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Structural and functional changes in the zebrafish (Danio rerio) skeletal muscle after cadmium exposure

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Abstract

This report describes the alterations induced by an environmentally realistic concentration of cadmium in skeletal muscle fibre organization, composition, and function in the teleost zebrafish. Results demonstrate that the ion induces a significant quantitative and qualitative deterioration, disrupting sarcomeric pattern and altering glycoprotein composition. These events, together with a mitochondrial damage, result in a significant reduction in swimming performance. In conclusion, the evidence here collected indicate that in presence of an environmental cadmium contamination, important economic (yields in fisheries/aquaculture), consumer health (fish is an important source

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Compliance with ethical standards The experiments were carried out in compliance with ethical provisions established by the EU Directive 2010/63/EU for animal experiment and in accordance with the “Guideline for Animal Experimentation” of the Italian Department of Health. They were organized to minimize stress and numbers of animals used and were approved by the Animal Care Review Board of the University of Naples Federico II.

of proteins), and ecological (reduced fitness due to reduced swimming performance) consequences can be expected.

Keywords

Behavioral tests; Cadmium; Lectins; Toxicity; Ultrastructure; Ucrit

Introduction

Over the past 30 years, increasing attention has been directed to the problem of heavy metal bioaccumulation in aquatic organisms. Tissue concentrations of many metals have now been measured in different invertebrate (Zhang et al. 2009; Shue et al. 2014) and vertebrate species (Liu et al. 2012; Ninomiya et al. 2004; Walker et al. 2014; Lavery et al. 2009). Among these metals, cadmium (Cd) is a ubiquitous contaminant and exposure to it and build up via the food chain is a threat for all organisms (ATSDR 2008; Lei et al. 2011; Leung et al. 2011). In fish, bioaccumulation is dose dependent and tissue specific with the highest concentrations occurring in the liver, followed by the kidney, gill, and muscle (Andreji et al. 2006, 2012; Cao et al. 2012). Cadmium induces a multitude of adverse effects primarily related to the generation of free radicals (Cao et al. 2012; Valko et al. 2005) with cell structure and function affected through altered mitochondrial activity (Toury et al. 1985; Ooi and Law 1989; Liu et al. 2013) and gene expression (Gonzalez et al. 2006).

Relatively little is known about the mechanism of muscle damage by cadmium in fish. It has been reported to interfere with fiber excitation-contraction (Mould and Dulhunty 1999), to upregulate different protooncogenes (Gonzalez et al. 2006) or decrease protein levels (Pretto et al. 2014), to modify enzymatic activity (Mendez-Armenta et al. 2011), and to deplete glycogen reserves (Cicik and Engin 2005). In embryos, it affects fiber patterning (Hen Chow and Cheng 2003). Indirect effects, via the vascular system, have also been proposed (Prozialeck et al. 2008).

This report describes alterations induced by an environmentally realistic concentration of cadmium in skeletal muscle fiber organization, composition, and function. Our results demonstrate both quantitative and qualitative deterioration in cadmium-contaminated fish muscles. This may present economic (yields in fisheries/aquaculture), consumer health (fish is an important source of proteins), and ecological (reduced fitness due to reduced swimming performance) consequences if widespread cadmium contamination of our natural resources continues.

Materials and methods

Zebrafish maintenance and intoxication with cadmium chloride

Adult male fish (average wet weight 1.25 ± 0.14 g), originated from a local pet shop and inbred for several generations, were maintained under standard conditions, in 30-L aquaria. At the time of treatment with $CdCl_2$, they were randomly allotted to three groups (18 fish per group). The first group was left untreated (experimental control), the second was exposed to 0.3 mg/L $CdCl_2$ (typical of concentrations found in the Sarno River, DePippo et

al. 2006), and the third group to 3 mg/L $CdCl_2$.Treatments were carried out for 30 days, under static condition (no $CdCl_2$ addition during the experimental period). No mortality was recorded during treatment.

The experiments were carried out in compliance with ethical provisions established by the EU Directive 2010/63/EU for animal experiment and in accordance with the "Guideline for Animal Experimentation" of the Italian Department of Health. They were organized to minimize stress and numbers of animals used and were approved by the Animal Care Review Board of the University of Naples Federico II.

Light microscopy

On day 15 or 30 of treatment, the animals were anesthetized with MS222 and sacrificed by decapitation. Lateral skeletal muscles were dissected and processed for light microscopy.

Tissues were fixed in Bouin's solution and processed for wax embedding according to routine protocols. Sections were stained with hematoxylin-eosin, eosin, toluidine blue, or Mallory's trichrome to show general morphology.

Periodic acid–Schiff (PAS) was used to highlight mucin, glycogen, and glycoproteins (Simoniello et al. 2013). Sections were oxidized in 0.5 % periodic acid solution for 10 min, rinsed in double-distilled water, and stained with Schiff's reagent in the dark for 45 min. The reaction was blocked by repeated washing in 2.5 % sodium bisulphite in 0.05 N HCl.

Glucid residues were analyzed by staining sections with FITC-lectins (Motta et al. 2005). LEA (*Lycopersicon esculentum* agglutinin, tomato) was used for N-acetyl-glucosamine $(glcNAc)_3$ and UEA-1 (*Ulex europaeus* agglutinin) for L-fucose and LCA (*Lens culinaris* agglutinin) for α-linked mannose residues. Slides were washed in PBS (0.2 M, pH 7.2–7.4) for 45 min and incubated with lectins at a concentration of 10 mg/mL in PBS for 2 h at room temperature in a moist chamber in the dark. After rinsing in 0.5 % BSA in PBS, binding sites were visualized under a UV light. Labeling was defined as positive or negative by the same observer. Negative controls were prepared by incubating slides with the lectins and the specific competing sugar or by omitting the lectin in the reaction to check for autofluorescence.

Oil red O (3 mg/mL) was used to stain neutral lipids present in lipid droplets and fiber membranes (Koopman et al. 2001).

Electron microscopy

On day 15 or 30 of treatment, the animals were anesthetized with MS222 and sacrificed by decapitation. Lateral skeletal muscles were dissected and processed for electron microscopy.

Electron microscopy was performed as previously described (Avallone et al. 2015). Briefly, muscle samples were fixed in 2.5 % glutaraldehyde and 4 % paraformaldehyde in 0.1 M PBS and post-fixed in 1 % osmium tetroxide. They were washed in 0.1 M PBS pH 7.4, at 4 °C, dehydrated in ascending series of ethyl alcohol, and then embedded in Epon. Semi-thin (1.5 mm) sections were cut for light microscopic observations. Sections were stained with

1 % toluidine blue in 1 % sodium tetraborate buffer. Ultrathin (50–80 nm) section were cut and stained with 3 % uranyl acetate in 50 % ethyl alcohol and with 2.6 % lead citrate. These sections, loaded on 200-mesh grids, were observed in a Philips EM 208S transmission electron microscope at 100 kV.

Protein purification, SDS-PAGE, blotting, and staining

Protein purification was done as described by Simoniello et al. (2010). Briefly, muscles were homogenized in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 % Triton X-100, 0.1 % sodium deoxycholate, 10 mM EDTA), analyzed by SDS-PAGE, and stained with Coomassie blue or with PAS to highlight glycoproteins (Motta et al. 2005). For the latter case, gels were fixed in 50 % methanol and thoroughly rinsed in 3 % acetic acid. Oxidation was carried out in periodic acid (7 g/L) in diluted (50 ml/L) acetic acid for 3 min. Gels were rinsed in distilled water, stained with Schiff reagent, and destained with methanolic acetic acid (Trivedi et al. 1983).

Carbohydrate residues were also stained with biotinilated lectins. Gels were blotted onto nitrocellulose paper (Motta et al. 2013), washed in PBS (pH 7.3), rinsed in 0.3 % BSA in PBS, and stained with Ponceau red. Membranes were then washed with a solution containing UEA-1 or LEA lectins (15 μg/mL in PBS) overnight. After washing in PBS for 30 min, membranes were exposed to the ABC complex (Dako, 1:1000 in PBS) for 30 min, rinsed in PBS, and developed with DAB and urea.

Swimming performance

Swimming performance was assessed by measurement of four parameters: routine activity, oxygen consumption, maximal aerobic sustained swimming speed (Ucrit), and escape response. Routine activity mainly involves aerobic slow-twitch red muscle while the escape response is mainly anaerobic activity involving fast-twitch white muscle (Rome 2000; Domenici 2011). Swimming at Ucrit is mainly aerobic with some recruitment of white fibers (Rome 2000). These investigations were conducted on three groups (control, $CdCl_2$ 0.3 mg/L, and 3 mg/L) of ten animals each. Each group was fasted for 24 h to avoid post-prandial effects on animal activity (Secor 2011).

Routine respiratory oxygen consumption (rMO_2) and routine activity were determined simultaneously, as the two parameters are usually highly correlated (Lucas and Priede 1992). The fish rMO_2 was measured in a closed system as described by Uliano et al. (2010); routine activity was evaluated from video recordings as the number of turns per animal per minute while in the respiratory chamber (Uliano et al. 2010).

Ucrit was determined in a swimming tunnel designed by M2M Engineering (Naples, Italy) according to Brett (1964). During adaptation time (about 40 min), water speed was set at 4 BL/s. Ucrit was determined following a stepwise increase in water speed until the fish were exhausted. Each step was 1 BL/s higher and lasted for 10 min (Tierney 2011). Ucrit was calculated using the classical Brett equation (Ucrit = Vp + [Vi * (Tf/Ti)], where Vi = increment of the swim speed, Vp = penultimate speed to which the fish swims before the exhaustion, Tf = time passed between the last increase of speed and the exhaustion, and Ti = time between the two increments of speed).

For the escape response test, animals were placed in a small circular tank (Fig. 6d) in a quiet room, with diffused lighting to reduce external interferences. After 15 min of acclimation, the test was started. A black band representing the threatening object was rotated around the tank, 35 times for 1 min and then hidden from vision (rest phase) with a white panel for 10 min. The procedure was repeated five times taking care to rotate the band clockwise and counterclockwise alternately. All experiments were video recorded and video clips were analyzed for the behavioral effects. The escape response was considered significantly reduced when it was 30 % less than that seen in control animals.

Statistical analyses

Data on swimming performance are presented as means ± SE of five determination. A two-way ANOVA was used to compare means ($p < 0.05$), with Tukey's post hoc test. Statistics was performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA).

Results

Light microscopy analyses

In control zebrafish, muscle fibers are well organized, compact, and regularly arranged (Fig. 1a–c). In the trunk, the lateral band of red muscle is clearly recognizable, just below the skin (Fig. 1a), and at higher magnification, both white (Fig. 1b) and red fibers (data not shown) show a regular and compact arrangement of myofilaments. The reticular organization of connective fibers separating myosepta muscular masses is distinct and well defined (Fig. 1d).

After cadmium treatment (15 and 30 days), fibers in white muscles appear disorganized and partially resorbed (Fig. 1e); myosepta lose their net-like framework and connective fibers are stretched and arranged almost parallel to each other (Fig. 1e–f). Higher magnification shows that myofibrils are resorbed under 0.3 mg/L (Fig. 1g) and 3 mg/L (Fig. 1j–k) treatments. Significantly, at the lower concentration, the white (Fig. 1h) and red (Fig. 1i) fibers show different degrees of damage with the former being most severe.

Transmission electron microscopy analyses

TEM micrographs of control muscles reveal the regular striated arrangement of the sarcomeres (Fig. 2a). Muscles in fish treated for 30 days with 0.3 mg/L $CdCl_2$ (Fig. 2d–e) show areas with disrupted sarcomeric pattern with loose A and I bands in the disorganized and degenerated myofibrils. However, muscles from animals receiving the higher dose, 3 mg/L $CdCl_2$, have less pronounced damage. The fibers do not degenerate but remain very compact and aligned (Fig. 2h). Occasional areas with ill-defined A and I bands and Z lines are visible and their myofilaments are thin and misaligned (Fig. 2i).

At higher magnification (Fig. 2b), the terminal cisternae of the sarcoplasmic reticulum in control muscles can be clearly distinguished. They are associated with the T tubule to form the triad at the level of the Z line. The sarcomeres appear with characteristic architecture, the I band containing thin electron light filaments and the A band with thick electron dense filaments. There are well-defined M lines and H bands.

With 0.3 mg/L $CdCl_2$ treatment, the terminal cisternae appear dilated and the myofibrils disassembled or misaligned, especially in the central region, along the M line (Fig. 2f). The Z lines have lost definition and myofilaments are massed and often randomly oriented. However, with 3 mg/L $CdCl_2$, the sarcomere architecture remains almost unchanged but the Z line appears thickened and in some areas, the myofilaments are clearly loosely formed (Fig. 2j).

Mitochondria in treated fibers have disorganized cristae, vesicular (Fig. 2g) or dilated (Fig. 2k) as compared to the control mitochondria (Fig. 2c).

Effects of cadmium on muscle fiber lipid and carbohydrate content and protein profiles

Normal lipid content in tissues from control animals is shown in Fig. 3a. Muscles from animals treated with cadmium show a significant decrease in neutral lipid content compared to those of controls, and this is evident from day 15, at both the lower (Fig. 3b) and higher (data not shown) doses.

A similar situation is observed for carbohydrate content. PAS staining is intensely and homogeneously distributed on all control fibers (Fig. 3c–d). This then disappears from both treatment groups by day 15 (Fig. 3e) so that on day 30, no staining is observed (Fig. 3f) except on myosepta and skin mucous cells that were used as positive controls (Fig. 3e–f).

FITC-lectins show that the abundance of carbohydrates in control fibers is greatest in the peripheral cytoplasm (Fig. 4a–c). Treatments with cadmium (0.3 or 3 mg/L, 15 or 30 days) does not induce significant changes in the presence and distribution of mannose, recognized by the lectin LCA (data not shown), but causes an increase in N-acetyl-glucosamine (GlcNAc) which is recognized by the lectin LEA (Fig. 4d) and Lfucose, recognized by the lectin UEA-1 (Fig. 4e). In both cases, fluorescence appears more intense and more diffused with respect to the controls. Negative controls are unstained (data not shown) and not auto-fluorescent (Fig. 4f).

Changes in carbohydrates observed after FITC-lectin staining were characterized by staining blotted proteins with biotinilated lectins. Cadmium treatment (0.3 mg/L $CdCl_2$) results in changes in muscle protein content at all molecular weights as clearly seen in the Coomassie gels (Fig. 5a). The marked decrease in intensity of the band at about 200 kDa, possibly corresponding to myosin heavy chain (Neti et al. 2009), seems particularly relevant. Staining with PAS (Fig. 5b) confirms the disappearance of the 200-kDa band and also shows a decrease of a glycoprotein around 10 kDa. Staining of blots with biotinilated lectin UEA-1 (Fig. 5c) or LEA (Fig. 5d) confirms the significant changes in glycoprotein pattern between the control and cadmium-treated samples at almost all molecular masses.

Swimming performance

The decrease seen in lipid and glycogen content in muscle fibers prompted the question of how treated fish perform in swimming. Routine activity is significantly reduced by the treatment (Fig. 6a). Interestingly, this reduction was not accompanied by a parallel reduction of oxygen consumption (Fig. 6b) which is significantly reduced compared to that of controls only after 30 days in the 0.3 mg/L group. Also, the Ucrit, which is mainly supported by red

muscle, is significantly reduced in the0.3 mg/L group only after 30 days of treatment (30 % reduction), (Fig. 6c).

Behavioral tests (Fig. 6e) demonstrate that control animals escape (anaerobic activity mainly supported by white muscle) from the threatening object in the first two trials and that their performance is progressively reduced, falling below 50 % of normal by the fifth trial. Animals treated with 0.3 mg/L behave as controls in the first two trials and significantly reduce their performance by ignoring the threat object in 50 % of passages during the third trial. The animals treated with 3 mg/L already ignore the threatening object in 50 % of the passages in the second trial.

Discussion

Though the evidence indicates that the muscles accumulate cadmium at a low rate with respect to other target organs (de Conto Cinier et al. 1997), our cytological observations clearly show extensive early morphocytological damage. Profound fibers disorganization is evident after 15 days of exposure and persists apparently unchanged at 30 days. There is no evidence of recovery through the treatment is carried out under static condition with only a single treatment at the start of the experimental period.

Our observations indicate that cadmium causes structural disorganization, a disassembly of muscular myofibrils in particular. Why this occurs is not clear. The hypothesis that cadmium induces an acceleration of the physiological process of fibril replacement is not sustainable since resorption occurs in the inner but not outermost part of the fiber where turnover usually occurs (Neti et al. 2009). Another hypothesis is that cadmium has acted by altering mRNA expression (Papa et al. 2014) or calcium balance with activation effects on enzymes involved in myofibril degradation such as calpains (Koohmaraie 1992; Goll et al. 2008) or caspase 3 (Du et al. 2004).

An alternative hypothesis is that cadmium has interfered with glycosylation, thus impairing myofibrillar assembly in functional contractile filaments. Glycation of myosin in particular is a widely studied event and its relevance in contractile function is well documented (Ramamurthy et al. 2001); in effect, our data show both qualitative and quantitative alterations in (glyco)protein patterns. Other evidence that glycation might be the cause of the poor fiber organization is the observation that glycogen stores become depleted. This is a wellknown effect of cadmium exposure (Cicik and Engin 2005), modifying myosin function in a dose-dependent manner (Ramamurthy et al. 2003).

The structural alterations we observed seem to have only a small impact on maximal aerobic swimming performance. However, the effects in terms of physical activity and behavior appear to be significant: the routine activity and the escape response we have measured following cadmium exposure clearly indicate that treated animals are lethargic and tire quickly. This may be explained by the altered mitochondria structure and compromised glycogen and neutral lipid reserves (Pierron et al. 2007). The observed swimming effects may also derive from direct cadmium interference on glycolytic enzymes, lactate dehydrogenase, hexokinase, and phosphofructokinase as reported (Almeida et al. 2001;

Ramırez-Bajo et al. 2014). It can be hypothesized that, in these animals, extra energy required to maintain swimming performance is mobilized by protein catabolism (El Naga et al. 2005). This would explain, at least partly, the observed myofibril resorption.

Another point to be addressed is why in treated *Danio* the observed decrease in swimming activity is not accompanied by the expected parallel decrease in oxygen consumption (Lucas and Priede 1992). This lack of correlation may be explained suggesting that the reduced muscle metabolic rate is in part counterbalanced by metabolic stimulation in other tissues, due to the activation of significant repair and/or remodeling processes. These would occur especially in primary target organs such as the liver or kidney (Cretì et al. 2010), within 2–4 weeks of exposure (Reimschuessel 2001; Cherian and Kang 2006).

The effects observed on muscles are surprisingly more evident in fish treated with the lower, environmental dose of cadmium. A possible explanation is that the higher dose had a stronger effect on stimulating detoxification processes. This negative correlation between response and dose has been observed in other organs (the retina, for example, Avallone et al. 2015) but not in a different animal model (lizard ovaries, retina, and brain; Simoniello et al. 2011). Usually, it is accepted that non-essential metals are efficiently detoxified at low concentration while, at higher concentrations, they would “spill over” to more sensitive cell compartments becoming toxic. The evidence collected here and by Giguere et al. (2006) indicate that this is not always the situation.

In conclusion, it is clear from our observations that cadmium has a harmful effect on muscle fiber structure and function. The comparatively more toxic effect seen at the typical “environmental” dose suggests that the effects of pollution might be more severe than expected. Muscle quality and quantity are essential for behaviors such as prey capture and escape from predators; being essential for survival, the ecological implications of degraded swimming function for fish fitness are obvious.

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Abbreviations

MS222	Tricaine methanesulfonate
PAS	Periodic acid–Schiff
LEA	*Lycopersicon esculentum* agglutinin
UEA-1	*Ulex europaeus* agglutinin-1
LCA	*Lens culinaris* agglutinin

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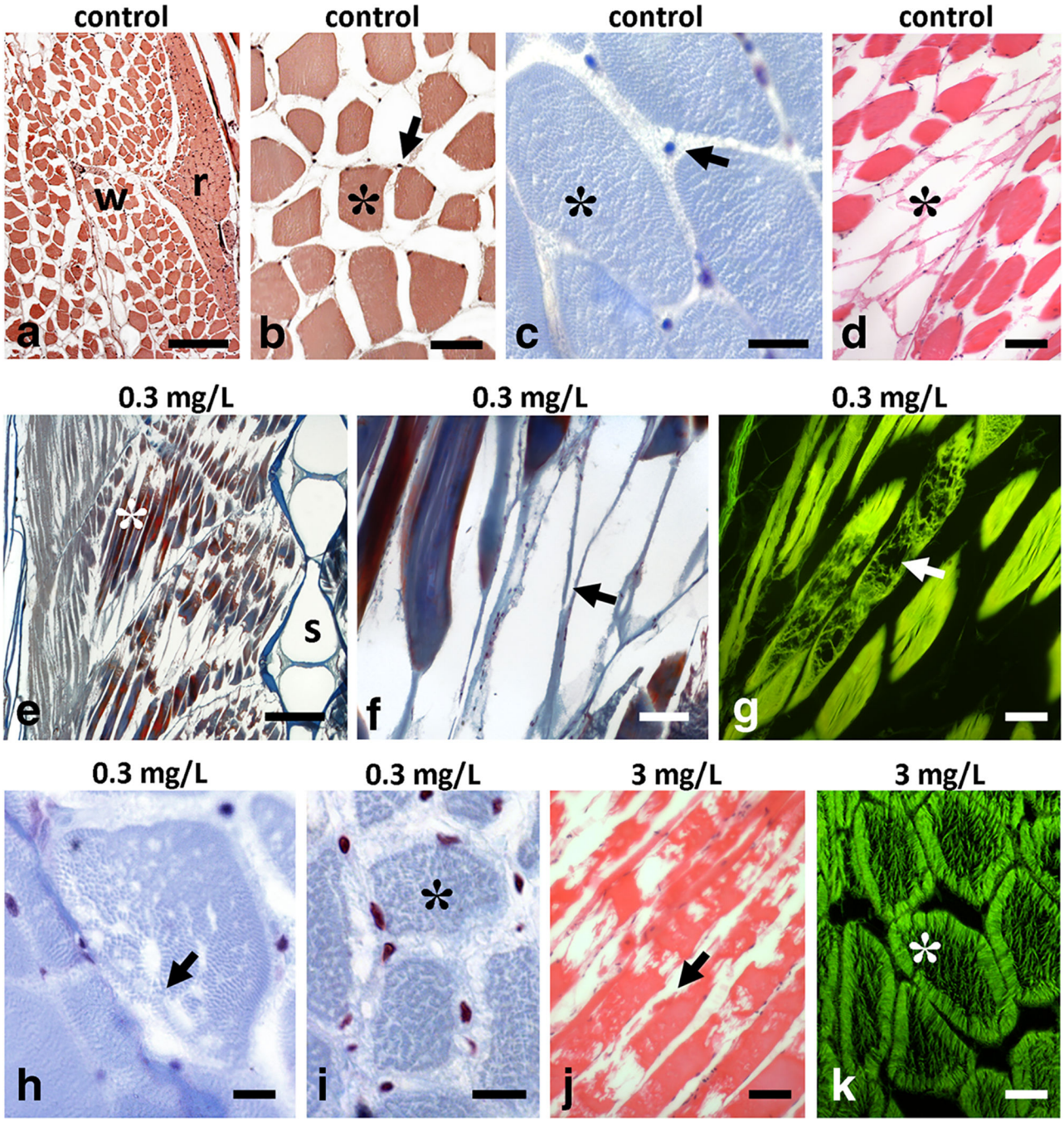

Fig. 1.

Effects of cadmium (30 days) on axial muscle structure in Danio rerio. **a**–**d** Control, **e**–**k** $CdCl_2$ treatment. **a** White (*w*) and red (*r*) muscle with different fiber compaction. **b** Detail of white fibers (*asterisk*) with marginal nuclei (*arrow*). **c** Detail of a white fiber cell with regularly distributed fibrils (*asterisk*); marginal nuclei (*arrow*). **d** Myoseptum formed by a net of endomesial connective fibers (*asterisk*). **e** Altered axial fibers (*asterisk*) close to the spinal cord (*s*). **f** Detail of the myoseptum with stretched and poorly anastomosed connective fibers (*arrow*). **g** Fibers with resorbed myofibrils (*arrow*). **h** Cross section of a white muscle

fiber showing discontinuous myofibrillar disposition (*arrow*). **i** Cross section of red muscle, with myofibrils arranged in bundles (*asterisk*). **j** White fibers with resorbed myofibrils (*arrow*). **k** Detail of fibers in cross section; resorption is more evident in the inner part of the cell (*asterisk*). **a**, **b**, **d**, **j** Hemalum-eosin staining. e–f Mallory-Galgano's trichromic stain. **c**, **h**, **i** Toluidine blue staining. g, k Eosin staining observed under UV light. *Bars*: **a**, **e** 200 μm; **b**, **d**, **f**, **g**, **j** 50 μm; **c**, **i**, **k** 25 μm; **h** 10 μm

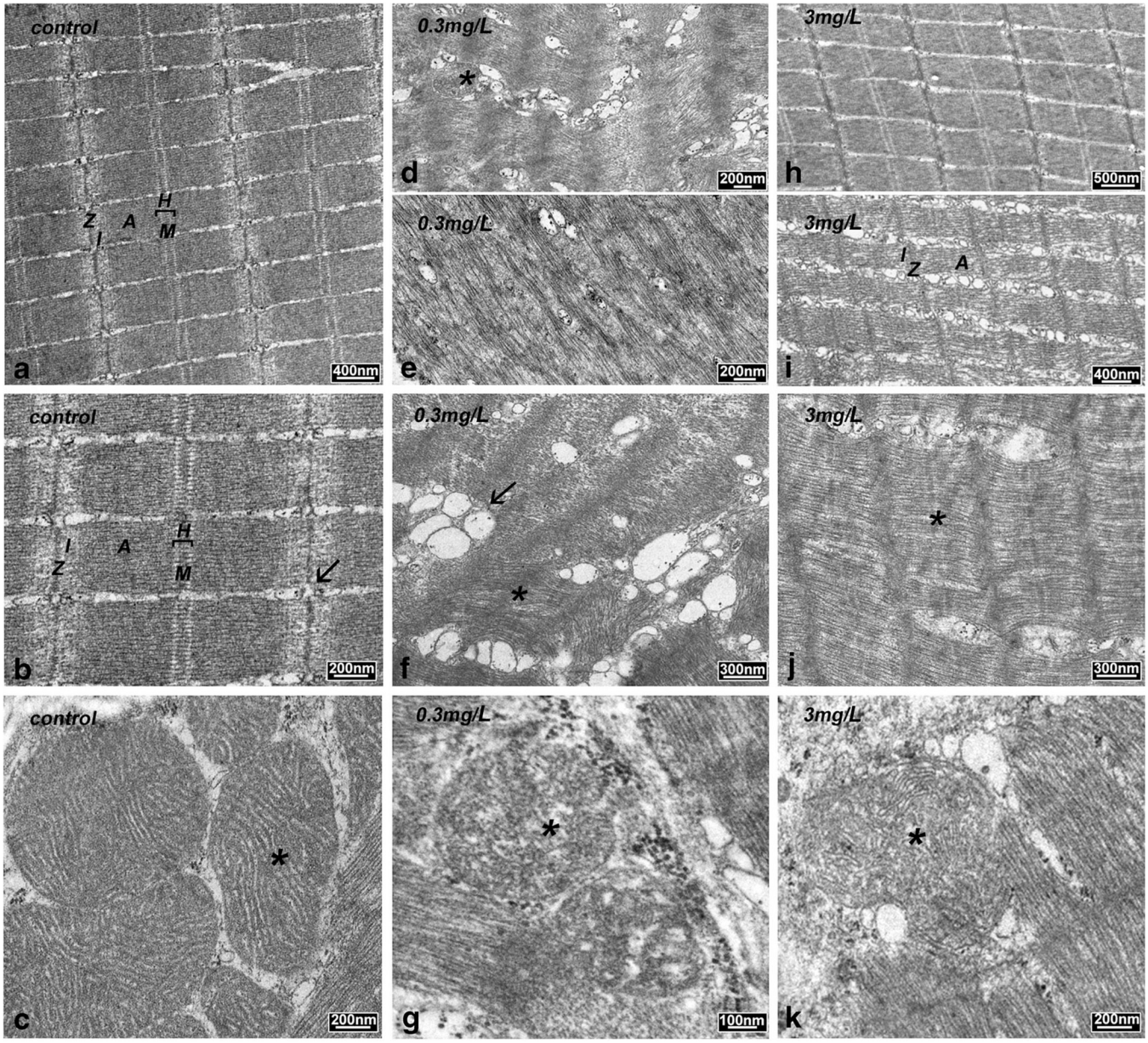

Fig. 2.

Effects of cadmium (30 days) on skeletal muscle ultrastructure in Danio rerio. **a**–**c** Control and **d**–**g** cadmium treated 0.3 mg/L or **h**–**k** 3 mg/L. a The sarcomeres are regularly organized. Z lines (*Z*), electron light filaments of the I band (*I*), electron dense filaments of the A band (*A*), as well as the M lines (*M*) and H bands (*H*) are clearly shown. **b** Higher magnification of (*a*), terminal cisternae (*arrow*) are evident. **c** Mitochondria with properly organized cristae (*asterisk*). **d** Disorganized myofibrils, with loose A and I bands and mitochondria (*asterisk*). **e** Disrupted sarcomeric pattern. **f** Higher magnification showing the dilated terminal cisternae (*arrow*) and the disassembled myofibrils (*asterisk*). **g** Mitochondria with vesicular cristae (*asterisk*). **h** Fibers do not degenerate but very compact and aligned. i Muscle fibers show relaxed myofilaments and thickened Z lines after treatment. **j** Higher

magnification shows relaxed myofilaments (*asterisk*). **k** Mitochondria with dilated cristae (*asterisk*)

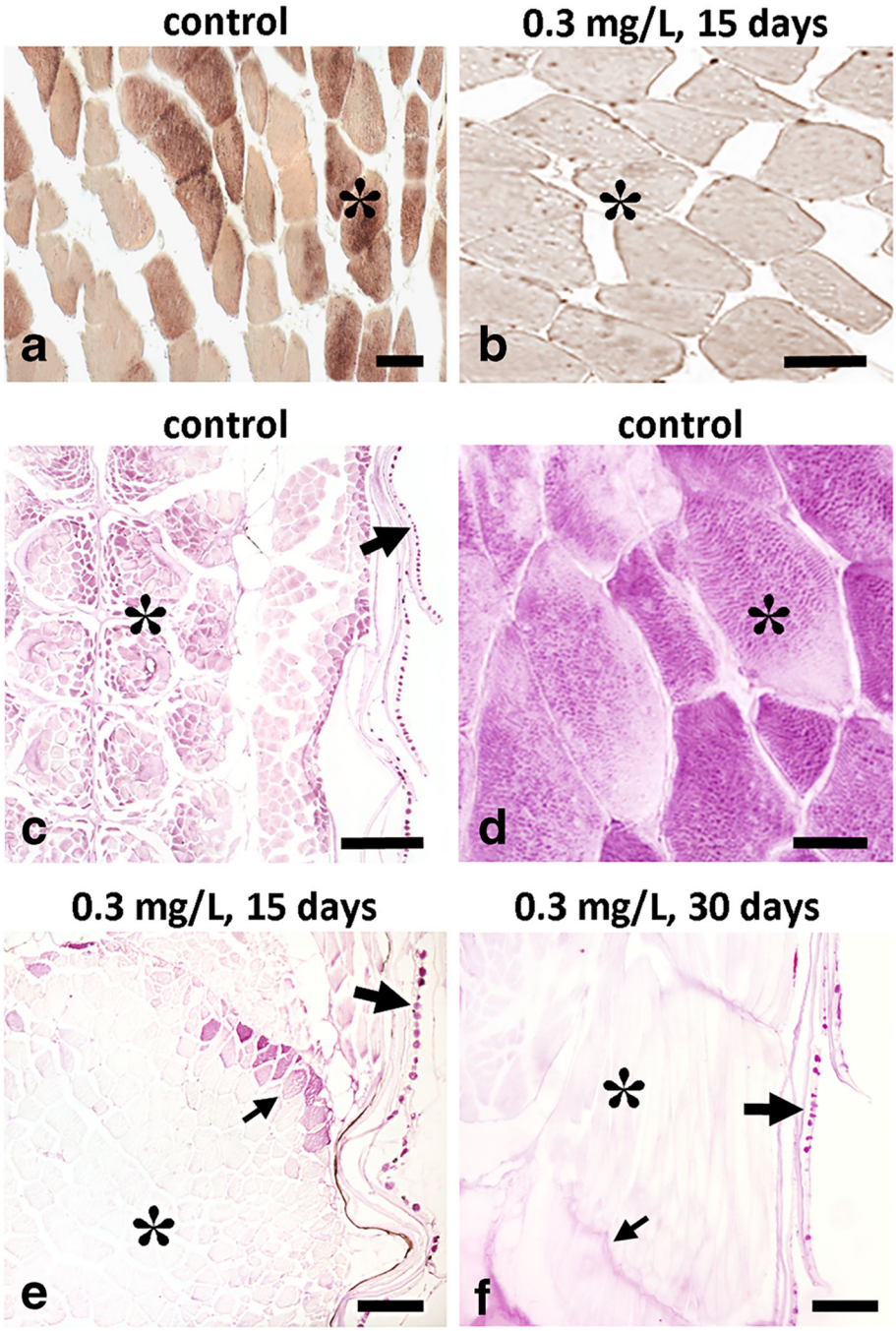

Fig. 3.

Effects of cadmium on **a**, **b** neutral lipid and **c**–**f** carbohydrate content in Danio rerio muscle fibers. **a** Muscle fibers (*asterisk*) from the control animal are intensely stained indicating considerable lipid content. **b** Muscle fibers (*asterisk*) from treated animals show almost no uptake of stain, indicating little lipid content. **c** Positive staining of fibers (*asterisk*) in control animals. Stained skin mucous cells (*arrow*). **d** More detailed view of fibers (*asterisk*). **e** Almost complete absence of stain in treated fibers (*asterisk*), occasional positive fibers (*small arrow*) and stained skin mucous cells (*large arrow*). **f** Unstained fibers (asterisk) and

stained skin mucous cells (*arrow*) and myosepta (*small arrow*). **a**, **b** Red oil O staining. **c**–**f** PAS staining. Bars: **a**, **b** 50 μm; **c** 300 μm; d 25 μm; **e**–**f** 150 μm

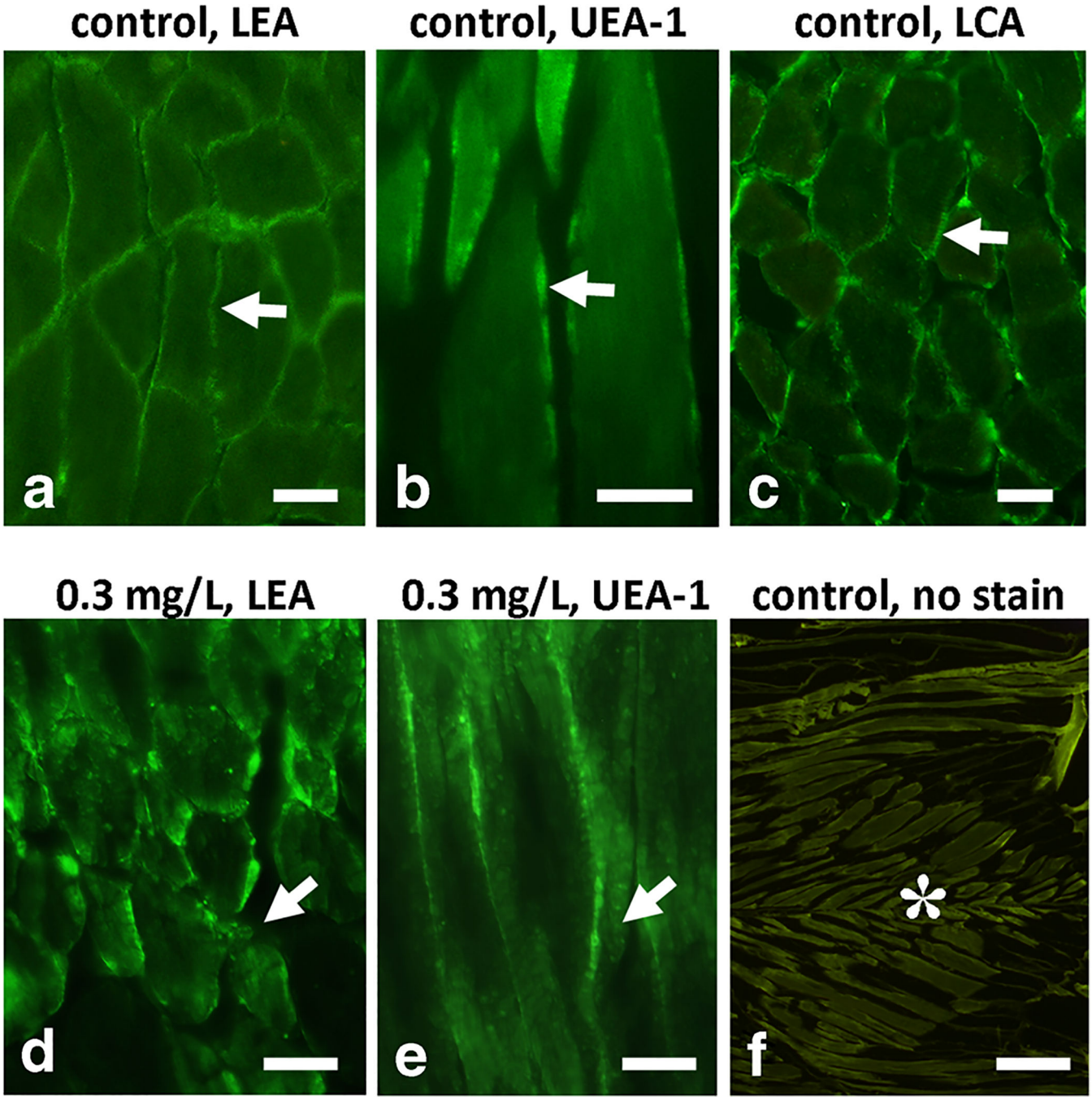

Fig. 4.

Effects of cadmium on carbohydrate content in Danio rerio muscle fibers: FITC-lectin staining. Muscle fibers from control animals (**a**–**c**) show fluorescent peripheral cytoplasm (*arrows*). Staining after 15-day cadmium exposure (**d**, **e**) is more intense and diffuse (*arrows*): an increase in N-acetyl-glucosamine $(GlcNAc)_3$ (**d**) and L-fucose (**e**) is evident. Negative control is unstained and not auto-fluorescent (*asterisk*) (**f**). Bars: 50 μm (**a**–**e**), 200 μm (**f**)

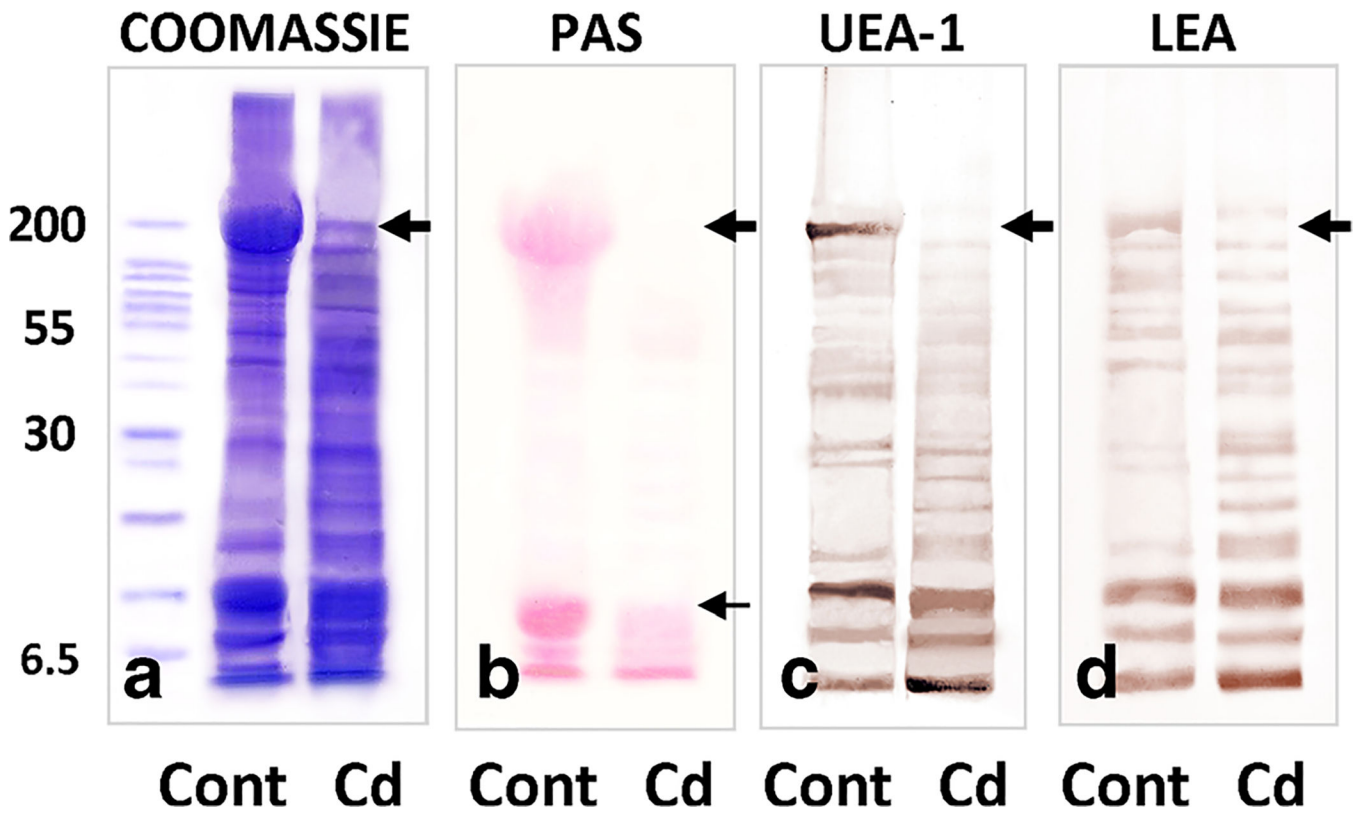

Fig. 5.

Effects of cadmium (0.3 mg/L, 15 days) on protein and carbohydrate content in Danio rerio muscle fibers. a Coomassie blue stained gel shows a significant difference between protein pattern in controls (*Cont*) and treated (*Cd*) muscles. Notice in particular the reduction of the 200-kDa band (*arrow*). **b** PAS staining of gel shown in (a) confirms the disappearance of the 200-kDa band (*arrow*) and demonstrates the disappearance of a 10-kDa band (*small arrow*). **c**, **d** Staining with the two lectins demonstrates that cadmium induces changes in the distribution of fucose (UEA-1) and N-acetyl-glucosamine (LEA) residues

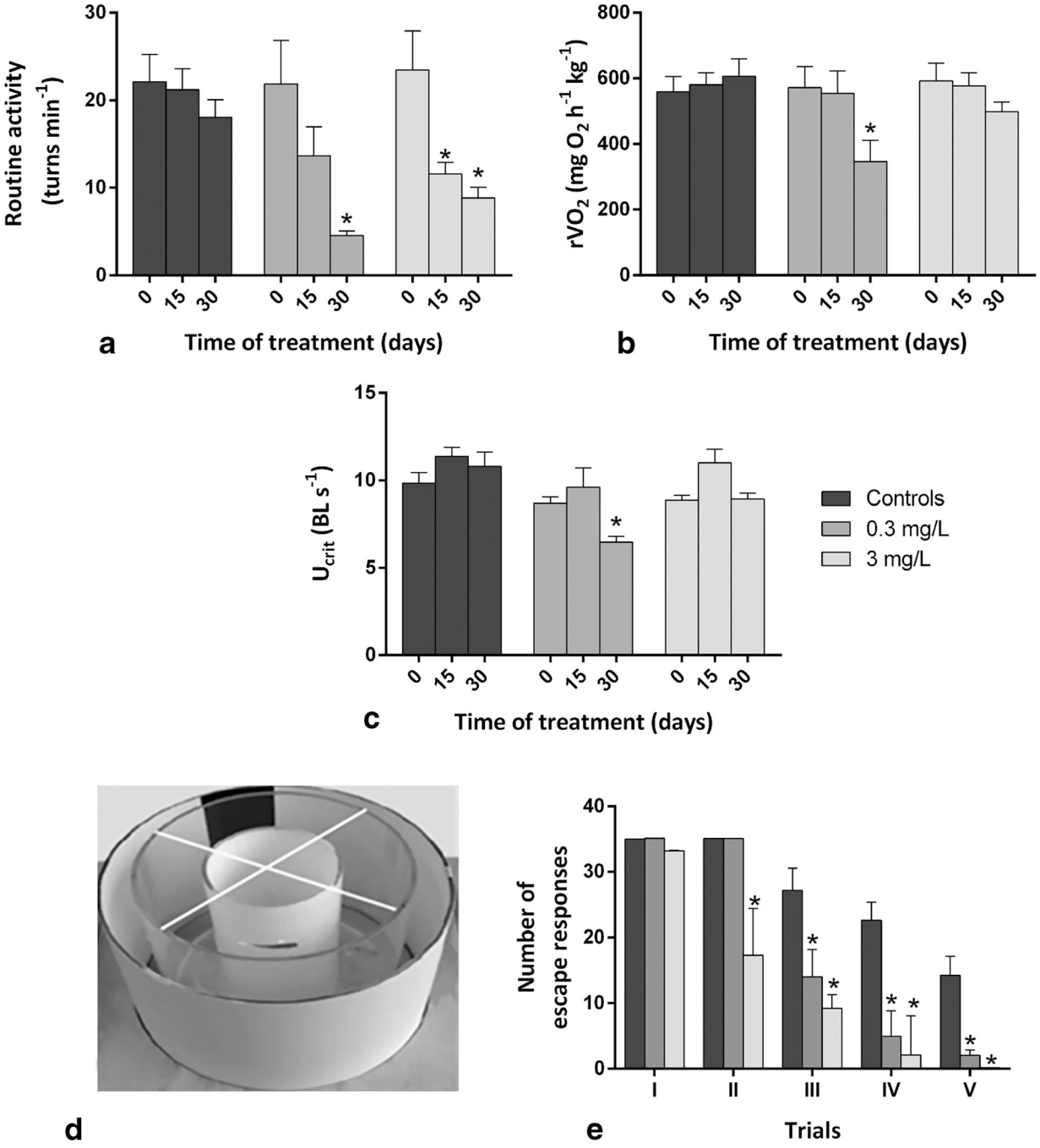

Fig. 6.

Effects of cadmium on the swimming performance of *Danio rerio*. **a** Spontaneous activity under routine conditions. *Asterisks* indicate values that are significantly different from control values (two-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). **b** Routine oxygen consumption shows no significant effect compared to controls of both time and dose of treatment except that after 30 days in the 0.3 mg/L group (two-way ANOVA followed by Tukey's post hoc test, $p < 0.05$). **c** Ucrit values: animals treated with 0.3 mg/L cadmium have significantly lower values after 30 days of treatment (two-way ANOVA followed by

Tukey's post hoc test, $p < 0.05$). **d** Tank used in the escape response test. The black band rotates, clockwise or counterclockwise, inducing an escape response by the fish. e Escape responses measured after trials. Treated animals show significantly reduced performance starting from trial II (3 mg/L) or III (0.3 mg/L) (twoway ANOVA, followed by Tukey's post hoc test, $*p < 0.05$)