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Original
Medium-term feasibility of the management of the invasive crayfish Procambarus clarkii with the sterile males release technique / Manfrin, C., Giglio, A., Pallavicini, L., Zampa, L., Vecchiet, L., Caputi, A., Chiandetti, C., Beorchia, A., Vidimari, R., Giulianini, P.G.. - In: PEST MANAGEMENT SCIENCE. - ISSN 1526-498X. - 77:5(2021), pp. 2494-2501. [10.1002/ps.6280]

Availability:
This version is available at: 11368/3004611 since: 2021-12-24T13:18:12Z
Publisher:

Published
DOI:10.1002/ps.6280
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12 October 2026

arTs Università degli Studi di Trieste
Archivio della ricerca – postprint

Medium-term feasibility of the management of the invasive crayfish *Procambarus clarkii* with the sterile males release technique

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Abstract

BACKGROUND: The sterile male release technique (SMRT) is a useful method applied for controlling invasive and pest species. However, the use of X-rays can lead to negative effects on the survival and health conditions of sterilized males.

RESULTS: This study was set up to evaluate the functional integrity of physiological, morphological and behavioural responses in males of the red swamp crayfish, *Procambarus clarkii* (Girard, 1852), exposed to a dose of 40 Gy of ionizing radiation. Concerning physiological responses, the results showed that the irradiation dose, tested at 5, 12, 28, 35, 65, 99, 132 and 193 days after treatment, has no effects on glycaemic and plasmatic total protein levels measured as biomarkers for general stress indexes. Nevertheless, the significant reduction of circulating haemocytes and the basal levels of phenoloxidase (PO) activities recorded in 40-Gy irradiated crayfishes indicate that the exposure shrinks their capability to mount a rapid non-specific response, and higher levels of plasmatic total PO activity indicate the ability to compensate and maintain an inducible response. Histological analyses performed at the end of the experiment showed no morphological damage in the testicular acini of irradiated males. Moreover, behavioural responses to two different water stimuli (vaporization and jet), measured at 15 and 45 days after the irradiation, were not modified in exposed crayfishes compared to the control group.

CONCLUSIONS: These results confirm the validity of SMRT on young males when the breeding season is less than 4 months but exposure to X-ray should be repeated at mid-breeding season when temperatures allow a longer breeding season.
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Keywords: *Procambarus clarkii*; X-ray; ionizing radiation; haemocytes; testis; habituation

1 INTRODUCTION

The red swamp crayfish, *Procambarus clarkii* (Girard, 1852), is an invasive alien species spread worldwide with significant economic and ecologic impacts.[1,2] It is listed among the invasive species of Union concern (EU Regulation n. 1143/2014) and DAISIE's '100 of the Worst' invasive species (http://www.europe-aliens.org) and, since 2015, it has been among the nine European invasive alien species impacting on more than four threatened species (IUCN Red List categories CR, EN, VU), as it affects five European native species.[3] A large number of previous studies investigated the life cycle, physiology and reproductive behaviour of *P. clarkii*, mainly to find new approaches to limit its diffusion in freshwater environments.[4,5]

Containment and management of highly invasive crayfish such as *P. clarkii* are issues that still require effective solutions. Several methods have been applied to control the spread of invasive crayfish,[4] but innovative techniques are still required, since none of the extensively applied approaches have so far given compelling results or are suitable for all the environments colonized by invasive crayfish.

Autocidal methods, such as the sterile male release technique (SMRT),[6,7] and the use of sex pheromone to interfere with crayfish reproduction[8] along with biocidal methods[9] are among the most intriguing and newest techniques applied for controlling invasive crayfish. The SMRT is based on the release into the environment of sterile males able to compete with untreated reproductive males for mating partners. This technique has already been successfully applied to sterilise insects for integrated pest management[10] and is known as the sterile insect technique (SIT).[11] The main parameter to achieve an optimal goal is the irradiating dose, which must be tightly tailored to ensure a high degree of sterility and low damage to somatic cells in order not to affect the reproductive behaviour of animals and ensure their competition for mating

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with wild-type males. The aspects to be taken into consideration for effective applicability of the SMRT are (i) the vitality of the males after irradiation, evaluating physiological and behavioural parameters, and (ii) the efficacy of the postirradiation damage of the testicular tissue that guarantees the effectiveness of the sterilization procedure. The dose needed for sterilization in arthropods can reach 300 Gy in the case of Lepidoptera.[11] Sterilization by radiation is already used in some commercial crustaceans, such as *Penaeus japonicus*,[12] *Palaemonetes pugio*,[13] and *Macrobrachium rosenbergii*,[14] with X-ray doses ranging from 10^{12} to 640 Gy.[15]

The SMRT was initially tested in the laboratory by Aquiloni *et al*.,[6] who followed up males of *P. clarkii* irradiated with 20 Gy at 1, 5, 10, 21, 30, 45 and 365 days after irradiation and compared the gonadosomatic index (GSI), seminiferous tubule length and number of pyknotic elements in histological sections. The irradiated males differed from controls for GSI and tubules length at 21, 30, 45 and 365 days after irradiation but significant differences in histological sections were recorded at 1, 5, 10, 21, 30 and 45 but not at 365 days after irradiation. The dose of 20 Gy did not alter the mating ability of *P. clarkii* males. Subsequently, in combination with intensive trapping, SMRT was proved to be particularly effective in closed waterbodies, such as at the lake of Casette Venchiaredo (Pordenone, Friuli Venezia Giulia, Italy), where the *P. clarkii* population was reduced by 87% after 2 years of activity.[7]

In a previous study,[16] extensive damage to the germinative tissues was observed in red swamp crayfish males that underwent 40 Gy of ionizing radiation after 30 days from the initial exposure without affecting crayfish vitality. In addition, short-term X-ray effects were also evaluated on circulating haemocytes of *P. clarkii*, after 20 days from irradiation of 40 Gy, leading a decrease of about 80% of circulating haemocytes in exposed animals and an increase in the percentage of semigranular and granular haemocytes.[17]

The detection of these short-term effects, following the application of the SMRT, paved the way to further investigate medium-term effects to determine the outcomes and time scale of X-ray exposure, and, more importantly, to analyse the survival, immune competence and gonadal damage in irradiated males in the medium-term to evaluate the applicability and potentiality of this technique in contexts where the breeding season is several months long.

Here medium-term effects of SMRT were evaluated in *P. clarkii* males after about 6 months (193 days) from the irradiation. Physiological, histological and behavioural parameters were considered to assess the state of irradiated animals. The glycemia and the plasmatic total proteins were measured as biomarkers for general stress assessment in crustaceans.[18] Total haemocyte counts (THCs) and phenoloxidase (PO) activities were evaluated as biomarkers for the immune competence in crustaceans.[19] The histological damage of gonadal tissues in the testis was analysed and compared with already reported information collected on a short-time scale.[16] Furthermore, the effects of X-ray were evaluated under behavioural aspects as well. We investigated whether a pervasive form of learning like habituation could be affected by X-ray irradiation. Habituation, the decrement to an irrelevant repeated stimulation that is not accounted for by sensory or motor fatigue, reveals how the nervous system learns to ignore innocuous stimuli for an efficient interaction with the environment.[20] It is a complex form of learning, and has a profound impact on other cognitive processes.[21,22] With this in mind, we assessed habituation and recovery of habituation in the crayfish to two different water stimuli, with the aim of evaluating the responsiveness of the animals and the integrity of the corresponding learning mechanism in two moments after the treatment. A disruption of either habituation or recovery would demonstrate that SMRT also affects crayfish cognitive functions.

Our work aimed to evaluate the survival and the health conditions of irradiated *P. clarkii* to improve our knowledge of the applicability of the SMRT in the field.

2 MATERIALS AND METHODS

2.1 Ethical notes

The experiments comply with the current laws of Italy, the country in which they were done. No specific permits are required for studies that do 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.

2.2 Animal collection and maintenance

Adult males (carapace length 48.69 ± 4.34 mm, $n = 29$, 19 exposed and 10 as control) were collected in February 2017 from the artificial canal inside the Bonifica del Brancolo (Brancolo's reclamation area, 45°46′ N, 13°30′ E, GO, Italy). In the laboratory, each animal was kept separated in pierced containers placed in the same glass tank (capacity 120 L) filled with tap water to expose them at the same conditions to avoid cannibalism. Prior to the start of the experiment they were acclimated to tank conditions for a week. For the entire period of the study, experimental individuals were maintained under a 12:12 h light/dark cycle at room temperature (20.07 ± 0.77 °C) and fed *ad libitum* twice a week with crayfish pellets (Sera Granular). Water was changed twice a week.

2.3 Irradiation of animals

Nineteen males were irradiated with a dose of 40 Gy (40Gy) in accordance with Piazza *et al*.[16] The crayfish were placed in a transparent polypropylene box with dimensions $19.5 \times 16.5 \times 9.5$ cm and thickness of 2 mm and exposed to X-rays in a clinical linear accelerator (Siemens Mevatron MX2) with a 4-MeV electron beam 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 20 min of exposure. The irradiation was carried out on 17 March 2017 at the Trieste Hospital (Fisica Sanitaria). A control group (CTRL) of 10 males was kept under the same conditions. Haemolymph was withdrawn after 5, 12, 28, 35, 65, 99, 132 and 193 days post treatment by mean of disposable syringes (1 mL, needle 25 G). Haemolymph withdrawal (about 200 μL/each animal) was performed through the dorsal abdominal artery. Plasma was isolated from haemocytes through centrifugation ($10\,000 \times g$ for 1 min) and stored at −20 °C for physiological analyses. At the end of the experiment, crayfish from both the CTRL and 40Gy groups were sacrificed and their testes dissected for histological analysis.

2.4 Glycaemia and total proteins

Glycaemia levels, as an indicator of generic stress, were measured using the Glucose Colorimetric Assay Kit (Cayman Chemical Company), following the manufacturer's instruction. Glycaemic levels were spectrophotometrically determined at 492 nm (Infinite 200 PRO NanoQuant, Tecan). The glucose concentration in each sample was calculated using a second-order polynomial curve, constructed on the basis of the absorbance and concentration measurements of the standards. Total proteins were evaluated

through the Nanodrop 2000 (Thermo Fisher Scientific) by analysing a dilution 1:5 of the initial plasma collected.

2.5 Total haemocyte count

THCs were performed in both CTRL and 40Gy groups at each time point after treatment, as specified in the previous section. Fifty microlitres of haemolymph were collected from each animal and haemocytes were counted using a Bürker haemocytometer (Brand GmbH).

2.6 Basal and total plasmatic phenoloxidase activities

Phenoloxidase (PO) activity was spectrophotometrically monitored as the formation of dopachrome from 3, 4-dihydroxy-L-phenylalanine (L-DOPA; Sigma-Aldrich).[23] For the determination of basal PO, 20 μL of plasma was taken and mixed with 180 μL of L-DOPA (3 mg mL^{-1} in Phosphate-buffered saline) in a microtiter plate. For the determination of total plasmatic PO (pPO) enzyme activity, 30 μL of plasma was added to 30 μL of sodium dodecyl sulfate (SDS, 1 mg mL^{-1}) that chemically activates PO from its inactive zymogen, prophenoloxidase (proPO).[14,24] The haemolymph–SDS mixture was incubated for 5 min at room temperature and 20 μL was mixed with 180 μL of L-DOPA (3 mg mL^{-1} in PBS) in a microtiter plate. The basal and total phenoloxidase enzyme activity at 20 °C was recorded at 492 nm for 60 min at 5 min intervals using a plate reader (Infinite 200 PRO NanoQuant, Tecan). All samples were assayed in duplicate. The enzyme activity was measured as the slope (absorbance *vs* time) of the reaction curve during the linear phase of the reaction. The slope of the reaction curve at V_{max} was plotted as absorbance per microlitre of plasma per minute.

2.7 Histological analysis

At the end of the experiment, testicular damage and spermatogenic series were evaluated in six specimens from both the 40Gy and CTRL groups by means of measurements of acini diameter in semithin sections of fixed testis. Gonads were fixed in modified SPAFG solution[25] (0.8% paraformaldehyde, 2.5% glutaraldehyde and 7.5% saturated aqueous solution of picric acid in 0.1 mol L^{-1} phosphate buffer saline, pH 7.4, with 1.5% sucrose). Samples for light microscopy 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 Embed 812-Araldite mixture (Electron Microscopy Sciences). A Pabisch TOP Ultra 150 was used to cut semi-thin resin sections (1 μm) which were stained with toluidine blue and examined with an Olympus BX50 (fluorescence microscope); images were acquired with a digital Olympus E-P1 camera. For each crayfish, the area of 180–386 acini from sections at five different levels of testes were measured. Subsequently, the equivalent diameters from the measured area were calculated. The analysis of the images was performed with the open-source program ImageJ.[26]

2.8 Experimental observations of habituation

2.8.1 Apparatus and stimuli

The experimental set-up consisted of a darkened rectangular glass tank equipped with a computer and a flat LCD screen placed within the maintenance room. The screen, placed outside one of the shorter walls of the tank, projected a white background that provided asymmetrical illumination within the tank. All around the tank, a black curtain prevented heterogeneous illumination and interference by the experimenter moving in the outer environment. On the opposite wall, two water sprayers were fixed to administer the stimuli. A further semicircular partition in poliplack confined the movements of the animal to the lighter part of the tank. The tank was empty to maximize crayfish reaction to the experimental stimuli. The behaviour was video-recorded from above at a constant speed of 29 fps to allow offline scoring. Two different water stimuli, each lasting 1 s, were used: vaporization of water (VAP) and a jet of water (JET). The two stimuli never directly addressed the body of the animal and were directed instead toward the wall tank with the white personal computer screen.

2.8.2 Procedure

On the day of testing, one crayfish at a time was left free to explore the rectangular tank for 10 min before starting with the stimuli presentation, which were administered when the crayfish reached the centre of the illuminated wall. If the crayfish did not start exploration of the environment in the next 20 min, it was discarded from the sample. The water stimulus was presented, and the crayfish was given about 2 min before the next trial occurred. This procedure was repeated five consecutive times with VAP (habituation) and one further time with JET (recovery of the response). The dependent measure was the intensity of a defensive response, comparable to the one we observed in previous works on *P. clarkii*[27,28] and operationalized as the number of frames per second to lift the body and to widely open the chelae up.

The original sample of crayfish was tested before the treatment started (T0, baseline) and then it was divided in two groups by randomly assigning 19 animals to the experimental group (40Gy) and 10 to the control group (CTRL). Each group was tested for habituation with the VAP and for response recovery with the JET in two moments. The first test occurred 15 days after the treatment (T1); the second test occurred 45 days after the treatment, i.e. 30 days after the first test (T2). All videos were scored blindly with respect to the group assignment of animals and a subset of 10% of the videos was analysed by a further independent observer, blind to the scope of the work and the group assignment of animals, to ascertain the interrater reliability.

2.9 Statistical analysis

Statistical analyses were performed using R version 4.0.0 software.[29] Differences between irradiated males and controls at a given time in THCs, glycaemia, plasmatic total proteins, basal and total PO activities and in testicular acini diameter were assessed by nonparametric statistics since the null hypothesis of the Shapiro–Wilk for normality and/or the Levene test for the homogeneity of variance among groups could not be rejected. To compare overall data for all the parameters measured between treated and control groups, the Mann–Whitney-Wilcoxon test was used. To compare between treated and control groups at different times of the experiment we used the Kruskal–Wallis test and *post hoc* Dunn pairwise comparisons with Bonferroni correction. Each single set of measurements at any time for the two groups was considered independent. The box-and-whiskers plots were drawn with the geom_boxplot command (ggplot2 R package).

The effects of irradiation on survival were assessed using the treatment as variable. Both exponential and Weibull parametric models with censoring were chosen because some individuals outlived the experiment.

The means of acinar equivalent diameters of three animals for each group were compared by the Mann–Whitney–Wilcoxon test.

All values are reported as mean ± standard error (SE) in the text. Differences were considered significant at P value ≤0.05.

Habituation was assessed in T0 with an overall Friedman test, and in T1 and T2 with a Mann–Whitney U test. Differences from the beginning to the end of the habituation session, generalization to the new stimulus and recovery of the response between consecutive testing sessions were assessed with Wilcoxon signed rank test. We reported the Bayes factor (BF) in support for the alternative hypothesis over the null hypothesis, showing that our study had enough power to detect all the effects, rather than being insensitive because of a too small sample, instead of simply having too few subjects to be sensitive.

3 RESULTS

3.1 X-ray effects on physiological markers

To evaluate differences between 40Gy irradiated males and CTRL ones at different time points, glycaemia (Fig. 1) and plasmatic total proteins (Fig. 2) were measured as generic stress indexes and THCs (Fig. 3) and basal and total plasmatic PO activities (Fig. 4(A),(B), respectively), were evaluated as immune competence indexes.

The glycaemic levels in the haemolymph were the most stable parameter, and they did not differ between the 40Gy (2.91 ± 0.24 mg dL^{-1}) and CTRL crayfish (3.23 ± 0.32 mg dL^{-1}), except for a few outliers in both groups. No significant difference was recorded between the CTRL and the 40Gy animals at different time points and within the CTRL and 40Gy groups among the different time points (Fig. 1).

The overall total plasmatic proteins measured in both CTRL (22.53 ± 3.36 mg mL^{-1}) and 40Gy crayfish (23.70 ± 2.88 mg mL^{-1}) did not show significant differences between groups at the different time points. Values were higher at 5, 12, 28 and 35 days post irradiation and lowered at 65, 99, 132 and 193 after the treatment (Fig. 2).

As far as immune competence was concerned, the THCs of CTRL animals (2 450 702 ± 136 616 haemocytes mL^{-1}) were not significantly different than those of 40Gy animals (1 017 956 ± 79 387 haemocytes mL^{-1}), taking into account all the values of the experiment. THCs at different times for the two groups showed significant differences (Kruskal–Wallis, $P < 0.001$). In particular, significant differences between CTRL and 40Gy crayfish were recorded at 12 (Dunn pairwise comparison, $P < 0.01$), 28 ($P < 0.05$) and 65 ($P < 0.05$) days after the treatment (Fig. 3).

The basal plasmatic PO activities of CTRL crayfishes (1.82e-3 ± 1.07e-4 abs μL^{-1} min^{-1}) were not significantly different than the 40Gy ones (1.54e-3 ± 7.08e-4 abs μL^{-1} min^{-1}) (Fig. 4(A)). No significant differences were recorded between CTRL and 40Gy crayfish at different days after the treatment (Fig. 4(A)).

The total plasmatic PO activities of CTRL crayfish (1.97e-2 ± 1.54e-3 abs μL^{-1} min^{-1}) were not significantly different than the 40Gy ones (1.41e-2 ± 1.13e-3 abs μL^{-1} min^{-1}) taking into account all the values of the experiment (Fig. 4(B)). No significant differences were recorded between CTRL and 40Gy crayfish at different days after the treatment (Fig. 4(B)).

3.2 Survival

Eight crayfishes survived 193 days, the end of the experiment, five from the 40Gy group and three from the CTRL group. Within the experiment period, 21 crayfish died with an average time of 44.21 ± 4.15 days for 40Gy irradiated males and 82.71 ± 19.47 days for CTRL males. The Kaplan–Meier survival analysis with constant risk indicated that the average death age for the 40Gy group was 113.14 days and for CTRL was 165.42 days. No significant statistical difference occurred in survival rate between the two experimental groups ($P = 0.33$) (Fig. 5).

3.3 Gonadal histology

After 193 days of irradiation, the acini showed good development and no sign of apoptotic features was present (Fig. 6(B)). Moreover, acini at all maturation stages were detectable in the testicular sections of 40Gy crayfish with a gradual maturation from distal

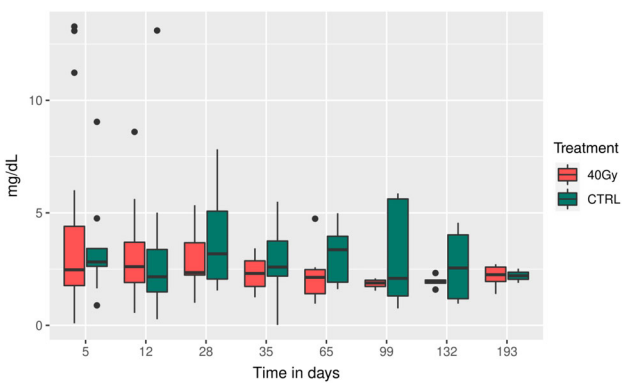

Figure 1. Glycaemia levels of irradiated (40Gy) and control (CTRL) crayfish at different time points.

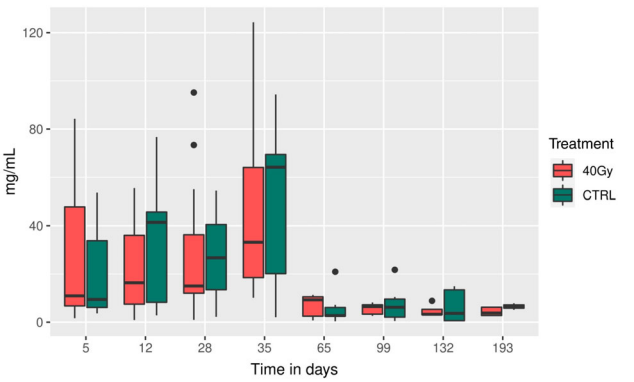

Figure 2. Total plasmatic proteins of irradiated (40Gy) and control (CTRL) crayfish at different time points.

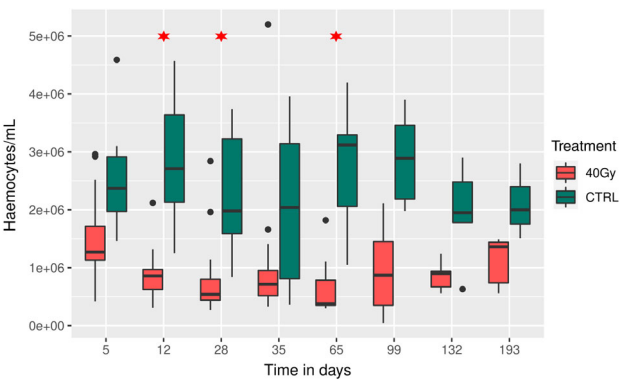

Figure 3. Boxplot of total haemocyte counts (THCs) of irradiated (40Gy) and control (CTRL) crayfish at different time points. Significant differences at a given time point between the two groups are indicated by asterisks (*).

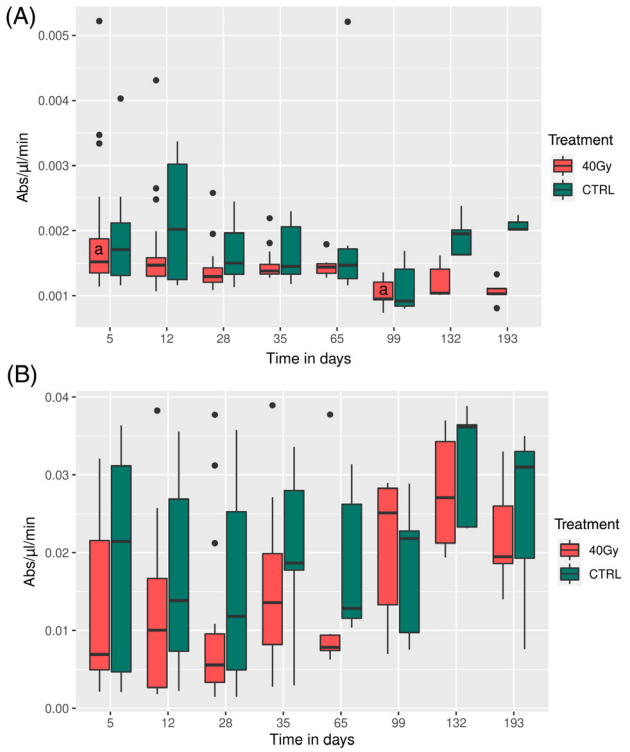

Figure 4. A boxplot of the basal (A) and total (B) plasmatic phenoloxidase (PO) activities of irradiated (40Gy) and control (CTRL) crayfish at different time points. The letter 'a' indicates a significant difference between 5 and 99 days after irradiation in 40Gy crayfish.

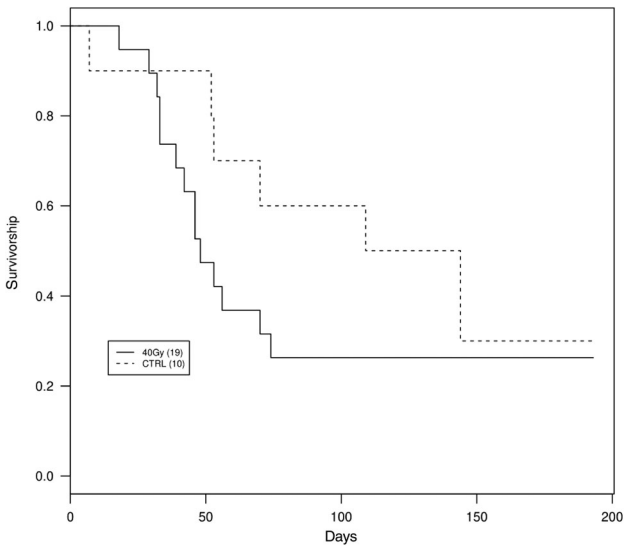

Figure 5. Kaplan–Meier survival distributions of irradiated (40Gy) and control (CTRL) crayfish by death event (days).

germinative zone like in CTRL testes (Fig. 6(A),(B)). Acini containing secondary spermatocytes in the meiotic metaphase and free spermatozoa were seen in section for the 40Gy testis (Fig. 6(B)). The equivalent diameters of the testicular acini of the CTRL crayfish (140.53 ± 1.39 µm) were larger than the 40Gy ones (118.09 ± 8.29 µm) (Fig. 6(C)), but no significant differences in acini size were observed.

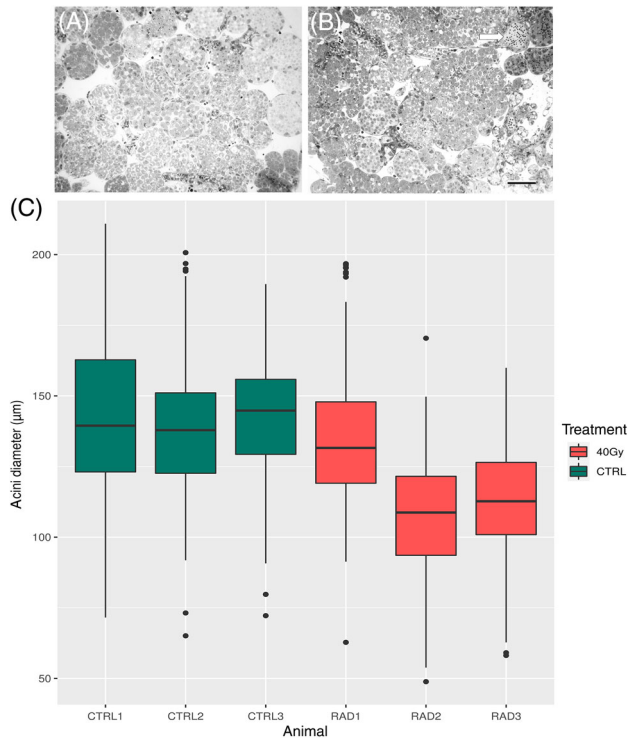

Figure 6. Semi-thin section (1 µm, toluidine blue) of testicular acini of (A) control (CTRL) and (B) irradiated (40Gy) males of *Procambarus clarkii*. (C) Boxplots of acinar equivalent diameters of three CTRL and three 40Gy crayfish. (B) The acini of 40Gy crayfish at different maturation stages are present and mature free spermatozoa in a collecting tubule are evident (white arrow). II SC, secondary spermatocytes. Calibration bar = 100 µm.

3.4 Habituation test

From the original sample of 29 crayfish, one animal was discarded because it did not provide any response in T0. The remaining 28 crayfish showed habituation to the VAP stimulus from the first to the fifth trial ($\chi^2_4 = 34.72$, $P < 0.001$, $BF_{10} = 2.695e+6$); the intensity of the reaction was lower in trial 5 than in trial 1 ($Z = 4.12$, $P < 0.001$, $BF_{10} = 1661$). As shown in Fig. 7(A), the crayfish also showed response specificity from the fifth to the sixth trial when the JET was presented ($Z = 1.94$, $P = 0.05$, $BF_{10} = 2.456$), indicating that during the first five trials the animals did not reduce the response due to motor fatigue because they continued in their exploratory activity while learning to ignore the irrelevant water stimulus, and that stress related to the unusual condition of being in a dry environment was not a factor at play.

Fifteen days after the x-ray irradiation, in T1, the group was made up of 23 crayfish (13 40Gy and 10 CTRL). By this time, crayfish of both groups showed spontaneous recovery since the response level in trial 1 of T1 was comparable to that in trial 1 before the treatment (40Gy: $Z = 1.73$, $P = 0.08$; CTRL: $Z = 1.07$, $P = 0.28$). Both 40Gy and CTRL crayfish performed the same during the five trials (T1: $P = 0.85$; T2: $P = 0.40$; T3: $P = 0.78$; T4: $P = 0.24$; T5: $P = 0.14$); however, a direct comparison between trial 1 and trial 5 resulted in a significant difference for CTRL ($Z = 2.6$, $P = 0.009$, $BF_{10} = 3.853$) but not 40Gy ($Z = 1.43$, $P = 0.15$), suggesting that habituation occurred in the CTRL group only. Both groups showed generalization of learning because none of them increased the response to the new stimulus at the sixth trial (trial

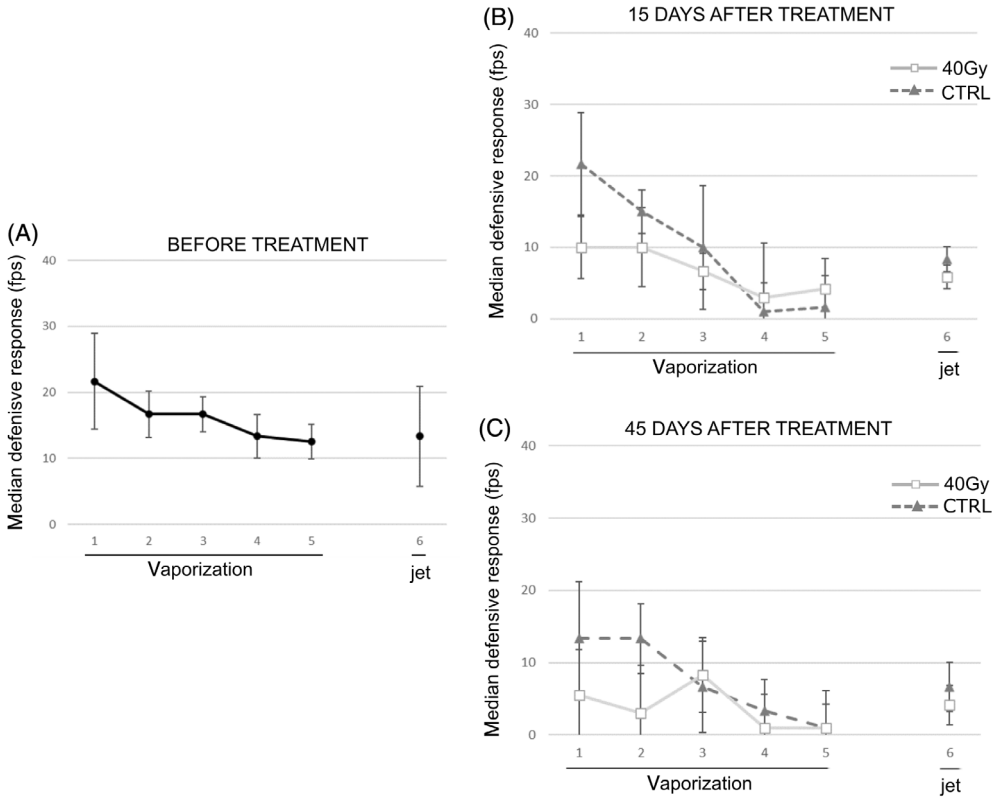

Figure 7. (A) The median intensity of the defensive response of the crayfish before assignment to experimental groups and treatment. After the repeated presentation of water vaporization (trials 1–5) crayfish alertness habituated and then recovered (trial 6) when a jet of water (novel stimulus) was presented. (B) The defensive response at T1, 15 days after treatment, for both irradiated (40Gy) and control (CTRL) crayfish. (C) The defensive response at T2, 45 days after the treatment, for both irradiated (40Gy) and control (CTRL) crayfish. Error bars represent the standard error of the median.

5 *vs* 6; 40Gy: Z = 1.16, $P = 0.25$; CTRL: W = 1.12, $P = 0.26$) as shown in Fig. 7(B). The analysis conducted after 30 days, at T2, on 19 animals (10 40Gy and 9 CTRL) revealed that all crayfish showed spontaneous recovery since the response level in trial 1 of T2 was comparable to that in trial 1 before the treatment (40Gy: Z = 0.76, $P = 0.45$; CTRL: Z = 1.01, $P = 0.31$). Both 40Gy and CTRL crayfish performed the same during trials 1, 2, 4, 5 (T1: $P = 0.49$; T2: $P = 0.20$; T4: $P = 0.77$; T5: $P = 0.33$) with the exception of trial 3 (T3: $P = 0.04$) in which 40Gy responded with higher intensity. A direct comparison between trial 1 and trial 5 resulted in a significant difference for both 40Gy and CTRL (40Gy: Z = 2.1, $P = 0.04$, $BF_{10} = 3.752$; CTRL: Z = 2.37, $P = 0.02$, $BF_{10} = 18.640$), indicating that habituation occurred for all crayfish. Both 40Gy and CTRL showed generalization of learning because none of them increased the response to the new stimulus at the sixth trial (5 *vs* 6; 40Gy: Z = 0.36, $P = 0.72$; CTRL: Z = 1.4, $P = 0.16$) as shown in Fig. 7(C).

4 DISCUSSION

In the view of the application of SMRT for containing *P. clarkii* populations we investigated the effects of X-ray exposure of 40Gy on the survival of males at the medium term (193 days) to determine if the X-ray treatment can hamper crayfish welfare. Despite the great importance of crustaceans in aquatic ecosystems, little is known about the biological effects of radiation on this taxon and the majority of papers concern the effect of ionizing radiation in the context of radiological protection of the environment (for a review see Fuller *et al.*[30]). A very different point of view evaluates the effects of radiation on the sterility of animals without compromising their viability, either for commercial purposes[12] or for the control of populations of harmful arthropods as, for example, against the vector of malaria *Anopheles arabiensis*.[31]

General indices of stress, as the glycemia and the plasmatic total proteins levels, were evaluated and did not show significant differences between control and irradiated crayfish in our study. The lower values of plasmatic proteins registered from day 65 in both control and irradiated crayfish are likely linked to laboratory conditions, since the decrease was recorded in both groups. As far as immune competence is concerned, the THCs of irradiated animals were significantly lower than those of the controls until 99 days after treatment, but not from 132 days till the end of the experiment. The decline in THC means that the antibacterial potential is probably reduced following X-ray exposure, though a partial cellular recovery of irradiated crayfishes was observed from 132 days onward. The reduction in THC could be an effect on the hematopoietic organs or due to an infiltration of the haemocytes into the tissues under stressful conditions.

A strong reduction in circulating haemocytes was observed in *P. clarkii* after 20 days from X-ray exposition of 40 Gy,[17] in *Crangon crangon* exposed to contaminated harbour dredgings, reared in mesocosms,[32] in crayfish haemocytes activated by lipopolysaccharides,[33] and correlated to the pesticide exposure with a dose close to LD_{50}.[34]

The humoral immunity measured by basal and total PO activities highlighted significant differences at 132 and 193 days after irradiation only for basal PO but not for total PO, which

maintained high values during all the experiments, demonstrating good humoral competence of irradiated crayfish that probably compensates for the lower cellular one. This is further supported by survival analyses, which reported a longer average time at death for control animals but no significant difference between the two groups during the experiment period. The survival at the end of the experiment was low for both the control and irradiated groups (<30%) but this is not surprising for adult males of a species with short lifespan maintained at a rather high temperature of 20 °C. The maximum longevity computed for *P. clarkii* in the largest Italian wetland (Central Italy) is 4 years.[35] The most surprising result of the present work is that after 193 days from X-ray treatment no significant differences in the acini diameters were recorded between irradiated and control crayfish, and the histology confirmed a recovered spermatogenesis and lack of signs of cellular degeneration. After 1 month from irradiation at the same dose extensive gonadal damage was described[15] and our results demonstrate that less than 193 days are sufficient to repair the irradiation damage or, at least, that the surviving staminal spermatogonia were able to restart a functional spermatogenesis. The possibility of compensating and recovering a few surviving cells has been demonstrated within the pancrustaceans on larvae of *Drosophila melanogaster* irradiated with 35 Gy. In this experiment, irradiation killed 60–75% of the cells of the imaginal discs, but the few surviving ones grown in adult indistinguishable in terms of dimensions between controls and irradiated.[36] In detail, the enzymatic phenoloxidase and prophenoloxidase activities, glycaemia and protein concentration in the haemolymph of the irradiated specimens did not significantly vary in comparison to the controls. However, the values of the total haemocyte count of the treated animals were always significantly lower than controls, indicating a decrease in cellular immunity activity certainly due to exposure to X-rays. A decrease in the total protein concentration and an increase of the PO activity since 65th day from the experiment for all the crayfishes could be likely linked to laboratory conditions or physiological seasonal variations.

Results on acini measurements did not show significant variations between the two groups, and this indicates a recovery by irradiated crayfish from the initial damage inflicted by the X-rays. Similarly, a dose of 35.08 Gy on larvae of *D. melanogaster* led to a 60–75% of the imaginal disc cells death, but the surviving larvae when grown became indistinguishable from other adults in terms of dimensions between controls and irradiated, indicating a replacement of dead cells and therefore a recovery.[36]

The resulting behavioural patterns were not significantly modified in exposed crayfish compared to the control group. Before the treatment, crayfish reduced the intensity of a behavioural response to an irrelevant stimulus (like a water vaporization) in five trials and showed specificity of this response when a different stimulus was presented (such as a jet of water). This result defines unequivocally the pattern of observed behaviour as habituation, disambiguating it from motor fatigue, and demonstrates that initially the two stimuli are perceived as different by the crayfish. Fifteen days after the treatment started, crayfish showed a spontaneous recovery of the response, which means that the memory of the stimulus was at least partially lost. By this time, a transient effect of the manipulation was measured in irradiated crayfish which maintained a constant response to the irrelevant stimulation. However, all crayfish did not show response specificity, demonstrating their ability to generalize learning across different stimuli. The performance by this time is likely showing that irradiated animals were still recovering from the treatment; indeed, the initial pattern of response was also completely re-established for irradiated crayfish when the stimuli were administered again after 30 days, meaning that learning was not altered by the treatment and generalization was still possible.[27,28]

In conclusion, our data confirm the validity of SMRT for managing *P. clarkii* in confined basins with the indication of irradiating large males a few days before the reproductive season considering life expectancy of about 80 days after treatment. Our findings document that, despite significant damage to the immune cell component, the humoral prophenoloxidase activities do not differ between irradiated and control animals. The results demonstrate for the first time that the survival of irradiated animals is not significantly different from control ones under experimental conditions 6 months after irradiation. The initial damage observed after 30 days,[16] together with recovery after about 6 months, does not make SMRT ineffective, but time factor must be taken into account. The time from X-ray exposition to release in target sites should not exceed 10 days when the reported gonadal damage is already present.[16] Thus, in light of the results presented here, the SMRT should be used only once at the beginning of the breeding season on young males when the breeding season is less than 4 months but should be repeated at mid-breeding season when temperatures permit a longer breeding season.

ACKNOWLEDGEMENTS

We are extremely grateful to Massimo Zanetti, Marco Presello, Romero Iacuzzo and Renzo Zanel (Ente Tutela Patrimonio Ittico, Friuli Venezia Giulia, Italy) for all sampling activities. The authors thank Dr Paolo Bertoncin (Servizio di Microscopia Elettronica, Università di Trieste) for his helpful suggestions and skilful technical support.

COMPETING INTERESTS

No competing interests declared.

FUNDING

No funds to be declared.

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