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Comet assay in *Aegla platensis* (Decapoda: Anomura) using a non-lethal hemolymph field sampling for in situ monitoring of freshwater genotoxicity

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Abstract

This study aimed to apply the comet assay on *Aegla platensis* crabs as a suitable non-destructive approach for in situ monitoring of freshwater genotoxicity. Animals were captured during four sampling periods in a stream under minor anthropogenic impacts in Southern Brazil. Crabs were captured with a hand net, then the hemolymph samples were collected, and the animals were released into the stream after a 20-min recovery time. Hemolymph samples were transported to the laboratory and used to perform the alkaline comet assay. Results showed an intermediate level in the DNA damage index (range 107.3–165.0 arbitrary unit). No significant differences were observed among the different sampling periods. Hemolymph was successfully used as a non-lethal source of biological samples, and the comet assay using *A. platensis* proved to be a feasible approach for genotoxicity studies.

Keywords Genotoxicity · Biomarkers · Comet assay · Crustaceans · In situ monitoring

Introduction

DNA damage biomarkers are valuable tools for evaluating acute and chronic effects on aquatic organisms exposed to genotoxic substances (Jha 2008; Dhawan et al. 2009). Among the several genotoxicity tests, the comet assay (single-cell gel electrophoresis) was carried out in several invertebrate species for both genetic toxicology and environmental biomonitoring (Lee and Steinert 2003; Jha 2008; Glei et al. 2016; Gajski et al. 2019). The comet assay technique, named after the comet-like appearance of the cellular DNA after electrophoresis, has been widely accepted as quite simple, sensitive, reliable, rapid, and low-cost assay for the detection of DNA damage (Gajski et al. 2019).

Crustaceans are currently employed as bioindicator organisms for environmental pollution monitoring because they constitute one of the main groups in freshwater ecosystems and usually are easily sampled in field evaluation (Astley et al. 1999; Brown et al. 2004; Martín-Díaz et al. 2007; Di Donato et al. 2016). The general abundance and high biomass of freshwater crabs in aquatic ecosystems, combined with their dominant detritus-shredding role, makes them potentially very important to the dynamics of nutrient recycling in rivers. In addition, freshwater crabs are integral components of food webs in tropical aquatic ecosystems and provide food for a wide range of predators (Cumberlidge et al. 2009).

Aeglids are freshwater anomuran crustaceans with potential for being used as a bioindicator for the assessment of water quality in South American freshwater ecosystems, where they represent the most widely distributed macroinvertebrate group (Bond-Buckup et al. 2008). These animals present benthic habits, and are found in streams, rivers, and ponds, usually, under rocks, leaves, and other detritus (Noro and Buckup 2002). In addition, it has been reported that aeglids are tolerant to poor water quality (Strieder et al. 2006). In Southern Brazil, *Aegla platensis* is easily found and captured all year long, which makes this species a bioindicator candidate for biomonitoring studies (Bond-Buckup and Buckup 1994; Borges et al. 2018). Recently, *A. platensis* were used as bioindicators to evaluate the correlation between the level of oxidative stress biomarkers with the metals present in the stream sediments and with land uses of three hydrographic basins (Borges et al. 2021).

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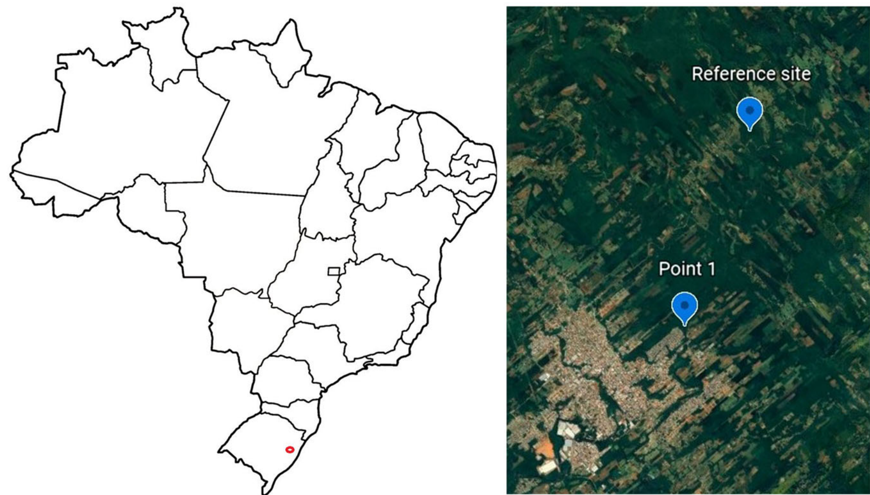

Fig. 1 **Location of study area.** **A** Sampling location in the state of Rio Grande do Sul, Southern Brazil. **B** Satellite image of the study area showing sampled sites (Google Earth®)

Although genotoxicity biomarkers have been developed in many marine invertebrates (Halsband et al. 2021; Sahlmann et al. 2019), less attention has been paid to freshwater invertebrates, particularly to crustaceans (Lacaze et al. 2010). Crustaceans possess an open circulatory system, where nutrients, oxygen, hormones, and cells (hemocytes) are distributed in the hemolymph (Vazquez et al. 2009). Three types of circulating hemocytes are usually recognized: hyalinocytes (the smallest cells without evident granules), semigranulocytes (containing small granules) and granulocytes (with abundant cytoplasmic granules) (Bauchau 1981). In this context, hemolymph of crustaceans is a highly valuable tissue for ecotoxicological evaluation, and assessment of environmental stressors due to ease of sampling and sensitive response to many disorder states (Lewbart et al. 2006), including DNA damage (Gajski et al. 2019; Di Donato et al. 2020).

Considering genotoxicity studies in literature, no reports were found using *Aegla* genus. Since ecological conditions differ widely between different parts of the world, test systems must be developed that are relevant for different regional conditions (Breitholtz et al. 2006). In addition, in situ studies have the advantages of incorporating many of the natural fluctuating environmental conditions and integrating toxicity mixtures naturally present in the field (Rodrigues and Pardal 2014). Thus, considering the importance of *Aegla* crabs in the South American aquatic ecosystems, the purpose of this study was to evaluate the feasibility of the comet assay using *Aegla platensis* for in situ biomonitoring studies.

Materials and methods

Sampling site and animal collection

Sampling was performed in August, October, and December 2012, and in January 2013. Crabs were captured in Feitoria Stream, in the municipality of Dois Irmãos (29°35'26.51"S and 51°4'16.64"O), in the state of Rio Grande do Sul, Southern Brazil (Fig. 1A). The sampling site (point 1, Fig. 1B) is under minor anthropogenic impacts, and it is located where water is collected to public supply (upstream the urban area). In addition, a sampling was carried out in a site further away from the urban area and close to the source of the stream, where the environment is more preserved (reference site, Fig. 1B). Due to difficulties in accessing the site, sampling was carried out only in December. *A. platensis* individuals (8–12 mm carapace length; Fig. 2) were collected from the bottom of the stream, and from below rocks manually and/or with a hand net (30 × 50 cm with a 60 cm deep mouth opening, and 1 mm mesh).

Fig. 2 Specimen of *Aegla platensis*

Hemolymph sampling

Hemolymph samples from each animal were collected in the field using heparinized needles and 1 mL syringes which were inserted at the base of pereiopods (according to images in Kankamol and Salaenoi 2018). The maximum volume of hemolymph collected from an animal was approximately 100 µL. After sampling, crabs were kept in aerated water for

20 min for recovery and released in the stream (a pilot study in laboratory with three animals did not show mortality until 6 days after hemolymph sampling). Hemolymph samples were transferred into microtubes protected from light and kept on ice during transport to the laboratory. The hemolymph samples were taken to the laboratory within 2 h after collection.

Comet assay

Comet assay was performed according to the protocol described by Singh et al. (1988) with some modifications. For each animal, 10 µL of hemolymph was added to 90 µL of low-melting agarose, then, these were placed on a slide pre-coated with normal melting point agarose. A coverslip was immediately added, and the gel was allowed to solidify at 4 °C for 10 min. After solidification, the coverslip was gently removed, and the slides were placed in cold freshly prepared lysis solution [2.5 M NaCl, 100 mM EDTA, 10 mM Tris, pH 10.2, to which 1% Triton X-100, and 10% dimethyl sulfoxide (DMSO) had been added]. The lysis solution (detergents in a hypersaline buffer) removes membranes and other cellular material, leaving protein-depleted nuclei with supercoiled DNA (called "nucleoids"). The high-alkaline-pH solution (with NaOH) disrupts the hydrogen bonding that holds the DNA strands together and converts certain nucleobase lesions into DNA strand breaks (Møller et al. 2020). After 24 h the slides were placed in an electrophoresis box filled with fresh electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) at 4 °C for 25 min to allow DNA unwinding. Electrophoresis was performed at 1 V/cm, and 300 mA for 25 min. The steps previously described were all carried out under red light to avoid induction of DNA damage. After electrophoresis, slides were neutralized with 0.4 M Tris (pH 7.5), air dried for 24 h, fixed (15% trichloroacetic acid, 5% zinc sulfate, and 5% glycerol), and stained with silver nitrate (Nadin et al. 2001). In order to evaluate DNA damage, 100 cells per individual were analyzed under light microscope at a magnification of ×400. All slides were analyzed by the same observer. The determination of damage can be carried out either through visual scoring of cells (after classification into different categories based on tail length and shape) or by using different commercially available or public domain software (which automatically recognize the extent of damage) (Kumaravel et al. 2009). It is generally accepted that visual scoring is as comparable as image analysis (García et al. 2004; Kumaravel et al. 2009; Azqueta et al. 2011; Andem et al. 2013). In the present study, nucleoids (comets) were visually scored into five classes, from undamaged (class 0) to completely damaged (class IV), according to their tail length. Based on the arbitrary values assigned to the different classes (from class 0 = 0 to class IV = 4), a genetic damage index (DI) was calculated as the sum of nucleoids observed in each damage class multiplied by the value of the respective class [(number of cells in class 0 × 0) + (number of class I cells × 1) + (number of class II cells × 2) + cells (number of class III cells × 3 cells) + (number of class IV cells × 4)] (Collins et al. 1995). Therefore, the DI of each animal ranged from 0 (all cells undamaged) to 400 (all cells maximally damaged) arbitrary units.

Statistical analysis

Differences among sampling periods were compared using ANOVA test. Comparison between point 1 and reference site was performed using the Mann–Whitney test. The significance level considered was $p \leq 0.05$. Analyses were carried out using the Statistical Package for the Social Sciences (SPSS) 25.0 for Windows.

Results

The Comet assay was performed successfully with the hemolymph of all sampled crabs. Mortality was not observed during the 20-min recovery period. The results of the comet assay are presented in Table 1. Both undamaged and damaged cells, belonging to the five DNA damage classes (0–IV), were observed in the four sampling periods. These results show that the lysis, as well as the electrophoresis conditions, was suitable (Fig. 3). The DNA damage of field-collected *Aegla* crabs clearly exhibited a great interindividual variability in the same sampling period, as evidenced by the high standard deviation values. DNA damage index varied from 107.3 ± 62.8 to 165.0 ± 43.5. No significant differences were observed among different sampling periods. In addition, no differences were observed in damage index between point 1 and reference site (165.0 ± 43.5 vs. 163.8 ± 73.6, respectively; $p = 0.96$).

Table 1 Damage index (mean ± SD, in arbitrary unit) estimated by the comet assay using the hemolymph of *A. platensis* captured in point 1 of the Feitoria stream, Southern Brazil

Sampling period	Number of animals	Damage index
August 2012	6	152.0 ± 27.7
October 2012	7	131.3 ± 28.7
December 2012	9	165.0 ± 43.5
January 2013	8	107.3 ± 62.8
*p**		0.07

**p* value of the ANOVA test to compare differences among sampling periods

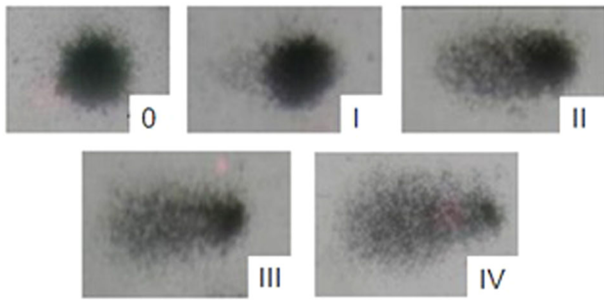

Fig. 3 DNA damage classes (0–IV) observed in the comet assay using the hemolymph of *A. platensis*

Discussion

Concerning the use of comet assay in field-collected crustaceans, Ternjej et al. (2014) reported significantly higher levels of DNA damage in *Gammarus balcanicus* individuals captured in an area under the influence of a gypsum mining in Croatia, when compared to animals captured in a reference location. Davanso et al. (2013), while investigating DNA damage in *Goniopsis cruentata* crabs captured in the Ceará River estuary (Brazil), also observed a significant decrease in DNA integrity in animals from an impacted area, in comparison with animals from a pristine environment. In the Antarctic region, Rocha et al. (2015) applied the comet assay to assess the damage to the DNA of hemocytes of the crustacean amphipods *Gondogeneia antarctica*. The damage to the DNA of animals captured in shallow waters near the fuel tanks and sewage treatment outflow was significantly higher than that of the control site. Faria et al. (2010), using the alkaline DNA precipitation assay, found that DNA damage in crayfish (*Procambarus clarkii*) was related to the level of pollution from different locations on the Ebro River in Spain. A limitation of the present study is that *Aegla platensis* have never previously been used in genotoxicological studies and the basal levels of DNA damage expected to be found in reference sites is unknown. The sampling carried out in a well-preserved site did not show differences compared to point 1. However, only one sampling is not enough to know the baseline levels of DNA damage of a species. This should be accessed in further studies. The lowest levels of hemocytes DNA damage found in *A. platensis* was 107.3 ± 62.8. In the studies in which DNA damage of crustacean has been assessed by visual scoring the baseline ranged from 106.1 ± 22.7 to 112.3 ± 10.7 in sperm of *Palaemon longirostris* (Erraud et al. 2019a), 55.8 ± 12.0 to 65.5 ± 9.3 in sperm of *Palaemon serratus* (Erraud et al. 2019b), and around 50.0 in the hemocytes of *Xiphopenaeus kroyeri* (da Silva Rocha et al. 2012). However, it is quite difficult to make inter-study comparison due to differences related to the sensitivity of the biological model, the comet assay protocol or the parameter used for expressing DNA damage (Esteves et al. 2020). It is, therefore, essential to define the values of the basal DNA damage for *A. platensis*.

Haemocytes are cells that can easily be collected. They are closely exposed to pollutants through their role in various defense mechanisms, and often chosen as single-cell suspensions in genotoxicity assessments using the Comet assay in invertebrates (Di Donato et al. 2016, 2020). In the present study, haemocytes of *A. platensis* were used in a comet assay for the first time to evaluate the feasibility of this species as bioindicator for genotoxic contamination. The chosen methodology for this study was considered adequate since the hemolymph sampling in the field allowed the animals to be released back in the stream. Hemolymph sampling, being non-lethal, has been recommended as a practical sampling method for animals that have been obtained from wild (Gruber et al. 2014; Jussila et al. 2015). Furthermore, sampling the animals in the field prevents a possible interference in the DNA damage rates caused by the stress conditions during transport of live animals to the laboratory. In situ studies have the advantages of incorporating many of the natural fluctuating environmental conditions and integrating toxicity mixtures naturally present in the field (Rodrigues and Pardal 2014).

The alkaline version of the Comet assay, which was used in this study, can detect a large array of DNA damage, such as double- and single-strand breaks, alkali-labile sites, and cross-links (Jha 2008; Dhawan et al. 2009; Glei et al. 2016). The importance of the comet assay is due to the relevance of understanding the effects of pollutant-induced genotoxicity and the risk imposed to the biota and surrounding human populations, especially due to the acknowledged link between DNA lesions and carcinogenicity (Martins and Costa 2015).

Taking the four sampling periods under consideration, the results obtained showed an intermediate level of DNA Damage Index (mean = 138.9 ± 40.6; in a range lying between 0 and 400 arbitrary units). The evaluation of DNA damage of *A. platensis* captured in different locations is necessary to determine the potential environmental risks of the site studied. In addition, the great observed interindividual variability in the DNA damage must be further assessed considering season, sex, body size, reproductive status, and other biotic and abiotic factors. This leads to the conclusion that further work is needed on the genotoxic responses of this species before it can be considered for biomonitoring studies.

Since the response of freshwater crustaceans to genotoxic agents appears to be different when compared to vertebrates, Di Donato et al. (2016) suggest that ecologically relevant crustaceans should be used more widely as potential bioindicators. In addition, there is increasing evidence on the pivotal role of aquatic invertebrates in the assessment of the impact of pollutants on the environment. Its potential use to replace fish bioassays, which offers

ethical advantages, has already been widely studied (Rodrigues and Pardal 2014).

The species used in this study has never previously been used in genotoxicological studies, and, despite the limitations that have already been mentioned, the comet assay on *A. platensis* proved to be a feasible and practical in situ approach for the genotoxic evaluation of freshwater ecosystems. There are more than 30,000 classified crustacean species worldwide, which differ in physiology, morphology, life-strategies, etc. and consequently also differ in both acute and chronic sensitivity to pollutants. Therefore, the interspecies comparisons in ecotoxicological studies presents shortcomings (Breitholtz et al. 2006). In this context, genotoxicity assessment should consider the genotoxic activity in ecologically relevant crustacean species.

Conclusion

The use of hemolymph as a source of cells for the comet assay was successfully attained, opening the possibility of a non-lethal and less invasive sampling method for *Aegla* as well as other crab species. The comet assay using *A. platensis* proved to be a feasible approach for monitoring of freshwater genotoxicity.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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Author contributions LBS designed the research; MRP and AG performed data collection and analysis; AG wrote the first draft; all authors reviewed the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Consent for publication All authors consent for publication.

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