

# Self-limiting outbreak of crayfish plague in an *Austropotamobius pallipes* population of a river basin in the Abruzzi region (central Italy)

Riccardo Caprioli\*, Daniele Cargini, Maurilia Marcacci, Cesare Cammà, Carla Giansante, Nicola Ferri

Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise 'G. Caporale', Via Campo Boario, Teramo 64100, Italy

**ABSTRACT:** Crayfish plague, caused by the oomycete *Aphanomyces astaci*, is a serious disease of European freshwater crayfish and has eliminated entire populations in several European countries. In September 2011, mortality was observed among the *Austropotamobius pallipes* population of a river basin in the Abruzzi region (central Italy), and *A. astaci* DNA was detected by PCR in dead crayfish. A systematic survey was carried out to evaluate the spread and the effects of the plague in the river basin. The source of the outbreak remained unknown since North American crayfish species, which frequently act as subclinical carriers of the infection, were not detected in the area. The *A. pallipes* population disappeared from a river stretch of ~1 km, where *A. astaci* infection was detected in dead crayfish. However, apparently unaffected crayfish were still present upstream of that area as well as in a tributary that joined the brook in the apparently depopulated stretch. *A. astaci* infection was not detected in dead individuals collected in the upstream area and tributary. A follow-up visit conducted in the following season showed the presence of *A. pallipes* in the river stretch hit by the plague. In this outbreak, the spread of the infection could have been limited by a low density of the crayfish population and by the geographic conformation of the river basin, which includes a dense network of small tributaries, characterized by high flow velocity and low water temperature. In this particular setting, crayfish plague outbreaks can remain undetected. This underlines the importance of active monitoring programs aimed at the prompt recognition of both episodes of mortality and the presence of non-indigenous crayfish species.

**KEY WORDS:** *Aphanomyces astaci* · Whiteclawed crayfish · Molecular detection

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## INTRODUCTION

Crayfish plague is an acute disease of freshwater crayfish caused by the oomycete *Aphanomyces astaci*. European crayfish species are highly susceptible to the disease, which can cause mass mortalities and has severely affected many crayfish populations throughout the continent (Edgerton et al. 2004, OIE 2009). Conversely, North American crayfish species, such as *Pacifastacus leniusculus*, *Procambarus clarkii*, and *Orconectes limosus*, have a low susceptibility for the disease and can act as subclinical carriers of the infection

(Oidtmann et al. 2002, 2006, OIE 2009). These non-indigenous crayfish are widely distributed in Europe (Holdich et al. 2009) and have contributed to the spread of the crayfish plague in areas populated by native European species (Bohman et al. 2006, Kozubíková et al. 2011a, Vrålstad et al. 2011). Large outbreaks of *A. astaci* infection among indigenous crayfish populations have been reported in many European countries, often associated with the presence and spread of non-indigenous crayfish species (Diéguez-Urbeondo et al. 1997, Bohman et al. 2006, Kozubíková et al. 2008, Vrålstad et al. 2011, Pârvulescu et al. 2012).

\*Email: r.caprioli@izs.it

Little is known about the presence and the distribution of the crayfish plague in Italy, although the first outbreak in Europe probably occurred in the Po river valley in northern Italy in the 1860s (Alderman & Polglase 1988, Edgerton et al. 2004). Several episodes of crayfish population mortalities were reported during the last century, but the involvement of *Aphanomyces astaci* has never been confirmed (Gherardi 1999). The first documented outbreak of crayfish plague in Italy occurred in 2009 (Cammà et al. 2010) and involved the white clawed crayfish *Austropotamobius pallipes*, which is the most common native species in Italy (Scalici et al. 2009, Aquiloni et al. 2010). The populations of 3 brooks in the watershed of the river Trigno, located in the Molise region (central Italy), were involved, and *A. astaci* infection was demonstrated in moribund crayfish (Cammà et al. 2010). Another outbreak of *A. astaci* infection occurred in another brook of the same river basin in 2010 and was notified to the World Organization for Animal Health (WOAH), formerly Office International des Epizooties (OIE) ([www.oie.int/wahis\\_2/temp/reports/en\\_fup\\_000009757\\_20100927\\_163756.pdf](http://www.oie.int/wahis_2/temp/reports/en_fup_000009757_20100927_163756.pdf)). In both episodes, field investigations did not detect the presence of North American crayfish species in the river basin involved, and the origin of the outbreaks remain unknown.

In the summer of 2011, an episode of crayfish plague affected the *Austropotamobius pallipes* populations in a river basin located in the Abruzzi region, which is adjacent to the Molise region where the 2009–2010 outbreaks had occurred. The Abruzzi outbreak involved a limited geographic area, and field investigations failed to reveal the presence of non-indigenous crayfish species in the area. In the present paper, we describe the characteristics of this outbreak and discuss the possible sources as well as the factors that may have contributed to limiting its spread.

## MATERIALS AND METHODS

### Background of the outbreak

During the summer of 2011, 8 brooks belonging to 4 separate river basins of the Abruzzi region (central Italy) were regularly visited for monitoring the *Austropotamobius pallipes* populations and for the collection of individu-

als to be used as broodstock, in the framework of a regional conservation and management program of the natural crayfish populations. On 6 September 2011, a dead *A. pallipes* individual was found in the Zingano brook (42.58494° N, 13.47921° W; altitude 838 m), located in the Vomano river basin, together with a few other apparently healthy-looking individuals. Once delivered to the laboratory, the dead crayfish tested positive for *Aphanomyces astaci* DNA using the real-time PCR method described below, with a cycle threshold (Ct) of 24. This finding led to a survey specifically dedicated to the detection of dead or diseased crayfish in the Vomano river basin.

### Survey sites and sample collection

The Zingano brook is a small watercourse located in the protected area of the *Gran Sasso e Monti della Laga* National Park. The river stretch involved in the outbreak is situated between 2 sites of community importance (IT7120201 and IT7110202), and previous monitoring activities carried out by our institute between 2009 and 2011 had shown the presence of a medium density population of *Austropotamobius pallipes* (unpubl. data). The survey concerned a total of 8 sites, 3 located upstream and 5 downstream of the place where the *Aphanomyces astaci*-positive crayfish had been collected, defined as 'Point 0' (Fig. 1, Table 1). Four sites were located on the Zingano brook, 3 on right-bank tributaries (Vaccaroli,

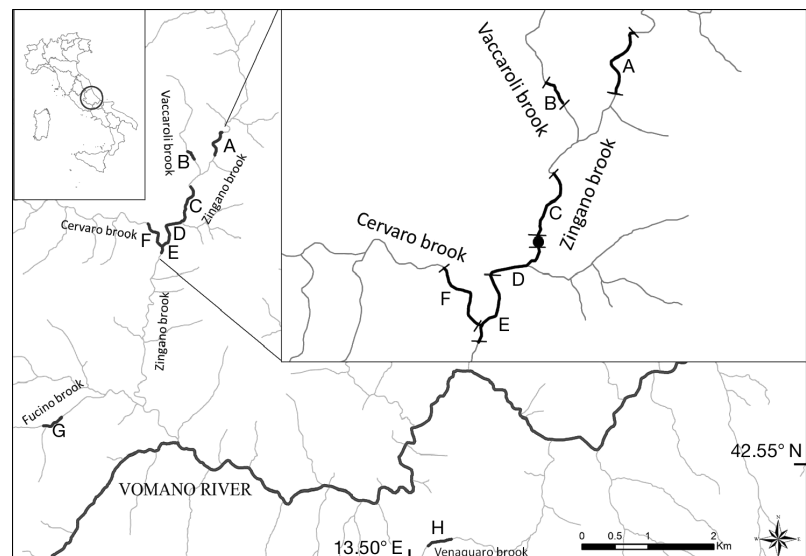


Fig. 1. Map of the Vomano river basin, with the location of the sampling sites on the Zingano brook (Sites A, C, D, and E) and the other tributaries (Sites B, F, G, and H). (●) Location where the first *Aphanomyces astaci*-positive crayfish was detected (Point 0)

Table 1. Length and characteristics of the river stretches selected as survey sites and their upstream or downstream positions with respect to the place where the first *Aphanomyces astaci*-positive crayfish was detected (Point 0). Stream flow velocity: from 0 (still water) to 5 (rapid water)

Survey site	Brook	Stretch length (km)	Distance of the initial point of the stretch from Point 0 (km)	Altitude (m)	Stream flow velocity (0 to 5)	Temperature at sampling (range, °C)
<b>Upstream sites</b>						
A	Zingano	0.5	2	985	2	10.8–13.1
B	Vaccaroli	0.15	0.9	935	3	14.2
C	Zingano	0.5	0	846	2	11.1–16.8
<b>Downstream sites</b>						
D	Zingano	0.3	0	831	1	12.3–17.8
E	Zingano	0.45	0.3	818	1	15.7–18.8
F	Cervaro	0.4	0.6	824	3	15.5
G	Fucino	0.2	4.5	819	3	14.8
H	Venaquaro	0.5	15.5	716	4	11.6

Cervaro, and Fucino brooks) and 1 on the Venaquaro brook, a lower right-bank tributary of Vomano River (Fig. 1). These sites represented the stretches of brook that were accessible for survey activities and as close as possible to Point 0. Each site was characterized in terms of geo-morphological features of the brook (Table 1), and the temperature of the water at sampling was recorded. The stream flow velocity was expressed as a scale ranging from 0 (still water) to 5 (rapid water). The distance from Point 0 ranged from 0 to 2 km for the upstream sites and from 0 to 15.5 km for the downstream sites. The total length of the monitored river stretches was 3 km. No artificial dam or natural waterfalls higher than 50 cm were present in the stretch of the Zingano brook included in the survey.

The survey was conducted between 8 September and 5 October 2011 and was interrupted when the water temperature fell below 11°C and heavy rains made it difficult to survey the rivers. Each sampling visit was carried out during the day, for the optimal detection of dead and dying crayfish, and at night, using a flashlight, to better evaluate the presence of apparently unaffected individuals. Crayfish were collected by handpicking, and the species and sex of each individual was determined. To estimate the density of the living crayfish population in the survey sites, the average number ( $n$ ) of apparently healthy crayfish (individuals with a general status and behavior that did not indicate acute crayfish plague and with apparent absence of damaged or melanized areas in the exoskeleton) observed along a 50 m river stretch during each visit was recorded. Densities were classified as high ( $n > 10$ ), medium ( $6 < n < 10$ ), low ( $2 < n < 5$ ), very low ( $n < 2$ ), and 'no observed crayfish' (when no living crayfish were observed along the whole site). The healthy crayfish were

immediately released, while freshly dead or dying individuals were inspected macroscopically for any signs of infection (damaged or melanized areas in the exoskeleton) and brought to the laboratory fresh cooled. All of the surveying equipment (boots, nets, and buckets) were cleaned and carefully disinfected with 10 % sodium hypochlorite after each visit.

The area just upstream and downstream of Point 0 (Sites C and D) was visited again in June 2012 to evaluate the presence of crayfish in the brook, using the same methodology as the 2011 sampling visits.

### Laboratory diagnosis of *Aphanomyces astaci* infection

Laboratory diagnosis of *Aphanomyces astaci* infection was carried out by detection of the pathogen DNA directly from the soft abdominal cuticle of crayfish by real-time PCR. The PCR method developed by Vrålstad et al. (2009) and based on the amplification of a unique sequence motif of *A. astaci* in the internal transcribed spacer 1 (ITS1) was used, with slight modifications. Total DNA was extracted from the soft abdominal cuticle of freshly dead crayfish using the Maxwell™ 16 Tissue DNA Purification kit (Promega) according to the manufacturer's instructions. Real-time PCR was carried out on a 7900HT Fast Real-time PCR System (Applied Biosystems), and raw data were analyzed using the SDS 2.4 software program (Applied Biosystems). The reaction protocol described by Vrålstad et al. (2009) was modified as follows: 20 s at 95°C, followed by 45 cycles of 1 s at 95°C and 20 s at 58°C. The cycle threshold (Ct) level was fixed at the same middle exponential position for all runs, and a cut-off value of Ct 41 was used to define a positive

signal. All of the samples as well as a no-template control (NTC) were run in triplicates. The presence of *A. astaci* DNA was confirmed by PCR amplification and sequencing of a 569 bp fragment of the ITS region, using the method described by Oidtmann et al. (2006) and recommended by OIE (2009). The PCR product was purified with the QIAquick PCR Purification Kit (Qiagen), sequenced using BigDye Terminator v3.1 (Applied Biosystems), and subsequently analyzed on a 3130 XL Genetic Analyzer (Applied Biosystems). Assembly of the raw sequence data was conducted in Contig Express (Vector NTI suite 9.1, Invitrogen), and consensus sequences were submitted to a BLAST search (<http://blast.ncbi.nlm.nih.gov/>).

## RESULTS

Between 8 September and 5 October 2011, a total of 19 sampling visits were carried out in the 8 survey sites. An additional follow-up visit was made in June 2012. No crayfish species other than *Austropotamobius pallipes* were observed, and the number of dead or moribund crayfish recorded in each visit is re-

ported in Table 2 together with the results of the PCR assays for the detection of *Aphanomyces astaci* DNA and the information on the presence and density of living crayfish.

Dead or dying crayfish were mainly observed at the sites (D and E) immediately downstream of Point 0, the place where the first *Aphanomyces astaci*-positive crayfish had been collected. Sites D and E covered a 750 m stretch of the Zingano brook. In general, no adverse effects were observed in the fish or aquatic macroinvertebrates living in the sites where dead crayfish were observed.

Of the 13 dead crayfish observed at Sites D and E on 15 September, 6 were collected for PCR analyses, while the remaining 7 individuals showed a decay too advanced to be evaluated for gross pathology and tested by PCR. All 6 of the samples tested positive for the presence of *Aphanomyces astaci* DNA, and the ITS sequences scored 100% identity to the published sequences of *A. astaci* detected in the Molise region in 2009 (GU174502). Two sequences representative of the positive samples from Sites D and E were released in GenBank (JX961602 and JX961603). None of the *A. astaci*-positive individuals had melanized areas in the cuticle.

Table 2. Survey for the detection of crayfish plague in the Vomano river basin. The density of the *Austropotamobius pallipes* populations and the number of dead crayfish recorded in each sampling visit are reported together with the number of individuals positive in the *Aphanomyces astaci* real-time PCR, out of those suitable for testing. Densities were classified as high ( $n > 10$ ), medium ( $6 < n < 10$ ), low ( $2 < n < 5$ ), very low ( $n < 2$ ), and 'no' (no living crayfish observed). The real-time PCR cycle threshold (Ct) values of the positive crayfish are reported. \*No signal in RealTime PCR

Site	Brook	Date (2011)	Observation of living crayfish density	No. of dead crayfish	No. of <i>A. astaci</i> -positive crayfish (Ct values)
A	Zingano	26 Sep	Medium	2	0/2*
		5 Oct	Low	0	–
B	Vaccaroli	27 Sep	Medium	0	–
C	Zingano	8 Sep	Medium	0	–
		12 Sep	Medium	2	0/2*
		13 Sep	Medium	0	–
		25 Sep	Medium	4	0/4*
		4 Oct	Low	0	–
		21 Jun 2012	Medium	0	–
D	Zingano	8 Sep	Very low	0	–
		13 Sep	Very low	0	–
		15 Sep	Very low	11	4/4 (21, 22, 19, 21)
		25 Sep	Very low	0	–
		4 Oct	No	2	2/2 (20, 22)
E	Zingano	21 Jun 2012	Low	0	–
		9 Sep	No	0	–
F	Cervaro	15 Sep	No	2	2/2 (21, 19)
		9 Sep	Low	0	–
G	Fucino	21 Sep	Low	0	–
		10 Sep	No	0	–
H	Venaquaro	3 Oct	Low	1	0/1*

Based on the PCR results, the Italian competent authorities reported the outbreak to WOAHP on 26 September ([www.oie.int/wahis\\_2/temp/reports/en\\_imm\\_0000011063\\_20110926\\_140614.pdf](http://www.oie.int/wahis_2/temp/reports/en_imm_0000011063_20110926_140614.pdf)).

The early visits at Site D showed also the presence of apparently healthy individuals, although the population density was classified as 'very low'. No living crayfish were observed during the visit carried out at Site D on 4 October, when 2 dead crayfish were collected and proved positive for *Aphanomyces astaci* DNA. The river stretch including Sites D and E was characterized by a slow current flow and by the presence of large pools. The water temperature at Site E on 15 September was 18.8°C, higher than temperatures recorded at the other sampling sites, which ranged between 11 and 16°C (Table 1). Only living and apparently unaffected crayfish were observed during 2 visits at Site F, which included the final 400 m stretch of the Cervaro brook, a right-side tributary that joins the Zingano brook at the level of Site E (Fig. 1).

Further downstream, the rapid water flow and the steep and bushy river banks made access to the Zingano brook too difficult: therefore, the following survey, Site G, was located 4.5 km from Point 0, at the confluence of another right-side tributary. No crayfish were observed at Site G, while a low density population was recorded at Site H, located on a right-side tributary of the Vomano River, the main water course of the river basin (Fig. 1). At this site, ~15 km from Point 0, only 1 dead crayfish was found, but no *Aphanomyces astaci* DNA was detected in the soft abdominal cuticle sample.

A total of 8 sampling visits were carried out at the 3 sites located up to 2 km upstream of Point 0. At 2 sites (A and C) on the Zingano brook and at the site on the Vaccaroli right-side tributary (B), a medium density of living crayfish was recorded in September. Altogether 8 dead individuals were collected from these sites in the same period, but the *Aphanomyces astaci*-specific PCR assay was negative for all tested samples. The density of the crayfish population was lower in October, with a higher prevalence of male and juvenile individuals.

Six brooks located in 3 other river basins of the region (Tronto, Tordino, and Aterno-Pescara) were regularly visited during the survey period, but no relevant episodes of mortality were observed.

The follow up visit carried out in June 2012 at the sites immediately upstream and downstream of Point 0 (Sites C and D) showed the presence of *Austropotamobius pallipes* populations with medium and low densities, respectively (Table 2).

## DISCUSSION

Crayfish plague is one of the main causes of mass mortalities in native European crayfish populations (Holdich et al. 2009), such that *Aphanomyces astaci* has been classified among the world's 100 worst invasive non-indigenous species (Lowe et al. 2004). This is the first report of crayfish plague in the Abruzzi region, and its underlying causes remain unknown. The episode occurred in a river basin located ~200 km north of the area in the adjacent Molise region, where documented outbreaks of the disease occurred in 2009 (Cammà et al. 2010) and 2010. It is difficult to establish any link with the Molise episodes since no connecting waterways exist between the 2 river basins. It is well known that long-distance transmission of *A. astaci* zoospores may occur through the transfer of infected crayfish, fish, fishing gear, or water (Kozubíková et al. 2008, OIE 2009). However, these activities are not carried out in the Zingano brook, a small watercourse located in a protected area. The Vomano River is re-stocked with trout downstream of its confluence with the Vaccaroli brook (Site H), but the fish stocks used are produced locally.

In European countries, outbreaks of crayfish plague in indigenous crayfish populations have often been associated with the concomitant presence of well-established populations of North American crayfish (Diéguez-Urbeondo et al. 1997, Bohman et al. 2006, Kozubíková et al. 2008, OIE 2009, Vrålstad et al. 2011, Pârvulescu et al. 2012). These populations are frequently infected with *Aphanomyces astaci* without development of clinical disease and act therefore as lifelong carriers of the pathogen (OIE 2009). In this outbreak, the transmission of the pathogen from carrier American crayfish is unlikely since all the investigations failed to reveal the presence of any crayfish species other than *Austropotamobius pallipes* in the river basin involved in the outbreak and in 3 other river basins of the region. In Italy, the presence of North American crayfish species has been reported in several areas (Holdich et al. 2009, Scalici et al. 2009, Aquiloni et al. 2010). *Procambarus clarkii* is the most widespread species, and a population of *A. astaci*-positive carrier crayfish was recently described in a stream located ~300 km north-west of the present outbreak area (Aquiloni et al. 2011). The presence of *P. clarkii* in one of the 4 provinces of the Abruzzi region has been mentioned in 2 review articles (Barbaresi & Gherardi 2000, Aquiloni et al. 2010). However, this finding has not been confirmed in recent years, despite the monitoring of the



regional crayfish populations carried out between 2003 and 2006 in the framework of a Life Program Project (LIFE03/NAT/IT/000137 2006) and by our group since 2009 (R. Caprioli unpubl. data). Even if we cannot exclude the possibility that small populations of non-indigenous crayfish may have gone undetected, alternative sources can be considered for this outbreak. In this respect, it is important to note that the presence of *A. astaci* infection has already been described in populations of the European narrow clawed crayfish *Astacus leptodactylus* living in areas where the presence of non-indigenous American species have never been reported, such as Turkey (Harlioglu 2008, Svoboda et al. 2012, Kokko et al. 2012) and the Danube Delta (Schrimpf et al. 2012).

In our case, *Aphanomyces astaci* might have persisted in the *Austropotamobius pallipes* population of the river basin as a chronic infection. This condition has already been reported for other European species, such as the noble crayfish *Astacus astacus* (Jussila et al. 2011, Viljamaa-Dirks et al. 2011) and *A. leptodactylus* (Svoboda et al. 2012). Jussila et al. (2011) and Viljamaa-Dirks et al. (2011) showed that, in Scandinavian lakes, *A. astaci* survived for several years supported by both weak and robust populations of the noble crayfish. Viljamaa-Dirks et al. (2011) also showed that the *A. astaci* strain involved belonged to genotype As (Huang et al. 1994), which has never been reported in North American crayfish species and appears to be less aggressive than the other 4 genotypes described so far (Kozubíková et al. 2011b).

The capability to coexist with *Aphanomyces astaci* for long periods of time has also been reported for *Astacus leptodactylus*. In Turkey, recent studies based on real-time PCR detection have confirmed that the pathogen is able to persist in local crayfish populations (Kokko et al. 2012, Svoboda et al. 2012). In particular, Kokko et al. (2012) showed a 95% prevalence of *A. astaci* DNA in the populations of 2 lakes. Despite this high prevalence and the presence of animals showing signs of infection, both the crayfish populations were capable of forming productive stocks, prompting the authors to hypothesize a partial resistance adaptation in the host and/or an evolution toward a lower virulence of the parasite. A similar situation was reported for a Danube Delta area, where Schrimpf et al. (2012) demonstrated that a substantial proportion (32%) of the local *A. leptodactylus* population was infected by *A. astaci*. Only some individuals showed signs of infection, like melanized spots, and no mass mortalities of crayfish

were reported from the region for several decades. Therefore, the authors considered the possibility that the crayfish plague may have persisted in the Danube Delta as a chronic infection from an old plague wave in the 19th century. The possibility that the crayfish plague may persist in *Austropotamobius pallipes* populations as a chronic subclinical carrier status clearly deserves further study.

In most cases, the introduction of *Aphanomyces astaci* in susceptible populations of European native crayfish species has caused high levels of mortality within short periods of time, often leading to the elimination of the entire populations impacted by the plague (Kozubíková et al. 2008, OIE 2009). The episode of crayfish plague described here, conversely, involved only a limited area of the river basin and appeared to be self-limiting, despite the fact that the species involved, *Austropotamobius pallipes*, is considered to be highly susceptible to *A. astaci* infection. The *A. pallipes* population disappeared from the river stretch where *A. astaci* infection was detected, but apparently unaffected crayfish were still present upstream of that area as well as in a tributary that joins the Zingano brook in the middle of the apparently depopulated river stretch. Consistently, the few dead individuals found in the upstream sites were negative in the *A. astaci*-specific PCR, although we cannot rule out the possibility that analysis of additional tissues could have revealed more positive individuals, based on recent investigations by Vrålstad et al. (2011) and Schrimpf et al. (2012). In other plague outbreaks, survival of crayfish populations upstream of the infection wave has been favored by the presence of artificial barriers, like pond dams (Kozubíková et al. 2008). In the case of the Zingano brook, no artificial dam was present in the river stretch included in the survey. However, a natural waterfall ~0.5 m high was located within Site C, just upstream of Point 0, and could have limited the upstream movement of *A. astaci* infected crayfish from Site D.

Follow-up visits conducted in the following season showed the presence of *Austropotamobius pallipes* in the river stretch hit by the crayfish plague. Interestingly, a similar situation was observed in the plague outbreak that occurred in 2009 in the neighboring Molise region (Cammà et al. 2010). Several hypotheses can be advanced to explain the limited spread of the infection in both the episodes. It is well known that the dispersal of crayfish plague through a river is faster when the population of susceptible crayfish is abundant, while the spread can be slower and evidence of mortality less dramatic in the presence of low crayfish densities and low water temperatures

(Alderman et al. 1987, OIE 2009). The crayfish population densities of both the river basins involved in the 2009–2010 and 2011 outbreaks were not high, and in the absence of a population of non-indigenous crayfish acting as long lasting reservoir of *Aphanomyces astaci*, this feature may have hindered the spread of the infection. Also, the geographic conformation of the river basins involved in the outbreaks may have influenced the favorable outcome. Very fast rates of plague spread have been reported for large water bodies, such as lakes (Bohman et al. 2006, Vrålstad et al. 2011) or large rivers like the Danube (Pârvulescu et al. 2012). In such settings, *A. astaci* infection can spread rapidly downstream, particularly at summer water temperatures, and river stretches of over 50 km may lose all their crayfish in a few weeks after the first observed mortality (OIE 2009). The river basins of the Abruzzi and Molise regions involved in the outbreaks include only rivers of limited dimensions, with a dense network of tributaries. This could have favored the survival of crayfish that were present in the highest reaches of tributaries that were not reached by spores. In addition, most of the Abruzzi and Molise watercourses are mountain brooks characterized by high flow velocity and low water temperature. Consistently, the river stretch more involved with the plague was characterized by the presence of large pools with slow current flow, which favored a rise of the water temperature to 19°C, the highest amongst those recorded during the survey.

Finally, it is possible that the *Aphanomyces astaci* strain involved in this outbreak was not highly pathogenic toward *Austropotamobius pallipes*. It has been reported that established older *A. astaci* strains that have been present in Europe for many years could be less aggressive than newer strains introduced with North American crayfish since the 1960s (Huang et al. 1994, OIE 2009, Kozubíková et al. 2011b). These strains usually belong to Group A (genotype As), which has only been reported in European crayfish and probably represents the genotype that caused the first massive wave of crayfish plague in the 19th century and has persisted in Europe since then (Huang et al. 1994, Kozubíková et al. 2011b). The other 4 known genotypes were presumably introduced later on, with their American crayfish hosts (Kozubíková et al. 2011b). The apparent absence of non-indigenous crayfish species in the area suggests that this could also be a possible explanation of the reduced impact of this outbreak. Unfortunately, the *A. astaci* strains responsible for this episode were not isolated and were not available for comparative studies.

In conclusion, this outbreak (1) confirms that crayfish plague can occur in areas where North American species are not apparently present, (2) poses the question of a possible *Aphanomyces astaci* subclinical infection status in *Austropotamobius pallipes* populations, and (3) suggests that in river basins mainly constituted by mountain brooks with high flow velocity, waterfalls, and low water temperature, the disease might not have a rapid, dramatic impact on the native crayfish populations. The absence of mass mortalities, together with the difficult access to the river banks, makes it possible that crayfish plague outbreaks can remain undetected in this particular setting. This underlines the importance of implementing active monitoring programs of the *A. pallipes* populations, aiming at the prompt recognition of episodes of mortality and presence of non-indigenous species as well as at the evaluation of the possible subclinical *A. astaci* carrier status of the crayfish.

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Editorial responsibility: Grant Stentiford,  
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