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Facultative symbiosis of *Oophila amblystomatis* (Chlorophyceae) with amphibian eggs and embryos

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ABSTRACT

Literature data on facultative mutualistic (symbiotic) relationships between amphibian embryos and unicellular green algae have been considered. Such symbioses may be important for the survival and dispersal of some tailed and anuran amphibians. The green amphibian clutches had been found in North America, Europe and Japan. The algae proliferate quite intensively within the jelly egg capsules of several species from the families Ambystomatidae and Hynobiidae (Caudata) and Ranidae (Anura). A unique symbiosis between the green alga *Oophila amblystomatis* and the salamander *Ambystoma maculatum* has been recently described. In this case a part of the symbiotic algal population from the egg capsule penetrates into embryonic tissues and cells. The intracellular algae display signs of stress and undergo a metabolic shift from oxidative metabolism to fermentation. A preliminary hypothesis about vertical transmission of the symbionts in *Ambystoma* has been suggested.

Keywords : Amphibian embryos, *Oophila amblystomatis*, symbiotic algae.

" Often the algal growth, especially within the innermost jelly envelope, is so dense that it is difficult to see the developing embryo" (Gilbert 1942, p. 215).

At the end of XIX century (Orr 1888), the occurrence of unicellular green algae within the egg capsules of the spotted salamander *Ambystoma maculatum* Shaw from North America was discovered. The algal species was named *Oophila amblystomatis* Lambert ex Printz, 1927. P.W. Gilbert (1942) first carried out its detailed morphological description (motile or nonmotile cells with a diameter of 6–30 µm, spherical or ovoid). He also suggested a hypothesis on the symbiotic relationships between ambystomatid embryos and unicellular green algae.

Further analysis of this facultative symbiosis of *O. amblystomatis* with *A. maculatum* (reviewed in Kerney 2011, Desnitskiy 2017) demonstrated that for the process of photosynthesis the algae use carbon dioxide which was produced as a result of amphibian embryo respiration. The symbionts also consume the nitrogenous waste products of embryonic metabolism. The alga, in turn, produces oxygen at light and this is important for the acceleration of salamandrid embryonic development. Besides, the algae may protect the embryos from the harmful effect of ultraviolet radiation as well as exclude the growth of microbial pathogens within the egg capsule. In addition, it has been recently shown (Hale *et al.* 2017) that the occurrence of the algal symbionts is especially important for the host embryos in worm, shallow waters. The concentration of dissolved oxygen declines under such conditions, but the demand for the oxygen is high.

The green clutches of amphibians may be found preponderantly in USA and Canada (reviewed in Kerney 2011, Desnitskiy 2017, Melo Clavijo *et al.* 2018). Many populations of *A. maculatum* harbour cells of *O. amblystomatis* within the egg capsules during breeding seasons. On the other hand, sometimes similar green clutches (with the algae of the genus *Oophila* Lambert ex Printz) may be also found in the populations of several other North American amphibian species: the northwestern salamander *Ambystoma gracile* Baird, the Jefferson salamander *A. jeffersonianum* Green, the tiger salamander *A. tigrinum* Green, the northern red-legged frog *Rana aurora* Baird & Girard and the American wood frog *R. sylvatica* LeConte. A molecular-phylogenetic analysis (18S rDNA sequencing) of symbiotic algae from the egg capsules of four North American amphibian species (the salamanders *A. maculatum* and *A. gracile* and the frogs *R. aurora* and *R. sylvatica*) has been carried out (Kim *et al*. 2014), but it remains unknown so far whether these symbionts represent one or several closely related taxa.

In other parts of the world, though, very few finds of green amphibian clutches have been made. Several Japanese populations of the black salamander *Hynobius nigrescens* Kuro Sansho-uo harbour symbiotic green alga *Oophila* sp. in the egg capsules (Muto *et al*. 2017). The first find of amphibian green clutch in Europe was made by Czech phycologist H. Ettl (1961), but neither location nor frog species were reported. The symbiont was identified by that author as the volvocine unicellular alga *Chlamydomonas gloeophila* var. *irregularis* Ettl. Later, the green clutches of the agile frog *Rana dalmatina* Bonaparte and the common brown frog *R. temporaria* Linnaeus were found in Austria (Baumgartner *et al*. 1996). The green clutches of *R. dalmatina* were also reported from Poland (Bonk *et al*. 2012). It should be remarked that the symbiotic algae were not specified in these two studies. Finally, the green clutches of *R. temporaria* have been recently found in Northwest Russia by Boldina (2018). The egg capsules usually harbour the *Chlamydomonas debaryana* Goroschankin. In some frog clutches, however, Boldina identified several other unicellular symbionts (including other green algae and even euglenoids and diatoms).

It is appropriate to note in this connection that the huge genus *Chlamydomonas* Ehrenberg is closely related to the genus *Oophila* (Kim *et al*. 2014, Nakada *et al*. 2019). Both genera belong to one and the same green algal order, which may be called today Chlamydomonadales (Nema *et al*. 2019) or Volvocales (Muto *et al*. 2017, Nakada *et al*. 2019). Therefore, placing *Oophila* in the group of nonmotile coccoid green algae as was recently suggested (Shubert and Gärtner 2015) seems unreasonable. According to the current views (Nema *et al*. 2019, p. 23), "*O. amblystomatis* appears to be paraphyletic with numerous other chlamydomonad algae".

The unexpected data have been recently obtained on several populations of *A. maculatum* from Canada and USA (Kerney *et al*. 2011, Graham *et al*. 2013, Bishop and Miller 2014, Small *et al*. 2014). During the periods of embryo cleavage and gastrulation, the symbiotic bi-flagellated algae (presumably haploid vegetative cells) proliferated only within the egg capsules. However, at the early neurula and subsequent developmental stages the symbionts were also detected (by means of time-lapse photography and fluorescence microscopy) within embryonic cells and tissues. By the end of gastrulation – beginning of neurulation, an active algal bloom and especially active proliferation of the bi-flagellated cells occurred within the egg capsule near the blastopore, through which the symbionts penetrated into the embryo. Such a bloom in the blastopore area might be associated with an increased concentration of ammonia produced in this part of the embryo. At later stages of embryogenesis, the algae were seen in epidermis, neural tube, optic cup, cranial mesenchyme, presumptive lens and some other rudiments. R. Kerney and colleagues suggested that "the *Oophila* endosymbiosis is initiated by induced phagocytosis rather than an "aggressive" invasion driven by motility of the alga" (Kerney *et al*. 2017, p. 210).

The algal DNA was detected within the embryos at the stage of pharyngula as well as in the older embryos of *A. maculatum* (Kerney et al. 2011). Nevertheless, not all symbionts enter the embryo: numerous algal cells remained within the egg capsule up to larval hatching and carried out photosynthesis there. At the tail bud stage they gradually lost motility and transformed into non-flagellated capsule membrane-bound cells (presumably resting zygotes, which later might survive unfavorable conditions in water) (Bishop and Miller 2014). On the other hand, the light did not reach the algal cells, which were inside the opaque embryo. The transfer of organic photosynthetic products to the embryos occurs mainly from the exosymbiotic algae which reside in the egg capsules, but not from the endosymbionts which penetrated into cells and tissues (Graham *et al*. 2014). The endosymbiotic algae display hallmarks of cell stress and undergo a shift from an oxidative metabolism to a fermentive metabolism. By contrast, the embryonic salamander cells do not display strong stress responses such as autophagy or apoptosis (Burns *et al.* 2017, Ball and Cenci 2017). It was also shown that the endosymbionts may utilize the host embryo glutamine as a primary nitrogen source.

Usually, it was not possible to detect the algae microscopically in the tissues of feeding larvae (Kerney *et al*. 2011). But later (Kerney *et al*. 2017) it was shown that occasionally a few algal cells persisted for many months after hatching. Algal 18S rDNA was amplified from reproductive tissues of adult female salamanders, and encysted algal cells were found inside the egg capsules of freshly laid eggs. This fact favors a hypothesis about the transmission of encysted algae from one generation to the next. It should be also noted that it was not possible to detect *Oophila* within embryonic cells and tissues of *R. sylvatica* (Kerney 2011) or *A. gracile* (Kerney *et al*. 2019). In these cases the algal symbionts were detected only within the egg capsules. Interestingly, *A. gracile* derived algae are able to enter *A. maculatum* host tissues under experimental conditions. In reciprocal experiments, however, *A. maculatum* derived algae never enter the embryonic tissues of *A. gracile* (Kerney *et al*. 2019).

In concluding, it remains still unclear whether *Oophila* penetrates into the amphibian egg capsules already within female reproductive system or from surrounding water after spawning. A free-living *O. amblystomatis* has been recently found in ponds where breed (often simultaneously) *A. maculatum* and *R. sylvatica* (Lin and Bishop 2015). There is some reason to believe that the alga is widely distributed as a free-living organism, though the details of its life cycle are poorly studied and there are very few fresh morphological data. The complete life cycle of *Oophila* is not yet formally described. On the other hand, the vertical transmission of *O. amblystomatis* in *A. maculatum* is also not yet fully proven. To date, no *Oophila* cells (instead of DNA) have been identified in the adult reproductive structures of *A. maculatum* (Kerney *et al.* 2017).

Finally, it is appropriate to note that we know surprisingly little about the green flagellate *Oophila*, the facultative symbiont of amphibian eggs and embryos, compared to the vast information about its close relative, free-living *Chlamydomonas* (e.g., Goodenough *et al.* 2007, Blaby *et al.* 2014, Cross and Umen 2015, Umen 2018). Nonetheless, now it is possible to maintain *O. amblystomatis* under laboratory culture conditions (Rodrigues-Gil *et al*. 2014, Burns *et al*. 2017, Kerney et al. 2019) and so it is hoped that this unusual alga will slightly open up some of its secrets related to the symbiotic lifestyle.

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