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ORIGINAL ARTICLE

Expansion of the marbled crayfish in Slovakia: beginning of an invasion in the Danube catchment?

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ABSTRACT

The marbled crayfish, Procambarus fallax *f.* virginalis*, is a taxon widely available in the aquarium pet trade, which has been introduced to open waters in several European countries and in Madagascar. Recent studies confirmed this parthenogenetically reproducing crayfish as a high-risk invasive species, and vector of the crayfish plague pathogen,* Aphanomyces astaci*. It has been first discovered in Slovakia in 2010, but the status of the local population was not studied since then. Due to enlarged sampling area around the first report and one locality, where we presupposed the crayfish occurrence, we identified three new marbled crayfish populations in Slovakia. Two populations are located critically close to the Váh River, a major tributary of the Danube River; one of them being directly connected to the Váh River* via *a side channel during occasional floods. The third established marbled crayfish population was found at the mouth of a thermal stream flowing into the Nitra River, a tributary of the Váh River. In this stream, crayfish coexist with exotic fish and gastropod species of aquarium origin. We presume that the reported localities may serve as a source for further expansion of the marbled crayfish in the mid-part of the Danube catchment. Floods, active dispersal (including overland), passive dispersal by zoochory or anthropogenic translocations are among the major drivers facilitating the marbled crayfish colonization. We have not detected the crayfish plague pathogen in any of the studied populations. However, if spreading further, the marbled crayfish will encounter established populations of crayfish plague carriers in the Danube River, in which case they may acquire the pathogen by horizontal transmission and contribute to spread of this disease to indigenous European crayfish species.*

Key words: Aquarium pet trade; crayfish plague; freshwater crayfish; Procambarus fallax *f.* virginalis*; species introductions.*

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INTRODUCTION

Biological invasions and human impacts (*e.g.*, habitat destruction and pollution) are the major factors negatively influencing global biodiversity (Sala *et al*., 2000; Dudgeon *et al*., 2006). One of the important introduction pathways of potential invaders is the global pet trade, of which aquatic organisms represent a great portion (Padilla and Williams, 2004). The problem of releases of aquarium fish in both freshwater and marine environments has been recognized for a long time (*e.g.*, Courtenay and Stauffer, 1990; Semmens *et al.*, 2004), but scantly addressed. More recently, establishment of ornamental crayfish populations received attention, particularly in Europe. Rising density of human population and increasing socio-economic conditions favor the chances of crayfish releases (Perdikaris *et al*., 2012; Chucholl, 2014) and some of the species kept in aquaria become established in the wild (Holdich *et al*., 2009; Kouba *et al*., 2014). Native European crayfish species are challenged by the ever increasing number of newly introduced alien crayfish and the risks associated with them (particularly disease transmission), which substantially complicates their population recovery and conservation (Peay and Füreder, 2011; Capinha *et al*., 2013).

One of the emerging crayfish invaders in European freshwaters is the marbled crayfish, also known as Mamorkrebs, a parthenogenetically reproducing form of *Procambarus fallax* (Hagen, 1870) (Martin *et al.*, 2010a) discovered originally in the aquarium trade (Scholtz *et al.*, 2003). Marbled crayfish are widely available undemanding pets, frequently sold both in brick and mortar shops and online (Chucholl, 2013, 2015; Faulkes, 2013; Mrugała *et al.*, 2015; Lipták and Vitázková, 2015). Due to its asexual mode of reproduction, marbled crayfish can overpopulate a home aquarium in a short time. Such situation often leads to sale or disposal of redundant individuals by aquarium holders (Patoka *et al*., 2014a).

In the wild, marbled crayfish were first recorded in

Germany in 2003 (Marten *et al*., 2004). Since then, their presence was reported from various European countries, including the Netherlands, Italy, Slovakia, Croatia, and even Sweden (summarized in Chucholl *et al*., 2012; Kouba *et al*., 2014; Samardžić, 2014), and the crayfish got apparently well established in Madagascar (Jones *et al*., 2009; Kawai *et al*., 2009). The first reliable record of an established population in Central Europe had been reported in 2010 from southwestern Germany (Chucholl and Pfeiffer, 2010), and by 2012 at least six established marbled crayfish populations were known in Europe (Chucholl *et al*., 2012). Moreover, the marbled crayfish has been recently confirmed as a vector of the crayfish plague pathogen, *Aphanomyces astaci* (Keller *et al.*, 2014; Mrugała *et al.*, 2015), which is responsible for substantial population declines and local extinctions of native European crayfish species (for review, see Holdich *et al*., 2009). The presence of the marbled crayfish in natural ecosystems may, therefore, facilitate the spread of this disease and thus affect native European crayfish species if they get into contact with an infected carrier.

The marbled crayfish had been first detected in Slovak surface waters in 2010, when more than 150 individuals were collected from a small gravel pit near the village Koplotovce (Janský and Mutkovič, 2010). The aim of our study was to evaluate the present status of the marbled crayfish in Slovakia, and its potential for further spread and impact. We report additional sites with the established marbled crayfish populations, in which we assessed the population structure. Furthermore, we tested the collected animals for the potential presence of the crayfish plague pathogen, *Aphanomyces astaci.*

METHODS

Study sites

The Slovak Republic is located in the heart of Europe, and its lowland regions are characterized by a continental climate with warm summers and cold winters. All three studied sites with marbled crayfish populations are located in the southwestern part of the country at relatively low elevations (Fig. 1; Tab. 1).

Koplotovce site

This is the first site from which the marbled crayfish was first reported in Slovakia (Janský and Mutkovič, 2010), it comprises seven adjacent groundwater-fed gravel pits (Fig. 1) ranging in area from 1600 m^2 to 21,600 m^2. The gravel pits freeze over in winter (with the bottom temperatures not exceeding 4°C), while the epilimnion warms up to 23-25°C in summer. Two of these pits (one being the site of the first marbled crayfish record for Slovakia) are privately owned. The area of the gravel pits is separated from the adjacent Váh River by an embankment that provides protection from the occasional floods. The pits have fluctuating water level and varying depth (up to

Fig. 1. The marbled crayfish (*Procambarus fallax* f. *virginalis*) occurrence in Slovakia. Black lines in the central map indicate country borders, blue lines indicate the river network, and the orange rectangle represents privately owned and thus inaccessible sites. Red stars represent newly discovered marbled crayfish populations, while the yellow star represents the original site of the first record in the country.

2-3 m), and are partially overgrown with macrophytes. Although isolated under standard hydrological and meteorological conditions, all gravel pits get periodically interconnected following excessive rainfall (last such events occurred in 2006 and 2010). The pits are frequently visited fishing grounds, seasonally restocked with fish.

Leopoldov site

It is represented by a single large gravel pit (surface area ca. 130,600 m^2), connected during floods with a side channel (Drahovský kanál) of the Váh River (last such event occurred in 2010). Depth of the gravel pit varies, reaching 5-7 m at its southern and 4 m in its northern section (Fig. 1). The water temperature regime of this gravel pit is similar to those in Koplotovce. The site is a frequently visited fishing ground, seasonally restocked with fish.

Opatovce site

It is a thermal stream flowing through Opatovce nad Nitrou, a small village next to the popular thermal spa town Bojnice. The water temperature in the stream varies little during the year, ranging from 29 to 31°C; pH values increase from 7.15 in the middle section to 8.30 at the stream mouth (Májsky 2007). The stream (ca. 1 m wide) empties into the Nitra River (ca. 8 m wide), a tributary of the Váh River. The stream bed is formed by concrete blocks, and the stream banks are continuously lined with dense vegetation.

Field work

We failed to get an access to the privately owned gravel pit in Koplotovce to inspect the original site of the marbled crayfish record. Therefore, the adjoining gravel pits were surveyed. A pilot study at the Koplotovce site was carried out in three gravel pits and one adjoining periodical pool on 15 May 2014. The survey was performed manually with small hand-held net and with 30 fishmeat-baited crayfish traps. The traps were left overnight and collected in the morning. Subsequently, the Koplotovce site was visited on 6 September 2014. Two gravel pits (previously sampled on 15 May 2014) were investigated with electrofishing equipment. In the larger gravel pit (ca. 17,000 m^2), the sampling was conducted along ca. 40 m of the shore; the smaller adjoining pit (ca. 1600 m^2) was surveyed for 10 min in a 10 m long shore area. Finally, an additional survey with a standardized sampling effort was carried out on 17 October 2014 in one of the pits where crayfish had been recorded during a previous visit. The animals were collected with a small hand-held net for 45 min in a shore area approximately 30 m long.

The Leopoldov site was inspected for three days on 16, 17 and 18 September 2014. On this occasion, crayfish were mainly observed and photographed. The collection of crayfish took place on 18 October 2014, with the same effort as during the last-mentioned samping at the Koplotovce site (*i.e.*, by manual search with a hand-held net for 45 min along an approx. 30 m long shore area).

The thermal stream in Opatovce was visited for the first time on 19 September 2013, when the site was inspected for crayfish by electrofishing. Subsequently, on 17 October 2014, crayfish individuals were sampled as in the gravel pits by a small hand-held net for 45 min along an approx. 30 m long stream section.

Upon capture, the carapace length of crayfish individuals was measured. The numbers of juveniles carried by females obtained at the Koplotovce site were counted; brood sizes of females from the Leopoldov site were roughly estimated from available photographs. Subsequently, all individuals were stored in 96% ethanol for further analyses.

Molecular analyses

From each locality, up to 16 specimens (as given in Tab. 1) were used for screening for the presence of the crayfish plague pathogen. From each crayfish, we dissected the whole soft abdominal cuticle, the tail fan, and two joints of walking legs (in individuals smaller than 4.5 cm all basal joints with legs). Furthermore, we inspected the crayfish for the presence of melanized spots, potentially indicating an immune response to pathogens; if these were observed, the respective body part was included in the analysis.

Tab. 1. Details on the sampled localities with the marbled crayfish (*Procambarus fallax* f. *virginalis*) populations in Slovakia, and on collected crayfish individuals.

Sampling site	River basin	Type of water body	Coordinates		Elevation (m)	Sampling date	Collected crayfish	CL (mm)	Crayfish tested for *A. astaci*
			Latitude (N)	Longitude (E)					
Koplotovce	Váh	Gravel pit	48°28’11”	17°48’15”	141	6 Sep 2014	11	13.3-44.8	6
						17 Oct 2014	10	5.6-24.3	10
Leopoldov	Váh	Gravel pit	48°27’02”	17°47’11”	140	18 Oct 2014	21	5.4-30.9	12
Opatovce	Nitra	Thermal stream	48°46’01”	18°34’39”	254	17 Oct 2014	38	7.4-35.7	12

CL, carapace length.

The genomic DNA was extracted with the DNeasy tissue kit (Qiagen) from up to 50 mg of the mix-tissue samples ground beforehand in liquid nitrogen (as in Kozubíková *et al*., 2009). The molecular detection of *A. astaci* was performed with the TaqMan minor groove binder quantitative polymerase chain reaction (qPCR; after Vrålstad *et al.*, 2009) as described in Svoboda *et al*. (2014). Additionally, the identity of the crayfish species was investigated by sequencing of a 648 bp long fragment of the mitochondrial gene for the cytochrome c oxidase subunit I (COI) from one crayfish individual per population. We used the universal primer pair LCO1490/HCO2198 (Folmer *et al*., 1994), following the protocols described in Mrugała *et al*. (2015).

RESULTS

Three new established populations of the marbled crayfish have been confirmed in Slovakia. As expected for this parthenogenetically reproducing taxon, all captured individuals were females. At the Koplotovce site, only exuviae of a single crayfish individual was found on 15 May 2014, despite the overnight use of crayfish traps and manual sampling effort. Five adult and six medium-sized (carapace lenght (CL) 10-20 mm) individuals were caught at this site on 6 September 2014 (Fig. 2A). Three mature females, captured on 6 September 2014, carried 372, 412 and 455 juveniles, respectively. The fourth female lost some of the offspring during manipulation, and thus carried only 81 juveniles at the time of counting. Ten more marbled crayfish were collected on 17 October 2014 (Fig. 2B), in the survey with a standardized sampling effort. Two mature marbled crayfish females with eggs were photo-documented at the Leopoldov site on 16 September 2014. Although the egg numbers were not counted, the assessment of photographs suggests that both brood sizes reached at least 300 eggs. Furthermore, young individuals were observed on 17 and 18 September 2014. During the survey on 18 October 2014, 21 medium-sized (CL 5-15 mm) individuals were collected (Fig. 2C). The crayfish were found mainly in the leaf litter accumulated at the banks of the gravel pit.

At the Opatovce site, four crayfish individuals were collected on 19 September 2013 and further 38 crayfish were caught during a standardized sampling on 18 October 2014 (Fig. 2D). Three ornamental fish species, the guppy (*Poecilia reticulata*), the Mozambique tilapia (*Oreochromis mossambicus*) and the convict cichlid (*Amatitlania nigrofasciata*), were observed at the site as well. Moreover, the stream bottom substrate was dominated by a dense population of the red-rimmed melania (*Melanoides tuberculata*), an alien gastropod frequently

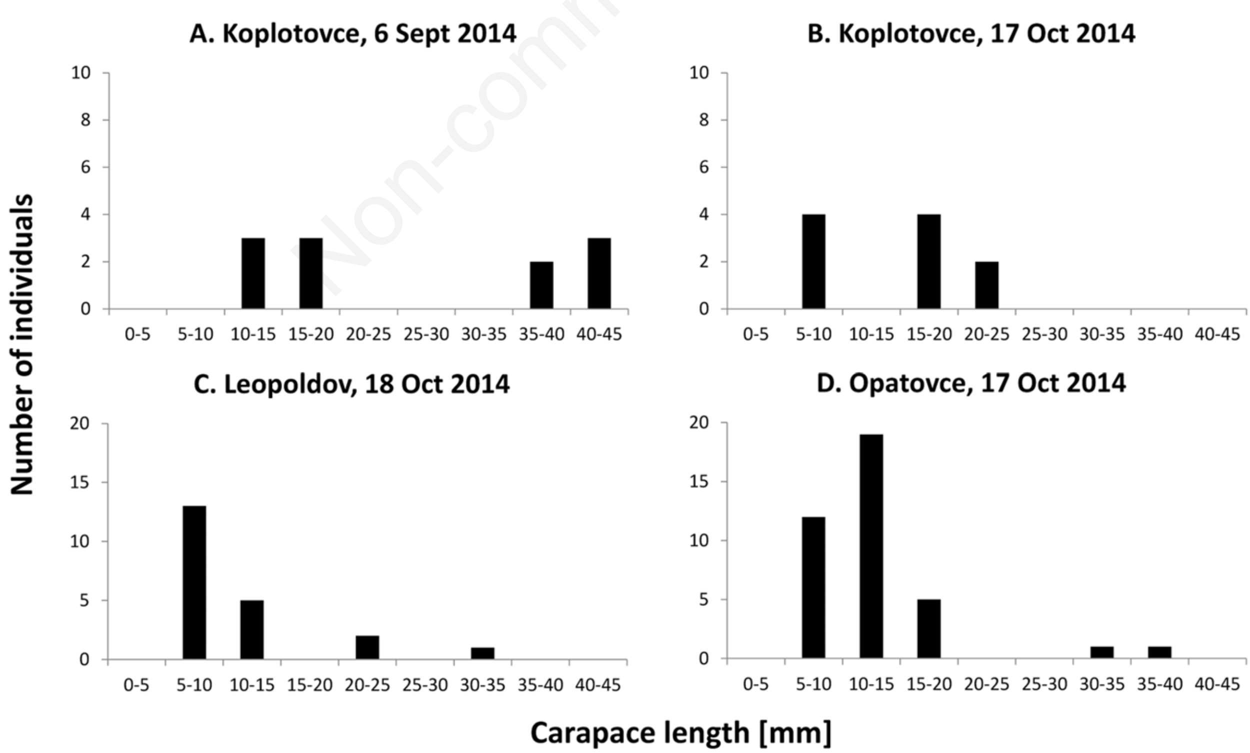

Fig. 2. Body size distribution (expressed as carapace length) of the marbled crayfish (*Procambarus fallax* f. *virginalis*) from the inspected Slovak crayfish populations.

kept in aquaria. All four crayfish individuals sampled on 19 September 2013 were transferred alive to laboratory for breeding; two individuals died soon but the two others were still alive in January 2015. By that time, both surviving females had reproduced five times, approximately every three months. First reproduction of their offspring was observed at the age of 6 months, in synchrony with the maternal generation.

The DNA barcoding confirmed the morphological identification of captured crayfish as *P. fallax* f. *virginalis*. All obtained COI fragments matched completely the publicly available reference sequences of the marbled crayfish from GenBank (acc. nos. KC107813, HM358011, JF438007; Martin *et al.*, 2010a; Filipová *et al.*, 2011; Shen *et al.*, 2013). No traces of *Aphanomyses astaci* DNA were detected in any of the analyzed marbled crayfish.

DISCUSSION

The marbled crayfish presence in Central Europe is an excellent example of successful introductions of an ornamental species. Although original prognoses questioned its survival in the wild, especially in temperate climate (Martin *et al.*, 2010b), it is now recognized as an established invader both in Europe and in Madagascar (Jones *et al*., 2009; Kawai *et al*., 2009; Chucholl *et al*., 2012; Kouba *et al*., 2014). Due to its parthenogenetic reproduction strategy, theoretically no more than one individual is needed to establish a viable population (Scholtz *et al*., 2003). Based on our data and on findings from Germany (Chucholl and Pfeiffer, 2010; Chucholl *et al.*, 2012), it is evident that the species survives and successfully reproduces in Central European climatic conditions. Its overwinteringt ability, with successful survival at 2 to 3°C for three months, was also confirmed experimentally (Veselý *et al*., 2015). As the marbled crayfish is widely available in the aquarium pet trade in Europe, this raises concerns of its further introductions (Chucholl, 2013, 2014; Patoka *et al*., 2014b).

However, even if no new marbled crayfish populations become established in near future in Slovakia or adjoining countries, the already known Slovak populations are an obvious threat, as they may serve as the foothold for the spread of this species in the Danube basin. Several North American invasive crayfish are known for their considerable capacity for active migration and colonization. For example, survival potential of a desiccation for up to several hours has been documented for the congeneric red swamp crayfish *Procambarus clarkii* as well as for the signal crayfish *Pacifastacus leniusculus* (Banha and Anastácio, 2014). This may promote crayfish passive dispersal over long distances, but also allows crossing of terrestrial barriers to new suitable habitats. Active overland dispersal has been recently documented for the spiny-cheek crayfish *Orconectes limosus* (Puky, 2014), and living marbled crayfish have been also observed out of water, over 100 m from a lake (Chucholl *et al*., 2012). Moreover, water birds may possibly serve as the translocation vectors for crayfish. Small juveniles of the red swamp crayfish were reported to climb to mallard feet, remain there for several minutes and survive on air for up to three hours (Águas *et al*., 2014).

If the marbled crayfish manages to successfully colonize rivers in the Danube basin (which seems likely as the population in the thermal stream in Opatovce is not separated from the Nitra River by any barrier, and the other populations are in a close vicinity of the Váh River and its side channel), the species' relatively fast dispersal can be expected unless restricted by environmental factors. The colonization potential of invasive crayfish can be dramatic, spreading downstream and even upstream in a considerable speed (Bubb *et al*., 2004). A good example is the colonization of the Danube River by the spiny-cheek crayfish in Hungary and adjoining countries (Puky and Shád, 2006; Pârvulescu *et al*., 2012; Lipták and Vitázková, 2014). The expansion of marbled crayfish may be further enhanced by passive dispersal along the rivers, in particular downstream by currents and floods. Single individuals of sexually reproducing crayfish invaders, when dispersing over long distances, are highly unlikely to establish a population unless a mated mature female or female with a clutch survives the translocation (note, however, that it remains unclear under which conditions facultative asexual reproduction, reported for spiny-cheek crayfish, takes place; Buřič *et al*., 2011). However, due to the obligate asexual reproduction of the marbled crayfish, this taxon is not limited by the Allee effect at very low population densities, and even dispersal of juvenile individuals may allow their subsequent reproduction in newly colonized sites. Floods (such as those occurring in the Váh basin in 2006 and 2010) may thus not only allow the crayfish to spread from the gravel pits to the river system, but also facilitate their rapid downstream dispersal.

Thermal streams, both fed by natural warm springs and those thermally polluted by human activities (*i.e.*, cooling water from industry), represent a specific category of habitats that may support introductions of ornamental aquatic species in temperate regions (Emde *et al*., 2016). Many of such species are elsewhere limited by the low water temperatures and are unable to proliferate outside the thermal streams; however, some of them tolerate a wide range of temperatures and may disperse successfully out of these sites of introduction. Numerous cases of establishment of aquarium crustaceans, in particular crayfish, have been documented in such habitats across Europe. The red swamp crayfish in a thermal stream in Austria (Petutschnig *et al.*, 2008) and the tropical redclaw crayfish, *Cherax quadricarinatus*, in an oxbow lake in Slovenia (Jaklič and Vrezec, 2011) seem so far restricted to thermal waters. In Germany, establishment of two aquarium shrimp species, one of which may tolerate also lower temperatures, has been documented in a stream fed by cooling water from a coal power plant (Klotz *et al*., 2013). In case of the marbled crayfish in a thermal stream in Slovakia, the temperature does not seem a limiting factor (as apparent from the other established marbled crayfish populations in Central Europe; Chucholl *et al*., 2012). Furthermore, the relatively fast current of the thermal stream can facilitate crayfish movement into the Nitra River.

Juveniles observed in autumn, and the presence of medium-sized individuals in our samples (Fig. 2), indicate at least two seasonal clutches of the marbled crayfish in studied sites. It is estimated that under the laboratory conditions, the marbled crayfish can complete up to seven reproduction cycles during its lifespan of 2 to 3 years, and the generation time is about 6-7 months (Vogt, 2010). The amount of juveniles increases with each cycle in relation to size increase of the maternal individuals (Vogt, 2011), and may reach very high values for large females. Under laboratory conditions, Vogt (2011) reported the maximum number of 427 juveniles in one clutch. Some field-collected individuals were nevertheless even more fecund: one female from Madagascar studied by Jones *et al.* (2009) carried approximately 530 eggs (see Fig. 2 in Jones *et al*., 2009), and Chucholl and Pfeiffer (2000) reported as many as 724 eggs in a single marbled crayfish clutch from a German population. Thus, 455 juveniles carried by one marbled crayfish from the Koplotovce site do not seem to be exceptional, even under Central European conditions, and this number confirms a substantial reproduction potential of this invasive species.

The ability of the marbled crayfish to act as an *A. astaci* vector deserves considerable attention as well. Although no *A. astaci* infection was detected in our study, a complete absence of the pathogen cannot be ascertained. Infected marbled crayfish have been already confirmed in the aquarium pet trade, laboratory cultures, as well as in the wild (Keller *et al*., 2014; Mrugała *et al*., 2015), and genotyping of the pathogen suggested that the species got infected by horizontal transmission from another species (Mrugała *et al*., 2015). If the marbled crayfish successfully colonizes the Danube, it is thus likely that it will acquire the infection from the spiny-cheek crayfish, confirmed to carry the crayfish plague pathogen in this river (Kozubíková *et al*., 2010; Pârvulescu *et al*., 2012). Due to the marbled crayfish potential to rapidly expand its range, it is possible that it might spread the infection also into habitats that the other American species has not reached yet.

CONCLUSIONS

The presence and potential spread of the marbled crayfish in Slovak freshwaters represents a threat not only to the native astacofauna but potentially also to other aquatic biota. Fast growth, early maturation, high fecundity and parthenogenetic reproduction strategy combined with a capacity for competition with other crayfish species (Jimenez and Faulkes, 2010) and an ability to spread crayfish plague pathogen (Keller *et al*., 2014; Mrugała *et al*., 2015), characterize a very successful invader. Given the fact that the species is widely available in the aquarium trade and already introduced to several locations in Europe, a management aiming to prevent further expansion is crucial.

This situation increases the pressure on local public and environmental agencies to promote adequate preventive actions, as the lack of proper education may promote translocations and introduction of the crayfish to new waterbodies, and thus contribute substantially to the marbled crayfish further colonization of the Danube catchment. The socioeconomic drivers increase the likelihood of species introductions, particularly in areas with high gross domestic product and high human population density (Perdikaris *et al*., 2012; Chucholl, 2014), such as the Vienna-Bratislava region and nearby Budapest metropolitan area in Hungary. Thus, our findings of established marbled crayfish might not be the last from this region. We believe that public education focusing on the mechanisms and consequences of crayfish spread, along with the development of more intensive regulation of ornamental trade, should constitute a basis of any management action. Furthermore, it should be supported by a further research evaluating marbled crayfish impacts on the native communities and habitats, and eventually, a development of the effective elimination means of alien crayfish from the natural environments.

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