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Managing of *Procambarus clarkii* by X-ray sterilisation of males: Cytological damage to gonads☆

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ABSTRACT

Procambarus clarkii is an invasive alien species spreading worldwide. It is therefore mandatory to find new methods to manage this species since traditional techniques are not sufficient for this purpose. The present study investigates gonad damage induced by different doses of ionising irradiation: 20, 40 and 60 Gy. Testis were analysed after 10 and 30 days by means of light, scanning and transmission electron microscopy. Control unirradiated testes present an acinar structure with a well-defined germinative cells maturation from the distal proliferative zone to the proximal stalk of the lobes whilst, in irradiated testes, induced apoptosis of germinative and accessory cells and a high level of vacuolisation inside the acini were identified, progressively increasing in accordance to Gy dosage and time after exposure. We determined the dose of 40 Gy as the best compromise: it causes an extensive damage to germinative tissues without affecting crayfish vitality, differing from 60 Gy. From an applicative point of view, this dose reduces the efforts, in terms of cost and time, for the application of SMRT.

1. Introduction

The red swamp crayfish *Procambarus clarkii* (Girard; 1852), native to the Southern United States and now present worldwide (Penn, 1954; Barbaresi and Gherardi, 2000 Yue et al., 2008), with the exception of Australia and Antarctica, represents a highly invasive and dangerous alien species. In Europe, it was first imported into Spain in 1972 (Ackefors, 1999), while in Italy it has been present in several northern and central areas of the Country since '90s (Gherardi et al., 1999). The Sterile Male Release Technique (SMRT) has been chosen as part of a strategy to control the spread of the red swamp crayfish in Friuli Venezia Giulia (Italy). This technique is based on the release into the environment of sterile males which are sexually active and able to compete with untreated males for mating partners. The SMRT technique has been deemed reliable because it exclusively acts on target species without interacting with existing biomes, whilst alsobeing safe for human health (Holdich et al., 1999; Lodge et al., 2006). Sterilization by radiation has already been used in some commercial crustaceans such as *Penaeus japonicus* (Sellars and Preston, 2005), *Palaemonetes pugio* (Rees, 1962) and *Macrobrachium rosenbergii* (Lee, 2000). However, no extensive investigations have been carried out on the protocols used in radiation treatments and the methods for quantifying histological and behavioral damage. A previous study on the sterilization of *P. clarkii* male by X-rays defines the methodological protocol for the irradiation and the evaluation of the damage induced within crayfish, and demonstrates that the dose of 20 Gy induces a reduction of 43% in the number of offspring, while sexual behaviour is not altered (Aquiloni et al., 2009). Therefore, using the same protocols, we have decided to test different doses of X-rays – 20 Gy, 40 Gy and 60 Gy – in comparison with a control group, to assess the cytological effects that higher doses of radiation generate on the testes of males of *P. clarkii*.

The male reproductive system in crustaceans is composed of testis and *vasa deferentia* that lead to the paired gonophores. In the *vasa deferentia*, spermatozoa merge into spermatophores, which are structures specialized in transferring spermatozoa to females' body and placing them in internal or external storage receptacles (Krol et al., 1992). The male reproductive system of *P. clarkii* has not yet been fully investigated. In fact, just one study has been car-

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ried out on this issue, namely that of Moses (1961a,b). In detail, this analysis focuses on the development of spermatozoa. The literature shows other studies generally concerning the reproductive system of decapods, such as species of the Astacidae (Erkan et al., 2009; Rotllant et al., 2012) and Cambaridae (Word and Hobbs, 1958; Moses, 1961a,b; Wielgus-Serafinska, 1973). Our work aims to describe the male reproductive system in wild *P. clarkii* from the control group, quantifying the effects of different radiation doses on testisin order to improve the applicability of the SMRT.

2. Materials and methods

2.1. Animal collection and housing conditions

About 400 adult crayfish were collected in May 2013 from Cascine di Tavola and Ombrone Lake (Tuscany, Italy) before the reproductive season. Once in the laboratory, they were kept, sexes apart, at a density of 15 m^2 in plastic tanks (80 × 60 × 60 cm) containing 48 L of still tap water and halved terracotta pots as shelters. For the entire period of the study (May 2013–October 2013), experimental individuals were maintained under a 12:12 light/dark cycle at room temperature (20 °C) and fed ad libitum with live *Calliphora* sp. larvae. Water was changed every two days. Hard-shelled crayfish with both claws and all appendages intact were selected and periodically tested for their responsiveness to sexual partners.

2.2. Irradiation of males

A total of 120 males were randomly divided into four groups of 30 males each: a control group (hereafter, C), in which males were subjected to the same manipulation of the other groups but not irradiated, and 3 treatment groups at 20, 40 and 60 Gy (hereafter, 20, 40 or 60), respectively, in which males were exposed at different radiation doses. The minimum dose of 20 Gy was chosen in accordance with Aquiloni et al. (2009) who was the first one to describe the effects of such a dose in the same species. The irradiation was carried out at the beginning of July at the Careggi Hospital (Florence, Italy). Specimens belonging to the same experimental group were treated together. During the irradiation, crayfish were maintained in a plastic tank (17 × 29 × 36 cm) with 10 L of tap water and covered with a sheet of Plexiglas (thickness: 2 cm). A clinical linear accelerator (Philips S175) with a 6 MeV electron beam was used to generate X-rays yielding 2 Gy min^{-1} at 100 cm from the target (40 × 30 cm), so that the treatment doses were achieved with different time exposures (10 min for the 20 Gy treatment, 20 min for the 40 Gy treatment and 30 min for the 60 Gy treatment). After the treatment, crayfish were kept isolated for two weeks in individual aquaria (25 × 20 × 20 cm), each containing a shelter (a halved terracotta pot), and were observed daily to assess possible alterations in their general activity.

2.3. Histological procedure

On the 10th day after the treatment, we dissected 5 males of the control group and 5 of the 20 Gy group, after being anesthetized with ice for one hour. The same operation was also repeated on the thirtieth day but we dissected 5 males of each group (C, 20 Gy, 40 Gy, 60 Gy). Dissections were used for gonad analyses. Testes were subsequently fixed in modified SPAFG solution (Ermak and Eakin, 1976) (0.8% paraformaldehyde, 2.5% glutaraldehyde and 7.5% saturated aqueous solution of picric acid in 0.1 M phosphate buffer saline, pH 7.4, with 1.5% sucrose). Samples for light microscopy and transmission electron microscopy (TEM) were post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in ethanol (50%, 70%, 95% and absolute) and propylene oxide, and finally embedded in Epon 812-Araldite mixture (Electron Microscopy Sciences, Fort Washington, PA). For optical microscopy, Pabisch TOP Ultra 150 was used to cut resin semi-thin sections (1 μm) which were stained with toluidine blue and examined with Olympus BX50; images were acquired with a digital Olympus E-P1 camera. The analysis of the images was performed with the open-source program ImageJ. For transmission electron microscopy, ultra-thin sections (120 nm) were cut with a Leica Ultracut UTC Ultratome, stained with uranyl acetate and lead citrate, and examined with a Philips EM201 electron microscope at 100 kV; images were acquired with a Veleta – 2k × 2k side-mounted TEM CCD Camera (Olympus, Germany) provided with an iTEM imaging platform and saved in TIF format. For scanning electon microscopy (SEM) analysis, the samples were dehydrated in a graded 50–100% ethanol series, sputter coated with gold in a Edwards S150A apparatus (Edwards High Vacuum, Crawley, West Sussex, United Kingdom), and examined with a Leica Stereoscan 430i scanning electron microscope (Leica Cambridge Ltd., Cambridge, United Kingdom). For the terminology of testis histology we used the one proposed by Hobbs et al. (2007).

2.4. Morphometric data and statistical analysis

For each group (control, 20 Gy at 10 days after irradiation, 20 Gy, 40 Gy and 60 Gy at 30 days after irradiation) major and minor diameters of 250 acini from sections at 5 different testes were measured. Subsequently, the average of two diameters was calculated. All statistical analysis were performed using R version 2.3.1 software (R Development Core Team 2011). The diameters of the acini of control and irradiated animals were checked for normality with the Shapiro–Wilk test and the homogeneity of variance among groups was checked with the Bartlett test. The differences were assessed by non-parametric statistics, Kruskal–Wallis test and pairwise comparisons using Wilcoxon test with Bonferroni correction, since the null hypothesis of the Shapiro–Wilk and/or the Bartlett test could not be rejected. The boxplot were drawn with the boxplot command. Measurements are expressed as mean ± standard error.

2.5. Ethical note

The experiments comply with the current laws of Italy, the Country in which they were done. No specific permits were required for the studies that did not involve endangered or protected species. Individuals were maintained in appropriate laboratory conditions to guarantee their welfare and responsiveness. After the experiments were completed, crayfish were sacrificed by hypothermia.

3. Results

P. clarkii males present a tri-lobed testis which is located in the cephalothorax, placed ventral to the pericardium. The mature testis has a pair of cephalic lobules, extending in the lateral cephalic region above the posteroventral lobes of the hepatopancreas, and a median caudal lobule located dorsal to the midgut. Lobes are connected through the fusion of their stalks from which emerge the paired *vasa deferentia* which extend caudolaterally over the dorsal surface of the caudal lobes of the hepatopancreas, then descending to the gonopores. In dorsal view, the right *vas deferens* is more developed than the left one, which appears thinner and atrophic. Each lobule has an acinar structure and resembles a bunch of grapes, where berries are its acini and the stalk corresponds to its protubules (distal) and collecting tubules (proximal) (for an exhaustive description of the testicular acinar structure of Cambaridae see Hobbs et al., 2007).

The SEM analysis shows that the testes of animals irradiated by 20 Gy, 40 Gy, 60 Gy are smaller than those of the control group. In addition, the medial-sagittal fractures of testicular lobes show a