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Species-specific differences in long-chain n-3 essential fatty acid, sterol, and steroidal ketone production in six heterotrophic protist species

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ABSTRACT: We investigated the capability and species-specific differences in long-chain n-3 essential fatty acid (LCn-3EFA), sterol, and steroidal ketone production of 6 heterotrophic protists: 3 thecate dinoflagellates (*Cryptoperidiniopsis brodyi*, *Pfiesteria piscicida*, and *Luciella masanensis*), 1 athecate dinoflagellate (*Amphidinium longum*), 1 herbivorous ciliate (*Strombidinopsis* sp.), and 1 bacterivorous ciliate (*Uronema* sp.) by feeding them algae (*Rhodomonas salina* or *Dunaliella tertiolecta*) or bacteria. The 3 thecate species did not convert algal sterols to other usual and common sterols. Instead, they produced sterols and steroidal ketones, such as dinosterol, dinostanol, dinosterone, and dinostanone, usually found in autotrophic dinoflagellates when fed *R. salina* or *D. tertiolecta*, both of which do not contain them. The *A. longum, Strombidinopsis* sp., and *Uronema* sp. did not bioconvert dietary sterols to other sterols or produce sterols and steroidal ketones. *Pfiesteria piscicida* and *L. masanensis* grown on the LCn-3EFA-deficient alga *D. tertiolecta* and *Uronema* sp. were capable of producing the long-chain n-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are essential for organisms at higher trophic levels. The bacterial prey of *Uronema* sp. lacked EPA, DHA, and LCn-3EFA precursors. Although the nutritional values of the sterols and steroidal ketones produced by the 3 thecate dinoflagellates are not known, the contribution of EPA and DHA by 2 of them and the bacterivorous ciliate are noteworthy. To further understand the intermediate roles of heterotrophic protists and their essential nutrient contribution in planktonic food webs, it is necessary to examine more species, particularly those newly discovered and isolated.

KEY WORDS: Heterotrophic dinoflagellates and ciliates · Sterols and steroidal ketones · Algae · Long-chain n-3 fatty acids · Eicosapentaenoic acid · Docosahexaenoic acid

INTRODUCTION

Both marine and freshwater pelagic systems are characterized by a wide variety of autotrophic and heterotrophic protists. They are the primary energy and nutrient sources for their immediate omnivorous grazers, the freshwater dominant metazoan, *Daphnia*, and the marine dominant metazoan, copepods. Microzooplankton, dominated by heterotrophic ciliates and dinoflagellates, feed on diverse prey, including phytoplankton of various sizes (pico-, nano- and micro-algae), bacteria, detrital particles, and eggs of mesozooplankton, such as copepods, cladocera and rotifers (e.g. Jeong et al. 1999, Sherr & Sherr 2002, Klein Breteler et al. 2004), and they themselves are prey for metazoans such as copepods and daphnids. There is growing evidence that they are major consumers of primary (phytoplankton) production in the open oceans, coastal areas, and oligotrophic lakes (Weisse 1993, Callieri & Stockner 2002, Tillmann 2004, Putland & Iverson 2007). As intermediates, they not only assimilate and repackage the biomass and nutrients of their algal prey, but they also upgrade their biochemical constituents, including essential lipids, the long-chain n-3 polyunsaturated fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and sterols, for subsequent use at higher trophic levels

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(Klein Breteler et al. 1999, Bec et al. 2003, 2006, Broglio et al. 2003, Tang & Taal 2005, Veloza et al. 2006, Chu et al. 2008a). EPA and DHA are essential for many animals, since they either cannot synthesize them de novo or cannot synthesize them in sufficient amounts to meet their requirements (Ackman et al. 1980, Sargent et al. 1987, 1989). The importance of EPA and DHA as membrane components and in maintaining proper physiological functions for marine organisms at higher levels of the trophic hierarchy are well recognized (Bell et al. 1995, 1999, Navarro et al. 1997). DHA is particularly important for visual and neural development (Bell et al. 1999). Similarly, sterols are not only important membrane components, but are also involved in a wide range of physiological functions and are critical for growth and reproduction (Finkelstein & Cass 1967, Bruckdorfer et al. 1969, Demel et al. 1972, Ederington et al. 1995, Crockett 1998). Thus, 'trophic upgrading' by heterotrophic protists via production of essential lipids at the phytoplankton–heterotrophic protist interface improved the nutritional quality of primary producers for their metazoan grazers such as copepods and *Daphnia* and, subsequently, significantly enhanced their predator's survivorship, growth, and fecundity (Klein Breteler et al. 1999, Tang & Taal 2005, Martin-Creuzburg et al. 2005, 2006, 2008, Bec et al. 2006, Veloza et al. 2006).

'Trophic upgrading' is believed to be one of the key factors in determining pelagic community production and structures. Also, the contribution of essential lipids by heterotrophic protists as trophic intermediaries may affect the nutritional status of the pelagic system as a whole. However, 'trophic upgrading' studies have been focused on only a few species, particularly the 2 estuarine/marine dinoflagellate species *Oxyrrhis marina* and *Gyrodinium dominans* (Klein Breteler et al. 1999, Tang & Taal 2005, Veloza et al. 2006, Chu et al. 2008a,b). The efficiency and significance of essential lipid production and the associated biochemical processes used by heterotrophic protists via 'trophic upgrading' have not been quantified until recently (Chu et al. 2008a,b, Lund et al. 2008). The contribution of essential lipids by heterotrophic protists via 'trophic upgrading' to pelagic food webs may be quantitatively significant (Chu et al. 2008a, Lund et al. 2008). When fed the EPA- and DHA-deficient alga *Dunaliella tertiolecta*, *O. marina* and *G. dominans* both produced significant quantities of EPA and DHA (Chu et al. 2008a). Both protists contained much higher levels of DHA compared to the good food quality alga *Rhodomonas salina* (Chu et al. 2008a). Also, as intermediates, these heterotrophic protists bioconverted dietary sterols derived from their algal diets to produce new sterol species. They increased the amount of cholesterol, a critical precursor for many physiologically functional biochemicals in higher animals, at the phytoplankton–zooplankton interface available to higher trophic levels relative to zooplankton feeding on algae directly (Chu et al. 2008b).

Moreover, the outcome of trophic interactions between heterotrophic protists and phytoplankton appears to vary with the predator–prey makeup. Results of our recent studies on 'essential lipid upgrading' and the associated biochemical processes in *Oxyrrhis marina* and *Gyrodinium dominans* suggest species-specific differences in 'essential lipid upgrading' and that the final products could vary with the biochemical composition of the algal prey (Chu et al. 2008a,b, Lund et al. 2008). Also, some heterotrophic protists can produce EPA and DHA from carbon sources other than algae (Lund et al. 2008). Different heterotrophic protist species may have different metabolic modes and utilize different pathways in modifying dietary lipids and/or in synthesizing essential lipids de novo. Furthermore, 'trophic upgrading' may not be a universal phenomenon among heterotrophic protists, perhaps limited to only certain species. For instance, no evidence was noted for EPA, DHA, and sterol synthesis in a ciliate (*Strombidium sulcatum*) studied by Klein Breteler et al. (2004). Studies by Martin-Creuzburg et al. (2005, 2006) also showed that the 2 freshwater ciliates *Colpidium campylum* and *Cyclidium* sp. did not synthesize sterols, but produced tetrahymanol and hopanoids instead when they were fed sterol-free cyanobacteria.

Therefore, there is a need to examine a greater variety of heterotrophic protists for their 'essential lipid upgrading' capabilities to clearly define their ecological importance in regulating the carbon transfer efficiency and essential lipid production at the phytoplankton–zooplankton interface. In this context, we experimentally investigated the capability and species-specific differences in long-chain n-3 essential fatty acid (LCn-3EFA), sterol, and steroidal ketone production, as well as the bioconversion of dietary sterols for 6 different heterotrophic protists, which included 3 thecate heterotrophic dinoflagellates (*Cryptoperidiniopsis brodyi*, *Pfiesteria piscicida*, and *Luciella masanensis*) isolated in the early and mid-1990s from estuaries on the mid-Atlantic coast of the USA (Steidinger et al. 1996, 2006, Mason et al. 2007), 1 athecate heterotrophic dinoflagellate (*Amphidinium longum*), 1 herbivorous ciliate (*Strombidinopsis* sp.), and 1 bacterivorous ciliate (*Uronema* sp.).

MATERIALS AND METHODS

Algal and protist cultures. The chlorophyte *Dunaliella tertiolecta* (CCMP 1320) and the cryptophyte *Rhodomonas salina* (CCMP 1319) were obtained from the Provasoli-Guillard National Center for Culture of Marine

Phytoplankton (CCMP) and were cultured in *f*/2 medium prepared from artificial sea water (ASW) of salinity 20. *R. salina* and *D. tertiolecta*, which differ in both fatty acid and sterol profiles (Goad et al. 1983, Patterson et al. 1992, Zelazny et al. 1995, Klein Breteler et al. 1999, Veloza et al. 2006, Chu et al. 2008a,b), were used as prey in the feeding experiments of the heterotrophic protists. *D. tertiolecta* lacks EPA and DHA, but contains high levels of potential precursors to EPA and DHA — e.g. α-linolenic acid, 18:3(n-3), and 18:2(n-6). The alga *R. salina* contains both EPA and DHA (Jonasdottir 1994, Lacoste et al. 2001, Veloza et al. 2006, Chu et al. 2008a). The major sterols in *D. tertiolecta* are ergosterol (Δ5,7,22-C28:3; 24-β-methylcholesta-5-7-22-trien-3β-ol; 45 to 49%), 7-dehydroporiferasterol (Δ5,7,22-C29:3; 24-α-ethylcholest-5, 7, 22 trien-3β-ol; 29 to 31%), and fungisterol (Δ7-C28:1; 24β-methylcholesta-7-en-3β-ol; 21 to 26%), and *R. salina* contains mainly brassicasterol (Δ5,22-C28:2; 24-α-methylcholesta-5,22-dien-3β-ol; ≅99%) and <2% cholesterol (Δ5-C27:1; cholest-5-en-3β-ol) (Klein Breteler et al. 2004, Chu et al. 2008b). *R. salina* and *D. tertiolecta* cultures used in feeding experiments were grown in 1 l round bottom flasks with aeration, in a walk-in environmentally controlled room at 19°C with a 12 h light:12 h dark cycle. The medium was refreshed in all algal culture flasks every 3 to 5 d. Exponential/log phase cultures of *R. salina* and *D. tertiolecta* were used for all of the feeding experiments.

The thecate heterotrophic dinoflagellate *Cryptoperidiniopsis brodyi* (CCMP 2782) was obtained from the CCMP, and the thecate heterotrophic dinoflagellate *Pfiesteria piscicida* (CCMP 2091) was obtained from Dr. Wayne Litaker at the NOAA Laboratory, Beaufort, North Carolina. The thecate heterotrophic dinoflagellate *Luciella masanensis* (NC Lucy, P27; Mason et al. 2007), an isolate from the New River Estuary, North Carolina, USA, was obtained from Dr. Patricia Tester at the NOAA Laboratory in Beaufort, North Carolina. Both *P. piscicida* and *L. masanensis* were re-isolated at the Virginia Institute of Marine Science (VIMS), and the re-isolated heterotrophic protists were used for our study. The athecate heterotrophic dinoflagellate *Amphidinium longum* was obtained from Dr. Suzanne Strom's laboratory at the Shannon Point Marine Center, Western Washington University. Dr. Strom's group identified this dinoflagellate to the species level and described it as a heterotroph (www.ac.wwu.edu/~stromlab/cultures.htm; Strom et al. 2003). *Strombidinopsis* sp. was obtained from Dr. George McManus's laboratory at the University of Connecticut at Avery Point. The bacterivorous ciliate *Uronema* sp. was acquired from Dr. David Caron's laboratory at the University of Southern California.

Feeding experiments. To determine the capability and species-specific differences in LCn-3EFA, sterol, and steroidal ketone production, as well as the bioconversion of dietary sterols of the heterotrophic protists, *Pfiesteria piscicida* and *Luciella masanensis* were fed either *Rhodomonas salina* and/or *Dunaliella tertiolecta*. Since *Cryptoperidiniopsis brodyi*, *Strombidinopsis* sp., and *Amphidinium longum* did not grow on the alga *D. tertiolecta* (our observation through several feeding trials for each of the above-listed heterotrophic protist species), they were fed only *R. salina* and then harvested for sterol analysis to determine whether bioconversion of dietary sterols occurs. Whenever possible, true replicates of similar culture age, heterotrophic protist density, and algal prey density were used for fatty acid and sterol analyses. However, due to difficulty in cultivation, and, very often, a lack of synchronicity among cultures (i.e. replicate culture flasks differed in protist and prey densities or in the time it took for cultures to reach levels of high protist density with low prey density), when it was not possible to generate 3 heterotrophic protist replicates of comparable age, cell density, and remaining algal prey content, harvested cultures were pooled prior to sample collection and then divided into 2 (*P. piscicida* fed *D. tertiolecta* for fatty acid profile analysis) or 3 replicates (*A. longum* fed *R. salina* for sterol analysis, *L. masanensis* fed *D. tertiolecta* for fatty acid analysis, and *Uronema* sp. for qualitative sterol analysis) containing similar numbers of both heterotrophic protists and unconsumed prey. Previous studies have shown that sterol and fatty acid contents in heterotrophic protist cultures can vary greatly with age and among culture flasks, but much less so in qualitative fatty acid and sterol composition (Chu et al. 2008a,b). Prior to collection, cell densities of heterotrophic protists and unconsumed algal prey in the cultures (pooled or non-pooled cultures) were determined at the time of harvesting by direct cell count of fixed cells with a 1% Lugol's solution in a 1.0 ml Sedgewick Rafter chamber using an inverted microscope (Nikon, phase contrast, ELWD 0.3), unless stated otherwise. Cells were then collected on acetone-rinsed GF/F filters and stored in glass screw-cap test tubes at −80 or −20°C no more than 4 wk prior to total lipid extraction and sterol and fatty acid analysis.

Expt 1 examined the ability of the 3 thecate heterotrophic dinoflagellate species *Cryptoperidiniopsis brodyi*, *Pfiesteria piscicida*, and *Luciella masanensis* to produce sterols and LCn-3EFA. *P. piscicida*, *L. masanensis*, and *C. brodyi* were cultured in L1 medium prepared from natural sea water (NSW) of salinity 33 diluted to a salinity of 12 with sterile MilliQ water prior to the addition of L1 medium components (https://ccmp.bigelow.org/node/82). All cultures were maintained on either *Rhodomonas salina* or *Dunaliella tertiolecta* for >2 mo. *P. piscicida* was fed either *R.*

salina at a prey:predator ratio of 10:1 or *D. tertiolecta* at a prey:predator ratio of 5:1 every 3 to 5 d. *L. masanensis* was fed either *R. salina* at a prey:predator ratio of 10:1 or *D. tertiolecta* at a prey:predator ratio of 5:1 every 7 to 10 d. *C. brodyi* was fed only *R. salina* at a prey:predator ratio of 10:1 every 3 to 5 d. All of the culture flasks were kept in the dark to minimize algal growth and maintained at room temperature (20 to 21°C). To offset the increase in salinity due to the feeding of algae cultured in medium of higher salinity, an appropriate amount of sterile MilliQ water was added to the heterotrophic protist–algal mixture after each feeding to maintain a salinity of 12. Prior to harvest, heterotrophic protist cultures were not fed for 3 to 12 d, depending on the grazing rate of the heterotrophic protist species, to ensure that cultures were prey depleted or near prey depleted. Cell densities of the heterotrophic protists and remaining algal prey were determined at the time of harvesting as described earlier.

Expt 2 examined the ability of the heterotrophic dinoflagellate *Amphidinium longum* to produce sterols. The athecate dinoflagellate *A. longum* was cultured in sterile filtered sea water of salinity 30 with dilute trace metal additions (Gifford 1985, Strom et al. 2003), and maintained on *Rhodomonas salina* for 3 mo at 17°C without light. The heterotrophic protists were fed *R. salina* at a prey:predator ratio of 20:1 every 7 d, and the medium was refreshed once a week. Prior to harvest, the heterotrophic protists were not fed for 1 wk, to reduce prey levels in the culture. At the time of harvest, cultures were pooled from different flasks, split into 3 replicates with equal volume and similar cell density, and then collected as described earlier.

Expt 3 examined the ability of the herbivorous ciliate *Strombidinopsis* sp. and the bacterivorous ciliate *Uronema* sp. to produce LCn-3EFA and sterols. *Strombidinopsis* sp. was cultured in Petri dishes (150 × 25 mm) in *f*/20 medium of salinity 33 made from 50× concentrate of Guillards (*f*/2) marine water enrichment solution without silicate (Gifford 1985) and fed *Rhodomonas salina* at a predator:prey ratio of 1:400 for >2 mo. Cultures were kept in an incubator set to 19°C without light. *Strombidinopsis* sp. was not fed for 3 d prior to harvest for sterol analysis. At the time of harvesting, cell densities of *Strombidinopsis* sp. were determined by directly counting live cells of 3 aliquots of 5 ml each taken from each replicate culture Petri dish, using a stereo microscope (Olympus SZ×12, DF PLAPO 1×PF, Japan). Then prey-depleted *Strombidinopsis* sp. cultures (n = 3) were collected on acetone-rinsed GF/F filters and stored at −80°C for later sterol analysis.

The scuticociliate *Uronema* sp. was cultivated in 0.001% bacto-yeast solution prepared with 0.22 µm filtered sea water of salinity 33. The bacto-yeast solution contained a wheat grain as a food source to sustain the natural assemblage of bacteria. Cultures were maintained at room temperature (≅20°C) and ambient light levels. *Uronema* sp. cultures were maintained in 70, 250 and 1000 ml flasks and refreshed with new medium every 3 d.

For qualitative sterol analysis, *Uronema* sp. from several culture flasks was pooled. To separate *Uronema* sp. from bacteria, the culture was first sieved through a 35 µm filter to remove larger pieces of debris, and then the filtrate containing ciliates was concentrated by centrifugation at 300 × *g* at 10°C for 30 min. The ciliate pellets were washed twice with sterile seawater (33 ppt) to further reduce the bacterial biomass (Lores et al. 1998). The washed ciliate pellets were then resuspended in sea water, cell density was determined, and the suspension was divided into 3 equal volumes. Ciliates were then collected as described previously. For fatty acid analysis (qualitative and quantitative), 3 replicates of ciliate samples (2.5×10^6 ciliates replicate^{-1}) were collected.

Since the ciliate pellets were not completely free of bacteria, to distinguish the sterol and fatty acid profiles of *Uronema* sp. from its bacterial prey, we qualitatively characterized the fatty acid and sterol composition of bacteria contained in the ciliate pellets. Cultures containing bacteria exclusively were obtained by serial dilutions of fresh *Uronema* sp. subcultures, and the diluted cultures were incubated at room temperature (≅20°C) next to a window for 11 d in the same medium used for cultivation of *Uronema* sp. At the time of harvest, bacterial cultures were pooled and split into 6 equal volumes, each of which was concentrated into 1 ml by centrifugation at 2000 × *g* at 10°C for 30 min for total lipid extraction (n = 6 replicates, 3 replicates for sterol analysis and 3 replicates for fatty acid analysis).

Total lipid extraction and sterol and fatty acid analyses. Lipids were extracted from the GF/F filters containing harvested cells (algae, hetrotrophic dinoflagellates and ciliates, or bacteria) by the method of Bligh & Dyer (1959). Total lipid samples were analyzed for sterol content and composition according to a modification of the method described by Soudant et al. (1996, 1998). Briefly, total lipid samples were transferred to 10 ml glass vials with 0.5, 1.0, or 1.5 µg 5-α-cholestane added as an internal standard and evaporated to dryness in a warm water bath with nitrogen. Then esterified sterols contained in the total lipids were hydrolyzed to free sterols by adding 2.0 ml sodium methoxide (0.5 M CH_3ONa in MeOH) to the vial and placed in a shaker for 1.5 h at room temperature after vortexing. After hydrolysis, sterols were recovered and extracted with 1 ml hexane. The hexane fraction containing the free sterols was washed with

2 ml of water to remove any traces of MeONa prior to being analyzed by gas chromatography-flame ionization detection (GC-FID) and gas chromatography-mass spectroscopy (GC-MS). A Varian Saturn 2000 GC-MS-MS ion trap (Varian Analytical Instruments) was used for sterol qualitative analysis. The GC-MS was equipped with a Varian 3800 CP gas chromatograph (GC). A J&W DB-5 (60 m × 0.32 mm; 0.25 µm film thickness) fused silica column was installed in the GC, and helium was used as the carrier gas at a constant flow rate of 1.6 ml min^{-1}. The injector temperature was 320°C. All analysis used the following column temperature program: initial temperature of 75°C holding for 1 min then programming at 15°C min^{-1} to a final temperature of 350°C holding for 9.67 min. The ion trap was held at 220°C, the manifold at 80°C, and the transfer line was 320°C. The GC-MS-MS was operated in the electron ionization (EI) mode over a mass range of 50 to 650 m/z at 0.77 s $scan^{-1}$. The identification of sterols and assigned structures was based on comparison of the mass spectra derived from the analyzed free sterols and their trimethylsilyl (TMS) derivatives, retention times to those of commercial authentic standards (e.g. cholesterol and brassicasterol), available spectra in NIST05 and Wiley07 mass spectral libraries, and/or comparison of their spectra with published GC-MS spectra (Mansour et al. 1999, Leblond & Chapman 2002, Leblond & Chapman 2004). Sterol standards used for identification include cholesterol, dihydrocholesterol (C27:0; cholesta-3β-ol), brassicasterol, desmosterol (Δ5,24-C27:2; cholesta-5,24-dien-3β-ol), campesterol (Δ5-C28:1; 24-α-methylcholest-5-en-ol), ergosterol, stigmasterol [Δ5,22-C29:2; 24-β-ethylcholest-5,24(28)-dien-3β-ol], β-sitosterol (Δ5-C29:1; 24-β-ethylcholesta-5-en-3β-ol), and fucosterol [Δ5,24(28)-C29:2; 24-β-ethylcholest-5,24(28)-dien-3β-ol]. To prepare TMS derivatives, samples containing free sterols derived from heterotrophic protists were dried completely under a stream of nitrogen; then, 40 µl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) (99%, Sigma-Aldrich Co.) was added to each sample vial. Vials containing sample and BSTFA were capped and heated at 80°C overnight. After cooling to ambient temperature TMS derivative samples were dried under nitrogen to completely remove the remaining BSTFA, then re-suspended in hexane and analyzed by GC-MS by the same methods used for underivitized free sterol samples. The elution order and major ions of TMS derivatives were compared to those reported by Leblond & Chapman (2004) for *Pfiesteria piscicida*. Steroidal ketones in TMS-derivitized samples were identified by their lack of ions associated with a TMS residue, since ketones are not derivitized by BSTFA.

A Varian 3800 GC-FID (Varian Analytical Instruments) equipped with a J&W DB-5 (60 m × 0.32 mm; 0.25 µm film thickness) fused silica column was employed for sterol quantitative analysis using 5-α-cholestane as an internal standard. Injection port and detector temperatures were both 320°C. Helium was used as a carrier gas at a flow rate of 2 ml min^{-1}, and the flow rates of air and hydrogen were 300 and 30 ml min^{-1}, respectively. The column temperature was programmed from an initial temperature of 75°C with a 1 min hold to 350°C at 15°C min^{-1} followed by a hold at 350°C for 9.67 min. The quantity of each identified sterol was calculated based on the relative response of cholesterol to the internal standard 5-α-cholestane.

To analyze fatty acid content and composition, total lipid extracts derived from algae, heterotrophic dinoflagellates and ciliates, or bacteria were spiked with 20 µg of the 23:0 internal standard and derivitized to fatty acid methyl esters using methanolic BF_3 (Metcalfe & Schmitz 1961). The fatty acid methyl esters (FAMEs) were extracted with carbon disulfide (Marty et al. 1992), which was evaporated under a stream of nitrogen. Samples were then redissolved in hexane for GC/FID and GC/MS analysis. FAME analysis was conducted on a Varian Model 3300 GC (Varian Analytical Instruments) equipped with a FID, using a DB-WAX capillary column (25 m × 0.32 mm; 0.2 µm film thickness; J&W Scientific). The column was temperature programmed from 60 to 150°C at 30°C min^{-1} and from 150 to 220°C at 2°C min^{-1}; injector and detector temperatures were 230 and 250°C, respectively; the flow rates of compressed air and hydrogen were 300 and 30 ml min^{-1}, respectively. Helium was used as the carrier gas (1.5 ml min^{-1}). Identification of FAMEs was based on the comparison of their retention times with those of authentic standards and was confirmed by GC/MS. The quantity of each FAME was calculated based on the internal standard.

Carbon content measurements of heterotrophic protists and algae. Algae and heterotrophic protist carbon contents were determined previously (Chu et al. 2008a) using a procedure modified from Smith et al. (2000). Briefly, algae and heterotrophic protists were filtered through precombusted (450°C for 2 h) Whatman GF/F filters under low vacuum. The filters, inside precombusted glass tubes, were placed in a desiccator containing a Petri dish filled with concentrated HCl for 16 h to remove inorganic carbonates; afterward, they were capped with combusted aluminum foil and dried at 60°C. Samples were analyzed using a Carlo-Erba Model EA 1108 elemental analyzer for flash combustion. Filter blanks were precombusted GF/F filters that were analyzed with the same procedures as the samples, and these values were subtracted from the final carbon values; subsequently, algal and protist carbon contents were calculated using carbon–cell conversion factors (pg C $cell^{-1}$).

RESULTS

Data were quantified and presented as: (1) nanograms of sterol or steroidal ketone per milligram of heterotrophic protist carbon (ng sterol or ketone mg^{-1} C) and micrograms of fatty acid per milligram of heterotrophic protist carbon (µg fatty acid mg^{-1} C) and (2) the percent (%) of total sterols, total steroidal ketones, or total fatty acids. For heterotrophic protist cultures that contained some unconsumed algal prey, heterotrophic protist sterol and fatty acid contents were corrected by subtraction of the fatty acid and sterol contents of the algal prey calculated based on the previously determined fatty acid and sterol content per algal prey cell (*Dunaliella tertiolecta* or *Rhodomonas salina*) (Chu et al. 2008a,b) from the total fatty acid and sterol content of the culture. The formula for calculating micrograms of fatty acid (FA) per milligram carbon or nanograms of sterol or ketone per milligram carbon is:

µg fatty acid mg^{-1} heterotrophic protist carbon = [(µg heterotrophic protist FA + µg unconsumed algal FA) – (µg algal FA per million algal cells × no. of million algal cells in sample)] ÷ mg heterotrophic protist carbon in sample

The above formula was used to calculate the nanograms of sterol or steroidal ketone per milligram heterotrophic protist carbon by replacing nanograms with micrograms and FA with sterol or steroidal ketone.

Expt 1: ability of *Pfiesteria piscicida*, *Luciella masanensis*, and *Cryptoperidiniopsis brodyi* to produce sterols and LCn-3EFA

Sterol profiles of *P. piscicida*, *L. masanensis*, and *C. brodyi* fed the alga *Rhodomonas salina*

As indicated earlier, the alga *R. salina* contains mainly brassicasterol (≅99%) and <2% cholesterol (Klein Breteler et al. 1999, Chu et al. 2008b). The sterol profiles of the 3 thecate dinoflagellate *P. piscicida*, *L. masanensis*, and *C. brodyi* cultures, which had been maintained on *R. salina* for 2 mo, differed significantly from their algal prey. In addition to sterols (i.e. dinosterol, dinostanol, and lophenol), usually found in autotrophic and some heterotrophic dinoflagellates, different C28, C29 and C30 sterols that were not present in the algal prey and 4 steroidal ketones were found in the 3 thecate heterotrophic protists. Two unidentified C29 sterols (C29:0a and C29:0b), a series of unidentified C29 sterols with 1 double bond (C29:1a, C29:1b, C29:1c) and 1 unidentified C30 sterol with 1 double bond (C30:1) were present in the 3 thecate heterotrophic protists, after feeding on *R. salina* (Table 1). The sterols of 2 *P. piscicida* isolates (CCMP 1834 and 1921) fed on a *Rhodomonas* sp. (CCMP 768) have been analyzed (Leblond & Chapman 2004). The sterols C29:1b, C29:0b, and C30:1 are likely the sterols tentatively identified by them as 4α,24-dimethyl-5α-cholest-22E-en-3β-ol, 4α,24-dimethyl-5α-cholestan-3β-ol, and 4α,23,24-trimethyl-5α-cholest-24(28)-3β-ol, or 4α,23,24-trimethyl-5α-cholest-8(14)-3β-ol, respectively, based on the comparison of their spectra derived from the TMS derivatives of sterols with those GC-MS spectra/values published by Leblond & Chapman (2004). The identification of dinosterol (4α,23,24-trimethyl-5α-cholest-22E-en-3β-ol), dinostanol (4α,23,24-trimethyl-5α-cholestan-3β-ol), and lophenol (4α-methyl-5α-cholest-7-en-3β-ol), and the 4 steroidal ketones, C29:0 (4α,24-dimethyl-5α-cholestan-3-one), C30:1 ketone [4α,23,24-trimethyl-5α-cholest-8(14)en-3-one], dinosterone (4α,23,24-trimethyl-5α-cholest-22E-en-3-one), and dinostanone (4α,23,24-trimethyl-5α-choleststan-3-one) was based on the comparison of their GC-MS spectrum fragmentations with previously reported GC-MS spectra (Mansour et al. 1999, Leblond & Chapman 2002, 2004). The identification of a C30:0 ketone, which eluted after dinostanone, was based on its mass spectrum fragmentation patterns, molecular weight, retention time, and lack of TMS derivative in TMS-derivitized samples. The steroidal ketones, C29:0, C30:1 ketone, dinosterone, and dinostanone, are present in *P. piscicida* and *L. masanensis*. *C. brodyi* contains only dinosterone, C29:0, and C30:1 ketone. The C30:0 ketone was detected only in *L. masanensis*.

Although generally the sterol profiles of the 3 thecate heterotrophic dinoflagellates were qualitatively similar, the relative abundance of individual sterols varied among species (Table 1). While dinosterol (Δ22-C30:1) was the most dominant sterol in *Pfiesteria piscicida* (≅43%) and *Luciella masanensis* (≅29%), the unidentified C29:0b sterol was the most abundant in *Cryptoperidiniopsis brodyi* and accounts for ≅30% of the total sterols. The second most abundant sterols in these 3 thecate dinoflagellate species were the unidentified sterol C30:1 in *P. piscicida* (≅25%) and *C. brodyi* (≅24%) and dinostanol in *L. masanensis* (≅25%). Cholesterol was absent in both *P. piscicida* and *L. masanensis*, but present in *C. brodyi* (1.2%). Brassicasterol was absent in *L. masanensis*, but both *P. piscicida* and *C. brodyi* contained low levels of this sterol, ≅3.0 and ≅6%, respectively. All of the 3 thecate species contained low levels of lophenol, approximately 3 to 8% (Table 1). There were very small amounts of the sterol Δ22-C28:1 present in all 3 thecate dinoflagellates (≅0.1 to 1.7%). The sterol C28:0 was present in both *P. piscicida* (0.5%) and *L. masanensis* (7.0%), but was not detected in *C. brodyi.* The amounts of unidentified C29 sterols (C29:0a and C29:0b) and C29 sterols with 1

Table 1. Sterol content (ng mg^{-1} C) and profiles of the 3 thecate heterotrophic dinoflagellates *Pfiesteria piscicida*, *Luciella masanensis*, and *Cryptoperodiniopsis brodyi* maintained on the alga *Rhodomonas salina* for >2 mo. All values are means (±SD) of 3 replicates. PP: *P. piscicida* (n = 3, 2.0×10^6 cells); LM: *L. masanensis* (n = 3, 1.25×10^6 cells, 9.86×10^5 cells, 9.86×10^5 cells); CB: *C. brodyi* (n = 3, 4.0×10^6 cells). Cell carbon contents: PP = 74.0 ± 3.0 pg C $cell^{-1}$; CB = 167.0 ± 2.8 pg C $cell^{-1}$; LM = 320.0 ± 14.06 pg C $cell^{-1}$, determined using a Carlo-Erba Model EA 1108 elemental analyzer. Cholesterol = Δ5-C27:1 (cholest-5-en-3β-ol); brassicasterol = Δ5, 22-C28:2 (24-α-methylcholesta-5, 22-dien-3β-ol); lophenol = Δ7-C28:1(4α-methyl-5α-cholest-7-en-3β-ol); dinosterol = Δ22-C30:1 (4α, 23, 24-trimethyl-5α-cholest-22E-en-3β-ol); dinostanol = C30:0 (4α, 23, 24-trimethyl-5α-cholestan-3β-ol); C29:0 ketone = 4α, 24-dimethyl-5α-cholestan-3-one; dinosterone = 4α, 23, 24-trimethyl-5α-cholest-22E-en-3-one; C30:1 ketone = 4α, 23, 24-trimethyl-5α-cholest-8(14)-en-3-one; dinostanone = 4α, 23, 24-trimethyl-5α-cholestan-3-one (Mansour et al. 1999, Leblond & Chapman 2002, 2004); C30:0 ketone*, a ketone eluted after dinostanone; its identification is based on its mass spectral fragmentation and molecular weight. ND: not detected

Carbon no.	Sterol	*Pfiesteria piscicida* (ng mg^{-1} C)	(%)	*Luciella masanensis* (ng mg^{-1} C)	(%)	*Cryptoperidiniopsis brodyi* (ng mg^{-1} C)	(%)
C_{27} sterols	Cholesterol	ND	ND	ND	ND	178.8 ± 99.2	1.2 ± 0.5
C_{28} sterols	Brassicasterol	456.6 ± 42.9	2.6 ± 0.3	ND	ND	932.6 ± 132.0	6.2 ± 0.4
	Δ22, C28:1	9.1 ± 1.7	0.1 ± 0.0	84.5 ± 37.1	1.7 ± 0.7	45.9 ± 5.3	0.3 ± 0.1
	Lophenol	495.3 ± 176.3	2.8 ± 1.0	391.8 ± 224.6	8.0 ± 4.4	493.4 ± 151.9	3.3 ± 1.1
	C28:0	87.6 ± 4.9	0.5 ± 0.0	339.2 ± 111.0	7.0 ± 2.5	ND	ND
C_{29} sterols	C29:1a (unidentified)	782.8 ± 21.9	4.5 ± 0.1	ND	ND	805.7 ± 82.1	5.4 ± 0.1
	C29:1b (unidentified)	ND	ND	ND	ND	337.8 ± 96.9	2.3 ± 0.8
	C29:1c (unidentified)	155.6 ± 9.5	0.9 ± 0.0	533.7 ± 105.7	11.0 ± 2.1	182.6 ± 23.5	1.2 ± 0.0
	C29:0a (unidentified)	87.0 ± 44.7	0.5 ± 0.2	123.2 ± 90.7	2.6 ± 1.9	17.7 ± 7.7	0.1 ± 0.0
	C29:0b (unidentified)	2927.7 ± 156.9	16.7 ± 0.3	413.4 ± 114.0	8.5 ± 2.1	4343.5 ± 549.0	28.9 ± 1.6
C_{30} sterols	Dinosterol	7481.3 ± 291.4	42.6 ± 0.8	1398.0 ± 232.1	28.7 ± 4.0	3545.5 ± 341.7	23.6 ± 0.3
	Dinostanol	631.8 ± 17.0	3.6 ± 0.1	1207.5 ± 224.3	24.9 ± 5.2	486.4 ± 55.0	3.3 ± 0.4
	C30:1 (unidentified)	4446.8 ± 185.0	25.3 ± 0.4	369.3 ± 103.0	7.6 ± 2.3	3631.6 ± 437.8	24.2 ± 1.0
Total sterols		17561.7 ± 643.8	100	4915.2 ± 110.5	100	15001.5 ± 1561.9	100
Steroidal ketones	C29:0 ketone	9.8 ± 3.9	0.6 ± 0.2	104.7 ± 78.2	5.2 ± 3.7	405.3 ± 32.0	12.4 ± 0.4
	Dinosterone	670.6 ± 50.7	38.1 ± 1.3	551.7 ± 92.9	27.9 ± 5.8	994.3 ± 81.0	30.3 ± 0.7
	C30:1 ketone	960.2 ± 117.5	54.3 ± 2.2	423.6 ± 135.8	21.4 ± 7.2	1885.1 ± 220.1	57.3 ± 0.9
	Dinostanone	123.4 ± 11.6	7.1 ± 1.2	737.1 ± 144.1	36.8 ± 4.7	ND	ND
	C30:0 ketone*	ND	ND	174.3 ± 94.7	8.7 ± 4.6	ND	ND
Total ketones		1764.0 ± 154.5	100	1911.9 ± 58.4	100	3284.7 ± 331.0	100
Total		19325.7 ± 797.3	100	6851.9 ± 126.9	100	18286.2 ± 1890.9	100

double bond (C29:1a, C29:1b, and C29:1c) varied among the 3 thecate dinoflagellate species. The most abundant unidentified C29 and C29:1 sterols were C29:0b in *C. brodyi* (≅30%) and *P. piscicida* (≅17%) and C29:1c in *L. masanensis* (≅11%). The unidentified C29:1a and C29:1b sterols were not detected in *L. masanensis*. The most abundant ketone in *P. piscicida* and *C. brodyi* was the C30:1 ketone (54 to 57%). *L. masanensis* contained the highest level of the ketone dinostanone (≅37%), but it had less dinosterone (≅29%) and C30:1 (≅21%) ketone than both *P. piscicida* and *C. brodyi*. The ketone C29:0 ranged from ≅0.6% in *P. piscicida* to ≅12% in *C. brodyi*. The C30:0 ketone, which eluted after dinostanone, was detected only in *L. masanensis* (≅9%). Among the 3 tested thecate species, *P. piscicida* had the highest amounts of total sterols and *C. brodyi* had the highest level of total ketones. The content of sterols was approximately 10-, 1.4-, and 5-fold of the steroidal ketone content in *P. piscicida, L. masanensis*, and *C. brodyi*, respectively.

Sterol profiles of *Pfiesteria piscicida* and *Luciella masanensis* fed the alga *Dunalliella tertiolecta*

Similarly to *P. piscicida* and *L. masanensis* fed the alga *Rhodomonas salina*, the sterol profiles of *P. pisci-*

Table 2. Sterol content (ng mg^{-1} C) and profiles of the 2 thecate heterotrophic dinoflagellates *Pfiesteria piscicida* and *Luciella masanensis* maintained on the alga *Dunaliella tertiolecta* for >2 mo. All values are means (±SD) of 3 replicates. PP: *P. piscicida* (n = 3, 1.0×10^7 cells); LM: *L. masanensis* (n = 3, 1.6×10^5 cells, 2.1×10^5 cells, 3.5×10^5 cells). Cell carbon contents: PP = 74.0 ± 3.0 pg C $cell^{-1}$, LM = 320.0 ± 14.06 pg C $cell^{-1}$, determined using a Carlo-Erba Model EA 1108 elemental analyzer. Fungisterol = Δ7-C28:1; 24β-methylcholesta-7-en-3β-ol; (Mansour et al. 1999, Leblond & Chapman 2002, 2004) for definitions of lophenol, dinosterol, dinostanol, dinosterone, C29:0 ketone and C30:1 ketone, see Table 1

Carbon no.	Sterol	*Pfiesteria piscicida*		*Luciella masanensis*	
		(ng mg^{-1} C)	(%)	(ng mg^{-1} C)	(%)
C_{28} sterols	Lophenol	843.7 ± 38.1	7.1 ± 0.7	1244.2 ± 321.2	12.9 ± 2.4
	C28:0	150.9 ± 15.5	1.3 ± 0.1	164.7 ± 28.5	1.7 ± 0.2
	Fungisterol	520.9 ± 41.4	4.4 ± 0.2	416.4 ± 30.3	4.4 ± 0.3
C_{29} sterols	C29:1c (unidentified)	1331.9 ± 55.4	11.2 ± 0.2	2784.3 ± 66.9	29.3 ± 1.8
	C29:0a (unidentified)	166.3 ± 13.1	1.4 ± 0.2	1135.0 ± 84.8	11.9 ± 0.9
	C29:0b (unidentified)	248.0 ± 24.8	2.1 ± 0.2	124.2 ± 29.8	1.3 ± 0.2
C_{30} sterols	Dinosterol	4593.1 ± 179.8	38.7 ± 0.7	1384.6 ± 88.4	14.6 ± 1.0
	Dinostanol	683.3 ± 197.2	5.7 ± 1.4	668.9 ± 61.5	7.0 ± 0.2
	C30:1 (unidentified)	3345.8 ± 221.2	28.1 ± 0.4	1604.4 ± 109.3	16.9 ± 0.9
Total sterols		11883.8 ± 658.6	100	9526.7 ± 650.9	100
Steroidal ketones	C29:0 ketone	108.5 ± 43.7	9.9 ± 3.7	101.4 ± 15.8	13.0 ± 2.3
	Dinosterone	324.7 ± 47.7	29.6 ± 3.0	185.0 ± 27.0	23.8 ± 4.3
	C30:1 ketone	572.2 ± 63.9	52.4 ± 5.7	366.0 ± 34.6	46.8 ± 2.2
	Dinostanone	88.3 ± 10.1	8.1 ± 1.0	128.1 ± 28.4	16.3 ± 2.8
Total ketones	100	1093.3 ± 76.3	100	780.5 ± 36.6	100
Total	100	12918.0 ± 652.9	100	10307.2 ± 623.5	100

cida and *L. masanensis* differed significantly from those of its algal prey *D. tertiolecta* (Table 2). The major sterols in *D. tertiolecta* are ergosterol, 7-dehydroporiferasterol, and fungisterol (Chu et al. 2008b). However, fungisterol was the only algal sterol present in *P. piscicida* (4.4 ± 0.2%) and *L. masanensis* (4.4 ± 0.3%). Most of the sterols and steroidal ketones found in *P. piscicida* and *L. masanensis* fed *R. salina* were present in these 2 species when they were fed *D. tertiolecta*. The principal sterols in *P. piscicida* were dinosterol (≅39%), the unidentified C30:1 (≅28%), the unidentified C29:1c (≅11%), and lophenol (≅7%). The principal sterols in *L. masanensis* were the unidentified C29:1c sterol (≅29%), the unidentified C30:1 sterol (≅17%), dinosterol (≅15%), lophenol (≅13%), and dinostanol (≅7%). The order of abundance of steroidal ketones in *P. piscicida* is: 30:1 ketone (≅52%) > dinosterone (≅30%) > C29:0 ketone (≅10%) > dinostanone (≅8%). The order of abundance of steroidal ketones in *L. masanensis* is: C30:1 ketone (≅47%) > dinosterone (≅24%) > dinostanone (≅16%) > C29:0 ketone (≅13%). *P. piscicida* had higher levels of both total sterols and steroidal ketones than *L. masanensis*. The content of sterols was approximately 11-fold higher than the steroidal ketone content in *P. piscicida*. In *L. masanensis*, the content of sterols was approximately 12 times higher than the content of steroidal ketone. The unidentified C29:1a sterols found in *P. piscicida* fed *R. salina* were not seen in this species when it was fed *D. tertiolecta*. Similarly, the unidentified C30:0 ketone found in *L. masanensis* fed *R. salina* was not detected in this species when it was fed *D. tertiolecta*.

Fatty acid composition of *Pfiesteria piscicida* and *Luciella masanensis* fed the alga *Dunalliella tertiolecta*

The fatty acid composition of the 2 thecate heterotrophic dinoflagellate species was qualitatively similar, but the quantity of individual fatty acids differed between the 2 species (Table 3). *P. piscicida* is higher in total fatty acid content than *L. masanensis*. The dominant saturated fatty acid in both species was palmitic acid 16:0 followed by 14:0 and 18:0. The monounsaturated C16 and C18 fatty acids in these 2 dinoflagellate species included 16:1(n-9), 16:1(n-7), 18:1(n-11), 18:1(n-9), 18:1(n-7), and 20:1 (n-9 + 11). The protist *L. mansanensis* contained the highest level of 18:1(n-9) (31.3 ± 0.6%), while *P. piscicida* had the highest proportion of 18:1(n-7) (10.1 ± 2.8%). Monounsaturated C16 fatty acids and C18 and C20 diunsaturated fatty acids were minor components. Polyunsaturated n-3 fatty acids accounted for 35.3 ± 7.54 and 26.2 ± 0.6% of the total fatty acids and consisted of 16:3(n-3), 16:4(n-3), 18:3(n-3), 18:4(n-3), 20:5(n-3) (EPA), and 22:6(n-3) (DHA). Both *P. piscicida* and *L. masanensis* had much higher DHA than EPA (Table 3). There were

Table 3. Fatty acid content (µg mg^{-1} C) and composition of 2 thecate dinoflagellates *Pfiesteria piscicida* and *Luciella masonensis* maintained on the EPA- and DHA-deficient alga *Dunaliella tertiolecta* for >2 mo. Cell carbon contents: *P. piscicida* = 74.0 ± 3.0 pg C cell^{-1}; *L. masonensis* = 320.0 ± 14.06 pg C cell^{-1}, determined using a Carlo-Erba Model EA 1108 elemental analyzer. Fatty acids reported comprised at least 0.5% of total fatty acids in one or more heterotroph species. *P. piscicida* values are means (±SD) of 3 replicates, 2 of which were derived from pooled cultures (2.0×10^7 cells replicate^{-1}). *L. masonensis* values are means (±SD) of 3 replicates derived from pooled cultures (3.0×10^6 cells replicate^{-1}). ND: not detected; FAME: fatty acid methyl esters

FAME	*Pfiesteria piscicida* (µg mg^{-1} C)	(%)	*Luciella masonensis* (µg mg^{-1} C)	(%)
14:0	14.9 ± 6.1	5.0 ± 0.3	10.3 ± 0.4	7.8 ± 0.1
14:1	7.9 ± 3.7	2.6 ± 0.3	2.0 ± 0.1	1.5 ± 0.0
Iso 15:0	3.5 ± 0.9	1.2 ± 0.1	0.7 ± 0.1	0.6 ± 0.0
15:0	3.4 ± 1.2	1.2 ± 0.0	1.0 ± 0.1	0.7 ± 0.0
15:1a	2.4 ± 0.6	1.0 ± 0.0	0.7 ± 0.1	0.5 ± 0.1
15:1b	1.0 ± 0.2	0.4 ± 0.1	1.1 ± 0.2	0.8 ± 0.1
16:0	48.3 ± 17.2	16.4 ± 0.1	17.3 ± 1.1	13.2 ± 0.4
16:1(n-9)	2.4 ± 0.4	0.9 ± 0.3	0.7 ± 0.2	0.5 ± 0.2
16:1(n-7)	6.6 ± 1.1	2.4 ± 0.4	1.6 ± 0.1	1.18 ± 0.03
16:2a	2.8 ± 0.4	1.1 ± 0.4	0.8 ± 0.2	0.6 ± 0.2
16:2b	1.6 ± 0.8	0.6 ± 0.3	1.4 ± 0.1	1.1 ± 0.1
17:0	2.1 ± 1.0	0.7 ± 0.1	0. 9 ± 0.3	0.7 ± 0.2
16:3(n-3)	3. 6 ± 1.6	1.2 ± 0.1	0.5 ± 0.2	0.4 ± 0.1
16:4(n-3)	11.7 ± 11.9	3.4 ± 2.4	2.2 ± 0.3	1.6 ± 0.2
18:0	11.9 ± 2.4	4.2 ± 0.6	5.9 ± 0.6	4.5 ± 0.3
18:1(n-11)	ND	ND	3.2 ± 0.3	2.4 ± 0.2
18:1(n-9)	20.3 ± 2.9	7.4 ± 2.0	41.2 ± 2.0	31.3 ± 0.6
18:1(n-7)	27.8 ± 2.0	10.1 ± 2.8	4.2 ± 0.2	3.2 ± 0.1
18:2(n-6)	4.9 ± 2.4	1.8 ± 1.1	1.0 ± 0.5	0.8 ± 0.4
18:3(n-3)	52.6 ± 43.2	16.2 ± 7.6	7.2 ± 0.3	5.5 ± 0.2
18:4(n-3)	0.5 ± 0.7	0.2 ± 0.3	0.8 ± 0.2	0.6 ± 0.1
20:1(n-9+11)[a]	0.2 ± 0.4	0.1 ± 0.2	1.4 ± 0.8	1.0 ± 0.6
20:2(n-9)	5.9 ± 3.8	2.0 ± 1.3	0.5 ± 0.4	0.4 ± 0.3
20:5(n-3)	3.1 ± 1.4	1.0 ± 0.2	1.4 ± 0.5	1.1 ± 0.4
22:6(n-3)	36.9 ± 4.6	13.2 ± 2.8	22.4 ± 0.7	17.0 ± 0.7
Total n-3	108.4 ± 61.8	35.3 ± 7.54	34.4 ± 0.7	26.2 ± 0.6
Total	293.7 ± 103.1	~95	131.1 ± 4.5	~100

[a]The fatty acids 20:1 (n-9) and 20:1(n-11) eluted together

some small amounts of odd-chain saturated (iso-15:0, 15:0, 17:0) and unidentified monounsaturated C15 (15:1a, 15:1b) and diunsaturated C16 (16:2a, 16:2b) fatty acids present in both species.

Expt 2: ability of *Amphidinium longum* to produce sterols and/or bioconvert dietary sterols

GC-MS analysis of both free and TMS-derivitized sterols of the heterotrophic dinoflagellate *A. longum* revealed a total of 3 sterols present in the samples (Table 4). Two of these were identified as cholesterol and brassicasterol based on their fragmentation patterns and retention times relative to authentic standards. The third sterol had a molecular ion of 426 as a free sterol and 498 as a TMS-derivitized sterol, which is consistent with it being a C30:2 sterol. Lack of known standards and matching spectra in available GC/MS spectral libraries precluded a more detailed characterization of this sterol. Cholesterol, brassicasterol, and the C30:2 sterol represent 63.4 ± 0.8, 16.2 ± 0.9, and 19.8 ± 0.5% of the total sterols, respectively.

Table 4. Sterol profile of the heterotrophic protist *Amphidinium longum* fed *Rodomonas salina*. All values are means (±SD) of 3 replicates derived from pooled culture (*A. longum* cells replicate^{-1} = 2.0×10^6 with a predator:prey ratio of 2:1). Cell carbon contents of *A. longum* = 228.8 ± 5.0 pg C cell^{-1}, determined using a Carlo-Erba Model EA 1108 elemental analyzer

Sterols	*Amphidinium longum* (ng mg^{-1} C)	(%)
Cholesterol (C27:1)	8024.1 ± 139.2	63.4 ± 0.8
Brassicasterol (C28:2)	2055.2 ± 174.0	16.2 ± 0.9
C30:2 (unidentified)	2587.3 ± 95.2	19.8 ± 0.5
Total	10611.4 ± 226.4	~100

Expt 3: ability of *Strombidinopsis* sp. and *Uronema* sp. to produce sterols and LCn-3EFA

Strombidinopsis sp.

The ciliate *Strombidinopsis* sp. had a similar sterol profile as its algal prey. Only brassicasterol (≅100%) was found in *Strombidinopsis* sp. after it was maintained on *Rhodomonas salina*, which contains ≅99% brassicasterol, for >2 mo; no cholesterol was detected in this ciliate species. Unfortunately, *Strombidinopsis* sp. did not replicate when it was maintained on the alga *Dunaliella tertiolecta*. Thus, we were unable to assess its ability to produce EPA and DHA.

Uronema sp.

Sterol composition. The GC-MS analysis of free sterol and TMS-derivitized samples of the bacterivorous ciliate *Uronema* sp. revealed no sterols.

Only 3 unknown, hopanoid-like compounds were present in the samples. The TMS-derivitized forms of these compounds had very long retention times relative to the internal standard, 5α-cholestane, and the sterol standards. Their spectra contained a prominent 191 ion. The most abundant of the 3 compounds also contained a prominent 189 ion. Long relative retention times and the presence of prominent 191 and 189 ions are characteristic of hopanoids, which are produced by many ciliates under conditions where dietary sterols are lacking (Ten Haven et al. 1989, Harvey & McManus 1991). Since there are no commercially available standards and no good spectral matches with published spectra or spectral libraries, we were unable to provide a more concise identification of these compounds other than they appear to be hopanoid-like.

Fatty acid profile. The bacterial prey fatty acid composition was dominated by saturated fatty acids such as 16:0, 18:0, and 20:0, which account for approximately 60% of the total fatty acids and contained several fatty acids characteristic of bacteria (e.g. the odd-chain fatty acids 15:0, 17:0, and 19:0) and odd/even-branch-chain fatty acids (e.g. iso- and anteiso-15:0 and -17:0, iso-19:0, and iso-16:0). Surprisingly, the bacterivorous ciliate *Uronema* sp. contained several polyunsaturated fatty acids, including the LCn-3EFAs EPA and DHA (Table 5). The fatty acids 18:3(n-3), 18:4(n-3), EPA, and DHA accounted for approximately 5, 4, 5, and 5% of the total fatty acids, respectively. *Uronema* sp. also contains minor quantities of 16:3(n-3) (≅0.5%), 16:4(n-3) (≅0.9%), 18:2(n-6) (≅0.9%), and 18:3(n-6) (≅0.3%). The level of the fatty acid 18:1(n-7), a monounsaturated fatty acid usually found abundantly in bacteria, was quite high (≅22% of the total fatty acids) compared to that of its bacterial prey (≅8%).

Table 5. Fatty acid compositions of the ciliate *Uronema* sp. and its bacterial prey. Values are means (±SD) of 3 replicates (2.5×10^6 cells replicate^{-1}). Cell carbon contents of *Uronema* sp. = 177.9 ± 2.1 pg C cell^{-1}, determined using a Carlo-Erba Model EA 1108 elemental analyzer. FAME: fatty acid methyl esters, ND: not detected

FAME	*Uronema* sp. (μg mg^{-1} C)	*Uronema* sp. (%)	Bacterial prey (%)
14:0	2.4 ± 0.1	2.9 ± 0.2	2.5 ± 0.4
14:1	2.1 ± 0.2	2.6 ± 0.2	ND
15:0	1.5 ± 0.1	1.8 ± 0.1	3.1 ± 1.7
Iso15:0	1.7 ± 0.1	2.1 ± 0.1	1.6 ± 0.7
Ant15:0	ND	ND	0.9 ± 0.3
16:0	10.8 ± 0.8	13.2 ± 0.6	24.8 ± 1.6
Iso16:0	ND	ND	1.0 ± 0.5
16:1(n-9)	3.9 ± 0.5	4.8 ± 0.1	0.8 ± 0.2
16:1(n-7)	4.6 ± 0.6	5.6 ± 0.2	0.2 ± 0.3
16:2	1.5 ± 0.1	1.9 ± 0.2	ND
16:3(n-3)	0.4 ± 0.0	0.5 ± 0.0	ND
16:4(n-3)	0.7 ± 0.1	0.9 ± 0.1	ND
17:0	0.8 ± 0.0	1.0 ± 0.1	6.1 ± 4.4
Iso17:0	1.3 ± 0.2	1.6 ± 0.4	4.3 ± 2.6
Ant17:0	1.5 ± 0.1	1.9 ± 0.1	3.5 ± 1.3
18:0	6.0 ± 0.8	7.3 ± 0.7	24.2 ± 8.4
18:1(n-9)	0.7 ± 0.6	0.9 ± 0.8	0. 7 ± 0.6
18:1(n-7)	18. 5 ± 2.7	22.3 ± 1.0	8.2 ± 5.7
18:2(n-6)	0.7 ± 0.1	0.9 ± 0.1	ND
18:3(n-6)	0.21 ± 0.04	0.3 ± 0.0	ND
18:3(n-3)	3.9 ± 0.6	4.8 ± 1.1	ND
18:4(n-3)	3.7 ± 3.2	4.2 ± 3.7	ND
19:0	ND	ND	1.4 ± 0.7
Iso19:0	ND	ND	1.0 ± 0.4
20:0	ND	ND	11.2 ± 7.3
20:01	0.9 ± 0.3	1.1 ± 0.3	3.4 ± 2.1
20:2	ND	ND	1.3 ± 0.5
20:4(n-6)	1.4 ± 0.1	1.7 ± 0.0	ND
20:5(n-3)	4.0 ± 0.5	4.9 ± 0.1	ND
22:5(n-3)	Trace	Trace	ND
22:6(n-3)	4.3 ± 0. 5	5.2 ± 0.3	ND
Total	77.7 ± 9.7	~94	100

DISCUSSION

The sterol profiles of the 3 thecate heterotrophic dinoflagellate species fed either *Rhodomonas salina* or *Dunaliella tertiolecta* shared some similarities qualitatively. Sterols and steroidal ketones commonly found in Dinophyceae were present in all 3 thecate species. They all contained some of the characteristic sterols and steroidal ketones, such as dinosterol, dinostanol, lophenol, dinosterone, and dinostanone, detected in other autotrophic marine dinoflagellates and 1 heterotrophic dinoflagellate species that have been analyzed (Mansour et al. 1999, 2003, Volkman et al. 1999, Leblond & Chapman 2002, 2004). Unlike other reported auto- and heterotrophic species these 3 thecate heterotrophic species produced a series of unidentified C29 saturated and monounsaturated sterols and an unidentified C30:1 sterol; in addition, an unidentified C30:0 ketone was found in *Luciella masanensis*. However, as indicated earlier, some of the unidentified C29 unsaturated and monounsaturated sterols and the C30:1 sterol could be those tentatively identified by Leblond & Chapman (2004). The sterol profiles of the 3 thecate heterotrophic species fed *R. salina* and *D. tertiolecta* are relatively similar to those found in the 2 *Pfiesteria piscicida* isolates fed a *Rhodomonas* sp. (Leblond & Chapman 2004).

Previously, we found increases in cholesterol, a dominant sterol in crustaceans and higher animals, in the 2 heterotrophic protists *Oxyrrhis marina* and *Gyrodinium dominans* when they were fed *Rhodomonas salina* and they produced a significant amount of this sterol when they were fed *Dunaliella tertiolecta*, which contains no cholesterol (Chu et al. 2008b). No cholesterol was detected in either *Pfiesteria piscicida* or *Luciella masanensis* when they were fed *D. tertiolecta*. The ability to synthesize cholesterol de novo or convert

dietary sterols to cholesterol and other sterols may be limited to certain species of heterotrophic protists, such as *O. marina* and *G. dominans* (Chu et al. 2008b). The cholesterol detected in *Cryptoperidiniopsis brodyi* fed *R. salina* was probably accumulated from its algal prey and/or derived from the remaining algal cells. Incorporation of fungisterol, one of the sterols contained in the algal prey *D. tertiolecta*, was also found in all 3 thecate heterotrophic species.

The sterols and ketones found in the 2 *Pfiesteria piscicida* isolates fed *Rhodomonas* sp. are believed to have come from de novo synthesis by the heterotrophic protist, since the *Rhodomonas* sp. contained only brassicasterol (Leblond & Chapman 2004), but none of the 4α-methyl-substituted sterols and steroidal ketones were present in the 2 heterotrophic protist isolates. Similarly, results of our study on the 3 thecate heterotrophic dinoflagellate species lead us to believe the same: the 3 thecate heterotrophic dinoflagellates are capable of producing a series of sterols and steroidal ketones that were not detected in their algal prey, *Rhodomonas salina* and *Dunaliella tertiolecta*. Some of the 4α-methyl sterols and steroidal ketones found in our study and the study by Leblond & Chapman (2004) have been previously identified and reported in other autotrophic dinoflagellates (e.g. Piretti et al. 1997, Mansour et al. 1999, 2003, Volkman et al. 1999, Leblond & Chapman 2004). Exactly how dinosterol and dinostanol and other 4α-methyl-substituted sterols and steroidal ketones are made is not completely clear. The pathways leading to the formation of the ring system of dinosterol and, subsequently, dinosterol via side-chain desaturation, saturation, and alkylation and then dinostanol by direct reduction of dinosterol or perhaps from the cationic intermediate peridinosterol (Mansour et al. 2003) have been proposed and discussed (Withers et al. 1979, Giner 1993, Mansour et al. 2003, Leblond & Chapman 2002, 2004). The diversity of sterols found among dinoflagellates and in the class of Dinophyceae is believed to be the result of a series of reduction/desaturation, isomerization, and alkylation/dealkylation reactions (Leblond & Chapman 2002, Mansour et al. 2003).

Dinosterol and dinostanol are widely distributed among the class Dinophyceae, particularly in photosynthetic species (Mansour et al. 1999, 2003, Leblond & Chapman 2002, 2004), and dinosterol was previously considered as a representative sterol 'marker' for the class Dinophyceae. Qualitative analysis of the heterotrophic dinoflagellate *Amphidinium longum* did not reveal the production of dinosterol, only brassicasterol and cholesterol, which were probably accumulated and/or derived from its algal prey *Rhodomonas salina*, and a C30:2 sterol were detected. This sterol may possibly be a 4α-methyl Δ8-14 sterol, as this sterol has been described in other species of *Amphidinium* (Withers et al. 1979, Kokke & Spero 1987). We did not detect dinosterol either in *Oxyrrhis marina* and *Gyrodinium dominans* when they were fed *R. salina* or *Dunaliella tertiolecta* (Chu et al. 2008b). Also, in a recent survey of sterol composition of marine dinoflagellates, this sterol was found in only about 50% of the 40 surveyed dinoflagellate species (Leblond & Chapman 2002).

The only sterol detected in the ciliate *Strombidinopsis* sp. was brassicasterol, a dominant sterol in the algal prey *Rhodomonas salina*. This suggests that this ciliate species is incapable of bioconverting dietary sterols and/or synthesizing sterols de novo; thus, sterol composition in this ciliate is dependent on diet. The study by Klein Breteler et al. (2004) also revealed the inability of the marine ciliate *Strombidium sulcatum* to modify dietary sterols and fatty acids. Possibly, marine ciliates differ somewhat from their counterparts, freshwater ciliates, some of which have been shown to 'upgrade' the biochemical constituents of their diets (Boechat & Adrian 2005, Martin-Creuzburg et al. 2005, 2006). The heterotrophic ciliates *Colpidium campylum* and *Cyclidium* sp. were able to upgrade the food quality of their cyanobacterial prey by producing compounds functionally equivalent to sterols, and they subsequently enhanced the growth and reproduction of their predator *Daphnia* (Martin-Creuzburg et al. 2005, 2006).

Hopan-3β-ol has been identified in several marine ciliates (Harvey & McManus 1991). Only high molecular weight sterol-like compounds, presumably hopanoids, were detected in the bacterivorous ciliate *Uronema* sp. We did not detect tetrahymanol, which has been suggested to be a specific marker for marine ciliates that feed on bacteria.

As we previously found in the 2 heterotrophic dinoflagellates *Oxyrrhis marina* and *Gyrodinium dominans* (Chu et al. 2008a), our results provide evidence that the 2 thecate heterotrophic dinoflagellates *Pfiesteria piscicida* and *Luciella masanensis* and the bacterial ciliate *Uronema* sp. were capable of producing the LCn-3EFAs EPA and DHA, when they were maintained on EPA- and DHA-deficient diets, the alga *Dunaliella tertioleca*, or bacteria. The detection of EPA and DHA and their potential precursors, 18:3(n-3) and 18:4(n-3), in *Uronema* sp. is interesting, though the quantities of these fatty acids are relatively small compared to the high content of the bacterial fatty acid 18:1(n-7) accumulated in the ciliate. The mechanisms and biochemical processes involved in the production of EPA and DHA in *P. piscicida*, *L. masanensis*, and *Uronema* sp. are not known. As with *O. marina* and *G. dominans* (Lund et al. 2008), apparently *Uronema* sp., did not produce EPA and DHA via elongation and desaturation of dietary n-3 fatty acids, since no n-3 fatty acids

were present in its bacterial prey. *O. marina* and *G. dominans* produced EPA and DHA de novo (Lund et al. 2008). Instances of de novo synthesis of EPA and DHA in heterotrophic protist species other than *O. marina* and *G. dominans* have also been reported (Barclay et al. 1994, Zhukova & Kharlamenko 1999, De Swaaf et al. 2003a,b, Bec et al. 2006). Interestingly, all the heterotrophic protists that were able to grow on alga deficient in EPA and DHA (i.e. *D. tertiolecta*) were capable of producing these fatty acids. Unfortunately, the difficulty of growing *Cryptoperidiniopsis brodyi*, *Amphidinium longum*, and *Strombidinopsis* sp. on *D. tertiolecta* to sufficient biomass for fatty acid analysis precluded determining whether or not they are able to make EPA and DHA.

In conclusion, the 3 thecate heterotrophic dinoflagellate species *Pfiesteria piscicida*, *Luciella masanensis*, and *Cryptoperidiniopsis brodyi* did not convert dietary sterols to other usual and common sterols, as previously reported for *Oxyrrhis marina* and *Gyrodinium dominans* (Chu et al. 2008b). Instead, they produced sterols and ketones similar to those found in the autotrophic dinoflagellates that have been analyzed and in the other 2 *P. piscicida* isolates (e.g. Piretti et al. 1997, Mansour et al. 1999, 2003, Volkman et al. 1999, Leblond & Chapman 2004). They all contained the typical dinoflagellate sterols, e.g. dinosterol and dinostanol, and steroidal ketones, e.g. dinosterone and dinostanone. The heterotrophic dinoflagellate *Amphidinium longum* and the herbivorous ciliate *Strombidinopsis* sp. were incapable of either bioconversion of dietary sterols or producing sterols and steroidal ketones. Similarly, the bacterivorous ciliate *Uronema* sp. did not produce any sterols. These results demonstrate the differences in bioconversion of dietary sterols and/or sterol production among the above tested heterotrophic species (the 3 thecate dinoflagellate vs. the athecate dinoflagellate *A. longum* and the 2 ciliate species). However, all the heterotrophic protist species that were able to grow on the EPA- and DHA-deficient alga *Dunaliella tertiolecta* and the bacterivorous ciliate species were capable of producing them. The bacterial prey of *Uronema* sp. lacked not only EPA and DHA, but also their potential precursors. Although the nutritional values of the sterols and steroidal ketones produced by the tested thecate species in the present study are not known and 4α-methyl-substituted sterols may not be readily assimilated by copepods (Talbot et al. 2000), the contribution of the LCn-3EFAs EPA and DHA by 2 of the thecate species and the bacterivorous ciliate are noteworthy. To further understand the intermediate roles of heterotrophic protists and their abilities in producing essential nutrients, examination of more species, particularly those newly discovered and isolated, is required.

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