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Prospects for Sterane Preservation in Sponge Fossils from Museum Collections and the Utility of Sponge Biomarkers for Molecular Clocks

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Abstract

The sponge biomarker hypothesis argues that 24-isopropylcholestanes preserved in Neoproterozoic-age rocks are "molecular fossils" left behind by marine sponges. Despite genetic and geologic support for this hypothesis, 24-isopropylcholestane has never been reported from a sponge body fossil. This lack of direct evidence regarding the source of sponge biomarkers through deep time leaves unanswered questions, such as whether their biosynthesis evolved once in sponges or multiple times across different lineages. In this study, we analyzed 10 sponge fossils from the Yale Peabody Museum of Natural History collections in pursuit of evidence of sterane biomarkers. We failed to recover 24-isopropylcholestane and instead found a near-identical sterane profile across all samples. This result indicates a combination of little to no sterane preservation in the fossils themselves, coupled with anthropogenic hydrocarbon contamination during their collection and storage. However, signals from bacterial biomarkers (hopanes) were more diverse across samples and consistent with a priori expectations, meaning that we cannot rule out the possibility that at least part of the hydrocarbon signal is syngenetic. We suggest that future attempts to extract biomarker hydrocarbons from sponge fossils be performed on freshly collected and specially prepared field samples. Despite the fact that demosponges or their ancestors still present the most likely source of Neoproterozoic 24-isopropylcholestanes, multiple evolutionary scenarios are consistent with current genetic and biomarker evidence: the "sponge biomarker" could represent an evolutionary novelty in demosponges, or a trait that evolved deeper in the animal tree. We therefore continue to affirm the validity of the sponge biomarker hypothesis but caution against using Neoproterozoic 24-isopropylcholestanes as a calibration point for sponges in molecular clocks. Instead, we recommend using it as a reference point for comparison, as scenarios where crown-group demosponges radiate after the Neoproterozoic remain inconsistent with the geologic record.

Keywords

Demosponges, sterols, 24-isopropylcholestane, 24-isopropylcholesterol

Introduction

Unusual steranes in the geologic record have been interpreted as molecular signatures left behind by marine sponges, which are the principal producers of such compounds in modern oceans. This "sponge biomarker" hypothesis, if correct, offers the oldest evidence for metazoan life and has become an important concept in Precambrian paleontology and in the calibration of molecular clocks (Peterson et al. 2008; Sperling et al. 2010; Erwin et al. 2011; Parfrey et al. 2011; Briggs and Summons 2014). Modern sponges produce a variety of sterols with atypical modifications (e.g., C_{26}/C_{27} side-chain methylation, cyclopentane ring addition), and such compounds may be responsible for some steranes that dominate the Late Proterozoic (Brocks et al. 2016). The best-known example of a sponge biomarker is the 30-carbon sterane 24-isopropylcholestane, a molecule found in abundance in certain Neoproterozoic to Early Cambrian rocks approximately 650 to 540 million years old. Isopropylcholestane is a diagenetic product of 24-isopropylcholesterol and related sterols (hereafter abbreviated as 24-ipc), which are principally produced by a clade of sponges known as demosponges (Love et al. 2009). Although demosponge species producing 24-ipc are distributed across all four major branches of the Demospongiae, most demosponges do not produce this sterol. It is therefore unclear whether the potential to synthesize 24-ipc is an ancient demosponge trait or one that has evolved independently in multiple lineages.

More detailed knowledge of demosponge sterols through deep time would provide essential data for distinguishing between these two evolutionary scenarios. However, despite growing evidence for the sponge biomarker hypothesis, 24-isopropylcholestane has never been identified in a sponge body fossil. Instead, these biomarkers have been recovered from geologic sediments that have accumulated organic compounds derived from many organisms. It is unclear whether individual body fossils should be expected to contain enough 24-isopropylcholestane to be detectable through deep time, particularly because 24-ipc represents less than 1% of total sterols in some demosponges (Calderón et al. 2004). Still, identifying sterols in taxonomically identifiable sponge fossils is critical to determining which group(s) of demosponges were the major producers of 24-ipc in the past.

To address whether sterane biomarkers can be detected in sponge body fossils, we analyzed 10 samples from the collections in the Division of Invertebrate Paleontology of the Peabody Museum of Natural History, Yale University, New Haven, Connecticut (YPM IP). We chose taxonomically and temporally diverse fossils and tested them for their sterane repertoires. Ultimately, we failed to find evidence of 24-isopropylcholestane and instead recovered a common sterane signature across samples. We suspect that our result represents a combination of very low levels of sterane preservation, coupled with hydrocarbon contamination resulting from collection and archival practices. Our data suggest that future attempts to extract biomarkers from sponge fossils should be performed on fresh field samples, and reinforces the uncertainty that remains regarding when 24-ipc biosynthesis evolved in demosponges.

Materials and Methods

Sample Selection

We queried the Yale Peabody Museum catalog for demosponge fossils that have been identified at least to the genus level. Fossils were selected to represent a range of ages and locations, and because this was a destructive procedure, we ensured that multiple examples of similar specimens remained in the collection. Ten sponge fossils were ultimately chosen, ranging in age from Ordovician to Cretaceous (see Table 1). As controls, we also tested two nonsponge fossils (a brachiopod and a bivalve) from sponge localities.

Sample Preparation

All aqueous solutions were precleaned using dichloromethane (DCM). Surface etching was performed to remove the outer layer of each demosponge fossil. Each sample was submerged in a 6 N hydrochloric acid (HCl) solution for 20 seconds and then washed at least four times with water. The remaining material was placed in a 60 °C oven until dry. The HCl/water supernatant underwent liquid-liquid extraction using DCM (three times total). The DCM extract was dried under nitrogen gas, reconstituted in hexane and analyzed by metastable reaction monitoring

TABLE 1. Fossils analyzed in this study. *Abbreviation:* Yale Peabody Museum of Natural History, Invertebrate Paleontology (YPM IP).

Catalog number	Scientific name	Stratigraphy	Locality	Year accessioned
YPM IP 003695	*Astylospongia inornata*	Devonian	New York, USA	1888
YPM IP 003695	*Astylospongia inornata*	Devonian	New York, USA	1888
YPM IP 123341	*Hindia fibrosa*	Devonian	New Brunswick, Canada	1905
YPM IP 123664	*Hindia* sp.	Devonian	New Brunswick, Canada	1908
YPM IP 165563	*Spongia* sp.	Ordovician	Tennessee, USA	1930
YPM IP 165907	*Spongia*? sp.	Cretaceous	Mississippi ,USA	1930
YPM IP 165917	*Spongia* sp.	Cretaceous	Kent County, England	1930
YPM IP 180318	*Anthaspidella* sp.	Ordovician	Tennessee, USA	1930–1950
YPM IP 180663	*Astylospongia* sp.	Silurian	Tennessee, USA	1930
YPM IP 181493	*Eospongia varians*	Ordovician	Quebec, Canada	1957
YPM IP 181493	*Eospongia varians*	Ordovician	Quebec, Canada	1957
YPM IP 203999	*Archaeoscyphia minganensis*	Ordovician	Newfoundland, Canada	1910
YPM IP 533356	*Meristella arcuate* (brachiopod)	Devonian	New York, USA	1888
YPM IP 200110	*Actinoceramus sulcatus* (bivalve)	Cretaceous	Kent County, England	1930

(MRM) using a Micromass AutoSpec-Ultima (Waters Corp., Milford, Massachusetts; www.waters.com) coupled with an Agilent Technologies 6890N Gas Chromatography system (Agilent Technologies, Santa Clara, California; www.agilent.com/chem). An oil standard rich in 24-isoproylcholestanes from Baghewala, India (Peters et al. 2005; S. Bhattacharya, S. Dutta and R.E. Summons, in review), was analyzed as a positive control. The brachiopod and bivalve samples were not subjected to surface etching but were briefly washed in methanol, DCM and hexane before going directly to crushing.

The interiors of the demosponge fossils were analyzed independently of the surfaces. After drying, each sample was crushed in a puck mill using a SPEX 8510 Shatterbox (SPEX SamplePrep, Metuchen, New Jersey; www.spexsampleprep.com/shatterbox). Between each crushing, the puck mill was cleaned by grinding 20 g of fired quartz sand, followed by sequential sonication in methanol, DCM and hexane for 10 minutes each. Several times during the process, 20 g of fired quartz sand was crushed using the same procedure as the fossils, providing a control for possible laboratory contamination. For the first fossil processed (YPM IP 165907), we used sonication-assisted extraction. For the remaining fossils and sand blanks, lipids were isolated from 10 g of powdered material using accelerated solvent extraction (ASE). These extracts are referred to as "bitumen I" in the text that follows.

Two samples (YPM IP 165563 and YPM IP 181493) were subjected to an additional analysis of the mineral-bound lipids (bitumen II) using the methods of Luo et al. (2015). The bitumen I–extracted residue was digested by the slow addition of 6 N HCl until no further chemical reaction (CO_2 production) was observed. This solution was diluted with H_2O, and then lipids were isolated by liquid-liquid extraction using DCM as described previously. We refer to these extracts as the "bitumen II liquid" in the text that follows. Given the low content of siliceous minerals, hydrogen fluoride dissolution was not performed. Instead, the HCl residues from each sample were transferred directly to a glass centrifuge tube, and lipids were extracted by sonication in 9:1 (volume-to-volume ratio) DCM/methanol for 10 minutes. After centrifugation, supernatants were recovered, and the extraction was repeated two additional times. These samples were dried down, reconstituted and analyzed as described previously. We refer to these extracts as the "bitumen II residues" in the text that follows.

TABLE 2. The amount of material removed through the surface etching procedure. *Abbreviation:* Yale Peabody Museum of Natural History, Invertebrate Paleontology (YPM IP).

Catalog number	Weight (g)	Weight after etching (g)	Sample loss from etching (%)
YPM IP 165907	10.17	9.16	9.93
YPM IP 181493	63.18	54.64	13.52
YPM IP 003695	57.7	57.12	1.01
YPM IP 165917	25.88	25.03	3.28
YPM IP 123664	35.67	34.6	3.00
YPM IP 123341	27.38	23.33	14.79
YPM IP 203999	92.43	83.77	9.37
YPM IP 165563	259.55	253.46	2.35
YPM IP 180318	144.76	139.43	3.68
YPM IP 180663	37.74	36.72	2.70

Results

Because the fossils examined have been repeatedly handled in nonsterile conditions, we performed HCl etching to separate the organic compounds on the fossil surfaces from their interiors. The amount of surficial material removed from each fossil varied from 3% to nearly 15% of total mass (Table 2). This variation serves as a rough proxy for the amount of carbonaceous material in each demosponge fossil.

MRM spectra show the sterane distributions across our samples (Figure 1). All fossils have a distinct chromatographic profile compared with the fired sand negative controls (Figure 1B), indicating that contamination during lipid extractions was negligible. However, all fossils—including the brachiopod and bivalve samples—have nearly identical sterane profiles when compared with each other (Figure 1D). These results are consistent regardless of how the samples were prepared (sonication in contrast to ASE) or the amount of carbonaceous material in each fossil (Table 2). The signal is dominated by a high relative concentration of diasteranes (asterisks in Figure 1C), whereas regular sterane peaks (Figure 1A and bars in Figure 1C) are present in the fossils at low levels compared with the positive control. Many of the surface extracts show no detectable signal, but when they do, this signal is indistinguishable from that yielded by the interior. There was no detectable signal in the bitumen II liquid or residue from sample YPM IP 165563. We did recover a signal from the residue extract in sample YPM IP 181493, but it was indistinguishable from bitumen I.

In addition to steranes, we also looked for the presence of hopanes (Figure 2A), a class of bacterial lipids that share a biosynthetic kinship to steroids (Rohmer et al. 1984). Although the MRM peaks are similar across demosponge samples, their abundance varies (Figure 2B–G). Some hopanes are detectable in the sand blank, but at a much lower abundance than the fossils, indicating that contamination during the extraction process was not an issue (Figure 2G). The isomer ratio of C_{31} hopanes in all samples is below 60%, suggesting that the thermal maturity of these fossils falls within the oil window (Figure 2B; Peters et al. 2005). The C_{27} Ts/Tm (triterpene stable/triterpene maturable) hopane ratio (Figure 2F) also increases through the oil window, but this ratio is heavily influenced by lithology as well as maturity, and could be indicative of an extraneous hydrocarbon source. The ratios of 2- and 3-methyl hopanes are broadly consistent with Phanerozoic averages (Figure 2C and E, respectively; Knoll et al. 2007; Rohrssen et al. 2013). As in the case of the sterane data, there is no detectable hopane signature in the bitumen II of YPM IP 165563. In contrast, the bitumen II residue from YPM IP 181493 shows a distinct, albeit weaker, signature compared with the bitumen I (Figure 2B–G).

Discussion

The data recovered in this study offer potentially conflicting evidence, making it difficult to interpret the significance of our failure to recover 24-isopropylcholestane. The hopane data are consistent with Phanerozoic averages from

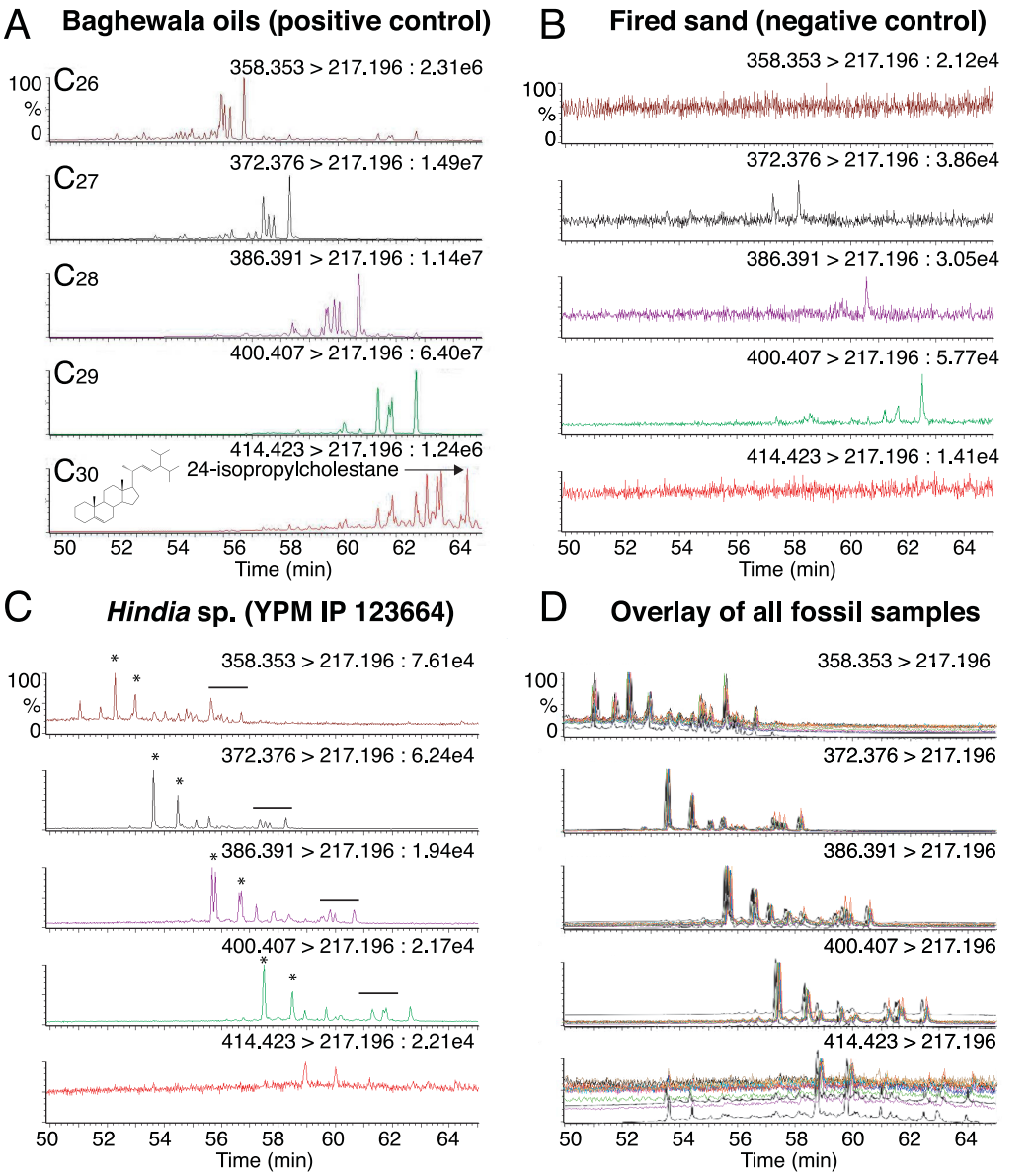

FIGURE 1. Metastable reaction monitoring sterane data from this study. Each sample has been separated into five panels based on mass spectra peaks, elucidating candidate C_{26}–C_{30} steranes. **A,** Baghewala oil standard from Punjab, India (S. Bhattacharya, S. Dutta and R.E. Summons, in review). This sample is rich 24-ispropylcholestane, which is identified at the bottom of the panel. **B,** Results from one of the fired sand negative controls. **C,** Results from one of the bitumen I extractions. Asterisks illustrate disteranes, and bars illustrate common steranes. **D,** Overlay of all bitumen I extractions, demonstrating that the hydrocarbon signature is shared across all 12 fossils.

marine marl and show expected variation between samples (Subroto et al. 1991). The bitumen II signal in YPM IP 181493 should be particularly robust against secondary contamination, and the fact that it is distinct from that of bitumen I supports its authenticity. In contrast to the hopane data, it is very unlikely that our common sterane signature represents an authentic or syngenetic signal. Although the high diasterane/sterane ratios are diagnostic for organic matter derived from shales (Rubinstein et al. 1975), such a uniform signal across samples is highly unlikely

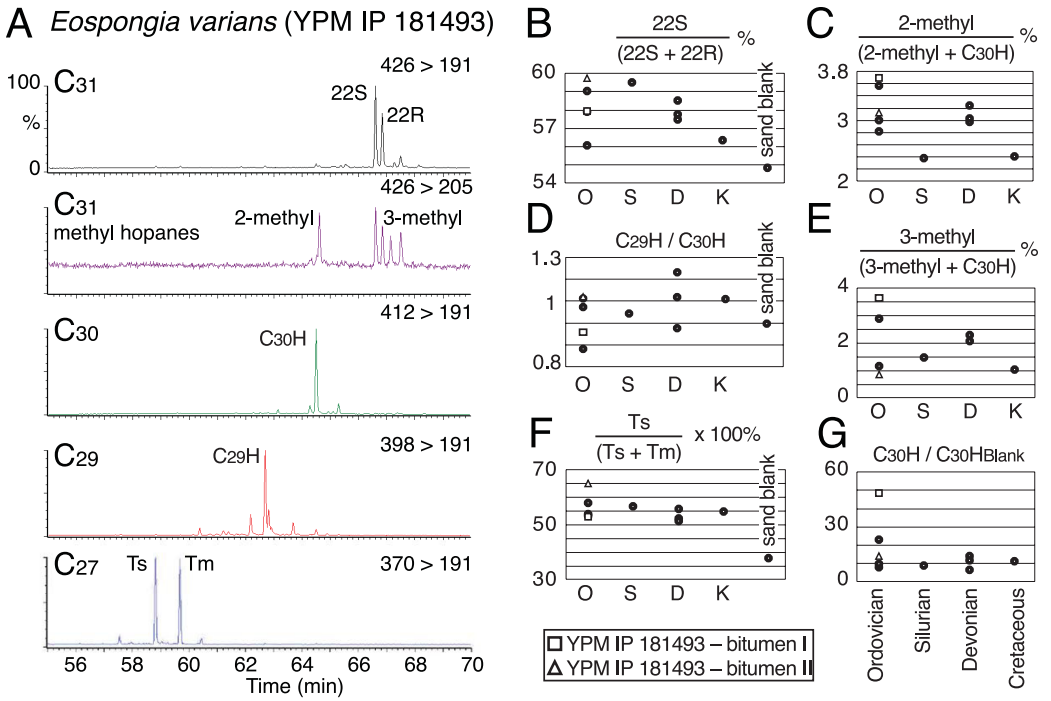

FIGURE 2. Overview of hopane data from demosponge fossils analyzed in this study. **A,** Hopane metastable reaction monitoring spectrum from one of the fossils. All samples have similar peaks, but their relative abundance varies. **B–G,** Plots showing important hopane abundance ratios, with samples binned according to their geologic age. The squares and triangles in these plots distinguish bitumen I and bitumen II, respectively, from sample YPM IP 181493. Sample YPM IP 165907 is not included in these plots, because a different technique was used to isolate its lipids (see "Materials and Methods"). **B,** The ratio of 22S to 22R C_{31} hopanes, with the sand blank included for comparison. **C,** The ratio of the abundance of 2-methyl C_{31} hopane to the dominant C_{30} hopane in each sample. **D,** The ratio of the abundance of the primary C_{29} hopane to the primary C_{30} hopane. **E,** The ratio of the abundance of 3-methyl C_{31} hopane to the C_{30} hopane in each sample. **F,** The ratio of Ts to Tm C_{27} hopanes, with the sand blank included for comparison. **G,** The ratio of the abundance of the primary C_{30} hopane in each sample to the sand blank. C_{31} methyl hopanes were not detectable in the sand blank and are not included in (C) and (E).

given that the fossils were collected from such divergent locations and strata. Instead, our results point to secondary contamination prior to hydrocarbon extraction. One demonstrated source of contamination is aerosols bearing fossil fuel residues (Illing et al. 2014). Laboratories and other facilities with high rates of air exchange are particularly prone to this problem. The fossils examined in this study were collected between 1888 and 1957, and the Museum's storage facilities would have been exposed to environmental aerosols over such extended periods of time. This hypothesis is more likely if the original concentration of preserved biomolecules was low or absent. The Yale Peabody Museum's Invertebrate Paleontology collections are now stored in new cabinets (Delta Designs, Ltd., Topeka, Kansas; www.deltadesignsltd.com) with baked polyester powder coatings that are nonreactive and solvent free, and the room doors and compactors are fitted with silicone gaskets to maintain microenvironments and prevent damage from particulates, insects and light. All specimens are held in acid-free archival quality paper specimen trays, and numbers are written on all accompanying papers and directly on the specimens with micropigment archival ink. Such developments are relatively recent, however, and environmental hydrocarbon contamination in geologic materials—particularly those housed in museum collections—remains a major issue in biomarker research (Gold et al. 2014; French et al. 2015). We recommend that future attempts to cor-

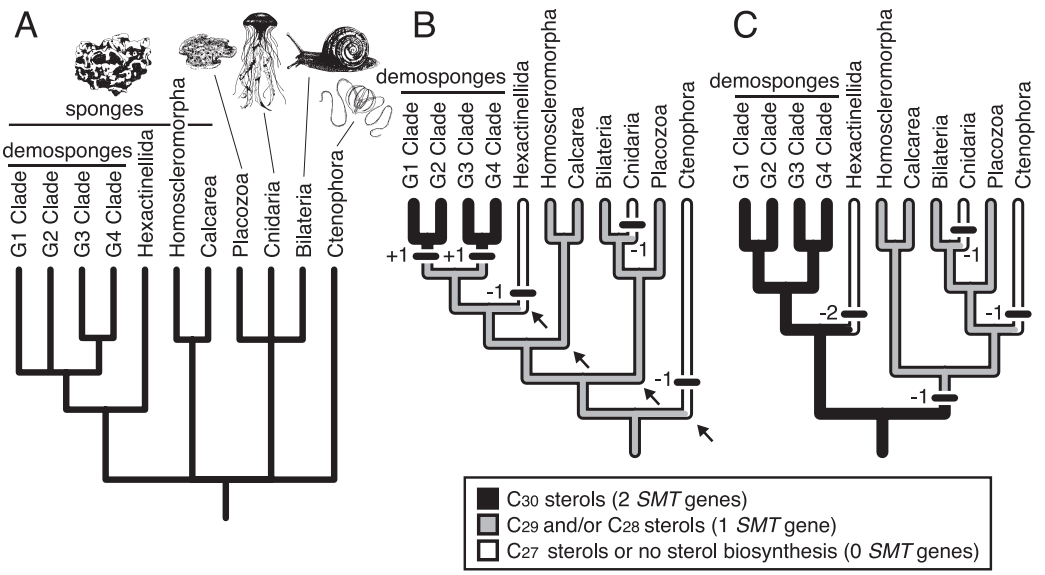

FIGURE 3. Phylogenetic uncertainty in *SMT* evolution and its relationship to sponge biomarkers. **A,** "Consensus" tree of animals based on Erwin et al. (2011), Hill et al. (2013), Pisani et al. (2015) and Whelan et al. (2015). **B,** An evolutionary scenario in which sponges are monophyletic (Pisani et al. 2015; Whelan et al. 2015), ctenophores are basal (Whelan et al. 2015) and independent *sterol 24-C-methyltransferase* (*SMT*) gene duplications occurred in the two major demosponge lineages (Gold et al. 2016). **C,** An evolutionary scenario in which sponges are paraphyletic (Erwin et al. 2011), ctenophores are part of the Eumetazoa (Pisani et al. 2015) and the *SMT* gene duplication occurred prior to the evolution of crown-group demosponges (Gold et al. 2016). Character states are based on Gold et al. (2016), with the following additions: gene presence in Bilateria is based on an *SMT* gene from *Capitella telata* (National Center for Biotechnology Information accession number ELU07827.1); gene absence from Placozoa is based on a query of the *Trichoplax adherans* genome; gene absence in Hexactinellida is based on a query of the *Aphrocallistes vastus* transcriptome, combined with evidence that hexactinellids do not synthesize sterols de novo (Blumenberg et al. 2002). Some of the animal images in this figure were modified under the creative commons agreement from the OpenLearn Tree of Life project (OpenLearn: Tree of Life 2009).

relate sponge biomarkers with body fossils be performed on fossiliferous outcrops or fresh cores, where greater yields of biomarkers are anticipated, and precautions against contamination can be taken at every stage from collection to analysis.

These inconclusive results highlight how much is still unknown regarding the evolution of sterol biosynthesis in demosponges. A recent genetic study suggests that demosponges that biosynthesize 24-ipc evolved this ability—at least in part—through the duplication of the sterol 24-C-methyltransferase (*SMT*) protein (enzyme commission number 2.1.1.41; Gold et al. 2016). However, this study was unable to determine whether the gene duplication occurred once in the demosponge stem lineage or twice in the demosponge crown. Thus, it is not clear whether 24-isopropylcholestane is best interpreted as a biomarker for demosponges or for their ancestor. Such uncertainty is exacerbated by ongoing controversies regarding the evolutionary relationships of early branching animals (Pisani et al. 2015; Whelan et al. 2015), particularly whether sponges are monophyletic (Erwin et al. 2011; Pisani et al. 2015). Here (Figure 3) we illustrate two (of many) possible evolutionary scenarios that are consistent with current hypotheses of animal relationships, combined with what is known about *SMT* evolution. In the first scenario (Figure 3B), 24-ipc biosynthesis is a novel capability that evolved after the divergence of crown-group demosponges; its presence in Neoproterozoic rocks indicates that at least four other animal lineages (marked by arrows) had also evolved by this time. In the second scenario (Figure 3C), 24-ipc is a conserved trait of demosponges, extending deep into the ancestral stem lineage. In this scenario, the "sponge" biomarker could be indicative of stem-

group animals, implying that no crown-group animal lineage was necessarily present during early 24-isopropylcholestane deposition in the Neoproterozoic. Increased genomic data and improved phylogenetic tools will likely help distinguish between these competing scenarios in the near future. Such research may also elucidate the genetic underpinnings behind other unusual sponge sterols, and continued study of eukaryotic biomarker distributions from Neoproterozoic rocks (with appropriate thermal maturity) coupled with molecular clocks of gene evolution may reveal additional sponge biomarkers besides 24-ipc. However, until then, the interpretation of "sponge biomarkers" in the context of animal evolution remains ambiguous.

Given the issues discussed previously, we suggest proceeding with caution when using the "sponge biomarker" as a calibration point in molecular clocks. Although there is growing evidence to attribute Neoproterozoic 24-isopropylcholestanes to sponges (reviewed in Gold et al. 2016), it remains unclear whether this sterane indicates crown-group demosponges, stem-group animals or some clade nested in between. Incorrect interpretations could introduce significant biases into molecular clock studies if this biomarker is used as a calibration point. As alternative calibration points, we recommend using 535 Ma-old hexactinellid spicules for crown-group sponges (Antcliffe et al. 2014) and the 600 Ma-old occurrence of *Eocyathispongia qiania* for stem-group sponges (Yin et al. 2015). Instead of providing a calibration point, we suggest that sponge biomarkers are used as a check on the results of molecular clock analyses: because 24-ipc production almost certainly evolved in demosponges by the time the major sublineages diverged, any clock estimate that places this event significantly later than the Neoproterozoic is inconsistent with the geologic record. Because recent molecular clock estimates for the origin of sponges seem consistent with the biomarker record, even when biomarkers are not used as a calibration point (Sperling et al. 2010; Erwin et al. 2011; Gold et al. 2015), we do not anticipate the need for any major revision of the timescale for sponge origins. Instead, the congruence between molecular clocks and the geologic record of 24-isopropylcholestanes supports our preferred interpretation of this molecule as a demosponge signature, even if its precise evolutionary history awaits further discovery.

Acknowledgments

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