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Astaxanthin in *Calanus pacificus*: Assessment of pigment-based measures of omnivory

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Abstract

We investigated the suitability of the pigments astaxanthin and canthaxanthin as biomarkers for measuring the contribution of microzooplankton prey to copepod diets. Identification and quantification of pigments of the marine planktonic copepod *Calanus pacificus* and heterotrophic protists were made using reverse-phase HPLC with a photodiode array detector. Astaxanthin content in the body tissues of *C. pacificus* declined significantly within 2 h after the onset of starvation. The esterified and unesterified pigment fractions behaved differently, with the unesterified fraction initially increasing in starved animals. Tissue astaxanthin content of *C. pacificus* increased by 50% in 24 h when fed the cryptomonad alga *Rhodomonas* sp. Such rapid changes in copepod body tissue pigments in response to starvation or feeding would lead to large errors in pigment-based measures of omnivory. Furthermore, neither astaxanthin nor canthaxanthin could be detected in any extract of six species of cultured and one species of field-collected heterotrophic marine protists. Although a variety of carotenoids were found in protist extracts, no single pigment was common to all heterotrophic protists.

Omnivory by copepods has been measured with laboratory cultures (Stoecker and Sanders 1985; Wiadnyana and Rassoulzadegan 1989) as well as with incubations with field-collected prey assemblages (Gifford and Dagg 1991; Ohman and Runge 1994; Fessenden and Cowles 1994). These studies demonstrate that many copepods are omnivorous and will selectively ingest microzooplankton over phytoplankton. An immunochemical approach was introduced by Ohman (1992); however, methods which quantify the contribution of microzooplankton to copepod diets without manipulating the predators or their prey have been generally lacking.

Kleppel et al. (1988) suggested that the carotenoid pigments astaxanthin and canthaxanthin could be used as biomarkers for the presence of animal prey in zooplankton diets in a manner analogous to the use of chlorophyll *a* and other algal pigments in quantifying copepod herbivory (e.g. Mackas and Bohrer 1976; Head and Harris 1994). The approach relies on the following assumptions: that astaxanthin and canthaxanthin are uniquely of "animal" origin and are widely distributed among potential prey; that these pigments remain identifiable once ingested by consumers; that the pigments originating from consumer tissues and prey tissues can be distinguished; and that there is a consistent relationship between prey astaxanthin-canthaxanthin content and measures of prey biomass such as organic C or N. Here we address the third and fourth assumptions in detail.

Acknowledgments

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Copepod herbivory can be quantified by measuring algal pigments in whole copepods because copepod tissue pigments can be distinguished from algal pigments in the guts. Measuring the astaxanthin and canthaxanthin in copepod gut contents is not as straightforward because astaxanthin is the dominant pigment in copepod body tissues (Hairston 1976; Goodwin 1984*b*; this study). The contribution of copepod tissue pigments must be subtracted from whole-animal extracts to obtain gut pigments by difference. The tissue pigment correction has been determined by starving control animals for several hours to evacuate their guts, then subtracting the remaining pigment in these controls from field samples (Kleppel et al. 1988). The difference is assumed to be the amount of astaxanthin present in the guts when the animals were captured. This method requires that copepod body pigments do not change upon starvation. If copepod tissue pigments were to change appreciably during starvation, then estimates of astaxanthin in the gut contents would be incorrect.

We tested this assumption of starvation-independence of tissue pigments in the marine copepod *Calanus pacificus* Brodsky. In addition, to better define the relationship between astaxanthin, canthaxanthin, and the organic C content of microzooplankton prey (Kleppel et al. 1988; Kleppel and Lessard 1992), we analyzed the pigment content of seven species of heterotrophic protists representative of many taxa found in marine plankton assemblages.

Methods

Field collections—Copepods were collected in oblique net hauls from 80-m depth at a site 1.5 km offshore (near 32°52′N, 117°16′W) of the Scripps Institution of Oceanography. Some samples were immediately filtered onto 150-μm-mesh Nitex and frozen in the field in liquid N_2. The rest were diluted into filtered seawater (15°C) and

transported to a laboratory, where individual adult *C. pacificus* females were sorted for experiments.

Pigment variability in the field was determined from copepods collected in the Southern California Bight near Pt. Conception (~34°N, 120°W) on two CalCOFI (California Cooperative Oceanic Fisheries Investigations) cruises. These animals were collected in the upper 70 m in 7-min hauls with a 0.70-m-diameter, 202-μm-mesh net on a bongo frame. Collections were made on 23–24 April 1992 (cruise 9204, stations 82.47, 83.55 and 80.60; SIO Ref. 92-20) and 12 July 1992 (cruise 9207, stations 83.42, 82.47, 80.51, 80.55; SIO Ref. 93-13). Animals were quickly frozen in liquid N_2 at sea, then transferred to a freezer at −80°C ashore until removed for pigment extraction.

We selected the cryptomonad alga, *Rhodomonas* sp., as prey for copepod feeding experiments because, unlike diatoms or dinoflagellates, *Rhodomonas* lacks pigments that coelute with astaxanthin or canthaxanthin, thus simplifying pigment identification. Because *Rhodomonas* contains neither astaxanthin nor canthaxanthin, we could also be assured that any such pigments in copepod extracts originated from copepod body tissues and not gut contents.

To further confirm which pigments came from copepod gut contents and which from body tissues, we dissected the intestinal tract from *C. pacificus* females and extracted and analyzed the gut contents and gut-free bodies separately. Small pieces of frozen samples were defrosted and *C. pacificus* intact females were sorted out for dissection. Guts were removed by slowly and evenly tugging on the last urosomal segment with microforceps while holding the body in place with another microforceps. The entire gut often popped out as an intact sac. The remaining urosomal segment was then removed from the end of the gut and combined with the rest of the body tissues. We analyzed gut contents and gut-free bodies of two lots of 20 adult female *C. pacificus* that had been fed at the high *Rhodomonas* concentration in the lab and three lots of 20 adult females collected and frozen in the field on CalCOFI cruises.

Feeding experiments—We tested whether well-fed *C. pacificus* had qualitatively or quantitatively different pigment composition compared to starved animals. Copepods were incubated in lots of 20 individuals in 1-liter polycarbonate bottles containing either filtered seawater (Whatman GF/F; starved treatment) or filtered seawater augmented with *Rhodomonas* (fed treatment). Bottles were secured to a plankton wheel and rotated at 1 rpm. Experiments were conducted on 4–5 August 1994 and 7–8 October 1994. On 4 August, two lots of animals were starved for 30 h while two additional lots were fed for 24 h at an initial concentration of 30,000 *Rhodomonas* cells ml^{-1}. The incubations began at 1700 hours and were run at 19°C with a 14 : 10 L/D cycle synchronized to field conditions. On 7 October, the animals were either starved for 36 h postcollection, fed at an initial *Rhodomonas* concentration of 30,000 cells ml^{-1} (high food concentration) for 24 h, or fed at an initial concentration of 5,000 cells ml^{-1} (low food concentration) for 24 h (three lots per treatment). These incubations began at 2300 hours and were run at 15°C with a 12 : 12 L/D cycle.

Incubations were terminated by collecting the animals on Nitex mesh, then freezing them in liquid N_2. The sample was later defrosted; species, stage, and abundance were confirmed; and intact adult *C. pacificus* females were transferred to a 47-mm GF/C filter. The filters were wrapped in aluminum foil and stored at <-73°C until pigment extraction.

Pigment decay rates in body tissues—Experiments assessed the time-course of change in tissue pigment of well-fed adult female *C. pacificus* upon starvation. Plexiglas cylinders with 333-μm-mesh bottoms were suspended within 3.8-liter glass jars filled with a solution of 30,000 *Rhodomonas* cells ml^{-1}. Copepods (40–60 individuals) were added to the cylinders. The jars were agitated occasionally to prevent settling of the food. After 24 h, the copepods were transferred to filtered seawater to begin starvation, then recovered and frozen in liquid N_2 at specified time intervals. On 30 September 1994, samples were collected after 0, 2, and 4 h of starvation. On 21 November 1994, samples were taken after 0, 6, 8, and 24 h of starvation. Both experiments were run at 15°C with a 12 : 12 L/D cycle.

Culturing of heterotrophic protists—Cultures of heterotrophic protists were grown at temperatures from 15 to 21°C with low, indirect light or in darkness. In addition to *Rhodomonas,* we found that the cyanobacterium *Synechococcus* sp. (WH 7803) and the heterotrophic bacterium *Vibrio natriegens* (ATCC 14048) also lacked pigments that coeluted with astaxanthin or canthaxanthin in our chromatographic system. Using these organisms as food allowed us to test for the presence of astaxanthin and canthaxanthin in herbivorous and bacterivorous microzooplankton without separating predator from prey. We also cultured the tintinnid ciliate *Favella* sp. on the dinoflagellate *Heterocapsa triquetra.* In this case, predator and prey were separated upon harvesting by screening through a 63-μm mesh. The ciliates were then resuspended in filtered seawater until their vacuoles had cleared. Inspection showed that there were essentially no residual prey and negligible *Favella* mortality with this procedure. We also collected the heterotrophic dinoflagellate *Noctiluca scintillans* in net hauls from the Scripps pier. *Noctiluca* was resuspended in filtered seawater for 24 h before extraction.

Triplicate cultures of each species were filtered onto GF/C filters and frozen at <-73°C until extracted. Subsamples were taken immediately before filtration and preserved in acidic Lugol's solution (final concn, 2%). After at least 48 h (Ohman and Snyder 1991), the abundance of microzooplankton in each culture was determined by microscopic counts. Linear dimensions were measured for randomly selected cells from each culture. These were converted to biovolume using the geometric formulae given by Kovala and Larrance (1966). Biovolumes were

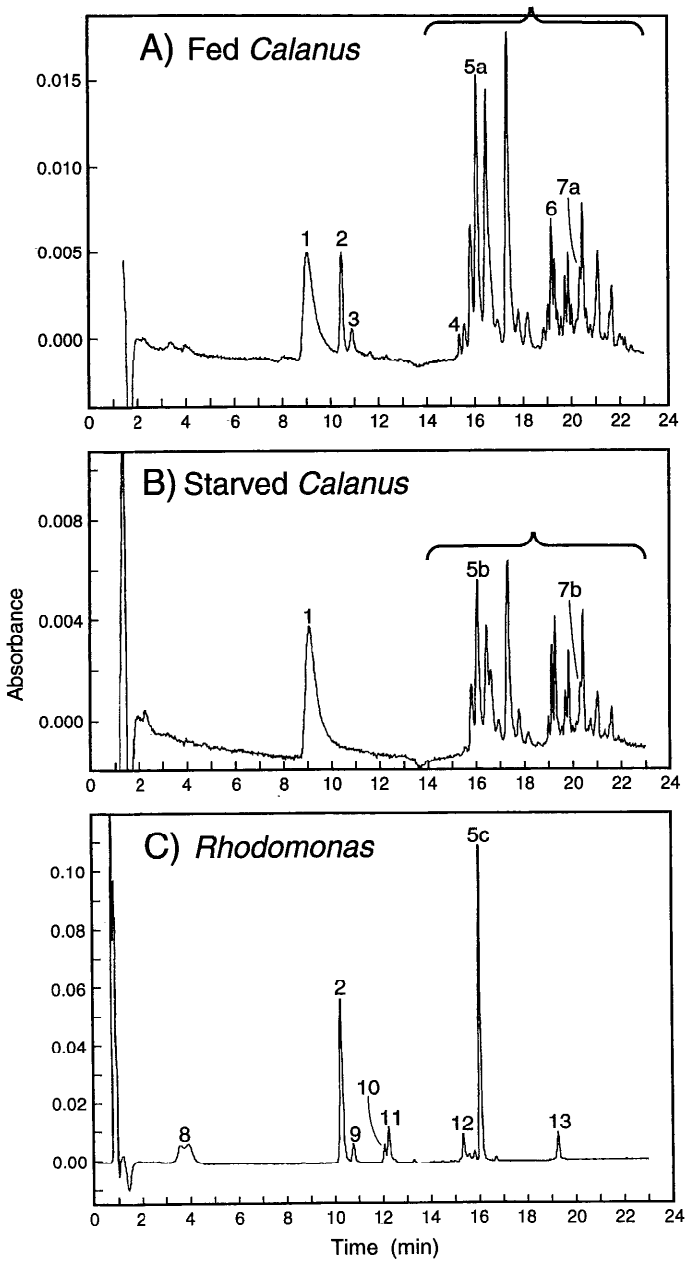

Fig. 1. Chromatograms from (A) *Calanus pacificus* adult females fed *Rhodomonas* sp. for 24 h, (B) *C. pacificus* adult females starved for 8 h, and (C) a pure culture of *Rhodomonas* sp. Numbered peaks are identified as follows: 1—unesterified astaxanthin; 2—alloxanthin; 3—4—similar to alloxanthin; 5a—Chl *a* and astaxanthin ester; 5b—astaxanthin ester only; 5c—Chl *a* only; 6—unidentified carotenoid; 7a—pheophytin *a* and astaxanthin ester; 7b—astaxanthin ester only; 8—Chl *c* (*c*1 + *c*2); 9–12—similar to α-carotene; 13—α-carotene. Peaks 5a and 7a show coelution of two pigments. Braces encompass all of the unnumbered peaks in panels A and B with retention times from 14 to 23 min that were identified by their absorption spectra as astaxanthins. Absorption spectra of pigments given in Fig. 2 legend.

multiplied by 0.21 pg C μm^{-3} to determine cellular C content (Ohman and Snyder 1991).

Pigment extraction and analysis—Samples of copepods, algae, or microzooplankton on 47-mm GF/F filters were freeze-dried until only 0.1 ml of water remained in the filter (as determined by weight). We did not detect any difference between samples which were freeze-dried and samples which were not freeze-dried because the latter were collected on smaller (25 mm) filters that already had low water content. Filters were extracted in 1 ml of 100% HPLC-grade acetone in a glass tissue homogenizer tube, then, after standing at least 0.5 h at −20°C, were ground for 30–60 s while in an ice bath. Samples were further extracted for at least 4 h at −20°C, then centrifuged for 5 min. Subsamples of 50–700 μl from the supernatant were mixed with Milli-Q H_2O (two parts extract: one part H_2O) immediately before injection.

All samples were analyzed on a C_{18} column-based system with a Rainin Dynamax cartridge column (10 cm, 4.6 mm, 3 μm), HPLC-grade solvents (A—85% methanol, 15% 0.5 M aqueous ammonium acetate; B—methanol; C—acetone), and the following linear tertiary gradient system derived from Goericke and Welschmeyer (1992) (time, %A, %B, %C): (0 min, 100, 0, 0), (5 min, 50, 50, 0), (7 min, 50, 50, 0), (11 min, 0, 100, 0), (20 min, 0, 20, 80), (22 min, 0, 20, 80), (24 min, 100, 0, 0). Flow rate was always 1.5 ml min^{-1}. Pigments were quantified at 440 nm and spectrally characterized on a Waters 991M photodiode array detector system. Pigments were identified by absorption spectra. Algal pigment standards for reference spectra were derived from extracts of a variety of cultured phytoplankton. Astaxanthin standard was obtained from Hoffman-La Roche (as Carophyll Pink) and canthaxanthin standard was obtained from Fluka Chemie AG. The detector response was linear through the range of pigment content in our samples ($r^2 \geq 0.99$). The extinction coefficient of astaxanthin from Davies (1976) was used for all peaks identified as astaxanthin esters. Our smallest detectable peaks contained the equivalent of 3–4 ng of pigment, corresponding to 0.15–0.2 ng of astaxanthin per copepod.

Results

Astaxanthin pigments of C. pacificus—A single peak was identified as unesterified astaxanthin in chromatograms of adult female *C. pacificus* (Fig. 1A, B) based on its absorption spectrum (Fig. 2B) and retention time. Numerous other peaks were found, all with absorption spectra characteristic of astaxanthin (*see Fig. 2C, I*) but longer retention times. These peaks were most likely astaxanthin esters (cf. Hairston 1976), although some could also have been intermediate compounds in astaxanthin metabolism or esters of canthaxanthin; astaxanthin and canthaxanthin have very similar absorption spectra. No evidence of unesterified canthaxanthin was ever found. We refer to all peaks with astaxanthinlike absorption spectra as "astaxanthins" and will distinguish between unesterified

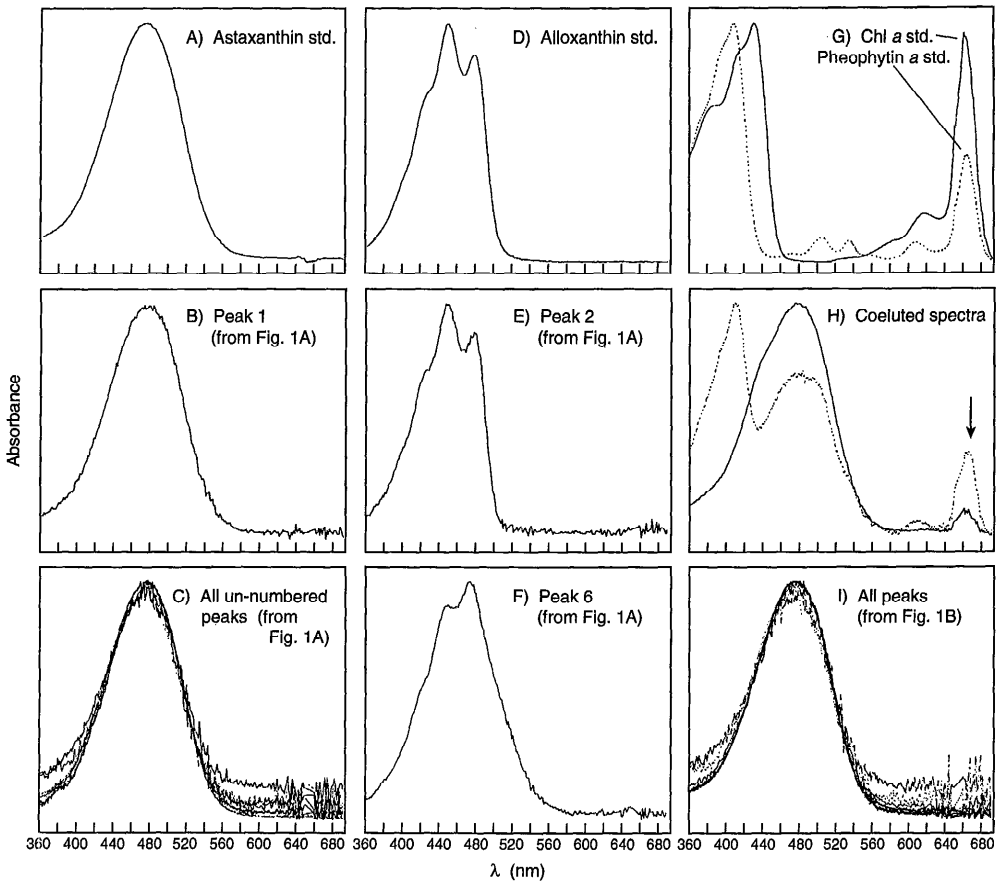

Fig. 2. Examples of pigment absorption spectra from pigment standards and selected peaks in Fig. 1. Vertical axes are standardized so that all spectra are scaled similarily. A. Unesterified astaxanthin standard. B. Unesterified astaxanthin from peak 1 (Fig. 1A). C. Overlain spectra from all unnumbered peaks in Fig. 1A with absorbance > 0.001 absorbance units (AU). All these peaks were identified as astaxanthins. D. Alloxanthin from *Rhodomonas* sp. E. Alloxanthin from peak 2 (Fig. 1A). F. Unidentified carotenoid from peak 6 (Fig. 1A). G. Chl *a* (solid line) and pheophytin *a* (dashed line) standards. H. Spectra from peaks 5a and 7a (Fig. 1A) which demonstrate the coelution of two pigments. Dashed line (peak 5a)—Chl *a* and astaxanthin ester; solid line (peak 7a)—pheophytin *a* and astaxanthin ester. Arrow indicates characteristic absorbance peak of Chl *a* and related pigments. I. Overlain spectra from all peaks in Fig. 1B with absorbance > 0.001 AU, all of which were identified as astaxanthins.

astaxanthin and a group of esterified astaxanthins (i.e. astaxanthins with longer retention times).

Whole animal extracts of experimentally fed copepods also contained the algal pigments alloxanthin and chlorophyll *a*, which must have originated from ingested *Rhodomonas* (Fig. 1C, cf. 2D with 2E and 2G with 2H). Pheophorbides (which include pheophorbide *a*, pheophorbide *a'* and pyropheophorbide *a*) and pheophytins (which include pheophytin *a*, pheophytin *a'* and pyropheophytin *a*) resulting from the digestive breakdown of chlorophyll *a* were present in the extracts of fed animals (Fig. 1A, 2H). However, chlorophyll *a* breakdown products were not always apparent in chromatograms analyzed at 440 nm because their absorption is low at this wavelength. Some peaks had absorption spectra characteristic of carotenoids other than astaxanthin but could not be identified with certainty (e.g. Fig. 2F). By comparison, starved copepods (Fig. 1B) contained only astaxanthins (Fig. 2I), indicating that only astaxanthins were present once the guts were evacuated.

Further demonstration that astaxanthins were localized in body tissues and not in gut contents is given by the results of the gut dissections (Fig. 3A–E). Figure 3A shows a chromatogram from *C. pacificus* frozen in the field (collected at 0339, 24 April 1992). The chromatogram is similar to Fig. 1A; it contains unesterified astaxanthin, many other astaxanthins, and several algal pigment peaks, indicating the animals had recently fed. Analysis of the dissected gut contents of *C. pacificus* from the same sample is shown in Fig. 3B. The peaks in Fig. 3B correspond to the nonastaxanthin peaks in Fig. 3A with only three exceptions (peaks 20, 21, and 22). Figure 3A and B were

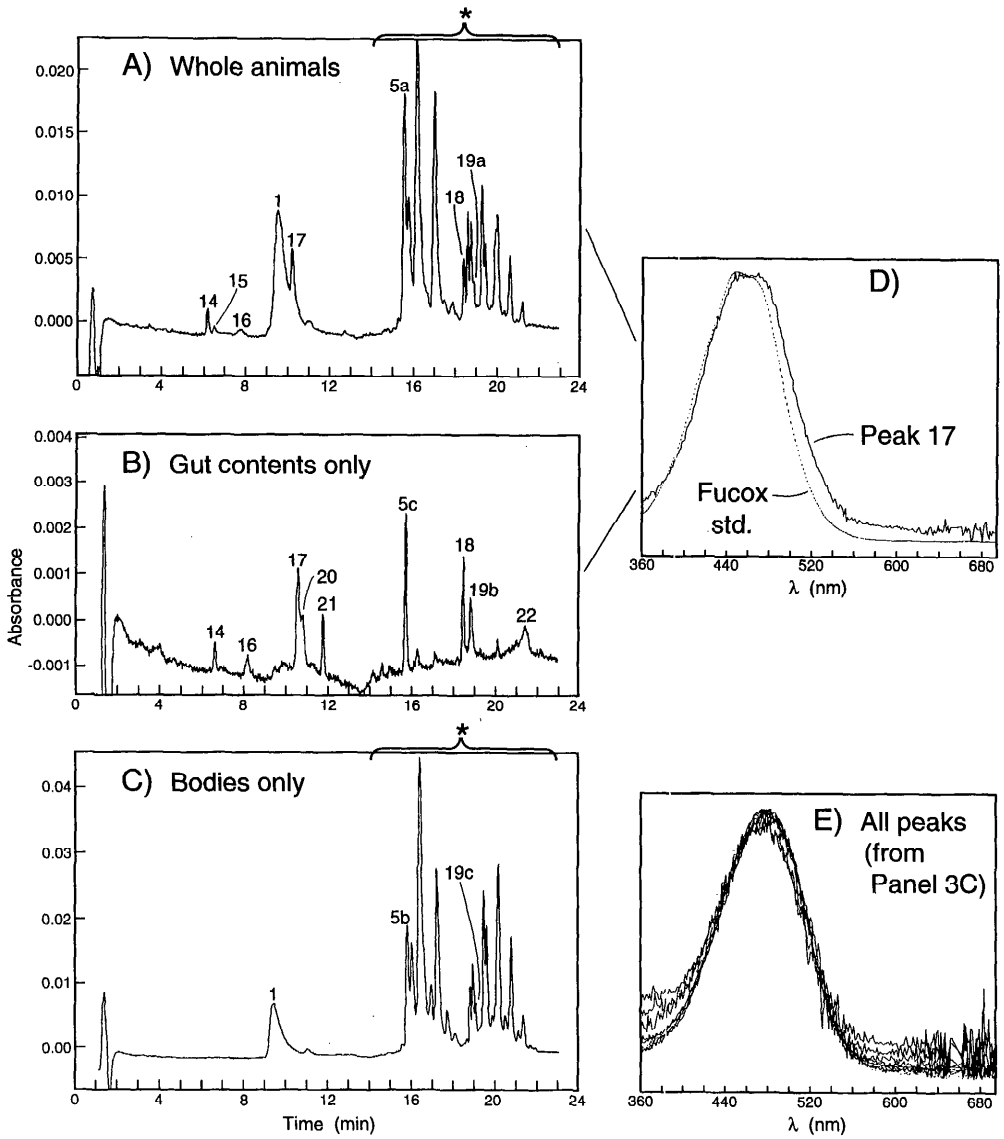

Fig. 3. Chromatograms from adult female *Calanus pacificus* collected in the field. A. Intact animals. B. Dissected gut contents only. C. Bodies only (tissues remaining after gut dissection). Peak identifications: 1—unesterified astaxanthin; 5a—Chl *a* and astaxanthin ester; 5b—astaxanthin ester only; 5c—Chl *a* only; 14–15—unidentified carotenoid (different from 6); 16—pheophorbide *a*; 17—unidentified carotenoid; 18—pheophytin *a* and an unidentified carotenoid; 19a—pheophytin *a* (probably pheophytin *a'*) and astaxanthin ester; 19b—pheophytin *a* (*a'*) only; 19c—astaxanthin ester only; 20–22—unidentified. The unidentified carotenoids in peaks 14, 15, 17, and 18 have similar absorption spectra resembling fucoxanthin. Braces encompass all of the unnumbered peaks in panels A and C with retention times from 14 to 23 min that are astaxanthins. D. Absorption spectrum from peak 17 (of panels A and B; solid line) compared to fucoxanthin from a diatom (dotted line). Both have a smaller λ_{max} and broader peak than astaxanthin. E. Overlain spectra of all peaks in panel C with >0.001 AU, all of which were identified as astaxanthins (cf. Fig. 2C, I).

prepared from two different lots of 20 copepods collected in the field, so it is not surprising that there is not 100% overlap. Despite an inability to conclusively identify all peaks in chromatograms of gut contents, analysis of absorption spectra for each peak established that guts never contained astaxanthins. On the other hand, extracts of gut-free body tissues contained astaxanthins exclusively (Fig. 3C). Body tissue extracts had ~20% less total astaxanthin per copepod than the corresponding intact animals. Gut content extracts never contained astaxanthins,

so we attribute this decrease to loss of tissue and lipids during dissection rather than to gut content removal per se.

Adult female *C. pacificus* that had been fed a high concentration of *Rhodomonas* cells for 24 h had significantly higher content of astaxanthins than starved animals (Fig. 4, two-sample t-test, $P < 0.002$ for each case). There was no significant difference between the starved treatment and the low food treatment (two-sample t-test, $P > 0.2$). After 24 h at the high food concentration, the astaxanthins per copepod had increased by ~50% over the initial value of animals frozen immediately upon collection in the field (Fig. 4, far right bars). This demonstrates that astaxanthin content increased rapidly during feeding. Therefore, differences between starved and fed copepods were partly due to a buildup of pigments in fed animals.

During starvation experiments, total astaxanthins declined (Fig. 5A; Jonckheere's test for ordered alternatives, $P < 0.001$). A 20–30% decrease was found during the first 4 h. The esterified and unesterified astaxanthin fractions behaved differently, especially during early starvation. Although the esterified fraction decreased more rapidly than total astaxanthins (Fig. 5B), the unesterified fraction increased through the first 8 h of starvation, then decreased by 24 h of starvation (Fig. 5C). In well-fed copepods and those which had been starved for 24 h, unesterified astaxanthin was ~10% of the esterified fraction, while in copepods in the initial phase of starvation, unesterified astaxanthin was ~20–25% of the esterified astaxanthin (Fig. 5D).

In our field collections, total astaxanthin content of individual adult female *C. pacificus* ranged from 11.4 to 92.4 ng ind.$^{-1}$ (17 analyses from eight independent samples). The range of astaxanthin content of animals from our experiments (11.8–34.9 ng ind.$^{-1}$) lies within the range of values encountered in the field, albeit on the low end. As with the experimental animals, the unesterified astaxanthin of copepods in the field collections was only 10–20% of the esterified fraction. Unesterified canthaxanthin was not detected. *C. pacificus* copepodid stage 5s from one of the CalCOFI samples averaged 15.0 ± 2.8 (±1 SD, $n = 4$) ng astaxanthin ind.$^{-1}$, while the adult females from the same sample averaged 34.3±3.8 ($n = 4$) ng astaxanthin ind.$^{-1}$, a factor of 2.28 difference (Mann-Whitney U-test, $P < 0.05$). This difference demonstrates the necessity of separating developmental stages in pigment analyses.

Pigments of heterotrophic protists—No astaxanthin or canthaxanthin was detected in any analyses of the species and diets of the seven species of heterotrophic protists tested (Table 1). Three species were selected to illustrate this result because the chromatograms were simple enough to display the absorption spectrum of each peak. An oligotrich ciliate, *Strombidium* sp. (Fig. 6A), and a hypotrich ciliate, *Euplotes* sp. (Fig. 6B), were cultured on the bacterium *V. natriegens*; *N. miliaris* (Fig. 6C) was collected from the field. A chromatogram with pure astaxanthin and canthaxanthin standards is shown for comparison of retention time and absorption spectra (Fig. 6D). Chromatograms from other species were more complicated, typically consisting of tens of peaks. Some of these peaks could be attributed to prey pigments or breakdown products of prey pigments. However, the absorption spectra of most pigments in heterotrophic protist extracts did not match known pigments and could not be conclusively identified.

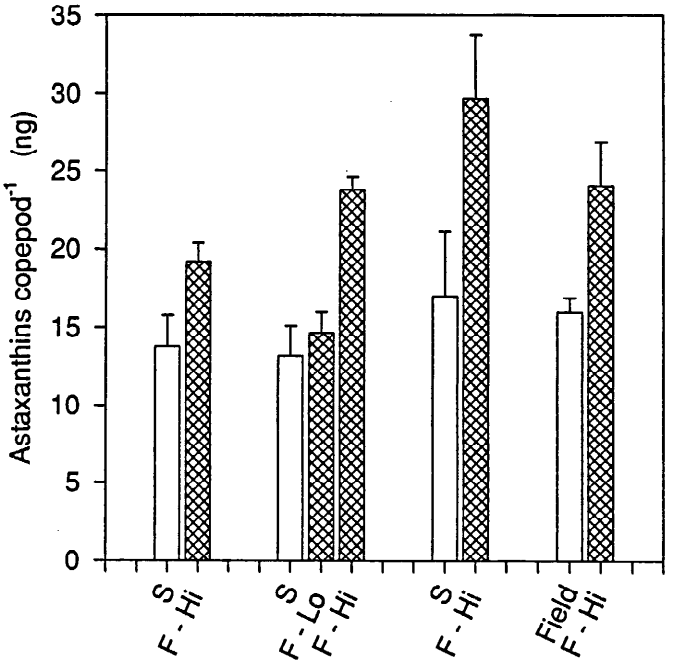

Fig. 4. The difference between starved (S) and fed (F) adult female *Calanus pacificus*. Fed animals were fed for 24 h at either Hi (30,000 *Rhodomonas* cells ml^{-1}) or Lo (5,000 *Rhodomonas* cells ml^{-1}) prey concentration. Starting from the left, S animals were starved 30, 36, and 24 h. The last set of bars compares the initial astaxanthin content of animals in the field to animals from the same sample following 24 h in the high food concentration. Each bar is the mean (±1 SD) of three samples (except the first set where $n = 2$).

The *Noctiluca* extract (Fig. 6C) contained a small Chl *a* peak despite separation from potential prey items for nearly 24 h. This pigment may have been retained in the *Noctiluca* cells or there may have been some contaminating algae, although we inspected the sample microscopically before harvesting and found no prey carryover.

Discussion

We have demonstrated that the copepods in our experiments contained astaxanthins exclusively in their body tissues and not in their gut contents. Changes in total astaxanthin during starvation could therefore be attributed to changes in body tissue astaxanthins and not to gut evacuation. Our experiments showed that the tissue pigment content of *C. pacificus* is dynamic. Significant

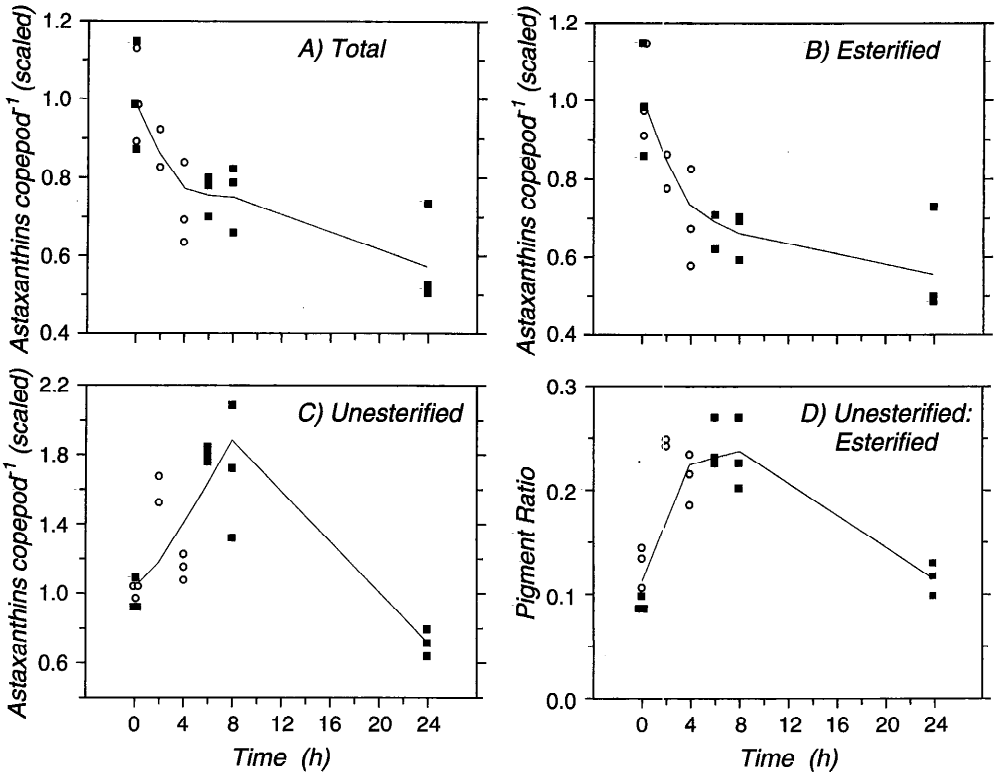

Fig. 5. The effect of starvation on astaxanthin content. Copepods were fed *Rhodomonas* for 24 h, then starvation was initiated at $t = 0$. Data from 30 September—○; data from 21 November—■. The amount of astaxanthin in each sample was scaled to the average value at $t = 0$ from the respective date to make the two experiments comparable. A. Total astaxanthin (=esterified + unesterified): 30 September at $t = 0$, 24.1 ng ind.$^{-1}$; 21 November at $t = 0$, 29.7 ng ind.$^{-1}$. B. Esterified astaxanthin only: 30 September at $t = 0$, 21.3 ng ind.$^{-1}$; 21 November at $t = 0$, 27.2 ng ind.$^{-1}$. C. Unesterified astaxanthin only: 30 September at $t = 0$, 2.7 ng ind.$^{-1}$; 21 November at $t = 0$, 2.5 ng ind.$^{-1}$. D. The ratio of unesterified to esterified astaxanthin. The lines drawn through the data represent the LOWESS fit to all points (Cleveland 1979).

changes can occur on time scales of a few hours to a day and are related to the recent feeding history of the animal. These results imply that subtracting the astaxanthin content of starved copepods from field-collected animals significantly overestimates the astaxanthin in gut contents. Because the rate of decrease of tissue astaxanthins is highest during the initial phase of starvation, this bias would be greatest if the copepods had been feeding prior to collection. The effect of starvation would therefore have varied with time of day in the study of Kleppel et al. (1988).

Quantification of astaxanthin in *C. pacificus* is difficult because the majority of the pigment is esterifed and elutes over a broad range of retention times. The esters can easily be confused with algal pigments unless a means of pigment identification, independent of retention time (such as absorption spectra), is available. The bias from misidentification is not constant because the relative proportions of the esterified and unesterified pool change during starvation.

Total astaxanthin content in *C. pacificus* females in our field samples ranged over nearly an order of magnitude. This range is too large to be explained by between-sample variation in individual copepod body mass. It more likely reflects differences in the recent feeding history of the copepods from different samples.

Difficulty in identifying all astaxanthin peaks may explain the discrepancy between our estimate of the astaxanthin content of *C. pacificus* and those given by Kleppel et al. (1988; ~1.5–8 ng ind^{-1} for the same species). Had we neglected astaxanthin esters, our values for astaxanthin content would have been underestimated by ~80–90%. This would give us values very similar to those of Kleppel et al. (1988). The inclusion of some preadult stages ("late copepodites") in their samples would also have contributed to low values. Our estimates are also higher than two other reported measurements of astaxanthin content for the genus *Calanus* (~1.5–13.5 ng ind.$^{-1}$, Fisher et al. 1952; ~1.3–5.5 ng ind.$^{-1}$, Fisher et al. 1964; both for *C. finmarchicus*). However, in those two studies, the copepods were immersed in boiling water for 1–2 min before storage. Immersion in boiling water was intended

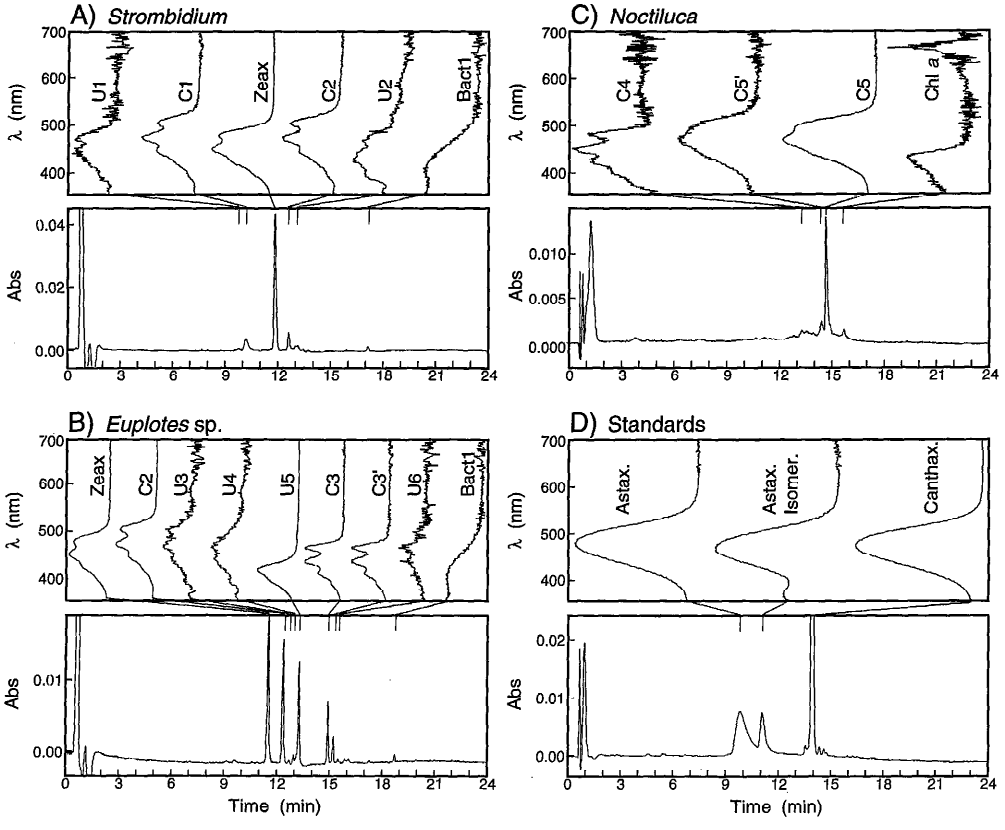

Fig. 6. Chromatograms from three species of heterotrophic protists and pigment standards. A. *Strombidium* sp. B. *Euplotes* sp. C. *Noctiluca scintillans.* D. Astaxanthin and canthaxanthin standards (the canthaxanthin peak extends to 0.12 AU). U—Unidentified pigment; C—unidentified carotenoids; Bact—an unidentified pigment found in *Vibrio natriegens*; Zeax—zeaxanthin; Chl *a*—chlorophyll *a*; Astax—astaxanthin; Canthax—canthaxanthin. C3′ and C5′ have essentially identical spectra (but smaller peaks) as C3 and C5.

to "fix" the pigment in its tissue of origin; however, the procedure may have caused degradation of astaxanthin. Other studies on copepod pigments have reported astaxanthin contents for other genera that are comparable or higher than ours. Hairston (1980) gave a value of 5 μg pigment (mg dry body wt)$^{-1}$ for brightly colored *Diaptomus nevadensis,* which is ~250 ng ind.$^{-1}$ (using dry weights from Hairston 1979). Ohman et al. (1989) found 93 ng pigment ind.$^{-1}$ for stage 5 and 164 ng ind.$^{-1}$ for adult female *Neocalanus tonsus,* a species which has ~3× the body mass of *C. pacificus.*

Hairston (1980) reported only a small decrease in the astaxanthin content of the freshwater copepod *D. nevadensis* after a week of starvation. The stability of pigment content in *Diaptomus* contrasts with the rapid changes in astaxanthin content of *Calanus* that we found. Kleppel et al. (1985) also demonstrated rapid changes in body pigments of *Acartia tonsa* which correlated with recent feeding history (their figure 2). These results may indicate that astaxanthin has a different function in copepods with different lifestyles. *Diaptomus* may use astaxanthin as a photoprotectant because it lives in alpine lakes where it could experience high light levels. However, this function seems unnecessary for a vertically migrating, deep-living, oceanic copepod such as *Calanus.* Because the majority (~80–90%) of astaxanthins in *Calanus* are esterified to lipids and because astaxanthin has been shown to protect lipids from attack by peroxide radicals (Terao 1989), we suggest that one of the roles of astaxanthin in *Calanus* and similar copepods is to provide antioxidant protection to unsaturated storage lipids.

Neither astaxanthin nor canthaxanthin was detected in extracts of heterotrophic protists. Although some peaks in the protist extracts could be identified as prey pigments or breakdown products of prey pigments, many pigments remained unidentified. Nevertheless, none of these unknowns had absorption spectra that matched astaxanthin or canthaxanthin. There was some overlap in pigments found in different species. For example, both the oligotrich ciliate *Strombidium* sp. and the hypotrich ciliate *Euplotes* sp. (both fed on *V. natriegens*) had large peaks with retention times and absorption spectra identical to the algal carotenoid zeaxanthin. This pigment was also found when the same species of *Strombidium* was raised on *Rhodomonas* sp. (data not shown), yet the pigment was not detected in either prey, *Rhodomonas* or *V. na-*

Table 1. Diet, cell biovolume (μm^3), number of cells, and total carbon per sample (ng) of heterotrophic protist species tested for the presence of astaxanthin and canthaxanthin. *Noctiluca scintillans* was collected in the field; hence its diet is unknown. Neither astaxanthin nor canthaxanthin were detected in any analyses of heterotrophic protist extracts.

Species	Prey	Cell bio-volume	Total No. cells sample^{-1}	Total C sample^{-1}
Strombidium sp.	*Vibrio natriegens*	18,650	160,000	6.27×10^5
			165,000	6.46×10^5
			160,000	6.27×10^5
Strombidium sp.	*Rhodomonas* sp.	25,000	93,500	4.91×10^5
			95,800	5.03×10^5
Favella sp.	*Heterocapsa triquetra*	275,000	3,430	1.98×10^5
			3,790	2.19×10^5
			2,970	1.72×10^5
Gymnodinium sp.	*Rhodomonas* sp.	1,086	68,000	1.55×10^4
	Synechococcus sp.		74,000	1.69×10^4
			49,250	1.12×10^4
Scuticociliate No. 1	*V. natriegens*	600	115,000	1.45×10^4
			208,000	2.62×10^4
			68,000	8.57×10^3
Scuticociliate No. 1	*Synechococcus* sp.	2,150	450,000	2.03×10^5
			206,000	9.30×10^4
			200,000	9.03×10^4
Uronema marinum	*V. natriegens*	1,760	794,000	2.93×10^5
			1,215,000	4.50×10^5
			700,000	2.59×10^5
Euplotes sp.	*V. natriegens*	33,000	53,000	3.67×10^5
			27,000	1.87×10^5
			54,000	3.74×10^5
Noctiluca scintillans	Unknown	5,840,000	1,165	1.43×10^6
			5,825	7.14×10^7
			1,165	1.43×10^6

triegens. However, comparison of the *Noctiluca* extract with the other taxa analyzed demonstrates that no single pigment is found in all heterotrophic protists.

Astaxanthin and canthaxanthin were previously reported in several of the same genera of heterotrophic protists we studied (Kleppel and Lessard 1992). Sufficient organic C was present in our extracts to have detected astaxanthin and canthaxanthin had they been present in the organic C : pigment ratios given by Kleppel and Lessard (1992). Because Kleppel and Lessard lacked a means of pigment identification independent of retention time and they experienced problems with coelution of pigments, they may have had difficulty identifying astaxanthin and canthaxanthin (Kleppel pers. comm.). Even if the species of heterotrophic protists used by Kleppel and Lessard did contain astaxanthin and canthaxanthin, our results nevertheless demonstrate that the pigments are not universally present in heterotrophic protists. Thus, in reference to the assumptions listed in our introduction, astaxanthin and canthaxanthin do not seem to be widely distributed among animal prey of copepods. We also note that astaxanthin and canthaxanthin can be found in some green algae and some cyanobacteria (Goodwin 1984*a*) and are therefore not uniquely of "animal" origin.

We conclude that the use of astaxanthin and canthaxanthin as biomarkers for copepod predation on heterotrophic protists is precluded both by the rapid changes in tissue pigments of *Calanus* and by the lack of these pigments in the species of heterotrophic protists examined. The dynamic nature of astaxanthin within copepod body tissues make it a poor choice as a biomarker even for potential prey (such as copepod eggs and nauplii) that do contain astaxanthin. Further difficulties arise because of the variability of astaxanthin content within and between copepod developmental stages in the field. The adaptive significance of such pigments remains to be fully explored.

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