

STEROL BIOSYNTHESIS

De novo phytosterol synthesis in animals

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Sterols are vital for nearly all eukaryotes. Their distribution differs in plants and animals, with phytosterols commonly found in plants whereas most animals are dominated by cholesterol. We show that sitosterol, a common sterol of plants, is the most abundant sterol in gutless marine annelids. Using multiomics, metabolite imaging, heterologous gene expression, and enzyme assays, we show that these animals synthesize sitosterol de novo using a noncanonical C-24 sterol methyltransferase (C₂₄-SMT). This enzyme is essential for sitosterol synthesis in plants, but not known from most bilaterian animals. Our phylogenetic analyses revealed that C₂₄-SMTs are present in representatives of at least five animal phyla, indicating that the synthesis of sterols common to plants is more widespread in animals than currently known.

Sterols are lipids that play essential roles in all multicellular eukaryotes. Their distribution and synthesis differs across eukaryotic kingdoms. Fungi and plants mainly synthesize sterols with 28 to 29 carbon atoms (C₂₈ and C₂₉) called ergosterols and phytosterols (1, 2), whereas animals predominantly produce the C-27 sterol cholesterol. These interkingdom differences reflect the complex evolutionary history of sterol synthesis. Phylogenetic analyses suggest that most enzymes for the biosynthesis of plant, fungal, and animal sterols were present in the last eukaryotic common ancestor (LECA) (3, 4), with the distribution observed in most extant eukaryotes evolving through multiple events of enzyme losses and different pathways for sterol synthesis.

Cholesterol differs from C₂₈ and C₂₉ sterols by only one methyl or ethyl group at position C₂₄. This alkylation is catalyzed by C-24 sterol methyltransferase (C₂₄-SMT), an enzyme widely distributed in plants, microbial eukaryotes, and fungi but not known from nearly all animals (5, 6). The exceptions are some marine sponges and the marine annelid *Capitella teleta* (7, 8). Marine sponges have an unusual sterol composition enriched in highly branched alkylated sterols (9–12), of which some are synthesized by C₂₄-SMT homologs (13). As with most other enzymes required for sterol synthesis, an ancestral C₂₄-SMT was likely present in the LECA (3) and is assumed to have been lost early in animal evolution following the diver-

gence of sponges, explaining why eumetazoans are not able to produce C₂₈ and C₂₉ sterols (6, 8).

Sitosterol is the main sterol in the marine gutless annelid *Olavius algarvensis*

O. algarvensis belongs to a group of gutless marine annelids found worldwide, mainly in coral reef and seagrass sediments. These annelid worms lack a digestive system and are obligately associated with bacterial endosymbionts that provide them with nutrition (14–17). As part of our ongoing research on the *O. algarvensis* symbiosis, we analyzed the metabolome of single worm individuals using both gas chromatography mass spectrometry and high-performance liquid chromatography mass spectrometry. These analyses revealed an unusual sterol composition, with sitosterol accounting for most of the sterols detected (60%), and the remainder consisting of cholesterol (Fig. 1A and fig. S1). This was unexpected, as cholesterol generally dominates the sterol pool in bilaterians, often making up more than 90% of the total sterol content (18, 19). Sitosterol is abundant in most plants but among bilaterians has only been reported as the most abundant sterol in a few phytoparasitic nematodes that are not capable of de novo sterol synthesis (20–22). In these plant parasites, it is unclear whether their sitosterol is only present in the nematode gut content or incorporated into their cells and tissues. The absence of a gut in *O. algarvensis* excludes sterol contamination from plant matter in the digestive tract.

O. algarvensis sterols have an isotopic composition that is distinct from their environment

We next asked whether *O. algarvensis* could acquire its sterols from the environment through passive diffusion. Chemical analyses of porewater collected in the vicinity of seagrass meadows—the habitat of many gutless annelids (including *O. algarvensis*)—revealed that sterols were present in the environment in concentrations sufficient to sustain the growth

of small sterol-auxotrophic invertebrates (supplementary text and fig. S2). Therefore, we further investigated the origin of sterols in *O. algarvensis* by analyzing the carbon isotopic signature ($\delta^{13}\text{C}$) of sterols in the worms, their environment (which includes the seagrass *Posidonia oceanica*) and the porewater of the sediments these worms live in. Carbon isotopic signatures are used to reveal carbon sources and their paths through the food web. As a rule, the bulk $\delta^{13}\text{C}$ values of animals reflect their dietary sources [0.5 per mil (‰) to 2 ‰ difference] (23, 24), but sterols are typically depleted in ^{13}C relative to bulk biomass by as much as 5 ‰ to 8 ‰ (25, 26). Results from gas chromatography isotope ratio mass spectrometry (GC-IRMS) with single metabolite resolution showed that sitosterol in the seagrass and porewater had $\delta^{13}\text{C}$ values ranging from –30 ‰ to –15 ‰ (Fig. 1E and supplementary text). The sterols in *O. algarvensis*, as well as those in another co-occurring gutless annelid species, *Olavius ilvae*, had much lower $\delta^{13}\text{C}$ values: –38 ‰ to –36 ‰ for sitosterol and –40 ‰ to –31 ‰ for cholesterol (Fig. 1E). The difference in the isotopic signature of sterols in both *Olavius* species and their environment excludes that these worms acquired sterols from their environment, and instead indicates an endogenous origin. *O. algarvensis*, as all other *Olavius* and *Inanidrilus* species, derives all its nutrition from its chemosynthetic bacterial symbionts, and this is reflected in its bulk isotopic composition with $\delta^{13}\text{C}$ values of –30.6 ‰ (27). The ^{13}C -depleted signatures of both cholesterol and sitosterol by 1 ‰ to 10 ‰ compared with bulk biomass in *O. algarvensis* and *O. ilvae* led us to hypothesize that these animals synthesize both sterols de novo, using carbon derived from their chemosynthetic symbionts.

Bacterial symbionts are not the source of sitosterol in *O. algarvensis*

Having shown that sitosterol in *O. algarvensis* and *O. ilvae* did not originate from the uptake of plant sterols from their environment, we next investigated whether their bacterial symbionts were the source of this C₂₉ sterol. The bacterial symbionts of these worms form a thick layer between the cuticle and the epidermis of the animal (Fig. 1B). To localize the distribution of sterols in *O. algarvensis*, we used two high spatial resolution metabolite imaging techniques. Time-of-flight secondary ion mass spectrometry (TOF-SIMS) data revealed that, at a spatial resolution of 0.4 μm , both sitosterol and cholesterol were uniformly distributed throughout the animals' tissues (Fig. 1, C and D). We found no evidence for a tissue-specific distribution of these two sterols—that is, there was no correlation between symbiont location and sitosterol distribution. These findings are supported by a second mass spectrometry imaging method, matrix-assisted laser

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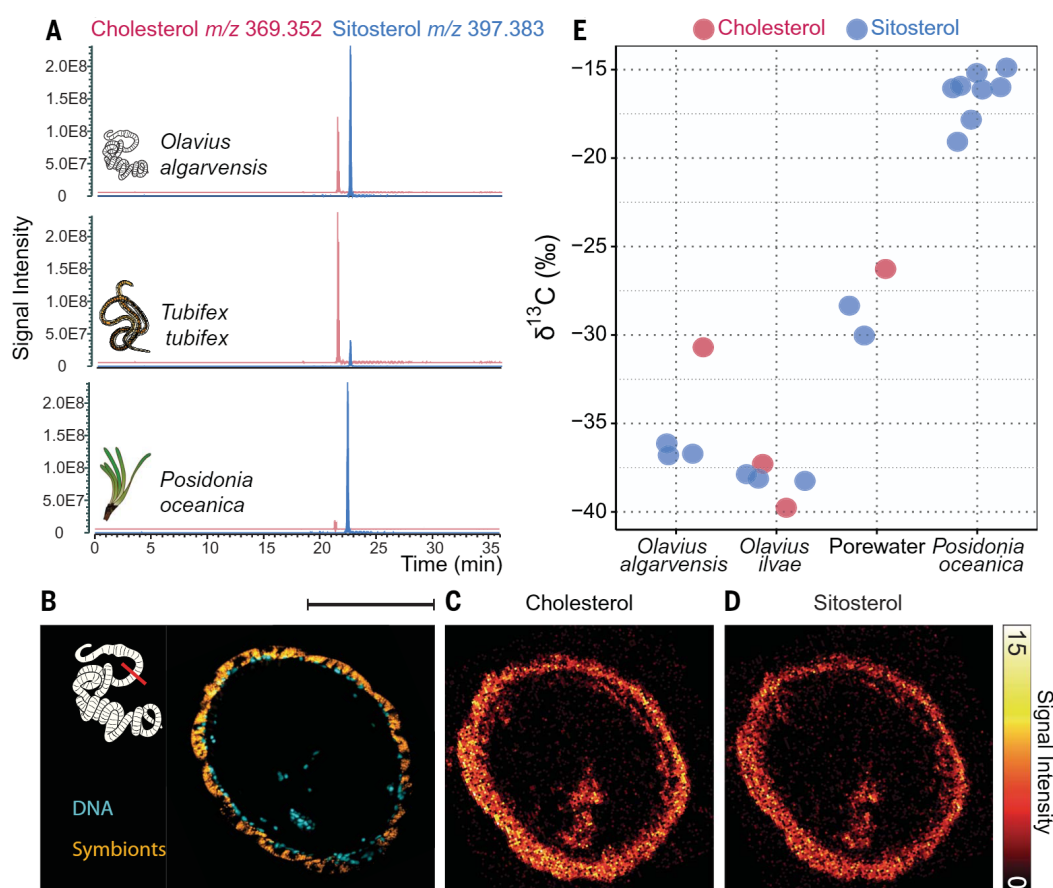
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Fig. 1. *Olavius algarvensis* has an unusual sterol profile dominated by sitosterol, a common plant sterol. (A) Extracted-ion chromatograms (XIC) of cholesterol

[[M-H₂O+H]⁺ C₂₇H₄₅ at m/z 369.352 (red)] and sitosterol [[M-H₂O+H]⁺ C₂₉H₄₉ at m/z 397.383 (blue)]. The XICs were generated from lipid extracts of (from top to bottom): the gutless marine annelid *O. algarvensis*, the freshwater annelid *Tubifex tubifex*, and the seagrass *Posidonia oceanica*. (B) Chemosynthetic symbiotic bacteria are located just below the cuticle of *O. algarvensis*. 16S rRNA fluorescence in situ hybridization (FISH) image of a cross section through a worm showing the symbionts in yellow (general eubacterial probe) and host nuclei in blue (DAPI). (C and D) Distribution of sitosterol and cholesterol in *O. algarvensis* measured by TOF-SIMS (C) Summed intensity of cholesterol ions (m/z 369.38, 385.34, 401.35) measured with TOF-SIMS. (D) Summed intensity of sitosterol ions (m/z 397.47, 383.37, 413.45) as measured by TOF-SIMS. (E) The ¹³C isotopic composition of sterols in gutless annelids differed from that of the neighboring seagrass (*P. oceanica*) and sediment porewater. Scale bar in (B) to (D) 100 μm.



desorption/ionization mass spectrometry imaging (MALDI-2-MSI), of cross and longitudinal sections at a spatial resolution of 5 μm. The MALDI imaging data of longitudinal worm sections confirmed a uniform distribution of sitosterol and cholesterol throughout the animal (figs. S3 and S4) and the identity of these sterols (table S1). This homogeneous sterol distribution suggests that the bacterial symbionts are not the source for sitosterol in *O. algarvensis*.

As a second approach to investigate whether the bacterial symbionts are the source of sitosterol, we sequenced and assembled the genomes of the *O. algarvensis* symbionts and screened them for enzymes involved in de novo sterol synthesis. These analyses revealed that the symbionts, as in most bacteria, do not encode enzymes involved in de novo sterol synthesis (for more details see materials and methods).

***O. algarvensis* encodes and expresses enzymes involved in sitosterol synthesis that overlap with those of cholesterol synthesis**

Having ruled out a symbiotic origin and an environmental source of sitosterol in *O. algarvensis*, we next investigated whether the animals themselves can synthesize this typical plant sterol. To identify and characterize the biosynthetic pathways involved in sterol production, we sequenced and assembled the genome of

O. algarvensis and analyzed metatranscriptomic and metaproteomic data to search for enzymes involved in de novo sterol synthesis. The host possessed the full enzymatic toolbox required for cholesterol and sitosterol synthesis. For cholesterol synthesis, the genome of *O. algarvensis* encodes homologs of 11 enzymes known to be involved in the synthesis of this sterol (fig. S5). The intron-exon structure of these genes confirms their eukaryotic origin and excludes bacterial contamination (Fig. 2A and table S2). The cholesterol biosynthesis pathway, starting with squalene, is a series of 10 connected enzymatic reactions encoded by 11 genes (fig. S5 and table S3). Homologs of all enzymes were transcribed (11 out of 11 enzymes) and 5 out of 11 proteins were detected in the proteome of *O. algarvensis* (fig. S5 and tables S4 and S5), indicating active expression of the genes involved in cholesterol synthesis. Phylogenetic analysis allowed us to assign each homolog to an ortholog group and thus to a potential function (figs. S6 to S15). Collectively, these data show that *O. algarvensis* has all the enzymes required for de novo cholesterol synthesis, which in combination with the isotopic signature of their cholesterol suggests that these annelids are able to synthesize cholesterol.

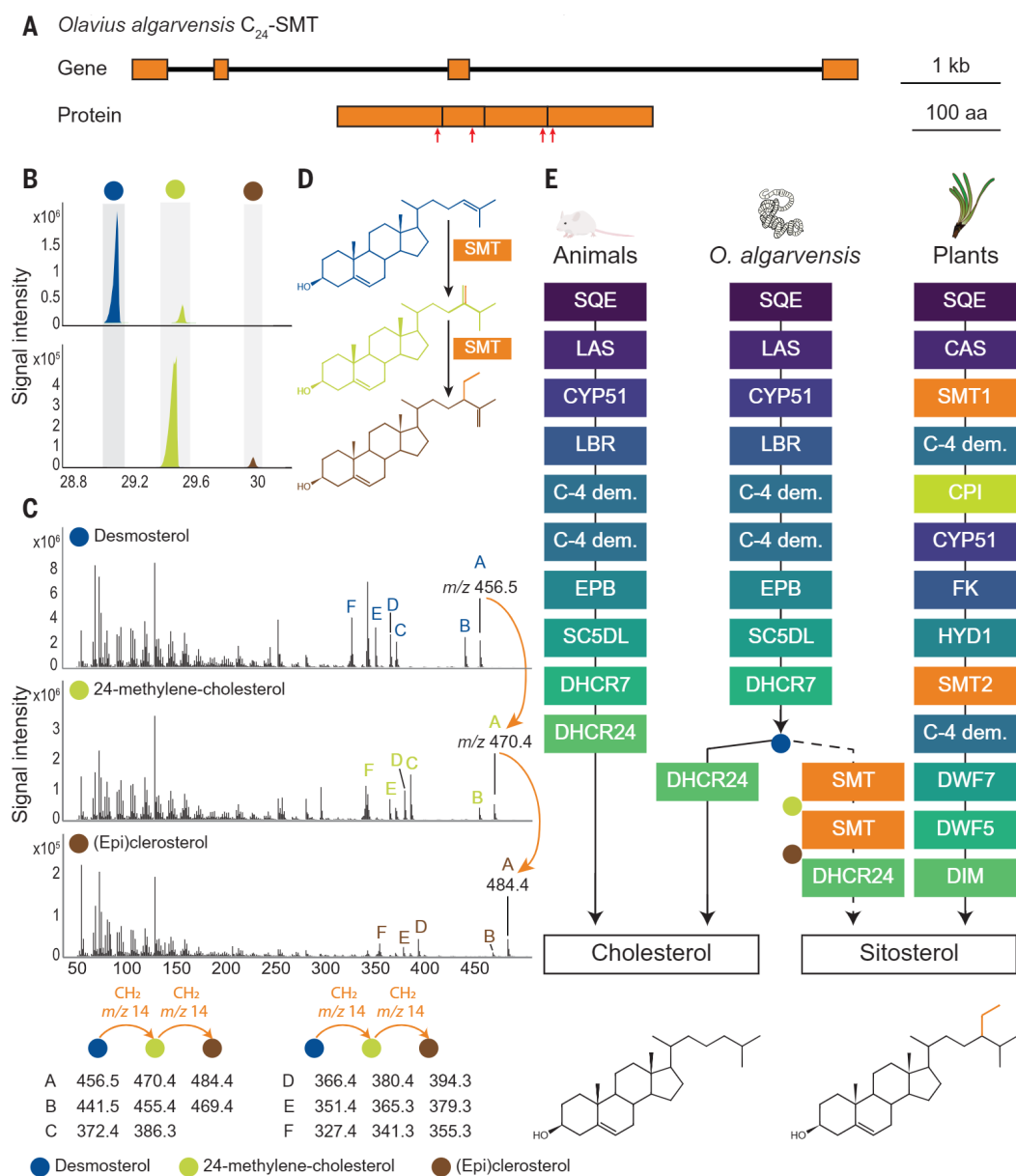
Notably, our analyses also identified a homolog of C₂₄-SMT in the genome of *O. algarvensis*, an enzyme essential to sitosterol synthesis in

plants (Fig. 2). As described above, most bilaterians are assumed to lack C₂₄-SMT. C₂₄-SMT catalyzes the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to the sterol side chain and is essential for the biosynthesis of sitosterol and other C₂₈-C₂₉ sterols commonly found in plants and fungi. As with the cholesterol synthesis genes, the intron-exon structure of the putative C₂₄-SMT gene confirmed its eukaryotic origin and excluded bacterial contamination (Fig. 2 and table S2). The putative C₂₄-SMT gene is a 1071-base pair (bp) open reading frame (ORF) encoding a 356-amino acid polypeptide and contains all the conserved residues characteristic of C₂₄-SMT as well as the 4 conserved signature motifs responsible for substrate binding (fig. S16) (28–31). We identified the C₂₄-SMT gene in *O. algarvensis* transcriptomes and proteomes, confirming that these animals express this enzyme (Fig. 2 and tables S4 and S5). Our findings suggest that the *O. algarvensis* C₂₄-SMT gene encodes a functional enzyme involved in sitosterol metabolism.

The *O. algarvensis* C₂₄-SMT homolog is bifunctional and consecutively transfers methyl groups to sterol intermediates

Two methylation reactions are required for the final steps of sitosterol synthesis, one that adds a methyl group at C-24 and one at C-28.

Fig. 2. *Olavius algarvensis* encodes and expresses a C_{24} -SMT that catalyzes two consecutive methylations, using desmosterol as the first and 24-methylene-cholesterol as the second substrate. (A) The *O. algarvensis* C_{24} -SMT gene consists of four exons, separated by three introns. The exons form a 1071-bp open reading frame encoding a 356 amino-acid polypeptide. The four conserved regions of the enzyme are highlighted by red arrows. (B) Chromatograms of enzymatic assays with desmosterol (top) and 24-methylene-cholesterol (bottom) as substrates. *O. algarvensis* C_{24} -SMT, after overexpression in *E. coli*, added a methyl group to the side chains of desmosterol and 24-methylene-cholesterol. In the first methylation step, desmosterol, an intermediate of cholesterol synthesis, was methylated to produce 24-methylene-cholesterol (C_{28} sterol). In the second methylation, 24-methylene-cholesterol was methylated to produce a C_{29} sterol, most likely (epi)clerosterol (C_{29} sterol). (C) Mass spectra of the different substrates and methylated products from the enzymatic assays. Sterol intermediates differ by the number of methyl groups (CH_2 at m/z 14) attached to their side chain. The side chain of desmosterol is not methylated, 24-methylene-cholesterol has a methyl group at C-24, and (epi)clerosterol has two methyl groups at C-24 and C-28. The substrates and methylated products were identified by MS, retention time, and comparison with standards. The fragmentation pattern suggests that the methyl groups were added to the side chain of the sterols. The stereochemistry of the methyl groups was not determined. (D) Structural representation of the two methylation steps in *O. algarvensis*. (E) Comparison of the enzyme used in the proposed sterol synthesis pathways in *Olavius* to the canonical cholesterol and sitosterol synthesis pathways (similar enzymatic reactions are colored similarly). The first six steps are common to both cholesterol and sitosterol synthesis pathways. This trunk pathway branches off after the synthesis of desmosterol. For sitosterol synthesis, desmosterol is first methylated by C_{24} -SMT to 24-methylene-cholesterol, which is then methylated in a second, consecutive step by C_{24} -SMT to (epi)clerosterol. (Epi)clerosterol is reduced to sitosterol by a sterol C_{24} -reductase (DHCR24, DIM). Squalene monooxygenase (SQE), oxidosqualene cyclase (LAS, CAS), sterol 14 demethylase (CYP51), sterol 14-reductase (LBR, FK), C-4 demethylation (C-4 dem.), Sterol Δ^7 - Δ^8 isomerase (EBP, HYD1), sterol 5-desaturase (SC5DL, DWF7), sterol Δ^7 reductase (DHCR7, DWF5), and C-24 sterol methyltransferase (C_{24} -SMT, SMT1, SMT2).



These reactions can be catalyzed by the same or different enzymes (6, 13, 32–34) (supplementary text). Because *O. algarvensis* encodes a single C_{24} -SMT homolog, we hypothesized that this enzyme mediates both C-24 and C-28 methylation.

To test this hypothesis, we overexpressed *O. algarvensis* C_{24} -SMT in *Escherichia coli* and examined its enzymatic activity, substrate preferences, and products by assaying crude protein extracts with the methyl-donor SAM and different sterol substrates. The heterologously expressed C_{24} -SMT from *O. algarvensis*

was not able to methylate classical plant sterol substrates (table S6). However, the enzyme was able to methylate zymosterol and desmosterol, two intermediates of the cholesterol biosynthetic pathway. When incubated with either of these sterol substrates and SAM, the *O. algarvensis* C_{24} -SMT produced a methylated sterol product (C_{28}) (Fig. 2 and figs. S17 to S20). Zymosterol was methylated to fecosterol and desmosterol to 24-methylene-cholesterol. The shift in retention times and changes in mass spectra of the products indicated that a methyl group was added to their sterol side

chain, likely at the C_{24} -position (Fig. 2, and figs. S19 and S20). These results suggest that the cholesterol and sitosterol synthesis pathways overlap in *O. algarvensis*, as the two C_{24} -SMT substrates, zymosterol and desmosterol, are intermediates produced in the second half of the animal cholesterol synthesis pathway (Fig. 2).

After confirming the first methylation step at C-24, we next searched for potential substrates for the second methylation step at C-28. This second methylation is essential as sitosterol is a C_{29} compound, characterized by the

presence of an ethyl group on its C-24 position. To test our hypothesis that both of these methylations are catalyzed by the *O. algarvensis* C₂₄-SMT, we selected the product of the first methylation, 24-methylene-cholesterol, as well as campesterol, as potential substrates for the second methylation (fig. S21). Only 24-methylene-cholesterol, but not campesterol, was methylated by the *O. algarvensis* C₂₄-SMT, producing a C₂₉ sterol compound, most likely (epi)clerosterol (Fig. 2 and fig. S22). 24-methylene-cholesterol is the product of the methylation of desmosterol, providing evidence to support our hypothesis that in *O. algarvensis*, the C-24 and C-28 methylations are catalyzed by the same enzyme and occur consecutively. That is, the *O. algarvensis* C₂₄-SMT first methylates desmosterol at C-24 to produce the C₂₈ sterol 24-methylene-cholesterol, and then adds a second methyl group to 24-methylene-cholesterol at C-28, to produce the C₂₉ sterol (epi)clerosterol. (Epi)clerosterol differs from sitosterol by the presence of a double bond at position C-25(26 or 27). This double bond is most likely removed by delta(24)-sterol reductase (DHCR24), which was expressed based on its presence in *O. algarvensis* transcriptomes and proteomes (figs. S5 and S15). Our results provide evidence for an animal C₂₄-SMT that catalyzes the two methylation steps needed to synthesize sitosterol from a cholesterol intermediate, revealing a previously unknown pathway for C₂₈-C₂₉ sterol synthesis in animals (Fig. 2).

C₂₄-SMT homologs are widespread in annelids

Having demonstrated the activity of an animal C₂₄-SMT homolog that enables *O. algarvensis* to synthesize sitosterol de novo, we asked whether other gutless annelids also encode functional C₂₄-SMTs. To answer this question, we analyzed the sterol contents of six additional gutless annelid species. All six species had lipid profiles similar to that of *O. algarvensis*, with sitosterol as their major sterol (table S7 and supplementary text).

In addition to lipid profiling, we screened the transcriptomes of nine *Olavius* and *Inanidrilus* species and found that all expressed a C₂₄-SMT homolog, including *O. ilvae* (table S8). Moreover, heterologous gene expression analyses confirmed the C-24 methylation ability of a C₂₄-SMT homolog from another gutless annelid, *O. clavatus*. The C₂₄-SMT from *O. clavatus* is also a bifunctional sterol methyltransferase, capable of methylating zymosterol, desmosterol, and 24-methylene-cholesterol (figs. S17, S18, and S22).

We next asked whether C₂₄-SMT homologs are present in other annelids. We screened published transcriptomes and genomes and identified C₂₄-SMT homologs in 3 deep-sea gutless tubeworm species and in 17 gut-bearing annelid species from marine, limnic, and terrestrial environments (table S9 and fig. S23). Despite the presence of C₂₄-SMT homologs in

these annelids, the published sterol profiles of annelids—including four gut-bearing species analyzed in this study—were dominated by cholesterol (table S9 and Fig. 1A). However, sitosterol as well as other C₂₈ and C₂₉ sterols accounted for a considerable proportion of total sterols (15 to 30%) in some of these species, including the vent and seep tubeworms *Riftia pachyptila* and *Paraescarpia echinospica* (table S9). These deep-sea siboglinid annelids are only distantly related to *Olavius* and *Inanidrilus*, but also lack a gut and gain all of their nutrition from their chemosynthetic symbionts (35). The sterol contents of these tubeworms are dominated by cholesterol and desmosterol, but the C₂₈ sterol campesterol as well as other C₂₈ and C₂₉ sterols make up as much as nearly one-third of their sterol contents (36–38). Our discovery of C₂₄-SMT homologs in these deep-sea annelids suggest that these tubeworms are able to synthesize C₂₈ and C₂₉ sterols as well. Further support that annelid C₂₄-SMT homologs methylate sterols was recently shown for the enzyme encoded by the nonsymbiotic annelid *C. teleta* (39). Taken together, these results indicate that functional C₂₄-SMT homologs are widespread in the annelid phylum and that the presence of this gene is not restricted to hosts that harbor symbiotic bacteria.

C₂₄-SMT homologs are present in at least five animal phyla: Cnidaria, Porifera, Rotifera, Annelida, and Mollusca.

To assess the broader distribution of C₂₄-SMT homologs in animals, we performed protein searches against public databases (see materials and methods for details). Hits were found in six additional animal phyla: sponges, cnidarians, rotifers, mollusks, nematodes, and chordata (supplementary text and table S10). For the latter two, we concluded they are not animal C₂₄-SMTs for the following reasons. Among the chordata, we found a single homolog in a metatranscriptome from a fruit bat. It had 99.1% identity to sequences from a plant and fell in a clade with these, suggesting that this C₂₄-SMT is a contamination (supplementary text). The nematode sequences belonged to a group of SMTs called C-4 sterol methyltransferases (C₄-SMTs) that are specific to nematodes (40, 41). Our phylogenetic analyses showed that these nematode C₄-SMTs are not closely related to the monophyletic clade of C₂₄-SMTs from plants, fungi, microbial eukaryotes, sponges, cnidarians, rotifers, annelids, and mollusks (Fig. 3B).

C₂₄-SMTs are widespread across the tree of life

We reconstructed the evolutionary relationships of animal C₂₄-SMTs from sponges, cnidarians, rotifers, mollusks, and annelids and compared these with previously described C₂₄-SMTs from bacteria, microbial eukaryotes,

fungi, and plants (Fig. 3A). Our phylogenetic analyses revealed that C₂₄-SMTs are widespread across the tree of life and fall into nine well-supported clades (Fig. 3B, A to I). These clades were not congruent with the phylogeny of bacteria and eukaryotes, indicating a complex evolutionary history for C₂₄-SMTs, which we discuss below. Many eukaryotes had more than one C₂₄-SMT homolog, with members of some groups such as the choanoflagellates and cnidarians encoding as many as four homologs that belonged to different, phylogenetically distant clades (Fig. 3C). Most animal C₂₄-SMTs clustered in seven clades (Fig. 3A and figs. S24 to S27), with each clade consisting of animal sequences only. The exceptions were animal C₂₄-SMTs that fell on single, long branches that formed sister lineages to other eukaryotic clades (Fig. 3A, supplementary text, and figs. S25 and S26).

For the animal C₂₄-SMTs, two of the seven clades (2.F and 3.F) contain homologs from sponges and annelids whose C-24 methylation function has been experimentally verified [this study and (13, 39)]. The five remaining clades consist of predicted C₂₄-SMTs, but their high sequence homology to verified C₂₄-SMTs together with conserved protein domains suggest that these homologs also methylate sterol intermediates at C-24.

The complex evolutionary history of C₂₄-SMTs

In two animal phyla, Annelida and Rotifera, we observed strong congruence between their C₂₄-SMT homologs and their evolutionary history (Fig. 3D and figs. S27 and S28). For annelids, in addition to the gutless *Olavius* and *Inanidrilus* sequences from our study, we found C₂₄-SMT homologs in nine annelid orders from limnic, terrestrial, and marine environments. Annelid C₂₄-SMTs fell within two sister clades, one from Errantia and the other from Sedentaria, corresponding to two major subgroups of Annelida (Fig. 3D). The only exception was *Megasyllis neponica*, which belongs to the Errantia but has a C₂₄-SMT that falls in the Sedentaria (Fig. 3D). Congruence was also visible within the two annelid clades (Fig. 3D and fig. S28). These results suggest that C₂₄-SMTs from extant annelids evolved through direct inheritance from a common ancestor. Similarly, the rotifer C₂₄-SMT clade consisted largely of sequences from the Bdelloidea, a phylogenetically well-defined class within the phylum Rotifera (supplementary text). Within this clade, the C₂₄-SMT topology corresponded well to the phylogeny of bdelloid rotifers, consistent with direct inheritance driving the evolution of this gene within this animal phylum as well (fig. S27).

At longer evolutionary time scales, there was no congruence between C₂₄-SMT homologs and the evolutionary history of the lineages they came from, not even between closely related

phyla. For example, Annelida and Mollusca are more closely related to each other than to Porifera, yet their C_{24} -SMTs often clustered with those from Porifera. The lack of congruence at deeper nodes of the C_{24} -SMT tree with the evolutionary history of eukaryotes is also visible in the interspersed phylogeny

of C_{24} -SMT homologs from fungi, plants, and animals throughout the tree (Fig. 3A). The consistent incongruencies throughout most of the C_{24} -SMT tree are hard to reconcile with direct inheritance from a common eukaryotic ancestor. Repeated, independent events of lateral gene transfer (LGT) may provide a

more likely explanation for the C_{24} -SMT tree topology.

LGT is widespread and well-studied in bacteria, but less is known about LGT within extant eukaryotic lineages. Recent evidence from high quality genome sequencing suggests that LGT may play a more important role in

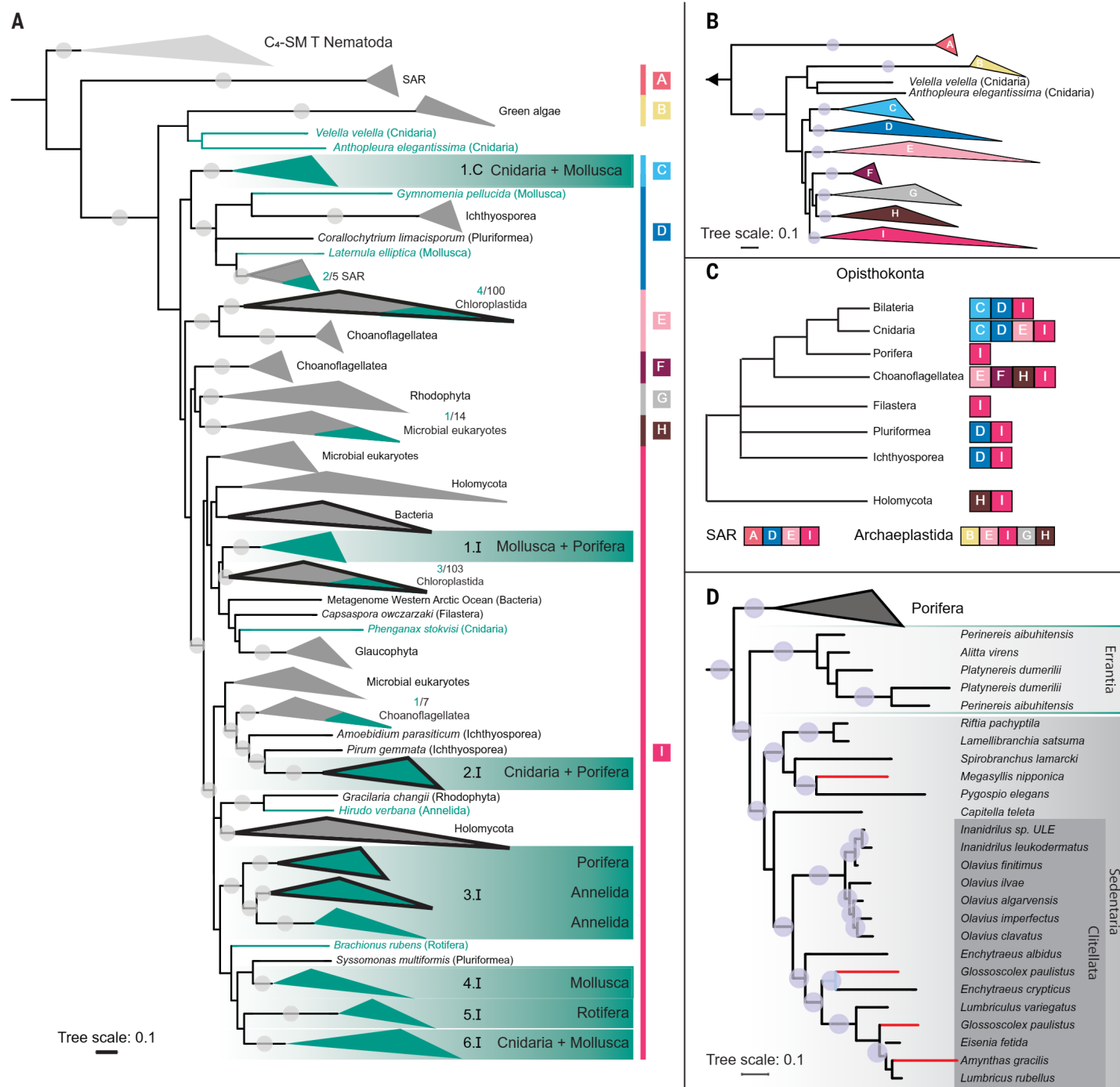


Fig. 3. C_{24} -SMT homologs are widely distributed across the tree of life including at least five animal phyla. (A) Maximum likelihood amino acid tree of eukaryotic and bacterial C_{24} -SMTs. Sequences were clustered at 90% identity. Nodes with ultrafast bootstrap (UFB) values >95% are shown with gray circles. Nematode C_4 -SMTs were used as an outgroup. The homologs found in animal datasets are highlighted in green. Clades with experimentally

verified C_{24} -SMTs are outlined in black. **(B)** Collapsed version of the tree shown in (A). C_{24} -SMTs fall into nine well-supported clades (UFB >95%). **(C)** Most eukaryotic clades had several phylogenetically distinct homologs. **(D)** Detailed view of the Annelida [clade 3.I from (A)]. The phylogeny of annelid C_{24} -SMTs largely corresponds to their evolutionary history, exceptions are shown in red.

eukaryotes than previously assumed (42–45). LGT within eukaryotes is assumed to be most commonly mediated by viruses and bacteria, particularly in species that are intimately associated with beneficial or pathogenic microorganisms (46). However, we ruled out LGT mediated by associated bacteria in the gutless *Olavius* and *Inanidrilus*, as we exhaustively searched their symbiont metagenomes for C₂₄-SMTs. LGT from bacteria to eukaryotes of C₂₄-SMTs is also unlikely to have played a role in the more recent evolution of other animals, as bacterial C₂₄-SMTs were not closely related to those from animals, with the exception of sponges (Fig. 3A). Alternatively, LGT mediated by viruses could explain how genes are transferred between eukaryotes (47). Independent of the precise route for LGT across eukaryotes, our study provides evidence for rampant LGT of gene homologs within the animal kingdom and suggests that the acquisition of C₂₄-SMTs provides animals with a strong selective advantage.

Conclusions

Although cholesterol is commonly the most abundant sterol in animals, through studies of non-model organisms and technological advances, evidence is growing that C₂₈ and C₂₉ sterols are also often present, sometimes in considerable amounts (48–51). Our findings highlight the value of reconsidering the source of C₂₈ and C₂₉ sterols in both extant animals and the fossil record and determining whether these originated from the animal's diet as is often assumed, or if they were synthesized by the animals themselves. It is also timely to reconsider the widespread use of the term phytosterols for C₂₈ and C₂₉ sterols, as we now know that microbial eukaryotes, plants, and animals can synthesize these lipids.

How can we explain the unusually high abundance of sitosterol in gutless *Olavius* and *Inanidrilus*? Studies have shown that C₂₈ and C₂₉ sterols can be incorporated into animal membranes (52, 53) and provide beneficial effects when added to animal diets or cell lines. For example, they act as cholesterol-lowering agents, have anti-tumor, anti-inflammatory, antibacterial, and antifungal properties (54, 55), and modulate interactions between bacterial pathogens and eukaryotic hosts (56). Therefore, the anti-inflammatory and antibacterial properties of sitosterol, as well as its ability to protect animal cells against toxins that target cholesterol (57), might play a role in the symbiosis between *Olavius* and *Inanidrilus* and their chemoautotrophic symbionts, by preventing the symbionts from entering the host cytoplasm. Changes in sterol composition also affect the fluidity and permeability of membranes, and these physical changes in turn affect many cellular processes. For example, sitosterol has been shown to enhance mitochondrial energy

metabolism in a mouse cell line (58) and might enable *Olavius* and *Inanidrilus* to gain more energy under low oxygen concentrations in their environment. Our findings highlight the need for studies that elucidate the physiological and ecological roles of sitosterol in animals. *Olavius* and *Inanidrilus* are valuable model systems for studying the impact of C₂₈ and C₂₉ sterols on animal membrane properties in vivo and furthering our understanding of the roles sterols play in eukaryotic cells.

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SUPPLEMENTARY MATERIALS

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Materials and Methods
Supplementary Text
Figs. S1 to S29
Tables S1 to S16
References (65–90)
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