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Research Article

Improving trapping effectiveness for controlling the red swamp crayfish *Procambarus clarkii*

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Abstract

In Tuscany (Central Italy), a population of the invasive alien red swamp crayfish *Procambarus clarkii* is present in Lake Romena, close to a national park, and threatens the conservation of the native white-clawed crayfish *Austropotamobius pallipes* complex. A field study was conducted to reduce the abundance of the *P. clarkii* population through intensive trapping activities and improve the effectiveness of catches using three different types of traps: two wire mesh traps (cylindrical and rectangular) and artificial refuge traps. The study also aimed at assessing the composition of the lake animal community, particularly the presence of crayfish predators (using eDNA), and the potential spread of *P. clarkii* outside the lake. The control activities conducted over two trapping seasons in 2022–2023 led to a decrease of at least 50% in the abundance index (Catch Per Unit Effort) of the crayfish population. Cylindrical traps caught more individuals, especially larger ones and males, with artificial refuge traps capturing relatively more females and smaller individuals. eDNA sampling highlighted the presence of a diversified community, mainly composed of alien species, and some crayfish predators (e.g. fish). The surveys conducted in the surrounding areas revealed the presence of *P. clarkii* downstream of the lake. Control activities using different types of traps should be maintained to decrease further the *P. clarkii* population abundance, while additional management activities should be carried out to halt the spread of the species outside the lake in order to prevent its further ecological impacts.

Key words: alien species, Artificial Refuge Trap, wire mesh trap, Central Italy, bycatch, crustacean

Introduction

Freshwater ecosystems provide support for several important ecosystem services, such as water supply, water quality control, habitat provision, erosion prevention as well as food supply, maintenance of biodiversity and climate regulation (Kaval 2019). However, they are highly vulnerable to biological invasions whose effects can be exacerbated by climate change (Francis 2012; Gallardo et al. 2016; Reid et al. 2019; Salis et al. 2023). Managing populations of some aquatic invasive alien species (e.g. crayfish, freshwater mussels, and many fish) often remains an unsolved problem (García-de-Lomas et al. 2020). This is due to the lack of effective eradication and control methods compatible with the maintenance of ecosystem services (e.g. water supply for humans, livestock or wildlife) and preservation of native species (Dana et al. 2019). Additionally, the huge number of established populations in a variety of environments makes management very costly and not always effective or possible.

The red swamp crayfish *Procambarus clarkii* (Girard, 1852) is the most widespread invasive alien crayfish species in Italy where it was introduced in the 1980s for aquaculture purposes (Tricarico and Zanetti 2023). Established populations are reported in 18 out of the 20 Italian regions, including the islands of Sardinia and Sicily. The preferred habitat of *P. clarkii* is lentic environments, such as swamps and marshes also subject to strong seasonal fluctuations and temporary desiccation, although the species can colonize any types of aquatic environments, including brackish and cave waters (Mazza et al. 2014; Souty-Grosset et al. 2016; Dörr et al. 2020; Nota et al. 2024). Through its omnivorous and opportunistic feeding habits, *P. clarkii* can exert negative ecological impacts on the invaded ecosystems, predating and consuming aquatic macroinvertebrates, amphibian larvae, fish eggs, and aquatic plants (e.g. Souty-Grosset et al. 2016). Moreover, it actively digs, especially in silty-clayey soils, increasing water turbidity (which reduces light penetration and consequent primary productivity) and instability of banks leading to collapse (Souty-Grosset et al. 2016; Bendoni et al. 2024). *Procambarus clarkii* is included in the List of invasive alien species of Union Concern of the EU Regulation 1143/2014 and therefore its management is mandatory. However, when the species is widespread as in Italy, site prioritization for management actions is recommended, especially in closed or confined environments within or close to protected areas.

Tuscany (Central Italy), one of the first reports of the species in Italy (Tricarico and Zanetti 2023), has been widely colonized by *P. clarkii*, especially in the alluvial plain areas. Since 2015, the species has been reported in Lake Romena (Mazza et al. 2017), which is located in the hilly area of the Pratovecchio-Stia municipality in Northeastern Tuscany. Here, the species represents a threat to the biodiversity of the Foreste Casentinesi Monte Falterona and Campigna National Park. Indeed, this population of *P. clarkii* is the only one reported in the area and is close to the National Park (about 5 km away) that hosts several native species of conservation concern. These include the white-clawed crayfish *Austropotamobius pallipes* complex (Lereboullet, 1858), the Apennine yellow-bellied toad *Bombina pachypus* (Bonaparte, 1838), and the northern spectacled salamander *Salamandrina perspicillata* (Savi, 1821). *Procambarus clarkii* can be a vector of the crayfish plague caused by the oomycete *Aphanomyces astaci* (Schikora) and lethal to native crayfish, whose spores can potentially be transported by fish, birds, and non-disinfected fishing gears, boots and nets (Souty-Grosset et al. 2016). Episodes of plague have already been reported within the National Park (Tobia Pretto, pers. comm.). *Procambarus clarkii* can also carry the chytrid fungus *Batrachochytrium dendrobatidis* (Longcore, Pessier & D.K. Nichols 1999), which causes chytridiomycosis – a disease that affects amphibians with almost always lethal consequences (Souty-Grosset et al. 2016; Scheele et al. 2019). The control of *P. clarkii* is therefore necessary to avoid a further population decline of the native crayfish present in the National Park, which is already threatened by habitat fragmentation, climate change, poaching, and other invasive alien species such as introduced salmonids and the American raccoon *Procyon lotor* (Linnaeus, 1758) (Mazza et al. 2011; Boncompagni et al. 2021; Tricarico et al. 2021). To this end, to protect its biodiversity, the National Park promoted and supported a management activity on *P. clarkii*.

The main aim of the present work was to reduce the population abundance of *P. clarkii* in Lake Romena through intensive trapping activities carried out from May to September in 2022 and 2023. The secondary aim was to evaluate the effectiveness of different types of traps to improve the efficacy of control activities. Finally, traditional monitoring activities with traps were carried out to evaluate whether the species was spreading outside of the lake and getting closer to the National Park, while sampling using environmental DNA was performed for a preliminary identification of the potential predators of *P. clarkii* and composition of the lake community (invertebrates and vertebrates).

Materials and methods

Study area

Field activities were conducted at Lake Romena (N 43.769796, E 11.707523), Pratovecchio-Stia, Arezzo Province (Northeastern Tuscany), Italy, 520 m a. s. l. Lake Romena is an artificial water body used for sport fishing (perimeter: 500 m; area: 13000 m^2; max depth: 10 m; Alessandro Volpone, pers. comm.), with a tributary and an outflowing stream (Fosso Camboffoli). The lake substratum is a mix of silt and boulders, and the banks are covered for approximately three-quarters by trees (mainly *Quercus* sp. and *Ostrya* sp.) and for the remaining quarter by cattail (*Typha* sp.). In some parts, the banks are steep, almost vertical. The lake is used for irrigating the surrounding agricultural fields during the summer, and this can lead to a significant decrease of water level, leaving some crayfish burrows exposed (e.g. from May to September 2023, the water level dropped by 125 cm). Due to the easy accessibility of the area, there are other alien species in this lake, probably introduced for fishing or else dumped by people. Some of these species are notable invaders, such as the American pond slider *Trachemys scripta*

(Thunberg in Schoepff, 1792) and fishes (e.g. largemouth bass *Micropterus salmoides* (Lacepède, 1802)). Some species were caught during the monitoring activities, others were identified through eDNA analysis carried out in 2022 (see below).

Control and monitoring activities

Control activities were performed in 2022 and 2023, from May to September (the species' maximum activity period), during one session per month lasting five–six days (in the first day traps were set up) for a total of 47 trapping nights using 78 traps at maximum per night. To evaluate the effectiveness of different types of traps and thus improve the efficacy of control activities, two types of traps with bait were used per each session: 40-43 wire mesh double entrances cylindric traps (30 × 60 cm; mesh: 12 mm), and 20–28 wire mesh double entrances rectangular traps (25 × 25 × 50 cm; mesh: 5 mm), more suitable for capturing smaller individuals but with a less narrow entrance. Traps were positioned semi-submerged to avoid the death of non-target species captured (such as amphibians and reptiles). Traps were baited with cat food (a perforated can containing 100 g). Following Green et al. (2018), 10 Artificial Refuge Traps (ARTs) made up of 7 PVC pipes 170 mm long, attached to an aluminium plate, were employed. Pipes of three different diameters were used: 50 mm (one pipe), 40 mm (three pipes) and 32 mm (three pipes). In this way, the ARTs were capable of capturing 7 individuals maximum (very rarely two crayfish enter the same pipe). The ARTs mimic burrows and their use is recommended for catching young or small individuals and females with eggs, which usually avoid the traditional traps and often remain in their burrows (Green et al. 2018). The ARTs without any bait were located near the banks where crayfish usually dig their burrows. All the traps were placed along banks in accessible sites and regularly distanced (average 10 m).

For convenience, the study area was divided into three sectors: sector L, corresponding to the main lake; sector E, to the outflowing stream; sector I, to the tributary (Figure 1A). In 2022, due to low water level, it was possible to conduct the trapping activities in sector E only in May, and never in sector I. In 2023, water levels allowed traps to be placed in sectors E and I only in May and June. The number and location of traps changed over time, following variation in water level, consequently changing the accessibility to the lake that, however, never dried. Traps were checked and bait changed daily (in the morning). Sometimes traps were moved, by people or wildlife, and therefore their efficacy and use were not optimal. These were marked as 'disturbed' (114 times in total) and were relocated correctly in the original place.

The number, sex, and size of all trapped individuals were annotated. Cephalothorax length (CL, from the tip of the rostrum to the posterior edge of the carapace) was measured using a caliper of 0.1 mm of precision, although measurements were rounded to 0.5 mm. Following Inghilesi et al. (2014), trapped crayfish were divided in two size classes: large CL ≥ 35 mm

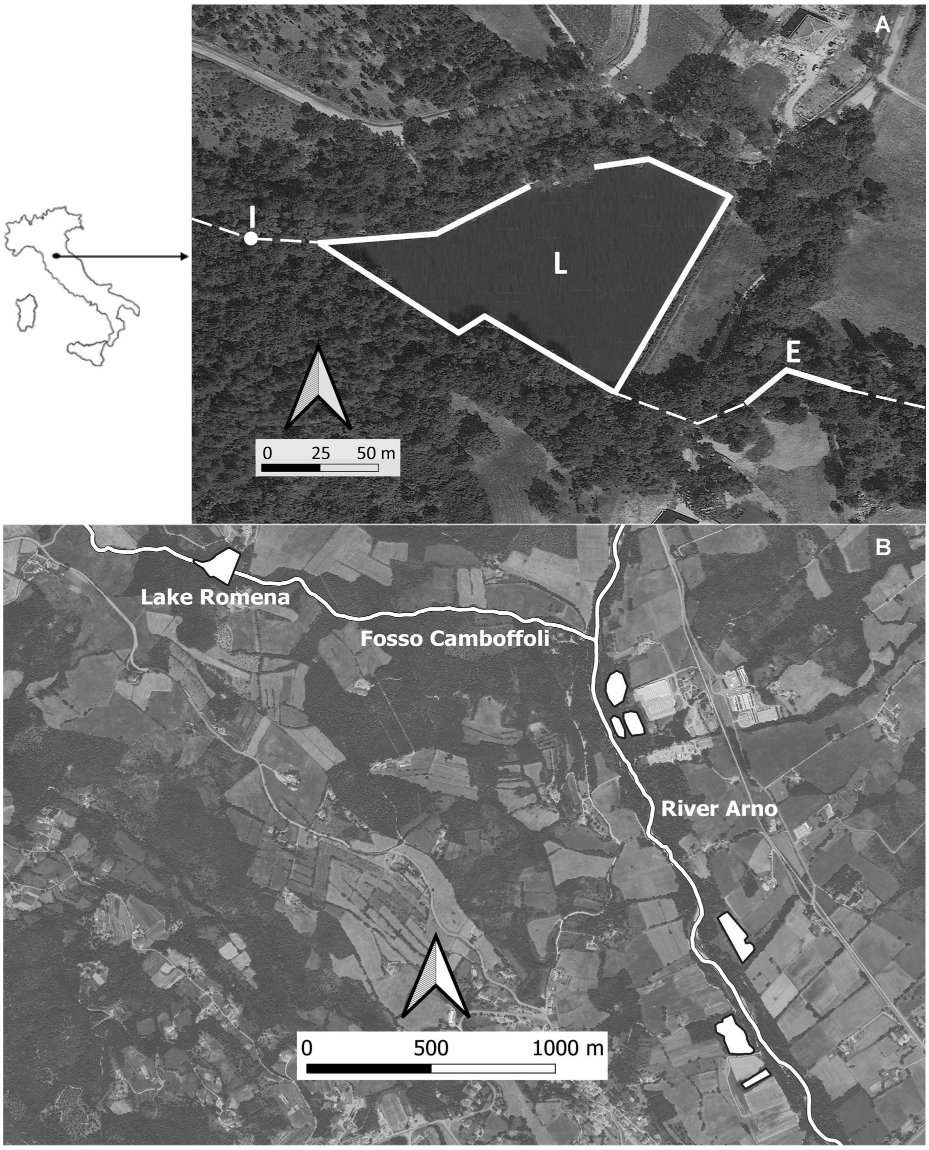

Figure 1. (A) The three sectors of Lake Romena sampled in the control activities: L = main lake; I = tributary sector; E = outflowing stream Fosso Camboffoli. (B) The six lakes monitored downstream of the confluence between Fosso Camboffoli and the River Arno. Top left Lake Romena.

and small CL < 35 mm. Reproductive status of females was also checked (glair glands and egg presence). Sex-ratio was calculated as the ratio of males to females. Some individuals were found preyed upon inside the trap, and their size or sex, or both, were not identifiable; hence, they were classified as 'not determined' (23 individuals in total). Trapped animals were humanely killed, in accordance with national legislation (Tricarico and Zanetti 2023), stored in the fridge at 4 °C for 24 hours, and then in the freezer for one week. All non-target species individuals found in the traps were also recorded and released following agreement with the lake owner. Daily surface water temperature was measured with a thermometer at three different points of the lake.

To assess the potential spread of the species in the area, in 2022 and 2023, monitoring activities were carried out in the water bodies connected or close to the lake. Particularly, Fosso Camboffoli (downstream of the sector E), Fosso delle Pillozze, the nearest stream that flows to the River Arno, and Fiumicello stream (inside the town of Pratovecchio) were monitored. Furthermore, downstream of the confluence of Fosso Camboffoli with River Arno, six lakes were monitored for 1-2 days using the same cylindrical traps described above and baited with cat food (Figure 1B).

eDNA

As it was not possible to conduct fish sampling activities, eDNA sampling, performed on 25/05/2022, was carried out at five equidistant points within the lake (Supplementary material Figure S1), generated using OruxMaps® (https://www.oruxmaps.com). For each site, 0.5 L of water was filtered in the field following Thomas et al. (2020). At the beginning and end of the eDNA sampling campaign, deionized, distilled, and autoclaved water was filtered as a negative filtration control, or filtration blank. The protocol used for eDNA filtration was adopted from Laramie et al. (2015). The filters, once wrapped in silver paper and stored in a cooler bag cooled by eutectic plates, were transported to the Department of Biology at the University of Pisa, where they were preserved in a freezer at −80°C.

Environmental DNA was extracted from the filters following the ‘DNeasy Blood & Tissue’ extraction protocol (QIAGEN), except for the final elution step, where the volume was halved. Extractions were performed under a biological safety cabinet, previously sterilized using UV light. For this phase, half of the filter was used, and the leftover was stored at −80°C. Additionally, DNA was extracted from an unused filter as a negative extraction control, or extraction blank. Subsequently, all extractions were quantified using the Qubit™ dsDNA HS Assay Kits (Invitrogen™) quantification kit. The DNA samples chosen and used in the amplification reactions were as follows: five extracted eDNA samples belonging to the five sampling sites, two negative filtration controls, and the negative extraction control and deionized water for the Polymerase Chain Reaction (PCR) negative control. Samples were amplified with two different set of primers, one amplifying a 97 bp ca., fragment of the 12S rRNA mitochondrial gene and specific for vertebrates (Riaz et al. 2011) and the other amplifying a fragment of 142 bp ca., belonging to the COI gene, specific for macro-invertebrates (Leese et al. 2021). A sample of DNA extracted from tissues of the European hedgehog (*Erinaceus europaeus* (Linnaeus, 1758), order Eulipotyphla) for 12S and the water scorpion (*Nepa cinerea* (Linnaeus, 1758), order Hemiptera) for COI was used as the positive control.

The PCR reactions, performed in triplicate for the two molecular markers, were carried out in a final volume of 20 μL per sample, using a thermocycler (T100 Thermal Cycler, Bio-Rad), with the following thermal cycles: 10 minutes of initial denaturation at 95°C, followed by 35 cycles of 95°C for 30 seconds, 50°C of annealing for one minute, 30 seconds at 72°C of elongation for COI marker, and 40 cycles of 95°C for 30 seconds, 47°C for one minute, and 30 seconds at 72°C for 12S rRNA. Final elongation was at 72°C for seven minutes for both markers. Once the outcome of PCR reactions was confirmed via 2% agarose gel electrophoresis, the five amplified eDNA samples were pooled for each of the three replicates. The PCR negative controls and extraction controls for the three PCR replicates were respectively pooled into two pools, while two pools were generated with the most contaminated filtration blank (calculated with DNA quantification see results). The pools were purified using the Euroclone spinNAker GEL & PCR DNA purification kit (Euroclone), and purification was checked and analyzed using 2% agarose gel electrophoresis. Then, quantification was performed using the Qubit™ dsDNA HS Assay Kits (Invitrogen™) kit. Notwithstanding the lack of evidence of successful amplification through PCR of all negative controls (no bands on gel electrophoresis), blanks were carried on during the following steps.

Sequencing of environmental DNA samples was performed at GENEWIZ from Azenta Life Sciences. For this project, Illumina Novaseq 6000 paired end 250 sequencing technology was utilized. Once obtained, reads were denoised, merged and chimeras were removed trough DADA2 (–p-chimera-method ‘consensus’). Then, a QIIME2 customized pipeline (Cananzi *et al.* 2022) was used to taxonomically assign Amplicon Sequence Variants (ASVs). Databases selected for each marker were BOLDfor COI (Ratnasingham and Hebert 2007) and CALeDNA database for 12S rRNA (Meyer et al. 2021). Taxonomic assignments were manually reviewed using BLAST (Altschul et al. 1990) (basic local alignment search tool, NCBI). Species sequences detected in any negative control were removed from the analyses.

Data analysis

Statistical analyses were performed with the RStudio software (R version 4.3.2, 2023-10-31 ucrt) at a significance level (α) of 0.05. Catch per Unit Effort (CPUE) index (calculated as total number of caught crayfish per trap per day) was used to estimate population abundance and compare it over time and among trapping methods. A decrease in CPUE was considered an indicator of successful control activities. From the calculation of CPUE, the so-called disturbed traps were removed. Normality of the data was checked using the Shapiro-Wilk test. Linear regressions (R^2) were performed between time/CPUE and temperature/CPUE, and temporal linear regressions were applied for both sex- and size-ratio (calculated as the ratio of large to small individuals) for the three trap types. Three-way ANOVA and Tukey *post hoc* test were used for comparing the CPUE by the factors trap, sex, and size. Data of CPUE were subject to the natural logarithmic transformation for the ANOVA.

Results

Control and monitoring activities

Overall, a total of 4621 crayfish were captured: 1724 in 2022 and 2897 in 2023. In total, 4603 crayfish were trapped within the lake (99.61% of the total catches; mean CL: 40.91 mm and sex-ratio: 1.38), 17 individuals were captured in sector E (0.37% of the total catches; mean CL: 26.06 mm and sex-ratio: 0.21) and only one female with a CL of 34 mm was caught in sector I (0.02% of the total). Laboratory analyses for the crayfish plague conducted on 60 individuals captured in 2022 showed the absence of this disease.

The overall CPUE was 1.37: 1.11 in 2022 and 1.6 in 2023. The trend in monthly CPUE was similar for both years, with higher values in June and lower values in September (Figure 2A). No statistically significant linear trend was found between time and CPUE ($R^2 = 0.003$, df = 1,8, $P = 0.89$). The average water temperature changed during the two years. Thus, 2022 was a particularly hot and dry year from the beginning of summer, where in 2023 there was abundant rainfall in May. Similar to time intervals in months, CPUE was not linearly related to the temperature ($R^2 = 0.08$, df = 1,8, $P = 0.43$).

The CPUE values differed per trap type, with cylindrical traps capturing more than the other traps, and per size, with large individuals being more trapped than the small ones (Figure 2B). Three-way ANOVA found only a significant interaction between trap and size: cylindrical and rectangular traps almost always captured more large individuals, whereas in ARTs the two size classes were more balanced (Table 1). The Tukey *post hoc* test showed a significant difference between cylindrical traps and ARTs ($P < 0.001$) and between rectangular traps and ARTs ($P < 0.05$), but not between cylindrical and rectangular traps ($P > 0.05$). No significant interaction was found among trap, size, and sex, or between trap and sex, even if there was a tendency to have more males in cylindrical and rectangular traps (Table 1). Tukey multiple comparison for the combination trap/size showed the highest CPUE for cylindrical traps capturing large individuals followed by CPUE of rectangular traps capturing large individuals (Figure 2C).

Higher sex-ratio values, hence more males, were found in September and more balanced ratios in May for both years (Figure 3A). June showed the highest value of ratio between sizes, meaning larger individuals being captured (Figure 3B). The temporal linear regressions (six in total) applied for both sex and size ratio for each trap type did not show any significant results (P always > 0.05). In total, 162 individuals of non-target species, including fish, amphibians, and reptiles, were captured (Table 2). The CPUE of non-target species was higher for cylindrical traps (0.07) than the rectangular ones (0.03).

The monitoring activities highlighted the presence of *P. clarkii* outside the lake. In the tributary, sector I, only one crayfish was found in 2023, whereas in the outflowing stream, sector E, 21 crayfish were found (and 21 others were seen) during both the 2022 and 2023 seasons. Monitoring in the six lakes on the right and left hydrographic side of the River Arno, downstream of the confluence with Fosso Camboffoli, confirmed the presence of the species in two lakes with the capture of 12 crayfish.

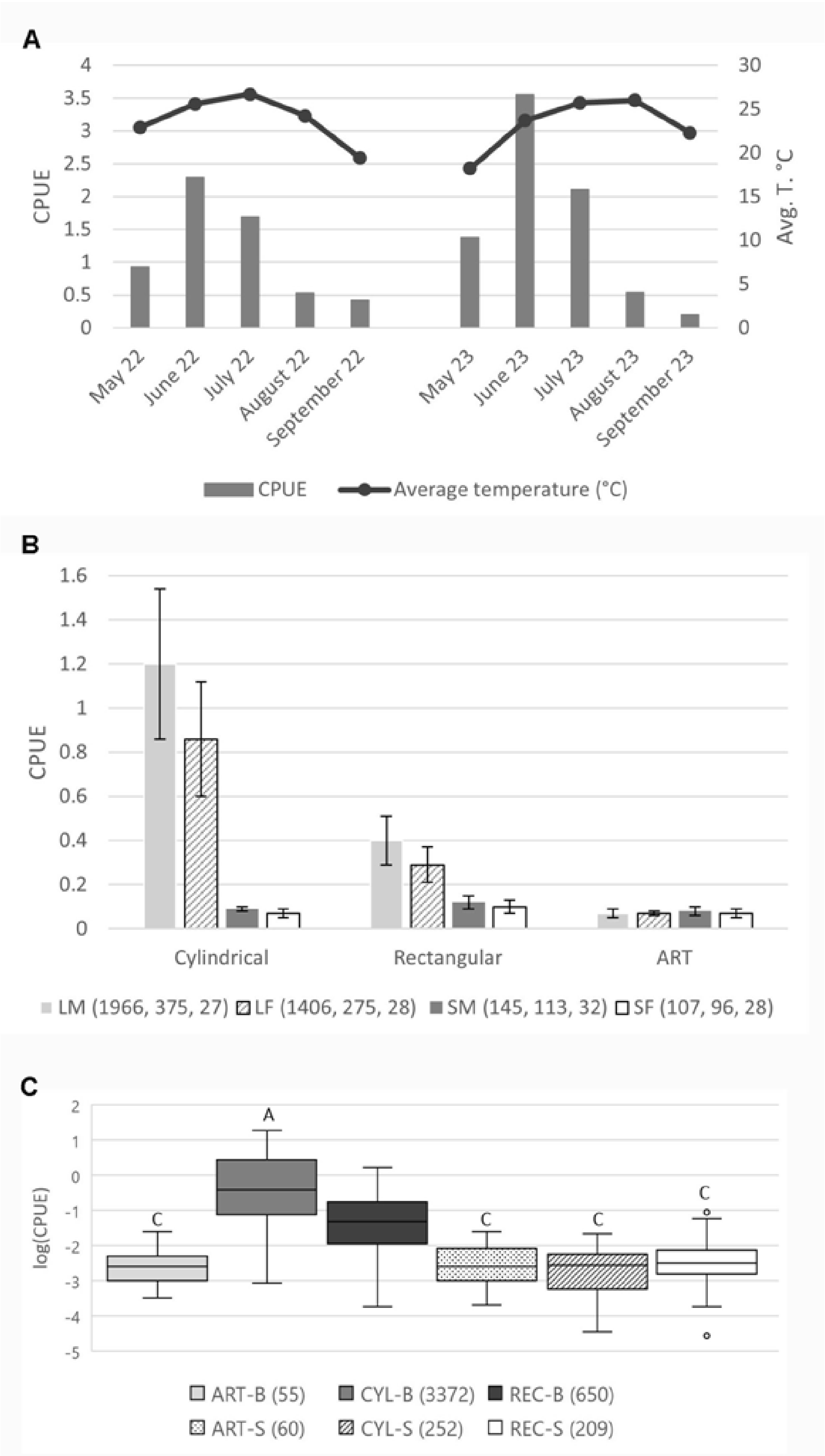

Figure 2. (A) CPUE and average temperature per month. (B) CPUE per large males (LM), large females (LF), small males (SM) and small females (SF) of *Procambarus clarkii* by trap types. Bars represent means ± S.E. (C) log(CPUE) boxplots per trap type (CYL: cylindrical, REC: rectangular) and size of *Procambarus clarkii* (L: large, CL ≥ 35 mm and S: small, CL < 35 mm). N is indicated in the legend. Letters over boxplots indicate the hierarchy after Tukey post hoc tests.

eDNA

For both molecular markers, the amplified and purified eDNA samples yielded good DNA concentrations (Table S1). For vertebrates, the negative filtration control performed after the eDNA samples returned a high DNA

Table 1. Comparison of CPUE per trap, sex, and size of *Procambarus clarkii* using the three-ways ANOVA. Significant p-values (p) are indicated in bold.

	Df	Sum Sq.	Mean Sq.	F	p
Trap	2	19.83	9.92	13.28	**< 0.001**
Sex	1	2.84	2.84	3.81	0.05
Size	1	42.75	42.75	57.23	**< 0.001**
Trap * sex	2	0.21	0.11	0.14	0.87
Trap * size	2	25.68	12.84	17.91	**< 0.001**
Sex * size	1	0.11	0.11	0.15	0.7
Trap * sex * size	2	0.02	0.01	0.01	0.99
Residuals	102	76.2	0.75		

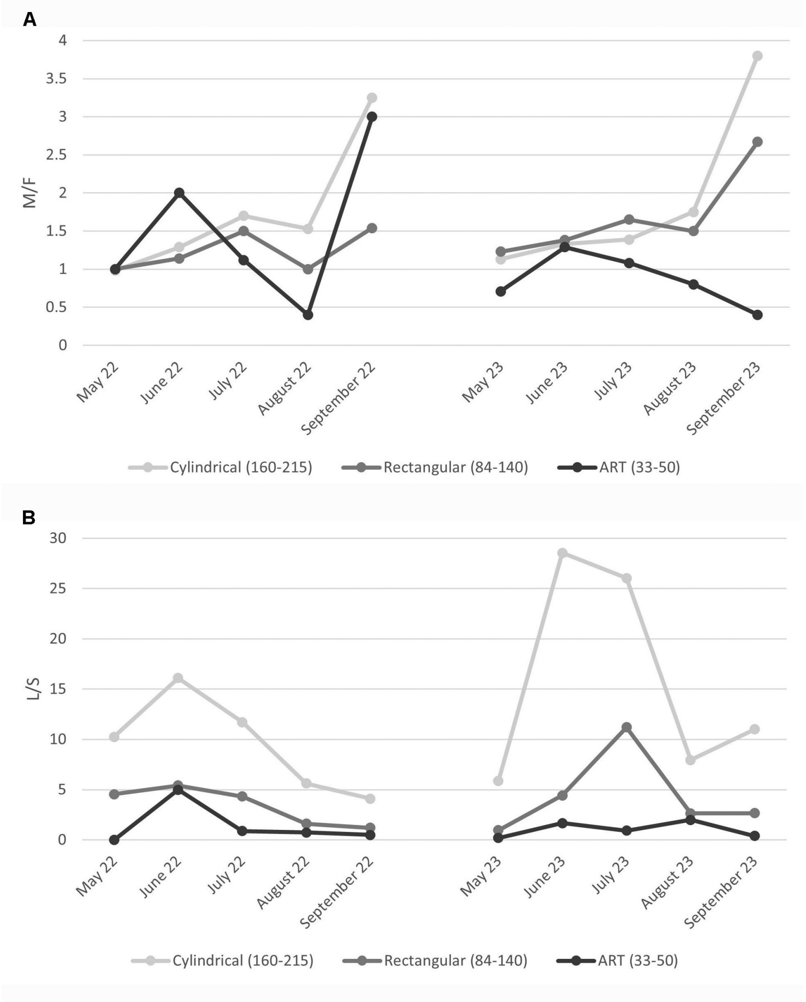

Figure 3. (A) Sex-ratio (M/F) of *Procambarus clarkii* by trap type per month. M=male; F=female. (B) Size-ratio (L/S) of *P. clarkii* by trap type per trap month. L: large, CL ≥ 35 mm and S: small, CL < 35 mm. Number of traps, minimum and maximum, indicated in the legend.

content, probably because the 12S rRNA marker used, targeting vertebrates, also amplified *Homo sapiens* (Linnaeus, 1758). The sequencing of eDNA samples (excluding negative controls) returned an average of 46k reads ca, reduced to 25k ca after denoising (see Table S2 for detailed per sample denoising statistics).

Regarding the results of the COI metabarcoding essay, obtained 20 taxonomic units were obtained from the eDNA samples (Table S3). Among these, 10% were assigned at the family level, 55% at the genus level, and 35%

Table 2. Number (and percentage) of individuals per non-target species, (A): alien species.

Species	N (%)
Largemouth bass (*Micropterus salmoides*) (A)	81 (50.0)
Perch (*Perca fluviatilis*) (A)	63 (38.9)
Pond slider (*Trachemys scripta*) (A)	8 (4.9)
Water frogs (*Pelophylax* sp.)	4 (2.5)
Common toad (*Bufo bufo*)	3 (1.9)
False map turtle (*Graptemys pseudogeographica)* (A)	2 (1.2)
Italian stream frog (*Rana italica*)	1 (0.6)
Total	162 (100)

at species level. In this case, many assignments stopped at the genus level, largely due to the limited literature information available on macro-invertebrates. 70% of the obtained samples were associated with the insects, 20% with crustaceans, and 10% with arachnids. In general, the macroinvertebrates biodiversity depicted is coherent to the expectancies of a lentic environment such as that one of Lake Romena (e.g. Lake Sibolla in Tuscany, Inghilesi et al. 2017).

For the 12S rRNA marker, 22 taxonomic units were obtained from the eDNA samples (Table S4). In this case, unlike the COI marker, no taxonomic units were associated at the family level, whereas 19% were attributed at the genus level, and 81% at the species level. In terms of vertebrates, there was much more literature available, which helped exclude any misassignments. 14% of the obtained taxa were associated with birds, amphibians, and reptiles, 23% with mammals, and 35% with fish.

Various alien species were found, such as the mosquitofish *Gambusia holbrooki* (Girard, 1859), the grass carp *Ctenopharyngodon idella* (Valenciennes in Cuvier and Valenciennes, 1844), the common carp *Cyprinus carpio* (Linnaeus, 1758), and three American genera of sliders, namely *Graptemys*, *Trachemys*, both caught during the control activities, and *Pseudemys*. Finally, largemouth bass *Micropterus salmoides* was detected and caught several times in the lake.

Discussion

The two trapping sessions, probably coupled with the predation activity of the fish found in the lake, led to a decrease in the crayfish population abundance. Even if CPUE was higher in 2023 (1.6) than in 2022 (1.11), a CPUE value of 0.2 was recorded in September 2023. This was 50% lower than September 2022 (0.42) and 80% lower than May 2022 (0.92), indicating a possible success in the control activities. The ongoing trapping activities besides the finding of tadpoles, which were never recorded in the previous years, seem to confirm a declining trend in the population (CPUE of August 2024: 0.13) and a potential positive effect on some native species. Cylindrical and rectangular traps as well as ARTs had a different but complementary effectiveness on CPUE. Unfortunately, the monitoring activities showed that *P. clarkii* spread outside the lake, mainly downstream, potentially increasing the threat to the native species and ecosystems.

The control activities in Lake Romena confirmed the presence of an established population of *P. clarkii*, although not particularly abundant. Indeed, in other areas of Tuscany and Italy with similar environments highly invaded by the species, higher CPUE values have been found using the same method during the same months (e.g. Paduletta di Ramone and Sibolla, Tuscany: average CPUE 18.48 and 3.66 respectively: Tricarico et al. 2015; Sardinia CPUE > 19: Chessa et al. 2010). Of note, in both years the highest catches occurred in June, indicating that this period probably represents the peak of the species activity in the area, despite suitable average temperatures for the species having occurred since May (22.9 °C in 2022 and 18.2 °C in 2023).

The most efficient traps were found to be the cylindrical ones. As expected, the absence of a bait (usually a strong attractant for crayfish) and the smaller size of the ARTs led to lower catches (they can host up to seven individuals). Rectangular traps have captured more than ARTs but less than cylindrical ones, possibly due to the larger entrance, which not only facilitates the entry of individuals but also their escape.

Overall, the number of males captured was higher than females and increased over time. In fact, the sex-ratio shifted from a nearly balanced condition between the two sexes, as observed in May 2022 (0.98) and 2023 (1.14), to a maximum imbalance in favour of males in September 2022 (2.68) and 2023 (2.67). This could be attributed to the reproductive state of females, which are less catchable as they tend to remain in burrows with eggs (Gherardi et al. 1999; Gherardi et al. 2011; Tricarico et al. 2015). In Italy, reproduction typically occurs in July–August, but sometimes even in September, depending on area and temperature (Tricarico and Zanetti 2023). Indeed, a female with eggs was caught in September 2022, with a cylindrical trap. Consistent with Green et al. (2018), ARTs were the only traps type with the sex-ratio values lower than 1, indicating a higher number of captured females compared to males. In accordance to Ilhéu et al. (2003), females seem to be more successful in occupying a shelter, and their reproductive status can also increase their competitive ability over males (Figler et al. 2005).

In general, as expected, cylindrical and rectangular traps captured more large individuals than small ones, where the opposite was true for ARTs. As observed in other monitoring and control activities using cylindrical traps, smaller individuals usually tend to avoid entering a trap with other conspecifics, especially if they are larger, to avoid the risk of being cannibalized (Chessa et al. 2010; Tricarico et al. 2015; Green et al. 2018; De Palma-Dow et al. 2020). ARTs, by mimicking a potential refuge, result to be more attractive to females and small individuals (Green et al. 2018), which is useful in increasing the CPUE effectiveness for these categories and complementary to traditional traps.

eDNA analyses and bycatches highlighted the presence of a rich animal community composed by several alien and invasive alien species, including known predators or competitors of *P. clarkii* such as the fish *M. salmoides*, *Perca fluviatilis* (Linnaeus, 1758) and *C. carpio* (Reynolds 2011; Souty-Grosset et al. 2016). The first two species can predate on medium–small sized crayfish, less catchable by traditional traps, whereas the latter one can compete with crayfish for food (Jackson et al. 2012). Notably, notwithstanding the huge presence of fish, three species of amphibians were detected with eDNA, with only one (*Rana italica* (Dubois, 1987)) being caught with traps. This presence may be due to DNA traces reaching the lake from upstream tributaries or to the opportunistic crossing of adult individuals, whereas the reproduction of these species in the lake may be possible but limited to areas which are difficult to reach for predatory fish and also crayfish. The presence of *P. lotor*, which was not detected by eDNA and whose traces were not found in 2022, was reported in the area in 2023, when numerous footprints were found during the control activity sessions – an individual handling a trap was also camera-trapped. Moreover, a relatively high number of disturbed traps, especially in 2023, could be attributed to *P. lotor* activity given fresh footprints found near the traps themselves. *Procion lotor* has been reported to predate the native crayfish *A. pallipes* complex in the nearby National Park (Tricarico et al. 2021). Up to now, no signs of predation have been found on *P. clarkii*, probably for the less accessibility of this species in the lake with steep banks compared to the small and less deep streams where the native crayfish is present. However, the hypothesis that *P. lotor* could also predate individuals of *P. clarkii* cannot be completely discarded. It is also noteworthy to note the absence of DNA traces of any bird predators of *P. clarkii*, such as species belonging to the family Ardeidae, has not often been reported in the area. Concerning invertebrates detected with eDNA, the predominance of arthropod diversity may be due to the selection of primers, designed to improve the detection of freshwater arthropods (Leese et al. 2021).

Monitoring activities revealed that *P. clarkii* has spread outside the lake, as 17 individuals were found in the lake's outflowing stream. Their smaller size (mean CL: 26.06 mm) compared to the one of the lake individuals (mean CL: 40.91 mm) suggests that they may have been carried away by the current due to the steep slope of the upper part of the outflowing stream. Despite the temporary nature of this water course (already dry in June 2022 and July 2023), the risk of the species invading the downstream areas is high as it can dig burrows and be more sedentary to overcome critical conditions (e.g. Gherardi et al. 2000, 2002). The outflowing stream reaches the River Arno 1.5 km further downstream where the species is not currently reported, even if it was found in two of the six lakes sampled along this river (the origin of this nucleus is under investigation). Conversely, upstream of the lake, in the tributary, only one crayfish was found, probably dispersed from the lake population. However, the lower water temperature (15° C) less preferred by the species suggests that *P. clarkii* is unlikely to spread upstream.

Conclusions

An integrated management approach is always recommended to control alien crayfish populations (Gherardi et al. 2011; Stebbing et al. 2014; Manfrin et al. 2019; García-de-Lomas et al. 2020). Alternative but complementary methods can tackle the species' different life stages and sexes, improving the effectiveness of control activities and leading to a significant decrease through time. Moreover, long-term control efforts are necessary to reduce substantially a population and prevent its recovery and compensatory responses (Gherardi et al. 2011). In accordance with Green et al. (2018), using different traps could be more helpful and cost effective that using one type of trap alone. Moreover, the presence of natural predators can enhance the effectiveness of trapping activities. Their presence can be assessed also using the eDNA technique, when traditional sampling cannot be carried out, as was the case of the present study.

Even if the results obtained in two seasons of trapping were promising, control activities should continue to monitor and control further the abundance of *P. clarkii* in the lake and its potential threat to the native species. Luckily, the crayfish plague seems to be absent up to now, but another screening is necessary, and precautionary and biosecurity measures should always be adopted by people visiting and working at the lake (e.g. check, clean, and dry protocols) to avoid the potential spread of this disease to native crayfish in the nearby areas of the National Park. In contrast, the species is already spreading in the area. To this end, other monitoring activities should be carried out to assess more accurately its distribution and contain its spread before reaching larger water bodies where control activities would be more difficult.

Authors' contribution

MM: sample design and methodology; data analysis and interpretation; investigation and data collation collection; writing – original draft. BM: investigation and data collation collection; writing – original draft. SF: investigation and data collation.MO: investigation and data collation. AR: investigation and data collation. GC: sample design and methodology; data analysis and interpretation; investigation and data collation; writing – original draft. AC: sample design and methodology; data analysis and interpretation; investigation and data collation. GP: sample design and methodology; data analysis and interpretation; investigation and data collation; writing - review and editing. ET: research conceptualization; sample design and methodology; data analysis and interpretation; investigation and data collation; ethics approval; writing - review and editing.

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Supplementary material

The following supplementary material is available for this article:

Figure S1. Aerial photo of Lake Romena, which shows the points where samples were taken for environmental DNA.
Table S1. The amplified eDNA concentration (ng/μL) of the eDNA pools.
Table S2. The ASVs (Amplicon Sequence Variants) obtained through the dada2 pipeline.
Table S3. The macroinvertebrate taxonomic units detected via eDNA.
Table S4. The vertebrate taxonomic units detected via eDNA.

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