

■ Preserved macroscopic polymeric sheets of shell-binding protein in the Middle Miocene (8 to 18 Ma) gastropod *Ecphora*

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Abstract

doi: 10.7185/geochemlet.1501

The genus *Ecphora* of Muricid gastropods from the mid-Miocene Calvert Cliffs, Maryland is characterised by distinct reddish-brown colouration that results from shell-binding proteins associated with pigments within the outer calcite (CaCO_3) portion of the shell. The mineral composition and robustness of the shell structure make *Ecphora* unique among the Neogene gastropods. Acid-dissolved shells produce a polymeric sheet-like organic residue of the same colour as the initial shell. NMR analysis indicates the presence of peptide bonds, while hydrolysis of the polymeric material yields 11 different amino acid residues, including aspartate and glutamate, which are typical of shell-binding proteins. Carbon and nitrogen elemental and isotopic analyses of the organic residue reveals that total organic carbon ranges from 4 to 40 weight %, with $11 < \text{C}/\text{N}_{\text{at}} < 18$. Isotope values for carbon ($-17 < \delta^{13}\text{C} < -15\text{‰}$) are consistent with a shallow marine environment, while values for nitrogen ($4 < \delta^{15}\text{N} < 12.2\text{‰}$) point to *Ecphora*'s position in the trophic structure with higher values indicating predator status. The preservation of the pigmentation and shell-binding proteinaceous material presents a unique opportunity to study the ecology of this important and iconic Chesapeake Bay organism from 8 to 18 million years ago.

Received 22 September 2014 | Accepted 1 December 2014 | Published 20 January 2015

Introduction

Ecphora is one of the most distinctive mollusks of the Neogene of North America. It is characterised by striking reddish-brown to tan colouration and typically 3 or 4 prominent costae (Fig. 1A). *Ecphora* represents an extinct group of Murex

gastropods that lived from the late Oligocene to the Pliocene (Carter *et al.*, 1994; Ward and Andrews, 2008). *Ecphora* in its various species and subspecies span nearly 10 Ma of time along Calvert Cliffs, Maryland within the Calvert, Choptank and St. Marys Formations. This genus is unique in the context of the Calvert Cliffs fauna, in that pronounced colouration is preserved in the sculpted calcitic (CaCO_3) outer shell layer, as opposed to the white aragonitic (also CaCO_3) nacreous inner shell layer (Fig. 1C). Most other mollusk shells of the Calvert Cliffs are composed principally of aragonite and occur as white chalky shells, or are completely leached out of some beds (Shattuck, 1904; Vokes, 1957; Petuch and Drolshagen, 2010). By contrast, *Ecphora* found *in situ* invariably maintains its rich red-brown colour, which fades gradually to light tan when exposed to sunlight. This striking colouration is preserved in specimens as old as 18 Ma and suggests the possibility that biomolecular fossils of shell-binding proteins and associated pigments might be preserved.

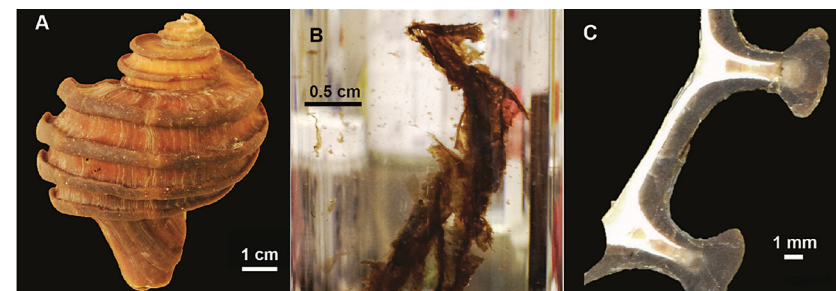


Figure 1 A. Image showing characteristic colouration and shape of *Ecphora gardnerae germonae*; St. Marys Formation, Little Cove Point member, Driftwood Beach, Calvert County MD. B. Polymeric protein-rich residue from the calcitic portions of dissolved *Ecphora* shell from a St. Marys Formation, Driftwood Beach specimen. This material gives the distinctive colour to the shells. C. A polished cross-section of two costae from *Ecphora gardnerae germonae* reveals the colored calcitic outer layer and white aragonitic inner layer.

Protein and polysaccharides are known to be important components of mollusk shell architecture; they form the organic matrix upon which the calcite and/or aragonite crystallises (Marin *et al.*, 2008; Mann, 2001; Weiner *et al.*, 1984; Dove, 2010). The organic shell-binding matrix forms sheets ~30 nm in thickness between which the minerals crystallise (Mann, 2001). Mollusks also commonly incorporate carotenoids derived from their diet to add shell pigmentation. Carotenoids are a common class of tetraterpenoid pigments in both plants and animals with the colour arising from conjugated carbon bonds in their C_{40} hydrocarbon chains (McGraw, 2006). Carotenoids are reactive and photosensitive molecules as demonstrated by their tendency to fade in the presence of light and oxygen, but they may be stabilised by binding to proteins to make carotenoid-protein complexes (Wade *et al.*, 2009). In this study we characterise organic residues dissolved from *Ecphora*'s outer calcitic shell, including element and isotopic composition and molecular identities.

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Materials and Methods

Specimens studied. Specimens were collected *in situ* from eroding bluffs from a wide range of stratigraphic and geographic areas in Southern Maryland (Fig. S-1). All fossils are mid-Miocene (8 to 18 Ma) in age from the St. Marys, Choptank and Calvert formations. We examined 17 specimens of several *Ecphora* species (from beds 4 to 9, 10, 12, 14, 17 and 21 to 23; Table S-1). The beds or zones of Calvert Cliffs were originally designated to further subdivide the formations and members of the Calvert Cliffs sedimentary sequence (Shattuck, 1904).

Specimen preparation. Shells were mechanically separated from the matrix and subsequently cleaned using distilled water to remove residual matrix. Calcitic costae, representing the thickest portions of the *Ecphora* shells (Fig. 1C), were mechanically removed and cleaned, and ~2 g of each shell were placed in a glass vial. Calcite dissolution was achieved with ~60 ml of 10 % HCl leaving behind an organic-rich polymeric residue (Fig. 1B). We ultrasonicated the residue with 20 ml of distilled water three times to de-acidify the solution. We pipetted water from the vials to concentrate the residue, which was then frozen at -19 °C before freeze drying. Two grams of raw shell material yielded 1 to 57 mg of coloured organic residue with an average of ~6 to 8 mg (Fig. 1B).

Scanning electron microscopy. We obtained SEM images and qualitative analyses on fragmental and polished specimens, as well as fragments of the polymeric residue, which were coated with iridium for analysis to prevent interference of carbon and nitrogen detection. We employed a JEOL 6500F SEM with an Oxford silicon drift energy dispersive detector (SDD-EDS). We used a 15 kV, 1 nA beam to obtain secondary electron images and elemental maps. Analyses were uncorrected for irregular specimen morphology so analytical so results are semi-quantitative.

NMR analysis. ¹³C Solid state NMR was performed using a Chemagetics Infinity Nuclear Magnetic Resonance spectrometer with a magnetic field of 7 Tesla. ¹H-¹³C cross polarisation was performed using a ramped contact pulse amplitude. The ¹H excitation (90°) pulse length was 4 μs, the contact time was optimised at 4.5 ms, and high-power ¹H decoupling was performed with RF power ($w_1/2\pi$) of 65 kHz. Magic angle sample spinning at a frequency ($w_R/2\pi$) of 11.5 kHz sufficiently averages the chemical shielding anisotropy and helps minimise ¹H-¹³C dipolar coupling, leading to well resolved ¹³C solid state NMR spectrum of the *Ecphora* organic residue. The number of acquisitions was 100,000 with a recycle delay of 1 s. The resultant spectrum is referenced to tetramethyl silane (TMS, defined as 0 ppm).

Amino acid analysis. The *Ecphora* organic residues were reacted in 6 N HCl at 100 °C for 24 hours to hydrolyse any intact protein to free amino acids. The solutions were separated from unreacted organic residue and dried under N₂. Standard derivitisation of amino acids for analysis using GC-MS involved esterification with 2-propanol and acetyl chloride at 110 °C for one hour. The solutions were then evaporated to dryness under N₂ to remove unreacted 2-propanol and acetyl chloride. The free amine group was then acylated using trifluoroacetic acid

in methylene chloride at 110 °C for one hour. Following evaporation to dryness, the derivitised amino acids were resuspended in dichloromethane for analysis using gas chromatography and mass spectrometry (GC-MS).

GC-MS analysis employed an Agilent 6890 gas chromatograph with a 30 metre 5 % phenyl polydimethylsilicone chromatographic column. Compound detection was performed using an Agilent 5973 mass spectrometer. A simple thermal program starting at 50 °C and ramping to 300 °C at a rate of 5 °C/min was sufficient to separate all compounds of interest. The identities of individual amino acids are confirmed based on comparison with their elution times and mass fragmentation with that of pure amino acid standards. No attempt was made to identify many of the unknown molecules present but inspection of the fragment radical cation masses does provide some insight into their origin.

Isotopic analysis. Bulk C and N elemental and isotopic compositions were determined on ~0.2 mg samples loaded into silver or tin capsules of known weight and stored in a dry N₂ flushed oven at 50 °C for at least 12 h. All analyses were conducted using a CE Instruments NA 2500 series elemental analyser (EA) linked to a Thermo Fisher Delta V Plus mass spectrometer by ConFlo III interface. Samples were introduced directly from an autosampler into the EA, where they were combusted with ultrapure O₂ at 1020 °C in a quartz oxidation column containing chromium (III) oxide and silvered cobalt (II, III) oxide. The resulting gases, CO₂ and N₂ mixed with zero-grade He as the carrier gas, were separated prior to isotopic analysis. Both N₂ and CO₂ samples were analysed relative to internal working gas standards. Acetanilide (C₈H₉NO) was analysed at regular intervals to monitor the accuracy of the measured isotopic ratios (±0.2 ‰ for δ¹³C and ±0.3 ‰ for δ¹⁵N) and elemental compositions.

Results and Discussion

Results from several analytical techniques are all consistent with the remarkable preservation of protein-rich, polymeric shell-binding material and associated pigments in specimens as old as 18 Ma. Four lines of evidence support this conclusion.

Dissolved calcitic portions of *Ecphora* yield significant amounts (≥ 3 mg per gram) of a polymeric substance with an orange-brown colour similar to that of the *in situ* fossil shell material. Microscopic and SEM imaging reveals that this residual material forms thin (≤ 30 nm) flexible sheets with maximum dimensions to ~ 1 cm (Fig. 2) – characteristics consistent with modern molluscan shell-binding proteins (Marin *et al.*, 2008; Mann, 2001; Weiner *et al.*, 1984; Dove, 2010). In addition, SEM images and analyses of broken shell material reveal thin organic sheets visible on fractured surfaces of the calcitic (but not aragonitic) portions of *Ecphora* shells. All EDS spectra reveal carbon; some spectra also indicate excess carbon from the carbon tape substrate that the samples were adhered to for SEM analysis.



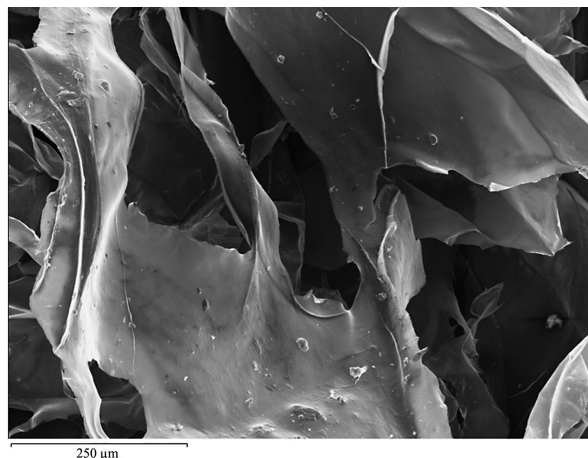


Figure 2 Scanning electron micrograph of the polymeric residue from dissolved *Ecphora* from a St. Marys Formation, Driftwood Beach specimen. The sheets are an estimated 10 nm thick. These polymeric sheets represent protein-rich shell-binding organic compounds which were recovered from calcite shell material dissolved in HCl.

The ^{13}C NMR analysis of the polymeric substance reveals a spectrum with prominent features associated with intact peptide and polysaccharide (Fig. 3). It is evident from the NMR data that approximately 70 % of the carbon is accounted for by peptide carbonyl, polysaccharide glycosidic carbon, carbohydrate secondary alcohols and aliphatic carbon associated with protein. The remaining ~ 30 percent of the carbon is aromatic/olefinic and most likely represents diagenetic alteration products of sugars with amino acids, i.e., so called melanoidins. These data indicate, in general, a very high degree of molecular preservation of the protein-polysaccharide composite that constitutes the major organic component within the calcareous shell. However, not all small organic molecules are diagenetic derivatives. Some might be pigments, organic acids (fatty acids), etc. that were originally part of the organic composite.

Hydrolysis of the organic *Ecphora* residue from a St. Marys Formation shell reveals at least 11 different amino acids derived from hydrolysis of preserved protein (Fig. 4), including aspartate and glutamate, which are typical of shell-binding proteins. Note that most of the molecular species observed in Figure 4 are not derived from hydrolysis of protein; rather the majority of compounds are complex acid-functionalised degradation products, many likely from sugars liberated through the partial hydrolysis of polysaccharide that subsequently were partially degraded to a complex array of small oxygen functionalised molecules. A large number of unidentified molecular species contain nitrogen, as which is evident from the abundant even mass fragment radical cations. These molecules most likely represent various altered amino acids. As for the reactions that yield

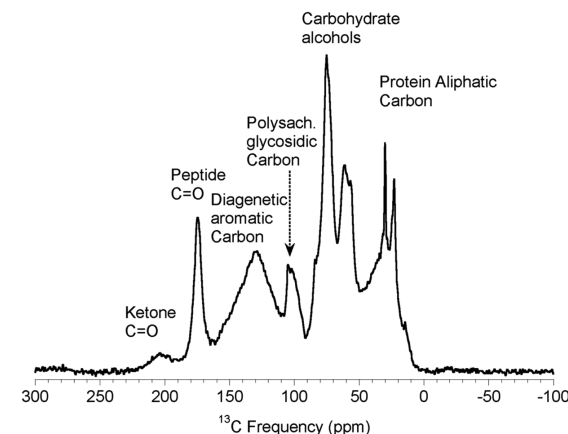


Figure 3 ^{13}C Solid-state Nuclear magnetic resonance spectrum of the organic residue obtained from *Ecphora* through acid demineralisation. Note that approximately 70 % of the carbon is represented by apparently well preserved protein and polysaccharide. The analysed sample came from the St. Marys Formation.

these compounds, the ^{13}C solid state NMR data (Fig. 3) reveal that ~30 % of the carbon in the residue exists in a complex aromatic/olefinic organic solid. Diagenetic reactions between free amino acids and sugars lead to the formation of so called melanoidin organic solids. The complex and largely unidentified N- and O-containing molecules observed in Figure 4 most likely represent a complex suite of products of sugar-amino acid reactions. It is possible that some of these molecules formed during the hydrolysis stage of the amino acid preparation; i.e., some if not all of these molecules could be neoformed. Whereas, the detection of intact amino acids (Fig. 4) is evidence for at least intact fragments of protein, their relatively low yield cannot be used as a quantitative measure of the relative quantity of intact protein.

Elemental and isotopic analyses of the residue from the shells (Fig. S-2; Table S-2) reveal total organic carbon of 4 to 40 %, with $11 < \text{C:N} < 18$. Pure protein C:N values typically range from 3 to 5. Higher C:N values for organic matter of this age could be indicative of diagenetic effects in which N could be lost from amino acids, the formation of melanoidins from carbohydrates or the original inclusion of indigenous polysaccharides. Percent carbon values < 10 clearly are composed primarily of non-organic matter (3 out of 17 samples), whereas those with %C values > 30 are almost pure organic matter. Isotope values for carbon ($-20 < \delta^{13}\text{C} < -14.6\text{‰}$) are consistent with the shallow marine environment where these organisms lived. Isotopic compositions for nitrogen ($4 < \delta^{15}\text{N} < 12.2\text{‰}$) are consistent with *Ecphora*'s known role as a predator of other mollusks. The range of values for both carbon and nitrogen isotopic compositions cannot be easily explained by preservation quality because there is no relationship between %C, %N, or C:N with either $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$. Another possible explanation for the range is that *Ecphora* occupied many different trophic levels. A third explanation might be that baseline conditions for the Chesapeake Bay varied during the 10 million year time span during which these organisms lived. Either way we interpret the



individual analyses, the organic matter elemental and isotopic compositions are very similar to those from modern marine invertebrates. We conclude, therefore, that essentially intact shell-binding proteins have been preserved for up to 18 Ma.

Gastropod pigmentation is known to come from a wide range of molecules including melanin, tetrapyrroles, ommochromes, sclerotin and pterins (Hollingworth and Barker, 1991). Nevertheless, the presence of carotenoid pigmentation in extant gastropods (Shadidi and Brown, 1998), coupled with the observed photosensitivity of the chromophore, is consistent with a protein-bound carotenoid.

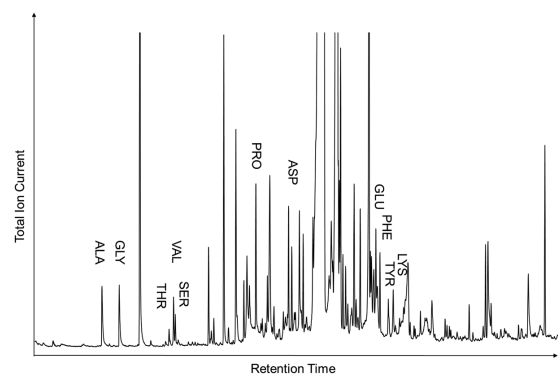


Figure 4 A mass chromatogram of the derivatised (to form isopropyl esterified and N-trifluoroacetylated derivatives) products of an acid hydrolysed *Ecphora* organic residue. In addition to a wide range of small molecules, numerous amino acids are observed derived from hydrolysis of protein associated with the pigmented residue. The analysed sample came from the St. Marys Formation.

Conclusions

A number of fossil shells and bones have yielded evidence for ancient protein. For example, brachiopod fossils from the Jurassic and Silurian were shown to have some preserved proteinaceous material (Joep, 1967); *Pecten* fossils from the Pleistocene through the Jurassic preserved amino acids and proteinaceous material (Akiyama and Wyckoff, 1970); the shell matrix protein, dermatopontin, has been reported from 1,500 year old snail fossils (Sarashina *et al.*, 2008); and the bone protein, osteocalcin, has been recovered and sequenced from bison fossils more than 55,000 years old (Nielsen-Marsh *et al.*, 2002). In this context, intact proteinaceous shell-binding material in 8 to 18 Ma *Ecphora* represents some of the oldest and best-preserved examples of original protein observed in a fossil shell.

An important implication of this study is that mineral-bound proteins and amino acids can be effectively protected from degradation. The similar reported preservation of Cretaceous osteocalcin in fossilised dinosaur bone (Muyzer, 1992) underscores the importance of mineral-molecule interactions in enhancing the stability of otherwise reactive organic molecules. Such protective chemisorption onto mineral surfaces could have played important roles in Earth's near-surface environments since the Hadean Eon and must therefore be considered in any analysis of the possible inventory of prebiotic biomolecules (Hazen, 2006).

An exciting opportunity presented by the discovery of intact Miocene shell-binding proteins is the possibility of amino acid sequencing and phylogenetic analysis through 10 million years of gastropod evolution. Similar analysis of bone osteocalcin has been successfully applied to the phylogenetic analyses of North American grazing mammals (Nielsen-Marsh *et al.*, 2002). Similar identification and sequencing of *Ecphora* proteins could provide unprecedented insight into muricid evolution.

Acknowledgements

We are grateful to Roxanne Bowden, Derek Smith and Andrew Steele for technical assistance in the analyses of our samples. We would also like to thank the reviewers for their helpful insights and suggestions. Work was supported in part by the NASA Astrobiology Institute, the Deep Carbon Observatory, the Hazen Foundation, and the Carnegie Institution of Washington.

Editor: Bruce Watson

Additional Information

Supplementary Information accompanies this letter at <http://www.geochemicalperspectivesletters.org/article1501>

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Cite this letter as: Nance, J.R., Armstrong, J.T., Cody, G.D., Fogel, M.L., Hazen, R.M. (2015) Preserved macroscopic polymeric sheets of shell-binding protein in the Middle Miocene (8 to 18 Ma) gastropod *Ecphora*. *Geochem. Persp. Let.* 1, 1-9.

References

- AKIYAMA, M., WYCKOFF, R.W.G. (1970) The total amino acid content of fossil *Pecten* shells. *Proceedings of the National Academy of Science of the USA* 67, 1097-1100.
- CARTER, J.G., ROSSBACH, T.J., ROBERTSON, K.J., WARD, L.W. (1994) Morphological and microstructural evidence for the origin and early evolution of *Ecphora* (Mollusca: Gastropoda). *Journal of Paleontology* 68, 905-907.
- DOVE, P.M. (2010) The rise of skeletal biomineralization. *Elements* 6, 37-42.
- HAZEN, R.M. (2006) Mineral surfaces and the prebiotic selection and organization of biomolecules (Presidential Address to the Mineralogical Society of America). *American Mineralogist* 91, 1715-1729.
- HOLLINGWORTH, N.T.J., BARKER, M.J. (1991) Colour pattern preservation in the fossil record: Taphonomy and diagenetic significance. In: Donovan, S.K. (Ed.) *The Processes of Fossilization*. Columbia University Press, New York, 105-119.



- JOPE, M. (1967) The protein of brachiopod shell-II. Shell protein from fossil articulates: Amino acid composition. *Comparative Biochemistry and Physiology* 20, 601-605.
- MANN, S. (2001) *Biomineralization: Principles and Concepts in Bioinorganic Materials Chemistry*. Oxford University Press, New York.
- MARIN, F., LUQUET, G., MARIE, B., MEDAKOVIC, D. (2008) Molluscan shell proteins: Primary structure, origin, and evolution. *Current Topics in Developmental Biology* 80, 209-276.
- MCGRAW, K.J. (2006) Mechanics of carotenoid-based coloration. In: Hill, G.E., McGraw, K.J. (Eds.) *Bird Coloration: Mechanisms and Measurements*. Harvard University Press, Cambridge, MA, 177-242.
- MUYZER, G., SANDBERG, P., KNAPEN, M.H.J., VERMEER, C., COLLINS, M., WESTBROEK, P. (1992) Preservation of the Bone Protein Osteocalcin in Dinosaurs. *Geology* 20, 871-874.
- NIELSEN-MARSH, C.M., OSTROM, P.H., GANDHI, H., SHAPIRO, B., COOPER, A., HAUSCHKA, P.V., COLLINS, M.J. (2002) Sequence preservation of osteocalcin protein and mitochondrial DNA in bison bones older than 55 ka. *Geology* 30, 1099-1102.
- PETUCH, E.J., DROLSHAGEN, M. (2010) *Molluscan Paleontology of the Chesapeake Miocene*. CRC Press.
- SARASHINA, I., KUNITOMO, Y., IJIMA, M., CHIBA, S., ENDO, K. (2008) Preservation of the shell matrix protein dermatopontin in 1500 year old land snail fossils from the Bonin islands. *Organic Chemistry* 39, 1742-1746.
- SHADIDI, F., BROWN, J.A. (1998) Carotenoid pigments in seafoods and aquaculture. *Critical Reviews in Food Science and Nutrition* 38, 1-67.
- SHATTUCK, G.B. (1904) Geological and paleontological relations, with a review of earlier investigations; the Miocene deposits of Maryland. In: *Miocene*. Maryland Geological Survey, The Johns Hopkins Press, 1-543.
- VISAGGI, C.C., GODFREY, S.J. (2010) Variation in composition and abundance of Miocene shark teeth from Calvert Cliffs, Maryland. *Journal of Vertebrate Paleontology* 30, 26-35.
- VOKES, H.E. (1957) *Miocene fossils of Maryland*, Bulletin 20. Maryland Geological Survey, The Johns Hopkins Press, 1-85.
- WADE, N.M., TOLLENAERE, A., HALL, M.R., DEGNAN, B.M. (2009) Evolution of a Novel Carotenoid-Binding Protein Responsible for Crustacean Shell Color. *Molecular Biology and Evolution* 26, 1851-1864.
- WARD, L.W. (1992) Molluscan Biostratigraphy of the Miocene, Middle Atlantic Coastal Plain of North America. *Virginia Museum of Natural History, Memoir No. 2*, Martinsville, VA, 159.
- WARD, L.W., ANDREWS, G.W. (2008) Stratigraphy of the Calvert, Choptank, and St. Marys Formations (Miocene) in the Chesapeake Bay Area, Maryland and Virginia. *Virginia Museum of Natural History, Memoir No. 9*, Martinsville, VA, 170.
- WEINER, S., TRAUB, W., PARKER, S.B. (1984) Macromolecules in mollusc shells and their functions in biomineralization [and Discussion]. *Philosophical Transactions of the Royal Society of London B* 304, 425-434.

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■ Supplementary Information

The Supplementary Information includes:

- Figures S-1 and S-2
- Tables S-1 and S-2



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Figure S-1 Map showing locations of *Ecphora* samples collected (Table S-1). Drum Cliff (DC), Broomes Island (BI), Kenwood Beach (KB), Randle Cliff (RC), Plum Point (PP), Parkers Creek (PC), Windmill Point (WP), Driftwood Beach (DB) (modified from Visaggi and Godfrey, 2010).

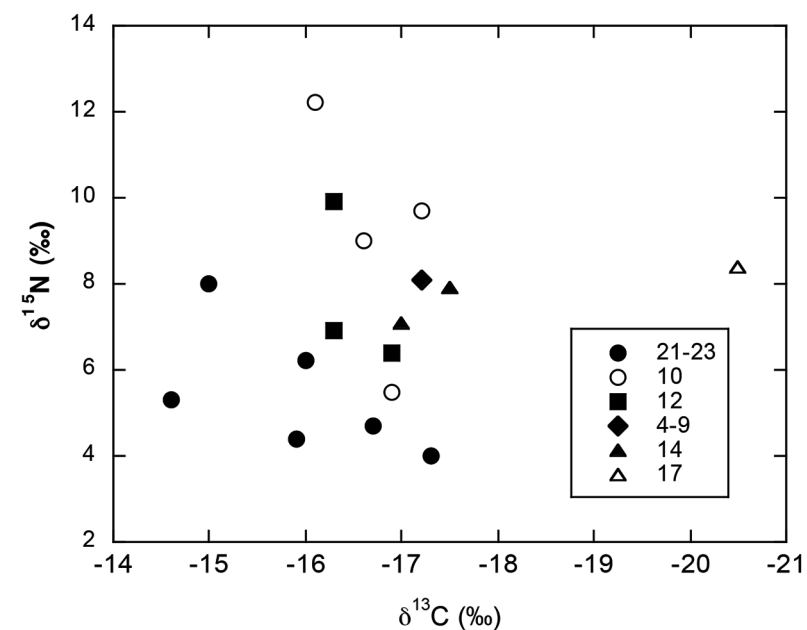


Figure S-2 Carbon and nitrogen isotopes indicate a marine environment with terrestrial influence. They also indicate a shift in trophic level over time. Numbers correspond to bed.



Table S-1 Sample information.

Sample Number	Formation	Bed*	Genus [†]	Species [†]	Collection Date(s)
DB 1-1	St. Marys	21-23	<i>Ecphora</i>	<i>gardnerae germonae</i>	6/1/2012 – 6/13/2012
DB 2-1	St. Marys	21-23	<i>Ecphora</i>	<i>gardnerae germonae</i>	6/1/2012 – 6/13/2012
DB 4-1	St. Marys	21-23	<i>Ecphora</i>	<i>gardnerae germonae</i>	6/1/2012 – 6/13/2012
WP 1-1	St. Marys	21-23	<i>Ecphora</i>	<i>gardnerae germonae</i>	6/1/2012 – 6/13/2012
WP 3-1	St. Marys	21-23	<i>Ecphora</i>	<i>gardnerae germonae</i>	6/1/2012 – 6/13/2012
WP 4-1	St. Marys	21-23	<i>Ecphora</i>	<i>gardnerae germonae</i>	6/1/2012 – 6/13/2012
PP 1-1	Calvert	10	<i>Ecphora</i>	<i>tricostata</i>	8/4/2012
PP 2-1	Calvert	10	<i>Ecphora</i>	<i>tricostata</i>	8/4/2012
PP 3-1	Calvert	10	<i>Ecphora</i>	<i>tricostata</i>	8/4/2012
PP 5-1	Calvert	10	<i>Ecphora</i>	<i>tricostata</i>	8/4/2012
PC 1-1	Calvert	12	<i>Ecphora</i>	<i>meganae</i>	8/11/2012
PC 2-1	Calvert	12	<i>Ecphora</i>	<i>meganae</i>	8/11/2012
PC 3-1	Calvert	14	<i>Ecphora</i>	<i>meganae</i>	8/11/2012
RC 1-1	Calvert	4-9	<i>Ecphora</i>	<i>tricostata</i>	8/13/2012
BI 1-1	Calvert	14	<i>Ecphora</i>	<i>meganae</i>	8/13/2012
KB 1-1	Calvert	14	<i>Ecphora</i>	<i>meganae</i>	8/3/2012
DC 1-1	Choptank	17	<i>Ecphora</i>	<i>meganae</i>	8/13/2012

Note: *Ecphora* samples with their localities and collection dates (Fig. S-1).

[†] Identification based on Ward (1992).

* The bed numbers are taken from Ward and Andrews (2008).



Table S-2 Results of Isotopic Analysis.

Sample Number	$\delta^{15}\text{N}$ (‰)	Wt.% N	$\delta^{13}\text{C}$ (‰)	Wt.% C	C/Nat
DB 1-1	4.0	3.07	-17.3	32.45	12.35
DB 2-1	4.4	4.48	-15.9	46.36	12.07
DB 4-1	4.7	3.98	-16.7	40.37	11.83
WP 1-1	6.2	1.85	-16.0	17.84	11.28
WP 3-1	8.0	0.60	-15.0	6.52	12.64
WP 4-1	5.3	2.86	-14.6	28.58	11.66
PP 1-1	9.7	0.49	-17.2	7.60	18.25
PP 2-1	9.0	1.23	-16.6	16.65	15.84
PP 3-1	12.2	1.12	-16.1	14.99	15.65
PP 5-1	5.5	1.24	-16.9	15.72	14.74
PC 1-1	6.4	1.73	-16.9	20.81	14.07
PC 2-1	6.9	1.81	-16.3	22.45	14.45
PC 3-1	9.9	2.18	-16.3	27.24	14.55
RC 1-1	8.1	0.62	-17.2	8.31	15.73
BI 1-1	7.9	2.95	-17.5	36.30	14.33
KB 1-1	7.1	0.61	-17.0	8.51	16.17
DC 1-1	8.4	1.72	-20.5	21.59	14.65

Note: Carbon and nitrogen weight percent, isotopic measurements, and C/N for 17 *Ecphora* samples. See Figure S-1 for sample number abbreviations.

