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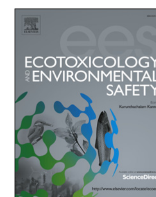

Myofibrilar functional dysregulation in fish: A new biomarker of damage to pesticides

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ABSTRACT

Endosulfan (ES) modifies the ultrastructure of skeletal muscle fibers and causes changes to the swimming behavior of fish. The objectives of the present work were to evaluate, in fishes of *Australoheros facetus*, 1) the integrity of myofibrils (Mf) by the analysis of SDS-PAGE profiles, and 2) the functionality of Mf through the microscopically monitoring of the contraction and changes in Mg^{2+} Ca^{2+}- ATPase and Mg^{2+}(EGTA) -ATPase activities. As expected, after the addition of the contraction buffer, control fish Mf contracted. On the contrary, Mf from fish exposed at 0.5 µg/L ES showed a partial contraction and none of the fish exposed at 10 µg/L ES contracted. As judged by its high Mg^{2+} Ca^{2+} ATPase activity and low Mg^{2+} (EGTA) ATPase activity, control Mf showed good functionality. In Mf from fish exposed to 0.5 and 10 µg/L ES the activities of these enzymes were similar, suggesting denaturation or degradation of some component of tropomyosin–troponin complex. SDS-PAGE patterns of Mf from fish exposed to ES showed degradation of the myosin heavy chain and of tropomyosin. Similar values of lipid peroxidation (TBARS) were found in both control and exposed Mf, suggesting that lipid oxidation was not be related to the above-mentioned changes. The observed effects expand the knowledge of ES action in muscles and could be used as biomarkers of damage in fishes exposed to organochlorine compounds like the insecticide endosulfan.

1. Introduction

Some pesticides generate neurotoxic effects in aquatic organisms. To evidence these effects, the enzyme acetylcholinesterase (AChE) and perturbations in the swimming behavior of fish are the biomarkers commonly used in ecotoxicology. The AChE activity removes the neurotransmitter acetylcholine (ACh) from the synaptic cleft through hydrolysis (Kumar et al., 2016), mainly in organisms exposed to organophosphorus and carbamate pesticides. Inhibition of AChE activity correlates with a decrease in swimming behavior -a second type of biomarker- in fishes exposed to high concentrations of organophosphorus pesticides (Bonansea et al., 2016) and to sublethal concentrations of the organochlorine endosulfan (ES) (Ballesteros et al., 2009). However, there is not always a direct relationship between both AChE activity and behavior (Ballesteros et al., 2017). In this sense, Da Cuña et al. (2011) showed no changes in this enzyme but hypo-activity and erratic swimming in *Cichlasoma dimerus* exposed to ES, suggesting that alterations in the swimming activity can be caused through other pathways. As an alternative, we propose new biomarkers (contraction of isolated myofibrils and ATPase activity) to explore if ES directly alters the structure and functionality of the muscle tissues.

Myofibrils are the functional units of skeletal muscle. Muscle contraction occurs by cyclic movements of myosin heads on thin filaments produced by the mechanical force generated by conformational changes in tropomyosin- troponins complex after Ca^{2+} capture. The energy necessary to produce these movements is obtained by ATP hydrolysis in the presence of Mg^{2+} and Ca^{2+}, reaction catalyzed by the Ca^{2+}ATPase present in myosin heads (Roura, 1992). In this way, normally contraction of muscle occurs when neurotransmitter ACh produced by nervous stimulation induces membrane depolarization and releases Ca^{2+} from the reticulum sarcoplasmic. Relaxed muscle takes place in the absence of nervous stimulus. In this condition, Ca^{2+} ions return to reticulum sarcoplasmic membrane spending energy to overcome the concentration gradient. On the other hand, a significant higher activity of the Mg^{2+} Ca^{2+}- ATPase than Mg^{2+} (EGTA) –ATPase enzymes evidence the normal functioning of the tropomyosin- troponins complex while if it is deregulated there is no difference between these activities. Depending on fish species, its nutritional and gonadal status, Mg^{2+} Ca^{2+}- and Mg^{2+} (EGTA)- ATPase activities in healthy fish muscle range between 0.20 and 1.0 µmol P/ mg protein. min and 0.05–0.4 µmol P/

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mg protein. min, respectively (Roura et al., 1995; Pagano et al., 2004; present work).

Mishra and Shukla (1995) demonstrated that ES exerts marked ultrastructural alterations in the skeletal muscle fibers of the fish, that correlate with an inhibitory effect on electron transport and affects ATP synthetase complex leading to impairment in mitochondrial bioenergetics. More recently, ultrastructural changes (breakage, collapse of Mf, disorganized myosin and actin filaments) in muscle tissues have been described in fish exposed to other synthetic compounds (Lee et al., 2014).

Considering the effects of ES on – the ultrastructure of the skeletal muscle fibers (Mishra and Shukla, 1995), – the swimming behavior (Ballesteros et al., 2009; Maynart Pereira et al., 2012), and that ES generates oxidative stress increasing the levels of thiobarbituric acid reactive substances (TBARs) able to react with proteins, lipids and nucleic acid producing denaturation and aggregation of biopolymers (Tironi and Tomas, 2000), the objectives of the present work were to evaluate, in fishes of *Australoheros facetus*, 1) the integrity of myofibrils (Mf) by the analysis of SDS-PAGE profiles, and 2) the functionality of Mf through the microscopically monitoring of the contraction and changes in Mg^{2+} Ca^{2+}- ATPase and Mg^{2+}(EGTA) -ATPase activities.

2. Material and methods

2.1. Fish exposure

Adult fish were collected in non- anthropized freshwater bodies around Mar del Plata (Buenos Aires Province, Argentina, 37° 53´ South, 57° 59´ West) and acclimatized for 2 months to laboratory conditions in 140 L tanks. Healthy specimens with approximately the same size (mean total length (± SD): 10.1 ± 1.4 cm; mean weight (± SD): 24.1 ± 11.4 g) were selected.

Two concentrations of ES were tested: 0.5 and 10 µg/L. These concentrations were selected according to previous studies of ES in *A. facetus* in which sublethal effects were detected (Crupkin et al., 2013). Both concentrations are environmentally relevant since values from 0.004 to over 2 µg/L ES have been reported in Argentina (Silva et al., 2005; Gonzalez et al., 2012), and higher than 100 µg/L in other countries like USA (Mersie et al., 2003).

A negative control containing tap water with 50 µl/L of DMSO (representing a concentration of 0.004%, the same concentration of DMSO used in ES treatments) was included. A solution of endosulfan (6, 7,8, 9, 10, 10 – hexachlor – 1, 5, 5, 6, 9, 9- exahydro – 6,9 – metane-2,4,3 –benzo (e) dioxatiepin -3-oxide) (70:30 alpha-: beta- isomers) was used. We prepared two stock solutions by diluting alpha- endosulfan (Riedel-de Haën, CAS 959-98-8) or beta-endosulfan (Riedel-de Haën, CAS 33213-65-9) in dimethyl sulfoxide-DMSO (Mallinckrodt). The concentrations of the stock solutions were 1600 mg/L for alpha-ES and 800 mg/L for beta-ES. Afterwards, we prepared the exposure medium using the following amounts of both isomers in tap water in order to obtain a relation according to commercial formulas (alpha-isomer 70% and beta-isomer 30%). For the treatment of 5 µg/L: 22 µl alpha-ES from a 1/10 dilution of the stock, plus 18.7 µl beta-ES from a 1/10 dilution of the stock were used. For the treatment of 10 µg/L: 44 µl alpha-ES from a 1/10 dilution of the stock, plus 7.5 µl beta-ES from a 1/2 dilution of the stock were used. The acute static experiment was conducted using one glass tank of 30 L per treatment, containing six fish per treatment (negative control, 0.5 and 10 µg/L). The concentrations of endosulfan in the exposure medium were quantified using a GC- ECD, data previously reported in Crupkin et al. (2013).

The experiments were conducted in an aquarium illuminated with fluorescent lamps setting 12:12 light: dark periods. Mar del Plata city tap water was used for the experiments. The water temperature was of 18 °C, pH of 8.2 ± 0.2, mean total hardness of 270.2 mg/L CaCO3 and mean alkalinity of 160 mg/L CaCO3. All fishes were fed during acclimation with a commercial food (pellet Shulet composed by 45% protein, 2% lipids, 3% fiber, 1.95–2.99% Ca, 1–1.4% P), but were kept starved during the exposure to ES.

All fish were sacrificed after an exposure period of 24 h by transecting the spinal cord using a fresh razor blade. This procedure was evaluated and approved by the Animal Ethical Committee at the National University of Mar del Plata (CICUAL/UNMDP) (OCA 146/15). Muscle was dissected, weighed and immediately stored for few hours at 4 °C before its analysis.

2.2. Extracts and measurement of biomarkers in muscle tissue

2.2.1. Isolation of myofibrils

The isolation of the myofibrils were prepared according to Yasui et al. (1975), with a little modification of the extraction and purification buffer: 20 mM Tris-maleate, 100 mM KCl, pH 7, without Triton X-100 because it inactivates contractile proteins (Roura et al., 1992). Muscle subsamples of 4 g were mechanically shredded and homogenized using 30 mL standard buffer standard (0,1 M KCL-20 mM Tris-maleate, pH = 7.0) at 4 °C during 3 min in a "Virtis 45" homogenizer. The homogenate was centrifuged at 850*g* during 15 min in a refrigerated centrifuge (HERMLE Z36HK). The pellet was resuspended in the same buffer and the centrifugation and resuspension was repeated two times more.

Protein concentration was determined in aliquots of myofibril suspensions by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

Contraction of isolated myofibrils was monitored microscopically, at ambient temperature, by Phase Contrast Microscopy OLYMPUS CX31 RTSF, according to the procedure previously used by Huxley (1983). Two determinations per sample (n = 6 per treatment) were done. A volume of 20 µl of the extract was poured on a slide and it was covered with a coverslip. A volume of 10 µl of contraction buffer containing: 50 mM KCl, 5 mM MgSO4, 1 mM ATP, 0,1 mM Ca^{2+} and 20 mM imidazol, pH = 7 was incorporated by capillarity. Myofibril contraction was registered by photography with Digital Panasonic DMC LZ8 camera with a maximum zoom (5 ×), before and 1–3 min after addition of contraction buffer. Two pictures per sample were taken. The time was registered using a chronometer. The time started (time = 0) with the addition of the buffer and finished after 3 min. In order to confirm the no contraction in samples from fishes exposed to ES, 2 min more were waited.

2.2.2. Myofibrillar ATPase activity

The activities of Mg^{2+} Ca^{2+}- ATPase and Mg^{2+}(EGTA) -ATPase are related to the actin/myosin interaction in the actomyosin complex in presence and absence of calcium ions respectively. The Mg^{2+} ATPase and Mg^{2+} ethylene glycol tetra acetic acid (EGTA) ATPase activities of myofibrils were measured at 37 °C in a 30 mM Tris-maleate buffer (pH 7) according to Roura (1992). For the inhibition of myofibrillar Mg^{2+}-ATPase in the absence of Ca^{2+} ions, 0.5 mM EGTA was used. Specific conditions for each enzyme were 0.12 mg/mL of protein, 0.75 mM ATP, 60 mM KCl, 2 mM $MgCl_2$ and 0.1 mM $CaCl_2$ for the Mg^{2+} Ca^{2+} ATPase. For the Mg^{2+} (EGTA) ATPase activity a mix of 0.25 mg/mL of protein, 0,75 mM ATP, 60 mM KCl, 2 mM $MgCl_2$ and 0.5 mM EGTA were used. A blank to evaluate the ATP hydrolysis was performed, consisting of the same mix than for enzymes but replacing the sample by distilled water. A final incubation volume of 3 mL was used in all cases. Incubation times were 4 min for both enzymes and for the blank. Reactions were stopped by addition of 1 mL of cold 40% trichloroacetic acid (TCA) solution (at 10% final concentration). Liberated phosphorous coming from the ATPsic activity was determined according to the method of Chen et al. (1956). The reactive was composed by 2 volumes of bidistilled water plus 1 vol of 10% ascorbic acid, 1 vol of 6 N sulfuric acid and 1 vol of 2.5% ammonium molybdate. An aliquot of 2 mL of reactive plus 2 mL of the sample were mix and incubated 1.5 h at 37 °C. Released inorganic phosphorus from the ATP hydrolysis was quantified by

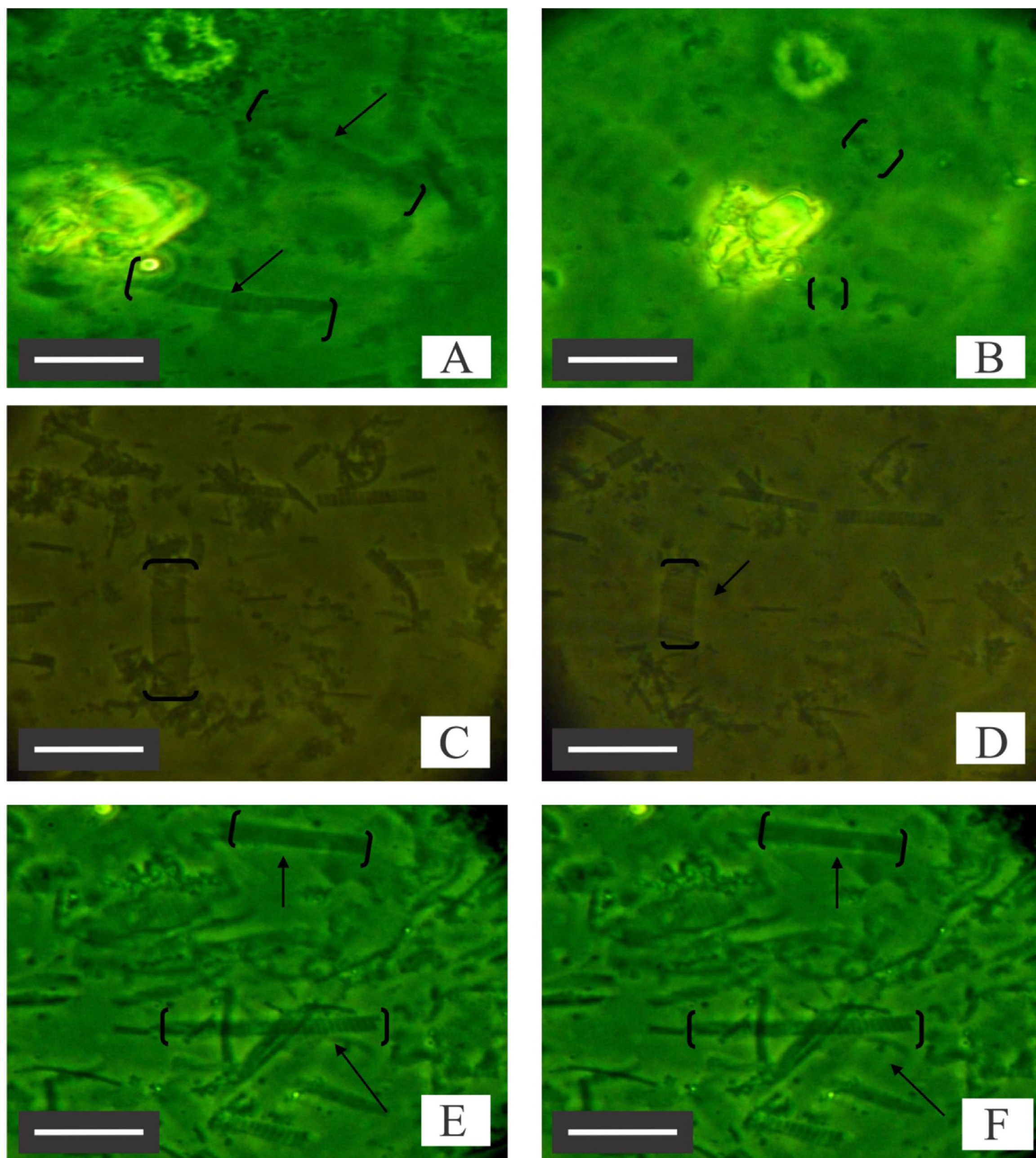

Fig. 1. Contractile function of myofibrils in muscle of *Australoheros facetus* (500 ×, Bar = 10 µm). A: Control samples without contraction buffer, B: Control samples 2 min after adding contraction buffer, C: Fishes exposed to 0.5 µg/L ES, without contraction buffer, D: Fishes exposed to 0.5 µg/L ES, 3 min after adding contraction buffer. E: Fishes exposed to 10 µg/L ES, without contraction buffer, F: Fishes exposed to 10 µg/L ES, 5 min after adding contraction buffer.

reacting with the ammonium molybdate giving a blue phosphomolybdate complex, measured using a spectrophotometer at 820 nm against the blank. The reactive blank consisted of 2 mL of reactive plus 2 mL of bidistilled water. A standard curve of inorganic phosphorus was done from a stock solution of 2 mg/mL by preparing the corresponding dilutions. The enzymatic activity was expressed as micromol of inorganic phosphorous released/ min/ mg protein at 37 °C.

2.2.3. *Integrity of myofibrilar structure*

The SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) of myofibrils was performed in 10% gels using a Micro-Slab (Sigma Chemical Co., St. Louis, MO) according to the procedure described by Laemmli (1970). Briefly, samples were denatured by heating at 100 °C during 5 min in buffer (Tris HCl 1 M, pH 6.8, SDS 10%, glycerol 40%, β-mercaptoethanol, 1%, bromophenol blue 0.05%). The amount of protein loaded on the gel was 30 µg for each sample, to obtain a linear response with protein concentration Crupkin et al. (1988). The electrophoresis was performed at constant voltage (100 V) for 50 min. In order to define bands corresponding to the areas of the troponins and for the calculation of MHC/A ratios, some gels were run for 80 min (data not shown). Then, the gels were fixed using a solution of ethanol: phosphoric acid: distilled water (50: 3: 47) overnight. After that, the gels were rinsed three times in Neuhoffs solution. Finally, they were stained with Coomasie Brillant Blue G250, 1 g/L, (BioRad) overnight and stored in 4% acetic acid until analysis. The mobility–molecular weight curve was calibrated with standards of molecular weights (range: 20–250 kDa, Sigma) as well as a sample of myofibrils from *Merluccius merluccius* previously characterized.

2.2.4. Densitometric analysis

The protein patterns were scanned and analyzed using the ImageQuant TL v2005 program. To quantify the possible degradation of MHC two different analyses were performed. The first one was the quantification of the relationship between the intensities of MHC and A bands, and the calculation of the myosin–actin ratio, by dividing myosin heavy chain area by actin area (Crupkin et al., 1988). The second one was the quantification of protein mark in the inter-band region between the MHC and the A bands. In addition, the quantification of protein mark in the inter-band region between the TM and Tn bands were performed in order to quantify the possible degradation of TM. However, because the upper edge of TM band was diffuse it was not possible to determine neither the intensity of this band nor the TM/actin ratio. The mean grey quantification of the interband- regions was performed using an 8-bit image (ImageJ 1.51d). In an intensity grey scale from 0 to 255, the 0 value corresponded to black color while the 255 value corresponded to white color. Thus, values close to 0 indicated more intermediate bands while values close to 255 indicated fewer amounts of bands.

2.2.5. Lipid peroxidation

The formation of thiobarbituric acid reactive substances (TBARs) was measured according to the procedures of Oakes and Van Der Kraak (2003). The content of TBARs in the supernatant was measured spectrophotometrically (λAbs: 532 nm) and expressed as nanomoles per mg of fresh tissue using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

2.3. Statistical analysis

Normality and homogeneity of variances were verified by Shapiro–Wilk and Levene tests, respectively. For most of the studied parameters a one-way ANOVA test was applied followed by the post hoc LSD test. In the case of ATPase activities the non-parametric Kruskal–Wallis and Mann - Whitney *U* tests were applied (Zar, 2010). Differences in all tests were considered significant at $p < 0.05$.

3. Results

The results of the contractile function of Mf from control and fish exposed during 24 h to 0.5 µg/L and 10 µg/L ES are shown in Fig. 1. The six Mf samples from control fish contracted within 3 min after addition of contraction buffer (Fig. 1A1, A2). For the same time, only three of the six analyzed Mf samples from fish exposed at 0.5 µg/L ES showed a partial contraction (Fig. 1B1, B2) while none of those from fish exposed at 10 µg/L ES contracted, even when the observation time was extended for two additional minutes (Fig. 1C1, C2). Contraction of Mf can be observed in the live streaming video (Supplementary material)

Supplementary material related to this article can be found online at http://dx.doi.org/10.1016/j.ecoenv.2018.03.096.

As can be seen in Fig. 2 both Mg^{2+} (Ca^{2+})- and Mg^{2+}(EGTA) ATPase activities of Mf from fish exposed either to 0.5 or 10 µg/L ES were similar to those of their respective controls ($p > 0.05$). Significant differences between Mg^{2+} (Ca^{2+}) and Mg^{2+}(EGTA) ATPase activities were only observed in control Mf ($p < 0.05$).

The SDS-PAGE profiles of Mf from control and fish exposed to 0.5 and 10 µg/L ES are shown in Fig. 3. The characteristic polypeptidic bands of myofibrillar proteins of fish are present in both control and exposed Mf: myosin heavy chain (MHC) (200 kDa), actin (A) (45 kDa), tropomyosin (TM) (37 kDa), troponin (Tn) (32 kDa) and myosin light chains (25 and 21 kDa) (Fig. 3A).

In addition, an increment of protein mark in the inter- band region between MHC and A can be seen in the Mf from 0.5 and 10 µg/L ES exposed fishes (Fig. 3, Table 1). Fig. 4 A shows that MHC/A ratios in Mf from fish exposed to 0.5 and 10 µg/L ES were lower than those obtained in Mf of control fish ($p < 0.05$). An increment of protein mark in the inter-band region between TM and Tn also can be seen in 0.5 and 10 µg/L ES exposed Mf (Fig. 3, Table 1).

No significant differences were observed in TBARS content in muscle of *A. facetus* exposed to 0.5 and 10 µg/L ES with respect to controls ($p > 0.05$) (Fig. 4B).

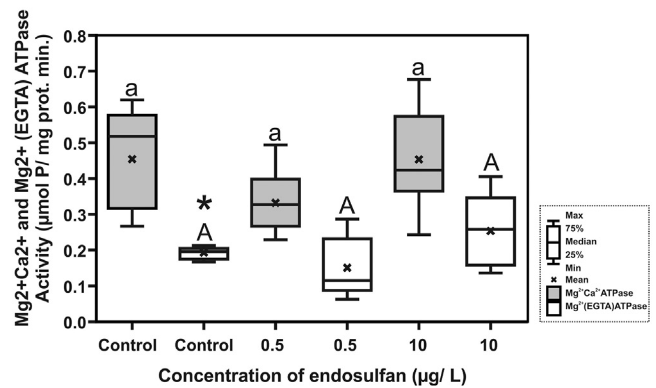

Fig. 2. Myofibrilar enzyme activities in *Australoheros facetus* exposed to 0.5 and 10 µg/L ES. Black box: Mg^{2+} Ca^{2+} ATPase. Different lowercase letters indicate differences among treatments ($p > 0.05$). White box: Mg^{2+} *EGTA* ATPase. Different capital letters indicate differences among treatments ($p > 0.05$). *: Differences between both enzymes ($p > 0.05$).

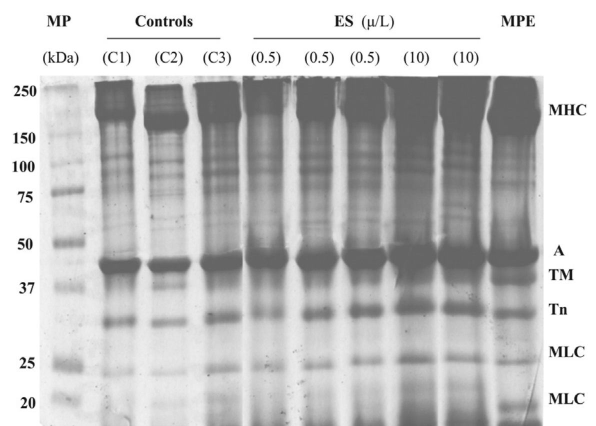

Fig. 3. A typical gel from n = 2 gels prepared per treatment showing the SDS-PAGE pattern of myofibril in *Australoheros facetus* exposed to endosulfan. MPE: Characterized bands in myofibrils of *Merluccius merluccius*. MP: Indicator of molecular weight.

Table 1
Densitometric analysis of myofibrilar proteins in muscle of *Australoheros facetus* exposed to endosulfan. Mean grey quantification of the interband- regions using an 8-bit image.

Quantified Region	Treatment (µg/L)	Area (px^2)	Grey intensity (0 – 255)
Between MHC and A bands	Negative control	6400	156 ± 29
	0.5	6400	122 ± 11
	10	6400	89 ± 14
Between TM and Tn bands	Negative control	920	203 ± 17
	0.5	920	171 ± 6
	10	920	128 ± 31

Intensity grey scale from 0 to 255, 0 value corresponded to black color and 255 value corresponded to white color. MHC: myosin heavy chain, A: actin, TM: tropomyosin, Tn: troponin.

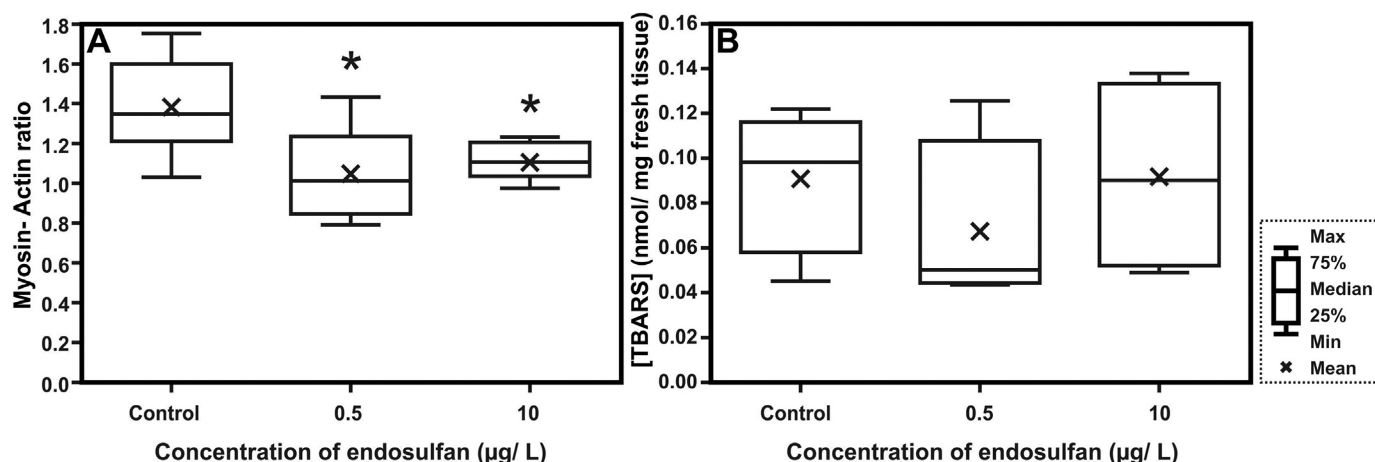

Fig. 4. Myosin heavy chain –actin (MHC-A) ratio in myofibrils of *Australoheros facetus* exposed to 0.5 and 10 μg/L ES (A). Thiobarbituric acid reactive substances (TBARS) in muscle of *Australoheros facetus* exposed to 0.5 and 10 μg/L ES (B). *: significantly different to control ($p < 0.05$).

4. Discussion

Phase contrast microscopy experiments demonstrate good contractile function in all the Mf of control fish and the absence of contraction in all Mf of fish exposed at 10 μg/L ES. These results suggest that some of structural proteins responsible for the contractile function are either denatured or degraded after exposition of fish at high concentrations of ES. Because only three of the six Mf samples from fish exposed at 0.5 μg/L ES contracted, the results obtained with this concentration of ES are not conclusive. On the other hand, the higher concentration tested showed clearly toxic effects. Preliminary studies on *A. facetus* showed erratic swimming behavior when exposed to 10 μg/L after 24 h (Crupkin, 2013). In other species diminished swimming motility was observed and associated to the inhibition of muscle AChE activity after 24 h exposure to ES (Ballesteros et al., 2009). Chronic exposure to other contaminants as well, for example to Cd, have revealed disruption of the sarcomeric pattern in the skeletal muscle and reduction in swimming performance (Avallone et al., 2015).

We propose that the insecticide ES might directly interact with some myofibrillar proteins involved in some step of the contractile mechanism. This hypothesis is based on the fact that it is already known that ES could reach muscle tissues. Although ES concentration in *A. facetus* muscle was not determined in the present work, bioaccumulation of ES in others fish species has been demonstrated (Ballesteros et al., 2011). Therefore, accumulation of ES in muscle of *A. facetus* would be expected when the concentration of ES in water increases 20 times (from 0.5 to 10 μg/L).

The activities of Mg^{2+} and Mg^{2+} (Ca^{2+}) ATPase in Mf are indicative of the actin–myosin interactions in the presence of endogenous or exogenous Ca^{2+} ions, respectively (Benjakul et al., 2002; Pagano et al., 2004). The Mg^{2+} (EGTA) ATPase activity is indicative of the integrity of the tropomyosin–troponin complex (Benjakul et al., 1997). Both Mg^{2+} Ca^{2+} and Mg^{2+} (EGTA) ATPase activities of control Mf from *A. facetus* muscle were similar to those reported for carp (*Cyprinus carpio*) (Seki and Narita, 1980), pre- spawned hake (*Merluccius hubbsi* Marini) (Roura and Crupkin, 1995) and pre- spawned flounder (*Paralichtys patagonicus)* myofibrils (Pagano et al., 2004). It is widely accepted that functional Mf is characterized by high Mg^{2+} Ca^{2+} ATPase activity and low Mg^{2+} (EGTA) ATPase activity. Therefore, the significant differences between these activities observed in control Mf demonstrate its good functionality. Conversely, the absence of significant differences between these activities in Mf from fish exposed to 0.5 and 10 μg/L ES suggest either denaturation or degradation of some component of tropomyosin –troponin complex as was demonstrated in other species under other stressors by Benjakul et al. (1997) and Pagano et al. (2004). SDS-PAGE patterns of control myofibrils from *A. facetus* showed the characteristic polypeptidic bands of myofibrillar proteins of fish (Benjakul et al., 2002; Pagano et al., 2004). The increase of protein mark in the inter- band region between MHC and A of 150–75 kDa as well as in the inter-band region between TM and Tn might be due to an increment in proteolytic activity induced by ES, which led to degradation of either MHC or cytoskeletal proteins or proteins of the tropomyosin- troponin complex. *In vivo* degradation of both MHC and cytoskeletal proteins, from pre-spawned hake (*Merluccius hubbsi*) muscle, was previously reported (Roura, 1992; Pagano et al., 2005). On the other hand, it is widely accepted that fish myosin has less thermal stability and resistance to proteolysis than fish actin. For this reason, it is common to show the changes in each component in SDS-PAGE profiles in reference to actin. In Mf from fish exposed at 0.5 and 10 μg/L ES, MHC/actin ratios were lower than those of the controls. These results suggest that MHC degradation could be, at least in part, responsible for the increment of the protein mark in the inter- band region between MHC and A.

Fish myofibrils and myosin contain lipids that bind to proteins and produce important effects on their properties (Busalmen et al., 1995; Pagano et al., 2005). These lipids of Mf are necessary for the contractile function of fish muscle (Roura et al., 1992). Under a lipid peroxidation condition, polimerization, insolubilization and rupture of polypeptidic chains in Mf of rabbit (Li and King, 1999) and *"in vitro"* denaturation and aggregation of major myofibrillar proteins from salmon (*Peudopercis semifaciata)* have been reported (Tironi and Tomas, 2000). In this way, we analyzed the TBARs content but the changes observed in the contraction of Mf from *A. facetus* exposed to ES were not accompanied by an increment of TBARs, suggesting that lipid oxidation was not involved in these changes. On the other hand, oxidation of proteins of Mf involved in the contraction could be the cause. The oxidation of sulfhydryl groups on the head portion presumably resulted in decreased Ca^{2+} ATPase and Mg^{2+}- Ca^{2+} -ATPase activities, whereas oxidation of sulfhydryl groups on the tail portion possibly caused increased Mg^{2+}-EGTA-ATPase activity (Benjakul et al., 2002). However, in *A. facetus* the absence of differences in the enzymes activities between Mf exposed to ES and its respective controls suggest that a protein oxidative mechanism was not involved in the loss of myofibril contraction induced by ES. Therefore, the mechanism that caused the alterations found in the structural and functional properties of Mf remains unclear. Recent evidence shows that even from the embryonic development ultrastructural changes (breakage, collapse of Mf, disorganized myosin and actin filaments) in muscle tissues could take place (Lee et al., 2014) and also genes related to the muscle organization could be altered in presence of neurotoxic contaminants (Kluver et al., 2011). Further studies

will be necessary to know if there was a direct effect of ES or its metabolites on the Mf.

Finally, we demonstrated for the first time the loss of contractile function associated with the degradation of myofibrilar proteins from the regulator tropomyosin–troponin complex in fishes exposed to ES. Therefore, we propose the evaluation of the contractile function and activity of both enzymes tested together as new biomarkers.

Acknowledgements

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The VIDEO caption is: Contraction of myofibrils in control samples of the fish *Australoheros facetus*.

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