J. Limnol., 2014; 73(1): 17-26
DOI: 10.4081/jlimnol.2014.707

ORIGINAL ARTICLE

The effects of pool sediments on the egg morphology of Neotropical *Eulimnadia* (Branchiopoda: Limnadiidae)

Nicolas RABET,[1,2*] David MONTERO,[3] Sébastien LACAU[2]

[1]Université Pierre et Marie Curie - UMR BOREA Biologie des Organismes et Ecosystèmes Aquatiques, MNHN-CNRS-UPMC-IRD, 61 rue Buffon, 75005 Paris, France; [2]Laboratório de Biossistemática Animal, Universidade Estadual do Sudoeste da Bahia, UESB/DEBI, BR 415, Km 03, 45700-000 Itapetinga, Brazil; [3]CNRS FR 2482 Institut des Matériaux de Paris Centre, Université Pierre et Marie Curie, 11 Place Marcelin Berthelot, 75231 Paris, France
*Corresponding author: rabet@mnhn.fr

ABSTRACT

Studying the species distribution of Limnadiidae, a family of crustaceans, in environments favourable to their growth and reproduction is made difficult by their extremely short life cycle and the transient existence of adult forms. However, the eggs of these branchiopods are highly resistant to environmental extremes and persist in the soil of humidified and dried pools. We therefore studied the capacity to detect populations of Limnadiidae and identify them at the species level using egg morphology. We investigated influences of pool sediments on Eulimnadia *(clam shrimp) egg morphology and asked whether we could rely on this morphology after various lengths of time in the soil to recognise species. We studied sediment collected from temporary pools in four Neotropical areas: Minas Gerais and Bahia (Brazil), French Guiana, and Martinique. These samples represented diverse geographical regions and climates and allowed us to study the four most widely distributed* Eulimnadia *species in the Neotropics:* E. colombiensis*,* E. cylindrova*,* E. geayi*, and* E. magdalensis*. Our results indicated that soil abrasion is superficial and does not affect the general shape of the eggs (cylindrical or spherical). However, details of egg ornamentation can be severely affected by the combined effects of erosion and filling with sediments, which can lead to difficulties in species identification. In the particular case of the spherical eggs of* E. magdalensis*, this species cannot be unequivocally identified without clean eggs collected from adults after breeding or from eggs recently deposited in the field. This work and the investigative methods described herein should facilitate identification of* Eulimnadia *species and promote further study of Limnadiidae crustaceans in the field.*

Key words: clam shrimps, resting egg, morphology, Brazil, French Guiana, Martinique.

Received: February 2013. Accepted: July 2013.

Non-commercial use only

INTRODUCTION

Clam shrimps of the genus *Eulimnadia* Packard 1874, are small crustaceans in the Limnadiidae family (Branchiopoda, Spinicaudata). These animals are generally about 0.5-1 cm in length and typically live in different types of ephemeral pools. They are able to survive in these transient habitats thanks to their rapid development and reproductive cycles and their highly adapted desiccation-resistant eggs (or cysts), which they deposit in the sediments of these pools. Once the pools dry out, their eggs remain viable through seasonal dry periods and hatch when the pools are refilled during the rainy season. In the Neotropical region, 12 species of *Eulimnadia* have been reported (Martin and Belk, 1989; Brendonck *et al.*, 1990; Brtek, 1997; Pereira and Garcia, 2001; Rabet, 2010; Rabet *et al.*, 2012) and six are known to be widely distributed (Pereira and Garcia, 2001; Rabet *et al.*, 2012). Collection of adults is usually possible only when the pools are first filled because these organisms have a very short life cycle, frequently no more than 20 days (Weeks *et al.*, 1997; N. Rabet, personal data). The rapid life cycle of these animals partially explains why they are rarely reported and suggests a need to develop new methods of detecting and studying them in the field.

A particularly promising method consists in using egg morphology to identify *Eulimnadia* species and study their distribution (Brendonck *et al.* 2008). The eggs of *Eulimnadia* species have different shapes that include spherical, cylindrical, and twisted forms. These variations are associated with details of egg ornamentation such as crests, ridges, furrows, and large depressions (Rabet, 2010). The homogeneity and fibre thickness of the alveolar layer – which is visible in sections of the egg shell – also varies according to species (Rabet *et al.*, 2012). All these characters have proven to be useful tools for studying *Eulimnadia* taxonomy (Belk, 1989; Rabet 2010; Rabet *et al.*, 2012). In large branchiopods, species determination generally requires to use additional characters shared by adult forms. In the case of genus *Eulimnadia*, the characters shared by their eggs have a special taxonomic interest. Indeed, in this genus most of the species are distinguished only by their superficial egg morphology (Belk, 1989; Martin, 1989; Martin and Belk, 1989; Pereira and Garcia, 2001; Rabet, 2010), which is unusual in branchiopods. However, all previous taxonomical analyses of eggs were conducted using eggs collected from fixed animals and the effects of different pool soils were not been studied.

In order to investigate the possibility of studying the distribution of *Eulimnadia* species using eggs collected in the field, we sampled sediments from pools harbouring *Eulimnadia* populations in several distinct Neotropical regions. In these regions, *Eulimnadia* are found with high frequency in favourable biotopes. We selected four areas: one each in Minas Gerais (Brazil), South Bahia (Brazil), French Guiana, and Martinique. Using *E. colombiensis* Roessler, 1989*, E. cylindrova* Belk, 1989*, E. geayi* Daday de Deés, 1926, and *E. magdalensis* Roessler, 1990 we tested the ability to use extracted eggs directly for identification and we then proposed a general strategy for identifying and studying new populations of Limnadiidae.

METHODS

Identification of favourable pools for collecting sediments

To detect new populations of *Eulimnadia,* we performed a systematic sampling of sediment (at depths up to 5 cm) in all types of pools. Alternatively, limited numbers of selected pools were analysed to save resources. When the pools were dry, temporary pools were identified by the presence of a depression, local modification of the vegetation and/or desiccation features. In these cases, dry sediment was collected from the centre of the former pool using a small spade. When the pools were filled but contained no adults and no shield remainders could be found, the temporary character of the pool was investigated by examining the aspect and configuration of the pool, the adjacent vegetation, and the depth of the pool which was reconciled with the local climate and recent rainfall. The presence of anostracans or other spinicaudatans was also a good indicator of a temporary pool. Damp sediments were collected from the centre and border of the pool and dried in the sun as rapidly as possible.

Sample storage and further utilisation

Dried soil samples were mechanically mixed by hand to homogeneity and then stored in closed plastic bags. The alveolar layer of resting eggs in the genus *Eulimnadia* is developed enough to enable the eggs to float after they are dry, which allows them to be easily separated from the sediment (Rabet *et al.*, 2012). To harvest eggs, 200 g of dried soil was mixed with 1.2 L of demineralised water in a 1.5 L plastic water bottle. The bottle was shaken vigorously six times for 1 min each over the course of 1 h to dissociate eggs from soil components. The supernatant containing floating eggs was filtered through progressively sized sieves (Hobby Artemia Sieve Combination - mesh size: 900, 560, 300, 180 and finally 80 µm; Reefphyto Ltd., Bristol, UK) to isolate the eggs from other floating debris. Eggs were identified under a stereomicroscope according to their size (100-200 µm in diameter), forms, and superficial ornamentations, and then separated for further analysis. Isolated eggs were used directly as needed. Some samples were conserved in 70% ethanol or re-dried on lamella. With this method we estimated that it was possible to detect *Eulimnadia* eggs if 5-10 eggs were present in 200 g of soil.

Breeding from the eggs

Adult specimens were collected directly from the field (Buritizeiro-Minas Gerais pool) or obtained from cultures derived from field-collected soil (other pools). For direct breeding of cultured adults, 200 g of pool soil was mixed with 12 L of distilled water in a standard aquarium. We also recycled material used for egg observations after mixture of eggs, supernatant, and soil in a final volume of 12 L. Tanks were maintained at 28°C and under constant light. Animals were fed on TetraMinBaby (Tetra GmbH, Melle, Germany). When adults developed, typically 5-7 days after hatching, they were fixed in 4% formalin and preserved in 70% ethanol.

Scanning electron microscopy

Eggs obtained from adults (removed from below the carapace with a fine needle and stored in 70% ethanol) or isolated from soil samples were dried and mounted onto scanning stubs using carbon conductive tape and then coated with a conductive layer (40 nm) of gold-palladium using a Polaron SC7640 sputter coater. Scanning electron microscopy (SEM) was performed on a JEOL 6100 SEM (JEOL Ltd., Tokyo, Japan). Eggs were opened using a fine tungsten needle to allow examination of the shell and the egg contents. The number of ornamented depressions on *E. magdalensis* eggs was estimated by methods used previously for anostracans (Timms and Lindsay, 2011).

Study sites

We studied four areas with various local conditions (Fig. 1).

Martinique (Saint Anne). Populations of *Eulimnadia* were detected only in trail freshwater pools in the dry forest in relatively isolated places near the sea. The surfaces of temporary pools in this area are less than 10 m^2 and normally less than 20-30 cm deep. Pools were filled by substantial rainfall generally during the rainy season but dried rapidly after the rain ceased. Ponds created for livestock – which were deeper, longer lasting, and common in this area – were not inhabited by *Eulimnadia* but

Non-commercial use only

rather by the anostracan *Streptocephalus similis* Baird, 1852.

Minas Gerais, Brazil (Buritizeiro). Temporary pools in this area are large (≥100 m^2), relatively deep (>50 cm), and can persist several months during the rainy season. Besides two *Eulimnadia* species (*E. colombiensis* and *E. geayi*), which can coexist in these pools, the anostracan *Dendrocephalus thieryi* Rabet, 2006 is also frequently present. The substrate of these pools is clayey.

Bahia, Brazil (Barrolândia). Populations of *Eulimnadia* were found in small pools which were frequently less than 10 m^2 and no more than 100 m^2 in size, and were located in windfall of the Atlantic forest. The pools have sandy substrates and can be found after heavy rains at different times of the year.

French Guiana (Sinnamary). Little information was available on these pools except that they had very clayey substrates and were in humid savannah.

Non-commercial use only

Fig. 1. Neotropical distribution of *Eulimnadia* species used in this study. Crosses represent *E. colombiensis*; squares indicate *E. magdalensis*; triangles show E. *geayi*; and circles with horizontal bar stand for *E. cylindrova*. Populations are indicated after simplifications, following Roessler (1995), Brendonck *et al.* (1990), Pereira and Garcia (2001), Rabet (2010), MacKay and Williams (2011), and from this study. Arrows with labels indicate the populations from which samples were collected for use in this study, *i.e.* Saint Anne (1), Sinnamary (2); Barrolândia (3); Buritizeiro (4).

Material examined

Eulimnadia colombiensis *Roessler, 1989*

Buritizeiro, Minas Gerais, Brazil (17°22’46’’S, 44°57’23’’W): dry pool soil was collected on 11 February 2008 by Leandro Braga Godinho; adults (LBSA-GBr38) were collected on 7 July 2008 by Leandro Braga Godinho.

Sinnamary, French Guiana (5°23’7’’N, 52°59’35’’W): wet pool soil was collected on 5 August 2007 by Michaël Manuel and Alexandre Alié; dry pool soil was collected on 10 December 2008 by Benoît Ferrere.

Eulimnadia cylindrova *Belk, 1989*

Saint Anne, Martinique (14°27’37’’N, 60°48’58’’W): wet and dry pool soil and adults were collected on 30 July 2003 by Nicolas Rabet.

Eulimnadia geayi *Daday de Deés, 1926*

Buritizeiro, Minas Gerais, Brazil (17°22’4’’S, 44°57’23’’W): dry pool soil was collected on 11 February 2008 by Leandro Braga Godinho; adults (LBSA-GBr37) were collected on 7 July 2008 by Leandro Braga Godinho.

Eulimnadia magdalensis *Roessler, 1990*

Barrolândia, Bahia, Brazil (16°06’20’’S, 39°12’24’’W): dry pool soil was collected on 19 June 2008 by Sébastien Lacau and Marc Pignal.

RESULTS

Morphology of the eggs collected from adults

E. magdalensis eggs are spherical and ornamented by a large depression with a flat bottom and high ridges (Fig. 2F-H). At each ridge intersection, a spiniform projection is present (Rabet *et al.*, 2012). The estimated number of depressions varies between 25 and 33 (average=29.38; standard deviation=2.67; N=8).

E. colombiensis eggs are cylindrical with flat ends and an inflated border. Ridges extend parallel along the length of the cylinder (Fig. 3D and 3G). At one end, a prominent ridge of varying height may be present (Tab. 1).

E. cylindrova eggs are cylindrical with a flat end and a moderately inflated border (Fig. 3I, Tab. 1).

E. geayi eggs too are cylindrical but with an extremity that is frequently wide and domed, while the opposite end is flat resulting in a pentagonal shape (Fig. 3B). The ridges are parallel along the length of the egg (Rabet *et al.*, 2012).

Effect of stay in pool sediment

The general morphology of the eggs, cylindrical or spherical, was always clearly recognisable (compare Fig. 2A-C with 2F; 3A with 3B; 3C with 3D; 3E and 3F with 3G; and 3H with 3I). However, the fine ornamentation was clearly affected by exposure to pool sediment. Ridges were eroded, while rows and depressions were frequently filled by sediment (Figs. 2A-C, 3A, 3C, 3E and 3H). In the case of *E. colombiensis,* parts of the inflated border were frequently broken (Fig. 3E and 3F). Several degrees of abrasion were observed in *E. magdalensis* producing various aspects of the egg (Fig. 2A-C). The intersection of the ridges was always eroded and ridges were more or less distinguishable from the depression (Fig. 2A-E). In cases of substantial abrasion, the surface had become porous and the alveolar layer was visible at the surface of the egg (Fig. 2E). However, the internal part of the egg

Tab. 1. Measurements for cylindrical *Eulimnadia* eggs with flat ends.

	E. colombiensis Buritizeiro	*E. colombiensis* Sinnamary	*E. cylindrova* Saint Anne
Minimum diameter	145±6.9 (138-157); 7	133±22.1 (108-150); 3	139±5.7 (134-151); 8
Maximum diameter at the level of the inflated border	183±20.9 (162-224); 7	212±16.0 (193-230); 3	165±5.0 (154-179); 8
Maximum to minimum diameter ratio	1.25±0.09 (1.15-1.49); 7	1.62±0.19 (1.31-1.82); 3	1.19±0.04 (1.00-1.29); 8
Egg length without crests	104±14.4 (81-123); 7	93±18.0 (69-107); 3	106±13.1 (83-129); 8
Egg length with crests	178±17.0 (148-194); 7	185±20.3 (164-205); 3	157±8.5 (143-171); 7
Ratio of length with crests to length without crests	1.75±0.36 (1.22-2.32); 7	2.04±0.5 (1.54-2.55); 3	1.53±0.24 (1.3-1.87); 7
Ratio of length without crests to diameter at the level of the inflated border	1.79±0.31 (1.45-2.41); 7	2.31±0.31 (2.12-2.67); 3	1.57±0.21 (1.39-1.98); 8

Data is presented as mean±standard deviation in µm (range); number of specimens.

Non-commercial use only

shell remained unchanged in section (Fig. 2C and 2I; Rabet *et al.*, 2012).

Variability of the Neotropical pools inhabited by *Eulimnadia*

The pools inhabited by *Eulimnadia* in the areas we studied had diverse characteristics but were always temporary and located on sandy or clayey substrates. Some pools were larger than 1000 m^2, as in Sinnamary and Buritizeiro, but others were also smaller than 10 m^2, as in Barrolândia and Saint Anne. The duration of inundation was a function of the local climate and could be relatively short, as in Barrolândia or Saint Anne (frequently less than one month), or relatively long (generally several months), as in Buritizeiro (no such information was available for the savannah pool of Sinnamary). In Buritizeiro, two *Eulimnadia* species cohabited with the anostracan *Dendrocephalus thieryi* Rabet, 2006. In other pools, only one species of *Eulimnadia* was detected in the absence of anostracans. No anostracans were found in Sinnamary or Barrolândia, but in Saint Anne the anostracan *Streptocephalus similis* was found in deeper pools with long inundation times.

DISCUSSION

Distribution and habitats of Neotropical *Eulimnadia*

Eulimnadia populations were found in temporary

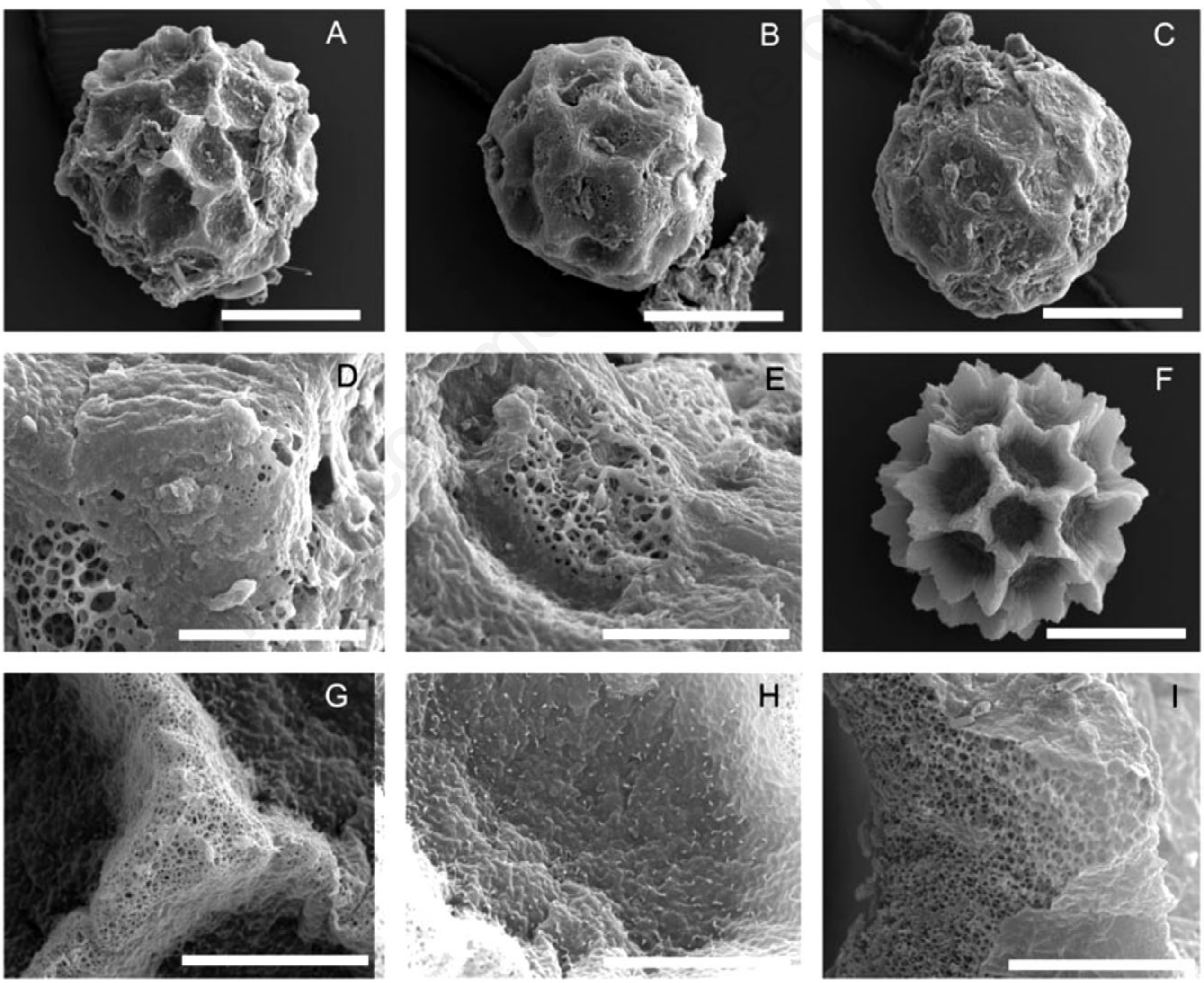

Fig. 2. Morphological effects of pool sediment on the egg morphology of *E. magdalensis* from Barrolândia (Bahia, Brazil). Eggs extracted from soil (A-E and I) or collected from adult animals after breeding (F-H). A-C) three degrees of egg abrasion; magnification of a soil-extracted egg, with D) detail of the eroded ridge intersection, and E) detail of the eroded depression with the emergence of the alveolar layer; F) Magnification of eggs collected from animals after breeding, showing G) detail of the spiniform projection of the ridge intersection, and H) detail of the depression; I) shell section. Scale bars: 100 µm (A-C,F); 20 µm (D,E,G-I).

pools similar to those already described for all species of this family (Timms, 2010; Rogers *et al.*, 2012) and we consider them as a good indicator of this type of habitat. In our investigation, the geological nature of the substrate varied from clayey to sandy. Indeed, the genus seems to be replaced by *Metalimnadia* in rock pools in the Neotropical region (Mattox, 1952; Pereira and Garcia, 2001). The size, depth and inundation time for *Eulimadia* habitats can vary, but global ecological data for this genus are relatively scarce and need further investigation. The climates vary and can have well-defined dry seasons, such as in Cerrado or the dry lands of the Caribbean islands, or be consistently humid, such as the Amazonian or Atlantic forest climates. The records of *E. colombiensis* in humid savannah pools of French Guiana are in agreement with the humid climate conditions indicated for this species in Colombia (Roessler, 1995). French Guiana completes the known distribution of this species with several reports from Brazil (Rabet *et al.*, 2012), Colombia, and Venezuela (Roessler, 1989, 1995; Pereira and Garcia, 2001). For *E. cylindrova* in Martinique, this is the first report in the West Indies, as previously it was only known in Venezuela, the Galapagos islands, the USA, and Mexico (Belk, 1989; Brendonck *et al.*, 1990; Pereira and Garcia, 2001).

Extraction of branchiopod debris and eggs from pool soil

Adults *Eulimnadia* are frequently absent from inundated pools but investigation of the pool sediment can provide good information for the effective presence of these species (Brendonck *et al.*, 2008). For example, adult exuviae and resting eggs have previously been used to identify different species in Saharan pools (Beladjal and Mertens, 2003). Fecal pellets and eggs of *Artemia* were also found in ancient salt lake sediments (27,000 to 200,000 years old) (Clegg and Jackson, 1997; Djamali *et al.*, 2010). Different traces of *Lepidurus articus* were also found in various paleo-sites in the British isles and in Northern France (Bennie, 1896; Mitchell, 1957; Morrison, 1959; Ponel, 1994). This species is now restricted to the Artic climate and such findings provide a good indication that this type of conditions existed in these areas during glaciations. For spinicaudatans, exuviae or dry animals found in the soils of pools can be used to determine the families and genera but not the individual species that inhabited the pools. Fortunately, in the case of *Eulimnadia*, Limnadiid eggs have ornamental features which are often species specific (Timms, 2010; Belk, 1989; Martin, 1989; Martin and Belk, 1989; Rabet, 2010; Rabet *et al.*, 2012). In addition, eggs can be recovered from pool sediment by following several techniques specific to the type of eggs, the density of which may be higher or lower than that of water after the first drying (Mura, 1992).

Taxonomical remarks

Egg measurements and dimension ratios should only be carried out on clean eggs because the presence of sediment and/or erosion can significantly affect these parameters. *E. colombiensis* eggs exhibit shell (Rabet *et al.*, 2012) and general shape variability (present study). The ratios of the large and small diameters were greater in eggs from French Guiana than in those from South Bahia suggesting that the border of the eggs from French Guiana was more inflated. Similarly, the ratios of the egg length without crest to the diameter at the inflated border and the ratios of the egg length with the crest to the length without the crest were both greater in eggs from French Guiana than in those from Minas Gerais. At the present time, we cannot assign a taxonomical value to these characters, but can only suggest that more studies be carried out on *Eulimnadia* species with cylindrical eggs.

Identification of species from eggs extracted from pool sediments

The direct determination of species from resting eggs extracted from pool sediment, in the absence of adult specimens, was performed previously for *Chirocephalus* populations detected in the centre of the Sahara (Beladjal and Mertens, 2003). In this case, the reference description of egg morphology in the literature was from eggs collected directly from adults and not from extracted eggs (Mura, 1986; Rabet, 2010). The principal impediment to the direct determination of species from extracted eggs is the effect of the sediment on the egg morphology. In fact, egg surfaces can be significantly modified according to the time spent in the sediment. Thus, the fine ornamentation may be more or less modified by abrasion and sediment (Figs. 2 and 3). If we use the most current determination key based on egg morphology proposed by Rabet (2010), it is possible to specifically identify cylindrical eggs with flat ends. Indeed, egg ridges and their obliquity are distinguishable despite the effects of sediments. The more or less inflated border is also observable in extracted eggs which allowed us to distinguish *E. cylindrova* and *E. colombiensis/E. belki* (Fig. 3C-G). In the case of cylindrical eggs with one domed end, identification is more problematic and confusion with *E. alluaudi* is possible (Fig. 3A and 3B). However, this species was described from Madagascar and has not yet been collected in the Neotropical region (Rabet, 2010). Spherical eggs can be distinguished by their superficial ornamentation (Fig. 2). This type of egg is frequent in *Eulimnadia* and shared by at least 20 species (Rabet, 2010; Babu and Nandan, 2010). Identification is based on the size and shape of the depressions, which can be recognised in extracted eggs, and on the shape of the ridges and the bottom of the depression, which cannot be discerned in such eggs. In

Non-commercial use only

our study, if we had used the current identification key, we likely could have confused *E. magdalensis* extracted eggs with those of *E. diversa* Mattox, 1937 collected from adults [compare Fig. 2B and 2C with Belk (1989)]. Although *E. diversa* Mattox, 1937 has not yet been collected in the Neotropical area, it is present in North America and might be discovered in the Neotropics in the future. If we had limited the application of the key to the shape and size of depression, we could not have distinguished *E. magdalensis* from at least 10 other species: *E. marplesi* Timms and McLay, 2005; *E. chaperi* Simon, 1886; *E. magdalensis*; *E. diversa* Mattox, 1937; *E. agassizi* Packard, 1874; *E. compressa* (Baird, 1860); *E. dalhi* Sars, 1896; *E. garreti* (Richters, 1882); *E. similis* Sars, 1900; *E. azisi* Babu and Nandan, 2010 (Rabet, 2010; Babu and Nandan, 2010). In this case, breeding from eggs recovered from soil or from new samples in the pool is necessary to obtain reproductive adults which clean eggs can provide specific characters from.

The texture of the alveolar layer and fibre thickness of the egg shell is variable among *Eulimnadia* species. These variations were recently used as new taxonomic characters (Rabet *et al.*, 2012). Regardless of the abrasion level of the shell, the alveolar layer is not affected by the soil and the

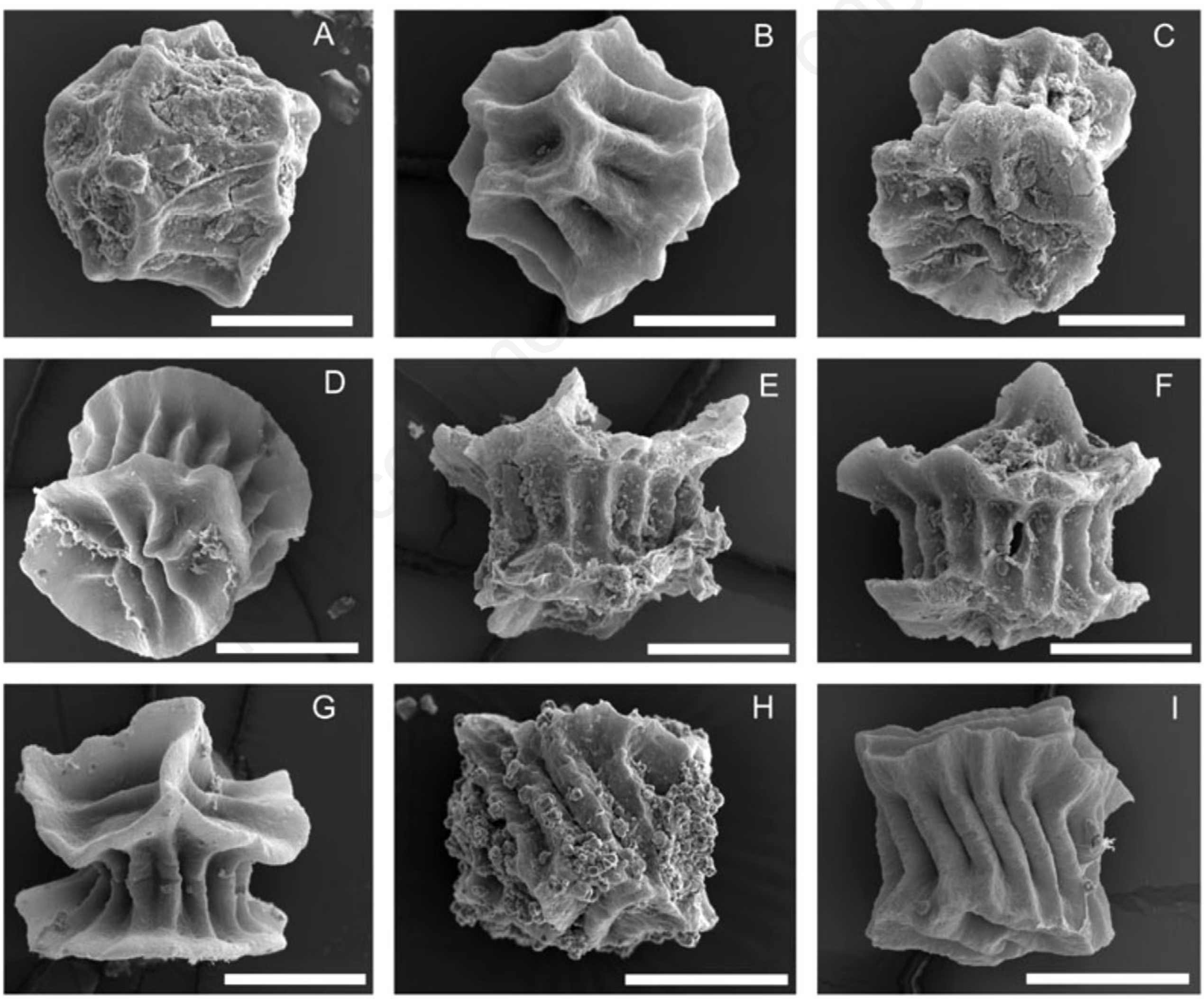

Fig. 3. Morphological effects of pool sediment on the egg morphology of three *Eulimnadia* species. *E. geayi* eggs from Buritizeiro (Brazil) extracted from the soil (A) or sampled from collected adults (B). *E. colombiensis* eggs from Buritizeiro (Brazil) extracted from soil (C) or sampled from the adults after breeding (D); and from Sinnamary (French Guiana) extracted from soil (E,F) or sampled from adults after breeding (G). *E. cylindrova* eggs from Saint Anne (Martinique) extracted from soil (H) or sampled from adults after breeding (I). Scale bars: 100 µm.

study of egg sections may assist in the identification of soil-extracted eggs. However, these structures have been completely described for only three species (Rabet *et al.*, 2012).

General strategy for studying limnadiid populations

In searching for new limnadiid populations, the first step consists in identifying temporary pools (Fig. 4). From the geological and climate contexts, pools can be first selected by analysis of geographical data, such as maps, aerial or satellite photographs, territories accessible in Google Earth (http://www.google.co.uk/earth/), and French territories accessible in Geoportail (http://www. geoportail.gouv.fr/accueil). In areas with well-defined dry seasons, the field research performed at the end of the dry season is the best period to identify temporary pools, which are frequently dry. Prospection at the beginning of the rainy season (after no more than 20 days) can allow direct collection of adult *Eulimnadia*. Collection at the end of the rainy season or at the beginning of the dry season is less dependable. If the pools are not dried yet the probability of finding adult *Eulimnadia* is low owing to their short life cycle. In this case, only the local context and the associated fauna can help to identify favourable temporary pools. When the pool is dry, a sample of dry soil can be collected and easily stored. If the pool is still filled with water, the soil collected should be rapidly dried because eggs will die rapidly in moist samples. Egg extraction can then be performed on dried samples following the protocol reported here. Species determination should be first attempted with extracted eggs. Several dozen eggs are necessary so that two species can be sympatric (as in the Buritizeiro samples reported here). These eggs can be used later for breeding or used directly in polymerase chain reaction to analyse the DNA sequence (Moorad *et al.*, 1997; Duff *et al.*, 2007). We recommend completing the first taxonomical analysis based on extracted eggs with eggs collected from adults after breeding or collected while sampling the pool at a favourable time (ideally 10 days after substantial rainfall).

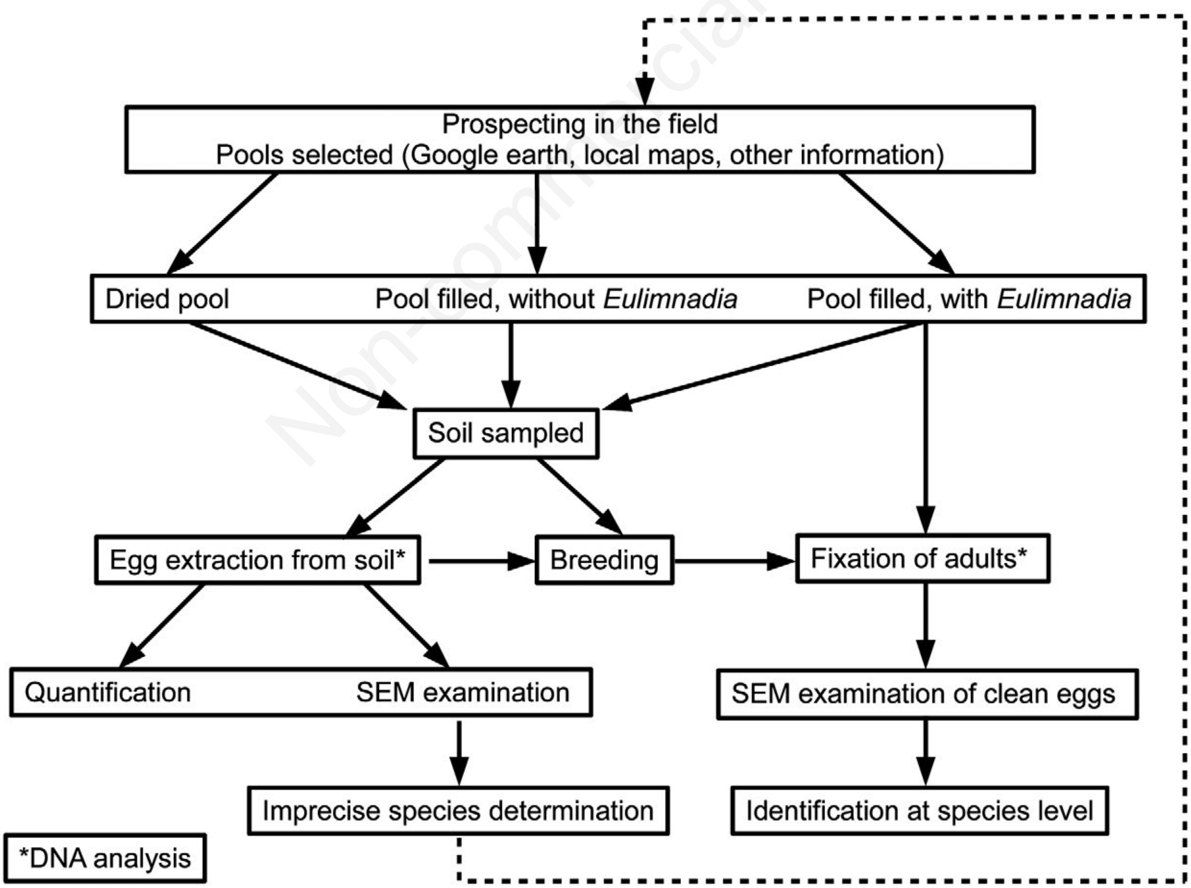

Fig. 4. General strategy for studying limnadiid populations. *Eulimnadia* adults are infrequently collected because their life cycle is short and their habitats are frequently dry. The presence of this genus can be easily revealed by extracting eggs from pool sediments which can be used in ecological and phylogenetic studies and for preliminary taxonomical analyses. *Possibility to do DNA analysis.

Non-commercial use only

Egg shell function

One egg shell function is to protect the embryo, particularly during the drought periods (Belk, 1970; Dumont and Negrea, 2002). In addition, the well-developed alveolar layer considerably diminishes the density of the egg after desiccation and is considered to be an important flotation mechanism (Morris and Afzelius, 1967; Gilchrist, 1978). This feature is common to all limnadiid species and is frequently present in tropical and sub-tropical species of large branchiopods. This floatability is probably important for dissemination and then for the functioning of the egg banks. Egg ornamentation has been suggested to provide protection against predation (Dumont *et al.*, 2002). However, from results reported here, this putative function in *Eulimnadia* would be most important just after laying. Indeed, in our study, once the eggs were exposed to or mixed with sediment, the folds and spines were clearly eroded. Although exposure to pool sediments caused reductions in egg shell thickness, such reductions were relatively minor and concerned only the most superficial structure of the egg (*i.e.* ridges or crests). Egg viability appeared to be generally unaffected by reduced egg shell thickness because we obtained larvae from uncrushed eggs abraded to different degrees. Similar observations have been reported for resting eggs of rotiferans, suggesting that damage induced by the environment affects viability only in cases of extreme abrasion (Garcia-Roger *et al.*, 2005). However, egg crushing by pressure is lethal, and the strength required to obtain such an effect for Anostraca is fairly low (Hathaway *et al.*, 1996). Thus, egg viability is probably more affected by crushing than abrasion during the time spent in the pool soil.

CONCLUSIONS

We have demonstrated that the species present in *Eulimnadia* populations cannot be identified efficiently using eggs extracted directly from pool sediments, especially for those species that have spherical eggs (*e.g. E. magdalensis*). However, the egg extraction method using water is a rapid way to find new *Eulimnadia* populations and obtain a first taxonomic determination. Such eggs can be used for molecular studies, such as barcoding or phylogenetic analyses, and can also be counted to understand the properties of egg bank. Following our analysis in the Neotropical region, we propose a global strategy for studying *Eulimnadia* populations that comprises several imperative steps: pool detection, soil extraction, and isolation of resting eggs from breeding or from newly deposited field samples obtained in favourable conditions. This approach can be included in a taxonomical or ecological context and may encourage new studies of this crustacean group, which is widely distributed but not frequently reported due to its particular environmental requirements.

ACKNOWLEDGMENTS

We thank our soil collectors and the people who helped us in the field: Marc Pignal, Michaël Manuel, Alexandre Alié, Eric Gallerne, Benoit Ferrere and Leandro B. Godinho. Particular thanks to Kurt Liittschwager for improving the text of the manuscript.

REFERENCES

Babu KKS, Nandan SB, 2010. Two new clam shrimp species (Crustacea: Branchiopoda: Spinicaudata) from Kerala, India. Zootaxa 2713:55-64.

Beladjal L, Mertens J, 2003. Interstitial remains for fauna reconstruction of desert pools using fairy shrimps as example (Anostraca, Crustacea). J. Crustacean Biol. 23:60-68.

Belk D, 1970. Functions of the conchostracan egg shell. Crustaceana 19:105-106.

Belk D, 1989. Identification of species in the Conchostracan genus *Eulimnadia* by egg shell morphology. J. Crustacean Biol. 9:115-125.

Bennie J, 1896. Artic plant-beds in Scotland. Ann. Scot. Nat. Hist. 17:53-56.

Brendonck L, Rogers DC, Olesen JJ, Weeks S, Hoeh R, 2008. Global diversity of large branchiopods (Crustacean; Branchiopoda) in freshwater. Hydrobiologia 595:167-176.

Brendonck L, Thiéry A, Coomans A, 1990. Taxonomy and biogeography of the Galapagos Branchiopod faune (Anostraca, Notostraca, Spinicaudata). J. Crustacean Biol. 10:676-694.

Brtek J, 1997. Checklist of the valid and invalid names of the "large branchiopods" (Anostraca, Notostraca, Spinicaudata and Laevicaudata), with a survey of the taxonomy of all Branchiopoda. Slovak National Museum ed., Bratislava: 66 pp.

Clegg JS, Jackson SA, 1997. Significance of cyst fragments of *Artemia sp.* recovered from a 27,000 year old core taken under the Great Salt Lake, Utah, USA. Int. J. Salt Lake Res. 6:207-216.

Djamali M, Ponel P, Delille T, Thiéry A, Asem A, Andrieu-Ponel V, de Beaulieu JL, Lahijani H, Shah-Hosseini M, Amini A, Stevens L, 2010. A 200,000-year record of the brine shrimp *Artemia* (Crustacea: Anostraca) remains in Lake Urmia, NW Iran. Int. J. Aqu. Sci. 1:14-18.

Duff RJ, Benvenuto C, Branch TL, Weeks SC, 2007. DNA extraction from resting cysts of the clam shrimp *Eulimnadia texana* (Banchiopoda: Spinicaudata: Limnadiidae). J. Crustacean Biol. 27:154-157.

Dumont HJ, Nandini S, Sarma SSS, 2002. Cyst ornamentation in aquatic invertebrate: a defence against egg-predation. Hydrobiologia 486:161-167.

Dumont HJ, Negrea SV, 2002. Introduction to the class branchiopoda. Guides to the identification of the microinvertebrates of the continental waters of the world. Backhuys Pub., Oegstgeest: 398 pp.

Garcia-Roger EM, Carmora MJ, Serra M, 2005. Deterioration patterns in diapausing egg banks of *Brachionus* (Müller, 1786) rotifer species. J. Exp. Mar. Biol. Ecol. 314:149-161.

Gilchrist BM, 1978. Scanning electron microscope studies of the egg shell in some Anostraca (Crustacea: Branchiopoda). Cell Tissue Res. 193:337-351.

Hathaway SA, Sheehan DP, Simovich MA, 1996. Vulnerability

Non-commercial use only

of branchiopod cysts to crushing. J. Crustacean Biol. 16:448-452.

MacKay SE, Williams DD, 2011. Invertebrate colonization of the surface and deep groundwaters of a small oceanic island (Barbados, West Indies). Trop. Zool. 24:1-48.

Martin JW, 1989. *Eulimnadia belki*, a new clam shrimp from Cozumel, Mexico (Conchostraca: Limnadidae), with a review of central and south American species of the genus *Eulimnadia*. J. Crustacean Biol. 9:104-114.

Martin JW, Belk D, 1989. *Eulimnadia ovilunata* and *E. ovisimilis*, new species of clam shrimps (Crustacea, Branchiopoda, Spinicaudata) from South America. P. Biol. Soc. Wash. 102:894-900.

Mattox NT, 1952. A new genus and species of Limnadiidae from Venezuela (Crustacea: Conchostraca). J. Wash. Acad. Sci. 42:23-26.

Mitchell GF, 1957. Late-glacia finds of *Lepidurus articus* (Pallas) in the British isles. Nature 180:513.

Moorad JA, Mayer MS, Simovich MA, 1997. Extraction of DNA from anostracan cysts (Crustacea, Branchiopoda) for use in RAPD-PCR analyses. Hydrobiologia 359:159-162.

Morris JE, Afzelius BA, 1967. The structure of the shell and outer membranes in encysted *Artemia salina* embryos during cryptobiosis and development. J. Ultra. Mol. Struct. R. 20:244-259.

Morrisson MES, 1959. *Lepidurus articus* in the Irish late-glacial. Nature 184:739.

Mura G, 1986. SEM morphological survey on the egg shell in the italian Anostracans (Crustacea, Branchiopoda). Hydrobiologia 134:273-286.

Mura G, 1992. Preliminary testing of Anostraca from Italy for use in freshwater fish culture. Hydrobiologia 241:185-194.

Pereira G, Garcia JV, 2001. A review of the clam shrimp family Limnadidae (Branchiopoda, Conchostraca) from Venezuela, with the description of a new species. J. Crustacean Biol. 21:640-652.

Ponel P, 1994. [Les fluctuations climatiques au pléniglaciaire würmien déduite des assemblages d'Arthropodes fossiles à la Grande Pile (Haute-Saône, France)]. [Article in French]. C. R. Acad. Sci. Paris 319:845-852.

Rabet N, 2010. Revision of the egg morphology of *Eulimnadia* (Crustacea, Branchiopoda, Spinicaudata). Zoosystema 32:373-391.

Rabet N, Godinho LB, Montero D, Lacau S, 2012. Exploration of the egg shell structure of three Neotropical *Eulimnadia* species: a new insight into genus taxonomy (Crustacea: Branchiopoda: Spinicaudata). Stud. Neotrop. Fauna Environ. 47:221-226.

Roessler EW, 1989. [Estudios sobre los entomotráceos de Colombia III. Estudio taxonómico de un nueva especie colombiana *Eulimnadia colombiensis* (Arthropoda, Crustacea, Conchostraca)]. [Article in Spanish]. Caldasia 16:58-65.

Roessler EW, 1995. Review of Colombian Conchostraca (Crustacea): morphotaxonomic aspects. Hydrobiologia 298:253-262.

Rogers DC, Rabet N, Weeks SC, 2012. Revision of the extant genera of Limnadiidae (Branchiopoda, Spinicaudata). J. Crustacean Biol. 32:827-842.

Timms BV, 2010. A revision of the Australian endemic clam shrimp genus *Limnadopsis* Spencer and Hall (Crustacea: Branchiopoda: Spinicaudata: Limnadiidae). Rec. Aust. Mus. 61:49-72.

Timms BV, Lindsay S, 2011. Morphometrics of the resting eggs of the fairy shrimp Branchinella in Australia (Anostraca: Thamnocephalidae). P. Linn Soc. N. S. W. 133:53-70.

Weeks SC, Marcus V, Alvarez S, 1997. Notes on the life history of the clam shrimp, *Eulimnadia texana*. Hydrobiologia 359:191-197.

Non-commercial use only