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Managing the invasive crayfish *Procambarus clarkii*: Is manual sterilisation the solution?

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Abstract

1. Management of invasive alien crayfish is challenging, as once established their eradication or control is difficult, even impossible in some areas. Sterile male release technique has been previously assessed in crayfish with encouraging results, however, the methods have not demonstrated the complete sterility of released competitive males. The present study explores whether manual removal of male gonopods, i.e. the appendages responsible for sperm transfer, as a sterilisation technique, might affect male competitiveness and sexual behaviour as well as reproductive potential in the red swamp crayfish *Procambarus clarkii*.
2. Under controlled laboratory conditions, we analysed the agonistic and sexual behaviour of 64 treated and 64 control males both coupled with a female in single pairs, and 40 treated and 40 control males together with 80 females in a natural-like social context.
3. Removal of gonopods partly altered sexual behaviour, affecting duration of copulation and competitiveness in treated males. However, male readiness to initiate sexual interaction with females was not affected by the treatment. Treated males needed to invest more in agonistic interactions with females to successfully dominate a female for the copulation to take place. Females coupled with treated males did not produce any offspring, compared to females coupled with control males. Treated males were able to regenerate removed gonopods, even if sometimes only partially or malformed. Females that mated with 11 treated males with regenerated gonopods did not produce any juveniles.
4. Although treated males managed to mate with females and impair their reproductive capability under the laboratory conditions, shorter copulation and elevated number of abdominal extensions were observed in treated couples. This indicates that males and/or females are able to sense the lack of gonopods and/or lack of the contact. We believe that female receptivity after an initial mating requires further investigation. Assessment of receptivity in an experimental setting where females are provided with refuges (e.g. burrows) would help us to elucidate whether there is a compensation for unsuccessful copulation. More research is needed on underlying biological mechanisms to better assess male competitiveness, technique effectiveness and limits of technique application.

KEYWORDS
biological control, gonopod, non-indigenous, pleopod, sterile male release technique

1 | INTRODUCTION

For centuries, inland aquatic ecosystems have been subject to biological invasions (Havel, Kovalenko, Thomaz, Amalfitano, & Kats, 2015; Tricarico, Junqueira, & Dudgeon, 2016). Today, with increasing globalisation, invasive alien species (IAS) are becoming an even greater threat to biodiversity of inland waters because of their capability to strongly affect the functions and services offered by these ecosystems across the globe (Aquiloni et al., 2010; Strayer, 2010). It is thus crucial to prevent new introductions and to control or eradicate already established IAS in order to halt and mitigate their negative impacts on ecosystems.

The red swamp crayfish, *Procambarus clarkii*, is considered one of the 100 worst invaders in Europe and is the most widely introduced crayfish today (Francesca Gherardi, 2006; Lodge et al., 2012; Souty-Grosset et al., 2016). It inhabits a wide variety of freshwater habitats, causing extremely negative impacts on ecosystems and ecosystem services such as reductions in valued edible native species, wide changes in ecological communities and increased costs to agriculture and water management (Souty-Grosset et al., 2016).

A number of control methods have been tested to control *P. clarkii* and to mitigate its multiple impacts (Francesca Gherardi, Aquiloni, Diéguez-Uribeondo, & Tricarico, 2011). Previous attempts to control invasive crayfish have shown that there is no definitive methodology for the control (Freeman, Turnbull, Yeomans, & Bean, 2010; Stebbing, Longshaw, & Scott, 2014). For this reason, investments in the development of innovative control techniques remain important. An ideal control technique should maximise control efficacy and minimise economic and environmental management costs (Caffrey et al., 2014).

Among available techniques, there is the sterile male release technique (SMRT), a highly species-specific and environmentally safe technique, that has successfully been applied in insects (Klassen & Curtis, 2005) and sea lamprey (Twohey et al., 2003). Undoubtedly most successful SMRT programme resulted with eradication of the New World screwworm *Cochliomyia hominivorax* in the USA, Mexico, and Central America (Klassen & Curtis, 2005). Additional advantages of SMRT are that the method ensures effective control even at low IAS densities and it could easily be used in integrated pest management. In the traditional form of SMRT males are sterilised by a radiation source in the laboratory and then released in nature (Stebbing et al., 2014). The sterile males then mate with wild females, thus impairing their reproductive capability. To be efficient, treated males should be able to compete with untreated males for mating and display behaviour typical of untreated males (Whitten & Mahon, 2005). The potential of X-ray irradiation as SMRT for controlling population of *P. clarkii* has been assessed (Aquiloni et al., 2009; Aquiloni & Zanetti, 2014; Duse, 2015). Using a dose of 20 and 40 Gy, up to 57% sterility was achieved without behavioural changes in treated males. A higher dose was tested (60 Gy), but it caused an alteration of mating behaviour of treated males, making it not recommendable for application in the field.

Stebbing et al. (2014) suggested manual removal of the gonopods, specialised male appendages used in reproduction to facilitate sperm transfer, as a form of sterilisation. Based on preliminary experiments with signal crayfish *(Pacifastacus leniusculus)* the authors concluded that this technique could achieve almost 100% male sterilisation, without effect on male competitiveness within the population or to find a mate. Thus far, the potential of this manual sterilisation technique has not been assessed in *P. clarkii*. The main concern of control programmes that integrate SMRT is that released sterile males successfully compete with the wild males, are able to find a mate, compete for it and copulate. Careful assessment of the males' competitiveness through behavioural experiments is thus crucial to evaluate efficacy of treatments.

The main aim of the present study was to assess the potential of manual sterilisation as SMRT against *P. clarkii*. For the sterilisation technique to be effective, it should not affect the competitiveness of treated males and should lead to a decrease in female reproductive output. Previous research showed that a sterilised male may be less competitive compared to a wild male, therefore it may mate less frequently, or with fewer females, or for a shorter duration; or it may take more time, compared to control males, to start mating (Calkins & Parker, 2005; Mazza et al., 2016). We hypothesised that manual sterilisation treatment could produce successful sterile males (1) without affecting males' ability to compete within the population and to mate normally (2). Manual removal of gonopods in *P. clarkii* will hinder the sperm transfer during the copulation act resulting with no eggs being fertilised and no juveniles being produced (3). To examine hypothesis (2) we compared precopulatory and copulatory behaviour of control and manually sterilised males, exposed to females. Hypotheses (1) and (3) were examined by assessment of reproductive output data. Data on moulting and gonopod regeneration were also evaluated to get an indication of potential longevity of the sterilisation treatment.

2 | METHODS

2.1 | Collection of animals and holding conditions

To eliminate any factor that could induce an obvious bias to our experiments (e.g. mutilations, moult stage), only sexually mature males (Form I; Huner, Lindqvist, & Könönen, 1988) and mature females in good condition (no mutilations or visible diseases, symmetric chelae) were used. A total of 504 crayfish were selected from those collected using baited traps from Sibolla lake and Ramone marsh (Tuscany, Italy) in April and May 2016 and 2017, before the onset

of reproduction (210 males and 182 females in 2016; 79 males and 33 females in 2017). Once the sex was determined in the laboratory, crayfish were separated by sex in PVC tanks, provided with refuges and maintained in a natural light/dark cycle at room temperature (28°C), and fed three times a week *ad libitum* with carrots. Water was changed weekly. Only hard-shelled, intact individuals were used for the experiments.

Prior to experiments the cephalothorax length (from the tip of the rostrum to the posterior edge of the carapace), and the width and length of both chelae were measured to the nearest 0.1 mm using a Vernier calliper. After 3 weeks of acclimation, crayfish were controlled for their sexual responsiveness by observing the behaviour of temporary couples, randomly composed by a male and female. Following Aquiloni and Gherardi (2008), if a male tried to turn the female over for copulation, during 30 min of observation, the male was defined as sexually responsive, and then separated from female before copulation took place. Overall, 208 sexually responsive males were paired with 208 females (80 males and 80 females for Experiment 1—Round 1 in 2016; 80 males and 80 females for Experiment 2 in 2016; and 48 males and 48 females for Experiment 1—Round 2 in 2017). The selected crayfish had a mean carapace length (±*SE*) of 45.39 ± 0.3 mm for males, and of 44.94 ± 0.3 mm for females. They were individually marked on their carapace with a waterproof nontoxic paint and kept in isolation in opaque plastic aquaria (24 × 14×15 cm) for at least 2 weeks, which is sufficient time to reset any previous social experience (Aquiloni, Gonçalves, Inghilesi, & Gherardi, 2012).

2.2 | Experimental design

Following the technique of Stebbing et al. (2014), half of the males were randomly subjected to removal of gonopods (treated males) and half were left intact (control males) but subject to similar manipulation as treated males without cutting gonopods. Gonopods were manually removed with scissors 48 hr before the experiment took place. Preliminary observations showed that treated and control males behaved naturally, and that 48 hr were enough for the animals to recover from the treatment.

2.2.1 | Experiment 1: single pair

Round 1

In June 2016, 40 treated (*T*) and 40 control (*C*) males were individually paired with one female each. Following Aquiloni et al. (2012), each pair was kept in an experimental container (a circular opaque PVC container, d = 30 cm, h = 17 cm) initially separated by an opaque PVC divider for 5 min to acclimatise. The experiment started with the removal of the divider and consisted of video-recording crayfish behaviour for 30 min for subsequent analysis. One observer, blinded to the treatment being watched, analysed video-tapes recording the following parameters: (1) latency time (i.e. the time elapsed between the removal of the separator and the moment in which first interaction occurs); (2) duration and type of interaction (i.e. sexual interactions: attempts, copulations; agonistic interactions: threats, weak and strong interactions); (3) abdominal (tail) movements: during copulation, male abdominal extensions were noticed in some couples and counted to compare them between control and treatment. An abdominal movement index (number of abdominal extensions per duration of copulation in minutes) was calculated for subsequent comparisons of (4) who initiated and who ended the interaction.

Following Gherardi & Daniels (2003) and Gherardi & Pieraccini (2004), agonistic interaction was classified as a simple threat (i.e. when chelipeds were raised above the plane of the carapace), weak contacts (i.e. simple touch, gentle pushing, and antenna taps) and strong contacts (i.e. crayfish exchanging chela strikes, intensively pushing each other, or interlocking their chelae). Any male attempt to grasp the female and turn her over was accounted as an attempt for copulation, while copulation started when the male turned the female holding it by the claws and when they were in copulation position (ventral parts in contact). Since our focus was to evaluate the agonistic and sexual competitiveness, data for agonistic interactions were separated by intensity, as high intensity interactions (strong contacts) and low intensity interactions (threats + weak contacts), following Gherardi & Daniels (2003), while attempts and copulations were assessed together as sexual behaviour. The majority of male–female interactions prior to copulation fell into low intensity agonistic interactions.

After the experiment, each couple was kept in the same aquarium for five consecutive days, a time sufficient for mating. Then, each female was isolated in an individual aquarium (24 × 14 × 15 cm) to allow spawning and hatching. After spawning, the number of eggs laid by each female was assessed by visual counts. The aborted eggs were counted and removed from the aquarium to prevent infection. The number of offspring was also recorded as soon as hatching took place. Treated males were monitored after the experimentation to assess moulting and gonopod regeneration.

Round 2

All females were maintained in the laboratory until the subsequent year: females that moulted once after copulation in 2016 were considered virgins and taken into consideration for the new run in 2017. Although female decapods show high variation in duration and capacity to store sperm, *P. clarkii* females are able to store sperm until they moult (Conde & Domínguez, 2015), when the *annulus ventralis* or sperm receptacle is shed along with its content. Besides females from Round 1, an additional 79 males and 33 females were collected from the field in May 2017. New collected females, that moulted in the laboratory and whose exoskeletons were hardened in the time of preparation for the experiment, were considered when selecting females for Round 2. All animals were subject to the identical procedures as in 2016. For the new round of experiments, we also selected 24 control males and 24 treated males, all collected in 2017. Selected males were paired with one female each. Reproductive output was assessed as in Round 1. To better assess behaviour, we prolonged observation time to 1 hour.

To test functionality of regenerated gonopods of treated males from Round 1, the remaining virgin females were size-matched with sexually responsive treated males with regenerated gonopods from Round 1 (n = 11). Treated and control males were also kept after experiment to assess moulting and gonopod regeneration.

From 80 females used in the Round 1, three died during the experiment (two *C*, one *T*) and an additional 15 died before the end of the hatching season (eight *C* and seven *T*) and most of them, except for the females that died in the experiment, had laid and lost eggs or had eggs hatching prior to deceasing. From 48 females used in Round 2, three *C* and three *T* females died during the experiment and an additional 6 in weeks after experiment (two *C* and four *T*). None of these females had eggs hatching prior to their decease. From 11 females paired with treated males which regenerated gonopods from Round 1, three died in 48 hr after experiment.

2.2.2 | Experiment 2: social context

Following Aquiloni & Zanetti (2014), in July 2016 ten groups of a balanced sex ratio, with 16 individuals (four treated males + four control males + eight females of similar size: maximum difference in cephalothorax length: 5–6%; width of both chelae: 2%; length of both chelae: 6%) were observed for 60 min interacting in circular arenas (d = 100 cm, h = 35 cm), containing 40 L of water, without shelters, with a population density comparable to that in the wild (20 individuals/m^2) (Gherardi et al., 1999). The 10 groups were video recorded using a digital camera (Sony HDR-CX240E). One observer, blinded to the treatment being watched, analysed video recordings for the following parameters: (1) duration and type of interaction (sexual and agonistic interactions; see above for definitions); (2) initiator, receiver, and interrupter (if interruption occurred) of each interaction. Interruptions indicated any interactions between couples that were interrupted by the involvement of a third animal. Treated males were maintained after experiment to assess gonopod regeneration.

2.3 | Statistical analyses

Data were first tested for normality and homogeneity of variance using the using the Kolmogorov–Smirnov and Levene tests, respectively, and transformed when necessary and possible. Since many data sets did not meet the assumptions for parametric tests, even after transformation, nonparametric tests were used. Statistical analyses were performed with SPSS version 21.0 (IBM SPSS Inc. 2016) and R version 3.3 (R Core Team 2016). The level of significance under which the null hypothesis was rejected is $\alpha = 0.05$.

2.3.1 | Experiment 1: single pair

The Mann–Whitney test (statistic: *U*) or Student *t* test (statistic: *t*) were used to compare control (*C*) and treatment (*T*) pairs. Kruskal–Wallis test (statistic: *H*) followed by post hoc test was applied for comparison among control, treated and regenerated males. The parametric data were reported in the text and figures as a mean value ± standard error while for nonparametric data median values and first and third interquartile were given.

Generalised linear models with Poisson regression and log link function (statistic: Z) were used to compare the proportion of sexual interaction in total interaction initiated by each male between control and treatment and Round 1 and 2, as the experimental time was different between Round 1 (30 min) and Round 2 (60 min). The number of total interactions and number of males initiated male-female sexual interactions was considered and used to calculate the proportion of sexual interactions in total interactions initiated by each male.

2.3.2 | Experiment 2: social context

NETDRAW within UCINET (Borgatti, 2002) was used for visualising matrices of all the types of interactions. Matrices were composed by the number of interactions (initiated/received) per individual. We selected male emission of social interactions, which is an individual based measure, for closer assessment of competitiveness and we focused on the parameters that directly indicate competitiveness, readiness to initiate: (1) male to male agonistic interaction; (2) male–female sexual interaction (attempts and copulation); (3) male–female agonistic interaction; and (4) numbers of female partners males attempted to copulate and/or copulated with. Interaction interruptions caused by males were not considered here as indicators of competitiveness because they represented only 8.67% of the total interactions, and there was no significant difference in frequency of interruptions initiated by *C* or *T* males (U = 464.5, n = 62, p = 0.826). Data on general activity of males, represented as total number of social interactions initiated by each male and numbers of sexual and agonistic interactions initiated by the same male, were used to evaluate differences in behaviours between the control and treated males. Zero inflated generalised linear model with Poisson regression and log link functions was used (statistic: W), with group as a random effect and number of each interaction per type and total number of interactions as dependent variables.

A further evaluation of treated male competitiveness was performed by computing the isolation index, defined as ISI = (WW - SW)/(WW/SW) (modified from Calkins & Parker, (2005) where WW means wild males mated with wild females and SW means sterilised males mated with wild females). This index ranges from –1 (complete negative assortative mating, i.e. in our case, all females mated with treated males) through 0 (random mating) to 1 (complete positive assortative mating, i.e. all females mated with control males). An ISI above 0.5 is considered a cause of concern as it suggests that treated males are not effective in competing with control males for females (Calkins & Parker, 2005).

TABLE 1 Comparison of number (*n*) and duration (s) of total interactions and duration (s) of different types of interactions between control (*C*) and treatment (*T*) pairs of *Procambarus clarkii* in *Round 1* (2016) and *Round 2* (2017) using Mann–Whitney test (*U*) or Student *t* test. The table reports medians (with first and third interquartile) for nonparametric data and mean values ± standard error for parametric data; significant *p* values are in bold.

	Round 1: 2016					Round 2: 2017				
Parameter	***U/t***	***n/df***	***p***	***C***	***T***	***U/t***	***n/df***	***p***	***C***	***T***
Total interactions (s)	−0.419	78	0.677	695.53 ± 67.64	T: 658.18 ± 58.16	−0.676	46	0.503	1622.17 ± 205.10	1791.92 ± 145.07
Latency (s)	799.5	80	0.996	141.5 (49.3–505.3)	156.5 (43.3–403.8)	213.5	48	0.124	197 (83.3–299.8)	131.5 (23–246.8)
Attempt (s)	−0.274	29	0.786	30.5 ± 6.5	33.29 ± 7.8	40	21	0.314	38 (22–54)	67.5 (23–114.25)
Copulation (s)	1.403	14	0.182	653 ± 167.6	366.4 ± 81.2	3.147	11	**0.009**	2055 ± 458.1	475.8 ± 68
Low intensity agonistic (s)	53.7	75	0.706	562.3 ± 53.7	591.3 ± 54.4	184	48	**0.032**	600 (398.5–1225.5)	1285 (534.8–2006.8)
High intensity agonistic (s)	40	23	0.118	3 (1–9)	11 (2–19.5)	128	33	0.8	261 (24–426)	191.5 (60.5–624.5)
Total interaction (*n*)	661.5	80	0.182	13.5 (9.25–21.75)	18.5 (11–25.5)	173.5	48	**0.018**	22 17.75–26.75)	27.5 (23–37)
Abdominal movement index (*n*/min)	8.5	14	**0.015**	0.21 (0.11–0.7)	2.02 (1.54–2.60)	9	11	**0.004**	0.18 (0.1–0.78)	1.97 (1.37–2.59)

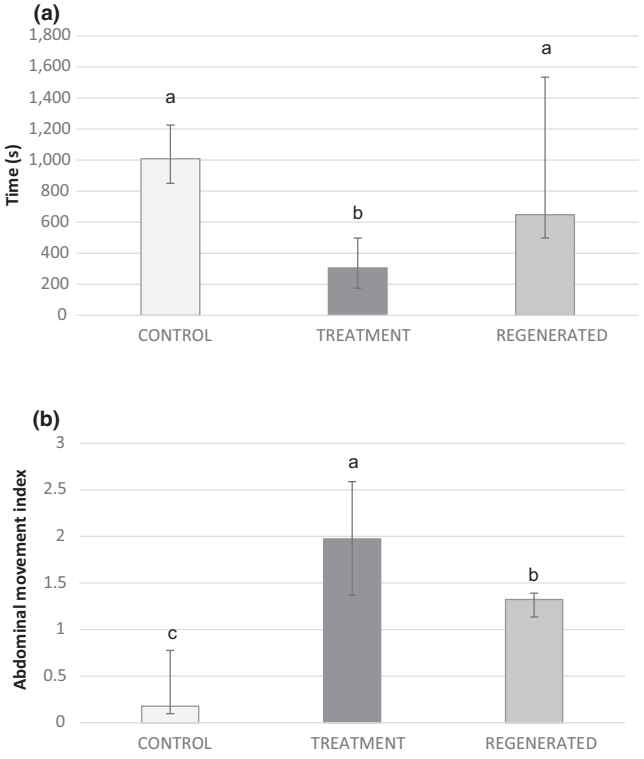

FIGURE 1 Comparison of copulation duration (a), and abdominal movement index (b), between control and treatment males of *Procambarus clarkii* from *Round 2* and males with regenerated gonopods. Letters over bars indicate post hoc hierarchy

3 | RESULTS

3.1 | Male behaviour

In Round 1 of Experiment 1 (2016), control and treatment pairs did not show any significant difference for duration of total interactions, duration of latency or number of interactions (Table 1). Likewise, the treated and control pairs did not differ in terms of duration of considered interaction types (Table 1). Frequency of males attempting at least one copulation ($U = 761.5$, $n = 80$, $p = 0.664$; out of 16 males attempting copulation in 40 *C* couples, 13 males attempted once, two twice, and one three times; while out of 15 males in 40 *T* couples, 12 males attended once, three twice, and none three times), and frequency of copulation ($U = 760{,}0$, $n = 80$, $p = 0.579$: $C = 9$, $T = 7$, all males copulated only once) showed also to be equal between control and treatment. Treated males showed a higher abdominal movement index compared to control ones (Table 1).

In Round 2 of Experiment 1 (2017), control and treatment showed no significant difference for most behavioural variables, such as duration of total interactions, latency, attempts, and high intensity agonistic interactions (Table 1). The number of interactions, by contrast, showed significant difference between *C* and *T*, with more interactions observed in treatment couples (Table 1). In treatment couples, low intensity agonistic interactions lasted longer, while duration of copulation was shorter compared to control pairs (Table 1). Neither frequency of males attempting copulation ($U = 278.5$, $n = 48$, $p = 0.827$; from

11 males attempting copulation in 24 *C* couples, six males attempted once, three twice, and two three times; from 10 males in 24 *T* couples, five males attempted once, three twice, and two three times), nor frequency of copulation (U = 271.0, n = 48, p = 0.653; from seven males copulating in 24 *C* couples, three males copulated once and four twice; from six males in 24 *T* couples, four males copulated once, two twice) showed significant difference between *C* and *T*. In total, 11 females copulated with control males and eight females copulated with treated males during video recording. Similarly to 2016, the abdominal movement index showed significantly different values between control and treated males (Table 1).

There was no significant difference in the proportion of sexual interaction initiated by control and treated males in both Round 1 and Round 2 (U = 759.0, p = 0.639 for Round 1, and U = 260.5, p = 0.532 for Round 2). When both Round 1 and Round 2 were compared for the proportion of sexual interaction in total interactions with a Poisson regression, there was a significant difference between the rounds (Z = 3.050, Pr(>|z|)=0.002), but not between *C* and *T* (Z = −1.793, Pr(>|z|) = 0.073).

Behaviour of males with regenerated gonopods differed from behaviour of treated and control males (Figure 1). Duration of copulation was equivalent to control males but significantly longer than in treated males (H = 9.44, df = 2, p = 0.009; control = regenerated>treated). Mean abdominal movement index was significantly different than both control and treatment (H = 13.75, df = 2, p = 0.0014; treated> regenerated>control).

In Experiment 2 from a total of 80 males across all 10 groups, only 50% of males were involved in sexual interactions and only 17.5% copulated during the video recording. Only 85 attempts, 50 attempts initiated by 24 control males and 35 initiated by 16 treated males, and 14 copulations, eight initiated by eight control males and six by six treated, were recorded. All males participated in agonistic interactions.

Generalised linear models showed significant difference only for the total interaction among treated and control males and readiness to start low intensity agonistic male to female interaction (Table 2). Readiness to attempt copulation with females or males, and number of copulations, did not differ between *C* and *T* (Table 2). Heterosexual attempts accounted for 86% of all male-initiated attempts (*C*: 91%; *T*: 80%). Neither frequency of males attempting copulation with females (U = 189.5, n = 40, p = 0.942) nor the number of females/males attempted copulation with (U = 192.0, n = 40, p > 0.999) showed significant difference between *C* and *T* (Table 3). Treated males showed somewhat smaller values in frequency of attempts and number of the partners. While 24 control males attempted copulation 50 times with altogether 44 females, only 16 treated males attempted copulation 35 times with a total of 28 females.

Isolation index, with the value of 0.1428, confirmed random mating. Control males mated eight times, with only one female each, while treated males mated six times, also with one female each. From 14 females who mated, 13 mated once and only one female mated twice, first with a treated and then with a control male. Duration of copulation in treated males again was significantly shorter (t = 4.410, df = 12, p = 0.001; *C*: 660 ± 64.74 and *T*: 299 ± 36.61). Homosexual attempts accounted for 14% of all attempts initiated by males (*C*: 9%; *T*:20%). No difference between *C* and *T* was showed for the frequency of males attempting copulation with males (U = 9, n = 10, p = 0.453).

3.2 | Reproductive output

In Round 1 of Experiment 1, no significant differences in the number of females having eggs and juveniles were observed between control and treatment pairs (eggs: χ^2 = 2.360, n = 76: *C*: 34, *T*: 32, p = 0.193; juveniles: χ2 = 0.059, n = 66, p = 0.495; Table 4).

From 37 females observed in Round 2 of Experiment 1, 14 out of 21 from control and 4 of 16 from treatment pairs had extruded eggs (χ^2 = 6.311, n = 37, p = 0.020; Table 4) and only 5 females from control group had juveniles. From 11 females paired with treated males having regenerated gonopods from Round 1, seven mated during video recording (one three times, four twice and two once). From eight surviving females, only one extruded and then lost eggs.

3.3 | Moulting and gonopod regeneration

By April 2018, 47 males from Round 1 never moulted, 21 moulted once, 11 moulted twice and only one three times (Figure 2a). The majority of moulted males (76.5 %, 26 males) regenerated all four gonopods (Figure 2b). In some cases, regenerated appendages were different in appearance from initial gonopods or gonopods of control males (Figure 3).

In Round 2, by April 2018 no treated male moulted, while altogether nine control males moulted, proving a significant difference in moulting frequency between control and treatment males (χ^2 = 14.378, n = 43, p < 0.05).

4 | DISCUSSION

The present work had the purpose of investigating the potential of manual removal of male gonopods as a sterilisation technique. During our evaluation we found that male crayfish were successfully sterilised (hypothesis 1) and that the technique was efficient in reducing sperm transfer, fertilisation and juvenile production (hypothesis 3). The results clearly showed that treated males were less competitive and did not mated normally (hypothesis 2).

4.1 | Changes in male precopulatory behaviour

Red swamp crayfish display a sequence of behaviours during male–female encounters prior to copulation, as described by Ameyaw-Akumfi (1981).On the first encounter chelae contacts are observed and short fights may also arise, finishing with male dropping its chelae in *refusal* to fight in majority of cases, while females that continue aggressive attacks have to be defeated by the male, in order to

TABLE 2 Comparison of number of total interaction and of different types of interactions between control (*C*) and treated (*T*) males of *Procambarus clarkii* using generalised linear models with Poisson regression. The table reports estimates and standard errors, Wald χ^2 values and Pr(>χ^2) values associated with those estimates. Median (with first and third interquartile) for nonparametric data is also presented for *C* and *T*; significant *p* values are in bold

	C versus *T*			
Parameter	**W**	**Pr(>χ^2)**	***C***	***T***
Total interaction (*n*)	515.3	**2.20E-16**	24.5 (13–40)	28 (15.3–43.5)
Heterosexual attempt (*n*)	0.254	0.615	1 (0–2)	0 (0–1)
Homosexual attempt (*n*)	0.8146	0.367	0 (0–0)	0 (0–0)
Copulation (*n*)	0.284	0.594	0 (0–0)	0 (0–0)
Low intensity agonistic male to male (*n*)	0.204	0.651	6 (2–10)	6 (3–11.8)
Low intensity agonistic male to female (*n*)	6.004	**0.014**	14.5 (6–22.8)	14 (8–24.8)
High intensity agonistic male to male (*n*)	0.18	0.672	1 (0–3)	2 (1–4)
High intensity agonistic male to female (*n*)	0.727	0.394	1 (0–2.8)	1 (0–2)

TABLE 3 Comparison of number of total heterosexual attempts and female partners between control (*C*) and treated (*T*) males of *Procambarus clarkii*

Attempts (*n*)	***C***	***T***	**Female partners (*n*)**	***C***	***T***
1	10	7	1	12	8
2	7	4	2	7	4
3	4	2	3	3	4
4	2	2	4	1	0
5	0	0	5	1	0
6	1	1	6	0	0
Total	24	16	Total	24	16

display non-aggressive postures on the next encounters. Later on, the male moves closer to the female in resting position, displaying movements of appendages for grooming, making antennule-to-antennule contacts. Next, the male may turn sideways, displaying his side presentation and, if the female remains passive, he will begin to mount her and later tries to turn her into the copulating position. Even relatively minor changes in the precopulatory and copulatory behaviour could reduce the male chances for successful copulation. Precopulatory behaviour has several functions in the animal world. It can reduce aggressive tendencies in either of the mates—the appeasement hypothesis—so that male and female can come close together and copulate; or it can favour sexual activity in individuals initially not interested in this activity–the arousal hypothesis (Barlow & Green, 1970). The sequence of interactions which follows the initial encounter between a male and a female decreases the aggressive tendencies and/or increases the sexual tendencies of the female, and occurrence of the mating evidences the success of such a display.

This whole process, from the first encounter until copulation takes place, can take up to 3–6 hours in *P. clarkii* (Ameyaw-Akumfi, 1981). In our experiments, treated males engaged in more and longer low intensity agonistic interactions than did control males. The degree of aggression and persistence are also major factors in other decapods, e.g. in lobsters, *Homarus americanus* (Waddy & Aiken, 1990), precopulatory behaviour, where male lobsters must become dominant over the female in order to continue with sexual interactions. During these encounters, crayfish release chemical cues by urine, signalling their motivation and physiological state. More readiness to initiate agonistic interactions, shown in treated males, is linked to an elevated aggressive state (Breithaupt & Eger, 2002), presumably induced by gonopod removal. Individuals with a high aggressive state release urine (which contains hormonal metabolites) for longer durations than crayfish with low aggression indices during precopulatory agonistic interactions. Those males are avoided by females in dense populations, in order to reduce the likelihood of suffering injuries from aggressive interactions (Berry, 2008). Therefore, it is likely that treated males, due to revealing of their aggressive state, had to initiate more interactions and invest more time to dominate the female and gain opportunity to mate.

TABLE 4 Comparisons for reproductive output between control (*C*) and treatment (*T*) pairs of *Procambarus clarkii* in Round 1 (2016) and Round 2 (2017) using Mann–Whitney test (*U*). The table reports medians (with first and third interquartile); significant *p* values are in bold

Reproductive output	**Round 1: 2016**					**Round 2: 2017**				
	U	***n***	***p***	***C***	***T***	***U***	***n***	***p***	***C***	***T***
Eggs	652	76	0.469	427.5 (246.5–551.25)	190 (125–250)	78.5	37	**0.003**	130 (50–595)	35 (6.5–59.75)
Juveniles	525	66	0.801	500 (270–550)	100 (50–250)	18	18	0.179	175 (75–500)	0 (0–0)

In some arthropods, the occurrence of minor variation in sensory cues (e.g. visual, auditory, chemical and mechanical) increases the ability of females to discriminate between wild versus treated males and usually results with relatively low rates at which wild females will accept courtship overtures of treated males (Lance, McInnis, Rendon, & Jackson, 2000). Although sterile males equally readily attempted to approach females and succeeded to persuade females to mate with them, they had to be more persistent to appease or arouse the female. It is disadvantageous for males to engage in lengthy energy demanding courtships and it demonstrates lesser competitiveness of treated males (hypothesis 2).

4.2 | Changes in male copulatory behaviour

Treated males displayed an elevated number of abdominal extensions during copulation. Slow muscles (tonic) are responsible for slow changes in abdominal position, such as the ones that we observed, and usually can be elicited by the loss of contact between one of the walking legs and a supporting substrate (Page, 1981). Presence of the same mechanism when contact between gonopod and *annulus ventralis* was not achievable, possibly indicates that males are able to sense the lack of gonopods and/or lack of the contact. Bauer (1996) suggested that in penaeoid shrimp, *Sicyonia dorslis*, the petasma functions as the male sensory and stimulatory device used to prod and touch females. The petasma is composed of endopods of pleopods 1 and appendices masculinae and used to connect to the genitalia, adjusting the copulatory position and in the same time providing the information about the condition of the male. A similar mechanism could exist in the red swamp crayfish, and both males and females would be able to detect a lack of gonopods and/or lack of gonopod–*annulus ventralis* contact. The lack of a contact could lead to a less stable copulatory position and induce abdominal extensions (males trying to adjust position) and a shorter copulation time.

Even though an equal number of treated and control males copulated with females, copulating position of treated males was less stable, and copulation was shorter in duration, demonstrating again lesser competitiveness (hypothesis 2). Under natural conditions, *P. clarkii* females hide in burrows immediately after successful copulation and stays hidden until the juveniles reach independence (Aquiloni et al., 2009; Thiel, 2007). In experimental conditions, with no refuges present, only one female copulated twice, the first time with a treated and second with a control male and this way compensated for potentially unsuccessful copulation. This observation is particularly interesting in relation to observed abdominal extensions during the copulation with treated males and raises the question if this movement is just a reflex reaction or part of a more complex mechanism, and if so, why more crayfish did not compensate for unsuccessful copulation with treated males. Changes in male behaviour and lesser competitiveness can be acceptable in the absence of compensatory processes and for this reason female receptivity after an initial mating requires further investigation.

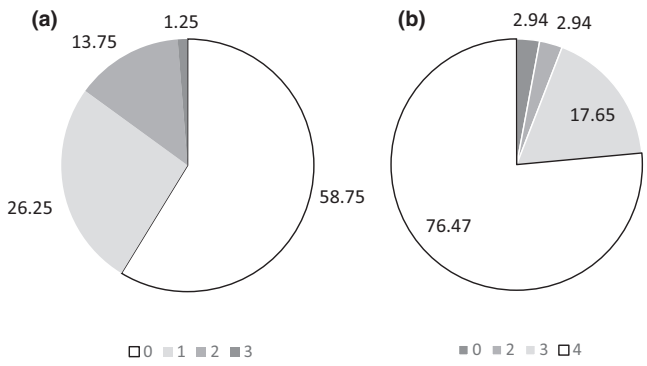

FIGURE 2 (a) Number of moults for male individuals of *Procambarus clarkii* from *Round 1* (0 = 47 individuals, 1 = 21, 2 = 11, 3 = 1) by April 2018 and (b) number of regenerated gonopods (0 = 1 individual, 2 = 1, 3 = 6, 4 = 26) by April 2018

4.3 | Moulting and gonopod regeneration

The majority of the treated males (*c.* 60%) did not moult by the onset of the new reproductive season. Adult crayfish usually moult twice

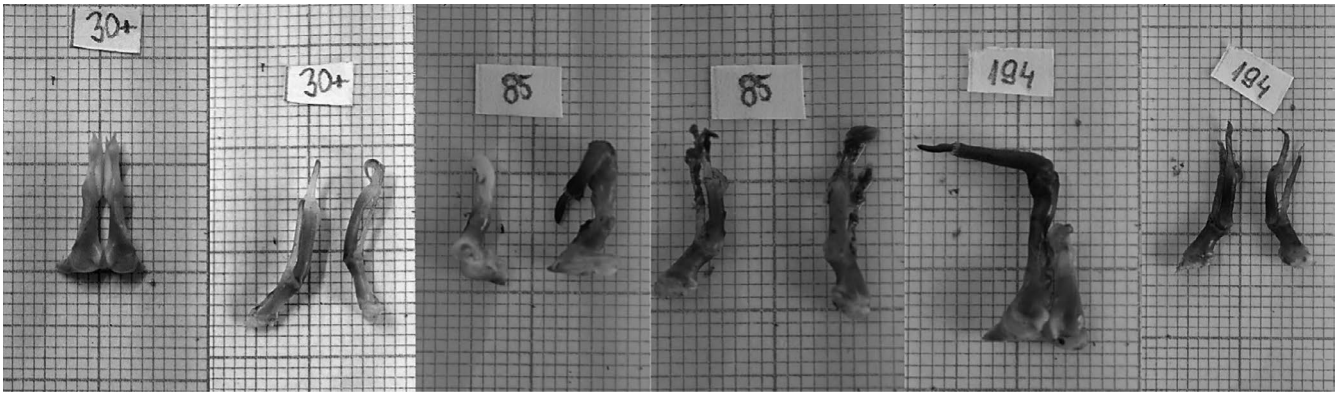

FIGURE 3 First and second pairs of *Procambarus clarkii* gonopods; first and second pairs of gonopods from control male (30+); and first and second pairs of regenerated gonopods from treated males (85 and 194) are regenerated gonopods from treated males. Right first gonopod of male marked with number 85 and left first gonopod of male marked with number 85 structurally resemble walking legs

a year, in late spring and early fall (Jegla, 1966; Barki, Levi, Hulata, & Karplus, 1997), and removal of multiple limbs and consequent damage accumulation could have stimulated the attainment of the terminal moult in older animals (Juanes & Smith, 1995). (Buřič, Kouba, & Kozák, 2010) found that for males close to maximum size, absence of moulting arises naturally, because of the size reached, since growth, as a function of the number of moults decreases with increasing size and age, that also positively influences the survival of large males in the population. The initial values of carapace length of the treated non-moulting males were not higher than those in treated moulted males, therefore lack of moulting is possibly due to the treatment. The majority of males that moulted regenerated all four missing gonopods in the first moult after treatment; however, many regenerated gonopods appeared malformed and virgin females that mated with those males did not produce offspring, suggesting that regenerated gonopods were not functional. All regenerated gonopods were photographed and changes in gonopod regeneration will be assessed elsewhere. Age of maturity in crayfish is temperature dependent and *P. clarkii* can reach sexual maturity in <3 months in their native range in Louisiana, USA (Goyert, 1978) and live up to 4–5 years (Scalici & Gherardi, 2007), even if the average is 2 years. Our study showed that, when applied to large adult males, the technique remained effective for almost 2 years and that regardless of moulting and regeneration treated males stayed sterile. If applied to smaller adults, it is possible that, due to increase moulting frequency, males could manage to regenerate gonopod functionality. Further studies are needed to confirm or disprove this. Assessment of the size-related technique efficiency would also be a logical continuation of the technique assessment before application in the field, that would help us standardise the method and ensure maximum effectiveness when used for management of *P. clarkii*.

4.4 | Reproductive output

In Round 1 of the single pair experiment, females who mated with control and treated males did not show any significant differences in extruding eggs and producing juveniles. This can be explained by the experimental setup, despite having collected crayfish before the expected onset of the reproductive season, we believe that not all females used in Round 1 where virgin (e.g. some crayfish may already have copulated or had sperm still stored from a previous season). Females of *P. clarkii* could have extruded the eggs fertilised with the sperm of males from previous season or extruded unfertilised eggs if they had not copulated during the experiment.

In Round 2 of the same experiment, in which only virgins were used, only half of the females observed mating with the treated males extruded eggs and none had juveniles hatching. Also, only one of 11 females paired with sexually responsive treated males that regenerated gonopods, extruded eggs. The extruded eggs from this female were cannibalised and abandoned few days after being extruded. The absence of hatching in females that mated with treated males may be related to the treatment and the lack of sperm transfer.

Fertilisation in *P. clarkii* occurs when eggs are being extruded. Eggs pass over the sperm that got deposited during copulation in the *annulus ventralis* (Saad & Hassan, 2010). During copulation male gonopods must get inserted to gonopores to enable transfer of the sperm from gonopore to the gonopods of the male and into *annulus ventralis* of the female (Yazicioglu, Reynolds, & Kozák, 2016). Females can extrude eggs even if they did not mate (Duse, 2015). Unmated females reabsorb eggs or extrude eggs at the end of the reproductive season (Aquiloni & Gherardi, 2008). Females also can discriminate between fertilised and unfertilised eggs and abandon or cannibalise the clutch to reduce the risk of taking care of unfertilised eggs. Although equal number of females mated with control and treatment males, significant difference in egg extrusion between control and treatment demonstrates that egg extrusion and sequential loss of eggs were probably related to the lack of fertilisation. Due to the lack of sperm transfer between treated males and females, females that mated with the males without gonopods or with regenerated gonopods displayed behaviour similar to unmated females.

5 | CONCLUSIONS

One of the main challenges in the development of SMRT is to reach the highest possible level of sterility in males without affecting their competitiveness. When constraints such as poorer competitiveness occurs, it can contribute to an increased cost of the SMRT programme, which must commit to sterilising and releasing more sterile males than would be required if released males were equal to wild males in their mating propensity and capability (Whitten & Mahon, 2005). Our research showed contrasting results: manual sterilisation lead to a complete male sterility and a decrease in female reproductive output, but also partly altered their precopulatory and copulatory behaviour. Minor differences in behaviour between control and treated males can be translated into poorer competitiveness but is not critical for the application of the technique if females do not actively choose control males to mate with, and if they do not compensate for copulation with treated males. It has been suggested that, in conditions where burrows are available, the *P. clarkii* females hide in them immediately after copulation, preventing them from copulating again (Aquiloni et al., 2009). This could mean that the competitiveness of the treated males was altered, without influencing the effectiveness of the technique, since males still managed to fulfil their duty to mate with females and impair juvenile production. Elevated number of abdominal extensions and shorter copulation observed in treated couples, by contrast, indicates that males and/or females may sense the lack of gonopods and/or lack of the contact. We believe that assessment of female receptivity after an initial mating in different experimental setting- provided with refuges- should be the next step to be taken in our research. Focusing on understanding biological mechanisms that cause abdominal extensions and female post copulative behaviour will help us in better assessment of competitiveness and potential effectiveness of the technique.

Sterile male release technique has been successfully used for almost 70 years against insect pests, but it will require substantial changes to be applied against crayfish species. In SMRT in insect management programmes, used insects are usually reared in laboratory conditions, and then sterilised and released in large numbers. By contrast, crayfish should be taken from the target population to avoid an immediate increase in the density of the impacting individuals (Aquiloni et al., 2009) and sterilised at the field site (Stebbing et al., 2014). Previous control attempts showed that the key for greater level of control is targeting different life stages by using different control techniques. In sea lamprey management programme, SMRT show to be more successful when combined with trapping in integrated pest management (Aquiloni et al., 2009). Single control method can still achieve good results when applied on the small population and isolated waterbody. Based on Aquiloni & Zanetti (2014) experience with SMRT, that used irradiation as sterilisation technique, 2 years of application of SMRT in Lake Casette, Italy, resulted in 87% reduction of *P. clarkii* population. In crayfish management these examples in literature are rare and using available traditional techniques to compliment SMRT will more likely lead to the best results.

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CONFLICT OF INTEREST

All authors certify that they have no affiliations with or involvement in any organisation or entity that could be considered as source of conflict of interest in the subject matter or materials discussed in this manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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