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Sterols of the Syndinian Dinoflagellate *Amoebophrya* sp., a Parasite of the Dinoflagellate *Alexandrium tamarense* (Dinophyceae)

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ABSTRACT. Several harmful photosynthetic dinoflagellates have been examined over past decades for unique chemical biomarker sterols. Little emphasis has been placed on important heterotrophic genera, such as *Amoebophrya*, an obligate, intracellular parasite of other, often harmful, dinoflagellates with the ability to control host populations naturally. Therefore, the sterol composition of *Amoebophrya* was examined throughout the course of an infective cycle within its host dinoflagellate, *Alexandrium tamarense*, with the primary intent of identifying potential sterol biomarkers. *Amoebophrya* possessed two primary C_{27} sterols, cholesterol and cholesta-5,22Z-dien-3β-ol (*cis*-22-dehydrocholesterol), which are not unique to this genus, but were found in high relative percentages that are uncommon to other genera of dinoflagellates. Because the host also possesses cholesterol as one of its major sterols, carbon-stable isotope ratio characterization of cholesterol was performed in order to determine whether it was produced by *Amoebophrya* or derived intact from the host. Results indicated that cholesterol was not derived intact from the host. A comparison of the sterol profile of *Amoebophrya* to published sterol profiles of phylogenetic relatives revealed that its sterol profile most closely resembles that of the (proto)dinoflagellate *Oxyrrhis marina* rather than other extant genera.

Key Words. Algae, *Amoebophyra* sp., dinoflagellate, lipid, sterol biomarker.

PAST examinations of dinoflagellate sterols have focused on two principal areas: (1) the search for biomarkers that can be used to track dinoflagellates in the environment, and (2) the fundamental biochemistry underlying sterol biosynthesis. Of these two, the former has received the most attention by far, with the vast majority of studies centered on sterol biomarkers in free-living photosynthetic dinoflagellates. For example, numerous studies have focused on dinosterol, a 4-methyl sterol that is rarely found in other classes of algae, and is produced by many, but not all, dinoflagellates. Hence, dinosterol is often considered indicative of the class Dinophyceae as a whole, and has been used as a biomarker to track both recent and ancient dinoflagellate blooms (Volkman 1986, 2003; Volkman et al. 1998). Other sterols, like those produced by the closely related, harmful, non-dinosterol-producing species, *Karenia brevis*, *Karenia mikimotoi*, and *Karlodinium micrum*, may provide a level of specification beyond that of dinosterol (Giner, Faraldos, and Boyer 2003; Leblond and Chapman 2002). It is also assumed that free-living, photosynthetic species such as these synthesize their sterols de novo, although little is known about the biosynthetic steps and genes involved (Leblond and Chapman 2002).

Heterotrophic dinoflagellates, by contrast, have not received the same level of attention given to photosynthetic species in the search for potential sterol biomarkers. Despite the fact that free-living heterotrophic dinoflagellates play a very important role in the environment (Jeong 1999), the search for biomarkers has covered only a limited number of free-living species to date. Moreover, the examination of dinoflagellates grown under mixotrophic conditions [see Stoecker (1999) for a review on mixotrophy in dinoflagellates], has not been attempted. The two most extensively studied heterotrophic species, *Crypthecodinium cohnii* and *Pfiesteria piscicida*, have both been found to produce sterols, including dinosterol, through what appears to be de novo synthesis, as in photosynthetic dinoflagellates (Giner et al. 1991; Leblond and Chapman 2004; Withers et al. 1978, 1979). Therefore, the use of dinosterol as a ''general marker'' for dinoflagellates appears to extend to some heterotrophic members as well.

Unlike *C. cohnii* and *P. piscicida*, several heterotrophic dinoflagellates are non-free-living, obligately intracellular parasites of other organisms (Coats 1999; Park, Yih, and Coats 2004). For example, members of the genus *Amoebophrya* spend a large portion of their life cycle residing within other dinoflagellates that serve as hosts (Coats and Park 2002; Fritz and Nass 1992). Infections render the host incapable of dividing, and eventually lead to lysis of the host cell as the newly formed parasite emerges. The free-living, dispersive stage consists of tiny zoospores with familiar dinoflagellate features. High infection rates reported in some areas suggest that *Amoebophrya* spp. may control their host populations (Coats and Bockstahler 1994; Coats et al. 1996; Nishitani and Chew 1984; Taylor 1968). Despite a growing resurgence in interest with this organism, nothing is known about either the presence of lipid biomarkers or the fundamental lipid biochemistry of this parasitic genus.

The recent isolation and cultivation of *Amoebophrya* sp. in *Alexandrium tamarense* offers a tractable system to address questions regarding the sterol composition, lipid biochemistry, and metabolism of the parasite. For instance, because of the obligate dependence of *Amoebophrya* upon host cell resources, it is unclear whether it has the ability to synthesize sterols, or if it must incorporate host sterols. In particular, because development of the parasite occurs within another host dinoflagellate in much the same way as a lytic virus, it is of interest to determine whether dinosterol and other lipids produced by the host are incorporated by the parasite.

The purpose of this work was therefore threefold. Firstly, we sought to examine whether *Amoebophrya* possesses any useful sterol biomarkers. Secondly, we sought to examine sterol production throughout the infective cycle of *Amoebophrya* sp. as it parasitized *Alexandrium tamarense* to determine whether it utilizes intact host sterols. Thirdly, we sought to use the sterol profile of *Amoebophrya* to compare its sterol biochemistry to close relatives at the base of the dinoflagellate lineage.

MATERIALS AND METHODS

Cultures. *Alexandrium tamarense* (SPE10-1 from the laboratory of D. Anderson, Woods Hole Oceanographic Institution),

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isolated from Salt Pond (Eastham, MA), was grown in modified filtered f/2 medium and maintained under conditions described by Anderson et al. (1999). Cultures were kept at 15 °C to match the field conditions during which the parasite was isolated. Host growth was monitored using in vivo cellular fluorescence (Model 10-AU Fluorometer, Turner Designs, Sunnyvale, CA).

Amoebophrya sp. was maintained by adding a few milliliters of 4-day-old, late stage cultures to fresh, 20-ml cultures of uninfected *A. tamarense* SPE10-1 in 50-ml tissue culture flasks. The cultures were kept under the same conditions as the hosts. The status of the infection was monitored by observing the flask under an inverted microscope with flourescein isothiocyanate illumination.

Infection experiment. Parasite dinospores were harvested at 4 days by gravity filtering cultures through an 8-μm Nucleopore filter and collecting the cells into sterilized Erlenmeyer flasks. A small aliquot was preserved with glutaraldehyde (1.2% v/v) and counted under epifluoresence using a Fuchs-Rosenthal haemocytometer. The initial host concentration was determined by preserving aliquots in Utermöhl solution (Utermöhl 1958) and counting with light microscopy using a Sedgewick-Rafter chamber. For the infection experiment, *Amoebophrya* sp. dinospores were added to 200 ml of *A. tamarense* (SPE10-1 at 11,000 cells/ml) in 2-L tissue culture flasks to yield a 1:8 host to parasite ratio. The flasks were maintained under the same conditions as before, and were harvested at several points in the infection cycle: (1) mid-infection = 2 days, (2) late infection prior to emergence = 3 days, (3) emergence = 4 days, and (4) aged dinospores = 6 days. Uninfected hosts (no parasites) were collected at the end of the experiment as a control.

At each of the first two time points (Days 2 and 3), 2 flasks (400 ml) were combined. To determine the parasite prevalence and host abundance, small aliquots were preserved with Ca-buffered formalin (5% v/v final concentration) and counted under epifluorescence. Cell count data are shown in Table 1. The cultures were then pelleted using a bench-top centrifuge at 3,200 *g* for 5 min at 4 °C. The pellets were then combined and frozen in liquid nitrogen. This procedure was followed for the host-only control.

After emergence at Days 4 and 6, the dinospores were collected by gravity filtration through an 8-μm Nucleopore filter. The cells were pelleted using a Sorval RC5 Superspeed refrigerated centrifuge at 1,580 *g* for 15 min at 4 °C. The pellets were combined and frozen in liquid nitrogen.

Lipid extraction and fractionation. Extraction of lipids from two cell pellets per time point was performed according to a modified Bligh and Dyer extraction (Guckert et al. 1985). The total lipid extracts were separated into five component lipid fractions on columns of activated Unisil silica (1.0 g, 100–200 mesh, activated at 120 °C, Clarkson Chromatography, South Williamsport, PA). The following solvent regime was used to separate lipids according to polarity, with fraction 5 eluting the most polar, and, therefore, likely charged, lipids (Leblond and Chapman 2000): (1) 12 ml methylene chloride (sterol esters), (2) 15 ml 5% v/v acetone in methylene chloride with 0.05% v/v glacial acetic acid (free sterols, tri- and diacylglycerols, and free fatty acids), (3) 10 ml 20% v/v acetone in methylene chloride (monoacylglycerols), (4) 45 ml acetone [glycolipids, including monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and sulfoquinovosyldiacylglycerol (SQDG)], and (5) 15 ml methanol with 0.1% v/v glacial acetic acid (polar lipids, including phospholipids and non-phosphorus-containing lipids). Fractions 3–5 were not addressed in this study.

Table **1.** Cell counts (cells/ml) during the infection of the dinoflagellate *Alexandrium tamarense* by the dinoflagellate *Amoebophrya* sp.

Time point (Day #)	Sample	*A. tamarense* cells	*Amoebophrya* sp. dinospores
2	Mid-infection	1.3×10^4	
3	Late infection	1.4×10^4	7.5×10^4
4	*Amoebophrya* emergence	9.4×10^3	3.8×10^6
6	Aged *Amoebophrya*		9.0×10^6
6	Host-only control	2.4×10^4	

Derivatization and gas chromatography/mass spectrometry (GC/MS) characterization of sterols. Derivatization of sterols as their trimethylsilyl (TMS) ethers found in the sterol ester and free sterol fractions was performed according to the methodology utilized by Leblond and Chapman (2002). Mass spectrometry analysis was performed on a Finnigan Magnum GC/MS using the same conditions as described in Leblond and Chapman (2002) with the exception that the final hold temperature was 300 °C rather than 310 °C. The structure of sterol **1** (see Results and Discussion) was tentatively confirmed using the approach of Brassell and Eglinton (1981) by examining the TMS derivatives of hydrogenated free sterols from fraction 2 of day-4 *Amoebophrya*. Briefly, saponified free sterols were dissolved in 2 ml of ethyl acetate and exposed to 10% w/v palladium on activated carbon and H_2 gas for 5 h at room temperature.

Gas chromatography/isotope ratio mass spectrometry (GC/IRMS) of sterols. Carbon isotopic compositions of cholesterol and dinosterol were determined by GC-combustion isotope ratio mass spectrometry on a Finnigan Delta$^+$-XL mass spectrometer (ThermoFinnigan, Austin, TX) at the University of Florida Wetland Biogeochemistry Laboratory. This continuous-flow system consists of a HP6890 gas chromatograph connected to the mass spectrometer via a GC-C III interface. Compounds were chromatographically separated on the GC and then combusted at 940 °C in a ceramic oxidation reactor to form CO_2 for $\delta^{13}C$ measurements. Three pulses of pre-calibrated standard CO_2 gas (referenced to Pee Dee belemnite) were injected via the GC-C III for each sample. An internal standard, 5α-cholestan-3-one (Aldrich Chemical, St. Louis, MO), was included in each sample with a $\delta^{13}C$ value of -25.9 ± 1.9 per mil (‰, average of nine runs). The oven program was 50 °C increasing to 300 °C at 4.0°/min with helium at a constant flow of 2 ml/min; a HP-5 column was used in the GC.

Bulk isotope ratio measurements. Harvested day-4 and day-6 *Amoebophrya* cells were acidified to pH 2 with hydrochloric acid to remove carbonates, and were then lyophilized to a dried powder. Samples were run on the Finnigan Delta$^+$ XL mass spectrometer using a Costech Instruments (Valencia, CA) elemental analyzer inlet system. Samples were standardized to National Institute of Standards and Technology (NIST)-traceable sucrose and expressed in delta units relative to Pee Dee belemnite.

RESULTS

Overall, 13 sterols were found in this survey (Table 2). A comparison of sterols found as free sterols (fraction 2) vs. sterols found as sterol esters (fraction 1) shows that the distribution was approximately 50/50 for uninfected host (host alone), and the mid- and late infection stages (Table 3). As the infection progressed into the emergence and aged stages, this distribution shifted drastically, with the vast majority of sterols being found as free sterols (Table 3).

The similarity in the distribution of sterols in the uninfected host, the mid-, and late infection stages is reflected in the similarity of the free sterol composition of these three stages. The dominant sterols in these three stages were the C_{27} sterol,

Table **2.** Sterols found during the infection of the dinoflagellate *Alexandrium tamarense* by the dinoflagellate *Amoebophrya* sp.

Carbon #	Compound #	Suggested structure	Mol. weight[a]	Retention time (min)	RRT[b]
C_{27}	1	Cholesta-5,22Z-dien-3β-ol (*cis*-22-dehydrocholesterol)	456	32.12	0.88
	2	Cholest-5-en-3β-ol (cholesterol)	458	33.13	1.00
	3	5α-Cholestan-3β-ol (cholestanol)	460	33.35	1.03
C_{28}	4	Unknown C_{28} sterol with one double bond	472	35.13	1.24
	5	24-Methylcholesta-5,24(28)-dien-3β-ol (24-methylenecholesterol)	470	35.45	1.28
	6	24-Methyl-5α-cholestan-3β-ol	474	35.50	1.29
C_{29}	7	23,24-Dimethylcholesta-5,22E-dien-3β-ol	484	36.20	1.37
	8	Unknown C_{29} sterol with one double bond	486	36.22	1.37
	9	23,24-Dimethyl-5α-cholest-22E-en-3β-ol	486	36.55	1.41
	10	Unknown C_{29} sterol with two double bonds	484	37.38	1.51
	11	Unknown C_{29} sterol with one double bond	486	37.78	1.56
	12	4,24-Dimethyl-5α-cholestan-3β-ol	488	38.68	1.67
C_{30}	13	4α,23,24-Trimethyl-5α-cholest-22E-en-3β-ol (dinosterol)	500	39.70	1.79

[a]Molecular weight of sterols as their trimethylsilyl ether derivatives.
[b]Relative retention time with respect to cholesterol calculated according to Jones et al. (1994).

cholest-5-en-3β-ol (cholesterol, compound **2**), and the C_{30} sterol, 4α,23,24-trimethyL-5α-cholest-22E-en-3β-ol (dinosterol, compound **13**, Table 4). Minor amounts of several other common dinoflagellate sterols were also found.

Progression of infection into the emergence and aged stages coincided with a shift in sterol composition (Table 4). Although cholesterol remained a dominant sterol, dinosterol disappeared, and a C_{27} sterol (compound **1**) identified as cholesta-5,22Z-dien-3β-ol (*cis*-22-dehydrocholesterol) comprised approximately 34% in both stages. Production of minor amounts of compound **1** (approximately 2%) began in the late infection stage. The emergence and aged stages were also characterized by the disappearance of a number of sterols (the C_{27} compound **3**, C_{28} compound **6**, and C_{29} compounds **8** and **10–12**) found in earlier stages. These sterols were replaced by the C_{28} compound **5** and C_{29} compound **7** (each approximately 6%). The C_{29} compound **9** was present in small amounts in all stages of infection. In general, the above mentioned trends also held true for sterols found as sterol esters (Table 5).

In order to determine whether *Amoebophrya* has the ability to synthesize cholesterol rather than taking it from the host, carbon stable isotope ratio ($\delta^{13}C$) characterization was performed on it at each sampling point (Table 6). The $\delta^{13}C$ values for the uninfected host and mid-infection samples were similar at approximately $-31‰$. As the infection progressed, the $\delta^{13}C$ became less depleted, with the aged samples having an approximate value of approximately $-23‰$. This indicated that *Amoebophrya* may have synthesized its own cholesterol and did not obtain it from the host. Characterization of $\delta^{13}C$ values for cholesterol pushed the lower limit for sensitivity of the instrument; there was not enough material available to obtain reliable $\delta^{13}C$ values for any other sterols found in *Amoebophrya*. Bulk isotope ratio measurements for emergence and aged samples of *Amoebophrya* gave values of $-17.9‰$ and $-17.8‰$, respectively.

Table **3.** Inter-fraction distribution of sterols during the infection of the dinoflagellate *Alexandrium tamarense* by the dinoflagellate *Amoebophrya* sp.

Sample	% of Total Sterols	
	Free Sterol Total Area	Sterol Ester Total Area
Host Alone-1	44.1	55.9
Host Alone-2	56.1	43.9
Mid-infection-1	43.3	56.7
Mid-infection-2	55.3	44.7
Late infection-1	46.8	53.2
Late infection-2	52.9	47.1
Amoebophrya emergence-1	94.5	5.5
Amoebophrya emergence-2	80.4	19.6
Aged *Amoebophrya*-1	96.6	3.4
Aged *Amoebophrya*-2	95.0	5.0

DISCUSSION

In examining the sterol composition of *Amoebophrya*, three major questions arise. (1) Are there any sterols that can serve as biomarkers? (2) Are sterols synthesized or taken directly from the host? (3) Does the sterol composition of this genus reflect that of its phylogenetic relatives?

In order for a sterol to serve as a biomarker, it should be of limited distribution, and it should generally be a dominant sterol in the organism (i.e. sterols at a trace level rarely serve as useful biomarkers because they are difficult to detect). Cholesterol, one of the two dominant sterols in *Amoebophrya*, is by no means unique to this organism and is not a biomarker. Tentative identification of compound **1** was performed according to the hydrogenation approach of Brassell and Eglinton (1981); there was not enough biomass to perform more definitive NMR characterization. In their work, trimethylsilyl ether (TMS) derivatives of 5α-cholestan-3β-ol and 27-*nor*-5α-cholestan-3β-ol, the hydrogenation products of cholesta-5,22Z-dien-3β-ol and 27-*nor*-24-methylcholest-5,22E-dien-3β-ol (occelasterol), respectively, were separable on a capillary column (whereas the TMS derivatives of the unhydrogenated sterols themselves are not). GC/MS analysis of TMS derivatives of the hydrogenation products of free sterols from day-4 cells revealed the presence of one sterol that was inseparable from the TMS derivative of authentic 5α-cholestan-3β-ol in a coinjection. We therefore concluded that sterol **1** was cholesta-5,22Z-dien-3β-ol. As Klein Breteler et al. (1999) did not distinguish between sterol **1** and 27-*nor*-24-methylcholest-5,22E-dien-3β-ol in *Oxyrrhis marina*, it is not possible at this point to determine whether sterol **1** is a possible *Amoebophrya* biomarker, or if it is shared with *O. marina* (see below). Other dinoflagellates have been observed to produce 27-*nor*-24-methylcholest-5,22E-dien-3β-ol (Thomson et al. 2004 and references therein). The remaining sterols observed in the emergence and aged stages are minor, and several of these are also found in other dinoflagellates.

Although the majority of the sterols produced by *Amoebophrya* in the emergence and aged stages are not found in the host, the most abundant sterol, cholesterol, is found throughout all infective stages and in uninfected host. It was therefore necessary to determine whether it was produced by *Amoebophrya* or taken directly from the host. The shift in the $\delta^{13}C$ value for cholesterol

Table **4.** Relative percentages of sterols found as free sterols during the infection of the dinoflagellate *Alexandrium tamarense* by the dinoflagellate *Amoebophrya* sp.

Carbon #	Compound #	Host		Mid-		Late		Amoeb.			
		Alone-1	Alone-2	Inf.-1	Inf.-2	Inf.-1	Inf.-2	Emer.-1	Emer.-2	Aged-1	Aged-2
C_{27}	1					1.5	2.0	34.5	35.3	33.5	32.0
	2	57.3	55.2	19.5	56.7	58.0	58.1	48.2	46.8	50.6	51.3
	3	Tr	1.2	Tr	1.1	1.1	1.2				
C_{28}	4										
	5							5.8	6.5	6.4	5.7
	6	Tr	Tr	Tr	Tr	1.4	1.6				
C_{29}	7							6.4	6.6	5.7	6.4
	8	Tr	Tr	Tr	Tr	Tr	Tr				
	9	1.1	1.1	3.8	1.6	1.4	1.5	3.6	3.5	3.8	4.6
	10	1.1	1.2	2.0	1.0	1.2	1.3				
	11	2.0	2.4	4.5	2.3	2.8	2.4				
	12	Tr	Tr	Tr	Tr	Tr	Tr				
C_{30}	13	34.0	30.1	70.2	34.5	29.4	29.7	1.6	Tr	Tr	

Tr, trace amount less than 1%; *Amoeb*., *Amoebophrya*; Emer., emergence; Inf., infection.

throughout the infective cycle indicates that it was not taken from the host, and that *Amoebophrya* may have the ability to synthesize it. As the *Amoebophrya* infection of *A. tamarense* progressed, the $\delta^{13}C$ value for cholesterol became less depleted and approached that of dinosterol. The $\delta^{13}C$ values for cholesterol in the emergence and aged stages were offset $-6.9‰$ and $-5.0‰$ compared with bulk cell material. These values are similar to the offset of $-4.2‰$ observed for the sterols of *Gymnodinium simplex* by Schouten et al. (1998), and indicate that sterol synthesis in *Amoebophrya* proceeds similarly to at least one other dinoflagellate with respect to carbon isotope fractionation.

Although one cannot rule out dinosterol as some sort of direct precursor to cholesterol, there are currently no known enzymatic steps in dinoflagellates that would remove the 4-methyl group of dinosterol, modify its side chain, and introduce a Δ^5 unsaturation to convert it to cholesterol. In addition, the $\delta^{13}C$ data for cholesterol suggest that other *Amoebophrya* sterols are likely synthesized by *Amoebophrya* itself as well. This isolate of *Amoebophrya* sp. from *A. tamarense* has the ability to infect other species of the genus *Alexandrium*, as well as other dinoflagellate genera (e.g. *Scrippsiella trochoidea*, *Heterocapsa triquetra*, *Prorocentrum micans*; MRS., unpubl. data). These alternative hosts may have different sterol profiles from *A. tamarense*. Therefore, a future study may be to examine how the parasite's sterol profiles change in these hosts compared with changes when infecting *A. tamarense*.

To consider whether the sterol composition of this genus reflects that of its phylogenetic relatives, one must examine the relationship of *Amoebophrya* to other dinoflagellates. The genus *Amoebophrya* is found within the dinoflagellate order Syndiniales along with the genus *Hematodinium*, a parasite of oysters (Saldarriaga et al. 2004). Based on rDNA-based phylogeny (Gunderson, Goss, and Coats 1999; Janson et al. 2000; Saldarriaga et al. 2003, 2004) and morphological differences with dinokaryotic dinoflagellates [summarized by Saldarriaga et al. (2004)], these two genera are considered early branches in the dinoflagellate lineage along with the (proto)dinoflagellate, *O. marina*, and the non-dinoflagellate genera *Perkinsus* and *Parvilucifera*, two members of the Perkinsida. In an evolutionary model put forth by Saldarriaga et al. (2004), these genera were the ancestors of all other dinoflagellates, with members of the Gymnodiniales being the next major group to arise and members of the Gonyaulacales being one of the last groups to arise.

At present, no published studies exist for the sterol composition of *Hematodinium* and *Perkinsus*, but Klein Breteler et al. (1999) have observed that *O. marina* produces either sterol **1** or 27-*nor*-24-methylcholest-5,22E-dien-3β-ol (occelasterol) and cholesterol as dominant sterols when it is fed the green alga, *Dunaliella*. The

Table **5.** Relative percentages of sterols found as sterol esters during the infection of the dinoflagellate *Alexandrium tamarense* by the dinoflagellate *Amoebophrya* sp.

Carbon #	Compound #	Host		Mid-		Late		Amoeb.			
		Alone-1	Alone-2	Inf.-1	Inf.-2	Inf.-1	Inf.-2	Emer.-1	Emer.-2	Aged-1	Aged-2
C_{27}	1					3.2	3.2	22.7	21.8	33.9	31.6
	2	74.5	74.0	72.7	76.5	64.6	69.3	42.8	44.1	66.1	68.4
	3	1.7	2.0	2.2	2.0	3.3	3.8				
C_{28}	4	1.2	1.1	Tr	Tr	1.5	1.4				
	5						5.8	13.8	10.6		
	6	2.2	1.9	1.9	1.6	6.1					
C_{29}	7					Tr	Tr	8.1	6.2		
	8	Tr	Tr	Tr	Tr						
	9	1.3	1.3	1.3	1.2	1.5	1.6	12.6	11.1		
	10										
	11	1.1	1.2	1.3	1.2	1.1	1.1		6.2		
	12	1.5	1.3	1.6	1.2	2.9	3.0				
C_{30}	13	15.5	14.9	17.7	14.7	15.9	10.3				

Tr, trace amount less than 1%; *Amoeb*., *Amoebophrya*; Emer., emergence; Inf., infection.

Table **6.** Carbon isotope ratios ($\delta^{13}C$ in units of ‰) of cholesterol and dinosterol found as free sterols during the infection of the dinoflagellate *Alexandrium tamarense* by the dinoflagellate *Amoebophrya* sp.

Compound #	Host		Mid-		Late		Amoeb.			
	Alone-1	Alone-2	Inf.-1	Inf.-2	Inf.-1	Inf.-2	Emer.-1	Emer.-2	Aged-1	Aged-2
2	− 31.2	− 31.0	− 30.1	− 32.2	− 25.2	− 25.9	− 25.1	− 24.5	− 23.9	− 21.6
13	− 20.8	− 19.8	− 20.9	− 22.6	− 21.1	− 23.8				

Amoeb., Amoebophrya; Emer., emergence; Inf., infection.

sterol composition of *Amoebophrya* thus may closely resemble that of its closest studied neighbor, *O. marina*. Neither *Amoebophrya* nor *Oxyrrhis* produces dinosterol, the sterol most commonly associated with dinoflagellates, or any other 4-methyl sterols often found in photosynthetic and other heterotrophic dinoflagellates. Future studies are needed to address whether *Hematodinium*, *Perkinsus*, and *Parvilucifera* are also similar in sterol composition, and furthermore, how the sterol composition of these presumed ancestors relates to the evolution of sterol biosynthesis in more recently evolved dinoflagellates.

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