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Life history determinants of the susceptibility of the blood alga *Haematococcus* to infection by *Paraphysoderma sedebokerense* (Blastocladiomycota)

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ABSTRACT

Haematococcus pluvialis is currently cultivated at large scale for its ability to produce high amounts of the high value keto-carotenoid astaxanthin when encysted. Mass cultivation of this species is threatened by the destructive blastocladialean fungus, *Paraphysoderma sedebokerense* Boussiba, Zarka and James, responsible for the fast collapse of *Haematococcus* populations. Given the difficulty of maintaining pathogen-free production systems and the lack of treatment options, the selection and development of resistant *Haematococcus* strains could potentially present an effective and efficient method to control infection.

In the present work, we examined the host specificity of *P. sedebokerense* (strain PS1) through quantitative phenotyping of 44 *Haematococcus* strains in a laboratory-controlled infectivity assay. We determined the growth and photosynthetic activity of strains in the presence and absence of PS1 over time (using Chl *a* in vivo fluorescence) and quantified the degree of infection through the intensity of fluorescence after staining with Wheat Germ Agglutinin (WGA)-Fluorescein, which labels PS1 without interfering with *Haematococcus*. The measurements were converted into three infectivity proxies, allowing comparisons amongst strains. Eventually, microscopy was performed to check the life stage of *Haematococcus* upon infection.

Strains of *Haematococcus* clearly exhibited different levels of susceptibility against PS1 as determined by the three proxies. These were not related to phylogenetic background, nor the sampling origin of the strains. Amongst ten strains with low susceptibility, five occurred as flagellated state cultures, while others were palmelloid and/or aplanospore dominated. In addition, in a long-term selection experiment, we showed that susceptibility to PS1 of a highly sensitive *H. pluvialis* strain decreased through the dominance of flagellated phenotypes over several generations of infection.

While providing considerable expansion of the relation between PS1 and *Haematococcus* our study opens the possibility for selection and development of resistant strains.

1. Introduction

The green unicellular green alga, *Haematococcus pluvialis* Flotow (Chlorophyceae), is bi-flagellated and motile under optimal growth conditions. When exposed to adverse conditions, it transforms into a non-motile palmelloid cell which further evolves into a resting cyst or aplanospore with a tough cell wall, accumulating large amounts of the oxygenated carotenoid astaxanthin. These different life stages exhibit radically different phenotypes [1–3]. Aplanospores of *H. pluvialis* are so far, the best known natural producers of astaxanthin, a high valued carotenoid in cosmetic, nutraceutical and animal feed industries. Since astaxanthin is a powerful coloring agent with strong anti-oxidant capacity, *H. pluvialis* is highly demanded and cultivated at large scale currently by over sixteen international companies [4]. Although numerous grazers, pathogens and parasites are challenging mass culture of *H. pluvialis* worldwide [5], the blastocladialean fungus *Paraphysoderma sedebokerense* Boussiba, Zarka and James (nom. prov.) [6] (hereafter called *PS*) is believed to be one of the most serious hurdles [7]. It has

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been discovered independently, in several production facilities over the world, and was found responsible for reduced astaxanthin productivity as well as frequent culture collapses [5,8,9]. *PS* is noticeably a problem of persistent nature in culture facilities, since it may, similar to chytrid fungi, produce thick-walled cysts that withstand disinfection [10]. Its complex life cycle is currently not fully understood [11,12].

Amongst all the disinfection methods tested so far [13,14], only the use of H_2O_2 was proven to successfully eliminate *PS* [15]. Nonetheless, the use of such stringent sterilization techniques may be expensive, labor intensive or have severe environmental impacts [16]. Integrated pest management strategies have therefore been proposed as the most efficient long-term strategy for control of parasites in production facilities [10,17], where the cultivation of resistant strains, limited use of chemical agents, and the development of biological control systems are combined to limit the shortcomings of each individual strategy. In this respect, research has targeted the search and development (through classical mutagenesis or genetic engineering techniques) of disease-resistant strains [10].

Chytrids, close relatives of the Blastocladiales, have been widely studied for their interaction with phytoplankton. Species can be extremely variable in their host specificity [18], with some being generalists while others are specialists, infecting one or very few host species [19]. In contrast; the host specificity of the Blastocladiales has been poorly studied. Species belonging to *Physoderma*, the sister genus of *Paraphysoderma* are obligate parasites of phanerogams. In this genus, host specificity was long used as an important character to delineate taxa, resulting in the designation of generally one single host per species [20], e.g. *Physoderma dulichii* specific to the threeway sedge, *Dulichium arundinaceum* [21]. Only few cross-inoculation trials were performed to study *Physoderma* host ranges, revealing either wide host ranges [20,22] or limited host ranges [23]. Being the only genus of the Blastocladiales infecting algae, *Paraphysoderma* members exhibit considerable variation in host specificity, at least from the still rather limited available literature. So far, four strains of *PS* have been isolated from separate geographic locations. A strain of *PS* (TJ-2007a) from Israel was found highly specific to *Haematococcus* given its capacity to infect twenty different strains of *H. pluvialis* [5]. The same strain was able to infect other green algal genera yet without complete culture crashes [5,8]. Strain FD61 also identified as *PS*, was found in an outdoor biofuel production facility in New Mexico, US, infecting *Scenedesmus dimorphus*, where it resulted in complete population crashes [11,24]. Two additional strains, identified as *PS*, were discovered in open raceway ponds in Arizona, US (JEL821) [15] and outdoor cultures in Portugal (PS1) [12], in both cases infecting *H. pluvialis*. Although true host ranges of these strains remain unexplored, the heterogeneity in these reports suggest that strains of *PS*, though possessing identical SSU 18S ribosomal RNA sequences, might considerably differ in their infectivity behavior.

In most cases, host range studies are performed at the species level [25–28] yet specialization may also occur within species or populations. Intraspecific variation in infectivity is well described in pathogens affecting terrestrial plants but has only been poorly explored amongst algal lineages [19,29–33]. Within the Blastocladiales, it was reported that varieties of corn could differ in their susceptibility to brown spot (*Physoderma zeae-maydis*) and the selection and self-fertilization of plants resistant to the fungus was successful [34]. Intraspecific variation in susceptibility to *PS* amongst *Haematococcus* strains has never been reported, yet a preference of *PS* for aplanospores and palmelloid cells of *Haematococcus* was demonstrated, while flagellated cells were not susceptible to infection [5]. Given the lack of methodologies for *PS* detection and quantification, no comparative studies on *Haematococcus* have been performed thus far. Better understanding the causality of this specialism together with the identification of drivers of variation in susceptibility to *PS* are both key goals towards prediction and control of disease outbreaks in mass cultivation facilities.

In this study, we wanted to better comprehend the causality and drivers of specialism in the *Haematococcus* - PS1 interaction, through the establishment of a quantitative phenotyping method by which the relative susceptibility of strains of *Haematococcus* was tested, in an attempt to possibly identify resistant strains. Specifically, we examined in vitro infectivity profiles by *PS* on 44 *Haematococcus* strains [35], which we graded for susceptibility to *PS* through the use of different infectivity proxies: maximal reduction percentage (*Red.* $_{max.}$), the percentage of survival (*Survival*) and the density of infected cells (*Dens.* $_{inf.}$). Alongside, a parallel screening was performed to explore resistance on a large number of strains by categorizing infection through direct visual observation of 143 *Haematococcus* strains. Finally, a 17-month selection experiment was set up to test the long-term response of a highly susceptible *H. pluvialis* strain (C1) in co-culture with PS1. A selection towards flagellated phenotypes upon prolonged exposure to infection with *PS* was observed.

2. Material and methods

2.1. Biological material

The strain PS1 (described in [12]) was propagated on a clonal isolate of *H. pluvialis*, hereafter named C1, generated in-house from the strain SCCAP K-0084 (Scandinavian Culture Collection of Algae & Protozoa, http://www.sccap.dk). During the experimental period, PS1 was kept infective through the addition of infected PS1-C1 culture (5 mL) to uninfected exponential culture of C1 (10 mL), every four days. Infected cultures were placed in an incubator at 25 °C and continuously illuminated with 22 ± 4 μmol photons $m^{-2} s^{-1}$. C1 was kept exponential by the addition of Bold Basal Medium modified with 3-fold nitrogen (BBM-3N), [36] every four days, under following growth conditions: 23 °C, using a 16:8 h light:dark regime and a light intensity of 20 ± 2 μmol photons $m^{-2} s^{-1}$.

Details on the isolation and cultivation methods of the different *Haematococcus* strains used in this experiment can be found in [35]. In total, 60*H. pluvialis* including nine additional strains from four different culture collections (CCAP, NIES, SAG and SCCAP), 7*H. rubens*, 40*H. rubicundus* and 36*H.* sp. (which were not assigned because not sequenced) strains were infected (Table A.1, Appendix). A selection of 44 strains was made, selecting generally, two random strains per sampled location for the quantitative infectivity assay.

2.2. Algal growth conditions

All strains were grown from stock conditions (6 °C) in BBM-3N at 23 °C, using a 16:8 h light:dark regime and a light intensity of 20 ± 2 μmol photons $m^{-2} s^{-1}$ and further prepared and acclimated as in [37], with the exception that where specified, 48 or 96 cell culture multiwell plates were used (Greiner Bio-One) with a working volume of 1 mL or 0.5 mL respectively.

For the infectivity assay, each strain was prepared in four replicates for infection (I, $n = 4$) and two controls (C, $n = 2$). The experiment was started after 15 days of cultivation, ensuring that all strains were stationary. Each replicate culture was harvested prior to setting up the experiment and washed twice with BBM-3N without phosphate. Two treatments were generated from one culture, one control (C) and one infected (I) and each was brought to an initial F_0 value of 0.0043 (settings, 1-1-2 as measured by PAM fluorometer, see below) through the addition of BBM-3N without phosphate (1 mL, 48 multiwell plates), corresponding to a final cell density of approximately 1550 *Haematococcus* cells mL^{-1}.

The broader screening of strain susceptibility was performed on 143 strains of *Haematococcus*, in this case, strains were infected in 96 multiwell plates (0.5 mL working volume).

The long-term selection experiment was performed for 17 months, on strain C1 cultured in industrial medium based on Algal medium, as