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Lipids of the sea anemone Anthopleura elegantissima: season and symbiont affect content and fractionation

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LIPIDS OF THE SEA ANEMONE ANTHOPLEURA ELEGANTISSLIMA: SEASON AND SYMBIONT AFFECT CONTENT AND FRACTIONATION

by

Monica R. Ponce-McDermott

Accepted in Partial Completion

Of the Requirements for the Degree

Master of Science

Kathleen L. Kitto, Dean of the Graduate School

ADVISORY COMMITTEE

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MASTER’S THESIS

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M. Ponce-McDermott
November 19, 2012
LIPIDS OF THE SEA ANEMONE ANTHOPLEURA

ELEGANTISSIMA: SEASON AND SYMBIONT AFFECT
CONTENT AND FRACTIONATION

A Thesis

Presented to

The Faculty of

Western Washington University

In Partial Fulfillment

Of the Requirements for the Degree

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ABSTRACT

*Anthopleura elegantissima*, the common Pacific sea anemone, can host two very different algal endosymbionts: zooxanthellae (*Symbiodinium muscatinei*) and zoochlorellae (*Elliptochloris marina*). The photosynthetic carbon provided by the symbionts supplements the host’s heterotrophic feeding, with zooxanthellae potentially translocating five times more carbon to the host than do zoochlorellae. We developed a method to measure lipid levels of zooxanthellate and zoochlorellate *A. elegantissima* in different seasons, focusing on non-polar lipids that are important components of gonad and gametes. In July 2009 and January 2010, zooxanthellate and zoochlorellate *A. elegantissima* were collected from the same habitat at the same tidal height. Non-polar lipids were extracted and separated into classes by thin layer chromatography. A semi-quantitative analysis of the chromatography plates allowed a determination of how lipid identity and quantity varied seasonally and by the symbiotic state of the anemone. Anemones collected in July were, on average, 41% larger than those collected in January. Total non-polar lipid content showed no significant seasonal pattern, but cholesterol and palmitic acid (a fatty acid) were present in greater proportions in all January anemones. Zooxanthellate anemones had significantly more non-polar lipid than zoochlorellate anemones in both seasons, and a significantly greater percentage of their lipid was composed of cholesterol and palmitic acid. The winter decrease in body mass likely resulted from increased rates of fission, metabolism of stored lipids, a decrease in food availability, and seasonally reduced translocation of photosynthetic products from the symbionts to their hosts. The reduction in symbiont-derived products may impact the ability of the host anemone to direct resources to development of gonad tissues. Zoochlorellae, which translocate less carbon to their hosts under most conditions, also appear to translocate different products. These differences would be magnified if *A. elegantissima* switch from zoochlorellate to zooxanthellate under the conditions of increasing temperature predicted in global climate change scenarios. Whether these changes ultimately increase or decrease fitness and whether such changes will also be seen in other symbiotic cnidarians (e.g., corals), that switch symbionts under environmental stress, are important questions that deserve additional attention.
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INTRODUCTION

*Anthopleura elegantissima* is the most abundant intertidal anemone along the Pacific coast of North America (Hand 1955, Kozloff 1993, Blanchette et al. 1998) ranging from Alaska to Southern California (Hand 1955). This critical member of the intertidal communities commonly reaches a size of 4-5 cm in diameter and can be found on sandy beaches or rocky shores (Hand 1955). *Anthopleura elegantissima* can occur as solitary individuals or form dense aggregations of clones (Francis 1973, 1979) and can be important spatial dominants (Dayton 1971). Because of its dominance in various intertidal communities, *A. elegantissima* is a significant contributor to primary production (Fitt et al. 1982), and an influential component of the coastal ecosystem.

The primary productivity of *A. elegantissima* is due to the specialized symbiotic relationship it forms with three distinct endosymbionts. Zooxanthellae, the brown dinoflagellates *Symbiodinium californium* and *S. muscatinei* (LaJeunesse and Trench 2000), occur in anemones located in higher intertidal habitats and more southern latitudes while zoochlorellae, the green alga *Elliptochloris marina* (Letsch et al. 2009), occur more frequently in lower intertidal habitats and more northern latitudes (Secord and Augustine 2000). Due to its ability to host multiple symbionts individually or concurrently, *A. elegantissima* provides a unique opportunity to study symbiosis, focusing on the influence of the symbionts on the ecology of the host.

As the symbiotic host, *A. elegantissima* provides shelter and nutrients for the algal symbionts (Lewis and Smith 1971, Blanquet et al. 1979, Schwarz et al. 2002) while the symbionts provide the animal with photosynthetic products (Muscatine and Hand 1958, Trench 1971a, b). The translocated carbon potentially supplements nutrients obtained by
the anemone through heterotrophic feeding. However, the symbionts differ in their value as symbiotic partners. In the Puget Sound, *Symbiodinium muscatinei* the zooxanthellae, potentially translocate five times more carbon to the host anemone than do zoochlorellae, *E. marina* (Verde and McCloskey 1996). *Anthopleura elegantissima* hosting zooxanthellae could, therefore, have an important advantage over those hosting zoochlorellae (Muller-Parker et al. 2001, Bergschneider and Muller-Parker 2008). This hypothesis has not been directly tested. Exploring lipid levels of anemones in different symbiotic states may clarify whether more translocated carbon equates to more energy reserves in the form of lipids for potential growth and reproduction in anemones hosting *S. muscatinei*.

Lipids in cnidarians are important constituents of mucus production (Crossland et al. 1980), membrane components (Mason 1972) and energy storage that may be called upon during bleaching events (Grottoli et al. 2004). Study of the energetics of *A. elegantissima* have mainly focused on energy budgets (Zamer and Shick 1987), prey absorption efficiencies (Zamer 1986), and seasonal contribution of symbionts to host respiration (Verde and McCloskey 2007) rather than on the lipids themselves. To better understand the energetics of *A. elegantissima*, it is necessary to consider factors that influence lipid levels and, therefore, energy availability and potential reproductive output.

Jennison (1979b) investigated the lipids of *A. elegantissima*, in central California where only zooxanthellate anemones occur. He measured the non-polar lipid content, referred to in his work as total lipid, of the anemones and found that large individuals had higher total lipid content than did small individuals, but that percent lipid levels were consistent regardless of anemone size. The total lipid content was high enough that it
could not be entirely structural, and Jennison (1979b) hypothesized that part of the lipid content was used for energy stores or reproductive materials. One month of starving the anemones had no effect on their lipid levels, suggesting that the anemones were not using the lipids for nutrition, at least in the short term. Total lipid content, however, varied with the reproductive condition of the anemone, with an increase in total lipid from early winter to summer followed by a spawning event and rapid lipid decline from summer to early fall. This indicates that lipid content could be a good index of reproductive condition in *A. elegantissima*. Sex of the anemone did not influence lipid levels, indicating that both male and female anemones were using lipids to build gametes.

Our goal of measuring the lipids of *A. elegantissima* in different seasons, comparing individuals in different symbiotic states, and relating those features to patterns of gametogenesis and reproduction should indicate how those factors influence anemone fitness. Based on the different contributions of the two algae (Verde and McCloskey 1996), we predict that symbiont complement will affect lipid levels of *A. elegantissima*, with higher lipid content in zooxanthellate anemones. We also predict that the lipid levels of *A. elegantissima* will vary seasonally with highest levels in the summer. The seasonal effect may be more pronounced than those reported by Jennison (1979b) due to increased seasonality experienced by anemones in Puget Sound waters.

Because it was unknown what lipids were present in the *A. elegantissima* we sampled, a general lipid survey using Thin Layer Chromatography (TLC) was necessary. TLC provided a quick and efficient way to survey the lipid classes. Due to our interest specifically in reproduction, we focused our analyses on non-polar lipid classes that appear to be a primary component of energy storage in marine invertebrates, larvae and
eggs (Holland 1978; Hill-Manning and Blanquet 1979; Moran and Manahan 2003, 2004; Byrne et al. 2008).
METHODS

Collection of anemones

*Anthopleura elegantissima* for this study were collected from Swirl Rocks, approximately 450 m southeast of Lopez Island, WA (48°25’06”N 122°50’58”W, Fig. 1) on July 6, 2009 and January 27, 2010. The July samples were collected during a low tide by carefully removing the anemones from the rock surface with a small spatula. In January, low tides occur at night and collecting the anemones during daylight hours required SCUBA. Divers collected the anemones again by gently loosening them from the rocks to which they were attached. The collection location supports a large population of *A. elegantissima* in both zoochlorellate and zooxanthellate forms.

Anemones were procured from a north-facing, nearly vertical crevice in the rocks. Presumably because of different exposures to sunlight, each side of the crevice had a different form of *A. elegantissima*. Specimens on the northeast wall were green, hosting *E. marina*. Those on the northwest wall were brown, hosting *S. muscatinei*. Specimens were all collected from the same approximate tidal height (between +0.35 m and +0.50 m) where presumably the anemones received similar planktonic and benthic prey.

*Anthopleura elegantissima* were collected and placed in individual Ziploc bags with seawater and stored in a cooler for transport back to Shannon Point Marine Center in Anacortes, Washington. Forty-four anemones (21 zoochlorellate, 23 zooxanthellate) were collected in July and fifty-four (27 zoochlorellate, 27 zooxanthellate) were collected in January.

In the lab, anemones were placed individually in glass specimen dishes (5 cm diameter) that were then submerged in a flow-through sea table for one week.
Figure 1. A) Map of *A. elegantissima* collection site on Swirl Rocks. B) Rock crevice on Swirl Rocks with zoochlorellate anemones on the left and zooxanthellate anemones on the right.
Illumination during this period was natural sunlight coming through large north facing windows.

_Symbiont density_

To determine symbiont identity and density, anemones were cut into approximately equal halves. One half was not needed at this stage and was frozen at -70°C for possible future analysis. The other half was weighed then homogenized in 5µm filtered seawater. Homogenate volume was measured and the samples were well-mixed before 1.5 ml was taken for symbiont counts. Another 1.5 ml sample was taken for protein analysis and a third sample of approximately 10-20 ml was taken for lipid analysis. These samples were all stored at -70°C until they could be processed (Fig. 2).

One previously frozen 1.5 ml homogenate sample was used to determine symbiont density and complement of each anemone. The samples were well-mixed then placed in a hemacytometer on a compound microscope. Algal cells were identified and counted within a known volume until approximately 100 cells were enumerated. Four replicate samples of each anemone homogenate were counted. Algal cell counts from a known hemacytometer volume afforded calculation of algal densities within each anemone. The two algal symbionts could be easily distinguished because _E. marina_ are 8-10 µm in diameter and green while _S. muscatinei_ are 10-13 µm in diameter and brown. Algal densities were later normalized to dry weight of the anemone to yield the number of algal cells per µg anemone dry weight.
Figure 2. Flowchart illustrating processing of anemones, extraction of lipids, and TLC separation of lipid classes.
Protein analysis

To permit comparison with other studies in which algal densities were expressed as number of algal cells per mg protein, protein content of each anemone was also determined (Fig. 2). Protein content of the 1.5 ml homogenate samples was measured using the Lowry et al. (1951) method, with a standard of bovine serum albumin (BSA). Sample absorbances were measured on a Spectronic 20D spectrophotometer.

L lipid extraction

In preparation for extracting lipids, the 10-20 ml homogenate samples of the anemones were thawed and their volumes were determined. If volumes of anemone homogenate were deemed insufficient for lipid analysis (<10-20 ml), the unprocessed half of the anemone was homogenized and portions of the 2 homogenates were combined. This procedure was necessary for 40 of the anemones collected in January. The original homogenate sample volume and the added homogenate volume were recorded, allowing for accurate determination of total non-polar lipid and protein content. Homogenate samples were then hard frozen at -70°C and lyophilized. The freeze dried holobiont (anemone and algae) material was scraped from the vials and weighed on a microbalance. These dry weights were later used to normalize non-polar lipid content to anemone dry weight. After weighing, samples were stored in a desiccator until they could be processed (within 30 days).

To determine total non-polar lipid content of the anemones, lipids were extracted from the dried homogenate samples using the Folch et al. (1957) method. A mixture of chloroform-methanol (2:1) was added to each vial to a final volume 20x the volume of
homogenate. The vials were gently vortexed then agitated for 15 minutes to separate the lipid.

The well-dispersed samples were filtered with a glass funnel and filter paper (VWR Grade 417, 9 cm). Filtered extract volumes were measured and the extracts were poured into previously weighed glass centrifuge tubes. A mixture of 0.9% NaCl solution was added to each tube to a final volume of 5x the volume of solvent to enhance separation of the lipids from the non-lipids. The tubes were capped and moderately vortexed. To isolate the non-lipid and the lipid containing phases, the samples were centrifuged at 581 x g for 5 minutes.

The upper non-lipid material phase was removed by aspiration and discarded. The lower chloroform phase, which contained the lipids, was washed three times with 0.5 ml of Reagent Type I water. The upper phase was again removed and discarded with each wash. After washing, the remaining phase containing the neutral lipids was evaporated to dryness under a stream of N\textsubscript{2} gas (Fig. 2). Weight of the extracted dry lipid was determined by weighing the sample remaining in the centrifuge tube on a microbalance then subtracting the previously determined weight of the tube. The percent of holobiont dry weight consisting of lipid was calculated from the weighed lipid volume and the lyophilized holobiont weight (\((\text{weight of the dry lipid extract/weight of the dry holobiont homogenate}) \times 100\)).

*Method development for lipid separation*

Identifying general classes of anemone and algal lipids required standards for comparison. The standards, chosen based on their success with the anemone *Metridium*
senile (Hill-Manning and Blanquet 1979), were dl-α-palmitin (a monoglyceride, Fig. 3A),
glyceryl 1,3-dipalmitate (a diglyceride, Fig. 3B), glyceryl tripalmitate (a triglyceride, Fig. 3C),
cholesteryl oleate and cholesteryl palmitate (cholesterol esters, Fig. 3D and 3E respectively),
cholesterol (Fig. 3F), and palmitic acid (a fatty acid, Fig. 3G). Cholesteryl oleate was later chosen over cholesteryl palmitate as the cholesterol ester standard for its better separation with the modified solvent system and for its lower elution on the TLC plate.

To create stock solutions, the dry lipids purchased from Sigma-Aldrich were weighed and dissolved in a chloroform-methanol (1:1) mixture, producing concentrations of 10 µg/µl. New stock standards were created at the start of separation and quality control checked against previous standards made during method development.

To create standard curves for comparison with the unknown lipid solutions, stock lipid standards (dl-α-palmitin, glyceryl 1,3-dipalmitate, cholesterol, and palmitic acid) were combined to produce a 4-lipid mixed standard containing 2.5 µg/µl of each. Additional dilutions were then prepared from the mixed concentration standard using the chloroform-methanol (1:1) solution to create 1.25 µg/µl and 0.625 µg/µl concentrations. Standard curves, relating absolute intensity of the lipid spot to lipid content, were created using the original stock (10 µg/ µl) and the dilution series (2.5 µg/µl, 1.25 µg/µl and 0.625 µg/µl).

A second mixed standard, containing cholesteryl oleate and glyceryl tripalmitate, was created by preparing stock solutions of each, then combining and diluting them to 5.0 µg/µl, 2.5 µg/µl, 1.25 µg/µl and 0.625 µg/µl concentrations. Standard curves were again created. The 4-lipid mixed standards were adopted for the monoglyceride, cholesterol,
Figure 3. Chemical structures of non-polar lipids used as standards during TLC analysis: dl-α-palmitin (monoglyceride), glyceryl 1,3-dipalmitate (diglyceride), glyceryl tripalmitate (triglyceride), cholesteryl oleate and cholesteryl palmitate (cholesterol esters), cholesterol, and palmitic acid (fatty acid).
fatty acid, and diglyceride assays. The 2-lipid mixed standards were adopted for the triglyceride and cholesteryl ester assays. New mixed standard solutions were created after a month of service and quality control checked against previous standards as necessary.

We were unable to successfully separate all six lipid classes with the Freeman and West (1966) method, necessitating an adaptation to enhance separation of lipids on the TLC plates. We maintained two solvent systems as in Freeman and West (1966), but we modified Solvent System II by adjusting from diethyl ether, hexane in proportions of 6:94 to diethyl ether, hexane, and acetic acid in 6:94:3 proportions. This change in the solvent system enhanced separation of the lipids from the origin and from each other, and allowed complete elution before the solvent front reached the end of the plate. The solvent systems were allowed to separate lipids on the plates simultaneously in separate tanks. Solvent System I was used for the more polar monoglyceride, cholesterol, fatty acid, and diglyceride. Solvent System II was used for the less polar triglyceride and cholesteryl ester. Solvent systems were placed into service for a maximum of 4 plate processings after which new solvents were made and quality control checked against standards.

Following the exact protocol of Freeman and West (1966) would have required TLC plates 20 cm wide x 40 cm high. Because the available glass development tanks were only 25 cm high, the plates were cut to dimensions of approximately 20 cm wide x 20 cm high. Using two distinct solvent systems allowed for these shorter plates to adequately separate the lipids.

Freeman and West (1966) visualized lipid spots by brightening fluorescence with a 1% aqueous solution of Ultraphor and examining the plates under UV light. We chose
instead to stain the plates with a 10% phosphomolybdic acid (PMA) solution. Of the staining techniques we tested, PMA staining was the only method that produced spots whose intensity did not fade as the plates aged. After staining, the plates were charred at 120°C for 30 minutes.

*Lipid separation*

In preparation for lipid separation and determination, the dry holobiont extracts were dissolved in 1500 µl of chloroform-methanol (1:1), vortexed thoroughly and transferred to clean labeled vials (Fig. 2). Samples were then stored at -70°C until they could be processed (within 5 months).

To start a series of TLC separations, fresh liquid stock solutions of each standard were created. The mixed standard solutions were then prepared according to the solvent system being adopted (e.g., Solvent System I used the 4 lipid mixed standard and Solvent System II used the 2 lipid mixed standard). Fresh solvent systems were also prepared and quality control checks were performed to ensure proper calibration.

The TLC was performed on silica-gel G 250 µm plates (Analtech, Inc.) The glass plates were marked with a dull pencil to designate lanes and the origin for spotting the homogenate and standard samples (15 mm from the bottom edge). The plate adsorbent was activated prior to a separation by heating the plates at 120°C for 15 minutes.

The appropriate mixed standard (1.25 µg/µl of the 4 mixed standard for Solvent System I and 2.5 µg/µl of the 2 mixed standard for Solvent System II ) was applied to the first and last lanes on each plate and the unknown holobiont lipid solutions (2 replicate samples per anemone) were spotted in the remaining lanes. Samples from each anemone
were spotted in duplicate lanes on plates in each of the 2 solvent systems to create replicate tests for all 6 lipids of interest for each anemone. Spots were 10 µl in volume and were placed at the origin of each lane. After spotting, the plates were immediately heated at 120°C for 15 minutes to evaporate acetic acid, reducing the risk that the acid would change the polarities of the solvent systems. The plates were then air cooled for 5 minutes in a fume hood. The spotted, prepared plates were finally placed in the glass development tanks containing the appropriate solvent system. The solvent front was permitted to migrate to within 0-10 mm of the end of the plate, which took between 60 and 120 minutes depending on the amount of solvent in the tank, the freshness of solvent, and the solvent system being utilized.

Once the solvent front had reached the end point, the plates were removed from the tanks and permitted to dry in the fume hood for 5 minutes. The plates were then heated at 120°C for 30 minutes and air cooled for 5 minutes. Lipid spots were visualized by spraying the plates with a PMA stain, air drying them for 5 minutes then heating them at 120°C for 30 minutes. The plates cooled before being scanned at 300 dpi on a RICOH Aficio MP C4501 and saved as PDFs.

Algae processing

Extra homogenate from the anemones collected in January was employed to measure lipid content of the symbiotic algae extracted from the anemones (July samples did not have enough material). To have sufficient material for analysis, pools of algae were created. Two pools of zoochlorellae were created by combining homogenate from 9 and then 12 zoochlorellate anemones. Two pooled zooxanthellae samples were similarly
created with 8 and 11 zooxanthellate anemones. The pooled homogenate volumes were measured, and then the algae were separated from the animal tissue by centrifuging the samples at 1500 x g for 3-4 minutes. The supernatant was poured off and the algal pellet was resuspended in 5 µm filtered seawater. This process was repeated three times to thoroughly remove all anemone tissue. The final pellet of algae was resuspended in 15 ml of 5 µm filtered seawater.

A sample (1 ml) of the algal mixture was removed and frozen at -70°C for later cell counts. The remaining well-mixed 14 mls were placed in labeled vials and stored at -70°C until they could be extracted, dried, and weighed as previously described to determine algal percent dry weight due to total non-polar lipid. Sample lipid fractions were then separated with TLC.

*Photo-analysis*

Holobiont lipid profiles were semi-quantitatively analyzed using Adobe Photoshop CS2. Scanned images of the TLC plates were converted to 72 dpi and grayscale mode. The images were then inverted to make the white lipid spots stand out against a dark background. Contrast was increased (+50) and the image enlarged by 200%.

With the lasso tool we circled each lipid spot. Identifying the boundaries of the spots required some judgment. To ensure there was no bias in the process, plates were identified only by number and anemone samples were run in random order. Once a spot had been outlined, its mean gray level (average darkness of the spot) and number of pixels (total size of the spot) were recorded from the Photoshop histogram window. The product of these 2 values gave a measure of the absolute intensity of each spot. The
measure tool was then employed to determine the distances between 1) the origin of the spot and the solvent front and 2) the origin of the spot and the center or most intense area of the spot at its final location. These distance measurements (retention times) permitted calculation of retention factors (Rf).

Absolute intensity and Rf were calculated for all lipid spots in all lanes on every plate. Final absolute intensity values were calculated by averaging the 2 replicate lanes. However, if a clear error occurred in a lane (e.g., a sample ran off the plate), a single value was adopted. Standard Rf values and the shape of the spots verified the identity of the lipids represented by each spot.

Data analysis
Because temperature, development time, and solvent age produced variability in development of lipid spots on the TLC plates, it was necessary to adjust for those differences. To do so, we made standard quality control (QC) plates by separating all standards individually, which created reference spots. New QC plates were generated each time new standards or solvent systems were made and these were accepted as the references for all plates produced with those standards or solvent systems. The absolute intensities of all reference spots from standard QC plates were calculated as described above.

The anemone and algae plates were grouped into batches that corresponded to each new set of standards, mixed standards, and solvent systems. The standards on the QC plates and on the plates with anemones were then used to calculate a relative intensity
correction factor (RICF):

\[
\frac{\text{Absolute intensity of a Lipid Mixed Standard on QC check plate}}{\text{Absolute intensity of a Lipid Mixed Standard on a anemone plate}} = RICF
\]

The RICF standardized the darkness of lipid spots on each plate.

Anemones and algae were matched with the appropriate RICF from the mixed standards for each lipid fraction. The anemone or algae absolute intensities were then multiplied by the RICF to calculate the corrected absolute intensities (CAI) for each lipid fraction of each anemone or algae on each plate.

With the lipid standard curves we converted CAI to lipid concentration. The linear regression (forced thru zero) from the standard curves, granted calculation of the lipid concentrations for each lipid of each anemone. Homogenate volumes could then be employed to calculate lipid content of the entire anemone. Freeze dried weights were similarly extrapolated to the whole anemone allowing for lipid/dry weight normalization.

Two-way ANOVAs tested for significant differences in holobiont dry weight, holobiont percent total non-polar lipid, and holobiont percent lipid fractions as a function of symbiont (E. marina or S. muscatinei) and season (July-summer and January-winter). In addition, we tested for differences in the extracted algae themselves. Elliptochloris marina and S. muscatinei cell densities, percent total non-polar lipid, and percent lipid fractions were each compared with one-way ANOVAs. Since only algae from anemones collected in January were tested, there was no way to look for seasonal effects.

Levene’s tests verified homogeneity of variances prior to running all analyses and data were transformed if the assumption was violated. A log transformation was necessary for the cholesterol and palmitic acid data. A square root transformation homogenized variances for the zoochlorellae density data. In one case, holobiont percent
total non-polar lipid, transformation could not stabilize variances so $\alpha$ was adjusted to 0.025 as recommended by Keppel and Wickens (2004). All statistical analyses were conducted using SPSS version 19.0.
RESULTS

Solvent systems

TLC, using a dual solvent system, was an effective way to assay lipids in A. elegantissima. Solvent System I successfully separated the lipids from the origin (Fig. 4A). The dl-α-palmitin resolved distinctly from the other lipid classes, but only one of the anemones examined contained dl-α-palmitin so no further analysis of that lipid was performed. Palmitic acid and cholesterol were not resolved as completely. Occasionally these two lipids had similar Rf values and the palmitic acid spot often produced a tail. These features made identification and quantification challenging in some cases. However, using the standard plates for reference and comparing the mixed standards on each plate yielded proper identification and accurate quantification. Glyceryl 1,3-dipalmitate did not resolve as one spot but as two. However, this did not affect the analysis since none of the anemones tested produced lipid spots in this range, indicating that 1) the anemones did not contain glyceryl 1,3-dipalmitate or 2) our method did not successfully extract or separate this compound.

Solvent System II clearly separated the lipids glyceryl tripalmitate and cholesteryl oleate from the origin and from each other (Fig. 4B). The two lipid classes had very different Rf values, which made identification and quantification easy. Unknown lipids developed close to the glyceryl tripalmitate in some anemone samples and in the standards, but the Rf values of the standards allowed identification of the glyceryl tripalmitate. The cholesteryl oleate resolved near the end of the plate and occasionally coincided with the solvent front. However, this was not an issue in the final analysis since no anemones contained cholesteryl oleate.
Figure 4. TLC plates demonstrating lipid separation in two solvent systems. A) Plate developed in Solvent System I. The standards applied in lanes across the plate, from left to right, were (1) dl-α-palmitin, (2) palmitic acid (fatty acid), (3) cholesterol, (4) glyceryl 1,3-dipalmitate, (5 and 6) a mixture of (1), (2), (3) and (4). B) Plate developed in Solvent System II. The standards applied in lanes across the plate, from left to right, were (1) glyceryl tripalmitate, (2) cholesteryl oleate, (3 and 4) a mixture of (1) and (2).
Holobiont dry weight

Comparison of holobiont dry weights showed that the anemones collected in July were, on average, 41% larger than those collected in January. ANOVA showed that this difference was statistically significant ($F_{1, 82} = 207.7, p < 0.01$; Fig. 5). However, there was no significant difference in size of zooxanthellate and zoochlorellate anemones ($F_{1, 82} = 3.04, p = 0.08$) and there was no significant season by symbiont interaction ($F_{1, 82} = 0.33, p = 0.56$).

Symbiont density

Densities of symbionts did not change significantly between July and January in either zooxanthellate or zoochlorellate anemones when measured as cells per $\mu$g anemone dry weight ($F_{1, 44} = 0.47, p = 0.49$, and $F_{1, 38} = 0.02, p = 0.87$ respectively; Fig. 6A-B). Symbiont densities overall tended to be slightly higher in zoochlorellate anemones. Results were very different when densities were measured as cells per $\mu$g protein; symbiont densities of both zooxanthellate and zoochlorellate anemones were statistically different across seasons ($F_{1, 44} = 7.75, p < 0.01$, and $F_{1, 39} = 7.27, p = 0.01$ for zooxanthellate and zoochlorellate anemones respectively; Fig. 7A-B). On average, both zooxanthellate and zoochlorellate anemones had approximately 33% more cells per $\mu$g protein in January than they did in July. Overall cell densities were almost twice as high in zoochlorellate anemones as they were in zooxanthellate individuals.
Figure 5. Holobiont freeze dried weight of zoochlorellate and zooxanthellate *A. elegantissima* collected in July and January. Uppercase letters over the bars signify statistically significant differences between seasons. Number of replicates is shown as numbers within the bars. Standard errors are shown.
Figure 6. A) Symbiont density based on holobiont dry weight for July and January zooxanthellate and B) July and January zoochlorellate *A. elegantissima*. Standard errors are shown.
Figure 7. A) Symbiont density based on holobiont protein for July and January zooxanthellate and B) July and January zoochlorellate *A. elegantissima*. Uppercase letters over the bars signify statistically significant differences between seasons. Standard errors are shown.
**Holobiont lipid profile**

Analysis of percent holobiont dry weight consisting of non-polar lipid showed no statistically significant seasonal pattern ($F_{1, 82} = 0.63, p = 0.43$; Fig. 8). However, there was a trend toward slightly higher lipid levels and more anemone to anemone variability in the July samples. Symbiotic state had a significant effect on lipid content ($F_{1, 82} = 4.29, p = 0.04$). Zooxanthellate anemones had significantly more non-polar lipid than did zoochlorellate anemones in July and January. There was no significant season by symbiont interaction ($F_{1, 82} = 0.77, p = 0.38$).

Our standard curves for the lipid fractions showed good $r^2$ values so applying absolute intensity as a measure of lipid content was appropriate (Fig. 9-11). Cholesterol and palmitic acid were present in most of the anemones tested, with nearly identical patterns for the two lipid classes. A greater proportion of the non-polar lipid was composed of these 2 fractions in January than it was in July ($F_{1, 82} = 23.6, p < 0.01$ for cholesterol and $F_{1, 82} = 11.3, p < 0.01$ for palmitic acid; Figs. 12 and 13). Measured as the percent of total non-polar lipid, the content of both more than doubled on average between July and January. Zooxanthellate anemones consistently had a higher percentage of their lipid composed of cholesterol and palmitic acid than did zoochlorellate individuals ($F_{1, 82} = 4.52, p = 0.03$ for cholesterol and $F_{1, 82} = 8.66, p < 0.01$ for palmitic acid). There was no season by symbiont interaction for either fraction (cholesterol: $F_{1, 82} = 1.70, p = 1.95$ and palmitic acid: $F_{1, 82} = 1.30, p = 0.25$).

Patterns of glyceryl tripalmitate content were similar to those of the other lipid fractions with, on average, a near doubling of the percentage of total non-polar lipid.
Figure 8. Holobiont non-polar lipid content as a percentage of dry weight for zoochlorellate and zooxanthellate *A. elegantissima* collected in July and January. Lowercase letters over the bars signify statistically significant differences between the zoochlorellate and zooxanthellate anemones. Standard errors are shown.
Figure 9. Standard curve of cholesterol. The line represents a linear regression of the points. Absolute intensity is a measure of the mean darkness of the lipid spot per area of the spot.
Figure 10. Standard curve of palmitic acid (FA). The line represents a linear regression of the points. Absolute intensity is the mean darkness of the lipid spots per area of the spot.
Figure 11. Standard curve of glyceryl tripalmitate (TG). The line represents a linear regression of the points. Absolute intensity is the mean darkness of the lipid spots per area of the spot.
Figure 12. Holobiont cholesterol content as a percentage of total non-polar lipid for zoochlorellate and zooxanthellate *A. elegantissima* collected in July and January. Uppercase letters over the bars signify statistically significant differences between seasons and lowercase letters over the bars signify statistically significant differences between the zoochlorellate and zooxanthellate anemones. Standard errors are shown.
Figure 13. Holobiont palmitic acid (FA) content as a percentage of total non-polar lipid for zoochlorellate and zooxanthellate *A. elegantissima* collected in July and January. Uppercase letters over the bars signify statistically significant differences between seasons and lowercase letters over the bars signify statistically significant differences the zoochlorellate and zooxanthellate anemones. Standard errors are shown.
composed of glyceryl tripalmitate between July and January ($F_{1,82} = 7.88, \ p < 0.01$; Fig. 14). However, there was no statistically significant difference between anemones in the two symbiotic states ($F_{1,82} = 0.96, \ p = 0.32$). Nor was there a sufficient season by symbiont interaction ($F_{1,82} = 1.21, \ p = 0.27$). Absolute weights of holobiont total non-polar lipid and lipid fractions are given in Table 1 for comparison with the work of others.

*Symbiont lipid profile*

Because measurements of lipid in the holobiont included the contribution of both the animal and the algal parts of the symbiosis, and we were primarily interested in the lipids of the animal alone, it was important to measure the lipid content of the extracted symbionts alone to see how they contributed to the lipid profile of the holobiont. This was done only for the January samples.

Percent of the algae alone composed of non-polar lipid was not significantly different for zooxanthellae and zoochlorellae ($F_{1,2} = 0.32, \ p = 0.62$; Fig. 15), nor was there a significant difference in the content of cholesterol ($F_{1,2} = 0.56, \ p = 0.83$; Fig. 16A) or palmitic acid ($F_{1,2} = 0.80, \ p = 0.46$; Fig. 16B). Only glyceryl tripalmitate was significantly different between the 2 species, with zooxanthellae containing significantly more than zoochlorellae ($F_{1,2} = 230.3, \ p < 0.01$; Fig. 16C). This was a surprise since glyceryl tripalmitate was the only lipid fraction not statistically different between the holobionts.
Figure 14. Holobiont glycercylin tripalmitate (TG) content as a percentage of total non-polar lipid for zoochlorellate and zooxanthellate *A. elegantissima* collected in July and January. Uppercase letters over the bars signify statistically significant differences between seasons. Standard errors are shown.
Table 1. Lipid composition of zoochlorellate and zooxanthellate *A. elegantissima* collected in July and January based on dry weights. Mean values are shown with standard deviations in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Zoochlorellate <em>A. elegantissima</em></th>
<th>Zooxanthellate <em>A. elegantissima</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>July</td>
<td>January</td>
</tr>
<tr>
<td>Dry Weight (mg)</td>
<td>3084.4 (476.7)</td>
<td>1839.3 (375.7)</td>
</tr>
<tr>
<td>Total Non-polar Lipid (mg)</td>
<td>90.9 (68.9)</td>
<td>41.6 (14.3)</td>
</tr>
<tr>
<td>Cholesterol (mg)</td>
<td>0.7 (0.5)</td>
<td>2.1 (2.5)</td>
</tr>
<tr>
<td>Palmitic Acid (mg)</td>
<td>0.7 (0.5)</td>
<td>0.9 (1.0)</td>
</tr>
<tr>
<td>Glyceryl Tripalmitate (mg)</td>
<td>1.1 (0.8)</td>
<td>1.9 (1.8)</td>
</tr>
<tr>
<td>n</td>
<td>17</td>
<td>23</td>
</tr>
</tbody>
</table>
Figure 15. Symbiont non-polar lipid content as a percentage of dry weight for January zoochlorellae (ZC) and zooxanthellae (ZX). Number of replicates is shown as numbers within the bars. Standard errors are shown.
Figure 16. Symbiont cholesterol (C) content as a percentage of total non-polar lipid for January zoochlorellae (ZC) and zooxanthellae (ZX) and B) Symbiont palmitic acid (FA) content as a percentage of total non-polar lipid for January zoochlorellae and zooxanthellae and C) Symbiont glyceryl tripalmitate (TG) content as a percentage of total non-polar lipid for January zoochlorellae and zooxanthellae. Lowercase letters over the bars signify statistically significant differences between symbionts. Standard errors are shown.
Due to low sample size and limited statistical power, these results should be interpreted cautiously. In general, however, they suggest that there are no large differences in lipid profiles of the 2 symbionts with the exception of glyceryl tripalmitate.
DISCUSSION

*Anthopleura elegantissima* lives in an environment of extreme variability. Seasonality can be strong in the temperate part of its range, where it experiences large temporal changes in temperature, day length, and prey availability (Bingham 1997, Dimond et al. 2011). We saw clear impacts of that seasonality on anemone body mass, with a large winter decrease in holobiont dry weight. Average holobiont dry weight dropped by 41% between summer and winter, possibly due in part to a decrease in the zooplankton and small invertebrates it normally eats (Sebens 1981a). Dimond et al. (2011) reported that chlorophyll levels (a proxy for abundance of potential planktonic prey) dropped approximately 75% between summer and winter in the waters from which our anemones were collected. Because *A. elegantissima* relies heavily on heterotrophy (Hiebert and Bingham 2012), a lack of prey could have a significant impact on body weight as the animal metabolizes resources to meet energy demands amidst a decreased prey field (Fitt and Pardy 1981, Ruiz et al. 1992).

A decrease in *A. elegantissima* body size could also result from fission (Sebens 1983), which can be induced by reduced prey availability. Sebens (1980) found that starvation in the laboratory induced fission of *A. elegantissima*. He also found that rates of fission in populations from the San Juan Islands increased in the winter (Sebens 1983) and suggested that fission may be a strategy utilized by the anemones to increase surface area for prey capture during a period of low food availability. Therefore, it is possible that the decrease in size we observed was simply a result of anemone fission. However, if the decrease in body weight was due entirely to fission, we would expect no change in the lipid content as a percent of dry weight between summer and winter. In fact, the total
non-polar lipids of *A. elegantissima* tended to decrease in winter and there was less anemone-to-anemone variability. This decrease in lipid suggests that the weight loss we saw may have been partially a result of fission, but exaggerated by the metabolism of stored lipids.

A winter decrease in all lipids (polar and non-polar) per individual has been seen for the scleractinian coral *Goniastrea aspera* from Japan (Oku et al. 2003), while a decrease in the non-polar lipid alone during the winter months has been reported in *A. elegantissima* in California (Jennison 1979b) and the anemone *Metridium senile* from Massachusetts (Hill-Manning and Blanquet 1979). Decreases in lipid due to partial seasonal metabolism of energy reserves could be made worse by a reduction in the amount of fixed carbon, carbohydrate and lipid translocated from the symbionts to the host (Zamer and Shick 1989, Anthony et al. 2002). Bergschneider and Muller-Parker (2008) found that the productivity of zooxanthellae and zoochlorellae extracted from *A. elegantissima* dropped by 83.6% and 59.3%, respectively, during the winter months. In addition, changes in the quality of light, like those associated with seasonal changes, influence the photosynthetic rates of different marine algae causing differential synthesis of biochemical components (Wallen and Geen 1971). The lack of light particularly in wavelengths necessary to fuel photosynthesis could lead the symbionts to switch from autotrophy to heterotrophy, leaving the symbionts to draw from the nutritional reserves of the host and become energy sinks rather than an energy sources (Steen 1986, Stat et al 2008). This could become important if the anemone is in a season when available nutrient resources are being directed to development of gonad in preparation for the reproductive season.
Anthopleura elegantissima develop gonads from early winter to summer and spawn in the summer and early fall (Ford 1964, Jennison 1979a, b, Sebens 1981b). Gamete production in preparation for spawning increases energetic requirements, generally leading to a build-up of lipids prior to the summer/early fall spawning events (Jennison 1979a, b, Sebens 1981b). Such an allocation of lipid to gonad and gamete production has been reported for a number of invertebrate species (e.g., Vijayaraghavan and Easterson 1974, Kozhina et al. 1978, Wenne and Polak 1989, Ward 1995). In our study, the total non-polar lipid levels appeared lower in the winter, which is what we would predict if the anemones had recently spawned and were in the early stages of gonad development. Microscopic examination of the anemones in January revealed no identifiable gonads. In contrast, every A. elegantissima in July was reproductive, with well-developed gonad tissue (B. Bingham, pers. com.).

Even as total non-polar lipid decreased in the winter anemone samples, all constituent lipids present, when measured as a percent of total non-polar lipid, increased. This indicates that the anemones were not metabolizing these particular lipid fractions at this time, perhaps instead reserving them to build gonad tissue. Kozhina et al. (1978) showed that eggs of the sea urchin Strongylocentrotus intermedius contained triglycerides, fatty acids and cholesterol while the sperm contained cholesterol. These are the lipid classes, represented by glyceryl tripalmitate, palmitic acid, and cholesterol, respectively, that we saw increasing in the winter samples. If the anemones were actively metabolizing stored lipids in winter to meet metabolic demands, they were fractions other than those we measured.
Elevated levels of cholesterol, triglyceride and fatty acid in marine invertebrates have been variously attributed to energy reserves, mucus production, adaptation to colder temperatures, and to gonad and gamete production (Bergmann et al. 1956, Patton et al. 1977, Hill-Manning and Blanquet 1979, 1980, Oku et al. 2003). These particular lipid components increased and decreased in similar ways in *A. elegantissima*, suggesting they all could be connected to the reproductive cycle of this anemone. We saw an increase in percent cholesterol and fatty acid in our winter samples similar to that reported for the coral *G. aspera* (Oku et al. 2003). A winter increase in cholesterol may be related to synthesis of membrane components for gametes (Hill-Manning and Blanquet 1980) while a summer decrease could be partially due to somatic tissue growth as reported in the scleractinian coral *Tubinaria reniformis* (Treignier et al. 2008). Hill-Manning and Blanquet (1980) showed that palmitic acid, the fatty acid we measured, is used to synthesize triglycerides in the anemone *M. senile*. They reported an increase in the percent triglyceride in *M. senile* during the winter (Hill-Manning and Blanquet 1979) similar to what we observed in *A. elegantissima*. If these lipid fractions are involved in gamete production and gametes, we would expect their levels to be highest in the summer prior to spawning. However, this was not the case. If the *A. elegantissima* are using these fractions for reproduction in the summer it may be partially for metabolic energy, which would explain the summertime decrease.

Independent of the seasonal changes we saw in lipid content and composition, we also saw differences related to the symbiotic condition of the anemones. The lipid profiles for zooxanthellate and zoochlorellate anemones differed even though the anemones came from the same location (within 50 cm of each other) and the same tidal
height. Presumably, all individuals had access to the same food resources and experienced similar tidal exposure. Despite the similarity in their location, however, their light environment was sufficiently distinct to create differences in the symbiotic state of the anemones on opposite sides of the crevice from which they were collected. These differences were reflected in the clear separation of zooxanthellate individuals on the western side of the crevice from zoochlorellate individuals on the eastern side. As reported by Sebens (1983), anemones in cracks can experience microhabitat differences sufficient to affect their ecology.

Zooxanthellate anemones in our study had significantly more total non-polar lipid and a higher percentage of their lipid was composed of cholesterol, fatty acid and triglyceride: lipids associated with energy storage and reproduction. Increased levels of lipid in zooxanthellate anemones may be due to slight differences in irradiance levels, which have been shown to influence the quantity of lipids in the symbiotic anemone Anemonia viridis (Harland et al. 1992). Fitt and Pardy (1981) reported that symbiotic A. elegantissima in the light can maintain their lipid reserves even when they are starved. However in the dark anemones can maintain their lipid stores only when fed heterotrophically. Increased light levels would provide the symbionts with more fuel for photosynthesis, and with the higher productivity of zooxanthellae, which can be 2.5x more productive than zoochlorellae (Bergschneider and Muller-Parker 2008), can potentially fill a significantly greater proportion of their hosts metabolic needs (Verde and McCloskey 2007).

The lipid levels in a cnidarian can be affected by direct translocation of lipids from the symbionts, not requiring additional processing by the host. Blanquet et al.
(1979) found palmitic acid to be a major fatty acid by mass in both partners of the *A. elegantissima* holobiont and they found evidence that saturated fatty acids are translocated directly from zooxanthellae to host. Harland et al. (1991) proposed that synthesis of triglycerides in the anemone *Anemonia viridis* is done by zooxanthellae, which translocate the products directly to the host for storage. Similar measurements have not yet been made for zoochlorellae. If lipids are being translocated directly from symbiont to host, symbionts as different as zooxanthellae (dinoflagellates) and zoochlorellae (chlorophytes) could be providing not only different amounts of lipid, but also different kinds of lipids.

Lipid changes reported here for zooxanthellate and zoochlorellate anemones could have been partially due to the lipid content of the symbionts themselves. Our direct comparisons of the lipids in zooxanthellae and zoochlorellae extracted from their hosts lacked statistical power due to limited replication. However, even with limited power, we saw clear differences in the amount of triglyceride in zooxanthellae and zoochlorellae, with zooxanthellae having a higher percentage of this important storage lipid. Cnidarian zooxanthellae are known to synthesize triglycerides (Patton et al. 1977, Blanquet et al. 1979, Harland et al. 1991), but no study of *E. marina* lipid synthesis has yet been done. The individual lipids we measured accounted for only 15% of the total non-polar lipid in our samples. Recovery of all the lipids may have revealed very different patterns. There is a clear need to identify and quantify the other lipids present in these animals and their symbiotic partners.

The lipid characteristic of an *A. elegantissima* holobiont is influenced by the identity of the symbionts it contains, but it is likely due to the translocation of products
from the symbionts and not simply to differences in the lipid content of the zooxanthellae and zoochlorellae themselves. Blanquet et al. (1979) found that *S. muscatinei* make up only about 7.7% of the dry weight of *A. elegantissima*. Though no similar data are available for *E. marina*, the values are probably similar. It is unlikely that weight fractions this low are sufficient to produce the differences we saw in holobiont lipids. Furthermore, some of the patterns in the lipids of zooxanthellae and zoochlorellae were opposite those seen in their hosts. The differences in holobiont lipid patterns appear to occur independent of differences in the lipids of the symbionts.

Our lipid measurements suggest that zoochlorellate *A. elegantissima* are at a disadvantage relative to zooxanthellate individuals. Zooxanthellate hosts have higher total lipid content, presumably as a result of either increased metabolic costs of zoochlorellae or as a result of poor efficiency of lipid translocation from zoochlorellae. These differences will undoubtedly be magnified as the anemones are exposed to increasing temperatures like those predicted under a scenario of global climate change (IPCC 2007), since the photosynthetic capacity of zoochlorellae is much more affected by high temperatures compared to that of zooxanthellae (Engebretson and Muller-Parker 1999, Verde and McCloskey 2001). Zooxanthellate anemones under all conditions appear to have more lipids available for metabolism and reproduction.

Scleractinian corals have the ability to shift symbionts under conditions of temperature stress (Mieog et al. 2007, Jones et al. 2008), replacing symbionts vulnerable to high temperature to more thermally tolerant clades. It has been suggested that this capacity increases the potential of the host to survive extreme conditions. It is less clear how such a change might affect function of the holobiont in the form of energy exchange,
growth and reproduction. Our results suggest that reported switches in the symbionts of
A. elegantissima (Bates 2000) can have a significant effect in lipid profiles. Whether
those changing profiles ultimately result in increasing or decreasing fitness and whether
such changes will also be seen in other symbiotic cnidarians are important questions that
deserve additional attention.
References


