# Why do worms need cholesterol?

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**Cholesterol is a structural component of animal membranes that influences fluidity, permeability and formation of lipid microdomains. It is also a precursor to signalling molecules, including mammalian steroid hormones and insect ecdysones. The nematode *Caenorhabditis elegans* requires too little cholesterol for it to have a major role in membrane structure. Instead, its most probable signalling functions are to control molting and induce a specialized non-feeding larval stage, although no cholesterol-derived signalling molecule has yet been identified for these or any other functions.**

Sterols are crucial for the function of most eukaryotic cells and for the structure of their membranes. Many cellular processes are directly or indirectly dependent on them (Fig. 1). Prominent examples of their importance include the role of cholesterol — the major sterol in animal cells — in atherosclerosis and the many physiological roles of steroid hormones. Biophysical studies have demonstrated that sterols in cellular membranes influence their physicochemical properties, including fluidity and ion permeability[1]. Cholesterol, together with glycosphingolipids, is also proposed to organize membrane microdomains (also called 'rafts'), which provide platforms for protein sorting or signal transduction[2]. In addition to this structural role in the membrane, cholesterol is essential for various signalling processes. It is a precursor of important classes of physiologically active compounds that include steroid hormones, oxysterols, ecdysones (in insects), vitamin D and bile acids. Furthermore, in *Drosophila melanogaster*, cholesterol influences intercellular signalling through its covalent attachment to signalling proteins such as Hedgehog, altering their diffusion and transport[3]. The importance of sterols is emphasized by animals' investment in their synthesis. Almost one hundred proteins are employed to synthesize, modify, transport or degrade sterols, consuming many equivalents of ATP. However, no energy is returned after their catabolism or excretion from the organism[1].

In animals, cholesterol is synthesized or taken up from food. It is then transported to specific destinations, incorporated into appropriate membranes, metabolized into active derivatives that function within or outside of membranes before finally being degraded or excreted.

Much is known about these processes in mammals, but the nematode *Caenorhabditis elegans* is starting to become a valuable new model for studying organismal orchestration of cholesterol metabolism and function. The genetics of the worm is well established and the complete genome sequence provides the opportunity to disrupt all genes that might regulate steroid function, either by double-stranded RNA interference (RNAi) or by selection of targeted deletion or point mutations. An excellent example of the success of such a strategy in *C. elegans* is the use of genome-wide gene disruption by RNAi to identify many new genes involved in fat storage[4,5].

Worm survival requires exogenous cholesterol (see below). This makes it possible to combine biochemical analysis of labelled sterols that have been fed to worms with gene-disruption experiments and phenotypic analysis to address how cholesterol functions. So, does cholesterol regulate membrane structure as it does in other organisms? If not, what replaces cholesterol in these functions? Are there signalling pathways that depend on cholesterol? What are they and how do they work? Why are dietary steroids modified by the worm? Answers to these questions for an entire organism will surely reveal conserved functions known in other animals, but may also reveal novel functions not yet recognized in other animals and perhaps distinct to worms.

Yeast, plants and mammals have complex biosynthetic pathways for sterols that comprise more than thirty enzymes (Fig. 2). By contrast, worms cannot synthesize sterols *de novo*[6]. In their natural environment, they depend on exogenous sources, such as plant or fungal remnants, or animal faeces. *C. elegans* express predicted homologues of the enzymes that produce the initial intermediates of the mammalian sterol biosynthetic pathway up to farnesyl pyrophosphate, but cannot complete the pathway to form sterols (Fig. 2). The isoprenoid farnesyl pyrophosphate is a branching point to the biosynthesis of vital classes of molecules, such as ubiquinone or dolichol. In addition, it is used to modify small GTPases and *C. elegans* can synthesize farnesyl pyrophosphate, ubiquinone and dolichol. However, it cannot synthesize either squalene or lanosterol, the key metabolites of sterol biosynthesis in yeast, plants and mammals[7]. When fed to worms, squalene or lanosterol do not substitute for cholesterol. In fact, only the final products of the plant and mammalian biosynthetic pathways (that is, ergosterol, sitosterol, cholesterol or their close precursors) can fully support worm growth. Consistent with these experimental observations, bioinformatic analysis of the worm genome shows that genes for squalene synthase and squalene cyclase are missing. A comprehensive comparative

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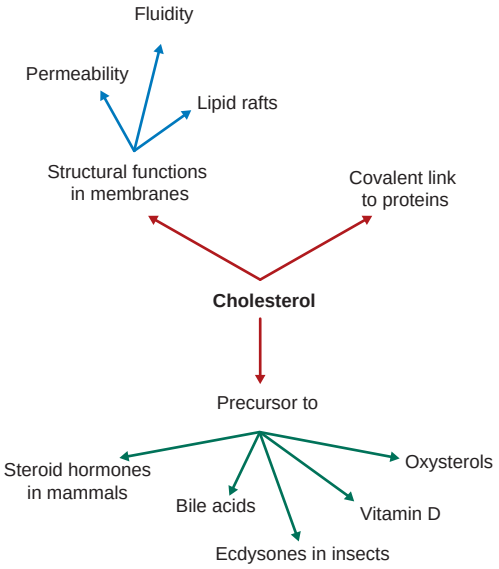


**Figure 1** Cholesterol has numerous functions in eukaryotic cells. Cholesterol has structural functions in membranes (blue arrows), can be covalently linked to proteins and can be a precursor to hormones and other biologically active compounds (green arrows).

genomic analysis of all the genes involved in cholesterol uptake and metabolism still needs to be performed. However, more information on *C. elegans* genes that are likely to be involved in sterol pathways is available from the Kyoto Encyclopedia of Genes and Genomes (http://www.genome.ad.jp/kegg/kegg2.html).

Despite the lack of enzymes for *de novo* sterol synthesis, nematodes can modify externally added yeast, plant or mammalian sterols by introducing double bonds, reducing or isomerizing them and by dealkylating the side chain[7]. Moreover, metabolic studies and lipid analysis have revealed a nematode-specific modification of sterols: methylation of the ring at the fourth position (Fig. 2)[8]. This is remarkable because in mammals, a complex series of almost twenty steps is used for the opposite reaction — to demethylate lanosterol at the fourth position — and no enzymes are known in mammals that can restore these methyl groups. The presence of enzymes for such specific modifications of cholesterol suggests that a pathway might exist to produce a hormone. However, efforts over the past fifty years to identify and purify steroid hormones or ecdysones in nematodes, which are essential signalling molecules in mammals and insects, have been unsuccessful. This seems particularly surprising as the *C. elegans* genome contains nearly 260 potential nuclear hormone receptors, many of which are homologous to steroid-binding receptors in other organisms[9]. So, what are the ligands for these receptors if they are not steroids or ecdysones?

Obviously, the first step in investigating the cholesterol requirement should be to ask what the consequences of sterol depletion are. This has proved surprisingly difficult, and the literature has conflicting descriptions of how cholesterol depletion affects the worm. It is now clear that this is because the worm requires very little cholesterol, so considerable effort must be made to remove contaminating sterols from agar and growth medium. Worms are routinely grown in the laboratory on agar plates seeded with bacteria. Standard agar plates are supplemented with 5 $\mu g\ ml^{-1}$ cholesterol (Brenner conditions)[10]. Just omitting sterols from agar has a weak effect on development and growth[11,12]: some larvae fail to shed the old cuticles properly during molting, gonad development is aberrant, and movement is uncoordinated. However, worms can still propagate for many generations. Thus, the trace amounts of sterols in agar (as well as in bacteria that have been grown on media with yeast extracts) seems to be sufficient to support worm growth. Indeed, a uniform phenotype was obtained only by both replacing agar with agarose extracted with organic solvents[11], and by using bacteria grown on defined media[13,14]. Under these (presumably cholesterol-free) conditions, the first generation of worms could develop from eggs to adults without the presence of external cholesterol, although these adults had a reduced brood size. The second generation, however, stopped growth uniformly at early larval stages. Adding cholesterol back to these larvae reversed this arrest and the worms matured into fertile adults[14]. Remarkably, transfer of arrested larvae to plates containing as little as 2.5 $ng\ ml^{-1}$ cholesterol (2,000 times less than at Brenner conditions) for 1 h is sufficient to induce arrested larvae to resume growth and to mature into fertile adults (T.K., unpublished observations).

The depletion experiments show that although absolutely necessary for worm growth, sterols are required only in tiny amounts. Quantification of sterol levels show that they are much less abundant in lipid extracts of several nematodes than in those of mammals[15]. From total lipids extracted from *C. elegans* embryos, cholesterol (when normalized to phosphatidylcholine) is approximately 20 times less abundant as it is in mammalian cell membrane lipids. Can such a low amount of sterols be sufficient for a structural role in nematode membranes? Typically, cholesterol constitutes approximately 20% of the total lipid content of animal cell plasma membranes, and the effect of cholesterol on membrane permeability is significant only at similarly high concentrations[1]. For example, in artificial or cellular membranes, ordered microdomains can be formed only if the concentration of cholesterol is greater than 10% (refs 2, 16, 17). So, if cholesterol were uniformly distributed in *C. elegans* membranes, there would be too little to have a major membrane-modifying function in most cell membranes. What, then, replaces the structural functions of cholesterol in most worm membranes?

The distribution of sterols in *C. elegans*, however, is not uniform, and it may affect membrane structure in a subset of cells. Using either the fluorescent analogue dehydroergosterol, which can replace cholesterol and support worm growth, or the antibiotic filipin, which binds to sterols, it has been shown that only a small subset of cells are labelled: the apical surface of the gut, excretory gland cell, pharynx, sensory amphids, nerve ring, sperm and oocytes[14,18]. The membranes of these cells must have specific properties that allow cholesterol accumulation, and these membranes may have altered physical properties and raft-like microdomains. Surprisingly, in spite of the small amount of cholesterol in worm membranes, the caveolin-1 protein behaves as a *bona fide* raft protein[19]. Caveolin is found in the floating fraction of worm membranes after detergent extraction, suggesting that rafts can form in the absence of cholesterol. Recent studies of lipid mixtures in large unilamellar vesicle membranes show that phase separation can occur in the absence of cholesterol, but that adding cholesterol alters phase boundaries and lipid mobility[16,17]. So,

although cholesterol could mediate membrane structure and raft function in a subset of cells, other cell types may assemble rafts through another mechanism.

Nematodes are not the only animals with little cholesterol in their membranes. Although *Drosophila* require sterols and rely on ecdysones to control molting, cultivated insect cells can grow indefinitely with only a trace amount of exogenous sterol[20]. Remarkably, in *Drosophila*, proteins similar to those regulating cholesterol levels in mammals are used instead to regulate enzymes that synthesize saturated fatty acids and phosphatidylethanolamine[21,22]. *C. elegans* also contains an essential gene that is a homologue of the key gene in this pathway, *SREBP* (sterol regulatory element-binding protein)[4,5]. So, in worms, as in *Drosophila*, this gene probably regulates phophatidylethanolamine levels, not cholesterol.

Whatever the structural roles of cholesterol in worm membranes, recent investigations of cholesterol uptake indicate that the transport mechanisms in *C. elegans* are similar to those in mammals. Using a photoactivatable analogue of cholesterol, it has been shown that cholesterol is transported into oocytes by yolk proteins, known as vitellogenins[18]. These proteins are similar in sequence to apolipoproteins, constituents of the cholesterol-carrying low-density lipoprotein (LDL) particle[23]. In addition, the receptor for these vitellogenins, RME-2, is a new member of the LDL receptor superfamily, supporting the similarity between endocytosis of LDL and that of yolk proteins[24]. Vitellogenins cannot be the only transporters of cholesterol in *C. elegans*, however, as cholesterol uptake by hermaphrodite larvae starts before yolk protein expression, and males do not express vitellogenins but still accumulate cholesterol in sperm.

If cholesterol has only a minor structural role in worm membranes, then its primary role may be in signalling. As noted above, no specific signalling molecules derived from cholesterol, steroid hormones or ecdysones, have yet been identified. However, there are several strong arguments to suggest that cholesterol is involved in signalling processes. It has been found that an enantiomer (mirror symmetric image) of cholesterol cannot substitute for cholesterol to support worm growth and reproduction[13]. The biophysical properties of the enantiomer are identical to natural cholesterol, so it should substitute for any structural role of cholesterol in membranes. So the observation that this enantiomer does not support growth could be explained if its altered conformation prevents specific interaction with an enzyme that mediates steroid hormone production. Additional suggestive evidence that cholesterol may be required for steroid hormone production comes from bioinformatic analysis of the *C. elegans* genome[25], showing that there are approximately 80 cytochrome P450s and six 17 oestradiol dehydrogenases — candidates for modifying cholesterol to form steroid hormones. However, only one of these cytochrome P450s is predicted to be found in mitochondria. In mammals and insects, mitochondria are the site of steroid hormone and ecdysone biosynthesis. So, if worms are using these cytochrome P450s to make steroid hormones,

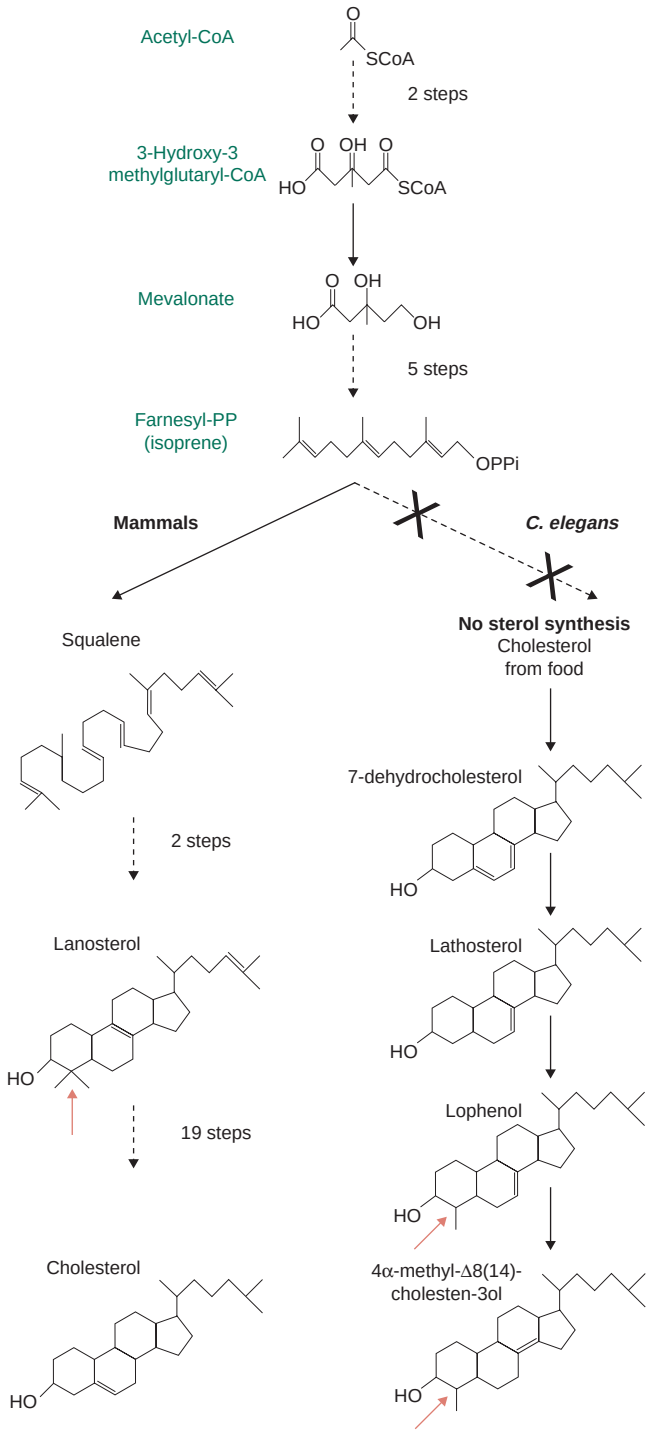


**Figure 2** Nematodes can modify, but not synthesize, sterols. Intermediates synthesized by both mammals and nematodes are shown in green. Red arrows show the fourth position of sterol ring that is demethylated in mammals and is methylated in *C. elegans*.

these may have a distinct site of synthesis. Another possible function for cholesterol in signalling is as a covalent linkage to signalling proteins; a precedent for this comes from the attachment of cholesterol to Hedgehog in *Drosophila* (Fig. 1). Indeed, *C. elegans* contains approximately ten genes that encode proteins similar to the carboxyl terminus of Hedgehog — the part of the protein involved in autoprocessing and covalent attachment of cholesterol[3,26]. So far, no evidence of cholesterol attachment to these proteins has been reported, although one of them has been shown to be essential for molting[27].

The processes that are most likely to be influenced by steroid hormone signalling are the dauer-formation pathway and molting. Formation of a dauer (enduring) larva is an alternative to the normal life cycle of the worm, similar to insect 'diapause', which enables the worm to survive inhospitable environments. Many genes can mutate to cause constitutive dauer larvae formation or to prevent dauer larvae formation[28]. One of them, *daf-9,* has a strong similarity to several cytochrome P450s that are involved in steroid metabolism in mammals[29,30]. The null mutation of *daf-9* results in constitutive dauer formation. Moreover, *daf-12*, a gene that functions downstream of *daf-9* and executes dauer larvae formation, is a putative nuclear hormone receptor[31,32]. Perhaps DAF-9 is an enzyme that produces a steroid hormone to regulate DAF-12. Additional evidence that steroids might function in dauer formation comes from the observation that deletion of two mammalian Niemann-Pick C homologues in *C. elegans* results in spontaneous dauer larvae formation[33]. The most pronounced cellular abnormality in Niemann-Pick C disease is the alteration of intracellular cholesterol homeostasis. In NP-C cells, cholesterol is sequestered in lysosomal compartments and this alteration is thought to reflect a defect in the Golgi-mediated efflux of unesterified cholesterol from lysosomes to the endoplasmic reticulum. All this supports, but does not prove, the idea that *C. elegans* could produce a steroid hormone, which could be a signal for dauer larvae formation.

The possible involvement of cholesterol in molting is based on an analogy with other animals. Molting in insects is regulated by ecdysones — polyhydroxylated sterols derived from cholesterol — which function through nuclear hormone receptors. Disruption of CHR3 (nhr-23), a *C. elegans* homologue of the *Drosophila* orphan nuclear receptor that is induced by ecdysone, results in defective shedding of the old cuticle[34,35]. Analysis of the *C. elegans* genome does not reveal a homologue of the ecdysone receptor (EcR) itself. However, homologues of EcR have been found in some parasitic nematodes[36]. A mutant of *lrp-1*, a homologue of the mammalian gp330/megalin protein, shows similar defects in shedding of the cuticle and this phenotype became more severe after cholesterol depletion[11]. Among other functions, megalin in mammals is involved in the uptake of a cholesterol derivative, vitamin D, by kidney absorptive cells[37]. As with the dauer pathway, the role of cholesterol and sterol signals in molting remains speculative. Careful biochemical studies to purify and identify the active hormones are necessary to establish whether they are indeed sterols.

Pharmacological strategies represent an alternative approach for identifying signalling pathways that rely on cholesterol, seeking compounds that substitute for cholesterol to support growth or alter dauer formation. Compounds similar to cholesterol can substitute for these functions, but other related compounds tested cannot, including pregnolonone, vitamin D, bile acids, steroid hormones and ecdysones[14,30]. Such negative results could mean that worms either cannot take up these compounds, or that they do not substitute for some distinctive worm-specific compound. Alternatively, these results could mean that if cholesterol has both an essential structural role for a few cells, as well as a hormonal role in signalling, then substitute compounds or products of cholesterol metabolism might be unlikely to do both. An example of this last possibility is that methylated sterols (see Fig. 2), which cannot support worm growth alone, can replace cholesterol if they are combined with a minute quantity of cholesterol[14].

At present, we have more questions than answers about the role of cholesterol in *C. elegans.* As most worm cell membranes contain little or no cholesterol, what replaces the structural functions ascribed to cholesterol in other animal cell membranes? Do other molecules in nematodes take over the regulation of membrane fluidity and permeability? Do *C. elegans* membranes contain lipid rafts that are formed in the absence of cholesterol? Answers to these questions are of a general importance, as they may identify new roles for cholesterol in mammalian and other cell membranes.

Understanding the role of cholesterol in signalling depends critically on careful biochemical analysis to identify cholesterol-derived signalling molecules. If these can be found, they will tell us whether there are signalling pathways that depend on cholesterol and why worms modify dietary steroids. Then the combination of biochemistry, disruption of candidate genes and analysis of phenotypes should establish how these signalling pathways function in the entire organism. Comparison of these pathways with other invertebrates, plants and mammals may help us to understand whether there are as yet undiscovered roles for cholesterol and how the role of sterols has evolved. □

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