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Sterol production and phytosterol bioconversion in two species of heterotrophic protists, *Oxyrrhis marina* and *Gyrodinium dominans*

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Abstract:

The kinetics and efficiency of sterol production and bioconversion of phytosterols in two heterotrophic protists *Oxyrrhis marina* and *Gyrodinium dominans* were examined by feeding them two different algal species (*Rhodomonas salina and Dunaliella tertiolecta*) differing in sterol profiles. *R. salina* contains predominantly brassicasterol (≅99%) and <2% cholesterol. The major sterols in *D. tertiolecta* are ergosterol (45–49%), 7-dehydroporiferasterol (29–31%) and fungisterol (21–26%). *O. marina* fed *R. salina* metabolized dietary brassicasterol to produce 22-dehydrocholesterol and cholesterol. *O. marina* fed *D. tertiolecta* metabolized dietary sterols to produce cholesterol, 22-dehydrocholesterol, brassicasterol and stigmasterol. *G. dominans* fed either *R. salina* or *D. tertiolecta* metabolized dietary sterols to make cholesterol, brassicasterol and a series of unknown sterols. When protists were fed *R. salina*, which contains cholesterol, the levels of cholesterol were increased to a magnitude of nearly 5- to 30-fold at the phytoplankton-heterotrophic protist interface, equivalent to a production of 172.5 ± 16.2 and 987.7 ± 377.7 ng cholesterol per mg *R. salina* carbon consumed by *O. marina* and *G. dominans*, respectively. When protists were fed *D. tertiolecta*, which contains no cholesterol, a net production of cholesterol by the protists ranged from 123.2 ± 30.6 to 871.8 ± 130.8 ng per mg algal C consumed. Cholesterol is not only the dominant sterol, but a critical precursor for many physiologically functional biochemicals in higher animal. As intermediates, these heterotrophic protists increase the amount of cholesterol at the phytoplankton–zooplankton interface available to higher trophic levels relative to zooplankton feeding on algae directly.

INTRODUCTION

Microzooplankton, dominated by ciliates and heterotrophic dinoflagellates, hold an important position in both freshwater and marine food webs because of their nutritional and energetic contributions to consumers at higher trophic levels. Unlike heterotrophic protists, which are a significant food source to crustacean zooplankton in nature, pico-plankton and nano-plankton less than 10 μm usually cannot be directly consumed by some mesozooplankton such as calanoid copepods (Ohman and Runge 1994, Merrell and Stoecker 1998, Bollens and Penry 2003, Gifford et al 2007). Heterotrophic protists are important grazers of autotrophic phytoplankton of various sizes (pico-, nano- and micro-algae) and themselves are prey for metazoans such as copepods and daphnids. Thus, they provide a critical link for channeling energy and nutrients from the base of the pelagic food web, via the microbial loop, to higher trophic levels. As intermediates, some heterotrophic protists not only assimilate/repackage nutrients from nano-and pico-plankton for subsequent use by zooplankton grazers and enhance the carbon transfer efficiency at the phytoplankton-zooplankton interface, but upgrade the biochemical constituents of their algal food for subsequent use at higher trophic levels by producing essential lipids that are absent in their algal diets (Klein Breteler et al. 1999; Bec et al. 2003; Broglio et al. 2003; Tang and Taal 2005;Veloza et al. 2006; Bec et al. 2006; Chu et al. 2008).

Some essential lipids such as the long chain polyunsaturated fatty acids, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA), and sterols (Gurr et al. 2002) are not only major constituents of structural lipids, but are precursors of physiologically

active metabolites that are involved in many biological and biochemical processes. In aquatic food webs, phytoplankton is the primary producer of these essential components. On the other hand, crustaceans, including copepods and higher consumers, are incapable of synthesizing some of these essential components and thus rely on dietary sources for these compounds. Trophic upgrading of food deficient in essential lipids by heterotrophic protists has been documented in both marine (e.g., Klein Breteler et al. 1999; Tang and Taal 2005; Broglio et al. 2003; Veloza et al. 2006; Chu et al. 2008) and freshwater ecosystems (Bec et al. 2003; Boechat and Adrian 2005; Martin-Creuzburg et al. 2005a; Bec et al. 2006). While studies of "trophic upgrading" in freshwater ecosystems emphasize sterol production and dietary sterol modification as being important (Bec et al. 2006), in marine ecosystems it has been most strongly linked to long chain n-3 essential fatty acid production (Chu et al. 2008; Lund et al. 2008). The long chain n-3 essential fatty acid (LCn-3EFA) production by two marine heterotrophic protists, *Oxyrrhis marina* and *Gyrodinium dominans*, fed an alga (*Dunaliella tertiolecta*) deficient of the LCn-3EFAs, EPA and DHA have been recently quantified (Chu et al. 2008). The contribution, particularly of DHA, by these two heterotrophic protists to the pelagic food webs appears to be quantitatively and ecologically significant and comparable to or higher than three representative species of algae (*Chaetoceros calcitrans*, *Tetraselmis suecica*, and *Rhodomonas salina*) that also contain LCn-3EFAs.

However, sterols are vital components of all eukaryotes (Volkman 2003) and generally account for ~0.1% of wet weight (Ackman 1989). Sterols are involved in a wide range of physiological functions and are critical for growth and reproduction (Finkelstein and Cass 1967; Bruckdorfer et al. 1969; Demel et al. 1972; Ederington et al. 1995; Crockett 1998). The essential

nature of sterols to the functioning of biological membranes makes them an important dietary component for organisms, such as calanoid copepods and Daphnia (Klein Breteler et al. 1999; Bec et al. 2006; Martin-Creuzburg et al. 2005b, 2008) that lack the ability to synthesize them de novo (Ballantine et al. 1980; Goad et al. 1983; Kanazawa 2001). Copepods are by far the most abundant animal group of marine plankton on which the ocean food chain depends. They hold the key for upward carbon and nutrient transfer since they are the main consumers of auto-and hetero-trophic protists. Along with LCn-3EFAs, dietary sterol content and composition could be potential limiting factors for egg production in copepods (Hasset 2004). It has been demonstrated that not only sterol content, but also sterol composition are critical in supporting the growth and reproduction of the freshwater dominant herbivores, the genus *Daphnia* (Von Elert et al. 2003, Martin-Creuzburg and Von Elert 2004, Martin-Creuzburg et al. 2005a; 2005b; Bec et al. 2006).

The ability of herbivorous heterotrophic protists to synthesize or modify sterols may be critical in determining copepod nutrition. Copepod survivorship, fecundity and offspring's survival are all adversely affected by diets low or deficient in LCn-3 EFAs and sterols (Klein Breteler et al. 1999, 2004, 2005; Broglio et al. 2003; Tang and Taal 2005; Veloza et al. 2006). Moreover, the nutritional quality, such as the LCn-3 EFAs and sterol content of the copepods, is an important factor controlling the development and growth and recruitment of fish (St. John and Lund 1996; St. John et al. 2001). To our knowledge, there are only three studies suggesting that marine heterotrophic protists can synthesize sterols from sterol-deficient diets (Harvey et al. 1997; Klein Breteler et al. 1999; Adolf et al. 2007). Although the heterotrophic protist *Oxyrrhis marina* has been shown to synthesize sterols and/or modify those present in its algal diet (Klein Breteler et al. 1999, 2005), its efficiency in sterol production and the ecological significance of

the production have not been quantified. Additionally, there may be species-specific differences in sterol production and dietary sterol modification among heterotrophic protists. To assess the sterol production and bioconversion of phytosterols by heterotrophic protists and their quantitative and potential ecological significance, we first examined the kinetics, efficiency and quantity of sterol production of two heterotrophic protists, *Oxyrrhis marina* and *Gyrodinium dominans,* fed two different algal species differing in sterol profiles.

MATERIALS AND METHODS

Algal and protist cultures

The chlorophyte *Dunaliella tertiolecta* (CCMP 1320) and the cryptophyte, *Rhodomonas salina* (CCMP 1319) were obtained from Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP) and cultured in f/2 culture medium prepared from artificial sea water (ASW) of salinity 20. *R. salina* and *D. tertiolecta,* which differ in sterol profiles (Goad et al. 1983; Patterson et al. 1992; Zelazny et al. 1995; Klein Breteler et al. 1999**),** were used as the prey for feeding experiments with the heterotrophic protists, *Oxyrrhis marina* and *Gyrodinium dominans*. Both *R. salina* and *D. tertiolecta* cultures used in feeding experiments were grown in 1-L round bottom flasks with aeration in a walk in environmental room at 19 °C with a 12h light:12h dark cycle. Medium was refreshed in all algal culture flasks every 3-5 days. Exponential/log phase cultures of *R. salina* and *D. tertiolecta* were used for all of the feeding experiments and they were sampled for total lipid extraction and sterol analysis, prior to feeding them to the protists.

The heterotrophic dinoflagellates *Oxyrrhis marina* Dujardin and *Gyrodinium dominans* Hulbert were obtained from the Shannon Point Marine Center and cultured in f/2 medium prepared from ASW of salinity 20 and fed solely on either *R. salina* or *D. tertiolecta* cultures. Both *O. marina* and *G. dominans* cultures used for feeding experiments were maintained in 300 ml bottles on a rotating plankton wheel at 1.5 rpm in a walk-in environmental room at 19 ˚C in the dark.

Experiments

I. Determination of the ability of *Oxyrrhis marina* and *Gyrodinium dominans* to produce and modify dietary sterols

1) Growth and sterol profiles of *Oxyrrhis marina* and *Gyrodinium dominans* fed the alga *Rhodomonas salina*

To determine the kinetics (production per unit time) and efficiency (production per unit of carbon ingested) of sterol production and/or dietary sterol modification by the heterotrophic protists, *O. marina* and *G. dominans,* eighteen 300 ml glass bottles containing 1.5 x 10^5 prey-depleted *O. marina* or *G. dominans* cells in f/2 medium of salinity 20, were inoculated with 1.5 x 10^7 *R. salina* cells (predator:prey = 1:100; n=18 bottles) and kept in the dark at 19°C on a rotating plankton wheel at 1.5 rpm. Prey-depleted *O. marina* and *G. dominans* were obtained by only feeding them 3 and 4 days, respectively, with *R. salina* in a predator:prey ratio of 1:100, prior to being used for feeding experiment and sampled for cell counts and lipid analysis.

Usually *O. marina* grazed down its algal prey by day 3 and *G. dominans* ingested almost 95% its food by day 4. However, to ensure that all prey had been consumed and metabolized 1 ml of samples (n=3) were taken to examine with an inverted microscope (Nikon, Phase contrast, ELWD, 0.3, Japan) (Chu et al. 2008) using a 1 ml Sedgewick-Rafter counting chamber.

At the beginning of the feeding experiment, 3×10^5 *O. marina* or *G. dominans* cells (n=6), 1.5×10^7 *R. salina* cells (n=6), and samples containing both 1.5×10^5 prey-depleted *O. marina* or prey-depleted *G. dominans* cells and 1.5×10^7 *R. salina* cells (n=6) were harvested on acetone-rinsed GF/F filters for initial sterol profiles. Then, six bottles of cultures containing *O. marina* and *R. salina* or *G. dominans* and *R. salina* were collected on acetone rinsed GF/F filters 1, 2, and 3 days for *O. marina* fed *R. salina* and 1, 2, and 4 days for *G. dominans* fed *R. salina*, after inoculation (n=6 bottles for each sampling date) for sterol analysis. For each sampling date, three 1 ml aliquots per bottle were taken for triplicate cell counts, which were determined by direct counting of cells stained with a 1% Lugol's solution in a 1.0 ml Sedgewick Rafter counting chamber using an inverted microscope (Nikon, Phase contrast, ELWD, 0.3, Japan). Then the volume of the remaining culture in each bottle was determined prior to filtration and used along with the cell counts to calculate the number of prey and predator cells in each bottle. Prey-depleted protists, algal prey, and protist-algal mixed cultures were harvested using gravity filtration, and gentle water aspiration was used only if needed. Collected sample filters were stored in glass screw cap test tubes at -80°C for no more than 4 weeks prior to lipid extraction and sterol analysis.

2) Growth and sterol profiles of *Oxyrrhis marina* and *Gyrodinium dominans* fed the alga *Dunaliella tertiolecta*

In the feeding experiments, in which *O. marina* and *G. dominans* were fed *D. tertiolecta*, the experimental conditions, predator (*O. marina or G. dominans*) and prey (*D. tertiolecta*) ratio (predator:prey = 1:100), sample replications and dates of sampling were identical to those described above for the *O. marina* and *G. dominans* fed *R. salina* experiments. As in the *O. marina* and *G. dominans* fed *R. salina* experiments, prey-depleted *O. marina* and *G. dominans* were obtained by only feeding them 3 and 4 days, respectively, with *D.tertiolecta* in a predator:prey ratio of 1:100, prior to being used for feeding experiment and sampled for cell counts and lipid analysis.

II. Total lipid extraction and sterol analysis

Lipids were extracted from the GF/F filters containing harvested cells (algae, protist, or protist-algal mixture; n=6 for initial and each sampling) by the method of Bligh and Dyer (1959). Sterols usually account for only ≤ 10% of the protist and algal total lipids, based on our preliminary analyses. Thus, to ensure sufficient lipid quantity for sterol detection and quantitation, two replicates of the six replicate extracted lipid samples were pooled based on their cell number to generate 3 replicate lipid samples with similar cell numbers. 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 2.3 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 hr at room temperature after vortexing. After hydrolysis, free 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-liquid chromatography/flame ionization detection (GC/FID) and gas-liquid chromatography/mass spectroscopy (GC/MS). A Varian Saturn 2000 GC/MS/MS ion trap (Varian Analytical Instruments, Walnut Creek, CA) was used for sterol qualitative analysis. The GC/MS was equipped with a Varian 3800 CP gas chromatograph (GC). A J&W DB-5 (60mX0.32mmX0.25µm film) fused silica column (Folsom,CA) was installed in the GC and helium was used as the carrier gas at a constant flow rate of 1.6 ml $minute^{-1}$. The injector temperature was 320ºC. All analyses used the following column temperature program: initial temperature of 75ºC holding for 1 minute then programming at 15ºC min^{-1} to a final temperature of 350ºC holding for 9.67 minutes. The ion trap was held at 220ºC, the manifold at 80ºC and the transfer line was 320ºC. The GC/MS/MS was operated in the electron ionization (EI) mode over a mass range of 50-650 m/z at 0.77 sec $scan^{-1}$. Identification of sterols was based on the comparison of their retention times relative to authentic standards, mass spectra of authentic standards, and available spectra in NIST05 and Wiley 07 mass spectral libraries. Sterol standards used for identification include cholesterol (Δ5-C27:1), dihydrocholesterol (C27:0), brassicasterol (Δ5,22-C28:2), desmosterol (Δ5,24-C27:2), campesterol (Δ5-C28:1), ergosterol (Δ5,7,22-C28:3), stigmasterol (Δ5,22-C29:2), β-sitosterol (Δ5-C29:1) and fucosterol (Δ5,24(28)-C29:2). The identification of 22-dehydrocholesterol (Δ5,22-C27:2), fungisterol (Δ7-C28:1) and 7-dehydroporiferasterol (Δ5,7,22-C29:3) was established based on mass spectra described in

literature (Fig. 1) (Gealt et al. 1981; Patterson, et al. 1992). The location of the double bond in the identified dehydrocholesterol is in C22 rather than C7 since the retention time of it is apart from the retention time of the 7-dehydrocholesterol standard and its fragmentation pattern is different from the one of 7-dehydrocholesterol standard. Several unknown sterols were present in the heterotrophic protist, *Gyrodinium dominans* after feeding on the alga, *Rhodomonas salina* or *Dunaliella tertiolecta*. These unknown sterols were tentatively identified based on mass spectra as: C29:0, C29:1a, C29:1b, C29:2, C30:0, C30:1, and C30:2. No authentic standards or published spectra were found that provided unambiguous identification. A Varian 3800 GC/FID (Varian Analytical Instruments, Walnut Creek, CA) equipped with a Restek RTX-65 capillary column (Crossbond 65% diphenyl-35% dimethylpolysiloxane; 15 m X 0.25 mm; 0.25 μm film thickness (RESTEK, Bellefonte, PA, USA) was employed for sterol quantitative analysis using using 5-α-cholestane as an internal standard. Injection port and detector temperatures were 280°C and 310°C, respectively. Helium was used as a carrier gas at a flow rate of 2ml min^{-1} and the flow rates of air and hydrogen were 300 and 30 ml min^{-1}. The column temperature was programmed from an initial temperature of 50 to 240°C at 20°C min^{-1}, then 240 to 300°C at 4 °C min^{-1} followed by a hold at 300°C for 10 min. The quantity of each identified sterol was calculated based on the relative response of cholesterol to the internal standard 5-α-cholestane.

III. Carbon content measurements of heterotrophic protists and algae

Algal and protist carbon contents were determined previously (Chu et al. 2008) using a procedure modified from Smith et al. (2000). Briefly, algae and protists were filtered through precombusted (450 ºC for 2 hr) 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 6 hr to remove inorganic carbonates; afterward they were capped with combusted aluminum foil and dried at 60 ºC. 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 the same as samples and these values were subtracted from the final carbon values and then algal and protist carbon contents were calculated using carbon-cell conversion factors (pg C/cell).

IV. Statistical analyses

The significance of the changes in contents (ng) of individual sterols and total sterols (ng) in culture bottles over time were analyzed by analysis of variance (1-way ANOVA). When results were significant ($p < 0.05$), Tukeys test was employed to discriminate the means.

RESULTS

I. Sterol profiles of *Rhodomonas salina*, *Dunaliella tertiolecta*, prey-depleted *Oxyrrhis marina* and *Gyrodinium dominans* inocula used for the feeding experiments

The algal prey *R. salina* contains predominantly brassicasterol (Fig.1; Δ5,22-C28:2; ≅ 99%; Table 1) and a small amount of cholesterol (Fig.1; Δ5-C27:1; ≅ 1-1.4%, Table 1). Both of these two sterols have a Δ5 double bond. The identified brassicasterol is believed to be

epibrassicasterol [24-α-methylcholesta-5,22-dien-3β-ol] (Goad et al. 1983, Bec et al. 2006). The major sterols in *D. tertiolecta* are ergosterol (Fig. 1, Δ5,7,22-C28:3), 7-dehydroporiferasterol (Fig.1; Δ5,7,22-C29:3) and fungisterol (Fig.1; Δ7-C28:1). Ergosterol, 7-dehydroporiferasterol and fungisterol account for approximately 45-49%, 29-31% and 21-26%, respectively for the total sterols (Table 2).

The prey-depleted *O. marina* that had been last fed *R. salina* 3 days prior to being used for feeding experiments, had a similar sterol profile to its algal prey, containing both brassicasterol (≅59%) and cholesterol (≅ 41%, Table 1). There were also trace amounts of 22-dehydrocholesterol (Fig. 1) and stigmasterol (Δ5, 22-C29:2, Fig. 1). The sterol composition of the prey-depleted *G. dominans* that had been last fed *R. salina* 3 days previously differed from its algal prey. In addition to cholesterol (≅ 6%) and brassicasterol (3%), the prey-depleted *G. dominans* contained unknown sterols [C29:0 (≅ 14%), C29:1 (A) (≅ 12%), C29:1 (B) (≅ 25%), C29:2 (≅ 29%), C30:0+ C30:1+C30:2 (≅ 12%)] that were not detected in *R. salina* (Table 1) and trace amounts of 22-dehydrocholesterol (Δ5, 22-C27:2) and desmosterol (Δ5, 24-C27:2). The sterols C29:1a and C29:1b are most likely stereoisomers at one carbon position, or differ in the location of the double bond. The sterol C29:1b is tentatively identified as 24-ethyl cholest-22-en-3β-ol based on fragmentation pattern compared to a published spectrum (Ghosh 1998).

The sterol profiles of prey-depleted *O. marina* and *G. dominans* that had last been fed with *D. tertiolecta* 3 and 4 days prior to being used for feeding experiments, differed significantly from their algal prey, particularly *G. dominans* (Table 2). Both protists contained sterols which were not detected in *D. tertiolecta*. Other than those found in the diet (ergosterol,

fungisterol, and 7-dehydroporiferasterol), *O. marina* contained cholesterol (≅ 53%), stigmasterol (≅ 11%), brassicasterol (≅ 7%) and 22-dehydrocholesterol (≅ 7%). Similarly, *G. dominans* contained cholesterol (≅ 5%), C29:0 (≅ 16%), C29:1a (≅ 7%), C29:1b (≅ 11%), C29:2 (≅ 36%), C30:0+ C30:1+C30:2 (≅ 12%) and trace amounts of stigmasterol.

II. Growth and sterol profiles of *Oxyrrhis marina* and *Gyrodinium dominans* fed the alga *Rhodomonas salina*

1) Predator-prey dynamics of *Oxyrrhis marina* and *Gyrodinium dominans* fed the alga *Rhodomonas salina*

Rhodomonas salina was rapidly ingested and consumed by *O. marina*. By the day 1 sampling *R. salina* concentrations dropped from the inoculation density of 50,000 cells ml^{-1} to less than half (23,651±3,689 cells ml^{-1}) while *O. marina* increased more than two-fold (1,109 ± 82 cells ml^{-1}) and reached 4,073 ± 209 cells ml^{-1} by day 3 (Fig. 2A). Similarly, *G. dominans* ingested and consumed *R. salina*, though rates of ingestion and replication were much slower than *O. marina* (Fig. 3A). The cell concentration of *G. dominans* reached 3,903± 482 cells ml^{-1} at day 4.

2) Changes in sterol contents and profiles in *Oxyrrhis marina* and *Gyrodinium dominans* fed the alga *Rhodomonas salina*

A) *Oxyrrhis marina* fed *Rhodomonas salina*

Total sterol and brassicasterol content decreased over the course of the feeding experiment (Table 3, Fig. 2B). The decline in the quantity of brassicasterol coincided with an increase in cholesterol as the protists replicated (Fig. 2A, 2B). The amount of cholesterol increased, in *O. marina -R. salina* cultures, from the initial 115.6 ± 11.1 ng to 452 ± 31.5 ng at day 3, while brassicasterol decreased from the initial 5,463.2 ± 361.6 ng to 1,636.8 ± 57.3 ng. There were also trace amounts of 22-dehydrocholesterol present. The decrease of brassicasterol accounted for the decrease of total sterol over time.

B) *Gyrodinium dominans* fed *Rhodomonas salina*

As *G. dominans* grazed down *R. salina* and proliferated, the quantity of brassicasterol declined significantly over time, from the initial 8,044.2 ± 105.2 ng to 893.4 ± 101.6 ng at day 4 (Table 4). In contrast, as the protists proliferated, cholesterol and the unknown sterols C29:1a, C29:1b, C29:2, C29:0 and C30:2 +C30:1+C30 increased in abundance over time (Table 4, Fig. 3A, 3B). The amount of cholesterol augmented significantly at the end of the feeding experiment, from the initial 77.9 ± 21.8 ng to 1,916.6 ± 702.0 ng at day 4, while 22-dehydrocholesterol decreased significantly from the initial 93.9 ± 4.4 ng to a trace amount at day 4. Total sterol content increased slightly, but not significantly, over time. There was a trace amount of desmosterol, which was not detected in the algal prey, *R. salina*, but present in the prey-depleted *G. dominans* (Table 1).

III. Growth and sterol profiles of *Oxyrrhis marina* and *Gyrodinium dominans* fed the alga *Dunaliella tertiolecta*

1) Predator-prey dynamics of *Oxyrrhis marina* and *Gyrodinium dominans* fed the alga *Dunaliella tertiolecta*

As previously reported (Chu et al. 2008), *O. marina* quickly grazed down *D. tertiolecta* (Fig. 4A). The pattern of grazing and growth of *G. dominans* was similar to *O. marina*, but much slower than *O. marina* (Fig. 5A). The cell densities of *O. marina* and *G. dominans* were 2,767 ± 326 cells ml^{-1} at day 3 and 2,194 ± 146 cells ml^{-1} at day 4, respectively.

2) Changes in sterol contents and profiles in *Oxyrrhis marina* and *Gyrodinium dominans* fed the alga *Dunaliella tertiolecta*

A) *Oxyrrhis marina* fed *Dunaliella tertiolecta*

The sterol species, ergosterol, fungisterol, and 7-dehydroporiferasterol, derived from *D. tertiolecta* decreased over time as *O. marina* proliferated (Table 5, Fig. 4A, 4B). 22-dehydrocholesterol and brassicasterol increased from the initial 103.6 ± 11.2 ng and 87.0 ± 16.6 ng to 1,934.6 ± 123.6 ng and 689.8 ± 28.1 ng respectively at day 2, corresponding to 19 and 9 fold increases, then decreased to 1,220.7 ± 56.7ng and 355.2 ± 30.6 ng respectively, at day 3 (Table 5). Similarly, stigmasterol increased to 316.9 ± 18.8 ng from the initial 276.7 ± 28.9 ng at day 1, then decreased afterward, to 120.9 ± 3.8 ng at day 3. The content of cholesterol remained

static over the course of the feeding experiment (Table 5, Fig. 4B). Total sterol content increased from the initial 3,383.1 ± 168.3 ng to 4,059.9 ± 114.4 ng at day 1 and 4,059.0 ± 208.5 at day 2 then decreased to 2,854.4 ± 66.9 ng (Table 5). The increases and decreases of 22-dehydrocholesterol, brassicasterol and stigmasterol probably accounted for the up and down of total sterol content. Cholesterol, stigmasterol, brassicasterol, and 22-dehydrocholesterol, which were not found in *D. tertiolecta*, but present in prey-depleted *O. marina* (Table 2) and in the protist during feeding, must be produced by the protist via bioconversion of phytosterols or de novo synthesis.

B) *Gyrodinium dominans* fed *Dunaliella tertiolecta*

Total sterol content did not change significantly until day 4. By day 4 (Table 6), total sterol content had increased significantly from the initial 4,194.3 ± 665.9 ng to 9,450.5 ± 390.3 ng (Table 6). Cholesterol levels fluctuated over time and increased significantly from the initial 85.0 ± 23.3 ng to 978.6 ± 155.7 ng at day 1 then decreased to 113.4 ± 36.3 ng at day 2, then increased back to a level similar to day 2 at day 4 (892.7 ± 121.2 ng). Similarly the amount of fungisterol increased significantly at day 1, then decreased to a level similar to day 0 at day 2, but at day 4 it increased back to a level similar to day 1. Sterols including unknown sterols that were present in prey depleted *G. dominans* cultures, but not found in *D. tertiolecta* cultures increased in parallel with *G. dominans* proliferation (Fig. 5A, 5B): brassicasterol (from the initial 135.0 ± 38.4 ng to 695.3 ± 87.9 ng at day 4), C29:0 (from the initial 157.7 ± 68.4 ng to 1,304.7 ± 119.1 ng) C29:1a (from the initial 338.1 ± 243.9 ng to 937.3 ± 29.9 ng at day 4), C29:1b (from the initial 661.2 ± 18.4 to 1,074.1 ± 100.9 ng at day 4), C29:2 (from the initial 166.6 ± 144.7 to

1779.6 ± 203.6 ng at day 4), and C30:2+C30:1+C30:0 (from the initial 284.0 ± 40.9 ng to 688.9 ± 185.3 ng). In contrast, ergosterol, one of the major sterols present in *D. tertiolecta*, decreased by almost half of its initial value at day 4. The amount of 7-dehydroporiferasterol did not change significantly over time. Trace amounts of stigmasterol were also present.

IV) Efficiency of cholesterol production (ng cholesterol per unit of carbon ingestion) by heterotrophic protists at the phytoplankton-heterotrophic protist interface

Sterols produced by both *O. marina* and *G. dominans* include cholesterol and brassicasterol. Cholesterol was produced when the heterotrophs were fed either *R. salina* or *D. tertiolecta*, but brassicasterol was only produced when *D. tertiolecta* was the algal prey. Additionally, *O. marina* produced 22-dehydrocholesterol when fed *D. tertiolecta* and *G. dominans* produced seven unknown 29 and 30 carbon sterols when fed either *R. salina* or *D. tertiolecta*. All other sterols detected in the feeding experiments either decreased over time or fluctuated in abundance. Since cholesterol was the only sterol produced by both heterotroph species regardless of algal prey species and it is by far the most abundant sterol in marine organisms at higher trophic levels the production efficiencies of cholesterol by *O. marina* and *G. dominans* were determined.

Cholesterol production by heterotrophic protists was calculated using carbon content values derived from direct measurement (Table 7). Production efficiency was defined as the ng sterol present in each culture bottle at the end of a feeding experiment minus the ng of sterol contained in the inoculum and in unconsumed algae normalized to the mg of algal carbon

consumed by the heterotrophic protist [production efficiency = (final ng sterol – (inoculum ng sterol + ng unconsumed algal sterol)) ÷mg algal C consumed]. Both protist species appear to be able to produce cholesterol either via bioconversion of dietary sterols or de novo synthesis. When *G. dominans* and *O. marina* were fed with *R. salina*, which contains mainly brassicasterol, both protists assimilated and/or produced significant amounts of cholesterol, probably, at the expense of brassicasterol (Tables 3 and 4). Calculation according to carbon contents derived from direct algal measurement are equivalent to a production of 987.7 ± 377.7 ng and 172.5 ± 16.2 ng cholesterol per mg *R. salina* carbon consumed by *G. dominans and O. marina*, respectively. Compared to the cholesterol concentration (cholesterol per mg algal C) in *R. salina*, the levels of cholesterol were "upgraded" to a magnitude of nearly 5 to 30 fold at the phytoplankton-heterotrophic protist interface. Feeding the alga, *D. tertiolecta*, which contains no cholesterol, to *G. dominans* and *O. marina*, a net production of cholesterol by *G. dominans* and *O. marina* is 871.8 ± 130.8 ng and 123.2 ± 30.6 ng per mg *D. tertiolecta* carbon consumed, respectively (Table 7).

DISCUSSION

As indicated earlier, sterols, or closely related compounds, are important nutrients for all eukaryotic organisms. Sterol requirements for the genus *Daphnia*, the dominant metazoan grazer in freshwater habitats, and copepods, the dominant metazoan grazer in marine environments have been addressed by several studies (Klein Breteler et al. 1999, 2004, 2005; Crockett and Hassett 2005; Martin-Creuzburg et al. 2005a, 2005b, 2006, 2008; Bec et al. 2006). Not only is dietary sterol content one of the crucial factors in determining food quality for Daphnia (Von

Elert et al. 2003; Martin-Creuzburg and Von Elert 2004; Martin-Creuzburg et al. 2005b), but the sterol species present in the diets also appears to be critical for the cladoceran's survival, growth and reproduction, although sterol-like compounds such as tetrahymanol and hopanoids could serve as sterol surrogates and partially release *Daphnia* fed on a sterol-deficient diet from sterol limitation (Martin-Creuzburg et al. 2005a). As with their freshwater counterpart, Daphnia, dietary essential fatty acids and sterols are essential and could limit the reproductive output and somatic growth of copepods (Klein Breteler et al. 1999, 2004, 2005; Crockett and Hassett 2005). Thus, algal sterol content and quality are potential limiting factors regulating/controlling the dynamic of metazoan grazer populations such as Daphnia and copepods, thereby determining the structure of aquatic food webs. However, sterol- limited growth and production could be ameliorated by insertion of an additional intermediary trophic level. As trophic links, the heterotrophic ciliates, *Colpidium campylum* and *Cyclidium* sp., and the heterotrophic nanoflagellate, *Paraphysomons* sp. were able to upgrade the food quality of their prey by producing essential fatty acids, sterols or sterol-like compounds and subsequently enhance the growth and reproduction of their predator, Daphnia (Martin-Creuzburg et al. 2005a, 2006, 2008; Bec et al. 2006). The presence of sterols in the nanoflagellate *Paraphysomons* sp. was credited for the improved growth and increased fecundity in *D. magna* fed the nanoflagellate previously maintained on cyanobacteria which are deficient in sterols (Bec et al. 2006). Similarly, feeding the 2 copepods, *Temora longicornis* and *Pseudocalanus elongatus* the heterotrophic protist, *O. marina* previously fed with *Dunaliella* sp., which contains low level of sterol and is deficient in LCn-3 EFA, significantly enhanced survivorship and development of copepod young nauplius larvae relative to a copepod diet of *D. tertiolecta* alone (Klein Breteler et al. 1999). Increased sterol content and production of new sterol species and LCn-3 EFAs by *O. marina* fed

Dunaliella sp. is believed to be the key factor contributing to the maturity improvement in the two tested copepod species.

However, presently the quantitative significance of sterol upgrading and/or repacking by heterotrophic protists in both freshwater and marine ecosystems is unclear. The effectiveness and quantitative significance of sterol production and phytosterol bioconversion at the phytoplankton-heterotrophic protist interface has not been determined. The present study is the first investigating/examining the kinetics and efficiency of sterol production and bioconversion of phytosterols by heterotrophic protists and their quantitative significance. Additionally, although *O. marina* seems able to synthesize sterols and/or modify those present in its diet (Klein Breteler et al. 1999; Adolf et al. 2007), it is uncertain if other species have similar capabilities. There is a need to investigate if sterol synthesis and/or modification are a widespread phenomenon among heterotrophic protists and its ecological effects. No evidence was noted for sterol synthesis in a ciliate (*Strombidium sulcatum*) studied by Klein Breteler et al. (2004).

Consistent with previous findings (Goad et al. 1983; Klein Breteler et al. 1999; Leblond and Chapman 2004), the algal prey *R. salina* contains mainly brassicasterol (≅ 99%) and a very small amount of cholesterol (≅ 1.0-1.4%). Klein Breteler et al (1999) reported 98% brassicasterol and 2% cholesterol in *R. salina*. The identified brassicasterol in the present study is believed to be epibrassicasterol (Goad et al. 1983; Bec et al. 2006). Three major sterols, 7-dehydroporiferasterol (38-50%), ergosterol (28-36%) and fungisterol (14-22%) were noted in *D. tertiolecta*. Previous studies also noted high levels of 7-dehydroporiferasterol (46%) and ergosterol (22%) in *D. tertiolecta* **(**Patterson et al. 1992; Zelazny et al. 1995). On the other hand,

in another two studies, only fungisterol (trace amount and 0.7 µg mg C^{-1}) and trace amounts of Δ7-C29:1, Δ7,24(28)-C28:2 and Δ7,24(28)-C29:2 were found in *D. tertiolecta* (Klein Breteler et al. 1999; Klein Breteler et al. 2004). However, these last two studies did not utilize authentic sterol standards for identification and did not provide detailed information about how sterols were identified using GC/MS, thus making it difficult to compare our results with theirs.

Our results demonstrate clearly that the two examined protists, *Oxyrrhis marina* and *Gyrodinium dominans,* are capable of producing new sterols species via either bioconversion of dietary sterols or de novo synthesis. The sterol profiles of both heterotrophic protist species do not completely reflect their algal diets. It is interesting to note the variety of sterols, both in quantity and quality, found in the protists but not in the algal prey after feeding with the two algal species. Considering that *R. salina* contains almost exclusively brassicasterol (99%) but low levels of cholesterol (1-1.4%), the amounts and percentage of cholesterol found in the two protists after feeding with this alga (Tables 1, 3 and 4) are noteworthy. The dramatic decrease of brassicasterol concomitant with the increase of cholesterol in the two protist species during the feeding experiments suggest that protists selectively and effectively assimilated cholesterol and probably catabolized brassicasterol for energy. Alternatively they might have converted brassicasterol to 22-dehydrocholesterol by dealkylation (removal of the methyl group from C-24) and then to cholesterol by hydrogenation (hydrogenation of the double bond at C22). Similarly, when the two protist species were fed with *D. tertiolecta,* which contains primarily ergosterol, 7-dehydroporiferasterol, and fungisterol, other sterols not found in the prey appeared in the predators (Tables 2, 5 and 6). These sterols include cholesterol, stigmasterol, brassicasterol, 22-dehydrocholesterol, and desmosterol. Additionally, several C29 and C30 unknown sterols were

found in *G. dominans* fed with either *R. salina* or *D. tertiolecta*. In a previous study, cholesterol, dehydrocholesterol, brassicasterol, stigmasterol and campesterol were also found in *O. marina* fed a *Dunaliella* sp. containing primarily fungisterol (Klein Breteler et al. 1999). However, the levels of the newly produced sterols either fluctuated (e.g., brassicasterol and stigmasterol in *O. marina* fed *D. tertiolecta*, Table 5) over time, or decreased at the end of the feeding experiments (e.g., 22-dehydrocholesterol in *G. dominans* fed *R. salina* and *O. marina* fed *D. tertiolecta*, Tables 4 and 5) except cholesterol and unknown sterols (in *G. dominans* fed either *R. salina* or *D. tertiolecta*, Table 4 and 6), which increased over time. Similarly, the levels of sterols derived from algal prey either fluctuated or decreased at the end of the experiment. There is no consistent pattern. These results suggest that coupling of assimilation and bioconversion of dietary sterols took place in protists using algae as an energy source.

Mechanisms for dietary sterol modification/bioconversion could include alkylation, dealkylation, hydrogenation and dehydrogenation (Goodwin 1980). It is possible that both *O. marina* and *G. dominans*: (1) convert dietary brassicasterol to 22-dehydrocholesterol by dealkylation of the methyl group at C24 and then to cholesterol by hydrogenation the double bond at C22; (2) convert the dietary ergosterol to brassicasterol by hydrogenation of the double bond at C7 and then to 22-dehydrocholesterol by dealkylation or to stigmasterol by alkylation at C24; (3) convert dietary brassicasterol to cholesterol by dealkylation and hydrogenation then to 22-dehydrocholesterol by desaturation; (4) alkylation of 22-dehydrocholesterol to brassicasterol; (5) convert the dietary 7-dehydroporiferasterol to stigmasterol via hydrogenation of the double bond at C7; and (6) though unlikely, synthesize cholesterol de novo, then converted it to 22-dehydrocholesterol via dehydrogenation (desaturation). Therefore, 22-dehydrocholesterol could

be an important metabolic intermediate if the bioconversion pathways described in 1-4 and 6 above do take place. While different biosynthetic routes of sterols in various organisms, including algae, have been recently reviewed (Bouvier et al. 2005), the mechanisms of bioconversion of the synthesized sterols to the other sterols and associated enzymes is unclear. Thus, at this point the pathways of phytosterol bioconversions and the physiological importance/significance of sterol bioconversion in heterotrophic protists are not known. The increased cholesterol levels in *O. marina* and *G. dominans* fed *R. salina* and the cholesterol-deficient *D. tertiolecta* suggest that these two protists species produce cholesterol via bioconversion of phytosterols. Further planned studies using stable isotope substrates will be required to determine whether or not these two protists synthesize cholesterol de novo. Results of our preliminary trials revealed that *O. marina* cannot synthesize cholesterol de novo using ^{13}C-labeled acetate provided in the medium. Our previous investigation suggest that *G. dominans* was unable to utilize acetate in the medium for growth or fatty acid synthesis (Lund et al. 2008).

It appears that there are species-specific differences in sterol metabolism. *O. marina* contains cholesterol, 22-dehydrocholesterol and brassicasterol when fed *R. salina* or *D. tertiolecta* (Tables 1, 2, 3, and 5), yet *G. dominans* contains cholesterol, brassicasterol, 22-dehydrocholesterol, demosterol and unknown sterols (C29:1a, C29:1b, C29:2, C29:0 and C30:2, C30:1, C30:0) when fed these same algal species (Tables 1, 2, 4 and 6). Very likely, each species utilizes different modes in sterol metabolism to modify dietary sterols and/or synthesize sterols de novo. *G. dominans* produced significant amounts of unknown sterols when it was fed *R. salina* or *D. tertiolecta.* Possibly, the unknown sterols C29:1a, C29:1b, C29:2, C29:0 and C30:2, C30:1 and C30:0 found in *G. dominans* are alkylation products of dietary sterols. For example, adding an ethyl group to cholesterol and a methyl group to brassicaterol would produce C29:1

and C29:2, respectively. Unknown C29 sterols with one or two double bonds were also noted in the parasitic syndinian dinoflagellate *Amoebophyra* sp. (Leblond et al. 2006). Dinosterol (Δ22-C30:1), a dominant sterol reported previously in both auto- and hetero- trophic dinoflagellates (Leblond and Chapman 2002), was not found in the *Amoebophyra* sp. (Leblond et al. 2006). We did not detect dinosterol in either *O. marina* or *G. dominans*.

Free sterols such as cholesterol are primarily used as membrane components and play a key role in regulating membrane permeability/fluidity (Finkelstein and Cass 1967; Bruckdorfer et al. 1969; Demel et al. 1972), osmotic sensing (Zelazny et al. 1995), and thermal tolerance (Crockett 1998). Cholesterol is not only an important membrane constituent in animals, but is also an important fundamental intermediate, a critical precursor for many physiologically functional biochemicals such as steroid hormones, bile salts, and vitamin D in higher animals and a precursor of the molting hormones, ecdysteroids in crustaceans (Fingerman 1987). Cholesterol is the dominant sterol in crustaceans and accounts for 90-95% of total sterols (Kanazawa 2001). Copepods may require optimal levels of cholesterol for membrane function, and cholesterol-enriched diet stimulated both egg production and hatching rates without altering the plasma membrane cholesterol content in the copepod *Acartia hudsonica* (Crockett and Hassett 2005). Thus, it is possible that under some conditions dietary cholesterol is a limiting factor for copepod production in nature. While some animals including mammals can synthesize cholesterol from acetate and mevalonate, crustaceans including calanoid copepods are incapable of synthesizing sterols de novo, thus dietary sources are essential. In both freshwater and marine environments, phytoplankton is the major source of sterols. Although copepods have been shown to selectively absorb specific dietary sterols (Prahl et al. 1984), the relative importance of

particular sterols to the growth and fecundity of calanoid copepods is currently unknown. Cholesterol is by far the most prevalent sterol in metazoans, thus this may be a significant factor in the nutritional value of heterotrophic protists relative to algae for marine zooplankton.

Since in the present study we did not feed the two studied protists, *O. marina* and *G. dominans* to copepods to investigate the benefit of "cholesterol upgrading" by them, the ecological significance of "cholesterol upgrading" and "phytosterol bioconversion" by these two protists remains to be verified. However, evidence of trophic upgrading of food quality by these two heterotrophic protists fed similar algal prey has been reported in our previous study (Veloza et al. 2006) and by other investigators (Tang & Taal 2005, Klein Breteler et al. 1999) by feeding them to copepods, though these studies focused on the impact of LCn-3 EFAs, but not sterols. Sterol production and bioconversion of dietary sterols by intermediate heterotrophic protists at the phytoplankton-zooplankton interface could be a critical issue and its ecological implication is worthy of further investigation.

In conclusion, results from the present study suggest that both *O. marina* and *G. dominans* are able to produce new sterol species not found in their diets either via bioconversion of phytosterols by alkylation (insertion of a methyl or an ethyl group), dealkylation (removal of a methyl or an ethyl group), dehydrogenation (insertion of a double bond) and hydrogenation (removal of a double bond) of dietary sterols, or de novo synthesis. The findings of effective assimilation of dietary cholesterol and bioconversion of phytosterols to cholesterol by the two tested protists may be ecologically important and has ecological implications when primary production is dominated by nano- and pico-plankton or algal species deficient in cholesterol.

Further study is needed to determine if cholesterol production by heterotrophic protists is a crucial factor in determining food quality for zooplankton in both marine and freshwater systems. Considering the physiological and biochemical roles of cholesterol and its dominance in living organisms, “cholesterol upgrading” by heterotrophic protists is likely important in nature.

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FIGURE LEGENDS

Figure 1 : Structures of major sterols founded in protists and the algae; Each sterol is represented by a body group and a side chain structure; a, b, c = body (R) structures ; 1 to 5 = side chain (SC) structures; Fungisterol = 1a ; Ergosterol = 2b ; 7-dehydroporiferasterol = 3b ; Brassicasterol = 2c ; Stigmasterol = 3c ; 22-dehydrocholesterol = 4c ; Cholesterol = 5c ; 29:1 = 3d (possibly).

Figure 2: (A) Predator-prey dynamic of *Oxyrrhis marina* fed *Rhodomonas salina* (n=6 bottles of cultures at each sampling date); (B) Changes of sterol profile and content in *Oxyrrhis marina* fed *Rhodomonas salina*. All values are mean ± SD of 3 replicates, each of which is derived from the pooled lipids of ⅄ culture bottles.

Figure 3: (A) Predator-prey dynamic of *Gyrodinium dominans* fed *Rhodomonas salina* (n=6 bottles of cultures at each sampling date); (B) Changes of sterol profile and content in *Gyrodinium dominans* fed *Rhodomonas salina*. All values are mean ± SD of 3 replicates, each of which is derived from the pooled lipids of 2 culture bottles. Unknown sterols: sum of 29:0, 29:1a, 29:1b, 29:2, 30:0, 30:1 and 30:2.

Figure 4: (A) Predator-prey dynamic of *Oxyrrhis marina* fed *Dunaliella tertiolecta* (n=6 bottles of cultures at each sampling date); (B) Changes of sterol profile and content in *Oxyrrhis marina* fed *Dunaliella tertiolecta*. All values are mean ± SD of 3 replicates, each of which is derived from the pooled lipids of 2 culture bottles.

Figure 5: (A) Predator-prey dynamic of *Gyrodinium dominans* fed *Dunaliella tertiolecta* (n=6 bottles of cultures at each sampling date); (B) Changes of sterol profile and content in *Gyrodinium dominans* fed *Dunaliella tertiolecta*. All values are mean ± SD of 3 replicates, each of which is derived from the pooled lipids of 2 culture bottles. Unknown sterols: sum of 29:0, 29:1a, 29:1b, 29:2, 30:0, 30:1 and 30:2.

Table 1. Sterol composition and content of *Rhodomonas salina* used as prey for two heterotroph feeding experiments and prey-depleted cultures of *Oxyrrhis marina* and *Gyrodinium dominans* previously maintained on *R. salina* for 3 and 4 days respectively. All values are mean ± SD of 3 replicates, each of which was derived from the pooled lipids of two culture bottles.

	O. marina		***R. salina***		***G. dominans***		***R. salina***	
Sterol	**ng**	**%**	**ng**	**%**	**ng**	**%**	**ng**	**%**
22-dehydrocholesterol	trace	--			trace	--		
Cholesterol	103.3±6.4	41.2±1.9	72.7±1.2	1.4	36.6±12.2	5.6±2.1	64.9±4.7	0.8±0.1
Desmosterol					trace	--		
Brassicasterol	147.2±4.5	58.8±1.9	5120.2±221.1	98.6	22.3±5.8	3.4±1.0	7920.1±258.3	99.2±0.1
Stigmasterol	trace	--						
*C29:0					90.0±6.2	13.6±0.6		
*C29:1a					79.6±11.9	12.1±1.6		
*C29:1b					161.7±10.0	24.5±0.6		
*C29:2					187.8±14.2	28.5±1.2		
*C30:0+*C30:1+ *C30:2					81.1±4.7	12.3±0.6		
Total	250.5±6.6		5192.9±225.2		659. ±124.1		7985.0±257.3	
#cells	$3x10^5$		$3x10^7$		$3x10^5$		$3x10^7$	

22-dehydrocholesterol = Δ5,22-C27:2; cholesterol = Δ5-C27:1; brassicasterol = Δ5,22-C28:2; desmosterol = Δ5,24-C27:2; stigmasterol = Δ5,22-C29:2; trace < 20ng. *Sterols are identified with number of carbon atoms and number of double bonds based on molecular mass determined by GC/MS analyses. The number of prey-depleted cells used for sterol analyses was twice the number used in the feeding experiments; values presented in the table have been normalized to the number of cells used for inoculations in feeding experiments (Tables 3 and 4).

Table 2. Sterol composition and content of *Dunaliella tertiolecta* used as prey for two heterotroph feeding experiments and prey-depleted cultures of *Oxyrrhis marina* and *Gyrodinium dominans* previously maintained on *D. tertiolecta* for 3 and 4 days respectively. All values are mean ± SD of 3 replicates, each of which was derived from the pooled lipids of two culture bottles.

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	O. marina		***D. tertiolecta***		***G. dominans***		***D. tertiolecta***	
Sterol	**ng**	**%**	**ng**	**%**	**ng**	**%**	**ng**	**%**
22-dehydrocholesterol	40.7±1.4	7.0±0.2			trace	--		
Cholesterol	307.4±7.8	53.2±1.3			56.7±7.6	5.3±0.7		
Desmosterol					trace	--		
Brassicasterol	41.2±4.3	7.1±0.8			trace	--		
Ergosterol	31.5±6.4	5.4±1.1	824.3±166.5	45.2±8.9	9.7±16.9	0.9±1.6	1447.7±101.8	48.6±1.7
Fungisterol	31.5±6.3	5.5±1.1	466.0±167.9	25.5±8.8	52.5±20.6	4.9±1.8	622.4±98.1	20.8±1.6
Stigmasterol	64.4±4.0	11.1±0.7			trace			
7-dehydroporiferasterol	61.0±6.2	10.6±1.1	530.6±163.8	29.3±9.5	80.5±8.6	7.5±0.7	911.5±88.8	30.6±2.4
*C29:0					168.3±1.9	15.7±0.8		
*C29:1a					72.5±8.9	6.7±0.7		
*C29:1b					122.4±18.7	11.4±1.6		
*C29:2					384.9±29.1	35.8±2.4		
*C30:0+*C30:1+ *C30:2					128.5±11.5	12.0±1.2		
Total	577.8±1.6		1820.9±40.5		1076.0±42.9		2981.6±247.1	
#cells	$3x10^5$		$3x10^7$		$3x10^5$		$3x10^7$	

22-dehydrocholesterol = Δ5,22-C27:2 ; cholesterol = Δ5-C27:1; brassicasterol = Δ5,22-C28:2; desmosterol = Δ5,24-C27:2; stigmasterol = Δ5,22-C29:2; ergosterol = Δ5,7,22-C28:3; fungisterol = Δ7-C28:1 and 7-dehydroporiferasterol = Δ5,7,22-C29:3; trace < 20ng. *Sterols are identified with number of carbon atoms and number of double bonds based on molecular mass determined by GC/MS analyses. The number of prey-depleted cells used for sterol analyses was twice the number used in the feeding experiments;

values presented in the table have been normalized to the number of cells used for inoculations in feeding experiments (Tables 5 and 6).

Table 3. Sterol composition and content (ng) of *Oxyrrhis marina* cultures fed *Rhodomonas salina*; all values are mean ± SD of 3 replicates, each of which was derived from the pooled lipids of 2 culture bottles.

Sterols (ng)	**Om-Rs Initial (Day 0)**	**Om-Rs Day 1**	**Om-Rs Day 2**	**Om-Rs Day 3**
22-dehydrocholesterol	trace	trace	trace	trace
Cholesterol	115.6±11.1[a]	145.8±59.5[a]	258.1±27.2[b]	452.0±31.5[c]
Brassicasterol	5463.2±361.6[c]	3863.9±1393.2[bc]	2869.6±159.0[ab]	1636.8±57.3[a]
Total	5578.7±368.8[b]	4009.8±1452.6[ab]	3127.7±167.7[a]	2088.8±26.2[a]
# *O. marina* (x 10^5)	3	5.5±1.9	23.10±0.4	24.5±0.3
# *R. salina* (x 10^5)	300	113.2±49.0	--	--
O. marina μg C	101	184.0±62.0	776.0±15.0	822.0±8.0
R. salina μg C	1950	736.0±318.0	--	--
Total μg C	2051	920.0±381.0	776.0±15.0	822.0±8.0

All values are mean ± SD (n = 3). Different letters denote significant differences at the $p < 0.05$ level. 22-dehydrocholesterol = Δ5,22-C27:2; cholesterol = Δ5 C27:1 and brassicasterol = Δ5,22-C28:2; trace < 20 ng.

Table 4. Sterol composition and content (ng) of *Gyrodinium dominans* cultures fed *Rhodomonas salina*; all values are mean ± SD of 3 replicates, each of which was derived from the pooled lipids of 2 culture bottles. .

Sterols (ng)	**Gd-Rs Initial (Day 0)**	**Gd-Rs Day 1**	**Gd-Rs Day 2**	**Gd-Rs Day 4**
22-dehydrocholesterol	93.9±4.4 [b]	87.5±5.9 [b]	91.8±6.5 [b]	trace [a]
Cholesterol	77.9±21.8 [a]	73.4±25.9 [a]	71.9±19.9 [a]	1916.6±702.0 [b]
Desmosterol	trace	trace	trace	trace
Brassicasterol	8044.2±105.2 [d]	6919.3±91.6 [c]	6149.7±81.4 [b]	893.4±101.6 [a]
*C29:0	104.8±21.2 [a]	224.5±89.4 [a]	323.4±166.9 [a]	823.5±140.0 [b]
*C29:1 (a)	85.6±7.5 [a]	357.7±33.8 [b]	686.1±35.1 [c]	1518.7±89.2 [d]
*C29:1 (b)	178.5±7.0 [a]	353.1±11.1 [b]	604.0±37.0 [c]	1650.6±126.5 [d]
*C29:2	64.3±9.8 [a]	269.9±21.3 [b]	454.7±23.1 [c]	1975.7±242.3 [d]
*C30:2 +*C30:1 + *C30:0	48.0±43.9	252.0±237.7	435.3±388.9	900.0±471.3
Total	8674.7±179.3	8537.5±296.2	8817.0±669.2	9678.5±873.2
#*G. dominans* (x 10^5)	3	4.0±0.1	6.2±0.1	23.2±1.0
# *R. salina* (x 10^5)	300	204.0±23.6	190.3±3.0	14.1±3.9
G. dominans μg C	85	112.0±3.0	174.0±3.0	655.0±28.0
R. salina μg C	1950	1326.0±154.0	1237.0±19.0	92.0±26.0
Total μg C	2035	1438.0±156.0	1411.0±22.0	747.0±53.0

All values are mean ± SD (n = 3). Different letters denote significant differences at the $p < 0.05$ level. 22-dehydrocholesterol = Δ5,22-C27:2; cholesterol = Δ5-C27:1; brassicasterol = Δ5,22-C28:2; desmosterol = Δ5,24-C27:2; trace < 20 ng. *Sterols are identified with number of carbon atoms and number of double bonds based on molecular mass determined by GC/MS analyses.

Table 5. Sterol composition and content (ng) of sterols in *Oxyrrhis marina* cultures fed *Dunaliella tertiolecta*; all values are mean ± SD of 3 replicates, each of which is derived from the pooled lipids of 2 culture bottles.

Sterol (ng)	**Om-Dt Initial (Day 0)**	**Om-Dt Day 1**	**Om-Dt Day 2**	**Om-Dt Day 3**
22-dehydrocholesterol	103.6±11.2 [a]	555.9±59.8 [b]	1934.6±123.6 [d]	1220.7±56.7 [c]
Cholesterol	515.1±96.8	606.7±189.1	536.8±22.6	657.8±35.4
Brassicasterol	87.0±16.6 [a]	678.4±118.8 [c]	689.8±28.1 [c]	355.2±30.6 [b]
Stigmasterol	276.7±28.9 [c]	316.9±18.8 [d]	174.9±20.5 [b]	120.9±3.8 [a]
Ergosterol	1085.0±154.2 [b]	947.3±45.8 [b]	630.9±51.0 [a]	499.7±19.1 [a]
Fungisterol	542.3±126.5 [b]	453.8±118.6 [b]	92.1±18.2 [a]	trace [a]
7-dehydroporiferasterol	773.5±190.7 [b]	500.8±110.3 [b]	trace [a]	trace [a]
Total	3383.1±168.3 [b]	4059.9±114.4 [c]	4059.0±208.5 [c]	2854.4±66.9[a]
#*O. marina* (x 10^5)	3	6.8±0.0	16.4±0.4	16.6±1.0
#*D. tertiolecta* (x 10^5)	300	32.7±2.7	-	-
O. marina μg C	101	230.0±1.0	550.0±13.0	558.0±34.0
D. tertiolecta μg C	1158	126.0±10.0		
Total μg C	1259	356.0±12.0	550.0±13.0	558.0±34.0

All values are mean ± SD (n = 3). Different letters denote significant differences at the $p < 0.05$ level. 22-dehydrocholesterol = Δ5,22-C27:2; cholesterol = Δ5-C27:1; brassicasterol = Δ5,22-C28:2; ergosterol = Δ5,7,22-C28:3; fungisterol = Δ7-C28:1; stigmasterol = Δ5,22-C29:2 and 7-dehydroporiferasterol = Δ5,7,22-C29:3; trace < 20 ng.

Table 6. Sterol composition and content (ng) of *Gyrodinium dominans* cultures fed *Dunaliella tertiolecta;* all values are mean ± SD of 3 replicates, each of which is derived from the pooled lipids of 2 culture bottles. .

Sterols (ng)	**Gd-Dt Initial (Day 0)**	**Gd-Dt Day 1**	**Gd-Dt Day 2**	**Gd-Dt Day 4**
22-dehydrocholesterol	33.0±57.2	154.2±138.7	148.0±24.7	115.8±84.6
Cholesterol	85.0±23.3 [a]	978.6±155.7 [b]	113.4±36.3 [a]	892.7±121.2 [b]
Desmosterol	trace	trace	trace	trace
Brassicasterol	135.0±38.4 [a]	141.0±126.8 [a]	519.6±235.2 [b]	695.3±87.9 [b]
Stigmasterol	trace	trace	trace	trace
Ergosterol	1064.5±155.5 [b]	439.6±91.4 [a]	344.4±133.9 [a]	503.7±20.5 [a]
Fungisterol	359.5±67.0 [a]	694.5±139.3 [b]	394.4±128.7 [a]	539.7±105.5 [ab]
7-dehydroporiferasterol	910.3±45.2	683.7±242.9	576.6±109.9	918.8±56.7
*C29:0	157.7±68.4 [a]	436.7±21.1 [b]	507.4±97.9 [b]	1304.7±119.1 [c]
*C29:1a	338.1±243.9 [a]	609.3±111.8 [ab]	671.4±162.3 [ab]	937.3±29.9 [b]
*C29:1b	661.2±18.4 [a]	739.9±69.7 [a]	730.1±220.6 [a]	1074.1±100.9 [b]
*C29:2	166.6±144.7 [a]	524.0±50.7 [a]	457.3±140.4 [a]	1779.6±203.6 [b]
*C30:2 +*C30:1 + *C30:0	284.0±40.9 [a]	471.6±141.8 [ab]	311.9±75.1 [a]	688.9±185.3 [b]
Total	4194.3±665.9 [a]	5873.1±1016.2 [a]	4774.5±1236.6 [a]	9450.5±390.3 [b]
#*G. dominans* (x 10^5)	3	4.3±1.5	7.0±0.2	13.2±0.8
#*D. tertiolecta* (x 10^5)	300	298.0±9.0	249.1±2.5	60.0±12.6
G. dominans μg C	85	120.0±42.0	198.0±4.0	372.0±20.0
D. tertiolecta μg C	1158	1150.0±34.0	962.0±10.0	231.0±49.0
Total μg C	1242.8	1270.0±76.0	1159.0±7.0	604.0±51.0

All values are mean ± SD (n = 3). Different letters denote significant differences at the $p < 0.05$ level. Gd = *Gyrodinium dominans*; Dt = *Dunaliella tertiolecta*; 22-dehydrocholesterol = Δ5,22-C27:2; cholesterol = Δ5-C27:1; brassicasterol = Δ5,22-C28:2; desmosterol = Δ5,24-C27:2; stigmasterol=Δ5,22-C29:2; ergosterol = Δ5,7,22-C28:3; fungisterol = Δ7-C28:1 and 7-dehydroporiferasterol = Δ5,7,22-C29:3; trace < 20 ng. *Sterols are identified with number of carbon atoms and number of double bonds based on molecular mass determined by GC/MS analyses.

Table 7. Cholesterol production (ng per mg algal C consumed; Mean ± SD, n=3) of *G. dominans* (Gd) at Day 4 and *O. marina* (Om) at Day 3 after feeding *R. salina* (Rs) or *D. tertiolecta* (Dt) and cholesterol content in their algal preys, *R. salina* and *D. tertiolecta*. All values are mean ± SD of 3 replicates.

	G. dominans **fed *R. salina*** **(Day 4)**	***O. marina*** **fed *R. salina*** **(Day 3)**	***G. dominans*** **fed *D. tertiolecta*** **(Day 4)**	***O. marina*** **fed *D. tertiolecta*** **(Day 3)**
ng cholesterol mg algal C^{-1} consumed	987.7±377.7	172.5±16.2	871.8±130.8	123.2±30.6
ng Rs cholesterol mg C^{-1}	33.3±2.4	37.3±0.6		
ng Dt cholesterol mg C^{-1}	-	-	-	-
# Gd x 10^5	23.2±1.0	-	13.2±0.7	-
# Om x10^5	-	24.5±0.3	-	16.6±1.0
# Rs x10^5	14.1±3.9	0	-	-
# Dt x10^5	-	-	60±12.6	0
*pg C $cell^{-1}$	282±47.9	336.1±45.1	282±47.9	336.1±45.1

* Chu et al. 2008.

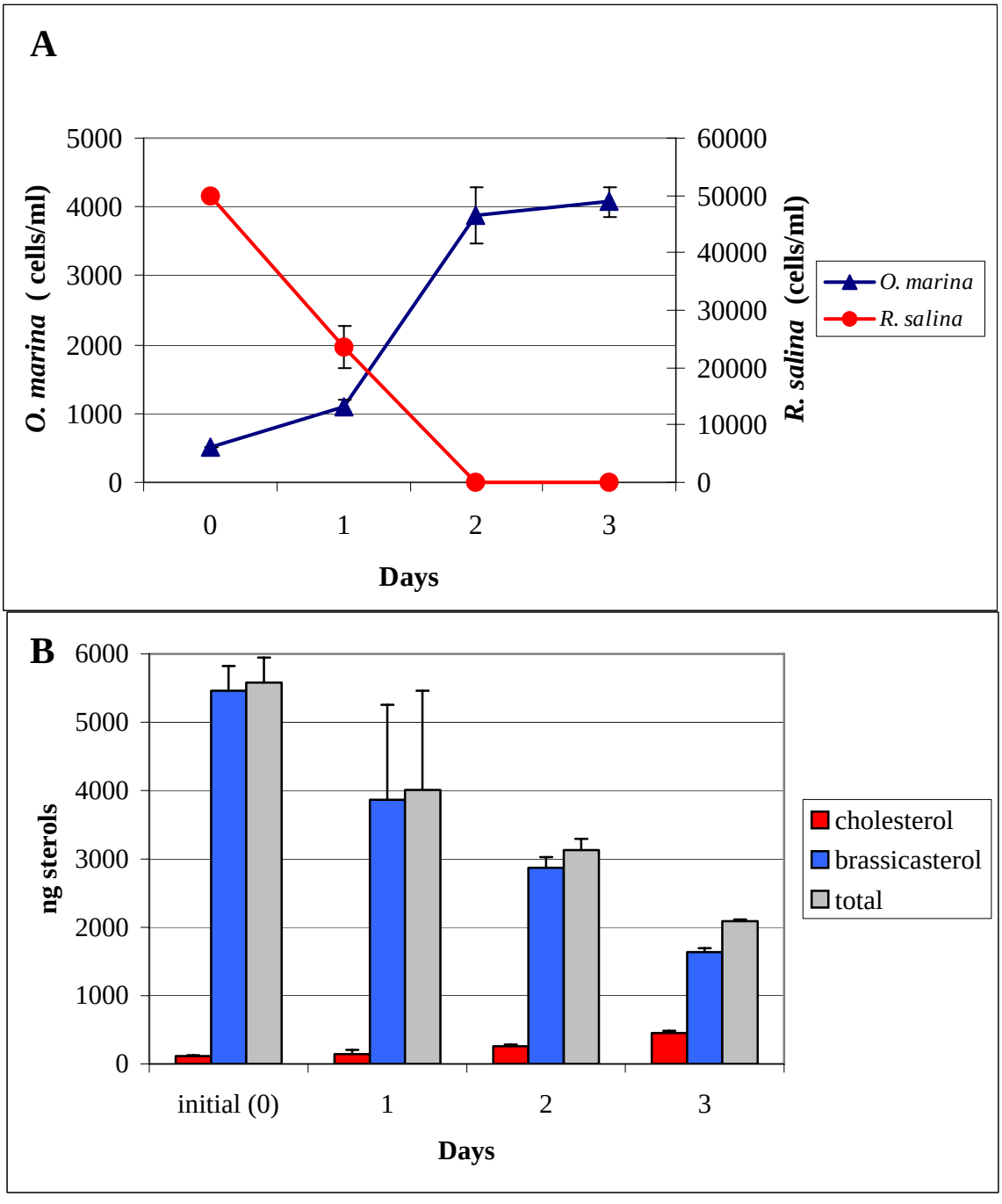

Figure2: (A) Predator-prey dynamic of *Oxyrrhis marina* fed *Rhodomonas salina* (n=6 bottles of cultures at each sampling date); (B) Changes of sterol profile and content in *Oxyrrhis marina* fed *Rhodomonas salina*. All values are mean ± SD of 3 replicates, each of which is derived from the pooled lipids of 2 culture bottles. .

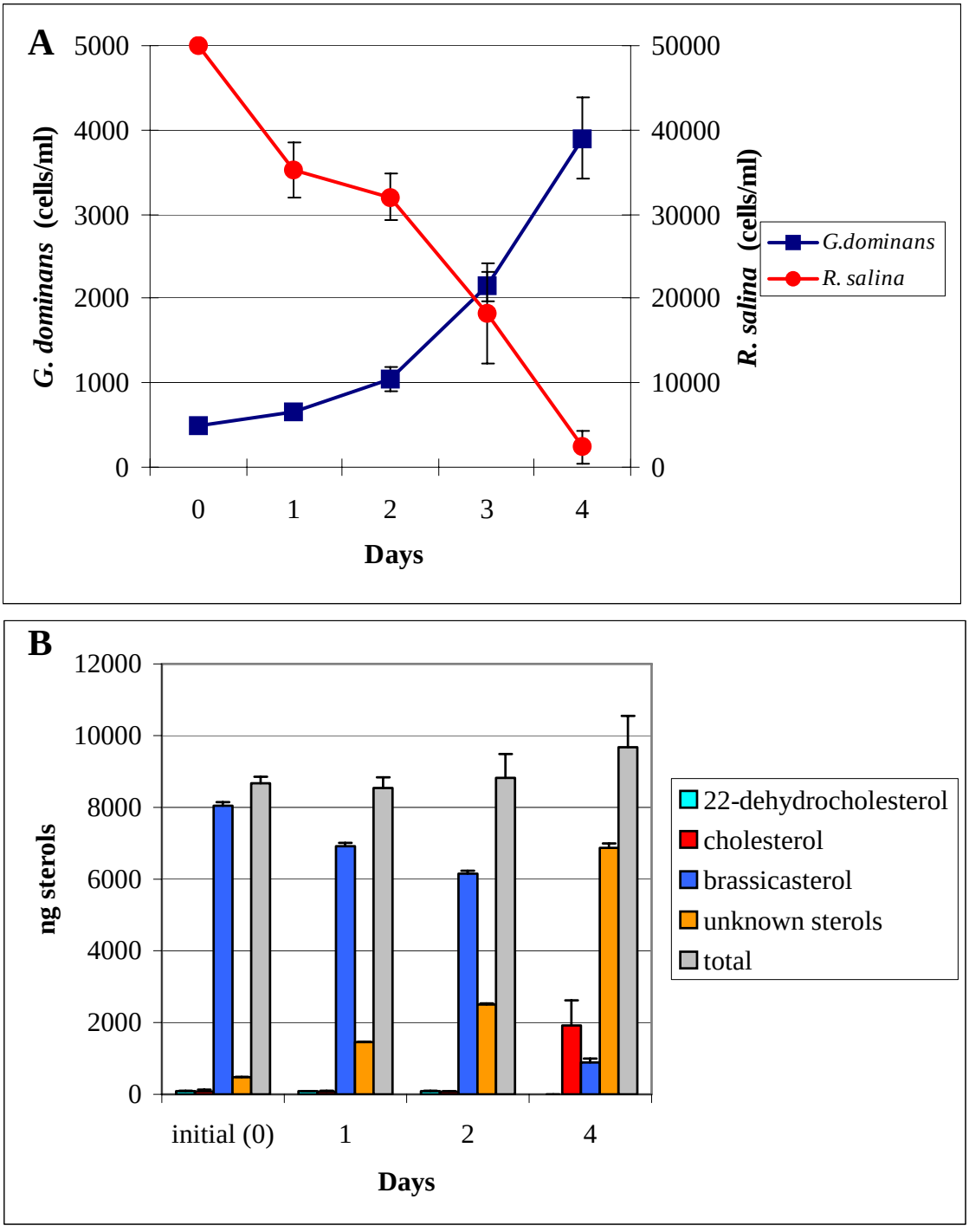

Figure 3: (A) Predator-prey dynamic of *Gyrodinium dominans* fed *Rhodomonas salina* (n=6 bottles of cultures at each sampling date); (B) Changes of sterol profile and content in *Gyrodinium dominans* fed *Rhodomonas salina*. All values are mean ± SD of 3 replicates, each of which is derived from the pooled lipids of 2 culture bottles. Unknown sterols: sum of 29:0, 29:1a, 29:1b, 29:2, 30:0, 30:1 and 30:2.

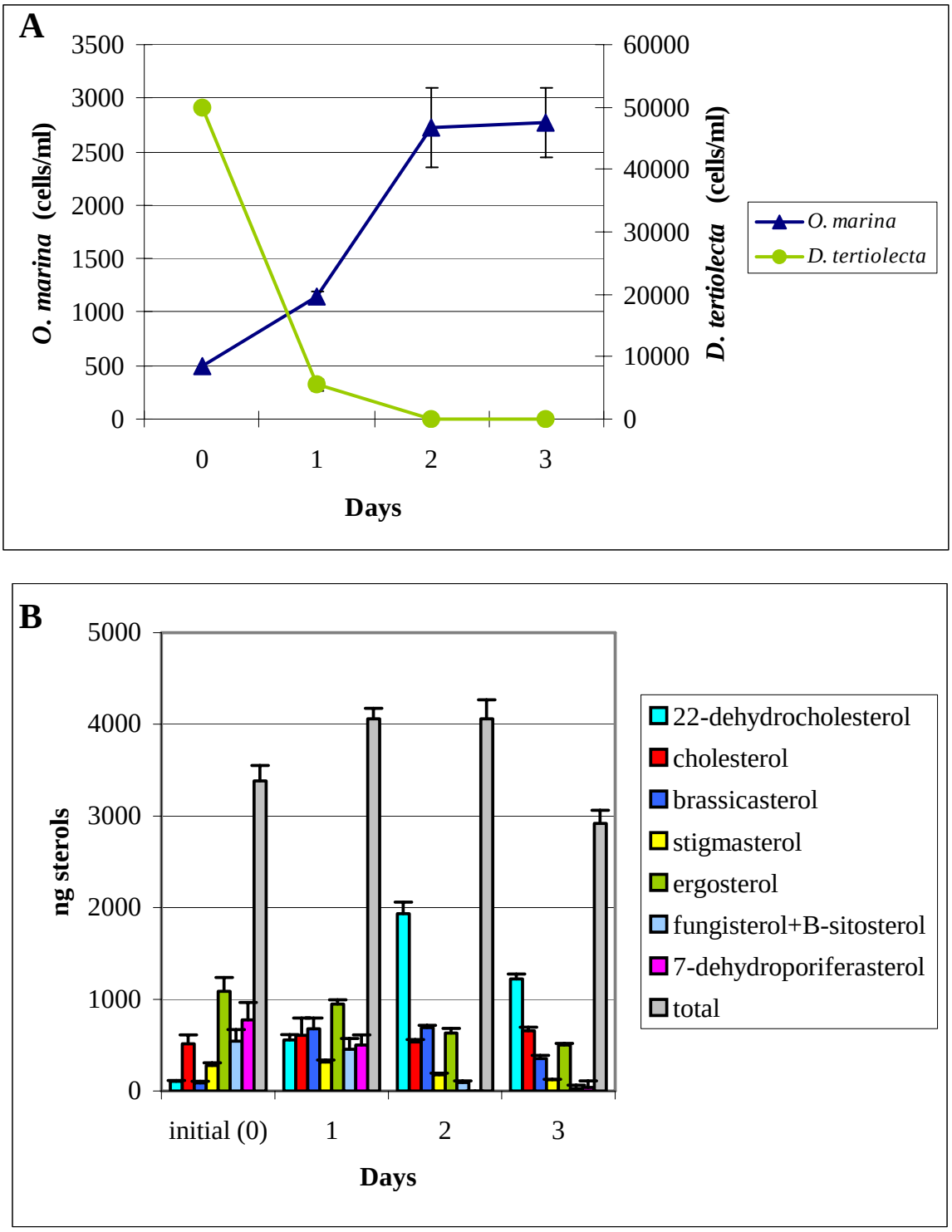

Figure 4: (A) Predator-prey dynamic of *Oxyrrhis marina* fed *Dunaliella tertiolecta* (n=6 bottles of cultures at each sampling date); (B) Changes of sterol profile and content in *Oxyrrhis marina* fed *Dunaliella tertiolecta*. All values are mean ± SD of 3 replicates, each of which is derived from the pooled lipids of 2 culture bottles.

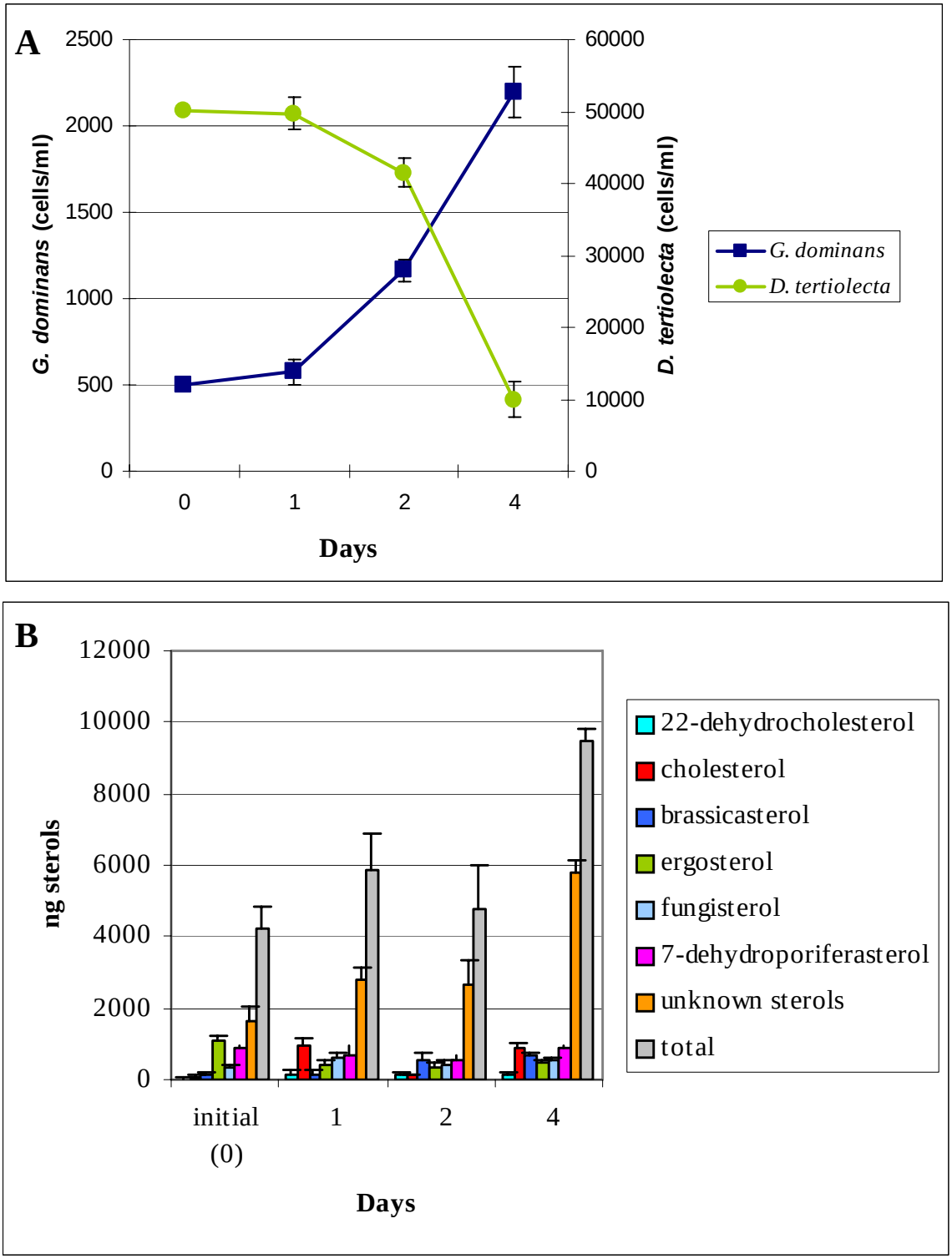

Figure 5: (A) Predator-prey dynamic of *Gyrodinium dominans* fed *Dunaliella tertiolecta* (n=6 bottles of cultures at each sampling date); (B) Changes of sterol profile and content

in *Gyrodinium dominans* fed *Dunaliella tertiolecta*. All values are mean ± SD of 3 replicates, each of which is derived from the pooled lipids of 2 culture bottles. Unknown sterols: sum of 29:0, 29:1a, 29:1b, 29:2, 30:0, 30:1 and 30:2.