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# Effect of Symbiotic State on the Fatty Acid Composition of *Anthopleura elegantissima*

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**EFFECT OF SYMBIOTIC STATE ON  
THE FATTY ACID COMPOSITION OF  
ANTHOPLEURA ELEGANTISSIMA**

by

Andrés J. Quesada

Accepted in Partial Completion  
Of the Requirements for the Degree  
Master of Science

Kathleen L. Kitto, Dean of the Graduate School

ADVISORY COMMITTEE

Chair, Dr. Brian L. Bingham

Dr. Katherina Schoo

Dr. Brooke Love

## **MASTER'S THESIS**

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Andrés J. Quesada,  
June 15, 2015

**EFFECT OF SYMBIOTIC STATE ON  
THE FATTY ACID COMPOSITION OF  
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A Thesis Presented to  
The Faculty of  
Western Washington University

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Of the Requirements for the Degree  
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June 2015

## ABSTRACT

*Anthopleura elegantissima*, the most abundant intertidal sea anemone on the Pacific coast of North America, naturally occurs in three distinct symbiotic states: zooxanthellate (hosting the dinoflagellate *Symbiodinium muscatinei*), zoochlorellate (hosting the chlorophyte *Elliptochloris marina*), and aposymbiotic (lacking symbionts). *S. muscatinei* is more productive during summer months and may translocate considerably more carbon to the sea anemone host than does *E. marina*. Quantitative and qualitative differences in the contribution of energy from the symbiont are believed to affect host lipid levels, influencing growth and reproductive output. To study the effect of symbiotic state on host lipids in *A. elegantissima*, we collected ten specimens in each symbiotic state from the same habitat, separated symbiont and host tissues, and compared the fatty acid profiles of the two symbionts themselves, compared the fatty acid profiles of anemones in the three symbiotic states, and compared the profiles of the symbionts to their host anemones. We found significant differences in the fatty acid profiles of *S. muscatinei* and *E. marina*, with docosahexaenoic acid (DHA), oleic acid, and  $\alpha$ -linolenic acid contributing most to the differences. DHA was abundant in *S. muscatinei*, while *E. marina* contained high levels of oleic and  $\alpha$ -linolenic acids. We found higher total fatty acid content in zooxanthellate hosts than in zoochlorellate hosts, supporting previous research suggesting that *S. muscatinei* is a more favorable symbiont. We also found that aposymbiotic sea anemones had significantly lower concentrations of total fatty acids than symbiotic hosts and overall lower levels of all fatty acids, including polyunsaturated fatty acids (PUFAs). Because all the anemones came from

the same habitat and should have had similar diets, and because cnidarians cannot synthesize PUFAs *de novo*, the depressed levels of fatty acids in aposymbiotic *A. elegantissima* suggest that these anemones deplete fatty acids faster due to the lack of symbiont photosynthate input. PUFAs play an important role in invertebrate growth and reproduction, so we expect symbiotic anemones to have higher fitness than aposymbiotic ones. Finally, although previous research suggests that symbionts translocate specific fatty acids to their cnidarian hosts, we found no evidence for fatty acid translocation in *A. elegantissima*. Our results reinforce the idea that hosting either type of symbiont increases the fitness of *A. elegantissima*, that hosting *S. muscatinei* may be more advantageous to the anemone, but do not support the hypothesis of fatty acid translocation between symbiont and host in this species.

## ACKNOWLEDGEMENTS

I would like to express my deepest appreciation and gratefulness to everyone who helped me complete this thesis. Brian Bingham provided exceptional guidance throughout the process. I am very thankful for his mentorship, kindness, understanding and relentless support. Katherina Schoo taught me how to conduct lipid analyses and exhibited selfless dedication to this project. Brooke Love participated in my thesis committee and made valuable observations that improved the manuscript. Jay Dimond taught me the methods to process sea anemones and generously shared his expertise on symbiosis. Charles Wandler and Erin Macri offered valuable assistance with the operation of the GCMS. Gene McKeen and Nathan Schwarck provided support with logistics, including laboratory use and sample collection.

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*Felix, qui potuit rerum cognoscere causas.*  
Fortunate [is he] who can know the causes of things.  
-Virgil

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## INTRODUCTION

Many cnidarians associate symbiotically with autotrophic, unicellular, dinoflagellate algae of the genus *Symbiodinium* (commonly called zooxanthellae), that provide them with photosynthetic products such as glucose, glycerol, amino acids and lipids (Yellowlees et al. 2008). Research suggests that the interaction between cnidarians and their endosymbiotic algae is not always mutualistic, but rather a continuum, ranging from parasitism to mutualism (Lesser et al. 2013). The nature of the relationship may be determined by the identity of the symbiont. For example, Mieog et al. (2009) found that growth, survival, and thermal tolerance of a tropical coral is determined by the *Symbiodinium* clade hosted. Another study showed that hosting clade A *Symbiodinium*, which fix less carbon than clade C, may reduce host fitness by not providing enough carbon to meet the host's nutritional requirements (Stat et al. 2008). Loram et al. (2007) found that sea anemones harboring clade A *Symbiodinium* incorporated more photosynthesis-fixed carbon into lipids and less into sugars and amino acids, compared to individuals hosting clade B symbionts. These quantitative and qualitative differences in the contribution of energy from the symbiont are believed to affect host lipid levels, in turn influencing host growth and reproductive output (Stimson 1987; Ward 1995; Leuzinger et al. 2003).

A growing body of evidence indicates that lipids play an important role in cnidarian-algal symbioses. For example, research has shown that *Symbiodinium* cells and cnidarian cells hosting *Symbiodinium* accumulate wax esters, triglycerides, sterols, phospholipids, and fatty acids in organelles called lipid bodies (Peng et al. 2011; Chen et al. 2011; Jiang et al. 2014; Pasaribu et al. 2014). Kopp et al. (2015) found that zooxanthellae cells in corals turn inorganic bicarbonate and nitrates into lipid droplets and glycogen. These photosynthates accumulate in the zooxanthellae

when exposed to light, showing that production of lipid droplets is linked to photosynthesis. The size and density of these lipid bodies is positively correlated with solar irradiation (Peng et al. 2012). Luo et al. (2009) suggest that “lipid trafficking” between symbiont and host lipid bodies plays a role in regulating the endosymbiosis. It is also believed that fatty acids produced by algal symbionts are translocated to their cnidarian hosts (e.g. Papina et al. 2003; Treignier et al. 2008; Imbs et al. 2014), with the presence of dinoflagellate fatty acid markers (i.e. fatty acids that can be synthesized by algae but not by animals) in cnidarian host tissue as evidence for translocation (Papina et al. 2003; Papina et al. 2007). Other authors have cited similarities in the relative proportions of fatty acids in zooxanthellae and host tissue as evidence for translocation (Treignier et al. 2008; Mortillaro et al. 2009; Imbs et al. 2011; Imbs et al. 2014). However, it should be noted that fatty acids and fatty acid markers can also be obtained through the diet, so the evidence for translocation to date remains inconclusive.

*Anthopleura elegantissima* is the most abundant intertidal sea anemone on the Pacific coast of North America and has been extensively used as a symbiosis research subject because individuals naturally occur in three distinct symbiotic states: zooxanthellate (hosting the dinoflagellate *Symbiodinium muscatinei*), zoochlorellate (hosting the chlorophyte *Elliptochloris marina*), and aposymbiotic (lacking symbionts) (LaJeunesse and Trench 2000; Lewis and Muller-Parker 2004). The two symbionts are taxonomically, ecologically, morphologically, and metabolically different. *E. marina* is negatively affected by high light and temperatures (Verde and McCloskey 2001; 2002), so zoochlorellate *A. elegantissima* are restricted to cooler, shaded sites, whereas zooxanthellate sea anemones occur in areas with more sunlight (Secord and Muller-Parker 2005). When in symbiosis with *A. elegantissima*, *E. marina* is about four times

more abundant and grows up to eight times faster than *S. muscatinei*, but has about half the volume, half the carbon content per cell, and less chlorophyll (Verde and McCloskey 1996; Bergschneider and Muller-Parker 2008). Research suggests that *S. muscatinei* is a more favorable symbiont, because its productivity during summer months is about 2.5 times greater than that of *E. marina* (Bergschneider and Muller-Parker 2008) and its population potentially translocates up to five times more carbon to the sea anemone (Verde and McCloskey 1996). However, Engebretson and Muller-Parker (1999) suggest that the total amount of carbon translocated to the host may be similar for both symbionts, mainly due to the greater density of *E. marina*. Hence it is unclear whether the greater productivity of *S. muscatinei* translates into higher rates of translocation and improved host performance. Nor is it known whether the two symbiont species transfer the same photosynthetic products to their hosts, which seems unlikely given their taxonomic differences.

The main goal of our study was to understand how symbiotic state affects fatty acid composition in *A. elegantissima*. Our specific approaches were, (i) to compare the fatty acid profiles of the symbionts *S. muscatinei* and *E. marina* themselves, (ii) to compare the fatty acid profiles of *A. elegantissima* in the three symbiotic states looking for differences in fatty acid concentration and composition that might produce fitness differences, and (iii) to compare the fatty acid profiles of the symbionts to their host sea anemones to determine similarities in fatty acid concentration and composition that may indicate translocation.

## METHODS

### Field sampling

Zooxanthellate, zoochlorellate, and aposymbiotic *A. elegantissima* were collected from Lawrence Point, Orcas Island on 26 June, 2014, in early summer, when studies demonstrate considerable differences in symbiont productivity due to high solar radiation and temperature (Bergschneider and Muller-Parker 2008). Ten specimens in each of the three symbiotic states were collected haphazardly from roughly the same tidal height (0 to +.5 m MLLW) within an area of approximately 30 m<sup>2</sup>. Presumably, sea anemones in this limited area at the same location and tidal height capture similar prey items, and might be expected to have similar lipid profiles if symbionts have no effect on the lipid composition of their host.

Specimens were collected by removing them from the rocky surface with a small spatula, placing them in a zip-top bag, and storing them in a bucket of seawater. The anemones were transported to Shannon Point Marine Center (SPMC), in Anacortes, WA where they were placed in individual glass dishes submerged in sea tables with running seawater. They were held for 96 h to allow them to empty their gut contents, ensuring that all fatty acids extracted were from the sea anemones themselves and not from ingested prey.

### Preparation of host and symbiont fractions

To permit comparison of the lipid profiles of symbionts and hosts, it was important to separate the algal symbionts from the sea anemone gastrodermal cells housing them. This was accomplished through homogenization and centrifugation. The sea anemones were cleared of any attached debris, then placed in a blender cup with 10-15 mL of filtered seawater and

homogenized for 30 seconds. The homogenate was poured into borosilicate glass test tubes and centrifuged for 2 minutes at high speed. This rendered a supernatant containing the host fraction and a pellet comprised of symbiont cells and a small amount of host material (mostly mesoglea). The supernatant was transferred to a new test tube and centrifuged again. This was repeated a third time to remove any remaining symbionts and the cleaned supernatant, containing only host cells, was pipetted into a 50 mL centrifuge tube and stored at -80°C.

A clean symbiont fraction was obtained by taking the pellet from the initial centrifugation through several washes and filtrations to remove any remaining host cells. The cells were resuspended by adding filtered seawater to the pellet and vortexing for 5 seconds. The mixture was centrifuged for 2 minutes at high speed and the supernatant, which contained small amounts of anemone mesoglea, was discarded. This procedure was repeated twice more to thoroughly wash the symbionts and remove remaining host material. The symbionts were resuspended in filtered seawater then forced through a filter syringe with a 100 µm Nitex mesh into a new test tube. The clean symbiont fraction was then transferred to a 15 mL centrifuge tube, and stored at -80°C. After being frozen for at least two days, both the animal fractions and cleaned symbionts were freeze dried at -50°C for 48 hours.

### Lipid extraction

Fatty acid extractions were based on a modification of the methods of Folch et al. (1957) and Bligh and Dyer (1959). Preliminary tests showed that approximately 20 mg of freeze dried tissue was optimal for our lipid extraction protocol and analyses. To initiate the extraction, 20-25 mg of freeze dried sea anemone (from a single individual) or symbiont (extracted from a single



anemone) was added to individual test tubes. The exact mass was noted to standardize the fatty acid concentrations in all samples to 20 mg. An internal fatty acid standard of nonadecanoic acid (19:0C) in a 2:1 (vol:vol) dichloromethane/methanol solution was added along with 4 mL of 2:1 dichloromethane/methanol. The mixture was flushed with nitrogen gas and placed in a freezer for 48 h at -80°C.

After 48 h, the samples were removed from the freezer and thawed. To precipitate any sediment from these samples, the test tubes were vortexed for 3-5 seconds and centrifuged at 3500 RPM for 10 min at 5°C. The solutions were then transferred to new test tubes, leaving any sediment behind. Immediately, 2 mL of 0.88 % KCl buffer was added to all test tubes, which were then shaken, vortexed, and centrifuged at 3500 RPM for 10 min at 5°C. The addition of the buffer resulted in two layers: an upper water-soluble fraction containing contaminants and a lower organic fraction containing the purified lipids. The top layer was discarded and the lower layer was transferred to a new test tube. The test tubes were quickly placed in a nitrogen evaporator (Organomation Associates N-EVAP) and the samples were evaporated using a steady flow of nitrogen gas. Once the sample was completely evaporated, 3 mL of methanolic-sulphuric acid was added and the samples were flushed with nitrogen gas. The test tubes were then placed in a heating block at 70°C to esterify the fatty acids. After 60 minutes, the test tubes were moved to a cooling rack, then 2 mL of hexane was added to wash the fatty acids from the methanolic-sulphuric acid and the test tubes were vortexed and centrifuged. This again produced two layers. The upper layer, which contained the fatty acid methyl esters, was transferred to a 2 mL vial that was placed in the nitrogen evaporator. As the samples were being evaporated with nitrogen gas, another 2 mL of hexane was added to the remaining layer, which was vortexed and centrifuged

again. The new upper layer was combined with the previous upper layer and the solution was evaporated completely in the nitrogen evaporator. Finally, 100  $\mu$ L of hexane was added, the vial was flushed with nitrogen gas, closed tightly, sealed with Teflon tape, and stored at  $-80^{\circ}\text{C}$  until analysis.

### Lipid analysis

All samples were analyzed in a Varian CP3800 Gas Chromatograph with a Saturn 2000 Ion Trap Mass Spectrometer. Fatty acid methyl esters (FAMES) were separated with an Agilent HP-88 capillary column with helium as the carrier gas. After injection at  $60^{\circ}\text{C}$ , the temperature was held for one minute then increased to  $150^{\circ}\text{C}$  at a rate of  $15^{\circ}\text{C}$  per minute, to  $170^{\circ}\text{C}$  at  $3^{\circ}\text{C}$  per minute, to  $190^{\circ}\text{C}$  at  $1^{\circ}\text{C}$  per minute, to  $240^{\circ}\text{C}$  at  $20^{\circ}\text{C}$  per minute, and finally held constant for 20 minutes. FAMES were identified via mass spectrometry by comparing their retention times with a Supelco 37 Component FAME Mix (Sigma-Aldrich) and verified with the NIST Mass Spectral Library. FAME concentrations were calculated by converting the area of the fatty acid peaks in the mass chromatogram to weight using the known nonadecanoic acid internal standard as a reference. The weights of arachidonic acid and erucic acid were pooled for the *S. muscatinei* and *E. marina* samples because the peaks could not be separated. One of the zooxanthellate sea anemone host samples was omitted from the analyses due to unreliable peaks.

### Statistical analyses

We used PAST 3.04 (Hammer et al. 2001) for all statistical analyses. Our first goal was to compare the fatty acid profiles of the symbionts *E. marina* and *S. muscatinei*. First, we used a

one-way ANOVA to determine if either symbiont contained significantly more total fatty acids. We then used MDS and paired-group cluster analyses to detect which samples were most similar. After MDS, we used an ANOSIM test to detect significant differences in fatty acid composition between the two symbiont species and a SIMPER test to detect which fatty acids contributed most to any differences. We used Euclidean distance as the distance metric and square root transformed the data to prevent the most abundant fatty acids from dominating the analyses. Following SIMPER, we used one-way ANOVA's or Mann-Whitney U tests (if data did not follow a normal distribution) to statistically compare the concentrations of fatty acids that contributed most to the separation of groups.

Our second goal was to compare the fatty acid profiles of hosts in the three symbiotic states (zooxanthellate, zoochlorellate, and aposymbiotic). As with the symbionts, we used one-way ANOVA's or Mann-Whitney U tests to compare total fatty concentrations, MDS and paired-group cluster analyses to detect differences among samples, ANOSIM tests to test treatment differences, then SIMPER to identify fatty acids that contributed most to any differences. The concentrations of fatty acids that contributed most to the separation between groups were statistically compared with one-way ANOVA's or Mann-Whitney U tests.

Our third goal was to compare the fatty acid profiles of the symbionts to their host sea anemones. We again used MDS to detect differences among samples and an ANOSIM test, this time to detect significant differences in fatty acid composition between symbionts and their hosts. This was done separately for *S. muscatinei* and their hosts, then for *E. marina* and their hosts. We then used Pearson correlation analyses and Spearman rank correlation analyses (if data did not follow a normal distribution) to look for linear relationships between the

concentrations of each fatty acid in the symbionts and in their hosts, which may be indicative of fatty acid translocation.

## RESULTS

### Fatty acid profiles of *Symbiodinium muscatinei* and *Elliptochloris marina*

Contrary to our expectations, *E. marina* had a significantly higher concentration of fatty acids than did *S. muscatinei* (one-way ANOVA,  $F_{1,18} = 10.06$ ,  $p = 0.005$ , Fig. 1), and MDS and cluster analyses showed a clear separation of *E. marina* and *S. muscatinei* based on fatty acid profiles (Figs. 2-3). ANOSIM showed that the differences were statistically significant ( $R = 0.96$ ,  $p < 0.05$ ) and SIMPER showed that cis-4,7,10,13,16,19-docosahexaenoic acid (DHA), oleic acid, and  $\alpha$ -linolenic acid contributed most to differences between the symbiont species (Table 1).

The overall most common fatty acids, oleic, palmitic, and  $\alpha$ -linolenic, were all more concentrated in *E. marina* than in *S. muscatinei* (Fig. 4). Oleic acid was over three times more concentrated and  $\alpha$ -linolenic over six times more concentrated in *E. marina*. Interestingly, DHA was over four times more concentrated in *S. muscatinei*, being detected in only in two samples of *E. marina*, but in all *S. muscatinei*. Myristoleic acid and behenic acid were detected only in *S. muscatinei*.

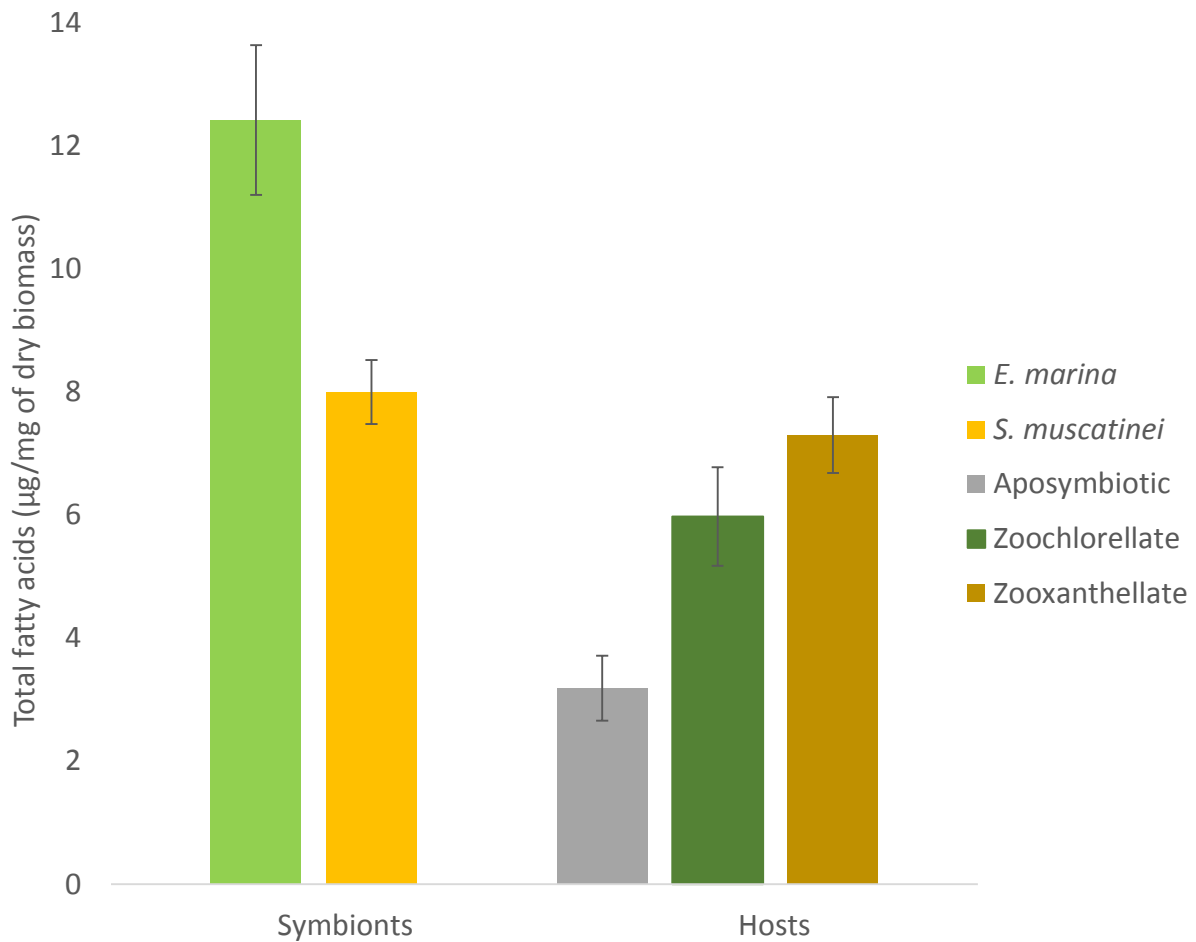


Figure 1. Total fatty acid concentration in *Symbiodinium muscatinei* and *Elliptochloris marina* extracted from *Anthopleura elegantissima* and in the animal tissue only of zooxanthellate, zoochlorellate, and aposymbiotic *Anthopleura elegantissima* from Lawrence Point, Orcas Island. Standard error bars are shown.

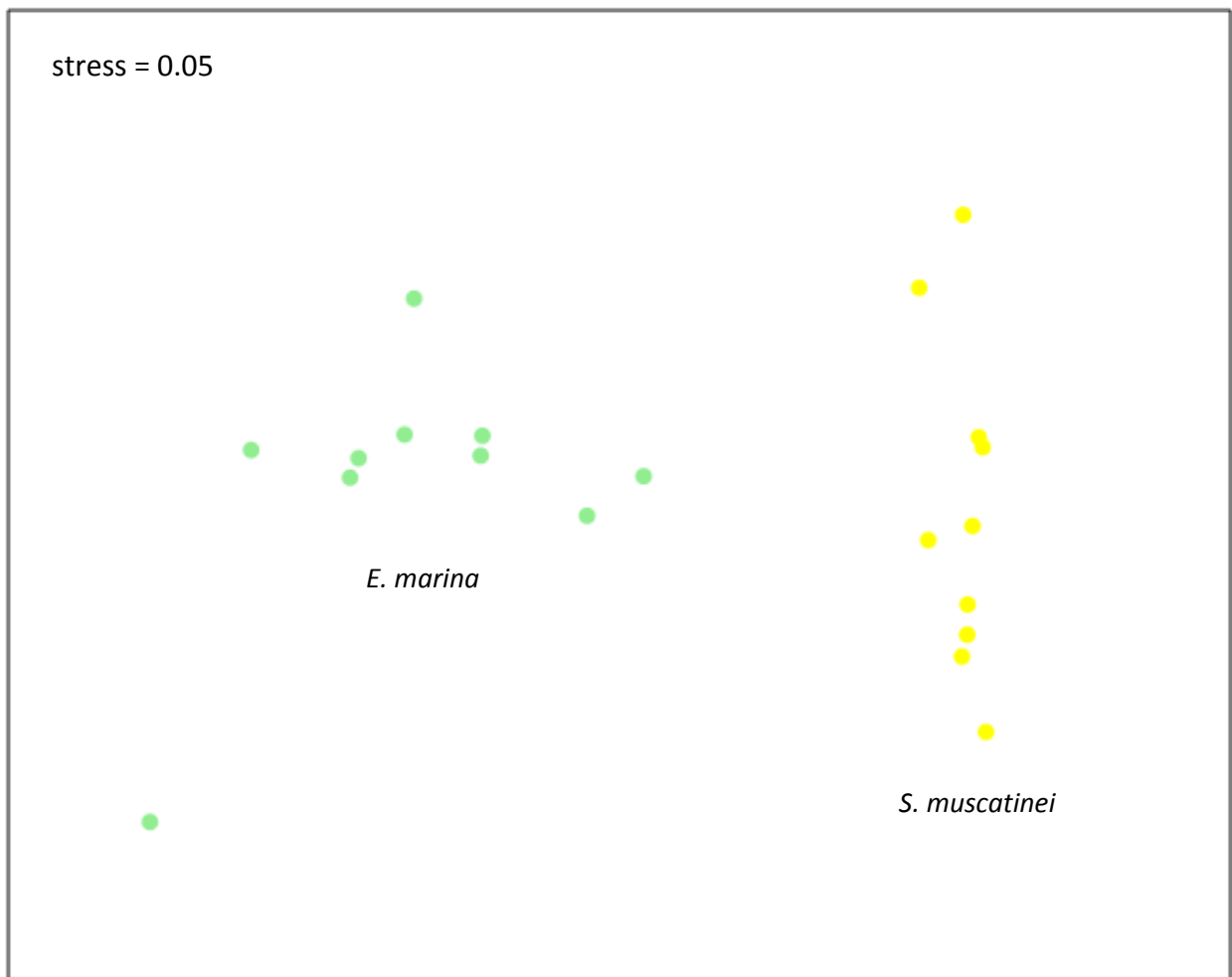


Figure 2. Non-metric Multi-Dimensional Scaling (MDS) scatter plot of the fatty acid profiles of *Symbiodinium muscatinei* (yellow) and *Elliptochloris marina* (light green) extracted from *Anthopleura elegantissima*.

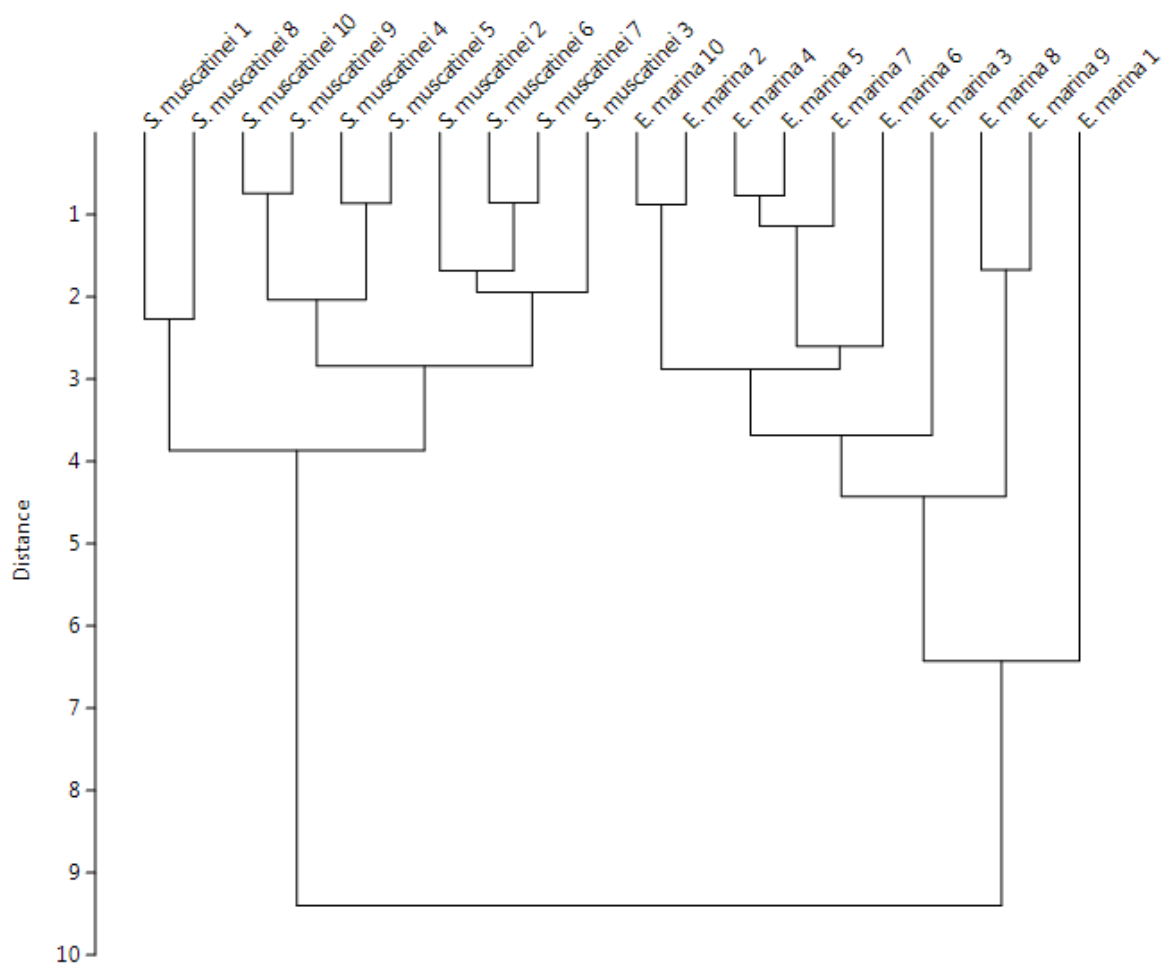


Figure 3. Cluster analysis of the fatty acid profiles of *Symbiodinium muscatinei* and *Elliptochloris marina* extracted from *Anthopleura elegantissima*. Replicate numbers are shown after the species names.

Table 1. Fatty acids contributing to the differences between *Symbiodinium muscatinei* and *Elliptochloris marina* extracted from *Anthopleura elegantissima* from Lawrence Point, Orcas Island, as indicated by SIMPER analyses. Significant statistical tests are reported.

<b>Groups compared</b>	<b>Fatty acid</b>	<b>Contribution (%)</b>	<b>F (p)</b>	<b>U (p)</b>	<b>df</b>
<i>S. muscatinei</i> vs. <i>E. marina</i>	DHA	35.8		0 (0.0001)	1,18
	Oleic	26.6		0 (0.0002)	1,18
	$\alpha$ -linolenic	22.7	68.25 (<0.0001)		1,18
	Myristic	3.8		0 (0.0001)	1,18
	Palmitic	3.1	6.71 (0.02)		1,18



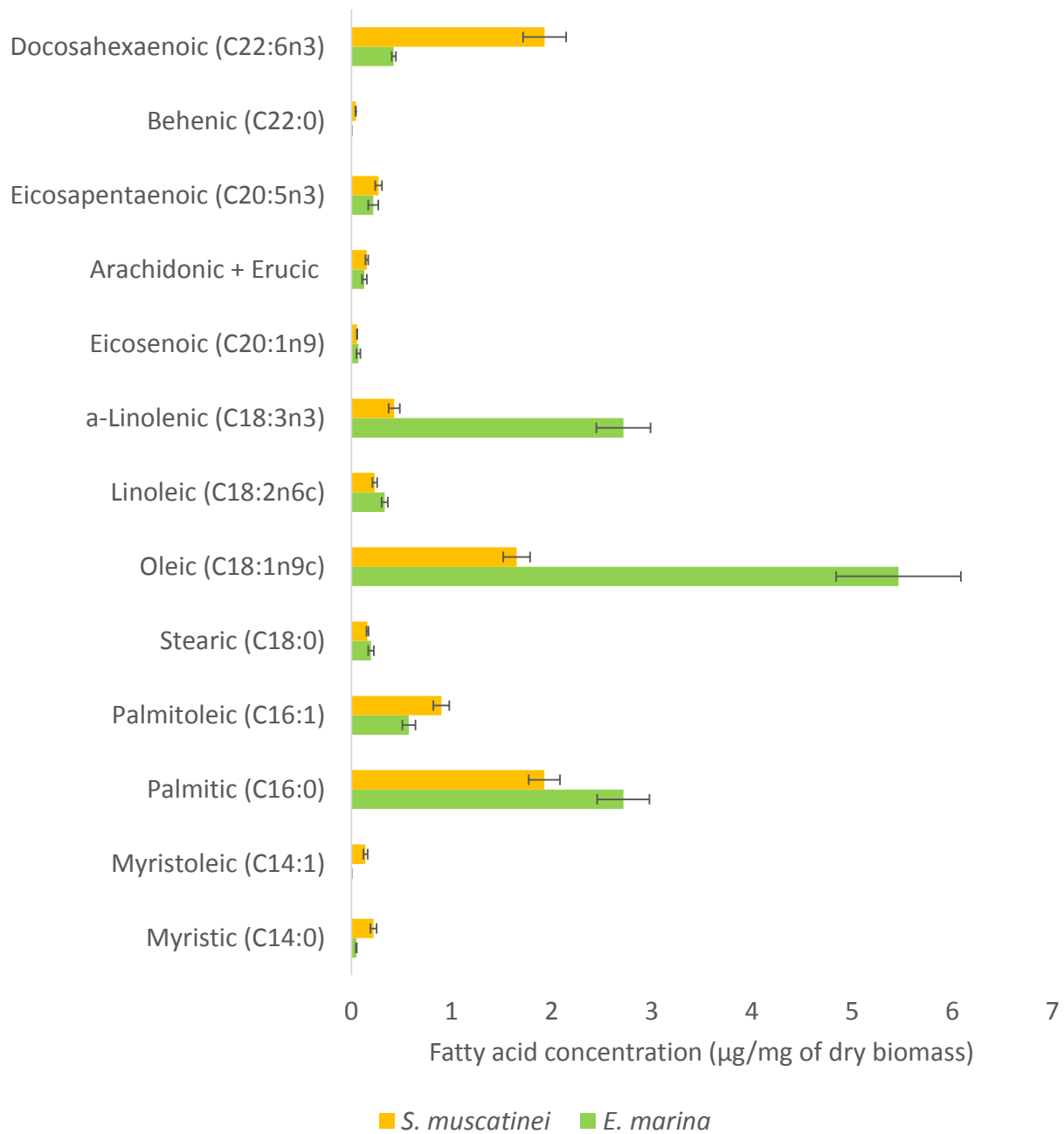


Figure 4. Fatty acid composition and concentrations in *Symbiodinium muscatinei* and *Elliptochloris marina* extracted from zooxanthellate and zoochlorellate *Anthopleura elegantissima* collected at Lawrence Point, Orcas Island. Fatty acids are ordered by length of the carbon chain. Standard error bars are shown.

### Fatty acid profiles of zooxanthellate, zoochlorellate, and aposymbiotic hosts

Fatty acid levels in aposymbiotic sea anemones were significantly lower than in zoochlorellate (one-way ANOVA,  $F_{1,18} = 7.87$ ,  $p=0.01$ ) and zooxanthellate hosts from which the symbionts had been removed (Mann-Whitney  $U_{1,17} = 2$ ,  $p=0.0005$ ). However, we found no significant differences between zoochlorellate and zooxanthellate sea anemones themselves (Fig. 1). Despite some overlap in the MDS and cluster analyses of samples from hosts in these three symbiotic states (Figs. 5-6), ANOSIM show significant differences between zooxanthellate and zoochlorellate hosts ( $R=0.24$ ,  $p<0.05$ ), between zooxanthellate and aposymbiotic hosts ( $R=0.75$ ,  $p<0.05$ ), and between zoochlorellate and aposymbiotic hosts ( $R=0.37$ ,  $p<0.05$ ). The main fatty acids contributing to the differences between hosts in different symbiotic states were linoleic acid, palmitoleic acid, elaidic acid, DHA, and EPA (Table 2).

Our data indicate that nearly every fatty acid was more concentrated in zooxanthellate than in zoochlorellate host tissues, and that aposymbiotic sea anemones had lower concentrations of every single fatty acid than hosts in either symbiotic condition (Fig. 7). In several cases, the concentration of fatty acids in symbiotic hosts doubled that of hosts lacking symbionts. Pentadecanoic acid was only detected in zooxanthellate hosts and  $\alpha$ -linolenic was only detected in zoochlorellate hosts, although both in very low concentrations. We did not detect myristic, palmitoleic, or linoleic acids in aposymbiotic anemones.

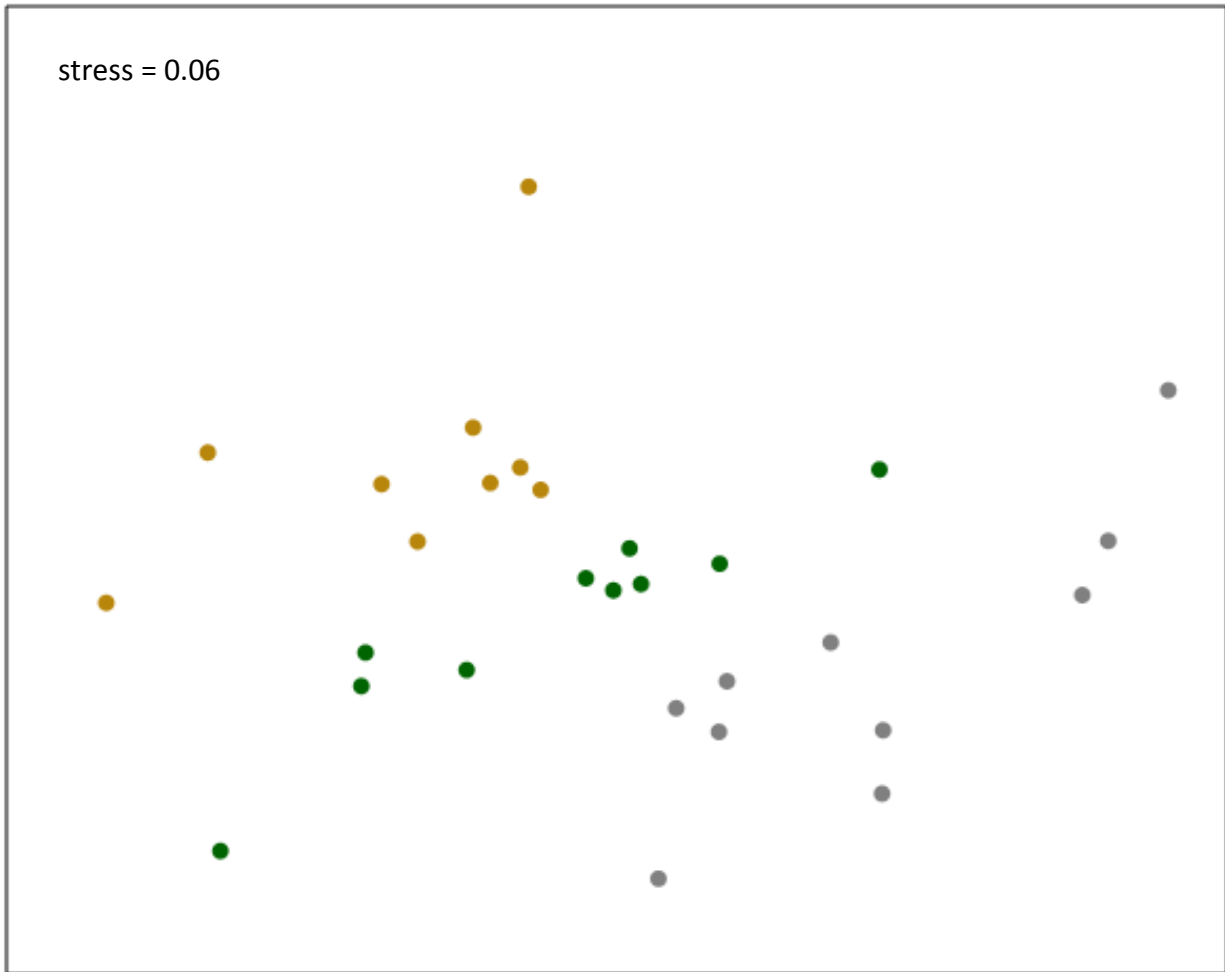


Figure 5. Non-metric Multi-Dimensional Scaling (MDS) scatter plot of the lipid profiles from *Anthopleura elegantissima* in the three symbiotic states: zooxanthellate (brown), zoochlorellate (green), aposymbiotic (grey). Data were for the animal portions alone with the symbionts removed.

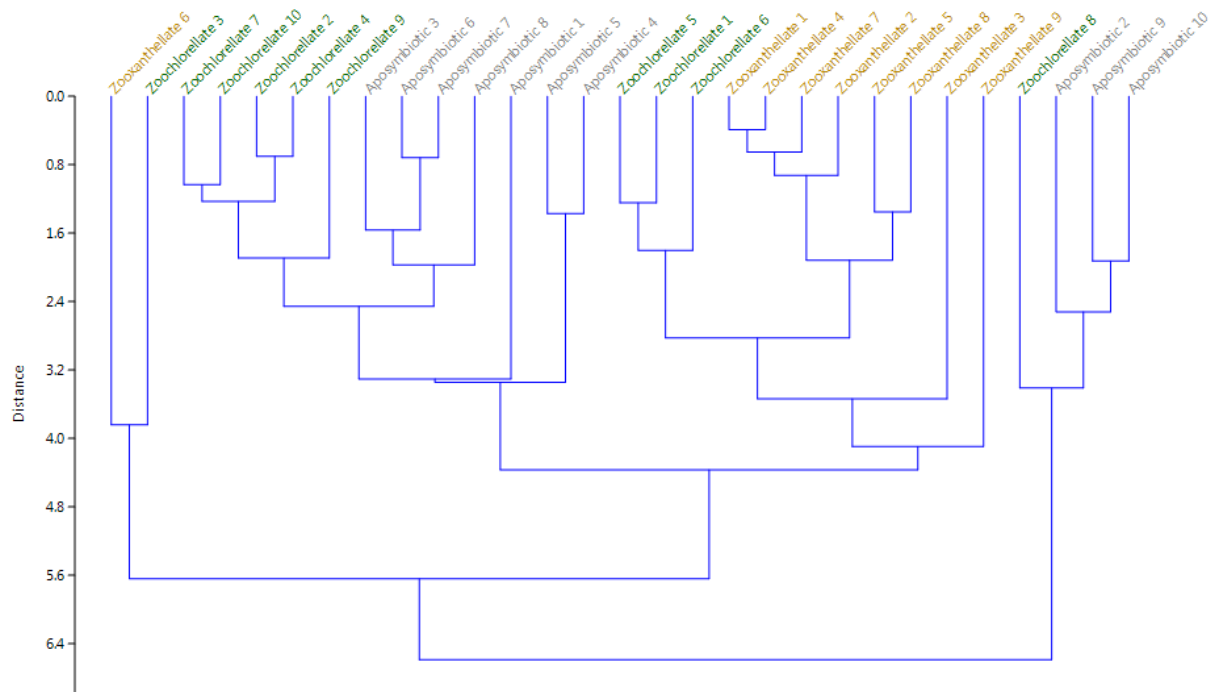


Figure 6. Cluster analysis of the lipid profiles from *Anthopleura elegantissima* in the three symbiotic states: zooxanthellate (brown), zoochlorellate (green), and aposymbiotic (grey).

Table 2. Fatty acids contributing to the differences between zooxanthellate, zoochlorellate and aposymbiotic *Anthopleura elegantissima* from Lawrence Point, Orcas Island, as indicated by SIMPER analyses. Statistical tests are reported.

Groups compared	Fatty acid	Contribution (%)	F (p)	U (p)	df
Zooxanthellate vs. zoochlorellate	Linoleic	13.9	38.73 (<0.0001)	0 (0.0002)	1,17
	Palmitoleic	10.8			
	EPA	9.9			
	DHA	9.7			
	Elaidic	7.5			
	Palmitic	7.4			
Aposymbiotic vs. zooxanthellate	Elaidic	20.8	87.23 (<0.0001)	0 (<0.0001)	1,17
	Palmitoleic	11.2			
	EPA	9.8			
	DHA	9.0			
	Linolelaidic	8.3			
	Oleic	7.2			
	Palmitic	7.1			
Aposymbiotic vs. zoochlorellate	Elaidic	16.7	38.69 (<0.0001)	0 (0.0001)	1,18
	EPA	14.1			
	DHA	13.8			
	Linolelaidic	8.3			
	Erucic	7.3			
	Palmitic	6.4			

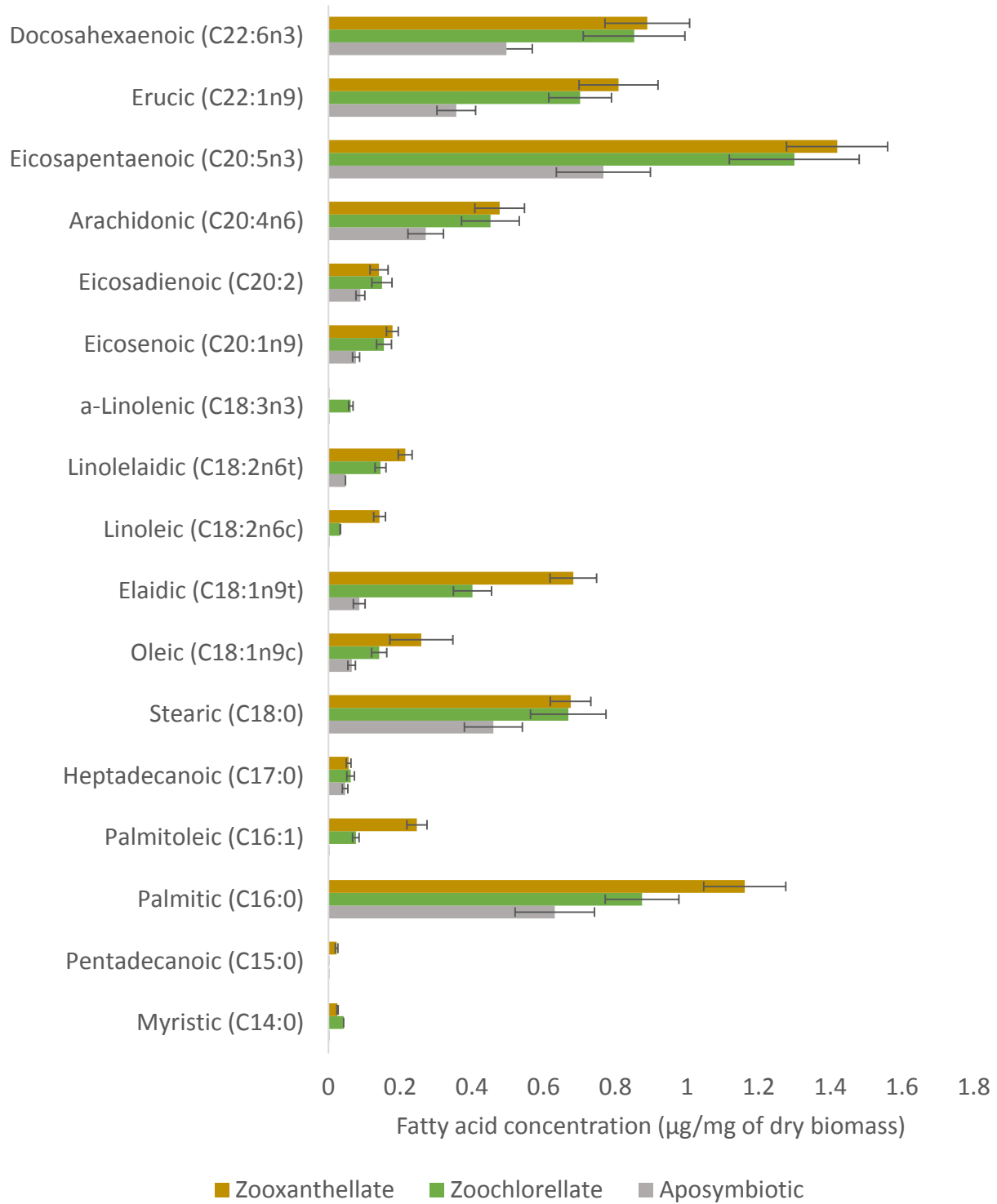


Figure 7. Fatty acid composition and concentration in animal tissues of zooxanthellate, zoochlorellate, and aposymbiotic *Anthopleura elegantissima* from Lawrence Point, Orcas Island. Fatty acids are ordered by length of the carbon chain. Standard error bars are shown.

### Fatty acid profiles of hosts and their algal symbiont

We found significant differences in the fatty acid profiles of zooxanthellate hosts and their symbiont *S. muscatinei* (ANOSIM,  $R=1$ ,  $p<0.05$ ) and between zoochlorellate hosts and their symbiont *E. marina* (ANOSIM,  $R=1$ ,  $p<0.05$ ). The MDS showed more similarity between zoochlorellate and zooxanthellate host tissues than between the *S. muscatinei* and *E. marina* (Fig. 8). Few fatty acids were found in high levels in both hosts and their symbionts (Figs. 9-10). Palmitoleic acid, oleic acid, and  $\alpha$ -linolenic acid were concentrated in the both symbionts, but scarce or absent in the hosts, whereas EPA, arachidonic, and erucic were concentrated in the hosts, but not in the symbionts. DHA was considerably more concentrated in *S. muscatinei* than in *E. marina*, but its levels were almost identical in zooxanthellate and zoochlorellate sea anemones.

We found a significant positive correlation between *S. muscatinei* and zooxanthellate hosts only in the concentrations of linoleic acid (Pearson,  $r=0.76$ ,  $p=0.02$ ). However, we found significant positive correlations in the concentrations of palmitic acid (Pearson,  $r=0.75$ ,  $p=0.01$ ), palmitoleic acid (Spearman,  $r=0.44$ ,  $p=0.04$ ), and oleic acid (Pearson,  $r=0.75$ ,  $p=0.01$ ) between *E. marina* and zoochlorellate hosts (Table 3).

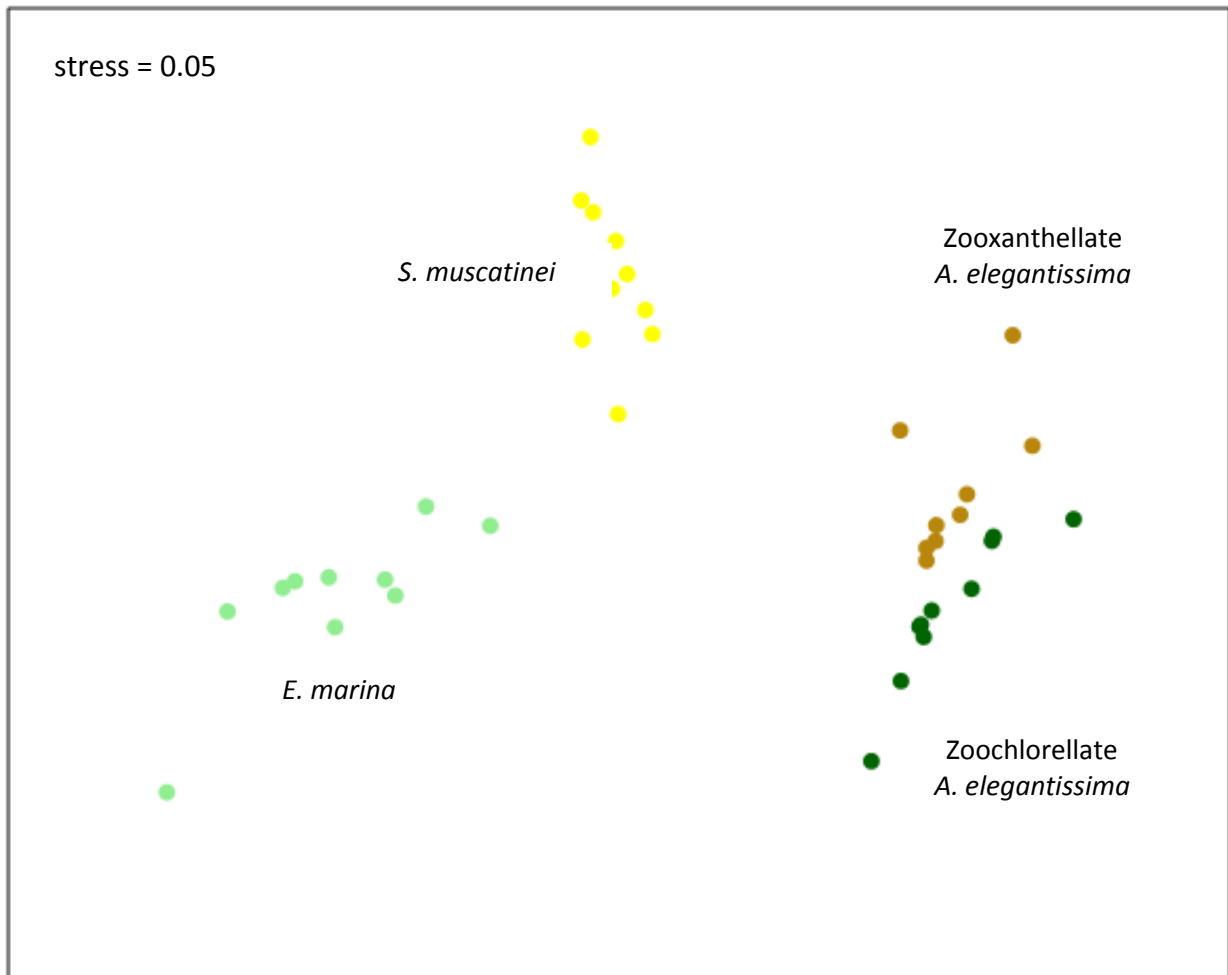


Figure 8. Non-metric Multi-Dimensional Scaling (MDS) scatter plot of the lipid profiles of zooxanthellate (brown) and zoochlorellate *Anthopleura elegantissima* (dark green) and their symbionts *Symbiodinium muscatinei* (yellow) and *Elliptochloris marina* (light green) from *Anthopleura elegantissima*.



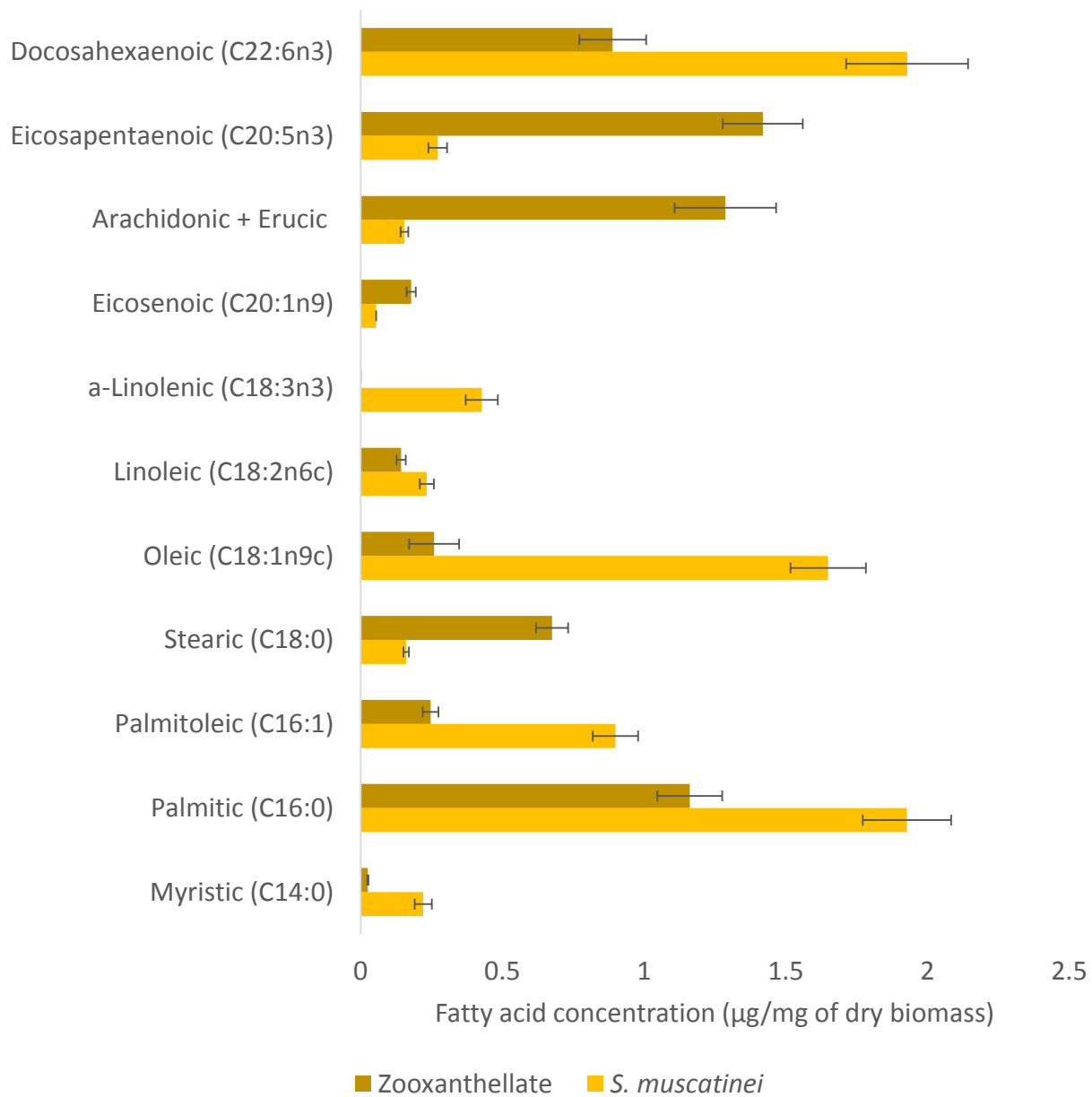


Figure 9. Fatty acid composition and concentration of *Symbiodinium muscatinei* and zooxanthellate *Anthopleura elegantissima* from Lawrence Point, Orcas Island. Fatty acids are ordered by length of the carbon chain. Standard error bars are shown.

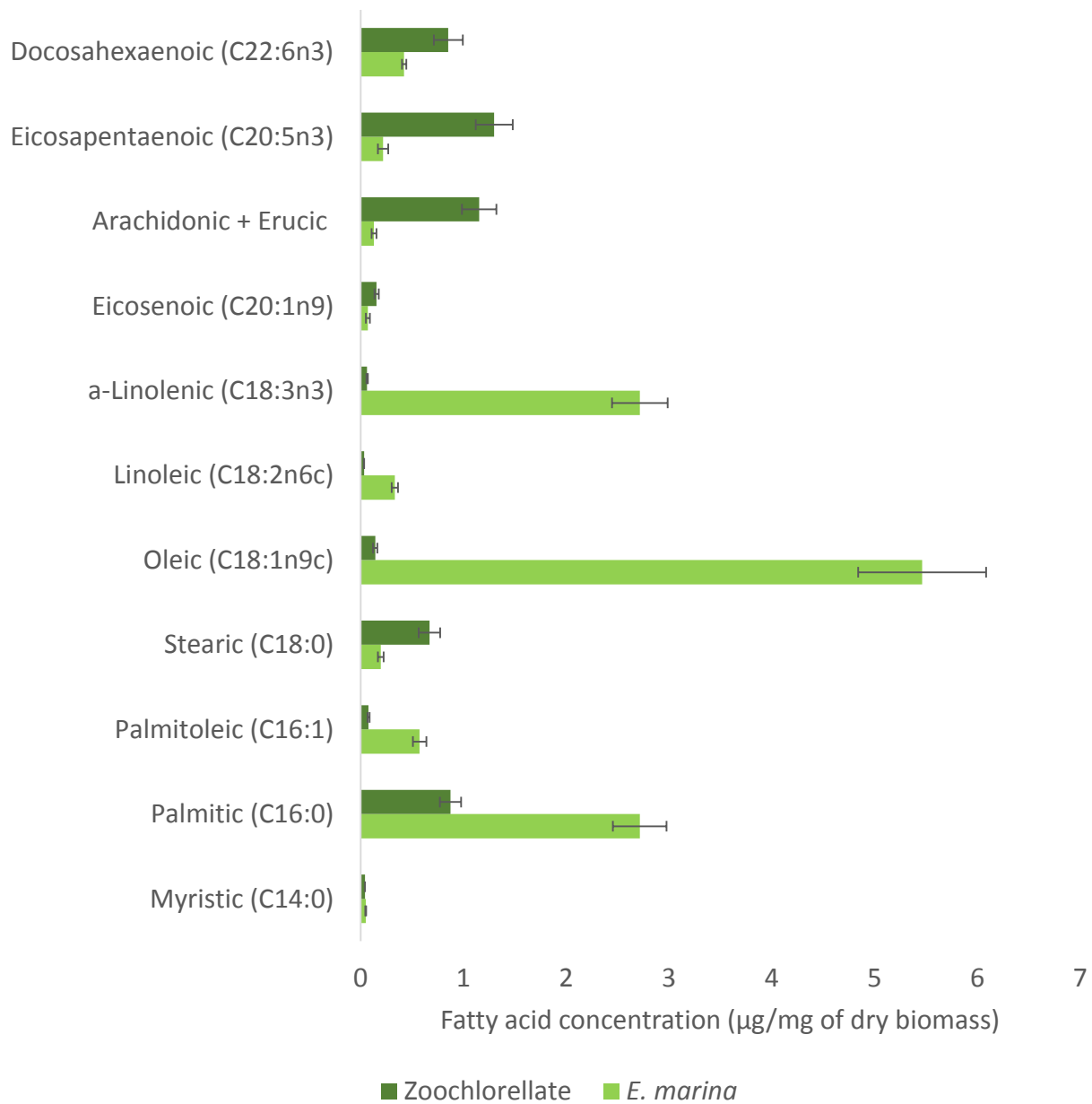


Figure 10. Fatty acid composition and concentration of *Elliptochloris marina* and zoochlorellate *Anthopleura elegantissima* from Lawrence Point, Orcas Island. Fatty acids are ordered by length of the carbon chain. Standard error bars are shown.

## DISCUSSION

### Fatty acid profiles of *Symbiodinium muscatinei* and *Elliptochloris marina*

Our findings that palmitic acid, docosahexaenoic acid (DHA), and oleic acid were the most abundant fatty acids in *S. muscatinei* support previous research. Multiple studies indicate that palmitic acid and DHA are the major fatty acids in *Symbiodinium* isolated from corals (Al-Moghrabi et al. 1995; Papina et al. 2003; Zhukova and Titlyanov 2006; Imbs et al. 2010). Other predominant fatty acids include stearic, stearidonic, palmitoleic, oleic,  $\gamma$ -linolenic, arachidonic, and eicosapentaenoic acids (Suzuki et al. 2003; Papina et al. 2003; Zhukova and Titlyanov 2006; Díaz-Almeyda et al. 2010; Kneeland et al. 2013; Imbs et al. 2014). Although stearidonic acid,  $\gamma$ -linolenic acid, and eicosapentaenoic acid (EPA) are considered marker polyunsaturated fatty acids for dinoflagellates (i.e. fatty acids synthesized by dinoflagellates but not by animals) (Imbs et al. 2014), we found low concentrations of EPA in *S. muscatinei* and did not detect stearidonic acid or  $\gamma$ -linolenic acid in any of our samples. Other fatty acids such as octadecapentaenoic acid and docosapentaenoic acid (DPA) have also been suggested as marker fatty acids, but were not detected in our samples, although their concentration in *Symbiodinium* is generally very low (Papina et al. 2003; Imbs et al. 2014).

Here we present the first fatty acid profile of *Elliptochloris marina*, which has high levels of oleic,  $\alpha$ -linolenic, and palmitic acids. As expected, *E. marina* has a fatty acid composition similar to other chlorophytes, although lipid profiles vary among species. *Tetraselmis* sp. has a similar fatty acid composition to *E. marina*, with oleic acid as the most concentrated form, followed by  $\alpha$ -linolenic acid and palmitic acid (Patil et al. 2007). In *Dunaliella* spp.,  $\alpha$ -linolenic acid and palmitic

acid comprise about 60% of the total fatty acids (Viso and Marty 1993), whereas *Chlorella* sp. has high concentrations of  $\alpha$ -linolenic, palmitic, hexadecatrienoic, and linoleic acids (Zhukova and Aizdaicher 1995). These findings agree with our own, although we did not detect hexadecatrienoic acid. Also *Dunaliella* spp. and *Chlorella* sp. have low levels of oleic acid, which was the most abundant fatty acid in our *E. marina* samples. DHA and palmitoleic acid are consistently either absent or found in low concentrations in chlorophytes (Viso and Marty 1993; Zhukova and Aizdaicher 1995; Wiltshire et al. 2000; Patil et al. 2007).

We found that DHA, oleic acid, and  $\alpha$ -linolenic acid contributed most to the differences between *S. muscatinei* and *E. marina*. DHA is typically absent in chlorophytes, but very concentrated in dinoflagellates (Viso and Marty 1993; Zhukova and Aizdaicher 1995), leading to its use as a dinoflagellate biomarker (e.g. Bachok et al. 2006). The high concentrations of oleic and  $\alpha$ -linolenic acids in *E. marina* may explain why it performs better at lower temperatures and why zoochlorellate anemones are more abundant in low intertidal habitats and higher latitudes (Secord and Augustine 2000). Cell membrane fluidity decreases at low temperatures, presumably affecting the function of membrane transport proteins and ion gradients (Murata and Los 1997; Reay et al. 1999). The incorporation of unsaturated fatty acids into cell membranes restores membrane fluidity, because the “kinks” in their carbon chains reduce cell membrane density (Hazel 1997). Unsaturated fatty acids in the thylakoid membranes of cyanobacteria protect them from photoinhibition caused by low temperatures (Gombos et al. 1992; Gombos et al. 1994) and enhance lipid diffusion across the membrane (Sarcina et al. 2003). Organisms will synthesize unsaturated fatty acids to cope with low temperatures. For instance, a drop in temperature causes *Nostoc* sp. (Cyanobacteria) from Antarctic lakes to up-regulate the activity of enzymes

that desaturate C18 fatty acids (Chintalapati et al. 2007). Lynch and Thompson (1984) found that shifting *Dunaliella salina* cultures from 30°C to 12°C led to an increase in oleic and  $\alpha$ -linolenic in the chloroplast phospholipids of the algal cells. Also, these fatty acids are more concentrated in macroalgae adapted to temperate waters than in macroalgae from warm waters (De Angelis et al. 2005; Colombo et al. 2006). However, a few studies suggest that chlorophytes accumulate key fatty acids such as oleic acid and palmitic acid during stress conditions, such as high irradiance, low temperatures, and nitrogen starvation (Mendoza et al. 1996; Mendoza et al. 1999; Zhekisheva et al. 2002). Whether the high levels of oleic acid we found in *E. marina* reflect their normal fatty acid composition, an adaptation to cold temperatures, or *in hospite* stress is unknown.

#### Fatty acid profiles of zooxanthellate, zoochlorellate, and aposymbiotic hosts

Although *A. elegantissima* is a temperate sea anemone, its overall fatty acid profiles match those of most tropical corals studied to date. Fatty acid content can be influenced by species, location, diet, and seasonality, but most corals have high concentrations of palmitic, stearic, elaidic, oleic, arachidonic, EPA, and DHA (Harland et al. 1993; Al-Moghrabi et al. 1995; Yamashiro et al. 1999; Oku et al. 2002; Bachok et al. 2006; Treignier et al. 2008; Imbs et al. 2010; Teece et al. 2011; Imbs 2013). Latyshev et al. (1991) analyzed the fatty acid composition of 14 calcifying coral species (with symbionts still in tissues) and found mainly palmitic, stearic, 18:1n9 (oleic and elaidic), arachidonic, EPA, and DHA. These findings agree well with our own, although most other studies did not detect erucic acid (or found it in very low concentrations) and found much lower concentrations of EPA.

The lipid profile of *A. elegantissima* can be explained by its diet and *de novo* fatty acid synthesis without requiring input from symbionts. Animals can synthesize saturated fatty acids and monounsaturated fatty acids (MUFAs) from acetyl-CoA or from precursor fatty acids such as palmitic acid via desaturations (the addition of a double bond to the carbon chain) and elongations (the addition of two carbons to the chain), whereas polyunsaturated fatty acids (PUFAs) with n-3 and n-6 desaturations cannot be synthesized *de novo* by most animals and must be acquired through the diet (Dalsgaard et al. 2003). We found high concentrations of saturated fatty acids and MUFAs such as palmitic, stearic, elaidic, and erucic in *A. elegantissima*, and high concentrations of PUFAs including arachidonic, erucic, DHA, and especially, EPA. *A. elegantissima* feeds mainly on zooplankton, invertebrate larvae, and intertidal invertebrates, including mytilid bivalves (Sebens 1981). Most of the PUFAs and other fatty acids found in high levels in *A. elegantissima*, especially palmitic, palmitoleic, stearic, EPA, and DHA, are also dominant in marine zooplankton (Dalsgaard et al. 2003; Lee et al. 2006; Treignier et al. 2008; Seemann et al. 2013). Mytilid bivalves are also very rich in palmitic, palmitoleic, EPA and DHA (Zandee et al. 1980; Karakoltsidis et al. 1995; Murphy et al. 2002; Passi et al. 2002). Erucic acid, which was very concentrated in our samples but not in the corals studied to date, is found in high levels in some species of copepods and present in some mytilids (Karakoltsidis et al. 1995; Dalsgaard et al. 2003).

Research shows that PUFAs play an important role in invertebrate physiology. Arachidonic acid and EPA accumulate in the eggs of a species of sea pansy during months preceding spawning, indicating their likely role in gonad maturation (Pernet et al. 2002). EPA has also been linked to growth and egg production in the freshwater cladoceran *Daphnia magna*

(Müller-Navarra et al. 2000). Similarly, DHA influences egg production in the calanoid copepod *Temora longicornis* (Evjemo et al. 2008). Thus, it seems likely that the greater concentrations of fatty acids in anemones hosting either symbiont would increase their fitness relative to aposymbiotic anemones.

Our finding that zooxanthellate hosts have greater total fatty acid concentrations than zoochlorellate hosts supports the idea that *S. muscatinei* is a better symbiont for the anemone. Previous research on *A. elegantissima* also suggests that zooxanthellate hosts have higher levels of non-polar lipids, cholesterol, and palmitic acid than do zoochlorellate hosts (Ponce-McDermott 2012). Interestingly, we found significantly greater concentrations of palmitic, palmitoleic, elaidic, and linoleic in zooxanthellate hosts than in zoochlorellate ones, but no differences in the PUFAs EPA and DHA. The former fatty acids can be synthesized by animals and their elevated concentrations in zooxanthellate hosts may be explained by greater contributions of (non-lipid) photosynthetic carbon from *S. muscatinei*. Carbon-containing photosynthates from *Symbiodinium* quickly accumulate into lipid droplets in the oral gastroderm of corals (Kopp et al. 2015). Also, corals synthesize lipids such as triglycerides and wax esters from glucose when exposed to light (Oku et al. 2003). Our results seem to confirm that *S. muscatinei* transfers more photosynthates to its host than *E. marina* does. Zooxanthellate hosts then use the additional carbon to synthesize fatty acids that are stored as triglycerides and wax esters, which are mainly composed of palmitic, stearic, and 18:1 (oleic/elaidic) acids in corals (Patton et al. 1983; Yamashiro et al. 1999).

The high concentration of fatty acids in zooxanthellate and zoochlorellate hosts compared to aposymbiotic sea anemones has three potential explanations, (i) fatty acid depletion by

aprosymbiotic anemones, (ii) variation in the diets of anemones in different symbiotic states, and (iii) translocation of fatty acids by symbiotic algae (discussed in the next section). Researchers have found significantly lower levels of wax esters and triacylglycerols in bleached corals (with low numbers of symbionts) than in healthy corals (Grottoli et al. 2004; Yamashiro et al. 2005). Corals store photosynthetic carbon from their symbionts as lipids to supply their daily metabolic needs (Bachar et al. 2007; Baumann et al. 2014) and start using up their triacylglycerol storage lipids immediately after bleaching (Rodrigues et al. 2008). Therefore, the lack of photosynthate input from symbionts may explain why aposymbiotic anemones had significantly depressed lipid levels. It is unlikely that feeding varied between anemones in different symbiotic states because we collected all sea anemones from the same site and at approximately the same tidal height. In addition, *A. elegantissima* symbiotic state has little effect on heterotrophic feeding (Hiebert and Bingham 2012), implying that the concentration of lipids acquired from the diet should be homogenous across symbiotic states. However, whether symbiont identity or presence influences host feeding should be further explored.

#### Fatty acid profiles of hosts and their algal symbiont

Several authors have suggested that algal symbionts translocate specific fatty acids to their hosts (Papina et al. 2003; Papina et al. 2007; Treignier et al. 2008; Mortillaro et al. 2009; Imbs et al. 2011). Although DHA is believed to be one of the fatty acids translocated, we found no correlation in its concentration in symbionts and hosts. We also found little difference in the levels of DHA between zooxanthellate and zoochlorellate hosts, even though this fatty acid was significantly more abundant in *S. muscatinei* than in *E. marina*. These findings suggest that DHA



is not translocated by the symbionts but is obtained by sea anemones through the diet or via synthesis from precursor fatty acids.

Our finding that the concentration of palmitic acid in *E. marina* is positively correlated with that in zoochlorellate hosts has two potential explanations. First, it is possible that *E. marina* translocates palmitic acid to its zoochlorellate host. Presumably, *E. marina* with greater concentrations of palmitic acid are able to transfer more of this fatty acid to their host, resulting in greater concentrations of palmitic acid in host tissues. It is also possible that *E. marina* translocate other photosynthetic products (e.g. glucose) to their host, rather than lipids. Kopp et al. (2015) showed that anemones quickly accumulate photosynthates from their symbionts as lipid droplets and glycogen granules. Zooxanthellae also accumulate carbon from photosynthesis as lipids (Kopp et al. 2015), so the concentration of palmitic acid in *E. marina* may simply reflect its photosynthetic efficiency and the concentration of this fatty acid in host tissues reflects how much photosynthates it receives from *E. marina*. Overall, we find no evidence for the translocation of fatty acids from algal symbionts to anemone hosts.

### Conclusions and future research directions

We show that the lipid composition of *S. muscatinei* from temperate *A. elegantissima* tracks that of zooxanthellae from tropical corals. We also present the first lipid profile of *E. marina*, which is rich in unsaturated fatty acids typically found in Chlorophytes. Whether the presence of these fatty acids gives *E. marina* greater resilience to colder temperatures and the extent to which the lipid profile of symbionts influences host ecology and life history are questions that warrant further research. We find that anemones hosting *S. muscatinei* have

greater concentrations of most fatty acids than do anemones hosting *E. marina*, suggesting that the former is a more favorable symbiont for the host. The difference in total fatty acids of anemones in these two symbiotic states is not significant, so it seems that even in the summer, the greater density *E. marina* in host tissues offsets the higher productivity of *S. muscatinei*, as suggested by Engebretson and Muller-Parker (1999). Our results suggest that sea anemones lacking symbionts are at a disadvantage, possessing significantly lower concentrations of lipids. Future studies of dynamics of multi-symbiont system, symbiont switching and bleaching should take care to analyze only host tissues and not host tissues still containing symbionts given differences in the lipid composition of each. Further research is needed on the role of fatty acids in cnidarian metabolism, development, and reproduction to shed light on how hosts are affected by symbiont loss events such as coral bleaching.

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