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Development of PVC Dispensers for Long-Lasting Release of Attractants for the Control of Invasive Crayfish Populations

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Abstract: Invasive alien species (IAS) are considered one of the major threats to biodiversity worldwide, thus requiring severe control strategies. Based on the promising results obtained in the field of insect pest management with polyvinyl chloride (PVC) for long-lasting release of attractants, the main aim of this study was to ascertain the efficacy of PVC/attractant dispensers also in the aquatic environment. Therefore, we developed PVC/food dispensers and evaluated their attractiveness, by means of behavioural bioassays on whole animals, over a 60-day period of continuous use towards the red swamp crayfish *Procambarus clarkii*, one of the top 100 worst IAS. The attractiveness in PVC of trehalose, leucine and taurocholic acid was also tested. Our results show that the PVC dispensers release the food and are strongly attractive for crayfish over a prolonged time, even if their effectiveness depends on the storage conditions: From 18 days when stored underwater at 23 °C up to 50 days when stored out of the water at −20 °C. Besides, trehalose, leucine and taurocholic acid in PVC resulted in reliable attractants for *P. clarkii*. The development of PVC dispensers for long-lasting release of attractants may help improve the efficiency of mass trapping strategies in the management and control of invasive crayfish.

Keywords: biodiversity; invasive alien species; population control strategies; polyvinyl chloride; *Procambarus clarkii*; olfaction; feeding behaviour

1. Introduction

We are currently witnessing a rapid and constant decline in ecosystem biodiversity worldwide, in terms of both the number and the variety of living organisms. Habitat deterioration, deforestation, overexploitation, pollution, and climate change are among the major threats to biodiversity [1–3]. The introduction of invasive alien species (IAS) outside their native ranges is now considered as one of the five most important causes of global environmental change, thus requiring severe population control strategies [4–6].

In particular, there is growing and renewed concern about the spread and the ecological impact of invasive crayfish throughout the world, because after their establishment they may become dominant in the aquatic community [5,7,8]. This is the case, for instance, of the red swamp crayfish *Procambarus clarki* (Girard, 1852) (Crustacea: Decapoda) that, following its use in both aquaculture and ornamental practices, has spread worldwide, becoming a generalist top consumer in the food web, and therefore,

is altering the structure of aquatic ecosystems [9–13]. In addition, it is a well-known carrier of the parasitic oomycete *Aphanomyces astaci*, the agent of the crayfish plague, therefore contributing to the decline of native crayfish [14]. Given its considerable ecological plasticity, resistance and aggressiveness, this species is now listed among the "100 of the worst" invasive species by the "Delivering Alien Invasive Species In Europe" project (DAISIE 2010).

In order to mitigate the ecological and economic impact, prevention of the ex-novo introduction of invasive crayfish would represent the most cost effective, desirable strategy [15]. However, prevention is difficult, so that eradication and control of invasive crayfish populations at low density levels are the two remaining management options [16]. None of the several attempts made in the last decades based on manual removal, such as mass trapping and electrofishing [17–19], release of potential fish predators [20], use of attractive chemicals like sex pheromones [21,22] or natural pesticides [23,24], proved definitive. In fact, in the absence of continuous catches, the populations always returned to the initial levels within a few reproductive seasons [7]. On the other hand, large-scale application of management strategies is likely associated to high costs due to long-term trapping or large personnel employment [5].

Like other invertebrates, crayfish heavily rely on chemical senses to produce a number of adequate behavioral responses, ranging from orientation to social communication, detection of predators, sex recognition, parental cues and, obviously, localization of food resources in their environment [25–33].

Such a specialized chemosensitivity represents, on the one hand, an evolutionary success for the interaction of crayfish with its environment, but also a potential Achilles' heel that may render crayfish vulnerable and potentially subject to population control strategies, provided that the attractive key compounds or suitable tools for their optimized release are known. Analogously, promising results were obtained in the field of insect pest management with the use of sex pheromones as attractants combined with the development of polyvinyl chloride (PVC) resin formulations capable of release of attractive compounds over a prolonged time; they were used in mating disruption or attraction to lethal source and/or mass trapping [34–37]. This knowledge may be transferred, at least in part, to design efficient strategies for the management of invasive crayfish.

On this basis, the main aim of this study was to ascertain if PVC dispensers might represent effective tools for the release of attractive compounds over a prolonged time also in the aquatic environment, so as to use them in the control of invasive crayfish populations.

To do this, we developed suitable dispensers consisting of a solid PVC substrate containing a highly appetitive commercial food as an attractant. We then tested how long they could be attractive when supplied over a 60-day continuous period of use towards the red swamp crayfish *P. clarkii*, used as a model of IAS. In order to ascertain if the attractive degree of the PVC/stimulus dispensers may be storage-dependent, they were stored under different modalities.

Furthermore, we tested the attractiveness degree in PVC of trehalose, leucine and taurocholic acid that were previously reported as stimulating and/or of potential behavioral significance for this and other crayfish species [38–44].

From an applied point of view, the discovery of tools for long-lasting release of suitable attractants may help improve the efficiency and the costs of mass trapping strategies aimed at better management and control of invasive crayfish populations.

2. Materials and Methods

2.1. Animal Collection and Rearing Conditions

All experiments were performed on wild, intermolt adult red swamp crayfish *P. clarkii* of both sexes, 35–40 mm in carapace length, collected using a backpack electrofishing unit (5.2–2.8 A, 230–400 V, 1300 W) at the Molentargius-Saline Regional Natural Park (Southern Sardinia, Italy) during the spring season of 2017–2018. After processing, the crayfish were kept in aerated and bio-conditioned (Aquasafe, Tetra, Melle, Germany) tap water (hereafter referred to as tap water) at 22–23 °C, 16 h light/8 h dark

photoperiodic regime, and fed with lettuce, squid or a highly appetitive commercial pellet food (Shrimps natural, SERA, Heinsberg, Germany) three times a week. Uneaten food was always removed within 1 h after delivery. Individuals were kept separate to avoid any reciprocal exposure of males and females and to prevent attacks or cannibalism.

2.2. Preparation of the PVC Substrate

The dispensers for prolonged release of crayfish attractants over time used in this study were prepared by coating a rigid support, typically the bottom of a Petri dish (diameter 35 mm), with a mixture containing an attractive stimulus suspended in a substrate of polyvinyl chloride (PVC; code 389293, Sigma-Aldrich, Milan, Italy). The PVC substrate was preliminarily prepared by dissolving the powdered PVC (125 mg/10 mL) in dichloromethane (CH_2Cl_2) and agitating on a magnetic stir plate at 60–70 °C until complete solubilization of PVC, which was typically a few minutes.

The chemical attractants of choice were then suspended in the PVC substrate when the latter was still liquid; 500 µL of the PVC/attractant mixture were then poured on the Petri dish and left to cool until solidification, typically 4–5 min. In this way, the attractant molecules remained trapped within the solid PVC matrix adhering to the Petri dish (Figure 1) and could be easily supplied to the crayfish for behavioural tests of chemical attractiveness.

Figure 1. Photograph of the PVC/attractant (food) dispenser used in this study.

2.3. Chemical Attractants

We tested finely hashed food, trehalose, leucine (all at 100 mg/mL) and taurocholic acid (10 mg/mL) suspended in liquid PVC, and PVC alone as a control. The disaccharide trehalose and the amino acid leucine are already known to stimulate the leg chemoreceptors (CRNs) and/or to be of behavioral significance for this and other crayfish species [38,39,41,42,44], while taurocholic acid has previously been reported as present in the feces of freshwater fishes [40] and as a potential chemical signal for territoriality and reproduction in freshwater habitats [43]. Taurocholic acid was also chosen for its stimulatory effect previously described on the leg CRNs in *P. clarkii* [44]. With the exception of the commercial pellet food, all other stimuli used were purchased from Sigma-Aldrich (Milan, Italy).

Blank PVC (attractant-free) dispensers were prepared by using the same procedure for the PVC substrate and were used as controls, both in the long-lasting trials with the PVC/food dispensers and in the experiments with trehalose, leucine and taurocholic acid.

2.4. Crayfish Bioassay and Supply Protocols of PVC/Attractant Dispensers

Crayfish were individually exposed to test compounds in Plexiglas® tanks (40 cm long × 30 cm wide × 15 cm deep) containing 15 L of tap water (22–23 °C). Animals were not fed for 24 h preceding the experiments. At the beginning of each test, crayfish were allowed to acclimatize until becoming motionless, typically within 15 min, then the PVC/attractant dispensers were added to the tank and each crayfish was allowed 10 min to respond. Trials were video-recorded for later analysis using a Samsung SMX-F34 (Samsung, Seoul, Korea) color digital camera mounted above the test tank. A PVC/attractant dispenser was considered attractive when crayfish started displaying searching behaviour and found the odour source within the established time, producing movements of the walking legs with dactyl probing and clasping of the dispenser associated with movement of the legs to the mouth, a typical food searching response according to Kreider and Watt [45] and Corotto et al. [42]. Observers were unaware of the stimulus identity. Two different groups of experiments were performed using the following protocols:

(1) In the experiments aimed at evaluating the attractive effectiveness of the PVC/food dispensers over time, dispensers of a same group were repeatedly supplied to the crayfish for 60 days, typically every 3–4 days. In order to ascertain if the attractive degree of the PVC/food dispensers might be storage-dependent, dispensers were divided into three different groups and, when not in use in the experimental tank, were stored under three different conditions for the entire duration of the trial: (1a) out of the water at 23 °C, (1b) out of the water at −20 °C and (1c) underwater, in a separate tank (volume: 4 L) at 23 °C. The last storage modality represents the most reliable and normal condition of use of the dispenser. Each of the 15 crayfish tested (Eight females and seven males) were repeatedly exposed to dispensers from each of the three different storage modalities, until the dispensers became unattractive. In addition to the attractiveness evaluation, we also considered the time the crayfish spent on the dispensers (two time options: less or more than 10 s) as a further index of its attractive strength.

(2) In the experiments aimed at ascertaining the attractiveness of trehalose, leucine and taurocholic acid in PVC, 25 crayfish (14 females and 11 males) were exposed to the compounds, supplied in random order. Each crayfish was supplied with each chemical only once. Also in this case, in addition to the evaluation of the attractiveness, we also considered the time the crayfish spent on the dispensers (two time options: less or more than 10 s) and the time it took the crayfish to find the dispensers (two time options: less or more than 5 min) as additional parameters defining their attractive strength.

2.5. Statistical Analysis

Repeated-measures ANOVA was used to assess the effectiveness of the dispensers at the three different storage modalities used (out of the water at 23 °C or at −20 °C or underwater at 23 °C for the entire duration of the tests) on the crayfish response across sex. Data are expressed as mean ± standard error (SE).

Since no differences related to sex were detected, data were then analyzed by only considering the three different storage modalities of dispensers, regardless of crayfish sex.

Data were checked for the assumptions of normality and sphericity (when applicable). When the sphericity assumption was violated, a Greenhouse-Geisser correction or Huynh-Feldt correction was applied in order to modify the degrees of freedom [46,47].

Post-hoc comparisons were conducted with the Tukey test, unless the assumption of homogeneity of variance was violated, in which case the Duncan's test was used. The Kolmogorov–Smirnov test (two sample K-S test) was employed to compare the distance between the time frequency distribution

of the PVC/food dispensers stored at the three different conditions. Statistical analyses were made using STATISTICA for WINDOWS (version 7.0; StatSoft Inc., Tulsa, OK, USA). p values < 0.05 were considered significant.

3. Results

3.1. Evaluation of PVC Effectiveness for Prolonged Release of Food as Crayfish Attractant over Time

After acclimatization in the experimental tank, crayfish became virtually motionless and, in the absence of dispensers, displayed only a basal level of antennular flicking or grooming activity. However, some dispensers caused in crayfish a stereotyped search strategy associated with a rapid walking phase within the animal arena, which culminated in prolonged inspections and leg-manipulation of the dish containing the PVC/food dispenser in the attempt to eat it. Blank PVC (control) dispensers in no case proved attractive to the crayfish, neither chemically nor in terms of possible indirect effects such as, for instance, responses of crayfish simply due to the addition of a novel object to the tank (Figure 2A).

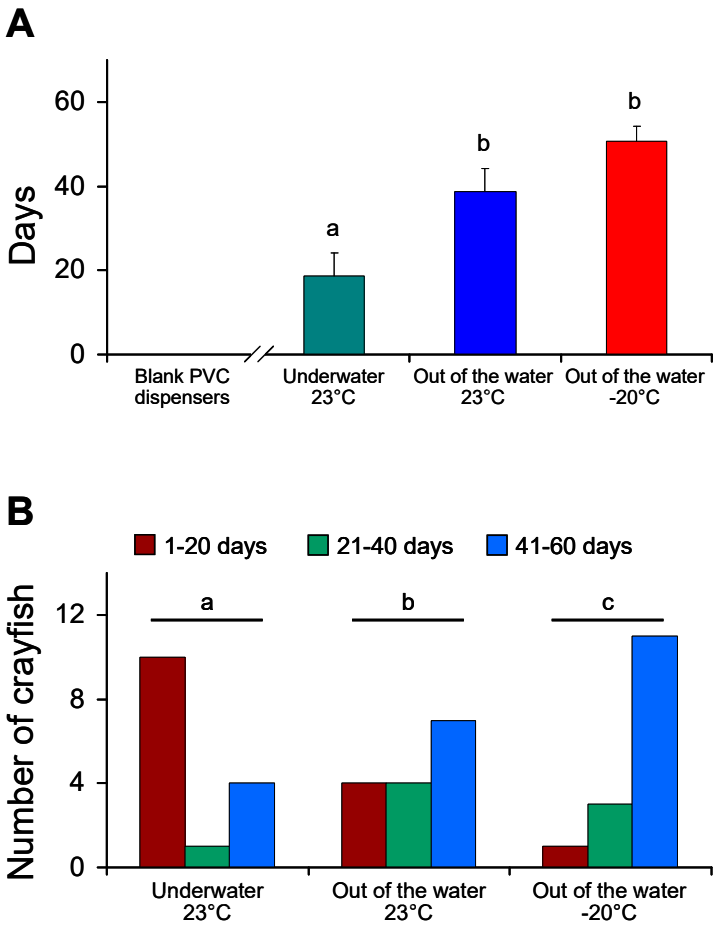

Figure 2. Attractiveness over time of the PVC/food dispensers stored under different conditions. (**A**) Mean values ± SE (vertical bars) of the days of attractiveness of the PVC/food dispensers stored underwater at 23 °C and out of the water at 23 °C or −20 °C. Data were obtained from 15 (eight females and seven males) crayfish. Bars followed by different letters are significantly different ($p < 0.05$; Tukey test subsequent to repeated-measures ANOVA). (**B**) Distribution histograms of the number of crayfish ($n = 15$) attracted by the PVC/food dispensers stored at the three different conditions for each of the three 20-day time intervals considered (1–20 days, 21–40 days and 41–60 days). Bars followed by different letters are significantly different ($p < 0.05$; Kolmogorov-Smirnov test (two sample K-S test)).

A preliminary repeated measures ANOVA revealed no significant interaction of the dispenser storage conditions across sex ($F_{(2,26)} = 1.3640$, $p = 0.27$). For this reason, data from males and females were pooled and analyzed by only considering the different dispenser storage conditions. In particular, the PVC/food dispensers were found to be attractive over a prolonged time, thus suggesting that the PVC substrate may ensure a long-lasting release of the included attractants, even if their duration differs as a function of the storage conditions adopted ($F_{(2,28)} = 13.124$, $p = 0.0001$; Figure 2A). When stored underwater at 23 °C, the dispenser PVC/food was attractive for the crayfish for 18.5 ± 5.5 days, but the duration was more than doubled (38.6 ± 5.6 days) when stored out of the water at 23 °C and greatly increased up to 50.7 ± 3.5 of the 60 days of the test when stored out of the water at −20 °C.

As shown in Figure 2B, the importance of the dispenser storage conditions was also confirmed by the fact that 10 crayfish out of 15 were able to sense the attractants within the first 20 days of use for the dispenser stored underwater at 23 °C, but 11 crayfish found the PVC/food dispenser attractive up to 40–60 days when it was stored out of the water at −20 °C. As for the last storage condition, nine crayfish sensed the PVC/food substrate for the entire time interval considered (60 days, data not shown). Despite the results highlighted by the Tukey test in Figure 2A, the K-S tests showed statistically significant differences among the three dispenser storage conditions (Figure 2B). The K-S test analyzing the cumulative time histogram frequency showed in any case *p*-values less than 0.05 (Underwater 23 °C vs. Out of the water 23 °C D = 0.6, $p = 0.009$; Underwater 23 °C vs. Out of the water −20 °C D = 0.6, $p = 0.002$; Out of the water 23 °C vs. Out of the water 20 °C D = 0.6, $p = 0.009$).

The attractiveness of stimulating the PVC/food dispenser was further confirmed by considering the time the crayfish spent on the dispensers after they found them (Figure 3). In fact, 71.4% of the attracted crayfish remained on the dispensers stored underwater at 23 °C for more than 10 s, while 80.1% and 89.3% were the percentages of crayfish that remained for more than 10 s on the dispensers stored underwater at 23 °C and −20 °C, respectively.

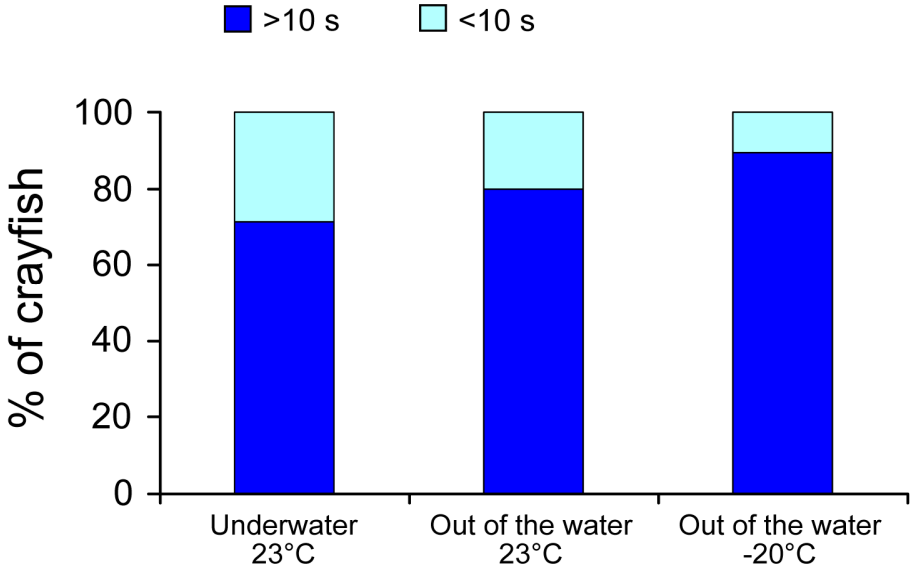

Figure 3. Attractiveness index of the PVC/food dispensers stored underwater at 23 °C and out of the water at 23 °C or −20 °C, evaluated by considering the time intervals (less or more than 10 s) the crayfish spent on the dispensers after they found them. Data were obtained from 15 (eight females and seven males) crayfish.

3.2. Attractiveness of Trehalose, Leucine and Taurocholic Acid Incorporated in the PVC Dispensers

The attractive effect of trehalose, leucine and taurocholic acid was evaluated, as compared to food and blank PVC, by supplying the different stimuli with the PVC dispenser method. All tested compounds exerted a certain degree of attractiveness on *P. clarkii*, even if to a different extent (Figure 4).

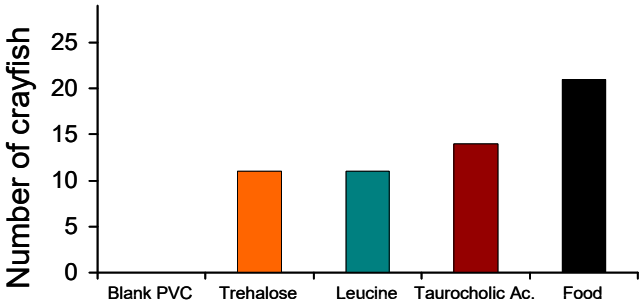

Figure 4. Attractiveness of trehalose, leucine and taurocholic acid in PVC, expressed as the number of crayfish that found the dispensers, as compared to food and blank PVC, within the established time (10 min). Data were obtained from 25 (14 females and 11 males) crayfish.

In fact, although the food revealed the most effective stimulus by attracting 21 out of the 25 crayfish tested, 14 of them were also attracted by taurocholic acid and 11 by both trehalose and leucine. More than half of the crayfish found the PVC/food dispensers in less than 5 min (Figure 5A); PVC dispensers with trehalose, leucine and taurocholic acid were found in the same time by 36–40% of the animals. Moreover, 68% of crayfish spent more than 10 s on the PVC/food dispensers (Figure 5B) in prolonged inspections and/or leg-manipulation of them trying to eat it. Only 28% and 32% of the animals spent more than 10 s on the PVC dispensers containing trehalose and taurocholic acid, respectively. PVC/leucine dispensers resulted in short visits and only 8% of the crayfish spent more than 10 s on these dispensers.

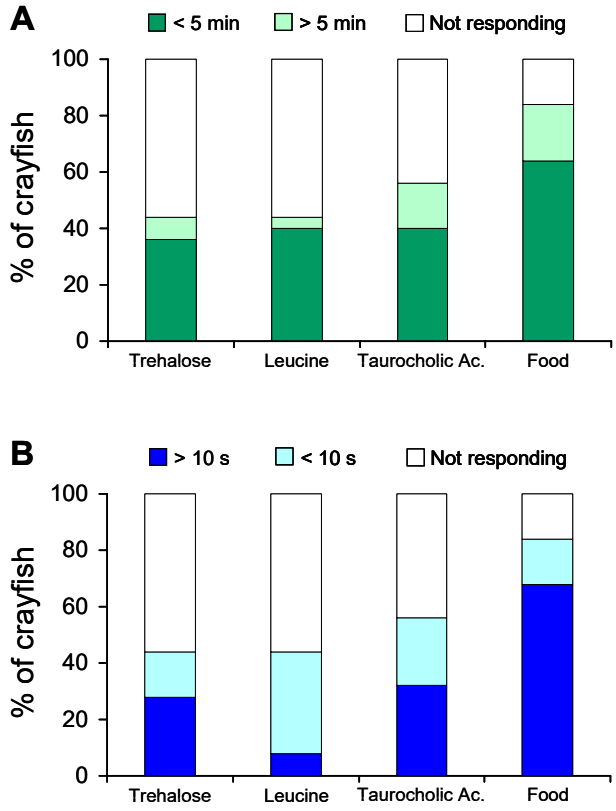

Figure 5. Attractiveness index of trehalose, leucine and taurocholic acid in PVC, expressed as (**A**) the time it took the crayfish to find the dispensers (less or more than 5 min) and (**B**) the time the crayfish spent on the dispensers (less or more than 10 s), as compared to food. Data were obtained from 25 (14 females and 11 males) crayfish.

In any case, the food resulted in the most effective stimulus, as it attracted a higher number of crayfish than the other tested compounds and most of the responding animals spent more than 10 s on it (Figures 4 and 5).

4. Discussion

4.1. Evaluation of PVC Effectiveness for Long-Lasting Release of Food as Crayfish Attractant over Time

Our results show that the PVC resin substrate we tested is capable of ensuring the release of suitable attractants for an aquatic organism like the invasive crayfish *P. clarkii* over a prolonged time. In fact, the dispensers we developed by including a highly appetitive commercial food as attractive stimulus inside a PVC solid matrix proved to be effective for more than 18 days when the dispensers were stored underwater at 23 °C. Moreover, the PVC/food dispensers resulted in being attractive up to 50 days when they were stored out of the water at −20 °C. In this respect, we had no doubt about the attractiveness of the commercial food; it is a mixture of a number of chemicals that, apart from its nutritional value, has been suitably formulated as to be as attractive as possible, in order to be quickly located and willingly consumed by crayfish. However, given its characteristics, the food tends to quickly dissolve in water and for this reason it is not useful to bait traps for long-term campaigns, unless it is embedded in a solid matrix like PVC for slow, long-lasting release.

In the past, promising results were obtained in the field of insect pest management by using attractants like sex pheromones combined with PVC resin formulations for their controlled, long-lasting release, and they were successfully employed for prolonged mating disruption, attraction to lethal source and/or mass trapping activities [34–37,48]. However, to the best of our knowledge, this is the first study reporting the efficacy of PVC as a tool for long-lasting release of attractants also in the aquatic environment, thus suggesting that this method may be also used in management and control strategies of invasive crayfish populations and/or other animals for which suitable attractants are chemically compatible and easily incorporable in the solid PVC matrix.

Obviously, in the case of insects, the advantage offered by the use of the PVC method is greatly enhanced by the knowledge and large-scale availability of the sex pheromones. In fact, given their high species specificity, they allow for the control of populations in a well targeted manner [49–52]. Unfortunately, this is not the case for crayfish yet, because despite decades of research on crayfish chemical communication consolidating the role of pheromones in sexual interactions, the numerous attempts aimed at isolating and characterizing them remained unsuccessful [21,28,29]. However, one of the advantages of our dispenser model is that it can be effective in long-lasting vehiculation of chemical attractants like food, as in the present study or any other compound of behavioural significance for the crayfish. It definitely represents a reliable tool for obtaining attractive baits for mass trapping practices because of the low cost of the chemicals used.

Further investigation is needed to better elucidate the physico-chemical properties of the PVC/attractant dispensers to extend their efficacy over time, especially underwater, which seems to be a less suitable medium for dispersion than air. We found that the effectiveness of our dispensers varied as a function of the storage conditions, being effective for a longer time when stored out of the water, even if the storage temperature also seemed to be an important factor. Moreover, determining the physico-chemical properties of the PVC/attractant dispensers may not be sufficient, because a number of other sometimes uncontrollable variables may influence their attractiveness, such as the intrinsic characteristics of the site where the PVC dispensers are used (stream speed, turbulence, depth and temperature of water, presence of waterfalls interrupting the smelling trace, positioning of baited traps, etc.). Obviously, the longer the duration of the attractiveness of the PVC dispensers, the more effective the trap campaigns where they are used may be, thus reducing the costs.

The effectiveness of the PVC dispensers is also suggested by the fact that in the case of a terrestrial animal like the insect *Lymantria dispar* the PVC dispensers still contained more than 75% of the attractant initially included in the PVC substrate, the sex pheromone (+) disparlure, after 16 weeks of use

(Leonhardt et al., 1993). It was also suggested that the release rate of the attractant may be optimized by increasing the attractant/PVC ratio, by enhancing the surface of molecular exchange with the environment and, possibly, by the solubility of the attractant molecules in the medium considered [37,48]. In this respect, our results were obtained by dispensing a small amount (500 µL) of the PVC/food mixture in only a few millimeters of the Petri dish surface. Therefore, using dispensers containing higher amounts of attractant and/or enlarged dispersal surface may increase the effectiveness of the dispensers itself in terms of both the action range and the duration over the time of the attractants.

Finally, since different animals may display different sensitivity levels for a same attractant during their life cycle, the use of the dispensers with a given attractant during the relative peak of sensitivity is a prerequisite to improve their efficacy over time.

4.2. Attractiveness of Trehalose, Leucine and Taurocholic Acid Incorporated in the PVC Dispensers

The present study also showed that other compounds like the disaccharide trehalose, the amino acid leucine and the bile acid taurocholic acid represent good attractants, even if to a lesser extent than the tested food, for the crayfish *P. clarkii*. Therefore, they may be used in association with the PVC method, thus confirming the validity of the PVC dispensers for the release of a potential wide range of attractive compounds. Both trehalose and leucine where previously reported to stimulate the chemoreceptor neurons in isolated legs of *P. clarkii* and to elicit behavioural responses in whole-animals [32,33,35], even if responses were detected using water solutions of these stimuli rather than the relative PVC substrate. The attractiveness of trehalose and leucine is consistent with the omnivorous feeding habits of the crayfish [30,53,54], that is known to be a generalist consumer of invertebrates, macrophytes, algae and detritus [9,55,56]. In this respect, the amino acid leucine represents a good protein food indicator for the carnivorous provision in the crayfish diet as it is commonly present at high concentrations in the tissue of a number of their potential animal preys [57], while carbohydrates are more likely indicators for the vegetarian diet counterpart [58,59]. In this respect, trehalose represents an anomalous carbohydrate; in fact, it chemically belongs to the class of sugars, but represents an indicator of a protein diet as it is a haemolymph sugar commonly present in the body fluid of invertebrates [60]. To the best of our knowledge, this is the first report of attractiveness exerted by taurocholic acid in crustaceans and, more specifically, in crayfish. At present the significance of this compound for *P. clarkii* is unknown, but it might represent a food indicator given its presence in the faeces of freshwater fishes [40], or a potential semiochemical for social and/or territorial interactions, as previously reported for freshwater habitats [43].

5. Conclusions

The spread and the ecological impact of invasive crayfish throughout the world represents a serious threat to biodiversity, requiring urgent demand for biotechnology aimed at population control that possibly meets two fundamental criterions: Great efficacy coupled with low cost.

Using the red swamp crayfish *P. clarkii* as a model of highly invasive species, the present behavioural investigation for the first time provides evidence on the effectiveness of PVC as a resin substrate capable of inclusion and long-lasting release of attractants also in the aquatic environment, similar to what has previously been reported for terrestrial organisms like insects. Besides, an attractive effect of stimuli of behavioral significance for crayfish, like trehalose, leucine and taurocholic acid dispensed in PVC is also reported. In this respect, as a follow-up of the present study, we shall evaluate the long-lasting effectiveness of the PVC dispenser method and the attractiveness of trehalose, leucine and taurocholic acid also in the field, in order to evaluate, under natural conditions, the promising results obtained in the laboratory.

On the whole, this study candidates the PVC/attractant dispenser model as a potentially novel, low cost tool for the development of attractive baits that may help improve the efficiency of mass trapping strategies in the management and control of invasive crayfish.

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Conflicts of Interest: The authors declare no conflict of interest.

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