Orsis 30, 2016 **41-49**

Emendation of *Paramoebidium avitruviense* (Mesomycetozoea) and peculiar observations about other gut endosymbionts associated with *Siphonoperla torrentium* (Plecoptera) nymphs

Laia Guàrdia Valle[1]

Reception date: 21 January 2016
Acceptance date: 28 April 2016
Publication date: 25 May 2016

Abstract

Aquatic macroinvertebrates harbor a broad spectrum of gut endosymbionts, among them trichomycetes species (including fungi and protozoan), which have been traditionally studied by mycologists. The protozoan *Paramoebidium avitruviense* lives in association with the nymphal stages of *Siphonoperla torrentium* (Chloroperlidae, Plecoptera). Important quantities of *P. avitruviense* thalli were obtained during a long-term study performed in Vallforners stream (Montseny Natural Park, Barcelona, Spain), allowing a revision and emendation of the species, particularly regarding the morphology of the cysts, which were previously misidentified. We also document for the first time the presence of *Smittium* (Harpellales, Kickxellomycotina) within the digestive system of *S. torrentium*, and provide a discussion for this unusual observation.

Keywords: aquatic macroinvertebrates; cross infestation; protozoa; stonefly; symbiosis; taxonomy trichomycetes.

Resum. *Esmena de* Paramoebidium avitruviense *(Mesomycetozoea) i observacions peculiars sobre altres endosimbionts intestinals associats a nimfes de* Siphonoperla torrentium *(Plecoptera)*

Els macroinvertebrats aquàtics són hostes d'una àmplia varietat d'endosimbionts aquàtics; entre aquests, els tricomicets (fongs i protozous), que han estat tradicionalment estudiats per micòlegs. El protozou *Paramoebidium avitruviense* viu associat amb les fases nimfals de *Siphonoperla torrentium* (Chloroperlidae, Plecoptera). En un estudi de monitoratge realitzat a la riera de Vallforners (Parc Natural del Montseny, Barcelona) es va obtenir un nombre important de tal·lus de *P. avitruviense*. Aquest fet ha permès fer una revisió i emanació de l'espècie, especialment per a les fases de cist, que anteriorment

1. Universitat Autònoma de Barcelona. Unitat de Botànica, Facultat de Biociències. 08193 Bellaterra. laia.guardia@uab.cat

ISSN 0213-4039 (imprès), ISSN 2014-9727 (en línia)

s'havien confós amb les d'una altra espècie pròxima. També s'aporten dades sobre la primera observació de *Smittium* (Harpellales, Kickxellomycotina) associat al sistema digestiu de plecòpters.

Paraules clau: macroinvertebrats aquàtics; infestació creuada; protozou; plecòpter; simbiosi; taxonomia; tricomicets.

Introduction

Aquatic macroinvertebrates harbor a broad spectrum of endosymbionts that develop many roles, some of which are not well understood. Among these, we found an ecological grouping of gut endosymbionts called the trichomycetes, including the order Asellariales, Harpellales (Fungi, Kickxellomycotina); Amoebidiales and Eccrinales (Mesomycetozoea). They were formerly included in the Class Trichomycetes, which is now recognized as polyphyletic (Hibbett et al., 2007). Most of these gut symbionts are thought to perform commensalistic associations (Lichtwardt et al., 2001), although mutualistic (by providing vitamins) (Horn & Lichtwardt, 1981) and parasitic relations (by perforating the gut, or infecting the ovarian cells and replacing the eggs by cists) (Sweeney, 1981; Lichtwardt et al., 2001; Lichtwardt, 2002; White, 2006) have been documented in few cases. Within the Amoebidiales, species of *Paramoebidium* inhabit the digestive tract, specifically the hindgut, of immature freshwater insects, including mayfly (Ephemeroptera) and stonefly (Plecoptera) nymphs and dipteran larvae. The genus *Paramoebidium* is poorly recognized from an ecological and biological perspective, with only 17 species currently accepted (Lichtwardt et al., 2001; Valle, 2014a, 2014b). During molt, mature thalli of *Paramoebidium* holocarpically reproduce by means of multiple amoeboid cells that crawl before encysting among the molted chitinous tissues of the host. The cysts produce a variable number of cystospores that will, after ingestion by the appropriate host, germinate within the hindgut to anchor, grow, and produce mature amoeba-forming thalli (Lichtwardt et al,. 2001; Valle, 2014a). Thus, the examination of exuvia provides a good source of material for the observation of cysts.

Paramoebidium avitruviense L.G. Valle, our target species (Fig. 1), is obligately associated within immature stages of the stonefly *Siphonoperla torrentium* Pictet (Valle, 2014a), a Chloroperlidae stonefly widely represented in Europe and distributed in the Northern regions of the Iberian Peninsula (Tierno de Figueroa et al., 2003). This species was recently described from the same site of the present study, and was also reported from proximal localities in the Montseny Massif and in the French Pyrenees (Valle, 2014a). The original description depicted cysts with cystospores which were misidentified, and thus the species is now emended to provide the actual description of these reproductive structures. *Paramoebidium avitruviense* was the first Mesomycetozoan observed within *S. torrentium* nymphs. In the present study we report the presence of the first Harpellid endosymbionts observed within this host.

Material and methods

A population of *S. torrentium* nymphs was monitored from February 2013 to October 2014 in Vallforners stream (41°43'37.66" N, 2°20'12.31" E, 530 m), a tributary of Mogent river (Besós basin) settled within the Montseny Natural Park (a UNESCO Biosphere Reserve, Barcelona, Spain). This stream feeds the Vallforners reservoir. The burrowing nymphs of *S. torrentium* were collected with a Surber sampler (500 µm mesh size) and by dragging aquatic nets (300 µm mesh size) in the interstitial zones of sand and pebble-rocks at the edges of the courses. The nymphs were collected, using plastic pipettes, from the net contents that were placed in white trays for rough sorting. Living nymphs were transported to the lab in jars on ice. Water temperature and pH were recorded for each collection date.

Each nymph was dissected on microscope slides to extract the gut using fine forceps, under a Leitz Wetzlar (Germany) stereomicroscope at 64x. The gut was placed on a clean slide with a water drop and opened with entomological needles. A cover slide was then placed on the drop. The hindgut content was observed under a Zeiss Axioscope (Germany) compound microscope equipped with a Jenoptik ProGresC3 digital camera at 200 and 400x, with water as mounting media. The presence of the endosymbiont *P. avitruviense* was verified, including vegetative thalli, cystospores or cysts. Shed molts (exoskeleton exuvia) were analyzed in the same way. Voucher slides containing *P. avitruviense* were preserved with Lactophenol cotton-blue and then sealed with clear fingernail polish (Lichtwardt et al. 2001). Samples of the hosts per date were preserved in 70% ethanol. All the slides are deposited in the BCB-Mycotheca, at the institutional address of the author.

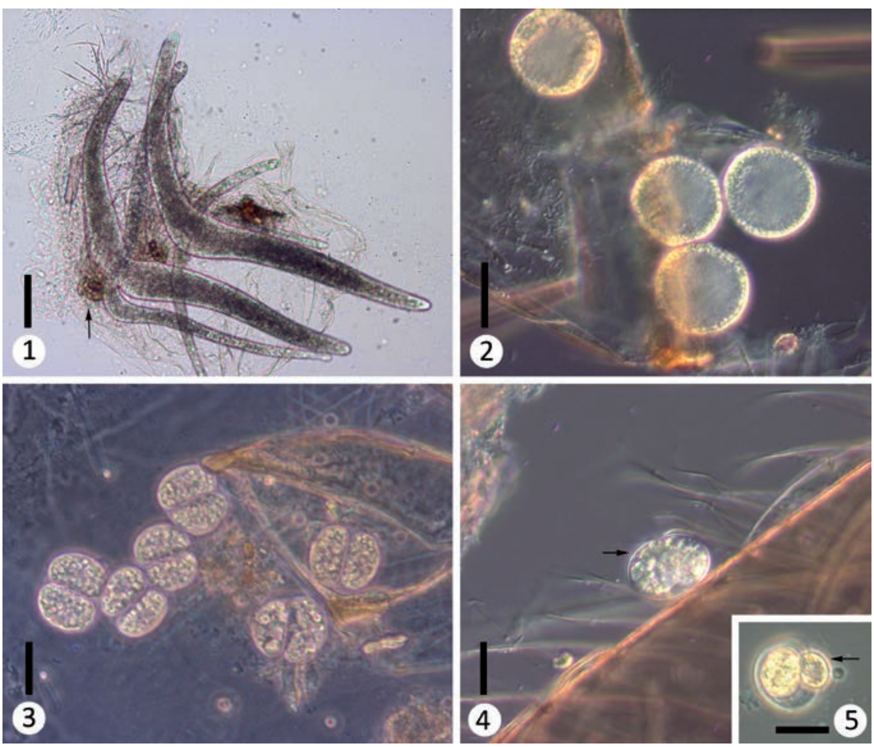
Results

Regarding the cysts of **P. avitruviense** *and species emendation*

Paramoebidium avitruviense was present in 36.8% of the hosts (*N*= 326), in the phases of thalli (Fig. 1), cysts (Figs. 2, 3, 5) and cystospores (Figs. 3-5). The thallial phases were common, but reproductive structures (cysts and cystospores) were only rarely observed (1.2% of the samples). Amoebae were not found. The observed cysts differed from those originally described by Valle (2014a), which most likely corresponded to the cysts of another species formed from amoebae sheltered in the shed exoskeleton of *S. torrentium*, what caused the confusion. The misidentified cysts possibly corresponded to *P. ecdyonuridae* L.G. Valle, a species associated with *Ecdyonurus* spp, (Heptageniidae, Ephemeroptera). The mature cysts and cystospores of *P. avitruviense* had a very distinct morphology and were easily separated from the cysts of other *Paramoebidium* species. An emendation of *P. avitruviense* to include the description of the cysts is provided. Amoebae of *P. avitruviense* were not observed and thus suppressed from the original description.

Paramoebidium avitruviense L.G. Valle in Mycologia 106: 483 (2014), *emend* L.G. Valle (Figs. 1-5)

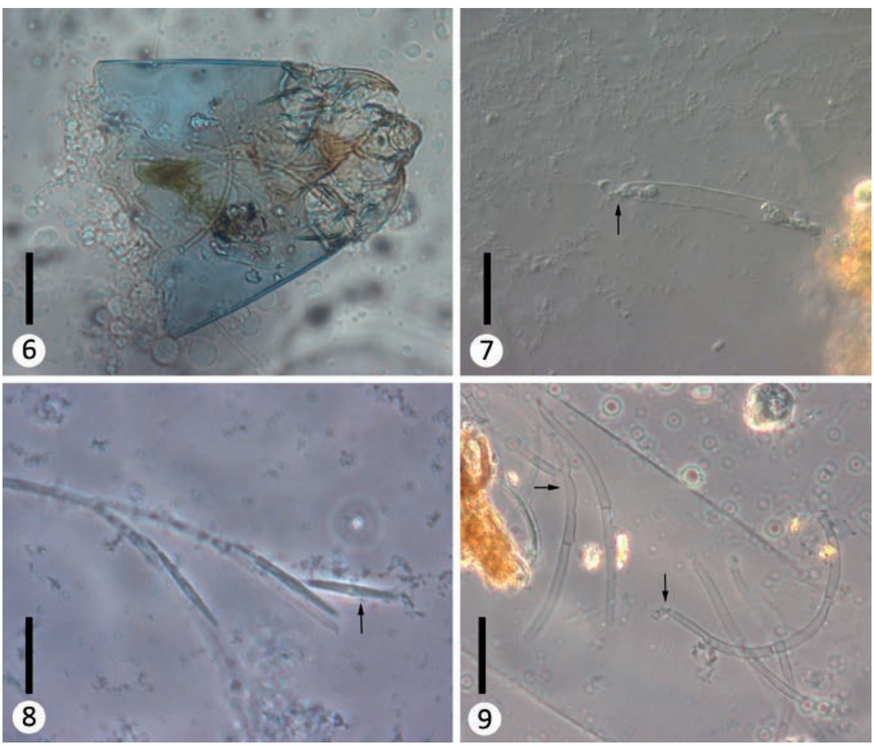
Thalli developing into two asymmetric lobes at initial stages, with the tips slightly oriented upwards. Mature thalli four-branched, with the two main branches (or lobes) at the dorsal side, the largest 300-400 × 25-30, the shorter 200-250 × 20-26 µm, and two smaller, secondary branches at the ventral side, also unequal in length, the largest reaching up to 100 × 24 µm, the smaller about 70 × 20 µm at maturity. Holdfast placed between the two ventral lobes at maturity, or ventrally between the two main branches at initial stages, being apparent, with a distal globose (13-24 µm diam) and often lobulated zone (Fig. 18, arrowhead), covered with holdfast material and an upper neck-like or constricted zone.



Figures 1-5. *Paramoebidium avitruviense*. 1: Various thalli removed from the gut of *Siphonoperla torrentium*, one holdfast indicated with the arrow. Scale bar = 50 µm. 2: Immature cysts in *Siphonoperla torrentium* shed exuvia. Scale bar = 25 µm. 3: Mature cysts with granular cystospores among *Siphonoperla torrentium* shed exuvia. Scale bar = 25 µm. 4: Released cystospore (arrow) with granular content, on *Siphonoperla torrentium* shed exuvia. Scale bar = 25 µm. 5: *Paramoebidium avitruviense*, detail of a cyst releasing a cystospore (arrow). Scale bar = 25 µm.

Amoebae not observed. Cysts spherical or slightly elliptical, 20-35 μm diameter, developing 2-3(-4) elliptical or sub-spherical cystospores 15-29 × 11-23 μm with a granular content (Figs. 2-5). Cysts and cystospores where observed only in shed molts. In the hindgut lining of *Siphonoperla* (Chloroperlidae, Plecoptera) nymphs.

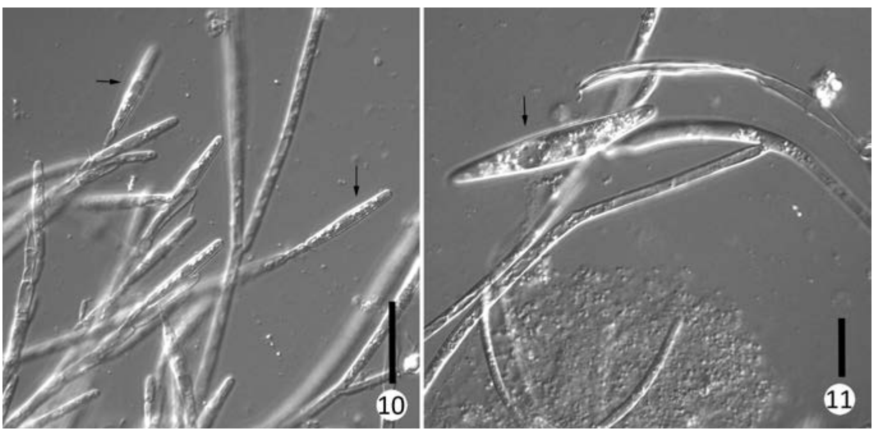
Specimens examined- SPAIN. CATALUNYA. Barcelona. Cànoves i Samalús, Vallforners stream (41°43'37.66" N, 2°20'12.31 E, 530 m asl), Montseny Natural Park. Microscope slides BCB-Tr-2285-2286 and BCB-Tr-2299-2300, all prepared from the shed hindgut exuvia of *Siphonoperla torrentium*, and collected respectively 17 Feb 2014 and 28 May 2014.



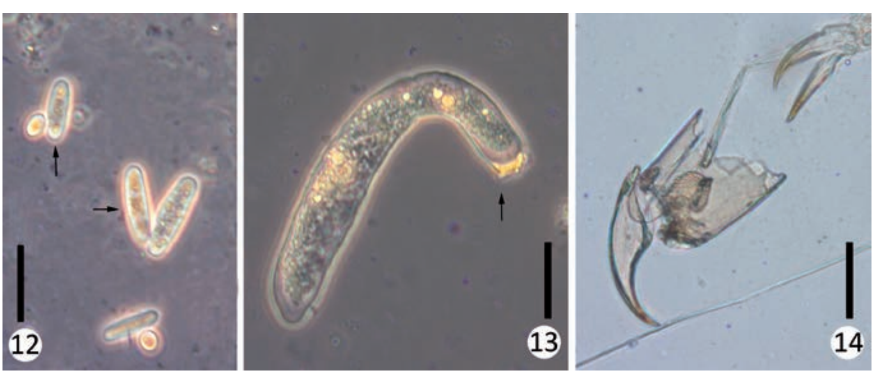
Figures 6-9. Gut content of *Siphonperla torrentium*. 6: Cephalic capsule of an ingested Chironomidae larva by a *Siphonoperla torrentium* nymph. Scale bar = 50 μm. 7: Thallus of *Stachylina* sp. (arrow) within the gut of an ingested Chironomidae larva by a *Siphonoperla torrentium* nymph. Scale bar = 25 μm. 8: Thallus of *Smittium* sp. bearing trichospores (arrow) within the gut of an ingested Chironomidae larva by a *Siphonoperla torrentium* nymph. Scale bar = 25 μm. 9: Various halli of *Harpella melusinae* (arrows) within the peritrophic matrix of an ingested Simuliidae larva by a *Siphonoperla torrentium* nymph. Scale bar = 25 μm.

***Observation of harpellid species in the gut of* Siphonoperla torrentium**

While analyzing the gut content of the nymph hosts, the presence of various species of Harpellid fungi attached to the gut lining of undigested or semi-digested preys were observed. Within ingested Chironomidae (non-biting midges) larvae (Fig. 6), *Stachylina* cf. *nana* Lichtw., *Stachylina* sp. (on the peritrophic matrix, midgut) (Fig. 7), and *Smittium* spp. (on the hindgut lining, Fig. 8) were observed. Within ingested Simuliidae (black fly) larvae, there were thalli of mature and immature *Harpella melusinae* Léger & Duboscq (peritrophic matrix, midgut) (Fig. 9) and *Genistellospora homothallica* Lichtw. (hindgut). In most cases, the trichospores (asexual sporangiospores of Harpellales) were apparently undamaged by the diges-



Figures 10-11. *Smittium megazygosporum* attached to *Siphonoperla torrentium* hindgut lining. 10: With trichospores (arrows). 11: With a zygospore (arrow). Scale bars = 25 µm.



Figures 12-14. Content ingested by *Siphonoperla torrentium*. 12: *Paramoebidium ecdyonuridae* cysts (arrows). 13: Young thallus of *P. ecdyonuridae* (arrow indicating the holdfast). Scale bars = 25 µm. 14: Fragments of an ingested *Ecdyonurus* nymph. Scale bar = 50 µm.

tive enzymes of *Siphonoperla,* even at the last portion of hindgut near the anal zone. However, the most notable observation was the presence of *Smittium megazygosporum* Manier & F. Coste mature thalli with trichospores and zygospores (sexual spores) (Figs. 10-11) attached to the hindgut lining of *S. torrentium* nymphs [BCB-Tr2280, 17 Feb 2014, trichospores and zygospores; BCB-Tr2294, 7 Apr 2014, trichospores]. Some of dissected nymphs also ingested cystospores of different *Paramoebidium* species, especially *P. ecdyonuridae* L.G. Valle, (Figs. 12-13), from the mayfly *Ecdyonurus* spp. which was also among the common ingested preys (Fig. 14). *Paramoebidium ecdyonuridae* was highly abundant at the study site and a profuse producer of cysts and cystospores (Valle, 2014a).

Discussion

About *P. avitruviense* cysts

The morphology of the cysts of *P. avitruviense* is very characteristic, and does not allow confusion with other species, once observed at maturity. Nonetheless, young cysts of *P. avitruviense* may be confused with those of other species, for lacking any distinctive characteristics. Cysts of *P. ecdyonuridae* are similar in size, although these may attain larger dimensions than *P. avitruviense* (up to 42 µm, according to Valle, 2014a). Seemingly, *P. inflexum* Léger & Duboscq produce cysts in the same size range, which can develop further attaining up to 50 µm diam (Léger & Duboscq, 1929; Duboscq et al., 1948). Once at maturity, the cysts of *P. avitruviense* become more distinctive, for the shape and number of cystospores inside. Cystospores of *P. ecdyonuridae* are more numerous and narrower (Valle, 2014a). Cystospores of *P. avitruviense* only resemble somewhat the cystospores of *P. cassidula* Strongman & M.M. White, the latter being kidney-shaped and smaller (Strongman & White, 2006) than those of *P. avitruviense*. The cysts *of P. avitruviense* were characterized by having few cystospores (2, rarely 4) in comparison with other prolific species found in the same site, such as *P. ecdyonuridae*, with up to 12 cystospores per cyst (Valle, 2014a). This outcome would most likely explain (at least in part) the lower prevalence of *P. avitruviese* (38.6%) in comparison with *P. ecdyonuridae* (79%, unpublished data from LGV).

About the observation of Harpellid species in the gut of S. torrentium

Notably, thalli of *Smittium megazygosporum* were found sporulating on the hindgut lining of *S. torrentium* whereas the common hosts of this fungus are Chironomidae larvae. Chironomidae larvae were found to be an important component in the diet of dissected *S. torrentium* nymphs. Licthwardt (1986) recorded *Smittium culisetae* from a pool crowded with dipteran hosts (Chironomidae, Culicidae and Ceratopogonidae), among which some Ephemeroptera nymphs where also present. One of the mayflies also harbored *Smittium culisetae*. This was a case of incidental cross infestation, which affected harpellid species with a wide host range, such as *S. culisaetae* (Lichtwardt et al., 2001). The occurrence of *Smittium megazygosporum* in *S. torrentium* represented the first record of a *Smittium* species within a plecopteran host, even being incidental. By the time of this cross infestation a high density of chironomidae hosts and a high prevalence of *Smittium* spp. were detected. The finding is noteworthy not only because the host is unusual, but also because *S. torrentium* has an opportunistic diet which includes a high portion of predated invertebrates (Rúa et al., 2011; Tixier et al., 2012; Valle & Tierno de Figueroa, 2015). Harpellales (as well as other trichomycetes, except *P. avitruviense*) are not supposed to be associated with predator hosts (Lichtwardt et al., 2001). However, *Smittium megazygosporum*, even incidentally, had the ability to grow and sporulate in the gut of opportunistic-predator hosts, meaning that there was not a critical physiological or anatomical factor limiting the development of the endosymbionts, and the limitations might be more related to the nutritional behavior. A similar conclusion was also noted by Tuzet et al. (1961) and Moss (1972) who intended to resolve whether the lack of infestation in different culicidae and chironomidae species in their natural habitats was caused by the lack of receptivity to the symbiont, or by other factors, such as environmental variables or host nutritional habits. They observed that nutritional habits were decisive in some cases for allowing spore ingestion and consequent gut infestation (Tuzet et al., 1961; Moss, 1972). Valle and Tierno de Figueroa (2015) also provided numeric evidences for the effect of the nutritional behavior and life history of *S. torrentium* on the prevalence of *P. avitruviense*. The authors concluded that the opportunistic behavior of the plecopteran, even at last nymphal phases with a highly carnivorous diet, allowed, aside with the predation, a range of shredder and gatherer activities. With the occasional feeding on the substrate and periphyton, ingestion of cysts and cystospores was possible (Valle & Tierno de Figueroa, 2015).

Just another case of a predator insect infested with a Harpellid species is documented in the literature. It was in Australia when Dr. Lichtwardt and Dr. Sweeney (in year 1983) observed one specimen of the predacious mosquito larva *Culex halifaxii* infested with *S. simulii* Lichtw. The hosts were kept in containers in the laboratory and fed with non-predaceous species of *Culex* infested with *Smittium simulii* and *S. culisetae* Lichtw. which provided the inoculum for the incidental cross infestation (Lichtwardt, 1986). The presence of other Harpellales attached to the gut lining of ingested invertebrates was merely circumstantial.

Acknowledgements

We are grateful to the students Maria Buendía, Marc Panisello, Nicolás Ortiz and Meritxell Tur, who kindly participated in some of the field surveys. We also thank Joaquin Peláez and Daniel Guinart from the office of the Montseny Natural Park who provided us the permits for developing the investigations in the Park. The investigation was supported by "MEC, Secretaría de Estado de Investigación, Desarrollo e Innovación" of the Spanish Government, grant CGL2009-07141.

Bibliographical references

Duboscq, O.; Léger, L.; Tuzet, O. 1948. Contribution à la connaissance des Eccrinides: les Trichomycètes. Arch. Zool. Exp. 86: 29-144.

Hibbett, D.S.; Binder, J.F.; Bischoff, M.; Blackwell, P.F.; Cannon, O.E.; Eriksson, S.; Huhndorf, T.; James, P.M.; Kirk, et al. 2007. A higher-level phylogenetic classification of the Fungi. Mycol. Res. 111: 509-547.

Horn, B.W.; Lichtwardt, R.W. 1981. Studies on the nutritional relationship of larval *Aedes aeqypti* (Diptera: Culicidae) with *Smittium culisetae* (Trichomycetes). Mycologia 73: 724-740.

Léger, L.; Duboscq, O. 1929. L'évolution des Paramoebidium, nouveau genre d'Eccrinides, parasite des larves aquatiques d'Insectes. Compt. Rend. Hebd. Sci. Paris 189: 75-77.

Lichtwardt, R.W. 1986. The Trichomycetes. Fungal Associates of Arthropods. Springer-Verlag. New York.

Lichtwardt, R.W. 2002. Trichomycetes: fungi in relationship with insects and other arthropods. In Seckbach J. (ed.). *Symbiosis*. Kluwer Academic Publishers, Netherlands, 515-588.

Lichtwardt, R.W.; Cafaro, M.J.; White, M.M. 2001. The Trichomycetes, fungal associates of arthropods. Revised Edition. Published on the Internet <www.nhm.ku.edu/~fungi>.

Moss, S.T. 1972. Occurrence, cell structure and taxonomy of the Trichomycetes, with special reference to electron microscope studies of *Stachylina*. Ph. D. Dissertation, University of Reading. 340 pp.

Rúa, J.; Garrido, J.; Tierno de Figueroa, J.M. 2011. Nymphal feeding habits of *Siphonoperla torrentium* (Plecoptera, Chloroperlidae) from North-Western Spain. Vie et Milieu - Life and Environment 6: 95-99.

Strongman, D.B.; White, M.M. 2006. New species of *Lancisporomyces*, *Orphella*, and *Paramoebidium*, endosymbionts of stonefly nymphs from stream in Nova Scotia, Canada. Can. J. Bot. 84: 1478-1495.

Sweeney, A.W. 1981. An undescribed species of *Smittium* (Trichomycetes) pathogenic to mosquito larvae in Australia. Trans. Brit. Mycol. Soc. 77: 55-60.

Tierno de Figueroa, J.M.; Sánchez-Ortega, A; Membiela Iglesias, P.; Luzón-Ortega, J.M. 2003. Plecoptera. In Ramos MA et al. (eds). Fauna Ibérica, vol. 22. Museo Nacional de Ciencias Naturales, CSIC, Madrid, 1-404.

Tixier, G.; Felten, V.; Guérold, F. 2012. Population characteristics and feeding habits of *Siphonoperla torrentium* subject to varying prey, predator, and competitor abundance across an acidification gradient. Freshwater Sci. 31: 191-204.

Tuzet, O.; Rioux, J.-A.; Manier, J.-F. 1961. *Rubetella culicis* (Tuzet et Manier 1947), Trichomycète rameux parasite de l'ampoule rectale des larves de Culicides (morphologie et spécificité). Vie et Milieu 12: 167-187.

Valle, L.G. 2014a. New species of *Paramoebidium* (trichomycetes, Mesomycetozoea) from the Mediterranean, with comments about the amoeboid cells in Amoebidiales. Mycologia 106: 481-490.

Valle, L.G. 2014b. Validation of the trichomycete *Paramoebidium chattoni* (Amoebidiales, Mesomycetozoea), a common and cosmopolitan black fly endosymbionts. Mycologia 106: 573-579.

Valle, L.G.; Tierno de Figueroa, J.M. 2015. Does nutritional behavior affect gut endosymbiont prevalence? The case of *Paramoebidium avitruviense* (Mesomycetozoa) within *Siphonoperla torrentium* (Plecoptera) nymphs. Limnologica 54: 58-65.

White, M.M. 2006. Evolutionary implications of a rRNA-based phylogeny of Harpellales. Mycol. Res. 110: 1011-1024.