Chapter 3.   The efficacy of male sterilisation in invasive signal crayfish Pacifastacus leniusculus: persistence and functionality in captive and wild conditions

In this chapter, data for ovigerous female CPUE, percentage total females captured and brood size has also been used in Chapter 5.

Aaron Hart assisted with the spermatophore placement experiments and the (controlled) counts of brood size (including sampling and husbandry).

Abstract

Management control methods for invasive crayfish have, to date, been of limited effectiveness, resulting in on-going invasions that have continued to have high ecological impacts. Although removals using trapping can be an effective management technique, traps tend to be selective in mainly capturing adults. As it has been suggested that management programmes that integrate methods to limit juvenile recruitment could lead to increased success, the efficacy of sterile male release techniques, based on the manual removal of male gonopods, was tested here in both captive and wild conditions by comparing the survival, gonopod regeneration rates and a range of reproductive metrics between sterilised and non-sterilised males. The results indicated that the survival of sterilised males was high, with their removed gonopods regenerating at sizes that were smaller than non-sterilised males in both controlled and wild conditions. In captive trials, sterilised males had significantly lower areas of spermatophore cover than non-sterilised males, and were less accurate in their placement. 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. The number of captive females that retained their clutches also did not differ significantly between those that reproduced with a sterilised versus non-sterilised male. In the wild, there was also no clear evidence to suggest that the use of SMRT over a six-year period had significantly reduced female brood sizes and clutch retention rates. Correspondingly, while this manual sterilisation technique was effective at altering the size and delivery accuracy of the regenerated gonopods, this did not appear to translate into reduced female reproductive success. Several reasons for this have been proposed and further work is required to understand why the positive responses observed under controlled conditions have not translated to the field trial.

3.1 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,* which is 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 in their management, as there is currently no definitive method for controlling or eradicating their populations, despite numerous methods having been trialled with varying degrees of success (Peay 2006; Hein et al. 2007; Gherardi et al. 2011, Stebbing et al. 2014). While baited funnel traps are frequently employed in their population control, these tend primarily to capture larger individuals that are usually sex-biased toward males. The use of artificial refuge traps can help overcome this through capturing a wider size range of crayfish that comprises a much higher proportions of females, although these traps are relatively ineffective at capturing juveniles (Green et al. 2018; Chapter 4). Consequently, for a control programme to succeed there is a need to target all size classes of a population, something most likely to be achieved via an integrated, multi-method approach (Stebbing et al. 2014). Given that

even the use of a range of trapping methods have not been successful at eradicating invasive crayfish populations, then they could be complemented with the application of more novel methods that act upon crayfish reproduction, such as the sterile male release technique (SMRT). This method has shown some potential for the control of invasive crayfish populations due to its action on inhibiting the production of progeny and thus potentially reducing recruitment success (Aquiloni et al. 2009).

Sterile male release techniques are generally considered a relatively successful method for the eradication of invertebrate pest species (Knipling, 1959; Klassen and Curtis, 2005), especially given it is inversely density-dependent and species-specific (Stebbing et al. 2014). Trials of the SMRT method on crayfish by Aquiloni et al. (2009) involved sterilisation of 40 wild males of invasive red swamp crayfish *Procambarus clarkii* that were then irradiated. This process reduced the size of the male testes and led to a reduction of 43 % in the production of crayfish offspring, as well as reducing the survival rates of offspring. However, the more widespread use of this method is limited, due to the costly process of capturing, irradiating and then returning males to the population (Aquiloni et al. 2009).

During copulation, male crayfish extrude spermatophores from the *vas deferens* via the anterior gonopods onto the ventral surface of the female, using all four gonopods to position the spermatophores close to the female's gonopore. The spermatophores closest to the gonopore have the highest chance of fertilising the eggs (McLay and Van den Brink 2016). Consequently, it has been postulated that the removal of the gonopods could result in functional sterilisation, with the spermatophore still being produced but with the male being unable to accurately place it close to the gonopore. Stebbing and Rimmer (2014)

tested this technique on *P. leniusculus*, achieving only one copulation from 20 pairings with sterilised males, which failed to deposit spermatophores anywhere on the female abdomen. Another study using this method reported that none of the 64 female *P. clarkii* that mated with sterilised males produced juveniles (Johović et al. 2019), although this species has internal fertilisation and thus is not directly comparable to *P. leniusculus.* This study also found the removal of gonopods in treated males shortened the duration of copulation, and that the sterilised males were equally ready to initiate sexual interaction as non-sterilised ones, but had to invest more effort into dominating the female prior to copulation (Johović et al. 2019).

The only known study using gonopod removal as its sterilisation method on *P. leniusculus* used a small sample size resulted in only one copulation (Stebbing and Rimmer 2014). Therefore, studies involving larger sample sizes that investigate the extent and accuracy of spermatophore cover, and follow this through to the brood hatch stage, are needed to test its ability to act as an effective SMRT. Moreover, there are considerable knowledge gaps on the persistence of gonopod removal, with Stebbing and Rimmer (2014) suggesting the removal technique would remain effective for approximately three years due to adults moulting annually, with gonopods regrowing at the rate of a damaged limb (i.e. 2 to 3 moult cycles; Westman et al. 1992). Other studies have, however, suggested that adult crayfish moult twice per year (Abrahamsson 1971), especially at lengths below 45 mm (Westman et al. 1992; Guan and Wiles 1999), so suggesting that regeneration will be faster. Johović et al. (2019) observed *P. clarkii* sterilised males moulted more frequently than non-sterilised, with most males regenerating all four missing gonopods after their first moult post sterilisation although many of the regenerated gonopods appeared malformed. This is consistent with regenerated crayfish limbs generally being

smaller than the original (Dunoyer 2020). Understanding the relationship between moulting and gonopod regeneration, and the nature of that regrowth, is thus key to determining sterilisation recovery rates and subsequent copulation effectiveness. Stebbing and Rimmer (2014) also detected increased mortality rates of sterilised crayfish (73 % survival versus 93 % survival of non-sterilised males), with gonopod removal potentially constituting an injury that affects the vitality of the individual, compromising its competitive abilities (e.g. for food, shelter and mates), but this remains untested with long-term data. There is thus a corresponding need to quantify the effect of *P. leniusculus* sterilisation on their survival rates and mating success, along with assessments of gonopod regeneration.

 The aim of this study, therefore, was to test how male *P. leniusculus* respond physically and functionally to gonopod removal as a sterilisation procedure by comparing relevant reproductive metrics with non-sterilised males and testing their effect on female reproductive success. Using complementary experimental trials in both captive and wild conditions, the objectives were to assess: (1) sterilisation survival and gonopod regeneration rates; (2) copulation effectiveness, as determined by spermatophore placement; and (3) frequency of ovigerous females and the resultant brood sizes produced between sterilised versus non-sterilised males. It was predicted that: (1) in sterilised males, regenerated gonopod lengths would be significantly smaller than in non-sterilised males and full regeneration would take up to two years; (2) the smaller (and potentially deformed) gonopods would result in significantly reduced copulation effectiveness in sterilised males; and (3) application of SMRT will result in reduced frequencies of ovigerous females and significantly lower brood sizes.

3.2   Methods

3.2.1   Sterilisation trials in captive conditions

Samples of *P. leniusculus* for use in laboratory trials were collected each September between 2016 and 2019. On each occasion, baited funnel traps were set overnight at a lake fishery in Dorset in the south of England and, following their lifting, captured animals were 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 of males sterilised by a range of methods (Table 3.1).

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. 30mm deep and had lengths of PVC pipe (L: 150 mm, D: 50 mm) added (ratios of > 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.

Table 3.1 Sterilisation methods 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)	24	Sept. 2019	42.2 ± 2.6	43.5 ± 3.0
2	Cutting whole gonopods	24	Sept. 2019	41.8 ± 1.4	42.8 ± 1.6
3	Pulling off whole gonopods	24	Sept. 2019	41.8 ± 1.0	42.5 ± 1.0
4	One year regeneration (trimmed once)	12	Sept. 2018	43.8 ± 3.7	49.5 ± 1.2
5	One year regeneration (trimmed twice)	7	Sept. 2016 (n=2)	45.8 ± 8.5	51.7 ± 9.1
			Sept. 2017 (n=5)		

3.2.2　Procedural survival and gonopod regeneration

The survival rates of sterilised versus non-sterilised males in controlled conditions were determined using 2019 samples, where survival of males to be used in experiments (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 1, 4 and 5 removed and photographed (DSLR camera on a horizontal mount with a ruler in frame). The gonopod area for groups 1, 4 and 5 were then measured using ImageJ (https://imagej.nih.gov/ij/download.html) 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 (Table 3.1), the extent of regeneration was standardised to the carapace length (CL) of each individual as adjusted gonopod area (mean area mm^2 / CL). Following testing for normality (Shapiro Wilk P = 0.65), differences between the groups were tested using ANOVA (adjusted post-hoc with Tukey's HSD).

Trial 1: 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 (Figure 3.1). The trials were completed between 17th and 25th October 2019 in nine clear plastic tanks (900 x 300 x 250 mm) that were located outdoors in ambient conditions and covered in black HDPE (high density polyethylene) sheeting. This sheeting was to maintain dark conditions, as crayfish are generally more active and, therefore, more likely to mate at night (Franke and Hörstgen-Schwark, 2015). Each tank was half-filled with dechlorinated tap water and allowed to settle to ambient temperature (12 to 14°C). One female

in glair was then 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 minutes. Across the trial, pairs were sized matched (as carapace length, CL) where possible (Table 3.1). Within groups 1 to 4, nine successful copulations per group were achieved, whilst only 5 animals from group 5 copulated.

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 to enable her subsequent identification. The location of spermatophore placement on each female was then measured. This involved 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 (Figure 3.1). The females were then placed in 200 L brood tanks specific to each male group (Table 3.1) 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). These metrics were quantified using three placement areas: (1) area 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. 3.1 c). 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 spermatophore cover then measured (as the total area and then for each of the three placement areas) using ImageJ. To standardise measurements across different individuals, spermatophore cover was expressed as the proportion of the area covered. Following testing for normality, 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') using a Mann Whitney U test.
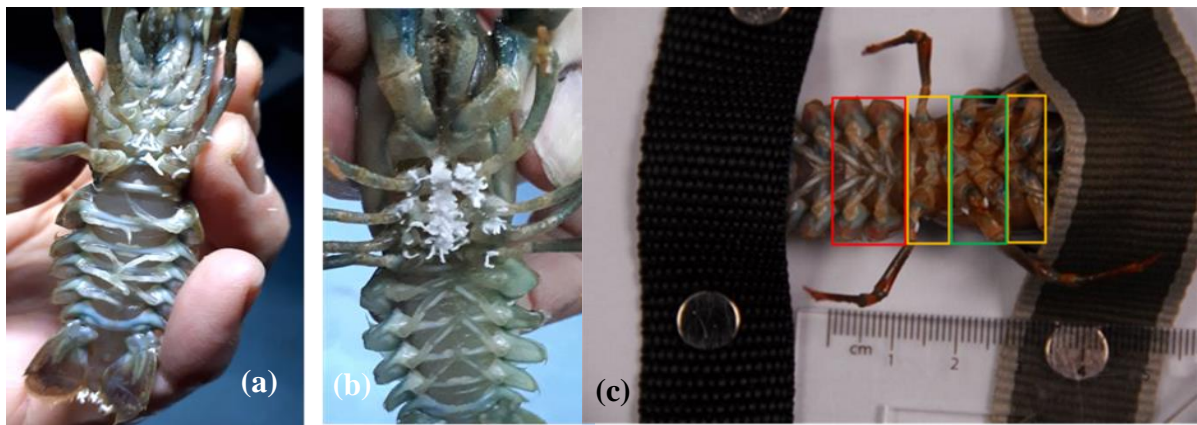

Figure 3.1 a) Typical spermatophore cover of (a) sterilised and (b) control male, where in (b) the majority of spermatophores are deposited around the egg pore (between the middle pairs of walking legs). C) Spermatophore placement areas 1 (green), 2 (yellow) and 3 (red) used for image analysis. Photos (a) and (b) N. Green, (c) A. Hart.

Trial 2: Clutch retention and brood size

This trial used the females from the spermatophore placement experiments, enabling testing of brood size versus spermatophore cover. The post-mating females were held in the 200 L brood tanks 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), 4 un-mated females (status marked by removal of one uropod) were added to Group 5, where only five copulations occurred, to ensure consistent densities. These crayfish were then held in the tanks until February 2020, with this time period 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, each individual was placed into their own tank (900 x 300 x 250mm), with their eggs 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 egg loss due to stress. The number of females failing to retain their clutch at this point was also recorded. All the females were then euthanised. In analyses, brood size (as egg number) was standardised to carapace length to account for differences between females (brood size / CL). Testing for differences in standardised brood size and clutch retention used Kruskal Wallis, Chi-squared goodness of fit and Fishers exact tests (as these data were not normally distributed). The five groups were then also tested as two groups ('sterilised' versus 'non-sterilised') in a Mann Whitney U test.

3.2.3   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 based on a mark-recapture exercise completed in a specific study area of the River Barle, Somerset, between September 2017 and October 2019 (Green et al. 2018; Chapter 4). This work formed part of a wider management programme in the study area, where trapping was completed between May and October of 2015 (Year 1) to 2020 (Year 6, Chapter 5). The initial male capture events were in baited or artificial refuge traps that were being deployed on the river on a weekly basis (Green et al. 2018), with all males that were sterilised between September of Year 3 and October of Year 4 also being tagged with a passive integrated transponder (PIT) tag (FDX-B; 7 x 1.35 mm; Loligo Systems, Denmark), providing each sterilised male with a unique tag code. Tags were inserted ventrally between the 2nd and 4th abdominal segment using a PIT tag implanter (Nightingale et al. 2017). Carapace length was then measured (nearest mm) using Vernier callipers and information on their moulting stage, damage status and capture location recorded. Moult was recorded when the crayfish exoskeleton gave way to a gentle squeeze, or when a gap had developed between the posterior end of the carapace and the first

abdominal segment. Damage status was categorised as: 1 = none or little damage (e.g. damage to < 2 legs, damaged antennae); 2 = moderate damage (e.g. damage to 1 claw, 3 to 4 legs); and 3 = major damage (e.g. to 2 claws, > 4 legs).

A total of 3055 males representing 13.6% of the total catch were sterilised and returned to the river close (within 5 m) to their capture location over the six year study period. 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 cutting them off 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 callipers). 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 (as total gonopod length) 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 combined 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 in 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, data were used from weekly trapping events (Green et al., 2018; Chapter 5). Although the study area covered 1500 m of river, subsequent analyses used only data from the central focal reach (1000m) 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 (Chapter 4). These metrics were measured for all female crayfish of ≥ 30mm CL captured within the study site in the five week period of the second week of May to the second week of June of each study year, this data and test results also being used in Chapter 5. The use of a minimum CL of 30 mm was based on the smallest ovigerous crayfish captured in all samples, the start date range was based on the latest start date for trapping each year, and the end date was based on the date of the last capture of an ovigerous female each spring. For brood size and the proportion of females > 30 mm CL

being ovigerous, all trapping occasions to mid-June each year were used. Testing of differences in ovigerous female relative abundance (as catch per unit effort (CPUE), weekly catch/ trapping effort), and the proportion of all females captured ≥ 30 mm CL that were ovigerous, were tested in generalised linear models. The proportion of all females captured ≥ 30 mm CL that were ovigerous used a negative binomial with log link GLM, where the covariates were water temperature recorded at 09.30 and the mean daily flow on each trapping occasion. The CPUE model assumed normal data distribution and again used temperature and flow as covariates. Brood size metrics were calculated using all ovigerous females caught in the spring of each year. For each ovigerous female, the embryos/juveniles were removed with a pair of tweezers and counted. Broods of <2 were excluded from counts, as single ova are frequently a relic after brood release (pers. obs.). Differences in brood sizes were then tested between years using a GLM (normal distribution), where brood size was the dependent variable, year was the independent variable, and the covariates were temperature flow and CL. The reported model outputs included the mean values of the dependent variables (as estimated marginal means ($\pm$ 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 (Environment Agency, 9.30am Pixton Hill) 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.

3.3 Results

3.3.1 Captive trials

Procedural 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 $F_{2,20} = 15.6$, $P < 0.01$). In the test, the significant differences were between the control and both treatment groups (both $P < 0.01$), but not between the two treatment groups ($P = 0.63$).

Trial 1: Copulation effectiveness as defined by spermatophore placement

The groups of sterilised males had lower areas of spermatophore cover than non-sterilised males (mean reduction overall: 49.3 %; mean reduction in cover between the middle two pairs of legs: 43.5 %) (Fig. 3.2). These reductions were all significantly different between the control and sterilised male groups (Kruskal Wallis tests: cut: $H = 3.17$, $P = 0.02$; pulled: $H = 2.9$, $P = 0.04$; trimmed twice: $H = 2.98$, $P = 0.03$). Sterilised males were also less accurate in their spermatophore placement, with an increase in spermatophore cover on the abdomen (46.7 %) and two outer pairs of legs (22.3 %; Fig. 3.2; 3.3). These differences were, however, only

significant between the control and the regeneration trimmed twice group (H = 12.48, P = 0.01).
Percentage spermatophore cover on the first two abdominal segments (indicating low placement accuracy) was generally higher than the control in all groups, except the trimmed twice group, with these differences significant (P < 0.05; Fig. 3.2). The data on spermatophore cover between the two outer pairs of legs varied between groups and with all differences being non-significant (P > 0.05; Fig. 3.3).

When treating the dataset as two groups (non-sterilised versus sterilised), overall percentage spermatophore cover was significantly higher for non-sterilised males (Mann Whitney U = 26.00, P < 0.01,). The percentage cover between the middle two pairs of legs was also significantly higher for non-sterilised males (U = 47.00, P = 0.01; Fig. 3.2), but there were no significant differences for cover on the abdomen (U = 194.5, P = 0.11; Fig. 3.2) or outer two pairs of legs (U = 172.00, P = 0.39; Fig. 3.3).

Trial 2: Clutch retention and brood size

Brood size was generally higher in the control than all sterilised groups, except the cut gonopod group (Fig 3.4), although the differences were not significant (Kruskal Wallis test: H (4) = 5.12, P = 0.28). When treated as two groups (sterilised versus non-sterilised), brood size for sterilised males was again higher than the control, but was not significantly different (Mann Whitney U Test: U = 105.00, P = 0.23; Fig. 3.4). Although the number of females retaining their clutch (i.e. retaining at least one fertile egg through winter until February) was lower in the groups that reproduced with sterilised males than unsterilized (Fig 3.5), the differences were again not significant: X^2 (1, N = 41) = 2.35, P = 0.12.
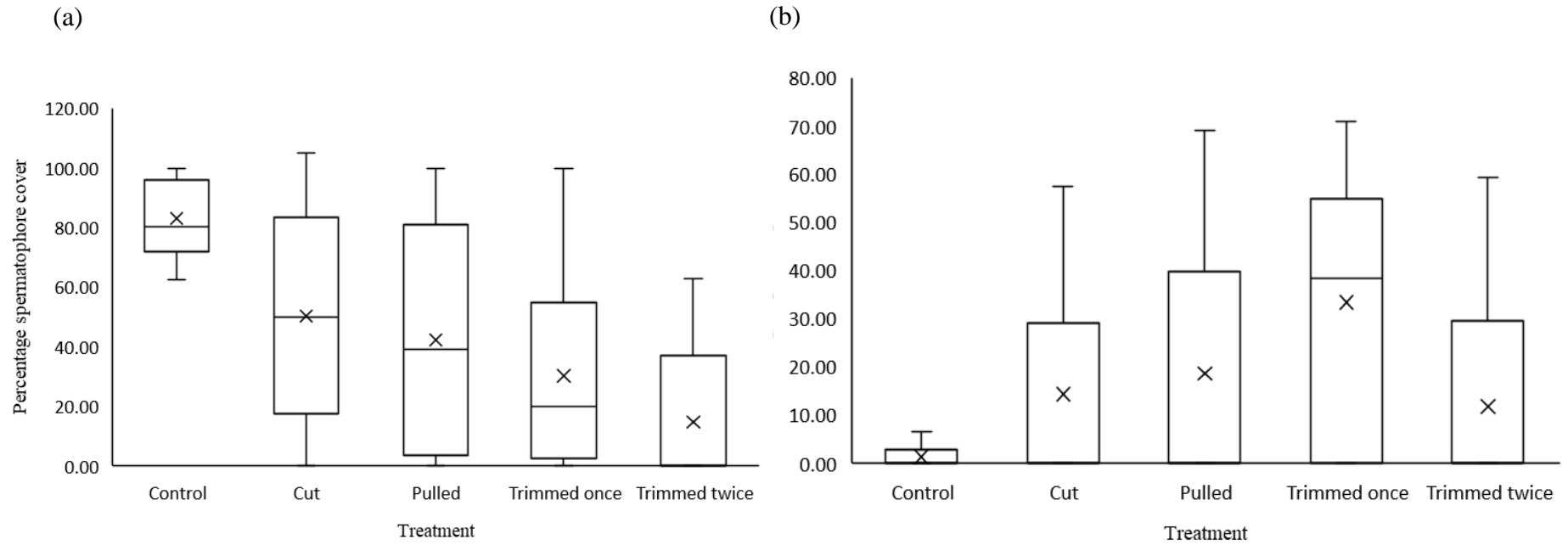
Figure 3.2. Boxplots revealing percentage spermatophore distribution between treatments for the middle (a), and abdomen (b) sections.

Horizontal lines mark the 10th, 25th, 50th, 75th and 90th percentiles of the data whilst x is the mean percentage spermatophore cover.
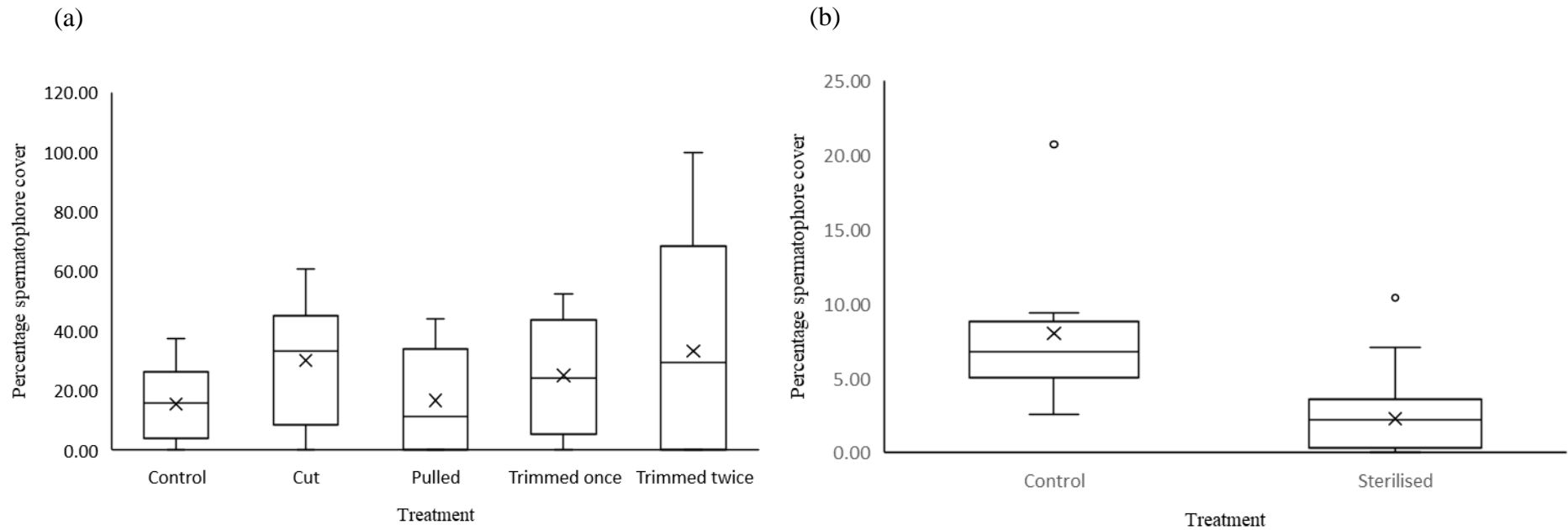
Figure 3.3. Boxplots to describe percentage spermatophore distribution for the outer (a) section between treatments whilst b) compares overall distribution between the two groups of males. Horizontal lines mark the 10th, 25th, 50th, 75th and 90th percentiles of the data, x is the mean percentage spermatophore cover, and clear circles are outlying data points.
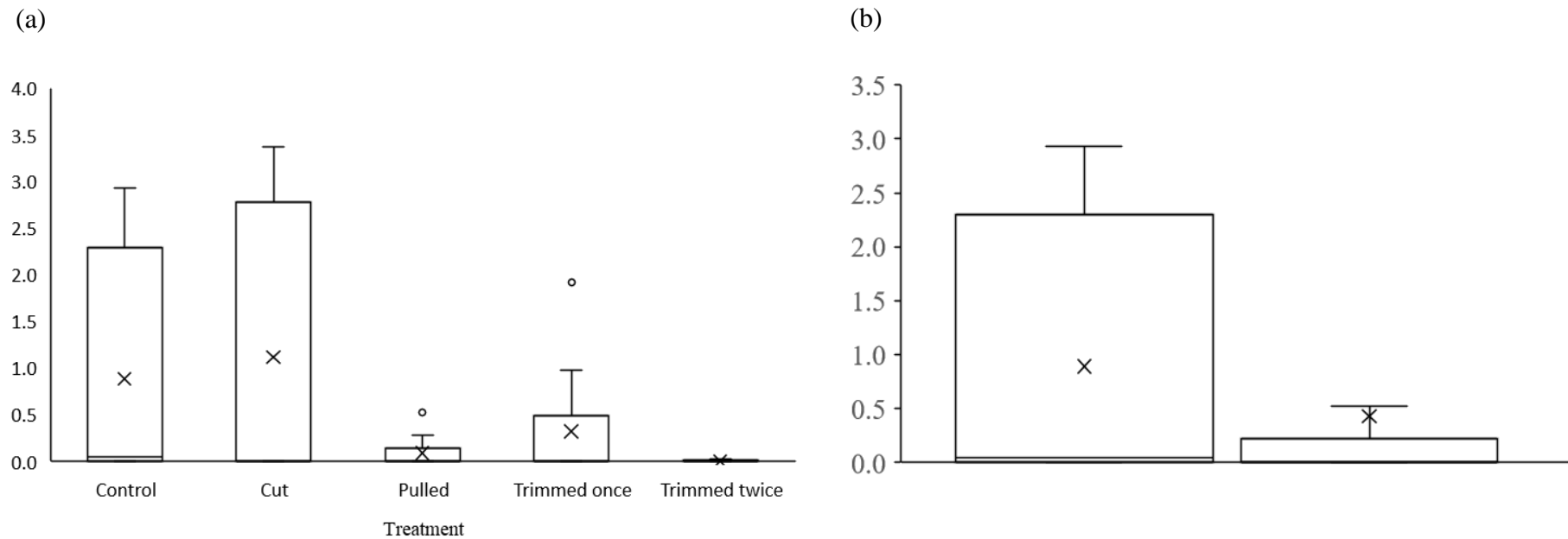
Figure 3.4. Counts of brood size (adjusted for the effect of carapace length) according to the experimental treatments (a) and as sterilised versus non-sterilised (b). Each plot communicates the median (solid line), interquartile range (boxes), 10th and 90th percentiles (error bars), mean (x) and outlier values (circles)
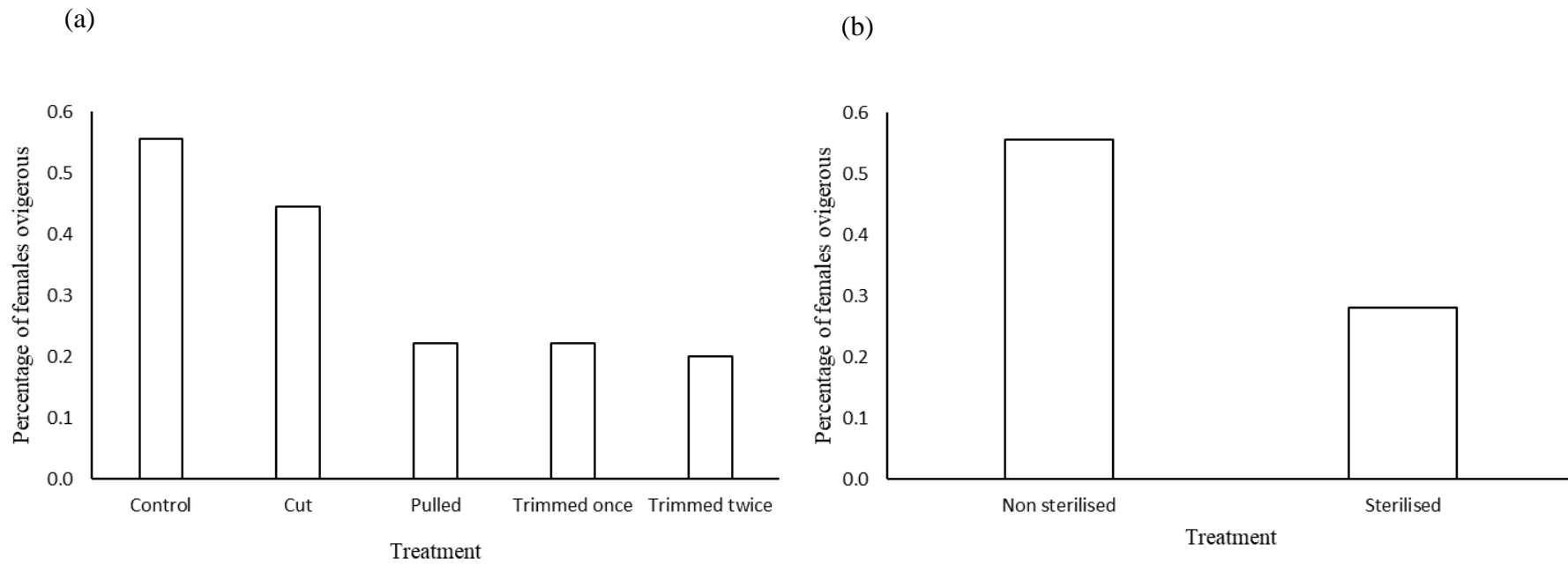
Figure 3.5. Percentage of female crayfish retaining embryos until spring according to (a) the experimental treatments, and (b) as sterilised versus non-sterilised

3.3.2   Trials in field conditions

Recapture rates and intervals

The time interval between the tagging of an individual and its final recapture was 11 to 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, with no relationship between increased damage and the time between capture and recapture (Mann Whitney U test = 156, P = 0.58).

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 (P < 0.01; Table 3.2, Figure 3.6). Both mean anterior and posterior gonopod lengths were also significantly larger in control versus sterilised males (P < 0.01 in both cases; Table 3.2). Regeneration lengths were more evenly balanced between anterior and posterior gonopods for the sterilised males, whereas in control males, the posterior gonopods were significantly larger (Table 3.2). Differences in mean gonopod regeneration between one and two seasons were not significant (GLM: Wald X^2 [1, N = 45] 1.66, P = 0.20, Figure 3.6).

There was no suggestion that the sterilisation procedure induced more frequent ecdysis in males. Of 2659 males of $\geq$ 40mm CL that were captured prior to sterilisation between

years 1 to 4 inclusive, 7.4 % were in ecdysis at point of capture. Over the same period, of 1128 recaptured sterilised males, 7.0 % were in ecdysis.

Brood size and ovigerous female abundance in the field

The CPUE of captured females ≥ 30mm that were ovigerous between the second week of May and third week of June each year increased between Years 1 and 2, but decreased thereafter (Fig. 3.7), with the GLM indicating that the differences were significant overall but not between years (Wald X^2 11.74, P = 0.04), and where the effects of temperature and flow as covariates were significant (P < 0.05). The proportion of females ≥ 30mm that were ovigerous also showed some significant changes over the study period (Wald X^2 = 21.41, P = <0.01), again with a declining pattern since Year 3 but no temporal significance (Fig. 3.7). Female brood size fluctuated over the study period, being significantly higher in Years 2 - 6 versus Year 1 (Wald X^2 = 12.35, P < 0.03; Fig. 3.7). The proportion of females retaining their clutches through the winter period was generally low, with annual means ranging from 1.2 ± 0.5 % in 2020 to 8.2 ± 3.0 in 2016.

Table 3.2 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.

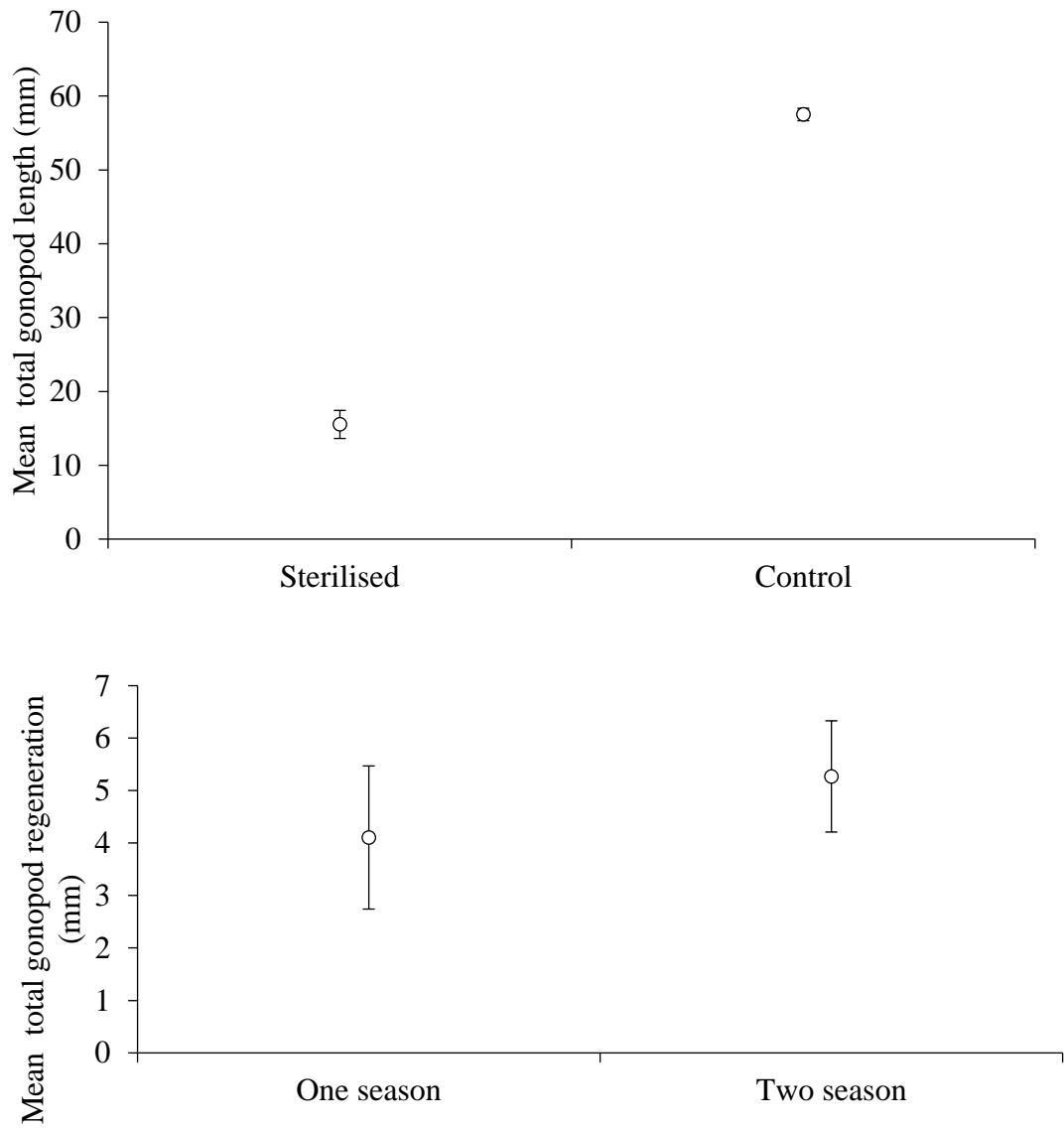| Gonopod length (mm)	Control (mm)	Sterilised (mm)	Model result
Mean total gonopod length	56.87 ± 5.68	$19.33 \ (\pm 11.55)$	Wald $X^2 = 1296.55$, P < 0.01
Mean total anterior	25.42 ± 4.75	$9.29 \ (\pm 5.85)$	Wald $X^2 = 1229.18$, P < 0.01
Mean total posterior	21.32 ± 3.25	$10.01 \ (\pm 6.61)$	Wald $X^2 = 1143.77$, P < 0.01

Figure 3.6 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.
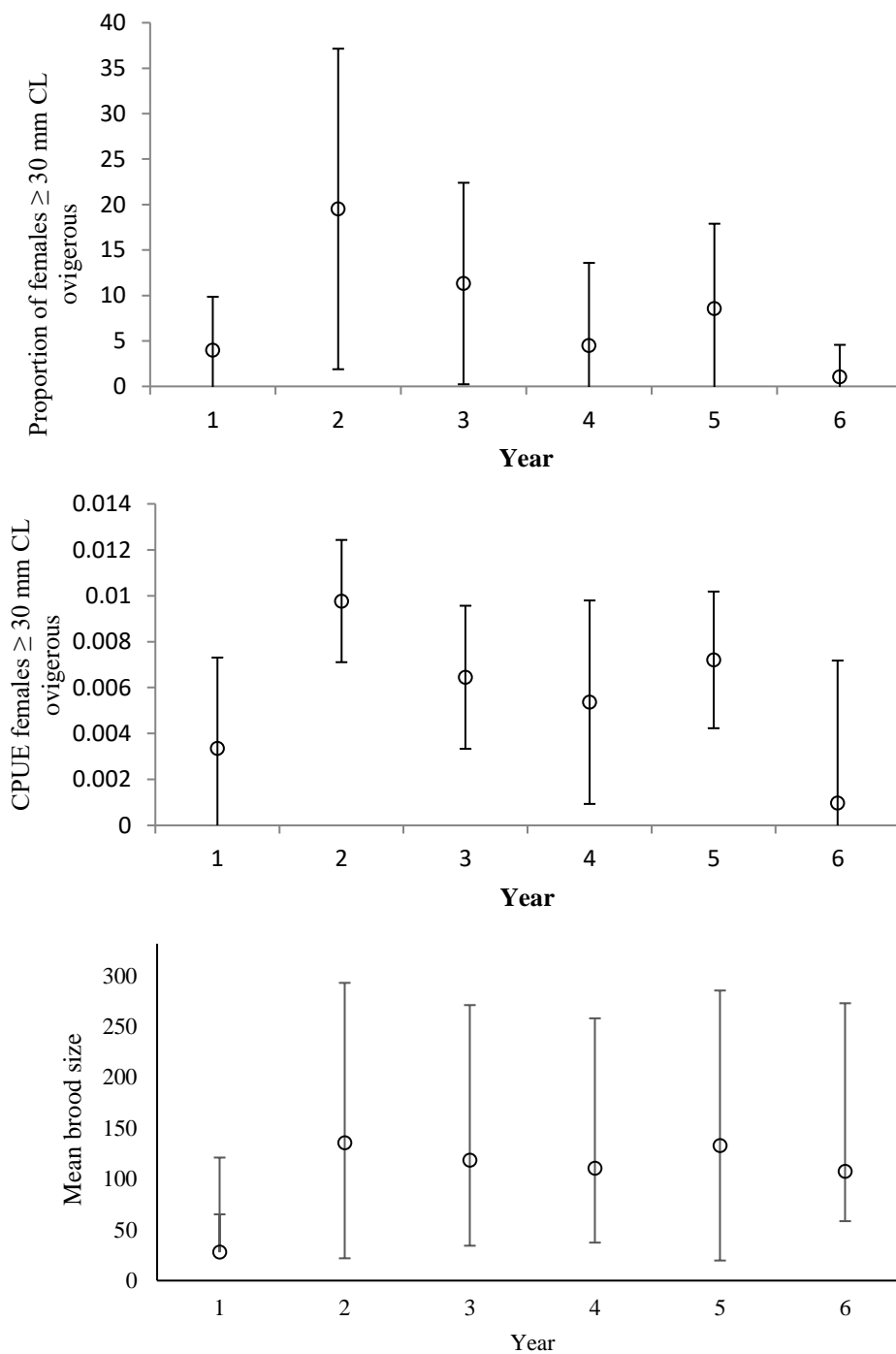
3.7. Proportion (as percentage) of females captured being ovigerous (as estimated marginal means from the best fitting GLM, top), Mean CPUE (middle) and mean brood size (bottom) at the River Barle study site Years 1 - 6. Error bars represent 95% confidence intervals.

3.4   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). Investigations here into SMRT on *P. leniusculus* enabled testing of its short-term (captive trials) and longer term (field trials) effectiveness. The results revealed 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 the gonopods were also deformed. Sterilised, tagged males that were released back into the field study site also resulted in some recaptures, indicating

some survived the SMRT process. Although gonopod regeneration was evident in these recaptured tagged males, their gonopod lengths also remained substantially and significantly smaller than non-sterilised males. This result concurs with Stebbing & 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.* (2017) who found no differences in survival or growth between tagged and untagged *A. pallipes.* Moreover, the study river is a relatively acidic upland river of low productivity, with it 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 & 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 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 per. 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, which potentially inhibited 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 90mm CL; Belchier et al. 1998), so the persistence of the sterilisation effects could arguably be longer, 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, Duperray *et al.* (2013) reported that the proportions of berried females captured following manual sterilisation of male *P. clarkii* in some French streams were related to the proportion of males sterilised. When only up to 3 % of catches comprised of 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 insufficiently low to significantly reduce the presence of ovigerous females in subsequent years.

In captive conditions, the 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. No relationship between gonopod size and spermatophore cover was identified, 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; Soutty-Grouset et al. 2018), but only if reduced spermatophore cover leads to reduced brood sizes. Indeed, the overall effectiveness of SMRT will not, however, be measured by male gonopod regeneration rates and the ability of the sterilised males to go on and have successful reproductions. It will instead be measured on its effectiveness in reducing recruitment success (Knipling 1959; Twohey et al. 2003). While recruitment success was not measured directly here, measures of female reproductive success were used. 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 clearly evident in the field data. This lack of reduced brood sizes could be an artefact of the timing of the field sampling period, as female *P. leniusculus* tend to hatch eggs between March and June in England (Guan & Wiles 1999, Holdich *et al.* 2014), whereas due to timing constraints the field data here were only able to be collected in May and June. Therefore, it is likely that the eggs of most females in the field site had already hatched prior to the start of the sampling period. This inference is supported by the annual mean percentage of sampled females that were ovigerous ranging between 1 and 8 %, concurring with Capurro *et al.* (2015), who found the percentage of female *P. leniusculus* caught being ovigerous in an oligotrophic lake in northern Italy declined from 73 % in December to 5 % in June. In contrast, Guan and Wiles (1999) found between 34 % and 59 % of females sampled over a three-year period were ovigerous in the River Great Ouse, England, but these animals were sampled at the end of April. The proportion of females spawning is related to environmental conditions (Reynolds et al. 1992), with

the River Barle system, being an upland, acidic, low productivity system compared with the mesotrophic lowland Great Ouse, likely to have a lower spawning rate regardless of timing. Nevertheless, the capture period used here was consistent between years and thus these inter-annual data were still valid for comparability, given that there was no evidence to suggest females mated by sterilised males would hatch any earlier in the year than those mated by non-sterilised males.

A 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 2020, with approximately 12000 *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 & Savolainen 2002, Parvulescu et al. 2015, Moorhouse & McDonald 2011a). It is thus possible that a reduction in fecundity 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.

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 (*Austropotamobius pallipes* and *A. italicus*) mated more frequently than smaller ones (Woodlock & Reynolds 1988; Gherardi 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 also in line with studies of other decapods. In addition, Woodlock & 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, with larger males also unable to mate effectively with small females (Woodlock & Reynolds 1988). 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. (2020) reporting that that individuals over 35 mm carapace length (CL) comprised between 1 and 5 % of a population versus 4 to 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 (Chapter 5). 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 more greater reproductive success. Finally, with the study site being an open river system, 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 invasions (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 six year period. Reasons for the apparent inability of SMRT to reduce female reproductive success were suggested as relating to small sample sizes in captive trials, and in the field low long-term survival rates of sterilised males, insufficient proportions of sterilised males in the population, and / or low reproductive efficiency in larger versus smaller males. As these could not be tested directly, they are suggested as requiring further work to understand why the technique did not result in reduced female reproductive success, especially as the technique still has potential to be more effective in more closed, lentic systems, and those of higher productivity where survival rates of the sterilised males could be higher and so the effectiveness of the sterilisation more persistent.