Hypoxia and anoxia effects on ADH and Hb in C. riparius

Hypoxia and anoxia effects on alcohol dehydrogenase activity and hemoglobin content in *Chironomus riparius* Meigen, 1804

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ABSTRACT

The metabolic effects of low oxygen content on alcohol-dehydrogenase (ADH) activity and hemoglobin (Hb) concentration were investigated in IV-instar larvae of *Chironomus riparius* (Diptera: Chironomidae) from an Italian stream. Two series of short-term (48 h) experiments were carried out: exposure to (1) progressive hypoxia (95 to 5% of oxygen saturation) and (2) anoxia (at <5% of oxygen saturation). In (1), Hb amount increased with increasing oxygen depletion up to a critical value of oxygenation (about 70% of oxygen saturation). Below this percentage, the Hb amount declined to values comparable with those present in the control. The respiration rate (R) remained almost constant at oxygen saturation >50% and decreased significantly only after 48 h of treatment (= <5% of oxygen saturation) reaching values <100 $molO_2$ $gAFDW^{-1}$ h^{-1}. ADH activity showed two phases of growth, within the first 14 h and over 18 h of exposure. Overall, we inferred that i) Hb might function as short-term oxygen storage, enabling animals to delay the on-set of anaerobiosis; and ii) alcoholic fermentation co-occurs for a short time with aerobic respiration, becoming the prevalent metabolic pathway below 5% of oxygen saturation (<1 mg L^{-1}). These considerations were supported also by results from anoxia exposure (2). In such condition, larvae were visibly stressed, becoming immobile after few minutes of incubation, and ADH reached higher values than in the hypoxia treatment (2.03±0.15 UADH mg $prot^{-1}$). Overall, this study showed a shift from aerobic to anaerobic activity in *C. riparius* larvae exposed to poorly oxygenated water with an associated alteration of ADH activity and the Hb amount. Such metabolites might be valid candidate biomarkers for the environmental monitoring of running waters.

INTRODUCTION

Chironomids (Diptera: Chironomidae) are the most widely distributed insect family in freshwaters, inhabiting nearly all types of aquatic or semi-aquatic habitats including tree holes, bromeliads, rotting vegetation, soil, sewage and artificial containers (Armitage *et. al*, 1995). Chironomids possess adaptations to a variety of environmental rigors (desiccation, anoxia, high temperature, freezing, eutrophication, chemical pollution), are ubiquitously distributed, easy to culture and with short life cycles (Ingersoll and Nelson, 1990; Choi and Roche, 2004). Thus they are an appropriate taxon to study the adaptive strategies necessary to survive environmental stress (Lencioni *et al.*, 2008), to monitor water quality as bioindicators (Rosenberg, 2005) and to test toxicity of chemicals in ecotoxicological assays (Choi, 2004). Within the family Chironomidae, a wide range of tolerance to hypoxia/anoxia is displayed. In particular, the genus *Chironomus* (subfamily Chironominae, tribe Chironomini) is very

tolerant towards low levels of dissolved oxygen (Frank, 1983; Hamburger *et al.*, 1994; Marziali *et al.*, 2006). Larvae inhabit the sediment of pools, profundal and sublittoral zones of lakes and river bottoms where they dwell in tubes and feed on detritus, encountering hypoxia or even anoxia (Jónasson, 1978; Sæther, 1979). *Chironomus* larvae are commonly known as "bloodworms" for the red colour of the body due to the hemoglobin (Hb) synthesized in the body fat then secreted into the hemolymph where it accumulates (Bergtrom *et. al.*, 1976). This "red" respiratory pigment is rarely present in invertebrates and restricted to some taxa within insects and crustaceans (Ha and Choi, 2008).

From an evolutionary point of view, the presence of Hb in invertebrates represents an adaptation to hypoxic environmental conditions, since this pigment helps to sustain aerobic metabolism under low-oxygen conditions (Weber and Vinogradov, 2001). Hb plays a role in transporting but also storing oxygen during periods of rest and feeding (Hoback and Stanley, 2001). From an ecotoxicological point of view, animals possessing Hb are often considered candidate sentinel species for water quality monitoring (Osmulski and Leyko, 1986), and Hb have considerable potential as a sensitive biomarker for environmental monitoring and risk assessment (Frank, 1983; Zebe, 1991). Hb is the most abundant protein in *Chironomus* larvae (Choi *et al.*, 2001), in which more than 30 cloned globin genes have been sequenced, and larvae synthesise up to 16 Hbs and 12 globin polypeptides (Weber *et al.*, 1985; Choi and Roche, 2004). *Chironomus* Hb has a very high affinity for oxygen that dissolves through the larval cuticle and saturates it. This process is favoured by the undulatory movements of the burrowing larvae to move water in their dwelling-tubes in the hypoxic mud (Weber, 1980; Lindegaard, 1995; Rossaro *et al.*, 2007). The large capability of larvae of different species of *Chironomus* (e.g., *C. plumosus* (Linnaeus 1758), *C. anthracinus* Zetterstedt 1860, *C. riparius* Meigen, 1804) to maintain at low oxygen concentrations an aerobic metabolism and consequently high levels of ATP, is associated to these extraordinary capability of their Hb to bind and store oxygen (Zebe, 1991; Penttinen and Holopainen, 1995).

Potentially, these high levels of ATP are maintained to support energy requirements for osmotic homeostasis, to filter-feed and resupply oxygen to the hemoglobin by flushing water through their burrows, to remove anaerobic end-products including lactate and alanine accumulated under hypoxia (Scholz and Zerbst-Boroffka, 1998). There are evidences that as hypoxia becomes severe, *C. riparius* larvae switch to alcoholic fermentation, so that in long-lasting anaerobic conditions, ethanol is the sole end-product of the glycogen degradation, and it diffuses into surrounding water (Redecker and Zebe, 1988; Zebe, 1991). This anaerobic pathway is a feature apparently unique to insects (Wilps and Schöttler, 1980; Redecker and

Zebe, 1988). The last reaction of the alcoholic fermentation (=oxidation of reduced nicotinamide adenine dinucleotide (NADH) coupled to reduction of acetaldehyde to ethanol) is catalyzed by the enzyme alcohol dehydrogenase (ADH).

The aim of this work was to study the effects of hypoxia and anoxia on the metabolism of *C. riparius* larvae through the measurement of Hb concentration in the hemolymph (associated to aerobic metabolism) and ADH enzymatic activity (associated to the anaerobic catabolism of carbohydrates). Although molecular and biochemical biomarkers have been frequently studied in *Chironomus* spp. (Choi, 2004), the effects of low oxygen content on hemoglobin concentration coupled with alcohol dehydrogenase activity were not previously reported for a single species of *Chironomus*.

METHODS

Animal collection and rearing

Experiments were conducted under laboratory conditions on a wild population of *C. riparius* living in a small lowland stream, Rio Gola, in NE-Italy (Trentino, 187 m asl. 46° 02' 20" N, 11° 07' 17" E). Larvae (all instars) were collected in early March 2010. Point measurements of water temperature (18.3°C), percent oxygen saturation (103%), dissolved oxygen concentration (9.4 mg l^{-1}), pH (8.0) and conductivity (2.45 mS cm^{-1}) were recorded in the field with a multiparametric probe Hydrolab Quanta in the time period from 11.00-13.00 h. Water level was of 5-10 cm and current velocity of 70 cm sec^{-1} recorded with an OTT propeller-flow meter. Granulometry was fine (<5 cm) and bottom was covered by algal mats (green algae and cyanobacteria) in the littoral zone.

The sampling site was located about 20 m upstream the confluence with the Adige River, few km downstream Trento city, in an area intensively planted with vineyards and apple-orchards. In all, 509 larvae (154 belonging to II instar and 355 to III-IV-instar) were collected with a 30 x 30 cm pond net (100 μm mesh size) and transported to the laboratory in plastic bags within 1 h after sampling. Rearing was performed in a aerated 34 L aquarium, filled with dechlorinated tap water maintained at 20 ± 2 °C by a refrigerator TECO TR15, with a natural photoperiod. A 5 cm-layer of natural substrate was placed on the bottom of the aquarium. The substrate consisted of stream mud dried in an oven at 60 °C for 48 h, sieved on 250 μm mesh and sterilized by UV radiation with a UV/White light transilluminator ® TFP-M/WL for 30 min. Adult midges were prevented to escape using wooden cages covered with 0.5 mm mesh size nylon net. Larvae were fed with fish food flakes (Tetra-Min®) every 2 days.

All the experiments were performed on IV-instar larvae (instar determination was performed using measurements of head capsule length according to McCauley, 1974) within three weeks after sampling.

Experimental design: progressive hypoxia exposure and anoxia incubation

One day before exposures, IV-instar larvae of *C. riparius* were randomly removed from the rearing aquarium and transferred, without mud, in 500 mL beaker (about 40 larvae per beaker), containing 200 mL of Hard Reconstituted Water (HRW) according to Lee *et al.* (2006). Larvae were kept for 24 h in a thermostatic room at 20 °C, with a 16L:8D photoperiod, at 95 ± 0.2 % of oxygen saturation without food nor mud supply to ensure empty guts (to avoid erroneously measuring oxygen consumption due to meal digestion). Three replicates of 1 larva each were used as control for each series of experiments. 111 replicates of 1 larva each were employed in progressive hypoxia (63) and anoxia (48) experiments. In both series of experiments, survival and biomarker concentration were detected after 0 (=control), 10, 14, 18, 24 and 48 h. The choice of a single control at t = 0 was done according to Forcella *et al.* (2007) who highlighted as even after a hypoxia treatment, in normoxia enzyme activity and metabolite concentrations reached the normal values after few hours of recovery.

Due to mortality or pupation of larvae in the two series of experiments, extra replicates were added (33 in all) to obtain a minimum number of replicates/h of 9. A total of 144 larvae were treated in the two series of experiments.

The larvae that moved spontaneously or that moved only following a tactile stimulus were considered alive.

Before exposure, the wet weight of each larva was determined with an analytical balance (0.0001 g of sensitivity). The mean weight of control larvae was 6.2±0.1 mg in progressive hypoxia and 6.3±0.2 mg in anoxia experiments.

As regards exposure to progressive hypoxia, 60 acclimatized larvae of similar weight (6.4±0.2 mg) were each placed in one 10 mL-Biological Oxygen Demand (BOD) Schott Duran® glass bottle filled with HRW with 100% oxygen saturation (=9.2 mg L^{-1}), without mud, closed taking care to avoid air bobbles. Bottles were immerged upside-down into a glass beaker filled with tap water pre-heated at 20 °C, to ensure the fixing of the glass stopper. Due to mortality and pupation observed at 24 and 48 h, extra replicates were added. At 10, 14 and 18 h, 12 replicates of 1 larva each were taken per each time of exposure; 9 of these replicates were used for the biomarker assays and 3 to measure oxygen consumption. At 24 h, a total of

19 replicates of 1 living larva each were taken; 13 of these replicates were used for the biomarker assays and 6 to measure oxygen consumption. At 48 h, 18 replicates of 1 living larva each were taken; 12 of these replicates were used for the biomarker assays and 6 to measure oxygen consumption.

As regards anoxia incubation, a 2 L- glass flask containing 1.5 L of HRW was flushed with pure N_2 for 30 min to establish anoxic conditions. Flushing was continued for 30 min after transferring the larvae and the flask was sealed with Parafilm®. Forty-five acclimatized larvae of similar weight (6.2±0.4 mg) were introduced in the flask, subdivided in five 20 mL-glass tubes (3 larvae/tube) without mud, each closed with a nylon gauze stopper (500 m mesh size). Exposures were carried out for 10, 14, 18, 24 and 48 h under constant temperature (20±1°C). At each experimental time the dissolved oxygen concentration was monitored with a WTW Oxi 330i oxygen probe: only if the concentration was higher than 0.5 mg L^{-1} (<5% of oxygen saturation), N_2 was gassed for some minutes. Due to mortality of three larvae at 48 h, 9 extra replicates were added for a total of 54 larvae treated. At 10, 14, 18 h and 24 h, a total of 9 replicates in all of 1 larva each were taken at each time of exposure for the biomarker assays. At 48 h, 15 replicates of 1 larva each were taken and used for the biomarker assays.

Oxygen consumption measurement

The oxygen consumption was measured using the closed-bottle method. Within the progressive hypoxia exposure experiments, on 3 replicate of each time series (6 at 24 and 48 h), the initial and final dissolved oxygen concentration in the experimental water was measured with Winkler's titration method (Winkler, 1888; Marsh and Manahan, 1999). The respiration rate was calculated using the following equation according to Lampert (1984), Hamburger and Dall (1990) and Brodersen *et al.* (2008):

$$\text{Respiration rate (R) (mol } g^{-1} h^{-1}) = (O_2 - O_1) \, AFDW^{-1} \, (t_2 - t_1)^{-1} \quad \text{(eq. 1)}$$

where:

O_2 (mol) = dissolved oxygen concentration (mg L^{-1}) at t_2 x 10/1000 x 31.25

O_1 (mol) = dissolved oxygen concentration (mg L^{-1}) at t_1 x 10/1000 x 31.25

AFDW (Ash-Free Dry Weight) (g) = 0.114 wet weight

$t_2 - t_1$ = time interval (h).

Hemoglobin and alcohol dehydrogenase assays

In both series of experiments, biomarker concentration was detected after 0 (= control), 10, 14, 18, 24 and 48 h on survived larvae.

Hemolymph Hb concentration was measured in single alive larvae using the cyano-methemoglobin procedure (Tentori and Salvati, 1981; Int Panis *et al.*, 1995). The larva was placed in 150 µL of saline solution (10 mM potassium phosphate buffer pH 7.4, containing 0.6% NaCl) in a concave glass leaning on ice. Hemolymph was extracted from each larva by making an incision in the second thoracic segment with a sterilized scalpel, and letting the hemolymph flow for about ten min as described by Choi *et al.* (2000) and Choi and Roche (2004). The hemolymph and the buffer were drawn into a 200 L micropipette, then frozen at -20°C. The remaining larval body was used for ADH detection (Choi *et al.*, 2000).

The amount of Hb in the extracted hemolymph was evaluate using the Drabkin's solution, (Drabkin's reagent SIGMA), added with the appropriate amount of water and Brij 35 detergent (Tentori and Salvati, 1981; Choi and Roche, 2004).

In a glass vial (Hellma Special Optical glass) 300 µl of Drabkin's solution and 30 µL of sample were added. A vial containing 300 µL of Drabkin's solution and 30 µL af milliQ water was used as control. Absorbance was measured with the spectrophotometer and Hb concentration was calculated with the following equation:

$$\frac{mgHb}{mL} = \frac{A_{540} \times d_f \times MW}{\varepsilon \times d} \qquad \text{(eq. 2)}$$

where:

A_{540}= Absorbance at 540 nm

d_f= dilution factor (= 11)

MW= molecular weight of the hemoglobin monomer (14300 Da)

ε = extinction coefficient (11 M^{-1} cm^{-1})

d = optical path (=1 cm).

Results were normalized on the sample total amount of proteins (mg Hb mg $prot^{-1}$), determined with the UV extinction method (Layne, 1957) to avoid errors due to different rate of exit of hemolymph in the different larvae during the extraction phase.

Each larva, after hemolymph extraction, was homogenized at 4°C in a hypotonic buffer (17 mM HEPES-Tris, pH 7.4, 100 mM mannitol), in a glass and Teflon Potter- IKA-WERK Rw 14H, two time 10-strokes at 2,000 rpm, separated by 2 min in ice (Forcella *et al.*, 2007). The

buffer volume (mL) was calculated as 9 times the larval initial weight expressed as g (Forcella *et al.*, 2004).

Crude homogenate was centrifuged at 4°C for 30 min (13,000 rpm). The resulting supernatant was stored at -20°C for further enzyme activity measurement. ADH was assayed according to Hanozet *et al.* (1976) at 340 nm with propionaldehyde.

The enzymatic activity was calculated with the following equation:

$$\frac{U}{mg} = \frac{\left(A/\min \right) \times V_t}{\varepsilon \times d \times V_S} \times \left(mg/mL \right)^{-1} \qquad \text{(eq. 3)}$$

where:

ΔA/min = absorbance variation per minute

V_t = final volume of the reaction mix (= 1000 μL)

V_s = volume of the sample added in the cuvette

ε = extinction coefficient of the NADH (6.3 mM^{-1} cm^{-1})

d = optical path (=1 cm)

mg mL^{-1} = protein concentration of the sample.

All assays were performed in duplicate at 30°C using a Cary3 Spectrophotometer, recorded on personal computer, and analysed by the Cary WinUV application software for Windows XP.

Activity was expressed in international units (UADH) and referred to protein concentration (UADH mg prot^{-1}). Total proteins were measured according to Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

All values expressed are mean ± SE. Means were compared by using the Mann-Whitney non-parametric U test to detect significant ($p < 0.05$) differences between treated and control groups. All analyses were run using STATISTICA$^{®}$ 12.0 computer package (StatSoft Inc. 2012).

RESULTS

Progressive hypoxia exposure

Survival was 100% except after 24 h and 48 h, when 20.8 % and 25% of treated larvae respectively died or pupated. Larvae remained active for the first 24h, 20% were undulating and 20% immobile after 48 h. Oxygen in the medium (% atm. saturation) was 95% in the

control, 88.1% after 10 h, 81.7% after 14 h, 70.4% after 18 h, 52.8% at 24 h and 4.4% after 48 h of treatment.

Fig. 1 shows the trend of Hb concentration and ADH activity in relation to progressive decrease in oxygen in the medium (% atm. saturation) with increasing treatment duration. The first significant increase in Hb was observed after 14 h of exposure (P=0.04), reaching a maximum after 18 h (0.663±0.032 mgHb mg prot^{-1}, P=0.01). Over time, its amount decreased to values significantly lower than the control after 2 days (P<0.001) (Fig. 1).

ADH activity increased progressively within the first 14h (P=0.03), when we recorded the maximum value (0.958±0.001 UADH mg prot^{-1}). The enzyme activity significantly decreased in the following 4-10 h (P=0.044 at 18h), but a significantly higher value, comparable with that measured at 14 h, was recorded after 48 h of treatment (P=0.02).

The respiration rate (R) increased slightly (P=0.06) within the first 14 h of treatment, reaching values of 309 ± 20 molO$_2$ gAFDW^{-1} h^{-1} at 81.7% of oxygen saturation but remained >270 molO$_2$ gAFDW^{-1} h^{-1} within values of oxygen saturation >50% (Fig. 1). R significantly decreased (P=0.03) only after 48 h of treatment (=<5% of oxygen saturation) reaching values < 100 molO$_2$ gAFDW^{-1} h^{-1}.

Anoxia incubation

Survival was 100% until 48 h when 25% of treated larvae died.

Larvae exhibited undulating movements within the first 10 min of incubation, then they stopped any movements and became immobile.

The total amount of hemoglobin increased significantly (P=0.04) in the first 10 h of incubation from 0.155 to 0.595 mg Hb mg prot^{-1}, then rapidly decreased (P<0.001) remaining on values ranging between 0.285 and 0.176, without significant differences between the four treatments (Fig. 2).

ADH activity significantly (P<0.001) increased in the first 14 h reaching its maximum value (2.028 ± 0.152 UADH mg prot^{-1}), then rapidly decreased (P<0.001) to values comparable with the control, and never increased later on even if the treatment continued (Fig. 2).

DISCUSSION

Considering the worldwide distribution of *Chironomus* larvae and their potential as sentinel organisms in environmental monitoring (Choi *et al.*, 1998), information about changes in their physiological condition and energy metabolism by natural stressors (i.e. physical stress or

toxicants) seem to be relevant (Choi *et al.*, 2001). *Chironomus riparius* is known to have an extraordinary adaptation capability due to the ability to shift to anaerobic metabolism when necessary, but also to keep activate aerobic metabolic pathways. As a consequence, this species can adapt quickly to the changing environmental conditions frequent in inland waters (Frank, 1983). Our data support these considerations. In *C. riparius* larvae we treated, hypoxia and anoxia induce hemoglobin synthesis and alter the specific activity of enzyme involved in carbohydrate catabolism such as alcohol-dehydrogenase. This means that in larvae exposed to decreasing oxygenation, the glycogen degradation is activated after the depletion of oxygen stored in Hb.

The trends observed for Hb and ADH under progressive hypoxia suggest in fact that Hb might function as short-term oxygen storage, enabling animals to retard the on-set of anaerobiosis, and that alcoholic fermentation is for a short time (14-24 h of incubation, about 84-53% of oxygen saturation) co-occurring, becoming the prevalent metabolic pathway at oxygen percentage lower than 5% (<1 mg L^{-1}). Other authors reported evidences that larvae of *C. riparius* can virtually maintain their normal respiratory rate until the oxygen content of the water reaches a critical limit at approximately 1 mgL^{-1} ($pO_2 = 15$ Torr), switching to anaerobic processes when oxygen content declines below 0.7 mgL^{-1} (Redecker and Zebe, 1988; Zebe, 1991). Jónasson (1978) reported as critical limit for aerobic respiration of 56% of oxygen saturation for larvae of *C. plumosus*.

Strong undulating body movements were observed only within the first 24 h of incubation to progressive hypoxia, the same kind of movements which are performed in natural conditions to irrigate their dwelling-tubes in the hypoxic mud to saturate the hemoglobin with oxygen (Hoback and Stanley, 2001). At this time of incubation, corresponding to an oxygen percentage of about 50%, the first suffering and dead larvae were found due to hypoxia stress. We excluded that starvation could have been the cause of death, according to previous studies that reported lethal times of 205 e 100 days in anoxia without food supply at 4°C respectively for *C. plumosus* and *C. anthracinus* larvae (Nagell and Landahl, 1978).

The respiratory rate (R) trend we measured is in accordance with that one observed by Hamburger *et al.* (1994) and Brodersen *et al.* (2008) in other Chironomini such as *C. anthracinus*, *C. riparius* and *C. hyperboreus* Staeger 1845. Nevertheless a slight (not significant) increase in the first hours of treatment resembling typical a oxy-stressor, R remained constant within the first 24 h of treatment with a significant decrease only below 50% of oxygen saturation suggesting for *C. riparius* larvae from the Rio Gola a oxy-regulator behaviour (i.e., it regulates and maintains a constant R within a % saturation interval)

(Brodersen *et al*., 2008). This trend demonstrates also the efficiency of chironomid Hb to capture oxygen from water (Brodersen *et al*., 2008). Only R measured after 48 h of incubation (=65 μmolO$_2$ gAFDW^{-1} h^{-1}) was lower than 100 μmolO$_2$ gAFDW^{-1} h^{-1}, as stated by Brodersen *et al*. (2008) for large chironomid larvae (*Chironomus*, *Stictochironomus* and *Procladius*) exposed for short periods of time (1 h) to progressive hypoxia. Conversely we found higher values of R after shorter expositions (e.g., 309 molO$_2$ gAFDW^{-1} h^{-1} after 14 h) comparable with those measured for smaller chironomid larvae such as *Micropsectra* described as oxy-stressor by Brodersen *et al*. (2008). The high oxygen consumption values we detected might also be associated to mud removing which occurred during the experiments (this is considering a strong stress for such tube-dwelling larvae; Jónasson, 1978).

In anoxic incubation experiments, metabolic adaptations occurred during the two days of treatments and alterations of biochemical parameters were evident after a few hours of anoxia exposure (larvae were found suffering just after few minutes of incubation). In these conditions, Hb and ADH both increased, reaching maximum value after 10 and 14 h of treatment respectively. After that, both decreased approximately to the values of the control. This trend was observed previously by Frank (1983) in *C. plumosus* where larvae tried to maintain as long as possible the aerobic metabolism even at oxygenation was constantly lower than 5% (<0.5 mg L^{-1}) as in our case. It has been calculated that in such conditions as much as 80% of the energy demand of *C. plumosus* gr. larvae is covered by the alcoholic fermentation of glycogen stores to ethanol (Scholz and Zerbst-Boroffka, 1998). Hamburger *et al*. (1994) reported that anaerobic metabolism represents from 3 (at 3 mgO$_2$ L^{-1}) to 40% (at 0.5 mgO$_2$ L^{-1}) of total energy production in *Chironomus* larvae (*C. anthracinus* can regulate its respiratory metabolism down to 2-3 mgO$_2$ L^{-1}). Choi *et al*. (2001) reported that at 1.6 mgO$_2$ L^{-1} a switch from aerobic to partial anaerobic metabolism occurred after 48 h of treatment. The trend we observed for ADH activity is comparable with findings by Forcella *et al*. (2007) in *C. riparius* and by Frank (1983) in *C. plumosus* IV-instar larvae maintained in anoxic conditions by N$_2$ insufflations. On the other hand, we measured higher values of enzyme activity (of about 57%) after the same time of exposure respect to Forcella *et al*. (2007) (about 1400 nmol min^{-1} mg prot^{-1} h in Forcella *et al*. (2007), 2637 μmol g dw^{-1} h^{-1} our result after 24 h) but similar to the ADH activity values reported by Frank (1983) (7127 μmol g dw^{-1} h^{-1} in Frank (1983) and 8163 μmol g dw^{-1} h^{-1} our result after 48 h). In anoxia conditions, larvae activated immediately strong undulating movements for few minutes then they stop their irrigating activity. This behavioural reaction was observed also by Walshe (1951) in *C. riparius* larvae exposed to less than 7% of oxygenation: they stopped almost immediately to

irrigate the tubes and their hemoglobin resulted partly deoxygenated. After two days of incubation at <1% of oxygen saturation, some larvae were found dead. Such oxygenation level are probably too low for *C. riparius*, that typically inhabit running waters polluted by sewage where oxygen saturation might be even approximately to 100% due to water turbulence (Frank, 1983) (in our case, the oxygen saturation of Rio Gola was 103%). In fact, within the genus *Chironomus*, *C. riparius* is not the most resistant species to low oxygen content. Specifically, Frank (1983) reported the following order to resistance: *C. plumosus* > *C. anthracinus* > *C. tentans* Fabricius 1805 > *C. riparius* > *C. piger* Strenzke 1956. Our data give new insight on autoecology of such species, confirming that anoxia represents anyway a stress condition to which physiological compensatory mechanisms are activated.

CONCLUSIONS

In accordance with findings and hypotheses tested in numerous previous studies (e.g., Hervant *et al.*, 1996; Choi *et al.*, 2001; Hermes-Lima and Zenteno-Savín, 2002; Ha and Choi, 2008), we confirm that IV-instar larvae of *C. riparius* can withstand hypoxic and anoxic conditions and that they undergo compensatory adjustments in the energy metabolism to maintain, in such conditions, physiological or biochemical functions at a normal (i.e. homeostatic) level, switching from aerobic to anaerobic pathways (consumption of energy-yielding substrates, e.g., glycogen). Notwithstanding Hg amount and ADH activity we measured did not respond linearly with the oxygen concentrations or the period of anoxia, Hb and ADH might be proposed as candidate biomarkers for the environmental monitoring of running waters even as alternative to the analyses of anaerobic end products to determine the anaerobic metabolism in chironomid larvae (Fields, 1983). The use of molecular endpoints (as done in an oriental river prawn by Sun *et al.*, 2014) might support these conclusions and clarify for example if the apparent changes in ADH activity are due to changes in expression of genes encoding the enzyme or are due to changes in existing ADH enzyme, giving more insights on such metabolites and their role in survive anoxia/hypoxia in aquatic insects.

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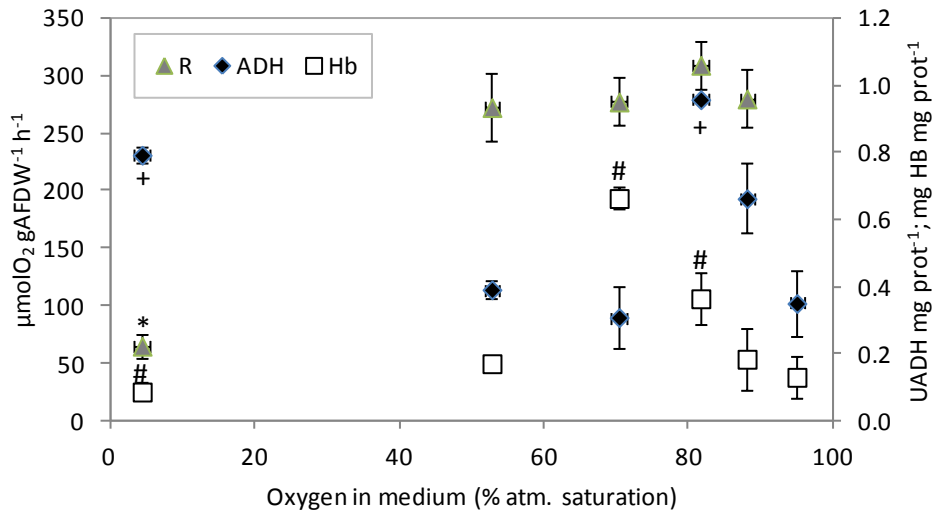
Fig. 1. Trend of ADH activity, Hb amount and oxygen consumption (R) (mean ± SE) in larvae exposed to progressive hypoxia. Statistically significant differences compared to control value (time zero) = *for R, #for Hb and + for ADH .
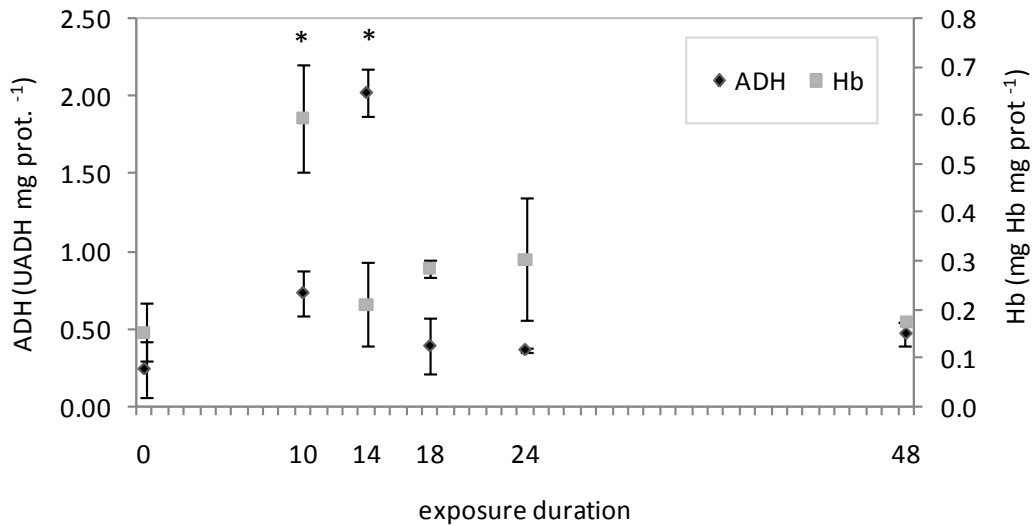

Fig. 2. ADH activity and Hb amount (mean ± SE) in larvae exposed to anoxia. *Statistically significant differences compared to control value (time zero).