https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

Effects of environmental cocaine concentrations on the skeletal muscle of the European eel (*Anguilla anguilla*).

Anna Capaldo[1*], Flaminia Gay[2], Marilena Lepretti[2], Gaetana Paolella[2], Stefania Martucciello [2], Lillà Lionetti [2,3], Ivana Caputo [2,3] and Vincenza Laforgia[1]

[1] Department of Biology, University of Naples Federico II, Via Cinthia, Edificio 7, 80126 Naples, Italy

[2] Department of Chemistry and Biology "A. Zambelli", University of Salerno, Via Giovanni Paolo II, 132 - 84084 Fisciano - Salerno, Italy

[3] ELFID (European Laboratory for Food-Induced Diseases), University of Naples Federico II, via Pansini 5, 80131 Naples, Italy

Running Title: Cocaine-induced eel muscle changes

Dr. Anna Capaldo: anna.capaldo@unina.it

Dr. Flaminia Gay: fgay@unisa.it

Dr. Marilena Lepretti: mlepretti@unisa.it

Dr. Gaetana Paolella: gpaolella@unisa.it

Dr. Stefania Martucciello: smartucciello@unisa.it

Prof. Lillà Lionetti: llionetti@unisa.it

Prof. Ivana Caputo: icaputo@unisa.it

Prof. Vincenza Laforgia: vincenza.laforgia@unina.it

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

Correspondence: Anna Capaldo.

Department of Biology, University Federico II.

Via Cinthia, Edificio 7, 80126 Naples, Italy.

Tel.: +39 081 2535173; fax: +39 081 679225.

(E-mails: anna.capaldo@unina.it; nevotta16@libero.it)

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

Abstract

This study evaluated the influence of environmental cocaine concentrations on the skeletal muscle of the European eel. Silver eels were exposed to 20 ng L^{-1} of cocaine for fifty days; moreover, control, vehicle control and two post-exposure recovery groups (three and ten days) were made. The eels general health conditions, the morphology of the skeletal muscle and some parameters indicative of the skeletal muscle physiology were evaluated: the muscle whole protein profile; the activities of cytochrome oxidase and caspase-3; the serum levels of creatine kinase, lactate dehydrogenase and aspartate aminotransferase. Cocaine-exposed eels appeared hyperactive but showed the same health status compared to the other groups. Their skeletal muscle, instead, showed serious injuries, still present ten days after the interruption of cocaine exposure. Except the expression levels of the main muscle proteins, that appeared unchanged, all the examined parameters showed alterations that continued ten days after the interruption of cocaine exposure. These results showed that, also at low environmental concentrations, cocaine heavily damaged the morphology and the physiology of the eel skeletal muscle. Considering the complex life cycle of the eel, involving a long reproductive migration needing a healthy skeletal tissue, and the condition of decline typical of this species, also caused by water pollutants, our results suggest that cocaine could threaten the ability of the eel to successfully migrate and reproduce. Moreover, since the skeletal muscle is the edible part of the eel, an effect of cocaine on the nutritional value of this tissue could be hypothesized.

Keywords: Eel caspase; Eel cytochrome oxidase; Eel muscle protein profile; Eel serum enzymes; Eel skeletal muscle; Environmental cocaine

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

1. Introduction

Many illicit drugs and their breakdown products are detected in surface waters (Daughton, 2011; Kasprzyk-Hordern et al., 2008; Loganathan et al., 2009; Pal et al., 2013; Rosi-Marshall et al., 2014; Zuccato et al., 2005, 2008) and in seawaters (Aligizakis et al., 2016; Seabra Pereira et al., 2016) throughout the world, due to both the enormous worldwide use of these substances, and an insufficient wastewater treatment. The environmental fate and ecological effects of the illicit drugs are not well understood; however, the first studies showed toxic effects to the aquatic organisms, as expected for a constant exposure to substances having strong pharmacological activities (Pal et al., 2013; Rehm et al., 2006). Indeed cytotoxic, genotoxic (Binelli et al., 2012), and sub-lethal (Parolini et al., 2013) effects were induced in the freshwater mussel *Dreissena polymorpha* by environmental cocaine, and cocaine metabolite benzoylecgonine, respectively. Moreover, alterations in the oxidative status, leading to increase of lipid peroxidation, protein carbonylation and DNA damage were observed after the exposure to environmental Δ-9-tetrahydrocannabinol (Parolini and Binelli, 2014). Since data about the effects of environmental illicit drugs on fish were lacking, we started a study aimed to evaluate and compare the effects on fish of the most frequent illicit drugs in surface waters. Our first studies, concerning the effects of environmental concentrations of cocaine on the European eel (*Anguilla anguilla*), selected as model species, showed that a chronic exposure induced the bioaccumulation of cocaine in its tissues (Capaldo et al., 2012) and alterations in its endocrine system (Gay et al., 2013). Since these results suggested histological changes as well, we also evaluated the condition of the peripheral tissues. Indeed the histological features may be considered suitable biomarkers in the evaluation of the health of fish exposed to contaminants (Camargo and Martinez, 2007; Capkin et al., 2009; Ebrahimi and Taherianfard, 2011; Fanta et al., 2003). Our first results, showing changes in the skin and the intestine, were presented in a

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

previous paper (Gay et al., 2016); in this paper, we describe our results concerning the skeletal muscle.

In the eels, as in most teleosts, the skeletal muscle has red and white muscle fibres, organized to form red and white muscles involved in two kinds of swimming activity. The red muscle, having aerobic, slow-contracting, fibres, is related to sustained activity, while the white muscle, having anaerobic, fast-contracting and fast-fatiguing fibres, is related to short, strong bursts of motion (Mumford et al., 2007; Tesch, 2003). The red muscle is confined to a zone beneath the lateral line whereas the white muscle makes up the bulk of the fish (Altringham and Ellerby, 1999). The skeletal muscle was chosen because it bioaccumulates cocaine in a large amount after a chronic exposure (Capaldo et al., 2012). Moreover, the peculiar life cycle of the European eel makes very interesting the study of the health condition of this tissue. Indeed, at the silver stage, the eel has to migrate across 6000 km without feeding to the spawning area of the Sargasso sea (Tesch, 2003; van Ginneken and Maes, 2005). This means that, in addition to sufficient energy reserves, the eel needs a healthy skeletal muscle and an efficient aerobic metabolism, to complete successful swimming migration. Finally, the European eel is an edible species, whose farming currently supplies more than the 80% of the world's consumption of the species (Nielsen and Prouzet, 2008). Since the skeletal muscle is the edible part of the eel, the study of the changes induced by the aquatic contaminants is informative from a human health point of view. The effects of the chronic exposure to cocaine were observed by evaluating the general health conditions of the eels, the general morphology of the skeletal muscle and a number of different parameters indicative of the skeletal muscle physiology: the muscle whole protein profile, as marker of the expression levels of the main muscle proteins (Fedorova et al., 2009); the cytochrome oxidase (COX) activity, as marker of the oxidative metabolism (Hovda et al., 1992); the caspase-3 activity as a marker of apoptosis activation, since the caspase-3 is the major executioner caspase in the apoptotic pathway (Cohen, 1997);

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

the serum levels of creatine kinase (CK), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST), well established biomarkers of damages to the skeletal muscle (Brancaccio et al., 2010).

2. Materials and methods

2.1. Chemicals

Cocaine free-base was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Ethyl 3-aminobenzoate, methanesulfonic acid salt 98% (MS-222) was purchased from Aldrich Chemical Corporation Inc. (Milwaukee, WI, USA).

2.2. Animals

Adult specimens of European eel (*Anguilla anguilla*) (38.85 ± 0.39 cm; 85.38 ± 1.60 g; mean ± s.d.) (silver eel stage), obtained from a local fish dealer, were acclimated to the laboratory for one month, in 300-L glass aquaria under a natural photoperiod, in dechlorinated, well-aerated tap water, with the following physicochemical conditions: salinity 0, ammonia < 0.1 mg L^{-1}, temperature 15 °C ± 1°C, pH 7.3 ± 0.2, dissolved oxygen 8.1 ± 0.5 mg L^{-1}; mean ± s.d., as previously described (Gay et al., 2016). The water was not recycled but renewed every 24 h**.** Since the eels during the silver stage undergo a natural starvation period, they were not fed. Fish exposure experiment was performed in accordance to EU Directive 2010/63/EU for animal experiments and authorized by the National Committee of the Italian Ministry of Health on in vivo experimentation (Department for Veterinary Public Health, Nutrition and Food Safety). Efforts were made to avoid animal suffering and minimize the number of specimens used. The animals were maintained in accordance with the institutional guidelines for care and use of laboratory animals.

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

150

151 **2.3. Experimental design**

152 After acclimatization, the eels from the aquaria were randomly divided into five groups
153 (control, vehicle control, cocaine exposed and two post-exposure recovery groups), each
154 containing ten specimens. Each group was kept in 300-L glass aquarium, under the previously
155 described conditions. In each aquarium the water was renewed every 24 h. The nominal
156 concentration of cocaine selected (20 ng L^{-1}) corresponded to the mean cocaine concentration
157 detected in surface waters (Castiglioni et al., 2006; Daughton, 2011; Mari et al., 2009; Pal et
158 al., 2013; Postigo et al., 2010; Zuccato et al., 2005, 2008; Zuccato and Castiglioni, 2009). A
159 stock solution of 0.006 mg mL^{-1} cocaine free-base in ethanol was prepared (Gay et al., 2016).
160 The treated group was exposed daily to a nominal concentration of 20 ng L^{-1} cocaine (1 mL of
161 the stock solution, administered directly into the aquarium, every 24 h, after renewing the
162 water). At the same time, a control group was only exposed to tap water and a vehicle control
163 group was exposed daily to the same concentration of ethanol as the treated group, in the same
164 conditions. The treatment lasted fifty days. To verify the recovery ability of the eels, two post-
165 exposure recovery groups were exposed to cocaine, as the treated group, and then deprived of
166 cocaine and exposed to tap water, respectively for three and ten days. The test was carried out
167 in triplicate. The eels were anesthetised with MS-222 at a concentration of 100 mg L^{-1} (Capaldo
168 et al., 2012; Gay et al., 2013, 2016), weighed and measured. Blood was collected from the
169 posterior cardinal vein with a 5 ml syringe, allowed to clot in Eppendorf tubes for 2-4 h,
170 centrifuged for 15 min at 2,000 g, and serum was collected and stored at –22 °C until assayed.
171 The animals were killed by decapitation immediately after collection of blood samples. From
172 each animal, blocks of superficial skeletal muscle tissue were removed from the lateral line
173 region and processed 1) for light microscopy, to evaluate the morphology of the skeletal muscle
174 and the sarcomeric actin filaments or 2) weighed, immediately frozen in liquid nitrogen and

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

stored at −80°C until the evaluation of proteins profile and the measurement of the activities of caspase-3 and COX.

2.4. Histology

To assess the general morphology, the samples of skeletal muscle were fixed in Bouin's solution, dehydrated in graded alcohols, cleared in Histolemon, embedded in Paraplast and cut into 6 μm serial sections. The sections were processed for routine histological analysis and stained with Mallory trichromic stain. To assess the diameter of the red and white fibres, samples of skeletal muscle of the specimens from each experimental group (ten samples for each experimental group) were cut transversely into 5 μm serial sections. The sections were stained by silver impregnation to highlight the reticular argyrophilic fibres (Bradbury and Gordon, 1980; Sheehan and Hrapchak, 1980), with the aid of a commercial kit (Bio-Optica s.p.a, Milan, Italy). Sections were de-waxed, pre-treated with trivalent iron and later treated with ammoniacal solution as silver source, based on the recommendations of the producer. Five sections for each sample tissue (250 sections in all), taken from the middle part of the sample, where the cross-section was largest, were examined. Observations and measurements were performed using a Zeiss Axioskop microscope (Carl Zeiss MicroImaging s.p.a., Milan, Italy). Images were captured with a camera attached to an IBM computer running the Kontron Elektronik KS 300 image analysis system (Carl Zeiss MicroImaging s.p.a., Milan, Italy) and Adobe Photoshop. The lesser diameter of the muscle fibre was used in calculations (Dubowitz, 1985). To assess the morphology of the sarcomeric actin filaments, the sections were de-waxed and stained with 0.1 mg ml^{-1} fluorescein isothiocyanate (FITC)-labelled phalloidin (Sigma-Aldrich, Milan, Italy) for 60 min at room temperature. After washing with PBS, stained sections were observed with an Axioskop 40 fluorescent microscope. Images were acquired and processed using the Axiovision software (Carl Zeiss MicroImaging s.p.a., Milan, Italy).

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

200

201 **2.5. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) of muscle**
202 **proteins**

203 Samples of skeletal muscle were homogenized in cold lysis buffer (50 mM HEPES, pH 7.4, 5
204 mM CHAPS, 5 mM dithiothreitol) by using the T10 basic ULTRA-TURRAX S10N-5G (two
205 cycles of two min each). Homogenates were then centrifuged at 16,000 g for 15 minutes at 4°C
206 to remove tissue debris. The supernatant was transferred into a new tube and protein content
207 was determined by the Bradford Protein Assay (Bio-Rad Laboratories, Milan, Italy). Proteins
208 were then separated on a 10% SDS–page and both a mini-chamber (gel dimension 8.3 cm x 7.3
209 cm) and a large-chamber (gel dimension 16 cm x 16 cm) from Bio-Rad Laboratories (Milan,
210 Italy) were used. In order to well resolve high molecular weight (HMW myosins) and low
211 molecular weight (actins and tropomyosin) proteins, 60 μg of proteins in the mini-chamber and
212 45 μg of proteins in the large-chamber, respectively, were loaded onto a 10% gel. Gels were
213 stained with a solution of Coomassie brilliant blue for 1h. Colorant excess was removed by
214 washing the gels with a destaining solution for 48 h. Gel images were acquired by using the
215 densitometer GS800 from Bio-Rad Laboratories (Milan, Italy).

216

217 **2.6. Cytochrome oxidase (COX) activity**

218 Cytochrome oxidase (COX) activity was measured polarographically with a Clark-type
219 electrode at 25°C in medium containing 30μM cytochrome c, 4μM rotenone, 0.5 mM
220 dinitrophenol, 10 mM Na-malonate, 75 mM HEPES, pH 7.4 (Lionetti et al., 2004). To detect
221 COX activity, 100 mg of skeletal muscle were diluted in Chappel and Perry medium (1mM
222 ATP, 100 mM KCl, 5 mM MgCl2, 1 mM EDTA, 5 mM EGTA, 50 mM HEPES pH 7.4)
223 containing Lubrol PX (225μg/mg protein) and incubated for 30 min in ice to unmask enzyme
224 activity. At the end of the incubation, COX activity in the whole homogenate was measured as

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

oxygen consumed in the presence of 4 mM ascorbate + 0.3 mM tetramethyl-p-phenylenediammine (TMPD) (Barrè et al., 1997).

2.7. Caspase-3 activity assay

Samples of skeletal muscle were homogenized and protein content was determined as above described for SDS-PAGE of muscle proteins. Caspase-3 activity was detected by the Colorimetric Caspase 3 Assay Kit (Sigma-Aldrich, Milan, Italy), according to the manufacturer-provided protocol. The assay is based on the hydrolysis of the synthetic tetrapeptide, acetyl-Asp-Glu-Val-Asp, labelled with p-nitroanilide, pNA). Proteins were incubated for 2 h at 37°C in a reaction mixture containing the labelled substrate (200 µM). Hydrolysis of the labelled substrate results in the release of free p-NA which has an absorbance at a wavelength of 405 nm. Caspase-3 activity was finally expressed as nmol of free pNA normalized for µgram of proteins and time (min). When requested the caspase-3 inhibitor Ac-DEVD-CHO was used at 20 µM.

2.8. Serum enzymes

Creatine kinase (EC 2.7.3.2; adenosine triphosphate: creatine N-phosphotransferase; CK); lactate dehydrogenase (EC 1.1.1.27; L-lactate: NAD^+ oxidoreductase: LDH) and aspartate aminotransferase (EC 2.6.1.1; L-Aspartate: 2-Oxoglutarate Aminotransferase, AST or AspAT; Glutamate Oxaloacetate Transaminase, GOT) were determined with the aid of commercial kits (CK-NAC FL; LDH FL (DGKG); GOT/AST FL; Chema-Diagnostica, Monsano, AN, Italy). The principle of the CK assay is as follows: CK catalyses the conversion of creatine phosphate and ADP to creatine and ATP. ATP and glucose are converted to ADP and glucose-6-phosphate by hexokinase. Glucose-6 dehydrogenase oxidizes glucose-6-phosphate to 6-phosphogluconate, reducing NADP to NADPH. The rate of conversion of NADP/NADPH,

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

monitored at 340 nm, is proportional to CK activity. N-acetl cysteine (NAC) is added as an activator of CK. The principle of the LDH assay is as follows: LDH catalyzes the conversion of pyruvate to L-lactate in presence of NADH, which is converted to $NAD^{+.}$ The rate of conversion of NADH/NAD^{+}, monitored at 340 nm, is proportional to LDH activity. The principle of the AST assay is as follows: AST catalyzes the transaminase reaction between L-Aspartate and α-ketoglutarate. The 2-Oxalacetate formed is reduced to malate in the presence of malate dehydrogenase (MDH). As the reactions proceed, NADH is oxidized to NAD^{+}. The disappearance of NADH per unit time is followed by measuring the decrease in absorbance at 340 nm. The changes in absorbance at 340 nm (ΔA/min) were measured by using the Smart Spec Plus Spectrophotometer (Bio-Rad Laboratories). The CK, LDH and AST activities were expressed as UI L^{-1}, multiplying the ΔA/min by the factor indicated from the producer. The sensitivity/limit of detection were 1UI L^{-1}, 31 UI L^{-1} and 0.463 UI L^{-1} for CK, LDH and AST, respectively.

2.9. Statistical analysis

The values were expressed as means ± standard error of mean (SE). All the data were first tested for normality and homogeneity of variance to meet statistical assumptions; the homogeneity of variance was assessed by the Bartlett test. The data were compared by one-way analysis of variance (ANOVA), followed by the Tukey-Kramer multiple comparison test. All statistical analyses were performed using commercial software (SigmaStat Version 4.0; SPSS); differences were considered significant when $P < 0.05$.

3. Results

3.1. General health condition

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

No mortality was observed throughout the experiment. The eels exposed to cocaine appeared hyperactive compared to the other groups; however, the exposed and the post-exposure recovery eels showed the same health status as the control and vehicle control eels.
Since no differences were observed between the control and the vehicle control eels in general health conditions and in all the parameters evaluated, the description of vehicle control results will be omitted.

3.2. General morphology

In the control eels, the red muscle lied as a wedge along the lateral line, just beneath the skin, whereas the white muscle formed the greatest volume of the body tissue (Fig. 1A). The red fibres appeared rounded and intensely stained (Fig. 1B); the white fibres larger and less intensely stained than red ones (Fig. 1C). Both red and white fibres were surrounded by a reticular connective tissue, more conspicuous in the red muscle (Figs. 1B, C). The white fibres were characterized by different diameters, giving the tissues a typical mosaic appearance; however, mainly large fibres were visible, as expected in the silver stage (Tesch, 2003) (Fig. 1C). In the sarcoplasm (the cytoplasm of the muscle fibre), the contractile apparatus of both types of fibres appeared compact, with myofibrils regularly aligned and parallel each other (Figs. 1B-D). With the silver impregnation technique, the connective reticular tissue appeared weakly argyrophilic in the red muscle (Fig. 1E) and deeply argyrophilic, black stained, in the white muscle (Fig. 1F). No stain was observed in the sarcoplasm of both types of fibres.

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

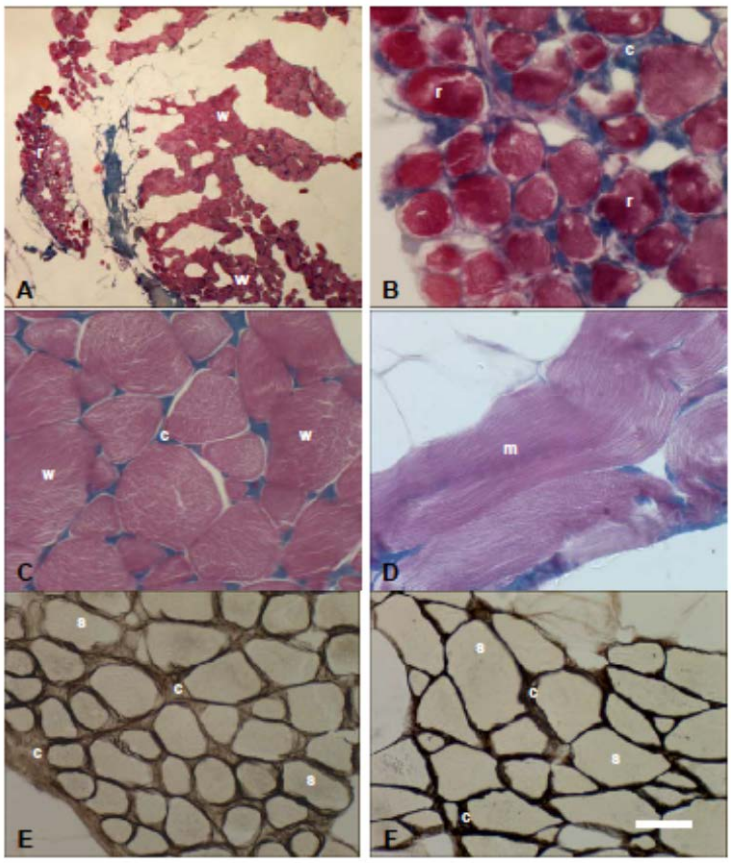
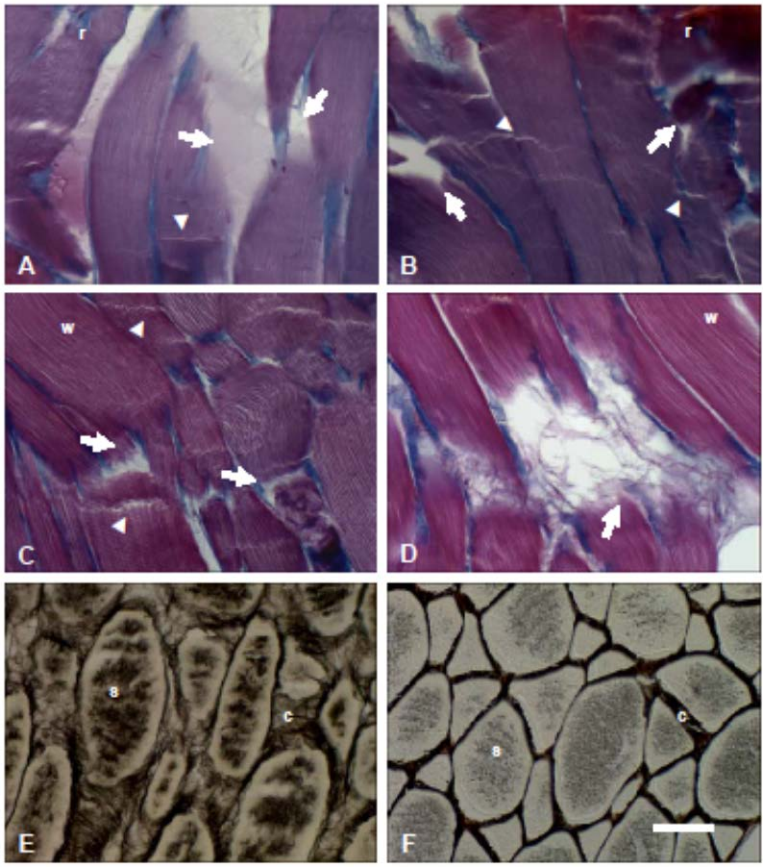


Figure 1. **Light micrographs of the skeletal muscle of *Anguilla anguilla*: control specimens.** (A, B, C, D: Mallory staining; E, F. silver impregnation). (A) The red (r) muscle lied as a wedge along the lateral line, whereas the white (w) muscle formed the greatest volume of the body tissue. (B, C) In transverse section, the red (r) fibres appeared rounded and intensely stained, and the white (w) fibres, larger and less intensely stained. The connective reticular tissue (c) was more abundant in the red muscle. The white fibres had different diameters; both types of fibres had a well organized contractile apparatus, showing, in longitudinal section (D), myofibrils (m) regularly aligned and parallel each other. (E, F) The connective reticular tissue (c) was weakly argyrophilic in the red muscle (E) and deeply argyrophilic, black stained, in the white muscle (F). No stain was observed in the sarcoplasm (s) of both types of fibres. Scale bar: a: 200 µm; b, c, d, e, f: 25 µm.

After the exposure to cocaine, both red (Figs. 2A, B) and white (Figs. 2C, D) fibres showed signs of injury as laceration and trasversal fragmentation. The silver impregnation showed that the sarcoplasm of both types of fibres appeared silver stained (Figs. 2E,F); the red fibres (Fig. 2E) appeared larger than control ones.

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357



311

312 **Figure 2**. **Light micrographs of the skeletal muscle of *Anguilla anguilla*: exposed specimens**. (A, B, C, D:
313 Mallory staining; E, F. silver impregnation). Both red (r) (A, B) and white (w) (C, D) fibres showed signs of injury
314 as laceration (arrow) and trasversal fragmentation (arrowhead). (E, F) In addition to the connective (c) tissue, the
315 sarcoplasm (s) of both the red (E) and white (F) fibres were silver-stained and argyrophilic. Scale bar: 25 µm.

316

317 Three days (Figs. 3A, B) and ten days (Figs. 3C, D, E) after the interruption of cocaine
318 exposure, both red (Figs. 3A, C) and white (Figs. 3B, D, E) fibres showed signs of injury and
319 breakdown. Especially the red fibres (Fig. 3A,C) appeared deeply altered showing signs of
320 swelling, rarefaction of the myofibrils and disorganization of the contractile apparatus. The
321 sarcoplasm of both types of fibres appeared still silver stained ten days after the interruption of
322 cocaine exposure (Fig. 3F).

323

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

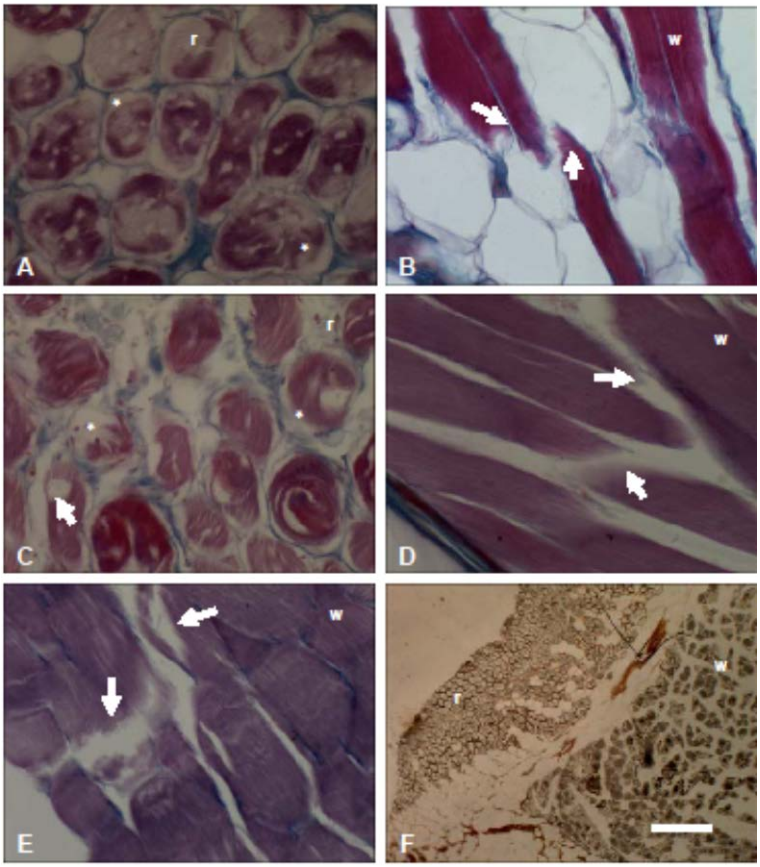


Figure 3. **Light micrographs of the skeletal muscle of *Anguilla anguilla*: post-exposure recovery three and ten days specimens.** (A, B, C, D, E: Mallory staining; F. silver impregnation). Three days (A, B) and ten days (C, D, E) after the interruption of cocaine exposure, both red (r) (A, C) and white (w) (B, D, E) fibres showed signs of injury and breakdown (arrow). (C). The red (r) fibres showed signs of swelling (asterisk), rarefaction of the miofibrils and disorganization of the contractile apparatus . (F) Ten days after the interruption of cocaine exposure, the sarcoplasm of both types of fibres appeared silver stained. Scale bar: a, b, c, d, e; 25 µm; f: 200 µm.

The assessment of the diameter of the red and white fibres evidenced an increase in the mean diameter of both types of fibres. This increase was always significant ($P < 0.05$) in the red fibres of the exposed and post-exposure recovery specimens , whereas in the white fibres became significant only ten days after the interruption of cocaine exposure (Table 1).

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

339 **Table 1**. Diameter (μm) of the red and white muscle fibers in control, exposed and post-exposure recovery three
340 and ten days specimens. Values are means ± SE of the mean. *: P < 0.05 vs. control.
341

	Red fibers (μm)	White fibers (μm)
Control	19.76 ± 5.90	24.41 ± 10.38
Exposed	*27.91 ± 15.19	26.72 ± 13.65
Post-exposure recovery 3 days	*29. 27 ± 10.05	30.35 ± 14.03
Post-exposure recovery 10 days	*33.55 ± 6.96	*33.37 ± 16.52

342
343
344 The assessment of the morphology of the sarcomeric actin filaments with FITC- phalloidin
345 (Fig. 4) confirmed previous observations. In control eels, actin filaments showed a regular
346 organization (Fig. 4A) whereas, after the exposure to cocaine, the filaments showed signs of
347 injury as laceration and transversal fragmentation (Fig. 4B). Three days (Fig. 4C) and ten days
348 (Fig. 4D) after the interruption of cocaine exposure, the alterations were still present.
349
350

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

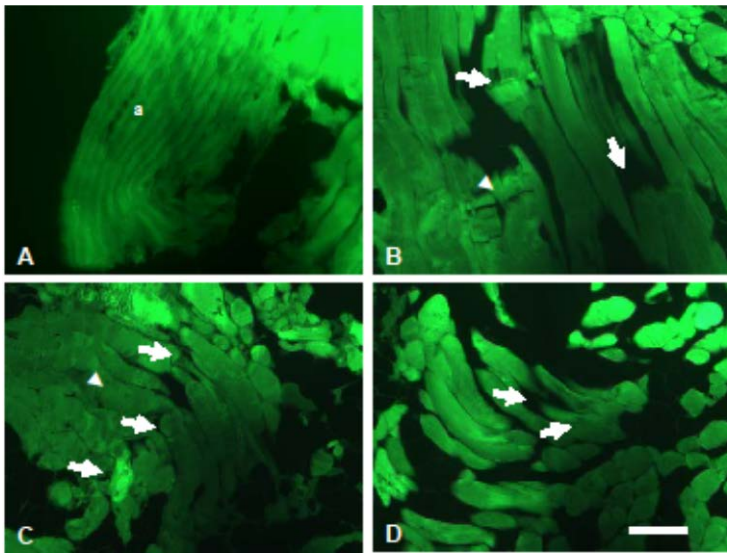
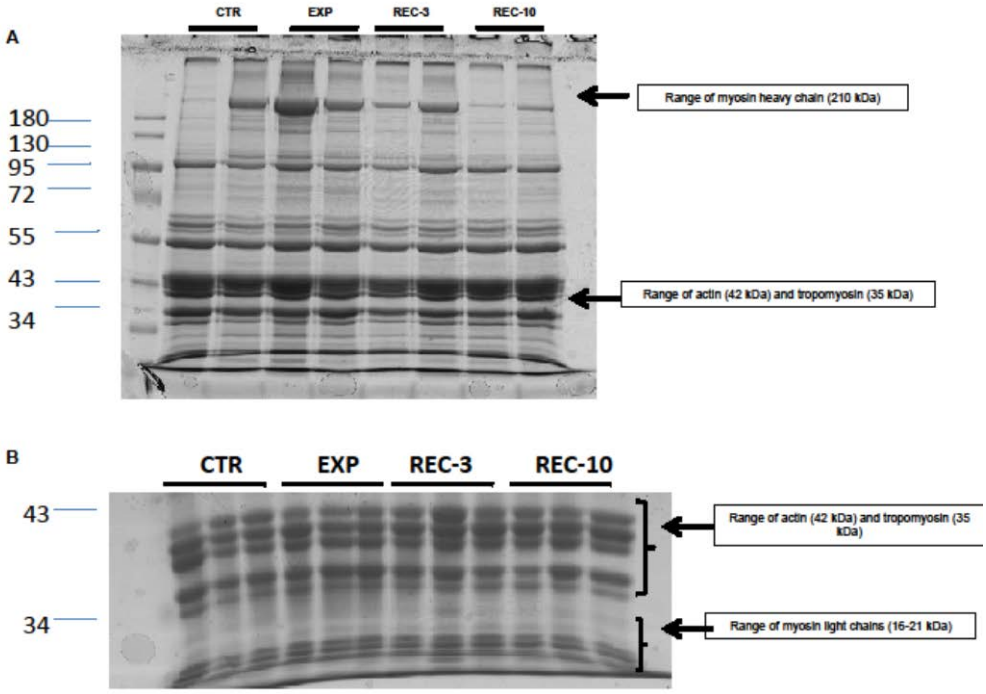


Figure 4. **Fluorescent micrographs of the skeletal muscle of *Anguilla anguilla*: FITC- phalloidin staining.** (A) Control specimens. Actin (a) filaments showed a regular organization (B) Exposed specimens. Actin filaments showed signs of laceration (arrow) and transversal fragmentation (arrowhead), still evident (C) three days after the interruption of cocaine exposure and (D) ten days after the interruption of cocaine exposure. Scale bar: a: 50 µm; b, c, d, e: 100 µm.

3.3. Muscle protein profile

No evident differences of protein bands belonging to the different experimental groups whether in the range of high molecular weight proteins (HMW myosins) (about 210 kDa) (Fig.5A) or in the range of low molecular weight proteins (actins and tropomyosins) (about 32-45 kDa) (Fig.5B) were observed. Sporadic differences of band intensities were only due to individual variability.

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357



364

365 **Figure 5**. **SDS-page representative profiles of proteins from samples of skeletal muscles from control (CTR),**
366 **exposed (EXP), post-exposure recovery three days (REC-3) and ten days (REC-10) specimens**. No
367 differences in both the ranges of high molecular weight (HMW myosins) (A) or low molecular weight (actins and
368 tropomyosins) (B) were observed.

369

370 ### 3.4. Cytochrome oxidase (COX) activity

371 Compared to control eels, COX activity slightly increased in cocaine-exposed eels. Significant
372 ($P < 0.05$) increases in COX activities were observed three days and ten days after the
373 interruption of cocaine exposure, respectively (Fig. 6).

374

375

376

377

378

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

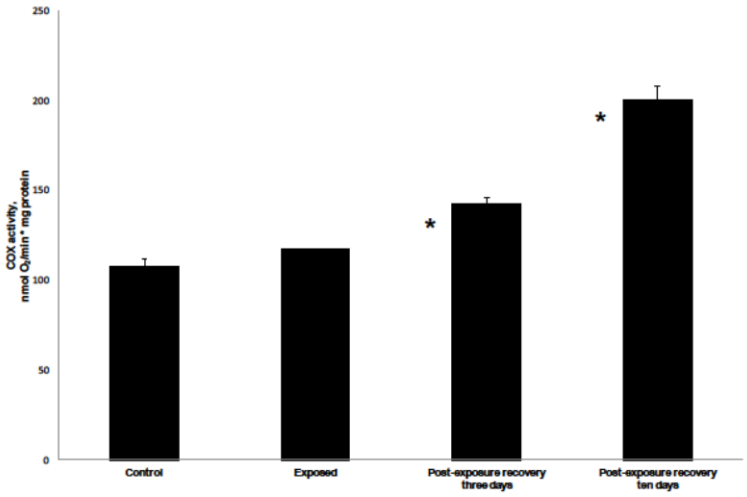


Figure 6. **Citocromo oxydase (COX) activity in control, exposed, post-exposure recovery three and ten days specimens.** Values are expressed as nmol O_2/min x mg protein. COX activity significantly increased three and ten days after the interruption of cocaine exposure. Values are mean ± SE of the mean . ***:** significantly ($P < 0.05$) different from the control values.

3.5. Caspase-3 activity

Compared to control eels, caspase-3 activity significantly ($P < 0.05$) increased in cocaine-exposed specimens, and further increased ($P < 0.02$) three days and ten days after the interruption of cocaine exposure (Fig.7A). To verify that the absorbances registered were due to specific caspase-3 activity, the caspase-3 inhibitor was used in representative samples from each experimental group. A significant ($P < 0.05$) reduction of the absorbances in all the samples was observed, as expected (Fig.7B).

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

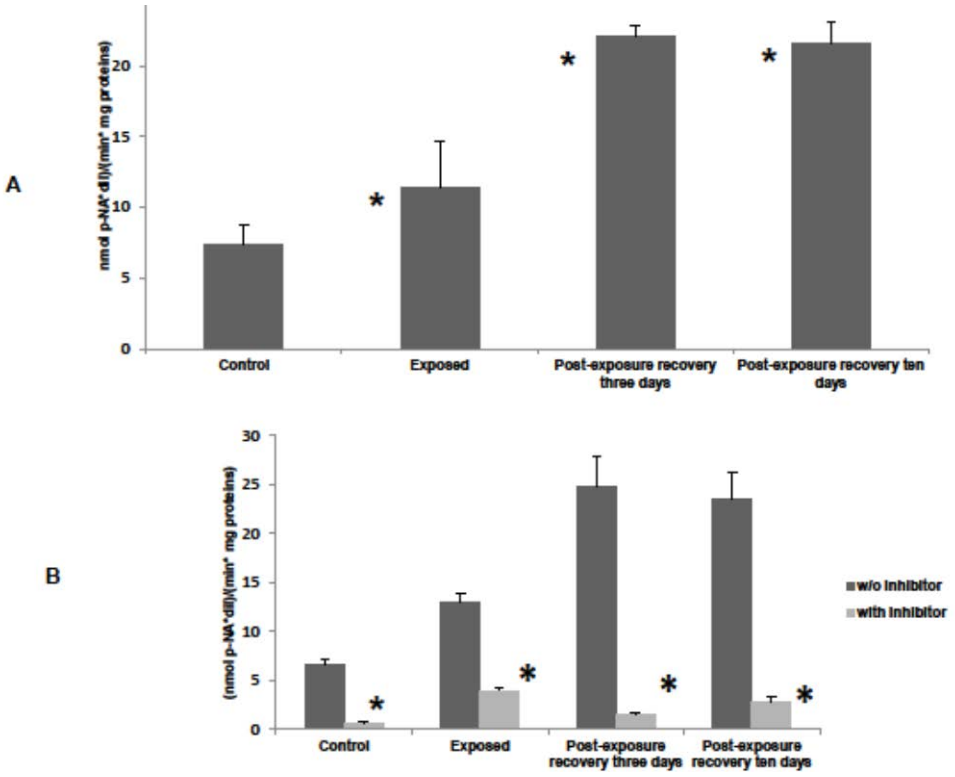


Figure 7. Caspase-3 activity in the skeletal muscle of control, exposed, post-exposure recovery three and ten days specimens. Values are expressed as nmol of free pNA normalized for μgram of proteins and time (min). (A) Caspase-3 activity significantly increased in cocaine-exposed and post-exposure recovery specimens. (B) In representative samples from each experimental group, the use of caspase-3 inhibitor reduced the registered absorbances of all samples. Values are the mean ± SE of the mean. *: significantly ($P < 0.05$) different from the control values.

3.6. Serum enzymes

After the exposure to cocaine, changes were observed in the serum levels of CK (Fig. 8A), LDH (Fig. 8B) and AST (Fig. 9). The serum levels of CK significantly ($P < 0.05$) increased, remained steady three days and further increased ten days after the interruption of cocaine exposure (Fig. 8A). The serum levels of LDH showed a gradual increase that became significant ($P < 0.05$) only ten days after the interruption of cocaine exposure (Fig.8B).

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

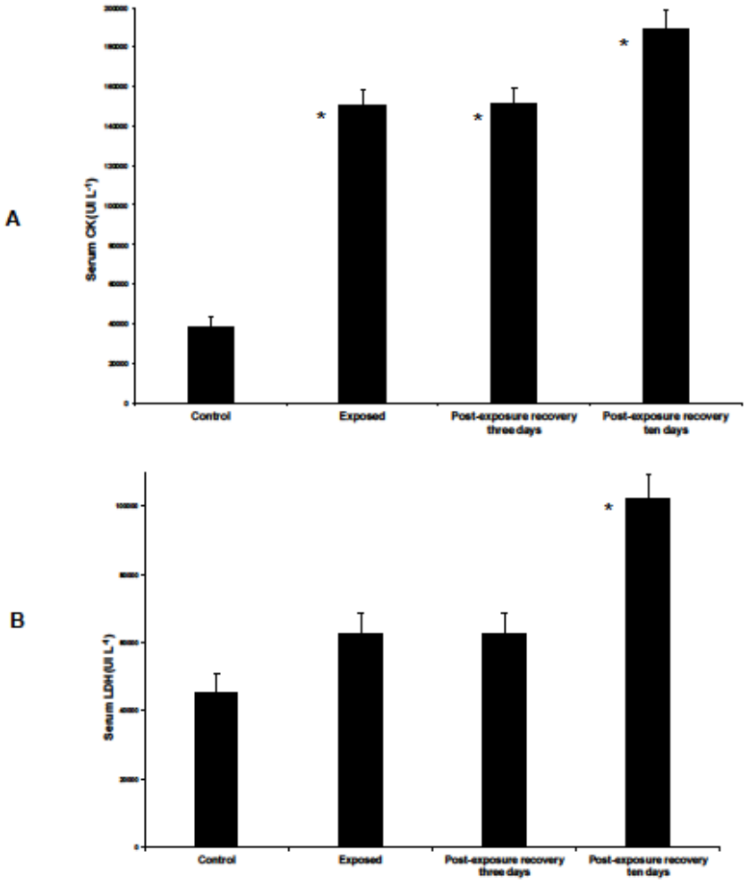
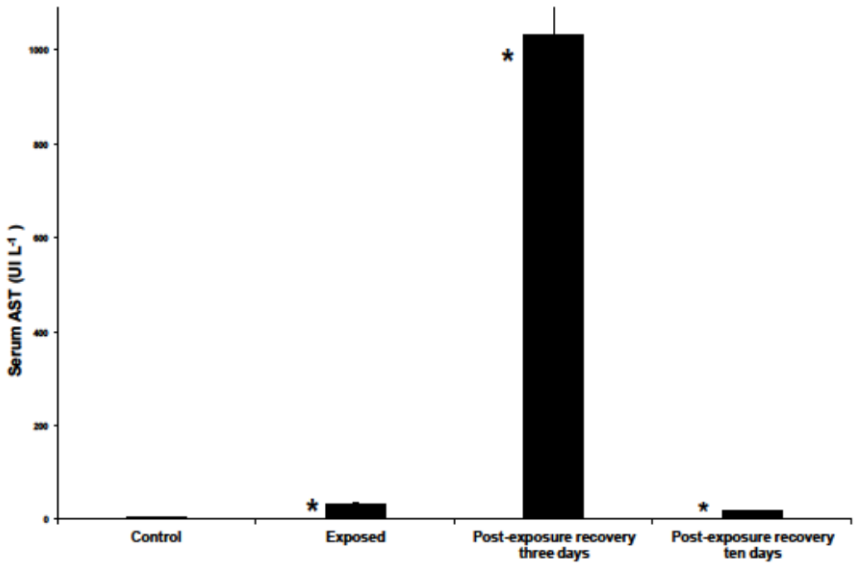


Figure 8. (**A) Serum creatine kinase (CK) and (B) serum lactate dehydrogenase (LDH) levels in control, exposed, post-exposure recovery three and ten days specimens**. Values are expressed as UI L^{-1}. (A) The serum CK levels significantly increased in the exposed specimens to be steady three days and further increase ten days after the interruption of cocaine exposure. (B) The serum LDH levels significantly increased only ten days after the interruption of cocaine exposure. Values are the mean ± SE of the mean. ***:** significantly ($P < 0.05$) different from the control values.

The serum levels of AST significantly ($P < 0.05$) increased after the exposure to cocaine and mainly three days after the interruption of cocaine exposure. The AST levels decreased ten days after the interruption of cocaine exposure, being however higher than control ones (Fig. 9).

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357



420

421 **Figure 9**. **Serum aspartate aminotransferase (AST) levels in control, exposed, post-exposure recovery three**
422 **and ten days specimens**. Values are expressed as UI L^{-1}. The serum AST levels significantly increased in the
423 exposed specimens to further hugely increase three days and to decrease ten days after the interruption of cocaine
424 exposure, when AST levels were however significantly higher than the control levels. Values are the mean ± SE of
425 the mean. ***:** significantly ($P<0.05$) different from the control values.

426

427 ## 4. Discussion

428 The results of the present study show, for the first time, that low environmental cocaine
429 concentrations heavily harm the skeletal muscle of the European eel, inducing alterations that
430 continue also after the interruption of cocaine exposure.

431

432 ### 4.1. General health condition

433 As regards the general health conditions of cocaine-exposed eels, they showed the same health
434 status of the other groups of eels, with the only exception of an increased locomotor activity.
435 This finding is consistent with the properties of cocaine, a psychomotor stimulant well-known
436 to produce motor stimulation and increase vigilance and alertness (Grilly and Salamone, 2012).
437 Hyperactivity was also observed in the zebrafish *Danio rerio*, but only during cocaine
438 withdrawal and not following a single administration of a range of cocaine doses (Lòpez-Patiño

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

et al., 2008). It is likely that such a difference could be related to the difference between the species examined, and/or the treatments performed (doses administered, acute *vs*. chronic administration etc.). In any case, these results indicate that also at the very low environmental concentrations, a chronic cocaine exposure has effects on the eels similar to what observed on humans (Grilly and Salamone, 2012).

4.2. General morphology and protein profile of the skeletal muscle

As regards the general morphology of the skeletal muscle, the routine histological analysis showed that a chronic exposure to environmental cocaine concentrations heavily damaged both the red and white fibres, that showed signs of injury as breakdown and swelling. These damages were still present ten days after the interruption of cocaine exposure, and were confirmed with the FITC-phalloidin staining technique, specific for the sarcomeric actin filaments, that appeared damaged both during the exposure and after the interruption of the cocaine exposure. The alterations observed in the skeletal muscle of *A. anguilla* look like those typical of rhabdomyolysis, a syndrome characterized by breakdown of muscle tissue, followed by dispersing into the circulatory system its intracellular components, as the enzymes CK, LDH, AST, the myoglobin and different electrolytes (Keltz et al., 2013; Khan, 2009). Our results, showing greatly increased serum levels of CK, LDH and AST in *A. anguilla* both during the cocaine exposure (CK, AST) and after its interruption (CK, LDH, AST) suggest that the morphological picture observed may be indicative of rhabdomyolysis. The development of rhabdomyolysis has been associated with a wide variety of diseases, injuries, medications, toxins, alcohol and drugs of abuse as opiates, amphetamines and other stimulants as cocaine (Brazeau et al., 1995; Keltz et al., 2013; Khan, 2009; Warren et al., 2002). It has been postulated that cocaine-induced rhabdomyolysis may occur through both a direct drug effect on the muscle and/or repeated ischemic events mediated by vasoconstrictor properties of cocaine

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

and its metabolites. Damage to the muscle tissue would result from the subsequent reperfusion and the generation of free radicals, not inactivated by radical scavenging systems in the muscle and associated vasculature. This in turn would damage the sarcolemma, the membrane enclosing the skeletal muscle fibres, leading to increased cytosolic enzyme release (Brazeau et al., 1995). It is possible that such mechanisms could take place also in *A. anguilla* during and after the interruption of cocaine exposure. Another effect of the disruption of sarcolemma is the leak into the circulation of large quantities of myoglobin, the cytoplasmic hemoprotein expressed solely in cardiac myocytes and in the skeletal muscle fibers (Ordway and Garry, 2004). If the myoglobin levels exceed the protein-binding capacity of the plasma, it can precipitate in the glomerular filtrate, causing renal tubular obstruction, direct nephrotoxicity, and acute renal failure (Khan, 2009). Our preliminary results, not yet published, have highlighted also renal damages in *A. anguilla* chronically exposed to cocaine, strengthening our hypothesis of a rhabdomyolysis syndrome.

An indirect confirmation of the structural damage of the skeletal muscle fibres come from the silver impregnation technique, that normally stains the argyrophilic reticular fibres of the connective tissue, but not the muscle tissue. During the cocaine exposure and after its interruption, both the red and white fibres appeared silver stained. The breakdown of the skeletal muscle fibres probably allowed silver to enter the sarcoplasm and bind to the cell structures. Since proteins bind silver ions with either a silver nitrate or a silver-ammonia complex solution (Blough et al., 1996; Chevallet et al., 2006), it is conceivable that the cell structures involved in the silver binding were the muscle proteins, even if it is difficult to understand which ones.

The increase in the mean diameter of the muscle fibres, as well, may be considered indicative of muscle damage. Indeed, it has been shown that the swelling of the muscle fibres can be the reaction of the muscle substance to a variety of injuries (Barer, 1947) and muscle

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

swelling is one of the clinical features of rhabdomyolysis (Nance et al., 2015). The red muscle appeared the most sensitive to cocaine, since the mean diameter of the red fibres increased in both the exposed eels and the post-exposure recovery eels. Instead, the mean diameter of the white fibres increased only ten days after the interruption of cocaine exposure. In silver eels, that have to perform a very long reproductive migration to the Sargasso sea, without feeding, the swimming activity is sustained by an increase in the activity of the slow red aerobic muscle. Indeed, in the silver stage, the volume of red muscle and the activity of aerobic enzymes, like COX, are increased compared to the yellow, non migrating, stage (Mortelette et al., 2010). It is possible that these features of the red muscle make it most sensitive to the vasoconstrictor properties of cocaine (Brazeau et al., 1995), compared to the fast white anaerobic muscle. Moreover, it is interesting to observe that the effect of cocaine on the skeletal muscle tissue was completely different from that on the smooth muscle tissue of the eel intestine. Indeed, cocaine, probably through the increase in plasma prolactin levels, restored the smooth intestinal musculature that in control eels showed signs of degeneration (Gay et al., 2016), whereas it seriously damaged the skeletal muscle, with an effect similar to what observed in humans (Brazeau et al., 1995; Keltz et al., 2013; Khan, 2009; Warren et al., 2002).

As regards the muscle protein profile, our analysis did not highlight differences among the different experimental groups, indicating that the chronic cocaine exposure did not change the expression levels of the muscles protein, whether in the range of high molecular weight (myosins) or in the range of low molecular weight (actins and tropomyosins). Cocaine is known to influence the cell cytoskeleton. In the rat nucleus accumbens cocaine increases actin cycling, indeed acute and withdrawal from repeated cocaine administration produces reversible and enduring elevations, respectively, in F-actin, through changes in the content or phosphorylation state of actin binding proteins, or reduced depolymerization and actin cycling (Toda et al., 2006). Moreover, cocaine increases the total protein content and the expression of

beta- myosin heavy-chain protein in the rat cardiac ventricular myocytes (Henning at al., 2000) and alters the protein profile of many different proteins, including cytoskeleton, of zebrafish embryos (Parolini et al., 2018). The lack of significant changes in the eel muscle protein profile after a chronic cocaine exposure could be due to species-specific differences and/or differences in the doses utilized. In any case, the effect of cocaine on of the eel muscle protein profile needs further studies to be better understood.

4.3. Cytochrome oxidase (COX) and caspase-3 activities

As regards the COX activity, our results showed an increase that became significant after the interruption of cocaine exposure. Cytochrome oxidase (COX), a well-known biomarker of the oxidative metabolism (Hovda et al., 1992), catalyzes the final step in the mitochondrial electron transfer chain (ETC), the reduction of oxygen to water. ETC plays a key role in the cell metabolism producing the most of cellular energy. The increase in COX activity observed in skeletal muscle homogenates could indicate an increase in specific mitochondrial enzyme activity and/or in mitochondrial protein mass. This increase could play an important role in the physiological adaptation to cocaine exposure by supporting the increased energy requirements for detoxification process. However, cocaine is known to induce mitochondrial dysfunction in primary cultures of rat cardiomyocytes (Yuan and Acosta, 1996) and in rat cortical neurons (Cunha-Oliveira et al., 2006), and to reduce COX activity in the rat prefrontal cortex (Vélez-Hernández et al., 2014). The contrast between these data and our finding could be due to the different animal/cellular models and/or to different dose and time of exposure to cocaine. Noteworthy, previous studies suggested that a chronic cocaine exposure leads to repeated ischemic events mediated by the vasoconstrictor properties of cocaine and its metabolites. The subsequent reperfusion and the generation of free radicals could induce rhabdomyolysis and/or muscle damage (Brazeau et al., 1995). Further studies showed, during the progression of

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

reperfusion injury, critical alterations in COX activity, in which three stages were proposed: an ischemic starvation phase, a reperfusion-induced hyperactivation phase, and a mitochondrial dysfunction phase. It was hypothesized that ischemia-induced stress alters the phosphorylation state of COX, that becomes hyperactive and leads to mitochondrial ROS generation during the beginning of reperfusion. In turn, ROS induce mitochondrial dysfunction during late reperfusion, and activate the intrinsic apoptotic pathway, committing the cell to death (Hüttemann et al., 2012). The increase in COX activity observed in eels after the interruption of cocaine exposure agrees with the hyperactivity of this enzyme typical of the ischemia/reperfusion injury, and could coincide with reperfusion-induced hyperactivation phase. This hypothesis should be confirmed by further experiments. In accordance with this hypothesis, the evaluation of the activity of caspase-3, the major executioner caspase in the apoptotic pathway (Cohen, 1997) showed a significant increase in both the exposed and the post-exposure recovery eels. Cocaine is known to induce caspase activation (Cunha-Oliveira et al., 2006) and apoptosis (Li et al., 2005; Su et al., 2003; Zhang et al., 1999) with a mechanism involving the release of cytochrome *c* from the mitochondria into the cytosol, and the subsequent activation of caspase-9 and caspase-3 (He et al., 2000). The increase in caspase-3 activity observed in the eels during the exposure and after the interruption of cocaine exposure agrees with this mechanism and further supports the hypothesis that the increase in COX activity could be associated with the ischemic/reperfusion injury.

4.4. Serum enzymes

As regards the serum levels of creatine kinase (CK), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST), our results showed that a chronic exposure to cocaine increased the levels of all these enzymes, even if with a different pattern. Indeed, the increases in CK and AST levels were always significant both during the cocaine exposure and after its

564 interruption, whereas the increase in LDH levels become significant only ten days after the
565 interruption of cocaine exposure. CK, LDH and AST are well-established serum markers of the
566 functional state of the muscle, and their increase may be an index of tissue injury (Brancaccio
567 et al., 2010). CK catalyzes the reversible reaction of creatine and ATP forming phosphocreatine
568 and ADP; it plays therefore a key role in energy homeostasis of cells and it is typical of tissues
569 with high energy demand as the skeletal muscle tissue, the heart, the brain and so on. LDH is
570 involved in the interconversion of pyruvate and lactate, with concomitant interconversion of
571 NADH and NAD, during the final reactions of glycolysis. It is present in the cytoplasm of all
572 the cells; since it is released during tissue damage, it is a marker of common injuries and
573 disease such as heart failure and muscle injury. AST catalyses the interconversion
574 of aspartate and α- ketoglutarate to oxaloacetate and glutamate, providing energy to the cells;
575 the enzyme is localized primarily in the skeletal and myocardial muscle, liver and erythrocytes
576 (Brancaccio et al., 2010; McLeish and Kenyon, 2005; Yousaf and Powell, 2012). The increase
577 in the serum levels of these enzymes observed both during the cocaine exposure (CK, AST)
578 and after its interruption (CK, LDH, AST) confirms the morphological observations of
579 structural damages to the skeletal muscle and could be indicative of a rhabdomyolysis
580 syndrome (Keltz et al., 2013; Khan, 2009;). However, considering that every enzyme has
581 different tissue-specific isoforms (Brancaccio et al., 2010) that were not evaluated, since only
582 the total amount of every enzyme was determined, the increases observed could be also
583 indicative of heart and/or liver damages. A correlation between muscle injury and the increase
584 in serum muscle enzymes levels was found also in the Atlantic salmon, in which increases in
585 serum CK and LDH levels, and CK levels, were associated with heart and skeletal muscle
586 inflammation (Yousaf and Powell, 2012), and with acute skeletal myopathy, respectively
587 (Rodger et al., 1991). Our results, showing an increase in the serum enzymes considered main

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

markers of muscle tissue injury, agree with these data and confirm the relationship between muscle damage and change in the serum levels of these enzymes.

4.5. Conclusion

In conclusion, our results showed that the low cocaine concentrations found in the fresh waters damaged the morphology and physiology of the eel skeletal muscle. Considering the life cycle of the eel, it is evident how much the environmental cocaine could threaten the reproductive fitness of this species. At the silver stage, the eel undertakes a swimming migration 6000 km long (Tesch, 2003; van Ginneken and Maes, 2005) needing sufficient energy reserves and an healthy skeletal muscle tissue, to be successfully completed. Our results suggest that cocaine, at environmental concentrations, could compromise both the sustained swimming, ensured by the slow red muscle, and the burst swimming, ensured by the fast white muscle. The European eel is under threat and in serious decline, due to many causes such as overfishing, habitat loss, presence of parasites, climate change, and water pollution (Dekker, 2003). Our results suggest that environmental cocaine could be considered another cause for the decline, as well.
Finally, the European eel is an edible species, whose farming currently supplies more than the 80% of the world's consumption of the species (Nielsen and Prouzet, 2008). Since the skeletal muscle is the edible part of the eel, the study of the changes induced by the aquatic contaminants is informative from a human health point of view. The eel muscle tissue bioaccumulates cocaine to a large extent (Capaldo et al., 2012); our results, showing severe damages induced from this drug in the muscle suggest also a potential consequence on the nutritional value of this tissue, although further studies are needed to verify this hypothesis.

5.Competing interests

The Authors declare no competing or financial interests.

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

6.Funding

This research received no specific grant from any funding agency in the public, commercial or not-for-profit sectors.

References

Aligizakis, N.A., Gago-Ferrero, P., Borova, V.L., Pavlidou, A., Hatzianestis, I. and Thomaidis N.S. (2016). Occurrence and spatial distribution of 158 pharmaceuticals, drugs of abuse and related metabolites in offshore seawater. *Sci. Tot. Environ*. 541, 1097-1105.

Altringham, J.D. and Ellerby, D.J. (1999). Fish swimming: patterns in muscle function. *J. Exp. Biol*. **202**, 3397-3403.

Barrè, H., Bailly, L. and Rouanet, J.L. (1997). Increased oxidative capacity in skeletal muscle from cold-acclimated ducklings: a comparison with rats. *Comp. Biochem. Physiol.* **88**, B5119-B5522.

Binelli, A., Pedriali, A., Riva, C. and Parolini, M. (2012). Illicit drugs as new environmental pollutants: Cyto-genotoxic effects of cocaine on the biological model *Dreissena polymorpha. Chemosphere* **86**, 906-911.

Barer, R. (1947). Observations on muscle-fibre structure. The swelling of muscle fibres by acids and alkalis. *J Anat*. **81**, 259-285.

Blough, E. R., Renn, E.R., Zhang, F. and Reiser, P.J. (1996). Enhanced electrophoretic separation and resolution of myosin heavy chains in mammalian and avian skeletal muscles. *Anal. Biochem*. **233**, 31-35.

Bradbury, P., and Gordon, K. C. (1980). Connective tissues and stain. In *Theory and Practice of Histological Techniques* (ed. J.D. Bancroft and A. Stevens), pp. 119-142. New York: Churchill Livingstone.

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

Brancaccio, P., Lippi, G. and Maffulli, N. (2010). Biochemical markers of muscular damage. *Clin. Chem. Lab. Med.* **48**, 757-767.

Brazeau, G.A., McArdle, A. and Jackson, M.J. (1995). Effects of cocaine on leakage of creatine kinase from skeletal muscle: in vitro and in vivo studies in mice. *Life Sci.* **57**, 1569-1578.

Camargo, M.M.P. and Martinez, C.B.R. (2007) Histopathology of gills, kidney and liver of a Neotropical fish caged in an urban stream. *Neotrop. Ichthyol.* **5,** 327-336.

Capaldo, A., Gay, F., Maddaloni, M., Valiante, S., De Falco, M., Lenzi, M. and Laforgia, V. (2012) Presence of cocaine in the tissues of the European eel, *Anguilla anguilla*, exposed to environmental cocaine concentrations. *Water Air Soil Pollut.* **223**, 2137-2143.

Capkin, E., Birinciogl, S. and Altinok, I. (2009) Histopathological changes in rainbowtrout (*Oncorhynchus mykiss*) after exposure to sublethal composite nitrogen fertilizers. *Ecotoxicol. Environ. Saf.* **72**, 1999-2004.

Castiglioni, S., Zuccato, E., Crisci, E., Chiabrando, C., Fanelli, R. and Bagnati, R. (2006). Identification and measurement of illicit drugs and their metabolites in urban wastewater by liquid chromatography-tandem mass spectrometry. *Anal. Chem.* **78**, 8421-8429.

Chevallet, M., Luche, S. and Rabilloud, T. (2006). Silver staining of proteins in polyacrylamide gels. *Nat. Protoc*. **1**, 1852-1858.

Cohen, G.M. (1997). Caspases: the executioners of apoptosis. *Biochem. J.* **326**, 1-16.

Cunha-Oliveira, T., Rego, A.C., Cardoso, S.M., Borges, F., Swerdlow, R.H., Macedo, T. and de Oliveira, C.R. (2006). Mitochondrial dysfunction and caspase activation in rat cortical neurons treated with cocaine or amphetamine. *Brain Res.* **1089**, 44-54.

Daughton, C.G. (2011). Illicit drugs: contaminants in the environment and utility in forensic epidemiology. In *Reviews of Environmental Contamination and Toxicology* (ed. D.M. Whitacre), pp. 59-110. Springer, New York.

Dekker, W. (2003). Did lack of spawners cause the collapse of the European eel, *Anguilla anguilla*? *Fish. Manage. Ecol.* **10**, 365-376.

Dubowitz, V., Dubowitz, V., Sewry, C., Dubowitz, V., Oldfors, A. and Sewry, C. (1985). *Muscle Biopsy*. *A practical approach.* London, UK: Bailliere Tindall.

Ebrahimi, M. and Taherianfard, M. (2011) Pathological and hormonal changes in freshwater fishes due to exposure to heavy metals pollutants. *Water Air Soil Pollut.* **217**, 47-55.

Ellerby, D.J., Spierts, I.L.Y. and Altringham, J.D. (2001). Slow muscle power output of yellow- and silver-phase european eels (*Anguilla anguilla l.*): changes in muscle performance prior to migration. *J. Exp. Biol.* **204**, 1369-1379.

Fanta, E., Rios, F.S., Romão, S., Vianna, A.C.C. and Freiberger, S. (2003) Histopathology of the fish *Corydoras paleatus* contaminated with sublethal levels of organophosphorus in water and food. *Ecotoxicol. Environ. Saf.* **54**, 119-130.

Fedorova, M., Kuleva, N. and Hoffmann, R. (2009). Reversible and irreversible modifications of skeletal muscle proteins in a rat model of acute oxidative stress. *Biochim. Biophys. Acta* **1792**, 1185-1193.

Gay, F., Maddaloni, M., Valiante, S., Laforgia, V. and Capaldo, A. (2013). Endocrine disruption in the European eel, *Anguilla anguilla*, exposed to an environmental cocaine concentration. *Water Air Soil Pollut.* **224**, 1579. https://doi.org/10.1007/s11270-013-1579-0

Gay, F., Ferrandino, I., Monaco, A., Cerulo, M., Capasso, G. and Capaldo, A. (2016). Histological and hormonal changes in the European eel (*Anguilla anguilla*) after exposure to environmental cocaine concentration. *J. Fish Dis.* **39**, 295-308.

Grilly, M.G. and Salamone, J.D. (2012). *Drugs, Brain and Behavior*. Boston, MA: Pearson.

He, J., Xiao, Y., Casiano, C.A. and Zhang, L. (2000). Role of mitochondrial cytochrome c in cocaine-induced apoptosis in coronary artery endothelial cells. *J. Pharmacol. Exp. Ther*. **295**, 896-903.

Henning, R.J., Silva, J., Reddy, V., Kamat, S., Morgan, M.B., Li, Y.X. and Chiou, S. (2000). Cocaine increases beta-myosin heavy-chain protein expression in cardiac myocytes. *J. Cardiovasc. Pharmacol. Ther.* **5**, 313-322.

Hovda, D.A., Chugani, H.T., Villablanca, J.R., Badie, B. and Sutton, R.L. (1992). Maturation of cerebral oxidative metabolism in the cat: a cytochrome oxidase histochemistry study. *J. Cereb. Blood Flow Metab*. **12**, 1039-1048.

Hüttemann, M., Helling, S., Sanderson, T.H., Sinkler, C., Samavati, L., Mahapatra, G., Varughese, A., Lu, G., Liu, J., Ramzan, R. et al. (2012). Regulation of mitochondrial respiration and apoptosis through cell signaling: cytochrome c oxidase and cytochrome c in ischemia/reperfusion injury and inflammation. *Biochim Biophys Acta*. **1817**, 598-609.

Kasprzyk-Hordern, B., Dinsdale, R.M. and Guwy, A.J. (2008). The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. *Water Res*. **42**, 3498-3518.

Keltz, E., Khan, F.Y. and Mann, G.(2013). Rhabdomyolysis. The role of diagnostic and prognostic factors. *Muscles Ligaments Tendons J*. **3,** 303-312.

Khan, F.Y. (2009). Rhabdomyolysis: a review of the literature. *Neth. J. Med*. **67**, 272-283.

Li., G., Xiao, Y. And Zhang, L. (2005). Cocaine induces apoptosis in fetal rat myocardial cells through the p38 mitogen-activated protein kinase and mitochondrial/cytochrome c pathways. *J. Pharmacol. Exp. Ther*. **312**, 112-119.

Lionetti, L., Crescenzo, R., Mollica, M.P., Tasso, R., Barletta, A., Liverini, G. and Iossa, S. (2004). Modulation of hepatic mitochondrial energy efficiency with age. *Cell. Mol. Life Sci.* **61**, 1366-1371.

Loganathan, B., Phillips, M., Mowery, H. and Jones-Lepp, T.L. (2009). Contamination profiles and mass loadings of macrolide antibiotics and illicit drugs from a small urban wastewater treatment plant. *Chemosphere* **75**, 70-77.

714 **Lòpez-Patiño, M.A., Yu, L., Cabral, H. and Zhdanova, I.V.** (2008). Anxiogenic effects of
715 cocaine withdrawal in zebrafish. *Physiol. Behav*. **93**, 160-171.
716 **Mari, F., Politi, L., Biggeri, A., Accetta, G., Trignano, C., Di Padua, M. and Bertol, E**.
717 (2009) Cocaine and heroin in waste water plants: a 1-year study in the city of Florence, Italy.
718 *Forensic Sci. Int.* **189**, 88-92.
719 **McLeish, M.J. and Kenyon, G.L.** (2005). Relating structure to mechanism in creatine kinase.
720 *Crit. Rev. Biochem. Mol. Biol*. **40**, 1-20.
721 **Mortelette, H., Amérand, A., Sébert, P., Belhomme, M., Calvès, P. and Moisan, C.** (2010).
722 Effect of exercise training on respiration and reactive oxygen species metabolism in eel red
723 muscle. *Respir. Physiol. Neurobiol*. **172**, 201-205.
724 **Mumford, S., Heidel, J., Smith, C., Morrison, J., Mac Connell, B. and Blazer, V.** (2007).
725 Fish Histology and Histopathology. U.S. Fish & Wildlife Service-National Conservation
726 Training Center https://nctc.fws.gov/resources/course-resources/fish-histology/
727 **Nance, R.J. and Mammen, A.L.** (2015). Diagnostic evaluation of rhabdomyolysis. *Muscle*
728 *Nerve* **51,** 793-810. doi:10.1002/mus.24606
729 **Nielsen, T. and Prouzet, P.** (2008). Capture-based aquaculture of the wild European eel
730 (*Anguilla anguilla*). In *Capture based aquaculture. Global overview* (ed. A. Lovatelli and P. F.
731 Holthus), pp. 141-168. Rome: FAO. FAO Fisheries Technical Paper No. 508.
732 **Norris, D.O.** (2007) *Vertebrate endocrinology*. Amsterdam, NL: Academic Press.
733 **Ordway, G.A. and Garry, D.J.** (2004). Myoglobin: an essential hemoprotein in striated
734 muscle. *J. Exp. Biol.* **207**, 3441-3446.
735 **Pal, R., Megharaj, M., Kirkbride, K.P. and Naidu, R.** (2013) Illicit drugs and the
736 environment-A review. *Sci. Total Environ.* **463-464**, 1079-1092.
737 **Parolini, M. and Binelli, A.** (2014). Oxidative and genetic responses induced by Δ-9-
738 tetrahydrocannabinol (Δ-9-THC) to *Dreissena polymorpha*. *Sci. Total Environ.* **468-469**, 68-76.

Parolini, M., Pedriali, A., Riva, C. and Binelli, A. (2013). Sub-lethal effects caused by the cocaine metabolite benzoylecgonine to the freshwater mussel *Dreissena polymorpha*. *Sci. Total Environ.* **444**, 43-50.

Parolini, M., Bini, L., Magni, S., Rizzo, A., Ghilardi, A., Landi, C., Armini, A., Del Giacco, L. and Binelli, A. (2018). *Environ. Pollut*. **232**, 603-614.

Postigo, C., de Alda, M.J.L. and Barcelò, D. (2010) Drugs of abuse and their metabolites in the Ebro river basin: Occurrence in sewage and surface water, sewage treatment plants removal efficiency, and collective drug usage estimation. *Environ. Int.* **36**, 75-84.

Rehm, J., Taylor, B. and Room, R. (2006) Global burden of disease from alcohol, illicit drugs and tobacco. *Drug Alcohol Rev.* **25**, 503-513.

Rodger, H.D., Murphy, T.M., Drinan, E.M. and Rice, D.A. (1991). Acute skeletal myopathy in farmed Atlantic salmon *Salmo salar*. *Dis. Aquat. Org*. **12**, 17-23.

Rosi-Marshall, E.J., Snow, D., Bartelt-Hunt, S.L., Paspalof, A. and Tank, J.L. (2015) A review of ecological effects and environmental fate of illicit drugs in aquatic ecosystems. *J. Hazard. Mater*. **282**, 18-25.

Seabra-Pereira, C.D., Maranho, L.A., Cortez, F.S., Pusceddu, F.H., Santos, A.R., Ribeiro, D.A., Cesar, A. and Guimarães L.L. (2016). Occurrence of pharmaceuticals and cocaine in a Brazilian coastal zone. *Sci. Tot. Environ*. **548-549**, 148-154.

Sheehan, D. C. and Hrapchak, B. B. (1980). *Theory and Practice of Histotechnology*. St Louis, MO: CV Mosby.

Su, J., Li, J., Li, W., Altura, B.T. and Altura, B.M. (2003). Cocaine induces apoptosis in cerebral vascular muscle cells: potential roles in strokes and brain damage. *Eur. J. Pharmacol*. **482**, 61-66.

Tesch, S.W. (2003) *The Eel.* Blackwell Science, Ltd.

763 **Toda, S., Shen, H.W., Peters, J., Cagle, S. and Kalivas, P.W.** (2006). Cocaine increases actin
764 cycling: effects in the reinstatement model of drug seeking. *J. Neurosci*. **26**,1579-1587.
765 **van Ginneken, V. and Maes, G.E.** (2005). The European eel (*Anguilla anguilla*, Linnaeus), its
766 lifecycle, evolution, and reproduction: a literature review. *Rev. Fish Biol. Fish.* **15**, 367-398.
767 **Vélez-Hernández, M.E., Padilla, E., Gonzalez-Lima, F. and Jiménez-Rivera, C.A.** (2014).
768 Cocaine reduces cytochrome oxidase activity in the prefrontal cortex and modifies its
769 functional connectivity with brainstem nuclei. *Brain Res*. **1542**, 56-69.
770 **Warren, J.D., Blumbergs, P.C. and Thompson, P.D.** (2002). Rhabdomyolysis: a review.
771 *Muscle Nerve* **25**, 332-347.
772 **Yousaf, M.N. and Powell, M.D.** (2012). The effects of heart and skeletal muscle inflammation
773 and cardiomyopathy syndrome on creatine kinase and lactate dehydrogenase levels in atlantic
774 salmon (*Salmo salar* L.). *Scientific World Journal* Article ID 741302.
775 http://dx.doi.org/10.1100/2012/741302
776 **Yuan, C. and Acosta, D.** (1996). Cocaine-induced mitochondrial dysfunction in primary
777 cultures of rat cardiomyocytes. *Toxicol.***112**, 1-10.
778 **Zhang, L., Xiao, Y. and He, J**. (1999). Cocaine and apoptosis in myocardial cells. *Anat. Rec*.
779 **257**, 208-216.
780 **Zuccato, E. and Castiglioni, S.** (2009) Illicit drugs in the environment. *Philos. Trans. R. Soc.*
781 *A* **367**, 3965-3978.
782 **Zuccato, E., Castiglioni, S., Bagnati R., Chiabrando C., Grassi P. and Fanelli, R.** (2008)
783 Illicit drugs, a novel group of environmental contaminants. *Water Res.* **42**, 961-968.
784 **Zuccato, E., Chiabrando, C., Castiglioni, S., Calamari, D., Bagnati, R., Schiarea, S. and**
785 **Fanelli, R.** (2005) Cocaine in surface waters: a new evidence-based tool to monitor community
786 drug abuse. *Environ. Health* **4**, 14-21.
787

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

788 **Figure legends**

789

790 **Figure 1**. **Light micrographs of the skeletal muscle of *Anguilla anguilla*: control**
791 **specimens.** (A, B, C, D: Mallory staining; E, F. silver impregnation). (A) The red (r) muscle
792 lied as a wedge along the lateral line, whereas the white (w) muscle formed the greatest volume
793 of the body tissue. (B, C) In transverse section, the red (r) fibres appeared rounded and
794 intensely stained, and the white (w) fibres, larger and less intensely stained. The connective
795 reticular tissue (c) was more abundant in the red muscle. The white fibres had different
796 diameters; both types of fibres had a well organized contractile apparatus, showing, in
797 longitudinal section (D), myofibrils (m) regularly aligned and parallel each other. (E, F) The
798 connective reticular tissue (c) was weakly argyrophilic in the red muscle (E) and deeply
799 argyrophilic, black stained, in the white muscle (F). No stain was observed in the sarcoplasm
800 (s) of both types of fibres. Scale bar: a: 200 µm; b, c, d, e, f: 25 µm.

801

802 **Figure 2**. **Light micrographs of the skeletal muscle of *Anguilla anguilla*: exposed**
803 **specimens**. (A, B, C, D: Mallory staining; E, F. silver impregnation). Both red (r) (A, B) and
804 white (w) (C, D) fibres showed signs of injury as laceration (arrow) and trasversal
805 fragmentation (arrowhead). (E, F) In addition to the connective (c) tissue, the sarcoplasm (s) of
806 both the red (E) and white (F) fibres were silver-stained and argyrophilic. Scale bar: 25 µm.

807

808 **Figure 3**. **Light micrographs of the skeletal muscle of *Anguilla anguilla*: post-exposure**
809 **recovery three and ten days specimens.** (A, B, C, D, E: Mallory staining; F. silver
810 impregnation). Three days (A, B) and ten days (C, D, E) after the interruption of cocaine
811 exposure, both red (r) (A, C) and white (w) (B, D, E) fibres showed signs of injury and
812 breakdown (arrow). (C). The red (r) fibres showed signs of swelling (asterisk), rarefaction of

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

813 the miofibrils and disorganization of the contractile apparatus . (F) Ten days after the
814 interruption of cocaine exposure, the sarcoplasm of both types of fibres appeared silver stained.
815 Scale bar: a, b, c, d, e; 25 µm; f: 200 µm.
816
817 **Figure 4**. **Fluorescent micrographs of the skeletal muscle of *Anguilla anguilla*: FITC-**
818 **phalloidin staining.** (A) Control specimens. Actin (a) filaments showed a regular organization
819 (B) Exposed specimens. Actin filaments showed signs of laceration (arrow) and transversal
820 fragmentation (arrowhead), still evident (C) three days after the interruption of cocaine
821 exposure and (D) ten days after the interruption of cocaine exposure. Scale bar: a: 50 µm; b, c,
822 d, e: 100 µm.
823
824 **Figure 5**. **SDS-page representative profiles of proteins from samples of skeletal muscles**
825 **from control (CTR), exposed (EXP), post-exposure recovery three days (REC-3) and ten**
826 **days (REC-10) specimens**. No differences in both the ranges of high molecular weight (HMW
827 myosins) (A) or low molecular weight (actins and tropomyosins) (B) were observed.
828
829 **Figure 6**. **Citocromo oxydase (COX) activity in control, exposed, post-exposure recovery**
830 **three and ten days specimens.** Values are expressed as nmol O_2/min x mg protein. COX
831 activity significantly increased three and ten days after the interruption of cocaine exposure.
832 Values are mean ± SE of the mean . ***:** significantly ($P < 0.05$) different from the control values.
833
834 **Figure 7. Caspase-3 activity in the skeletal muscle of control, exposed, post-exposure**
835 **recovery three and ten days specimens**. Values are expressed as nmol of free pNA
836 normalized for µgram of proteins and time (min). (A) Caspase-3 activity significantly increased
837 in cocaine-exposed and post-exposure recovery specimens. (B) In representative samples from

each experimental group, the use of caspase-3 inhibitor reduced the registered absorbances of all samples. Values are the mean ± SE of the mean. *: significantly ($P < 0.05$) different from the control values.

Figure 8. (**A) Serum creatine kinase (CK) and (B) serum lactate dehydrogenase (LDH) levels in control, exposed, post-exposure recovery three and ten days specimens**. Values are expressed as UI L^{-1}. (A) The serum CK levels significantly increased in the exposed specimens to be steady three days and further increase ten days after the interruption of cocaine exposure. (B) The serum LDH levels significantly increased only ten days after the interruption of cocaine exposure. Values are the mean ± SE of the mean. ***:** significantly ($P <0.05$) different from the control values.

Figure 9. **Serum aspartate aminotransferase (AST) levels in control, exposed, post-exposure recovery three and ten days specimens**. Values are expressed as UI L^{-1}. The serum AST levels significantly increased in the exposed specimens to further hugely increase three days and to decrease ten days after the interruption of cocaine exposure, when AST levels were however significantly higher than the control levels. Values are the mean ± SE of the mean. ***:** significantly ($P<0.05$) different from the control values.

Table 1. Diameter (μm) of the red and white muscle fibers in control, exposed and post-exposure recovery three and ten days specimens. Values are means ± SE of the mean. *: $P < 0.05$ vs. control.

https://doi.org/doi:10.1016/j.scitotenv.2018.05.357

864

	Red fibers (µm)	White fibers (µm)
Control	19.76 ± 5.90	24.41 ± 10.38
Exposed	*27.91 ± 15.19	26.72 ± 13.65
Post-exposure recovery 3 days	*29. 27 ± 10.05	30.35 ± 14.03
Post-exposure recovery 10 days	*33.55 ± 6.96	*33.37 ± 16.52

865