the
212 black nanoballs (immunolabelings) are localized in the cytoplasmic inclusion and the
213 nucleus (Figure 3 C) of hepatocytes of MC-LR treated fish, being particularly intense
214 in the lysis area (Figure 3 B). MC-LR is also distributed intensely in residual bodies
215 of the macrophage (Figure 3 D), whereas no immunolabeling is observed in the
216 control fish (Figure 3 A) or in the negative control slide (Supplementary Figure A and
217 B).

218 By light microscopy observation of the ovary of toxin-treated fish, clear
219 immunolabelings are detected in the connective tissue and some round cells with
220 above 10 μm in diameter, being probably the early stage of oocyte (Figure 2 E),
221 whereas the negative control slide does not display any positive signal (Figure 2 F),
222 being similar to the control fish in which no immunolabeling is observed (Figure 2 D).
223 Moreover, electron microscopy shows that immunogold labelings are not only present
224 in the gonadal somatic cells, but also distributed intensively in the chorion of oocytes
225 (Figure 3 G), being less intense in the yolk vesicles and cytoplasm of oocytes (Figure
226 3 H), whereas no immunolabeling is observed in either the control fish (Figure 3 E
227 and F) or the negative control slide (Supplementary Figure C).

228 On the light microscope, weaker immunolabelings are found in the testicular
229 connective tissue of a few toxin-treated male fish (Figure 2 H), whereas the negative
230 control slide does not display any positive signal (Figure 2 I), being similar to the
231 control fish in which no immunolabeling is observed (Figure 2 G). Immunogold
232 electron microscopy shows a remarkable labeling in some round unidentified
233 structures, being probably some degenerated germ cells (Figure 3 J), as well as in the
234 connective tissue of certain area nearby seminiferous tubules (Figure 3 K), whereas
235 no immunolabeling is observed in either the control fish (Figure 3 I) or the negative
236 control slides (Supplementary Figure D).

237 (Figure 2 and 3)

238 **4. Discussion**

239 MC-treated female and male fish in this study show the characteristic
240 hepatocellular changes and the depletion of glycogen reserve that have been observed
241 previously in fish (Djediat et al., 2011; Fischer et al., 2000; Fischer and Dietrich, 2000;
242 Marie et al., 2012; Mezhoud et al., 2008) and mice (Guzman and Solter, 2002;
243 Yoshida et al., 2001, 1998) acutely administered with MCs or MC-containing
244 cyanobacteria extracts. In fact, the observed liver damage in the present study is also
245 similar to some of those described in the fish after chronic intoxication with MCs
246 (Acuña et al., 2012; Qiao et al., 2016a; Trinchet et al., 2011). Trinchet and her
247 colleagues observed an increase in rough endoplasmic reticulum in hepatocytes of
248 medaka fish chronically exposed to MC-LR ($5 \mu\text{g.L}^{-1}$, 30 d), which is also found in
249 the male toxin-treated medaka in the present study. This could be associated with the
250 induction of detoxification process. Another chronic in-house study reported a
251 noticeable hepatic glycogen store depletion in medaka fish upon exposure to MC-LR
252 ($5 \mu\text{g.L}^{-1}$, 28 d) (Qiao et al., 2016a), which is consistent with the acute intoxication
253 effect of MC-LR observed here, and the increased energy needs for the MC
254 detoxification process might be the cause.

255 Severe liver damages are often accompanied by remarkable accumulation of MCs
256 in the liver. The present study shows an intense distribution of MC-LR in the lytic
257 hepatocyte of the toxin-treated medaka fish through the immunohistochemistry. MCs
258 have been reported to mostly localize in the cytoplasm of hepatocytes where they
259 disturb a sequence of phosphorylation/dephosphorylation-dependent biochemical
260 reactions, resulting in the disruption of multiple cellular processes, and consequently
261 causing the deformation of hepatocytes, apoptosis and lysis (Campos and
262 Vasconcelos, 2010). Furthermore, a high concentration of MC-LR is also observed in
263 the hepatic nucleus here, which is consistent with the results provided in a few
264 previous acute studies (Fischer et al., 2000; Guzman and Solter, 2002; Yoshida et al.,
265 1998). Protein phosphatases PP1 and PP2A, one of the main molecular “target” of
266 MCs, localize in both nucleus and cytoplasm (Shenolikar, 1994). Therefore, the

267 observed nuclear accumulation of MC-LR could be the consequence of the MC
268 specific antibodies binding to the MC-LR-PP1/PP2A adducts localized in the nucleus.

269 In the present study, MC-LR is also observed to be present in the gonad of
270 MC-treated fish. For females, MC-LR is localized in the connective tissue of ovary, as
271 well as in some immature and maturing oocytes. The presence of MC in the connective
272 tissue of fish ovary has been reported previously in common bream collected from
273 MC-contaminated lakes (Trinchet et al., 2013). It seems that MCs are transported
274 through the bloodstream into gonads, in which they can firstly accumulate in the
275 connective tissue, and then transported into various gonadal somatic cells, even
276 reproductive cells. Our results indeed exhibit an apparent distribution of MC-LR in
277 the oocyte of vertebrates with *in vivo* condition for the first time, and the toxin is clearly
278 localized in the yolk and the cytoplasm of the oocyte, being particularly highly
279 intensive in the chorion. Recently, one field study detected noticeable microcystin
280 variants in the egg-shell membranes and yolk of dead hatchlings of Nile crocodiles
281 (*Crocodylus niloticus*) by using liquid chromatography-high resolution mass
282 spectrometry (Singo et al., 2017). Through the immunochemistry, MC was once
283 reported to be present in the oocyte of snail upon exposure to MC-LR for 5 weeks
284 (Lance et al., 2010). It is unclear how MC-LR enters into oocytes since the oocyte
285 plasma membrane seems to lack the OATPs possessing MC-transport capability. It
286 can be assumed that MCs may be transported into oocytes as protein-bound forms
287 along with protein import during oogenesis, since the exchange of a big amount of
288 protein and other materials are occurring prior to complete chorion maturation.

289 For MC-treated male fish, immunogold electron microscopy reveals an intense
290 distribution of MC-LR in the connective tissue of the testis in some certain area, as
291 well as in some degenerated spermatocyte-like structure, which implies a potential
292 germ cell damage induced by MC exposure. The connective tissue of the testis
293 consists of massive fibers and various types of gonadal somatic cells, such as Leydig
294 cells and Sertoli cells. They are important functional cells in the testis, being
295 responsible for synthesizing and secreting androgens or transporting of nutrients into

296 the growing germ cells (Dietrich and Krieger, 2009). Therefore, the high
297 accumulation of MC-LR in the connective tissue and its subsequent toxic effect on
298 gonadal somatic cells may indirectly disturb spermatogenesis process, affecting the
299 reproductive process. Although Sertoli cell contributes to the formation of the
300 blood-testis barrier (BTB) which hinders the diffusion of MCs into the spermatogenic
301 microenvironment (Wan et al., 2013), a recent *in vitro* study reported that MC-LR can
302 cause disruption of the tight junction between Sertoli cells, and destroy BTB (Chen et
303 al., 2018, 2016), implying that MC-LR could enter the spermatogenic
304 microenvironment and subsequently induce apoptosis and degeneration in the
305 spermatocyte. This could be one explanation for the accumulation of MC-LR in some
306 degenerated spermatocyte-like structure observed here. Considerable testicular injury
307 and spermatocyte abnormality have been found in several species of vertebrate model
308 animals, such as mouse, rat and fish, upon exposure to MCs acutely or chronically (Li
309 et al., 2008; Su et al., 2016; X. Wang et al., 2013; Zhao et al., 2012; Zhou et al., 2013).
310 However, to date, according to *in vivo* studies using these vertebrate models, no
311 evidence regarding the incorporation of MCs into spermatocytes has been reported yet.
312 In the present study, MC-LR is not found in any clear spermatocytes of the
313 toxin-treated fish through the immunogold labeling technique, which is attributed to
314 the integrity of BTB and the organ distribution of the MC-transporting OATPs. The
315 identified OATPs that possess strong MC-transport capabilities are highly abundant in
316 the liver, but hardly known to be present in the cell membrane of reproductive cells
317 (Ho and Kim, 2018). However, two *in vitro* studies reported that MC-LR was able to
318 immigrate into isolated rat spermatogonia (L. Wang et al., 2013; Zhou et al., 2012).
319 Furthermore, in Zhou's study, at least 5 OATPs were detected at the mRNA level in
320 spermatogonia and their expression level was affected by MC-LR, implying that these
321 OATPs may involve in MC-transport into male reproductive cells. It seems that some
322 unidentified OATPs which possess MC-transport capabilities might be expressed in
323 male reproductive cells at a relatively low level. But the information regarding the
324 unidentified MC-transporting OATPs and their expression level on germ cells remains
325 very limited. Due to various physiological differences between female and male fish

326 (Qiao et al., 2016b), the toxic effects of MC on gonads of two genders of fish seem to
327 be different. One previous study demonstrated that female zebrafish are more
328 vulnerable than males to MC-LR exposure indicated by more cell apoptosis in ovary
329 compared with testis (Qiao et al., 2013). The present study also showed a clear
330 accumulation of MC in oocytes but not spermatocytes with *in vivo* condition, which
331 implies a consistent idea that MC exposure exerts more potent reprotoxic effects on
332 female fish than males.

333 It is worth to mention that the process of histological section preparation may
334 affect the result, since a fraction of MC-LR (mainly free MC-LR) can be removed
335 during the dehydrating process of the section preparation. The immunostaining here
336 indeed detects MC-LR-PP1/PP2A adducts mostly, due to the strong affinity of
337 MC-LR to protein phosphatases PP1/PP2A (Yoshida et al., 2001). Dmet-Asp and
338 D-Glu residues of MC-LR play important roles in forming the MC-LR-PP1/PP2A
339 adducts (Campos and Vasconcelos, 2010). Nevertheless, the present acute study
340 indeed shows a clear subcellular distribution of MC-LR in the gonad of the
341 toxin-treated fish, implying a direct effect of gonadal impairment on reproductive
342 function. However, in another in-house chronic study, MCs were not detected in the
343 ovary or testis of medaka fish following a balneation exposure to 5 $\mu\text{g}\cdot\text{L}^{-1}$ of MC-LR
344 for 30 days using the same immunolocalization techniques (Qiao et al., 2016a), which
345 suggests a more potent and indirect effect of liver dysfunction on fish reproduction
346 upon chronic low-dose exposure. Comparing these two studies, toxin concentration,
347 and exposure time account for the discrepancy. In the present study, 10 $\mu\text{g}\cdot\text{g}^{-1}$ bw of
348 MC-LR is a quite high concentration (LD₅₀ values of MC-LR by i.p. injection range
349 from 50 to 400 $\mu\text{g}\cdot\text{kg}^{-1}$ bw in different species of fish) (Testai et al., 2016), and the
350 short time of exposure (1 h) largely reduced the possible toxin excretion process
351 through liver detoxification, together leading to a sufficient quantity of toxin that
352 could access to gonad through blood stream and be detected by the
353 immunohistological method.

354

355 **5. Conclusion**

356 Our histological and immunohistochemical results reveal that both liver and
357 gonad are significantly affected by MC-LR exposure. An intense distribution of
358 MC-LR within hepatocytes along with a severe liver damage attests to the potent
359 hepatotoxicity of MC. The immunohistochemical results show that, besides being
360 accumulated in the hepatocytes, MC-LR is also found in the connective tissue of the
361 gonads, as well as in the reproductive cells (oocytes but not spermatocytes). This
362 finding constitutes the first observation of the presence of MC in the reproductive
363 cells (female, oocytes) in vertebrate model animals with *in vivo* condition. Both liver
364 and gonad play important roles in the reproductive process of oviparous vertebrates.
365 Our results of the present acute study, which provide a distinct subcellular localization
366 of MC-LR in the liver and gonad, contributes to a better understanding of the
367 potential reproductive toxicity of MC-LR at the histological level, favoring the
368 characterization of underlying mechanisms. Meanwhile, the penetration of MCs into
369 the reproductive cell suggests a possible transferring of MCs from adults into
370 offspring which could cause a big issue for the population of aquatic organisms. The
371 further investigation concerning this perspective is needed to advance our current
372 knowledge of the protection of aquatic organism populations, as well as human beings
373 from the widespread MCs in the freshwater body.

374

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382 administrative support.

383 **Author Contribution**

384 Q.Q., C.D., H.H., C.B., M.E. and B.M. conceived the experiments, Q.Q., C.D., H.H,
385 C.D., S.L.M. and B.M. conducted the experiments, Q.Q., C.D., H.H and B.M.
386 analyzed the results. All authors reviewed the manuscript.

387 The authors declare no competing financial interest.

388

389 **List of Supplementary materials:**

390 Supplementary material 1. Formaldehyde fixing solution (100 mL) provided by the
391 platform of ENVA

392 Supplementary material 2. Paraformaldehyde fixing solution (100 mL) provided by the
393 platform of MNHN

394 Supplementary Table. The body weight of individual fish in different treatment groups

395 Supplementary Figure. Representative photos of the negative control slide for the
396 liver and gonad of toxin-treated fish through immunogold electron microscopy.

397

398 **Figure descriptions:**

399 Figure 1. Representative photos of histological observation of medaka liver under a
400 light microscope.

401 A-D: resin sections (0.5 μm thick) of medaka liver with toluidine blue staining.

402 Female controls (A): abundant and distinct distribution of reserve vesicles (v);

403 Females exposed to 10 $\mu\text{g}\cdot\text{g}^{-1}$ bw of MC-LR (C): disintegration of the cord-like

404 parenchymal organization, rounded hepatocytes containing various small bubbles (b),

405 loss of reserve vesicles, nuclear membrane breakdown, chromatin concentration (c);

406 Male controls (B): abundant and distinct distribution of reserve vesicles (v); Males

407 exposed to 10 $\mu\text{g}\cdot\text{g}^{-1}$ bw of MC-LR (D): disintegration of the cord-like parenchymal

408 organization, swollen and rounded hepatocytes, nuclear chromatin condensation (c),

409 and dilation in endoplasmic reticulum (e) and loss of nuclei (n). E-H: paraffin sections

410 (4 μm thick) of medaka liver with PAS staining. Female (E) and male (F) controls:

411 abundant and distinct distribution of glycogen-reserve vesicles (red-purple); Females

412 (G) and males (H) exposed to 10 $\mu\text{g}\cdot\text{g}^{-1}$ bw of MC-LR: less glycogen-reserve vesicles

413 compared with the control group.

414

415 Figure 2. Representative photos of immunolocalization of MC-LR in the liver and
416 gonad of toxin-treated fish through light microscopy.

417 The liver (A), ovary (D), and testis (G) of control fish incubated with MC-LR
418 immunolabeling (MC10E7): no immunolabeling. The liver (B), ovary (E), and testis
419 (H) of the fish exposed to $10 \mu\text{g}\cdot\text{g}^{-1}$ bw of MC-LR incubated with MC-LR
420 immunolabeling (MC10E7), and revealed with peroxidase specific reaction of DAB.
421 For the liver, brown labelings of MC-LR are found in the cytoplasm and nuclei (n) of
422 hepatocytes, but not in the blood cell (h); For the ovary, brown labelings of MC-LR
423 are detected in the connective tissue and some round cells; For the testis,
424 immunolabelings are observed in the testicular connective tissue. The negative control
425 slides of the toxin-treated liver (C), ovary (F), and testis (I) incubated with only the
426 secondary antibody: no immunolabeling.

427

428 Figure 3. Representative photos of immunolocalization of MC-LR in the liver and
429 gonad of toxin-treated fish through immunogold electron microscopy.

430 The liver (A), ovary (E and F) and testis (I) of control fish: no clear immunolabeling.
431 The liver (B-D), ovary (G-H) and testis (J and K) of the fish exposed to $10 \mu\text{g}\cdot\text{g}^{-1}$ bw
432 of MC-LR, showing the immunolabeling of MC-LR indicated by the white arrow. For
433 the liver, immunolabelings of MC-LR are clearly observed in the lytic residual (B)
434 and nuclear (C) of hepatocyte and the macrophage (D), particularly in the residual
435 bodies (r); For the ovary, immunolabelings are found in the connective tissue (left)
436 and the chorion (c) of the oocyte (G, right), and less labelings are observed in the yolk
437 vesicle (y) and the cytoplasm (cy) of the oocyte (H). For the testis, labelings are
438 intensively distributed in some degenerated germ cell-like structures (J), and in the
439 connective tissue of certain area nearby seminiferous tubules (K).

440

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610

Figure 1

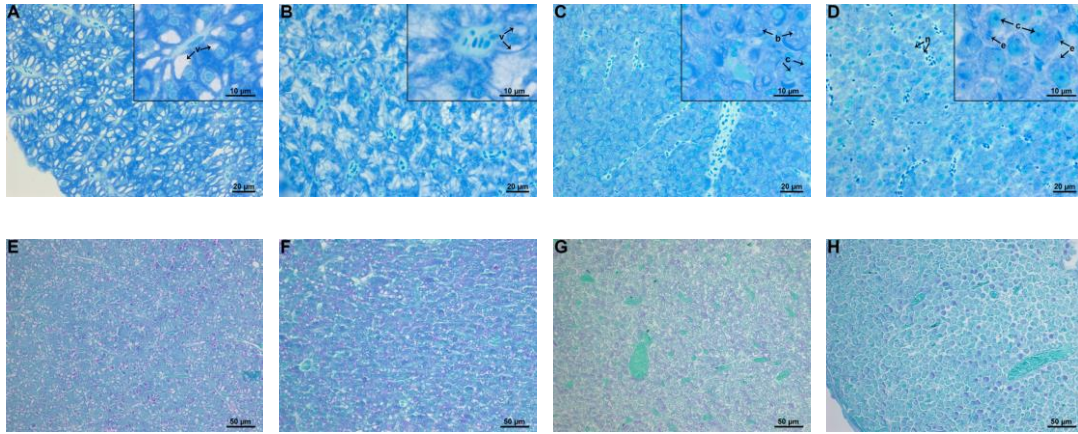


Figure 2

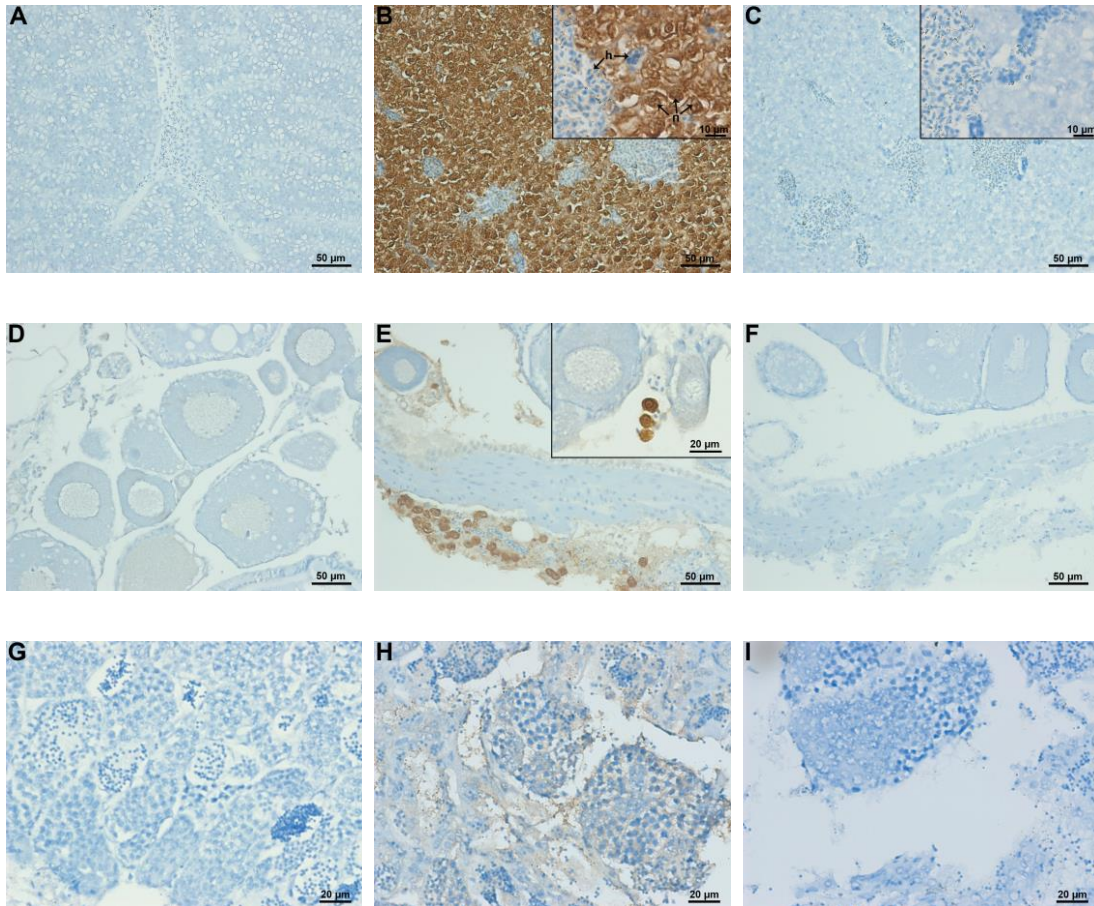
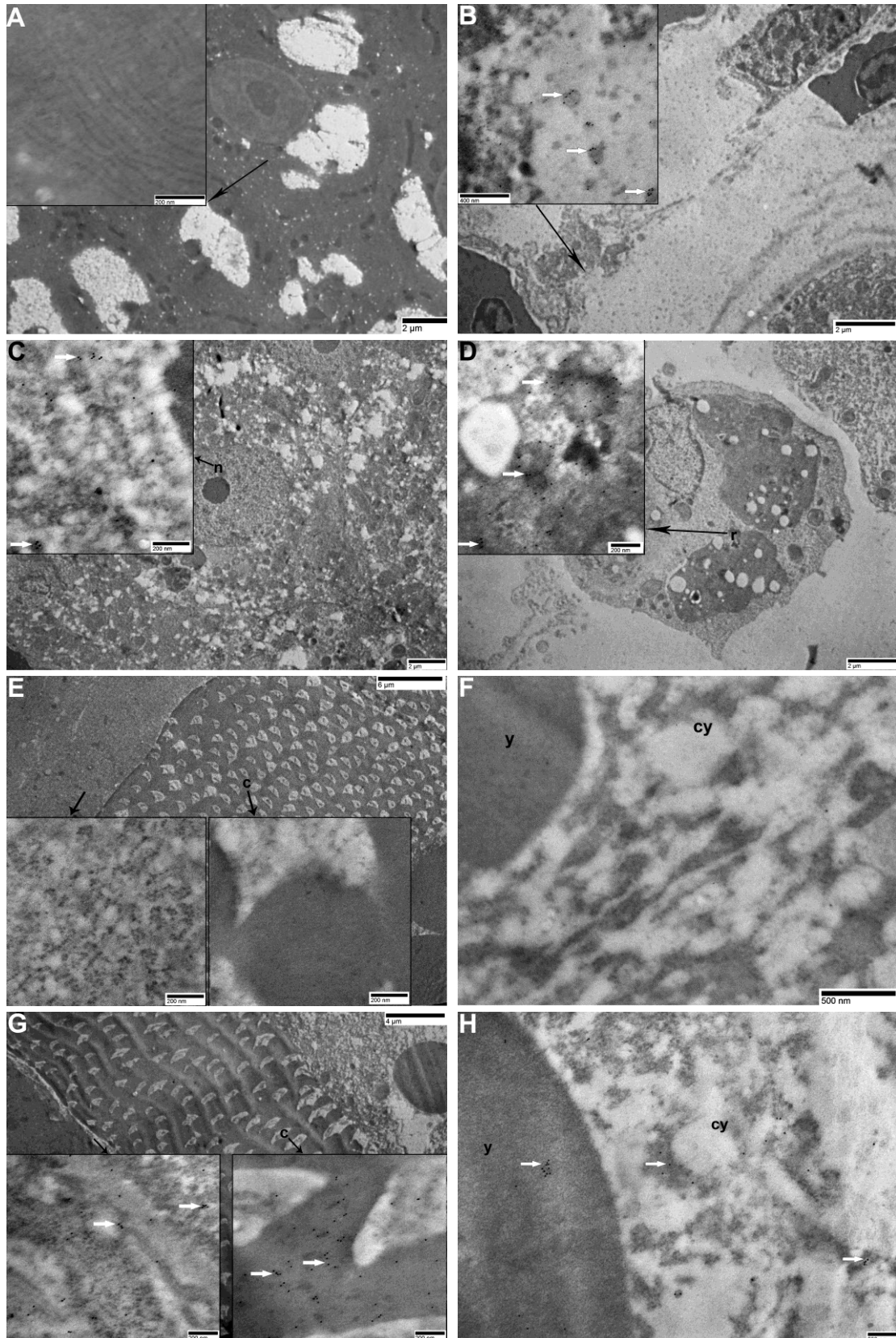


Figure 3



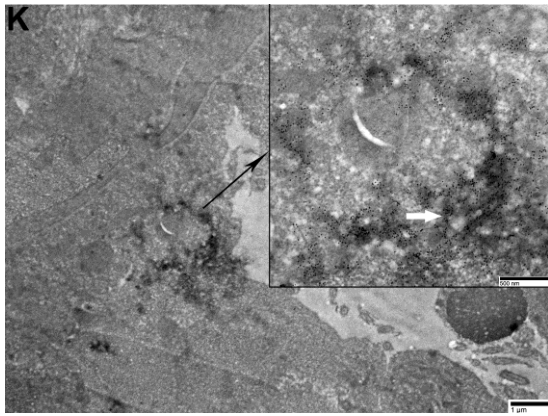
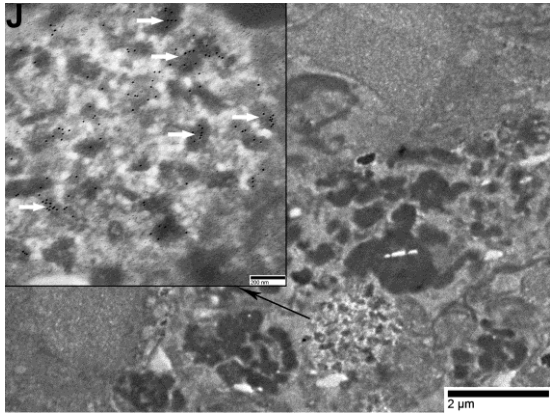
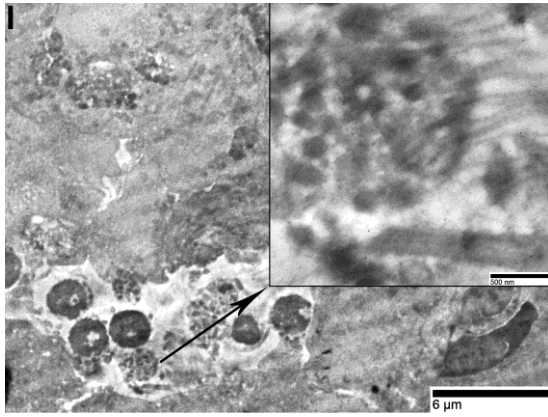


Table 1 Histological and immunohistochemical observation of each individual liver and gonad

Group	Fish ^a	Post-gavage	Histology (Liver)		Anti-MC-LR labeling			
			Light microscopy		Light microscopy ^d		Electron microscopy ^e	
			Liver damage ^b	Glycogen reserve (%) ^c	Liver	Gonad	Gonad	
10 $\mu\text{g}\cdot\text{g}^{-1}$ b.w. of MC-LR	F01	1 h	+	23%	89%	0.1%	++	
	F03	1 h	++	22%	79%	0.1%	+	
	F04	1 h	+	15%	76%	0.2%	++	
	F07	1 h	++	12%	97%	2.9%	++	
	F10	1 h	+	1%	95%	0.5%	n.o.	
	M02	1 h	+	0%	89%	0	++	
	M05	1 h	++	20%	26%	0.1%	++	
	M06	1 h	+	2%	15%	0.1%	n.o.	
	M08	1 h	+	26%	89%	0	+	
M09	1 h	+	6%	89%	0.7%	++		
Control	F12	1 h	0	22%	0	0	0	
	Non-toxin	F14	1 h	0	50%	0	0	0
		M13	1 h	0	16%	0	0	0
		M11	1 h	0	25%	0	0	0
	Non-gavage	F16	1 h	0	49%	0	0	0
	M15	1 h	0	47%	0	0	0	

^a F and M indicate female and male fish, respectively. ^b “++” indicates a severe liver damage, characterized by a large proportion of rounded hepatocytes, significant mitosis arrest, and obvious cell lysis and disjunction. “+” indicates a moderate liver damage, characterized by a large proportion of rounded hepatocytes, and a small area of cell lysis and disjunction. “0” indicates the normal liver structure. ^c indicates the percentage of glycogen reserve in the liver, the calculation formula: the area of the purple-red pixels \div the area of the whole image (1388 \times 1040 pixels) \times 100%. ^d indicates the percentage of brown immunolabelings in the liver and gonad, the calculation formula: the area of the brown pixels \div the area of the whole image (1388 \times 1040 pixels) \times 100%. ^e “++” indicates strong and widely distributed immunolabelings, “+” indicates only a few immunolabeling spots, “0” indicates no immunolabeling, and “n.o.” indicates not observed.