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A new alternative technique for sterilising invasive crayfish: removing female pleopods did not alter courtship pheromone release in signal crayfish

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Abstract – Invasive species require effective management, especially when population density is still low. Autocidal methods for controlling invasive species offer the advantages of being species-specific and inversely density dependent, without causing environmental changes. An ideal control technique should decrease numbers of juveniles, and, therefore, progressive population ageing. In crayfish, female pleopods can be removed to eliminate support for the attachment of newly fertilised eggs. The aim of this study was to investigate if pleopod removal affects the release of female sexual pheromones. An experiment was performed by exposing signal crayfish males to four waters conditioned by (1) mature females after cutting pleopods (treated), (2) untreated mature females, (3) sexually inactive females, and (4) control water. Males exposed to both treated and untreated mature female waters showed behavioural similarities and increased mating activity compared to males exposed to sexually inactive female or control waters. Removing female pleopods did not affect the release of courtship pheromones, so treated females were still able to attract males by misleading them into mating activity. When females spawn their eggs, they will be lost due to the missing pleopods. Therefore, this method might be considered to control invasive crayfish in management programmes.

Keywords: Behavioural ecology / bioassay / biological control / non-native species / swimmerets

1 Introduction

In recent years, deliberate or accidental introduction of non-native species has increased due to ongoing globalisation, free trade, and commercialisation (Chapman *et al.*, 2017). Biodiversity loss and community structure changes are among the many consequences of spreading invasive alien species. Freshwater ecosystems are more susceptible to the effects of invasive species than terrestrial ecosystems (Gherardi, 2007; Simberloff *et al.*, 2013; Reid *et al.*, 2019). This issue highlights the urgent need to manage the rapidly increasing numbers of non-native species globally.

Crayfish are the largest and among the longest-lived of the freshwater invertebrates, and some of the most widely distributed and invasive aquatic species (Souty-Grosset *et al.*, 2006). Invasive crayfish impact all levels of the aquatic ecosystem (Souty-Grosset *et al.*, 2016), for example by deteriorating macrophyte communities by grazing (Peters *et al.*, 2008; van der Wal *et al.*, 2013); reducing density and taxonomic richness of macrobenthic communities (Wilson *et al.*, 2004; Correia and Anastácio, 2008; Moorhouse *et al.*, 2014; Ercoli *et al.*, 2015); and adversely affecting benthic fish (Dorn and Mittelbach, 2004; Reynolds, 2011) and amphibian larvae (Cruz *et al.*, 2006). They also cause displacement and loss of native crayfish biodiversity (Manenti *et al.*, 2014) via competitive exclusion and disease transmission. Invasive crayfish species from North America may be lethal to European native crayfish populations by carrying the oomycete *Aphanomyces astaci* Schikora, 1906, responsible for the crayfish plague (Aquiloni *et al.*, 2011). Crayfish also impact river morphology and environment, affecting ecosystem services and regional economies (Lodge *et al.*, 2017;

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Manenti *et al*., 2019; Kouba *et al*., 2022). An emerging invader from the pet trade is the marbled crayfish (*Procambarus virginalis* Lyko, 2017), which is highly invasive due to its reproductive cycle by apomictic parthenogenesis and easy establishment of large populations from single animals (Gutekunst *et al*., 2018).

Due to the above-mentioned reasons, the implementation of management programmes to eradicate or control invasive crayfish is a priority for biodiversity conservation and assumes global importance. Unfortunately, there is no reliable methodology for the control of invasive crayfish to date, although several approaches involving mechanical, physical, biological, biocidal and autocidal techniques, or combinations of them, have considerable potential (Aquiloni *et al*., 2010; Cecchinelli *et al*., 2012; Peay *et al*., 2015; Manfrin *et al*., 2019; Krieg and Zenker, 2020; Green and Grosholz, 2021). Trapping activities have long been the main techniques to remove crayfish from the freshwater environment and manage invasive crayfish (Green *et al*., 2018). However, due to their elusive behaviour, juveniles do not usually enter traps, so adults constitute the dominant proportion of trapping catches, leaving juveniles to maintain population growth (Donato *et al*., 2018). Some crayfish species counterbalance exploitation activities with high breeding and survival rates due to increased availability of resources for juveniles (Gherardi *et al*., 2011). In noble crayfish, *Astacus astacus* (Linneus, 1758) populations, removing larger individuals reduced competition on smaller ones, thereby giving rise to larger populations (Skurdal and Qvenild, 1986). This effect on population structure may lead to earlier sexual maturity in young and small crayfish (Freeman *et al*., 2010).

In recent years, new methods have been tested to support or replace more traditional techniques, such as trapping, barriers, or biocide control (Hein *et al*., 2006; Peay and Dunn, 2014; Stebbing *et al*., 2014; Manfrin *et al*., 2019). Recently, the use of eDNA analyses of water samples facilitated detection of individuals in their early stages of the invasion process (Cai *et al*., 2017; Baudry *et al*., 2021; Greenhalgh *et al*., 2022), allowing prompt actions to be taken against alien species before their establishment. The use of sexual attractants or the release of sterile males to reduce the percentage of fertilized females have been proposed as a method to eradicate or control invasive crayfish populations already established in the invaded ecosystem (Piazza *et al*., 2015; Green *et al*., 2022). For instance, the Sterile Male Release Technique to control *Procambarus clarkii* (Girard, 1852) populations has already been investigated using ionising irradiation, resulting in a 43% reduction in the number of offspring (Aquiloni *et al*., 2009b).

The main requirement for successful eradication is removing enough individuals to achieve a density threshold (the Allee threshold), below which the population will cease to be self-sustaining and collapses (Keitt *et al*., 2001; Reynolds and Souty-Grosset, 2012). Hence, an ideal control technique should decrease eggs and juvenile production, and the population thus ages progressively until the density threshold is reached. Autocidal methods interfere with reproduction, offering advantages of being species-specific and inversely density dependent, without causing environmental contamination (Gherardi and Angiolini, 2004). Mechanical removal of male gonopods (responsible for sperm placement in Astacidae and Cambaridae families) has been effective at preventing a male from effectively depositing spermatophores, resulting in reduced female reproductive output (Stebbing *et al*., 2014; Johović *et al*., 2020). However, this sterilisation method also partly altered copulatory behaviour in red swap crayfish males (Johović *et al*., 2020). This approach was not successful in the field, where female reproductive success was unaffected over a seven-year period study in signal crayfish populations (Green *et al*., 2022).

A decrease in juvenile recruitment occurred in some crayfish populations affected by Eroded Swimmeret Syndrome (ESS), discovered recently in Fenno-Scandinavian signal crayfish populations (Edsman *et al*., 2015). This syndrome involves a multi-stage process responsible for the partial or complete erosion of female pleopods, appendages of abdominal segments that, in female crayfish, are involved in carrying eggs (Sandström *et al*., 2014). By eroding swimmerets, ESS weakens female reproductive rates, reduces clutch size, and therefore could affect crayfish population dynamics (Sandström *et al*., 2014; Jussila *et al*., 2021). Such a response makes it possible to envisage control methods based on female sterilisation by removal of pleopods to decrease reproductive success.

To date, no previous study has considered such an approach for the control of invasive crayfish. To be effective, the sterilisation technique should decrease female reproductive yield without affecting survival of sterilised females, development of glair glands involved in spawning, or mating behaviour.

During the mating period, males deposit spermatophores on the ventral surface or into the *annulus ventralis* of females. After release through ovipores, eggs are externally fertilised and attached to the pleopods for brooding (Reynolds, 2002). Many species have evolved a mate recognition system to ensure mating success, involving chemical, visual, behavioural and tactile stimuli (Aquiloni and Gherardi, 2008; Breithaupt *et al*., 2016). Receptive females emit urine-borne sex pheromones, which act like a signal that triggers mate search behaviour in males (Aquiloni *et al*., 2009a; Berry and Breithaupt, 2010).

Sterilising females through pleopod removal may affect mating behaviour; notably, whether females without pleopods are as sexually attractive as those with pleopods, thus initiating mating response from males. If pleopod removal causes stress, then females may no longer invest in mating, thus inhibiting the release of pheromones. Removal of pleopods must not affect mating, but only the ability of females to retain eggs. A prerequisite for this technique to be effective is that males do not discern treated females from untreated ones and still mate with them.

The aim of this study was to investigate whether pleopod removal affects female attractiveness for males in the invasive signal crayfish, *Pacifastacus leniusculus* (Dana, 1852). This species is ideal for testing the effects of pleopod removal, since, during the breeding season, sexual pheromone release by mature females to promote courtship and mating behavioural responses by males have already been described (Stebbing *et al*., 2003a). Moreover, since signal crayfish belong to the family Astacidae, the deposition of male spermatophores on the female ventral surface allows easy identification of female mating status (Yazicioglu *et al*., 2016). Therefore, if pleopod removal does not affect female mating

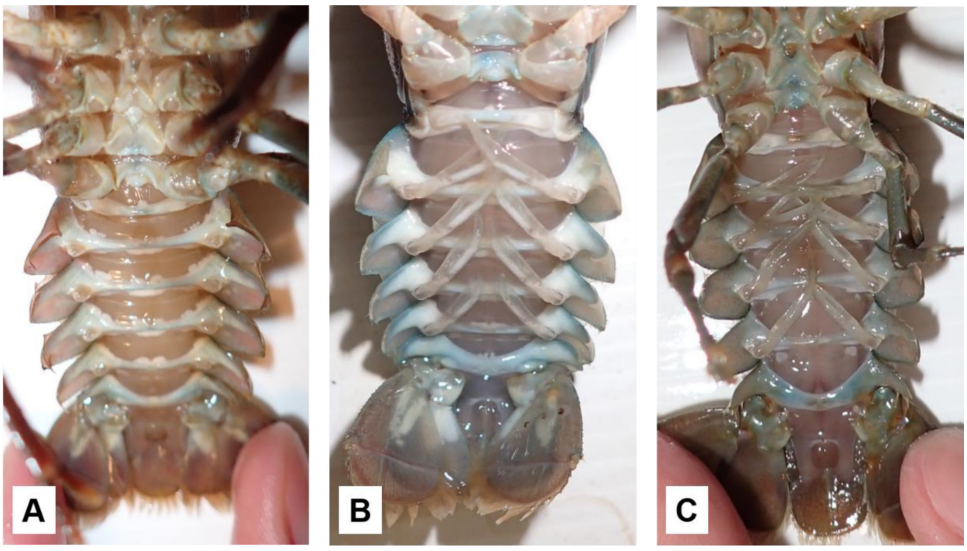

Fig. 1. Abdomen ventral view of signal crayfish females: A) treated mature female (TMF), after cutting the pleopods; B) control mature female (CMF), with pleopods; C) control sexually inactive female (CIF), with pleopods, but undeveloped glair glands.

behaviour, pheromone release by mature females should not be affected by treatment. We tested this hypothesis by exposing males to waters conditioned by females, after removing their pleopods or by untreated females, and comparing their responses.

2 Materials and methods

2.1 Study species

Signal crayfish was selected as the model species in this study for its invasive features, its well-known mating behaviour and pheromone releases during courtship (Stebbing *et al.*, 2003a), and its widespread distribution. Signal crayfish is endemic to the northwestern U.S.A. and southwestern Canada; it was first introduced into Japan in 1928 for use as food (Kawai *et al.*, 2002), and then into northern Europe (Sweden) in the 1960s to replace decreasing stocks of the native *A. astacus* (Souty-Grosset *et al.*, 2006). Currently, signal crayfish is widespread across Europe, with at least 29 invaded countries, and it is one of the most ecologically impactful invasive crayfish (Kouba *et al.*, 2014). Therefore, it is listed in the EU Regulation on Invasive Alien Species (Council of the European Communities, 2014).

2.2 Crayfish collection and holding conditions

In total, 180 signal crayfish (120 males and 60 females) were collected at the beginning of the breeding season, in October 2021, from five sites in the Valla stream in Northern Italy. Crayfish were collected by hand along the stretch from downstream (44.51971 °N, 8.3452 °E) to upstream (44.46341 °N, 8.3556 °E), during the night, when they are more active. Valla stream is the first known Italian stream ecosystem where signal crayfish successfully established, with the first report in 2009 (Candiotto *et al.*, 2010; Ghia *et al.*, 2017; Ercoli *et al.*, 2021). Once in the laboratory at the University of Pavia, crayfish were sexed and measured to record the cephalothorax length (from the tip of the rostrum to the posterior median edge of the cephalothorax) with a digital calliper (accuracy ± 0.1 mm). Then, each crayfish was individually marked on the cephalothorax using a waterproof pen. Crayfish were housed in separate sex groups in plastic tanks (50 × 35 × 33 cm), with multi-hole bricks as shelters, each containing 35 L of constantly aerated, dechlorinated tap water and maintained at room temperature (15–18 °C) under a natural light/dark cycle. Each tank was covered with a plexiglass layer and secured with weights to prevent crayfish escape. Crayfish were fed three times per week with carrots and peas. Acclimation lasted three weeks.

2.3 Conditioning of test water by females

After the first acclimation week, 32 females were assessed for sexual maturity by presence of well-developed glair glands (Taugbøl and Skurdal, 1989), with a cephalothorax length > 36 mm (Jussila *et al.*, 2021). From these females, 16 were randomly selected to remove their pleopods (treated mature females – TMF), and 16 were left intact as controls (control mature females – CMF). Pleopods were manually removed with scissors, cutting them at the base of the basipod (Fig. 1). A further control group of 16 females was selected for reproductive size, but with undeveloped glair glands (control sexually inactive females – CIF). Both CMF and CIF groups were subjected to similar manipulation as treated females, without removing pleopods. No significant difference in cephalothorax length was found among the groups (Kruskal–Wallis ANOVA test: $n = 48$, $\chi^2 = 4.55$, $df = 2$, $p = 0.103$).

Two weeks later, the three female groups were employed to prepare conditioned waters for the bioassay experiment (Stebbing *et al.*, 2003a). To provide a different stimulus for each male, and to control for potential individual female effects (*e.g.*, females releasing large or small quantities of pheromones), 25 different 4-female combinations were randomly generated for each group, employing most of the females 6–7 times and obtaining no more than four combinations with two out of four females being the same. Water was conditioned by

placing four females in a small tank containing 2 L of constantly aerated, dechlorinated tap water over a 24-hour period (Stebbing *et al.*, 2003a). To provide a non-informative stimulus, control water without crayfish was Calcium-enriched ($CaCO_3$ = 592 ppm *vs.* 124 ppm in tap water) and aerated over a 24-hour period. Then, water samples were collected from each tank, transferred into sterile 60 ml containers and stored at −20 °C for at least 24 hours (Belanger and Moore, 2006). Conditioned waters were brought to room temperature before use.

2.4 Bioassay

One hundred males were assessed for sexual maturity by presence of whitened gonopods (Reynolds, 2002) with a cephalothorax length >31 mm, as well as selected with both claws and undamaged antennae. They were randomly assigned to four experimental groups (n = 25 each group) in a way that no significant difference occurred in cephalothorax length among groups (Kruskal–Wallis ANOVA test: n = 100, χ^2 = 0.164, df = 3, p = 0.983). Before starting the experiment, each male was isolated in a single opaque tank (30 × 19 × 16 cm) and left to acclimate for at least 1 hour. Each tank contained 4 L of constantly aerated, dechlorinated tap water (aerated using a cylindrical aquarium air-stone, 2 cm in length, placed in the middle of one long side of the tank). The tank was then moved under a webcam set (Microsoft Life Cam HD-3000) with lateral curtains to simulate twilight, when crayfish become more active (Franke and Hörstgen-Schwark, 2015). A syringe containing 40 ml of conditioned water was attached to the air-stone via 50 cm of 5 mm-diameter silicone tubing. Five minutes after setting up, we started the trial. The experiment consisted of video-recording crayfish for 15 min before and after the introduction of the water treatment, and for 15 min afterwards. Conditioned water was injected into the test tank at a rate of about 4 ml/s. Following random sequences, all trials were run within one week during the breeding season, early November, in the afternoon, at 14–15 °C. Males were only used once, and the four groups were randomly assigned to each of the four stimulations: water conditioned by treated mature females (wTMF), control mature females (wCMF), sexually inactive females (wCIF), and Calcium-enriched water (wCCE). After each trial, the tanks were carefully washed and dried to remove any chemical stimuli left by crayfish from the previous trial. After the experiment, all males and females were killed by hypothermia in accordance with European and Italian laws on animal use in scientific research (Tricarico and Zanetti, 2023).

2.5 Data collection, video recording

Recording was managed by Debut Video Capture Software (NCH Software), setting quality to 960 × 720 pixels and 15 frames/s. The video-recordings were analysed using BORIS v. 7.12 (Behavioral Observation Research Interactive Software (Friard and Gamba, 2016, available at www.boris.unito.it), by an observer unaware of the treatment being watched, for the following four response variables: (i) time (seconds) of 'antennal flicking' (AntFlic); (ii) time (seconds) of 'crayfish moving' (CrayMov); (iii) frequency (number of times) of 'crayfish handling the air-stone' (Nstone); and (iv) time (seconds) of 'crayfish handling the air-stone' (Tstone). These behavioural responses might be inferred as increasing activity level by males triggered by reproductive female crayfish cues (in our study, release of pheromones). Therefore, two responses were measured as proxies for mating activity through the animals making contact with, and/or seizing, and/or mounting the air-stone (Stebbing *et al.*, 2003a). In crustaceans, antennae are mechanoreceptive organs and are highly chemosensitive, playing a role in the localisation of chemical stimuli, probably acquiring them from possible mates (Bruski and Dunham, 1990; Voigt and Atema, 1992). In decapod crustaceans, chemoreceptors on the first antennae are responsible for pheromone detection, and antennal flicking also supports molecule exchange at the receptor level (Breithaupt and Thiel, 2011). The second antennae are involved in interactive and exploratory behaviour; for instance, the animals perform highly intensive antennal movements while exploring a new aquarium to obtain information from the new environment (Zeil *et al.*, 1985; Basil and Sandeman, 2000). The behavioural response 'AntFlic' thus measured the basal activity of tactile localisation by motionless animals (Zeil *et al.*, 1985). Finally, the behavioural response 'CrayMov' measured increasing activity by the animal moving around the tank (Berry and Breithaupt, 2008). The time spent by each crayfish in behavioural responses, both before and after the addition of treatment, was calculated.

2.6 Statistical analysis

Behavioural responses were assessed by a random intercept linear mixed model (LMM) with a pair-wise comparison, to compare *ex-ante* to *ex-post* test water injection (measure) male behaviour. Behavioural variables were set as the response variable, each in a different model, while the measure (msr, before and after water injection), the treatment (trt, the three waters conditioned by females and the control water), and their interaction were the fixed effects. Male identity (id) entered the model as a random effect on the intercept to account for all other individual traits, which remain constant over the trial (*e.g.*, size, personality). Visual inspection of residual plots did not reveal any obvious deviations from homoscedasticity or normality for the response variables. Analyses were performed using the packages *lme4* (Bates *et al.*, 2023), *lmerTest* (Kuznetsova *et al.*, 2020), *interactions* (Long 2021), and *visreg* (Breheny and Burchett, 2020) in R ver. 4.1.3 (R core Team, 2022).

3 Results

All crayfish showed the basal activity of tactile localisation by AntFlic, both *ex-ante* and *ex-post* the injection treatment (Tab. 1). Similarly, all individuals moved before and after the stimulus injection, except for one male, which moved only after the treatment injection. Finally, all but 14 male crayfish handled the air-stone at least once.

The LMMs for handling the air-stone (both Nstone and Tstone) found highly significant effects in all predictors, especially for measure × treatment interactions, whereas the models for AntFlic found limited effects, as only the interaction was marginally significant (Tab. 2). Moreover,

Table 1. Descriptive statistics of the results of the behaviour of males (*ex-ante*) and the behavioural responses of males exposed (*ex-post*) to the treatment waters. Nstone: handling the air-stone as frequency (n), Tstone: time (s) of handling the air-stone as time, AntFlic: time (s) of antennal flicking, CrayMov: time (s) of crayfish moving. Sample size (N), means, standard deviation (SD), and ranges (minimum and maximum) are shown.

Behavioural response	*N*	Mean (SD)	Min-max
Nstone (n)	100	2.3 (2.47)	0–15
ex-ante	100	1.6 (1.74)	0–7
ex-post	100	3 (2.9)	0–15
Tstone (s)	100	27 (49.6)	0–394.8
ex-ante	100	11.9 (19.57)	0–117.4
ex-post	100	42.1 (64.12)	0–394.8
AntFlic (s)	100	771.4 (144.36)	154.3–940.0
ex-ante	100	778.3 (141.38)	154.3–918.4
ex-post	100	764.6 (147.68)	306.7–940.0
CrayMov (s)	100	251.5 (98.31)	0–552.5
ex-ante	100	252.3 (96.39)	0–438.3
ex-post	100	250.7 (100.68)	9–552.5

Table 2. Results for all the behavioural response variables as estimated by LMMs. msr: measure (before and after water injection); trt: treatment (the three waters conditioned by females and the control water); msr x trt: interaction. Nstone: handling the air-stone as frequency (n), Tstone: time (s) of handling the air-stone as time, AntFlic: time (s) of antennal flicking, CrayMov: time (s) of crayfish moving. Significant *p* values ($p < 0.05$) are highlighted in bold.

Behavioural response	Model	*F*	df	*p*
Nstone (n)	msr	35.92	1.96	**<0.001**
	trt	3.103	3.96	**0.030**
	msr x trt	7.270	3.96	**<0.001**
Tstone (s)	msr	27.33	1.96	**<0.001**
	trt	5.243	3.96	**0.002**
	msr x trt	5.896	3.96	**<0.001**
AntFlic (s)	msr	1.660	1.96	0.20
	trt	0.286	3.96	0.83
	msr x trt	2.746	3.96	**0.047**
CrayMov (s)	msr	0.035	1.96	0.85
	trt	0.531	3.96	0.66
	msr x trt	1.773	3.96	0.16

the LMMs for CrayMov did not detect any significant effect (Tab. 2). The interaction between measure and treatment was significant in the models for AntFlic and, notably, for handling the air-stone (for both frequency and time), whereas no significant effects were detected for CrayMov (Tab. 2).

Specifically, crayfish males exposed to both treated mature female (wTMF) and control mature female (wCMF) waters exhibited significantly increased activity of handling the air-stone for both counts (wTMF: $\beta = 2.6 \pm 0.4$, $t_{96} = 5.858$, $p < 0.001$; wCMF: $\beta = 2.1 \pm 0.4$, $t_{96} = 4.687$, $p < 0.001$; Fig. 2a) and times (wTMF: $\beta = 65.5 \pm 11.5$, $t_{96} = 5.679$, $p < 0.001$; wCMF: $\beta = 39.7 \pm 11.5$, $t_{96} = 3.438$, $p < 0.001$, Fig. 2b), but in the same way ($\beta < 22.3 \pm 12.6$, $t_{187.3} < 1.775$, $p > 0.08$; Figs. 2a, 2b). Indeed, no behavioural difference was observed in males exposed to sexually inactive female (wCIF: $\beta < 0.5 \pm 0.4$, $t_{96} < 1.172$, $p > 0.24$) or control (wCCE: $\beta < 5.7 \pm 11.5$, $t_{96} < 0.49$, $p > 0.62$) waters (Figs. 2a, 2b). Crayfish treated by wCMF significantly decreased AntFlic after the water injection ($\beta = 49.7 \pm 21.3$, $t_{96} = 2.338$, $p = 0.021$; Fig. 2c). However, no significant differences occurred between groups after water injection ($\beta < 65.3 \pm 41.1$, $t_{125} < 1.59$, $p > 0.11$; Fig. 2c).

All groups showed no significant difference for each behavioural response before the treatment injection ($\beta < 0.6 \pm 0.6$, $t_{150.2} < 0.867$, $p > 0.39$; Figs. 2a, 2b, 2c, 2d – '*ex-ante*'), and males exposed to both sexually inactive female (wCIF) and control (wCCE) waters exhibited no difference before and after the treatment ($\beta < 33.3 \pm 21.3$, $t_{96} < 1.567$, $p > 0.12$; Fig. 2).

4 Discussion

Our study showed evidence of strong behavioural similarities between signal crayfish males exposed to both treated mature female (wTMF) and control mature female (wCMF) conditioned waters. We recorded increased activity of

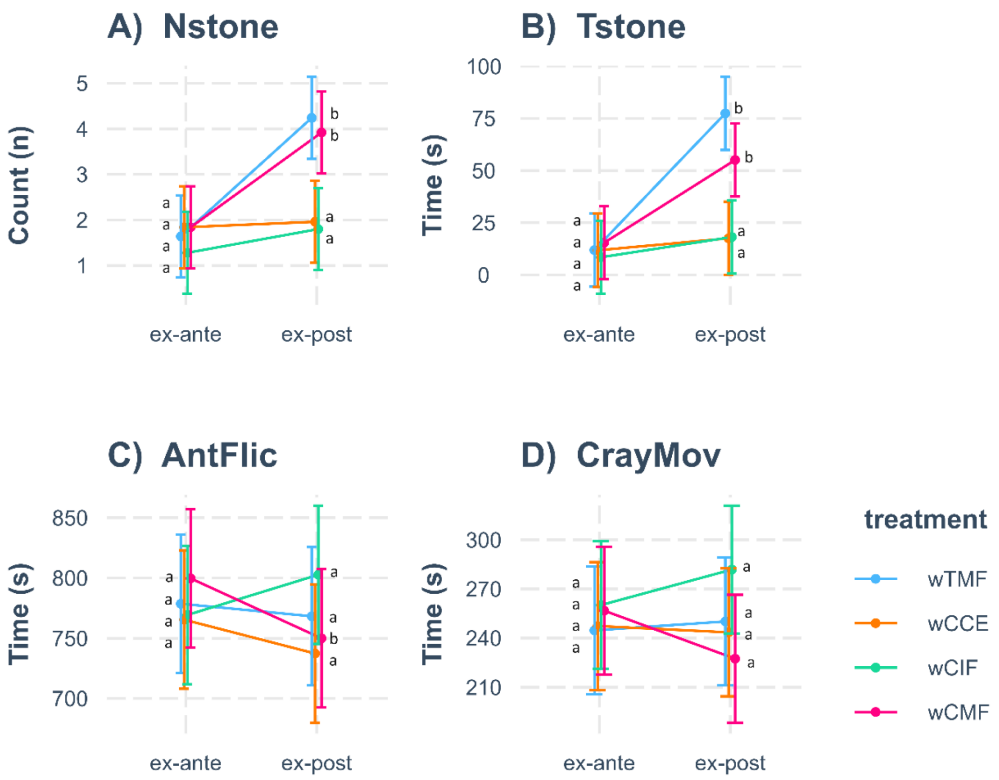

Fig. 2. Interaction plots illustrating the behaviour of males (*ex-ante*) and the behavioural responses of males exposed (*ex-post*) to the four treatment waters: Calcium-enriched water (wCCE). water conditioned by sexually inactive females (wCIF), by control mature females (wCMF) and by treated mature females (wTMF). (A) Nstone: handling the air-stone as frequency, (B) Tstone: handling the air-stone as time, (C) AntFlic: antennal flicking, (D) CrayMov: crayfish moving. Different letters (a-b) indicate statistically significant differences ($p < 0.05$); same letters (a-a or b-b) indicate no statistically difference.

handling the air-stone, as a proxy for mating behaviour, in both groups, compared to males exposed to sexually inactive female (wCIF) or control (wCCE) waters. These results provide the first, albeit indirect, support for the hypothesis that removing swimmerets does not affect courtship pheromone release by signal crayfish mature females.

Regarding the females employed in our study, we did not observe any crayfish deaths during the two-week period between pleopod removal and their use in preparing conditioned waters. Daily visual inspections also revealed no noteworthy differences in behaviour between the TMF and control groups in terms of movement or food consumption. Assessing any changes in female behaviour will be investigated further in future research.

Our findings confirmed that pheromones are released by mature female signal crayfish during the breeding season, which stimulate courtship and mating behaviour in males (Stebbing *et al*., 2003a). The behavioural variables considered in our study involved well-known and typical mating behaviours for signal crayfish. These variables allow distinctions between mating behaviour and other possible reactions, not related to reproduction, from males in the presence of female conditioned water, such as feeding or aggressive behaviour (Stebbing *et al*., 2003a). Mounting is an important pre-mating behaviour, as it prepares the male for spermatophore deposition. In recent studies, by delivering odour to males through an air-stone, crayfish increased 'handling' behaviours in response to mature female odours, but specific sexual behaviours could not be distinguished (Belanger and Moore, 2006). In our trials, no males deposited spermatophores on the surface of the air stone, despite the increase in mating behaviour. This pattern is consistent with observations made by Berry and Breithaupt (2008), whereas this behaviour was reported for two signal crayfish males by Stebbing *et al*. (2003a). A lack of significant differences in males tested with wCIF indicated that sexually inactive females do not release pheromones during the breeding season (no difference was observed compared to the wCCE group), and that the behaviour of males exposed to both wTMF and wCMF waters is related to reproduction.

Exploratory behaviour in decapod crustaceans is displayed by highly intensive antennae movements, to obtain information from new environments (Zeil *et al*., 1985; Basil and Sandeman, 2000). In our study, although males exposed to wCMF significantly decreased their antennal movement, they did not show different activity after treatment when compared to other male groups exposed to wTMF, wCIF and wCCE. This result could be explained by a more homogeneous reaction of males exposed to wCMF.

Organisms living in freshwater environments, where other cues may be limited (*e.g.*, visual), depend mainly on chemical signals, or in combination with environmental signals, for reproduction (Corkum and Belanger, 2007). Urine-blocking experiments demonstrated that female crayfish urine contains

sex-specific components, to which reproductive males respond (Corkum and Belanger, 2007; Berry and Breithaupt, 2008; Berry and Breithaupt, 2010). Sexual pheromones appear to be species-specific (*e.g.*, those of signal crayfish are repellent to white-clawed crayfish) and are crucial in attracting males and stimulating mating (Stebbing *et al*., 2003b). Increased activity characterises the mating period in crayfish, when sexually mature adults actively search for partners (Buřič *et al*., 2009). Both mating behaviour and egg laying in females are controlled by pheromones and influenced by environmental stimuli, such as water temperature and photoperiod (Dubé and Portelance, 1992; Yazicioglu *et al*., 2016). After fertilisation, the eggs attach to the pleopods of female for brooding (Reynolds, 2002).

In signal crayfish populations with a high prevalence of infection by ESS, a potential decrease in juvenile recruitment has been observed, since females affected by ESS are unable to retain all of their eggs with their remaining pleopods. Each lost or damaged pleopod would result in roughly 12.5% egg loss (Edsman *et al*., 2015). This estimate seems valid also for regenerated swimmerets following moults, since only undamaged swimmerets have been observed to successfully carry a full egg mass during incubation (Jussila *et al*., 2021). Based on observations of trends in signal crayfish populations affected by ESS, any modifications to pleopod morphology (either by disease or manual removal) would affect the ability of females to carry their eggs, which consequently will impact their spawning success. Therefore, the sterilisation of females could be an alternative, novel technique for controlling and decreasing invasive crayfish populations. Sterilised females must behave similarly to unsterilized ones to attract males by misleading them into mating activity. The result would be twofold: on one hand, females release eggs that will be lost into the environment due to the lack of physical support provided by the pleopods; or spermatophore release still occurs by the male. Although males can have multiple mates with females, more mating with sterilised females will lead to a decrease in the amount of spermatophore a male can utilise in future matings. Therefore, in the likely case that some females could not be sterilised, and if the probability of a male mating with sterilised or unsterilised females was similar, then the number of matings leading to offspring would decrease. Our results on female sterilisation also do not seem to have altered the mating behaviour of signal crayfish males, as instead appeared to occur in sterilised *P. clarkii* males, that also partly modified their precopulatory and copulatory behaviour, reducing the male chances for successful copulation (Johović *et al*., 2020).

However, the behavioural responses of males in bioassays have not always been consistent, but varied depending on maturity, as reported for the green crab *Carcinus maenas* (Linnaeus, 1758) (Fletcher and Hardege, 2009). Although in our study the use of only males for performing the assessment behaviour on water samples conditioned by females solved this issue, these aspects are worth further investigation. After testing the pheromone release from mature crayfish females sterilised by removing pleopods, and concurrently observing unvarying mating behaviour in males, the next step in our research will focus on female fecundity, pleopod regeneration rate, and mating experiments to evaluate the potential decreases in the number of eggs and juvenile recruitment. The proper functioning of pleopods is essential for the survival and development of crayfish eggs, as they are responsible for their cleaning and oxygenation. Without the support of female pleopods, eggs would disperse into the aquatic environment and become nonviable, lacking the necessary conditions for successful hatching (Reynold, 2002). Artificial incubation of crayfish eggs involves cylindrical containers with a slow upward water flow, which keeps the eggs suspended and well-oxygenated (Matthews and Reynolds, 1995).

One of the main requirements needed for successfully managing invasive species by sterilisation is to implement a technique that does not compromise their physiological condition, but rather maintains their natural behaviour.

5 Conclusions

Our study is the first known attempt to provide a basis for an alternative control method based on female crayfish sterilisation. It provides an initial assessment that females released courtship pheromones even after removing pleopods. This method could reduce female fertility, providing an effective, alternative method to control the spread of invasive crayfish populations. Compared to other, existing control methods, our approach also could apply to all crayfish species. Sterilisation of females would also provide an additional advantage for managing invasive crayfish belonging to the superfamily Parastacoidea, whose males do not have the gonopods, distinctive from Astacoidea (Riek, 1972; Horwitz, 1988). Furthermore, female sterilisation would especially target a highly invasive species, the marbled crayfish (*Procambarus virginalis* Lyko, 2017), which reproduces by apomictic parthenogenesis, facilitating the establishment of large populations from single animals (Gutekunst *et al*., 2018).

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Conflicts of interest

The authors declare no competing interests.

Author contribution statement

Conceptualisation: DG, GF, RS. Formal analysis: RS, DG, FE. Fieldwork and investigation: SM, DG, GF. PhD of DG Supervision: RS, FE. Writing original draft: DG, RS. All authors contributed and approved the final manuscript.

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