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Review

Evolution and biomineralization of pteropod shells

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We dedicate this work to the memory of Arie W. Janssen who devoted his life to studying pteropods and has been a great inspiration to us.

Keywords:
Biominerals
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Helical microstructure
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ABSTRACT

Shelled pteropods, known as sea butterflies, are a group of small gastropods that spend their entire lives swimming and drifting in the open ocean. They build thin shells of aragonite, a metastable polymorph of calcium carbonate. Pteropod shells have been shown to experience dissolution and reduced thickness with a decrease in pH and therefore represent valuable bioindicators to monitor the impacts of ocean acidification. Over the past decades, several studies have highlighted the striking diversity of shell microstructures in pteropods, with exceptional mechanical properties, but their evolution and future in acidified waters remains uncertain. Here, we revisit the body-of-work on pteropod biomineralization, focusing on shell microstructures and their evolution. The evolutionary history of pteropods was recently resolved, and thus it is timely to examine their shell microstructures in such context. We analyse new images of shells from fossils and recent species providing a comprehensive overview of their structural diversity. Pteropod shells are made of the crossed lamellar and prismatic microstructures common in molluscs, but also of curved nanofibers which are proposed to form a helical three-dimensional structure. Our analyses suggest that the curved fibres emerged before the split between coiled and uncoiled pteropods and that they form incomplete to multiple helical turns. The curved fibres are seen as an important trait in the adaptation to a planktonic lifestyle, giving maximum strength and flexibility to the pteropod thin and lightweight shells. Finally, we also elucidate on the candidate biomineralization genes underpinning the shell diversity in these important indicators of ocean health.

1. Introduction

Shelled pteropods, known as sea butterflies, are considered vulnerable to ocean acidification (OA) (Bednaršek et al., 2012b; Gazeau et al., 2013; Manno et al., 2017; Mekkes et al., 2021b). Pteropods spend their entire lives swimming and drifting in the open ocean, a major sink of anthropogenic CO_2 (Zeebe, 2012; Gruber et al., 2019). They build thin and lightweight shells of aragonite, a metastable polymorph of calcium carbonate that can be 50% more soluble in seawater than calcite (Mucci, 1983; Sun et al., 2015) and are major contributors to carbon and carbonate fluxes in the pelagic domain (Bednaršek et al., 2012a; Berner and Honjo, 1981; Buitenhuis et al., 2019). Over the last decade, shelled pteropods have been under scrutiny in OA research and were reported to decrease calcification rates and experience extensive shell dissolution in low pH conditions (Feely, 2004; Orr et al., 2005; Fabry et al., 2008; Comeau et al., 2009; Bednaršek et al., 2012b; Moya et al., 2016; Maas et al., 2018). Moreover, they were found to produce thinner shells across time in their natural environment (Roger et al., 2012; Howes et al., 2017), and along declines in aragonite saturation levels *in situ* (Mekkes et al., 2021a). Thus pteropods have been regarded as biological indicators to monitor the impacts of OA (Bednaršek et al., 2014, 2017; Manno et al., 2017), however, the extent of pteropod's vulnerability to OA remains a matter of debate. Structural studies have demonstrated that representatives of the species *Limacina helicina* can effectively repair their damaged shells by extensive thickening of the inner shell wall, and as long as the organic outermost layer of the shell – the periostracum – remains intact (Peck et al., 2016, Peck et al., 2018).

Recently, a phylogenomic approach combined with molecular clock dating demonstrated that the split of pteropods in shelled (sea butterflies, order Thecosomata) and non-shelled (sea angels, order Gymnosomata) groups was much older than predicted based on their fossil record, dating back to the early Cretaceous, 139.1 million years ago (Ma)

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(Peijnenburg et al., 2020). Peijnenburg et al. (2020) showed that all lineages including the modern fully, partially and non-shelled groups, were already present in the mid to the late Cretaceous. These lineages have thus persisted through periods of major environmental change and ocean acidification such as the Cretaceous Paleogene extinction event (K-Pg, 66 Ma) and the Paleocene-Eocene Thermal Maximum (PETM, 56 Ma), the most analogous event to the present day rises in CO_2 (Janssen et al., 2016; Peijnenburg et al. 2020).

Thus, pteropods may have been more resilient to global change than currently thought. While sea angels (shell-less as adults) evolved as specialized predators of sea butterflies during the Cretaceous, sea butterflies adapted to their planktonic life and responded to predation pressure by developing ever more complex, thin, lightweight, but also strong aragonitic shells. In fact, many open questions remain with regards to the evolution and biomineralization of pteropod shells. *How do pteropods make their shells? How did they adjust their shells to a wholly pelagic existence? How did their unique microstructures evolve? Which genes are involved in biomineralization? How did biomineralization genes evolve to give rise to shelled, partially shelled and unshelled species?*

Similarly to other molluscs, pteropods produce their shells through a biomineralization process biologically controlled at the molecular and cellular levels (Lalli and Gilmer, 1989). In molluscs, the mantle – the soft tissue directly in contact with the shell growth surface – is responsible for shell formation. Mantle cells activate biomineralization pathways and express biomineralization genes, including genes coding for ion transporters and shell matrix proteins (SMPs) (Marin and Luquet, 2004; Marin et al., 2007; Marin et al., 2012; Marin, 2020), among other categories. The SMPs together with sugars, polysaccharides, lipids and pigments, form an extracellular organic matrix that is secreted to the extrapallial space, at the interface between the shell and the mantle tissue, and directly induces crystal nucleation, growth, morphology and deposition (Addadi and Weiner, 1985; Mann, 1988; Albeck et al., 1996; Belcher et al., 1996; Samata et al., 1999; Kono et al., 2000). The process results in a stable and well-packed organo-mineral assemblage with 96–99% of mineral phase and up to 4% of organic matrix, depending on the mollusc species (Marie et al., 2009, 2011, 2013; Ramos-Silva et al., 2012). The exceptional mechanical properties of the shell biomaterials are due to: *(1)* the three-dimensional arrangement of crystal aggregates, *i.e.* shell microstructures, and *(2)* the occluded organic matrix (Wainwright et al., 1982; Weiner and Addadi, 1997).

The SMPs have long been recognized as key components of the biological control underlying shell formation (reviewed in Marin 2020). SMPs influence the modulation of calcium carbonate polymorphs, e.g. calcite *vs.* aragonite (Belcher et al., 1996; Falini et al., 1996; Thompson et al., 2000), crystal nucleation, growth and orientation (Belcher et al., 1996) as well as crystal morphology (Samata et al., 1999; Kono et al., 2000). Whether SMPs regulate other aspects of shell microstructures is still an open question (Marin, 2020).

To date, SMPs and biomineralization genes remain uncharacterized for pteropods, but a growing number of studies have focused on their shell microstructures. Pteropod shell microstructures have tremendous potential as environmental proxies (Howes et al., 2017), possess valuable taxonomic information that can be used by palaeontologists in combination with other morphological traits (Garvie et al., 2020) and constitute an inspirational source for the design of ultrathin and/or lightweight biomaterials with superior mechanical properties (Zhang et al., 2011; Teniswood et al., 2013). Here, we review the body-of-work on pteropod microstructures carried out over the decades while bringing new insights on the evolution of pteropod biomineralization. With pteropods having their evolutionary history resolved, it is timely to examine their shell morphologies and microstructures in this context and look for candidate biomineralization genes underpinning the shell diversity of these important ocean sentinels.

2. Diversity in pteropod shell microstructures

Pteropods are a monophyletic group within the euopisthobranch gastropods (Heterobranchia) (Klussmann-Kolb and Dinapoli, 2006; Zapata et al., 2014) (Fig. 1A). Apart from the pteropods, heterobranchs include only benthic species, some of which are commonly used as outgroups in pteropod phylogenies (Burridge et al., 2017; Peijnenburg et al., 2020) such as sea hares with internal shells (e.g. *Aplysia*), bubble snails (e.g. *Haminoea*), sea slugs and false limpets with limpet-like shells made of protein (e.g. *Tylodina*) (Jörger et al., 2010; Kano et al., 2016). Hence, pteropods are unique within the whole Heterobranchia subclass in that they have adapted to a strictly planktonic life cycle and encompass fully or partially aragonitic shelled as well as unshelled species (Lalli and Gilmer, 1989). The shell, if present, can be either left-coiled or uncoiled (bilaterally symmetrical). Although pteropods that are shell-less as adults also have a small aragonite shell in their larval stage, here we focus our attention on the pteropods with a calcified shell as adults.

2.1. Superfamily Cavolinioidea (suborder Euthecosomata, uncoiled shells)

The first detailed study on pteropod biomineralization was published in 1972 (Bé et al., 1972). Until that time pteropod shells had received little attention from biologists and palaeontologists, mostly because of their small size and extremely thin walls (<40 μm). Bé and co-workers proposed an exceptional microstructure, only found in pteropods, where aragonitic curved nanofibres coil helically around axes perpendicular to the shell surface (Fig. 1C and D). In their study, Bé et al. (1972) used specimens of *Cuvierina* (Euthecosomata, Cavolinioidea) and scanning electron microscopy (SEM) to describe four shell layers: apertural, inner and outer prismatic layers, and a main middle helical layer along the whole shell. The existence of the helical microstructure was further confirmed in other pteropod species, particularly in all the other members of the Cavolinioidea, including the genera *Creseis*, *Clio*, *Cuvierina*, *Cavolinia*, *Diacavolinia*, *Diacria, Styliola* and *Hyalocylis* (Rampal, 1975; Glaçon et al., 1994; Checa et al., 2016). Here, we provide new SEM images showing examples of these curved microstructures for the species *Diacria costata*, *Diacavolinia longirostris*, *Creseis acicula* and *Styliola subula* (Fig. 2).

Members of the Cavolinioidea have uncoiled bilaterally symmetrical shells with remarkably distinct shell shapes from other gastropods including straight cones, vases or more intricately shaped shells (see Fig. 2A, E, G and I for some examples). Besides curved fibres, Cavolinioidea shells also display other (thinner) shell layers, at external regions, made of the more common crossed lamellar (composed of straight fibres crossing at an angle, Fig. 1B) or prismatic microstructures (see also Bé et al., 1972; Checa et al., 2016; Glaçon et al., 1994; Rampal, 1975).

2.2. Superfamily Limacinoidea (suborder Euthecosomata, sinistrally coiled shells)

In contrast to Cavolinioidea, members of their sister group, Limacinoidea – including the genera *Limacina* and *Heliconoides*, have regularly coiled shells with varying heights (Fig. 3A and D). Species of the *Limacina* genus exhibit a crossed lamellar layer (middle) (Fig. 3C) between two thin prismatic layers (inner and outer) (Fig. 3B) in most of their shell sections, which can vary in thickness depending on the location (Rampal, 1975; Glaçon et al., 1994; Sato-Okoshi et al., 2010; Teniswood et al., 2016). Aragonitic crossed lamellar and prismatic microstructures are among the most common in molluscs and they have been described in detail for several species (Bøggild, 1930; Nakahara, 1981; Suzuki et al., 2011; Checa et al., 2012; Almagro et al., 2016; Böhm et al., 2016; Agbaje et al., 2017; Crippa et al., 2020). The curved fibres, usually seen as a distinctive trait of the Cavolinioidea (Lalli and Gilmer, 1989; Checa

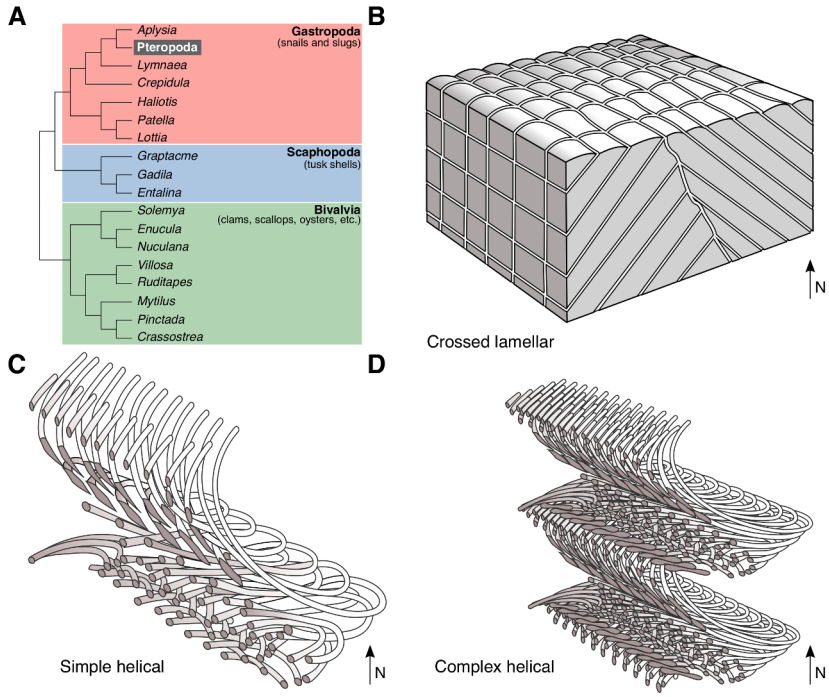

Fig. 1. Pteropods and schematic views of the main microstructures forming their shells. (A) Cladogram depicting the position of pteropods in relation to other molluscs based on the phylogenetic relationships described in Kocot et al. (2020) and Zapata et al. (2014). (B) Crossed lamellar: parallel rods or blades crossing at angles, perpendicular or oblique to the depositional surface. Adapted from Currey and Taylor (1974). (C) Simple helical: coiled helical fibres with one turn or less. (D) Complex helical: coiled helical fibres with more than one turn. Diameter and leading angle of the spirals are larger in the simple helical than in the complex helical. Fibre interlocking is not represented. Adapted from Li et al. (2015). N, normal direction, *i.e.,* perpendicular to the shell surface.

et al., 2016), are also present in Limacinoidea. In the genus *Limacina* they were reported exclusively at the apex (Rampal, 2017), which is the oldest part of the shell, produced at embryonic stages. In the genus *Heliconoides* (formerly known as *Limacina inflata*) curved fibres comprise the main layer of the shell (Rampal, 1975; Glaçon et al., 1994). Images E and F of Fig. 3. show the presence and details of the curved fibres in *Heliconoides inflatus* collected from the southern Atlantic Ocean. The fibres seem to form a helical structure similar to the one found in some cavolinioidean species (Fig. 2H and J-L). In addition, *Heliconoides* shells also have the crossed lamellar microstructure at external positions (Fig. 3G). Despite also having a coiled shell, *Heliconoides inflatus* specimens are morphologically quite distinct from *Limacina* species. They usually have less whorls, with the last whorl inflated and a wide aperture (Fig. 3D).

2.3. The genus Peracle (suborder Pseudothecosomata, sinistrally coiled shells)

Pseudothecosomes are still an understudied group of pteropods that is estimated to have diverged from the euthecosomes in the early Cretaceous (110.9 Ma) (Peijnenburg et al., 2020). The group includes fully shelled, for example, the genus *Peracle* with aragonitic coiled shells (Fig. 4.), but also partially shelled (e.g. *Cymbulia* with a gelatinous pseudoconch) and completely unshelled genera (e.g. *Desmopterus*). *Peracle* species have sinistral coiled shells with high but also lower spires, and a last whorl that is relatively large (Fig. 4A and E). The shell is often made of a main crossed lamellar layer and thin outer fibrous layers (Rampal, 1975) (Fig. 4B–D). The spire is thicker and can be made of multiple superimposed crossed lamellar layers aligned in different directions (Fig. 4G). Complex ornamentations on the shell surface are present in some species, for example, *P. reticulata* and *P. valdiviae* (Fig. 4F). In the latter, the ornament microstructure is made of first order elongated fibres (Fig. 4G and H). The crossed lamellar microstructure also comprises the main layer of other euopisthobranch (benthic) gastropods including *Aplysia* (Marin et al., 2018), *Akera* and *Haminoea* (Rampal, 1975).

3. Fossil record and evolution of curved fibres

It is interesting to note that there are at least two types of the helical microstructure clearly distinguishable in pteropods: one with one turn or less (here termed 'simple helical') and one with multiple turns (here termed 'complex helical') (Fig. 1C and D). The number of turns is correlated to shell thickness but other aspects, such as the lead angle of the helices and their diameters, also influence the number of turns. The coiled *Heliconoides* and the earlier diverging groups of Cavolinioidea, including the straight-shelled *Creseis* (~99 Ma), *Styliola* (~70 Ma) and *Hyalocylis* (~63 Ma), all have the simple version of the helical microstructure as the main shell layer (Fig. 1C), in which curved fibres form one or less helical turns and seem to have higher lead angles and diameters. In contrast, the more recently diverged clade comprising the genera *Diacria*, *Cavolinia*, *Diacavolinia*, *Clio* and *Cuvierina*, which originated ~58 Ma, in the late Paleocene (Fig. 6), has clearly developed further into a more sophisticated version of the structure, more compact, with smaller lead angles and diameters, and displaying more than one turn (Glaçon et al., 1994; Zhang et al., 2011; Li et al., 2015; Checa et al., 2016) (Fig. 1D). Also, *Limacina* species appear to have curved fibres but only located at the apex (Rampal, 2017). Curved crystals have also been described in the meroplanktonic larval shells of benthic gastropods, including the architectonicid *Philippia krebsii,* sacoglossans (a clade of small photosynthetic sea slugs) (Richter, 1976) and fissurellids (known as keyhole limpets) (Batten, 1975). Moreover, curved fibres have been depicted in the adult shell layers of other microgastropods including the holoplanktonic atlantid heteropods (sea elephants) (Batten and Dumont, 1976) and the scissurelids (little slit snails) (Batten, 1975). Batten (1975) and Batten and Dumont (1976) coined the term type-2 crossed lamellar structure to describe the layers found in benthic scissurelids, embryonic fissurellids and heteropods. As the name suggests, this microstructure was described as a modified version of the typical crossed lamellar structures (Carter et al., 1991) and was proposed to be a specific adaptation resulting from the thinness of the shell walls to provide a flexing function (Batten, 1975). In the type-2 structure described by Batten, both first order lamellae and second order rods may have curved or sigmoidal fibres, among other variations that blur the crossed lamellar aspect (Batten and Dumont, 1976). It would be

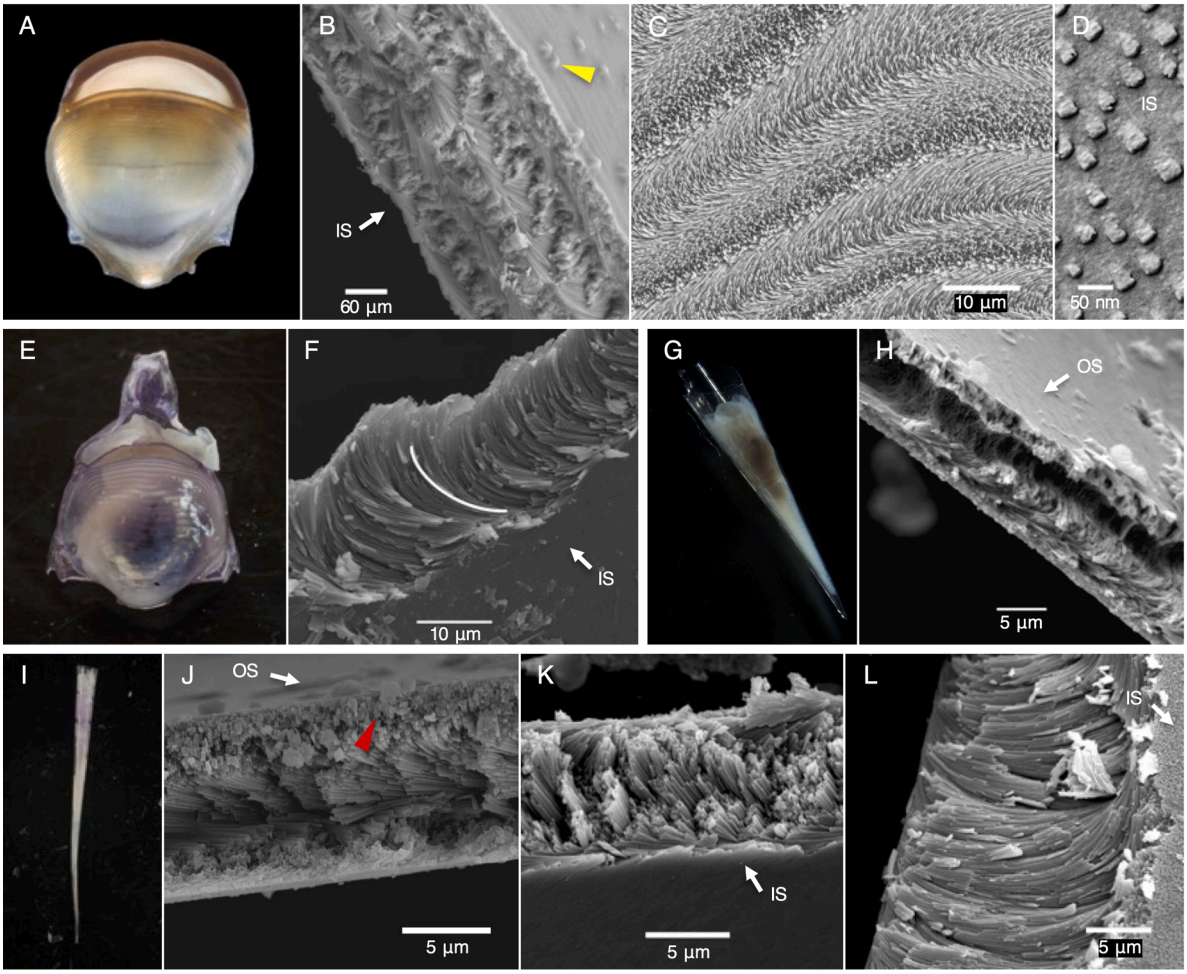

Fig. 2. Curved fibres in Cavolinioidea (Euthecosomata). (A) *Diacria costata*, (B) fresh cut in *D. costata* showing the main layer of helical fibres and ornaments on the shell surface (yellow arrowhead), (C) polished cut in *D. costata* highlighting the complex helical microstructure with multiple turns, (D) internal shell-growth surface showing the tips of the fibres, (E) *Diacavolinia longirostris*, (F) fresh cut in *D. longirostris*, (G) *Styliola subula* (H) fresh cut in *S. subula* showing the simple helical microstructure. (I) *Creseis acicula*, (J-K) fresh cuts in *C. acicula* showing simple helical microstructure and the interlocked texture (red arrowhead). (L) Oblique view from shell of *C. acicula* from Wall-Palmer et al. (2011). IS – inner surface, OS – outer surface. Information about sample origins is given in Tab. S1. Shell locations from which the SEM images were taken are represented in Fig. S1.

interesting to re-examine the curved fibres across these and other molluscan groups, in particular, at larval stages and in thin-shelled taxa, to compare their morphology with the pteropod helical microstructures.

Batten (1975) suggested that scissurelids were neotenously derived from fissurelids based on similar growth stages and on the presence of the type-2 crossed lamellar wall on the adult and embryonic stages, respectively (Batten, 1975). Similarly, neotenic origins have been proposed for pteropods, *i.e.* the larvae of a benthic gastropod became sexually mature and stayed in the water column for their entire lives (Bandel et al., 1984; Huber, 1993; Lemche, 1948). However, this hypothesis has been questioned by other authors (Jägersten, 1972; Lalli and Gilmer, 1989). In the transition from a benthic to a holoplanktonic life the gastropod foot (poda) – an adult feature – was decisive to evolve swimming wings (ptero-). In any case, the development of a shell layer made of curved fibres seems to be an adaptation not only related to a planktonic existence but also due to the thinness and lightweight nature of the shells (of a few micrometres). Curved structures with a helical shape have been observed at different scales in several biological systems, ensuring a favourable combination of strength and flexibility (Wainwright et al., 1982). The aragonitic helical microstructure allows for more suppleness over brittleness to the thin pteropod shells that must withstand varied hydrodynamic forces in the water column (Karakas et al., 2020).

The development of the curved fibres can be further elucidated by looking at the fossil record. To date, however, only a few descriptions of microstructures have been made for fossil pteropods (Rampal, 1975; Curry and Rampal, 1979; Rehfeld and Janssen, 1995; Garvie et al., 2020). With their thin aragonitic shells, pteropods are particularly sensitive to diagenesis, the process by which aragonite transforms into the more stable polymorph calcite, in ambient conditions and in the presence of water. The diagenetic process in molluscan shells is still far from understood (Milano and Nehrke, 2018) but it is known to blur or even destroy the original microstructures in molluscs (Janiszewska et al., 2018), including pteropods (Brachert and Dullo, 2000). In addition, pteropods are more susceptible to post-mortem dissolution in the water column, disappearing more rapidly from sediments than other planktonic calcifiers that produce calcite biominerals (Brachert and Dullo, 2000; Janssen and Peijnenburg, 2017; Manno et al., 2017). Hence, the occurrence of pteropods in rocks of Cretaceous age is likely to be underestimated (Janssen and Peijnenburg, 2017). Consequently, some fossil placements remain unclear. This is the case of the genus *Altaspiratella,* whose oldest fossils date back to the early Ypresian, but has long been proposed as the coiled ancestor of the genus *Creseis* (Cavolinioidea), and of the fossils *Camptoceratops* (Ypresian) and *Euchilotheca* (Ypresian and Lutetian) (Garvie et al., 2020; Janssen and Peijnenburg, 2017; Janssen and Peijnenburg, 2014). The curved fibres

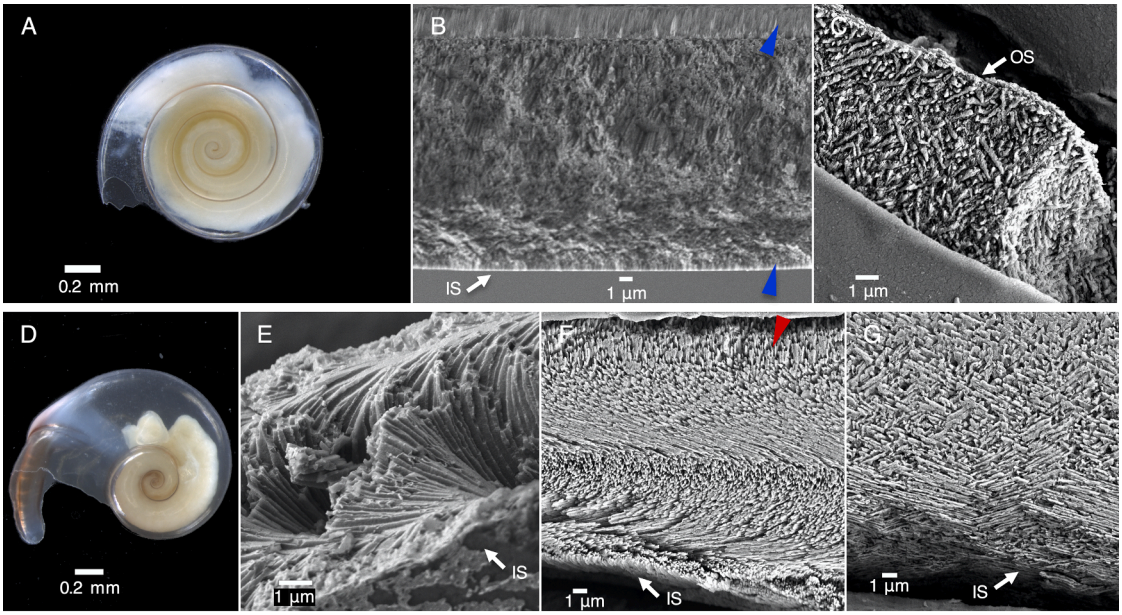

Fig. 3. Shell microstructures in Limacinioidea (Euthecosomata). (A) *Limacina helicina antarctica*, (B) fresh cut in *L. h. antarctica* showing the thicker crossed lamellar (middle) layer and two thinner fibrous outer and inner layers (blue arrowheads), (C) details of the crossed lamellar microstructure in a polished cut in *L. h. antarctica*, (D) *Heliconoides inflatus*, (E) fresh cut in *H. inflatus* showing the simple helical microstructure, (F) polished cut in *H. inflatus* highlighting the simple helical microstructure and the interlocked fibers (red arrowhead), (G) details of the crossed lamellar layer located at external positions in a polished cut in *H. inflatus*. IS – inner surface, OS – outer surface. Information about sample origins is given in Tab. S1. Shell locations from which the SEM images were taken are represented in Fig. S1.

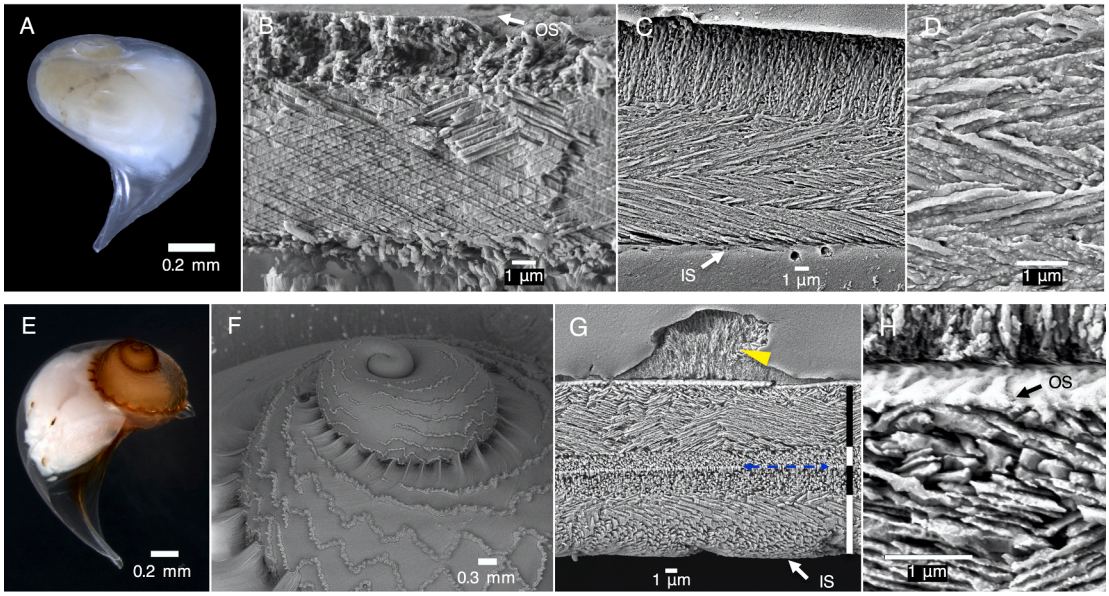

Fig. 4. Shell microstructures in the genus *Peracle* (Pseudothecosomata). (A) *Peracle moluccensis*, (B) fresh cut of *P. moluccensis* and (C) polished cut showing crossed lamellar and fibrous layers in the last whorl (D) details of the elongated crossed lamellar layer, (E) *Peracle valdiviae*, (F) details of the reticulate ornamentation in the spire of the shell of *P. valdiviae*, (G) polished cut at the spire revealing crossed lamellar layers with various aspects according to the orientation of the lamellae (length of each layer marked with black and white bar on the right), growth line (dashed line and blue arrow), and elongated fibres of the ornament on top (yellow arrowhead), (H) detail of the border between ornamentation and crossed lamellar layer. IS – inner surface, OS – outer surface. Information about sample origins is given in Tab. S1. Shell locations from which the SEM images were taken are represented in Fig. S1.

observed here for *Altaspiratella bearnensis* (Fig. 5A–C) would support this evolutionary path, placing *Altaspiratella* at the base of Euthecosomata (placement [1], Fig. 6). Yet, the coiled shell and fossil age, given the context of pteropod's evolutionary history, suggest that *Altaspiratella* could also be an ancestor of modern *Limacina* species. The fossil *Camptoceratops priscus*, whose shell is slightly coiled in an open helix (Fig. 5D), was proposed as an intermediate form between coiled species and uncoiled Creseidae (Janssen and Peijnenburg, 2014; Janssen and Peijnenburg, 2017), see placement [2] in Fig. 6. This fossil has curved fibres (Curry and Rampal, 1979) (see Fig. 5E and F). In addition, the younger fossil *Creseis cylindrica,* from the middle Eocene, with a shape resembling that of modern *Creseis*, has curved fibres (Garvie et al., 2020) as well as

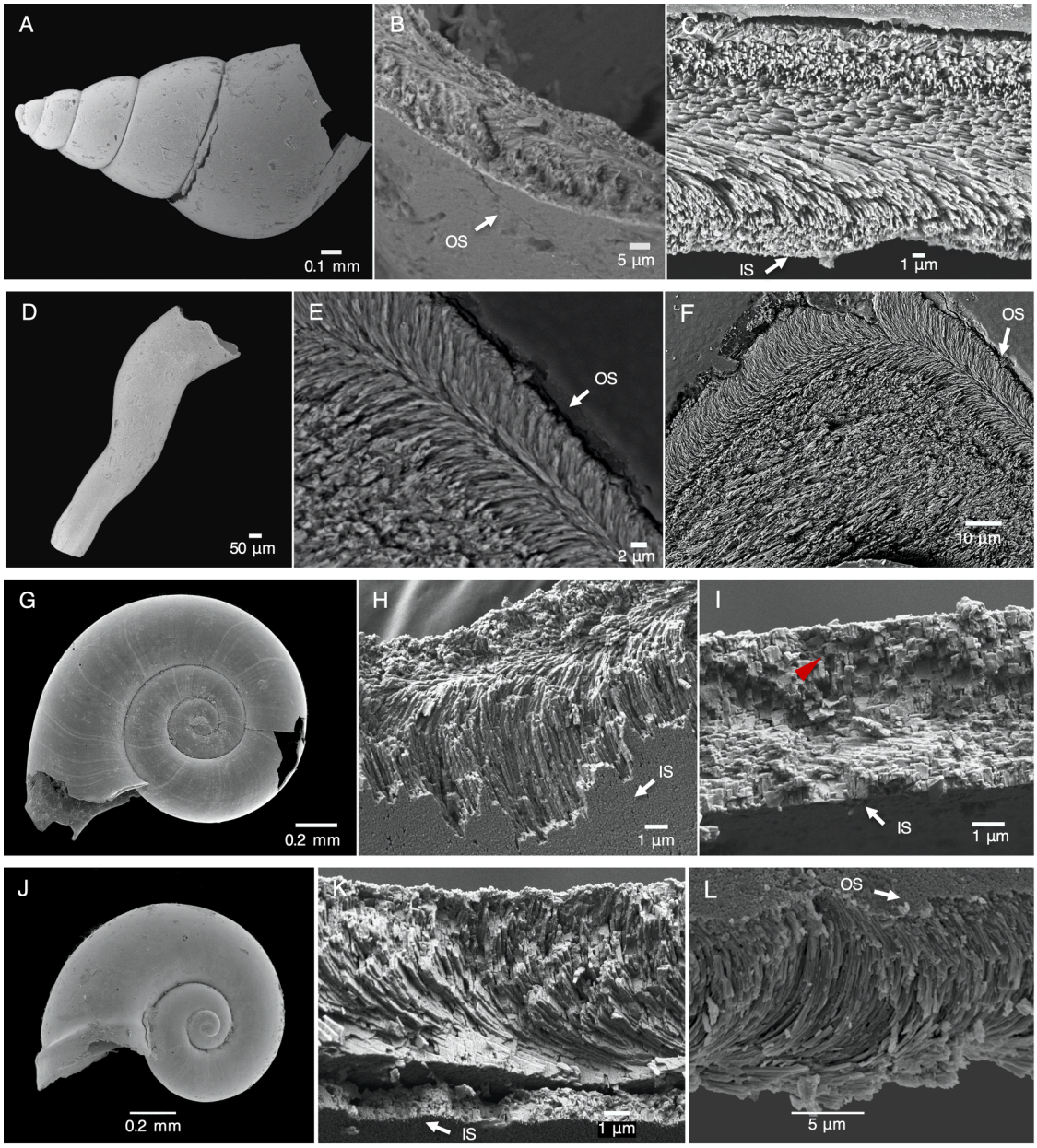

Fig. 5. Shell microstructures in fossil pteropods. (A) *Altaspiratella bearnensis* specimen (RGM.229304.a; Eocene, Ypresian) (B) fresh cut showing the curved microstructure of *A. bearnensis* (C) polished cut showing an outer crossed lamellar layer and the inner layer made of curved fibres (D) *Camptoceratops priscus* specimen (RGM.396617.a; Eocene, Ypresian) (E-F) polished cuts of *C. priscus shell* showing two layers, curved and confused, probably due to diagenetic processes (G) *Heliconoides mercinensis* specimen (RGM. 515524.a; Eocene, Ypresian) (H-I) fresh cuts showing curved fibres and the interlocking structure (red arrowhead) in *H, mercinensis* (J) *Heliconoides tertiaria* specimen (RGM.229472.a; Miocene, ~ Langhian) (K – L) fresh cuts showing curved fibres in *H. tertiaria* (RGM.229472.b). Information of sample origins is given in Tab. S1. Shell locations from which the SEM images were taken are represented in Fig. S1.

the Miocene *Vaginella depressa* (Curry and Rampal, 1979) and *Gamopleura melitenses* (Rehfeld and Janssen, 1995). Hence, the most likely scenario is that the last common ancestor of the Cavolinioidea (99.1 Ma) had a main shell layer composed of curved fibres. The same is true for the last common ancestor of the Limacinoidea (107.2 Ma) and, by extension, the entire Euthecosomata clade (110.2 Ma) because we find curved fibres in fossil *Heliconoides* (Fig. 5G–L) similar to the those observed in the modern *H. inflatus* (Fig. 3E and F), which are proposed to form a simple helical microstructure. These fossils include *Heliconoides tertiaria*, from the middle Miocene, and *H. mercinensis*, a pre-PETM (Paleocene-Eocene Thermal Maximum) species from the latest Paleocene/earliest Eocene transition (Janssen and Peijnenburg, 2017). Finally, there is also evidence of curved fibres in the whole shell of *Limacina pygmaea* from the Lutetian (middle Eocene) (Curry and Rampal, 1979) and in the apex of the extant *Limacina retroversa* (Rampal, 2017), which suggests that at some point, the genus *Limacina* has adapted the crossed lamellar microstructure as its main shell layer.

Fossil pteropods of Cretaceous age are extremely rare with only a single specimen of a *Heliconoides* known from the middle to late Campanian (Cretaceous, ~ 79–72 Ma), followed by a gap in the fossil record until the late Paleocene (reviewed in Janssen and Peijnenburg 2017). Yet, a recent study reported pteropod-like fossils from the late Cretaceous with a *Creseis*-like shape and a crossed lamellar microstructure (Garvie et al., 2020). If these specimens are later confirmed as pteropods, despiralisation must have happened more than once in pteropod evolution (Garvie et al., 2020; Peijnenburg et al. 2020).

4. From biomineralization to bioinspiration

4.1. Microstructures and their formation mechanisms

Of the three main types of microstructures found in modern pteropods – crossed lamellar, prismatic and helical – crossed lamellar is by far the most common among gastropods (Salinas and Kisailus, 2013). This structure is found in all pteropod lineages and encompasses the main shell layers in the genera *Peracle* and *Limacina* (Fig. 6). The crossed lamellar microstructure is composed of blades oblique to the depositional surface and crossing at an angle. It has a complex hierarchical structure with first-, second-, and third-order lamellae (Carter, 1989; Carter et al., 1991) (Fig. 1B). Due to its complexity and wide distribution among molluscs, many open questions remain on the fabrication of the crossed lamellae (Almagro et al., 2016). In one of the first structural studies on the gastropod *Strombus gigas*, Nakahara, (1981) identified envelopes of organic matrix where the crystals initially develop (Nakahara, 1981). More recently, using cryo-scanning electron microscopy on the aragonitic crossed lamellar layer of limpet shells (also Gastropoda), Suzuki et al. (2011) showed that the deposition of the first crystalline particles occurs in a thin granular layer on the growing surface of the mature crossed lamellae. The granules subsequently grow to form the crossing fibres in alternate orientations, typical of the mature crossed lamellar structure (Suzuki et al., 2011).

Prismatic microstructures are also found in many pteropod lineages, but only located at peripheral positions. These microstructures are structurally more simple and consist in adjacent, elongated crystalline units that grow in parallel without interlocking themselves (Carter, 1989; Carter et al., 1991). This general construction can have several variations among molluscs. In pteropods there are also different varieties that have not yet been described in detail (Glaçon et al., 1994; Teniswood et al., 2016) (e.g. Fig. 3B and Fig. 4G). Crystal competition and direct cellular activity have been proposed as the main drivers for the formation of the columnar prismatic microstructure (reviewed in Checa 2018). However, given their quite distinct morphologies, it is unlikely that the same mechanisms would apply to the pteropods prismatic layers.

The curved fibres are proposed to form a helical 3D structure consisting of nested coiled helical rods with helix axes oriented perpendicular to the depositional surface (Bé et al., 1972; Carter, 1989; Carter et al., 1991) (Fig. 1C and D). This structure is unique to pteropods, although other curved fibres have been found in the shells of planktonic veligers and micromolluscs (see above), they do not seem to form a helical assembly. The helical microstructure was first described by Bé and co-authors (1972) in *Cuvierina sp*. Four decades later, it became the subject of intense research with several studies describing the structural nature of the aragonite curved nanofibers. In 2011, Zhang and co-workers published a new characterization of the helical microstructure and its mechanical properties in *Cavolinia uncinata* (Zhang et al., 2011). The authors showed that shells of *C. uncinata* are made of tightly packed curved nanofibers, twisting clockwise and crystallographically aligned, with the coiling axis perpendicular to the shell surface. The nanofibers intergrow, resulting in an interlocking structure that produces irregular mosaic cross-sections (as marked in Fig. 2J and Fig. 5I with the arrowheads). Follow-up work in *Cuvierina* shells showed evidence for continuously crystalline fibres with preferred crystallographic growth directions that constantly adapt to the changes in curvature imposed by helical growth (Willinger et al., 2016). The authors argued that this preferential growth must result from strong biological control. Moreover, helical fibres were proposed to be surrounded by an organic-rich band, whose tips appear as micro elevations on the internal growth face of the shell (Checa et al., 2016) (Fig. 3D). These micro elevations were proposed as contact-points between shell growing edges and mantle cells, which directly determine the growth trajectories of the fibres (Checa et al., 2016). Because the tips are wider than the rest of the fibre once incorporated within the shell, Checa et al. (2016) suggested that this reduction in volume would make sense if the tip initially consisted of amorphous calcium carbonate and subsequently transformed into aragonite. Later, by Raman spectroscopy of living *Creseis acicula* it was indeed shown that the growing edge and newly formed parts of the

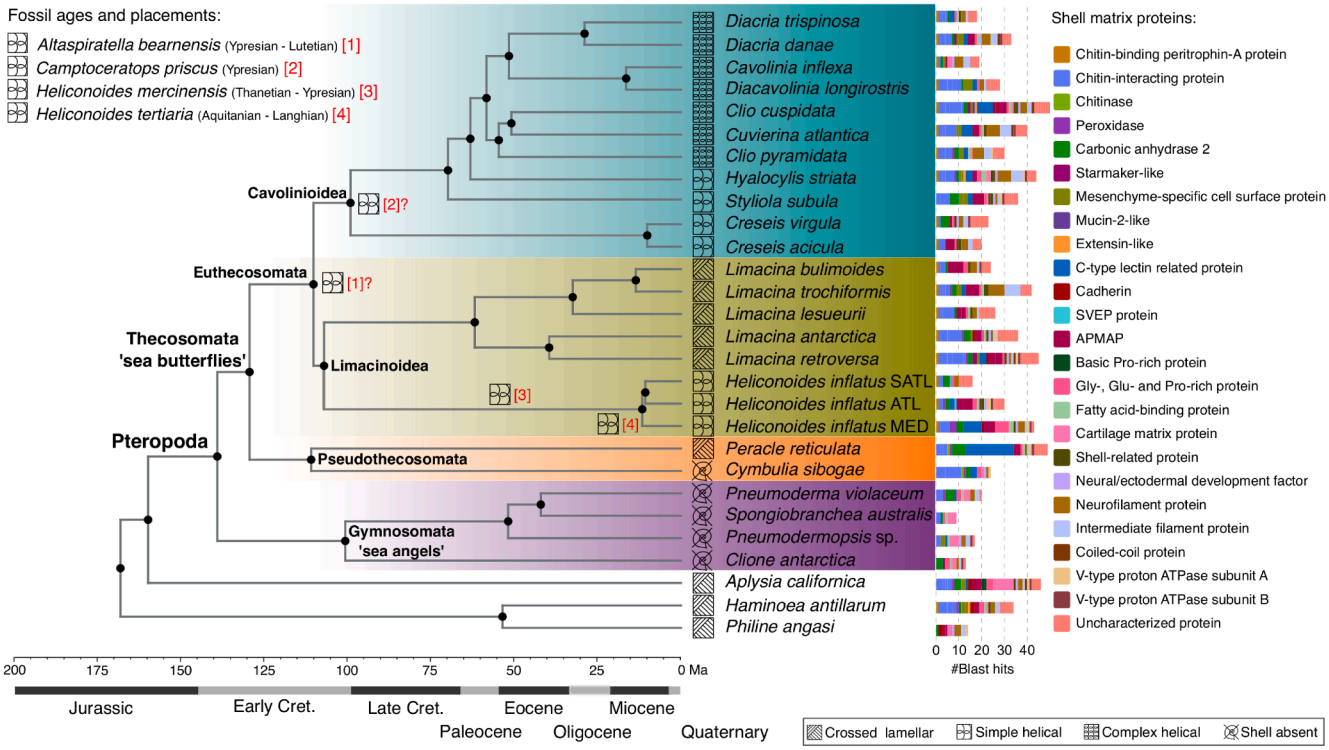

Fig. 6. Chronogram with the evolutionary relationships of pteropods (25 taxa and 3 outgroups) based on 200 concatenated genes, adapted from Peijnenburg et al. (2020). Gymnosomata (sea angels) are unshelled pteropods and the sister clade of the Thecosomata (sea butterflies). Sea butterflies include Euthecosomata (fully shelled) – comprising the superfamilies Cavolinioidea (uncoiled shells mostly made of curved fibres) and Limacinoidea (coiled shells mostly made of crossed lamellar or curved fibres) – and Pseudothecosomata (covering unshelled to fully shelled species with crossed lamellar microstructure). Fossils that were examined in this study are placed onto the branches of this phylogeny and their microstructures are indicated (top left). Microstructure of the shell main layer is indicated before each species name on the chronogram. See legend (bottom right) for explanation of the symbols used. (Right) Homology profiles of candidate SMPs in pteropods and outgroups. The transcriptomes from Peijnenburg et al. (2020) were used in a blast search against the shell proteomes from two panpulmonate gastropods: *Cepaea nemoralis* and *Lymnaea stagnalis* (Mann and Jackson, 2014; Herlitze et al., 2018). Homology searches were performed using BLASP (Cut-off: 35% id, 75% query coverage). Name of protein clusters are indicated on the right. Bar sizes are proportional to the number of blast hits. Methods are described in the supplementary file.

internal shell are made of a highly disordered aragonite precursor phase (Sibony-Nevo et al., 2019). From the disordered precursor the mineral matures into crystalline aragonite fibres (Sibony-Nevo et al., 2019). This formation strategy is common to other molluscs and calcifying organisms (Weiss et al., 2002; Addadi et al., 2003; Weiner et al., 2003, Weiner et al., 2009). Still, no consensus has been reached about whether the curved fibres are or not continuous crystals, and about the evidence that they form complete helices (Li et al., 2015, Willinger et al., 2016).

4.2. Shell properties for bioinspired design

Shell microstructures from benthic molluscs provide exceptional mechanical properties to the calcified shell and hold great potential in the design of novel bioinspired materials (Kamat et al., 2000; Munch et al., 2008; Morris et al., 2016; Connors et al., 2019). Likewise, the microstructures described here for pteropods can also inspire biomimetic studies since they underpin very thin, transparent and lightweight shells, with outstanding mechanical properties. Shells of *Limacina helicina antarctica* (with crossed lamellar microstructure, Fig. 3A–C) were shown to be quite resistant to local deformation with an average hardness of 2.30 GPa and modulus of 45.27 GPa by nanoindentation (Teniswood et al., 2013, 2016). There were no significant variations in these values from multiple positions across the shell because the majority of the sample has a crossed lamellar microstructure. In contrast, hardness and modulus in the cavolinioidean *Cavolinia uncinata* (Zhang et al., 2011) were significantly higher with values of 5.2 or 5.6 GPa and 85.9 or 51.5 GPa, respectively, depending if the measurements were taken parallel or transverse to the shell surface. These values and differences across the shell are as good as nacre and reflect the anisotropic mechanical properties provided by the well-packed curved fibres and their interlocked structure (Zhang et al., 2011). Anisotropic properties, *i.e.,* having different mechanical properties in different directions, have long been described in wood (Wainwright et al., 1982) and have also been reported in other biominerals due to their complex hierarchical microstructures, such as bone (Weiner and Wagner, 1998), tooth enamel (Zaytsev and Panfilov, 2015), nacre (Barthelat et al., 2006), chiton scales (Connors et al., 2019) and crustacean exoskeletons (Chen et al., 2008; Fabritius et al., 2009). Li and coauthors, using shells of the pteropod *Clio pyramidata*, have also demonstrated the shell anisotropic properties for this species and that the helical and interlocking nature of the fibres limits damage and retards crack propagation (Li et al., 2015). The shells of the Cavolinioidea are thus mechanically robust although being very thin and made of at least 95% of aragonite, a mineral that is naturally stiff and brittle (Zhang et al., 2011; Li et al., 2015). Pteropods undergo considerable vertical migrations everyday by sinking and swimming in the water column (Karakas et al., 2020). The improved mechanical properties of their shells make them well adapted to withstand variations from hydrodynamic forces.

Interlocking structures in nature have been sought to develop new multifunctional materials, which are both flexible and fracture resistant (Estrin et al., 2009; Connors et al., 2019; Zhu et al., 2020). Helical shapes have also been found to have a favourable effect on the overall mechanical properties of novel materials (Lapovok et al., 2017; Chang et al., 2021; Gao et al., 2021). The interlocked design of the aragonite fibres in pteropod shells combined with the helical structure is therefore a promising prototype in the field of biomaterials research, ensuring a good combination for high strength and stretchability. An exact *in vitro* reproduction of the structure can however be quite challenging since it is the product of mantle epithelial cells and particular organic molecules, notably SMPs, which are likely to provide a scaffold and are able to promote/prevent growth by adhering to particular crystal faces (Checa et al., 2016).

5. The quest for biomineralization genes

Due to the growing interest in pteropods as bioindicators of OA (Bednaršek et al., 2014, 2017; Manno et al., 2017), different species have been subject to studies on the effects of increasing CO_2 on calcification (Comeau et al., 2009; Moya et al., 2016; Howes et al., 2017; Maas et al., 2018; Mekkes et al., 2021a, Mekkes et al., 2021b). Differential gene expression has become a commonly used method to understand the observed physiological responses at a molecular level and has been performed in the straight shelled *Clio pyramidata* (Maas et al., 2015), the coiled *Limacina helicina* (Koh et al., 2015), *Limacina helicina antarctica* (Johnson and Hofmann, 2017; Johnson and Hofmann, 2020; Johnson et al., 2019; Bogan et al., 2020), *Limacina retroversa* (Maas et al., 2018, Maas et al., 2020), *Heliconoides inflatus* (Moya et al., 2016) and the unshelled species *Clione limacina* (Thabet et al., 2017). Some of the studies combine both molecular and physiological responses (e.g. by measuring calcification and respiration rates) and report shifts in the expression of biomineralization genes (reviewed in Strader et al., 2020). Identifying candidate biomineralization genes is thus a critical step for accurate interpretation of gene expression results with respect to calcification. In pteropods, annotation of biomineralization genes has been done by homology searches against a biomineralization database (Moya et al., 2016) or by using the mantle transcriptome and SMPs identified from the pacific oyster *Crassostrea gigas* as a reference (Maas et al., 2015; Johnson and Hofmann, 2017). However, biomineralization genes, most notably those coding for SMPs, show a complex evolutionary history, with both ancient and rapidly evolving genes being continuously recruited to shell formation (Marie et al., 2013; Arivalagan et al., 2017). Moreover SMP-coding genes have the tendency to expand, contract and rearrange in the genome (Kocot et al., 2016; Aguilera et al., 2017) giving rise to a pool of shared domains and lineage-specific novelties that hinder cross-species sequence comparisons. On one hand, domains in SMPs are not specific of biomineralization and are present in many other sequences, returning false positives in blast searches. On the other hand, molluscan SMPs are primarily identified from bivalves or gastropods that are phylogenetically distant from pteropods, resulting in lower detection of homologs for fast evolving genes in this group. To minimize these aspects while having an indication of candidate SMPs across the pteropods evolutionary tree, we searched the adult transcriptomes (Tab. S2) from 19 Euthecosomata (fully shelled), 2 Pseudothecosomata (1 pseudo-shelled, 1 shelled) and 4 Gymnosomata (unshelled) from Peijnenburg et al. (2020) plus 3 outgroups from Zapata et al. (2014) against a database of the SMPs identified in two heterobranch species by proteomics (Mann and Jackson, 2014; Herlitze et al., 2018). The two gastropods were the terrestrial grove snail *Cepaea nemoralis* and the freshwater great pond snail *Lymnaea stagnalis*, which are the most

closely related species to pteropods with a characterized shell proteome, also known as 'shellome' (Marin et al., 2013). The resulting homology profiles (Tab. S3) are visualized in the context of pteropod evolution (Fig. 6).

Overall, the transcriptomes of unshelled pteropod species have fewer hits (<20) with SMPs than the transcriptomes of the shelled species. The SMP 'Chitin-binding peritrophin-A' (light brown, Fig. 6) is present as a putative single gene in 18 out of 20 shelled pteropods and absent from all unshelled species (Tab. S3). Experiments with a recombinant Pif97, a pearl oyster SMP having one von Willebrand factor type A (vWA) domain and one Peritrophin A chitin-binding domain, have shown that the protein interacts with calcite crystals by becoming occluded and forming nanochambers within the crystal interior (Chang and Evans, 2015). The same study suggested that both the vWA and Peritrophin A chitin-binding domains would be directly involved in the protein–mineral interaction. Moreover chitin, a complex polymer of N-acetylglucosamine, is often an important component of shells (Goffinet and Jeuniaux, 1979) and it is therefore likely that other genes involved in chitin formation are expressed in shelled species. Most shelled species show the presence of multiple transcripts homologous to chitin-interacting proteins (see in blue, Fig. 6), while unshelled species have fewer blast hits (Tab. S3). One exception is the pseudothecosome *Cymbulia sibogae* which possesses 11 transcripts and at least 6 putative genes homologous to chitin-interacting proteins. This species lacks a calcified shell but is characterized by a gelatinous pseudoconch made of proteins and polysaccharides. The presence of multiple transcripts with chitin-interacting domains may indirectly suggest that chitin is a major component of this structure. Also the 'Adipocyte plasma membrane-associated like protein' (APMAP), which is one of the most abundant SMP in the land snail *Euhadra quaesita* based on a proteomics approach (Shimizu et al., 2019), is consistently found in the shelled pteropods but absent from the unshelled species (see APMAP in dark pink, Fig. 6). Similar homology profiles are also observed for the 'Shell-related protein' (dark green, Fig. 6) and several uncharacterized proteins (pink, Fig. 6), i.e. proteins that lack homology with known domains or functionally characterized sequences. By contrast, some SMPs have identical homology profiles across the dataset for shelled and unshelled species, for example, 'Carbonic anhydrase 2′ (green, Fig. 6), 'Mucin-2-like' (dark purple, Fig. 6), 'Gly-, Glu- and Pro-rich protein' (pink, Fig. 6), the 'Neurofilament protein' (light brown, Fig. 6) and the 'Intermediate filament protein' (light purple, Fig. 6). These proteins highlight the multifunctional aspects of some SMPs and might have harnessed other structural roles in non-shelled lineages, suggesting that extensive co-option events may underlie the evolution of shells in pteropods. Identification of *bona fide* SMPs from pteropods, however, is still missing but essential for understanding the molecular bases of their biomineralization and for quantifying the impact of environmental changes, such as ocean warming and acidification, on this process.

6. Conclusions

In this review we aimed to describe the striking diversity of pteropod shell microstructures, including prismatic, crossed lamellar and helical arrangements of aragonite fibres. We highlight that the helical microstructure, commonly viewed as a trait unique to the Cavolinioidea (uncoiled shells), is also present in species of the superfamily Limacinoidea (coiled shells). Moreover, there appear to be different levels of complexity of the helical microstructure, depending on the species. While the genus *Heliconoides* (Limacinoidea) and the early diverging lineages within Cavolinioidea (*Creseis*, *Styliola*, *Hyalocylis*) have a simple version of the structure – with one or incomplete helical turns – the more recent Cavolinioidea (*Clio*, *Diacria*, *Cavolinia*, *Diacavolinia*, *Cuvierina*) have a more compact helical assembly with more than one turn. Curved fibres, that seem to form the same helical assemblies, were also found in pteropod fossils, supporting the appearance of the curved microstructures at the base of the Euthecosomata. Older scientific literature reports the presence of curved crystals in other micromolluscs, in particular, in larval stages and in thin shelled taxa, suggesting that the crystal bending may be a specific adaptation for having thin, yet flexible shells. In the case of pteropods, these were essential features for a successful adaptation to a fully planktonic lifestyle, in which they drift, swim or sink, creating varying flow conditions around their shells.

To this date there is no direct identification of shell matrix proteins (SMPs) in pteropod species but, using the SMPs from the grove snail and the great pond snail as reference, we were able to determine some transcripts associated with shelled pteropods. Based on the homology profiles of SMPs between shelled and non-shelled species we identified the Chitin-binding peritrophin-A, the Adipocyte plasma membrane-associated like protein, the Shell-related protein and several uncharacterized proteins as being strong candidates for roles in pteropod biomineralization.

Shelled pteropods are seen as valuable bioindicators to monitor the impacts of ocean acidification. Identifying biomineralization genes and characterizing the diversity of shell microstructures in different pteropod species is thus an important piece of the puzzle in understanding their potential to adapt to ocean's changes.

CRediT authorship contribution statement

Paula Ramos-Silva: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing – original draft, Writing - review & editing. **Deborah Wall-Palmer:** Resources, Investigation, Writing - review & editing. **Ferdinand Marlétaz:** Data curation, Writing - review & editing. **Frédéric Marin:** Conceptualization, Methodology, Investigation, Writing - review & editing. **Katja T.C.A. Peijnenburg:** Conceptualization, Funding acquisition, Supervision, Resources, Data curation, 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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jsb.2021.107779.

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