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ORIGINAL ARTICLE

Are antioxidant capacity and oxidative damage related to biological and autecological characteristics in aquatic insects?

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ABSTRACT

*In this work, we study the oxidative state of nine taxa of aquatic insects [*Serratella ignita *(Poda, 1761),* Ephemera danica *Müller, 1764,* Crocothemis erythraea *(Brullé, 1832),* Dinocras cephalotes *(Curtis, 1827),* Perla bipunctata *Pictet, 1833,* Isoperla morenica *Tierno de Figueroa & Luzón-Ortega, 2011,* Notonecta maculata *Fabricius, 1794,* Gerris *sp., and* Hydropsyche *sp.] in order to determine the relationships between this state and the biological and environmental characteristics of the species studied. The studied taxa are all in the same life cycle stage (nymph/larva), and many of them have different maximum life span potentials. We assess the antioxidant capacity through the determination of the trolox-equivalent antioxidant capacity, the ferric-reducing/antioxidant power and the activity of the antioxidant enzymes catalase, DT-diaphorase, glutathione peroxidase, glutathione reductase, glutathione transferase, superoxide dismutase and glucose-6-phosphate dehydrogenase. Furthermore, to determine the oxidative damage, we examine thiobarbituric acid reactive substances, free malondialdehyde, protein-bound malondialdehyde, total hydroperoxides, and protein hydroperoxides. In summary, we can consider that having predatory feeding habits, having a long-life cycle and living in permanent streams with cold, well-oxygenated waters are related to a proper oxidative state in the insects that we studied. On the other hand, non-exclusive predator species living in temporary streams with warm and poorly oxygenated waters with a short life cycle have a worse oxidative state. Thus, the oxidative state of each taxa could be defined by an interaction of biological and autecological factors, for which the relative importance is difficult to assess.*

Key words: Antioxidant enzymes; feeding habits; freshwater insects; habitat; life cycle; oxidative state.

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INTRODUCTION

Ageing is a physiological process under a clear genetic regulation that happens, continuously and progressively, from birth to death of the organisms. This manifests in a set of molecular, genetic, cellular, tissular and organic alterations that affect the morphology, physiology and behaviour of organisms. The theory of ageing by free radicals of mitochondrial origin (Harman, 1956; Hulbert *et al*., 2007) is the most accepted theory for explaining the characteristics of ageing because mitochondria are an endogenous source of free radicals and all the cells forming the vital organs produce those radicals continuously in them. The production of reactive oxygen species (ROS) in isolated mitochondria of post-mitotic tissues is lower in long-lived animal species than in short-lived species (Ku *et al*., 1993; Barja, 2004; Dowling and Simmons, 2009; Monaghan *et al.*, 2009). On the other hand, when the maximum life span potential (MLSP) of a species is greater, the intensity of the oxidative attack to its mitochondrial DNA is lower (Richter *et al*., 1988; Barja, 2000; Barja and Herrero, 2000). Oxidative stress occurs when organisms have an imbalance between pro-oxidant and anti-oxidant molecules. Pro-oxidant molecules (free-radical oxygen species and other reactive species) trigger a cascade-like process, producing lipid hydroperoxides (LOOH) and protein hydroperoxides (PrOOH) and damaging biological membranes and other lipid- and protein-containing structures (Halliwell, 2007).

The majority of the studies about animal longevity that were initially conducted focused on antioxidant factors. One of the first hypotheses raised was that ageing could occur due to a decrease in antioxidant levels. Several studies have noted a lack of a uniform pattern in the variation of the cellular antioxidant concentrations with either individual age or the MLSP of a given species, and the results that were obtained depended on the antioxidant measured, the studied organ or the chosen species (Tolmasoff *et al*., 1980; Sohal *et al*., 1990; Mockett *et al*., 1999; Huang *et al*., 2000). In a study of two species of fish [rainbow trout, *Oncorhynchus*

mykiss (Walbaum, 1792) and sturgeon, *Acipenser naccarii* Bonaparte, 1836] we found much lower oxidative levels in the brain lipids of the sturgeon than in the trout (unpublished data). Consequently, we hypothesized that the greater longevity of the sturgeon was possibly related to a better oxidative state of its nervous tissue. Subsequently, Sanz *et al*. (2013) elucidated the relation between the greater MLSP of different fish species [*Squalius pyrenaicus* (Günther, 1868), *Cyprinus carpio* Linnaeus, *Carassius auratus* Linnaeus, 1758, *Luciobarbus sclateri* (Günther, 1868) and *Oncorhynchus mykiss*] and the lower oxidation of the brain. We also found a positive relation between the level of the antioxidant enzymes superoxide dismutase (SOD), the glutathione peroxidase (GPX), the glutathione transferase (GST) and the MLSP in the brain tissue. The importance of the protection of the high lipid content in the nervous tissue in the face of oxidative damage relates to the proper state of functions responsible for life and consequently, for the longevity of a given individual and the MLSP of a given species (Sanz *et al*., 2013). Moreover, the previous results obtained in different parameters indicative of oxidative status in four insects species in the nymphal stage of the aquatic insect order Plecoptera [*Perla marginata* (Panzer, 1799), *Guadalgenus franzi* (Aubert, 1963), *Isoperla morenica* Tierno de Figueroa & Luzón-Ortega, 2011 (under the name *I. curtata* Navás, 1924), and *I. grammatica* (Poda, 1761)] indicate that all of these species showed an important antioxidant activity and that the differences found among species seemed to be mainly due to the duration of the nymphal developmental period (Sanz *et al*., 2010).

Despite the different studies that were conducted, some authors still considered the role of oxidative stress as a determinant of the longevity of the organism to be an open question (Buffenstein *et al*., 2008; Page *et al*., 2010; Gil del Valle, 2011; Montgomery *et al*., 2011). A large number of environmental factors can induce oxidative stress in animals, including: oxygen (Hetz and Bradley 2005; Lushachack *et al*., 2005; Storey and Storey, 2010; Costantini, 2014; Hermes-Lima *et al*., 2015), temperature (Laurette *et al*., 2011; Jena *et al*., 2013) and feeding habits (Barbehenn, 2002, 2003; Kim *et al*., 2007; Martínez-Álvarez *et al*., 2005; Sanz *et al*., 2010) are directly related to ROS production (Ahmad, 1995). Oxidative stress is a strong evolutionary and ecological force (Costantini *et al*., 2010; Costantini, 2014).

In this work, we studied the oxidative state of nine taxa of aquatic insects to relate their antioxidant capacity and the oxidative damage with the characteristics of their environment, their biology and MLSP. Our main objective was to study aquatic insects under their natural conditions We chose a descriptive and correlational approach rather than a more experimental one (for example, controlling for environmental factors in the laboratory) to obtain more realistic information. The studied taxa from five different Hexapoda orders are *Serratella ignita* (Poda, 1761), *Ephemera danica* Müller, 1764 (Ephemeroptera), *Crocothemis erythraea* (Brullé, 1832) (Odonata), *Dinocras cephalotes* (Curtis, 1827), *Perla bipunctata* Pictet, 1833, *I. morenica* Tierno de Figueroa & Luzón-Ortega, 2011 (Plecoptera), *Notonecta maculata* Fabricius, 1794, *Gerris* sp. (Hemiptera), and *Hydropsyche* sp. (Trichoptera), all which are immatures (nymph/larva) and approximately in an intermediate stage of their development, many of them with different MLSPs. For each taxon, we assessed the antioxidant capacity through the determination of the trolox-equivalent antioxidant capacity (TEAC), ferric-reducing/antioxidant power (FRAP) and the activity of the antioxidant enzymes catalase (CAT), DT-diaphorase (DTD), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione transferase (GST), superoxide dismutase (SOD) as well as glucose-6-phosphate dehydrogenase (G6PDH). Furthermore, to determine the oxidative damage, we examined thiobarbituric acid reactive substances (TBARs), free malondialdehyde (FrMDA), protein-bound malondialdehyde (PrMDA), total hydroperoxides (LOOH) and protein hydroperoxides (PrOOH).

METHODS

Collection, locations and samplings

Specimens of nine aquatic insect taxa listed above were collected during the spring of 2014 from four different freshwater systems:

- River Despeñaperros (Sierra Morena, Jaén, Spain; 38°22′22.98″N, 3°30′26.25″W, 560 m asl), a slow-flowing Mediterranean seasonal stream with relatively warm water (9.6-17.9°C during the collection period) and a intermediate oxygen level. Collected taxa: *S. ignita* and *I. morenica*.
- River Castril (Sierra de Castril, Granada, Spain; 37°52′37.6″N, 2°45′26.1″W, 1040 m asl), a fast-flowing Mediterranean-type permanent stream with cold (9.3-12°C during the collection period) and well-oxygenated water. Collected taxa: *D. cephalotes*, *P. bipunctata* and *Hydropsyche* sp.
- Charca del Cañaveral (Sierra de Huétor, Granada, Spain; 37°14′24.6″N, 3°33′08.1″W, 990 m asl), a pond with warm (12.5-14.0°C during the collection period) and little oxygenated water. Collected taxa: *C. erythraea*, *N. maculata* and *Gerris* sp.
- River Aguas Blancas (between Sierra Nevada and Sierra de Huétor, Granada, Spain; 37°13′23.9″N, 3°24′30.3″, 1140 m asl), a permanent Mediterranean stream with cold (7.0-13.0°C during the collection period), well-oxygenated water. Collected taxon: *E. danica*.

All taxa were collected in the nymphal/larval stage of the life cycle and at approximately the same developmental

stage in order to compare organisms in the same physiological stage. They were sampled using a kick net (250 µm mesh size), immediately placed in liquid nitrogen and stored at -80°C until the laboratory analyses. The studied taxa were selected according to their different habitat preferences and life histories. Data on the biology of these taxa are reported in Tab. 1.

Treatment of samples

Enzymatic determinations were performed using the whole individuals. Animal samples (approximately eight animals per sample, eight replicates per species) were homogenized in ice-cold distilled water (1/9, w/v) with an electric homogenizer (Heidolph Instruments). The homogenates were centrifuged at 30000 g for 30 min at 4°C in a model 3 K30 Sigma centrifuge. After centrifugation, the supernatant was collected and frozen at -80°C until analysis. All enzymatic assays were performed at 25 ± 0.5 °C. Each sample was tested twice in 96-well microplates (UVStar®, Greiner Bio-one, Germany) using a PowerWavex microplate scanning spectrophotometer (Bio-tek Instruments, USA). The enzymatic reactions were started by the addition of the tissue extract, with the exception of the SOD reaction in which xanthine oxidase was used. The specific assay conditions were as follows.

Determination of antioxidant capacity

To assess the antioxidant capacity, the trolox-equivalent antioxidant capacity (TEAC) (Erel, 2004) and ferric-reducing/antioxidant power FRAP (Benzie and Strain, 1996) , as well as a set of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST), and DT-diaphorase (DTD), were measured (Sanz *et al*., 2014).

TEAC was measured according to Erel (2004). ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonic acid) in an acidic medium that is oxidized by hydrogen peroxide, turning an emerald-green colour. The reduction of this compound in the presence of antioxidants results in a loss of colour, measured at 595 nm, proportional to the total antioxidant capacity of the extract. Antioxidant activity refers to the equivalent of a water-soluble analogue of vitamin E (Trolox) used as a standard. The results are expressed in terms of µmol of Trolox-equivalent antioxidant capacity per litre of tissue extract (µM).

FRAP measures the antioxidant power of a sample according to its ability to reduce the ferric iron (Fe^{+3}) that is present in a complex with 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) to the ferrous form (Fe^{+2}). This test was performed in an acetic acid-sodium acetate buffer (pH of 3.4) containing TPTZ and $FeCl_3$. After 60 min of reaction, the absorbance was determined at a wavelength of 593 nm. The reference curve was constructed using ascorbic acid as a primary standard. The activities of the samples were expressed as ascorbic acid equivalent antioxidant capacity (AEAC: mg of ascorbic acid/100 g of extract).

The SOD (EC 1.15.1.1) activity was measured spectrophotochemically by the ferricytochrome C method using xanthine/xanthine oxidase as the source of superoxide radicals. The reaction mixture comprised 50 mM potassium phosphate buffer (pH of 7.8), 0.1 mM EDTA, 0.1 mM xanthine, 0.013 mM cytochrome c, and 0.024 IU mL^{-1} xanthine oxidase. One activity unit was defined as the amount of enzyme necessary to bring about a 50% inhibition of the ferricytochrome c reduction rate measured at 550 nm (McCord and Fridovich, 1969).

Tab. 1. Biological and autecological traits of the studied taxa.

Taxa	Main food source	Life cycle duration (years)	Habitat
Ephemeroptera			
*Serratella ignita**	Detritus, diatoms	1	Benthos
Ephemera danica°	Detritus, coarse organic matter	2	Benthos (burrower)
Odonata			
Crocothemis erythraea#	Prey	<1	Benthos
Plecoptera			
Dinocras cephalotes§	Prey	2	Benthos
Perla bipunctata^	Prey	2-3	Benthos
Isoperla morenica§	Detritus, diatoms, prey	1	Benthos
Hemiptera			
*Notonecta maculata***	Prey	≤1	Nekton
Gerris sp.°°	Prey	≤1	Neuston
Trichoptera			
Hydropsyche sp.**	Detritus and prey	≤1	Benthos

**López-Rodríguez* et al., *2009a;* °*López-Rodríguez* et al., *2009b;* #*Askew, 2004;* §*Tierno de Figueroa* et al., *2015;* ^*Tierno de Figueroa* et al., *2003 and unpublished data;* §*López-Rodríguez* et al., *2009c;* ***Tachet* et al., *2010;* °°*Pfenning and Poethke, 2006.*

The CAT (EC 1.11.1.6) activity was determined spectrophotochemically by measuring the decrease in the H_2O_2 concentration at 240 nm (Aebi, 1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH of 7.0) and 10.6 mM H_2O_2 (freshly prepared).

The GPX (EC 1.11.1.9) activity was measured following the method of Flohé and Günzler (1984). A freshly prepared glutathione reductase solution (2.4 U mL^{-1} in 0.1 M potassium phosphate buffer, pH of 7.0) was added to 50 mM potassium phosphate buffer (pH of 7.0), 0.5 mM EDTA, 1 mM sodium azide, 0.15 mM NADPH, and 0.15 mM cumene hydroperoxide (CumOOH). After the addition of 1 mM GSH (reduced glutathione), the NADPH-consumption rate was monitored spectrophotochemically at 340 nm.

The GR (EC 1.6.4.2) activity was assayed using methods described by Carlberg andMannervik (1975), with some modifications,by measuring the oxidation of NADPH at 340 nm. The reaction mixture consisted of 0.1 M sodium phosphate buffer (pH 7.5), 1 mM EDTA, 0.63 mM NADPH, and 0.15 mM GSSG.

The GST (EC 2.5.1.18) activity was determined by the method of Habig *et al*. (1974) adapted to a microplate. The reaction mixture comprised 0.1 M phosphate buffer (pH 6.5), 1.2 mM GSH, and 1.23 mM solution of 1-chloro-2,4- dinitrobenzene in ethanol, which was prepared just before the assay. The GST activity was monitored spectrophotochemically at 340 nm by the formation of glutathione-CDNB-conjugate.

The DTD (EC 1.6.99.2) activity was measured according to Sturve *et al*. (2005). The reaction mixture contained 50 mMTris–HCl (pH 7.3), 50μM DCPIP (2,6-dichlorophenol indophenol) and 0.5 m MNADH. The control reaction contained distilled water instead of the sample extract. The DTD activity is defined as the difference between the sample and the control in the DCPIP reduction reaction.

Except for SOD (the arbitrary units of which have been mentioned above), for every other enzymatic activity, one unit of activity is defined as the amount of enzyme required to transform 1 μmol of substrate/min under the aforementioned assay conditions (Sanz *et al*., 2010). To estimate the enzyme-specific activity, the soluble protein of the extracts was determined by a bicinchoninic acid protein assay reagent (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA) using bovine serum albumin as the standard.

For G6PDH (EC 1.1.1.49) the change in the absorbance of NADPH at 340 nm was monitored to determine the enzymatic activity (millimolar extinction coefficient, 6.22). The reaction mixture contained 50 mM imidazole–HCl buffer (pH of 7.4), 5 mM $MgCl_2$, 2 mM NADP, and 1 mM glucose-6-phosphate (Morales *et al.*, 1990). The enzyme activities are reported in milliunits (nmol of substrate transformed per minute) per mg of protein.

Determination of oxidative damage

To quantify the oxidative damage, the following lipid and protein peroxidation biomarkers were evaluated: free malondialdehyde (FrMDA), protein-bound malondialdehyde (PrMDA), lipid hydroperoxides (LOOH), protein hydroperoxides (PrOOH) (Grintzalis *et al*., 2013), and thiobarbituric acid-reactive substances (TBARs) (Buege and Aust, 1978). The reason we measure TBARs as a measurement of oxidative damage in a whole sample, together with FrMDA, PrMDA, LOOH and PrOOH measurements, is to determine the degree of oxidation in the three main fractions, *i.e*., aqueous, lipid and protein. Higher levels of oxidation in the aqueous fraction could be related with higher and more advanced oxidation of molecules, assuming that free MDA is the oxidized molecule in organisms. The sum of FrMDA, PrMDA, LOOH and PrOOH quantifies the total oxidative damage (TOD) and could be used as a tool for comparison of global oxidative damage.

At the first step, each sample was divided into two homogenates of 0.5 mL, which were mixed with 1 mL of chlorophorm:methanol (2:1) followed by vigorous vortexing (Nahita© vortex); then, 150 μl of 100% TCA (trichloroacetic acid) was added to the resulting mixture and briefly vortexed. After 20 min of incubation in an ice-water bath, the samples were centrifuged at 20000 *g* for 10 min. As a result of this process, three layers were formed: an upper aqueous layer, an intermediate protein disk, and a bottom chloroform layer. The aqueous layers of the two replicates of every sample were combined and stored at -20°C to use later for the FrMDA determination. The combination of the two chloroform layers was concentrated by a continuous flow of nitrogen and stored at -20°C for the LOOH assay. One of the resulting disks of protein was solubilized in 1500 μL NaOH 0.1 mM and incubated at 60°C for 30 min to hydrolyse PrMDA from the protein-MDA complex. This was immediately cooled in an ice-water bath and centrifuged at 20000 g for 5 min; simultaneously, the supernatant was collected, stored at -20°C and then used for PrMDA measurement. The other resulting disk of protein was solubilised in 1800 μL 0.1 M NaOH and neutralized with 200 μL 1 M HCl, stored directly at -20°C, and afterwards used for PrOOH measurement.

The same procedure was followed for both the LOOH and PrOOH determination. Firstly, the lipid pellet was dissolved in 2000 μL of absolute methanol for the LOOH assay. For both, the sample was incubated for 30 min with or without the presence of Fe^{+2}. The final concentrations of reagents for the +Fe sample were 92.5% methanol, 100 μM xylenol orange, 12.5 mM H_2SO_4, and 200 μM Fe^{2+}, whereas the final concentrations of reagents for the -Fe sample were 92.5% methanol, 100 μM XO, and 12.7 mM H_2SO_4. The absorbance was measured at 560 nm, and the net absorbance difference was calculated as (Sample+Fe) - (Blank reagent+Fe), from which the absorbance differ-

ence (Sample-Fe) - (Blank reagent-Fe) was subtracted. For LOOH, the net absorbance was converted to CumOOH concentration equivalents using a standard curve (0-20 µM) or converted to H_2O_2 concentration equivalents using a standard curve (0-40 µM) for PrOOH. The results were expressed as µmol equivalents CumOOH/mg protein of the original homogenate (LOOH) or µmol H_2O_2/mg protein equivalents of the PrOOH fraction (PrOOH).

For both PrMDA and FrMDA, the same procedure was followed. The samples were incubated with a final reagent concentration of 7% TCA, 0.21% TBA (thiobarbituric acid), and 1 mM BHA (butylhydroxyanisole) for the sample reaction or 7% TCA and 1 mM BHA for the sample blank for 20 min at 100°C. Then, the samples were cooled in an ice-water bath and centrifuged at 10000 g for 3 min. The absorbance of the supernatant was measured at 535 nm. The net absorbance (sample reaction - sample blank) was converted to MDA (malondialdehyde) concentrations from the corresponding standard curve, prepared in 0.1 M NaOH solution (0-15 µM MDA) for PrMDA determination or prepared in an extraction solution (100 mM phosphate buffer, 0.5 mM BHA, 33% methanol, 13.3% TCA) (0-40 µM) for the FrMDA assay. The results are expressed as µmol MDA/mg protein of the PrMDA fraction (PrMDA) or µmol MDA/mg protein of the original homogenate (FrMDA).

For all biochemical variables, two measurements were taken from each aliquot, and the average was used in the statistical analyses. All biochemical reagents, including substrates, coenzymes, and purified enzymes, were obtained from Roche (Mannheim, Germany), Sigma Chemical Co. (St. Louis, MO, USA) or Merck (Darmstadt, Germany). The lipid and protein peroxidation products in the samples, FrMDA and PrMDA, are expressed as µmol MDA, LOOH are expressed as µmol CumOOH equivalents and PrOOH, are expressed as µmol H_2O_2 equivalents. The values are expressed as per mg protein as determined using the BCA method. Furthermore, another index of oxidative damage widely used was measured in the same samples: the lipid-peroxidation levels were determined based on the malondialdehyde levels (MDA) generated as a product of lipid peroxide degradation. In the presence of thiobarbituric acid, MDA reacts to produce a coloured thiobarbituric acid-reacting substance (TBAR) that was measured at 535 nm and was expressed as nmol MDA per g tissue.

All biochemicals, including the substrates, the coenzymes, and the purified enzymes, were obtained from Roche or Sigma Chemical Co. All other chemicals were obtained from Merck and were of reagent grade. The plasma glucose, total lipid, triglyceride, and cholesterol levels were assayed using standard colorimetric tests (Labkit 30232, 30345, 30360, 30180, CHEMELEX S.A., Barcelona, Spain).

Statistical analyses

The results are expressed as the means ± SEM. The data were statistically analysed by a one-way analysis of variance (ANOVA, SPSS version 19.0 for Windows) to assess the significance of the differences between taxa. When these differences were significant ($P<0.05$), the taxa were compared using a Duncan's multiple range test (Duncan, 1955). Pearson's correlations were generated to examine the relationships among all of the variables.

Parametric tests were used because the data followed a normal distribution (Kolmogorov-Smirnov with $P>0.05$) and had homocedasticity (Levene test with $p>P.05$).

RESULTS

The soluble protein content in the body tissues was significantly lower in *I. morenica* and *E. danica.* In contrast, a higher lipid content was found in *I. morenica* (Tab. 2).

Antioxidant capacity

The highest values of trolox-equivalent antioxidant capacity (TEAC) were found in *I. morenica*, *P. bipunctata* and *C. erythraea* (Tab. 3), and the lowest values are found in *Hydropsyche* sp., *Gerris* sp. and *E. danica*.

Regarding FRAP (Tab. 3), the highest values were present in *S. ignita and C. erythraea*, and the lowest were present in *Gerris* sp., *D. cephalotes*, *P. bipunctata* and *E. danica*. The highest levels for each enzyme (Tab. 3) were recorded as follows: *N. maculata*, *Gerris* sp. and *E. danica* for SOD; *I. morenica* for CAT; *S. ignita* for GR; *D. cephalotes, P. bipunctata* and *I. morenica* for GPX; *P. bipunctata*, *I. morenica*, *N. maculata*, *Gerris* sp. and *C. erythraea* for GST; *I. morenica* for DTD, and *Gerris* sp. for G6PDH.

On the other hand, the lowest levels of each enzyme

Tab. 2. Protein and lipid-soluble content in the studied taxa of aquatic insects.

	Lipids (g/mL)	Proteins (mg/mL)
Serratella ignita	$0.097^{cd}\pm0.002$	$4.689^{bc}\pm0.073$
Ephemera danica	$0.078^{a}\pm0.001$	$3.186^{a}\pm0.257$
Crocothemis erythraea	$0.079^{a}\pm0.002$	$4.260^{ab}\pm0.643$
Dinocras cephalotes	$0.082^{ab}\pm0.000$	$5.961^{c}\pm0.180$
Perla bipunctata	$0.094^{bcd}\pm0.005$	$6.006^{c}\pm0.496$
Isoperla morenica	$0.102^{d}\pm0.007$	$3.089^{a}\pm0.072$
Notonecta maculata	$0.090^{abc}\pm0.001$	$5.872^{c}\pm0.611$
Gerris sp.	$0.086^{abc}\pm0.005$	$6.038^{c}\pm0.345$
Hydropsyche sp.	$0.086^{abc}\pm0.001$	$5.006^{bc}\pm0.427$

Values are mean ± SEM (N= 8). Superscripts letters indicate significant differences among different species (P< 0.05).

were found in the following: *S. ignita* for SOD; *S. ignita*, *N. maculata* and *C. erythraea* for CAT; *P. bipunctata*, *Gerris* sp., *E. danica* and *C. erythraea* for GR; *Hydropsyche* sp., *S. ignita* and *E. danica* for GPX; *D. cephalotes, S. ignita, E. danica* and *Hydropsyche* sp. for GST; *S. ignita*, *Gerris* sp., *E. danica* and *C. erythraea* for DTD; and *C. erythraea, I. morenica* and *E. danica* for G6PDH.

Oxidative damage

The highest values of TBARs were present in *S. ignita* and *E. danica*, while the lowest values were recorded in *D. cephalotes* (Tab. 4).

For PrMDA, the highest values were found in *I. morenica*, *S. ignita* and *C. erythraea*; however, in *D. cephalotes* and *E. danica,* PrMDA was not detected. The highest values of PrOOH were detected in *S. ignita* and *E. danica,* whereas in *D. cephalotes* and *P. bipunctata,* PrOOH was not detected. The highest value of FrMDA was present in *S. ignita*, and the lowest FrMDA values were found in *D. cephalotes*, *P. bipunctata* and *Hydrospyche* sp. The highest value of LOOH was found in *Hydropsyche* sp., and the lowest was found in *N. maculata*.

A strong positive correlation was found between TBARs and FrMDA (Pearson r=0.815; P<0.0001) and between TBARs and PrOOH (r=0.801; P<0.0001). Significant correlations were also found between FrMDA and PrMDA (r=0.740; P<0.0001), FrMDA and PrOOH (r=0.931; P<0.0001), and PrMDA and PrOOH (r=0.826; P<0.0001).

DISCUSSION

Antioxidant capacity

To accurately test the antioxidant state of the nine insect species, we used three separate techniques (TEAC, FRAP and antioxidant enzymes). We found a correspondence among the values obtained in TEAC and FRAP for the studied species. Moreover, the FRAP values were higher than the TEAC values for each species, except *S. ignita*, *N. maculata* and *Hydropsyche* sp. Although both techniques determined substances with antioxidant power, the determination of TEAC assumes the assessment of 53.0% -SH groups of proteins, 33.1% uric acid, 4.7% vitamin C, 2.4% bilirubin, 1.7% vitamin E and 5.2% others (glutathione, *etc*.) (Erel, 2004). However, FRAP assesses the capacity of a given substance to reduce ferric iron, and is also considered a measure of the total antioxidant power. It treats reducers and antioxidants equally; however, not all reducers that transform ferrous iron to ferric iron are antioxidants, and some antioxidants cannot produce that reaction, as is the case of glutathione, which can be determined by means of TEAC (Prior and Cao, 1999). Nevertheless, both techniques (TEAC and FRAP) can be

Tab. 3. Activity of some antioxidant enzymes and antioxidant potential in the studied taxa of aquatic insects.

	SOD (U/mg protein)	CAT (U/mg protein)	GPX (mU/mg protein)	G6PDH (mU/mg protein)	GR (mU/mg protein)	GST (mU/mg protein)	DTD (mU/mg protein)	TEAC (trolox equivalent µM)	FRAP ($FeSO_4$ equivalent µM)
Serratella ignita	$27.92^{a}\pm2.27$	$2.85^{a}\pm0.62$	$3.62^{a}\pm0.44$	$7.58^{d}\pm0.29$	$9.89^{d}\pm0.07$	$25.53^{a}\pm4.82$	$15.61^{ab}\pm0.82$	$612.40^{de}\pm6.74$	$1540.63^{d}\pm95.83$
Ephemera danica	$186.25^{d}\pm14.26$	$35.07^{bc}\pm4.69$	$6.89^{ab}\pm0.40$	$0.35^{a}\pm0.33$	$2.35^{bc}\pm0.33$	$13.47^{a}\pm3.75$	$15.57^{ab}\pm0.77$	$182.68^{a}\pm23.01$	$562.90^{a}\pm24.43$
Crocothemis erythraea	$133.43^{cd}\pm7.56$	$15.36^{ab}\pm1.22$	$16.17^{ab}\pm2.08$	$0.56^{a}\pm0.19$	$2.07^{ab}\pm0.31$	$74.43^{b}\pm8.56$	$11.49^{ab}\pm2.26$	$725.40^{f}\pm7.09$	$1377.43^{d}\pm64.51$
Dinocras cephalotes	$76.24^{b}\pm1.47$	$46.15^{bcd}\pm1.00$	$69.70^{fg}\pm4.55$	$5.32^{cd}\pm1.40$	$2.70^{bcd}\pm0.19$	$27.64^{a}\pm1.48$	$29.31^{cd}\pm0.99$	$515.75^{de}\pm26.26$	$615.63^{a}\pm53.89$
Perla bipunctata	$99.19^{bc}\pm20.94$	$48.33^{bc}\pm4.02$	$78.47^{g}\pm8.45$	$4.29^{bcd}\pm1.42$	$1.43^{a}\pm0.25$	$68.20^{b}\pm5.94$	$28.95^{cd}\pm3.58$	$657.56^{ef}\pm36.03$	$942.01^{b}\pm71.49$
Isoperla morenica	$106.83^{bc}\pm10.00$	$93.98^{d}\pm8.54$	$71.35^{g}\pm3.49$	$0.46^{a}\pm0.08$	$2.57^{bcd}\pm0.26$	$75.34^{b}\pm6.45$	$53.47^{e}\pm5.66$	$709.46^{f}\pm9.24$	$1141.32^{c}\pm14.50$
Notonecta maculata	$211.04^{fg}\pm25.86$	$6.47^{a}\pm0.05$	$24.25^{b}\pm4.93$	$2.39^{abc}\pm0.52$	$3.00^{a}\pm0.08$	$65.56^{b}\pm1.59$	$25.89^{bc}\pm4.24$	$570.10^{cd}\pm15.62$	$1466.32^{d}\pm59.63$
Gerris sp.	$198.35^{ef}\pm6.14$	$52.22^{bc}\pm4.13$	$27.10^{bc}\pm1.91$	$24.02^{e}\pm2.98$	$2.13^{ab}\pm0.09$	$66.65^{b}\pm7.42$	$20.76^{abc}\pm2.90$	$403.36^{b}\pm38.97$	$677.43^{a}\pm52.74$
Hydropsyche sp.	$87.58^{b}\pm4.98$	$42.97^{bc}\pm2.57$	$3.82^{a}\pm0.93$	$1.45^{ab}\pm0.13$	$2.84^{bcd}\pm0.33$	$20.73^{a}\pm5.62$	$38.86^{de}\pm4.40$	$405.82^{b}\pm25.76$	$1069.10^{bc}\pm19.56$

Values are mean ± SEM (N=8). SOD, superoxidedismutase; CAT, catalase; GPX, glutathioneperoxidase; G6PDH, glucose 6-phospahte dehydrogenase; GR, glutathionereductase; GST, glutathione S transferase; DTD, DT-diaphorase; TEAC, trolox equivalent antioxidant capacity; FRAP, ferric reducing antioxidant power. Superscripts letters indicate significant differences among different species (P< 0.05).

considered as indicative of some form of antioxidant capacity of the tissue from which they are determined. In fact, we find that the species *S. ignita*, *I. morenica*, *C. erythraea* and *N. maculata* are notable for a higher antioxidant power as indicated by both techniques (Fig. 1), and *Gerris* sp. and *E. danica* had the lowest capacity. If the sum of the antioxidant enzymes are considered together with the antioxidant capacity determined by TEAC and FRAP (TAC, Fig. 1), the same results were obtained.

Specimens of *S. ignita*, *I. morenica*, *C. erythraea* and

Tab. 4. Values of different parameters of oxidative damage in the studied taxa of aquatic insects.

	LOOH (µmoleqCumOOH/mg lipid)	FrMDA (µmolMDA/mg lipid)	PrMDA (µmolMDA/mg protein)	PrOOH (µmol eqH_2O_2/mg protein)	TBARs (µmolMDA/g tissue)
Serratella ignita	$0.048^{bc}\pm0.001$	$0.159^{d}\pm0.000$	$16.45^{c}\pm0.9$	$10.20^{d}\pm0.7$	$40.85^{e}\pm3.11$
Ephemera danica	$0.048^{bc}\pm0.002$	$0.070^{c}\pm0.003$	$5.24^{a}\pm0.00$	$6.66^{c}\pm0.54$	$32.51^{d}\pm2.27$
Crocothemis erythraea	$0.045^{bc}\pm0.003$	$0.078^{c}\pm0.003$	$7.09^{b}\pm0.63$	$3.17^{b}\pm0.21$	$17.12^{b}\pm0.61$
Dinocras cephalotes	$0.095^{d}\pm0.004$	$0.035^{a}\pm0.004$	$3.44^{a}\pm0.21$	n.d.	$11.08^{a}\pm1.01$
Perla bipunctata	$0.059^{c}\pm0.008$	$0.031^{a}\pm0.004$	$4.17^{a}\pm0.20$	n.d.	$21.61^{bc}\pm1.00$
Isoperla morenica	$0.036^{b}\pm0.005$	$0.077^{c}\pm0.003$	$16.98^{c}\pm1.2$	$5.58^{c}\pm0.32$	$16.61^{b}\pm1.71$
Notonecta maculata	$0.017^{a}\pm0.000$	$0.054^{b}\pm0.005$	$3.36^{a}\pm0.09$	$2.12^{a}\pm0.20$	$20.25^{bc}\pm1.02$
Gerris sp.	$0.043^{b}\pm0.008$	$0.055^{b}\pm0.005$	$3.56^{a}\pm0.37$	$3.06^{b}\pm0.10$	$17.83^{b}\pm1.73$
Hydropsyche sp.	$0.157^{e}\pm0.004$	$0.041^{a}\pm0.001$	$4.20^{a}\pm0.22$	$1.30^{a}\pm0.13$	$23.74^{c}\pm1.76$

Values are mean ± SEM (N= 8). LOOH, lipid hydroperoxides; FrMDA, free malondialdehyde; PrMDA, malondialdehyde associated to protein; PrOOH, protein hydroperoxides; TBARs, thiobarbituric acid reactive substances; n.d., non detectable. Superscripts letters indicate significant differences among different species (P<0.05).

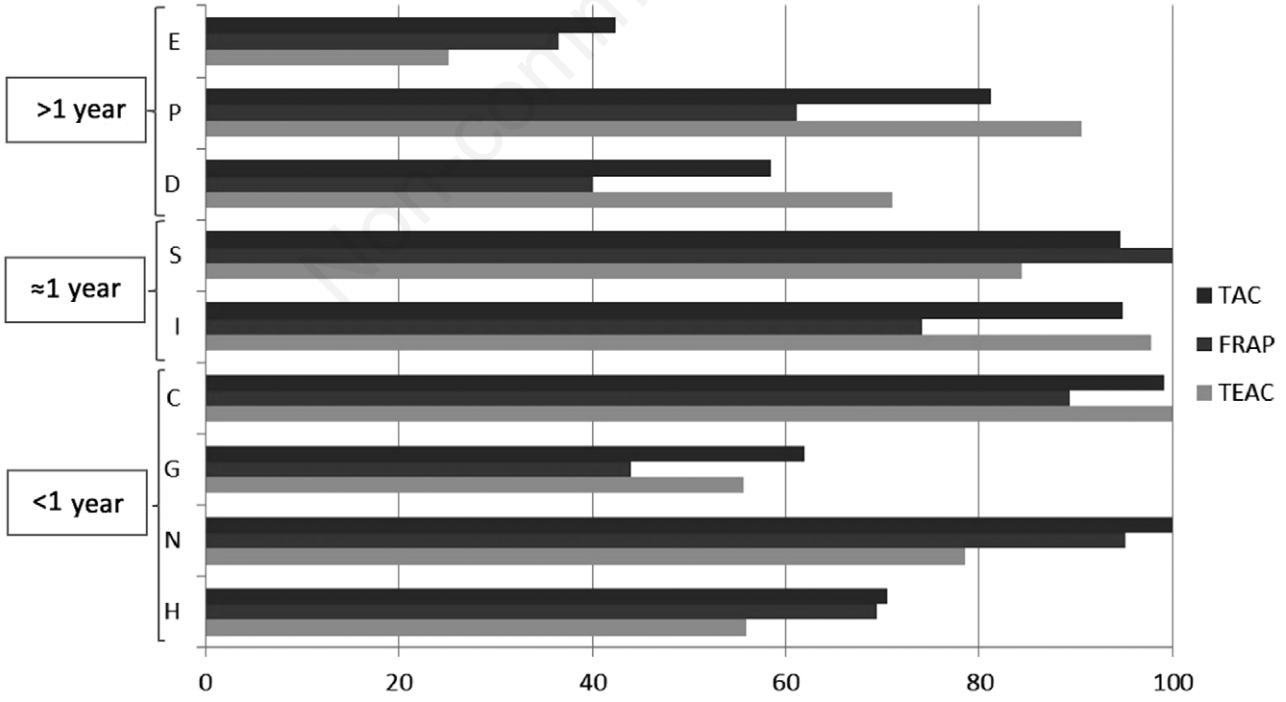

Fig. 1. Trolox-equivalent antioxidant capacity (TEAC), ferric-reducing/antioxidant power (FRAP) and total antioxidant capacity (TAC) for the studied taxa. The parameters are represented as the percentage in relation to the maximum value (100%) found in a taxon. E, *Ephemera danica*; P, *Perla bipunctata*; D, *Dinocras cephalotes*; S, *Serratella ignita*; I, *Isoperla morenica*; C, *Crocothemis erythraea*; G, *Gerris* sp.; N, *Notonecta maculata*; H, *Hydropsyche* sp.

N. maculata were collected from warm or relatively warm-waters habitats. It is known that temperature increases metabolic processes, as well requiring higher antioxidant protection due to increase in metabolism (Ahmad and Pardini, 1990). In contrast, *Gerris* sp. and *E. danica* had the lowest values of antioxidant capacity as previously mentioned, and both species occur neither in the water column nor on the riverbed (*Gerris* sp. lives on the surface of the water, and *E. danica*, a burrowing mayfly nymph, lives primarily in U-shaped tubes excavated in the riverbed, Tab. 1). It is possible that *Gerris* sp. has a lower energetic cost for locomotion than the other species, resulting in a slower metabolism. However, the oxygen content in the environment that *E. danica* usually inhabits is low (in comparison with other habitats in the same stream); consequently, this species should have a lower formation of free radicals. However, *E. danica*, unlike *Gerris* sp., also experiences important oxidative damage (Tab. 4). This and other currently unknown factors associated to its biology may cause this stress, such as the fact that *Gerris* sp. lives on the water surface where the oxygen level is permanently high if compared with aquatic environments. Moreover, it lives in a environment with rapid temperature changes. Thus, it is necessary to have a better antioxidant protection.

The remaining species (*Hydropsyche* sp., *P. bipunctata* and *D. cephalotes*) had a total antioxidant capacity half that of the previously mentioned species (Fig. 1). The occurrence of these three taxa in cold-water streams may explain the lower antioxidant capacity (Tab. 1). The antioxidant potential may be directly related with habitat temperature and with ecological characteristics (benthic, neustonic, etc.) of the habitat of each taxon measured.

The values obtained for TEAC and FRAP were comparable with those of TAEA (the sum of the enzymatic activities of SOD, CAT, GR, GPX, GST and DTD, Fig. 2), except for *S. ignita* in which the TEAC/TAEA and FRAP/TAEA relations are approximately four times higher than in the remaining species. This would indicate a higher proportion of metabolites with antioxidant power relative to the activity of the antioxidant enzymes in *S. ignita*. Although this species has a very low level of antioxidant enzymes, its GR activity is higher than for the other species (Tab. 3). This indicates that glutathione is important among the antioxidant metabolites due to the important role of GR in its regeneration. The activity of the G6PDH enzyme in this species is exceptional. This enzyme is associated with NADPH production and with the maintenance of GR activity. This higher proportion of antioxidant metabolites may be related to the diet of the nymphs, which feed on diatoms to a large extent (almost one third of their diet), together with detritus in the studied population (López-Rodríguez *et al*., 2009a). Their great availability in the sampled stream and the characteristics of these trophic resources relative to their antioxidant properties (Bertrand, 2010; Palatal *et al*., 2016) could promote a higher proportion of antioxidant metabolites relative to the antioxidant enzymatic potential with respect to the remaining species (Fig. 3).

The first studies conducted with insects reported that some antioxidant enzymes were absent in the organisms (Mathews *et al*., 1997). However, we found all the fundamental antioxidant enzymes are active in our studied aquatic insect species and that there are significant differences among these taxa. This fact has been previously noted also by Berra *et al*. (2004). In particular, the GPX activity in insects was reported for nymphs of different species of Plecoptera (Sanz *et al*., 2010, 2014). In these studies, the total GPX activity was assayed, including selenium-dependent and selenium-independent isoforms using a organic peroxide (cumene hydroperoxide) as the substrate. The same protocol was followed in the present study, which could explain the GPX activity that was manifested. In fact, the control reaction without the tissue extract confirmed a GPX activity proportional to the extract concentration. A recent study (Dias *et al*., 2016) on the GPX activity and the gene encoding the selenium-dependent isoform in the kissing bug, *Rhodnius prolixus* Stål, 1859, supports our results.

In our study, *N. maculata, Gerris* sp. and *E. danica*

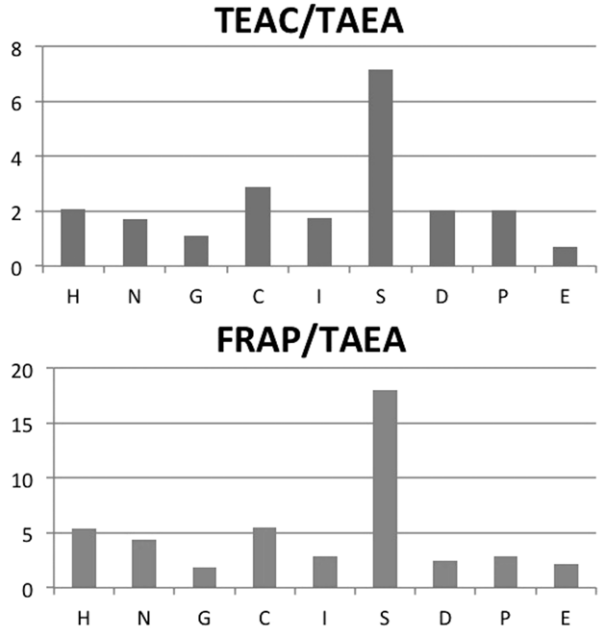

Fig. 2. Relation between TEAC (trolox-equivalent antioxidant capacity, upper graph) and FRAP (ferric-reducing/antioxidant power, down graph) with TAEA, total antioxidant enzyme activity. E, *Ephemera danica*; P, *Perla bipunctata*; D, *Dinocras cephalotes*; S, *Serratella ignita*; I, *Isoperla morenica*; C, *Crocothemis erythraea*; G, *Gerris* sp.; N, *Notonecta maculata*; H, *Hydropsyche* sp.

were notable for a SOD activity higher than that of the other studied species and *I. morenica* had high CAT, GPX, GST and DTD activity. All the other studied species have similar values for GST activity, with *S. ignita*, *D. cephalotes*, *Hydropsyche* sp. and *E. danica* had the lowest values. The species *I. morenica*, *P. bipunctata* and *D. cephalotes* had high GPX activity, and they are species with the intermediate or great lipid content. It is known that the GPX plays a protective role for lipids against oxidation (Brigelius-Flohé, 1999). *Serratella ignita* has high GR activity as mentioned before, and *Gerris* sp. is notable for its high G6PDH activity, an enzyme in the pentose phosphate pathway with a high reduction power responsible for several functions in the animal organism. Berra *et al*. (2004) noted that G6PDH activity is very low or undetectable in most taxa, including Plecoptera. In our work, the lowest values corresponded to *I. morenica*, *C. erythraea* and *Hydropsyche* sp., species belonging to three taxonomic groups, Plecoptera, Odonata and Trichoptera, respectively. Our study suggests that the differences in antioxidant enzymes found among distant related taxa are not associated with autecological traits.

Oxidative damage

To quantify the oxidative damage, we evaluated lipid and protein peroxidation biomarkers: free malondialdehyde (FrMDA), protein-bound malondialdehyde (PrMDA), total hydroperoxides (LOOH), and protein hydroperoxides (PrOOH) and TBARs (Tab. 4). We found a strong positive correlation between TBARs and FrMDA and between TBARs and PrOOH. There were also correlations between FrMDA and PrMDA, FrMDA and PrOOH, and PrMDA and PrOOH. This indicates that, except for LOOH, the remaining determinations used to indicate oxidative damage have values correlated among them. This allows accuracy in detecting actual oxidative damage.

Fig. 4 presents the sum of all the parameters of oxidative damage (TOD). The species *S. ignita* had the greatest oxidative damage, followed by *E. danica* and *I. morenica*. The lowest was found in *D. cephalotes*, followed by *P. bipunctata*. *Serratella ignita* and *I. morenica* exhibited a short, univoltine, life cycle (Tab. 1), and both were collected in the same stream with relatively well-oxygenated, warm water, and a temporal regime, i.e., a stream with a flow period of several months and a drought period during summer. They are also the species with the highest lipid content. In contrast, *D. cephalotes* and *P. bipunctata* are two long lived Plecoptera predator species (Tab. 1) that were collected from a well-oxygenated fast-flowing cold stream with a permanent hydrological regime. Both of these species have a intermediate body lipid content. Although these two species differ slightly in their oxidative state due to a possible mechanism of coexistence in the same habitat (Sanz *et al*., 2014), they are notable for their lower oxidative damage compared with the other studied species.

The accumulation of oxidative damage with ageing

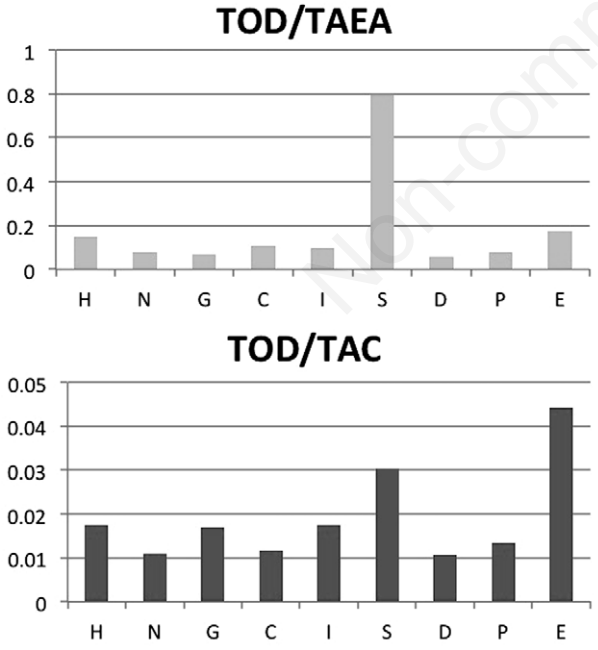

Fig. 3. Relation between TOD, total oxidative damage, with TAEA (total antioxidant enzyme activity, upper graph) and with TAC (total antioxidant activity, down graph). E, *Ephemera danica*; P, *Perla bipunctata*; D, *Dinocras cephalotes*; S, *Serratella ignita*; I, *Isoperla morenica*; C, *Crocothemis erythraea*; G, *Gerris* sp.; N, *Notonecta maculata*; H, *Hydropsyche* sp.

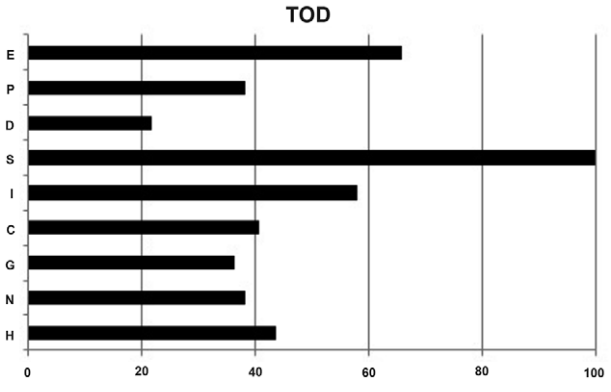

Fig. 4. Total oxidative damage (TOD) for the studied taxa. For each measurement, the species that shows the maximum level is represented as 100%, and the other species reflect the percentage value with respect to this maximum. E, *Ephemera danica*; P, *Perla bipunctata*; D, *Dinocras cephalotes*; S, *Serratella ignita*; I, *Isoperla morenica*; C, *Crocothemis erythraea*; G, *Gerris* sp.; N, *Notonecta maculata*; H, *Hydropsyche* sp.

has been mentioned by Farooqui and Farooqui (2012) and noted in previous works with fish and other organisms (Sanz *et al*., 2013). This could partially explain the maximum values of oxidative damage recorded in *S. ignita* and *I. morenica*, short-lived species with detritivorous and non-exclusively predator feeding habits, and the minimum values of *D. cephalotes* and *P. bipunctata*, long-lived, mainly predator species, as found in this study. The antioxidant machinery (enzymatic and non-enzymatic) in organisms keeps the oxidative species concentration in equilibrium. However, when antioxidant machinery cannot regulate the concentration of pro-oxidant substances, oxidative-stress levels rise (Halliwell and Gutteridge, 1995; Sies, 1997; Halliwell, 2007).

The results obtained in this study could indicate an influence of the life cycle duration, the environment where species live and the taxonomic group to which the species belong (and even of the feeding habits) on the oxidative state of each studied species. Thus, we could differentiate the following three groups:

1) Predator species with a long-life cycle that inhabit permanent streams with well-oxygenated, cold waters (*D. cephalotes* and *P. bipunctata*) have a relatively better oxidative state. Both species belong to the insect order Plecoptera. *Isoperla morenica* also belongs to the same order and has a high antioxidant potential; however, it also has high oxidative damage. Contrary to *D. cephalotes* and *P. bipunctata*, this species may act as an omnivore, have a shorter life cycle, and inhabit relatively warmer waters in a temporary stream (Tab. 1).
2) Species with non-exclusively predator feeding habits, from warm, temporal waters and with a short life cycle are those that have the greatest oxidative damage (*S. ignita* and *I. morenica*; Tab. 1). *Isoperla morenica* also has a high antioxidant power; thus, the balance between the total antioxidant capacity and the oxidative damage is similar to the other species, contrary to *S. ignita,* which is notable as the species with the poorest oxidative balance. *Ephemera danica* is a member of the first group based on its life cycle and the temperature of the habitat where it exists (Tab. 1), but the oxidative state of this species is similar to that of *S. ignita*. Although the oxidative damage of *E. danica* is lower than the one presented by *S. ignita*, its lower antioxidant capacity generates a high oxidative imbalance. Both species belong to the Ephemeroptera and are detritivorous.
3) The remaining species have an intermediate oxidative state between that of the previous groups. *Notonecta maculata*, *Gerris* sp. and *C. erythraea* were collected in a poorly oxygenated, warm-water pond and have a short life cycle (Tab. 1), although *Gerris* sp. inhabits on the water surface and is exposed to the oxygen concentration in the atmosphere. *Notonecta maculata* and *Gerris* sp. are Hemiptera. *Hydropsyche* sp. is a caddisfly (Trichoptera) with an intermediate oxidative state, and it is a species that mixes the biotic and abiotic characteristics of groups 1 and 2 (omnivorous, with a short life cycle and from the cold, well-oxygenated waters of a permanent stream).

CONCLUSIONS

In summary, predators with a long-life cycle and occurring in permanent streams with cold waters seem to have a proper oxidative state within the insects studied. In contrast, non-exclusively predator species living in temporary streams with warm and poorly oxygenated waters that have a short life cycle have a poorest oxidative state. Therefore, the oxidative state of each species would be defined by an interaction of biological and autecological factors. Future researches are needed to evaluate the relative importance of these factors.

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