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

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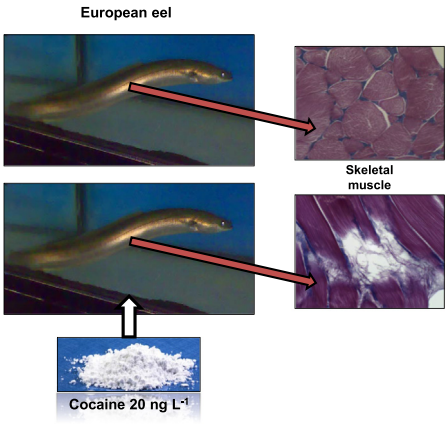
HIGHLIGHTS

- The effects of cocaine on the eel skeletal muscle were studied.
- Silver eels were exposed to environmental concentrations of cocaine.
- Morphology, caspase, COX, muscle protein profile and serum enzymes were studied.
- Cocaine altered the eel skeletal muscle morphology and physiology.
- Cocaine could hinder the reproductive migration of this species.

GRAPHICAL ABSTRACT



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ABSTRACT

The presence of illicit drugs in the aquatic environment represents a new potential risk for aquatic organisms, due to their constant exposure to substances with strong pharmacological activity. Currently, little is known about the ecological effects of illicit drugs. The aim of this study was to evaluate the influence of environmental concentrations of cocaine, an illicit drug widespread in surface waters, on the skeletal muscle of the European eel (*Anguilla anguilla*). The skeletal muscle of silver eels exposed to 20 ng L^{-1} of cocaine for 50 days were compared to control, vehicle control and two post-exposure recovery groups (3 and 10 days after interruption of cocaine). The eels general health, the morphology of the skeletal muscle and several parameters indicative of the skeletal muscle physiology were evaluated, namely the muscle whole protein profile, marker of the expression levels of the main muscle proteins; cytochrome oxidase activity, markers of oxidative metabolism; caspase-3, marker of apoptosis activation; serum levels of creatine kinase, lactate dehydrogenase and aspartate aminotransferase, markers of skeletal muscle damages. Cocaine-exposed eels appeared hyperactive but they showed the same general health status as the other groups. In contrast, their skeletal muscle showed evidence of serious injury, including muscle breakdown and swelling, similar to that typical of rhabdomyolysis. These changes were still present 10 days after the interruption of cocaine exposure. In fact, with the exception of the expression levels of the main

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muscle proteins, which remained unchanged, all the other parameters examined showed alterations that persisted for at least 10 days after the interruption of cocaine exposure. This study shows that even low environmental concentrations of cocaine cause severe damage to the morphology and physiology of the skeletal muscle of the silver eel, confirming the harmful impact of cocaine in the environment that potentially affects the survival of this species.

1. Introduction

Many illicit drugs and their breakdown products are detected in surface waters (Li et al., 2016; Pal et al., 2013; Rosi-Marshall et al., 2015) and in seawater (Aligizakis et al., 2016; Seabra-Pereira et al., 2016) throughout the world, for two main reasons: the enormous worldwide use of these substances and the variable efficiency with which they are removed from sewage effluent in sewage treatment plants, which is highly dependent upon the technology used (Pal et al., 2013). The environmental fate and ecological effects of illicit drugs are not well understood. However, despite the very low concentrations of these substances in surface waters (0.4 to 44 ng L^{-1} for cocaine), the first studies showed toxic effects to the aquatic organisms, as expected for a constant exposure to substances with strong pharmacological activity (Pal et al., 2013). 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 concentrations of cocaine, and the cocaine metabolite benzoylecgonine, respectively. Further studies showed that in zebrafish embryos environmental concentrations of cocaine induced cytotoxic and genotoxic effects (Parolini et al., 2017) and alteration of the protein profile of many different proteins, including cytoskeletal proteins (Parolini et al., 2018) and the impairment of skeletal muscle development in zebrafish larvae (Monaco et al., 2016). Since data on the effects of environmental concentrations of illicit drugs on fish were lacking, we started a study aimed to evaluate and compare the effects on fish of the most frequent illicit drug found in surface waters. Our first studies, concerning the effects of environmental concentrations of cocaine on the European eel (*Anguilla anguilla*), showed that chronic exposure induced the accumulation of cocaine in its tissues (Capaldo et al., 2012) and alterations in its endocrine system (Gay et al., 2013). Since these results suggested the presence of histological changes, we also evaluated the condition of the peripheral tissues. Indeed the histological features described in these studies may be considered as suitable biomarkers for the evaluation of the health of fish exposed to contaminants (Yancheva et al., 2016). Our first results, showing changes in the skin and the intestine, were reported in a previous manuscript (Gay et al., 2016). In this study, we describe the effects of cocaine exposure on skeletal muscle of the silver eel.

In 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 accumulates cocaine in large amounts after chronic exposure (Capaldo et al., 2012). Moreover, due to the peculiar life cycle of the European eel, the study of the health condition of this tissue is particularly interesting. Indeed, at the silver stage, the eel migrates across 6000 km of open sea without feeding to the spawning grounds of the Sargasso sea (Righton et al., 2016). This means that, in addition to sufficient energy reserves, the eel needs a healthy skeletal muscle and an efficient aerobic metabolism, in order to complete successfully its migration. Finally, the European eel is an edible species, and food resource (Arai, 2014). 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 chronic exposure to cocaine were observed by evaluating the general health of the eels, the general morphology of the skeletal muscle and a number of different parameters indicative of skeletal muscle physiology: the muscle whole protein profile, as a marker of the expression levels of the main muscle proteins (Fedorova et al., 2009); cytochrome oxidase (COX) activity, as a marker of oxidative metabolism (Lee and Hüttemann, 2014); caspase-3 activity as a marker of apoptosis activation, since caspase-3 is the major player in the apoptotic pathway (Brentnall et al., 2013); serum levels of creatine kinase (CK), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST), all well-established biomarkers of skeletal muscle damage (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

150 adult male specimens of the European eel (*Anguilla anguilla*), caught as glass eels and raised in farms (38.85 ± 0.39 cm; 85.38 ± 1.60 g; mean ± s.d.) (silver eel stage), were obtained from a local fish dealer. They were acclimatized to the laboratory for 1 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, which was not recycled, was 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 General Direction of Animal Health and Veterinary Drugs of the Italian Ministry of Health. Efforts were made to avoid animal suffering and minimize the number of animals used. The eels were maintained in accordance with the institutional guidelines for care and use of laboratory animals.

2.3. Experimental design

After acclimatization, the eels from the aquaria were randomly divided into five groups (untreated control, vehicle control, cocaine exposed and two post-exposure recovery groups), each containing ten specimens. Each group was kept in a 300-L glass aquarium, under the previously described conditions. In each aquarium the water was renewed every 24 h. The nominal concentration of cocaine selected (20 ng L^{-1}) corresponds to the mean cocaine concentration detected in surface waters (Li et al., 2016; Pal et al., 2013). A stock solution of 0.006 mg mL^{-1} cocaine free-base in ethanol was prepared (Gay et al.,

2016); the concentration of cocaine in the stock solution was verified by mass spectrometry. Three cocaine exposed groups received daily a nominal concentration of 20 ng L^{-1} cocaine (1 mL of the stock solution, administered directly into the aquaria, every 24 h, after renewing the water; the percentage ethanol concentration in the water was 0.000333%). The untreated control group was exposed daily only to tap water and the vehicle control group was exposed daily to the same percentage ethanol concentration (0.000333%) as the treated group, in the same conditions. The treatment lasted for 50 days. At the end of the exposure period, two of the three cocaine exposed groups (named post-exposure recovery groups) were deprived of cocaine and only exposed to tap water, for 3 and 10 days, respectively, in order to verify the ability of the eels to recover from the changes induced by cocaine exposure. The experiment was carried out in triplicate. All eels (30 specimens per treatment, 150 specimens in all) were anesthetised with MS-222 at a concentration of 100 mg L^{-1} (Gay et al., 2013, 2016), weighed and measured. Blood was collected from the posterior cardinal vein with a 5 mL syringe, allowed to clot in Eppendorf tubes for 2–4 h, centrifuged for 15 min at 2000*g*, and serum was collected and stored at −22 °C until assayed. The anesthetized animals were killed by decapitation immediately after collection of blood samples; all of them were males, as revealed by visual inspection of their gonads. From each animal, blocks of superficial skeletal muscle tissue were removed from the lateral line region and processed 1) for light microscopy, to evaluate the morphology of the skeletal muscle and the sarcomeric actin filaments or 2) weighed, immediately frozen in liquid nitrogen and 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), with the aid of a commercial kit (Bio-Optica s.p.a., Milan, Italy). Sections were de-waxed, pretreated with trivalent iron and later treated with ammoniacal solution as a silver source, based on the recommendations of the manufacturer. 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 et al., 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).

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

To highlight differences in muscle protein expression between untreated and cocaine exposed eels, total protein electrophoretic profiles were analysed by SDS-PAGE. Samples of skeletal muscle were homogenized in cold lysis buffer (50 mM HEPES, pH 7.4, 5 mM CHAPS, 5 mM dithiothreitol) by using the T10 basic ULTRA-TURRAX S10N-5G (two cycles of 2 min each). Homogenates were then centrifuged at 16,000*g* for 15 min at 4 °C to remove tissue debris. The supernatant was transferred into a new tube and protein content was determined by the Bradford Protein Assay (Bio-Rad Laboratories, Milan, Italy). Proteins were then separated on a 10% SDS–page and both a mini-chamber (gel dimension 8.3 cm × 7.3 cm) and a large-chamber (gel dimension 16 cm × 16 cm) from Bio-Rad Laboratories (Milan, Italy) were used. In order to resolve well high molecular weight (HMW myosins) and low molecular weight (actins and tropomyosin) proteins, 60 μg of proteins in the mini-chamber and 45 μg of proteins in the large-chamber, respectively, were loaded onto a 10% gel. Gels were stained with a solution of Coomassie brilliant blue for 1 h. Excess colorant was removed by washing the gels with a destaining solution for 48 h. Gel images were acquired by using the densitometer GS800 from Bio-Rad Laboratories (Milan, Italy).

2.6. Cytochrome oxidase (COX) activity

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

To determine whether cocaine exposure caused apoptosis in muscle cells, a caspase-3 activity assay was performed on muscle protein extracts from different untreated and cocaine-exposed specimens. Samples of skeletal muscle were homogenized and protein content was determined as described above 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 manufacturers 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 expressed as nmol of free pNA normalized for μg of proteins and time (min). When requested the caspase-3 inhibitor Ac-DEVD-CHO was used at 20 μM.

2.8. Serum enzymes

To assess the presence of skeletal muscle damage, the serum levels of some enzymes that are well established biomarkers of muscle damage were evaluated. 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 catalyzes 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, monitored at 340 nm, is proportional to CK activity. *N*-acetyl cysteine (NAC) was 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 the 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 1 UI L^{-1}, 31 UI L^{-1} and 0.463 UI L^{-1} for CK, LDH and AST, respectively.

2.9. Statistical analysis

The quantitative data on the diameter of the muscle fibres, COX and caspase-3 activities, and serum enzymes were subject to statistical analysis; the values were expressed as means ± standard error of mean (SEM). 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 $P < 0.05$.

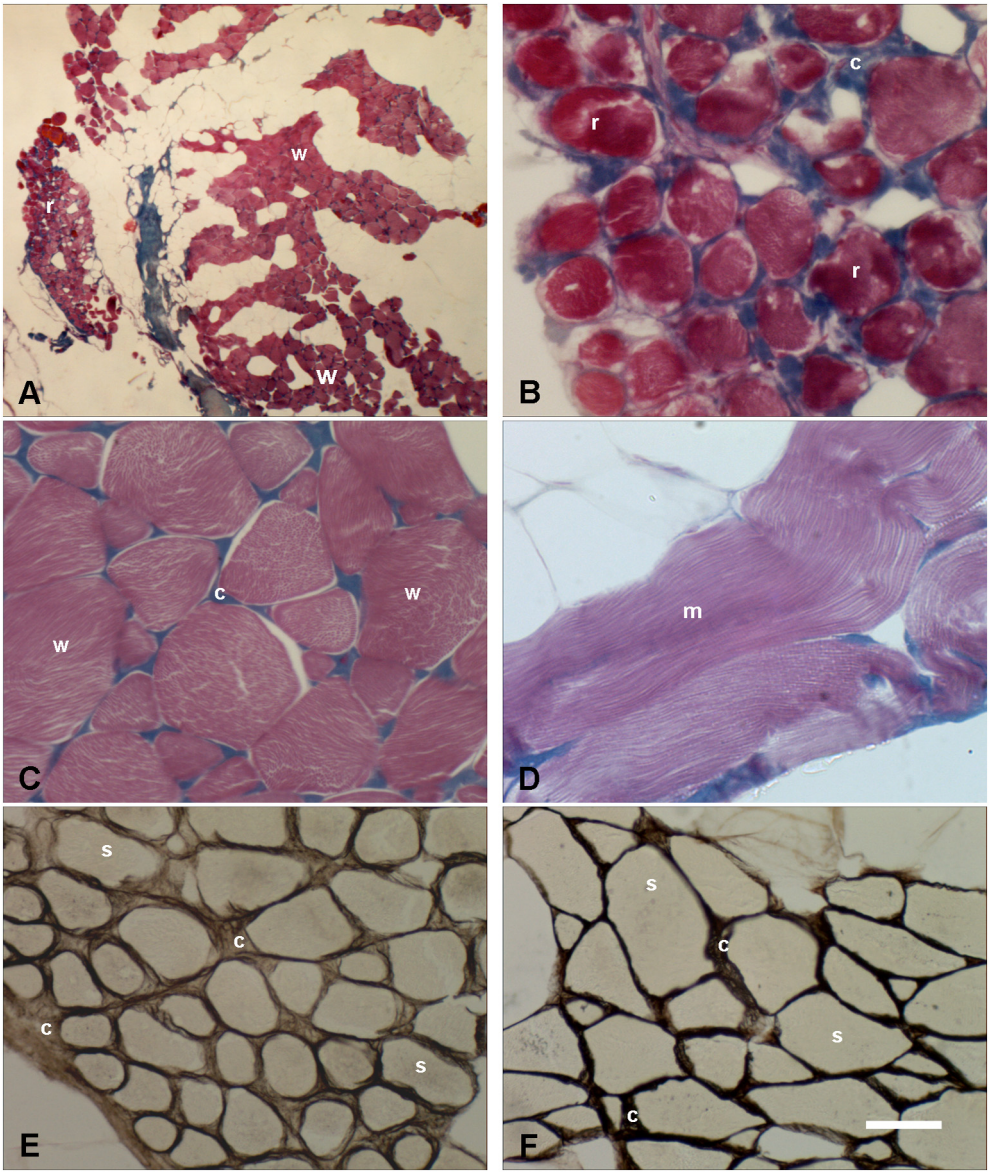

Fig. 1. Light micrographs of the skeletal muscle of *Anguilla anguilla*: control specimens ($n = 30$). (A, B, C, D: Mallory staining; E, F. silver impregnation). (A) The red (r) muscle lied as a wedge along the lateral line, whereas the white (w) muscle formed the greatest volume of the body tissue. (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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3. Results

3.1. General health condition

Throughout the experiment, all the specimens were monitored for clinical signs of disease; neither mortality nor signs of disease were observed. Every day of the treatment, after renewing the water and administering cocaine or vehicle, all the experimental groups were observed for 60 min, by five different observers. Eels exposed to cocaine appeared to be hyperactive, compared to the control animals, showing accelerated swimming. Since no differences were observed between the two control groups for any of the parameters evaluated, further description of vehicle-treated controls will be omitted.

3.2. General morphology

In the control eels, the red muscle formed a wedge along the lateral line, just beneath the skin, whereas the white muscle formed the greatest volume of body tissue (Fig. 1A). The red fibres appeared to be 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 that was more conspicuous in the red muscle (Fig. 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 to each other (Fig. 1B–D). After silver impregnation, 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 either type of fibre.

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

Three days (Fig. 3A, B) and 10 days (Fig. 3C, D, E) after the interruption of cocaine exposure, both red (Fig. 3A, C) and white (Fig. 3B, D, E) fibres showed signs of injury and muscle breakdown. In particular, the

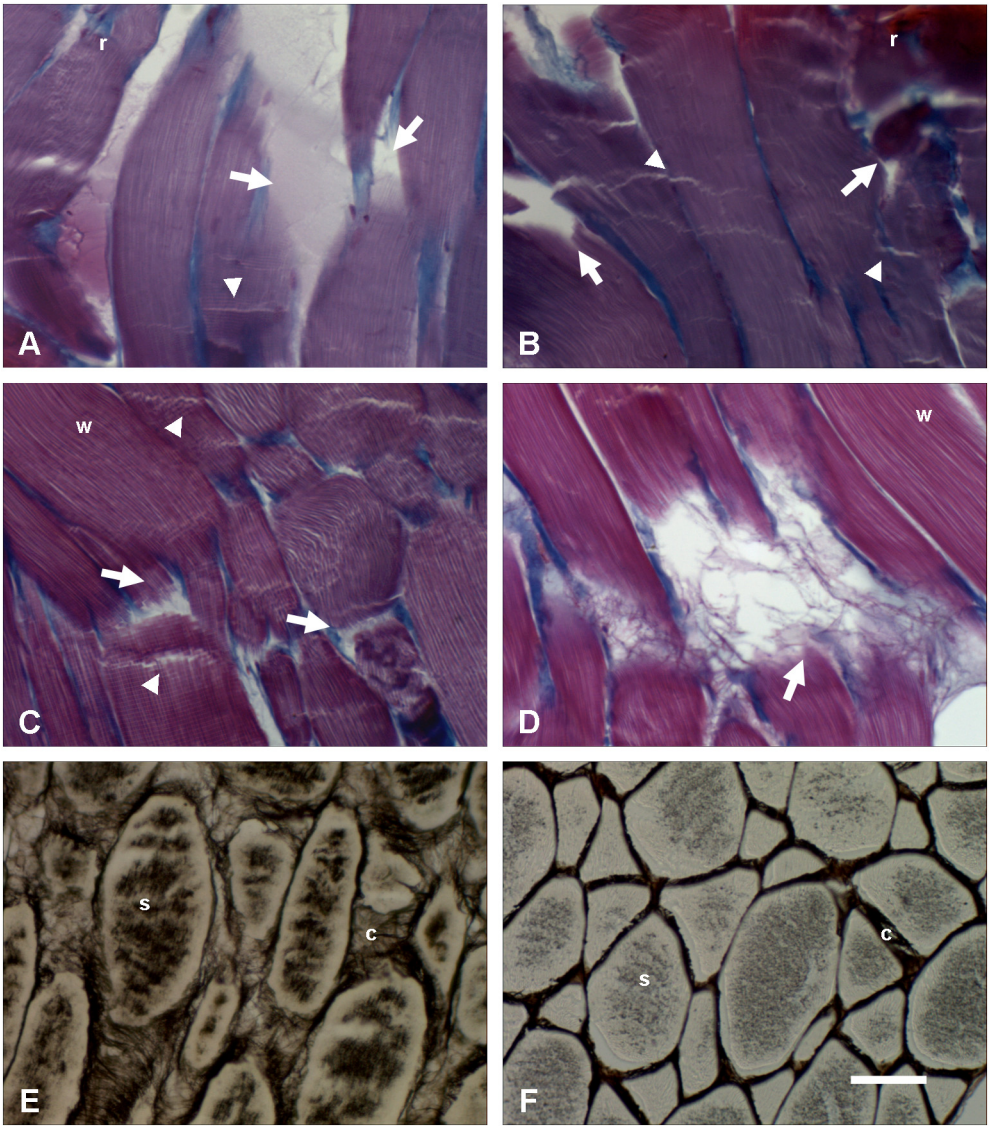

Fig. 2. Light micrographs of the skeletal muscle of *Anguilla anguilla*: exposed specimens ($n = 30$). (A, B, C, D: Mallory staining; E, F. silver impregnation). Both red (r) (A, B) and white (w) (C, D) fibres showed signs of injury as laceration (arrow) and transversal fragmentation (arrowhead). (E, F) In addition to the connective (c) tissue, the sarcoplasm (s) of both the red (E) and white (F) fibres were silver-stained and argyrophilic. Scale bar: 25 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

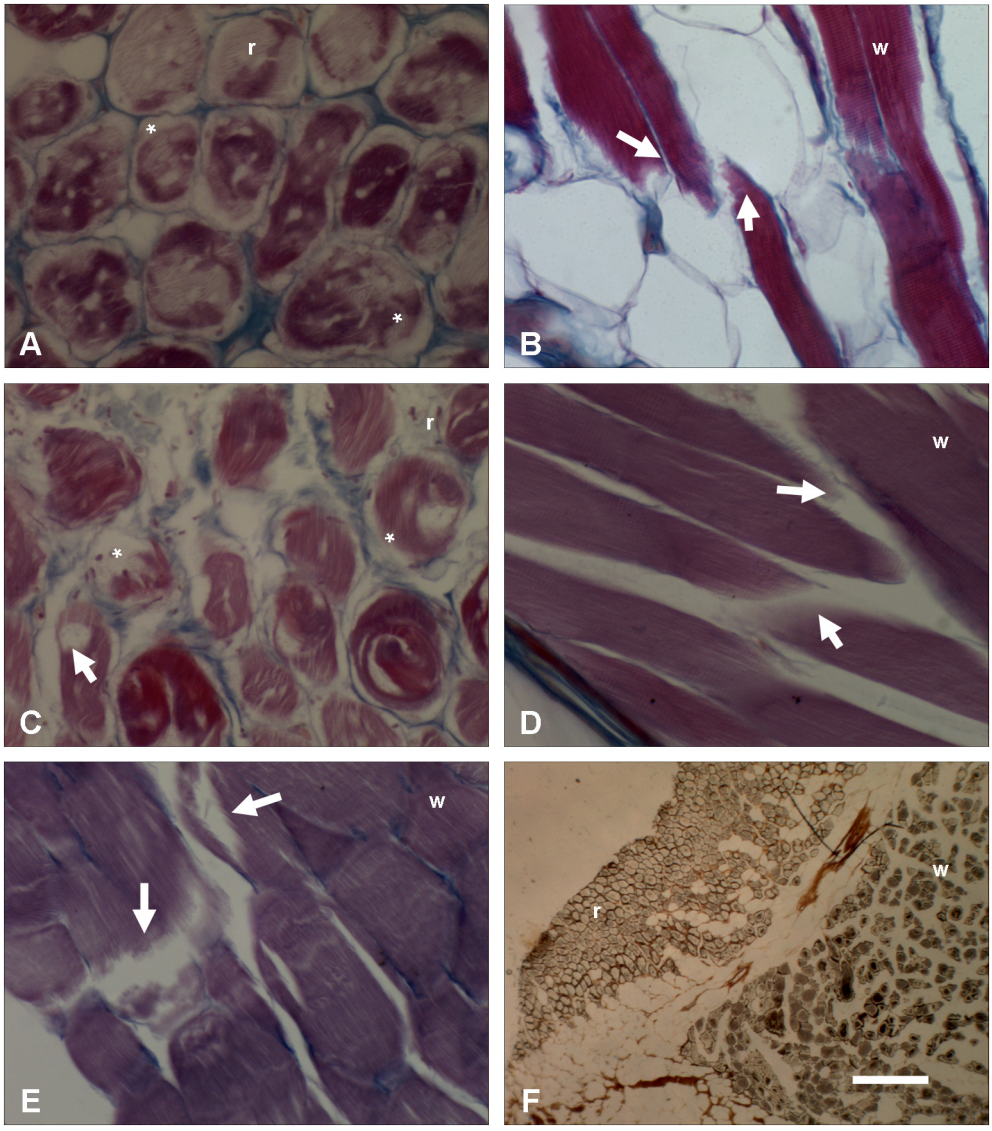

Fig. 3. Light micrographs of the skeletal muscle of *Anguilla anguilla*: post-exposure recovery three ($n = 30$) and 10 days ($n = 30$) specimens. (A, B, C, D, E: Mallory staining; F. silver impregnation). Three days (A, B) and 10 days (C, D, E) after the interruption of cocaine exposure, both red (r) (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 myofibrils and disorganization of the contractile apparatus. (F) Ten days after the interruption of cocaine exposure, the sarcoplasm of both types of fibre appeared silver stained. Scale bar: a, b, c, d, e; 25 μm; f: 200 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

red fibres (Fig. 3A,C) appeared to be more severely affected than white fibres, showing signs of swelling, rarefaction of the myofibrils and disorganization of the contractile apparatus. The sarcoplasm of both types of muscle fibre was still silver stained 10 days after the interruption of cocaine exposure (Fig. 3F).

Assessment of the diameter of the red and white fibres revealed an increase in the mean diameter of both types of muscle fibre. This increase was significant ($P < 0.05$) in the red fibres of the exposed and post-exposure recovery specimens, whereas in the white fibres differences in fibre diameter became significant only 10 days after the interruption of cocaine exposure (Table 1).

Assessment of the morphology of the sarcomeric actin filaments with FITC-phalloidin (Fig. 4) confirmed previous observations. In control eels, actin filaments showed a regular organization (Fig. 4A) whereas, after the exposure to cocaine, the filaments showed signs of injury, such as laceration and transversal fragmentation (Fig. 4B). Three days (Fig. 4C) and 10 days (Fig. 4D) after the interruption of cocaine exposure, the alterations were still present.

3.3. Muscle protein profile

An electrophoretic analysis of muscle protein composition in samples from different experimental groups was performed. SDS-page profiles did not show evident qualitative differences in protein bands between groups, either in the range of high molecular weight proteins (HMW myosins) (about 210 kDa) (Fig. 5A) or low molecular weight

Table 1
Diameter (μm) of the red and white muscle fibres in control ($n = 30$), exposed ($n = 30$) and post-exposure recovery three ($n = 30$) and ten ($n = 30$) days specimens. Values are means ± SE of the mean. *: $P < 0.05$ vs. control.

	Red fibres (μm)	White fibres (μm)
Control ($n = 30$)	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

proteins (actins and tropomyosins) (about 32–45 kDa) (Fig. 5B). Sporadic small differences of band intensities, which were not related to a specific experimental group, might be due to individual variability in protein abundance.

3.4. Cytochrome oxidase (COX) activity

Compared to control eels, COX activity was slightly increased in cocaine-exposed eels. Significant ($P < 0.05$) increases in COX activities were observed 3 days and 10 days after the interruption of cocaine exposure, respectively (Fig. 6).

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).

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 at 3 days and further increased 10 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 10 days after the interruption of cocaine exposure (Fig. 8B).

The serum levels of AST significantly ($P < 0.05$) increased after the exposure to cocaine and reached a maximum 3 days after the interruption of cocaine exposure. The AST levels then decreased 10 days after the interruption of cocaine exposure, remaining however higher than in controls (Fig. 9).

4. Discussion

The results of the present study show, for the first time, that low environmental cocaine concentrations damage the skeletal muscle of the European eel, inducing alterations that persist after the interruption of cocaine exposure.

4.1. General health condition

On the basis of personal observations, the cocaine exposed eels, compared to the control/vehicle ones, showed signs of an increased motor activity, namely accelerated swimming. This finding is consistent with the properties of cocaine, a psychomotor stimulant well-known to produce motor stimulation and increase vigilance and alertness (Grilly and Salamone, 2012). Hyperactivity was also observed in the zebrafish *Danio rerio*, but only during cocaine withdrawal and not following the single administration of a range of cocaine doses (Lòpez-Patiño 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 even at very low concentrations, chronic cocaine exposure has effects on eels that are similar to those observed in humans (Grilly and Salamone, 2012).

4.2. General morphology and protein profile of the skeletal muscle

With regard to the general morphology of the skeletal muscle, routine histological analysis showed that chronic exposure to environmental cocaine concentrations heavily damaged both red and white muscle fibres, both of which showed signs of injury, such as fibre breakdown

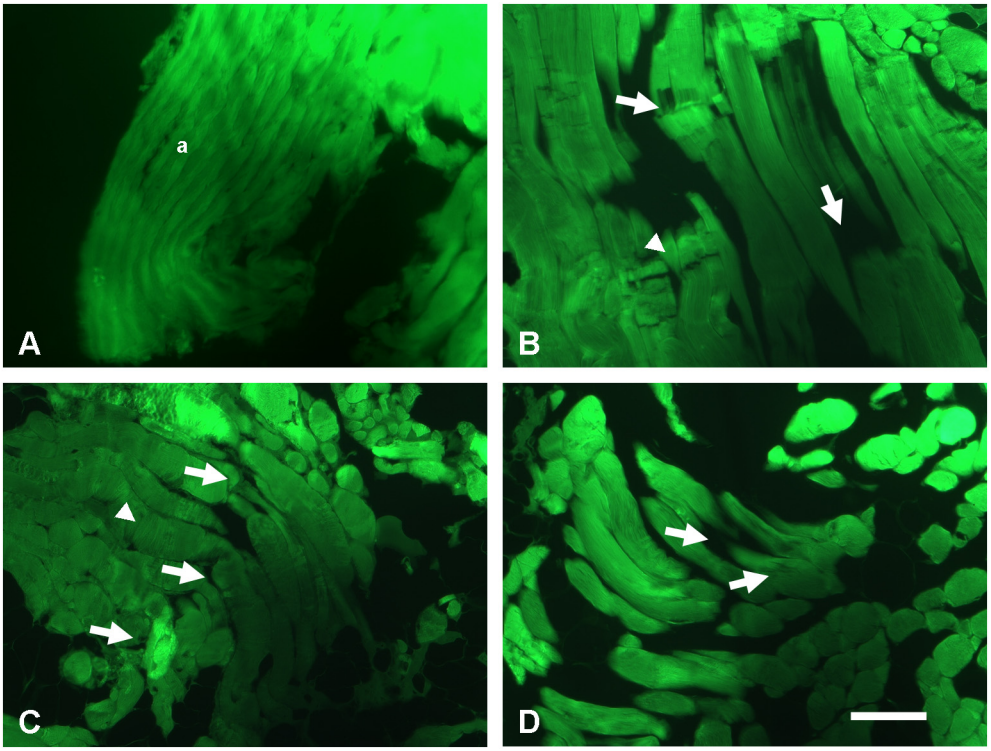

Fig. 4. Fluorescent micrographs of the skeletal muscle of *Anguilla anguilla*: FITC-phalloidin staining. (A) Control specimens ($n = 30$). Actin (a) filaments showed a regular organization (B) Exposed specimens ($n = 30$). Actin filaments showed signs of laceration (arrow) and transversal fragmentation (arrowhead), still evident (C) 3 days ($n = 30$) after the interruption of cocaine exposure and (D) 10 days ($n = 30$) after the interruption of cocaine exposure. Scale bar: a: 50 μm; b, c, d, e: 100 μm.

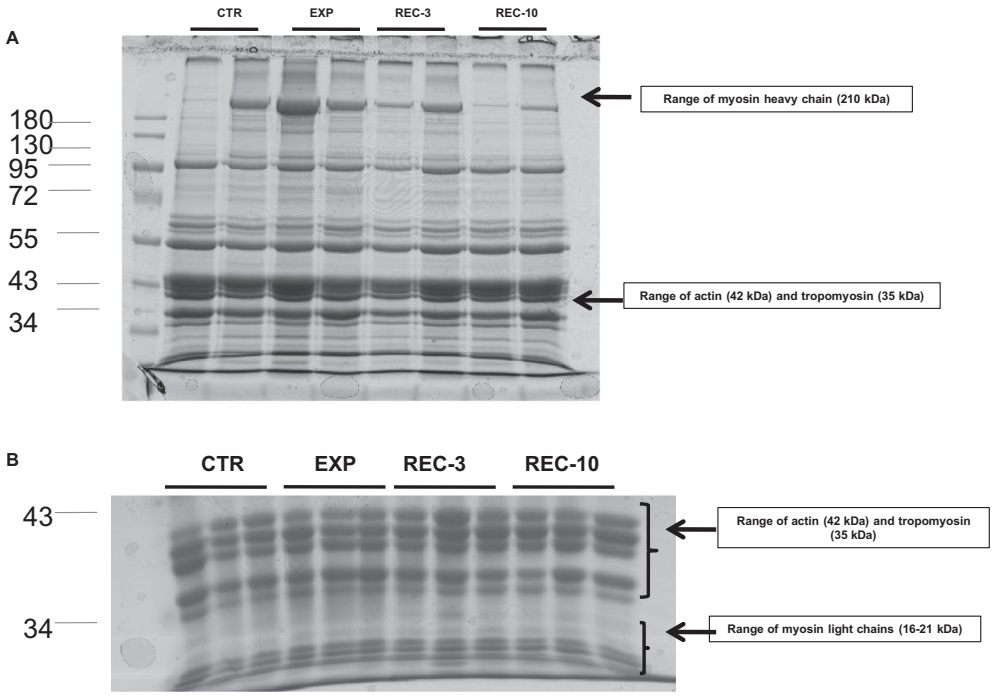

Fig. 5. SDS-page representative profiles of proteins from samples of skeletal muscles from control (CTR, $n = 30$), exposed (EXP, $n = 30$), post-exposure recovery three days (REC-3, $n = 30$) and ten days (REC-10, $n = 30$) specimens. No differences in the range of high molecular weight (HMW myosins) (A) or low molecular weight (actins and tropomyosins) (B) proteins were observed.

and swelling. This damage was still present 10 days after the interruption of cocaine exposure, and was confirmed by FITC-phalloidin staining, which revealed damage to the sarcomeric actin filaments, both during exposure and after the interruption of the cocaine exposure. The alterations observed were similar to those typical of rhabdomyolysis, a syndrome that is characterized by breakdown of the muscle fibres and dispersion into the circulatory system of the intracellular components, including the enzymes CK, LDH and AST, myoglobin and electrolytes (Keltz et al., 2013; Khan, 2009). Our finding of greatly increased serum levels of CK, LDH and AST in eels both during the cocaine exposure (CK, AST) and after its interruption (CK, LDH, AST) is reminiscent of rhabdomyolysis. The development of rhabdomyolysis has been associated with a wide variety of diseases, injuries, medications, toxins, alcohol and drugs of abuse such as opiates, amphetamines and other

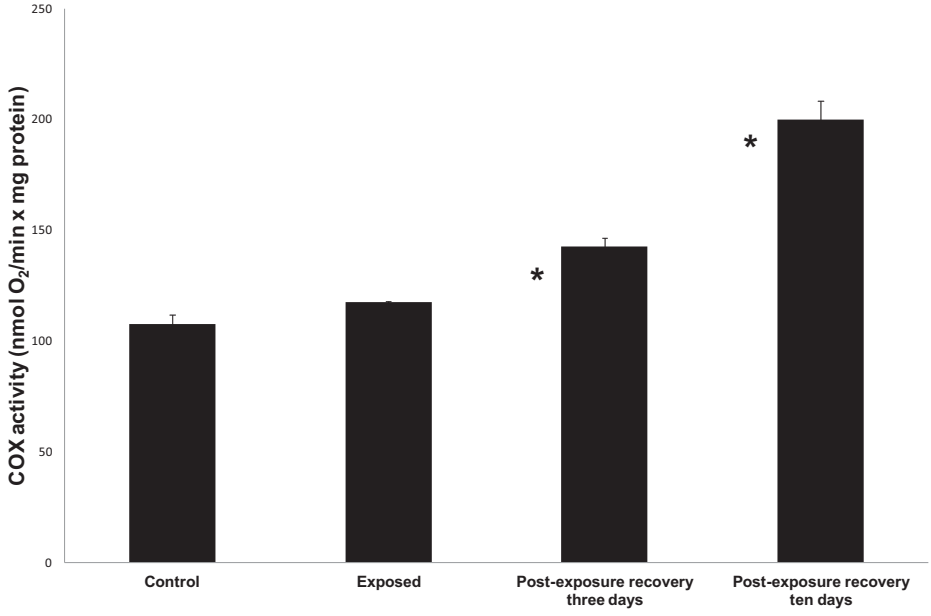

Fig. 6. Cytochrome oxydase (COX) activity in control ($n = 30$), exposed ($n = 30$), post-exposure recovery three ($n = 30$), and ten ($n = 30$) days specimens. Values are expressed as nmol O_2/min x mg protein. COX activity significantly increased three and 10 days after the interruption of cocaine exposure. Values are mean ± SE of the mean. *: significantly ($P < 0.05$) different from the control values.

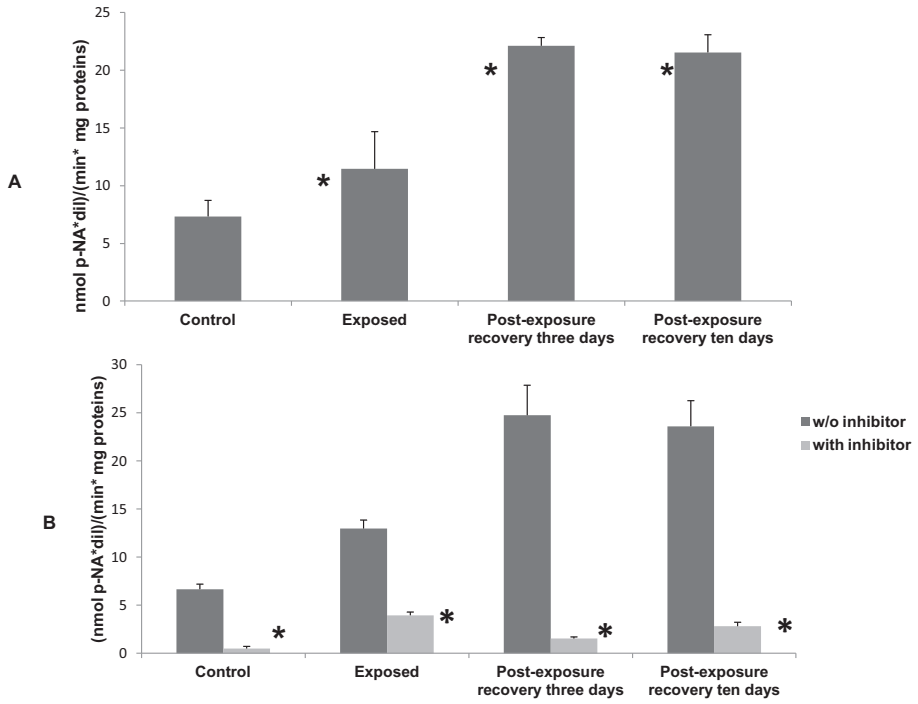

Fig. 7. Caspase-3 activity in the skeletal muscle of control ($n = 30$), exposed ($n = 30$), post-exposure recovery three ($n = 30$) and ten ($n = 30$) days specimens. Values are expressed as nmol of free pNA normalized for μg of proteins and time (min). (A) Caspase-3 activity significantly increased 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.

stimulants including cocaine (Brazeau et al., 1995; Keltz et al., 2013; Khan, 2009). 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 the vasoconstrictor properties of cocaine and its metabolites. Further damage to the muscle tissue results from the subsequent reperfusion and the generation of free radicals that escape the radical scavenging systems of the muscle and associated vasculature. This in turn damages 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 are activated in eels during and after the interruption of cocaine exposure. Another effect of the disruption of sarcolemma is leakage into the circulation of large quantities of myoglobin, a cytoplasmic hemoprotein expressed in cardiac myocytes and in skeletal muscle fibres (Ordway and Garry, 2004). If 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 have highlighted renal damages in *A. anguilla* chronically exposed to cocaine, strengthening our hypothesis of a cocaine-induced 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 (Chevallet et al., 2006), it is conceivable that the cell structures involved in the silver binding were the muscle proteins, although these are difficult to identify with certainty without specific staining techniques.

The increase in the mean diameter of the muscle fibres may be considered indicative of muscle damage. Indeed, it has been shown that the swelling of the muscle fibres is one of the clinical features of rhabdomyolysis (Alessandrino and Balconi, 2013; Nance and Mammen, 2015). The red muscle appeared to be 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 significantly increased only 10 days after the interruption of cocaine exposure. In silver eels, which 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 more 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 to that observed in smooth muscle tissue of the eel intestine (Gay et al., 2016). 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 that observed in humans (Brazeau et al., 1995; Keltz et al., 2013; Khan, 2009).

Our electrophoretic analysis of the muscle protein profile did not highlight qualitative differences between the different experimental groups. These findings indicate that chronic cocaine exposure does not influence expression of high molecular weight (myosins) or low molecular weight (actins and tropomyosins) muscle proteins. However, in the rat, 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 in F-actin, through changes in the content or phosphorylation state of actin binding proteins, or reduced depolymerization and actin cycling respectively (Toda et al., 2006). In contrast, cocaine increased the total protein content and the expression of beta-myosin heavy-chain protein in rat cardiac ventricular myocytes

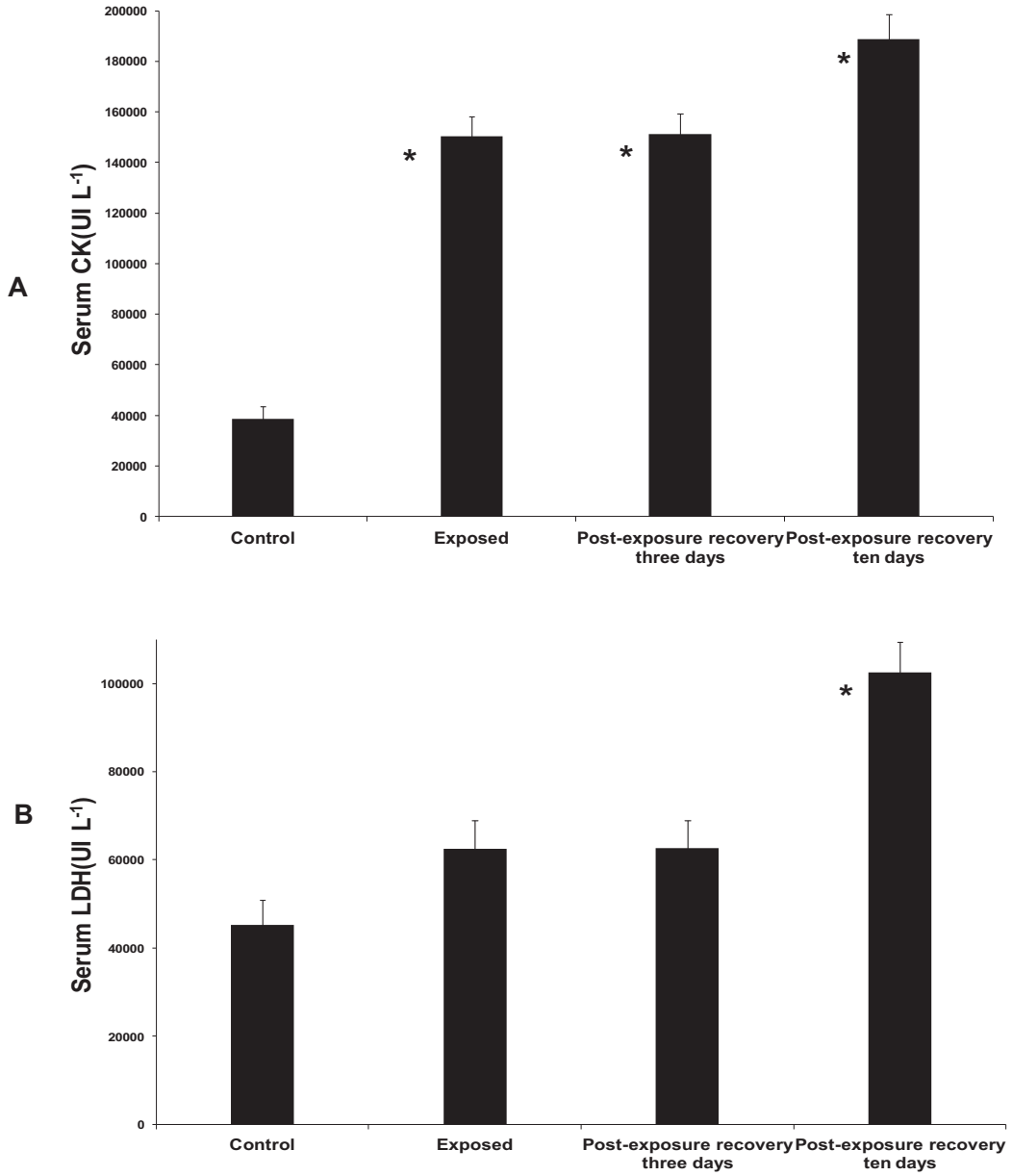

Fig. 8. (A) Serum creatine kinase (CK) and (B) serum lactate dehydrogenase (LDH) levels in control ($n = 30$), exposed ($n = 30$), post-exposure recovery three ($n = 30$) and ten ($n = 30$) days specimens. Values are expressed as UI L^{-1}. (A) The serum CK levels significantly increased in the exposed specimens to be steady 3 days and further increase 10 days after the interruption of cocaine exposure. (B) The serum LDH levels significantly increased only 10 days after the interruption of cocaine exposure. Values are the mean ± SE of the mean. *: significantly ($P < 0.05$) different from the control values.

(Henning et al., 2000) and it alters the protein profile of many different proteins, including cytoskeletal proteins in zebrafish embryos (Parolini et al., 2018). In any case, a better understanding of the effects of cocaine on the muscle protein profile of eels will require further studies.

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

Our results showed an increase in COX activity that became significant after the interruption of cocaine exposure. Cytochrome oxidase (COX), a well-known biomarker of oxidative metabolism (Lee and Hüttemann, 2014), catalyzes the final step in the mitochondrial electron transfer chain (ETC) and the reduction of oxygen to water. ETC plays a key role in cell metabolism producing most of the cellular energy. The increase in COX activity observed in skeletal muscle homogenates might indicate an increase in specific mitochondrial enzyme activity and/or in mitochondrial protein mass. This increase might play an important role in the physiological adaptation to cocaine exposure by supporting the increased energy requirements for the detoxification process. However, cocaine is known to induce cardiovascular mitochondrial dysfunction (Graziani et al., 2017) 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 doses and times of exposure to cocaine. Noteworthy are previous studies that suggest that 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 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

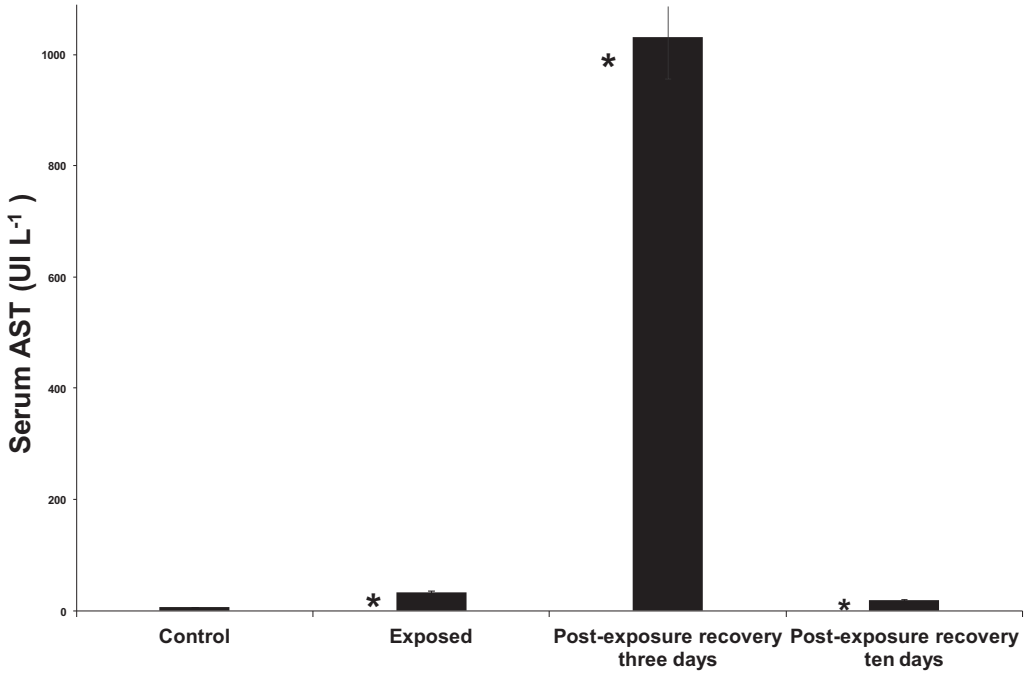

Fig. 9. Serum aspartate aminotransferase (AST) levels in control ($n = 30$), exposed ($n = 30$), post-exposure recovery three ($n = 30$) and ten ($n = 30$) days specimens. Values are expressed as UI L^{-1}. The serum AST levels significantly increased in the exposed specimens to further hugely increase 3 days and to decrease 10 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.

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 is consistent 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 (Brentnall et al., 2013) 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) 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 might be associated with the ischemic/reperfusion injury.

4.4. Serum enzymes

Our results showed that chronic exposure to cocaine increased the serum levels of creatine kinase (CK), lactate dehydrogenase (LDH) and aspartate aminotransferase (AST), albeit with a different pattern. Indeed, the increases in CK and AST levels were significant both during cocaine exposure and after its interruption, whereas the increase in LDH levels became significant only 10 days after the interruption of cocaine exposure. CK, LDH and AST are well-established serum markers of the functional state of the muscle, and their increase may be an index of tissue injury (Brancaccio et al., 2010). CK catalyzes the reversible reaction of creatine and ATP forming phosphocreatine and ADP; it plays therefore a key role in energy homeostasis of cells and it is typical of tissues with high energy demand, such as skeletal muscle, heart and brain. LDH is involved in the interconversion of pyruvate and lactate, with concomitant interconversion of NADH and NAD during the final reactions of glycolysis. It is present in the cytoplasm of all the cells and, since it is released during tissue damage, it is a marker of common injuries and diseases such as heart failure and muscle injury. AST catalyzes the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate, providing energy to the cells; the enzyme is localized primarily in the skeletal and myocardial muscle, liver and erythrocytes (Brancaccio et al., 2010; Yousaf and Powell, 2012). The increase in the serum levels of these enzymes observed both during the cocaine exposure (CK, AST) and after its interruption (CK, LDH, AST) confirms the morphological observations of structural damage to the skeletal muscle and could be indicative of a rhabdomyolysis syndrome (Keltz et al., 2013; Khan, 2009). However, considering that every enzyme has different tissue-specific isoforms (Brancaccio et al., 2010) that were not evaluated here, since only the total amount of every enzyme was determined, the increases observed could also be indicative of heart and/or liver damage. A correlation between muscle injury and the increase in serum muscle enzyme levels was also found in the Atlantic salmon, in which increases in serum CK and LDH levels, and CK levels, were associated with heart and skeletal muscle inflammation (Yousaf and Powell, 2012), and with acute skeletal myopathy, respectively (Rodger et al., 1991). Our results, showing an increase in the serum enzymes considered main 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, the results of this study showed that low cocaine concentrations damaged the morphology and physiology of the eel skeletal muscle. At the silver stage, the eel undertakes a swimming migration 6000 km long (Righton et al., 2016) needing sufficient energy reserves and a 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, including overfishing, habitat loss, presence of parasites, climate change and water pollution (Dekker, 2003). Our results suggest that

environmental cocaine concentrations contribute to the threat of water pollution to this susceptible species. Finally, the European eel is an edible species used as food resource (Arai, 2014). Since the skeletal muscle is the edible part of the eel, and bioaccumulates cocaine to a large extent (Capaldo et al., 2012), these results suggest the possibility that cocaine could be taken by humans with food, although further studies are needed to verify this hypothesis.

Competing interests

The Authors declare no competing or financial interests.

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