Sterol biosynthesis and phytosterol bioconversion in *Crassostrea gigas* larvae

Fiz da Costa[1,2], Margaux Mathieu-Resuge[1,3], Fabienne Le Grand[1], Claudie Quéré[1], Gabriel V. Markov[4], Gary H. Wikfors[5], Philippe Soudant[1*]

[1] Univ Brest, CNRS, IRD, Ifremer, LEMAR, F-29280 Plouzané, France

[2] Present address: Instituto Español de Oceanografía (IEO, CSIC). Centro Oceanográfico de Vigo. Subida a Radio Faro, 50, 36390 Vigo, Spain

[3] Univ Brest, Ifremer, UMR BEEP, F-29280 Plouzané, France

[4] Sorbonne Université, CNRS, Integrative Biology of Marine Models (LBI2M, UMR8227), Station Biologique de Roscoff (SBR), Roscoff, France

[5] Northeast Fisheries Science Center, NMFS, NOAA, 212 Rogers Avenue, Milford, CT, 06460, USA

[*]Corresponding author: *tel:+33298498623; fax: + 33298498645; e-mail address: soudant@univ-brest.fr*

Highlights

- A mass-balance approach compared dietary and larval sterol compositions.
- Pacific oyster larvae synthetized *de novo* sterols under low dietary sterol supply.
- Larval sterol biosynthesis was modulated by sterol dietary supply.
- Dietary sterols were bioconverted under all dietary conditions.
- 13 ng cholesterol larvae^{-1} is necessary to achieve competence for metamorphosis.

Abstract

Dietary sterols are important for bivalve larval growth and survival. Sterol incorporation in Pacific oyster *Crassostrea gigas* larvae has been studied here by means of a flow-through larval rearing technique allowing accurate quantification of microalgal ingestion. Two sets of experiments were done using two cultured microalgae: *Tisochrysis lutea* (T) and *Chaetoceros neogracile* (Cg) as mono- and bi-specific diets. Accumulation of tissue sterols in oyster larvae, in addition to those present in the diet, indicate that *C. gigas* larvae appear to have the ability to synthetize sterols *de novo* under low dietary sterol supply,

e.g., when fed the T diet. Sterol synthesis was dependent upon sterol dietary supply; larvae fed T exhibited greater sterol incorporation at the pediveliger stage than larvae fed TCg (2.7 *vs* 1.3-fold sterol incorporation compared to sterol ingestion, respectively). Larval sterol compositions under the different dietary regimes indicate likely bioconversion pathways modifying dietary sterols. Larvae fed T bioconverted dietary brassicasterol mainly to cholesterol *via* a 22-dehydrocholesterol intermediate. Brassicasterol was also actively synthetized in larvae fed T and TCg, even though it predominated in larvae fed T, suggesting a possible metabolic role of this sterol in *C. gigas* larvae. Apparent desmosterol synthesis under all experimental conditions suggests a role as a membrane component or as an intermediate in cholesterol synthesis. Our data also indicate that *C. gigas* larvae require approximately 13 ng cholesterol $larvae^{-1}$ to achieve competence for metamorphosis.

Keywords: oyster, larvae, sterol, synthesis, bioconversion.

Abbreviations

GC, Gas chromatography; GC-MS, Gas chromatography–mass spectrometry; Cg, *Chaetoceros neogracile*; LSD, Least Significant Difference; PAR, Photosynthetically active radiation; PVC, polyvinyl chloride; T, *Tisochrysis lutea*; TCg. *T. lutea* + *C. neogracile.*

1. Introduction

Sterols are required for the maintenance of cellular membrane fluidity (Dowhan and Bogdanov, 2002). Sterol *de novo* synthesis and bioconversion pathways in mollusks remain incompletely documented, with contradictory data even for the same species (for review see Knauer and Southgate, 1999). Sterols can be elongated by C_{24}-alkylation, and sterol breakdown can occur through C_{24}-dealkylation (Knauer and Southgate, 1999). Phytosterol alkylation has been demonstrated in oysters, such as *Crassostrea gigas* and *Crassostrea virginica* (Knauer et al., 1998; Teshima and Patterson, 1981). It is generally accepted that most bivalves lack the ability to dealkylate C_{29}-sterols (Teshima et al., 1979); however, other studies demonstrated dealkylation activities in bivalves, such as the dealkylation of [^{3}H]-

fucosterol (C_{29}) to desmosterol and cholesterol (both C_{27}) in *Ostrea gryphea* and *C. gigas* (Saliot and Barbier, 1973; Knauer et al., 1998).

To our knowledge, until now, the capacity for *de novo* sterol synthesis has only been studied in bivalves using radiolabeled dietary precursors (for review see Knauer and Southgate (1999) and Giner et al. (2016)). The study of Salaque et al. (1966) was performed with 2-[^{14}C]-mevalonate and CH_3-[^{14}C]-L-methionine and lasted for 66-70 h. Such a short experimental exposure may account for the observed lack of sterol incorporation into tissues. Similarly, Walton and Pennock (1972) observed no incorporation of sterols after 24 - 32 h with injected [^{14}C]-mevalonate in *Cardium edule* and *Mytilus edulis*. The study carried out by Saliot and Barbier (1973) with *O. gryphea* using seawater containing 3-[^{3}H]-fucosterol, however, found apparent cholesterol incorporation after 48 h. Napolitano et al. (1993), in their study of incorporation of dietary sterols by the sea scallop, *Placopecten magellanicus,* fed live microalgae for 1.5 months, documented low turnover rates of sterols between bivalve tissues, which may explain why results of the above studies appeared contradictory. Moreover, Knauer et al. (1998) suggested the importance of using sufficiently long incubation periods following administration of radiolabelled precursors to detect labeled sterols. Studies of longer duration are needed to study sterol bioconversion and synthesis more thoroughly. For example, sterol bioconversion was demonstrated, but the absence of *de novo* sterol synthesis was reported, in *Crassostrea gigas* spat fed [^{3}H]-labeled sterols encapsulated in gelatin-acacia microcapsules (GAM) (Knauer et al., 1998) for 7 days. Teshima and Patterson (1981) observed sterol synthesis and bioconversion after injection of 2-[^{14}C]-acetate and 2,3-[^{3}H]-lanosterol in *C. virginica* with incubation periods of 7 and 17 days. More recently, Giner et al. (2016) used ^{13}C-labeled phytosterols to supplement the unialgal diet *Rhodomonas* sp. to study the bioconversion of phytosterols to cholesterol by the Northern Bay scallop *Argopecten irradians irradians.* This study showed that scallops efficiently dealkylated Δ^5 C_{29} (24-ethyl) sterols to cholesterol, and the only C_{28} sterol that was dealkylated efficiently possessed the 24(28)-double bond. Taken together, the relatively few studies of sterol metabolism in bivalves support no clear consensus about the sterol metabolism abilities of bivalve mollusks. This study examined accumulation of tissue sterols in *C. gigas* larvae fed very precisely controlled microalgal diets that differed in sterol content and profile and proposes previously unreported sterol bioconversion and synthesis pathways in this bivalve species.

The flow-through bivalve larval rearing system used in the present study allows the maintenance of a constant food density in culture tanks and, accordingly, precise determination of microalgal

consumption, enabling calculation of microalgal sterol incorporation by larvae. A main advantage of our approach is consistent and precise provision of larvae with live microalgae, which contrasted with previous studies related to sterol bioconversion wherein sudden and episodic sterol additions were often done. Indeed, a number of previous studies were performed either with unfed animals, or supplied marked sterols encapsulated in GAM, dissolved in seawater, or injected (e.g. Saliot and Barbier (1973); Trider and Castell (1980); Teshima and Patterson (1981); Knauer et al. (1998)). Our method also allows the use of several phytosterols extending the limited variety delivered in other sterol metabolism studies (for review see Knauer and Southgate (1999)). Lastly, we examined a complete developmental stage (larval stage), contrasting with aforementioned literature. Our approach is, accordingly, more integrative and innovative.

2. Materials and methods

2.1. Microalgal culture

Two microalgae were cultured: *Tisochrysis lutea* (Tiso, formerly known as *Isochrysis* affinis *galbana*, named here T, volumetric size, 45 μm^3, strain CCAP 927/14) and *Chaetoceros neogracile* (named here Cg, 77 μm^3, UTEX LB2658). Microalgae were grown in 6-L glass carboys at 20-23°C under continuous illumination (180-220 µmol photons m^{-2} s^{-1} PAR) provided by cool-white fluorescent tubes. Seawater (salinity 34-35 ‰) was filtered to 1 µm, enriched with sterilized Conway medium (400 mg $NaNO_3$) (Walne, 1966), and then autoclaved. A 3% CO_2-air mixture was supplied to support growth and to maintain the pH within a range of 7.5-8.1. For culture of the diatom *C. neogracile*, sodium metasilicate (40 mg L^{-1}) was added as a silica source and salinity was reduced to 25 ‰ by addition of distilled water. Microalgae were harvested in late-logarithmic phase (after 3-4 days).

2.2. Broodstock conditioning and larval culture

Broodstock were collected from Aber Benoit (Brittany, France) and transferred to Argenton hatchery facilities for conditioning. Individuals were maintained in flow-through tanks at 19ºC for 2 months with a daily supply of a mixed diet of *T. lutea* and *C. neogracile* equivalent to 6% of oyster dry weight in dry weight of algae per day. Gonads of the broodstock were stripped for gamete harvesting,

using a pool of 11 males and 4 females. After gamete counting, fertilization was performed at a ratio of 50 spermatozoans per oocyte. Two hours later, the embryos were incubated in cylindro-conical tanks of seawater filtered to 1 µm at 22ºC. After 48 hr of incubation, the percentage of D-larvae was determined microscopically. Veligers were transferred to 5-L, translucent, methacrylate cylinders and reared in the flow-through system as described by da Costa et al. (2016). A continuous, filtered, UV-treated seawater flow of 0.87 ml min^{-1} was provided from the bottom of each experimental tank (100% tank water renewal per hour). Each diet was delivered by pumping from a reservoir, which was cleaned and filled with the appropriate feed daily, directly to the larval tanks down the seawater line. Seawater was 1-µm-filtered and UV-treated. Temperature was maintained at 25°C using a thermo-regulated automatic valve, and ambient salinity was 34‰. In each tank, aeration, provided from the bottom to maximize circulation of water within the tank, was set at 30 ml min^{-1}. The outlet of each tank was equipped with a beveled, polyvinyl chloride (PVC) pipe as a sieve to prevent larvae from escaping. Mesh sieve sizes of 40, 60 and 80 µm were used at the beginning of the experiment, on days 6 or 7, and day 10, respectively.

Two experiments were performed to study sterol assimilation by *C. gigas*. In the first, two single diets (T or Cg) and bi-specific diet (TCg) were compared. In this experiment, all groups of larvae were reared until they reached competency (19 days post-fertilization for the lowest group). A second, follow-up trial using only two diets (T and TCg) was performed to confirm and validate results observed in the first experiment (sterol synthesis and bioconversion). The second experiment lasted 15 days (i.e. until larvae fed TCg reached competency). Each experimental treatment consisted of three replicate tanks of larvae. Larvae were fed different single- and bi-specific microalgal diets at a constant concentration of 1500 μm^3 μl^{-1} at the exit of the rearing tanks, corresponding to ≈ 40 algal cells μl^{-1} (equivalent *T. lutea*: Rico-Villa et al. (2010)). Seawater at inlet and outlet of each experimental tank was sampled twice a day to determine phytoplankton consumption. Cell counts were done using an electronic particle counter (Multisizer 3) equipped with a 100-µm aperture tube. Survival was checked every other day for larval grazing determinations. Larvae were reared at a density of 100 and 50 larvae ml^{-1} for experiments 1 and 2, respectively, both densities leading to equivalent larval survival and growth in such flow-through systems (Petton et al., 2009).

2.3. Biochemical analysis

Samples of each microalgal diet (250-300 x 10^6 cells) were collected during all larval culture (diets were temporally replicated, n = 5 and n = 7 for experiments 1 and 2, respectively). Thus, food composition data represent means and variances of fatty acid measurements throughout larval rearing. Each larval replicate for all treatments was sampled at day 2 (D-shaped larvae, start of feeding), on day 8, and when the positive control (TCg) reached the pediveliger stage (day 16 or 15, for experiments 1 and 2, respectively). In the first experiment, larval samples were also collected when larvae fed other diets reached the pediveliger stage (day 18 or 19, for larvae fed T or Cg, respectively). Samples were washed with 3.5% ammonium formate to remove salt and collected on 450ºC pre-combusted GF/F glass fiber filters (Whatman, diameter 47 mm). Lipids were extracted in 6 ml chloroform-methanol (2:1, v/v) according to Folch et al. (1957), sealed under nitrogen, and stored at -20°C.

Sterols in microalgae and larvae were hydrolyzed with sodium methoxide (MeONa) for 90 min at room temperature as described by Soudant et al. (2000). The sterols were extracted in hexane and injected directly into the gas chromatograph (GC) Chrompak CP 9002 (Varian Inc., Walnut Creek, CA, USA) equipped with a Restek RTX65 fused silica capillary column (15 m x 0.25 mm, 0.25 µm film thickness) using an on-column injection system. Hydrogen was used as the carrier gas with a temperature gradient from 160 to 280 ºC. The sterols were identified by comparison of their retention times with commercial standards and in-house standard mixtures from marine bivalves, and cholestane was used as an internal standard. Sterol identification in larvae was also confirmed by GC mass spectrometry (GC-MS). A 1-mL aliquot of the total lipid extract was saponified and silylated as described in Mathieu-Resuge et al. (2019). Then, derived sterols were injected in a Shimadzu QP2010-SE GC-MS equipped with a Restek RTX65 column (30m x 0.25 mm ID, 0.25µm). The GC temperature was programmed as follows: from 60°C to 250°C at 60°C/min, then to 280°C at 2°C/min and maintained for 5 min. The GC was equipped with a splitless injector slated at 270 °C; whereas, the mass spectrometer was equipped with an ion source programmed at 250°C, and an interface at 250°C. Mass spectra were generated with the scan mode, from 50 to 1000m/z. Trivial and systematic names of the detected sterols were: 22-dehydrocholesterol (cholesta-5,22E-dien-3β-ol) ($C_{27}H_{44}O$), Dihydrocholesterol (cholestan-3β-ol), ($C_{27}H_{48}O$), Cholesterol (cholesta-5-en-3βol) ($C_{27}H_{46}O$), Brassicasterol (ergosta-5,22E-dien-3β-ol) ($C_{28}H_{46}O$), Desmosterol (cholest-5,24-dien-3β-ol) ($C_{27}H_{44}O$), Campesterol (campest-5-en-3β-ol) ($C_{28}H_{48}O$), 24-methylenecholesterol (24-methylcholesta-5,24-dien-3β-ol) ($C_{28}H_{46}O_2$), Stigmasterol (stigmasta-5,22E-dien-3β-ol) ($C_{29}H_{48}O$), 4-α-methyl poriferasterol (4-α-methyl-24-α-ethylcholesta-22-en-

182 3β-ol) ($C_{30}H_{52}O$), ß-Sitosterol (stigmast-5-en-3β-ol) ($C_{29}H_{50}O$), Fucosterol (24E-ethylidene-cholest-5-en-
183 3β-ol) ($C_{29}H_{48}O$) and Isofucosterol (24Z-ethylidene-cholest-5-en-3β-ol) ($C_{29}H_{48}O$).
184
185 *2.4. Data treatment and statistical analysis*
186
187 In the present work, we focused on sterol metabolism between day 8 and days 15-16 because the
188 first 5-6 days of larval life in *C. gigas* correspond to a period during which larvae feeding changes from
189 lecitotrophic to exotrophic. During the transition (mixotrophic) period, exogenous feeding is low (during
190 the gradual activation of digestive enzymes in early larvae), and larvae are still relying upon the energy
191 and material reserves contained within the embryo itself (Rico-Villa et al., 2009; Ben Kheder et al.,
192 2010). Moreover, total sterol contents decrease during the mixotrophic phase in bivalves, specially steryl
193 esters which are fully depleted (Soudant et al., 1998). Accordingly, both processes may mask sterol
194 bioconversion and synthesis. We also report sterol incorporation during the entire larval phase, between
195 day 2 and competence for metamorphosis (Experiment 1: day 16 for TCg; day 18 for T and day 19 for
196 Cg; and experiment 2: day 15 for TCg), to determine cholesterol requirement for the entire larval phase.
197 We compared the quantity of ingested sterols (ng $larvae^{-1}$) with the accumulated counterpart by
198 larvae between two sampling dates. For each experiment, sterol ingestion was calculated using mean
199 sterol composition for each diet (n = 5 or n = 7 for experiments 1 or 2, respectively) and microalgal
200 consumption for each diet treatment. When a sterol was present in larvae but absent from the diet, we
201 interpreted this to indicate that this sterol was of endogenous origin (through biosynthesis *de novo* or
202 bioconversion of another dietary sterol). When the accumulated amount of a sterol by larvae was above
203 100% of the ingested amount, we expected that this sterol had both exogenous and endogenous origins.
204 Data normality first was evaluated using the Shapiro-Wilk test, and then one-way analysis of
205 variance (ANOVA) for significant differences was performed using STATISTICA software (Stat Soft,
206 Inc., Tulka, OK, USA, version 12). Homogeneity of variances was checked by means of the Barlett test.
207 When necessary, *post hoc* analyses with the Least Significant Difference (LSD) test were applied to test
208 differences among treatments for the sterols ingested and incorporated. Percentage data were arcsine-
209 transformed to normalize variance (Sokal and Rohlf, 1995). Differences were considered statistically
210 significant if $P \leq 0.05$.
211

2.5. Comparative genomics analysis

To determine the known presence of sequences coding for enzymes involved in sterol metabolism, protein sequences for candidate Δ24 sterol reductase (DHCR24) were retrieved from previous studies (Desmond and Gribaldo, 2009; Belcour et al., 2020), and the dataset was completed by targeted BLAST searches on the NR database on NCBI. A maximum phylogenetic tree was built using the phyML version implemented in Seaview (Gouy et al., 2010), using the LG model with a gamma law and an approximate likelihood ratio test (aLRT) to assess branch robustness.

3. Results

3.1. Experiment 1: two single diets (T or Cg) and bi-specific diet (TCg)

Larvae receiving T and TCg incorporated more total sterols than their dietary consumption between day 8 and 16 (8.2 *vs* 3.5 and 17.1 *vs* 12.2 ng of total sterol incorporation *vs* ingested per larva in T and TCg respectively: Table 1). Larval sterol contents corresponded to 233 and 139% of total supplied sterol, respectively. In contrast, total sterols incorporated by larvae fed Cg were lower than sterols ingested (6.3 *vs* 11.3 ng of total sterol per larva: Table 1). Brassicasterol incorporation by larvae fed T and TCg exceeded brassicasterol ingested by larvae (6.6 *vs* 3.5 and 6.3 *vs* 4.9 ng of total sterol per larva in T and TCg, respectively: Table 1). Larvae fed Cg incorporated only 3.8 ng of cholesterol per larva of the 6.4 ng ingested, thus representing 58% assimilation (Table 1). Similarly, only a half of the 24-methylene-cholesterol ingested was incorporated by larvae receiving Cg (0.6 *vs* 1.0 ng per larva: Table 1). Incorporation of fucosterol in larvae fed Cg was even lower, 30% (Table 1). Cholesterol incorporated by larvae fed TCg was 1.5-fold the quantity supplied in the diet (6.8 *vs* 4.4 ng $larva^{-1}$: Table 1).

Larvae fed T accumulated cholesterol (0.4 ng $larva^{-1}$) when none was present in the diet, implying *de novo* synthesis. These larvae also appear to have synthetized significant quantities of desmosterol, β-sitosterol, 22-dehydrocholesterol, 24-methylene-cholesterol, campesterol, and stigmasterol (Table 1). Desmosterol was also presumably synthetized in both larvae receiving Cg and TCg (0.5 and 1.2 ng $larva^{-1}$, respectively, Table 1). Secondarily, larvae fed Cg produced brassicasterol and dihydrocholesterol, with 5-fold lower synthesis activity compared to desmosterol synthesis (Fig. 2B). Larvae fed TCg also accumulated a small amount of β-sitosterol (0.2 ng $larva^{-1}$: Table 1).

3.2. Experiment 2: two diets (T and TCg)

Total quantities of sterols accumulated within larvae fed T and TCg between days 8 and 15 were higher than the quantities ingested (5.9 *vs* 1.8 and 22.3 *vs* 13.7 ng of total sterol per larva in T and TCg, respectively: Table 2). Accumulation of total sterols by larvae fed T and TCg were 323 and 163% of the ingested sterol amounts. Similarly, brassicasterol accumulation by larvae fed T and TCg surpassed 2.7 and 1.8 times the quantities ingested, respectively (Table 2). Cholesterol was presumptively synthetized in larvae fed TCg (6.3 ng per larva ingested *vs* 9.3 ng per larva incorporated: Table 2). In contrast, incorporation of 24-methylene-cholesterol and fucosterol was lower than ingestion in larvae fed TCg (Table 2).

Larvae fed T, which supplied brassicasterol only, mainly synthetized cholesterol (0.3 ng $larva^{-1}$), and in a lesser extent desmosterol (0.2 ng $larva^{-1}$: Table 2). Desmosterol was actively synthetized *de novo* (no known pathway from brassicasterol) by larvae fed TCg (2.1 ng $larva^{-1}$: Table 2).

3.3. Sterol contents within competent larvae

At competence, total sterol accumulation substantially exceeded sterol ingestion in larvae fed T (17.0 *vs* 6.3 ng total sterol incorporation *vs* ingested per larva, data calculated between days 2 and 18; Table 3). Incorporation of brassicasterol was twice dietary ingestion in larvae receiving T (Table 3).

In contrast, at competency, larvae fed Cg incorporated only 66% of total sterol supplied in the diet (33.0 *vs* 21.3 ng total sterol per larva, calculated between days 2 and 19: Table 3).

Larvae fed Cg achieved competence on day 19, exhibiting 74% and 55% of cholesterol and 24-methylene-cholesterol incorporation, respectively (Table 3). Fucosterol was also poorly incorporated by larvae fed Cg (35% accumulation of ingested sterols (Table 3).

In competent larvae fed TCg in experiment 1, a higher incorporation of total sterols was also observed (18.4 *vs* 13.5 ng total sterol per larva at day 16: Table 3). Cholesterol and brassicasterol accumulation by larvae receiving TCg in experiment 1 were 148 and 129%, respectively (Table 3). Lower incorporations of 24-methylene-cholesterol and fucosterol were observed in larvae fed TCg in experiment 1 (73 and 51%, respectively between day 2 and day 16: Table 3). Similarly, larvae fed TCg in experiment

2 exhibited greater total sterol accumulation than sterol supply at competence (23.4 *vs* 15.0 ng total sterol per larva between day 2 and day 16: Table 3). In experiment 2, cholesterol and brassicasterol incorporation by larvae fed TCg was also greater than dietary supply (Table 3).

3.4. Comparative genomics analysis

A clear ortholog of the delta-24 sterol reductase widely conserved across eukaryotes was identified in oysters, as well as in two other molluscs (Figure 1). Therefore, there is a clear candidate for performing the bioconversion of sitosterol into isofucosterol and desmosterol into cholesterol (Figure 2). Using the same methodology, a search for other candidate enzymes to perform the dealkylation or 22-23 hydrogenation and dehydrogenation steps was also carried out on oyster candidate proteins but led to no conclusive result. In particular, no clear orthologue was found in the oyster predicted proteome for the sterol dealkylase described in the ciliate *Tetrahymena* (Tomazic et al., 2011), in spite of the presence of numerous proteins from the fatty acid hydroxylase superfamily in the Pacific oyster genome.

4. Discussion

In multicellular animals, cholesterol is an essential cell-membrane component. Vertebrate animals satisfy this requirement by *de novo* synthesis of cholesterol from simple precursors with what is known as the squalene-mevalonate pathway. Metabolic pathways that provide cholesterol in other animal phyla are less thoroughly understood. Phytophagous insects, perhaps the best-studied animal models outside vertebrates, have been shown to metabolically modify some but not all dietary phytosterols to cholesterol (Jing and Behmer, 2020). Bivalve mollusks, including important seafood species, have been studied less methodically. As hatchery production of shellfish seed for aquaculture becomes more widely practiced, a better understanding of what live-feed microalgae are necessary to constitute a complete diet for young stages is becoming more urgent. Sterol nutrition and metabolism of larval shellfish remains a topic in need of refined knowledge for effective expansion of shellfish farming.

This is the first study determining the incorporation of cholesterol and other dietary sterols using a flow-through larval rearing system in bivalves. This larval culture system allows accurate quantification of algal cells, and their component sterols, ingested by larvae. It represents a step forward for the study of

nutritional requirements of bivalve larvae, because the incorporation of individual sterols can be calculated using a mass-balance approach, and optimum levels of dietary sterols can be determined.

A key finding of this study is that, when *C. gigas* larvae were supplied with low-sterol diets (i.e., with T and TCg), they were able to partially bioconvert dietary phytosterols. Furthermore, total sterol accumulation in larvae fed T surpassed sterol ingested between day 8 and day 15-16 in both experiments (238 and 325% of total ingested sterol in experiments 1 and 2, respectively), implying *de novo* synthesis. Similarly, larvae fed TCg likely synthetized *de novo* sterols, because total sterol accumulation in larvae was 137 and 156% of total ingested sterols at competence (day 15-16) in experiments 1 and 2, respectively. Conversely, total sterol accumulation in larvae fed Cg was only 65% of total ingested sterols, possibly because higher dietary sterol supply by Cg than T (132 *vs* 17 fg cell^{-1} (da Costa et al., 2016). Teshima and Patterson (1981) reported that adult *Crassostrea virginica* biosynthesized *de novo* cholesterol, desmosterol, isofucosterol, and 24-methylenecholesterol from injected [2-^{14}C]-acetate through squalene, probably using the lanosterol pathway. Moreover, *Ostrea gryphea* was capable of incorporating sterols when 3-[^{3}H]-fucosterol propionate was dissolved in seawater (Saliot and Barbier, 1973). Conversion of [2-^{14}C]-mevalonate to cholesterol, desmosterol, 22-dehydrocholesterol, and 24-methylenecholesterol was tentatively demonstrated in *Mytilus edulis* (Teshima and Kanazawa, 1974). In contrast, *C. gigas* spat were unable to synthetize *de novo* sterols when fed gelatin-acacia microcapsules containing ^{3}H-squalene (Knauer et al., 1998). Sterol synthesis from [^{14}C]-acetate was not detected in *C. virginica* heart tissue culture (Holden and Patterson, 1991). These authors, however, did not exclude the possibility of sterol synthesis in other oyster tissues and/or life stages. Despite the inconsistent literature, our data suggest that sterol biosynthesis may take place in *C. gigas* larvae under low sterol dietary supply. We note that this synthesis may be species- and stage-dependent.

Larvae fed T accumulated significant amounts of cholesterol in both experiments (0.28-0.46 ng larva^{-1}), with this sterol absent from the microalgae fed. We attribute this to sterol bioconversion or synthesis *de novo* of sterol. Brassicasterol (C_{28}) can be bioconverted into cholesterol (C_{27}) in the heterotrophic protists *Oxyrrhis marina* and *Gyrodinium dominans* (Chu et al., 2008; Lund et al., 2009). Brassicasterol (C_{28}) is dealkylated to 22-dehydrocholesterol (C_{27}) by the removal of a methyl group at C24 and subsequently 22-dehydrocholesterol is hydrogenated at C22 to cholesterol (C_{27}) (Lund et al., 2009) (Fig. 2). 22-dehydrocholesterol was also detected in oyster larvae fed T, ranging from 0.1 to 0.2 ng larva^{-1}. Brassicasterol transformation to cholesterol in *Artemia salina* has also been reported (Teshima

and Kanazawa, 1972). Larvae fed Cg could have transformed cholesterol to brassicasterol by the reverse aforementioned pathway (Fig. 2), as suggested by Lund et al. (2009). Strikingly, the high level of brassicasterol accumulation in oyster larvae suggest that it is likely that brassicasterol was also synthetized *de novo* in larvae fed T (190-270% of incorporation) and TCg (130-182% of incorporation), even with a high dietary supply in T. Brassicasterol is synthetized in higher plants, microalgae, and fungi from squalene (Arnqvist et al., 2008; Pereira et al., 2010). More recently, *de novo* sitosterol synthesis has been demonstrated in some annelids (Michellod et al., 2023); however, to our knowledge brassicasterol synthesis was never previously reported in mollusks. Such brassicasterol synthesis in larvae fed T (i.e., diet lacking cholesterol) and TCg support the assertion of Voogt (1975) who highlighted the importance and possible role of brassicasterol in bivalves, which might replace cholesterol in some of its structural and/or physiological functions. Another possible explanation may be linked to membrane fluidity. Sterol chain length and unsaturation (bonded at C20) can influence membrane microviscosity (Soudant et al., 1998). Brassicasterol (28C5,22) created a more rigid structure in the membrane than cholesterol in the freshwater mollusk *Diplodon delodontus* (Irazu et al., 1984). Da Costa et al. (2016) reported that *C. gigas* larvae fed T actively synthetized *de novo* the essential fatty acids 20:4n-6 and 20:5n-3 and non-methylene-interrupted fatty acids from fatty acid precursors found in the diet. The increase in unsaturated fatty acids in the membranes of *C. gigas* larvae fed T may have increased the fluidity of membranes and thus the incorporation of brassicasterol in membranes may be a compensating mechanism to maintain homeostasis of membrane fluidity.

Fucosterol was supplied by *C. neogracile* in larvae fed Cg and TCg, but its incorporation was low after 16 days of larval development (27 and 51% in experiment 1, respectively). This could be attributable to fucosterol (C_{29}) bioconversion to cholesterol (C_{27}) by dealkylation at C24 as described in the oyster *Ostrea gryphea* (Saliot and Barbier, 1973) (Fig. 2). The isomers isofucosterol/fucosterol can be bioconverted to cholesterol by the scallop *Argopecten irradians* (Giner et al., 2016). In contrast to low fucosterol incorporation, desmosterol (C_{27}) appeared to be actively synthetized (1.3-2.2 ng $larva^{-1}$ in larvae fed TCg at day 15-16). Desmosterol is an intermediate in bioconversion of fucosterol to cholesterol (Saliot and Barbier, 1973). Desmosterol is reduced by Δ24-sterol reductase to cholesterol (Fig. 2), as demonstrated in the prawn *Penaeus japonicus* and the oyster *C. gigas* (Irazu et al., 1984; Knauer et al., 1998). In the present study, genomic analysis reveals a clear ortholog of Δ24-sterol reductase in oysters. Desmosterol was also actively synthetized in larvae fed Cg (0.6 ng $larva^{-1}$, between days 2 and 16) and T

(0.15 ng larva^{-1}, between days 2 and 15-16 in both experiments). Desmosterol synthesis observed under all experimental conditions suggests the importance of desmosterol as an intermediate in sterol bioconversion to cholesterol in *C. gigas* larvae. This agrees with the observation by Giner et al (2016) who found relatively large amounts of labeled desmosterol in all cases in which the test sterol was converted into cholesterol.

Assimilation of 24-methylene-cholesterol in larvae fed Cg was lower (55%) than those receiving TCg (73%) at competence. Bioconversion to cholesterol may contribute to relatively low 24-methylene-cholesterol level in TCg-fed larvae by the alkylation of desmosterol (C_{27}) to 24-methylene-cholesterol (C_{28}), as previously demonstrated in adult *C. gigas* (Knauer et al., 1998) (Fig. 2). Although it is generally accepted that most bivalves lack the ability to dealkylate C_{29}-sterols (Teshima et al., 1979), dealkylation of fucosterol to desmosterol was observed in *C. gigas* (Knauer et al., 1998) and *O. gryphea* (Saliot and Barbier, 1973).

Cholesterol incorporation in larvae fed Cg (74% of ingested cholesterol during the entire period of larval development), which is rich in cholesterol, is in accordance with general food-assimilation efficiency reported in bivalves, which is approximately 70% (Bayne et al., 1999). In larvae fed TCg, we observed incorporation of cholesterol exceeding 100% of ingested cholesterol in both experiments (139-148%). Consequently, its synthesis is likely, consistent with a lower dietary supply in the mixed diet TCg as compared to Cg alone. Our data allowed us to propose an optimal supply of 13 ng cholesterol per *C. gigas* larvae to reach competence. Further studies with varying cholesterol dietary contents, however, should allow establishment of more accurate cholesterol requirements. Moreover, this approach could also be used to determine any dietary requirements for other sterols.

Beyond the identification of the clear ortholog of Δ24-sterol reductase. Our attempts to confirm other sterol metabolic pathways within the genome of the Pacific oyster provided inconclusive results. Annotation efforts are ongoing, and we recommend that attention be given to sequences coding for enzymes involved in the specific pathways implied by our findings and those of others investigating sterol metabolism in bivalves.

In conclusion, the use of a flow-through larval rearing system coupled with a mass-balance approach allowed the calculation of incorporation and metabolic modification and synthesis of dietary sterols. This study represents a step forward in the knowledge of the incorporation of dietary components in bivalves and links to biological performance. The present work has established foundations for the

determination of essential and important biochemical components in bivalve larvae nutrition. Future studies using this methodology should be carried out to determine sterol requirements, limitation and synthesis. Moreover, the present technique could be coupled with [^{13}C]-labeled microalgae using GC-c-IRMS to investigate sterol bioconversion pathways in different bivalve species and life stages. Our findings suggest that *C. gigas* larvae are capable of accomplishing not only sterol bioconversion, but also *de novo* sterol synthesis, using a combination of well-conserved and lineage-specific enzymes. Cholesterol and brassicasterol synthesis were observed and desmosterol could be a metabolic intermediate in sterol synthesis or an important membrane component.

Acknowledgements

F. da Costa acknowledges the Fundación Juana de Vega (Spain) for the postdoctoral fellowship at Ifremer. Special thanks to Dr. René Robert who was the postdoctoral supervisor of F. da Costa during his stage at Ifremer. Recently retired, he refused to be one of the co-authors despite his active involvement in the work that lead to this manuscript. The authors would like to acknowledge the technical staff of the experimental mollusk hatchery of Argenton, B. Petton, C. Mingant, I. Quéau and L. Lebrun for assistance in broodstock conditioning, larval rearing and phytoplankton production. The authors would like to thank Jean Girard who participated in the implementation of the sterol analysis method in GC-MS.

Funding

This work was carried out during the UE funded ReproSeed project (FP7-KBBE 245119), which contributed to its functional outlays.

CRediT authorship contribution statement

Fiz da Costa: Writing - Original Draft, Conceptualization, Methodology; Investigation, Visualization, Formal analysis, Writing - Review & Editing; **Margaux Mathieu-Resuge:** Writing - Review & Editing, Validation; **Fabienne Le Grand:** Writing - Review & Editing, Validation; **Claudie Quéré:** Methodology; Investigation, Writing - Review & Editing; **Gabriel V. Markov:** Formal analysis, Writing - Review & Editing; **Gary H. Wikfors:** Writing - Review & Editing; **Philippe Soudant:** Supervision, Conceptualization, Validation, Writing - Review & Editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All data presented in the research are available from the corresponding author on request.

References

Arnqvist, L., Persson, M., Jonsson, L., Dutta, P.C., Sitbon, F., 2008. Overexpression of CYP710A1 and CYP710A4 in transgenic *Arabidopsis* plants increases the level of stigmasterol at the expense of sitosterol. Planta 227, 309-317. https://doi.org/10.1007/s00425-007-0618-8

Bayne, B.L., Hedgecock, D., McGoldrick, D., Rees, R., 1999. Feeding behaviour and metabolic efficiency contribute to growth heterosis in Pacific oysters [*Crassostrea gigas* (Thunberg)]. J. Exp. Mar. Biol. Ecol. 233, 115-130. https://doi.org/10.1016/S0022-0981(98)00125-7

Belcour, A., Girard, J., Aite, M., Delage, L., Trottier, C., Marteau, C., Leroux, C., Dittami, S.M., Sauleau, P., Corre, E., Nicolas, J., Boyen, C., Leblanc, C., Collén, J., Siegel, A., Markov, G.V., 2020. Inferring Biochemical Reactions and Metabolite Structures to Understand Metabolic Pathway Drift. iScience, 23, 100849. https://doi.org/10.1016/j.isci.2020.100849

Ben Kheder, R., Quéré, C., Moal, J., Robert, R., 2010. Effect of nutrition on *Crassostrea gigas* larval development and the evolution of physiological indices. Part A: Quantitative and qualitative diet effects. Aquaculture 305, 165-173. https://doi.org/10.1016/j.aquaculture.2010.04.022

Blary, A., Darnet S., Berna, A., Schaller, H., 2022. Phytosterol metabolism in plants and algae, time to dive into the unknown. Adv. Bot. Res. 101, 287-343. https://doi.org/10.1016/bs.abr.2021.09.006

Chu, F-L.E., Lund, E.D., Littreal, P.R., Ruck, K.E., Harvey, E., Le Coz, J-R., Marty, Y., Moal, J., Soudant, P., 2008. Sterol production and phytosterol bioconversion in two species of heterotrophic protists, *Oxyrrhis marina* and *Gyrodinium dominans.* Mar. Biol. 156, 155-169. https://doi.org/10.1007/s00227-008-1072-2

da Costa, F., Robert, R., Quéré, C., Wikfors, G. H., Soudant, P., 2015. Essential fatty acid assimilation and synthesis in larvae of the bivalve *Crassostrea gigas*. Lipids, 50(5), 503-511. https://doi.org/10.1007/s11745-015-4006-z

da Costa, F., Petton, B., Mingant, C., Bougaran, G., Rouxel, C., Quere, C., Wikfors, G.H., Soudant, P., Robert, R., 2016. Influence of one selected *Tisochrysis lutea* strain rich in lipids on *Crassostrea gigas* larval development and biochemical composition. Aquac. Nutr. 22(4), 813-836. https://doi.org/10.1111/anu.12301

Desmond, E., Gribaldo, S., 2009. Phylogenomics of sterol synthesis: insights into the origin, evolution, and diversity of a key eukaryotic feature. Genome Biol. Evol. 1, 364-381. https://doi.org/10.1093/gbe/evp036

Dowhan, W., Bogdanov, M., 2002. Functional roles of lipids in membranes. New Compr. Biochem. 36, 1-35. https://doi.org/10.1016/S0167-7306(02)36003-4

Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497-509. https://doi.org/10.1016/S0021-9258(18)64849-5

Giner, J.L., Zhao, H., Dixon, M.S., Wikfors, G.H., 2016. Bioconversion of ^{13}C-labeled microalgal phytosterols to cholesterol by the Northern Bay scallop, *Argopecten irradians irradians*. Comp. Biochem. Physiol. B, Biochem. Mol. Biol. 192, 1-8. https://doi.org/10.1016/j.cbpb.2015.11.003

Gouy, M., Guindon, S., Gascuel, O., 2010. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Mol. Biol. Evol. 27, 221–224. https://doi.org/10.1093/molbev/msp259

Holden, M.J., Patterson, G.W., 1991. Absence of sterol biosynthesis in oyster tissue culture. Lipids 26, 81-82. https://doi.org/10.1007/BF02544030

Irazu, C.E., Pollero, R.J., Brenner, R.R., 1984. Occurrence of a 22-2 nonmethylene interrupted dienoic fatty acid and its seasonal distribution among lipids and tissues of the fresh-water bivalve *Diplodon delodontus* from an isolated environment. Lipids 19, 649-655. https://doi.org/10.1007/BF02534524

Jing, X., Behmer, S.T., 2020. Insect sterol nutrition: physiological mechanisms, ecology, and applications. Annu. Rev. Entomol. 65, 251-271. https://doi.org/10.1146/annurev-ento-011019-025017

Knauer, J., Southgate, P.C., 1999. A review of the nutritional requirements of bivalves and the development of alternative and artificial diets for bivalve aquaculture. Rev. Fish. Sci. 7, 241-280. https://doi.org/10.1080/10641269908951362

Knauer, J., Kerr, R.G., Lindley, D., Southgate, P.C., 1998. Sterol metabolism of Pacific oyster (*Crassostrea gigas*) spat. Comp. Biochem. Phys. B 119, 81-84. https://doi.org/10.1016/S0305-0491(97)00269-1

Lund, E.D., Chu, F-L.E., Littreal, P.R., Ruck, K.E., Harvey, E., 2009. An investigation of the mechanisms for sterol synthesis and dietary sterol bioconversion in the heterotrophic protists *Oxyrrhis marina* and *Gyrodinium dominans*. J. Exp. Mar. Biol. Ecol. 374, 150-159. https://doi.org/10.1016/j.jembe.2009.04.018

Mathieu-Resuge, M., Kraffe, E., Le Grand, F., Boens, A., Bideau, A., Lluch-Cota, S. E., Racotta, I.S., Schaal, G., 2019. Trophic ecology of suspension-feeding bivalves inhabiting a north-eastern Pacific coastal lagoon: Comparison of different biomarkers. Mar. Environ. Res. 145, 155-163. https://doi.org/10.1016/j.marenvres.2019.02.016

Michellod, D., Bien, R., Birgel, D., Violette, M., Kleiner, M., Fearn, S., Zeidler, C., Gruber-Vodicka, H.R., Dubilier, N., Liebeke, M., 2023. De novo phytosterol synthesis in animals. Science 380, 520-526. https://doi.org/10.1126/science.add7830

Napolitano, G.E., Ackman, R.G., Silva-Serra, M.A., 1993. Incorporation of dietary sterols by the sea scallop *Placopecten magellanicus* (Gmelin) fed on microalgae. Mar. Biol. 117, 647-654. https://doi.org/10.1007/BF00349777

Pereira, M., Song, Z., Santos-Silva, L.K., Richards, M.H., Nguyen, T.T.M., Liu, J., de Almeida Soares, C.M., da Silva Cruz, A.H., Ganapathy, K., Nes, W.D., 2010. Cloning, mechanistic and functional analysis of a fungal sterol C24-methyltransferase implicated in brassicasterol biosynthesis. Biochim. Biophys. Acta - Mol. Cell Biol. Lipids 1801, 1163-1174. https://doi.org/10.1016/j.bbalip.2010.06.007

Petton, B., Le Souchu, P., Mingant, C., Robert, R., 2009. Small volume flow-through containers for bivalve larval rearing, in Aquaculture Europe 09. Trondheim, Norway. p. 491-492.

Salaque, A., Barbier, M., Lederer, E., 1966. Sur la biosynthèse des stérols de l'huitre (*Ostrea gryphea*) et de l'oursin (*Paracentrotus lividus*). Comp. Biochem. Phys. 19, 45-51. https://doi.org/10.1016/0010-406X(66)90545-7

512 Saliot, A., Barbier, M., 1973. Stérols en solution dans l'eau de mer: leur utilisation par les Invertébrés
513 marins. J. Exp. Mar. Biol. Ecol. 13, 207-214. https://doi.org/10.1016/0022-0981(73)90067-1
514 Rico-Villa, B., Bernard, I., Robert, R., Pouvreau, S., 2010. A Dynamic Energy Budget (DEB) growth
515 model for Pacific oyster larvae, *Crassostrea gigas.* Aquaculture 305, 84-94.
516 https://doi.org/10.1016/j.aquaculture.2010.04.018
517 Rico Villa, B., Pouvreau, S., Robert, R., 2009. Influence of food density and temperature on ingestion,
518 growth and settlement of Pacific oyster larvae *Crassostrea gigas*. Aquaculture 287, 395-401.
519 https://doi.org/10.1016/j.aquaculture.2008.10.054
520 Sokal, R.R., Rohlf, F.J., 1995. Biometry. The principles and practice of statistics in biological research.
521 Freeman, New York
522 Soudant, P., Le Coz, J-R., Marty, Y., Moal, J., Robert, R., Samain, J-F., 1998. Incorporation of
523 microalgae sterols by scallop *Pecten maximus* (L.) larvae. Comp. Biochem. Physiol. A 119, 451-
524 457. https://doi.org/10.1016/S1095-6433(97)00451-0
525 Soudant, P., Sanles, M.V., Quere, C., Le Coz, J.R., Marty, Y., Moal, J., Samain, J.F., Sorgeloos, P., 2000.
526 The use of lipid emulsions for sterol supplementation of spat of the Pacific oyster, *Crassostrea*
527 *gigas.* Aquaculture 184, 315-326. https://doi.org/10.1016/S0044-8486(99)00323-3
528 Teshima, S., Kanazawa, A., 1972. Bioconversion of the dietary brassicasterol to cholesterol in *Artemia*
529 *salina.* Bull. Jpn. Soc. Sci. Fish. 38, 1305-1310. https://doi.org/10.2331/suisan.38.1305
530 Teshima, S-I., Kanazawa, A., 1974. Biosynthesis of sterols in abalone, *Haliotis gurneri*, and mussel,
531 *Mytilus edulis.* Comp. Biochem. Phys. B 47, 555-561. https://doi.org/10.1016/0305-
532 0491(74)90004-2
533 Teshima, S-I., Kanazawa, A., Miyawaki, H., 1979. Metabolism of β-sitosterol in the mussel and the snail.
534 Comp. Biochem. Phys. B 63: 323-327. https://doi.org/10.1016/0305-0491(79)90256-6
535 Teshima, S-I., Patterson, G.W., 1981. Sterol biosynthesis in the oyster, *Crassostrea virginica.* Lipids 16,
536 234-239. https://doi.org/10.1007/BF02535022
537 Tomazic, M.L. Najle S.R., Nusblat A.D., Uttaro A.D., Nudel C.B., 2011. A novel sterol desaturase-like
538 protein promoting dealkylation of phytosterols in Tetrahymena thermophila. Eukaryotic Cell 3,
539 423-434. https://doi.org/10.1128/ec.00259-10
540 Trider, D.J., Castell, J.D., 1980. Effects of dietary lipids on growth, tissue composition and metabolism of
541 the oyster (*Crassostrea virginica*). J. Nutr. 110, 1303-1309.

https://doi.org/10.1093/jn/110.7.1303 Voogt, P.A., 1975. Investigations of the capacity of synthesizing 3β-sterols in mollusca - XIII. Biosynthesis and composition of sterols in some bivalves (Anisomyaria). Comp. Biochem. Phys. B 50, 499-504. https://doi.org/10.1016/0305-0491(75)90078-4

Walne, P.R., 1966. Large scale culture of larvae *Ostrea edulis* L. Fish. Invest. II 25, 1-52.

Walton, M.J., Pennock, J.F., 1972. Some studies on the biosynthesis of ubiquinone, isoprenoid alcohols, squalene and sterols by marine invertebrates. Biochem. J. 127, 471-479. https://doi.org/10.1042/bj1270471

552 **Figure captions**

553

554 Fig. 1 Maximum-likelyhood tree of eukaryotic candidate 24-sterol reductases.
555 The oyster candidate sequence is highlighted in bold, and groups with two other bivalve sequences, and
556 more widely with other animals (cnidarians and deuterostomes). Branch support values (aLRT) are shown
557 when superior to 0.95. Colour code is as follows: blue for animals, green for viridiplantae, red for red
558 algae, orange for haptophyte and brownish yellow for diatom.
559 **Fig. 2** Possible pathways for sterol bioconversion in *C. gigas* larvae. 1. Lund et al. (2009) in the
560 heterotrophic protists *Oxyrrhis marina* and *Gyrodinium dominans.* 2. Saliot and Barbier (1973) in the
561 oyster *Ostrea gryphea.* 3. Knauer et al. (1998) in the oyster *Crasssostrea gigas.* 4. Teshima and
562 Kanazawa (1973) in the prawn *Penaeus japonicus*. 5. Teshima (1983) in the prawn *P. japonicus*. 6. Giner
563 et al. (2016) in the Northern Bay scallop *Argopecten irradians.* 7. Blary et al. (2022) in photosynthetic
564 eukaryotes. In blue, the two possible places where the candidate sterol 24 reductase may act.

565

566