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

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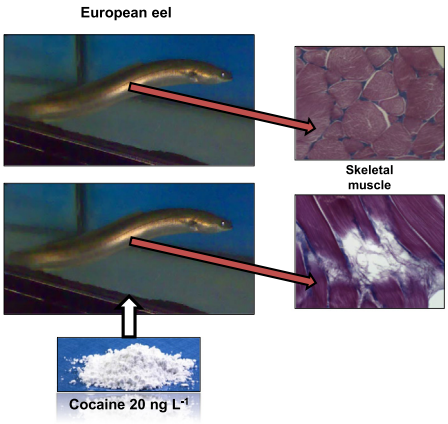
## HIGHLIGHTS

- 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.,