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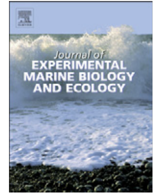

An investigation of the mechanisms for sterol synthesis and dietary sterol bioconversion in the heterotrophic protists *Oxyrrhis marina* and *Gyrodinium dominans*

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The ability of the marine heterotrophic protists *Oxyrrhis marina* and *Gyrodinium dominans* to synthesize sterols de novo and modify dietary sterols was investigated using ^{13}C-labeled substrates. De novo sterol synthesis of *O. marina* was determined by incorporation of ^{13}C acetate into the culture medium. For *G. dominans* which has low tolerance of acetate, a protozoan prey *Perkinsus marinus* that cannot synthesize sterols, was cultured with ^{13}C acetate then fed to *G. dominans*. Both heterotrophs utilized dietary ^{13}C to synthesize fatty acids de novo, but not sterols. The ability of *O. marina* and *G. dominans* to alkylate, saturate, and desaturate dietary sterols was tested using *P. marinus* incorporated with ^{13}C-labeled cholesterol as prey. *O. marina* did not modify the dietary ^{13}C-cholesterol, but *G. dominans* produced 5 labeled sterols (brassicasterol, C28:1, and unknown C28, C29 and C30 sterols) indicating that *G. dominans* has the ability to desaturate and alkylate dietary cholesterol. The ability of *O. marina* and *G. dominans* to dealkylate dietary sterols was tested by feeding them gelatin acacia microspheres (GAMs) containing ^{13}C-labeled brassicasterol. Neither heterotroph dealkylated brassicasterol to make cholesterol, but *G. dominans* alkylated and saturated brassicasterol to make 2 sterols (C29:1 and C30:0). The lack of dealkylation of brassicasterol by both protist species suggests problems with the substrate and/or delivery system since previous studies suggest that dealkylation of brassicasterol occurs when either species is fed algae containing this sterol.
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1. Introduction

Heterotrophic protists play an ecologically important role in both freshwater and marine environments by forming a trophic bridge between autotrophs, including nano, pico and microalgae, and mesozooplankton which graze both auto-and heterotrophs. As predators, heterotrophic protists consume, assimilate and repackage not only the biomass and nutrients of their prey, but, most importantly, upgrade their biochemical constituents, such as the long-chain n-3 essential fatty acids (LCn-3 EFAs), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), and sterols. These essential lipids, EPA, DHA and sterols, have a wide range of critical functions including being important structural components and precursors to bioactive molecules such as eicosanoids and steroids (Ackman et al., 1980; Sargent et al., 1987, 2002).

"Essential lipid upgrading" by heterotrophic protists has been documented in both freshwater (Bec et al., 2003; Boechat and Adrian, 2005; Martin-Creuzburg et al., 2005; Bec et al., 2006) and marine (Klein Breteler et al., 1999; Broglio et al., 2003; Veloza et al., 2006; Chu et al., 2008) ecosystems. In freshwater ecosystems sterol production and modification of dietary sterols has been most closely associated with essential lipid upgrading (Bec et al., 2006), while in marine systems essential lipid upgrading has been most closely linked to production of the LCn-3 EFAs DHA and EPA (Klein Breteler et al., 1999; Broglio et al., 2003; Veloza et al., 2006). However, bioconversion of dietary sterols has also been observed in marine hetrotrophic protists (Klein Breteler et al., 1999; Adolf et al., 2007; Chu et al., 2008). Two marine heterotrophic dinoflagellate species, *Oxyrrhis marina* and *Gyrodinium dominans*, have been recently shown to produce significant quantities of cholesterol compared to their algal prey (Chu et al., 2008). When these two protists were fed the alga *Rhodomonas salina*, which contains approximately 1% cholesterol and 99% brassicasterol, they produced 5 to 30-fold more cholesterol than was present in their prey (Chu et al., 2008). When these protists were fed *Dunaliella tertiolecta*, which contains no cholesterol, a net production of cholesterol was also noted (Chu et al., 2008). These findings suggest that heterotrophic protists are not passive accumulators of dietary sterols, but rather they modify dietary sterols and/or synthesize their own sterols de novo.

Essential lipids cannot be synthesized by higher marine metazoans, such as crustaceans and fish, in sufficient quantities to support growth and reproduction (Sargent et al., 1987, 2002). Thus, animals

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at higher trophic levels rely on their diet to provide the essential lipids they require. Studies of both marine and freshwater ecosystems have produced evidence that algae and cyanobacteria that are deficient in essential lipids will not support copepod or daphnid growth and reproduction (Klein Breteler et al., 1999; Broglio et al., 2003; Martin-Creuzburg et al., 2006; Bec et al., 2006; Veloza et al., 2006). However, protists such as ciliates and heterotrophic dinoflagellates can consume these algae and/or bacteria and, in turn, these protists as prey items of mesozooplankton support good growth, egg production and egg hatching rates (Klein Breteler et al., 1999; Broglio et al., 2003; Martin-Creuzburg et al., 2006; Bec et al., 2006; Veloza et al., 2006).

Although production of DHA and EPA by heterotophic protists in marine ecosystems is certainly an important component of trophic upgrading, several studies have also demonstrated the importance of dietary sterols to copepod nutrition (Ederington et al., 1995; Hassett, 2004; Crockett and Hassett, 2005). These findings raise the possibility that sterol production and/or modification by heterotophic protists may also be an important component of essential lipid upgrading. Copepods, the most abundant group of marine zooplankton, are a key component in marine pelagic ecosystems. Copepod abundance is a critical determinant of larval fish recruitment and subsequent production at higher trophic levels (Cushing, 1995; Rutherford et al., 1997). The predominant sterol in copepods and organisms at higher trophic levels is cholesterol, a Δ5,27 carbon sterol (Ballantine et al., 1980). Crustaceans require cholesterol both as a membrane component and as a precursor to ecdysteroids which are critical signaling molecules involved in molting (Fingerman, 1987).

Supplemental dietary cholesterol has been shown to increase egg production in three species of copepods when they were maintained on the diatom *Thalassiosira weissflogii* (Hassett, 2004), an algal species in which the predominant sterol is brassicasterol (Δ5,22–C28:2) (Klein Breteler et al., 2005). Additionally, the calanoid copepods *Temora longicornis* and *Pseudocalanus elongatus* do not grow or reproduce when fed *D. tertiolecta* which is deficient in cholesterol (Klein Breteler et al., 1999). Some crustaceans can modify C28 and C29 Δ5 sterols by dealkylation and saturation of double bonds to make cholesterol, (Teshima, 1970), but copepods lack the ability to remove Δ7 double bonds from dietary sterols (Prahl et al., 1984). Thus, production of sterols via modification and/or de novo synthesis by heterotrophic protists may be an important factor in regulating mesozooplankton production in marine ecosystems.

While mechanisms for EPA and DHA production in *O. marina* and *G. dominans* have been recently defined (Lund et al., 2008) and they have been shown to be capable of producing significant amounts of cholesterol (Chu et al., 2008), it is unclear whether these two species produce new sterol species via modification of dietary sterols or de novo synthesis. The ability of heterotrophic protists to synthesize sterols has potentially large ecological ramifications due to their role as trophic intermediates between autotrophs and mesozooplankton. If some heterotrophic protists have the ability to synthesize sterols, then the total amount of sterols available to higher trophic levels is not necessarily determined by autotroph production. If, on the other hand, heterotrophic protists cannot synthesize sterols then total sterol production is “fixed” at the autotroph level and the role of heterotrophic protists is limited to changing the types, but not amounts, of sterols available to higher trophic levels.

In order to determine if *O. marina* and *G. dominans* produce new sterol species via bioconversion and/or de novo synthesis, a new approach to the problem is required. The present study investigating the mechanism(s) of sterol metabolism by heterotrophic protists utilized a series of feeding experiments with stable isotope-labeled substrates to determine whether *O. marina* and *G. dominans* modify/bioconvert dietary sterols by alkylation, dealkylation, saturation and desaturation. Additionally, the ability of *O. marina* and *G. dominans* to produce sterols de novo was investigated.

2. Materials and methods

2.1. Determination of de novo sterol synthesis capabilities of *O. marina*

In a previous study (Lund et al., 2008) we demonstrated that *O. marina*, but not *G. dominans* could utilize a 2 carbon substrate, ^{13}C-labeled sodium acetate, present in the medium to produce fatty acids, including the essential fatty acid docosahexaenoic acid (DHA). To determine if *O. marina* is capable of using acetate to produce sterols de novo, prey-depleted *O. marina* previously maintained on *D. tertiolecta,* which contains no cholesterol or brassicasterol, were inoculated at a density of 1000 cells ml^{-1} into 300 ml culture bottles containing fresh medium with either sodium 1,2 ^{13}C acetate (Cambridge Isotopes, Andover, MA) or non-labeled sodium acetate at a concentration of 200 μg ml^{-1} ($n=3$).

Twenty-four hours after inoculation 3 × 1 ml aliquots were removed from each culture bottle for determining cell concentrations using a 1 ml Sedgewick–Rafter counting chamber. The cells in the remainder of each culture bottle were harvested on acetone-washed GF/F filters and stored at −20 °C for no more than 2 weeks prior to processing for sterol analysis.

Lipids were extracted from the GF/F filters containing harvested cells by the method of Bligh and Dyer (1959). Lipids from each sample were derivitized to produce free sterols and fatty acid methyl esters (FAMEs) 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 1.5 μg 5-α-cholestane added as an internal standard and evaporated to dryness under a stream of nitrogen. Esterified sterols and fatty acids contained in the total lipids were then hydrolyzed to free sterols or transmethylated to FAMEs, respectively, by the addition of 2.0 ml sodium methoxide (0.5 M CH_3ONa in MeOH) to the vial followed by vortexing and then shaking for 1.5 h at room temperature. Free sterols and FAMEs were recovered and extracted with 1 ml hexane. The hexane fraction containing the sterols and FAMEs was washed with 2 ml of water to remove any traces of sodium methoxide prior to being analyzed by gas chromatography/flame ionization detection (GC/FID) and gas–liquid chromatography/mass spectroscopy (GC/MS/MS).

To detect incorporation of ^{13}C-labeled acetate in sterols and FAMEs, samples were analyzed qualitatively and quantitatively by GC/MS/MS with a Varian 3800 gas chromatograph (Varian Analytical Instruments, Walnut Creek, CA) equipped with a Varian Saturn 2000 GC/MS/MS ion trap detector in electron ionization (EI) mode over a mass range of 50–650 m/z at 0.77 s scan^{-1} using a J&W DB-5 column (60 m × 0.32 mm × 0.25 μm film thickness). The carrier gas was helium with a constant flow rate of 1.6 ml min^{-1}. Injection port temperature was 320 °C, the ion trap was 220 °C, the manifold was 80 °C and the transfer line was 320 °C. The column was temperature programmed from an initial temperature of 75 °C with a 1 min hold then increased at 15 °C min^{-1} to a final temperature of 350 °C and a hold at 350 °C for 9.67 min. Samples were also analyzed by GC/FID using the same column and method as described for GC/MS/MS analysis. 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 Wiley07 mass spectral libraries. When authentic standards and/or published spectra were not available tentative identifications of sterols (# carbons, # double bonds) were made based on the molecular ion. The quantity of each identified sterol was calculated based on the relative response of cholesterol to the internal standard 5-α-cholestane. FAMEs were analyzed from the same chromatograms used for sterol analyses. FAMEs were identified by relative retention time and MS spectra of known standards and non ^{13}C-labeled controls.

2.2. Determination of de novo sterol synthesis capabilities of *G. dominans*

Because *G. dominans* cannot be maintained as an osmotroph (Lund et al., 2008) a different approach was utilized to test the ability of this

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