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**ANIMAL SCIENCE**

# Hydra-Amoeba system: a double infection with a lethal ending

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**Abstract:** Within each ecosystem, organisms and populations maintain a complex set of relationships. These interactions can determine the distribution area of a species and play an essential role in its evolution. Parasites are ubiquitous components of nature and have a high influence on various aspects of the biology and ecology of organisms, affecting the populations of their hosts and, therefore, their communities and ecosystems. Free-living amoebae are unicellular organisms that can be found in water, soil or air. Some species are of great importance in human health. In *Hydra*, there are several reports of *Hydramoeba hydroxena* infections. In this work we present a double parasitosis: two concatenated infectious periods in the host polyp of *Hydra vulgaris* and *Hydra vulgaris pedunculata* for three freshwater bodies in the province of Buenos Aires, Argentina. *Hydramoeba* sp. and *Acanthoamoeba* sp. unchain a series of anatomical lesions that in all cases cause the death of the polyps due to total disintegration. This finding becomes important at a sanitary level due to the appearance of *Acanthoamoeba* sp. in waters associated with human recreational activities; For the *Hydra* genus, the importance lies at an ecological and evolutionary level, considering the possible impact on its natural populations.

**Key words:** Amoeba, Argentina, hydra parasitism, shallow lakes.

## INTRODUCTION

Organisms live within an ecological community, which is defined as an assemblage of populations of at least two different species that interact directly and indirectly within a defined geographic area (Agrawal et al. 2007, Ricklefs 2008, Brooker et al. 2009). Species interactions form the basis for many ecosystem properties and processes and the nature of these interactions can vary depending on the evolutionary context and environmental conditions in which they occur (Lang & Benbow 2013). Interactions between species are part of the framework that forms the complexity of ecological communities and are extremely important in shaping community dynamics (Agrawal et al. 2007). In the particular case of parasites, these are ubiquitous components of nature and make up a relevant proportion of biodiversity, which in general remains little known. Their influence on various aspects of the biology and ecology of the organisms that harbor them can affect their populations and, therefore, their communities and ecosystems (Timi 2019).

In the genus *Hydra*, the association par excellence is endosymbiosis with algae of the genus *Chlorella* and species of the *viridis* group. In fact, this association is what determines the group as such, commonly referred to as the "green hydra" group. Other interactions that are common in hydra are epizoic. Observations of ciliates associated with hydras are as old as the discovery of the polyp itself (Trembley 1744, Rösel von Rosenhof 1755, Ehrenberg 1838,

Stein 1854, 1859). The best known are with *Kerona pediculus* and *Trichodina pediculus* (Ehrenberg 1838). Both protozoa appear not to damage polyps even when they are present in large numbers (Cavallini 1930, Coleman 1966). Rarer still are those phoretic relationships, such as those described by Stoks & De Bruin (1996) between *H. viridis* and *Anax imperator*; the report of Grabow & Martens (2000) between *Hydra* spp. and *Somatochlora metallica;* the finding of *Hydra* sp. on *Calopteryx* sp. by Shull et al. (2012); *Hydra* spp. on the abdominal segments of a *Leucorrhinia pectoralis* (Brochard & van der Ploeg 2014, Wildermuth & Martens 2019) and the report of Maynou & Martín (2021) about *Hydra* sp. and *Calopteryx virgo*.

A different case is that of the amoeba *Hydramoeba hydroxena*. Usually, this organism occupies the tentacular surface of the polyps. Unlike the ciliates, this organism produces a detriment to the health of its host, since it usually feeds on its tissues and sometimes causes fatal epidemics. (Stiven 1964, 1971, Page & Robson 1983, Page 1991). This host – pathogen system is found throughout many parts of the world (Stiven 1962). The *Hydra-Hydramoeba* system was useful because it was possible to raise large numbers of hydras and parasites in a relatively small space (Coleman 1966). The parasite is cosmopolitan, having been reported repeatedly from Europe, Asia and North America (Stiven 1971).

Entz (1912) recorded specimens of *Hydra oligactis* with several signals of degeneration. A microscopic examination discovered amoeba in the body wall, peristome, and tentacles and in the coelenteron of hydras. Entz observed that amoebas fed on the ectodermal and endodermal cells of the host. He considered the amoeba like a "food-robbers" since they also fed on preys which live in the coelenterons of hydras. This author labelled *H. hydroxena* it as an opportunist, attacking fresh-water hydras weakened by a state of depression. Many species of *Hydra* have been reported infected by the cosmopolitan parasite *H. hydroxena*. The experiments of Reynolds & Looper (1928), Itô (1949, 1950) and Rice (1960) take special care in elucidating the ecological and pathological relationships between these two organisms. This interaction has generated different opinions: Entz (1912) and Wermel (1925) agree that *Hydramoeba* is a commensal and not a parasite. However, Reynolds & Looper (1928) and Maxwell (1969) present evidence that said amoeba is a pathogenic parasite in *Pelmatohydra oligactis* and *H. graysoni*, respectively. Itô (1949, 1950), for his part, describes a similar parasitic relationship for *H. magnipapillata* and *H. japonica*, finding four different body forms for amoebas under different conditions. The latter author considers them as facultative parasites, as does Stiven (1964), since they can remain free from the host.

Free living amoeba (FLA) are unicellular organisms that are ubiquitous in natural ecosystems and can be found in large numbers in water, soil or air. These organisms play an important role in ecosystems, both as reducers and re-enders of bacterial biomass (Nagyová et al. 2010, Vaerewijck et al. 2014). Despite the large number of amoeba genera and species described in nature, only some species of the genus *Acanthamoeba* spp., and the species *Naegleria fowleri* and *Balamuthia mandrillaris*, are the ones that have the highest incidence in human infections (Marciano-Cabral et al. 2003, Farra et al. 2017, Javanmard et al. 2017, Visvesvara et al. 2007). Most FLA have two stages during their life cycle, the cyst form being the one that gives them great resistance to adverse environmental conditions. Even in reference to human health, these cysts resist most of the water purification treatments (Thomas et al. 2008, Loret & Greub 2010, Valster et al. 2010, Muchesa et al. 2014).

Likewise, the presence of amoeba in water for both consumption and agricultural or recreational use increases the possibility of infection of animals or food, this being another way of entering the food chain (Abdul Majid et al. 2017). In Argentina, most reports on FLA refer to cases of human infections. There are some records of environmental isolations for the provinces of Buenos Aires, Mendoza, Corrientes Córdoba and La Pampa (Gorodner et al. 2006, Lucchesi et al. 2010, Laconte et al. 2013, Gertiser 2015, Rojas et al. 2017), *Acanthamoeba* being the most frequently isolated genus (Gertiser 2015).

In thiswork we present a different case of hydra – amoeba system: a double infection with two genera of amoebae. Different degrees of morphological abnormalities were detected in *H. vulgaris* and *H. vulgaris pedunculata* collected in three different shallow lakes of Buenos Aires province. The identification of *Hydroamoeba* and *Acanthoamoeba* in those infected polyps, could be evidence the existence of two concatenated infective periods that in all cases cause the death of the host polypin a few days or even hours. The theory proposed here involves a first infective event by *Hydramoeba*. This infection would cause morphological damage to the host and therefore a detriment to its health. This "new infected host" becomes the perfect target for the opportunistic *Acanthoamoeba*. This second infectious event, added to the injuries caused by the first, would cause an irreversible failure in the polyp, leading to death by disintegration. This parasitosis is totally new for the *Hydra* genus. This finding raises several questions, not only in relation to the infective mechanisms, but also regarding the consequences that these parasites can cause in the natural populations of hydra. Although much remains to be elucidated, this first report constitutes the beginning of a line of investigation, so far unknown.

## MATERIALS AND METHODS

Infected polyps were detected in a seasonal sampling carried out in the following freshwater bodies located in the southeast region of the Pampa grasslands, Buenos Aires Province, Argentina: Nahuel Rucá Lake (37° 37' S, 57° 26' W; 0.60 m deep; 245 ha), Los Padres Lake (37° 56' S; 57° 45' W; 1.24 m deep; 216 ha) and La Brava (37°52' S, 57° 58' W; 4. 57 m; 400 ha) (Figure 1). The specimens were collected seasonally for two complete years: the first year from autumn 2013 until summer 2014 and the second, from autumn 2014 until summer 2015.

For the extraction of hydras, some substrates were collected where these organisms are usually attached. Each sample include: (1) the first 20 cm of the submerged portion of twenty stems of *Scirpus californicus*, (2) floating vegetation (different species depending of the annual season) and (3) submersed macrophyte *Ceratophyllum demersum*.

The samples was packaged and transported to the laboratory and conditioned in separated aquariums with water from the site and aerators. The samples kept at 20° ± 3 °C with a photoperiod of 12 hours of light and 12 hours of dark. In each sampling, the main limnological parameters were determined *in situ*: water temperature with manual thermometer and depth and transparency with a disc of Secchi. In addition, water samples were taken for analyses of dissolved oxygen (Winkler method) and pH (Table I).

The specimens were observed under a stereoscopic microscope. Those polyps with abnormalities or different morphological damage were isolated individually in Petri dishes with culture solution M (Lenhoff 1983).

To identify those structures "foreign" to polyps were made random squashes and analyzed under microscope. The diameter of 30

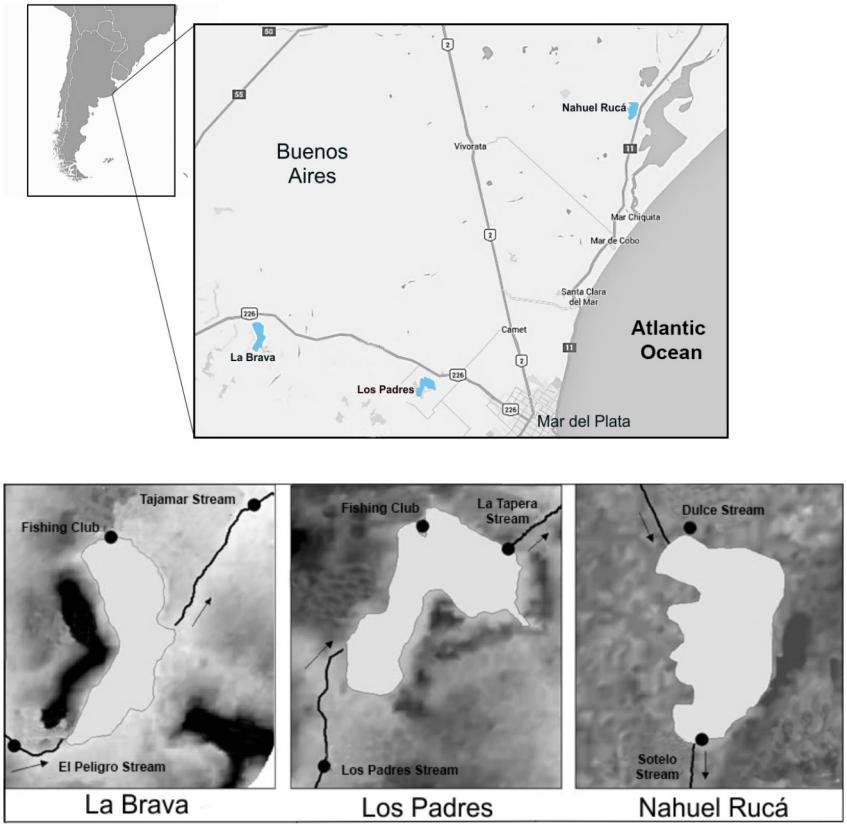


**Figure 1.** La Brava (37° 53´ S; 57° 58´ W), Los Padres (37° 56´ S; 57° 44´ W) and Nahuel Rucá (37° 40´ O; 57° 23 'W). Modified from Romanelli et al. (2013).

resistance cysts and pseudo cysts of amoeba (Figure 3) were measured with micrometric ocular at 1000x with immersed oil.

The following protocol was applied to two polyps of *H. vulgaris* and one of *H. vulgaris pedunculata* fixed in 70% alcohol:

### Solutions, reagents and buffers

-AmpONE ™ PCR Kit (GeneAll).
- Buffer TAE 50X: 2 M Tris-HCl pH 7.2, 50 mM EDTA pH 8, glacial acetic acid 57.1 ml and $H_2O$ up to 1 L.

### Culture Medium

- ANN (Non-Nutritive Agar)
- Bacto ™ Agar (20 g).
- Distilled water (1000 ml).
- Autoclave at 121 °C for 25 minutes and pour 15 ml into Petri dishes to solidify.

### Genomic DNA extraction

For the molecular identification of the isolates obtained, the Maxwell® 16 DNA purification and extraction kit was used according to manual No. TM284 (Promega). 1 to 2 ml of the culture was filled directly into the kit cartridges, one for each sample, and placed in the extraction device. The DNA was collected on the elution columns and was subsequently quantified using the DS-11 DeNovix® spectrophotometer. Once quantified, the samples were stored at -20 °C.

### Identification by PCR

In order to classify the amoebas found in the different samples at the molecular level, the Polymerase Chain Reaction or PCR was carried out. These amplification reactions were carried out in the Artik Thermal Cycler thermocycler (Thermo Scientific): for a final volume of 50 µl, 5

**Table I. Limnological parameters. Temperature (T°), depth, transparency (transp), pH and dissolved oxygen (DO) at each sampling site by station.**

| Station | Lagoon | T° (C°) | Depth (cm) | Transp. (cm) | pH | DO (mg/l) |
|---|---|---|---|---|---|---|
| Autumn 13 | **LB** | 4 | 40 | 10 | 8.5 | 9.0 |
| | **LP** | 17 | 105 | 20 | 8.8 | 5.7 |
| | **NR** | 12.5 | 55 | 20 | 8.5 | 4.0 |
| Winter 13 | **LB** | 9 | 60 | 15 | 8.5 | 9.3 |
| | **LP** | 10 | 30 | 10 | 8.9 | 7.0 |
| | **NR** | 9 | 30 | 5 | 8.3 | 7.1 |
| Spring 13 | **LB** | 19 | 55 | 20 | 8.3 | 8.7 |
| | **LP** | 11.5 | 40 | 8 | 8.5 | 11.5 |
| | **NR** | 19.5 | 65 | 5 | 8.2 | 6.2 |
| Summer 14 | **LB** | 20 | 35 | 20 | 8.3 | 5.8 |
| | **LP** | 22 | 10 | 10 | 8.8 | 9.3 |
| | **NR** | 18 | 6 | 3 | 8.1 | 6.0 |
| Autumn 14 | **LB** | 8 | 45 | 15 | 8.6 | 4.4 |
| | **LP** | 19 | 30 | 20 | 8.6 | 3.4 |
| | **NR** | 8 | 60 | 10 | 8.6 | 5.4 |
| Winter 14 | **LB** | 9.5 | 80 | 35 | 8.4 | 9.0 |
| | **LP** | 7 | 45 | 15 | 8.5 | 9.0 |
| | **NR** | 19 | 40 | 5 | 8.3 | 5.9 |
| Spring 14 | **LB** | 20 | 50 | 40 | 8.6 | 8.9 |
| | **LP** | 26 | 45 | 20 | 9.1 | 6.9 |
| | **NR** | 21.5 | 30 | 5 | 8.6 | 4.4 |
| Summer 15 | **LB** | 21 | 30 | 15 | 8.3 | 5.2 |
| | **LP** | 18 | 22 | 10 | 8.8 | 10.4 |
| | **NR** | 14.9 | 60 | 5 | 8.3 | 5.3 |

pmol of each primer, between 40 and 100 ng of AVL DNA and 0.25 units of Taq polymerase were used. The following universal amoeba primers were used:

Free-Living amoebas (Tsvetkova et al. 2004)

- Forward: 5´- CGCGGTAATTCCAGCTCCAATAGC - 3´

- Reverse: 3´- CAGGTTAAGGTCTCGTTCGTTAAC - 5´

- Program: 2 minutes at 95 °C; 40 cycles of 30 seconds at 95 ° C, 30 seconds at 50 °C and 30 seconds at 72 °C; and a final cycle of 7 minutes at 72 °C.

*Acanthamoeba* spp. (Schroeder et al. 2001)

- Forward: 5´- GGCCCAGATCGTTTACCGTGAA - 3´

- Reverse: 3´- TCTCACAAGCTGCTAGGGAGTCA - 5´

- Program: 5 minutes at 95 ° C; 35 cycles of 30 seconds at 95 °C, 30 seconds at 50 °C and 30 seconds at 72 °C; and a final cycle of 7 minutes at 72 °C.

The DNA fragments amplified by PCR were separated by electrophoresis in agarose gels immersed in 1X TAE buffer, using horizontally developed underwater cuvettes under a potential

difference of 5V / cm. The agarose percentages ranged between 0.8 and 2%, depending on the size of the DNA fragments to be separated. The molecular weight standard used was 100 bp, indicating the fragments from 150 to 1500 bp (PCRBIO Ladder IV). ATCC type strains were used as controls.

Samples were prepared by adding 1 µl of DNA loading buffer for every 2 µl of DNA sample. For DNA visualization, 0.02 µl of RealSafe (Biotein) was added to the gel for each ml of gel and observed through a UV light transilluminator at a wavelength of 260 nm. Images were processed by Chemi-Doc with ImageLab software.

### Genotyping of isolates

The determination of the genotype of the amoeba cultures was based on the analysis of the sequence of the 18s fragment of rRNA, a region of the gene of the small subunit of rRNA, as previously described (Booton et al. 2002, Corsaro & Venditti 2010).

The PCR conditions for DNA sequencing of the different isolated amoebae followed the same pattern as those used for their amplification. The sequencing was carried out in the Sequencing Service of the University of La Laguna and the sequences obtained were compared with the sequences available in GenBank to establish the genus and species of the new isolates.

## RESULTS

Three species of genus *Hydra* were identified in the sampled freshwater bodies: *H. vulgaris*, *H. vulgaris pedunculata* and *H. viridissima* for Los Padres and Nahuel Rucá. In La Brava, only found *H. vulgaris* and *H. vulgaris pedunculata*. Of 184 polyps collected, 16 were infected (Table II).

The infected species were: *H. vulgaris* collected in spring 2013 and summer 2015 for Nahuel Rucá, summer 2015 for Los Padres and winter 2013 for La Brava and *H. vulgaris*

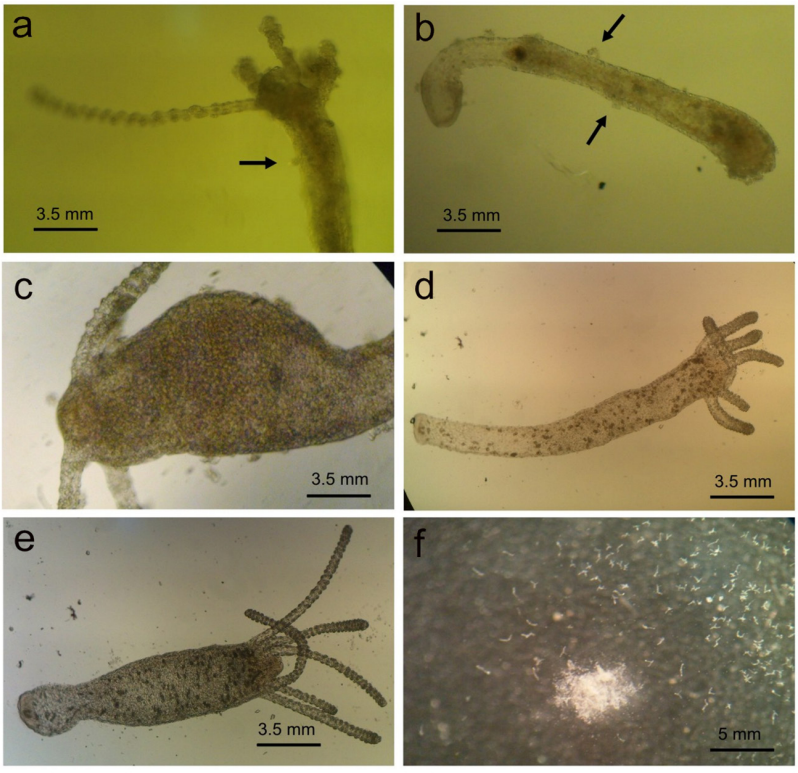


**Figure 2.** ***Hydra vulgaris*** **from Los Padres collected in summer 2015 with amoebas attached to its body and cut tentacles (a, b, c) bar: 3.5 mm.** ***H. vulgaris*** **from Nahuel Rucá collected in spring 2013 with dark clumps within their tissues (d, e) bar: 3.5 mm and** ***H. vulgaris pedunculata*** **from La Brava collected in winter 2013 completely disintegrated on the bottom of the Petri dish (f) bar: 5 mm.**

*pedunculata* collected in winter 2013 in La Brava and summer 2015 in Nahuel Rucá. As shown in Table II, *H. viridissima* did not show signs of infection.

The infected polyps showed different levels of anatomical lacerations and, already in a final phase, the death of the polyps.

For all cases, the deterioration was progressive. It was not possible to determine exactly which symptoms correspond to each infection, but in all cases, the result was the death of the polyp by desintegration. From the beginning of the infective process, the polyps showed a decrease in their mobility and certain morphological abnormalities.

In some cases some structures attached to its columns and tentacles could be observed (Figure 2a-c) and others, showed dark agglomerations inside their tissues (Figure 2d,e). Subsequently, the polyps presented almost all of their tentacles cut off and for most of the cases, a little more than 24 hours later, the specimen had completely disintegrated (Figure 2f).

Inspection of the squash revealed, in some polyps, large number of cysts and pseudo-cysts (the latter also called moribund stage) (Figure 3). Easily distinguishable from other structures, 30 cysts were chosen at random. The range was 6.61 – 10.63 µm and a mean of 8.07 ± 0.59 µm. In the case of the moribund or pseudo cysts phase, over 30 of these structures chosen at random too, the average diameter was 7.63 µm, with a minimum of 6.50 and a maximum of 9 µm.

In the three samples fixed in alcohol, the morphological analysis of the cysts and pseudo cysts together with the genotyping of the isolates, detecting the presence of two types of amoebas in the same host: the genus *Hydramoeba* sp. and *Acanthoamoeba* sp.

## DISCUSSION

Most biologists are familiar with the concept of amoebae as potential pathogens (Lorenzo-Morales 2010). The wide variety of FLA is amazing (Baquero et al. 2014). Despite the small group of amoebas that are pathogenic for humans, the severity of the pathologies produced is high and has been on the rise in recent years. FLA research, especially in water, has increased due to their ubiquity, as well as their pathogenicity on humans or their ability to interact with other types of organisms, such as bacteria and viruses, acting as transport vehicles (Abdul Majid et al. 2017). The increase in the finding of FLA in new environments where it was not believed that they could develop (Chavatte et al. 2016), their high survival and their ubiquity in the environment, raises the need to place more emphasis on research on the possible niches and ecosystems where these can be found and that can be the focus of infections for animals or humans (Armand et al. 2016).

**Table II. Total and infected polyps collected in three freshwater bodies of Buenos Aires province.**

| | ***pedunculata*** | | ***vulgaris*** | | ***viridis*** | |
|---|---|---|---|---|---|---|
| | **total** | **infected** | **total** | **infected** | **total** | **infected** |
| La Brava | 2 | 2 | 12 | 1 | 0 | 0 |
| Los Padres | 8 | 0 | 18 | 3 | 3 | 0 |
| Nahuel Rucá | 23 | 2 | 113 | 8 | 5 | 0 |
| Total | 33 | 4 | 143 | 12 | 8 | 0 |

The reported double amebiasis in *Hydra* is the first record of an association of this type for the genus. Until now, there are only data on the infection of polyps by *H. hydroxena*, which is located on the tentacles, prevents feeding of the infected specimens and also causes tissue damage (Entz 1912, Wermel 1925, Itô 1949, 1950, Rice 1960, Stiven 1964, Maxwell 1969).

Apparently, there are three ways in which a susceptible hydra becomes infected: first *Hydramoeba* tend to be passed directly from an infected to an uninfected host by contact between tentacles; second, the trail- ing tentacles of the susceptible hydra frequently come in contact with the sticky free *Hydramoeba* on the substrate, and third, *Hydramoeba* have been observed to migrate up the stalk of the host from the substrate (Stiven 1964).

Reynolds & Looper (1928) observed that during the early stages of infection, the amoebae are practically always more plentiful on the tentacles than on the body, but as the infection progresses, the parasites become rather generally scattered over the body-surface. On the other hand, the hydras, which were infected with amoebae, died and disintegrated within a relatively short time. The authors also found amoebae in the enteric cavity of sectioned infected hydras, possibly them carried in from the peristomal region on particles of food. It is also probable that they may be taken in on tentacles, which as has been reported above, are occasionally bitten off by the polyp himself (Reynolds & Looper 1928).

In our case, a first infective episode occurs caused by *Hydroamoeba* sp. In this first scenario, the host polyp begins to suffer deterioration in its morphology, a clear detriment to its health and probably, severe flaws in its defense system. The first effect of this parasitism is the impossibility of feeding because of the damage on the tentacles. Anomalies are initially observed on the tentacular surface, which, in more advanced stages of infection, can cause laceration of all of them. Another of the lethal effects, results in the inhibition or destruction of their reproductive capacities. These damages agree with those observed by other authors in polyps infected by *H. hydroxena* (Entz 1912, Wemell 1925, Reynolds & Looper 1928, Threlkeld & Reynolds 1929, Stiven 1962, 1964, Itô 1949, 1950, Maxwell 1969, Augustin

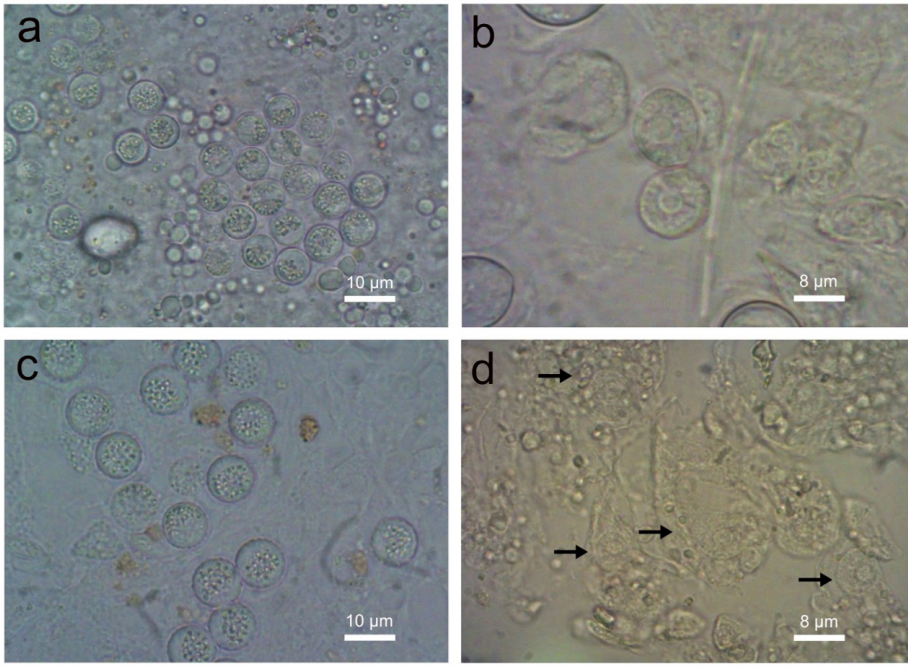


**Figure 3. (a) and (c) cysts observed in *Hydra vulgaris* from Nahuel Rucá in spring 2013, bar: 10 µm and (b) and (d) pseudo cysts or dying stage observed in *H. vulgaris* from Nahuel Rucá in summer 2015, bar: 8 µm.**

et al. 2010) and also with those reported for the freshwater jellyfish, *Craspedacusta sowerbii* Lankester. Payne (1924) reported the occurrence of amoeba in *C. sowerbii,* and concluded from his observations that the association is one of parasitism. In the experiments of Rice (1960), the base of the tentacles and the umbrella are the first parts of the body to be damaged by amoebas. Finally, just like in *Hydra*, when the infection is advanced, the damage affects the gonads and the central part of the coelenteron.

This new setting becomes the ideal for the next event: a second re-infection by *Acanthoamoeba* sp. This group of amoebas are free-living opportunistic parasites that pervade the entire environment and can be found in a wide diversity of habitats, like in tap, fresh and coastal waters, bottled mineral water, sewage and in soil, dust and air (Lorenzo-Morales et al. 2005, Omaña-Molina et al. 2013).

Together, both amoebas causing the death of the polyp, going through several previous stages of progressive deterioration, ending in a total disintegration of the same (Figure 2).

However, it is known that amoebae can cause the death of their host only under certain conditions or a combination of them. Its pathogenicity is usually limited not only by the physiological state of the host, but also by environmental conditions; the first state can change due to starvation (Reynolds & Looper 1928) and the second, due to the "soiling" of the waters. Another factor has been reported for *Hydramoeba*, detecting pathogenicity thresholds related to the aquatic concentrations of the hydrogen ion (Threlkeld & Reynolds 1929, Rice 1960). However, Itô (1948) concluded that the pathogenicity of Japanese *Hydramoeba* in two Japanese species of hydra, is not influenced markedly by differences in hydrogen-ion concentrations.

The experiments of Rice (1960) on *H. cauliculata* showed that the prevention of appreciable fouling by the daily change of water in cultures did not, in the absence of food, prevent the *Hydramoeba* from destroying the hydras. Conversely, the amoebae were unable to affect seriously the growth and reproduction of well-nourished hydras even in a fouled medium. These experiments show that the resistance of hydras decreases rapidly in the absence of food. This is why said author expresses that in this case, *Hydramoeba* should be considered as a facultative parasite of *H. cauliculata*. In our case, when infected polyps were identified and isolated in Petri dishes, they were deprived of food. These starvation conditions could favor the rapid evolution of the infection, which in just hours caused the death of the hosts.

Temperature also plays an important role in this interaction. Stiven (1964) observed increases in amoeba infections in hydras, correlated with increases in temperature. Bryden (1952) also detected a massive death of hydras by *Hydramoeba* and agrees that temperature was an essential protagonist in the infection. However, this correlation is not always direct between both variables; other factors may play important roles in favor of one or another organism (Stiven 1964); one of them, experimentally corroborated by the last author, was the sprouting rate of polyps.

In our studied environments, "water soiling" may have a certain relationship with their natural eutrophicity (Ringuelet 1962) and with levels of pollution or anthropic impact (Romanelli et al. 2013). These factors could cause the existence of favorable periods for the amoebae bloom that coexist with *Hydra*. The combination with the physiological state of the polyps, these infections would cause a more or less massive death of hydras, at least on a local scale in the collected substrates. Infected polyps were mostly reported for high temperature seasons (spring 2014 and

summer 2015 for Nahuel Rucá and Los Padres). For the particular case of La Brava, infected specimens were collected in winter 2013. This season is an exception, since the temperature recorded was lower than the rest (9°C compared to 18° and 14.9°C for summer 2015 in Los Padres and Nahuel Rucá respectively and 19°C for spring 2013 in Nahuel Rucá) (Table I). Although the temperatures recorded by Bryden (1952) and Stiven (1964) are above 25°C, the difference in the eutrophicity of the environments could be the key to other factors combined with temperature, favoring amoeba infections. For example, in our case, the highest number of infected specimens and with higher magnitudes of deterioration, were collected in Los Padres and La Brava, two of the three environments studied that present a greater degree of impact (Romanelli et al. 2013).

In contrast to vertebrates, which have developed a complex immune system that comprises fast innate immune responses and delayed, adaptive defense mechanisms, invertebrates rely exclusively on their innate immunity to defend themselves against potential pathogens. The innate immune system is well studied in bilaterian invertebrates like *Drosophila* or *Caenorhabditis* and largely relies on receptor-mediated pathogen recognition (Franzenburg 2013). In contrast to these ecdysozoan model organisms, *Hydra* neither possesses non-permeable barriers like exoskeletal or cutical structures, nor mobile phagocytes (Bosch et al. 2009). It is likely that the absence of this first defensive barrier makes the *Hydra* genus more prone than other genera to infections of this type.

Several authors have also reported a highest resistance by some species to this first *Hydramoeba* infection. Maxwell (1969) report that *H. graysoni and H. oligactis* died within three days; *Chlorohydra viridissima, H. pirardi* and *H. littoralis* died within four days and *H. pseudoligactis* died within five days. *H. vulgaris* was the most resistant to the parasite, surviving for 12 days. Reynolds & Looper (1928) and Stiven (1964) observed that green hydras are more resistant to the infection that *H. oligactis* and *H. pseudoligactis* respectively. These last observations agree with what were found here. While some specimens of the *vulgaris* group were found parasitized in Nahuel Rucá and Los Padres, the green hydras collected there did not show any signs of infection. Despite the few specimens collected, this absence of infection can be taken as a sign of highest resistance to the first attack caused by *Hydramoeba*.

*Acanthoamoeba* re-infection could then be crucial for polyps. Knowing that hydras can often tolerate *Hydramoeba* infections (Miller 1936, Stiven 1964), this second intrusion could finally be responsible for their death. This double infection adds a new environment in which *Acanthoamoeba* can develop, and given the importance that these amoebas have in human health, it is an important point to highlight, above all, considering that the three freshwater bodies sampled for this work, are directly linked to human activities. Nahuel Rucá is included in a private farmland. Although access to the body of water is not public, the lands associated with it are dedicated to raising cattle (Romanelli et al. 2013) that usually enter the lagoon, and even drink water from it. In the northern sector of La Brava, a residential zone has been developed, with important permanent population growth over the last years, with at least 300 residents. Close to Los Padres there is a residential village with a thousand families. In the last two ecosystems, large numbers of people use these areas for recreational activities and several tourism-related enterprises are also located along their basins (Romanelli et al. 2013).

In addition, these FLA can behave as "Trojan horses" since they can harbor endosymbionts such as bacteria and viruses. This association allows the endosymbiont to remain, resist and

spread in environments that are not ideal for its growth. Microorganisms remain alive inside, the former acting as disseminators (vectors) of the latter (Greub & Raoult 2004, Khan 2006, Marciano-Cabral et al. 2010). Inside they are protected from chemotherapeutic agents, from the host's immune system, from the adverse environment and, in addition, they can reproduce (Drozanski 1956, Proca-Ciobanu et al. 1975, Khan 2009).

*Acanthoamoeba* pathogenicity is a sum of multiple processes which must come together in time and space for the successful transmission of pathogens to a susceptible host, overcome hosts barriers and cause disease (Lorenzo-Morales et al. 2005). At this point, *Hydramoeba* seems to be the perfect nexus for *Acanthoamoeba* to invade this new host.

Second opportunistic re-infections by *Acanthoamoeba* have been reported, for example, for some sea urchins (Scheibling & Hennigar 1997, Clemente et al. 2014) causing massive deaths in their populations. However, in these cases, re-infection occurs after a first bacterial infective episode. On this ocassion, the role of the bacteria is similar to that of *Hydramoeba*: to cause a state of depression in the sea urchins that leaves them vulnerable to a second attack by *Acanthoamoeba*.

This double amebiasis not only becomes important at a sanitary level due to the finding of *Acanthoamoeba* in these bodies of water, but also at an ecological and evolutionary level for the *Hydra* genus. It is likely that the disappearance or reduction of local populations of hydras at certain times of the year may be directly linked to the combination of environmental factors that favor this double amebiasis.

Parasites exploit individual, population, and community biological characteristics of their hosts to ensure their transmission, survival, and the maintenance of viable populations. Consequently, knowledge of parasitic biology provides a set of tools for the interpretation of different aspects of the biology of their hosts, many of them related to estimates of biodiversity (Timi 2019).

According to the aforementioned, there are no doubts about the importance of this finding. To unravel the mechanisms of this double infection and the variables that influence it, directed sampling and some experiments in laboratories focused on this objective are necessary. However, this first report offers the starting point to expand studies of this nature.

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## Author contributions

MID carried out the sampling, cultivation and sample and data processing. FHA contributed to the data analysis, directing (or manage) the sampling and data collection, and JLM performed the typing and genotyping of the amoebae.