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Evidence of Holarctic distribution: common frog *Rana temporaria* eggs in Fennoscandia host photosymbiotic green algae (*Oophila* sp.)

Pontus F. Eriksson[1,2], **Ryan Kerney**[3], **River Larson-Pollock**[3,4], **Elliana Vickers**[3,5] and **Ane Timenes Laugen**[1,2]

[1]Department of Ecology, SLU-Swedish University of Agricultural Sciences, Uppsala, Sweden
[2]Department of Natural Sciences, University of Agder, Kristiansand, Norway
[3]Department of Biology, Gettysburg College, Gettysburg, PA, USA
[4]Molecular and Cell Biology and Genetics, Drexel University, Philadelphia, PA, USA
[5]Translational Biology, Medicine and Health, Virginia Tech, Blacksburg, VA, USA

Correspondence: Pontus F. Eriksson (pontusferiksson@gmail.com)

Oikos
2025: e10752
doi: 10.1111/oik.10752
Subject Editor: Karoline Ceron
Editor-in-Chief: Pedro Peres-Neto
Accepted 7 January 2025

Symbiosis is a commonly occurring phenomenon, with most plants and animals being holobionts, comprising of a host and its corresponding symbionts. It is, however, less common to have a symbiont only under a specific life stage and even more so between a photosynthesizing organism and a vertebrate animal. One such relationship is found between the unicellular green algae *Oophila amblystomatis* and the North American yellow-spotted salamander *Ambystoma maculatum*, which is so far the only known endosymbiotic relationship between an alga and a vertebrate. In this study we investigate the possible occurrence of *O. amblystomatis* in Fennoscandian egg clutches from the genus *Rana* using citizen science data. We establish that egg capsules of the common frog *Rana temporaria* in Sweden act as a host species for two subclades of the monophyletic genus *Oophila* on a microgeographical scale (< 10 km) using a phylogenetic analysis. Furthermore, we investigated a possible endosymbiotic relationship between *O. amblystomatis* and *R. temporaria* embryos with fluorescence microscopy, which showed no algal invasion of frog tissue or cells in vivo. The association with a known symbiont *Oophila* may hold ecological importance for ontogeny, individual fitness, behavior, population dynamics and evolutionary potential of *R. temporaria*. However, the reciprocal benefits to both partners have not been determined for any *Oophila*-bearing ranid. We discuss the possible Holarctic distribution of *Oophila* and consider the implications of the relationship between *R. temporaria* and *O. amblystomatis*.

Keywords: algae, amphibia, frog, *Oophila amblystomatis*, photosymbiosis, *Rana temporaria*

Introduction

Most plants and animals are holobionts, comprising of a host and its corresponding external and internal symbionts, which in most cases are bacteria (Bordenstein

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and Theis 2015, Colston and Jackson 2016). The hosts rely on interactions with their symbionts for crucial processes such as immune response (Colombo et al. 2015), gut differentiation and function (Fraune and Bosch 2010), and organogenesis (McFall-Ngai 2014). The in utero and in ovo microenvironment is considered sterile, or at least microbe-scarce in many vertebrates such as fish (Stephens et al. 2016), birds (Cook et al. 2005), reptiles (Trevelline et al. 2018), and mammals (Perez-Muñoz et al. 2017), as reviewed by Kerney (2021). However, it has been known for a long time that amphibian eggs can host a diversity of microbes surrounding healthy embryos (Orr 1888).

Photosymbioses require a photosynthetic microbe and a non-photosynthetic host (Cowen 1988). Algal symbioses are mutualistic relationships between a host and a photosynthesizing algal symbiont, such as corals (Baker 2003) or lichens (Spribille et al. 2016). Photosymbiotic interactions are present across several invertebrate taxa and a few chordates, as reviewed by Melo Clavijo et al. (2018). Some vertebrates have algal symbionts, which are mainly in ectosymbiotic relationships with algae (Yang et al. 2022), meaning that the alga does not enter the host in vivo.

Since the late 19th century, it's been known that eggs from the yellow spotted salamander *Ambystoma maculatum* of North America turn green during development (Orr 1888). The green hue results from *A. maculatum* having a mutualistic symbiotic relationship (Gilbert 1942, 1944) with a unicellular green alga called *Oophila amblystomatis* (Collins et al. 1905, Wille 1909, Bishop and Garbary 2024), hereafter referred to as *Oophila*. That *A. maculatum* embryos share a photosymbiotic mutualism with *Oophila* has been shown through exclusion experiments; the embryos hatch more synchronously (Gilbert 1942, 1944, Pinder and Friet 1994, Tattersall and Spiegelaar 2008) and exhibit lower mortality (Gilbert 1944, Marco and Blaustein 2000) with the algae present. This results from increased partial oxygen pressure in the intracapsular fluid containing *Oophila* (Bachmann et al. 1986, Pinder and Friet 1994). The algae gains a safe space for population growth (Kerney 2011, Bianchini et al. 2012, Bishop and Miller 2014) and may utilize nitrogenous waste from the embryo (Goff and Stein 1978, Graham et al. 2013, 2014, Small et al. 2014, but see Burns et al. 2020).

Ambystoma maculatum and *Oophila* exhibit the only known endosymbiotic relationship (the symbiont enters host tissue and cells) between an algae and a vertebrate (Kerney et al. 2011, Burns et al. 2017, Yang et al. 2022). *Oophila* also exhibit a mutualistic ectosymbiotic relationship with *A. maculatum* (Gilbert 1942) and *A. gracile* (Marco and Blaustein 2000). Furthermore, *Oophila* is present in egg clutches of *Amerana* (*Rana*) *aurora* (Kim et al. 2014), and *Boreorana* (*Rana*) *sylvatica* (Pinder and Friet 1994) in North America. *Oophila* are also present in the intracapsular fluid of a salamander *Hynobius nigrescens* endemic to Japan (Muto et al. 2017) and a European ranid, *Rana dalmatina* (Anslan et al. 2021). There are mentions of algae in eggs of *Rana temporaria*, but reports are scarce and limited to England (Savage 1961, p.14) and Austria (Baumgartner et al. 1996). Moreover, to the best of our knowledge no one has used molecular methods to establish which species of green algae coinhabits *R. temporaria* eggs.

Whether *Oophila* is a monophyletic genus has recently been contested (Correia et al. 2020). The consensus was that *Oophila* was a monophyletic genus with four subclades: I, II, III, IV (Kim et al. 2014), and a tentative fifth subclade: J1 (Muto et al. 2017). Later, the genus *Oophila* was divided into A and B clades by Nema et al. (2019) through their analysis of *A. maculatum* – associated green algae. Clade B, which contained the five subclades (I – IV + J1), was considered a 'pseudo-*Oophila*', while the paraphyletic clade A was considered *Oophila* (Nema et al. 2019). This partitioning into clade A and B relied on culture-based approaches, which failed to capture the dominant intracapsular growth of clade B *Oophila* (Jurga et al. 2020). However, 18s rDNA gene trees later verified that clade B comprised the monophyletic genus *Oophila* in North America, Europe and Japan based on wider sampling of all known amphibian hosts (Anslan et al. 2021, Vences et al. 2024). Moreover, a recent sequencing and phylogenetic analysis of the type material from *A. maculatum* that was collected in 1905 verified the initial taxonomic assignment of clade B as the monophyletic *Oophila amblystomatis* (Bishop and Garbary 2024).

The aims of this study were threefold. Firstly, to expand the possible geographic range of *Oophila* we searched citizen science databases for observations of algae in egg clutches from Fennoscandian amphibians of the genus *Rana*. Second, we used field collected eggs from three separate populations in Uppsala (59°N) to classify the algae observed in these laboratory reared egg clutches of *Rana temporaria* and determine if they are of the monophyletic genus *Oophila*. Third, to test for variation on a microgeographical scale we examined the host–symbiont relationship between algal subclades and host-species locality. We discuss the possible implications of this frog–alga relationship on amphibian ontogeny, individual fitness and population dynamics.

Material and methods

To explore the possible occurrence of *Oophila* across Fennoscandia we used citizen science observations between 2000 and 2023 in the form of 435 ranid egg clutch pictures registered in the Swedish Observation System (SLU Artdatabanken 2024), the Norwegian Observation System (Artsdatabanken 2024), and the Finnish Biodiversity Information Facility (FinBIF 2024); see the Supporting information for details. These were analyzed in search of the characteristic green egg capsules, or newly hatched embryos laying on top of a green mass of egg jelly (Fig. 1A), both of which indicates *Oophila* presence based on DNA metabarcoding studies (Jurga et al. 2020, Anslan et al. 2021, Vences et al. 2024).

To confirm the presence of *Oophila*, we collected egg clutches during spring 2018 from three sites (distance between site 1 – site 2: 5.3 km, site 2 – site 3: 5.3 km, site 1

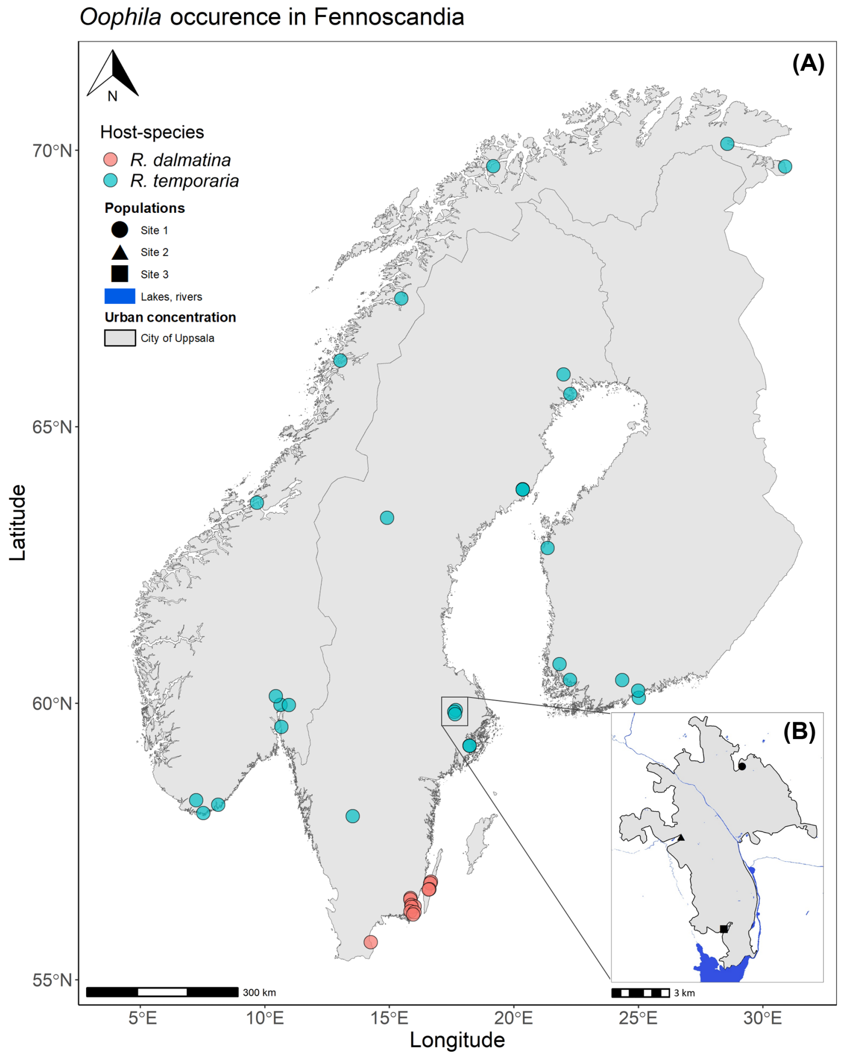

Figure 1. (A) Possible occurrence of unicellular green algae of the genus *Oophila* with corresponding host species in Fennoscandia based on pictures of egg clutches collected from citizen science data (Supporting information). (B) Sample sites where *Rana temporaria* egg clutches were collected during the spring of 2018 for phylogenetic analysis of intracapsular algae within the city limits of Uppsala, Sweden.

– site 3: 8.5 km) within the city limits of Uppsala (Fig. 1B, Supporting information). To minimize development of the embryos, they were transported in site-specific buckets with chilled pondwater for no longer than 30 min. All embryos were collected at Gosner stage 2 (Gosner 1960) and reared in opaque 0.75-litre vials containing reconstituted soft water (APHA 1985). To account for variance in algae population sizes, we collected concentric egg capsules with corresponding intracapsular fluid ($n = 25$) from six different clutches across different temperatures, collection dates and Gosner stages (Supporting information). To investigate embryonic cell invasion (indication of endosymbiosis), the embryos from the egg capsules were fixed in 10% neutral buffered formalin for fluorescence microscopy analysis (using a Leica MZ16F, DFC9000 sCMOS camera, Cy5 filter). There were considerably less visible algae in egg capsules (using a Nikon HR Plan APO 1x WD 54, Nikon SMZ1500 10x zoom microscope) from site 3. Therefore, more egg capsules were analyzed from site 3 compared to sites 2 and 1 (Supporting information).

To determine algal taxon and establish locality-subclade association through phylogenetic analysis we amplified the 18s rRNA gene using the primer set nu-SSU-0033-5' and nu-SSU-1768-3' (Kim et al. 2006). PCR products were TA ligated into pCR2.1 and transformed into One Shot TOP10 Chemically Competent *Escherichia coli* (Invitrogen). Transformed *E. coli* were plated onto LB agar plates containing 100 µg ml^{-1} carbenicillin and top-coated with 40 µl each of 40 mg µl^{-1} X-gal and 100 mM IPTG for blue-white screening. Streak-purified white colonies were screened by PCR with the original primers, inoculated in 3 ml of LB broth and 1 µg ml^{-1} ampicillin, and incubated overnight at 37°C with shaking. Broth cultures were stored in 15% glycerol at -80°C. The insert-containing plasmids were isolated from broth culture using QiaPrep Spin Miniprep Kit (Qiagen). Purified plasmids were screened with an EcoR1 digest (New

England Biolabs), and thirteen screened colonies were sent to GeneWiz (South Plainfield, New Jersey) for Sanger sequencing using universal M13 primers.

Sequences from 201 samples analyzed by Anslan et al. (2021) were aligned with thirteen *R. temporaria*-derived algal 18s rDNA sequences with MUSCLE ver. 3.8.425 (Madeira et al. 2022) and ClipKit to remove uninformative sites (Steenwyk et al. 2020). The 1763-bp alignment was used to infer a maximum likelihood tree using the general time reversible model with gamma distribution for evolutionary rate differences in RAxML-NG ver. 1.2.0. The gamma distribution allowed some sites to be unavailable (+I, 67.5% of sites), and gaps were treated as partially missing data (6.13%). A best maximum likelihood consensus tree was found following a tree search from 25 random and 25 maximum parsimony guide trees. Bootstrapping converged after 700 trees to a 3% threshold (Pattengale et al. 2010). Newly obtained sequences have been deposited to the GenBank (Supporting information).

Results

Of the 435 photographs investigated from the Fennoscandian citizen science databases, 44 contained signs of algae (Fig. 1A). Microscopy and phylogenetic analysis confirmed *O. amblystomatis* presence in the egg capsules of cultured *R. temporaria* eggs (Fig. 1B, 2A–C). The morphology of *Oophila* follows that of other chlamydomonads: the zoospores were spherical or oblong, between 5–10 μm in size (Fig. 2A), and the spherical diploid zygote was ca 20 μm in diameter (Fig. 2B). Moreover, we found that *Oophila* grows predominantly on the inner egg capsules of *R. temporaria* (Fig. 2C). We further postulate that *R. temporaria* embryos congregate on the disintegrating jelly masses and exhibit basking behavior until they start feeding (Fig. 2D–E). The fluorescence microscopy analysis of the frog embryos did not show any algae in vivo (642 nm excitation, 1 min exposure on DFC9000 cooled monochrome camera).

The maximum likelihood tree topology matched the best tree found by Anslan et al. (2021). Five *R. temporaria* sequences from sites 1 and 2 were nested within subclade I. However, this relationship was recovered with poor bootstrap support (32%, Fig. 3). The remaining eight *R. temporaria* sequences, all from site 3, were nested within subclade III, including samples from North American *A. maculatum* and *R. aurora* (Fig. 3).

Discussion

Interpreting citizen science data with appropriate caution, our data nevertheless suggest that *Oophila* is present across the entirety of Fennoscandia (Fig. 1A, Supporting information), with approximately ten percent of clutches containing visible green algae. Based on this study and mentions of algae within *R. temporaria* egg capsules from England (Savage 1961, p. 14), Austria (Baumgartner et al. 1996), and most recently from Norway (Vences et al. 2024) coupled with North American (Kim et al. 2014), Japanese (Muto et al.

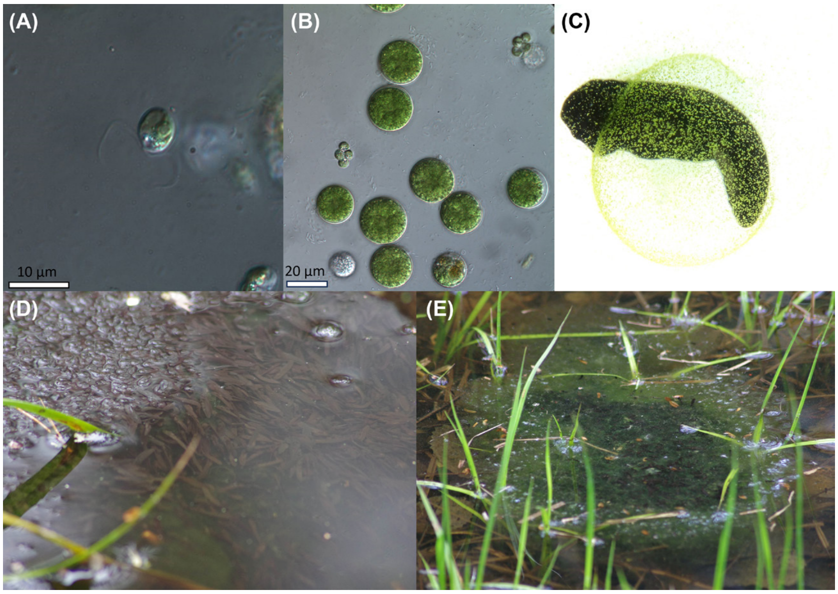

Figure 2. Unicellular green algae (*Oophila amblystomatis*) found in the intracapsular fluid and on the capsular membranes in *Rana temporaria* egg capsules. (A) Zoospore of *O. amblystomatis* with visual nucleus, pyrenoid, and whiplash flagellum. (B) Vegetative diploid zygote of *O. amblystomatis,* a pairing of four daughter cells (middle left), and another four-cell pairing sharing a cell wall (top right). (C) Hatching embryo of *Rana temporaria* (Gosner stage 19) with the algae mainly growing on the innermost capsular membrane. (D) Newly hatched *R. temporaria* embryos basking on communal disintegrating egg jelly (Gosner stage 20; non-feeding). (E) Common sight of post-hatched egg clutch of *R. temporaria* in study area teeming with *O. amblystomatis*.

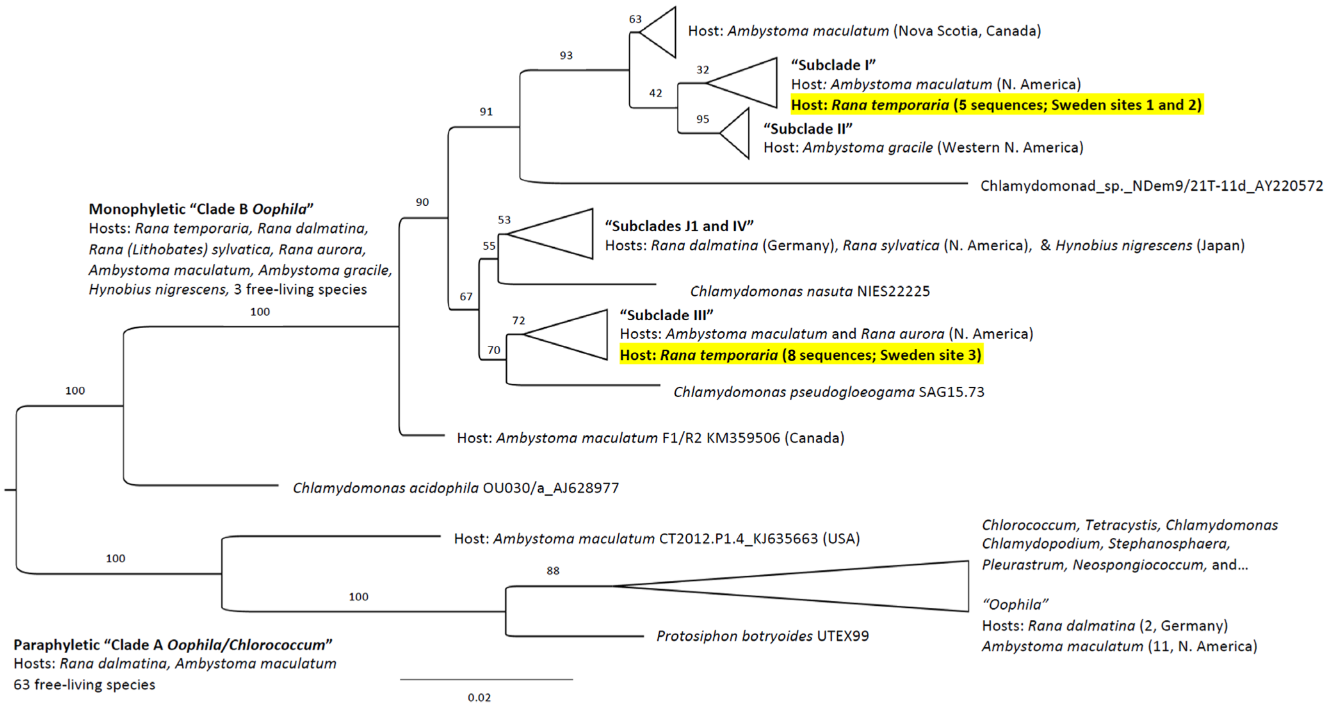

Figure 3. Maximum likelihood phylogeny of conserved 18s rDNA (1763 sites) of *Oophila* sp. and select green algae from Anslan et al. (2021) including the *Rana temporaria* samples from this study. The topology matches the best tree from Anslan et al. (2021). 'Hosts' indicates each algal sample's amphibian embryo host egg capsule. Subclade designations also follow Kim et al. (2014) and Anslan et al. (2021) with the Japanese hynobiid clade of Muto et al. (2017) collapsed into the wood frog clade IV (with weak bootstrap support – 53%). *Rana temporaria* derived *Oophila* fell within clade B subclades III and I. However, the placement within subclade I is based on weak bootstrap support (32%).

2017), and central European hosts (Anslan et al. 2021), *Oophila* likely has a Holarctic distribution.

Swedish *R. temporaria* hosts two different subclades (I and III) of the monophyletic unicellular green algae genus *Oophila* on a microgeographical scale (< 10 km, Fig. 1B, 3). All of the algal sequences collected from *R. temporaria* clutches were found within the monophyletic clade B, unlike several *R. dalmatina*-derived algal samples from Germany that nested in the paraphyletic clade A *Chloroccocum/Oophila* (Nema et al. 2019, Correia et al. 2020, Anslan et al. 2021, Conde et al. 2022). Fluorescent microscopy analysis of embryonic *R. temporaria* showed no *Oophila* in vivo. Our observations of intracapsular and capsular-adherent *Oophila* suggest that most algae transitioned into thick-walled zygotes which coincided with the hatching event of *R. temporaria* (Fig. 2B–C). This aligns with previous observations of *Oophila* in egg capsules of *A. maculatum,* which may use the amphibian egg as a breeding ground to transition life stages (Bishop and Miller 2014).

Historically, only *A. maculatum* have been known to host *Oophila* from subclade I (Kim et al. 2014, Anslan et al. 2021). However, our results showed that two of three populations of *R. temporaria* (site 1 and 2) host *Oophila* subclade I and one population (site 3) hosts *Oophila* subclade III (Fig. 3). While the subclade I sequences are placed with only weak bootstrap support, these results indicate that the invasion of *Oophila* in cells (endosymbiotic relationship) is not necessarily due to subclade I affiliation alone and likely requires reciprocal interactions between symbiont and host (Kerney et al. 2019).

The transmission of *Oophila* to the intracapsular fluid and egg capsules of amphibian eggs is still unknown. However, it seems that symbiont algae transfer to the host *A. maculatum* mainly from surrounding pond water, thus supporting horizontal transmission. The same study also showed that some eggs in autoclaved pond water had algae growth, which could be due to contamination or vertical transmission (Bishop 2022). Kerney et al. (2011) amplified algal 18s rDNA from the Wolffian ducts and oviducts of *A. maculatum*; there were also encysted algal cells found inside freshly laid egg clutches, which indicates possible vertical transmission. However, no evidence of transgenerational *Oophila* transfer exists (Burns et al. 2017). If horizontal transmission is the mode of transfer, the oviposition behavior and egg capsule composition may be important factors explaining which amphibian embryos host *Oophila*.

All amphibians mentioned in this study use mainly fish-free, ephemeral ponds early in the year as oviposition sites. These fast-drying ponds hold less oxygen than larger bodies of water and heat up faster. These ranids all oviposit singular globular egg clutches with numerous eggs (500–3000) as soon in the year as possible. *R. temporaria* and the congeneric *R.* (*Boreorana*) *sylvatica* can also exhibit communal egg-laying (Waldman 1982, Fog et al. 1997), where egg clutches are aggregated to form rafts that increase temperature (Seale

1982, Håkansson and Loman 2004) and restrict oxygen (Savage 1935). The salamanders (*Hynobius* and *Ambystoma*) share a similar egg structure, with clutches of singular eggs within an external egg sack (Salthe 1963). The sizeable globular egg masses and egg sack structures restrict oxygen convection and diffusion within the clutch (Pinder and Friet 1994). These circumstances of oviposition and egg structure indicate that if an amphibian has these types of eggs, oviposition behavior and phenology, they may benefit from a photosymbiotic relationship with *Oophila*. However, experimental evidence of a beneficial relationship between *Oophila* and its host embryos has only been shown in *A. maculatum* (Gilbert 1942) and *A. gracile* (Marco and Blaustein 2000).

Post-hatching behavior of *R. temporaria* is not well described in the literature. Loman (2009) mentions that newly hatched embryos cling to the disintegrating jelly and may utilize the algae present as an early food source. However, since they hatch early (Gosner stage 19–20, Fig. 2C–D), they cannot feed (Gosner 1960), and even though tadpoles are known to absorb nutrients through their skin, surrounding microbes seem to outcompete them (Savage 1961). After transitioning into tadpoles, the *Oophila* bloom concentrated on the egg jelly (Fig. 2E) could ensure early food availability as Loman (2009) suggested. It has been shown that food quality and availability during the first four days of the larval stages have significant implications for the age and size at metamorphosis in *R. temporaria* (Merilä et al. 2000), reinforcing the view that early-life nutrition has a significant impact on fitness later in life and probably on population and evolutionary scales as well.

The presence of symbiotic algae within *R. temporaria* egg capsules in Sweden, and a possible Holarctic distribution of *Oophila amblystomatis*, leads us to believe that effects of anthropogenic stressors such as pollutants might need to be reevaluated. Disease, climate change, and habitat loss/degradation (including pollutants), are the main driving forces for amphibian population declines in recent years (Luedtke et al. 2023). There is not only the direct effect of pollutants (e.g. atrazine, Olivier and Moon 2010; glyphosate, Luis et al. 2011) on fitness to consider but the indirect effects channeled through the associated algae. Furthermore, we do not know the eco-evolutionary implications of the relationship between *R. temporaria* and *O. amblystomatis*, whether this association is dependent on specific environmental factors, and how it affects adaptation to long-term environmental change.

Acknowledgements – We thank Göran Hartman, Eva Herlitz, Stina Drakare, and the entire phyto- and zooplankton laboratory at the Swedish University of Agricultural Sciences.

Funding – PFE was supported by the Oscar and Lili Lamm Memorial Foundation (EX2017-0006). RK was supported by the Gordon and Betty Moore Foundation Grant (GBMF5604 https://doi.org/10.37807/GBMF5604).

Permits – Spawn of *Rana temporaria* was collected by the dispensation from the Swedish preservation clause in the species protection regulation (2007:845) issued by the County Board of Uppsala, Ref: 522-1708-19. Date of decision: 2018-04-18.

Author contributions

Pontus F. Eriksson: Conceptualization (lead); Data curation (equal); Funding acquisition (equal); Investigation (equal); Project administration (supporting); Visualization (equal); Writing – original draft (lead); Writing – review and editing (equal). **Ryan Kerney**: Data curation (equal); Formal analysis (equal); Funding acquisition (equal); Methodology (equal); Project administration (equal); Resources (equal); Software (equal); Visualization (equal); Writing – review and editing (equal). **River Larson-Pollock**: Formal analysis (equal); Methodology (equal); Software (equal); Visualization (equal); Writing – review and editing (supporting). **Elliana Vickers**: Formal analysis (equal); Methodology (equal); Software (equal); Visualization (equal); Writing – review and editing (supporting). **Ane Timenes Laugen**: Conceptualization (supporting); Investigation (equal); Project administration (equal); Resources (equal); Writing – review and editing (equal).

Data availability statement

There are no additional data for this paper.

Supporting information

The Supporting information associated with this article is available with the online version.

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