Deciphering mollusc shell production:

Clark, Melody S.; Peck, Lloyd S.; Arivalagan, Jaison; Backeljau, Thierry; Berland, Sophie; Cardoso, Joao C. R.; Caurcel, Carlos; Chapelle, Gauthier; De Noia, Michele; Dupont, Sam; Gharbi, Karim; Hoffman, Joseph I.; Last, Kim S.; Marie, Arul; Melzner, Frank; Michalek, Kati; Morris, James; Power, Deborah M.; Ramesh, Kirti; Sanders, Trystan

Published in:
Biological Reviews

Publication date:
2020

BIOLOGICAL REVIEWS
Cambridge Philosophical Society
Biol. Rev. (2020), pp. 000–000.
doi: 10.1111/brv.12640

Deciphering mollusc shell production: the roles of genetic mechanisms through to ecology, aquaculture and biomimetics

Melody S. Clark[1*], Lloyd S. Peck[1], Jaison Arivalagan[2,3†], Thierry Backeljau[4,5], Sophie Berland[6], Joao C. R. Cardoso[7], Carlos Caurcel[8], Gauthier Chapelle[4], Michele De Noia[9,10†], Sam Dupont[11], Karim Gharbi[8], Joseph I. Hoffman[9], Kim S. Last[12], Arul Marie[2], Frank Melzner[13], Kati Michalek[12], James Morris[4], Deborah M. Power[7], Kirti Ramesh[13], Trystan Sanders[13], Kirsikka Sillanpää[14], Victoria A. Sleight[15], Phoebe J. Stewart-Sinclair[12], Kristina Sundell[14], Luca Telesca[16], David L. J. Vendrami[9], Alexander Ventura[11], Thomas A. Wilding[12], Tejaswi Yarra[1,8] and Elizabeth M. Harper[16]

[1]*British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley Road, Cambridge, CB3 0ET, U.K.*
[2]*UMR 7245 CNRS/MNHN Molécules de Communications et Adaptations des Micro-organismes, Sorbonne Universités, Muséum National d'Histoire Naturelle, Paris, France*
[3]*Proteomics Center of Excellence, Northwestern University, 710 N Fairbanks Ct, Chicago, IL, U.S.A.*
[4]*Royal Belgian Institute of Natural Sciences, Rue Vautier 29, Brussels, B-1000, Belgium*
[5]*Evolutionary Ecology Group, University of Antwerp, Universiteitsplein 1, Antwerp, B-2610, Belgium*
[6]*UMR 7208 CNRS/MNHN/UPMC/IRD Biologie des Organismes Aquatiques et Ecosystèmes, Sorbonne Universités, Muséum National d'Histoire Naturelle, Paris, France*
[7]*Centro de Ciencias do Mar, Universidade do Algarve, Campus de Gambelas, Faro, 8005-139, Portugal*
[8]*Ashworth Laboratories, Institute of Evolutionary Biology, University of Edinburgh, Charlotte Auerbach Road, Edinburgh, EH9 3FL, U.K.*
[9]*Department of Animal Behavior, University of Bielefeld, Postfach 100131, Bielefeld, 33615, Germany*
[10]*Institute of Biodiversity Animal Health and Comparative Medicine, University of Glasgow, Glasgow, G12 8QQ, U.K.*
[11]*Department of Biological and Environmental Sciences, University of Göteburg, Box 463, Göteburg, SE405 30, Sweden*
[12]*Scottish Association for Marine Science, Scottish Marine Institute, Oban, Argyll, PA37 1QA, U.K.*
[13]*GEOMAR Helmholtz Centre for Ocean Research, Kiel, 24105, Germany*
[14]*Swemarc, Department of Biological and Environmental Science, University of Gothenburg, Box 463, Gothenburg, SE405 30, Sweden*
[15]*School of Biological Sciences, University of Aberdeen, Zoology Building, Tillydrone Avenue, Aberdeen, AB24 2TZ, U.K.*
[16]*Department of Earth Sciences, University of Cambridge, Cambridge, CB2 3EQ, U.K.*

ABSTRACT

Most molluscs possess shells, constructed from a vast array of microstructures and architectures. The fully formed shell is composed of calcite or aragonite. These $CaCO_3$ crystals form complex biocomposites with proteins, which although typically less than 5% of total shell mass, play significant roles in determining shell microstructure. Despite much research effort, large knowledge gaps remain in how molluscs construct and maintain their shells, and how they produce such a great diversity of forms. Here we synthesize results on how shell shape, microstructure, composition and organic content vary among, and within, species in response to numerous biotic and abiotic factors. At the local level, temperature, food supply and predation cues significantly affect shell morphology, whilst salinity has a much stronger influence across latitudes. Moreover, we emphasize how advances in genomic technologies [e.g. restriction site-associated DNA sequencing

* Address for correspondence (Tel: +44 1223 221371; E-mail: mscl@bas.ac.uk).
†Current address.

(RAD-Seq) and epigenetics] allow detailed examinations of whether morphological changes result from phenotypic plasticity or genetic adaptation, or a combination of these. RAD-Seq has already identified single nucleotide polymorphisms associated with temperature and aquaculture practices, whilst epigenetic processes have been shown significantly to modify shell construction to local conditions in, for example, Antarctica and New Zealand. We also synthesize results on the costs of shell construction and explore how these affect energetic trade-offs in animal metabolism. The cellular costs are still debated, with $CaCO_3$ precipitation estimates ranging from 1–2 J/mg to 17–55 J/mg depending on experimental and environmental conditions. However, organic components are more expensive (∼29 J/mg) and recent data indicate transmembrane calcium ion transporters can involve considerable costs. This review emphasizes the role that molecular analyses have played in demonstrating multiple evolutionary origins of biomineralization genes. Although these are characterized by lineage-specific proteins and unique combinations of co-opted genes, a small set of protein domains have been identified as a conserved biomineralization tool box. We further highlight the use of sequence data sets in providing candidate genes for *in situ* localization and protein function studies. The former has elucidated gene expression modularity in mantle tissue, improving understanding of the diversity of shell morphology synthesis. RNA interference (RNAi) and clustered regularly interspersed short palindromic repeats - CRISPR-associated protein 9 (CRISPR-Cas9) experiments have provided proof of concept for use in the functional investigation of mollusc gene sequences, showing for example that Pif (aragonite-binding) protein plays a significant role in structured nacre crystal growth and that the *Lsdia1* gene sets shell chirality in *Lymnaea stagnalis*. Much research has focused on the impacts of ocean acidification on molluscs. Initial studies were predominantly pessimistic for future molluscan biodiversity. However, more sophisticated experiments incorporating selective breeding and multiple generations are identifying subtle effects and that variability within mollusc genomes has potential for adaption to future conditions. Furthermore, we highlight recent historical studies based on museum collections that demonstrate a greater resilience of molluscs to climate change compared with experimental data. The future of mollusc research lies not solely with ecological investigations into biodiversity, and this review synthesizes knowledge across disciplines to understand biomineralization. It spans research ranging from evolution and development, through predictions of biodiversity prospects and future-proofing of aquaculture to identifying new biomimetic opportunities and societal benefits from recycling shell products.

Key words: integrative biomineralization, calcification, calcium, skeleton, adaptation, phenotypic plasticity, ion channels, *Crassostrea*, *Pinctada*, *Mytilus*

CONTENTS

I. INTRODUCTION

Molluscs are amongst the most successful phyla with 70000–76000 currently described species and calculated estimates of up to 200000 species (Rosenberg, 2014). A major part of the success of the Mollusca is often claimed to be their biomineralized shells, although some groups, including most cephalopods (e.g. squid and octopus) and some gastropods (e.g. nudibranchs) have secondarily lost an external shell. Their biomineralized shells have also produced a long and rich evolutionary history in the fossil record. Yet, the heavily calcified shells in bivalves and gastropods are now seen as a potential vulnerability under future environmental change, which has fuelled research into molluscan biomineralization.

In recent years, the main focus on the consequences for molluscs under changing conditions has been on ocean acidification. The continuing reduction in seawater pH and associated changes in carbonate chemistry mean that skeletons of marine species are more energetically expensive to produce with consequences for the ability of molluscs to construct and maintain their calcium carbonate skeletons (Wittmann & Pörtner, 2013). However, numerous studies demonstrate that temperature, salinity and hypoxia can have greater impacts than acidification on animal energetics: altering metabolic rates, increasing nutritional requirements and affecting the food supply itself (e.g. Dickinson *et al.*, 2012; Clark *et al.*, 2013; Hiebenthal *et al.*, 2013; Telesca *et al.*, 2018, 2019). The majority of these observations are based on laboratory experiments, examining the effects of one or two (at most) stressors on molluscs. In reality, the marine environment is far more complex with multiple interacting biotic and abiotic drivers that can significantly impact animal homeostasis. It is difficult, if not impossible, to incorporate the wide range of environmental heterogeneity, natural fluctuations and dispersal opportunities over different spatial scales into laboratory experiments (Sanford & Kelly, 2011; Urban *et al.*, 2016). Thus, it is perhaps not surprising that as experimental protocols become more complicated, using, for example, long experimental timescales and multiple generations, significant resilience is being found within populations to altered environmental conditions, much more than was predicted 10 years ago (Clark, 2020). Whilst such experimental approaches have limitations for predicting future biodiversity patterns at assemblage and ecosystem levels, they have two major advantages. Namely, they can indicate differences in resilience among species and aid in uncovering the mechanisms underpinning molluscan responses to changing environments. The latter may include evaluations of cellular energetics, transport of calcium and the genes and proteins involved in shell production, thus contributing to fundamental knowledge on how molluscs build and maintain their shells.

It should be also emphasized that whilst ecological studies evaluating molluscs' resilience to climate change have taken centre stage over recent years, there are many other areas of research where a greater understanding of biomineralization is important. These include evolution of species and particular proteins, global carbon cycling and more applied areas including optimization of aquaculture practices, development of novel bio-inspired materials (biomimicry) and reuse of shells for societal sustainability (Clark, 2020). Irrespective of the research field, what the recent ecological and molecular-related studies highlight is that a full mechanistic understanding of shell production, even in a single species is an enormous task. Large knowledge gaps still exist in the fundamental understanding of how molluscs construct and maintain their shells, and indeed in how they produce so many diverse forms.

The aim of this review is to take a multidisciplinary approach synthesizing current knowledge on how molluscs produce shells. Starting with shell structures, it encompasses mineralogy, physiology, ecology and molecular biology as all these underpin successful mollusc shell production. Discussions centre on state-of-the-art knowledge of the molluscan genetic landscape (genes, proteins, population genetics and epigenetics) and methodologies to increase the understanding of gene functions associated with biomineralization. Examples concentrate on model species for which most knowledge exists, such as the commercially important oysters *Crassostrea* spp., pearl oysters *Pinctada* spp. and blue mussels *Mytilus* spp., with examples drawn from other bivalves and gastropods as appropriate. The final section briefly reviews socio-economic aspects with regard to future prospects for molluscs in adapting to environmental change, their sustainable exploitation for aquaculture, and provision of novel products for society, including biomimetic applications and how waste shell material contributes to the circular economy.

II. SHELL MORPHOLOGY: GENETIC ADAPTATION AND PHENOTYPIC PLASTICITY

(1) Shell structure

Molluscan shells comprise layers of hierarchically arranged biocomposite materials in which a stiff mineralized phase is embedded in a softer organic matrix. Although there is great diversity in shell microstructure and architecture, the basic shell production process is shared among shelled molluscs wherein a fleshy mantle secretes the calcareous shell onto a proteinaceous sheet, the periostracum. Most shell production

research has been on bivalves, so the following account uses this group to illustrate the processes involved.

The two laterally disposed bivalve shells enclose the viscera, and in the post-larval stages are secreted by the mantle lobes, which are thin layers of tissue underlying both valves (Fig. 1). The distal edge of each mantle lobe is divided into folds, typically three in most bivalves, although there are exceptions where the outer fold is duplicated (e.g. Waller, 1980; Harper & Morton, 1994) [the arrangement is similar in the shelled cephalopod *Nautilus* but for most gastropods the typical arrangement is only two marginal folds – see Stasek & McWilliams (1973)]. While the innermost and middle folds are chiefly concerned with water inflow and sensory functions, respectively, the outermost fold secretes the shell (Yonge, 1957, 1982; Ponder & Lindberg, 2020). The first shell material formed is the periostracum, a thin (from submicrons to tens of microns thick) layer of quinone-tanned proteins, mucopolysaccharides, and lipids, secreted by specialized cells in the periostracal groove which lies between the outermost and middle mantle folds (Saleuddin & Petit, 1983; Harper, 1997). The forming sheet, secreted in the periostracal groove, extends and thickens by secretion from the inner surface of the outer mantle lobe, then matures by tanning once the thickening is complete (Saleuddin & Petit, 1983) (Fig. 1). Ultimately, the periostracal sheet reflects dorsally, forming the shell edge. The periostracum's primary function is to define and enclose the space in which the shell is secreted. In taxa with a thin periostracum, it often decays or abrades and may not persist over older parts of the shell. However, in taxa with a thicker periostracum it may remain on outer shell surfaces, where it plays important secondary roles including protection from corrosion (Tevesz & Carter, 1980). Although some bivalves initially mineralize within the periostracum layer (Checa & Harper, 2010), calcareous shell is predominantly laid down onto the periostracum by the epithelium of the outer mantle surface. Typically, figures in scientific articles about shell formation show an apparently capacious void, the extrapallial space, between the outer mantle surface and the most recently secreted shell (e.g. Harper, 1997; Checa, 2000). However, the actual gap is very narrow, around only 100 nm in the bivalve *Neotrigonia* spp. (Checa *et al.*, 2014), housing a thin film of extrapallial fluid (Fig. 1).

Molluscs display an extraordinary diversity of shell microstructure, each shell comprising two or more layers with different microstructural (and sometimes mineralogical) arrangements (Fig. 2). The precise microstructural detail has considerable phylogenetic and functional significance (Taylor, Kennedy, & Hall, 1969, 1973). The mineralized component of any particular microstructural layer in fully formed shell is always either calcite or aragonite [although the unstable polymorph vaterite may occur pathologically in patches or repairs, e.g. Nehrke *et al.* (2012) and patches of calcite have been identified in the aragonitic shell layers of some individuals of chamid bivalves (Harper, 1998)]. There is also good palaeontological and phylogenetic evidence that aragonite is the ancestral molluscan state (Taylor, 1973; Vendrasco, Checa, & Kouchinsky, 2011; Wood & Zhuravlev, 2012). Three-dimensional arrangements of mineral units vary widely, and also occur over a wide range of scales (Fig. 2). These units may be multiple crystals (Taylor, 1973), with each crystal composed of nano-units or particles as revealed by atomic force microscopy (Dauphin, 2001). The mineralized units of calcite and aragonite microstructures fall into two broad categories: fibres (including prisms, gastropod nacre and granules of various types) and sheets (including bivalve and cephalopod nacre, foliae and crossed-lamellar structures) (Checa & Salas, 2017). Despite the apparent diversity of shell microstructures, they appear to share common controls of patterns

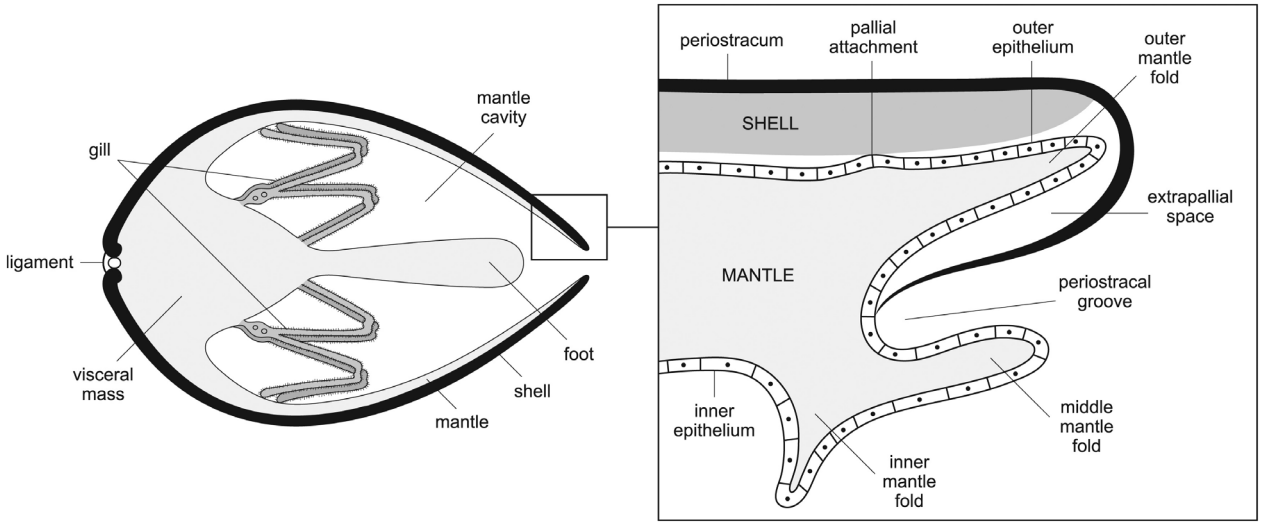

Fig 1. Schematic of bivalve morphology. (A) Generalized bivalve morphology showing the main internal features. (B) Close-up of growing shell edge showing different mantle folds and their relationship with the shell, periostracum and extrapallial space.

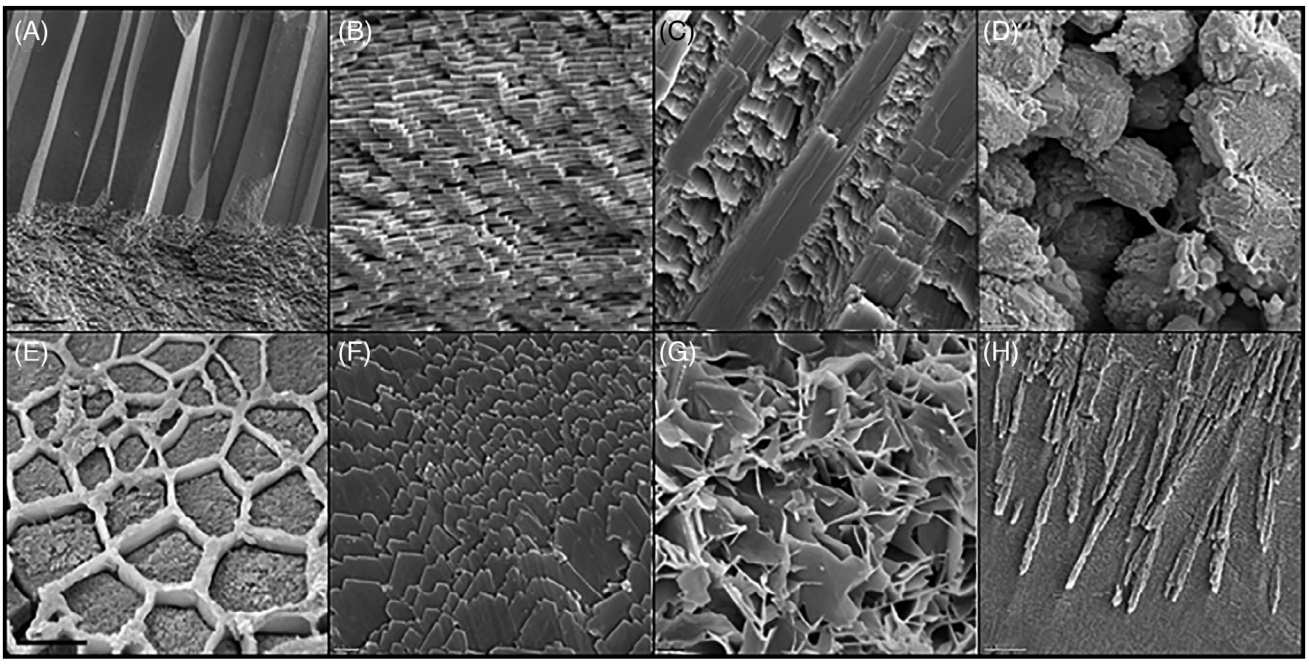

Fig 2. Scanning electron micrographs showing the diversity of bivalve shell microstructures. (A) Aragonite prisms and nacre in *Aspatharia pfeifferiana*. Scale bar = 50 μm. (B) Nacre (aragonite) in *A. pfeifferiana*. Scale bar = 10 μm. (C) Crossed-lamellar aragonite in *Ctenioides scabra*. Scale bar = 5 μm. (D) 'Homogeneous' aragonite in *Entodesma navicula*. Scale bar = 5 μm. (E) Calcite prisms over-etched showing thick organic envelopes in *Isognomon legumen*. Scale bar = 10 μm. (F) Foliated calcite in *Crassostrea gigas*. Scale bar = 2 μm. (G) Chalk (calcite) in *Ostrea edulis*. Scale bar = 5 μm. (H) Lamellar vaterite with acicular aragonite in *Corbicula fluminea*. Scale bar = 2 μm. All are fractured surfaces, except E and H, which are acid etched. Micrograph H taken by Max Frenzel.

of nucleation and growth. Surprisingly, many of the microstructures have evolved independently across or within different molluscan classes. For example, nacre has been separately acquired in bivalves, gastropods and cephalopods, and there are clear differences in the crystallographic detail among groups (Vendrasco *et al.*, 2011). Shell organic matrix is composed largely of proteins, acidic polysaccharides and chitin (Weiner & Dove, 2003). It is usually only a relatively small percentage of the shell (<5%, although it is 16% of the dry mass in certain unusual microstructures in Fan shells, *Pinna* spp.), but it appears to have extremely important roles in pre-determining the form of many shell microstructures. For example, in shell microstructures where there is a clear organic matrix the constituent crystalline units show strict morphological control and orientation whereas in those where there is little or no organic material, the growth of crystals is more reminiscent of simple inorganic spherulitic growths (Checa *et al.*, 2016), as discussed in Sections V and VI). Thus, shells are complex biocomposite materials, usually with their structure and composition under tight taxon-specific control, but which are further modified by habitat conditions.

(2) Shell phenotypic variability

Mollusc shell structure can be considerably influenced by a variety of abiotic and biotic factors. The intertidal zone can be particularly challenging, with the rigours of periodic emersion, desiccation and increased temperatures due to solar radiation significantly affecting shell morphologies, but also animal distributions and metabolic rates (Davies, 1966, 1969). Intertidal limpets generally exhibit taller, more ridged shells (providing more surface area for convective heat loss) compared with sub-tidal conspecifics (Harley *et al.*, 2009). Wave exposure also affects shell morphology: the nodulose form of the intertidal periwinkle *Tectarius striatus* (previously *Littorina striata*) inhabits sheltered areas, whilst the smooth form is common in wave-exposed areas (De Wolf, Backeljau, & Verhagen, 1998). Different shell morphologies have likewise been described in a related intertidal periwinkle *Littorina saxatilis* (Johannesson, 1986) and the flat tree oyster *Isognomon alatus* (Wilk & Bieler, 2009). In Antarctica, ice is an added environmental stress. In response to frequent brash ice impact, intertidal limpets *Nacella concinna* produce thicker, taller shells and the infaunal clam *Laternula elliptica* produces more robust shells in regions with high iceberg scour compared to protected sites (Hoffman *et al.*, 2010; Harper *et al.*, 2012).

Thicker shells are sometimes produced in response to predation threat, often sensed *via* chemical cues. The classic examples include intertidal snails and mussels that thicken shells in habitats where predatory crabs are present (e.g. Appleton & Palmer, 1988; Trussell & Smith, 2000; Trussell & Nicklin, 2002; Freeman & Byers, 2006). Chemical cues inducing thickening can be either from the predator and/or from damaged conspecifics (Appleton &

Palmer, 1988; Freeman, Meszaros, & Byers, 2009). The invasive mussel *Xenostrobus securis* produces thicker shells, stronger byssal attachments and heavier adductor muscles when drilling predators are present (Babarro, Vazquez, & Olabarria, 2016). Similarly, the blue mussel *Mytilus edulis* produces smaller, thicker shells with significantly larger adductor muscles in the presence of the predatory starfish *Asterias rubens* (Freeman & Byers, 2006). Interestingly, when exposed singularly to crabs (crushing predators) or to starfish (which prise apart the shell) *M. edulis* develops either a thicker shell or larger adductor muscles, respectively. When both cues are introduced at the same time, *M. edulis* did not express either predator-specific response, indicating that such responses are poorly integrated (Freeman *et al.*, 2009).

The altered shell morphologies produced in response to environmental factors are not necessarily uniform across all the component microstructures. There may be selective deposition of different shell layers according to the stimulus. For example, shell thickening in the periwinkle *Littorina obtusata* in response to predation was due to a 91% increase in the irregular prismatic calcite layer in new shell (Brookes & Rochette, 2007). In *Pecten maximus* scanning electron microscopy and atomic force microscopy revealed that weaker shells produced in suspended aquaculture systems compared with wild-caught animals were due to the development of modifications and abnormalities in different microstructure components over time (Grefsrud *et al.*, 2008). In fact, detailed monitoring found that changes in water chemistry, food and nutrient supply operating at very small spatial scales allowed microenvironments to be detected along single mussel ropes that impacted shell production (Michalek, 2019). At larger spatial scales *Mytilus* species studied across a 30° latitudinal range (3980 km spanning from the North Atlantic to the Arctic) identified temperature and food supply as the main drivers of mussel shape heterogeneity (Telesca *et al.*, 2018, 2019). Salinity, however, had the strongest effect on latitudinal patterns of shell shape. Salinity was also the major driver of shell deposition, organic content and microstructures (Fig. 3A, B). Mussels in low-salinity environments had thin shells and higher proportions of prismatic calcite (compared to aragonite) and organic matrix and a thicker periostracum. Mussels in high-salinity environments produced thicker shells with an increased proportion of the aragonitic nacreous layer, potentially providing enhanced mechanical protection against predators (Telesca *et al.*, 2019) (Fig. 3A, C, D). In addition, there was a strong interaction between decreasing salinity and increasing food supply, resulting in thicker periostraca, as a potential protection mechanism against the corrosive effects of low salinity (Telesca *et al.*, 2019) (Fig. 3D). Thus, many environmental factors significantly affect molluscan shell morphologies. These variant shell morphologies may be underpinned by genetic mechanisms.

(3) Phenotypic plasticity, adaptation and epigenetics

Variation in shell characteristics can often be attributed to multiple environmental factors (as detailed above). However, the relative contribution of each factor to altered shell morphology and the underlying genetic mechanisms is often unknown (e.g. Johannesson, 1986; Solas *et al.*, 2015). Two main genetic mechanisms may bring about this variation. The first is genetic determination of shell characteristics, with selection acting on standing genetic variation within populations and driving selection for characteristics more suited to the local conditions. The second is phenotypic plasticity, where a given genotype can produce different phenotypes in different environments. These mechanisms are not mutually exclusive and many studies have shown them acting in concert. Furthermore, epigenetic effects may fix favourable traits, at least temporarily, across generations. Potential genetic mechanisms have been investigated using several approaches, including controlled breeding, reciprocal transplant and common garden experiments.

Genetic crosses between either half-sib pairs or different ecotypes have demonstrated significant genetic underpinning of shell characteristics in, for example *Littorina* spp., *Bembicum vittatum* and *Nucella lapillus* (Boulding & Hay, 1993; Parsons, 1997; Guerra-Varela *et al.*, 2009), with the *Littorina* experiments also indicating significant genotype–environment interactions (Boulding & Hay, 1993). Adaptation of shell characteristics to altered conditions may be rapid. For example, *M. edulis* occur all along the North American New England coastline. In northern New England, *M. edulis* had been exposed to predation by the common crab *Carcinus maenas* for >50 years, but had never encountered the invasive predatory crab *Hemigrapsus sanguineus*. By contrast, southern New England *M. edulis* had been exposed to *C. maenas* for over 100 years and *H. sanguineus* for around 15 years (Freeman & Byers, 2006). When southern and northern New England *M. edulis* populations were experimentally exposed to *H. sanguineus*, shell thickening was weaker in northern populations that never been exposed to *H. sanguineus*. This difference was attributed to rapid adaptation (over 15 years) of the southern populations (Freeman & Byers, 2006). In recent laboratory experiments, adaptation of *M. galloprovincialis* to low pH was associated with changes in allele frequency (Bitter *et al.*, 2019). It was suggested that polygenic characters governed evolution to low pH and genotype–environment interactions released 'cryptic' genetic variation of fitness-related traits. Furthermore, three *M. edulis* generations grown in CO_2-enriched conditions revealed heritable components of calcification performance in early development (Thomsen *et al.*, 2017) and rapid adaptation in shell thickness to this corrosive environment occurred in *M. chilensis* (Guiñez *et al.*, 2017).

Reciprocal transplant experiments have been performed to test the prediction that shell shape and thickness are plastic traits, with transplanted animals adjusting their morphologies to those of native animals. Whilst experiments analysing shell shape characteristics in *Lymnaea stagnalis* indicated strong environmental control (Arthur, 1982), similar transplant experiments revealed both genetic control and phenotypic plasticity in the intertidal gastropods *Nodilittorina australis*, *Nucella lapillus* and *Acanthina monodon* (Yeap, Black, &

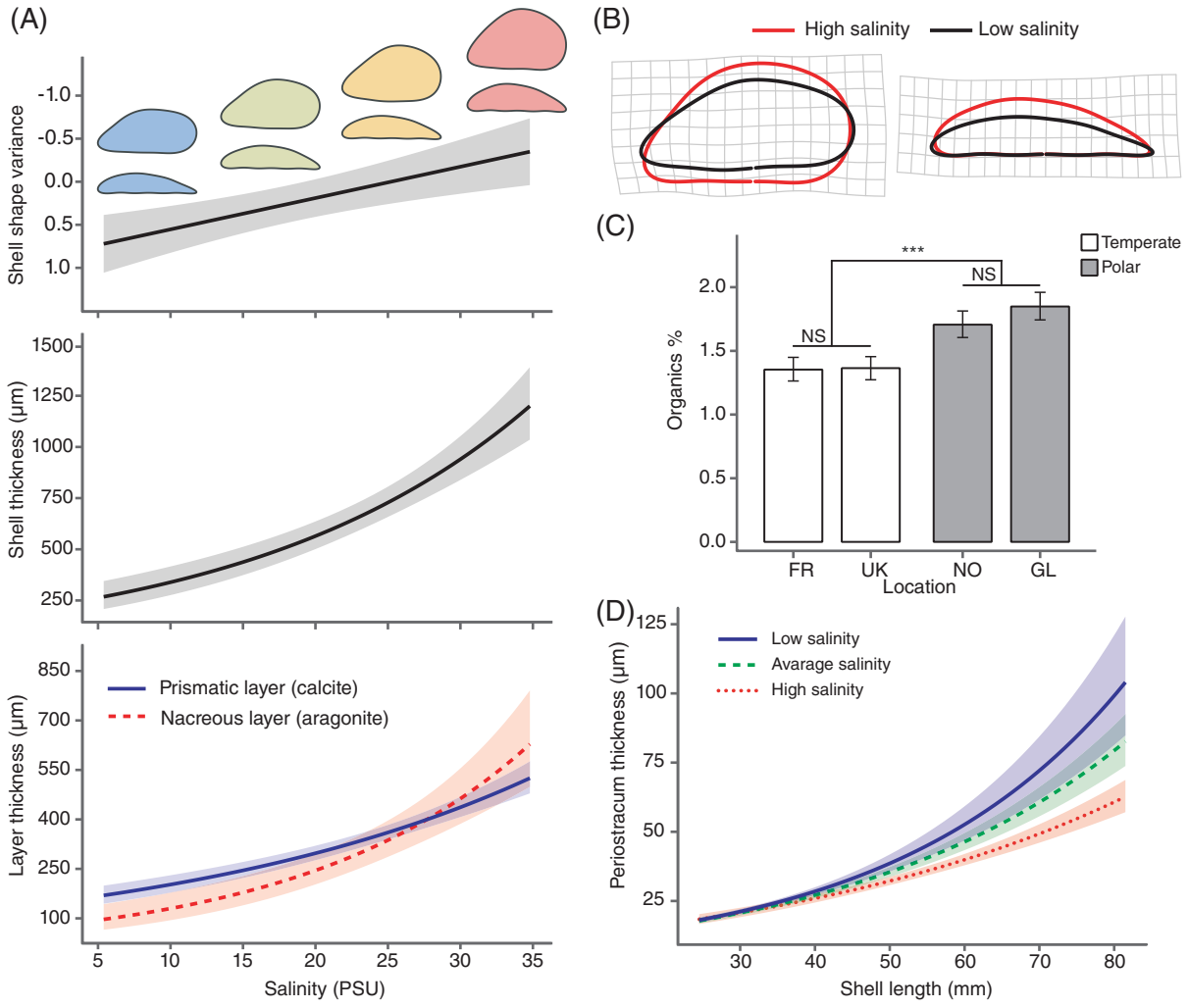

Fig 3. Influence of water salinity on *Mytilus* spp. shell shape, deposition and microstructure. (A) Top panel: shell shape variation of lateral and ventral shell views with salinity indicating formation of more elongated, narrower shells and more parallel dorsoventral margins with decreasing salinities. Middle panel: positive exponential increase of shell deposition (total thickness) with increasing salinity. Bottom panel: relationships between the thickness of prismatic (solid line) and nacreous (dashed line) layers, and salinity indicating a decreased proportion of prismatic calcite with increasing salinity and the deposition of relatively thicker aragonitic nacre at salinities >27.67 psu. Mean values (lines) and confidence intervals (shaded areas) are predicted while controlling for shell size (47.42 mm). (B) Deformation grids for both lateral and ventral *Mytilus* shell depicting the outline regions subject to different degrees of change and the bindings required to pass from the average shape under low and high salinity regimes. (C) Latitudinal variation of shell organic content within prismatic layers among shells from temperate [Brest, France (FR) and St. Andrews, United Kingdom (UK); open bars] and polar [Tromsø, Norway (NO) and Upernavik, Greenland (GR); solid bars] regions. Pairwise contrasts indicate significantly (***) higher proportions of organics (+29%) in high-latitude than low-latitude specimens with non-significant (NS) variation within climatic regions. Error bars indicate 95% CI. (D) Interacting effects of salinity and shell length on *Mytilus* periostracum. Periostracum thickness is modelled as a function of shell length for low (18.92 psu; solid line), average (25.52 psu; dashed line) and high (33.13 psu; dotted line) salinities. Predicted values (lines) and confidence intervals (shaded areas) indicate higher rates of exponential periostracal thickening with decreasing salinity. Redrawn from Telesca *et al.* (2018, 2019).

Johnson, 2001; Pascoal *et al.*, 2012; Solas, Sepulveda, & Brante, 2013). With common garden experiments, animals from different environments are cultivated in common conditions and expected to become more similar and lose the characteristics of their original environments (e.g. Pascoal *et al.*, 2012; Clark *et al.*, 2018). Again, a mix of responses occurred with these experiments in different species. Shell characteristics were genetically determined in *Littorina saxatilis* (Johannesson & Johannesson, 1996), but more likely resulted from phenotypic plasticity in the freshwater snail *Semisulcospira reiniana* (Urabe, 1998). Interestingly in transgenerational reciprocal transplant and common garden

experiments in *Nucella lapillus*, shell shape converged, suggesting synergy between maternal effects, genetic and plastic responses (Pascoal *et al.*, 2012). Shell shape also converged in freshwater snails in a multiple-generation experiment; however, there was a lag in convergence suggesting epigenetic effects (Gustafson *et al.*, 2014). This last experiment highlights one issue of using single-generation experiments. Complete roles of genetic factors involved in shell morphology may be masked by epigenetic effects, which can take several generations to diminish or change. Epigenetics can significantly modify responses, and the extent of epigenetic regulation in invertebrates is seriously underestimated (Suarez-Ulloa, Gonzalez-Romero, & Eirin-Lopez, 2015).

An early epigenetic study in molluscs using methylation-sensitive polymerase chain reaction (PCR) and bisulphite sequencing revealed extensive CpG methylation in *Crassostrea gigas* (Gavery & Roberts, 2010). To date, epigenetic studies in molluscs have predominantly involved methylation analyses, often studying developmental effects (Fallet *et al.*, 2020), although other mechanisms include remodelling of chromatin structure through chemical changes to histone proteins and regulation by small RNA molecules. Thus, epigenetic effects may result from a mix of mechanisms in natural environments (Suarez-Ulloa *et al.*, 2015). Epigenetic data on shell production in molluscs are limited. In the Antarctic limpet *Nacella concinna*, intertidal and subtidal individuals have significantly different shell masses, yet exhibit no significant population genetic differentiation (Hoffman *et al.*, 2010). Transplant and common garden experiments alongside measurements of methylation sensitive amplification polymorphisms ((MSAPs) or methylation-sensitive amplified fragment length polymorphisms (AFLPs)) revealed that these cohorts (intertidal and subtidal) had significantly different methylation patterns. However, these disappeared after 9 months in a common garden experiment, suggesting that at least some of their environmental acclimation was associated with reversible epigenetic effects through DNA methylation (Clark *et al.*, 2018). Similarly, methylation patterns correlated with adaptation to local habitats in the New Zealand mud snail (Thorson *et al.*, 2017).

The multiple factors involved in producing different shell characteristics and the contribution of each will almost certainly be species- and population-specific. Many experiments, whilst useful for demonstrating trait heritability, did not necessarily identify the genes responsible for morphological variation. Studies of morphological variation should consider the organism's genetic background along with gene flow between populations, as these analyses help to determine whether responses are mediated by phenotypic plasticity or adaptation (Michalek, 2019).

(4) Genetic background and gene flow among populations

For many years, marine environments were thought to be demographically open, especially for broadcast-spawning species with planktonic larvae (Hellberg, 2009). The genetic background and gene flow between morphologically conspecific mollusc populations has been evaluated using a variety of methods, including allozymes, random amplified polymorphic DNA (RAPD), AFLPs and microsatellites (De Wolf *et al.*, 1998; Hoffman *et al.*, 2010; Zieritz *et al.*, 2010; Pascoal *et al.*, 2012). These studies demonstrated that heterogeneity in shell shape and thickness in Antarctic limpets and freshwater mussels persist while no population differentiation could be demonstrated in neutral markers, suggesting that these characteristics may have a phenotypic plasticity component (Hoffman *et al.*, 2010; Zieritz *et al.*, 2010). The current low-cost sequencing techniques, such as restriction site-associated DNA sequencing (RAD-seq), which allow fine-scale population structure analysis, deliver resolutions not previously possible. As a result, significant population structure is being demonstrated, even in broadcast-spawning molluscs (Van Wyngaarden *et al.*, 2018; Bernatchez *et al.*, 2019; Vendrami *et al.*, 2019a, 2019b). Understanding population structure is essential for studying traits that likely result from phenotypic plasticity, local adaptation or mixtures of both.

This approach was exemplified in a recent shell morphology study in the great scallop *Pecten maximus* along the Northern Irish coast (Vendrami *et al.*, 2017). Nine populations were genotyped using microsatellite markers and single nucleotide polymorphisms (SNPs) (Vendrami *et al.*, 2017). Microsatellites revealed little genetic differentiation among populations, but using just five animals per site with RAD-Seq, Mulroy Bay (which has long been used for commercial farming of scallops) was separated from the eight other sites which contain wild unfarmed populations (Fig. 4A, B). Significant shell shape and colour differences were evident in the eight genetically undifferentiated populations, suggesting these traits have plastic components (Vendrami *et al.*, 2017) (Fig. 4C). However, in spite of these detailed SNP analyses, this study could not discount local adaptation or that shell shape and pigmentation were controlled by one or a small number of loci.

Furthermore, whilst highly sensitive at identifying population structure, RAD-Seq is limited with regards to identifying mechanisms underpinning traits because it analyses anonymous markers (short stretches of typically unannotated DNA). Although SNPs associated with environmental variables can be identified, for example temperature (Van Wyngaarden *et al.*, 2018; Vendrami *et al.*, 2019*b*), it is usually not possible to determine the causal underlying polymorphisms, limiting our understanding of mechanisms and functions (Van Wyngaarden *et al.*, 2018). Mapping SNPs produced by RAD-Seq onto reference genomes may enable the identification of genomic regions potentially under selection (Bernatchez *et al.*, 2019). However, for the vast majority of molluscs, reference genomes are not available. In this respect, the real advantage of RAD-Seq is in allowing fine-scale genome-wide mapping of genetic backgrounds and gene flow among populations, which can significantly impact experimental design when studying divergent shell shapes in conspecifics or closely related congenerics.

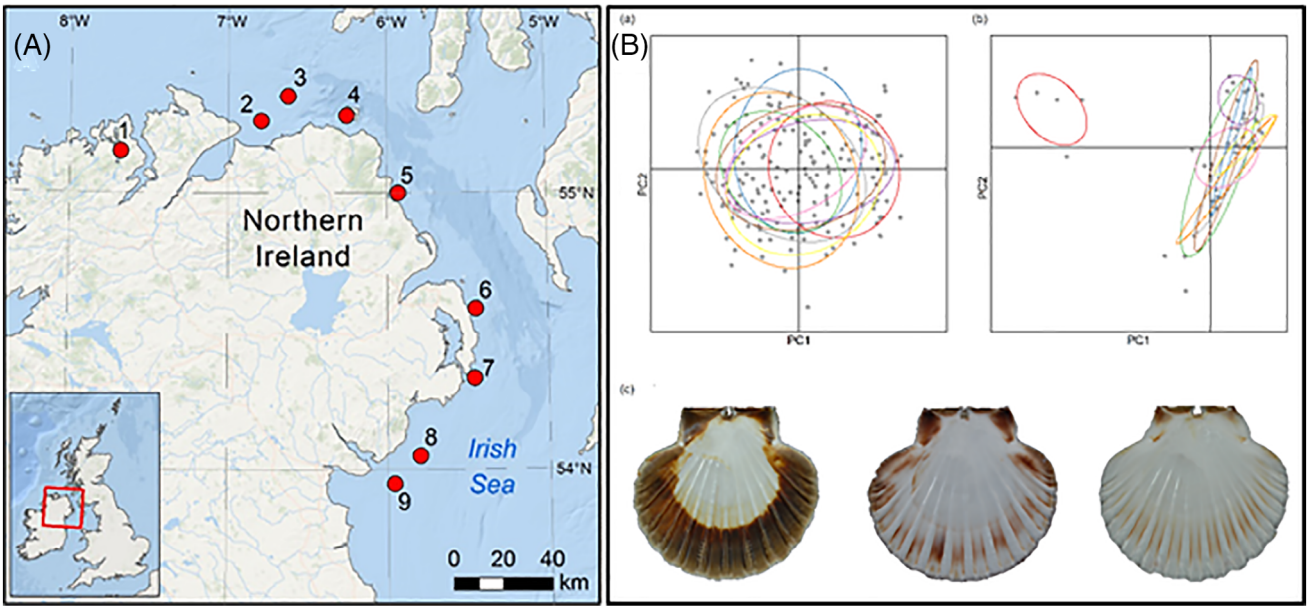

Fig 4. Genetic structure and morphological variation in scallops (*Pecten maximus*) from Northern Ireland. (A) Map of sites produced courtesy of Laura Gerrish, Esri, Garmin, GEBCO, NOAA NGDC and other contributors. (B) Panels (a) and (b) show scatterplots of individual variation in the first two principal components (PCs) derived from principal component analysis conducted on 180 individuals genotyped at 13 microsatellites and 45 individuals genotyped at 10539 single nucleotide polymorphisms (SNPs), respectively. Inertia ellipses are reported and colour-coded differently for each population. The outlier population in panel (b) is from Mulroy Bay (site 1 in A): site of the commercial scallop farm. Panel (c) shows phenotypic shell variation among three scallop shells from locations 1, 8 and 9, respectively. Data as reported by Vendrami *et al.* (2017).

III. THE COST OF MAKING A SHELL AND ENERGETIC TRADE-OFFS

(1) Costs

Shells comprise a major proportion of marine molluscan biomass. Typically >95% of a shell comprises calcium carbonate crystals and the energetic costs during construction derive from the metabolic costs of accumulating, transporting and precipitating these crystals. Separating these costs from routine metabolism is non-trivial due to the overlapping contributions of physiological processes in metabolic maintenance, soft tissue growth and calcification (Palmer, 1992). In the first experiments to quantify these costs, Palmer (1983, 1992) measured how much extra shell material was produced by groups of marine snails under particular sets of environmental conditions and the associated amount of food (energy) ingested in those conditions. The costs of calcification for *Nucella lamellosa* and *N. lapillus* were estimated by calculating the extra energy assimilated per unit of extra shell produced at a common rate of tissue growth after extracting the cost of the organic matrix. Using this approach Palmer (1992) estimated that producing 1 mg of calcium carbonate cost 1–2 J with the equivalent mass of proteinaceous organic matrix costing 29 J/mg. Similar calcification costs were estimated for oyster larvae (Waldbusser *et al.*, 2013) based on calculations of the energy demand of transmembrane ion transporters, thought to be important in molluscan calcification. These 1–2 J/mg costs were queried by Sanders *et al.* (2018) using energy budget calculations derived from scope for growth experiments of Baltic *Mytilus* spp. living at low salinity. The Sanders *et al.* (2018) calculations derived from experiments combining data from three different salinities, three feeding regimes and two temperatures. Their data indicated that shell production required much more energy than previously suggested. They calculated a cost of 10–55 J/mg calcium carbonate, which was 31–60% of available energy. However, they emphasized that these calculations were for low-salinity experiments and energetic costs decreased when salinity increased (17–55 J/mg for 6 psu and 10–14 J/mg for 11 and 16 psu). Therefore, estimating these costs for *Mytilus* mussels at full salinities (33 psu) could produce figures close to those of Palmer (1992), especially as there are extra costs experienced in low-salinity conditions. Solubility of calcification substrates decreases with salinity and therefore HCO_3^- extraction costs are higher at lower salinities, which would increase the costs of cellular transport of calcium ions *via* transmembrane ion transporters (Waldbusser *et al.*, 2013; Maar *et al.*, 2015; Sanders *et al.*, 2018) (see Section IV). In addition, shells of mussels in low salinities have different layer thicknesses and microstructures (increased prismatic layer) and thicker periostraca to protect against corrosive conditions (Telesca *et al.*, 2019). Since the periostracum is proteinaceous, such thickening adds an energetic burden (Palmer, 1992).

Although the organic matrix is only a small proportion of the shell, it is estimated that it is 22% of the cost of producing shells with 1.5% organic content, which increases to 50% with 5% organic material (Palmer, 1992). These costs confirmed previous shell regeneration experiments, in which rates of repair were inversely related to shell organic content (Palmer, 1983). Further examples of these costs include the organic content of new shell produced in *Littorina obtusata* when responding to predation threat, which was 21% lower compared with control animals as the priority was to produce a thicker shell to counteract the predation threat, irrespective of shell microstructure (Brookes & Rochette, 2007). Also limpet shells suffer erosion from being grazed by herbivores (often by conspecifics) feeding on their encrusting corallines and endolithic parasites (Day, Branch, & Viljoen, 2000). Although erosion rates were greater in *Cymbulla granatina* (previously *Patella granatina*) compared with *Scutellastra argenvillei* (previously *Patella argenvillei*), the latter produced more total shell mass annually. Day *et al.* (2000) suggested this was probably due to the higher organic content of *C. granatina* shells (3.58% compared with 1.75% for *S. argenvillei*). These observations highlight the potential long-term costs of erosion and parasite infestation that vary among species and infestation type. This has been estimated at 8–20% of total energy devoted to growth and reproduction in *C. granatina* and *S. argenvillei*, whilst infected *Haliotis tuberculata* produce 2–3 times thicker shells in response to parasitic infections of worms of the genus *Polydora* and the sponge *Cliona celata* (Peck, 1983).

Shell production costs also vary considerably with life-history stages. In mytilid mussels, larval skeletons have higher organic fractions (>10%) than adult shells (<5%), with higher relative calcification rates. This suggests higher costs of shell growth in early stages (Thomsen *et al.*, 2013). In addition, *Mytilus* spp. larval maintenance costs in low pH reflect enhanced calcification to compensate for increased dissolution rates (Ventura, Schulz, & Dupont, 2016). It is still unclear how much biotic and abiotic factors impact the energy invested into shell growth, and how this affects relative proportions of crystals and protein. Also, little is known about the consequential energetic trade-offs with regard to somatic and gonad growth when shell production costs increase (Trussell & Smith, 2000).

(2) Trade-offs

It is recognized that calcareous shell secretion and maintenance are more costly in colder waters, due to the increased solubility of calcium carbonate, making it more energetically expensive for the animal to extract (Palmer, 1983; Clarke, 1993; Watson *et al.*, 2012). However, a recent study demonstrated that high-latitude *Mytilus* spp. had thinner shells, but a higher shell organic content including production of a thicker periostracum (Telesca *et al.*, 2019). This increase in organic material should increase energetic costs associated with shell manufacture, but presumably the mussels benefit from reduced shell corrosion. Such costs are further exemplified in the invasive mussel *Xenostrobus securis* when exposed to predatory dog whelks, in which there is a trade-off between shell growth and tenacity. The presence of dog whelks induces *X. securis* to produce more proteinaceous byssus threads to increase tenacity, to the detriment of shell growth (Babarro *et al.*, 2016). The production of byssus threads can be very costly in some species requiring up to 44% of total carbon and 21% of total nitrogen uptake (Hawkins & Bayne, 1985). A further example is the Antarctic clam *Laternula elliptica*, which produces thicker shells in response to damage (most likely iceberg impact; Fig. 5). In this example, trade-offs were apparent, with the Antarctic species having higher proportions of soft tissue mass per unit shell, compared with lower latitude species despite the thicker shells. This was achieved by the Antarctic *Laternulas* spp. becoming more inflated in shape (Watson *et al.*, 2012). This trade-off of body size with shell thickness is also recognized in non-Antarctic species, the classic examples being littorinids. In these gastropods reduced body mass correlates with increased shell thickness but reduced linear shell growth (Trussell & Smith, 2000; Trussell & Nicklin, 2002; Brookes & Rochette, 2007). This results in a change in shape of the shell, which imposes additional constraints, as available living space for tissues is constrained by the shell architecture: the 'skeleton limitation' hypothesis (Palmer, 1981). Gastropod fecundity correlates with body size, and a possible consequence of size limitation is reduced fecundity (Trussell & Smith, 2000). Furthermore, predator cues may impact gastropod feeding. In the presence of predators, littorinids are more refuge-seeking and feed less, reducing energy available for shell growth (Trussell & Nicklin, 2002; Brookes & Rochette, 2007; Bourdeau, 2010). Thus, changes in shell characteristics need to be considered both in relation to environmental factors and the consequences for future sustainability (e.g. reduced fecundity).

IV. CELLULAR CALCIUM TRANSPORT

(1) Mantle tissue and calcium turnover

The basic mechanism for shell secretion in bivalve molluscs is regarded as common to all shell-bearing Mollusca, except the chitons, which build their multi-part shells, consisting of eight separate plates and girdle of spicules, within a cuticle (Kniprath, 1980; Haas, 1981). Final shell structure involves interactions between periostracum, specialized mantle epithelial cells, crystal nucleation events and proteinaceous scaffolds within the nanometre thick extrapallial region (Checa, 2000; Joubert *et al.*, 2010; Nudelman, 2015). Recent advances in molecular tools for molluscs now make it possible to examine these processes in more detail, and to begin identifying specific proteins involved in calcium transport.

Mantle tissue is responsible for calcium turnover and deposition in molluscan shells (Jodrey, 1953). Furthermore, using radioactively labelled sea water (^{45}Ca) and excised mantle strips, Jodrey (1953) demonstrated that calcium from

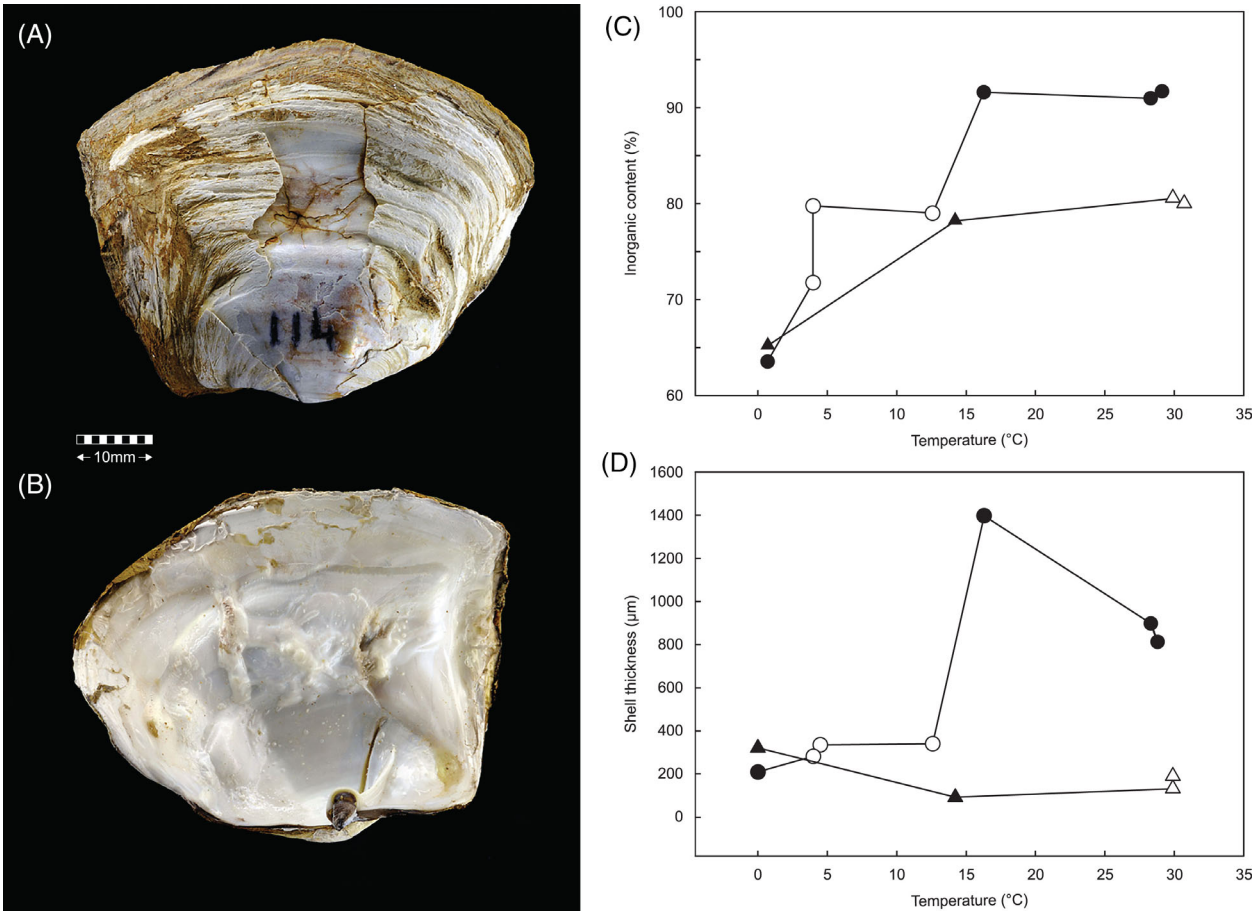

Fig 5. Variation in inorganic content and shell thickness with habitat temperature. (A) Exterior shell view of the Antarctic bivalve *Laternula elliptica* showing damage caused by iceberg impact. (B) Same shell interior showing damage extent and depth. (C) Graph showing total animal inorganic content (ash, % dry mass) of bivalves, genus *Laternula* (triangles) and gastropods, family Buccinidae (circles) with habitat temperature. Open symbols denote Northern hemisphere species and filled symbols are Southern hemisphere species. (D) Variation in shell thickness of standard-sized animals with temperature (symbols as described in C). Shell images by Pete Bucktrout, British Antarctic Survey, from Harper *et al.* (2012), reproduced under Creative Commons Attribution (https://creativecommons.org/licenses/by/3.0/us/). Data for graphs from Watson *et al.* (2012).

sea water was used for shell synthesis. Most calcium uptake for shell production in marine molluscs occurs *via* gill and mantle tissues, with the haemolymph used for transportation (Jodrey, 1953; Fan *et al.*, 2007; Li *et al.*, 2016; Sillanpää *et al.*, 2016). A small amount of calcium is rapidly turned over and it is thought that this small fraction is used to synthesize shell (Sillanpää *et al.*, 2016). This calcium primarily appears to be transported to the outer mantle epithelia in the ionic form, with small amounts bound to proteins and smaller ligands (Sillanpää *et al.*, 2016; Sillanpää, Sundh, & Sundell, 2018).

There is still considerable debate around how calcium is transported throughout the animal and transferred across outer mantle epithelia into the extrapallial space (Nudelman, 2015; Ramesh *et al.*, 2017; Sillanpää *et al.*, 2018). Several studies have shown haemocyte involvement in mineralization processes in *Crassostrea* spp., *Pinctada fucata* and *Haliotis tuberculata* (Mount *et al.*, 2004; Li *et al.*, 2016; Ivanina *et al.*, 2018). However, haemocyte contribution may be species specific. In *Crassostrea* spp., for example haemocytes are more involved in immune processes in *C. gigas* than in *C. virginica* where ion regulation and calcium transport appeared more prominent (Ivanina *et al.*, 2018). In epithelia, calcium ions might be transferred passively between cells through paracellular pathways (including septate junctions between the epithelial cells) and/or actively *via* transcellular routes (Sillanpää *et al.*, 2018). The latter mechanisms include calcium bound to specialized transport proteins, potentially involving haemocytes and intracellular vesicles, which may or may not contain amorphous calcium carbonate (Nair & Robinson, 1998; Addadi *et al.*, 2006; Li *et al.*, 2016; Ramesh *et al.*, 2017). Furthermore, contact between mantle cells and the growing edge might play a significant role in crystal nucleation, more so than the extrapallial fluid (Rousseau *et al.*, 2009; Marie *et al.*, 2012). There may also be differences in shell formation between larvae and

adults. No persistent amorphous calcium carbonate (ACC) phase was found in larval shell formation in six marine species (Kudo *et al.*, 2010; Yokoo *et al.*, 2011; Ramesh *et al.*, 2017, 2018), but roles for ACC in adult shell production are still debated. Much of this research relied on histochemical and ultra-high-powered microscopy techniques (Nudelman, 2015), but physiological studies can also provide important molecular evidence for functionality of the outer mantle epithelium, and intracellular calcium transport processes.

(2) Identification of ion channels involved in calcium transport across membranes

Ussing chamber methods (Ussing & Zerahn, 1951) developed to measure electrochemical properties of membranes and epithelia are now providing detailed insights into cellular calcium transport in molluscs. Electrophysiology of mantle epithelia in Ussing chambers has demonstrated both their calcium permeability, and their polarity (Coimbra *et al.*, 1988). Radioactive calcium ion ($^{45}Ca^{2+}$) experiments on *C. gigas* outer mantle epithelium allowed studies of both calcium transport kinetics and the identification of potential membrane proteins involved in that process. Passive transport across septate junctions occurs at rates expected from leaky epithelia, but the majority of transport occurs transcellularly. ^{45}Ca ion transfer kinetics across outer mantle epithelia indicate that 60% of calcium mobilization occurs *via* transcellular routes and at least two active transporters are involved, the other 40% occurs *via* paracellular routes, (Sillanpää *et al.*, 2018).

Selective use of $^{45}Ca^{2+}$ ions across *C. gigas* outer mantle epithelia in tandem with pharmacological inhibitors (Verapamil) indicates that calcium can enter outer mantle epithelia through L-type and T-type voltage-gated calcium channels located in the basolateral membrane (inner mantle and haemolymph side) (Sillanpää *et al.*, 2018). Similar inhibitory effects of Verapamil on calcium channels are present in other bivalve species (Roesijadi & Unger, 1993; Wang & Fisher, 1999). However, other membrane proteins also are involved in this process as Verapamil only reduced calcium transport by 20% *via* this route. In addition, due to Verapamil's general action, the particular genes involved could not be definitively identified. Calcium ion secretion from cells into extrapallial spaces across the apical membrane is mainly *via* calcium ATPases and Na^+/Ca^{2+} exchangers, as demonstrated by caloxin and ouabain experiments (Sillanpää *et al.*, 2018). The primary active membrane protein here was a plasma membrane calcium ATPase (PMCA), accounting for >50% of active calcium transport across epithelia, with indirect evidence for a secondarily active Na^+/Ca^{2+} exchanger (NCX) involved in this process (Sillanpää *et al.*, 2018). These experiments led to the development of a Ca^{2+} transfer model across outer mantle epithelia in *C. gigas* (Fig. 6). The activity of calcium ATPase proteins is energetically expensive in cells (McConnaughey & Whelan, 1997). Given the major contribution of calcium ATPases to calcium intracellular transport, the increased activity of these proteins under low-salinity environments is expected to contribute significantly to calcification costs in Baltic Sea blue mussels, limiting their distribution in this region (Sanders *et al.*, 2018; Thomsen *et al.*, 2018). Transcriptomic studies from carbonate manipulation experiments are providing a more complete list of the genes involved in cellular ion transport including Na^+/K^+ ATPases (NKAs) (De Wit *et al.*, 2018; Ramesh *et al.*, 2019). These new sequence data will facilitate the design and testing of tailor-made pharmacological inhibitors, the development of gene knockouts and potentially enable more accurate calculations to be made of the energetic costs associated with calcium transport.

V. MOLLUSC GENES AND PROTEINS ASSOCIATED WITH SHELL PRODUCTION

Early work on understanding the molecular structure of shells in the 1950s involved dissolving shells and separating proteins by chromatography, but it was not until 1996 that the first shell protein (nacrein-a) was finally characterized (Miyamoto *et al.*, 1996). For the next decade molecular data were limited and research largely focussed on cloning and characterization of individual genes and proteins. This changed dramatically with the development of molluscan expressed sequence tag (EST) libraries (e.g. Jackson *et al.*, 2006) and subsequently, from pyrosequencing, the first next generation sequencing (NGS) technology.

(1) Gene transcripts and proteins

In 2010, the first pyrosequencing results were published for molluscs (Clark *et al.*, 2010; Craft *et al.*, 2010). The latter study was the first specifically to describe the application of NGS to investigate molluscan calcification processes, using comparative genomics approaches on a mantle transcriptome. Transcriptomes catalogue expressed sequences and can be applied from single cells to whole organisms. In molluscs, research has largely concentrated on sequencing mantle tissue, because it secretes the proteins necessary for shell construction. To enhance discovery of biomineralization genes, mantle transcriptomes can be generated from shell damage-repair experiments (Clark *et al.*, 2013; Sleight *et al.*, 2015; Hüning *et al.*, 2016; Yarra, 2018). Responses vary with the site of damage compared to the tissue sampling site (Sleight *et al.*, 2015) and also whether mantle edge or inner mantle regions were targeted (Yarra, 2018). This approach, along with developmental studies on larvae raised in low-calcification-substrate conditions (e.g. De Wit *et al.*, 2018; Ramesh *et al.*, 2019) is significantly increasing knowledge of putative biomineralization genes. These approaches are particularly powerful when combined with analyses of expression modules and gene networks, such as correlative weighted *gene* co-*expression network* analysis (*WGCNA*) and programs such as the algorithm for the reconstruction of accurate cellular networks (ARACNe) (e.g. De

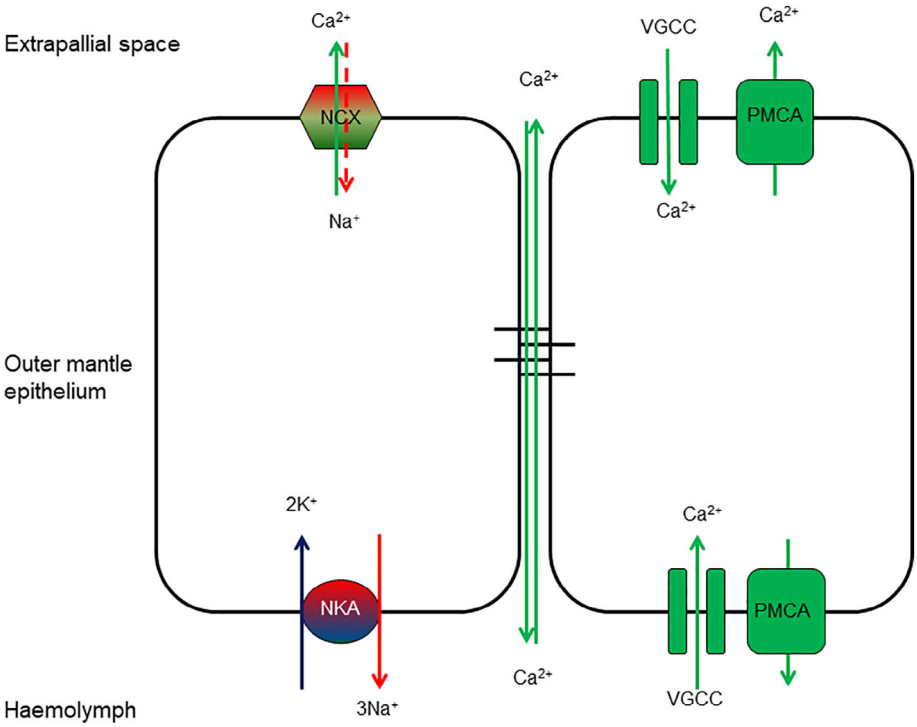

Fig 6. Proposed model of Ca^{2+} transfer across outer mantle epithelia of *Crassostrea gigas*. Ca^{2+} passively flows into outer mantle epithelium cells through basolateral voltage-gated Ca-channels (VGCCs). The Ca^{2+} is further actively secreted into the extrapallial space by apical ion channels, plasma membrane calcium ATPases (PMCAs) and Na^{+}/Ca^{2+} exchangers (NCXs), the latter driven by the trans-membrane sodium-gradient created by basal Na^{+} K^{+} ATPase (NKAs). Apical L-type voltage-gated calcium channels and basal plasma membrane calcium ATPases are also present, which suggests Ca^{2+} transfer in the opposite direction, when needed. Based on data from Sillanpää *et al.* (2018).

Wit *et al.*, 2018; Sleight *et al.*, 2020). These *in silico* techniques visualize gene interactions and provide major progress towards defining biochemical pathways in non-model species. They are particularly useful for identifying upstream control genes and incorporation of species-specific transcripts that have little or no annotation, into biochemical pathways (De Wit *et al.*, 2018; Yarra, 2018; Sleight *et al.*, 2020; Ramsøe, Clark, & Sleight, 2020).

(2) Mollusc genomes and comparative genomics

In 2012, a landmark paper in molluscan genomics described the first draft of the *C. gigas* genome (Zhang *et al.*, 2012). Since then, despite molluscs being amongst the most diverse and speciose phyla, they remain poorly represented in genome databases [e.g. the Genomes OnLine Database (GOLD; https://gold.jgi.doe.gov/index) and EnsemblMetazoa database (http://metazoa.ensembl.org/index.html)]. However, this situation should change soon with the Earth Biogenome Project (https://www.earthbiogenome.org/), which has the ambitious aim to sequence all eukaryotic species in the next decade and also programmes such as the Darwin Tree of Life (https://www.darwintreeoflife.org/). Until then mollusc genomes can be accessed far more easily than previously *via* transcriptomes and there is now a specific mollusc database of genomes and transcriptomes in 'ensembl' easy access format (https://molluscdb.org/).

(3) The evolution of shell production in molluscs

Comparison of these comprehensive gene data sets across bivalve and gastropod molluscs has revealed huge diversity in the mechanisms of shell production between even closely related species (Kocot *et al.*, 2016). This diversity became apparent in an early large-scale mollusc EST study in *Haliotis asinina*, where almost 85% of the secreted proteins were encoded by novel genes (Jackson *et al.*, 2006). Even transcriptomes from cells performing identical functions in different species (nacre-forming cells in the bivalve *Pinctada maxima* and the gastropod *H. asinina*) varied greatly in expressed gene sets. After removing housekeeping sequences, only 10% of genes from shell secretory cells were shared between the species (Jackson *et al.*, 2010). These large differences support the hypothesis of rapid and convergent evolution of nacre gene pathways in diverse molluscan lineages (Jackson *et al.*, 2010).

Rapid evolution of biomineralization pathways in molluscs was validated by the identification of species-specific large expansions in some gene families. Examples include tyrosinase, shematrin, chitinase-like protein, repetitive low complexity domain proteins (RLCDs), lysine (K)-rich mantle proteins

(KRMPs), carbonic anhydrase and calcitonin-like G-protein coupled receptors (GPCRs), all of which play roles in biomineralization (McDougall, Aguilera, & Degnan, 2013; Aguilera, Mcdougall, & Degnan, 2014; Le Roy *et al.*, 2014; Takeuchi *et al.*, 2016; Cardoso *et al.*, 2019). It is unclear why these gene families evolved in certain species and what functions the individual duplicated genes perform. Furthermore, the genes involved in shell production also comprise lineage-restricted proteins and unique combinations of co-opted ancient genes. In some cases, these genes result from domain shuffling, with expansion and loss of particular domains in genes such as RLCDs (Kocot *et al.*, 2016; Aguilera, Mcdougall, & Degnan, 2017). With such complexity, the question is whether conserved sets of genes or proteins involved in shell secretion exist in molluscs, that is a biomineralization toolbox.

(4) A conserved biomineralization tool box

In spite of the rapid evolution of many biomineralization genes, some genes, including carbonic anhydrase and RLCDs have long evolutionary histories within calcifying metazoans. Many of these 'ancient' genes have multiple functions for example carbonic anhydrase is important for acid–base physiology alongside biomineralization (Le Roy *et al.*, 2014; Murdock, 2020). Furthermore, recent proteomic studies on dissected shell microstructures in a single species showed that whilst there were different shell matrix proteins in each layer, common proteins were present throughout the shell (Marie *et al.*, 2012; Gao *et al.*, 2015).

Identification of a conserved biomineralization tool box within the Mollusca is impeded due to the lack of comprehensive data sets across species and most data originating from transcriptomes, where a gene is only represented if it is expressed. In addition, transcriptome and proteome content depend highly on the methods used to generate them (Arivalagan Immanuel, 2017). Two recent studies addressed some of these methodology issues by generating shell proteomes and mantle transcriptomes from the same individuals in species with differing mineralogies (Arivalagan *et al.*, 2017; Yarra, 2018) (Fig. 7). These investigations revealed several domains categorized as an evolutionarily conserved toolbox for shell biomineralization [tyrosinase, carbonic anhydrase (CA), chitin-binding (CB) and von Willebrand Factor A (VWA)]. Other domains were mineralogy specific [calcite: epidermal growth factor (EGF), fibronectin (FN3) and whey acidic protein (WAP); aragonite: laminin (Lam-G)] (Arivalagan *et al.*, 2017; Yarra, 2018) (Fig. 7). These data were substantiated by analyses comparing larval and adult biomineralization genes, which revealed substantial differences between life-history stages, albeit with conservation of CA, CB and VWA domains (Zhao *et al.*, 2018).

Transcriptome and proteome data sets are highly complementary. Transcriptomes have proven more effective than proteomes at identifying transcripts containing intrinsically disordered domains, including those for RLCD proteins, but proteomes proved that immune-related proteins were incorporated into shell materials. Previously their presence in transcriptomes was assumed to be an immune response or contamination from haemocytes (Arivalagan *et al.*, 2017; Yarra, 2018). Transcriptome studies also highlighted signalling molecules and especially transmembrane transporters, as important (Yarra, 2018; Ramesh *et al.*, 2019), some of which have been independently validated using physiological studies (Sillanpää *et al.*, 2018).

(5) Further levels of complexity: alternative splicing, isoforms and development

Beyond the tremendous complexity already revealed in molluscan biomineralization genes described above, there are

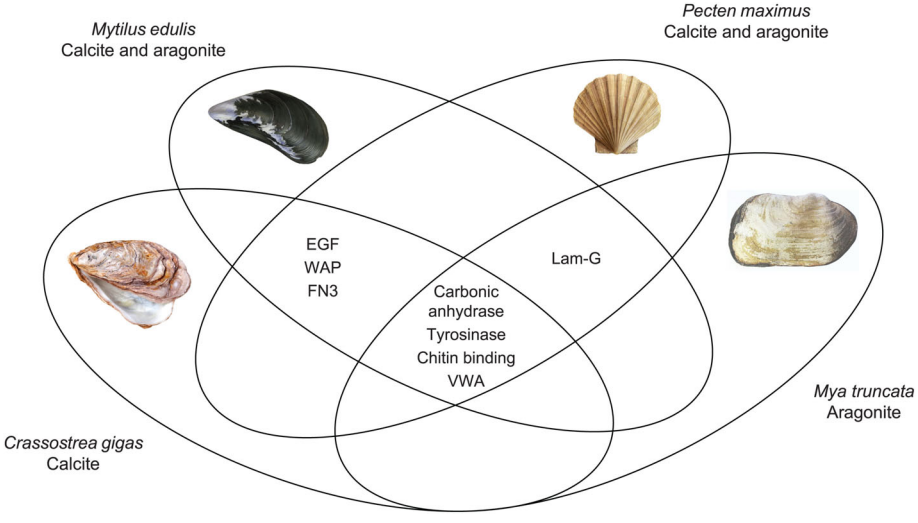

Fig 7. Venn diagram showing conserved protein domains in four bivalve species with different shell microstructures. Data from Arivalagan *et al.* (2017) and Yarra (2018) with annotation from Yarra (2018) (https://doi.org/10/cz2w). EGF, epidermal growth factor; FN3, fibronectin 3; Lam-G, laminin G; VWA, Von Willebrand factor A; WAP, whey acidic protein.

additional complications. Algorithms underpinning NGS transcriptome assembly programs enable gene isoform or alternatively spliced gene product discovery. Alternative splicing could produce isoforms with different functions or cellular locations, as recently shown with glycoprotein precursors in *Pinctada imbricata fucata* (Zhao *et al.*, 2016*a*). Comprehensive analysis of the shell proteome and tissue-specific transcriptomes of the snail *Lymnaea stagnalis* revealed several shell-forming genes associated with the crossed-lamellar microstructure. A significant number were alternatively spliced, depending on tissue origin and location (Herlitze *et al.*, 2018). A few *Lymnaea stagnalis* isoforms were solely found in either adults or larvae, suggesting a further level of biomineralization pathway sophistication (Herlitze *et al.*, 2018). Developmental delimitation in gene expression patterns has also been demonstrated in various bivalves (Kakoi *et al.*, 2008; Miyazaki *et al.*, 2010; Zhao *et al.*, 2018), suggesting this phenomenon is likely widespread. This is not surprising, as early biomineralization originates in different cells from adults. During early embryogenesis, biomineralization of the first shell (prodissoconch in bivalves and protoconch in gastropods) initiates in the shell gland as the mantle has yet to develop. The subsequent shell stage (prodissoconch II or protoconch II), is secreted by the juvenile mantle (Verdonk & Van Den Biggelaar, 1983). Larval shells of many species have similar aragonitic microstructures, whilst adults display a wide variety of species-specific crystal microstructures (Eyster, 1986). There also appears to be a species-specific element to this developmental delimitation (Herlitze *et al.*, 2018; Yarra, 2018; Zhao *et al.*, 2018; Carini *et al.*, 2019). However, since prodissoconch or protoconch production is an essential precursor to adult shell, understanding how one leads to the other and the interactions between different biomineralization pathways within the same individual is essential [the evo-devo approach (Jackson & Degnan, 2016)]. Studying larval developmental programs can provide deep insight into adult tissue morphology and structure, and how their functions differ among species (Jackson & Degnan, 2016). However, achieving a comprehensive understanding of biomineralization in adult molluscs is still a distant goal, particularly with regard to functional validation of genes involved in shell development.

VI. BIOMINERALIZATION GENES AND PROTEINS: FUNCTIONALITY

Most knowledge concerning the proteins essential for shell production has come from proteomic studies, where whole shells or specific shell layers have been powdered and proteins extracted (Joubert *et al.*, 2010; Marie *et al.*, 2012; Gao *et al.*, 2015; Arivalagan *et al.*, 2017). Any protein present within the shell probably has some role in producing the relevant microstructures. Indeed functional assays (Section VI.2) have demonstrated direct roles for many proteins in the calcification process. By contrast, transcriptomic data, largely obtained from mantle tissue, have demonstrated a particular gene's involvement through elevated expression levels using transcript counts or reverse transcription polymerase chain reaction (RT-PCR) (Miyazaki *et al.*, 2010; Fang *et al.*, 2012; Sleight *et al.*, 2015). The mantle secretes the shell, but it has many other functions; some proteins might be involved in $CaCO_3$ transport rather than in the shell structure itself. Thus, functional studies are key to deciphering the true role of these candidate biomineralization genes. Several techniques are available to provide greater functional characterization and evidence for participation in biomineralization pathways.

(1) Cellular localisation of gene expression

The mantle is a complex tissue (Harper, 1997) (Fig. 1). Whilst shell damage-repair experiments often take tissue for RNA extraction across all the mantle folds (e.g. Sleight *et al.*, 2015; Yarra, 2018), some have sampled with greater precision and shown discrete expression patterns, which were validated by *in situ* localization mapping (Gardner *et al.*, 2011). At the cellular level, similar investigations started in 1997, when the protein MSI30 was extracted from the nacreous layer of *P. imbricata fucata* and localized to the outer mantle epithelia (Sudo *et al.*, 1997). This was probably the first demonstration of mantle modularity using gene expression, although previous studies had identified regional differences in histochemical properties of mantle tissue (Timmermans, 1968). However, the generation of EST libraries and NGS data revealed the true extent of mantle gene expression modularity (e.g. Jackson *et al.*, 2006; Jackson, Worheide, & Degnan, 2007; Gardner *et al.*, 2011; Sleight *et al.*, 2016; Herlitze *et al.*, 2018). The most recent example was in *Lymnaea stagnalis*, in which 34 gene transcripts were localized by *in situ* hybridization across different developmental stages (trochophore larvae and juveniles) and in adult tissue. These data revealed six distinct expression zones in adult mantle and developmental stage-specific expression (Herlitze *et al.*, 2018). It is likely that even greater complexity and partitioning of mantle function will be revealed as more high-throughput *in situ* hybridization studies are conducted in different species in future. Such studies will play essential roles in assigning putative biomineralization functions to genes with no associated annotation, only mantle-specific expression (e.g. Sleight *et al.*, 2016).

Localization studies carried out at the proteomic level have also shown distinct partitioning of proteins between prismatic and nacre layers using mass spectrometry (Marie *et al.*, 2012). Use of antibodies raised to specific shell proteins has demonstrated that extracellular matrix proteins are associated with different crystal structures (calcite prisms or nacre) in *Pinctada* (Kong *et al.*, 2009; Fang *et al.*, 2012; Marie *et al.*, 2012). Some proteins (e.g. pearlin) have been localized to the interlamellar matrix of nacre aragonite tablets (Montagnani *et al.*, 2011), whilst others (e.g. caspartin) are present in continuous films at interfaces between prisms and surrounding insoluble sheets (Marin *et al.*, 2005).

The composition and location of extracellular matrices are being refined beyond the mass extraction of proteins from shells. This provides greater insight into crystal–protein matrix associations, and raises questions about how molluscs selectively secrete proteins to distinct microstructures. This clearly relates to the mantle zonation described above and the models discussed in Section VII.

(2) Protein activity associated with biomineralization

Despite many attempts over decades, only one immortalized molluscan cell line exists, from embryos of the snail *Biomphalaria glabrata*, but short-term cultures are still useful (Yoshino, Bickham, & Bayne, 2013). Calcium carbonate microcrystals have been produced during cell culture derived from mantle tissue of marine and freshwater pearl oysters (Samata *et al.*, 1994; Barik, Jena, & Ram, 2004; Gong *et al.*, 2008). Furthermore, during *in vitro* primary cell line culture proteoglycans and collagen have been synthesized in *Haliotis tuberculata* and nacrein protein in *P. imbricata fucata* (Poncet *et al.*, 2000; Gong *et al.*, 2008). Nacre is well known for inducing mineralization in human and mammalian bone-forming cells (Atlan *et al.*, 1997), but the osteoinductive compounds in nacre remain unidentified. The water-soluble matrix from nacre induced mineralization in mouse pre-osteoblast cell lines (Rousseau *et al.*, 2003, 2008). However, current evaluations centre on ethanol-soluble matrix fractions, which have demonstrated greater osteogenic activity, with mineralization observed in human osteoarthritis osteoblast and mouse preosteoblast cells after adding ethanol-soluble fractions from oysters (Zhang *et al.*, 2016). In a different functional assay, the chitinase-specific pharmacological inhibitor Allosamidin demonstrated a potentially important role for chitinase in shell production and shell remodelling. Larvae cultured with this inhibitor produced thinner shells and applying a chitinase solution to adult shells resulted in shell dissolution (Yonezawa *et al.*, 2016).

In a few examples, isolated shell proteins have induced calcification activity in cell cultures. For example, when a shell protein isolated from *P. imbricata fucata*, p10, was added to mammalian mineralogenic cell lines, alkaline phosphatase (an early biomarker of osteoblast differentiation) activity increased. Thus, p10 has a potential biomineralizing function (Zhang *et al.*, 2006). Mollusc genes can be inserted into expression vectors and transfected into human cell lines, enabling them to be assessed with heterologous ligands to test for functional similarity in molluscs and vertebrates. For example, the ligands, calcitonin (CALC) and parathyroid hormone-related protein (PTHrP), in vertebrates regulate calcium uptake and resorption, and osteoblast and osteoclast function. Recently, homologues of the vertebrate calcitonin system have been demonstrated in molluscs, with both multiple ligands and receptors (CALCRs) identified in *M. galloprovincialis* and *C. gigas* (Fig. 8). Relatively few mollusc CALCRs have been studied, but in both *M. galloprovincialis* and *C. gigas* these receptors respond to reduced salinity (Schwartz *et al.*, 2019; Cardoso *et al.*, 2020). Demonstrating that a specific CALCR with calcitonin mediates local regulation of Ca^{2+} transport in the mantle in *M. galloprovincialis* suggests that molluscs have a regulatory system triggered from sensory inputs that likely modulate biomineralization (Fig. 8) (Cardoso *et al.*, 2020). Using a different approach, a luciferase reporter assay for the putative biomineralization transcription factor *Pf-Sp8/9* demonstrated interactions between different shell proteins (Zheng *et al.*, 2016). Increasing concentrations of this transcription factor were added to human embryonic kidney cell lines containing constructs for pearlin, prisilkin-39 and KRMP promotors (known biomineralization genes). Activity of all three genes increased

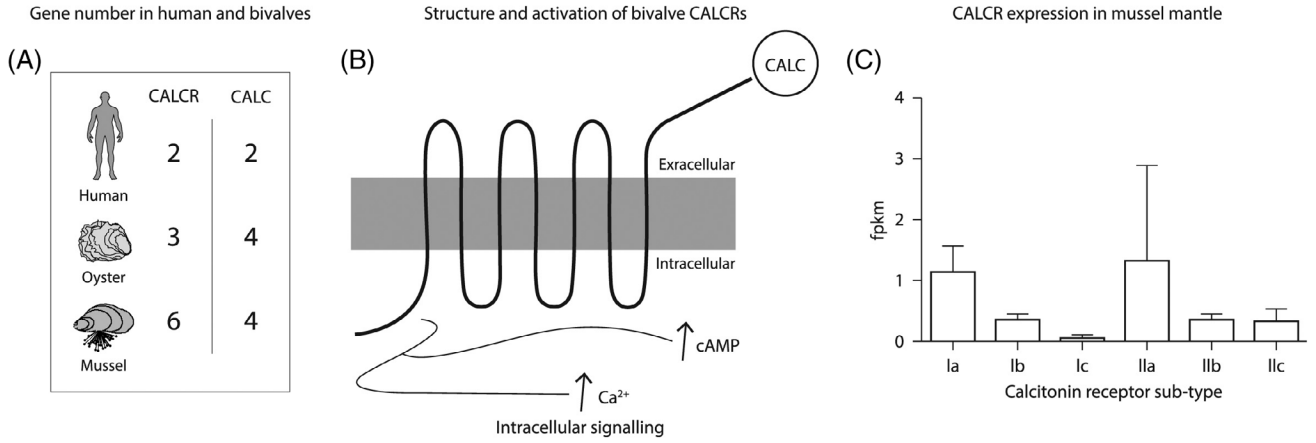

Fig 8. The bivalve calcitonin-like G-protein coupled receptor (GPCR) system. (A) Gene numbers of calcitonin-like receptors (CALCRs) and mature calcitonin-like peptide (CALC) in human, Pacific oyster (*Crassostrea gigas*) and blue mussel (*Mytilus galloprovincialis*). (B) Predicted structure of mussel CALCRs and activated intracellular signalling pathways in the presence of vertebrate (human and salmon) calcitonins and mussel calcitonin-like peptide. (C) Relative abundance (fragments per kilobase of transcript per million mapped reads; fpkm) of putative CALCR transcripts in the mussel posterior mantle edge transcriptome. Bars show mean data + standard error of the mean (SEM). Data from Cardoso *et al.* (2020).

linearly, indicating their control by *Pf-Sp8/9* (Zheng *et al.*, 2016). Thus, either homologous or heterologous *in vitro* assays using cell culture systems are providing potentially important functional information on shell proteins.

An additional approach examining *in vitro* crystallization does not use cell cultures, but aims to identify which proteins interact with $CaCO_3$ to form crystals and specific crystal microstructures. In some of the above studies, calcium precipitation assays used in tandem with cell culture experiments demonstrated the calcium binding properties of isolated proteins. For example, caspartin isolated from the *Pinna nobilis* prismatic layer strongly interacted *in vitro* with growing calcite crystals (Marin *et al.*, 2005). Its activity suggested that it was probably a calcium carbonate nucleator, but also that it constrained crystal growth and determined crystallographic orientation in the prism layer (Marin *et al.*, 2005). Prismalin-14 also exhibited calcium-binding properties, inhibiting calcium carbonate activity (Kong *et al.*, 2009; Suzuki *et al.*, 2009). Both p10 and a *Pinctada fucata* novel basic protein (PfN23) accelerated calcium carbonate nucleation *in vitro* and induced aragonite formation (Zhang *et al.*, 2006; Fang *et al.*, 2011). These *in vitro* studies usefully demonstrate calcium binding and uncover potential interactions between proteins and $CaCO_3$ precipitation and/or crystallization. This is especially useful for proteins with little sequence similarity to known proteins and with disordered structures rather than precise three-dimensional shapes (Amos & Evans, 2009). However, these *in vitro* crystallization reactions are outside the complex intracellular milieu and such experiments are predominantly employed by materials scientists aiming to engineer novel biomaterials and crystal surfaces with unique properties (e.g. Amos & Evans, 2009; Chang & Evans, 2015).

(3) Disruption of biomineralization to identify gene function

Gene knockout (or knock down) or gene editing techniques are very useful for demonstrating gene functions and interactions. To date, although there have been several successful examples in molluscs, the use of such technologies is not routine in this phylum. The earliest experiments concentrated on *P. imbricata fucata* and generally used double-stranded RNA interference (ds RNAi) to knock down target genes. The first such experiment partially knocked down Pif (aragonite-binding protein) messenger RNA (mRNA) and resulted in disordered growth of nacre crystals (Suzuki *et al.*, 2009). A subsequent experiment involving knockout of the *Pinctada fucata* homeobox containing transcription factor *msx* gene (PfMSX) also resulted in disordered nacre crystal growth (Zhao *et al.*, 2014). Furthermore, splice variants of the glycoprotein precursor (granulin/epithelin precursor) interact with the msx PfMSX to disrupt nacre formation, and the bone morphogenetic protein-2 (BMP-2) pathway, which is required to maintain normal vertebrate bone homeostasis, is involved in nacre formation (Zhao *et al.*, 2016a, 2016b). These studies therefore, established wider gene signalling and network connectivity involved in nacre formation in *P. imbricata fucata*. RNAi experiments also validated some unannotated gene effects on nacre formation (Fang *et al.*, 2011; Funabara *et al.*, 2018), expanding knowledge of biomineralization beyond 'the usual suspects', for example Pif, nacrein, p10. In a similar approach, using antibodies to disrupt the function of prisilkin-39 in *P. imbricata fucata*, led to dramatic morphological deformation of the inner shell layer (Kong *et al.*, 2009). Clustered regularly interspersed short palindromic repeats (CRISPR)-associated protein 9 (CRISPR/Cas9) was first used in *Crepidula fornicata* in 2014, demonstrating the knock-in of β-catenin in embryos (Perry & Henry, 2015), but its first use for knockout in molluscs was in 2019. This pioneering study in *Lymnaea stagnalis* reported that the *Lsdia1* gene set shell chirality from the one-cell stage in (Abe & Kuroda, 2019). This research demonstrated the utility of gene-editing technologies and how genes play key roles in three-dimensional shell structures. Given the popularity of CRISPR/Cas9 gene editing in non-mollusc species, this first use will very likely inspire more shell biomineralization functional studies.

VII. PRODUCING THE THREE-DIMENSIONAL STRUCTURE OF THE SHELL

Much evidence indicates that shell synthesis results from both biologically controlled (genetic and cellular activity, with proteins determining mineral phases, crystal shape and nucleation) and physical mechanisms (crystal competition, growth in confined spaces and self-organization) with interactions between the two (Checa, 2018). The balance between these mechanisms varies with microstructure, resulting in the wide variety of shell shapes and designs observed (Checa, 2018). From the biological side, shell protein content is important in crystal formation as are developmental pathways, such as that determined by the *Lsdia1* gene, which dictates dextral/sinistral coiling in snails (Abe & Kuroda, 2019). The modularity of mantle gene expression can also explain how such complexity in shell design could be developed (Herlitze *et al.*, 2018).

Understanding of such biological problems can be aided by theoretical studies embedded in mathematics and materials science (Gerber, 2017). In one mechanical model, shell growth was described as a function of local geometry and the mechanics of the aperture and mantle. Shell ornamentation, such as ribs on ammonite shells, could be explained from stresses and strains on the flexible mantle tissue during growth on the rigid shell aperture template (Moulton, Goriely, & Chirat, 2015). This was similar to a model using differential growth patterns, where varying proliferation rates and changing the mantle edge–cell division axis bias contributed to the production of different shell morphologies (Johnson, Fogel, & Lambert, 2019). Another mechanical model explained spine morphogenesis by the mechanical interaction of the secreting mantle edge and the calcified shell edge, which the mantle adheres to during shell growth (Chirat,

Moulton, & Goriely, 2013). This latter model accounted for the large diversity in spine structure by relatively small variations in the control parameters of the process (Chirat *et al.*, 2013). Thus, modelling mechanical processes can potentially explain shell morphology variation, although the underlying molecular mechanisms producing such forces remain elusive.

Other proposed models, including activator–effector and neural net models, are based on mathematical models developed by Turing (1952). The activator–effector model uses shell-surface pigment patterns to code for shell design (Fowler, Meinhardt, & Prusinkiewicz, 1992) and was recently used to investigate pigmentation in *Haliotis asinina* (Budd *et al.*, 2014). In this species, shell pigmentation occurs *via* prismatic layer secretory tubules likely underpinned by coordination of signals to individual cells. These are transmitted through the entire tubule, synchronizing pigment production and secretion within the shell (Budd *et al.*, 2014). Neural net models assume that neural-stimulated secretion controls cell growth, but neural nets can also encode information required for shell growth and pigmentation (Boettiger, Ermentrout, & Oster, 2009; Gong *et al.*, 2012). In these models, sensory cells read the pigmentation history and send this to a neural net, which uses this information to predict the next pigmentation increment required and instruct secretory cells accordingly, with direct feedback from output to input (Boettiger *et al.*, 2009; Gong *et al.*, 2012). These intra- and intercellular messages may include bioelectrical signalling, for which there is increasing evidence for an important role in cell-to-cell communication (Adams & Levin, 2013).

Another model provides potential links to materials science to explain crystal microstructure self-assembly. Zlotnikov & Schoeppler (2017) suggested that shell microstructures are formed *via* biologically controlled extracellular biomineralization, whereby cells create specific biochemical and physical settings that subsequently regulate mineral self-assembly without directly involving cells. Spontaneous chemical interaction then occurs between $CaCO_3$, the organic precursors and physical properties within the extracellular pallial space. This mechanism is similar to mineral deposition processes from colloidal chemistry (Zlotnikov & Schoeppler, 2017). Whether any of these models operate *in vivo* is not resolved and will be difficult to demonstrate, however, they provide theoretical frameworks to describe shell structure and a basis for further experimental design.

VIII. FUTURE PROSPECTS

(1) Molluscs in a changing world

Many factors are important in dictating species success or failure when environments change. These include generation times, population sizes, ability to adapt including genetics and phenotypic plasticity, number of propagules produced, dispersal capability, rate of environmental change and predator–prey interactions, amongst others (Peck, 2011; Urban *et al.*, 2016). In few molluscs are all, or even many, of these factors known. Many laboratory manipulation experiments suggest poor prospects for molluscan biodiversity, but more recent studies involving natural evaluations, longer-term experiments and multiple generations are producing more positive results. Molluscs may be more robust to changing environments than previously anticipated. Indeed molluscs fared comparatively well in previous extinction events compared with other phyla (Ros & Echevarria, 2011).

There is considerable genetic variability within many mollusc populations, which provides the material for natural selection and rapid adaptation and many traits have plastic characteristics (Section II.3). Certainly molluscs can thrive in some extreme situations, including at 20–30% undersaturation of calcium carbonate in the deep sea (Allen, 1978), around hydrothermal vents (Jollivet, 1996) and in shallower water CO_2 vents, such as at Ischia in the Tyrrhenian Sea (Langer *et al.*, 2014). There is also increasing recognition in a range of taxa including molluscs, that larvae can survive even fairly severe experimental conditions (albeit in depleted numbers) which provides the potential for the next generation (Ventura *et al.*, 2016; Thomsen *et al.*, 2017). These larvae, on maturation are potentially more resilient than their parents to the altered conditions, due to transgenerational plasticity (Ross, Parker, & Byrne, 2016). Parental genotypes are also critical, as very different outcomes for larval performance are produced depending on parental source, supporting the premise that resilient lines can be bred for aquaculture (e.g. Goncalves *et al.*, 2016). Laboratory experiments provide valuable evidence about mechanisms underlying responses to altered conditions, but results need context from evaluations in the natural environment, as their complexity is far greater than in experimental systems. Time series analyses of museum collections can provide valuable insights into responses to changing environments over historical timescales (relevant for the Anthropocene).

Evaluations of *Mytilus* shells from the Belgian coast have demonstrated increasing shell thickness over the past 112 years, whereas thinner shells would be predicted from experimental data (L. Telesca, L.S. Peck, T. Backeljau, M. Henig & E.M. Harper, in preparation). In this historical study, shell microstructure also varied with predator pressure from crabs, seagulls and the dog whelk *Nucella lapillus*. When the latter disappeared from local habitats in 1981, mussel shells developed with 13% less organic matrix and a 29% thinner periostracum (L. Telesca, L.S. Peck, T. Backeljau, M. Henig & E.M. Harper, in preparation). This exemplifies the complexity of mollusc shell responses to changing conditions and the need to understand biotic interactions, and responses to local conditions, including both triggers and compensatory mechanisms. This unexpected response to historical changes is supported by another historical study in a heavily calcified organism, the brachiopod *Calloria inconspicua*

(Cross, Harper, & Peck, 2018). *Calloria inconspicua* collected over the past 120 years showed no shell dissolution, but had a 3.4% increase in shell density, despite sea surface temperature increasing by 0.6°C (1953–2016) and pCO_2 increasing by 35.7 μatm (1 standard atm = 101325 Pascal) (1988–2016) (Cross *et al.*, 2018). Making accurate predictions of future mollusc biodiversity is difficult because the resilience of the mollusc itself is not the only factor to consider, as species responses are variable and each species exists within a complex ecosystem. There are indirect effects of changing conditions on feeding behaviour and food supplies (Sanders *et al.*, 2013; Mackenzie *et al.*, 2014), behaviour (Gunderson, Armstrong, & Stillman, 2016) and predator–prey dynamics (Beaty *et al.*, 2016; Donelan & Trussell, 2019). There are also altered disease risks and host–parasite interactions (Marcogliese, 2008).

Most of these factors also apply to aquaculture and predicting how aquaculture should, and can, develop globally is complex. Across the globe, molluscan aquaculture will likely suffer from environmental change impacts, the drivers of which will differ among nations (Fig. 9) (Stewart-Sinclair *et al.*, 2020). Aquaculture practices will need to evolve as conditions alter. Environmental change, for example temperature and pH, will not only have direct effects (Allison, Badjeck, & Meinhold, 2011; Frost *et al.*, 2012), but there will be indirect effects of emerging diseases, invasive species, etc. (Burge *et al.*, 2014). Monitoring environments over large spatial and temporal scales will be key for aquaculture practices to be modified accordingly (Allison *et al.*, 2011; Callaway *et al.*, 2012). This has already been particularly successful in the Pacific north-west shellfish industry. Mitigation measures including buffering sea water in header tanks and spawning stocks at key times has been very effective in a region that has been badly affected by periodic, and unpredictable, corrosive upwellings (Barton *et al.*, 2015). Furthermore, changing conditions may provide new opportunities for culturing warmer water aquaculture species in traditionally colder areas, such as around the UK and Ireland (Callaway *et al.*, 2012). Warmer waters can improve scallop recruitment and would expand the current range for culturing *C. gigas* oysters and potentially open up new areas for *Haliotis* abalone culture (Allison *et al.*, 2011; Callaway *et al.*, 2012; Goulden, 2018). Given current data, regional assessments that account for local conditions will likely be important for planning new aquaculture facilities (Michalek, 2019). Global assessments are more likely to indicate new aquaculture opportunities developing as species' ranges change (Shumway, 2011; Gjedrem, Robinson, & Rye, 2012).

(2) Mollusc shells for future innovation

Irrespective of future food security concerns, mollusc shells are increasingly recognized as valuable in themselves, as models for biomimetics or exploited in applied projects. Molluscs constitute one of the most diverse and widespread phyla that use calcium carbonate when manufacturing their

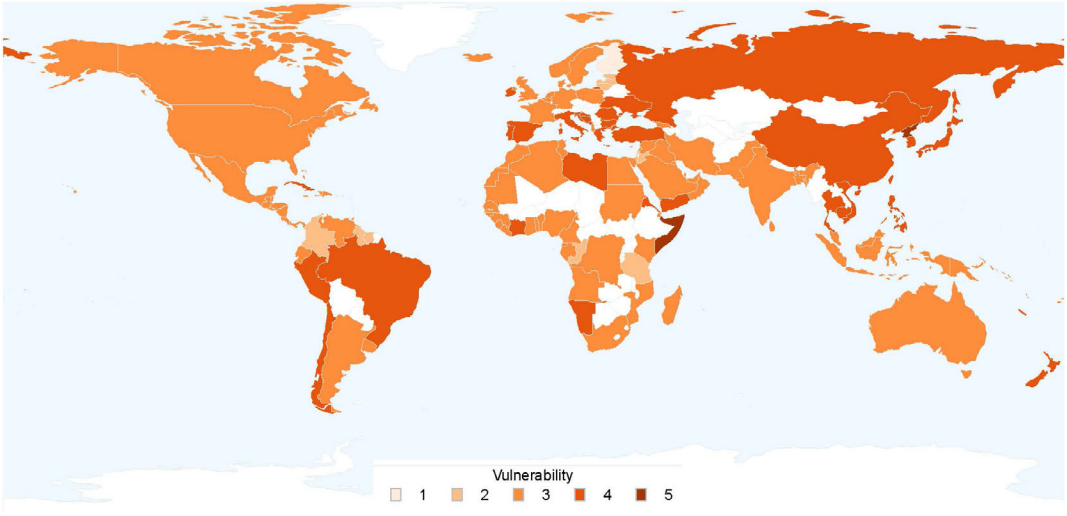

Fig 9. Overall predicted vulnerability to climate change for shellfish mariculture in coastal nations 2090–2100 using a vulnerability assessment model. Vulnerability (*V*) was measured as nation-specific mean for Exposure (*E*), Sensitivity (*S*), and Adaptive capacity (*AC*) sub-layers under the IPCC RCP8.5 'business as usual' scenario. These sub-layers comprised the following factors: exposure (*E*) was based on changes in sea surface temperature, depth of aragonite saturation horizon, primary production and occurrence of extreme weather events; sensitivity (*S*) evaluated species habitat range, production value as a normalized percentage of national gross domestic product, and nutritional dependence based on fish protein as a percentage of animal protein in the diet of each nation; adaptive capacity (*AC*) applied the Shannon index to industry diversity and governance using the World Bank world-wide governance indicator. The high sensitivity of developing nations was based largely on the narrow habitat tolerance of the cultivated species, whilst that of developing countries was due to the relatively high economic value of shellfish production with limited adaptive capacity due to the small number of species cultivated. Colours represent overall *V* score (1 = very low, 2 = low, 3 = moderate, 4 = high, 5 = very high), while white indicates absence of data. Adapted from Stewart-Sinclair *et al.* (2020).

protective shells and thus present great potential for novel bio-inspired materials (Currey, 1999). One particular example is in using nacre construction as a framework for body armour (Yadav *et al.*, 2016). Moreover, shells are produced at ambient temperature and pressure with water as a solvent. Hence, they offer very different, more sustainable ways (less energy usage, lower toxicity, etc.) to produce innovative $CaCO_3$ and related mineral-based materials (Nishimura, 2015; Xia, 2016). The production of hierarchical structures from nano- to metre-scales is, however, beyond current technologies and scalability is an issue (Mann, 2000). Thus, whilst understanding of biomineralization processes is incomplete, the self-assembly of nanocomposites remains a major challenge for bulk biomimetic materials, but one that offers great future promise (Morris *et al.*, 2016).

Alongside the inspiration given by shells in biomimetic industrial applications is the use of shells themselves. Sustainable expansion, or Blue Growth, is required and waste management in targeted sectors such as aquaculture is a key consideration. Shells comprise a significant proportion of the animal biomass and are usually discarded in aquaculture practices. There are increasing pressures for recycling and the promotion of circular economy principles (Hughes, 2017; Domenech & Bahn-Walkowiak, 2019). Much research highlights the importance of shells in marine environments, for example for reef restoration, which encourages mollusc cultivation and ecosystem development (e.g. Gutierrez *et al.*, 2003). Such studies could also be key for environmental change adaptation in facilitating natural ecosystem approaches for the protection of some low-lying coasts, under long-term sea-level rise (Bamber *et al.*, 2019; Oppenheimer *et al.*, 2019). Shells have been exploited in a wide variety of applications, including as a calcium source for laying birds, liming agents, in wastewater biofiltration and incorporation into construction aggregates (Morris, Backeljau, & Chapelle, 2019). Many more innovative shell uses could be exploited if various technical, regulatory and logistical bottlenecks can be overcome (Morris *et al.*, 2019).

IX. FUTURE DIRECTIONS

Future research will require interdisciplinary teams with broad knowledge bases fully to understand the complexities of shell design and production from the molecular level to physical shell performance. Greater genome sequencing will be required to enable the wider identification of the gene networks that control biomineralization. There is also the need to understand the role that unannotated genes play in this process, as many are often ignored in current studies but may be critical members of biomineralization pathways and fundamental drivers of skeletal structural diversity. Genomes also underpin the development of advanced molecular tools, for example in gene editing and probes for high-resolution microscopy. The latter requires resources including cell lines, which are virtually absent for molluscs, yet they are vital for gene manipulation studies and for single molecule particle tracking in live cells, which will enable comprehensive evaluations of calcium transport through cells. The development of model mollusc species is required to exploit the latest biophysical and biological techniques, more often used in medical research. Given molluscan diversity levels, a single species is unlikely to suffice and questions and requirements will dictate the mollusc models needed.

X. CONCLUSIONS

(1) Bivalves and gastropods demonstrate considerable inter- and intraspecific shell morphological variation, both inherent, and in response to environmental conditions.
(2) Variation in intraspecific shell characteristics can result from genetic adaptation with selection acting on standing population genetic variation and/or phenotypic plasticity. In some instances, adaptation can be rapid, taking place within a few generations.
(3) Epigenetics may modify phenotypic traits across generations.
(4) Shell characteristics and animal physiology are strongly defined by adaptation to local conditions, particularly temperature and food supply. Salinity exerts a stronger influence across latitudes, particularly on shell organic content and microstructures.
(5) Energetic trade-offs can occur when accommodating intraspecific variations in shell composition to different environmental conditions. Abiotic and biotic factors trigger regulatory processes that impact ongoing processes such as somatic growth, energy use and shell growth.
(6) Transport of $CaCO_3$ within cells is mediated by epithelial processes, which often involves ion channels. However, considerable debate continues as to how calcium is transported in cells.
(7) Current knowledge of biomineralization molecular pathways is largely restricted to the final proteins; there is much less information on upstream control sequences and gene networks associated with biomineralization and protein–protein interactions.
(8) At least some genes involved in biomineralization pathways have evolved rapidly. These comprise a mix of lineage-restricted proteins and unique combinations of co-opted ancient genes, which have gained additional functions through the acquisition of new domains.
(9) Alternative splice forms of genes provide added layers of complexity to biomineralization pathways. Alternative splice forms of the same genes associated with larvae and adults of the same species can differ dramatically.

(10) Recent gene editing using CRISPR/Cas9 in molluscs provides an important tool for advancing understanding of gene function and gene interactions.
(11) Despite mathematical model developments and the demonstration of transcript compartmentalization in mantle tissues, the complex variety of three-dimensional shell morphologies is largely unexplained to date.
(12) Prediction of future environmental change impacts on aquaculture is complex and needs to include indirect effects (parasites, emerging diseases, invasive species, etc.) alongside direct effects (temperature, ocean acidification, salinity, etc.) along with species interactions and assemblage/ecosystem-level effects.
(13) Historical studies indicate a greater resilience to chronic incremental climate change than has been predicted using short-term experimental evaluations.
(14) Regional assessments, accounting for local habitat conditions, will be more accurate for planning new aquaculture facilities, whilst global assessments will better indicate where new aquaculture opportunities may emerge as species' ranges alter under climate change.
(15) Selection experiments indicate that sufficient standing genetic variation exists in molluscs to enable breeding of lines more resilient to future conditions.
(16) Exploitation *via* biomimetics of shell microstructures and properties in materials science and recycling shell waste for a circular economy represent important areas where mollusc research could potentially realize societal gains.

XI. ACKNOWLEDGEMENTS

This manuscript was partly funded by the European Union Seventh Framework Programme [FP7] ITN project 'CACHE: Calcium in a Changing Environment' (www.cache-itn.eu) under REA grant agreement 605051. The authors thank everyone involved in this project including Mark Blaxter, Xushuai Zhang, Yan Wang-Duffort, Nina Fox, Rita Pereira, Nicola Munro, Elaina Ford, Lucy Gonzalez, Christina Chatzela, Rachel Ramirez, our EU Project Officer Giuliana Donini and EU Mid-Term Review Expert Guy Duke. Also, many thanks to our Project Partners: the Association of Scottish Shellfish Growers, specifically Nick Lake, Janet Brown and Walter Speirs, Coastal Research and Management, Kiel especially Peter Krost, and our External Experts Professor Catherine Boyen, Station Biologique de Roscoff and Professor Mike Thorndyke. We also thank Laura Gerrish and Jamie Oliver (British Antarctic Survey) for drawing figures. Additional funds were provided by Fundação para a Ciência e a Tecnologia (FCT) through project UID/Multi/04326/2019 and the European Marine Biological Research Infrastructure Cluster-EMBRIC (EU H2020 research and innovation program, agreement n° 654008) and a Natural Environment Research Council Studentship (Project Reference: NE/J500173/1) to V.A.S.

XII. REFERENCES

Abe, M. & Kuroda, R. (2019). The development of CRISPR for a mollusc establishes the formin Lsdia1 as the long-sought gene for snail dextral/sinistral coiling. *Development* **146**(9), dev180778.

Adams, D. S. & Levin, M. (2013). Endogenous voltage gradients as mediators of cell-cell communication: strategies for investigating bioelectrical signals during pattern formation. *Cell and Tissue Research* **352**(1), 95–122.

Addadi, L., Joester, D., Nudelman, F. & Weiner, S. (2006). Mollusk shell formation: a source of new concepts for understanding biomineralization processes. *Chemistry-A European Journal* **12**(4), 981–987.

Aguilera, F., Mcdougall, C. & Degnan, B. M. (2014). Evolution of the tyrosinase gene family in bivalve molluscs: independent expansion of the mantle gene repertoire. *Acta Biomaterialia* **10**(9), 3855–3865.

Aguilera, F., Mcdougall, C. & Degnan, B. M. (2017). Co-option and *de novo* gene evolution underlie molluscan shell diversity. *Molecular Biology and Evolution* **34**(4), 779–792.

Allen, J. A. (1978). Evolution of deep-sea protobranch bivalves. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **284**(1001), 387–401.

Allison, E. H., Badjeck, M.-C. & Meinhold, K. (2011). The implications of global climate change for molluscan aquaculture. In *Shellfish Aquaculture and the Environment* (ed. S. E. Shumway), pp. 461–490. John Wiley & Sons Inc., Hoboken.

Amos, F. F. & Evans, J. S. (2009). AP7, a partially disordered pseudo c-ring protein, is capable of forming stabilized aragonite *in vitro*. *Biochemistry* **48**(6), 1332–1339.

Appleton, R. D. & Palmer, A. R. (1988). Water-borne stimuli released by predatory crabs and damaged prey induce more predator-resistant shells in a marine gastropod. *Proceedings of the National Academy of Sciences of the United States of America* **85** (12), 4387–4391.

Arivalagan Immanuel, J. R. R. (2017). *Insights from Shell Proteome: Biomineralization and Environmental Adaptation in Bivalves*. PhD thesis. Museum Natural D'Histoire Naturelle, France.

Arivalagan, J., Yarra, T., Marie, B., Sleight, V. A., Duvernois-Berthet, E., Clark, M. S., Marie, A. & Berland, S. (2017). Insights from the shell proteome: Biomineralization to adaptation. *Molecular Biology and Evolution* **34**(1), 66–77.

Arthur, W. (1982). Control of shell shape in *Lymnaea stagnalis*. *Heredity* **49**, 153–161.

Atlan, G., Balmain, N., Berland, S., Vidal, B. & Lopez, E. (1997). Reconstruction of human maxillary defects with nacre powder: histological evidence for bone regeneration. *Comptes Rendus de L Academie des Sciences Serie III-Sciences de La Vie-Life Sciences* **320**(3), 253–258.

Babarro, J. M. F., Vazquez, E. & Olabarria, C. (2016). Importance of phenotypic plastic traits on invasion success: response of *Xenostrobus securis* to the predatory dogwhelk *Nucella lapillus*. *Marine Ecology Progress Series* **560**, 185–198.

Bamber, J. L., Oppenheimer, M., Kopp, R. E., Aspinall, W. P. & Cooke, R. M. (2019). Ice sheet contributions to future sea-level rise from structured expert judgment. *Proceedings of the National Academy of Sciences of the United States of America* **116**(23), 11195–11200.

Barik, S. K., Jena, J. K. & Ram, K. J. (2004). $CaCO_3$ crystallization in primary culture of mantle epithelial cells of freshwater pearl mussel. *Current Science* **86**(5), 730–734.

Barton, A., Waldbusser, G. G., Feely, R. A., Weisberg, S. B., Newton, J. A., Hales, B., Cudd, S., Eudeline, B., Langdon, C. J., Jefferds, I., King, T., Suhrbier, A. & Mclaughlin, K. (2015). Impacts of coastal acidification on the Pacific northwest shellfish industry and adaptation strategies implemented in response. *Oceanography* **28**(2), 146–159.

Beaty, L. E., Wormington, J. D., Kensinger, B. J., Bayley, K. N., Goeppner, S. R., Gustafson, K. D. & Luttbeg, B. (2016). Shaped by the past, acting in the present: transgenerational plasticity of anti-predatory traits. *Oikos* **125**(11), 1570–1576.

Bernatchez, S., Xuereb, A., Laporte, M., Benestan, L., Steeves, R., Laflamme, M., Bernatchez, L. & Mallet, M. A. (2019). Seascape genomics of eastern oyster (*Crassostrea virginica*) along the Atlantic coast of Canada. *Evolutionary Applications* **12** (3), 587–609.

Bitter, M. C., Kapsenberg, L., Gattuso, J. P. & Pfister, C. A. (2019). Standing genetic variation fuels rapid adaptation to ocean acidification. *Nature Communications* **10**(1), 5821.

Boettiger, A., Ermentrout, B. & Oster, G. (2009). The neural origins of shell structure and pattern in aquatic mollusks. *Proceedings of the National Academy of Sciences of the United States of America* **106**(16), 6837–6842.

Boulding, E. G. & Hay, T. K. (1993). Quantitative genetics of shell form of an intertidal snail - constraints on short-term response to selection. *Evolution* **47**(2), 576–592.

Bourdeau, P. E. (2010). An inducible morphological defence is a passive by-product of behaviour in a marine snail. *Proceedings of the Royal Society B-Biological Sciences* **277** (1680), 455–462.

Brookes, J. I. & Rochette, R. (2007). Mechanism of a plastic phenotypic response: predator-induced shell thickening in the intertidal gastropod *Littorina obtusata*. *Journal of Evolutionary Biology* **20**(3), 1015–1027.

Budd, A., Mcdougall, C., Green, K. & Degnan, B. M. (2014). Control of shell pigmentation by secretory tubules in the abalone mantle. *Frontiers in Zoology* **11**, 62.

Burge, C. A., Eakin, C. M., Friedman, C. S., Froelich, B., Hershberger, P. K., Hofmann, E. E., Petes, L. E., Prager, K. C., Weil, E., Willis, B. L., Ford, S. E. & Harvell, C. D. (2014). Climate change influences on marine infectious diseases: implications for management and society. In *Annual Review of Marine Science* (Volume **6**, eds C. A. Carlson and S. J. Giovannoni), pp. 249–277. Palo Alto, California: Annual Reviews.

Callaway, R., Shinn, A. P., Grenfell, S. E., Bron, J. E., Burnell, G., Cook, E. J., Crumlish, M., Culloty, S., Davidson, K., Ellis, R. P., Flynn, K. J., Fox, C., Green, D. M., Hays, G. C., Hughes, A. D., et al. (2012). Review of climate change impacts on marine aquaculture in the UKand Ireland. *Aquatic Conservation-Marine and Freshwater Ecosystems* **22**, 389–421.

Cardoso, J. C. R., Ferreira, V., Zhang, X., Anjos, L., Felix, R. C., Batista, F. M. & Power, D. M. (2019). Evolution and diversity of alpha-carbonic anhydrases in the mantle of the Mediterranean mussel (*Mytilus galloprovincialis*). *Scientific Reports* **9**, 10400.

Cardoso, J. C. R., Felix, R. C., Ferreira, V., Peng, M. X., Zhang, X. & Power, D. M. (2020). The calcitonin-like system is an ancient regulatory system of biomineralization. *Scientific Reports* **10**, 7581.

Carini, A., Koudelka, T., Tholey, A., Appel, E., Gorb, S. N., Melzner, F. & Ramesh, K. (2019). Proteomic investigation of the blue mussel larval shell organic matrix. *Journal of Structural Biology* **208**, 107385.

Chang, E. P. & Evans, J. S. (2015). Pif97, a von Willebrand and Peritrophin biomineralization protein, organizes mineral nanoparticles and creates intracrystalline nanochambers. *Biochemistry* **54**(34), 5348–5355.

Checa, A. G. (2000). A new model for periostracum and shell formation in Unionidae (Bivalvia, Mollusca). *Tissue & Cell* **32**(5), 405–416.

Checa, A. G. (2018). Physical and biological determinants of the fabrication of molluscan shell microstructures. *Frontiers in Marine Science* **5**, 353.

Checa, A. G. & Harper, E. M. (2010). Spikey bivalves: intra-periostracal crystal growth in Anomalodesmatans. *The Biological Bulletin* **219**(3), 231–248.

Checa, A. G. & Salas, C. (2017). Treatise online no 93: Part N, Revised, Volume 1, Chapter 3. Periostracum and shell formation in the Bivalvia. https://journals.ku.edu/treatiseonline/article/view/6650

Checa, A. G., Salas, C., Harper, E. M. & Bueno-Perez, J. D. (2014). Early stage biomineralization in the periostracum of the 'living fossil' bivalve *Neotrigonia*. *PLoS One* **9**(2), e90033.

Checa, A. G., Macias-Sanchez, E., Harper, E. M. & Cartwright, J. H. E. (2016). Organic membranes determine the pattern of the columnar prismatic layer of mollusc shells. *Proceedings of the Royal Society B-Biological Sciences* **283**(1830), 20160032. https://doi.org/10.1098/rspb.2016.0032.

Chirat, R., Moulton, D. E. & Goriely, A. (2013). Mechanical basis of morphogenesis and convergent evolution of spiny seashells. *Proceedings of the National Academy of Sciences of the United States of America* **110**(15), 6015–6020.

Clark, M. S. (2020). Molecular mechanisms of biomineralization in marine invertebrates. *Journal of Experimental Biology* **223**(11), jeb206961.

Clark, M. S., Thorne, M. A. S., Vieira, F. A., Cardoso, J. C. R., Power, D. M. & Peck, L. S. (2010). Insights into shell deposition in the Antarctic bivalve *Laternula elliptica*: gene discovery in the mantle transcriptome using 454 pyrosequencing. *BMC Genomics* **11**, 362.

Clark, M. S., Thorne, M. A. S., Amaral, A., Vieira, F., Batista, F. M., Reis, J. & Power, D. M. (2013). Identification of molecular and physiological responses to chronic environmental challenge in an invasive species: the Pacific oyster, *Crassostrea gigas*. *Ecology and Evolution* **3**(10), 3283–3297.

Clark, M. S., Thorne, M. A. S., King, M., Hipperson, H., Hoffman, J. I. & Peck, L. S. (2018). Life in the intertidal: cellular responses, methylation and epigenetics. *Functional Ecology* **32**(8), 1982–1994.

Clarke, A. (1993). Seasonal acclimatization and latitudinal compensation in metabolism - do they exist. *Functional Ecology* **7**(2), 139–149.

Coimbra, J., Machado, J., Fernandes, P. L., Ferreira, H. G. & Ferreira, K. G. (1988). Electrophysiology of the mantle of *Anodonta-cygnea*. *Journal of Experimental Biology* **140**, 65–88.

Craft, J. A., Gilbert, J. A., Temperton, B., Dempsey, K. E., Ashelford, K., Tiwari, B., Hutchinson, T. H. & Chipman, J. K. (2010). Pyrosequencing of *Mytilus galloprovincialis* cDNAs: tissue-specific expression patterns. *PLoS One* **5**(1), e8875.

Cross, E. L., Harper, E. M. & Peck, L. S. (2018). A 120-year record of resilience to environmental change in brachiopods. *Global Change Biology* **24**(6), 2262–2271.

Currey, J. D. (1999). The design of mineralised hard tissues for their mechanical functions. *Journal of Experimental Biology* **202**(23), 3285–3294.

Dauphin, Y. (2001). Nanostructures of the nacreous layers in recent cephalopod shells. *Palaeontologische Zeitschrift* **75**(1), 113–122.

Davies, P. S. (1966). Physiological ecology of *Patella*. I. the effect of body size and temperature on metabolic rate. *Journal of the Marine Biological Association* **46**, 447–658.

Davies, P. S. (1969). Physiological ecology of *Patella*. III. Desiccation effects. *Journal of the Marine Biological Association* **49**, 291–304.

Day, E. G., Branch, G. M. & Viljoen, C. (2000). How costly is molluscan shell erosion? A comparison of two patellid limpets with contrasting shell structures. *Journal of Experimental Marine Biology and Ecology* **243**(2), 185–208.

De Wit, P., Durland, E., Ventura, A. & Langdon, C. J. (2018). Gene expression correlated with delay in shell formation in larval Pacific oysters (*Crassostrea gigas*) exposed to experimental ocean acidification provides insights into shell formation mechanisms. *BMC Genomics* **19**, 160.

De Wolf, H., Backeljau, T. & Verhagen, R. (1998). Spatio-temporal genetic structure and gene flow between two distinct shell morphs of the planktonic developing periwinkle *Littorina striata* (Mollusca : Prosobranchia). *Marine Ecology Progress Series* **163**, 155–163.

Dickinson, G. H., Ivanina, A. V., Matoo, O. B., Portner, H. O., Lannig, G., Bock, C., Beniash, E. & Sokolova, I. M. (2012). Interactive effects of salinity and elevated CO_2 levels on juvenile eastern oysters, *Crassostrea virginica*. *Journal of Experimental Biology* **215**(1), 29–43.

Domenech, T. & Bahn-Walkowiak, B. (2019). Transition towards a resource efficient circular economy in Europe: policy lessons from the EU and the member states. *Ecological Economics* **155**, 7–19.

Donelan, S. C. & Trussell, G. C. (2019). The effects of embryonic experience with predation risk vary across a wave exposure gradient. *Ecosphere* **10**(4), e02676.

Eyster, L. S. (1986). Shell inorganic composition and onset of shell mineralization during bivalve and gastropod embryogenesis. *The Biological Bulletin* **170**(2), 211–231.

Fallet, M., Luquet, E., David, P. & Cosseau, C. (2020). Epigenetic inheritance and intergenerational effects in mollusks. *Gene* **729**, 144166.

Fan, W. M., Li, C. Z., Wang, X., Gong, N. P., Xie, L. P. & Zhang, R. Q. (2007). Cloning, characterization and expression analysis of calcium channel beta subunit from pearl oyster (*Pinctada fucata*). *Journal of Bioscience and Bioengineering* **104**(1), 47–54.

Fang, D., Xu, G. R., Hu, Y. L., Pan, C., Xie, L. P. & Zhang, R. Q. (2011). Identification of genes directly involved in shell formation and their functions in pearl oyster, *Pinctada fucata*. *PLoS One* **6**(7), e21860.

Fang, D., Pan, C., Lin, H., Lin, Y., Zhang, G., Wang, H., He, M., Xie, L. & Zhang, R. (2012). Novel basic protein, PFN23, functions as key macromolecule during nacre formation. *Journal of Biological Chemistry* **287**(19), 15776–15785.

Fowler, D. R., Meinhardt, H. & Prusinkiewicz, P. (1992). Modeling seashells. In *Siggraph 92 : Conference Proceedings. Computer Graphics* (Volume 26, ed S. Cunningham), pp. 379–387.

Freeman, A. S. & Byers, J. E. (2006). Divergent induced responses to an invasive predator in marine mussel populations. *Science* **313**(5788), 831–833.

Freeman, A. S., Meszaros, J. & Byers, J. E. (2009). Poor phenotypic integration of blue mussel inducible defenses in environments with multiple predators. *Oikos* **118**(5), 758–766.

Frost, M., Baxter, J. M., Buckley, P. J., Cox, M., Dye, S. R. & Harvey, N. W. (2012). Impacts of climate change on fish, fisheries and aquaculture. *Aquatic Conservation-Marine and Freshwater Ecosystems* **22**(3), 331–336.

Funabara, D., Ohmori, F., Kinoshita, S., Nagai, K., Maeyama, K., Okamoto, K., Kanoh, S., Asakawa, S. & Watabe, S. (2018). *Screening for Genes Participating in the Formation of Prismatic and Nacreous Layers of the Japanese Pearl Oyster Pinctada fucata by RNA Interference Knockdown. Biomineralization*, pp. 383–390. Springer Singapore, Singapore.

Gao, P., Liao, Z., Wang, X. X., Bao, L. F., Fan, M. H., Li, X. M., Wu, C. W. & Xia, S. W. (2015). Layer-by-layer proteomic analysis of *Mytilus galloprovincialis* shell. *PLoS One* **10**(7), e0133913.

Gardner, L. D., Mills, D., Wiegand, A., Leavesley, D. & Elizur, A. (2011). Spatial analysis of biomineralization associated gene expression from the mantle organ of the pearl oyster *Pinctada maxima*. *BMC Genomics* **12**, 455.

Gavery, M. R. & Roberts, S. B. (2010). DNA methylation patterns provide insight into epigenetic regulation in the Pacific oyster (*Crassostrea gigas*). *BMC Genomics* **11**, 483.

Gerber, S. (2017). The geometry of morphospaces: lessons from the classic Raup shell coiling model. *Biological Reviews* **92**, 1142–1155.

Gjedrem, T., Robinson, N. & Rye, M. (2012). The importance of selective breeding in aquaculture to meet future demands for animal protein: a review. *Aquaculture* **350**, 117–129.

Goncalves, P., Anderson, K., Thompson, E. L., Melwani, A., Parker, L. M., Ross, P. M. & Raftos, D. A. (2016). Rapid transcriptional acclimation following transgenerational exposure of oysters to ocean acidification. *Molecular Ecology* **25** (19), 4836–4849.

Gong, N. P., Li, Q., Huang, J., Fang, Z., Zhang, G. Y., Xie, L. P. & Zhang, R. Q. (2008). Culture of outer epithelial cells from mantle tissue to study shell matrix protein secretion for biomineralization. *Cell and Tissue Research* **333**(3), 493–501.

Gong, Z. Q., Matzke, N. J., Ermentrout, B., Song, D., Vendetti, J. E., Slatkin, M. & Oster, G. (2012). Evolution of patterns on *Conus* shells. *Proceedings of the National Academy of Sciences of the United States of America* **109**(5), E234–E241.

Goulden, M. (2018). *Portland Harbour – A High Level Review of Aquaculture Opportunities (PDF). portland-Port.Co.UK (Report)*. MacAlister Elliott & Partners Limited, Lymington.

Grefsrud, E. S., Dauphin, Y., Cuif, J. P., Denis, A. & Strand, O. (2008). Modifications in microstructure of cultured and wild scallop shells (*Pecten maximus*). *Journal of Shellfish Research* **27**(4), 633–641.

Guerra-Varela, J., Colson, I., Backeljau, T., Breugelmans, K., Hughes, R. N. & Rolan-Alvarez, E. (2009). The evolutionary mechanism maintaining shell shape and molecular differentiation between two ecotypes of the dogwhelk *Nucella lapillus*. *Evolutionary Ecology* **23**(2), 261–280.

Guiñez, R., Toro, J. E., Krapivka, S., Alcapan, A. C. & Oyarzun, P. A. (2017). Heritabilities and genetic correlation of shell thickness and shell length growth in a mussel, *Mytilus chilensis* (*Bivalvia:Mytilidae*). *Aquaculture Research* **48**(4), 1450–1457.

Gunderson, A. R., Armstrong, E. J. & Stillman, J. H. (2016). Multiple stressors in a changing world: the need for an improved perspective on physiological responses to the dynamic marine environment. In *Annual Review of Marine Science* (Volume **8**, eds C. A. Carlson and S. J. Giovannoni Palo Alto), pp. 357–378. California: Annual Reviews.

Gustafson, K. D., Kensinger, B. J., Bolek, M. G. & Luttbeg, B. (2014). Distinct snail (*Physa*) morphotypes from different habitats converge in shell shape and size under common garden conditions. *Evolutionary Ecology Research* **16**(1), 77–89.

Gutierrez, J. L., Jones, C. G., Strayer, D. L. & Iribarne, O. O. (2003). Mollusks as ecosystem engineers: the role of shell production in aquatic habitats. *Oikos* **101**(1), 79–90.

Haas, W. (1981). Evolution of calcareous hard parts in primitive molluscs. *Malacologia* **21**, 403–418.

Harley, C. D. G., Denny, M. W., Mach, K. J. & Miller, L. P. (2009). Thermal stress and morphological adaptations in limpets. *Functional Ecology* **23**(2), 292–301.

Harper, E. M. (1997). The molluscan periostracum: an important constraint in bivalve evolution. *Palaeontology* **40**, 71–97.

Harper, E. M. (1998). Calcite in chamid bivalves. *Journal of Molluscan Studies* **64**, 391–399.

Harper, E. M. & Morton, B. (1994). The biology of *Isognomon legumen* (Gmelin, 1791) (Bivalvia: Pterioida) at cape d'Aguilar, Hong Kong, with special reference to predation by muricids. In *The Malacofauna of Hong Kong and Southern China III* (ed. B. Morton), pp. 405–425. Hong Kong University Press, Hong Kong.

Harper, E. M., Clark, M. S., Hoffman, J. I., Philipp, E. E. R., Peck, L. S. & Morley, S. A. (2012). Iceberg scour and shell damage in the Antarctic bivalve *Laternula elliptica*. *PLoS One* **7**(9), e46341.

Hawkins, A. J. S. & Bayne, B. L. (1985). Seasonal variation in the relative utilization of carbon and nitrogen by the mussel *Mytilus edulis*: budgets, conversion efficiencies and maintenance requirements. *Marine Ecology Progress Series* **25**, 181–188.

Hellberg, M. E. (2009). Gene flow and isolation among populations of marine animals. *Annual Review of Ecology Evolution and Systematics* **40**, 291–310.

Herlitze, I., Marie, B., Marin, F. & Jackson, D. J. (2018). Molecular modularity and asymmetry of the molluscan mantle revealed by a gene expression atlas. *Gigascience* **7** (6), giy056.

Hiebenthal, C., Philipp, E. E. R., Eisenhauer, A. & Wahl, M. (2013). Effects of seawater pCO_2 and temperature on shell growth, shell stability, condition and cellular stress of Western Baltic Sea *Mytilus edulis* (L.) and *Arctica islandica* (L.). *Marine Biology* **160**(8), 2073–2087.

Hoffman, J. I., Peck, L. S., Hillyard, G., Zieritz, A. & Clark, M. S. (2010). No evidence for genetic differentiation between Antarctic limpet *Nacella concinna* morphotypes. *Marine Biology* **157**(4), 765–778.

Hughes, R. (2017). The EU circular economy package - life cycle thinking to life cycle law? In *24th Cirp Conference on Life Cycle Engineering. Procedia CIRP* (Volume 61, eds S. Takata, Y. Umeda and S. Kondoh), pp. 10–16.

Hüning, A. K., Lange, S. M., Ramesh, K., Jacob, D. E., Jackson, D. J., Panknin, U., Gutowska, M. A., Philipp, E. E. R., Rosenstiel, P., Lucassen, M. & Melzner, F. (2016). A shell regeneration assay to identify biomineralization candidate genes in mytilid mussels. *Marine Genomics* **27**, 57–67.

Ivanina, A. V., Borah, B. M., Vogts, A., Malik, I., Wu, J. Y., Chin, A. R., Almarza, A. J., Kumta, P., Piontkivska, H., Beniash, E. & Sokolova, I. M. (2018). Potential trade-offs between biomineralization and immunity revealed by shell properties and gene expression profiles of two closely related *Crassostrea* species. *Journal of Experimental Biology* **221**, 183236.

Jackson, D. J. & Degnan, B. M. (2016). The importance of evo-devo to an integrated understanding of molluscan biomineralisation. *Journal of Structural Biology* **196**(2), 67–74.

Jackson, D. J., Mcdougall, C., Green, K., Simpson, F., Woerheide, G. & Degnan, B. M. (2006). A rapidly evolving secretome builds and patterns a sea shell. *BMC Biology* **4**, 40.

Jackson, D. J., Worheide, G. & Degnan, B. M. (2007). Dynamic expression of ancient and novel molluscan shell genes during ecological transitions. *BMC Evolutionary Biology* **7**, 160.

Jackson, D. J., Mcdougall, C., Woodcroft, B., Moase, P., Rose, R. A., Kube, M., Reinhardt, R., Rokhsar, D. S., Montagnani, C., Joubert, C., Piquemal, D. & Degnan, B. M. (2010). Parallel evolution of nacre building gene sets in molluscs. *Molecular Biology and Evolution* **27**(3), 591–608.

Jodrey, L. H. (1953). Studies on shell formation. III. Measurement of calcium deposition in shell and calcium turnover in mantle tissue using the mantle-shell preparation and Ca^{45}. *The Biological Bulletin* **104**, 398–407.

Johannesson, B. (1986). Shell morphology of *Littorina-saxatilis olivi* - the relative importance of physical factors and predation. *Journal of Experimental Marine Biology and Ecology* **102**(2–3), 183–195.

Johannesson, B. & Johannesson, K. (1996). Population differences in behaviour and morphology in the snail *Littorina saxatilis*: phenotypic plasticity or genetic differentiation? *Journal of Zoology* **240**, 475–493.

Johnson, A. B., Fogel, N. S. & Lambert, J. D. (2019). Growth and morphogenesis of the gastropod shell. *Proceedings of the National Academy of Sciences of the United States of America* **116**(14), 6878–6883.

Jollivet, D. (1996). Specific and genetic diversity at deep-sea hydrothermal vents: an overview. *Biodiversity and Conservation* **5**(12), 1619–1653.

Joubert, C., Piquemal, D., Marie, B., Manchon, L., Pierrat, F., Zanella-Cleon, I., Cochennec-Laureau, N., Gueguen, Y. & Montagnani, C. (2010). Transcriptome and proteome analysis of *Pinctada margaritifera* calcifying mantle and shell: focus on biomineralization. *BMC Genomics* **11**, 613.

Kakoi, S., Kin, K., Miyazaki, K. & Wada, H. (2008). Early development of the Japanese spiny oyster (*Saccostrea kegaki*): characterization of some genetic markers. *Zoological Science* **25**(5), 455–464.

Kniprath, E. (1980). Ontogenetic and plate field development in two chitons, *Middendorffia* and *Isochiton*. *Roux's Archives of Developmental Biology* **189**, 97–106.

Kocot, K. M., Aguilera, F., Mcdougall, C., Jackson, D. J. & Degnan, B. M. (2016). Sea shell diversity and rapidly evolving secretomes: insights into the evolution of biomineralization. *Frontiers in Zoology* **13**, 23.

Kong, Y., Jing, G., Yan, Z., Li, C., Gong, N., Zhu, F., Li, D., Zhang, Y., Zheng, G., Wang, H., Xie, L. & Zhang, R. (2009). Cloning and characterization of Prisilkin-39, a novel matrix protein serving a dual role in the prismatic layer formation from the oyster *Pinctada fucata*. *Journal of Biological Chemistry* **284**(16), 10841–10854.

Kudo, M., Kameda, J., Saruwatari, K., Ozaki, N., Okano, K., Nagasawa, H. & Kogure, T. (2010). Microtexture of larval shell of oyster, *Crassostrea nippona*: a FIB-TEM study. *Journal of Structural Biology* **169**(1), 1–5.

Langer, G., Nehrke, G., Baggini, C., Rodolfo-Metalpa, R., Hall-Spencer, J. M. & Bijma, J. (2014). Limpets counteract ocean acidification induced shell corrosion by thickening of aragonitic shell layers. *Biogeosciences* **11**(24), 7363–7368.

Le Roy, N., Jackson, D. J., Marie, B., Ramos-Silva, P. & Marin, F. (2014). The evolution of metazoan alpha-carbonic anhydrases and their roles in calcium carbonate biomineralization. *Frontiers in Zoology* **11**, 75.

Li, S. G., Liu, Y. J., Liu, C., Huang, J. L., Zheng, G. L., Xie, L. P. & Zhang, R. Q. (2016). Hemocytes participate in calcium carbonate crystal formation, transportation and shell regeneration in the pearl oyster *Pinctada fucata*. *Fish & Shellfish Immunology* **51**, 263–270.

Maar, M., Saurel, C., Landes, A., Dolmer, P. & Petersen, J. K. (2015). Growth potential of blue mussels (*M-edulis*) exposed to different salinities evaluated by a dynamic energy budget model. *Journal of Marine Systems* **148**, 48–55.

Mackenzie, C. L., Ormondroyd, G. A., Curling, S. F., Ball, R. J., Whiteley, N. M. & Malham, S. K. (2014). Ocean warming, more than acidification, reduces shell strength in a commercial shellfish species during food limitation. *PLoS One* **9**(1), e86764.

Mann, S. (2000). The chemistry of form. *Angewandte Chemie-International Edition* **39**(19), 3393–3406.

Marcogliese, D. J. (2008). The impact of climate change on the parasites and infectious diseases of aquatic animals. *Revue Scientifique Et Technique-Office International Des Epizooties* **27**(2), 467–484.

Marie, B., Joubert, C., Tayale, A., Zanella-Cleon, I., Belliard, C., Piquemal, D., Cochennec-Laureau, N., Marin, F., Gueguen, Y. & Montagnani, C. (2012). Different secretory repertoires control the biomineralization processes of prism and nacre deposition of the pearl oyster shell. *Proceedings of the National Academy of Sciences of the United States of America* **109** (51), 20986–20991.

Marin, F., Amons, R., Guichard, N., Stigter, M., Hecker, A., Luquet, G., Layrolle, P., Alcaraz, G., Riondet, C. & Westbroek, P. (2005). Caspartin and calprismin, two proteins of the shell calcitic prisms of the Mediterranean fan mussel *Pinna nobilis*. *Journal of Biological Chemistry* **280**(40), 33895–33908.

McConnaughey, T. A. & Whelan, J. F. (1997). Calcification generates protons for nutrient and bicarbonate uptake. *Earth-Science Reviews* **42**(1–2), 95–117.

McDougall, C., Aguilera, F. & Degnan, B. M. (2013). Rapid evolution of pearl oyster shell matrix proteins with repetitive, low-complexity domains. *Journal of the Royal Society Interface* **10**(82), 20130041. https://doi.org/10.1098/rsif.2013.0041.

Michalek, K. (2019). *Scottish Mussel Culture in the Natural Environment: Observations and Implications for Industry*. PhD thesis. University of Aberdeen, UK.

Miyamoto, H., Miyashita, T., Okushima, M., Nakano, S., Morita, T. & Matsushiro, A. (1996). A carbonic anhydrase from the nacreous layer in oyster pearls. *Proceedings of the National Academy of Sciences of the United States of America* **93**(18), 9657–9660.

Miyazaki, Y., Nishida, T., Aoki, H. & Samata, T. (2010). Expression of genes responsible for biomineralization of *Pinctada fucata* during development. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **155**(3), 241–248.

Montagnani, C., Marie, B., Marin, F., Belliard, C., Riquet, F., Tayale, A., Zanella-Cleon, I., Fleury, E., Gueguen, Y., Piquemal, D. & Cochennec-Laureau, N. (2011). Pmarg-Pearlin is a matrix protein involved in nacre framework formation in the pearl oyster *Pinctada margaritifera*. *Chembiochem* **12**(13), 2033–2043.

Morris, J. P., Wang, Y., Backeljau, T. & Chapelle, G. (2016). Biomimetic and bio-inspired uses of mollusc shells. *Marine Genomics* **27**, 85–90.

Morris, J. P., Backeljau, T. & Chapelle, G. (2019). Shells from aquaculture: a valuable biomaterial, not a nuisance waste product. *Reviews in Aquaculture* **11**(1), 42–57.

Moulton, D. E., Goriely, A. & Chirat, R. (2015). The morpho-mechanical basis of ammonite form. *Journal of Theoretical Biology* **364**, 220–230.

Mount, A. S., Wheeler, A. P., Paradkar, R. P. & Snider, D. (2004). Hemocyte-mediated shell mineralization in the eastern oyster. *Science* **304**(5668), 297–300.

Murdock, D. J. E. (2020). The 'biomineralization toolkit' and the origin of animal skeletons. *Biological Reviews*. https://doi.org/10.1111/brv.12614.

Nair, P. S. & Robinson, W. E. (1998). Calcium speciation and exchange between blood and extrapallial fluid of the quahog *Mercenaria mercenaria* (L.). *The Biological Bulletin* **195** (1), 43–51.

Nehrke, G., Poigner, H., Wilhelms-Dick, D., Brey, T. & Abele, D. (2012). Coexistence of three calcium carbonate polymorphs in the shell of the Antarctic clam *Laternula elliptica*. *Geochemistry Geophysics Geosystems* **13**, Q05014.

Nishimura, T. (2015). Macromolecular templates for the development of organic/inorganic hybrid materials. *Polymer Journal* **47**(3), 235–243.

Nudelman, F. (2015). Nacre biomineralisation: a review on the mechanisms of crystal nucleation. *Seminars in Cell & Developmental Biology* **46**, 2–10.

Oppenheimer, M., Glavovic, B., Hinkel, J., Van De Wal, R., Magnan, A. K., Abd-Elgawad, A., Cai, R., Cifuentes-Jara, M., Deconto, R. M., Ghosh, T., Hay, J., Isla, F., Marzeion, B., Meyssignac, B. & Sebesvari, Z. (2019). Chapter 4: sea level rise and implications for low lying islands, coasts and communities. In *IPCC Special Report on the Ocean and Cryosphere in a Changing Climate*, 1st Edition (ed. H. O. Portner), pp. 4–169. IPCC, Monaco.

Palmer, A. R. (1981). Do carbonate skeletons limit the rate of body growth. *Nature* **292** (5819), 150–152.

Palmer, A. R. (1983). Relative cost of producing skeletal organic matrix versus calcification – evidence from marine gastropods. *Marine Biology* **75**(2–3), 287–292.

Palmer, A. R. (1992). Calcification in marine mollusks - how costly is it. *Proceedings of the National Academy of Sciences of the United States of America* **89**(4), 1379–1382.

Parsons, K. E. (1997). Contrasting patterns of heritable geographic variation in shell morphology and growth potential in the marine gastropod *Bembicium vittatum*: evidence from field experiments. *Evolution* **51**(3), 784–796.

Pascoal, S., Carvalho, G., Creer, S., Rock, J., Kawaii, K., Mendo, S. & Hughes, R. (2012). Plastic and heritable components of phenotypic variation in *Nucella lapillus*: An assessment using reciprocal transplant and common garden experiments. *PLoS One* **7**(1), e30289.

Peck, L.S. (1983) *An Investigation into the Growth and Early Development of the Ormer, Haliotis Tuberculata* L. PhD thesis. University of Portsmouth, UK.

Peck, L. S. (2011). Organisms and responses to environmental change. *Marine Genomics* **4**(4), 237–243.

Perry, K. J. & Henry, J. Q. (2015). CRISPR/Cas9-mediated genome modification in the mollusc, *Crepidula fornicata*. *Genesis* **53**(2), 237–244.

Poncet, J. M., Serpentini, A., Thiebot, B., Villers, C., Bocquet, J., Boucaud-Camou, E. & Label, J. M. (2000). *In vitro* synthesis of proteoglycans and collagen in primary cultures of mantle cells from the nacreous mollusk, *Haliotis tuberculata*: a new model for study of molluscan extracellular matrix. *Marine Biotechnology* **2**(4), 387–398.

Ponder, W. F. & Lindberg, D. R. (2020). *Biology and Evolution of the Mollusca*, Edition (Volume **1**), p. 900. CRC Press, Boca Raton.

Ramesh, K., Hu, M. Y., Thomsen, J., Bleich, M. & Melzner, F. (2017). Mussel larvae modify calcifying fluid carbonate chemistry to promote calcification. *Nature Communications* **8**, 1709.

Ramesh, K., Melzner, F., Griffith, A. W., Gobler, C. J., Rouger, C., Tasdemir, D. & Nehrke, G. (2018). *In vivo* characterization of bivalve larval shells: a confocal Raman microscopy study. *Journal of the Royal Society Interface* **15**(141), 20170723. https://doi.org/10.1098/rsif.2017.0723.

Ramesh, K., Yarra, T., Clark, M. S., John, U. & Melzner, F. (2019). Expression of calcification-related ion transporters during blue mussel larval development. *Ecology and Evolution* **9**(12), 7157–7172.

Ramsøe, A., Clark, M. S. & Sleight, V. A. (2020). Gene network analyses support subfunctionalization hypothesis for duplicated *hsp70* genes in the Antarctic clam. *Cell Stress & Chaperones*. https://doi.org/10.1007/s12192-020-01118-9.

Roesijadi, G. & Unger, M. E. (1993). Cadmium uptake in gills of the mollusk *Crassostrea-virginica* and inhibition by calcium-channel blockers. *Aquatic Toxicology* **24** (3–4), 195–206.

Ros, S. & Echevarria, J. (2011). Bivalves and evolutionary resilience: old skills and new strategies to recover from the P/T and T/J extinction events. *Historical Biology* **23**(4), 411–429.

Rosenberg, G. (2014). A new critical estimate of named species-level diversity of the recent Mollusca. *American Malacological Bulletin* **32**(2), 308–322.

Ross, P. M., Parker, L. & Byrne, M. (2016). Transgenerational responses of molluscs and echinoderms to changing ocean conditions. *ICES Journal of Marine Science* **73**(3), 537–549.

Rousseau, M., Pereira-Mouries, L., Almeida, M. J., Milet, C. & Lopez, E. (2003). The water-soluble matrix fraction from the nacre of *Pinctada maxima* produces earlier mineralization of MC3T3-E1 mouse pre-osteoblasts. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **135**(1), 1–7.

Rousseau, M., Boulzaguet, H., Biagianti, J., Duplat, D., Milet, C., Lopez, E. & Bedouet, L. (2008). Low molecular weight molecules of oyster nacre induce mineralization of the MC3T3-E1 cells. *Journal of Biomedical Materials Research Part A* **85A**(2), 487–497.

Rousseau, M., Meibom, A., Geze, M., Bourrat, X., Angellier, M. & Lopez, E. (2009). Dynamics of sheet nacre formation in bivalves. *Journal of Structural Biology* **165**(3), 190–195.

Saleuddin, A. S. M. & Petit, H. P. (1983). The mode of formation and the structure of the periostracum. In *The Mollusca. Volume 4: Physiology, Part 1* (eds S. ASM and K. M. Wilbur), pp. 199–234. Academic Press, Cambridge, MA.

Samata, T., Somiya, H., Horita, C. & Akera, S. (1994). SEM observation of microcrystals developed over black secretion on the cultured tissue of the pearl oyster *Pinctada-fucata*. *Fisheries Science* **60**(3), 343–344.

Sanders, M. B., Bean, T. P., Hutchinson, T. H. & Le Quesne, W. J. F. (2013). Juvenile king scallop, *Pecten maximus*, is potentially tolerant to low levels of ocean acidification when food is unrestricted. *PLoS One* **8**(9), e74118.

Sanders, T., Schmittmann, L., Nascimento-Schulze, J. C. & Melzner, F. (2018). High calcification costs limit mussel growth at low salinity. *Frontiers in Marine Science* **5**, 352.

Sanford, E. & Kelly, M. W. (2011). Local adaptation in marine invertebrates. In *Annual Review of Marine Science, Vol 3. Annual Review of Marine Science* (Volume **3**, eds C. A. Carlson and S. J. Giovannoni), pp. 509–535. Palo Alto, California: Annual Reviews.

Schwartz, J., Realis-Doyelle, E., Dubos, M. P., Lefranc, B., Leprince, J. & Favrel, P. (2019). Characterization of an evolutionarily conserved calcitonin signalling system in a lophotrochozoan, the Pacific oyster (*Crassostrea gigas*). *Journal of Experimental Biology* **222**(13), 201319.

Shumway, S. E. (2011). *Shellfish Aquaculture and the Environment*. John Wiley & Sons, Hoboken.

Sillanpää, J. K., Ramesh, K., Melzner, F., Sundh, H. & Sundell, K. (2016). Calcium mobilisation following shell damage in the Pacific oyster, *Crassostrea gigas*. *Marine Genomics* **27**, 75–83.

Sillanpää, J. K., Sundh, H. & Sundell, K. S. (2018). Calcium transfer across the outer mantle epithelium in the Pacific oyster, *Crassostrea gigas*. *Proceedings of the Royal Society B-Biological Sciences* **285**(1891), 20181676. https://doi.org/10.1098/rspb.2018.1676.

Sleight, V. A., Thorne, M. A. S., Peck, L. S. & Clark, M. S. (2015). Transcriptomic response to shell damage in the Antarctic clam, *Laternula elliptica*: time scales and spatial localisation. *Marine Genomics* **20**, 45–55.

Sleight, V. A., Marie, B., Jackson, D. J., Dyrynda, E. A., Marie, A. & Clark, M. S. (2016). An Antarctic molluscan biomineralisation tool-kit. *Scientific Reports* **6**, 36978.

Sleight, V. A., Antczak, P., Falciani, F. & Clark, M. S. (2020). Computationally predicted gene regulatory networks in molluscan biomineralization identify extracellular matrix production and ion transportation pathways. *Bioinformatics* **36** (5), 1326–1332.

Solas, M. R., Sepulveda, R. D. & Brante, A. (2013). Genetic variation of the shell morphology in *Acanthina monodon* (*Gastropoda*) in habitats with different wave exposure conditions. *Aquatic Biology* **18**(3), 253–260.

Solas, M. R., Hughes, R. N., Marquez, F. & Brante, A. (2015). Early plastic responses in the shell morphology of *Acanthina monodon* (*Mollusca, Gastropoda*) under predation risk and water turbulence. *Marine Ecology Progress Series* **527**, 133–142.

Stasek, C. R. & McWilliams, W. R. (1973). The comparative morphology and evolution of the molluscan mantle edge. *The Veliger* **16**, 1–19.

Stewart-Sinclair, P. J., Last, K. S., Payne, B. L. & Wilding, T. A. (2020). A global assessment of the vulnerability of shellfish aquaculture to climate change and

ocean acidification. *Ecology & Evolution*, **10**, 3518–3534. https://doi.org/10.1002/ece3.6149.

Suarez-Ulloa, V., Gonzalez-Romero, R. & Eirin-Lopez, J. M. (2015). Environmental epigenetics: a promising venue for developing next-generation pollution biomonitoring tools in marine invertebrates. *Marine Pollution Bulletin* **98** (1–2), 5–13.

Sudo, S., Fujikawa, T., Nagakura, T., Ohkubo, T., Sakaguchi, K., Tanaka, M., Nakashima, K. & Takahashi, T. (1997). Structures of mollusc shell framework proteins. *Nature* **387**(6633), 563–564.

Suzuki, M., Saruwatari, K., Kogure, T., Yamamoto, Y., Nishimura, T., Kato, T. & Nagasawa, H. (2009). An acidic matrix protein, Pif, is a key macromolecule for nacre formation. *Science* **325**(5946), 1388–1390.

Takeuchi, T., Koyanagi, R., Gyoja, F., Kanda, M., Hisata, K., Fujie, M., Goto, H., Yamasaki, S., Nagai, K., Morino, Y., Miyamoto, H., Endo, K., Endo, H., Nagasawa, H., Kinoshita, S., et al. (2016). Bivalve-specific gene expansion in the pearl oyster genome: implications of adaptation to a sessile lifestyle. *Zoological Letters* **2**, 3.

Taylor, J. D. (1973). The shell structure and mineralogy of the Bivalvia. II. *Lucinacea-Clavagellacea*, conclusions. *Bulletin of the British Museum (Natural History). Zoology Series* **22**, 225–294.

Taylor, J. D., Kennedy, W. J. & Hall, A. (1969). The shell structure and mineralogy of the *Bivalvia* introduction *Nuculacea trigonacea*. *Bulletin of the British Museum (Natural History) Zoology* **3**, 1–125.

Taylor, J. D., Kennedy, W. J. & Hall, A. (1973). The shell structure and mineralogy of the *Bivalvia* part 2 *Lucinaea clavagellacea* conclusions. *Bulletin of the British Museum (Natural History) Zoology* **22**(9), 253–294.

Telesca, L., Michalek, K., Sanders, T., Peck, L. S., Thyrring, J. & Harper, E. M. (2018). Blue mussel shell shape plasticity and natural environments: a quantitative approach. *Scientific Reports* **8**, 2865.

Telesca, L., Peck, L. S., Sanders, T., Thyrring, J., Sejr, M. K. & Harper, E. M. (2019). Biomineralization plasticity and environmental heterogeneity predict geographical resilience patterns of foundation species to future change. *Global Change Biology* **25**(12), 4179–4193.

Tevesz, M. J. & Carter, J. G. (1980). Environmental relationships of shell form and structure of unionacean bivalves. In *Skeletal Growth of Aquatic Organisms* (eds D. C. Rhoads and R. A. Lutz), pp. 295–322. Plenum Press, New York.

Thomsen, J., Casties, I., Pansch, C., Kortzinger, A. & Melzner, F. (2013). Food availability outweighs ocean acidification effects in juvenile *Mytilus edulis*: laboratory and field experiments. *Global Change Biology* **19**(4), 1017–1027.

Thomsen, J., Stapp, L. S., Haynert, K., Schade, H., Danelli, M., Lannig, G., Wegner, K. M. & Melzner, F. (2017). Naturally acidified habitat selects for ocean acidification-tolerant mussels. *Science Advances* **3**(4), e1602411.

Thomsen, J., Ramesh, K., Sanders, T., Bleich, M. & Melzner, F. (2018). Calcification in a marginal sea - influence of seawater Ca^{2+} and carbonate chemistry on bivalve shell formation. *Biogeosciences* **15**(5), 1469–1482.

Thorson, J. L. M., Smithson, M., Beck, D., Sadler-Riggleman, I., Nilsson, E., Dybdahl, M. & Skinner, M. K. (2017). Epigenetics and adaptive phenotypic variation between habitats in an asexual snail. *Scientific Reports* **7**, 14139.

Timmermans, L. P. M. (1968). Studies on shell formation in molluscs. *Netherlands Journal of Zoology* **19**(4), 413–523.

Trussell, G. C. & Nicklin, M. O. (2002). Cue sensitivity, inducible defense, and trade-offs in a marine snail. *Ecology* **83**(6), 1635–1647.

Trussell, G. C. & Smith, L. D. (2000). Induced defenses in response to an invading crab predator: an explanation of historical and geographic phenotypic change. *Proceedings of the National Academy of Sciences of the United States of America* **97**(5), 2123–2127.

Turing, A. (1952). The chemical basis of morphogenesis. *Philosophical Transactions of the Royal Society of London B* **237**, 37–72.

Urabe, M. (1998). Contribution of genetic and environmental factors to shell shape variation in the lotic snail *Semisulcospira reiniana* (*Prosobranchia : Pleuroceridae*). *Journal of Molluscan Studies* **64**, 329–343.

Urban, M. C., Bocedi, G., Hendry, A. P., Mihoub, J. B., Pe'er, G., Singer, A., Bridle, J. R., Crozier, L. G., De Meester, L., Godsoe, W., Gonzalez, A., Hellmann, J. J., Holt, R. D., Huth, A., Johst, K., et al. (2016). Improving the forecast for biodiversity under climate change. *Science* **353**(6304), aad846.

Ussing, H. H. & Zerahn, K. (1951). Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiologica Scandinavica* **23**(2–3), 110–127.

Van Wyngaarden, M., Snelgrove, P. V. R., Dibacco, C., Hamilton, L. C., Rodriguez-Ezpeleta, N., Zhan, L., Beiko, R. G. & Bradbury, I. R. (2018). Oceanographic variation influences spatial genomic structure in the sea scallop, *Placopecten magellanicus*. *Ecology and Evolution* **8**(5), 2824–2841.

Vendrami, D. L. J., Telesca, L., Weigand, H., Weiss, M., Fawcett, K., Lehman, K., Clark, M. S., Leese, F., Mcminn, C., Moore, H. & Hoffman, J. I. (2017). RAD sequencing resolves fine-scale population structure in a benthic invertebrate: implications for understanding phenotypic plasticity. *Royal Society Open Science* **4**(2), 160548. https://doi.org/10.1098/rsos.160548.

Vendrami, D. L. J., De Noia, M., Telesca, L., Handal, W., Charrier, G., Boudry, P., Eberhart-Phillips, L. & Hoffman, J. I. (2019a). RAD sequencing sheds new light on the genetic structure and local adaptation of European scallops and resolves their demographic histories. *Scientific Reports* **9**, 7455.

Vendrami, D. L. J., Houston, R. D., Gharbi, K., Telesca, L., Gutierrez, A. P., Gurney-Smith, H., Hasegawa, N., Boudry, P. & Hoffman, J. I. (2019b). Detailed insights into pan-European population structure and inbreeding in wild and hatchery Pacific oysters (*Crassostrea gigas*) revealed by genome-wide SNP data. *Evolutionary Applications* **12**(3), 519–534.

Vendrasco, M. J., Checa, A. G. & Kouchinsky, A. V. (2011). Shell microstructure of the early bivalve *Pojetaia* and the independent origin of nacre within the Mollusca. *Palaeontology* **54**, 825–850.

Ventura, A., Schulz, S. & Dupont, S. (2016). Maintained larval growth in mussel larvae exposed to acidified under-saturated seawater. *Scientific Reports* **6**, 23728.

Verdonk, N. H. & Van Den Biggelaar, J. A. M. (1983). Early development and formation of the germ layers. In *The Mollusca Vol. 3. Development* (eds N. H. Verdonk, J. A. M. Biggelaar and A. S. van den Tompa), pp. 91–122. Academic Press, Cambridge, MA.

Waldbusser, G. G., Brunner, E. L., Haley, B. A., Hales, B., Langdon, C. J. & Prahl, F. G. (2013). A developmental and energetic basis linking larval oyster shell formation to acidification sensitivity. *Geophysical Research Letters* **40**(10), 2171–2176.

Waller, T. R. (1980). Scanning electron microscopy of shell and mantle in the order Arcoida (Mollusca: Bivalvia). Smithson. *Contributions to Zoology* **313**, 1–58.

Wang, W. X. & Fisher, N. S. (1999). Effects of calcium and metabolic inhibitors on trace element uptake in two marine bivalves. *Journal of Experimental Marine Biology and Ecology* **236**(1), 149–164.

Watson, S.-A., Peck, L. S., Tyler, P. A., Southgate, P. C., Tan, K. S., Day, R. W. & Morley, S. A. (2012). Marine invertebrate skeleton size varies with latitude, temperature and carbonate saturation: implications for global change and ocean acidification. *Global Change Biology* **18**(10), 3026–3038.

Weiner, S. & Dove, P. M. (2003). An overview of biomineralization processes and the problem of the vital effect. In *Biomineralization. Reviews in Mineralogy & Geochemistry* (Volume **54**, eds P. M. Dove, J. J. DeYoreo and S. Weiner Chantilly), pp. 1–29. VA: Mineralogical Society of America.

Wilk, J. & Bieler, R. (2009). Ecophenotypic variation in the flat tree oyster, *Isognomon alatus* (*Bivalvia: Isognomonidae*), across a tidal microhabitat gradient. *Marine Biology Research* **5**(2), 155–163.

Wittmann, A. C. & Pörtner, H. O. (2013). Sensitivities of extant animal taxa to ocean acidification. *Nature Climate Change* **3**(11), 995–1001.

Wood, R. & Zhuravlev, A. Y. (2012). Escalation and ecological selectively of mineralogy in the Cambrian radiation of skeletons. *Earth-Science Reviews* **115**(4), 249–261.

Xia, Z. (2016). *Biomimetic Principles and Design of Advanced Engineering Materials*. John Wiley & Sons, Hoboken.

Yadav, R., Naebe, M., Wang, X. G. & Kandasubramanian, B. (2016). Body Armour materials: from steel to contemporary biomimetic systems. *Rsc Advances* **6**(116), 115145–115174.

Yarra, T. (2018) *Transcriptional Profiling of Shell Calcification in Bivalves*. PhD thesis. University of Edinburgh, UK.

Yeap, K. L., Black, R. & Johnson, M. S. (2001). The complexity of phenotypic plasticity in the intertidal snail *Nodilittorina australis*. *Biological Journal of the Linnean Society* **72**(1), 63–76.

Yokoo, N., Suzuki, M., Saruwatari, K., Aoki, H., Watanabe, K., Nagasawa, H. & Kogure, T. (2011). Microstructures of the larval shell of a pearl oyster, *Pinctada fucata*, investigated by FIB-TEM technique. *American Mineralogist* **96**(7), 1020–1027.

Yonezawa, M., Sakuda, S., Yoshimura, E. & Suzuki, M. (2016). Molecular cloning and functional analysis of chitinases in the fresh water snail, *Lymnaea stagnalis*. *Journal of Structural Biology* **196**(2), 107–118.

Yonge, C. M. (1957). Mantle fusion in the *Lamellibranchia*. *Publicazione della Stazione Zoologica di Napoli* **29**, 151–171.

Yonge, C. M. (1982). Mantle margins with a revision of siphonal types in the Bivalvia. *Journal of Molluscan Studies* **48**, 102–103.

Yoshino, T. P., Bickham, U. & Bayne, C. J. (2013). Molluscan cells in culture: primary cell cultures and cell lines. *Canadian Journal of Zoology* **91**(6), 391–404.

Zhang, C., Li, S., Ma, Z., Xie, L. & Zhang, R. (2006). A novel matrix protein p10 from the nacre of pearl oyster (*Pinctada fucata*) and its effects on both $CaCO_3$ crystal formation and mineralogenic cells. *Marine Biotechnology* **8**(6), 624–633.

Zhang, G., Fang, X., Guo, X., Li, L., Luo, R., Xu, F., Yang, P., Zhang, L., Wang, X., Qi, H., Xiong, Z., Que, H., Xie, Y., Holland, P. W. H., Paps, J., et al. (2012). The oyster genome reveals stress adaptation and complexity of shell formation. *Nature* **490** (7418), 49–54.

Zhang, G., Willemin, A. S., Brion, A., Piet, M. H., Moby, V., Bianchi, A., Mainard, D., Galois, L., Gillet, P. & Rousseau, M. (2016). A new method for

the separation and purification of the osteogenic compounds of nacre ethanol soluble matrix. *Journal of Structural Biology* **196**(2), 127–137.

Zhao, M., He, M. X., Huang, X. D. & Wang, Q. (2014). A homeodomain transcription factor gene, PfMSX, activates expression of Pif gene in the pearl oyster *Pinctada fucata*. *PLoS One* **9**(8), e103830.

Zhao, M., He, M. X., Huang, X. D., Wang, Q. & Shi, Y. (2016a). Functional characterization and molecular mechanism exploration of three granulin epithelin precursor splice variants in biomineralization of the pearl oyster *Pinctada fucata*. *Molecular Genetics and Genomics* **291**(1), 399–409.

Zhao, M., Shi, Y., He, M. X., Huang, X. D. & Wang, Q. (2016b). PfSMAD4 plays a role in biomineralization and can transduce bone morphogenetic protein-2 signals in the pearl oyster *Pinctada fucata*. *BMC Developmental Biology* **16**, 9.

Zhao, R., Takeuchi, T., Luo, Y.-J., Ishikawa, A., Kobayashi, T., Koyanagi, R., Villar-Briones, A., Yamada, L., Sawada, H., Iwanaga, S., Nagai, K., Satoh, N. & Endo, K. (2018). Dual gene repertoires for larval and adult shells reveal molecules essential for molluscan shell formation. *Molecular Biology and Evolution* **35**(11), 2751–2761.

Zheng, X. N., Xiang, L., Liang, J., Xie, L. P. & Zhang, R. Q. (2016). Pf-Sp8/9, a novel member of the specificity protein family in *Pinctada fucata*, potentially participates in biomineralization. *Journal of Structural Biology* **196**(2), 119–126.

Zieritz, A., Hoffman, J. I., Amos, W. & Aldridge, D. C. (2010). Phenotypic plasticity and genetic isolation-by-distance in the freshwater mussel *Unio pictorum* (*Mollusca: Unionoida*). *Evolutionary Ecology* **24**(4), 923–938.

Zlotnikov, I. & Schoeppler, V. (2017). Thermodynamic aspects of molluscan shell ultrastructural morphogenesis. *Advanced Functional Materials* **27**(28), 1700506.

(*Received 30 March 2020; revised and accepted 17 July 2020*)