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Review

Requirement of sterols in the life cycle of the nematode *Caenorhabditis elegans*

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Abstract

The nematode *Caenorhabditis elegans* represents an excellent model for studying many aspects of sterol function on the level of a whole organism. Recent studies show that especially two processes in the life cycle of the worm, dauer larva formation and molting, depend on sterols. In both cases, cholesterol or its derivatives seem to act as hormones rather than being structural components of the membrane. Investigations on *C. elegans* could provide information on the etiology of human diseases that display defects in the transport or metabolism of sterols.

Keywords: *Caenorhabditis elegans*; Cholesterol; Sterols; Dauer formation; Molting

Contents

1. Introduction

In this review we will discuss several functions of sterols and in particular of cholesterol in the life cycle of the nematode *C.elegans*. This organism is one of the well-established genetic models and many aspects of its development have been well characterized. Generation time of *C. elegans* is short (about 50 h at 25 °C) and large amounts of worms can be easily grown for biochemical studies. The optical transparency of *C. elegans* facilitates approaches of fluorescence imaging and photoaffinity labeling in the whole organism. A vast array of methods to genetically manipulate *C. elegans* allows to disrupt genes that might regulate steroid function, either by double-stranded RNA interference (RNAi) or by selection of targeted deletion or point mutations. The main advantage to use *C. elegans* as a model system for studies on sterol function lies on the fact that nematodes are auxotrophic for sterols. Transport and metabolism of exogenously added sterols can be easily studied by feeding worms with radioactive or fluorescent derivatives. By combining phenotypic analysis, gene disruption experiments and feed-

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ing of chemically modified sterols, the relationship between structure and function of sterols can be investigated.

Cholesterol plays multiple roles in the organism and the alterations of normal metabolism, transport and distribution frequently causes diseases. In most eukaryotic cells, cholesterol is a major constituent of the plasma membrane, controlling membrane fluidity and permeability [1,2]. It has been proposed that together with sphingolipids, cholesterol forms membrane "rafts" that are platforms for the regulation of signaling events [2,3]. Cholesterol is also found to be covalently attached to proteins of the Hedgehog family [4,5]. This modification of Hedgehog morphogens determines both their spreading through the tissue and signaling capability [5–7]. Furthermore, cholesterol is precursor for biologically active molecules such as steroid hormones, bile acids, insect ecdysones, oxysterols and vitamin D. Thus, cholesterol properties, metabolism and transport transverse many aspects of life. Data accumulated in the last years indicate that *C.elegans* can be used as an excellent model for studying all these facets of sterol function on the level of a whole organism.

2. Depletion of sterols in *C. elegans*

Already early studies have shown that *C. elegans* relies on exogenous sterols for its development and might not synthesize sterols de novo [8,9]. Under laboratory conditions, worms are grown either on bacterial lawns seeded on agar plates or in liquid bacterial co-culture and cholesterol is supplemented to agar plates/medium at a final concentration of 13 μM [9]. It looked as if to analyze the particular phenotype of sterol depletion by omitting sterols in medium, will be simple. However, this task appeared to be difficult because of the contaminating sterols: even vanishing amounts of these could lead to ambiguous results. For instance, when dietary cholesterol was omitted in medium, several generations of worms could still develop and only a mild reduction in brood size and decrease of growth rate were observed [10–12]. In addition, only a low percentage of embryonic lethality was detected and germ line cells showed abnormal proliferation and differentiation [10,12]. In other experiments, at suboptimal sterol concentrations, a fraction of animals displayed defects in shedding of the old cuticle during molting [13–15].

Unequivocal phenotypes of sterol depletion were observed only when agar was replaced with chloroform-extracted agarose and bacteria were grown in either ether-extracted peptone [12,16] or in defined minimal medium [11,17]. Remarkably, the first generation of worms derived from cholesterol fed mothers under strict cholesterol depletion conditions still developed to adulthood and gave progeny. However, these worms had a reduced brood size and growth and locomotion defects [11,12,16,17]. The fact that worms survive for one generation without externally provided cholesterol indicates that mothers provide their progeny with sterols. The latter was directly visualized with dehydroergosterol (DHE), a fluorescent analog of cholesterol (see below). In DHE-fed worms, significant labeling of embryos was detected [18].

In the second generation, strict sterol depletion caused larval arrest [11,17]. Detailed examination of these arrested larvae [11] revealed that they were similar in size and appearance to normal L2 larvae grown on cholesterol. However, the arrested larvae displayed features of dauer (enduring) larvae, which form in response to hostile conditions (see below). The arrested larvae had a double cuticle with the outer layer of a normal L2 larvae and the inner layer resembling the normal dauer larva cuticle.

Taken together, data from sterol depletion experiments point out that: (i) worms can survive on tiny amounts of sterols and (ii) cholesterol in worms is required for variety of processes, including proper molting, dauer formation and locomotion.

3. Distribution and transport of sterols in *C. elegans*

In humans, defects in cholesterol transport and distribution in the organism or within a cell are associated with a number of severe diseases (e.g. atherosclerosis, type C Niemann-Pick disease, obesity, Alzheimer's disease and others). In order to carry out its function in a particular cell of *C. elegans*, cholesterol should be taken up from the environment and transported to its destination. Thus, the worm can be used as a valid model to investigate molecular mechanisms of sterol transport, storage and excretion. Despite the vital role of sterols for the worm, these processes are still poorly understood. It is not even clear whether sterols are exclusively taken up by the digestive tract or also through the cuticle.

Only recently, the lack of methods for imaging cholesterol or for identification of cholesterol-binding proteins was overcome using fluorescent or photoactivatable analogs of cholesterol [18]. Dehydroergosterol (DHE) is a naturally occurring fluorescent analog of cholesterol [19,20] and was previously used to visualize sterol distribution in living cultured cells [21]. DHE can functionally replace cholesterol, since worms can be propagated on this sterol for many generations [18]. Surprisingly, when fed to worms, DHE accumulates only in a subset of cells rather than being distributed uniformly throughout the body. The accumulation was observed in pharynx, nerve ring, excretory gland cells, apical surface of gut, oocytes and spermatozoa [18]. In embryos, DHE displayed both cytoplasmic and membrane labeling. In addition, males showed a very strong labeling of spermatids, which suggested a possible role for sterols in sperm development or fertilization. The majority of sterols in embryos must be, however, supplied by the mother: when males were fed with DHE and mated with unlabeled hermaphrodites, embryos remained unlabeled.

Distribution of sterols in *C. elegans* was also investigated by staining of fixed worms with filipin, a polyene antibiotic that becomes fluorescent upon forming a complex with 3-hydroxysterols [12]. In general, the staining with filipin

was similar to DHE labeling. Again, only distinct cells of the organism were labeled. However, both methods of staining have caveats: DHE and filipin might not represent the distribution of all potential sterol-derivatives in the worm. The fluorescence of DHE is based on three conjugated double bonds in the ring structure (Δ-5, Δ-7 and Δ-9). Thus, the reduction of any double bond will render the derivative non-fluorescent. Filipin, on the other hand, stains only sterols with free 3-hydroxyl group [22] and thus can detect neither cholesterol esters nor putative sterol derivatives with 3-keto group nor steroids with a cleaved side chain. In addition, both approaches would not be able to detect sterols with low abundance. Nevertheless, DHE as well as filipin staining gave a general overview of the sterol distribution in *C. elegans*.

Through photoaffinity labeling and genetic studies several proteins have been identified that might be involved in the transport of sterols in *C. elegans* [15,18,23,24]. Using the photoactivatable probe, [^{3}H] photocholesterol [25], vitellogenins (also called yolk proteins) were identified as major cholesterol-binding proteins [18]. This finding draws a parallel between the transport of cholesterol in mammals via LDL-particles and the sterol transport in nematodes, since vitellogenins are homologous to apolipoproteins [26,27]. RME-2, a receptor for vittelogenins in oocytes, is a member of the LDL receptor superfamily [28]. Vitellogenins are produced in the intestine of L4 larvae and adult worms and secreted to the pseudocoelomic cavity [29]. From here they are taken up into oocytes by endocytosis. It was shown that *rme-2* mutants fail to transport a fluorescently tagged yolk protein into embryos and instead accumulated it in the body cavity [28]. Similarly, in *rme-2* mutants, very little DHE was transported into oocytes but instead a very strong labeling of the body cavity was observed [18]. However, there was little co-localization of DHE and vitellogenins within oocytes and it was postulated that after transport into oocytes cholesterol is released from the vitellogenin complex. This might be similar to the separation of cholesterol from LDL particles after uptake into late endosomes of mammalian cells. Interestingly, vitellogenins and *rme-2* are transcriptionally upregulated when worms are fed with azacoprostane [30], an inhibitor of Δ-24 reductase that catalyzes conversion of desmosterol to cholesterol [31,32].

As uptake of cholesterol in hermaphrodite larvae starts earlier than the expression of vitellogenins, it is clear that vitellogenins cannot be the only transporters of cholesterol in *C. elegans*. In addition, neuronal cells and spermatozoa, that accumulate significant amounts of DHE, are not able to accumulate vitellogenins. Also, males, that are strongly labeled with DHE do not express vitellogenins at all. Further studies might reveal which other proteins are involved in the transport of sterols in these cells or at earlier stages of development. Remarkably, *C. elegans* has several classes of potential fatty acid/sterol-binding proteins [33].

Genetic studies identified several other genes, including *lrp-1*, *ncr-1* and *ncr-2* related to sterol transport [15,23,34]. *lrp-1*, is a homologue of the mammalian gp330/ megalin related protein [15], whereas *ncr-1* and *ncr-2* are Niemann-Pick type C proteins [23,34]. The potential role of these proteins in the sterol transport will be discussed below in conjunction with the processes of molting and dauer-larva formation.

4. Sterol metabolism in *C. elegans*

What is known about the metabolism of sterols in *C. elegans*, apart that worms might not synthesize cholesterol de novo? A bioinformatical examination of the *C. elegans* genome confirmed the absence of homologues of squalene synthase and squalene cyclase, key enzymes required for the generation of lanosterol. This methylated sterol is a starting point for production of cholesterol, ergosterol or sitosterol in mammals, yeast and plants, respectively. Thus, it remains unlikely that nematodes produce sterols by a novel pathway not yet described in other phyla.

Worms are able to utilize as a main source different sterols bearing methyl groups or double bonds in different positions of the side chain and the 4-ring structure (sitosterol, ergosterol, cholesterol, 7-dehydrocholesterol, lathosterol, etc.) [8,35–37]. It should be mentioned, however, that they cannot propagate on sterols/steroids without a side chain (e.g. pregnolonone, progesterone [11]).

Although, the bulk of exogenously added sterol remains unchanged [35–37], *C. elegans* is able to carry out some modifications on them. This has been shown either by analyzing worm sterols by GC–mass spectrometry or by TLC analysis of worms fed with radioactive cholesterol [8,11,31,35–40]. In this way, several enzymatic modifications of cholesterol were identified and the major metabolic pathway in *C. elegans* was outlined [40]. Hereby, two modifications should be especially mentioned: the dehydrogenation of cholesterol at 7th position (production of 7-dehydrocholestrol) and methylation of the ring at fourth position. Remarkably, in mammals both two modifications occur preferably in the opposite direction. Accumulation of 7-dehydrocholestrol is observed in Smith-Lemli-Opitz syndrome, which is a genetic recessive deficiency in Δ-7 reductase required for the conversion of 7-dehydrocholesterol to cholesterol [41]. The most puzzling modification of sterols in worms is methylation of the 4th position in the A-ring [31]. Astoundingly, around 20 reactions are required in mammals, to produce cholesterol from lanosterol, a 4,4′- dimethyl sterol.

Do these cholesterol modifications by worms have a physiological significance? This question can only be answered by identification of enzymes involved in these reactions and by subsequent inactivation of their coding genes. As discussed below, the processes of dauer larvae-formation and molting require sterol-derived hormones and might involve the methylation of sterols. Bioinformatics analysis of the *C. elegans* genome revealed a number of potential enzymes that could be involved in the production of these active compounds. Hereby, more than 80 members of the cytochrome P450 family [42] and a number of short-chain dehydroge-

nases [43] may be of particular importance. Interestingly, one short-chain dehydrognenase was found to be the *let-767* locus. Mutants of this gene, a homologue of human 17-estradiol dehydrogenase, display phenotypes similar as observed during sterol depletion [14].

An important modification of cholesterol in mammals is its esterification with fatty acids. This process is tightly connected with the transport of cholesterol in the organism. Worms possess homologues of enzymes (ACAT and LCAT) that might produce cholesterol esters. Indeed, by radioactive labeling of worms as well as by GC–MS analysis significant amounts of sterol esters were detected [11,40]. However, it still remains unclear whether esterification reaction has similar physiological importance in worms as in mammals and whether it would be a prerequisite for sterol transport within the body.

5. Sterols and dauer larvae formation

The normal reproductive cycle of *C. elegans* includes four subsequent larval stages each separated by a molt. Under hostile conditions such as starvation or overcrowding *C. elegans* enters an alternative third larval stage called dauer (enduring, reviewed in [44]). Dauer larvae stop pharyngeal pumping of food and display a constricted gut. The dauer cuticle has a specific morphology and is resistant even to a harsh treatment with a detergent SDS. On the biochemical level, dauer larvae reduced the activity of the TCA cycle and predominantly use stored lipids and glycogen. In general, dauer formation includes global remodeling of gene expression [45] and an overall reduction in transcriptional activity [44].

A possible hormonal regulation of the dauer larva formation was initially proposed based on genetic studies. Mutations in a number of *daf* genes (*daf*, from dauer formation) cause either constitutive formation (Daf-c) of dauer larvae or prevent their formation (Daf-d) [44]. Three signaling pathways (TGF-beta, cyclic GMP and insulin-like IGF-1) control the formation of dauer larvae [44]. The molecular identification of two genes, *daf-12* and *daf-9*, suggested the existence of sterol related hormone that might integrate these pathways. DAF-12 is a nuclear hormone receptor required to execute dauer formation, since *daf-12* null mutants fail to form dauers and undergo reproductive development [46,47]. DAF-9 is a member of the cytochrome P450 family closely related to the mammalian CYP2 family, which might be involved in sterol metabolism [34,48–50]. DAF-9 is essential for reproductive development since *daf*-9 mutants constitutively form dauers. The double mutant of *daf-9 daf-12* cannot form dauer larvae indicating that *daf-9* acts upstream of *daf-12*. Taking together, a scenario was proposed where DAF-9 is an enzyme that produces a steroid hormone inhibiting DAF-12. In absence of the hormone activated DAF-12 triggers dauer larvae formation [48,50].

Direct evidence for the involvement of sterol-derived hormones in the process of dauer larvae formation is based on biochemical studies. As mentioned above, strict cholesterol depletion induces worms to enter the dauer formation pathway, although the dauer phenotype is not complete in cholesterol depleted conditions [11]. Biochemical analysis has shown that in the second generation of sterol-depleted worms, most residual sterols were methylated [11]. Furthermore, if worms were grown on lophenol, a 4-alpha methylated sterol, in the second generation they produced regular dauer larvae despite sufficient food and low population density. This raises the question whether lophenol itself actively induces dauer larvae formation and the methylation in the 4-alpha position is necessary for this process. This possibility was excluded by using 4-alpha fluorocholesterol, which cannot be methylated, but also induces dauer larvae formation. Thus, the methylation per se is not mandatory for dauer larva formation but rather the accessibility of the forth position in the ring for some other modification is required to allow reproductive development [11].

The effect of lophenol on *C. elegans* development allowed to purify the hormone (activity) required for reproductive development [11]. The rationale of this approach was to rescue lophenol induced dauer larvae formation by a substance derived from a lipidic worm extract. This activity was called gamravali (from *gamravleba* which means “reproduction” in Georgian). It is proposed gamravali derived from cholesterol acts to promote reproduction. The effect of internal signals that induce dauer formation upon starvation and overcrowding is to prevent gamravali production, thus promoting entry into diapause. According to this view, growth on lophenol resembles the absence of gamravali. The chemical structure of gamravali and the identity of enzymes involved in its synthesis still have to be elucidated. The former task, however, is demanding because of extremely low amounts of this substance in worms. Gamravali is more hydrophilic than cholesterol and its retention time on a HPLC-column indicates that it could be a polyhydroxylated sterol, lack the hydrophobic side chain or even containing a charged group [11]. Presently, it is also not clear where gamravali is synthesized and what are gamravali target cells.

The growth on lophenol, allowed to identify the steps at which gamravali was required. It was assumed that Daf-d mutants acting upstream of the gamravali-requirement will form dauer larvae on lophenol. Such mutants are *daf-22* (required for production of pheromone inducing dauer formation, [51]), *daf-6* (required for sensing of pheromone, [52]), *daf-10* (required in sensory neurons, [53]), *daf-3* (Smad transcription factor, [54]), *daf-5* (Ski, a modulator of TGF-beta signaling, [55]). In contrast, a null mutant of *daf-12* developed normally on lophenol, making DAF-12 candidate gamravali receptor. Somewhat different results were obtained with a mutant of *daf-16*, a FOXO Forkhead transcription factor. Here, on lophenol, neither reproductive adults nor regular dauers were observed [11]. It was previously shown that upon activation by IGF-1 or TGF-beta pathways, DAF-16 accumulates in the nuclei of the neurons in the pharynx, ventral cord and tail [56,57]. Similar accumulation of DAF-16 in the nuclei

occurred in lophenol induced dauer larvae [11]. However, in a *daf-12* null background, DAF-16 failed to accumulate in these nuclei, indicating that the activation of DAF-12 induced by the absence of gamravali leads to accumulation of DAF-16 in the nuclei of the neurons. Taken together, these results suggest that dauer formation is initiated by DAF-12 but complete dauer differentiation needs nuclear import of DAF-16 into neuronal nuclei.

One of the major efforts in the field is to link the biochemical search for sterol-derived hormones with genetic pathways and many questions remain unanswered so far. How is gamravali connected with the putative product of DAF-9? If and how do TGF-beta and insulin-like signaling pathways control the formation of gamravali or its degradation? Is gamravali synthesized cell-autonomously or is its (or cholesterol's) transport to target cells that is regulated? A promising approach in identification of sterol derived hormone(s) was the crude isolation of activity from a lipophilic extract of larvae rescuing reproductive development in *daf-9*, and partially in *daf-2* and *daf-7* mutant animals [58]. Surprisingly, an extract derived from *daf-12* mutants was more potent in rescuing the *daf-9* phenotype than extract from wild-type animals. It has been shown that *daf-12* is a positive regulator of *daf-9* expression [34,49]. There are indications that gamravali and the *daf-9*-rescuing activity are not identical. Firstly, in the double null *daf-9 daf-12* mutant strain no major changes of cholesterol metabolism were observed [11]. More importantly, all our attempts to rescue *daf-9* mutants with gamravali failed (TVK and EVE, data not published).

Another indication for the role of sterols in dauer formation process comes from studies of mutant strains *ncr-1* and *ncr-2*. Theses genes code for Niemann-Pick type C disease related proteins in *C. elegans*. *ncr-1; ncr-2* double mutant animals form dauer larvae constitutively, even under favorable conditions [23,24]. The expression pattern of *ncr-1* largely coincides with the cholesterol distribution described above, and is consistent with a function of *ncr-1* in bulk cholesterol trafficking. In contrast, the expression of *ncr-2* is limited to XXXL/R neurons and the somatic gonad [24]. XXXL/R cells are head neuroendocrine cells, ablation of which leads to constitutive dauer formation [48,59]. Remarkably, these cells also express high amounts of DAF-9 [48,59]. The constitutive dauer formation in *ncr-1; ncr-2* double mutant can be partially rescued by higher levels of cholesterol, suggesting that *ncr-1* and *ncr-2* control the cholesterol susceptibility and thus control the reproductive development. The fact that single *ncr-1* mutant as well as double *ncr-1; ncr-2* are sensitive to reduced cholesterol levels [23] and progesterone [24] implies that *ncr-1* is a more global regulator of cholesterol distribution in *C. elegans* while *ncr-2* is required more specifically in the dauer formation. Thus, future studies on *ncr-1* and *ncr-2* mutants could help to discriminate between different functions of cholesterol in worms and might reveal how homologues of these proteins in mammals regulate the intra- or inter-cellular cholesterol transport.

6. Sterols and molting

The cuticle is the worm's exoskeleton that is required to maintain the body shape and motility. In addition, it protects animal from the external environment [60]. The transition between larval stages is characterized by a molt in which a new cuticle is formed and the old is shed. Nematodes were suggested to be a part of the evolutionary clade Ecdysozoa, which contains all molting invertebrates, including insects [61]. The process of molting in *Drosophila* is governed by ecdysones, a group of polyhydroxylated sterol hormones. During each molt, ecdysone pulses trigger not only molting but also major morphological changes of larvae and pupae (reviewed in [62]). 20-hydroxyecdysone, the active form of ecdysone, binds to the nuclear hormone receptor EcR, that together with the protein ultraspiracle, regulates the transcription process. A bioinformatic comparison/analysis in *C. elegans* did not reveal any orthologs of EcR and ultraspiracle [63]. Moreover, except early reports [64], no molecules similar to ecdysones have been biochemically isolated from nematodes. Ecdysones supplied to worms exogenously have no effects and cannot substitute for cholesterol [11,50].

Many observations, however, point out that the molting process in *C. elegans* depends on sterols. One of the phenotypes described in cholesterol-depletion studies is incomplete shedding of the old cuticle [13–15]. As described above, larvae of the second generation grown without sterols have a double cuticle [11]. Furthermore, mutants displaying molting defects are sensitive to reduced cholesterol levels [14]. Mutations in *lrp-1*, the gp330/megalin homolog in *C. elegans*, show arrest at the molt between the third and the fourth larval stage [15]. In mammals, megalin is LDL receptor related protein and it was suggested to be required for renal uptake of vitamin D [65]. In *C. elegans*, Lrp-1 is expressed at the apical surface of hyp7, the polarized epithelium apically secreting cuticle, and it was proposed that Lrp-1 is involved in uptake of sterols/hormones in these cells [15].

In *Drosophila*, ecdysone pulses are transduced through stage-specific regulatory cascades of nuclear hormone receptors [62]. In *C.elegans*, a number of nuclear hormone receptors have been identified as *Drosophila* orthologous [63,66–69]. Based on this observation a conserved nuclear receptor "ecdysone cascade", controlling molting in *C. elegans* has been proposed [67]. However, only biochemical isolation of the molting hormone can answer whether this suggestion is correct. Isolation and chemical identification of a molting hormone remains a challenging task. Similar to gamravali, that regulates reproductive development, this hormone should be present at minute amounts in worms. A tempting speculation on the structure of the molting hormone(s) is that it is derived from 4-methylated sterols (see above). As mentioned above, *daf-12* can normally grow on lophenol for many generations without displaying defects in molting.

7. Sterols in signaling versus structural function in membranes and further perspectives

Studies on *C. elegans* have raised several fundamental questions regarding the role of sterols both on the level of the single cell and the whole organism. The major outcome of these studies was the finding that worms require very low amounts of sterols and that these are not uniformly distributed throughout the body. The amount of sterols deployed by mothers into embryos is sufficient not only for survival of the first generation but even for the embryonic development of about 130 embryos that reach the L2 stage in the second generation. It is very difficult to reconcile this observation with a widely accepted role for cholesterol in the maintenance of the plasma membrane's structural integrity. However, it is not excluded, that cholesterol plays a structural role in membranes in a subset of cells.

Are sterols dispensable for the functioning of the membrane? If yes, which other molecules substitute for it in regulating membrane permeability and fluidity. Also, the composition and formation of lipid rafts is thought to depend on membrane cholesterol, while in *C. elegans* other lipids might replace cholesterol. Furthermore, how are structural and signaling function of sterols related: are sterols used exclusively for the production of signaling molecules/hormones? Answers to these questions are important not only to understand the functioning of *C. elegans* membrane but also for all other species. Notably, in recent years the knowledge about sterol requirement of in mammals is also changing. For instance, mouse deficient for desmosterol reductase, the enzyme responsible for the reduction of 24th position in cholesterol, has no visible phenotype [70]. Thus cholesterol can be substituted by desmosterol in mammals.

There are two other topics that will challenge the field in coming years: (i) elucidation of mechanisms of sterol transport and (ii) the identification of sterol-derived hormones. The first of these could clarify the etiology of diseases such as atherosclerosis, type C Niemann-Pick disease, Alzheimer's disease and obesity. A promising starting point for the second task was provided by the partial purification of gamravali [11] and of the activity that rescues *daf-9* [58]. The final chemical identification of these compounds will require scaling-up of biochemical preparations and application of sophisticated analytical tools of micro mass-spectroscopy and NMR. In a complimentary approach, a combination of RNAi and biochemistry of potential enzymes involved in sterol modifications should delineate the sterol metabolism.

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