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Mechanical male sterilisation in invasive signal crayfish *Pacifastacus leniusculus*: persistence and functionality in captive and wild conditions

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Abstract – Management control methods for invasive crayfish remain of limited effectiveness, resulting in ongoing invasions of high ecological impact. As management programmes integrating methods to limit juvenile recruitment could reduce population abundances, the efficacy of a sterile male release technique (SMRT) based on the manual removal of male gonopods was tested here in captive and wild conditions by comparing the survival, gonopod regeneration rates and a range of reproductive metrics of sterilised versus non-sterilised males. Sterilised male survival was high, with their removed gonopods regenerating at sizes that were always smaller than those of non-sterilised males. In captive trials, while sterilised males showed significantly lower areas of spermatophore cover than non-sterilised, and less accuracy in placement, subsequent female brood size did not differ significantly between the two male groups. The number of females retaining their clutches also did not also differ significantly between these groups. Over a seven-year period in the wild, there was no evidence suggesting SMRT significantly reduced female brood sizes and clutch retention rates. Although mechanical SMRT altered the size and delivery accuracy of sterilised male gonopods, female reproductive success of invasive crayfish was unaffected. Several potential reasons for this failure of the technique were identified and require further research.

Keywords: Invasive crayfish control / sterile male release technique / biological invasion

1 Introduction

Numerous freshwater species have been introduced outside their native range, with their introductions and subsequent invasions in new regions being a powerful driver of native biodiversity loss in inland waters (Gherardi *et al.*, 2011). Crayfish, being omnivorous, mobile, long-lived and resistant to desiccation, have proved to be highly invasive around the world due to these traits enabling their rapid establishment following their introduction into new ecosystems (Nyström *et al.*, 1999). One of the most ecologically damaging invasive crayfish species is the North American signal crayfish *Pacifastacus leniusculus*, now widespread across Europe after being introduced for aquaculture in the 1970s (Holdich *et al.*, 2014; Mathers *et al.*, 2016). Their impacts include causing substantial declines in native crayfish populations through a combination of inter-specific competitive interactions and the transmission of crayfish plague *Aphanomyces astaci* (Holdich and Reeve, 1991).

A major challenge with invasive crayfish is their management, there being no definitive method for controlling or eradicating their populations (Peay, 2006; Hein *et al.*, 2007; Gherardi *et al.*, 2011; Stebbing *et al.*, 2014). Whilst baited funnel traps are frequently used in their population control, these primarily capture larger individuals, predominantly males. Artificial refuge traps can capture a wider size range of crayfish, including equal sex ratios, but are relatively ineffective at capturing juveniles (Green *et al.*, 2018). For a control programme to succeed in reducing population abundances, all size classes of a population need targeting, something most likely achieved using a multi-method approach (Stebbing *et al.*, 2014). As even the use of a range of trapping methods have not eradicated invasive crayfish populations, they could be complemented by the application of

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Table 1. Sterilisation methods (abbreviations in parentheses) for male signal crayfish *Pacifastacus leniusculus* in captive conditions and the mean lengths of both males and females used subsequently in their copulation trials.

Group	Sterilisation method	n	Sampling date(s)	Mean carapace length in trials (mm) Female	Male
1	Not sterilised (control/CTRL)	24	Sept. 2019	42.2 ± 2.6	43.5 ± 3.0
2	Cutting whole gonopods (cut/CWG)	24	Sept. 2019	41.8 ± 1.4	42.8 ± 1.6
3	Pulling whole gonopods (pulled/PWG)	24	Sept. 2019	41.8 ± 1.0	42.5 ± 1.0
4	One year regeneration (trimmed once/R1T1)	12	Sept. 2018	43.8 ± 3.7	49.5 ± 1.2
5	One year regeneration (trimmed twice/R1T2)	7	Sept. 2016 (n = 2) Sept. 2017 (n = 5)	45.8 ± 8.5	51.7 ± 9.1

more novel control methods, such as sterile male release techniques (SMRT) (Aquiloni *et al.*, 2009).

SMRT are considered a relatively successful method for eradicating invertebrate pest species (Knipling, 1959; Klassen and Curtis, 2005), especially as their actions are inversely density-dependent and species-specific (Stebbing *et al.*, 2014). Previous trials on invasive red swamp crayfish *Procambarus clarkii* involved male sterilisation using irradiation, which significantly reduced male testes size and juvenile production and survival (Aquiloni *et al.*, 2009). However, irradiation-induced gonadal damage in *P. clarkii* was subsequently shown to be repaired within 193 days of treatment (Manfrin *et al.*, 2021). Moreover, the widespread application of this method will be limited due to the costly process of capturing, irradiating and then returning males to the population (Aquiloni *et al.*, 2009). Correspondingly, it has been posited that the mechanical removal of the gonopods could result in a more cost effective and efficient male sterilisation method that reduces the extent and accuracy of spermatophore placement (Stebbing and Rimmer, 2014). This is because during copulation, male crayfish use their gonopods to place extruded spermatophores onto the ventral surface of the female. Crayfish spermatozoa are non-motile so it is assumed that spermatophores closest to the gonopore have the highest chance of fertilising ova (McLay and Van den Brink, 2016), although spermatozoa can be circulated during the secretion of glair, a highly viscous gel secreted by the female into which the ova are deposited (Yazicioglu *et al.*, 2016), and through movement of the female's pleopods (Niksirat *et al.*, 2014).

Initial testing of the effectiveness of gonopod removal as a SMRT on *P. leniusculus* resulted in only one copulation from 20 pairings with sterilised males, which failed to deposit spermatophores anywhere on the female abdomen (Stebbing and Rimmer, 2014). It was also fully effective in female *P. clarkii*, with sterilised males initiating mating as frequently as non-sterilised, but having to invest more effort in dominating the female and having shortened copulation times (Johović *et al.*, 2019). However *P. clarkii* have internal fertilisation so this study is not directly comparable to *P. leniusculus*. No study to date has followed this mechanical SMRT through to the brood hatch stage. Moreover, there remain considerable knowledge gaps on the persistence of gonopod removal. Stebbing and Rimmer (2014) suggested mechanical removal would be effective for approximately three years due to adults moulting annually, but moulting studies suggest this period will be shorter as adult crayfish can moult twice per year (Abrahamsson, 1971), especially at smaller sizes (Westman and Savolainen, 2002; Guan and Wiles, 1999). Sterilised male *P. clarkii* moulted more frequently than non-sterilised, with most regenerating all their gonopods after their first moult post-sterilisation, although many of these were malformed (Johović *et al.*, 2019). Stebbing and Rimmer (2014) also detected increased mortality rates of sterilised crayfish.

Given the uncertainties that remain in the long-term effectiveness of gonopod removal as a SMRT, especially in relation to its ability to reduce population abundances, this study aimed to understand how male *P. leniusculus* respond physically and functionally to gonopod removal through comparing relevant reproductive metrics with non-sterilised males and then testing the effects on female reproductive success. Experimental trials in captive and wild conditions assessed post-sterilisation survival and gonopod regeneration rates, copulation effectiveness (as accuracy of spermatophore placement), and frequency of ovigerous females and the resultant brood sizes produced between sterilised versus non-sterilised males. We posit: (1) regenerated gonopod lengths are significantly smaller in sterilised versus non-sterilised males; (2) the smaller (and potentially deformed) gonopods significantly reduce copulation effectiveness in sterilised males; and (3) application of SMRT in the wild will result in reduced frequencies of ovigerous females and significantly lower brood sizes.

2 Methods

2.1 Sterilisation trials in captive conditions

Samples of *P. leniusculus* for use in laboratory trials were collected each September between 2016 and 2019. Animals were captured from a lake fishery in Dorset (50°49′49″N, 001°56′17″W) in the south of England using baited funnel traps set overnight, with captured animals transferred to the laboratory where they were sorted by sex, with males being selected for sterilisation. Male crayfish collected between 2016 and 2018 were used in pilot studies to determine an effective sterilisation procedure. The basis of the sterilisation procedure was the removal of the gonopods by excising with scissors or pulling them out with tweezers (Green *et al.*, 2020). Work was also completed to successfully determine whether the crayfish survived trimming the regenerated gonopods on one and two occasions. This work then enabled the use of five groups of male crayfish to be used experimentally in 2019: a control group (not sterilised) and then four groups comprising males sterilised by a range of methods (Tab. 1).

The crayfish to be used in the trials were initially held in a secure outdoor area where, following sterilisation of males, they were housed in separate treatment/sex groups in filtered and aerated 200 litre black tanks. Each tank had a gravel substrate c. 30 mm deep with lengths of PVC pipe (L: 150 mm, D: 50 mm) added (>1:1 ratio of pipe to individuals) to act as refuges. To reduce the likelihood of intra-specific conflict, a maximum of twelve individuals were housed in each tank. The animals were fed on raw carrot every two days and the tanks cleaned weekly by syphoning the gravel. Each tank was covered with netting and secured with timber along the edges in order to prevent crayfish egress.

2.1.1 Post-sterilisation survival and gonopod regeneration

The survival rates of sterilised versus non-sterilised males in controlled conditions were determined using the 2019 samples, where the survival of males to be used in experiments (CTRL, CWG and PWG; $N = 72$: sterilised, $n = 48$, control: $n = 24$) were monitored between 24th September (date of collection/sterilisation) and 25th October 2019 (conclusion of experiments). To then assess gonopod regeneration, all males used in the mating experiments were euthanised post copulation, and the gonopods for groups CTRL, R1T1 and R1T2 removed and photographed (DSLR camera on a horizontal mount with a ruler in frame). These gonopod areas were then measured using ImageJ (Rueden *et al.*, 2017) to establish rates of regeneration as accurately as possible, and test these versus the control group. To test these differences in gonopod regeneration between the groups (Tab. 1), the extent of regeneration was standardised to the carapace length (CL) of each individual as adjusted gonopod area (mean area mm^2/CL). As the data were normally distributed (Shapiro Wilk $P = 0.65$), differences between the groups were tested using ANOVA (adjusted post-hoc with Tukey's HSD).

2.1.2 Copulation effectiveness as defined by spermatophore placement

The first trial on copulation effectiveness tested whether male sterilisation reduced the extent and accuracy of the spermatophore placement. Females were selected for experiments based on their glair (mating receptivity) status, *i.e.*, blueish/whitish colouration caused by the formation of glair glands. The trials were completed between 17th and 25th October 2019 in nine clear plastic tanks (900 × 300 × 250 mm) located outdoors in ambient conditions and covered in black HDPE (high density polyethylene) sheeting that maintained dark conditions, as crayfish are generally more active and, therefore, more likely to copulate at night (Franke and Hörstgen-Schwark, 2015). Each tank was half-filled with dechlorinated tap water and allowed to settle to ambient temperature (12–14 °C). One female in glair was placed into each tank and allowed to acclimatise for five minutes. A male was then introduced and the animals were left together until either copulation was concluded or for a maximum of 30 min. Across the trial, pairs were sized matched (as carapace length, CL) where possible (Tab. 1). The number of experiments per group varied, the aim being for every male held prior to 2019 to copulate, with the number of copulations from the 2019 groups being commensurate with that. Only nine of twelve males from the largest pre-2019 group (R1T1: 2018 trimmed once; Tab. 1) copulated whilst in Group 5 (R1T2: 2016 and 2017 trimmed twice; Tab. 1), all five of the 2017 but neither of the 2016 males copulated, resulting in nine copulations for all groups except for R1T2 which had five copulations. Some copulations were discarded where the female avoided spermatophore placement on her ventral surface by curling her abdomen up tightly, particularly when the male was larger than the female.

Where copulation had occurred, the male and female were separated and the males euthanised. The mated female was then marked with a reference number on her carapace for identification. The location of spermatophore placement on each female was then measured by immobilising the crayfish beneath a camera using straps placed across the abdomen and holding the chelae down with small magnets, with an image then captured (Fig. S1). The females were then placed in 200 L brood tanks specific to each male group (Tab. 1) and held until February 2020. Where copulation did not occur, the male and female were returned to stock tanks for a minimum of 24 hours before re-use.

Two metrics were used to measure the location of spermatophore placement: total cover (expenditure) and distribution (accuracy), and were measured in three placement areas: (1) between the 2nd and 3rd pair of walking legs adjacent to the ovipore; (2) area covered by the first and fourth pairs of walking legs; and (3) area of the first two abdominal sections (Fig. S1). All areas extended to the full width of the crayfish. In processing, all images were made black and white to display the spermatophores more fully, with the cover and distribution of spermatophores then measured (as the total area and then for each of the three placement areas) in ImageJ. To standardise measurements across different individuals, spermatophore cover was expressed as the proportion of the placement area covered. As the data were not normally distributed then differences between the treatment groups were tested using a Kruskal Wallis test; differences in the data were then also tested as two groups ('sterilised' versus 'non-sterilised') in a Mann Whitney *U* test.

2.1.3 Clutch retention and brood size

This trial used the females from the spermatophore placement experiments, held in the 200 L brood tanks post mating according to their male sterilisation group at a density of nine per tank. As crayfish density can affect female brood size (Celada *et al.*, 2005), four un-mated females (status marked by removal of one uropod) were added to Group 5, where only five copulations occurred, to provide consistent densities. These crayfish were then held in the tanks until February 2020, with this providing sufficient time for the loss of any unfertilised eggs (Guan and Wiles, 1999; Celada *et al.*, 2005). During this period, feeding and tank cleaning was undertaken weekly. Then, all individuals from each group were placed into their own tank (900 × 300 × 250 mm), with their embryos then removed using tweezers and placed into individual plastic pots for counting. This procedure was completed on the same day for all crayfish to minimise embryo loss due to stress. The number of females failing to retain their clutch at this point was also recorded. All the females were

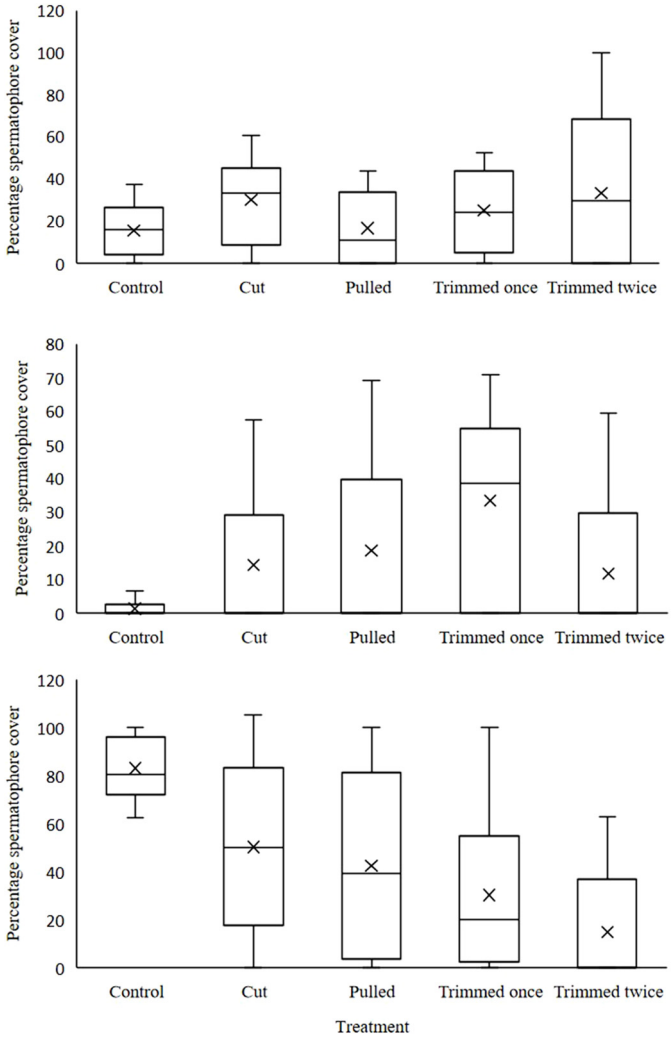

Fig. 1. Boxplots revealing percentage spermatophore distribution between treatments for the outer (top), abdomen (middle) and middle (bottom) sections. Horizontal lines mark the 10th, 25th, 50th, 75th and 90th percentiles of the data whilst *x* is the mean percentage. spermatophore cover.

then euthanised. In analyses, brood size (as number of embryos) was standardised to account for size differences between females (brood size/CL). Testing for differences in standardised brood size and clutch retention used Kruskal Wallis test, with the five groups were then also tested as two groups ('sterilised' versus 'non-sterilised') in Mann Whitney U and Chi-squared tests.

2.2 Sterilisation trials in field conditions: mark-recapture, gonopod regeneration and brood size

The trial to investigate the efficacy of male sterilisation in field conditions was completed in a specific study area of the River Barle, Somerset (51°060 24.200N; 3°390 32.200W; Green *et al.*, 2018). Trapping was undertaken between April and October of 2015 (Year 1) to 2021 (Year 7) using baited or artificial refuge traps that were being deployed on the river on a weekly basis (Green *et al.*, 2018). Throughout this period all males ≥40 mm CL were sterilised by either cutting or pulling of gonopods then returned to the river. In 2021, all males ≥30 mm CL were sterilised. All female crayfish and males <40 mm CL (30 mm in 2021) were euthanised. A total of 3832 (3055 Years 1–6; 777 Year 7) males were sterilised and returned to the river close (within 5 m) to their capture location over the study period.

The data for the gonopod regeneration experiments was based on a mark-recapture exercise carried out between September of Year 3 (2017) and October of Year 4 (2018), when all sterilised males were tagged at point of capture with a uniquely coded passive integrated transponder (PIT) tag (FDX-B; 7 × 1.35 mm; Loligo Systems, Denmark). Tags were inserted ventrally between the 2nd and 4th abdominal segments using a PIT tag implanter (Nightingale *et al.*, 2018). Carapace length was then measured (nearest mm) using Vernier calipers and information on moult stage, damage status and capture location recorded. Damage status was categorised as: 1 = none or little damage (*e.g.*, damage to < 2 walking legs, damaged antennae); 2 = moderate damage (*e.g.*, damage to 1 chela, 3 to 4 walking legs); and 3 = major damage (*e.g.*, to 2 chelae, > 4 walking legs). In Year 3, all tagged males ($n = 75$) were sterilised by pulling the gonopods off with a pair of tweezers; in Year 4 ($n = 301$), sterilisation involved a mixture of pulling and excising with a pair of scissors, with some ($n = 247$) subject to the use of both methods per individual by cutting the gonopods on one side and by pulling on the other. Subsequent crayfish trapping events in the study stretch resulted in the recapture of the sterilised males. Each trapped male crayfish was scanned for PIT tag presence and, following identification as a tagged recapture, the data recorded were their carapace length, damage and moult status, and capture location, plus the length of each gonopod if regeneration had occurred (using *Vernier calipers*). Logistical constrains in the field meant that measures of gonopod area could not be completed as per the captive trial and instead, gonopod length was used as the measure of their regeneration. Mean total gonopod regeneration (total gonopod length/4) for all gonopods, and total length of only the anterior gonopods and posterior gonopods, were determined for each animal. For the damage status, an additional metric was included (damage increment on recapture: 0 = no change; 1 = new or increased damage).

Reference values for mean gonopod length by CL of control males were derived by measuring gonopod length of a minimum of 10 non-sterilised males of each CL (to the nearest mm) that were also captured in traps. Mean values for each of the four gonopods were determined for each CL before combining into categories of all gonopod lengths, total anterior gonopod lengths, and total posterior gonopod lengths. The field regeneration data were combined in the same way, before differences in the gonopod lengths between the control and removal categories were tested in generalised linear models (GLMs). The GLMs used gonopod length as the dependent variable, status (sterilised or control) as the independent variable and CL as the co-variate. The extent of gonopod regeneration between seasons was then tested with a GLM as before, where season was used as the independent variable. In all GLMs, the reported outputs were estimated marginal means of the gonopod lengths of each category or season (±95%

confidence limits), and the significance of their differences according to linearly independent pairwise comparisons (with Bonferroni adjustment for multiple comparisons). The extent of regeneration from the sterilisation methods (cutting versus pulling) was then tested using a Mann Whitney U test; the damage increments were then tested against the total length of time between sterilisation and recaptures, again using a Mann Whitney *U* test.

To test the effect of sterilisation on brood size in the field, ovigerous female abundance and brood size data were used from the weekly trapping events. Although the study area covered 1500 m of river, subsequent analyses used only data from the central focal reach (1000 m) to reduce the effects of crayfish immigration from adjacent reaches, and only artificial refuge trap data were used as catches of ovigerous females in baited traps were negligible (Green *et al.*, 2018). The analysis used all female crayfish of ≥30 mm CL captured within this central reach between the first trapping event each spring (usually mid – late April depending on flow conditions) and the second week of June of each study year. The use of a minimum CL of 30 mm was based on the smallest ovigerous crayfish captured in all samples and the end date was based on the latest date of capture of an ovigerous female during the trial period. Testing of differences in ovigerous female relative abundance (as catch per unit effort (CPUE), weekly catch/ trapping effort) was tested in a generalised linear model and used ovigerous female CPUE as the dependent variable, year as the independent variable and covariates of temperature recorded at 09.30 on day of capture and mean daily flow (m^3/sec, UK Environment Agency data).

Female brood size metrics were calculated using all ovigerous females caught in the spring of each year excluding brood sizes <2 ($N = 150$), as single ova are frequently a relic after brood release (N. Green, pers. obs.). For each ovigerous female, the embryos/juveniles were removed with a pair of tweezers and counted and brood size standardised to CL consistent with the controlled experiments. Differences in standardised brood sizes were then tested between years using a linear GLM, where brood size was the dependent variable, year was the independent variable, and the covariates were temperature and flow. The reported model outputs included the mean values of the dependent variables (as estimated marginal means (±95% confidence limits) adjusted for the effects of the covariates) and the significance of their differences according to linearly independent pairwise comparisons (with Bonferroni adjustment for multiple comparisons). As winter water temperature potentially influences brood size and hatching date, annual winter temperature data were tested between years. As differences between years were not significant (ANOVA: $F_{3,104} = 1.3$, $P = 0.26$) then winter temperatures were not considered as influencing these data and were not considered further.

All statistical tests on data from the captive and field trials were completed in SPSS v.26 (IBM, 2019); use of parametric tests only followed after testing for normality (Shapiro Wilkes and Kolmogorov–Smirnov tests); non-parametric tests were always used where data were not normally distributed. Where error values are presented around means, they represent standard error unless stated, and results from multiple comparisons were adjusted using Bonferroni correction. Significance is reported as exact two-tailed unless stated and where t-tests are used, Levene's tests assume equal variance unless stated.

3 Results

3.1 Captive trials

3.1.1 Post-sterilisation recovery and gonopod area by sterilisation group

All males survived the sterilisation procedure in the 2019 trial, where the time between sterilisation and their euthanasia was between 23 and 31 days. For gonopod area, there was a significant difference in mean area (adjusted for carapace length) between the control group (mean 40.95 ± 16.9 mm^2) and the sterilised groups (trimmed once: mean 21.93 ± 12.44 mm^2; trimmed twice: mean 17.53 ± 12.68 mm^2) (ANOVA $F_{2,20} = 15.6$, $P < 0.01$), where the significant differences were between the control and both treatment groups (both $P < 0.01$), but not between the two treatment groups ($P = 0.63$).

3.1.2 Copulation effectiveness as defined by spermatophore placement

The groups of sterilised males had lower total cover of spermatophore than non-sterilised males (mean reduction overall: 49.3%; mean reduction in cover between the middle two pairs of legs: 43.5%; Fig. 1). These reductions were all significantly different between the control and sterilised male groups (Kruskal–Wallis tests: cut: $H = 3.2$, $P = 0.02$; pulled: $H = 2.9$, $P = 0.04$; trimmed once: $H = 2.6$, $P = 0.01$; trimmed twice: $H = 3.0$, $P = 0.03$). Sterilised males were also less accurate in their spermatophore placement, with increased spermatophore cover on the first two abdominal segments and two outer pairs of legs, whilst spermatophore placement for control males was concentrated in the middle section close to the ovipore (Fig. S1, Fig. 1). These differences were, however, only significant between the control and the regeneration trimmed twice group (Kruskal–Wallis: $H = 12.5$, $P = 0.01$). Percentage spermatophore cover on the abdomen (indicating poor placement accuracy) was generally higher than the control in all groups, except the trimmed twice group, with these differences significant ($H = 6.82$; $P < 0.05$; Fig. 1). The data on spermatophore cover between the two outer pairs of legs varied between groups and with all differences being non-significant ($H = 3.07$; $P > 0.05$; Fig. 1). When treating the dataset as two groups (non-sterilised vs sterilised), overall percentage spermatophore cover was significantly higher for non-sterilised males (Mann Whitney: $U = 26.0$, $P < 0.01$; Fig. 2). The percentage cover between the middle two pairs of legs was also significantly higher for non-sterilised males ($U = 47.0$, $P = 0.01$), but there were no significant differences for cover on the abdomen ($U = 194.5$, $P = 0.11$) or outer two pairs of legs ($U = 172.0$, $P = 0.39$).

3.1.3 Clutch retention and brood size

Brood size was generally higher in the control than all sterilised groups, except the cut gonopod group (Fig. 3), although the differences were not significant (Kruskal–Wallis test: $H = 5.1$, $P = 0.28$). When treated as two groups (sterilised versus non-sterilised), brood size for sterilised males was again higher than the control, but not significantly different (Mann

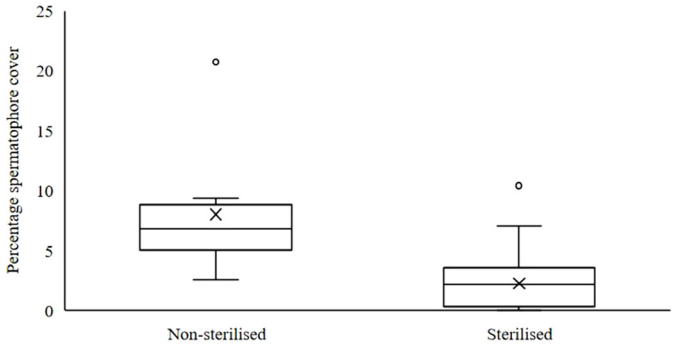

Fig. 2. Boxplot comparing overall percentage spermatophore distribution between sterilised and non-sterilised groups of males. Horizontal lines mark the 10th, 25th, 50th, 75th and 90th percentiles of the data, *x* is the mean percentage spermatophore cover, and clear circles are outlying data points.

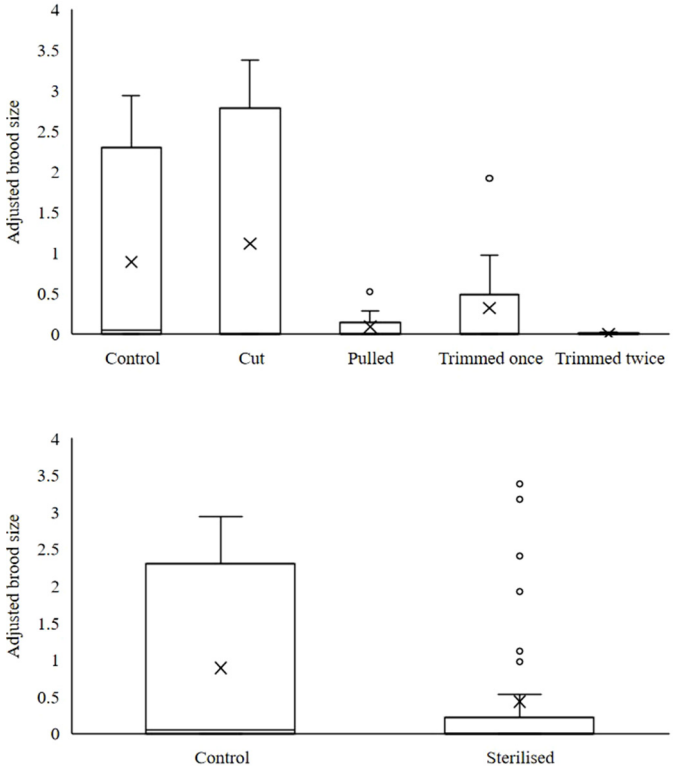

Fig. 3. Mean brood size (adjusted for the effect of carapace length) according to the experimental treatments (top) and as sterilised versus non-sterilised (bottom). Each plot communicates the median (solid line), interquartile range (boxes), 10th and 90th percentiles (error bars), mean (*x*) and outlier values (circles)

Whitney: $U = 105.0$, $P = 0.23$; Fig. 4). Although the number of females retaining their clutch was lower in the groups that reproduced with sterilised males than non-sterilised (Fig. 4), the differences were again not significant: X^2 (1, N = 41) = 2.35, $P = 0.12$ (Fig. 4).

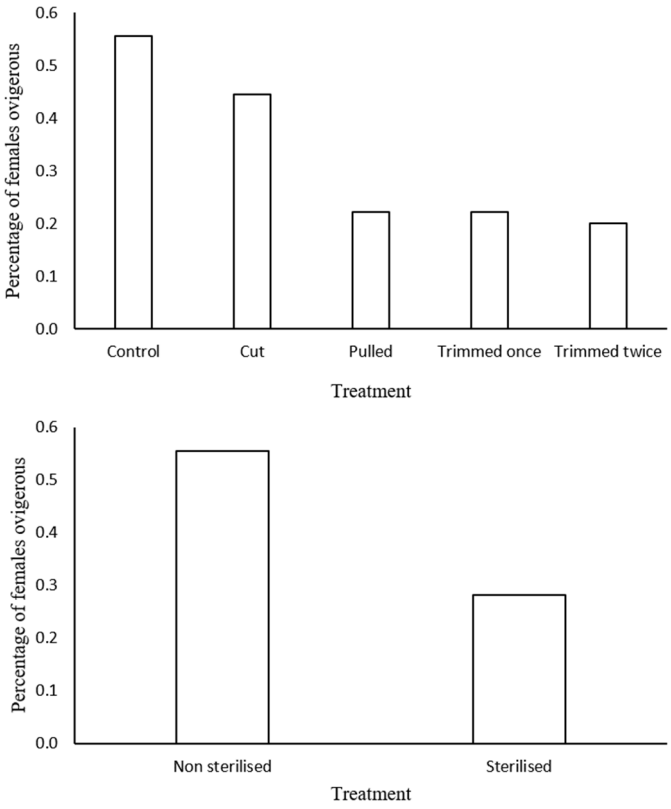

Fig. 4. Percentage of female crayfish retaining embryos until spring according to (top) the experimental treatments, and (bottom) as sterilised versus non-sterilised.

3.2 Field trials

3.2.1 Recapture rates and intervals

The time interval between the tagging of an individual sterilised male and its final recapture ranged between 11 and 778 days (mean 188 ± 28 days). Due to the seasonality of trapping, the recapture data were split into three groups: 'one season' (11 to 98 days; $n = 27$), 'two season' (98 to 364 days; $n = 17$), and 'three season': 410 to 778 days; $n = 1$). These data indicated that at least 56% of sterilised males survived the season in which they were tagged, 37% survived at least one winter and 7% at least two winters. Only 26.6% of recaptured tagged males experienced increased damage since sterilisation, there being no relationship between increased damage and the time between capture and recapture (Mann Whitney U test = 156.0, $P = 0.58$).

3.2.2 Gonopod regeneration

Mean total gonopod lengths of the recaptured sterilised males from all seasons (n = 45) were significantly smaller than reference values for control males (Wald X^2 = 1296.5; $P < 0.01$; Tab. 2, Fig. 5). Both mean anterior and posterior gonopod lengths were also significantly larger in control versus sterilised males (anterior: Wald $X^2 = 1239.2$; posterior: Wald $X^2 = 1143.8$; $P < 0.01$ in both cases; Tab. 2). Regeneration lengths were more evenly balanced between anterior and posterior

Table 2. Comparisons of gonopod lengths (all total, total anterior and total posterior) between non-sterilised and sterilised males in the River Barle study site, with the results of the generalised linear model testing differences in gonopod lengths where the effect of carapace length was a significant covariate in the model.

Gonopod length (mm)	Control (mm)	Sterilised (mm)	Test result
Mean total gonopod length	56.87 ± 5.68	19.33 (±11.55)	Wald $X^2 = 1296.6$, $P < 0.01$
Mean total anterior	25.42 ± 4.75	9.29 (±5.85)	Wald $X^2 = 1229.2$, $P < 0.01$
Mean total posterior	21.32 ± 3.25	10.01 (±6.61)	Wald $X^2 = 1143.8$, $P < 0.01$

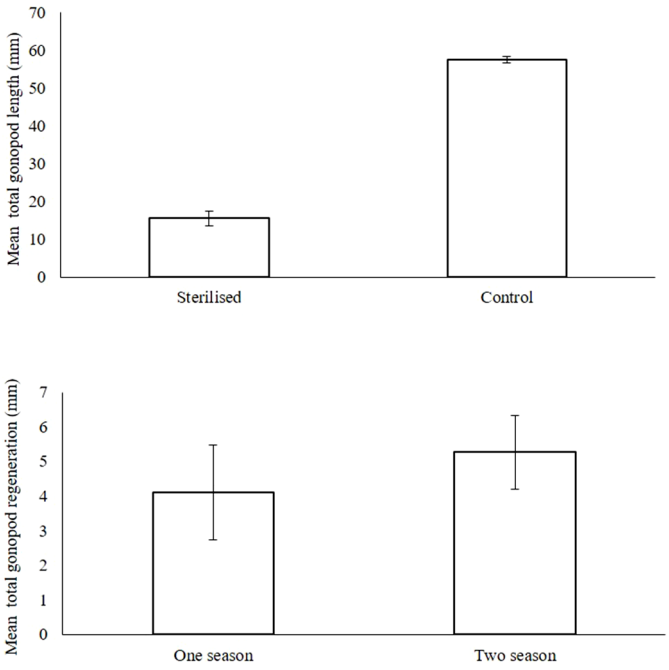

Fig. 5. Mean gonopod regeneration rates of sterilised males (as estimated marginal means, adjusted for the effects of carapace length) as gonopod length (top), where the comparison is with non-sterilised males, and regeneration between male crayfish recaptured after one and two winters in the River Barle study site (bottom). Error bars represent 95% confidence intervals.

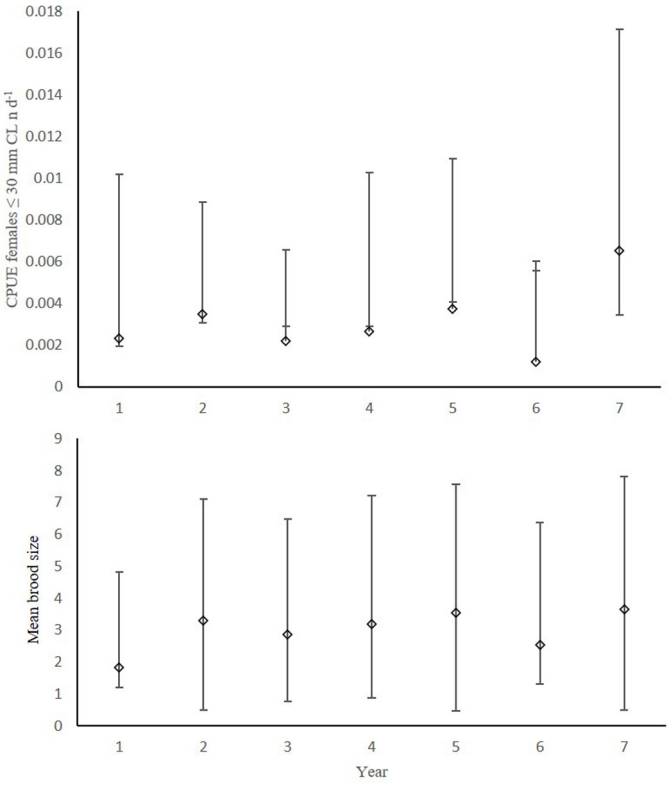

Fig. 6. Mean CPUE of females ≤30 mm CL captured being ovigerous (top) and mean adjusted brood size (bottom) at the River Barle study site Years 1–6 (as estimated marginal means from the best fitting GLM, top). Error bars represent 95% confidence intervals.

gonopods for the sterilised males, whereas in control males, the posterior gonopods were significantly larger (Tab. 2). Differences in mean gonopod regeneration between one and two seasons were not significant (GLM: Wald $X^2 = 1.7$, $P = 0.20$, Fig. 5). There was no suggestion that the sterilisation procedure induced more frequent moulting in males, with little difference between moult rates of males at point of sterilisation (7.4%) and point of recapture (7.0%).

3.2.3 Brood size and ovigerous female abundance in the field

The CPUE of captured females ≥30 mm that were ovigerous between the start of trapping and the third week of June each year fluctuated between Years (Fig. 6), with the GLM indicating that the differences were non-significant (Wald $X^2 = 4.8$, $P = 0.57$). Female brood size (standardised to CL) also fluctuated over the study period with the GLM being non-significant (Wald $X^2 = 12.0$, $P < 0.06$; Fig. 6).

4 Discussion

Sterile male release techniques have been posited as providing effective management techniques for reducing the recruitment success of populations of invasive species, especially crayfish (Aquiloni *et al.*, 2009; Stebbing and Rimmer, 2014). Here, investigations into SMRT on *P. leniusculus* enabled testing of its short-term (captive trials) and longer term (field trials) effectiveness. The results revealed that following the manual sterilisation of males, regenerated gonopods were always reduced in area (captive) and length (field), with this consistent with the prediction. In captive trials, sterilised males had significantly lower areas of spermatophore cover than non-sterilised males, and were more inaccurate in their placement, with this again as predicted. However, this did not result in captive females

that reproduced with sterilised males having significantly reduced brood sizes compared with those that reproduced with non-sterilised males, with the number of captive females that retained their clutches also not differing significantly between those that reproduced with a sterilised versus non-sterilised male. The field trial data also suggested that SMRT had not significantly reduced female brood sizes and clutch retention rates by the end of the study period, also disagreeing with prediction.

The application of manual sterilisation to male *P. leniusculus* did not appear to reduce their survival rates, with all sterilised males surviving the relatively short experimental periods in captive conditions. Their regenerated gonopod data revealed that when adjusted for the effect of carapace length, gonopod area was reduced, with the extent of the reduction increasing with the number of treatments. Moreover, the regeneration that was observed indicated that gonopod deformation, subsequent spermatophore cover and resultant brood sizes decreased with the number of treatments. Gonopod regeneration rates amongst recaptured tagged males in the wild also remained substantially and significantly smaller than non-sterilised males. This result concurs with Stebbing and Rimmer (2014), who suggested that complete gonopod regeneration would take up to 3 years. While gonopod regeneration was limited in the field site, a relatively low proportion of sterilised tagged males survived for more than one winter post-sterilisation. Although this might suggest low survival rates due to sterilisation, the trial did not also involve the capture, tagging and release of non-sterilised males, inhibiting assessments of natural versus sterilisation related mortality. The likelihood of tagging related mortality was considered low, as the methods used followed Nightingale *et al.*, (2018) who found no differences in survival or growth between tagged and untagged *Austropotamobius pallipes.* Moreover, the study river is a relatively acidic upland river of low productivity, with it being likely that the crayfish population consists of individuals with relatively limited lifespans and relatively high natural mortality rates. Indeed, while crayfish demographic data from other rivers and lakes indicates life spans of between 6 and 20 years (Belchier *et al.*, 1998; Guan and Wiles, 1999), it is suggested that males in the River Barle rarely attain ages above 6 years old. This is because males are observed from trapping results to reach 'large' size (40 mm CL) at age 3+ years old, with the mean size of that cohort being only 44.9 ± 4.7 mm, and where the maximum length was 64 mm CL, that being representative of the largest and therefore oldest individuals (N. Green, unpublished data). With an average of two moults per year and moult increment of 2–4 mm in this size class (N. Green, pers. obs.) then the majority of large males are unlikely to be above six years old.

The efficiency of SMRT in the field trials could have thus resulted from a relatively high proportion of the sterilised males 'dropping out' of the pool of sterile males within a short time period from natural causes, potentially inhibiting the efficacy of the technique at the population level. In lentic systems and in more productive rivers, signal crayfish have longer life spans and attain much larger sizes (in excess of 90 mm CL; Belchier *et al.*, 1998), so the persistence of the sterilisation effects could arguably be greater, increasing its effectiveness in the longer term. Additionally, the proportion of the sterilised males present in the population is likely to be important in determining the efficacy of the technique. For example, Basilico *et al.* (2013) reported that the proportions of ovigerous females captured following manual sterilisation of male *P. clarkii* in some French streams were related to the proportion of males sterilised. When less than 3% of catches comprised large males that were then sterilised and released, 46% of females were ovigerous the following year; when 20 to 30% of the catches were sterilised and released males, juvenile catches declined by 90% the following year. As in the River Barle study site the total catch rarely consisted of more than 13% sterilised males, then this proportion might be insufficient to significantly reduce the presence of ovigerous females in subsequent years.

The failure of SMRT in the field trial to reduce female reproductive success could also relate to the role played by large males in reproduction. Crayfish form dominance hierarchies (Fero *et al.*, 2007; Herberholz and Mc Curdy, 2007; Goessmann *et al.*, 2000) and it is widely assumed that large, dominant males conduct the majority of mating behaviour through preventing smaller males from copulating. Some studies support this, where large males of other crayfish species (*A. pallipes* and *A. italicus*) mated more frequently than smaller ones (Woodlock and Reynolds, 1988; Galeotti *et al.*, 2006). However, Rubolini *et al.*, (2007) found there was reduced investment in sperm production with increasing male size, suggesting senescence of reproductive performance with age, which is commensurate with studies of other decapods. In addition, Woodlock and Reynolds (1988) found 33% of large male *A. pallipes* (>40 mm CL) failed to copulate at all, with copulations taking longer than those of smaller males, and larger males also unable to mate effectively with small females (Woodlock and Reynolds 1988; N. Green, pers. obs.). Consequently, should smaller males be more reproductively active and successful than larger males, the selective application of SMRT here to relatively large (and potentially elderly) males might have inhibited its effectiveness in reducing female reproductive success. This is emphasised by smaller males being at least twice as abundant as larger males in most *P. leniusculus* populations with, for example, Chadwick *et al.* (2021) reporting that that individuals over 35 mm carapace length (CL) comprised between 1 and 5% of a population *versus* 4–12% for lengths between 26 and 34 mm CL. In the River Barle study site, trapping data from artificial refuge traps (which are less size biased than conventional funnel traps) from 2015 to 2020, revealed 66% of captured crayfish were 25 to 39 mm, with only 19% of 40 mm and above. Although there is no evidence of female *P. leniusculus* being promiscuous (Green *et al.*, 2020), the higher abundance of smaller males creates more mating opportunities for females and an increased likelihood of successful clutches. Furthermore, if the sterilisation of large males leads to increased fatality within that group, females may be more likely to mate with smaller and potentially more productive males, potentially leading to greater reproductive success.

In captive conditions, sterilised males had significantly lower spermatophore cover and placement accuracy than the control groups. The groups where gonopod regeneration had then been trimmed were also the poorest performing in the trials, and gonopod regeneration amongst sterilised males appeared to be malformed, smaller and less functional. These

results again infer that sterilisation should be an effective form of population control (Stebbing *et al.*, 2014; Manfrin *et al.*, 2019), but only if reduced spermatophore cover leads to reduced brood sizes. It has been assumed that the 'normal' placement of spermatophore close to the females ovipore is a prerequisite of successful mating (McLay and Van den Brink, 2016) since crayfish spermatozoa are non-motile. However, it is known that spermatozoa can circulate through the female's glair during spawning and through subsequent movement of the female's pleopods (Niksirat *et al.*, 2014; Yazicioglu *et al.*, 2016). The number of spermatozoa produced by *P. leniusculus* is likely to be high: Harlioğlu *et al.* (2012) found the mean sperm number for *Astacus leptodactylus* of 41-56 mm CL ranged from 4×10^8 to 8.5×10^9 sperm/distal vas deferens (DVD) section. Moreover, this study found that spermatophore distribution amongst sterilised males increased on the abdomen, an area in contact with glair and therefore with spermatozoa. Consequently, it is suggested that the sterilisation process still enables widespread fertilisation of the ova due to higher than anticipated levels of sperm circulating through the females' glair.

In captive trials, females that reproduced with sterilised males did produce smaller brood sizes compared with those that reproduced with non-sterilised males, with this consistent with the results of the limited number of studies completed on other crayfish species (Johovićh *et al*, 2019; Aquiloni *et al.*, 2009), however the differences were not significant. Furthermore, reduced brood sizes over time were not evident in the field data. This lack of reduced brood sizes could be an artefact of the constraints of the field sampling. Due to the study site being on an upland river, high flows often restricted access during spring and the ovigerous female/brood size dataset lacked consistency between years, resulting in relatively small sample sizes (CPUE: $n = 79$; brood size: $n = 150$). *P. leniusculus* tend to hatch eggs between March and June in England (Guan and Wiles, 1999, Holdich *et al.*, 2014), and inconsistent sampling in April and May could have missed large numbers of ovigerous females.

Another potential explanation for the lack of decline in mean annual brood sizes and the percentages of ovigerous females in the field trials is the role of population compensatory responses. The study population has been subject to weekly trapping between 2015 and 2021, with approximately 20,000 *P. leniusculus* having been removed. Crayfish populations respond to reduced density and greater food availability via increased growth (moulting) and fecundity (brood sizes and incidence of ovigerous females), coupled with migration into lower density areas (Hudina *et al.*, 2012; Westman and Savolainen, 2002; Parvulescu *et al.*, 2015; Moorhouse and McDonald, 2011). It is thus possible that a reduction in reproduction caused by the presence of sterilised males is being confounded by increased female fecundity as they respond to population reductions through trapping by increasing their reproductive investment. Furthermore, sterilised males could have emigrated and non-sterilised males immigrated into the site, given that large crayfish, particularly males, are known to be the most exploratory sex/age class, exhibiting nomadic behaviour (Bubb, 2004) and tending to lead population expansion (Hudina *et al.*, 2012).

To summarise, the captive trials indicated male sterilisation can reduce male reproductive performance through reduced spermatophore placement and placement accuracy. The captive trials also indicated that gonopod regeneration rates were slow and resulted in malformed gonopods, but following reproduction, did not result in lower female brood sizes. While the results on gonopod regeneration were similar in the field data, this also did not result in reduced female brood sizes, with the incidence of ovigerous females also not significantly reducing over time, despite over 3000 male crayfish being sterilised and released over a seven-year period. Potential reasons contributing to this apparent inability of SMRT to reduce female reproductive success were suggested as relating to small sample sizes, the relevance of spermatophore expenditure and accuracy to successful fertilisation, low long-term survival rates of sterilised males, insufficient proportions of sterilised males in the population, low reproductive efficiency in larger versus smaller males, capture efficiency of ovigerous females and/or compensatory responses. Closer investigation of these influences is necessary in order to understand why the technique did not result in reduced female reproductive success, especially as it still has potential to be more effective in more closed, lentic systems, and those of higher productivity where the persistence of sterilised males could be higher and so lead to greater effectiveness of the sterilisation technique.

Supplementary Material

The Supplementary Material is available at https://www.10.1051/kmae/2022014/olm.

Figure S1. Typical spermatophore cover of sterilised and control male.

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