The "Cheshire Cat" escape strategy of the coccolithophore *Emiliania huxleyi* in response to viral infection

Miguel Frada*†‡, Ian Probert*, Michael J. Allen§, William H. Wilson¶, and Colomban de Vargas*

*Station Biologique, Equipe EPPO-Evolution du Plancton et PaléoOcéans, Centre National de la Recherche Scientifique et Université Pierre et Marie Curie (Unité Mixte de Recherche 7144), Station Biologique, 29682 Roscoff, France; †Departamento de Geologia, Faculdade de Ciências, Universidade de Lisboa, Edificio C6, Campo Grande, 1749-016 Lisbon, Portugal; ¶Plymouth Marine Laboratory, Prospect Place, The Hoe, Plymouth PL1 3DH, United Kingdom; and §Bigelow Laboratory for Ocean Sciences, 180 McKown Point, P.O. Box 475, West Boothbay Harbor, ME 04575-0475

Communicated by Paul G. Falkowski, Rutgers, The State University of New Jersey, New Brunswick, NJ, August 6, 2008 (received for review June 9, 2008)

The coccolithophore *Emiliania huxleyi* is one of the most successful eukaryotes in modern oceans. The two phases in its haplodiploid life cycle exhibit radically different phenotypes. The diploid calcified phase forms extensive blooms, which profoundly impact global biogeochemical equilibria. By contrast, the ecological role of the noncalcified haploid phase has been completely overlooked. Giant phycodnaviruses (*Emiliania huxleyi* viruses, EhVs) have been shown to infect and lyse diploid-phase cells and to be heavily implicated in the regulation of populations and the termination of blooms. Here, we demonstrate that the haploid phase of *E. huxleyi* is unrecognizable and therefore resistant to EhVs that kill the diploid phase. We further show that exposure of diploid *E. huxleyi* to EhVs induces transition to the haploid phase. Thus we have clearly demonstrated a drastic difference in viral susceptibility between life cycle stages with different ploidy levels in a unicellular eukaryote. Resistance of the haploid phase of *E. huxleyi* provides an escape mechanism that involves separation of meiosis from sexual fusion in time, thus ensuring that genes of dominant diploid clones are passed on to the next generation in a virus-free environment. These "Cheshire Cat" ecological dynamics release host evolution from pathogen pressure and thus can be seen as an opposite force to a classic "Red Queen" coevolutionary arms race. In *E. huxleyi*, this phenomenon can account for the fact that the selective balance is tilted toward the boom-and-bust scenario of optimization of both growth rates of calcifying *E. huxleyi* cells and infectivity of EhVs.

eukaryotic life cycle | haplo-diploidy | marine viruses | host-parasite interaction | Red Queen

The coccolithophore *Emiliania huxleyi* (Lohmann) Hay and Mohler is one of the most abundant and widely distributed photosynthetic unicellular eukaryotes in modern oceans. Coccolithophores (Calcihaptophycidae, Haptophyta) produce composite skeletons of minute calcite platelets (the coccoliths) and, consequently, have been key contributors to both the oceanic carbon pump and the counterpump, and thus to the flux of CO_2 between atmosphere and oceans, since their origin in the Triassic (1). In this context, the impact of predicted anthropogenically induced ocean acidification on calcifying plankton is a subject of intense debate (2, 3). Coccolith-bearing *E. huxleyi* cells periodically develop extensive blooms covering wide coastal and midoceanic areas at high latitudes in both the northern and southern hemispheres. Termination of these blooms is accompanied by massive release of organic and inorganic matter to the water column, including detached coccoliths that reflect sunlight and are readily detectable in satellite images (4). Over the last decade, the role of large (≈175 nm) lytic coccolithoviruses (Phycodnaviridae), named *E. huxleyi* viruses (EhVs), in the regulation and termination of massive *E. huxleyi* blooms has been clearly established (5), to the extent that this system has become a case study in marine virology (e.g., refs. 6–8). Both *E. huxleyi* and EhV populations have been shown to be genetically diverse (9), with host succession suggested to follow "kill the winner" dynamics (10). However, recent observations show that the same *E. huxleyi* genotype blooms and is infected and decimated by the same EhV genotype over multiannual time scales (11).

What, then, is the selective advantage for the "winner" *E. huxleyi* clone(s) to bloom? Is there intense and permanent selection pressure for resistance to viral infection ["Red Queen" (RQ) dynamics]? And how is the high lytic virulence of EhVs sustained? These questions are fundamental to understanding the evolutionary ecology of this biogeochemically important species. The answers may be related to sex and life cycling, basic biologic features of unicellular eukaryotes that are typically ignored in oceanographic models addressing the ecology of planktonic functional groups. The fitness of many eukaryotic species may be based on their potential for alternation between variable life cycle phases and adaptation of each phase to different ecological niches (12, 13), including in terms of biological interactions. Current evidence suggests that coccolithophore life cycles are characterized by independent haploid and diploid phases displaying radically different morphologies (14, 15) and distinct physiologies (refs. 16 and 17; our unpublished data). In *E. huxleyi*, the life cycle comprises two main forms: the diploid (2*N*), nonmotile, coccolith-bearing phase that forms blooms, and the haploid (*N*) flagellated phase that possesses nonmineralized organic scales overlying the cell membrane (18, 19). This motile, noncalcifying, haploid stage is not easily amenable to identification by conventional microscope techniques and has been almost completely overlooked by biological oceanographers. Its ecological role and the importance of sexual cycling in *E. huxleyi* in the natural environment remain unknown. Flow cytometric surveys of mesocosm blooms of 2*N* *E. huxleyi* have revealed the onset, after virus-mediated bloom demise, of new active populations of cells with the same chlorophyll fluorescence signature but lower light-scattering values than 2*N* calcified cells (20, 21). We hypothesized that these new populations consisted of noncalcifying *N* cells, and that their presence reflected their resistance to infection by the viruses responsible for the decline of the 2*N* blooms.

In the present work, we explore the *in vitro* infectivity profiles of both the coccolith-bearing diploid and noncalcifying haploid life cycle phases of *E. huxleyi* by using multiple host and viral

Author contributions: M.F., I.P., and C.d.V. designed research; M.F. and I.P. performed research; I.P. and C.d.V. contributed new reagents/analytic tools; M.F., I.P., M.J.A., W.H.W., and C.d.V. analyzed data; and M.F., I.P., and C.d.V. wrote the paper.

The authors declare no conflict of interest.

See Commentary on page 15639.

‡To whom correspondence should be addressed. E-mail: frada@sb-roscoff.fr.

This article contains supporting information online at www.pnas.org/cgi/content/full/0807707105/DCSupplemental.

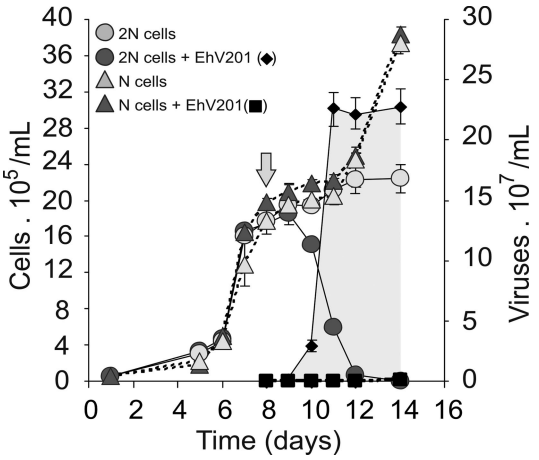

Fig. 1. The impact of EhV on the growth of diploid and haploid *E. huxleyi*. Growth curves of both life cycle stages of *E. huxleyi* (strain RCC1216) and the virus EhV201 are shown. The arrow indicates the day of virus addition (multiplicity of infection 0.2). Standard deviation bars are generally too short to be visible.

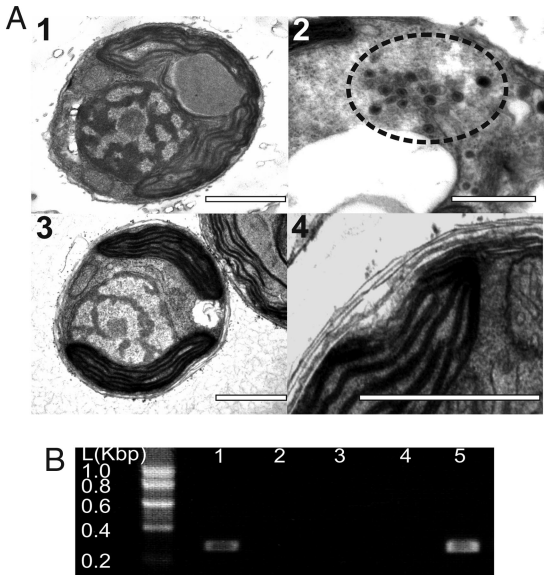

Fig. 2. Microscopy and genetic tests for the presence/absence of EhVs inside and/or on infected *E. huxleyi* haploid and diploid cells. (*A*) TEM: 1, healthy 2*N* cell before infection; 2, 2*N* cell at day 1 after infection, displaying newly formed viral particles (dashed circle); 3, *N* cell from an infected culture; and 4, detail of the *N* cell periplast, showing the presence of organic scales attached to the membrane and the absence of coccoliths and viruses. (Scale bars: 1 μm.) (*B*) PCR amplifications of the viral *MCP* gene at day 11 after infection (see Fig. 1). Agarose gel lanes: 1, positive control (EhV201 DNA extract); 2, *N* culture; 3, 2*N* culture; 4, *N* culture exposed to viruses; and 5, 2*N* culture exposed to viruses. Cells were carefully filtered and washed several times to remove free viral particles before DNA extractions.

strains and various experimental setups. The results show the critical significance of life cycling in the survival and ecological dynamics of *E. huxleyi*. They illustrate a previously unrecognized type of ecological and evolutionary interaction that opposes classical RQ host–pathogen dynamics and is potentially a fundamental force for the maintenance of sex and life cycling and for untying the constraints imposed by pathogens in the evolution of eukaryotic microbes.

Results

As a first approach, we qualitatively scanned the infectivity of the 15 available EhV strains against both life cycle stages of three different strains of *E. huxleyi* (RCC1216, RCC1249, and RCC1213). All 2*N E. huxleyi* strains were sensitive to five of the viral strains (EhV201, EhV202, EhV205, EhV207, and EhV208), whereas none of the *N* strains were affected. As no obvious differences in infectivity were observed between the *E. huxleyi* and EhV strains tested, the host–virus combination RCC1216:EhV201 was chosen for subsequent experiments.

We then monitored the growth (Fig. 1) and photosynthetic activity [supporting information (SI) Fig. S1] of both infected and noninfected *E. huxleyi* 2*N* and *N* phases. In 2*N* cultures, daily flow cytometry and fluorimetric measurements revealed 96% and 98% decreases of cell density and photosynthetic activity, respectively, within 3 days of virus inoculation into the growing culture. There was a concurrent increase in viral particle concentration from 3.5×10^5 to 23×10^7 viral particles per ml from days 8–14. In contrast, no differences were observed between infected and noninfected *N* cultures throughout the experiment, and virus concentration neither increased nor decreased. Visually, the 2*N* cultures became transparent, and deposits of cell debris were observed at the bottom of the culture flasks 3 days after infection (Fig. S2). To further verify the absence of infection of the *E. huxleyi* haploid phase, various viral densities and coexistence times were tested (Fig. S3). Haploid cultures that were previously used for infection experiments and fresh *N* cultures were incubated for 26 days with viruses at various multiplicities of infection (MOI). All cultures displayed similar growth curves, reaching typical concentrations of 15×10^5 to 20×10^5 cells per ml at day 10.

We then used transmission electron microscopy (TEM) and PCR of the gene coding for the EhV major capsid protein (MCP) (Fig. 2) to verify the absence of viral adsorption and production by haploid *E. huxleyi* suggested by flow cytometry. No viral capsids were detected inside or adsorbed to the *N* cells from virus-infected cultures in any of the multiple TEM preparations, whereas capsids were obvious in the cytoplasm of infected 2*N* cells as early as 1 day after virus addition (Fig. 2*A*). Furthermore, the *MCP* gene was easily detected by PCR of DNA extracts from filtered and washed infected 2*N* cells, whereas the *N* cells in contact with viruses never yielded positive amplifications (Fig. 2*B*).

Finally, we set up a series of 50-day experiments to test longer-term responses of 2*N* and mixed 2*N*–*N E. huxleyi* cultures to viruses. The infected 2*N* cultures crashed 5 days after infection (Fig. 3*A*). On day 6, a small new peak of cells ($\approx 4 \times 10^5$ cells per ml) was observed within the flow cytometric window defined for *N* cells (Fig. S4). Microscope observations revealed that these *E. huxleyi* cells possessed neither coccoliths nor flagella, resembling moribund noncalcified diploid cells. Thirty of these were sorted by flow cytometry into fresh, virus-free culture media, but none of them established a new growing population. Between days 7 and 23, cell density remained at a background level of 10 to 10^3 cells per ml. At day 24, however, a new population of *N* cells appeared, as observed by both flow cytometry (Fig. 3*A*) and light microscopy. The concentration of these actively swimming *N* cells reached a plateau after $\approx$10 days. In contrast, the noninfected 2*N* control culture declined after the plateau phase, with no sign of the presence of *N* cells (Fig. 3*B*).

Two additional long-term experiments were performed with both life cycle stages mixed in the same culture vessel. In the first of these experiments, the diploid and haploid stages were grown together in the presence of viruses (Fig. 3*C*). The population dynamics were similar to those in the experiment with 2*N* cells only, with the same peak of drifting, noncalcified cells at day 6, followed by a lag phase of very low cell densities, and the

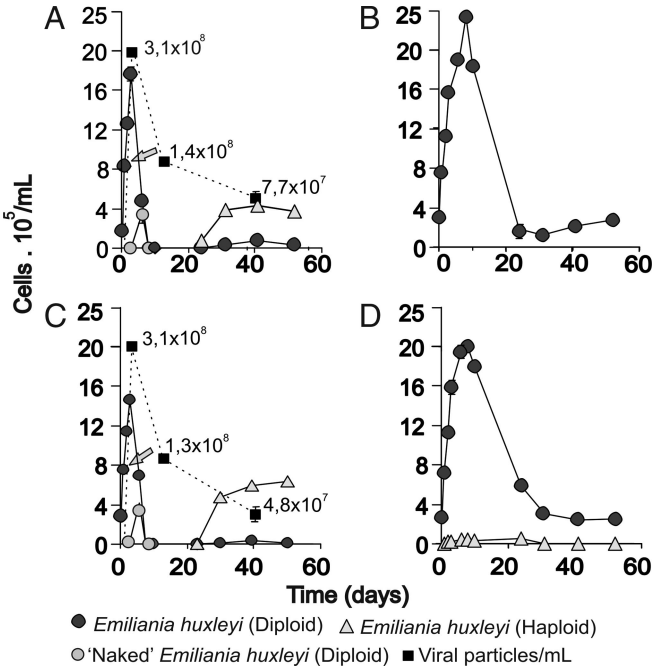

Fig. 3. Long-term infection assays of 2*N* and mixed 2*N*–*N* cultures of *E. huxleyi*. (*A*) 2*N* cells infected with EhV201 virus. (*B*) 2*N* cells without virus. (*C*) Mixture (100:1) of 2*N* and *N* cells infected with EhV201. (*D*) Mixture (100:1) of 2*N* and *N* cells without virus. 2*N* naked cells (due to coccolith loss after viral infection) were distinguished from *N* cells by their permanent immobility. The arrows indicate time of infection. Standard deviation bars are generally too short to be visible.

development of the *N* population by day 20. The population of *N* cells attained a higher density than in the assay that started with 2*N* cells only. In the last experiment, 2*N* and *N* cells were grown together without viruses. The shape of the 2*N* growth curve was identical to that of the culture without *N* cells (Fig. 3*B*), although of lower cell density. However, haploid cell density remained low and constant ($\approx 10^4$ cells per ml) during the first 24 days and then decreased to minimum values of $\approx 10^3$ cells per ml in the second half of the experiment. Controls with pure haploid cultures with or without viruses were conducted, and these displayed typical haploid cell growth curves (data not shown).

Discussion

Our data demonstrate that the noncalcifying haploid phase of the coccolithophore *E. huxleyi* is resistant to viruses that infect and lyse the diploid calcifying phase of the same species. This phenomenon was confirmed *in vitro* with multiple *E. huxleyi* and EhV strains. In all experiments using haploid *E. huxleyi* cultures, concentrations of free-floating viral particles were stable over time, and neither viral capsids nor viral DNA was detected by TEM or PCR (Fig. 2) within *N* cells in contact with viruses. This observation indicates the existence of a mechanism impeding the adsorption of viruses in *E. huxleyi* haploid cells. Differential susceptibility to viral infection has been reported between clones of the same ploidy level in a number of microalgal taxa (22), but there has been no previous clear demonstration of a drastic difference in viral susceptibility between life cycle stages with different ploidy levels in a marine protist. Our results further indicate that viral infection may trigger meiosis, or at least a shift from diploid to haploid populations, in *E. huxleyi* (Fig. 3). These observations have important implications for the ecology and evolution of both *E. huxleyi* and EhVs, and potentially all oceanic protists with sexual life cycles.

Mechanisms of Viral Resistance of *E. huxleyi* Haploid Cells. To initiate infection, viruses attach to host-specific cell surface receptors (23). Obvious phenotypic differences exist in the nature of the cell cover between life cycle phases in *E. huxleyi* (Fig. 2). One or several loose layers of interlocking coccoliths surround diploid cells, but these do not produce organic scales covering the cell membrane (commonly known as body scales and typical for most haptophytes). By contrast, haploid cells do not produce coccoliths, but their cell surface is covered by distinctively tightly packed body scales organized in overlapping layers (18). This haploid cell covering, characteristic of most noncalcifying members of the order Isochrysidales, may efficiently prevent viruses from coming into contact with putative receptor sites on the cell membrane. Alternatively, plasmalemma molecules recognized by EhV capsids may be modified or simply absent in haploid cells. Modification or loss of receptor molecules is the most common way in which bacteria develop resistance to bacteriophages (24), but these phenotypic differences typically result from mutation(s) of the genotype rather than differential gene expression, as would most likely be the case over the *E. huxleyi* life cycle. Note that the fact that the three haploid *E. huxleyi* strains tested proved resistant to viral infection makes it highly improbable that resistance results from a simple Mendelian allele segregation effect. The receptors to which viruses bind typically serve primary metabolic functions in the host, and hence receptor differences between life cycle phases would imply physiologic differentiation. In this context, a notable difference between *E. huxleyi* life cycle phases is calcium metabolism, which is known to be particularly intense in the calcifying 2*N* phase (25), but is presumably negligible in the noncalcifying *N* phase. Calcium often promotes the physical interactions between viruses and host receptors through a direct effect on the conformation of the viral capsid as, for example, in the hepatitis A virus in humans (26).

Other mechanisms of viral avoidance reported in marine protists include the extracellular production of viral inhibitors, like the cell wall sulfated polysaccharide produced by the red microalga *Porphyridium* sp. (27), the exudation of a protective extracellular polysaccharidic layer or aggregates that may trap viruses, as observed in the colony-forming haptophytes *Phaeocystis globosa* and *Phaeocystis pouchetii* (28, 29), or the secretion of dimethyl sulfide and acrylic acid, which are known to inhibit infection (30). However, such mechanisms do not seem to be relevant in the case of the *E. huxleyi* life cycle, as EhVs infected 2*N* cells in mixed 2*N*–*N* cultures as efficiently as in pure 2*N* cultures.

Viral Infection and Life Cycle Phase Transition in *E. huxleyi*. Preliminary indications suggest that life cycle phase switches in coccolithophores may be regulated by chemical (31) or physical (14) properties of the medium. Our data show that biotic interactions with viruses may also play a key role in directly triggering life cycle changes. Oxidative stress in response to viral infection could be the trigger for both the peak of dead *E. huxleyi* 2*N* cells at day 6 after infection and the sexual diploid-to-haploid transition observed in an initially pure 2*N* culture (Fig. 3*A*). As has commonly been observed in infected plant and animal cells, viral infection can induce elevated production of reactive oxygen species (ROS) in diploid *E. huxleyi* cultures (32). ROS and viral infection were found to be associated with the induction of metacaspases and programmed cell death (PCD) in diploid *E. huxleyi* (33), a phenomenon also demonstrated in other marine protists (34). However, cell death is not the exclusive response to oxidative stress in single-celled eukaryotes. ROS-induced PCD pathways are also clearly linked to the induction of life cycle phase transitions in phytoplankton. Cell cycle arrest and PCD are alternative responses to increased oxidative stress in the colonial green alga *Volvox carteri* (35), and developmental programs that lead to the concomitant formation of dead cells and

spores have been described in protists, such as the slime mold *Dictyostelium discoideum*, as well as in several prokaryotes (36).

An alternative explanation could be that *N* cells are produced regularly by meiosis in 2*N* cultures, but remain at very low concentrations such that they are effectively undetectable by microscopy or flow cytometry. In our mixed 2*N*–*N* culture experiment (Fig. 3*D*), diploid cells clearly out-competed haploid cells to the point that the growth dynamics of 2*N* populations were strikingly similar with or without addition of *N* cells. In such a case, the very rare *N* cells would provide a permanent inoculum for the potential emergence of a haploid population, and viral infection would indirectly promote succession of life cycle stages through elimination of the more competitive diploid phase.

Ecological Implications. The visible worldwide ecological success of the diploid stage of *E. huxleyi* has stimulated interest in the underlying physiologic mechanisms that allow these calcifying cells to form extensive blooms under certain conditions (4). Two of the more remarkable capacities of *E. huxleyi* 2*N* cells are their exceptionally high phosphate uptake and the fact that they do not exhibit photoinhibition of photosynthesis, even at very high light intensities (ref. 18 and references therein). These and other data help explain how the diploid *E. huxleyi* comes to dominate its phytoplankton competitors. However, in light of the growing body of evidence indicating the devastating impact of the highly infective EhVs on these blooms, the evolutionary advantages of this ecological strategy are not clear. Why has investment by *E. huxleyi* 2*N* of more resources into defense mechanisms at the expense of growth not been selected? Why has reduction of infectivity of EhVs to confer a clear selective advantage for blooming in their host clones not been selected?

The ability of haploid cells to escape viral infection can explain why the selective balance is tilted so far toward optimization of growth rates and infectivity in *E. huxleyi* 2*N* cells and EhVs, respectively. By increasing massively in density as a result of successive mitoses, more cells of a given 2*N* clone are likely to undergo meiosis, potentially as a direct response to viral infection. If newly formed *N* cells were susceptible to viral infection they would be rapidly decimated, because viral density is highest during bloom demise (5, 11), and because motile cells are theoretically more likely to encounter viral particles (37). Blooming would then have no selective advantage compared with a strategy of maintaining low background 2*N* cell concentrations. On the other hand, transformation into a haploid phenotype invisible to the virus provides an escape mechanism that ensures that the genes of an individual (or clone) are passed on to the next generation. It can be argued, therefore, that this phenomenon dictates positive selection for rapid growth and meiosis in the diploid host while imposing little negative selection pressure for high infectivity of the virus. It also leads to the prediction that *E. huxleyi N* cells do not simply act as gametes by mating at the first opportunity, as this would produce 2*N* cells susceptible to viral attack in an environment where viruses are still present in high concentrations. Rather, the noncalcifying haploid phase probably plays a prominent ecological role, dividing and migrating with the consequence of temporally and spatially displacing an eventual inoculum of novel 2*N* cells.

Evolutionary Significance: Red Queen or Cheshire Cat? Originally proposed by Van Valen (38), the metaphor of an evolutionary "arms race" has been widely used for describing various biotic interactions and termed Red Queen (RQ) dynamics in reference to the Red Queen's race in Lewis Carroll's *Through the Looking Glass*, in which the Red Queen states "it takes all of the running you can do, to keep in the same place" (39). The idea that genetic recombination through sex could be key in the evolutionary arms race between parasites and hosts was developed later by several authors (e.g., refs. 40–42). Our results indicate that viral infection of the diploid stage of *E. huxleyi* promotes sexual cycling, either directly by inducing meiosis and/or indirectly by removing the more competitive 2*N* cells. A classical RQ interpretation would be that this sexual cycling leads to increased diversity in the following diploid generation, and that among this diversity certain genotypes would be more resistant to viral attack, and therefore positively selected. The fact that EhVs typically infect a limited range of diploid *E. huxleyi* strains in culture (9) may support this interpretation.

However, the limited data available from natural populations do not indicate a constant and rapid turnover of coevolving *E. huxleyi* and EhV genotypes in the oceans. In our experiments, an EhV strain isolated from the North Atlantic was capable of infecting host strains originating from the Mediterranean Sea (RCC1249, RCC1213) and from the Tasman Sea off New Zealand (RCC1216). Rapid RQ dynamics would presumably have led to localized, genetically distinct subpopulations of host and virus, at least in terms of resistance and recognition genes, respectively. Furthermore, the *GPA* gene, encoding a highly polymorphic protein with calcium-binding motifs and used as a genotype marker in *E. huxleyi*, did not reveal variation of the dominant genotypes over multiannual mesocosm experiments in Norwegian fjords (11), suggesting the existence of an efficient strategy for survival of these genotypes between blooms.

The persistence of *E. huxleyi* strains over multiple years in the North Atlantic, the lack of biogeographic structuring in *E. huxleyi*–EhV infectivity patterns, and in general the maintenance of elevated growth rate and infectivity in *E. huxleyi* and EhV, respectively, argue against the existence of a classical, highly dynamic RQ equilibrium in this host–virus system. The invisibility of the host haploid stage to the virus circumvents, or at least drastically slows, the arms race. The period of respite experienced during one life cycle phase means that resources of the phase susceptible to viral attack can be focused on interactions with direct ecological competitors rather than on developing new arms against the virus. In keeping with the RQ metaphor taken from Lewis Carroll, we liken this theory to the strategy used by the Cheshire Cat in *Alice's Adventures in Wonderland* (43) of making its body invisible to make the sentence "off with his head" pronounced by the Queen of Hearts impossible to execute.

RQ and Cheshire Cat (CC) mechanisms, both of which act around a central eukaryotic process, sexual reproduction, should not be considered mutually exclusive. CC dynamics, which rely to some extent on separation of the sexual processes of meiosis and fusion in time and/or space, release the host from short-term pathogen pressure, thus widening the scope for the host to evolve in other directions. Evolution of genes conferring resistance to viral attack in the host and the counteractive evolution of new arms by the virus likely still occur, but over much longer time scales than classically inferred. *E. huxleyi* is a relatively young species (or species complex), having evolved from the Gephyrocapsids some 270,000 years ago (44), and thus represents an interesting model for assessment of the pace of host–virus coevolution and the interaction between the Cat and the Queen. CC dynamics could, of course, also apply to predator–prey interactions. Cases of phenotypic changes linked to predation pressure have been documented in protists (45); however, to our knowledge there are, as yet, no reports of phenotypic switches associated with ploidy changes under these conditions.

Concluding Remarks. Each milliliter of seawater is known to contain millions of viral particles, and the growing evidence of an enormous diversity of eukaryotic viruses (46) and parasites (47) suggests that each eukaryote species, and potentially each clonal strain in the oceans, has its own pathogen(s). Our data reveal a fundamental mechanism that may correlate the massive diversity of oceanic pathogens and the maintenance of dimor-

phic sexual life cycles in marine eukaryotes. Understanding these relationships involves more than mere intellectual curiosity on the coevolution of sex and viruses. In fact, eukaryotic marine protists are responsible for nearly half of global primary productivity and control most of the flux of matter between the atmosphere and the lithosphere (48). The fate of oceanic primary production, whether sedimented to the ocean floor or remineralized in surface waters, depends fundamentally on the physical properties of the eukaryotic cells themselves, which change dramatically as they differentiate over their life cycle. In the case of *E. huxleyi*, the impact of each life cycle phase on Earth System dynamics is obviously radically different: the diploid stage is responsible for a significant amount of planetary carbonate production and is thus heavily implicated in global climate regulation (4), whereas the haploid stage does not calcify and is probably mostly remineralized in surface waters. Understanding the genomic controls of pathogen resistance and ploidy-related phenotypic differentiation in oceanic protists will significantly advance assessment and prediction of their impact on biogeochemical cycles.

Methods

Strains and Culture Conditions. The *E. huxleyi* strains used in this study, RCC1216 (origin: Tasman Sea, New Zealand), RCC1249 (origin: Mediterranean Sea, Spain), and RCC1213 (origin: Mediterranean Sea, Italy) from the Roscoff Culture Collection, France (http://www.sb-roscoff.fr/Phyto/RCC), were originally initiated by micropipette isolation of a single diploid (coccolith-bearing) cell. After diploid-to-haploid life cycle transitions of a few cells in the original cultures, pure cultures of each phase were established from the mixed-phase cultures by single-cell micropipette isolation. Culture purity was verified by light microscopy before each experiment. Experiments were conducted in triplicate in K/2 (minus Si, minus Tris) medium at 18°C, 12:12 (light:dark) cycle and 85 $\mu E \cdot m^{-2} \cdot s^{-1}$ irradiance. The viral strains (EhV84, EhV86, EhV88, EhV163, EhV201, EhV202, EhV203, EhV204, EhV205, EhV206, EhV207, EhV208, EhV209, EhVv1, and EhVv2; ref. 6) were maintained at 4°C in K/2 medium and filtered through a 0.2-μm filter (Minisart; Sartorius) before utilization.

Flow Cytometry and Photosynthetic Activity Analyses. Enumeration of *E. huxleyi* cells and viruses was performed with a FACSCalibur flow cytometer (Becton Dickinson) equipped with an air-cooled laser providing 15 mW at 488 nm and with the standard filter setup. Haploid and diploid *E. huxleyi* cells in unfixed samples were identified on the basis of their chlorophyll fluorescence and side-scatter signatures (Fig. S4). Virus enumeration was performed according to Marie *et al.* (49). A FACSAria flow cytometer (Becton Dickinson) was used for sorting *E. huxleyi* haploid and diploid cells. The photosynthetic activity of cells was quantified by measuring variable fluorescence before and during a saturating light pulse (0.6 s, 470 nm, 1,700 $\mu mol \cdot m^{-2} \cdot s^{-1}$) with a PHYTO-PAM (Mess und Regaltechnik) using fresh samples incubated for 10 min in the dark before analyses. The quantum efficiency of photosystem II (F_v/F_m) was calculated by $F_v/F_m = (F_m - F_0)/F_m$, where F_0 is the minimum fluorescence and F_m is the maximum fluorescence of the dark-adapted sample during the saturating light pulse.

Infection Assays. The first experiment was designed to assess the response of the haploid and diploid phases of all *E. huxleyi* strains to infection with each of the viral strains. Exponentially growing cultures (200 ml; $\approx 2 \times 10^6$ cells per ml) of *E. huxleyi* strains were infected independently with each virus strain at a virus-to-cell ratio (MOI) of 0.2. Enumeration of algae and viruses and measurement of photosynthetic activity were performed daily. Controls without addition of viruses were performed in parallel. The *E. huxleyi* RCC1216 *N* and 2*N* strains and the EhV201 viral strain were chosen for further experiments.

The effect of addition of various viral concentrations on the haploid phase of *E. huxleyi* was then tested. Cultures of the haploid stage used in the previous infection experiment (including controls) were diluted 200 times in 200 ml of fresh K/2 (minus Si, minus Tris) medium and incubated in conditions previously described. After attaining $\approx 2 \times 10^6$ cells per ml, duplicates were inoculated with mois of 0.05, 1, and a control (no virus addition). Control cultures, which had never been in contact with viruses, were inoculated with MOIs of 0.5, 5, and a control. Samples for enumeration of algae and viruses were collected six and three times, respectively, at irregular intervals over a 26-day period.

Long-Term Infection Assays. To mimic a bloom situation based on the observations of Castberg *et al.* (20) and Jacquet *et al.* (21), 200-ml exponentially growing diploid cultures were inoculated with haploid cells in exponential growth at a 2*N*/*N* cell ratio of 100 ($\approx 1 \times 10^6$ 2*N* cells per ml and 1×10^4 *N* cells per ml), and infected with viruses at an MOI of 0.2. Noninfected diploid cultures with and without addition of haploid cells were used as controls. Populations of *N* and 2*N* cells of *E. huxleyi* and viruses were monitored for 50 days. In the initial stages of infection, cells from the samples were sorted by flow cytometry and cultured in 2 ml of K/2 (minus Si, minus Tris) medium.

DNA Extraction and PCR Amplification. To assess whether viruses were sticking to and/or penetrating *E. huxleyi* haploid cells, we attempted to PCR amplify the viral major capsid protein (MCP) gene ($\approx$300 kb; ref. 9) from infected haploid and diploid cells after multiple washes. Samples from *N* and 2*N* cultures were collected 2 days after viral addition. Free-floating viral particles were washed away with fresh medium by multiple filtrations of the cells on polyethersulfone membranes (0.45-μm pore size, GE Osmonics Labstore). Total DNA was then extracted from the cells on the filters (50). PCR amplifications with the primer pair MCP-F and MCP-R were performed according to Schroeder *et al.* (9). A pure extract of EhV201 DNA was used as a positive control.

Transmission Electronic Microscopy. For thin sectioning, the cells were fixed for 1.5 h in 4% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.2) with 0.25 M sucrose. Cells were then washed three times in 0.1 M sodium cacodylate containing decreasing concentrations of sucrose and were postfixed in 2% osmium tetroxide in 0.1 M sodium cacodylate for 1.5 h. After washing in distilled water, cells were dehydrated in a graded ethanol series and embedded in Epon resin. Sections were double stained with uranyl acetate followed by Reynold lead citrate. Observations were carried out on a JEOL JEM 1011 electron microscope.

ACKNOWLEDGMENTS. We thank the Plancton Océanique group at the Station Biologique de Roscoff, especially D. Marie, F. Le Gall, A. Pagarete, P. Von Dassow, and E. M. Bendif, for assistance with flow cytometry and algal cultures, and for informative discussions. We also thank J. Barcelos e Ramos for sharing observations. This work was supported by a Ph.D. fellowship awarded to M.F. by the Fundação Para a Ciência e para a Tecnologia, Portugal, and an Actions Thématiques et Incitatives sur Programme (ATIP) grant awarded to C.d.V. by the Centre National de la Recherche Scientifique, France. It is part of the pluridisciplinary project BOOM (Biodiversity of Open Ocean Microcalcifiers) funded by the French Agence Nationale de la Recherche, Grant ANR-05-BIODIV-004.

1. de Vargas C, Aubry MP, Probert I, Young J (2007) Origin and evolution of coccolithophores: From coastal hunters to oceanic farmers. *Evolution of Aquatic Photoautotrophs*, eds Falkowski P, Knoll AH (Elsevier Academic, New York), pp 251–281.
2. Iglesias-Rodriguez MD, *et al.* (2008) Phytoplankton calcification in a high-CO_2 world. *Science* 320:336–340.
3. Riebesell U, *et al.* (2000) Reduced calcification of marine plankton in response to increased atmospheric CO_2. *Nature* 407:364–367.
4. Tyrrell T, Merico A (2004) *Emiliania huxleyi:* Bloom observations and the conditions that induce them. *Coccolithophores: From the Molecular Processes to Global Impact*, eds Thierstein HR, Young JR (Springer, Heidelberg, Germany), pp 75–97.
5. Wilson WH, *et al.* (2002) Isolation of viruses responsible for the demise of an *Emiliania huxleyi* bloom in the English Channel. *J Mar Biol Assoc UK* 82:369–377.
6. Bratbak G, Egge JK, Heldal M (1993) Viral mortality of the marine alga *Emiliania huxleyi* (Haptophyceae) and the termination of the algal bloom. *Mar Ecol Prog Ser* 93:39–48.
7. Allen M, Martinez-Martinez J, Schroeder DC, Somerfield PJ, Wilson WH (2007) Use of microarrays to assess viral diversity: From genotype to phenotype. *Environ Microbiol* 9:971–982.
8. Wilson WH, *et al.* (2005) Complete genome sequence and lytic phase transcription profile of a coccolithovirus. *Science* 309:1090–1092.
9. Schroeder D, Oke J, Malin G, Wilson WH (2002) Coccolithovirus (Phycodnaviridae): Characterisation of a new large dsDNA virus that infects *Emiliania huxleyi*. *Arch Virol* 147:1685–1698.
10. Thingstad TF (2000) Elements of a theory for the mechanisms controlling abundance, diversity, and biogeochemical role of lytic bacterial viruses in aquatic systems. *Limnol Oceanogr* 45:1320–1328.
11. Martinez JM, Schroeder DC, Larsen A, Bratbak G, Wilson WH (2007) Molecular dynamics of *Emiliania huxleyi* and cooccurring viruses during two separate mesocosm studies. *Appl Environ Microbiol* 73:554–562.
12. Valero M, Richerd S, Perrot V (1992) Evolution of alternation of haploid and diploid phases in life cycles. *Trends Ecol Evol* 7:25–29.
13. Thornber CS (2006) Functional properties of the isomorphic biphasic algal life cycle. *Integr Comp Biol* 46:605–614.
14. Billard C (1994) Life cycles. *The Haptophyta Algae*, eds Green JC, Leadbeater BSC (Clarendon, Oxford, England), Vol 51, pp 167–186.

15. Houdan A, *et al.* (2004) Holococcolithophores-heterococcolithophores (Haptophyta) life cycles: Flow cytometry analysis of relative ploidy levels. *Syst Biodivers* 1:453–465.
16. Houdan A, Probert I, Van Lenning K, Lefebvre S (2005) Comparison of photosynthetic responses in diploid and haploid life-cycle phases of *Emiliania huxleyi* (Prymnesiophyceae). *Mar Ecol Prog Ser* 292:139–146.
17. Houdan A, Probert I, Zatylny C, Véron B, Billard C (2006) Ecology of oceanic coccolithophores. I. Nutritional preferences of the two stages in the life cycle of *Coccolithus braarudii* and *Calcidiscus leptoporus*. *Aquat Microb Ecol* 44:291–301.
18. Green JC, Course PA, Tarran GA (1996) The life-cycle of *Emiliania huxleyi*: A brief review and a study of relative ploidy levels analysed by flow cytometry. *J Mar Syst* 9:33–44.
19. Paasche E (2001) A review of the coccolithophorid *Emiliania huxleyi* (Prymnesiophyceae), with particular reference to growth, coccolith formation, and calcification-photosynthesis interactions. *Phycologia* 40:503–529.
20. Castberg T, *et al.* (2001) Microbial population dynamics and diversity during a bloom of the marine coccolithophorid *Emiliania huxleyi* (Haptophyta). *Mar Ecol Prog Ser* 221:39–46.
21. Jacquet S, *et al.* (2002) Flow cytometric analysis of an *Emiliana huxleyi* bloom terminated by viral infection. *Aquat Microb Ecol* 27:111–124.
22. Tarutani K, Nagasaki K, Yamaguchi M (2000) Viral impacts on total abundance and clonal composition of the harmful bloom-forming phytoplankton *Heterosigma akashiwo*. *Appl Environ Microbiol* 66:4916–4920.
23. Baranowski E, Ruiz-Jarabo CM, Domingo E (2001) Evolution of cell recognition by viruses. *Science* 292:1102–1105.
24. Bohannan BJM, Lenski RE (2000) Linking genetic change to community evolution: Insights from studies of bacteria and bacteriophage. *Ecol Lett* 3:362–377.
25. Brownlee C, Taylor AR (2004) *Coccolithophores: From the Molecular Processes to Global Impact*, eds Thierstein HR, Young JR (Springer, Heidelberg).
26. Bishop NE, Anderson DA (1997) Early interactions of hepatitis A virus with cultured cells: Viral elution and the effect of pH and calcium ions. *Arch Virol* 142:2161–2178.
27. Huheihel M, Ishanu V, Tal J, Arad S (2002) Activity of *Porphyridium* sp. polysaccharide against herpes simplex viruses in vitro and in vivo. *J Biochem Biophys Methods* 50:189–200.
28. Jacobsen A, Bratbak G, Heldal M (1996) Isolation and characterization of a virus infecting *Phaeocystis pouchetii* (Prymnesiophyceae). *J Phycol* 32:923–927.
29. Brussaard CPD, Mari X, Van Bleijswijk JDL, Veldhuis MJW (2005) A mesocosm study of *Phaeocystis globosa* (Prymnesiophyceae) population dynamics II: Significance for the microbial community. *Harmful Algae* 4:875–893.
30. Evans C, Malin G, Wilson W, Liss P (2006) Infectious titres of *Emiliania huxleyi* virus 86 are reduced by exposure to millimolar dimethyl sulfide and acrylic acid. *Limnol Oceanogr* 51:2468–2471.
31. Noel MH, Kawachi M, Inouye I (2004) Induced dimorphic life cycle of a coccolithophorid, *Calyptrosphaera sphaeroidea* (Prymnesiophyceae, Haptophyta). *J Phycol* 40:112–129.
32. Evans C, Malin G, Mills GP, Wilson WH (2006) Viral Infection of *Emiliania huxleyi* (Prymnesiophyceae) leads to elevated production of reactive oxygen species. *J Phycol* 42:1040–1047.
33. Bidle KD, Haramaty L, Barcelos e Ramos J, Falkowski P (2007) Viral activation and recruitment of metacaspases in the unicellular coccolithophore, *Emiliania huxleyi*. *Proc Natl Acad Sci USA* 104:6049–6054.
34. Vardi A, *et al.* (1999) Programmed cell death of the dinoflagellate *Peridinium gatunense* is mediated by CO_2 limitation and oxidative stress. *Curr Biol* 9:1061–1064.
35. Nedelcu AM, Michod RE (2003) Sex as a response to oxidative stress: The effect of antioxidants on sexual induction in a facultatively sexual lineage. *Proc R Soc London Biol Sci* 270:136–139.
36. Ameisen JC (2002) On the origin, evolution, and nature of programmed cell death: A timeline of four billion years. *Cell Death Differ* 9:367–393.
37. Brussaard CPD (2004) Viral control of phytoplankton populations–A review. *J Eukaryot Microbiol* 51:125–138.
38. Van Valen L (1973) A new evolutionary law. *Evol Theory* 1:1–30.
39. Carroll L [Dodgson CL] (1872) *Through the Looking-Glass and What Alice Found There* (MacMillan and Co., London); reprinted (2000) (Signet Classics, New York).
40. Bell G (1982) *The Masterpiece Of Nature: The Evolution and Genetics of Sexuality* (University of California Press, Berkeley).
41. Jaenicke J (1978) An hypothesis to account for the maintenance of sex within populations. *Evol Theory* 3:191–194.
42. Howard RS, Lively CM (1994) Parasitism, mutation accumulation and the maintenance of sex. *Nature* 367:554–557.
43. Carroll L [Dodgson CL] (1865) *Alice's Adventures in Wonderland* (MacMillan and Co., London); reprinted (2000) (Signet Classics, New York).
44. Thierstein H, Geitzenauer K, Molfino B, Shakleton N (1977) Global synchroneity of late Quarternary coccolith datum levels: Validation by oxygen isotopes. *Geology* 5:400–404.
45. Boraas ME, Seale DB, Boxhorn JE (1998) Phagotrophy by a flagellate selects for colonial prey: A possible origin of multicellularity. *Evol Ecol* 12:153–164.
46. Suttle CA (2007) Marine viruses–Major players in the global ecosystem. *Nat Rev Microbiol* 5:801–812.
47. Kim S, *et al.* (2008) Genetic diversity of parasitic dinoflagellates in the genus *Amoebophrya* and its relationship to parasite biology and biogeography. *J Eukaryot Microbiol* 55:1–8.
48. Falkowski PG, *et al.* (2004) The evolution of modern eukaryotic phytoplankton. *Science* 305:354–360.
49. Marie D, Brussaard CPD, Thyrhaug R, Bratbak G, Vaulot D (1999) Enumeration of marine viruses in culture and natural samples by flow cytometry. *Appl Environ Microbiol* 65:45–52.
50. Shaked Y, de Vargas C (2006) Pelagic photosymbiosis: rDNA assessment of diversity and evolution of dinoflagellate symbionts and planktonic foraminiferal hosts. *Mar Ecol Prog Ser* 325:59–71.