Sterol metabolism in the filasterean *Capsaspora owczarzaki* has features that resemble both fungi and animals

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Sterols are essential for several physiological processes in most eukaryotes. Sterols regulate membrane homeostasis and participate in different signalling pathways not only as precursors of steroid hormones and vitamins, but also through its role in the formation of lipid rafts. Two major types of sterols, cholesterol and ergosterol, have been described so far in the opisthokonts, the clade that comprise animals, fungi and their unicellular relatives. Cholesterol predominates in derived bilaterians, whereas ergosterol is what generally defines fungi. We here characterize, by a combination of bioinformatic and biochemical analyses, the sterol metabolism in the filasterean *Capsaspora owczarzaki*, a close unicellular relative of animals that is becoming a model organism. We found that *C. owczarzaki* sterol metabolism combines enzymatic activities that are usually considered either characteristic of fungi or exclusive to metazoans. Moreover, we observe a differential transcriptional regulation of this metabolism across its life cycle. Thus, *C. owczarzaki* alternates between synthesizing 7-dehydrocholesterol de novo, which happens at the cystic stage, and the partial conversion—via a novel pathway—of incorporated cholesterol into ergosterol, the characteristic fungal sterol, in the filopodial and aggregative stages.

1. Introduction

Sterols are essential membrane components of most eukaryotic cells, having fundamental structural and signalling functions. As structural components, sterols participate in regulating membrane fluidity and permeability barrier properties [1]. As signalling, sterols act directly as precursors of diverse hormones and bioactive metabolites, including steroids, bile salts and vitamin D in vertebrates [1], ecdysteroids in arthropods [2], dafachronic acids in nematodes [3], brassinosteroids in plants, and antheridiol and oogoniol in fungi [4]. Moreover, together with sphingolipids, sterols are involved in the formation of the lipid rafts, regions of reduced fluidity that selectively incorporate proteins involved in concerted functions such as cell-to-cell recognition, adhesion and communication [5,6]. Thus, sterols play a role in many cellular processes crucial for the development and homeostasis of multicellular organisms, being critical to cell-to-cell communication processes [7–11]. Indeed, alterations in sterol composition and biosynthesis produce several diseases [12].

Sterols form one of the most diverse families of organic molecules in nature. Interestingly, different eukaryotic groups are usually characterized by having a
Figure 1. Phylogeny of eukaryotes. Schema of an opisthokont phylogeny (after [19]). Genera mentioned in this study are indicated after the dashes. Dark and light green boxes represent the Opisthokonta and the Holozoa, respectively. LOCA, last opisthokont common ancestor.

particular type of sterol, or a reduced number of them, as the major sterol constituent of their membranes. For example, phytosterols (such as sitosterol, C29Δ5,7,22; stigmastanol, C29Δ5; stigmasterol, C29Δ5,7,22) are the typical sterols found in plants and green algae [13]. On the other hand, ergosterol (ergosta-5,7,22-trien-3-ol, C28Δ5,7,22) was historically considered a signature of fungi [14], also used as biomarker for the presence of fungi in the environment [15]. However, by superimposing sterol data available in the literature on a recent phylogeny of fungi, Weete et al. [14] suggested that the situation for fungi is more complex. Thus, while ergosterol is indeed the predominant sterol in the most derived fungal clades, cholesterol (cholesta-5,7,22-trien-3-ol, C27Δ5,7,22) and other Δ5 sterols, including 24-methyl and 24-ethyl sterols, are the major sterols in the membranes of the earliest diverging fungal clades, such as Chytridiomycota [14]. Therefore, it seems that the evolution of Holomycota (fungi and their close relatives) was accompanied by a trend from cholesterol-related sterols to ergosterol. Among animals, an opposite situation is observed. Cholesterol predominates in derived bilaterians such as vertebrates, tunicates, annelids, arthropods and molluscs, although other sterols might also be present [13,16,17]. However, early-branching deuterostomes such as echinoderms, and non-bilaterian animals such as cnidarians and poriferans, usually contain a extraordinarily large variety of sterol species, including ergosterol as well as other C24-alkyl sterols [16]. It is worth mentioning the case of sponges, in which more than 70 different sterol species, including ergosterol as the main sterol of the unicellular holozoans. In one study, it was proposed that ergosterol, like in fungi [24], is the main sterol of the unicellular Corallinophyta limacidosporum, the sister-group to Ichthyosporea (figure 1). However, the sterol identification was based only on the UV absorption spectra of sterol extracts from this species and compared to those obtained from a wild-type and a Δerg3 mutant (deficient in C5-desaturase) strains of the fungus Neurospora crassa [24]. In a different study, Kodner et al. [25] described the complete sterol profile of the choanoflagellate Monosiga brevicollis. The authors showed cholesterol-5,7,22-trien-3β-ol (C27Δ5,7,22) to be the major sterol constituent in M. brevicollis membranes and ergosterol as the second most abundant sterol component. In a more recent study, Gold et al. [26] explore the sterol composition of the choanoflagellate Salpingoeca rosetta, the filasterean Capsaspora owczarzaki and the ichthyosporeans Creolimax fragrantisima and Sphaeroforma arctica. The authors focused on sterol sidechain modifications, but they tried to determine whether 24-isopropylcholesterol—a precursor of the sterane side chain modifications, because they tried to determine whether 24-isopropylcholesterol—a precursor of the sterane metabolisms in the filasterean Capsaspora owczarzaki and the ichthyosporeans Creolimax fragrantisima and Sphaeroforma arctica. The authors reported S. rosetta to have only C27 sterols, hence lacking side chain alkylation, and showed ichthyosporeans C. fragrantisima and S. arctica to possess a wider range of sterols, including C27, C28 and C29 sterols. Moreover, the authors showed cholesterol to be the predominant sterol in C. owczarzaki, with trace amounts of ergosterol as well as a few other unidentified minor sterol components [26]. However, there is not yet a systematic analysis of the full repertoire of genes involved in sterol metabolism in unicellular holozoans. Moreover, it is unclear whether and how sterol metabolism is regulated throughout their life cycle.

To fill that gap, we here analysed in detail the sterol metabolism in the filasterean C. owczarzaki, a close unicellular relative of animals [23,28]. Capsaspora owczarzaki was described as a symbiont of the freshwater snail Biomphalaria glabrata from whose haemolymph the unicells were first isolated [29]. Capsaspora owczarzaki also offers us the possibility to analyse the dynamics of the sterol metabolism along its life cycle, which includes a filopodial stage, a cystic stage and a multicellular aggregative stage that potentially segregates an extracellular matrix [30]. To understand its sterol metabolism, we performed a bioinformatic survey to identify the gene repertoire of sterol metabolism and analysed their regulation along C. owczarzaki's life cycle. Moreover, we performed biochemical assays to characterize the different sterol synthesis pathways used by this species. We show that sterol metabolism in C. owczarzaki is differentially transcriptionally regulated during its life cycle. In the filopodial and aggregative stages, de novo sterol synthesis is repressed, and the cells use cholesterol from the media to produce ergosterol, with sterol neosynthesis restricted to the cystic stage. We also define a potential novel biosynthetic pathway from which C. owczarzaki cells produce ergosterol from cholesterol.

2. Results

2.1. Gene repertoire for sterol synthesis in Capsaspora owczarzaki

To have an idea of the gene repertoire for sterol metabolism in C. owczarzaki, we mined its genome sequence for genes
encoding enzymes involved in the canonical sterol biosynthetic pathways of animals, fungi and plants. We used protein basic local alignment search tool (BLAST [31]) with a set of amino acid sequences of characterized sterol synthesis proteins from *Homo sapiens*, *Saccharomyces cerevisiae* and *Arabidopsis thaliana* as queries. We identified clear orthologues for proteins involved in the ‘canonical’ ergosterol biosynthesis pathway typical of fungi (table 1 and figure 2), as well as other sterol synthesis genes that do not participate in this metabolic route. The presence of orthologues of the first two genes of the pathway, squalene monooxygenase (SMO) and oxidosqualene cyclase (OSC), suggests the capability of *C. owczarzaki* for de novo sterol synthesis. In the canonical sterol pathways, SMO produces squalene epoxide from

<table>
<thead>
<tr>
<th>gene description (vertebrate/yeast/plant)</th>
<th>protein ID</th>
<th>similarity to known sequences</th>
<th>Homo sapiens</th>
<th>Saccharomyces cerevisiae</th>
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<tr>
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<td>score (bits)</td>
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<tr>
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<td>—</td>
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*Orthologues of the Neverland/DAF-36 family of Rieske sterol C7(8)-desaturases are found in vertebrates, except mammals, as well as in some ciliate species [32,33].
Figure 2. Gene repertoire for sterol biosynthesis in unicellular holozoans. The canonical ergosterol synthesis pathway of fungi and cholesterol synthesis pathway of vertebrates are detailed (blue and red boxes, respectively), with the enzymes involved in each step indicated. Bullets indicate the presence of homologues for the corresponding enzymes in the C. owczarzaki and/or M. brevicollis genomes, for comparison. Coloured bullets indicate homologues of proteins that are considered exclusive either of fungal (blue) or vertebrate (red) clades. The Rieske sterol C7-desaturase is shown apart as it is not involved, as far as is known, in de novo sterol synthesis.
M. brevicollis (table 1), with some important differences with respect to choanoflagellates [19–21,23,28]. The predicted amino acidic sequence of the C. owczarzaki OSC shows T381, C/Q449 and V453. This, together with the phylogenetic analysis of squalene and oxidosqualene cyclases (electronic supplementary material, figure S1), strongly suggests that the sterol biosynthetic pathway in this species would begin with the cyclization of epoxidosqualene to lanosterol or cycloartenol by different orthologues can be mainly inferred based on the nature of the amino acid residues at the conserved positions 381, 449 and 453 (numbering relative to the H. sapiens OSC) [32]. Fungal and vertebrate lanosterol synthases possess T381, C/Q449 and V453, whereas Y381, H449 and I453 are signatures of cycloartenol synthases [32]. However, there are some exceptions to this rule; amino acid 453 is the only determinant of the enzyme’s specificity [13]. The predicted amino acidic sequence of the C. owczarzaki OSC possesses two. Second, while M. brevicollis and Salpingoeca rosetta each harbour one fungal-like Δ8, Δ7-isomerase (ERG2), C. owczarzaki possesses two ERG2 homologues as well as a putative protein belonging to the emopamil binding protein (EBP) family, characteristic of vertebrates and plants (table 1 and figure 2) [13]. Regarding the sterol reductase family of proteins (C7-, C14- and C24-reductases), no homologue of sterol C7-reductase is found in C. owczarzaki, which is in concordance with its phylogenetic position within the Opisthokonta, as a close unicellular relative of metazoans [19–21,23,28].

Homologues of the enzymes necessary to complete the downstream steps in the pathway were also identified (table 1), with some important differences with respect to choanoflagellates (figure 2). First, contrary to what was reported for M. brevicollis [25], a clear orthologue of cytochrome P450 sterol C22-desaturase is found in C. owczarzaki (table 1 and figure 2; electronic supplementary material, figure S1), strongly suggests that the sterol biosynthetic pathway in this species would begin with the cyclization of epoxidosqualene to lanosterol, which is in agreement with its phylogenetic position within the Opisthokonta, as a close unicellular relative of metazoans [19–21,23,28].

To understand the dynamic use of sterol synthesis genes in C. owczarzaki, we analysed their expression profiles, including the latest genes for the pre-squalene pathway of isoprenoid synthesis (isopentenyl diphosphate isomerase, farnesyl diphosphate synthase and squalene synthase), from the different C. owczarzaki life cycle stages using the data generated in [30] (figure 3). We found that the first four genes in the de novo synthesis pathway (i.e. squalene synthase, SMO, OSC and sterol C14-demethylase; square in figure 3), are exclusively expressed at the cystic stage, being repressed in the filopodial and aggregative stages. Interestingly, a few genes are strongly upregulated in the filopodial and/or aggregative stages with respect to the cystic stage (highlighted in bold in figure 3). These include sterol 24-C-methyltransferase (SMT), C22-desaturase, Δ24(28)-reductase and the Rieske sterol C7-desaturase. Among these, SMT and C22-desaturase are the most highly expressed in both stages, and are also completely repressed in cysts.

2.3. Gas chromatography–mass spectrometry profile of Capsaspora owczarzaki sterols

To have a complementary view, we analysed the sterol composition of C. owczarzaki membranes obtained from cultures at the filopodial and cystic stages, using gas chromatography–mass spectrometry (GC/MS) as described in Material and methods. Figure 4a and electronic supplementary material figure S3 show total ion chromatograms (TIC) of the acetylated derivatives from filopodial and cystic stage cells, respectively. No significant differences in sterol profiles were observed between filopodial and cystic stages. Sterol identifications were performed by comparison of the mass spectra obtained with those from commercial standards, published in the literature and/or available in the NIST mass spectral library. Mass spectral fragments of all the sterols identified in the chromatogram are listed in table 2. We identified four dominant compounds (1–4, figure 4a, b), with cholesterol, 2 (C27Δ5), being the major constituent, representing 84.17% of the total sterols in the extract. Three other peaks define the bulk of sterol products. In decreasing order of abundance, they were assigned as ergosterol, 4 (C29Δ5,7,22) (7.76%); 7,22-his-dehydrocholesterol, 1 (C27Δ5,7,22) (5.04%); and 7-dehydrocholesterol, 3 (C27Δ5,7) (2.04%). Although the peak corresponding to 1 partially overlaps with that of 2 under our conditions of analysis (figure 4a), both compounds were unambiguously identified by differentially subtracting their corresponding mass spectra. Minor peaks in the chromatogram represent 1% of the total of sterols identified and were tentatively assigned to choles-5,7,9(11),22-tetraene-3β-ol (peak 5, C27Δ5,7,9(11),22), 22-dehydrocholesterol (peak 6, C27Δ5,22), brassicasterol (peak 7, C29Δ5,22), ergosta-5,7,9(11),22-tetraene-3β-ol (peak 8, C30Δ5,7,9(11),22),
ergosta-5,7,22,24(28)-tetraen-3β-ol (peak 9, C_{28}D_5,7,22,24(28)) and campesterol (peak 10, C_{28}D_5) (table 2). Structures of all these compounds are detailed in electronic supplementary material, figure S3b. No early sterol precursor, namely lanosterol, nor any other 4,4 dimethyl sterol was identified in the profile.

2.4. Supplementation of cultures with radioactive precursors

In order to assess the possibility that the cholesterol present in the growth medium is effectively incorporated by the cells and can be further modified for the production of ergosterol, C. owczarzaki cultures were supplemented with [^{14}C]-cholesterol. After incubation for 4 days, confluent adherent cells were collected, and total lipids were extracted and saponified as described [34]. The non-saponifiable lipid fraction, containing mainly sterols, was analysed by reversed-phase high-performance thin-layer chromatography (HP-TLC) and revealed by autoradiography as detailed in Material and methods. An aliquot of [^{14}C]-cholesterol was used as standard, also ensuring the purity of the commercial radiolabelled sterol (lane I in figure 4c). Lanes II and III correspond to duplicates from two independent C. owczarzaki cultures. Besides the major cholesterol spot (R_f = 0.50), other spots corresponding...
to 7-dehydrocholesterol ($R_f = 0.53$), ergosterol ($R_f = 0.59$) and 7,22-bis-dehydrocholesterol ($R_f = 0.62$) were identified in the autoradiography (figure 4c). To further confirm the identity of the radioactive sterol products, unsaponified lipids from $C. owczarzaki$ cultures, supplemented with [14C]-cholesterol, were subjected to high-pressure liquid chromatography.
Figure 5. (a) Autoradiography from a silica G60 TLC plate of total lipids extracts obtained after growing C. owczarzaki cells supplemented with the radioactive precursor 1-14C-acetate. All lipid species synthesized de novo are labelled. The picture shows the region of the TLC plate which includes phospholipids, sterols and free fatty acids. Sterols produced de novo can be detected after 168 h (7 days) of incubation with the radioactive precursor (dashed square). The spot (asterisk) was their polarity, and all sterol species migrate together in a single spot. (b,c) shows that, whereas other lipid classes but potentially act on different substrates (figure 2). Similarly, to metazoans. A good example is the coexistence of the two different C24-reductases described above, ERG4-like (fungal) to non-homologous protein families, two homologues of that in electronic supplementary material, figure S4. However, the radioactivity profile showed a single peak at retention time 26.1 min, indicative of 7-dehydrocholesterol (not shown).

3. Discussion

3.1. Fungal and metazoan traits are combined in Capsaspora owczarzaki sterol pathways

Our data demonstrate that C. owczarzaki possesses a peculiar repertoire of sterol metabolism genes, including the complete canonical ergosterol pathway typical of fungi as well as other sterol-metabolism-related genes not involved in this pathway (table 1 and figure 2). This suggests that sterol metabolism in C. owczarzaki combines enzymatic activities that are usually considered either characteristic of fungi or exclusive to metazoans. A good example is the coexistence of the two different C24-reductases described above, ERG4-like (fungal) and DHCR24-like (metazoan), that perform similar reactions but potentially act on different substrates (figure 2). Similarly, C. owczarzaki possess three putative Δ8,7-isomerases belonging to non-homologous protein families, two homologues of (HPLC), as described in Materials and methods. We detected radioactive products that eluted at the same retention times as 7,22-bis-dehydrocholesterol (20.6 min), ergosterol (25.7 min) and 7-dehydrocholesterol (26.1 min; electronic supplementary material, figure S4). To investigate the capability of sterols neosynthesis in C. owczarzaki, 1/100 subcultures were initiated from 4-day cultures (formed by adherent cells under microscopic examination) supplemented with [14C]-acetate, and collected at different time points from 4 h up to 7 days post-supplementation. Total lipid extracts were prepared from those cultures and analysed by thin-layer chromatography on SilicaGel G60 plates and revealed by autoradiography (see Material and methods). In this system, lipids can be separated into families based on their polarity, and all sterol species migrate together in a single spot. Figure 5a shows that, whereas other lipid classes are produced from as early as 4 h post-incubation with the substrate, sterols were only detected after 7 days of incubation with the radioactive precursor (dashed square in figure 5a). This TLC system does not allow the separation of the different sterol species. So, in order to further identify the sterols produced de novo, the spot was scratched from the G60 TLC plate and then analysed by reverse phase HP-TLC using a series of standards as described in Material and methods (figure 5b,c). The relative position of each compound could be established by superimposing the autoradiogram with the corresponding HP-TLC plate revealed with Cu:phosphoric reagent (dashed black lines between figure 5b,c, and dashed white lines in figure 5c). Interestingly, as can be noted in lane III (figure 5b), the radioactive sterol species recovered from the cystic cells migrates at the same retention time as 7-dehydrocholesterol standard. Other, non-radioactive sterol species were also present in the sample (lane III’ in figure 5c), including cholesterol and the triple unsaturated derivatives 7,22-bis-dehydrocholesterol and ergosterol. To further characterize de novo synthesized sterols, the unsaponifiable lipids obtained from cells incubated for 7 days in the presence of the radioactive precursor [14C]-acetate, were analysed by HPLC as described in Materials and methods. The profile obtained by absorbance at 210 nm was identical to that in electronic supplementary material, figure S4. However, the radioactivity profile showed a single peak at retention time 26.1 min, indicative of 7-dehydrocholesterol (not shown).
fungal ERG2 and one homologue to the EBP family of proteins, typical of vertebrates and plants. However, phylogenetic analysis suggests that one of the two ERG2 paralogues could be the actual sterol isomerase of *C. owczarzaki*. On the one hand, the other parologue of this family groups in a different clade with the homologous sigma receptors, members of the opioid receptors family typical of vertebrates, which lacks sterol isomerase activity [36] (electronic supplementary material, figure S2a). On the other hand, the EBP homologue, besides conserving the amino acids known to be essential for isomerase activity of EBP's [37], groups to the EBP-like clade in a phylogenetic analysis (electronic supplementary material, figure S2b,c). EBP-like proteins lack sterol isomerase activity and their function is still unknown [37,38]. The identity of the real sterol Δ8,Δ7-isomerase in *C. owczarzaki* can be potentially confirmed by heterologous expression of the candidate genes in a Δerg2 yeast strain, but those experiments are beyond the aim of this paper.

Another interesting characteristic of this organism is the Rieske C7-desaturase, with no known orthologues in fungi and highly conserved in animals [34,35]. Among unicellular holozoans, orthologues of C7-desaturase were identified in *C. owczarzaki* and *S. rosetta*, but not in *M. brevicollis* [34] (table 1 and figure 2). Also in contrast to *M. brevicollis*, *C. owczarzaki* possesses an orthologue of the cytochrome P450 sterol C22-desaturase (table 1 and figure 2). This enzyme was postulated to be present in the common ancestor of fungi, animals and plants, and to have been subsequently lost in animals [39]. SMT orthologues are found in both *M. brevicollis* and *C. owczarzaki* genomes (figure 2). SMTs are responsible for the alkylation of the lateral chain of sterols at C24, and are widely conserved in fungi and plants. In animals, orthologues of SMT can be identified in sponges [26] and in the annelid *Capitella teleta*, but are not present in more derived metazoans known to synthesize cholesterol.

Thus, our data on *C. owczarzaki* suggest that the last opisthokont common ancestor had a complex repertoire of genes for sterol metabolism that would allow it to produce a variety of sterol structures. After the divergence of Holozoa and Holomycota, evolutionary canalization leads to derived fungi having ergosterol as the major sterol component of their membranes [14], presumably as an adaptation to live on land [40]. On the other hand, cholesterol predominates in membranes of vertebrates and other bilaterian animals [16].

From a functional point of view, taking into account the particular gene repertoire of *C. owczarzaki*, it is difficult to hypothesize a sterol synthesis pathway in which all these enzymes act in a logical order. For example, the action of Δ8,Δ7-isomerase and Rieske C7-desaturase would redound to sterol products having a double bond in position C7(8). Besides, the presence of both the non-homologous fungal-like and metazoan-like C24-reductases is intriguing. As is described in §3.2, the data hint to a tight regulation of sterol metabolism along the *C. owczarzaki* life cycle.

### 3.2. Dynamic use of sterol metabolism genes in *Capsaspora owczarzaki*

An important issue is how the different genes for sterol metabolism are orchestrated in these cell stages. The expression profiles of those genes that appear to be differentially transcriptionally regulated along the life cycle (figure 3) [30], together with the biochemical results, lead us to propose a model for sterol metabolism during the life cycle of *C. owczarzaki*. This model should be taken as a working hypothesis. We suggest that the cells alternate between two different metabolic pathways for sterol synthesis during the life cycle (figure 6). Thus, during the filopodial and aggregative stages (yellow background in figure 6), *C. owczarzaki* does not seem to produce sterols de novo. This is supported by the complete repression of the first genes necessary for de novo sterol synthesis (i.e. squalene synthase, SMO, OSC and C14-demethylase) during these stages (dashed square in figure 3b). Moreover, by supplementing *C. owczarzaki* cultures with the radioactive precursor [14C]-acetate, we further confirmed the absence of neosynthesized sterols during the first 96 h post-incubation with the substrate. At these time points, the cultures are mainly composed of filopodial/adherent and aggregative cells with no cystic cells observable under microscopic examination. On the other hand, after 7 days of incubation with the substrate, when most of the cells in the cultures are in cystic stage, de novo sterol synthesis was evidenced (figure 5a).

Our data show that *C. owczarzaki* cystic cells produce 7-dehydrocholesterol de novo (figure 5b). Sterol neosynthesis occurring at this stage would start with the production of lanosterol as the first cyclic product. Downstream steps of the pathway would, in principle, be the same as those of the cholesterol synthesis pathway of vertebrates, except for the absence in *C. owczarzaki* of a Δ7-reductase. In vertebrates, this enzyme is responsible of removing the C7(8) double bond from 7-dehydrodesmosterol (cholesta-5,7,24(25)-tri-en-3β-ol, C29Δ5,7,24(25)) producing desmosterol [13]. As an inescapable consequence of the lack of Δ7-reductase, the C7(8) double bond generated by Δ8,Δ7-isomerization in a previous step would remain in the final product leading to the production of 7-dehydrocholesterol instead of cholesterol (figure 6b).

Another source of 7-dehydrocholesterol in cysts could involve the action of the Rieske C7-desaturase on the cholesterol previously incorporated by the cells before encystment. This possibility is suggested by the relatively high level of expression of this gene in cystic cells (figure 3b). Rieske sterol C7-desaturases are highly conserved in animals [34,35], and have a crucial role in development and physiology in ecdysozoans as they catalyse the conversion of diet cholesterol into 7-dehydrocholesterol as the first step in the production of steroid hormones [41,42]. Whether *C. owczarzaki* further modifies 7-dehydrocholesterol for the production of more derived compounds with hormonal functions remains to be determined. At the moment, based on the evidence in favour of the production of 7-dehydrocholesterol de novo in cysts, we think that 7-dehydrocholesterol, even though it is a minor component of membranes, could be important for the physiology of the cystic cells. The presence of both C5(6) and C7(8) double bonds in the B-ring of sterols has been correlated with an increase in resistance to oxidative stress under desiccation in yeasts [40]. It was proposed that these conjugated double bonds allow the stabilization of sterol radicals by resonance, protecting membrane phospholipids against peroxidation [40]. Moreover, the C7(8) double bond is involved in the packing and overall rigidity of the plasma membrane [12,40,43]. Taking into account that *C. owczarzaki* cysts are assumed as a dispersal form [44], we then speculate that the presence of 7-dehydrocholesterol in the membrane is providing cysts with properties of robustness and resistance to dryness,
which are useful to face potential tough environmental conditions during dispersion out of the host.

### 3.3. A novel pathway for the conversion of cholesterol into ergosterol

Our data show that the filopodial and aggregative cells take advantage of the cholesterol available in the growth medium, which contains 10% fetal calf serum with a cholesterol concentration of approximately 5 mg ml⁻¹. More interestingly, the data strongly suggest that exogenous cholesterol is incorporated by these cells and partially transformed into ergosterol by a previously unknown metabolic pathway (figure 6a). More than 80% of incorporated cholesterol remains as such in the cell membranes, whereas the rest is converted into ergosterol (figure 4). Three modifications on the cholesterol moiety are necessary for this conversion: (i) the introduction of a double bond at C7(8) of the B ring, (ii) the introduction of a double bond at position C22(23) of the lateral chain, and (iii) the ramification of the lateral chain by addition of a methyl group at C24 (figure 3). Notably, the three genes coding for the enzymes necessary to carry on the abovementioned modifications (i.e. Rieske sterol C7-desaturase, sterol C22-desaturase and SMT) are strongly upregulated in the active stages (figure 3b).

Hence, the production of 7-dehydrocholesterol in C. owczarzaki is explained by the action of the Rieske sterol C7-desaturase on the serum-derived cholesterol (2 in figure 4). Similarly, the triple unsaturated derivative 7,22-bis-dehydrocholesterol (1) would be the product of the P450 sterol C22-desaturase acting on 3 (figure 4). As expected, no early sterol precursor, namely lanosterol nor any other 4,4-dimethyl sterol, was identified in the GC/MS profile, further reinforcing the lack of neosynthesis in these stages. Furthermore, we have demonstrated the production of [14C]-ergosterol (4), as well as the intermediary products [14C]-7-dehydrocholesterol (3) and [14C]-7,22-bis-dehydrocholesterol (1), by supplementing C. owczarzaki cultures with [14C]-cholesterol (2) (figure 4; electronic supplementary material, figure S4).

In the canonical ergosterol synthesis pathway of yeasts (figure 2 or figure 3a), the transfer of the methyl group from S-adenosyl-methionine to the sterol Δ24(25) unsaturated chain by SMT produces a sterol with a C24(28) methylene that is reduced in the last step of the pathway by the sterol Δ24(28)-reductase, ERG4 [45]. Concordantly, we identified...
a putative tetra-unsaturated sterol in the GC/MS profile that could correspond to ergosta-5,7,9(11),22-tetaenol (peak 5 in figure 4; C_{28}D_{5}) and brassicasterol (peak 7 in figure 4; C_{29}D_{5}) (table 2 and figure 4a; electronic supplementary material, figure S3) can be considered as intermediary products of alternative pathways by using the same set of enzymes, leading ultimately to the same end product: ergosterol (figure 6a). Another two tetra-unsaturated sterols detected in our analysis were identified as cholesta-5,7,9(11),22-tetraenol (peak 5 in figure 4; C_{28}D_{5}) and ergosta-5,7,9(11),22-tetraenol (peak 8 in figure 4; C_{29}D_{5}) (table 2 and figure 4e; electronic supplementary material, figure S3). Sterols of this series were previously reported in S. cerevisiae [48], nematodes [49], poriferans of the class Demospongiae [18,50] and Calcispongiae [51], and in the choanoflagellate M. brevicollis [25].

The capacity of C. owczarzaki to uptake and modify cholesterol is in consonance with its potential symbiotic lifestyle, as the host snail’s haemolymph is a rich source of sterols [52]. Commitment into one route or the other might depend on the opportunity of the cells to incorporate exogenous sterols. We hypothesize that de novo sterol synthesis in filopodial and aggregative cells could be inhibited by the cholesterol incorporated from the medium (figure 4a). A similar process occurs in Trypanosoma brucei, where de novo synthesis of ergosterol is severely repressed by blood cholesterol in mammalian stages of the parasite [41], although a basal synthesis persists, ensuring the presence of trace amounts of ergosterol as a signalling factor to spark cell proliferation [53,54]. In the ciliate Tetrahymena thermophila, the synthesis of the sterol surrogate tetrahymanol is completely repressed after the cells incorporate exogenous sterols [55]. The chance of importing sterols from the environment is also advantageous for these organisms and for C. owczarzaki.

In principle, only four enzymatic activities are involved in the production of ergosterol from cholesterol (figure 6a), compared with the more than 12 enzymatic steps required for ergosterol synthesis from squalene, as occurs in fungi [13]. Interestingly, the two alternative pathways described here are tightly controlled at the transcriptional level (figure 3b). Sterol import would be probably impeded in cystic cells, triggering the expression of genes for de novo synthesis of 7-dehydrocholesterol (figure 6b). The way the alternative pathways are controlled, and especially the peculiar subset of genes used by filopodial and aggregative cells, as well as their high levels of expression, suggest that ergosterol has an important function in the membranes of these cells. Taking into account the differential expression of the components of the integrin adhesome and signalling partners during these stages [30], proteins known to be associated with lipid rafts [56,57], it will be interesting to investigate the possible involvement of ergosterol in the formation of membrane domains, and hence on the activity of these proteins and their role in aggregative multicellularity.

4. Material and methods

4.1. Cell culture conditions

Capsaspora owczarzaki cells were grown axenically in 25 to 75 cm² flasks containing either 7 or 25 ml of ATCC 1034 medium of the following composition: 1% Bacto peptone, 1% yeast extract, 0.1% ribonucleic acid type VI from Torula yeast, 15 mg l⁻¹ folic acid, 1 mg l⁻¹ haemin, 10% fetal bovine serum, 2% phosphate buffer (18.1 g l⁻¹ KH₂PO₄. 25 g l⁻¹ Na₂HPO₄). Cultivation was carried out in a 23°C incubator. Adherent filopodiated cells were obtained by starting cultures of approximately 5 x 10⁶ cells ml⁻¹, after stationary incubation during 3–4 days. Aggregates formation was induced by initiating cultures of approximately 1.5 x 10⁶ cells ml⁻¹ and incubated with gentle agitation at 70 r.p.m. for 4–5 days. Floating cystic cells were obtained from 14-day-old stationary cultures, started in the same conditions as the adherent cultures.

4.2. Gene expression profiling from RNAseq data

Gene expression analysis was performed using the RNAseq dataset from C. owczarzaki obtained by Sebé-Pedrós et al. [30], publicly available at NCBI (http://www.ncbi.nlm.nih.gov/BIOSAMPLE/?term=txid599528[Organism:noexp]). Reads were aligned to the reference genome using TopHat [58] with default parameters. Significant differential expression was calculated by performing pairwise comparisons with DESeq [39] (threshold 1 x 10⁻⁵), EdgeR [60] (threshold 1 x 10⁻⁵), CuffDiff [58] (threshold 1 x 10⁻⁵) and NOISeq [61] (threshold 0.8). To be considered as differentially expressed, genes needed to be significant at least in three out of the four methods used.

4.3. Identification of sterols by gas chromatography–mass spectrometry

Cells from 4-day-old cultures (adherent) were collected by centrifugation at 3000 g for 5 min at 4°C, washed twice with 20 ml of 1X phosphate saline, and the lipids were extracted according to Bligh & Dyer [62]. The organic phase was evaporated to dryness under a N₂ stream, and the residue was resuspended in 50 µl of acetic anhydride and incubating for 40 min at 80°C. The samples were analysed by running through an SPB-1 column (30 m x 0.25 mm x 0.25 µm; Supelco) in a Shimadzu GC-2010 Plus gas chromatograph. The column was temperature programmed at 5°C °C min⁻¹ from 160 to 320°C and subsequently held for 10 min at 320°C. MS was carried out using a GCMS-QP2010 Plus mass detector, operated at an ionization voltage of 70 eV with a scan range of 20–600 atomic mass units. The retention
times and mass spectra of all new peaks obtained were compared with those of standards (Sigma-Aldrich), as well as those available in the literature and in the National Institute of Standards and Technology mass spectral library.

4.4. Whole-cell radiolabelling and analysis of sterols by thin-layer chromatography and high-pressure liquid chromatography

*Capsaspora owczarzaki* cultures were initiated from a 1/100 subculture from approximately 5 x 10^8 cells ml^-1 initial culture and incubated in ATCC 1034 medium at 23°C with the addition of either 5 μl [4-14C]-cholesterol or 10 μl [1-14C]-acetic acid (both with specific activity of 55 mCi mmol^-1; American Radiolabeled Chemicals Inc., St Louis, MO) to a final concentration of 2 or 25 μM, respectively. After the appropriate incubation time, cultures were collected by centrifugation, and lipids were extracted following the methodology of Bligh & Dyer [62] with some modifications. In short, 6 ml of a mixture of chloroform–methanol (1 : 2 by volume) was subjected to reverse-phase HP-TLC in Silicagel 60 RP-18 chromatography using BioMax MR film (Kodak). The retention factors (R_f) for the different compounds were determined with commercial standards from Sigma-Aldrich (cholesterol, 7-dehydrocholesterol and ergosterol) or, in the case of 7,22-bis-dehydrocholesterol, by using a sterol extract obtained from culturing *Tetrahymena thermophila* for 72 h in a medium supplemented with cholesterol [34]. Whole lipid extracts from cultures supplemented with [14C]-acetate were analysed in Silicagel 60 TLC plates (Merck Millipore) using a mixture of hexane–diethyl ether–acetic acid (40 : 9 : 1 by volume) as developing solvent, and revealed by autoradiography, whereas the non-radioactive sterols were identified by treating the HP-TLC plate with ethanol:Cu-phosphoric reagent 1:1 at 120°C for 20 min [63]. Sterols from [4-14C]-cholesterol or [1-14C]-acetic acid labelled cultures were subjected to HPLC using a Phenomenex Luna C18 column (5 μm, 250 x 4.6 mm). HPLC was developed at 40°C with acetonitrile/water (97 : 3% v/v) isocratically, with a flow rate of 1 ml per min.

Data accessibility. Gene expression analysis was performed using the RNAseq dataset from *C. owczarzaki* obtained by Sebe-Pedrós et al. [30], publicly available at NCBI 440 (http://www.ncbi.nlm.nih.gov/bioproject/?term=txid595528[Organism:noexp]). The remaining data supporting this article have been uploaded as part of the electronic supplementary material.

Authors’ contributions. S.R.N. and A.D.U. conceived, designed and coordinated the study; S.R.N., M.C.M and A.D.U. carried out the experimental work and collected the data; I.R.-T. provided new experimental data; S.R.N., A.D.U. and I.R-T. interpreted the data and helped draft the manuscript. All authors gave final approval for publication.

Competing interests. We declare we have no competing interests.

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