



1 **Complex molecular changes induced by chronic progestogens exposure in**
2 **roach, *Rutilus rutilus***

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18 Abstract

19 In our previous study, we measured 0.23–13.67 ng/L progestogens (progesterone,
20 drospirenone, levonorgestrel) in natural waters in the catchment area of the largest shallow
21 lake of Central Europe, Lake Balaton. Progestogen contaminations act as potent steroids with
22 mixed progestagenic, androgenic and mild estrogenic effects that is why our aim was to
23 investigate the morphological and molecular effects of mixture of progesterone, drospirenone,
24 and levonorgestrel in different exposure concentration in common roach, *Rutilus rutilus*.
25 Steroids (e.g. progestogens) and the protein deglycase DJ-1 chaperon molecule aim the same
26 target molecules in cells, therefore, we hypothesized that a relationship may exist between
27 progestogens and DJ-1. Furthermore, our other aim was to follow the changes of signal
28 molecules of different biological function due to progestogen treatment in serum and brain.

29 Adult roaches were exposed to 10, 50 and 500 ng/L of mixture of progestogen for 42 days and
30 their somatic indices (brain-somatic, liver-somatic, gonadosomatic and kidney-somatic) were
31 measured. Vitellogenin (VTG) expression (estrogen effect) or inhibition (androgen effect) in
32 fish is a widely used biomarker so we measured its changes in liver by ELISA. To determine
33 the quantity and to map the spatial distribution of DJ-1 chaperon protein the brain and liver
34 tissues were analyzed by ELISA and immunohistochemistry. Furthermore, we also studied
35 molecular alterations: a) in the serum by measuring cholesterol, low-density lipoprotein
36 (LDL), high-density lipoprotein (HDL) and triglyceride concentrations and b) in brain
37 homogenate using a cell stress array kit (26 protein).

38 The somatic index of liver and kidney significantly in all the treated groups, whereas the
39 gonadosomatic index of 500 ng/L treated group showed significant decrease compared to
40 control animals. VTG level increased significantly in 500 ng/L progestogen treated group.
41 Since the concentration of DJ-1 significantly increased in brain and liver in all progestogen
42 treatment groups, the DJ-1 protein could be able to a more sensitive marker than VTG. Serum
43 LDL and cholesterol levels of exposed fish were significantly decreased. DJ-1 was mediated
44 through the stimulation of the expression of LDL-receptor which facilitates reuptake
45 subsequently.

46 In summary, our observations unfolded new data about molecular alterations induced by the
47 combined action of environmental progestogens. In addition, the DJ-1 chaperon protein as a
48 possible biomarker helped to trace the abiotic chemical environmental contaminations, like
49 progestogens in the freshwater ecosystems.

50 Keywords: steroid hormones, progestogens, vitellogenin, DJ-1 protein, fish, roach

51 1. Introduction

52 The most extensively studied pharmaceutical pollutant is 17 α -ethinylestradiol (EE2). This
53 steroidal estrogen compound is used in synthetic oral contraceptives (SOC), and has been
54 shown to impair reproduction in fish even at concentrations below 1 ng/L (Caldwell et al.,
55 2008). Recently, another class of contraceptive pharmaceuticals has emerged into focus in
56 ecotoxicology: the progestogens. Progesterone (PRG) is an endogenous steroid hormone
57 involved in the female menstrual cycle, pregnancy and the embryogenesis of humans and
58 other species (Rodriguez et al., 2010). It plays important role in brain function as a
59 neurosteroid (Baulieu and Schumacher, 2000). Progestins are a group of molecules that have
60 effects similar to those exerted by PRG. In fish, the main natural progestin is 17 α ,20 β -
61 dihydroxy-4-pregnen-3-one (DHP). In females, DHP is responsible for final maturation of
62 oocytes (Nagahama and Yamashita, 2008), while in males it is involved in spermiation and
63 sperm motility (Tubbs and Thomas, 2009). Besides natural representatives of PRG analogs,
64 there are several synthetic progestins such as drospirenone (DRO), levonorgestrel (LNG) or
65 norethindrone (NET). The endogenous PRG and its synthetic analog progestins together are
66 generally referred to as progestogens. There are approximately 20 different progestogens used
67 in human and veterinary medicine, which all activate progesterone receptors (Sitruk-Ware and
68 Nath, 2010). Although, progestogens and their metabolites could also interact with other
69 steroid hormone receptors, exerting combinations of progestagenic, (anti)androgenic,
70 (anti)estrogenic, glucocorticoidogenic and anti-mineralocorticoidogenic effects (Africander et
71 al., 2011). Progestogens are widely used as SOCs. More than 100 million women use SOCs
72 yearly (Huezo, 1998). Progestogens or their metabolites are eliminated from the human body
73 mainly through the renal system and a remarkable amount is excreted unchanged or in the
74 form of active metabolites (Besse and Garric, 2009; Liu et al., 2011). These active agents
75 enter waste water treatment plants (WWTP) where the generally applied treatment process is
76 not suitable to eliminate progestogen contaminations of different origins perfectly (Can et al.,
77 2014; Liu et al., 2011).

78 The very highest concentrations (from several tens to often hundreds or thousands ng/L) are
79 reported in animal farm flush water, and associated runoffs and lagoons in China and in the
80 U.S. Lower concentrations are reported in WWTP influents and effluents. In WWTP influent
81 waters DRO, LNG, NET and PRG have mainly been measured in concentrations ranging
82 from a few to more than a hundred ng/L. In WWTP effluents, concentrations are generally
83 lower with an order of magnitude. In surface waters, which are more relevant from an
84 ecotoxicological point of view, the reported progestogen concentrations are approximately
85 similar to WWTP effluent concentrations. In rivers, streams, lakes and ground water, DRO,
86 LNG, NET and PRG are detected at concentrations of typically a few ng/L (Chang et al.,
87 2011; Fent, 2015; Liu et al., 2012; Liu et al., 2014; Orlando and Ellestad, 2014; Vulliet et al.,
88 2008; Yost et al., 2014). By analyzing freshwater samples from the catchment area of Lake
89 Balaton and River Zala, varying progestogens concentration 0.26-4.30 (DRO), 0.85-3.40
90 (LNG) and 0.23-13.67 (PRG) ng/L were detected (Avar et al., 2016).

91 There is increasingly high amount of evidence available about the toxic effects of individual
92 progestogen. Fecundity or eggs production of fathead minnows (*Pimephales promela*) were
93 dramatically decreased due to the 21 days long treatment by 22-596 ng/L NET. Plasma

94 concentration of the endogen 11-ketotestosterone in male and 17 β -estradiol in female fathead
95 minnows were significantly decreased after 21 days exposure to high concentration of NET
96 (Paulos et al., 2010). Toxicity studies reported that DRO (6.5 μ g/L) reduces fertility in adult
97 fathead minnows (Zeilinger et al., 2009). The disruption of the reproductive system occurred
98 in male roach (*Rutilus rutilus*) after exposure to 31 ng/L LNG (Kroupova et al., 2014). 6.5
99 ng/L LNG can already disrupt the androgen-dependent reproductive cycle in male three-
100 spined sticklebacks (*Gasterosteus aculeatus*) (Svensson et al., 2013; Svensson et al., 2014).
101 Bioaccumulation of progestogenic chemicals in the rainbow trout (*Oncorhynchus mykiss*) has
102 also been published (Liu et al., 2011). Being aware of the results of previously reported
103 toxicity experiments with we aimed to investigate the combined impact of progestogens on
104 the molecular and somatic level of a common fish (*Rutilus rutilus*). Our investigations include
105 a 10 ng/L treatment group, which represents a realistic level of contamination in freshwater.
106 According to literature data, the presence of protein deglycase DJ-1 chaperon molecule was
107 also described in medeka (*Oryzias latipes*) (Ansai et al., 2013; Li et al., 2006), zebrafish
108 (*Danio rerio*) (Bai et al., 2006; Bretaud et al., 2011), atlantic salmon (*Salmo salar*) (Shinbo et
109 al., 2006) and pike (*Esox lucius*) (Leong et al., 2010). Fish DJ-1 protein (189 AA) is
110 homologous with human DJ-1 which shows its evolutionary conserved appearance (Bai et al.,
111 2006; Bandyopadhyay and Cookson, 2004). This protein deglycase chaperon protein
112 possesses multiple functions in vertebrates. Besides antioxidant defense of DJ-1, it is also
113 involved in cell survival and proliferation, cytoprotection and gene transcription. There are
114 factors, such as nuclear E2-related factor 2 (NRF2) (Clements et al., 2006; Schultz et al.,
115 2014), polypyrimidine tract-binding protein-associated splicing factor (PSF) (Dong et al.,
116 2005; Xu et al., 2005) and low-density lipoprotein (LDL) receptor (Yamaguchi et al., 2012),
117 which are common target molecules for steroids and DJ-1 (see Fig.S1). We hypothesized that
118 steroids (progestogens) could elevate the level of DJ-1 and may also indirectly influence their
119 targeted receptors through DJ-1 (Fig.S1), therefore, we examined the relationship between
120 progestogens and DJ-1 protein in roach. Furthermore, using a cell stress array kit for
121 observation our other aim was to follow the changes of 26 signal molecules of different
122 biological function due to progestogen treatment.

123 2. Materials and Methods

124 2.1. Animals and chemicals

125 All procedures on fish were approved by the Scientific Committee of Animal
126 Experimentation of the MTA-ÖK Balaton Limnological Institute (VE-I-001/01890-10/2013).
127 Roaches were caught (collection permit: VE-I-001/01890-9/2013) in the Sárvíz stream
128 situated in the drainage system of Lake Balaton (N46.91266 E16.87705) in March 2014 and
129 were brought to the MTA-ÖK Balaton Limnological Institute, Tihany (the number of keeping
130 permit: VE-I-001/01890-3/2013). During our previous survey carried out on the drainage
131 system of Lake Balaton (Avar et al., 2016) we did not detect traceable progestogen
132 concentrations from this stream section. Therefore, we supposed that the individuals used for
133 this experiment avoided previous hormone expositions. Efforts were made to minimize the
134 suffering of animals. During acclimatization period, fish (n=90, weight: 18.5 \pm 4.5g, length:

135 95.0±7.3mm) were maintained in artificial Balaton water at 20 °C under conditions of 12 h
136 light, 12 h dark cycle in 200 L tanks, and were fed 3 times a week with vegetable-based fish
137 food (TETRA Werke).
138 Mixture of PRG (P0130, HPLC grade), DRO (SML0147, HPLC grade) and LNG (L0551000,
139 HPLC grade) were used for the treatments as progestogen agents (Sigma Aldrich).
140 Progestogens were dissolved in 0.5 M cyclodextrine (WXBB5296, 2-hydroxypropyl-β-
141 cyclodextrin, Sigma Aldrich) and added to fish tanks waters to reach 10-, 50-, 500 ng/L final
142 concentrations to each progestogens. Cyclodextrins (cyclic oligosaccharides) are mainly used
143 solubilizing agents to increase water-solubility of lipophilic compounds. LC-MS grade
144 methanol, acetonitrile and water were all obtained from VWR International (Hungary).

145 2.2. *Ethical permissions*

146 All procedures on fish were approved by the Scientific Committee of Animal
147 Experimentation of the MTA-ÖK Balaton Limnological Institute (VE-I-001/01890-10/2013).
148 Roaches were caught (collection permit: VE-I-001/01890-9/2013) in the Sárvíz stream
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150 were brought to the MTA-ÖK Balaton Limnological Institute, Tihany (the number of keeping
151 permit: VE-I-001/01890-3/2013).

152 2.3. *Progestogen exposure and tissues*

153 After one month acclimatization period, the healthy mixed gender fish were divided into 4
154 groups (n=10 fish/group in 50 L tank). Each experimental group was replicated 2 times
155 (method control). During the experiment, there was no significant difference between these
156 methodical replicates, so the total fish number per treatment (n=20) could be combined. Fish
157 of the 1st group were maintained under quiet and suitable conditions and used as a vehicle
158 control (100 µL, 0.5 M cyclodextrine/60 L water). Fish of the other three groups were
159 exposed for 42 days to 10, 50, 500 ng/L mixture of progestogen treatment. Water quality was
160 monitored at least twice a week in all aquaria. Mean ± SD values measured were:
161 temperature, 20.3 ± 2.4. °C; pH, 7.8 ± 0.2; dissolved oxygen, 90.4 ± 5.5%; conductivity,
162 748.4 ± 19.5 µS. The length and weight of all individuals were measured before and after
163 treatment period. At the end of 42 days exposure period all fish were anaesthetized with clove
164 oil and killed by decapitation. Their brain, liver, gonads and kidneys were removed on ice and
165 weighted. Somatic indices (brain-somatic index: BSI, liver-somatic index: LSI,
166 gonadosomatic index: GSI, kidney-somatic index: KSI) were determined (calculated as:
167 [tissue weight/body weight] × 100). All the analyzed tissue samples were taken at the same
168 time, so that factors, such as diurnal rhythm and photoperiod, which affect the expressions of
169 redox state-related factors could be minimized. Tissues were homogenized for ELISA or were
170 fixed in 4% paraformaldehyde for immunohistochemistry (IHC) experiments.

171 2.4. *Recovery measurement of progestogens*

172 Concentration of all progestins were measured in experimental tanks via an HPLC-MS
173 method according to (Avar et al., 2016). Hormone residues were concentrated on Strata C18-
174 E (1g/20 mL) SPE cartridges after filtration on Whatman glass microfiber (0.45 µm). The

175 SPE cartridges were conditioned with 15 mL methanol, equilibrated with 20 mL LC-MS
176 grade water, loaded with 770 mL filtrated sample, washed with 20 mL LC-MS grade water
177 and eluted with 15 mL methanol. After evaporation to dryness extracts were reconstituted in
178 50 μ L methanol. Five μ L was injected 3 times from each concentrated sample into the HPLC-
179 MS system (Dionex Ultimate 3000 UHPLC, Q-Exactive HRMS, Thermo Fisher Scientific).
180 Liquid chromatographic separation was carried out on a Kinetex 2.6 μ C18 100 \AA HPLC
181 column (100*2.1 mm) maintained at 40 $^{\circ}$ C. The mobile phase consisted of solvent A (0.01
182 v/v% formic acid in water) and solvent B (0.01% v/v% formic acid in acetonitrile). The flow
183 rate was 300 μ L/min. The mass spectrometer was equipped with a heated electrospray ion
184 source which was operated in positive ion mode. Spray voltage was set to 4.0kV. Capillary
185 temperature was 300 $^{\circ}$ C. Different HCD (Higher-Energy Collisional Induced Dissociation)
186 cell energies were applied for the fragmentation: 55 % by DRO and 45 % by PRG and LNG.
187 Data analysis was carried out with the software Thermo Xcalibur (version 2.2 SP1.48). For
188 quantitative analysis five point calibration curves were used in the concentration range of 0.5
189 to 750 ng/L. Correlation coefficients (R²) of the calibration curves were over 0.95. The limit
190 of detection (LOD) calculated from standard mixture was 0.01 (DRO), 0.01 (LNG) and 0.003
191 (PRG) ng/L while the limit of quantification (LOQ) was 0.11, 0.09 and 0.03 ng/L,
192 respectively. Recoveries of 100 μ g spiked standards were 76% (DRO), 81% (LNG) and 95%
193 (PRG). For measured concentrations of progestogens from experimental tanks, the
194 progestogen contents were checked weekly on the seventh day by HPLC-MS method. The
195 percent of mean recovery for each treating levels were always near or over 80% respect to
196 nominal concentrations for DRO, LNG and PRG. Based on these recovery data, the water in
197 all experimental tanks were changed and exposed with applied concentrations every weeks.
198

199 2.5. Sandwich ELISA measurements

200 2.5.1. Quantitative analysis of vitellogenin (VTG)

201 Female roach were used to the VTG measurement. Livers (n=3) were homogenized in ice
202 cold PBS (10 mg/100 μ L) with a glass homogenizer. The homogenate was sonicated to
203 further desintegrate cell membranes and the centrifuged for 15 minutes at 1500 \times g.
204 Supernatant was collected and stored at -80 $^{\circ}$ C prior to analysis.

205 Fish VTG ready to use ELISA Kit (MBS010726, MyBioSource) was applied for the
206 quantitative analysis. 50 μ L VTG standards (31.2, 62.5, 125, 250, 500, 1000 ng/mL) and 50
207 μ L fish liver samples were added to each well. 100 μ L HRP conjugate reagent was added to
208 the wells and incubated for 1 h at 37 $^{\circ}$ C. The plates were gently washed four times with wash
209 buffer. After that, 50-50 μ L Chromogen solution A and B reagent were added to each well
210 and mixes were incubated for 15 minutes at 37 $^{\circ}$ C then 50 μ L Stop solution was added to
211 cease the enzyme reaction. Finally, optical density was read with a micro plate reader
212 (Victor3 1420 multilabel counter, PerkinElmer) at 450 nm and VTG concentration of the
213 tissue was calculated.

214 2.5.2. Quantitative analysis of protein deglycase DJ-1

215 The procedure was used according to manual ensured by the company. Briefly, special 96-
216 well micro plates (DuoSet ELISA Kit-R&D systems, RD-DY8136-05) were coated with 0.8

217 $\mu\text{g/mL}$ capture antibody and the plates were washed three times with washing buffer.
218 Blocking was performed with reagent diluent while incubating at room temperature for 1
219 hour. After washing, 100 μl of the protein deglycase DJ-1 standards (0.313; 0.625; 1.25; 2.5;
220 5 ng/mL) were added to each well along with the 100 μL fish antigen samples ($n=6$, brain,
221 liver). Triplicates made on the plate for each sample were incubated at room temperature. 45
222 ng/mL detection antibody dissolved in reagent diluent was added and incubated at room
223 temperature for 2 hours. Incubation was followed by an additional washing step. HRP
224 conjugated detection antibody dissolved in reagent diluent (1:40) was added and incubated at
225 room temperature for 20 minutes in dark, according to the protocol. The bound HRP
226 conjugate was detected by adding 3,3',5,5'-Tetramethylbenzidine -one ready to use (Sigma
227 Aldrich), and incubated in a dark place at room temperature for 20 minutes. The presence of
228 the immune-complex was demonstrated by the development of blue colour and the enzymatic
229 reaction was stopped by additional 100 $\mu\text{L/well}$ 2N H_2SO_4 . Finally, the optical density
230 reading was taken with a micro plate reader (Victor3 1420 multilabel counter, PerkinElmer) at
231 450 nm, and corrected subtraction was used at 550nm for correcting imperfections on the
232 plate.

233 2.6. Immunohistochemistry (IHC)

234 Fixed tissues ($n=4$) from each group were incubated in 20 % glucose solution for 4 hours and
235 washed twice with PBS solutions for 10 minutes. The 12-14 μm roach liver tissue sections
236 were cut in a cryostat. After washing, the slides were incubated with mouse anti-PARK7/DJ-1
237 biotinylated primary antibody (0.45 $\mu\text{g/mL}$, R&D systems) for overnight at 4°C. The slides
238 were washed twice again with PBS then were incubated with avidin Alexa 488 in 1:1000
239 dilution for 2 hours at 4°C. After washing with modified Dulbecco PBS (DPBS), the nuclei
240 were stained with Hoechst (1 $\mu\text{g/mL}$ in DPBS) for 10 min at room temperature. The slides
241 were washed with DPBS and covered with fluorescent mounting medium. The stained tissue
242 was analyzed with a Leica DMI8 confocal microscope (BioMarker Ltd, Hungary).

243 2.7. Serum lipid measurements

244 One mL blood was collected from the anaesthetized fish ($n=4$ in each groups) with a closed
245 blood collection system without anticoagulant. Blood samples were centrifuged for 10
246 minutes at $1500 \times g$ and free cholesterol, HDL-, LDL-cholesterol and triglyceride were
247 measured by automatized enzymatic and colorimetric methods from serum using Roche
248 Integra 800 System (La-Roche Ltd.). Measurement were achieved according to the
249 instrument's manual.

250 2.8. Cell stress array kit

251 Serum from control and treated fish ($n=3$ in each groups) was collected after 42 days
252 progestogen treatment and was processed for cell stress array studies (Human Cell Stress
253 Array Kit, R&D Systems). Serum was mixed with PBS containing protease inhibitor cocktail
254 (Sigma Aldrich). Triton X-100 was added to reach the final concentration of 1%. Samples
255 were centrifuged at $10,000 \times g$ for 5 minutes to remove cell debris. We performed total
256 protein measurement to ensure the identical handling of all samples as follows. Array

257 membranes were blocked for 1 h and incubated with the reconstituted detection antibody
258 cocktail for another 1 h at room temperature. Then, the membranes were immersed in 1 mL of
259 samples at 2–8°C overnight on a rocking platform. After washing with buffer three times and
260 adding HRP-conjugated streptavidin to each membrane, we exposed them to a
261 chemiluminescent detection reagent (Amersham Biosciences, Hungary), then we put an X-ray
262 film on top of the membranes and developed them in a dark cassette. The developed films
263 were scanned and pixel intensities of the bands were determined by using Fiji ImageJ
264 software. Pixel intensities of the bands of interest were normalized to the control.

265 *2.9. Statistical analysis*

266 Statistical analysis was made using the IBM SPSS Statistics Version 20 (IBM Magyarország
267 Kft.) software. Differences between starting and final body lengths and weights such as in the
268 case of somatic indexes of different treatments were compared by non-parametric Kruskal-
269 Wallis tests. The differences in the amount of the VTG, DJ-1 protein, cholesterol, LDL-,
270 HDL-cholesterol and triglyceride between the experimental groups (control, 10-, 50-, 500
271 ng/L mixture of progestogen treated groups) were analyzed. Normality of the dataset was
272 checked using the Kolmogorov-Smirnov test, homogeneity of variances between groups was
273 tested using Levene's statistic. Analysis of variance was performed with independent-samples
274 T-probe and one-way ANOVA with Scheffe post hoc tests. Differences were considered
275 statistically significant at $p < 0.05$.

276 **3. Results**

277 *3.1. Total morphology*

278 A slight growth and small weight losses were detected during the 42 days exposure period in
279 all cases but these changes did not show significant differences. However, the data of somatic
280 index measurements revealed significant differences between treated and control groups
281 (Fig.1). The relative change in brain weight to the percentage of total body weight (BSI) did
282 not differ among the four groups (control: 0.581 ± 0.23 ; 10 ng/L: 0.720 ± 0.19 ; 50 ng/L:
283 0.646 ± 0.18 ; 500 ng/L: 0.704 ± 0.21), but the other three variables (LSI, GSI, KSI) showed
284 significant alterations due to the progestogen treatments. Fig.1 present that the value of LSI
285 (10 ng/L: 2.059 ± 0.23 ; 50 ng/L: 1.863 ± 0.46 ; 500 ng/L: 1.796 ± 0.76) and KSI (10 ng/L:
286 0.786 ± 0.06 ; 50 ng/L: 0.703 ± 0.08 ; 500 ng/L: 0.788 ± 0.08) significantly increased in treated
287 groups compared to the control value of LSI (1.088 ± 0.28) and KSI (0.563 ± 0.08) in roach.
288 However, there were no significant differences between the three treated groups. At the same
289 time, mean GSI value in the 500 ng/L group was only the half (2.184 ± 1.27) of the measured
290 and calculated values in the other three groups (control: 4.620 ± 1.19 ; 10 ng/L: 4.672 ± 1.02 ; 50
291 ng/L: 4.141 ± 0.62). Statistically significant differences were also observed between control
292 and treated as well as variously exposed groups suggesting a concentration dependent effect
293 of progestogens.

294 3.2. *Molecular changes*

295 The VTG level as a general biomarker was measured with ELISA. The result of these
296 experiments show that the mixture of progestogens induced an increase in the VTG level in
297 the liver of female fish (Fig.2). However, statistically significant (one-way ANOVA, $p < 0.05$)
298 increase was observed only in the 500 ng/L group (treated: 48.62 ± 7.21 and control:
299 33.65 ± 4.03 ng/mg tissue). Furthermore, the progestogen treatment had physiological effects
300 at lower concentrations (10 and 50 ng/L) also, which emerges the need to find a more
301 sensitive and/or effectiveness biomarker for tracing its disruption in fish. Brain and liver
302 samples were analyzed by sandwich ELISA to determine the level of the DJ-1 protein, as
303 possible indirect biomarker for steroid contamination. Fig.3A shows the progestogen induced
304 increase of DJ-1 in the brain samples derived from treated groups. In each treated group, the
305 increase of DJ-1 level (10 ng/L: 0.54 ± 0.13 ; 50 ng/L: 0.50 ± 0.07 ; 500 ng/L: 0.38 ± 0.08 ng/mg
306 tissue) was significant compared to control fish (0.20 ± 0.04 ng/mg tissue). Interestingly, the
307 largest change was observed at 10 ng/L concentration which attenuated with increasing
308 steroid concentrations. Fig.3B represents significant changes in the DJ-1 protein level of
309 treated groups (10 ng/L: 3.64 ± 0.30 ; 50 ng/L: 3.65 ± 0.69 ; 500 ng/L: 4.22 ± 0.45 ng/mg tissue)
310 compared to the control (0.54 ± 0.25 ng/mg tissue) in the liver. Contrary to the data obtained
311 on the brain an increasing tendency in the level of DJ-1 protein was observed with the
312 increasing steroid concentrations.

313 The IHC experiment demonstrates the spatial distribution of the DJ-1 protein (green colour)
314 visualized by confocal microscopy. Fig.4A represents a typical picture about control group
315 where similar sized immunopositive particles showed a homogeneous distribution in the
316 hepatic cells. We observed that in the 500 ng/L treated group (Fig.4B), the amount of the
317 immunoreactive DJ-1 protein increased slightly and it aggregated around the nuclei of the
318 cells (blue colour).

319 After that we performed the serum cholesterol, LDL-, HDL-cholesterol and triglyceride level
320 measurements (Fig.5). By comparing the control with the 500 ng/L steroid treated group we
321 observed that the serum cholesterol (control: 7.06 ± 1.55 ; 500 ng/L: 4.63 ± 0.60 mmol/L) and
322 LDL (control: 1.21 ± 0.57 ; 500 ng/L: 0.43 ± 0.29 mmol/L) levels were significantly decreased
323 due to treatment. However, there was no significant differences in the level of serum HDL-
324 cholesterol (control: 2.10 ± 0.43 ; 500 ng/L: 1.67 ± 0.98 mmol/L) and triglyceride (control:
325 1.85 ± 0.16 ; 500 ng/L: 2.06 ± 0.60 mmol/L).

326 Finally, the level of 26 proteins were measured parallel by a stress array kit before and after
327 progestogen treatment. These proteins are involved in various biological processes such as:
328 metabolic (FABP-1; PON1; PON3), apoptotic (BCL-2; Cytochrome c; Phospho-JNK Pan;
329 Phospho-p38a), antioxidative (PON2; Thioredoxin-1; SIRT2; SOD2), stress (HIF-1 alpha;
330 HIF-2alpha; Phospho-HSP27; HSP60; HSP70; NFKB1; Phospho-p53), cell differentiation
331 (ADAMTS1; Cited-2; Dkk-4; p21/CIP1; p27), catalytic (Carbonic anhydrase IX; IDO) or
332 anti-inflammatory (COX-2). We compared the serum derived samples of the control and 500
333 ng/L groups. The members of protein groups performing different physiological functions did
334 not show the same changes due to the mixture of progestogen exposure because proteins
335 showing decreased, unchanged or increased levels. Changes in the protein content were
336 summarized in Table 1. Noticeable decrease were observed in FABP-1 (-42.0%), NFKB1 (-

337 40.8%) and Dkk-4 (-63.7%) protein levels. The PON1 (-2.3 %), BCL-2 (13.8 %), Cytochrome
338 c (-4.8 %), Phospho-JNK Pan (-15.1 %), PON2 (-13.8 %), Thioredoxin-1 (-8.3 %), Phospho-
339 HSP27 (17.3 %), HSP60 (8.4 %), HSP70 (-13.0 %), Phospho-p53 (8.1 %), ADAMTS1 (16.1
340 %), Cited-2 (0.9 %), p21/CIP1 (-20.2 %), Carbonic anhydrase IX (28.4 %), IDO (23.7 %),
341 COX-2 (8.4 %) proteins could be identified but alterations were not remarkable. However, the
342 level of PON3 (43.3 %), Phospho-p38a (40.6 %), SIRT2 (482.6 %), SOD2 (81.5 %), HIF-1
343 alpha (35.4 %), HIF-2alpha (39.9 %), p27 (111.7 %) proteins were markedly increased.

344 4. Discussion

345 The effect of progestogen treatment showed some tendencies affecting the body length and
346 weight, however these data were not verifiable statistically. This observation could be
347 explained with the relatively short exposure period, and/or the relatively low number of
348 specimens used for the analyses. At the same time, the hormone exposure resulted in
349 significant changes in the somatic indices of several organs. The liver and the kidney showed
350 significant growth in the treated groups already at 10 ng/L concentration which was not
351 affected further by increasing progestogen concentration. In contrast to that the
352 gonadosomatic indices showed concentration dependent decrease which was significant at the
353 highest hormone concentration (500 ng/L). In a previous study, kidney hypertrophy were also
354 observed due to ≥ 40 ng/L LNG treatment on female sticklebacks (Svensson et al., 2013).

355 VTG genes are present in both female and male fish, but are normally only expressed in
356 mature females, which have high enough plasma 17 β -estradiol levels. VTG is suitable
357 biomarker to detect the estrogenic or androgenic substances (i.e. 17-alpha-ethynylestradiol,
358 17-beta-trenbolone, dihydrotestosterone) in fish where its level shows increases to estrogens
359 and decreases to androgens (Miracle et al., 2006; Runnalls et al., 2015; Shilling and Williams,
360 2000). But unfortunately, VTG shows an alternating level depending on the type of
361 progestogen and the applied concentrations. For example, using 55 ng/L DRO increases the
362 VTG level, but 55 ng/L DRO coadministered with 3.5 ng/L PRG decreases it in female
363 zebrafish. DRO treatment at the concentration of 553 ng/L decreases but 553 ng/L DRO and
364 33 ng/L PRG applied together increases the VTG level (Zucchi et al., 2014). Furthermore,
365 according to other studies 0.5 and 5 ng/L LNG had no effect but 25 ng/L did already cause a
366 significant increase, while 100 ng/L concentration significantly decreased the plasma VG
367 level in fathead minnow (Runnalls et al., 2015; Runnalls et al., 2013). We observed that the
368 mixture of DRO, LNG and PRG induced an increased VTG level in the liver of female roach
369 but its significant increase was observed only in the 500 ng/L group. Based on the above, we
370 suggested that due to variable effects of progestogens the VTG cannot be used as indicator to
371 reveal progestogens contamination.

372 There are several factors that are targeted by both the steroids and the DJ-1 chaperon protein.
373 Both DJ-1 and NRF2 were found to regulate androgen receptors (Niki et al., 2003; Schultz et
374 al., 2014; Takahashi et al., 2001). DJ-1 inhibits PSF, which normally has transcriptional
375 silencing activity and thereby stimulates the neuronal apoptosis (Xu et al., 2005).
376 Furthermore, it is observed that PSF negatively regulates the progesterone receptor (Dong et
377 al., 2005). In addition, DJ-1 has a neuroprotective effect due to its ability to stabilize the
378 NRF2 protein, the antioxidant transcriptional master regulator and thereby prevents cell

379 apoptosis induced by oxidative stress (Clements et al., 2006). Due to the progestogen
380 treatment the DJ-1 protein level increased significantly in the brain and liver of the fish,
381 although the concentration in the brain was substantially lower than in the liver. We also
382 observed a parallel decrease in the serum cholesterol and LDL levels in hormone treated
383 animals. The results suggest first the effects of DJ-1 in cholesterol homeostasis of fish. In DJ-
384 1-knockdown cells and DJ-1-knockout mice a reduced expression of the LDLR-gene was
385 observed and increase in the cholesterol and LDL serum level was reported (Dong et al.,
386 2005; Yamaguchi et al., 2012). It is suggested therefore that the decrease in the serum LDL
387 level induced by mixture of DRO, LNG and PRG and DJ-1 was mediated through stimulation
388 of the expression of LDLR, which facilitates reuptake subsequently. We hypothesized that
389 progestogens might also directly affect the expression of DJ-1 protein and thereby indirectly
390 stimulate LDLRs (see Fig.S1). Thus, the DJ-1 protein could be able to a more sensitive
391 indicator than VTG in progestogen contamination.

392 In the next experiments, using a Cell Stress Array kit we assessed the effect of mixture of
393 progestogens on the expression of 26 proteins. One group of proteins including PON3,
394 Phospho-p38a, SIRT2, SOD2, HIF-1 alpha, HIF-2alpha and p27 were upregulated upon
395 progestogen treatment. HIF-1 functions as a master regulator of cellular and systemic
396 homeostatic response to hypoxia by activating transcription of many genes, including those
397 involved in energy metabolism, apoptosis, and other genes whose products increase oxygen
398 delivery or facilitate metabolic adaptation (Iyer et al., 1998; Oldham et al., 2015; Semenza,
399 2010; Wang et al., 1995). The PON3 encoded protein is secreted into the bloodstream and
400 associates with HDL. The protein also rapidly hydrolyzes lactones and can inhibit the
401 oxidation of LDL. The observation was in accordance with the lowered level of LDL
402 measured from the serum (Shamir et al., 2005). Phospho-p38a is the mitogen-activated
403 protein kinase 14, the protein encoded by this gene is a member of the MAP kinase family.
404 MAP kinases act as an integration point for multiple biochemical signals, and are involved in
405 a wide variety of cellular processes such as proliferation, differentiation, transcription
406 regulation and development. This kinase is activated by various environmental stresses and
407 pro-inflammatory cytokines (Eckert et al., 2003; Grossi et al., 2014). SIRT2-like family
408 deacetylases are involved in the normal ageing process through their role in resistance to
409 cellular stress (Maxwell et al., 2011).

410 The NFKB1 and FABP-1 were downregulated, NFKB1 is a rapidly acting primary
411 transcription factor found in all cell types. It is involved in cellular responses to stimuli such
412 as cytokines and stress, and plays a key role in regulating the immune response to infection
413 (Baeuerle, 1991). The roles of FABP-1 include fatty acid uptake, transport, and metabolism. It
414 is binding cholesterol and enhances in vitro cholesterol transfer between the plasma
415 membrane and microsomes to stimulate cholesterol esterification by acyl-CoA cholesterol
416 acyltransferase in vitro. FABP1 levels may act as a circulating biomarker of adiposity and
417 insulin resistance related metabolic diseases (Huang et al., 2015). TG was independently
418 associated with serum FABP1 (Peng et al., 2015). Additionally, hypertriglyceridemia and low
419 HDL-cholesterol were significantly correlated to serum FABP1 levels after adjusting for age,
420 gender and BMI (Shi et al., 2012). It increases the fatty acid transport, lipogenesis and lipid
421 transport.

422 There were no changes in the protein levels of the HSP superfamily. Our results obtained
423 during chronic progestogen treatment are in accordance with those published earlier. It was
424 suggested that the HSPs adapt to the external environmental factors and although their levels
425 increase during an acute treatment, chronic exposure restores them to the normal interval
426 (Pechenino and Frick, 2009; Xie et al., 2014).

427 Our results show that mixture of progestogens cause significant morphological and molecular
428 changes in this model organism, despite the relatively short exposure time (42 days) and the
429 fact that the concentration of the progestogens in one of the treatment groups (10ng/L) was
430 relevant concentration range in the worldwide watercourses and water body.

431 5. Conclusion

432 Overall, some of the progestogens (e.g. DRO, LNG and PRG) has also been found in
433 freshwaters and drinking water, which is a warning sign that the current handling of
434 pharmaceuticals may lead to future health and environmental problems. Our analysis showed
435 that the mixture of progestogens directly affect the DJ-1, cholesterol, LDL levels and several
436 other proteins with various functions (e.g. PON3, Phospho-p38a, SIRT2, SOD2, HIF-1 alpha,
437 HIF-2alpha, p27 and NFKB1, FABP-1) implying the possibility of a direct connection
438 between them. It is suggested that the currently low (few ng/L) progestogen concentrations
439 already has biological risk in the catchment area of the largest shallow lake in Central Europe
440 both in present and future.

441 6. Authors contribution

442 The study was design by MG and PZ, and the experimental work was performed by MG, ZZ
443 and FI. Statistical analyses was made by ZZ and TP, with additional interpretation by MG.
444 The manuscript was written by MG, TP and PZ with feedback from LS and KT.

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451 9. Figure legend

452 Fig.S1 – Relationship between steroids, DJ-1 protein and their common target molecules. The
453 figure represents the possible co-effect to the metabolic balance (LDL reuptake from serum),
454 nuclear E2-related factor 2 (NRF2) and polypyrimidine tract-binding protein-associated
455 splicing factor (PSF) (Clements et al., 2006; Dong et al., 2005; Schultz et al., 2014;
456 Yamaguchi et al., 2012; Xu et al., 2005).

457 Fig.1 – Somatic index values of the control and different treated groups (Fig.1A–brain
458 somatic index, Fig.1B–liver somatic index, Fig.1C–gonadosomatic index, Fig.1D–kidney
459 somatic index). The asterisk (*) indicates significant differences between the control and
460 treated group at $P<0.05$ significance level. The hashmark (#) denotes differences between the
461 treated groups at $P<0.05$ significance level.

462 Fig.2 – Changes of VTG level due to the different concentration of progestogen exposure.
463 The vertical axis shows the concentration of VTG [ng/mg tissue], the horizontal axis
464 illustrates different groups (n=3). Significant difference was found between the control and
465 500 ng/L group, it is denoted with an asterisk (*) (SEM, $P<0.05$). Statistics were as follows:
466 one-way ANOVAs, $F(3)=8.22$, $P<0.01$, Scheffe post hoc test showed significant difference
467 between the control and 500 ng/L groups ($P<0.009$).

468 Fig.3 – Sandwich ELISA measurement of the DJ-1 protein concentration in *Rutilus rutilus*
469 brain (A panel) and liver (B panel). Vertical axis shows the concentration of DJ-1 protein
470 [ng/mg tissue], the horizontal axis illustrates different groups (n=6). The DJ-1 concentration
471 was significantly increased in treated groups compared to the control. The asterisk (*)
472 indicates significant differences between the control and treated groups with the following
473 significance levels: *** $P<0.001$, ** $P<0.01$, * $P<0.05$. In the brain the independent-samples
474 T-test showed significant difference between control and 10 ng/L groups [$t(10)=-3.08$,
475 $P<0.05$], control and 50 ng/L [$t(10)=-4.80$, $P<0.01$] ; control and 500 ng/L groups [$t(10)=-$
476 2.335 , $P<0.05$]. In the liver the independent-samples T-test showed significant difference
477 between the control and 10 ng/L groups [$t(10)=-11.47$, $P<0.01$], control and 50 ng/L [$t(10)=-$
478 6.41 , $P<0.01$]; control and 500 ng/L groups [$t(10)=-9.71$, $P<0.01$]. Significant differences
479 between treated groups were not observed neither in the brain nor in the liver.

480 Fig.4 – Confocal microscopic images of *Rutilus rutilus* liver tissue section. Blue (Hoechst)
481 represents the cell nuclei, green illustrates the spatial distribution of the DJ-1 protein detected
482 by immunohistochemistry. A panel shows the control group, B panel represents the 500 ng/L
483 steroid treated group. The pictures were taken using a 40X magnification, the enhanced areas
484 represent a 63X magnification. Scale bar represents 5 μm and 50 μm on 40- and 63X
485 magnification, respectively.

486 Fig.5 – The effect of steroid treatment on the serum lipid levels. The vertical axis shows the
487 level of serum parameters [mmol/L], the horizontal axis illustrates different groups (n=4).
488 White columns represent control values and black ones show the 500 ng/L group values. The
489 serum cholesterol and LDL-cholesterol were significantly decreased in the 500 ng/L group
490 compared the control. The asterisk (*) denotes significant differences between the control and
491 treated group at a $P<0.05$ significance level. No significant changes were observed between
492 the groups in the HDL-cholesterol and triglyceride levels. Statistics used were as follows: in
493 case of cholesterol and triglyceride the Levene's test showed unequal variances, so
494 independent-samples Mann-Whitney U test was used. Significant differences ($P<0.05$) were
495 observed between the control and 500 ng/L groups in cholesterol levels, but not in triglyceride
496 levels ($P=1.00$). In case of the other analytes independent-samples T-test was performed,
497 which showed significant difference in the LDL-cholesterol level between the control and the

498 500 ng/L groups [$t(7)=2.65$, $P<0.05$], but not in the levels of HDL-cholesterol [$t(7)=0.69$,
499 $P=0.52$].

500 Table 1 – Summary of the 26 measured proteins. We classified the proteins based on their
501 biological function (first two columns). The third column shows the sequence identity [%]
502 between the protein sequences of the human and *Danio rerio* derived from the on line BLAST
503 database. The last column represents the changes [%] in the serum triggered by the steroid
504 treatment compared to the control. The coefficient of variation (CV) was calculated by using
505 the following equation: $c_{\text{treated}}/c_{\text{control}}*100$.

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644

Table1

Biological function	Common Name	Pubmed Number	Sequence equality [%]	Ident [%]	CV [%]
Metabolism	FABP-1	P00505.3	99	65	-42,0
	PON1	P27169.3	99	49	-2,3
	PON3	NP000931.1	99	49	43,3
Apoptosis	BCL-2	P10415.2	99	68	13,8
	Cytochrome c	NP061820.1	100	85	-4,8
	Phospho-JNK Pan	AAC50127.1	99	87	-15,1
	Phospho-p38a	P46108.2	100	86	40,6
Antioxidant capacity	PON2	Q15165.3	94	55	-13,8
	Thioredoxin-1	AAF86466.1	97	63	-8,3
	SIRT2	AAK51133.1	94	66	482,6
	SOD2	AAH16934.1	100	85	81,5
Stress	HIF-1 alpha	Q16665.1	99	57	35,4
	HIF-2alpha	NP001421.2	63	55	39,9
	Phospho-HSP27	P04792.2	93	58	17,3
	HSP60	P10809.2	96	87	8,4
	HSP70	P08107.5	100	86	-13,0
	NFKB1	CAB94757.1	99	56	-40,8
	Phospho-p53	BAC16799.1	84	57	8,1
Cell differentiation, Proliferation	ADAMTS1	AAF15317.1	93	60	16,1
	Cited-2	P05549.1	100	56	0,9

	Dkk-4	Q9UBT3.1	90	51	-63,7
	p21/CIP1	Q9BXP2.1	87	38	-20,2
	p27	BAA25263.1	52	60	111,7
Catalitic enzymes	Carbonic anhydrase IX	EAW58359.1	93	38	28,4
	IDO	AEF30540.1	90	45	23,7
	COX-2	P35354.2	93	57	8,4

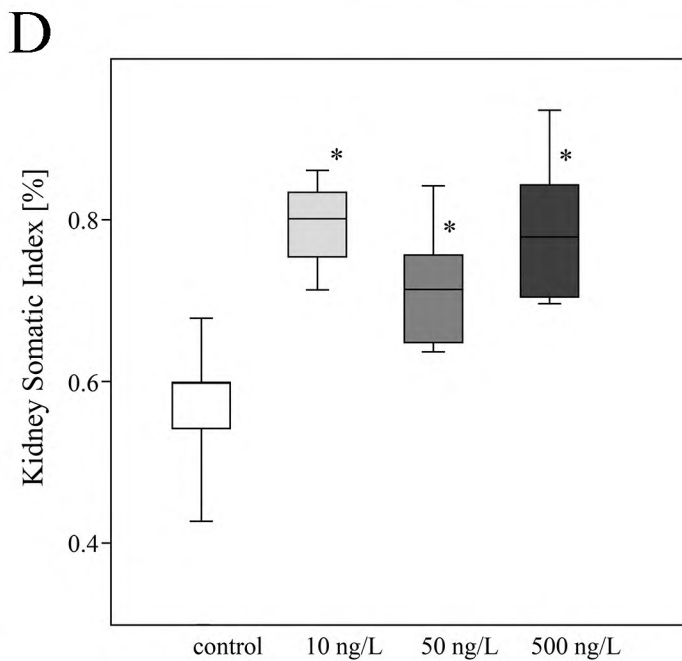
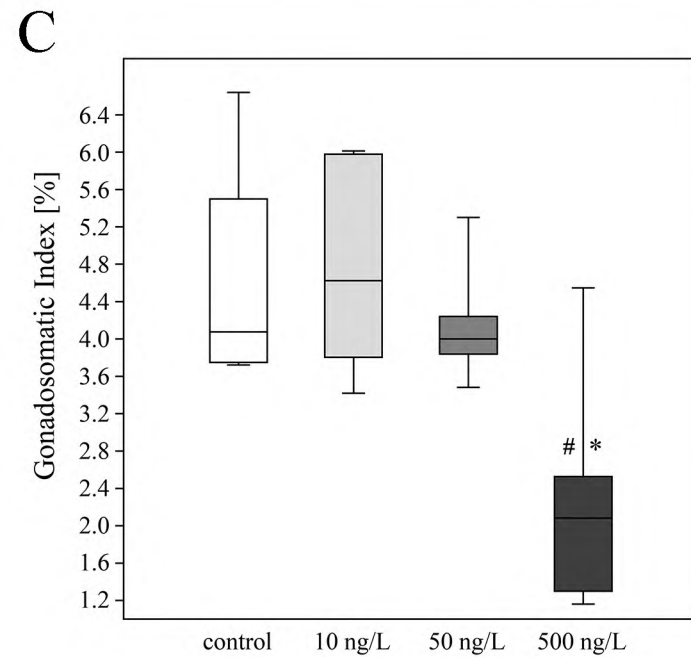
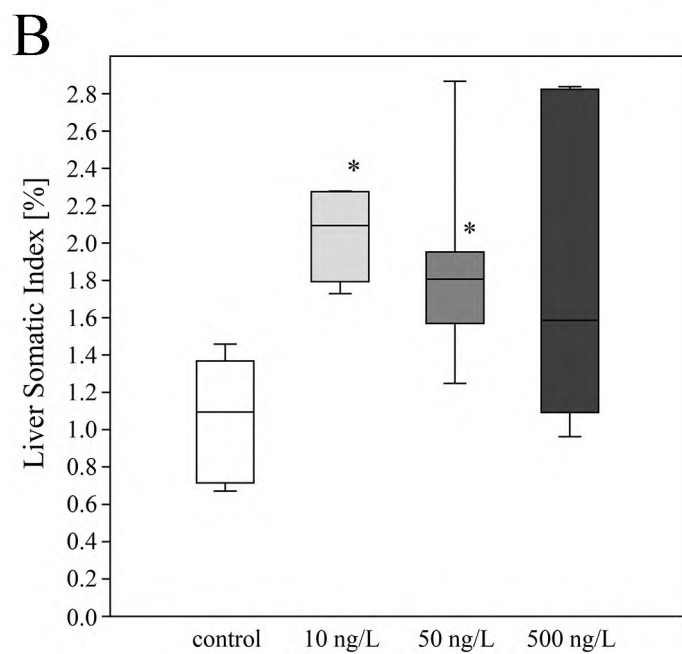
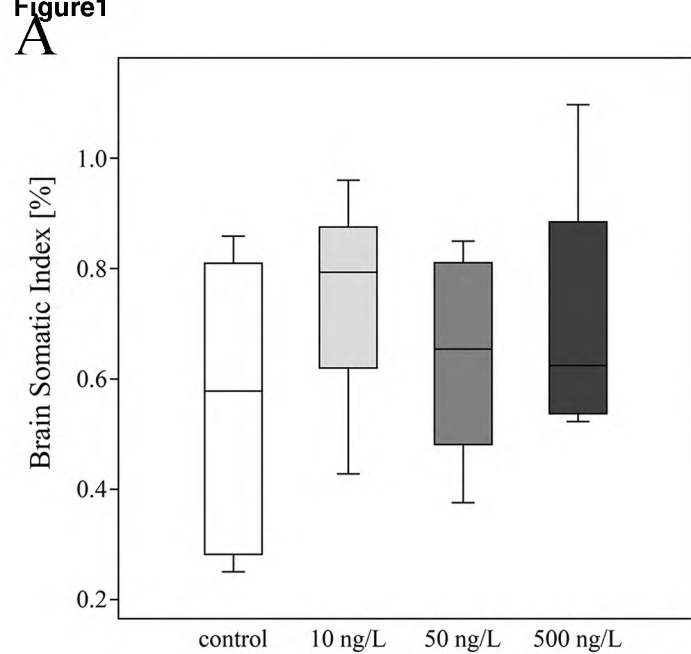
Figure 1

Figure 2

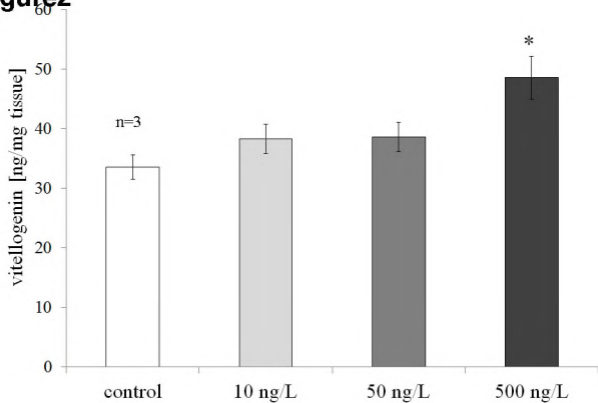
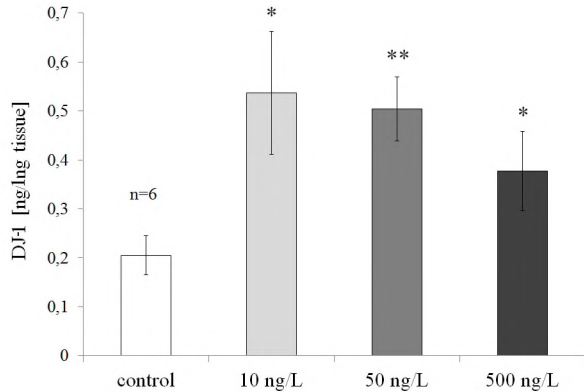
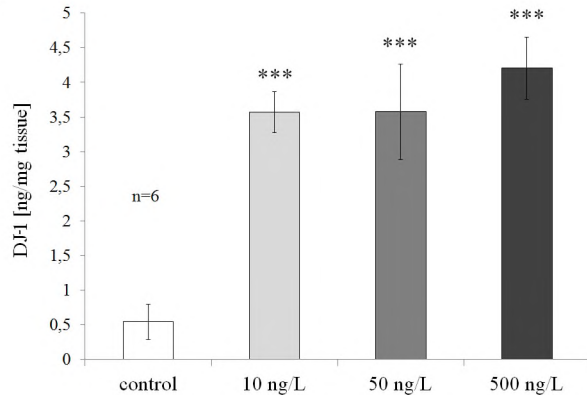


Figure 3
A



B



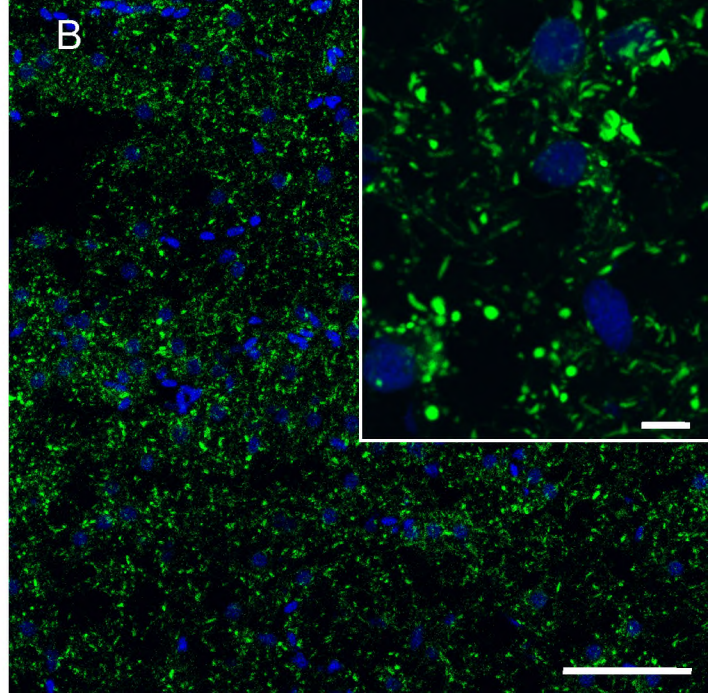
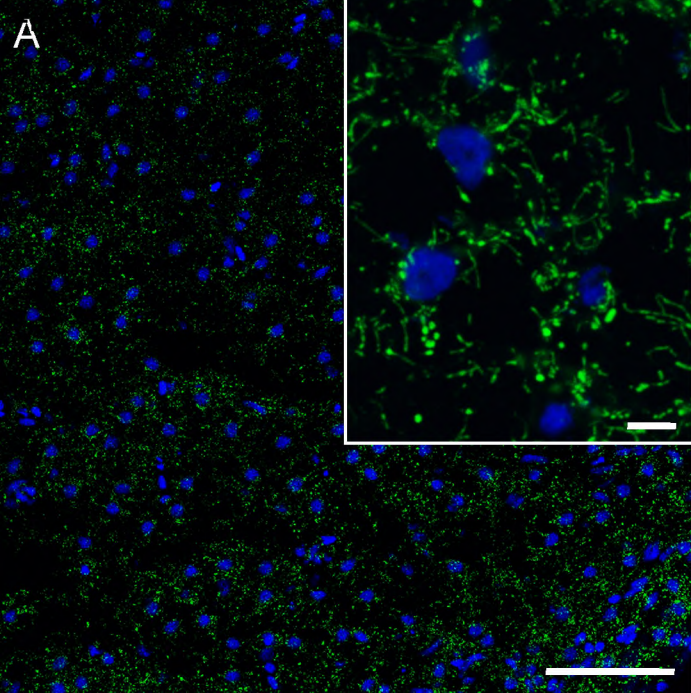


Figure 5

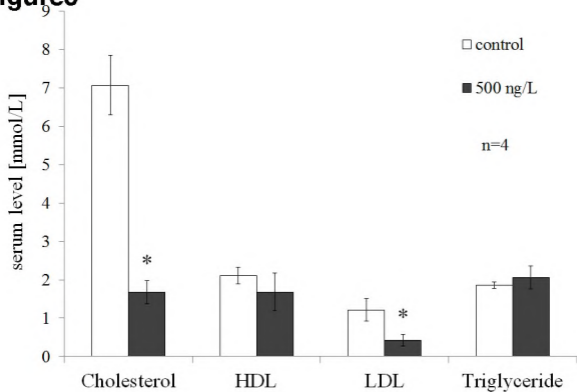


Figure S1

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