Mobilising molluscan models and genomes in biology

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Abstract

Molluscs are amongst the most ancient, diverse, and important of all animal taxa. Even so, no individual mollusc species has emerged as a broadly applied model system in biology. We here make the case that both perceptual and methodological barriers have played a role in the relative neglect of molluscs as research organisms. We then summarize the current application and potential of molluscs and their genomes to address important questions in animal biology, and the state of the field when it comes to the availability of resources such as genome assemblies, cell lines, and other key elements necessary to mobilising the development of molluscan model systems. We conclude by contending that a cohesive research community that works together to elevate multiple molluscan systems to 'model' status will create new opportunities in addressing basic and applied biological problems, including general features of animal evolution.

Introduction

Molluscs are globally important as sources of food, calcium and pearls, and as vectors of human disease. From an evolutionary perspective, molluscs are notable for their remarkable diversity: originating over 500 million years ago, there are over 70,000 extant mollusc species [1], with molluscs present in virtually every ecosystem. However, despite their biological, ecological (e.g. invasive species), economic (e.g. fisheries), and medical importance (e.g. schistosomiasis vector), critical steps towards understanding molluscan biology have been prevented by both general challenges associated with working with molluscs and specific challenges in genome sequencing and assembly. This has been compounded by the longstanding presumption that molluscs and related phyla (Figure 1) are not sufficiently important or of high-enough profile to be worthy of intense research focus, relative to studies on vertebrates or other invertebrates. Molluscs also often have large, highly repetitive, and heterozygous genomes [2, 3]. Together, these multiple challenges mean that as animal genome sequencing has increased in pace, well-assembled molluscan genomes (and associated resources like genome browsers; Table 1) have remained scarce, at least until very recently.

The relative neglect of research on molluscs, including the absence of targeted funds for molluscan genome sequencing, has been problematic for the collective effort. This challenge is especially evident now that highly contiguous genome assemblies are a starting point for much of modern biology. Of course, this is a very recent development; until the last decade or so, the genome assembly of the most commonly used research organisms was usually the last to be developed, well after lab culture was established, and tools such as transgenesis, cell-lineage tracing, etc, were made available.

Now, in an era when it is straightforward to sequence and assemble a genome, there is a risk that the genome is viewed as the end goal, and the corollary, that a genome on its own is sufficient to raise an animal to 'model' status. Like others, we would argue that a stricter definition of 'model' is more useful: a model organism is a species that is convenient for the study of a particular biological process, and for which there is sufficient infrastructure, and appropriate resources, to enable investigations [4-6]. Ideally, a model species should have a well characterised ecology (although this is rarely the case, and often comes last), be easily collected and amenable to lab-culture (Figure 2). As the system develops and grows,

the associated scientific community should adapt appropriate technologies and methods, especially CRISPR-Cas9, and benefit from access to resources such as biological databases (e.g. multiple-species genome browsers) and cell lines.

Regardless of how we – or others – define model taxa, breakthroughs in DNA sequencing technology and assembly approaches are finally allowing scientists to produce the first cost-effective assemblies of notable molluscan genomes, such as octopus [7], giant African land snail [8], and the deep-sea scaly foot snail [9]. As technology overcomes these challenges, molluscan models and genomes will be used to address broad questions in biology. A deepening of the basic knowledge of molluscs and their genomes will also provide the resources and tools needed to take key steps towards harnessing the unique biology of molluscs for human benefit and preserving important biodiversity.

Accordingly, our aim here is to provide a brief overview of the role that model molluscs (aka "research organisms" [10], or even "non-model model organisms" [4]) and their genomes have to play in molluscan biology, and animal biology in general. We illustrate some of the benefits but also address some of the most important issues that continue to limit further study and, ultimately, prevent human benefit. As there is insufficient space to comprehensively cover each member of this large set of concepts and taxa, we reference authoritative reviews on individual topics or species where appropriate [e.g. 2, 11-13].

Making a model mollusc

Scientists in the pre-genomic era of molecular biology came together to study a handful of more-or-less prescribed model animals, mainly vertebrates (e.g. chick, mouse) in the Deuterostomia and the nematode and fruitfly in the Ecdysozoa [6, 14]. These efforts led to the development of tools and resources specifically for these organisms, including infrastructure such as databases and strain collections, in addition to molecular toolkits, and extensive collections of reproducible techniques and methods [4]. In comparison, the third main animal group, the Lophotrochozoa (Figure 1), which includes molluscs and other diverse spiralian lineages such as the annelids, has been more or less ignored, especially with respect to tools, infrastructure and resources.

To this day the Lophotrochozoa does not contain a taxon around which a large research community has formed, with the possible exception of some platyhelminthes, especially the *Schistosoma* parasite (which ironically has to be maintained by vectoring through the snail intermediate host *Biomphalaria glabrata* [15]), and some other flatworms [10, 16]). The irony is that lophotrochozoans should be especially valuable in providing a powerful comparative framework to study animal evolution, because of their ancient divergence from the other groups (~500-700 Million years), their diverse body plans and in light of evidence for retention of an ancestral bilaterian gene repertoire relative to traditional Ecdysozoan models like flies and nematodes [17-19].

The consequent absence of appropriate tools and resources, including genomes and methods for transgenesis, has hindered progress in understanding molluscs relative to other groups. In parallel, while there are many meetings and conferences devoted to single model animals, there are few equivalent resources for molluscs.

While no single model species can address all possible biological questions, this fundamental limitation did not prevent the development of organisms like the fruitfly, nematode, yeast, and mouse into model systems to be applied to broad questions across biology. Why has no model mollusc taken its place amongst the other textbook model organisms? In our opinion, this is probably a historical contingency, in that yeast, flies, worms, etc. were relatively 'easy' to find and convenient to maintain. In comparison, many molluscs are more difficult to raise in laboratory conditions and have a relatively long or complex life cycle.

Whichever the explanation, the consequence is that scientists who study molluscs have typically taken a piecemeal approach, applying one or several related mollusc species in the context of a targeted question. For instance, octopus and other cephalopods are often used for studies on intelligence and consciousness [20, 21]. A much wider range of molluscs is used to study more general neurobiological questions. Another example comes from the focussed use of *Biomphalaria glabrata* in the context of research into snail-vectored trematode diseases like schistosomiasis [e.g. 22, 23]. Biomineralisation is also an active topic for which a variety of taxa are used, from marine species such as abalone [24] and oyster [25] to freshwater *Lymnaea stagnalis* pond snails [26] or *Cepaea* land snails [27]. Indeed, many mollusc species have been put forward as 'models' in the niche sense of the concept, to answer a limited range of biological questions [28-36], although these model prospects frequently lack infrastructure and appropriate resources to enable in-depth investigations [4-6].

Molluscs have thus lagged behind relative to other animal phyla, even when the former are being used to ask the same types of questions. The absence of genetic and genomic resources is also likely an important – and self-reinforcing – part of the problem. For example, studies on the shell polymorphism of the snail *Cepaea* were a crucial element towards establishing the role of natural selection in maintaining morphological variation, with the genus becoming a pre-eminent model for ecological genetics [37, 38]. However, the absence of a high-quality genome assembly and genetic tools means that the genes that drive the 'exuberant' variety of shell colours and patterns remain unknown in *Cepaea*, preventing further progress. By contrast, the precise mutation that defines a famous colour polymorphism in the peppered moth has been identified [39]. More broadly, the use of genomics to characterise Lepidopteran wing colour genes has led to an array of different research avenues [40] and many training opportunities for junior scientists. A similar example of a prominent mollusc held back by the absence of genomic resources is provided by the freshwater New Zealand snail *Potamopyrgus antipodarum*, a textbook model system for the evolution of sexual reproduction and host-parasite coevolution [41], for which a genomic assembly is nearly complete.

While model molluscs are individually valuable, the community of researchers associated with each system is generally too small to provide the resources or impetus needed to develop that system into a more broadly applicable model. The accompanying resources and tools are also generally missing (Table 1). This challenge even extends to the biomedically important snail vectors of human disease (including *B. glabrata*), for which the range of tools, resources, and applications is insignificant in comparison to the economic damage caused by the diseases. All of these problems are often compounded by the large and repetitive genomes that characterize many molluscan species (but not all, e.g., *Lottia gigantea* [42]).

There are nonetheless some mollusc species (or groups of related species) for which it has been argued that there is a critical mass of persons and resources to study a range of questions. In particular, Fodor et al. [13] make the case for the pond snail *Lymnaea stagnalis*. This species has long been a model for neurobiology, but in more recent years the genus has been used to study ecotoxicology [43], sexual selection [44], biomineralisation [26], parasitology [45, 46], and development [47]. However, while we do not doubt the relatively wide-ranging existing utility of the pond snail, development of further key resources would allow *L. stagnalis* to be applied powerfully to an even wider range of research questions. Equally, we would argue that it is better to strive to ensure that any tools and resources are effective or useful in a range of species (e.g. both *L. stagnalis* and *B. glabrata*), rather than groups competing for pre-eminence of their 'own' model system. These different groups can also come together and exchange knowledge and resources. A good example is provided by the newly formed "Spiraliabase", whose stated aim is to grow the community,

incorporating labs from around the world into an interactive and cohesive group that can plan future meetings, grants, and education efforts [48].

The take-home message is that model species, including but not limited to molluscs, should continue to be selected according to the biological question. At the same time, we should push for permanent and reusable data, resources, and web tools [49, 50], including high-quality contiguous genomes [7-9], portable genome browsers [51], and pipelines that can be used for other taxa [52].

The potential for genomics in molluscs

Molluscan models may be used to make inferences about other taxa that have not been studied in the same manner or are so seemingly unique [e.g. the scaly-foot snail; 9, transmissable cancers in bilvalves; 53] that they are thought to confer especially distinctive insights. There are also considerable potential commercial benefits, especially in aquaculture [54, 55]. More generally, there is a compelling argument that broad insights into the evolution of the Bilateria requires representatives from each of its three main groups, the Deuterostomia, Ecdysozoa, and Lophotrochozoa [42, 56, 57]. The importance of adequate representation is exemplified by the presumption in the early 2000s that the signalling gene *nodal* was a deuterostome innovation because it was absent in the ecdysozoan fruitfly and nematode. This inference turned out to be premature as it was made in the near absence of genomic resources for any lophotrochozoan taxon. In 2009, *nodal* was reported in the limpet *Lottia* as well as *Biomphalaria glabrata* [58], and subsequently in other lophotrochozoan phyla. The gene had evidently been lost during the evolution of the Ecdysozoa, with the lack of data for the Lophotrochozoa incurring a misleading interpretation.

We here provide a few key examples (Table 1; Figure 3) from some especially high-profile and potentially powerful systems, ranging from single species to diverse molluscan groups. Rather than trying to summarise the main research findings (for which directed reviews are a better source), our aim is to illustrate important research questions to which molluscs can be usefully applied and how the study of model molluscs and their genomes may impact upon on our understanding of this phylum and the much wider group of animal life. Despite recent progress, there is still very little genomic research on molluscs, with broader implications for (mis)understanding animal biology and missed opportunities regarding human benefits.

Blood-fluke planorb *Biomphalaria glabrata* – disease prevention

Snails are an important vector of human disease. The most well-known of these diseases is schistosomiasis, which sickens hundreds of millions and kills thousands of people every year [59]. Snails also are the source of several other food and water-borne diseases that are biomedically or agriculturally significant, such as opisthorchiasis and fascioliasis [60, 61]. To date the majority of relevant research on these diseases has tended to focus on the parasites and their interactions with humans. In comparison, the snails have received comparatively little attention despite a growing body of evidence that controlling the snail vectors is perhaps the most effective means to reduce the incidence of the disease [62].

Most disease-focused work to date has involved the snail *Biomphalaria glabrata*, intermediate host to the *Schistosoma mansoni* parasite. This snail is amenable to laboratory culture and is easy to raise. The *B. glabrata* resources available include a genome assembly [63, 64], a linkage map [65], several long-standing laboratory lines [some inbred; 66], and the only molluscan (and lophotrochozoan) immortal cell line [67, 68].

Much of the research on *B. glabrata* has aimed to characterize the snail immune system [69-71], with a long-term view to identify biological targets that may lead to the development of methods to block or prevent schistosome infection. Another avenue has been in

identifying loci that confer resistance to infection [22, 23, 63, 64], albeit usually partial, so that individuals might be bred that are wholly refractory to infection or transmission. This might be via conventional breeding and an understanding of Mendelian genetics, but it seems more likely (barring ethical issues [72]) that in the future, new technologies [73] such as CRISPR/Cas9 gene editing and gene drive techniques will provide a faster and more effective means of inserting and driving a resistance component through the population [64, 74].

Gene finding and mapping and genetic manipulation all require, or are benefited by, a well-assembled genome. While this resource is in place for *B. glabrata* and is leading to scientific advances [23, 64], the community lacks a chromosomal-level assembly, a set of re-sequenced *B. glabrata* laboratory lines, or wild isolates. A further important issue is that transgenic methods have not yet been applied to *B. glabrata*. Finally, there is little knowledge of the other snail species that are important intermediate vectors for schistosomes and other trematodes [61].

Great pond-snail *Lymnaea stagnalis* – development, biomineralisation, neurobiology, eco-toxicology, sexual selection

Pond snails have long been used to study molluscan development in general, and development of the shell in particular. More recently, pond snails have come to the fore in studies of biomineralization [26], neurobiology [75], eco-toxicology [43], and sexual selection [44]. In our own work, we have used inherited variation in the chirality of the body and the shell to understand the establishment of left-right asymmetry in snails and the conserved role of the formin gene in establishing chirality in bilaterians [47, 76-79].

Most of the recent advances have been made in the absence of a mature genome assembly. For example, although a fragmented genome assembly has been available since 2016 [47], and a well-assembled and annotated genome assembly is in progress (consortium led by Marie-Agnes Coutellec and funded by Genoscope), we mainly relied upon traditional BAC sequencing and linkage mapping [76] to identify the chirality gene, albeit aided by high-throughput sequencing methods. Others have used *L. stagnalis* transcriptome data (rather than genomic) to identify horizontal gene transfer between invertebrates and vertebrates, likely facilitated by host-parasite interactions [45]. Similarly, in the absence of a genome, peptide sequencing of seminal fluid was used to identify ovipostatin, a protein that suppresses egg mass production [80]. Subsequently, the genome sequence was used to identify the complete gene sequence of ovipostatin; gene expression data were used to understand the role of ovipostatin in reproduction [81].

One clear benefit of working with *L. stagnalis* is that the snails are straightforward to keep in the laboratory and can be raised in the thousands, either from controlled crosses or by self-fertilisation. In our work we undertook repeated rounds of self-fertilisation and full-sib mating to create highly inbred lines. One of these lines was then used to create the in-progress genome assembly and is also freely available to other labs.

Another advantage of using pond snails is that proof-of-principle experiments have shown that CRISPR/Cas9 methods are an efficient means to knock-down gene function in early embryos. In recent work, Abe and Kuroda [82] injected early *L. stagnalis* embryos with a CRISPR/Cas9 knock-down construct and then raised these embryos to hatching in glass capillaries, using the knock-down to provide definitive proof that a mutation in the formin gene is causative of changes in chirality [83, but see commentary: 84]. It is nevertheless unclear if this method will achieve wide uptake. A key issue, in addition to the skill and equipment needed for microinjection, is that *L. stagnalis* embryos do not readily develop outside of the egg capsule. This problem of embryo viability is likely general to many molluscs. Thus, like *B. glabrata*, *L. stagnalis* stands as a promising model mollusc that nevertheless faces substantial hurdles alongside a general lack of resources and methods.

New Zealand freshwater snail *Potamopyrgus antipodarum* – host-parasite coevolution, evolution of sex

The tiny prosobranch snail *Potamopyrgus antipodarum*, unusual in the frequent natural coexistence between obligately sexual and obligately asexual individuals, is a textbook model for host-parasite coevolution and the evolution of sex. John Maynard Smith [85] first promoted the system as one with perhaps uniquely high potential to apply to the study of sex. Curt Lively and collaborators discovered an important role for host-parasite coevolution in the maintenance of sex in at least in some *P. antipodarum* populations [e.g. 86, 87]. This work also raised a host of follow-on and still unanswered questions, including but not limited to the mechanisms driving the origin of new asexual lineages, the maintenance of sex in lakes without high frequency of coevolving parasites, and how asexual reproduction influences genomes and phenotypes.

Answering these questions in *P. antipodarum* requires genomic resources, which is what spurred us to start generating transcriptomes nearly ten years ago [88]. There now exist dozens of transcriptomes [89-91] and a forthcoming high-quality draft genome assembly (Table 1) as well as dozens of resequenced genomes [e.g. 92] representing the diversity of the species in its native range. These resources have been used to, for example, demonstrate evidence for accelerated mutation accumulation in the genomes of asexual *P. antipodarum* [92] and reconstruct the invasion route of destructive *P. antipodarum* populations that have colonized North America and Europe [93]. These new genomic resources have also revealed a very recent genome duplication in *Potamopyrgus* that has complicated genome assembly [94]. In the future, comparative analysis of patterns of nucleotide and structural evolution in these genomes could be to assess support for a host of major hypotheses for sex and host-parasite coevolution. Like most other molluscan systems, however, other important genomic tools (e.g. transgenesis, cell lines) await development.

Various bivalves and gastropods – biomineralisation

Molluscs are a powerful system in which to study the evolution and mechanics of biomineralization because of their high diversity and their highly complex, robust, and often patterned shells [11, 12, 95]. In this respect, bivalves in particular are also a subject of relatively intense study, both because of their important commercial applications and because a few bivalve species are highly invasive [54].

The general finding of biomineralization studies to date is that the majority of the proteins that are involved in making the shell are unique to each separate group, with only a low proportion shared across, for example, bivalves and gastropods [11, 24]. Accordingly, no single mollusc species has come to dominate the subject area. A diversity of models will ultimately be required to draw general conclusions and identify common patterns.

An early survey of genes involved in molluscan shell formation was based on an analysis of the oyster genome [25]. Because some of these shell proteins constitute important components of the extracellular matrix across metazoans, Zhang et al. [25] suggested that the organic matrix of the shell might share key similarities - but nevertheless still harbours some major differences - with the connective tissue of other animals. Wollesen et al. [96] made an analogous point with respect to the fact that characteristic brain regionalization genes in other animal lineages are expressed in the mantle of molluscs during development, suggesting that brain regionalization genes might have been co-opted into the shell patterning in molluscs. Other important findings include the fact that a large proportion of secreted proteins contain simple repetitive motifs, which might further promote the evolvability of the mantle secretome [11].

Nonetheless, despite substantial progress, considerable caution is required in making general inferences on biomineralization. In particular, most of the genomic studies on biomineralisation to date have involved either bivalves (*Crassostrea*, *Pinctada* oysters, and *Mytilus* mussels) or gastropods (*Haliotis*, *Lymnaea*), comprising just two of the eight of the major lineages of the Mollusca (Figure 1). As there is only one exception, using chitons [97], broad conclusions set in an evolutionary framework are premature.

A key aim for the future must be to understand the regulatory networks that lie at the core of shell formation and whether there is a conserved genetic 'toolbox' [12]. Prior gene expression studies in a range of species have revealed several conserved genes expressed in discrete zones within and around the developing shell, hinting at a conserved network [refs in 11, 12]. Broader sampling should include, for example, studies of the embryonic shells of a variety of taxa as well as polyplacophoran shell plates and test whether these structures have independent evolutionary or developmental origins. Only then may it be possible to make progress in understanding the means by which by gene interactions and deviations from regulatory networks contribute to the diversity of the shell patterning phenotype.

Slipper shell *Crepidula* – early development

The early development of molluscs is characterised by a spiral cleavage pattern, a form of development that unites several lophotrochozoan groups, including annelids, some flatworms, and most molluscs, but excluding cephalopods [98, 99]. Spiralian development has relatively few cell divisions before gastrulation, meaning that it is possible (in theory) to map the fate of each cell in the blastula.

The traditional focus of this area of research has aimed to understand the developmental process and the means by which diverse larval and adult body plans are produced. Most studies have been carried out in a relatively small group of model systems, frequently gastropods and bivalves [e.g. 36, 99, but not always, see 100], that were selected for reasons such as ease of production of embryos and an ability to access and manipulate them during early development.

The slipper snail *Crepidula fornicata* is perhaps the most high-profile species in the context of the study of molluscan development [35; alongside *Tritia (Ilyanassa) obsoleta*, see Table 1]. While the original research on the species was used to create the cell lineage nomenclature of spiral cleavage [101], recent work has produced high-resolution cell lineage fate maps, described the morphogenetic events during gastrulation, and provided important insight into the molecular basis of early development [102-104]. Notably, *C. fornicata* was also the first molluscan species in which CRISPR/Cas9 genome editing was demonstrated [105]. Most recently, the sister taxon the black-foot snail *Crepidula atrasolea* has come to the fore as a complementary model because it has a short life-cycle and is easy to rear through successive generations in closed aquaria [106, 107]. A further benefit is that the rearing methods that are being used are open source and economical and may thus be easily applied to other species [106]. Even so, it is not clear at this point whether *C. atrasolea* will come to be used by many research groups; whether this happens is likely dependent upon ease of culture and the tools that are made available.

Octopus and other cephalopods – intelligence, adaptive camouflage, vision, development

Cephalopods show several remarkable features that distinguish them from other molluscs, including camera eyes, high intelligence, and absence of the stereotyped spiral cleavage pattern. As the "first intelligent beings on the planet" [Brenner, quoted in 21], cephalopod models therefore have the potential to offer special insight into an especially wide range of biological questions [108]. Cephalopod genome assemblies and functional genomic

methods that have been developed in parallel offer a powerful means to study a wide range of sophisticated adaptations that evolved in cephalopods, independently of similar traits in vertebrates.

The first cephalopod genome assembly [7] revealed that while the octopus developmental and neuronal gene set is roughly the same as that found across other invertebrates, there have been large expansions in two gene families, the protocadherins and a zinc-finger transcription factor family. Both of these gene families also independently expanded in vertebrates. The same study also showed that messenger RNA editing plays a major role in generating diversity in proteins involved in neural function. Broadly similar results have since been reported from other cephalopods [109]. Most recently of all, CRISPR/Cas9 gene editing was used to knock out a pigmentation gene in the longfin bobtail squid [110].

Nonetheless, much of the potential in co-opting genomics into the study of cephalopods otherwise remains largely unrealised. For instance, while molluscs have perhaps the greatest diversity in eye structure of all animals [111], there are few genomic comparative studies that span the wide diversity of molluscs/lophotrochozoans [112] and their light-sensing systems [e.g. in scallops 113]. Instead, even recent studies continue to use a gene-by-gene approach [114, 115] rather than taking advantage of genome-era resources.

A potential challenge facing cephalopod researchers is an apparent disconnect between the genomics and behaviour-focussed studies. As an example, a key recent work hypothesised that intelligence in cephalopods and some vertebrates might have evolved through similar processes, yet there was no discussion of ‘genomics’ in the work [20]. Perhaps the barrier is a lack of interdisciplinarity, alongside challenges in devising methods that use genomics to experimentally test hypothesized genotype-phenotype relationships with respect to intelligence, consciousness, etc. A transcriptome atlas of the brain would provide a powerful starting point [116]. Subsequent work could be modelled on studies in vertebrates, promoting a comparative approach to understand the cellular and genetic innovations that underpin cephalopod brain expansion [117]. More broadly, a framework that compares cephalopods to vertebrates, and also to other molluscs (e.g., *Aplysia*, *Lymnaea*) with relatively simple neuronal systems could provide important steps towards our understanding of the evolution of complex cognition.

A final issue is that experiments involving cephalopods may require extra guidelines and permissions than typical for other invertebrates [e.g. 118]. These additional restrictions are obviously appropriate for the welfare of these cephalopod models, but will also likely incur extra costs and time, as well as potentially limiting the nature of experimentation.

Cone snails – bioactive compounds for human benefit

Cone snails have long attracted scientific interest because of potency of the venom that they use to immobilise their prey. The requirement for cone snail venom peptides to be simultaneously potent and specific to their molecular targets means that these peptides are both of interest to physiologists and are bioactive compounds that may be used for medical benefit. The remarkable diversity of conotoxins means that prospecting studies will certainly lead to new therapeutic applications [119, 120].

To date, proteomic and transcriptomic studies have revealed that individual cone snail species use hundreds of different peptides [e.g. 121, 122, 123], with the total conotoxin repertoire across all ~10,000 venomous species in the Conoidea estimated around 100,000 distinct molecules [124]. Although significant progress has been made – including the first commercially available conoidean venom peptide drug used to treat chronic pain [125] – the study of conotoxins, or ‘venomics’, is underdeveloped relative to the potential academic and commercial gains. Despite profound medical and thus commercial potential, the majority of the genomic resources for cone snails are limited to transcriptomes, alongside some partial

genomes [126, 127]. The existing resources have been adequate from a basic bioprospecting perspective, but more refined studies will benefit from well-assembled genomes and the development of a common model species.

One example of an advance that can come from the availability of these resources is with respect to a better understanding of conopeptide post-translational modifications, which are carried out by enzymes that are themselves of biomedical interest [121]. Characterizing these interactions will be facilitated by the development of a model species with a genome assembly and associated resources. The high diversity of conopeptides also demands that scientists prospect across an unusually wide range of species, providing a strong case for generating genomic data and other tools from a wide variety of species.

What's missing from molluscan models?

Now that molluscan genome assemblies are relatively straightforward to produce and becoming commonplace, it is worthwhile to consider the barriers that remain towards using these genomic resources to understand the biology of molluscs and beyond. Some of these barriers are taxon-specific or new, while other challenges are long-standing and of much wider relevance. For the latter, solutions might be especially likely to be transformative in enabling molluscan research.

Accessible transgenesis

In most phyla outside of the Lophotrochozoa, the advent of gene editing, particularly via the CRISPR/Cas9 system, has transformed our ability to study the basics of animal biology. Gene editing has also provided powerful means to implement solutions to important human problems [105] such as biological control of disease vectors [72, 128]. By contrast, CRISPR/Cas9 has been used in only a few occasions in molluscs over five years [82, 105, 129, 130]. Similarly, RNA interference has seen some successes (e.g., [131]), especially in bivalves, but has still not received wide take-up [54, 55, 132, 133].

These tools might be scarce at least in part because of the continuing lack of research focus and funds directed towards molluscs. However, it is also the case that technical challenges play a role, especially in terms of vector delivery and successful culture of genetically modified embryos. In the current iteration, gene editing is "efficient" in molluscs [e.g. 110], but only once the vector is successfully *delivered* into the embryo. In our view, the continuing problem with using these methods in many molluscan species is that the delivery tends to be technical and highly skilled and thus low throughput.

We believe that these barriers can be overcome with a few relatively straightforward solutions. The mollusc community should invest in the development of viral vectors (e.g. virus pseudotyped with the Vesicular Stomatitis Virus G protein, with suitable promoters and polyadenylation sequences) that are able to directly deliver constructs to molluscan cells or embryos, obviating the requirement for low through-put injection or electroporation, and the removal of the embryo from any protective membranes. For example, packaged lentiviral vectors are able to deliver transgenes efficiently across a broad host range [134-136], have been widely used in gene therapy for the last twenty years or so, and are optimized to give high levels of expression and integration.

Presently, we are not aware of experiments that have established that the same vectors will also infect snail cells. The VSVg protein binds to the LDL receptor and to other members of this family [137], which is widespread in metazoan organisms including snails. It is therefore reasonable to suppose that VSVg pseudotyped viruses will transfect snail cells. The technology already exists; it just needs to be adapted for use in molluscs.

Transgenic methods are a necessary complement to genomics; there are limited means to gain proof of gene function if there is no quick and straightforward method to up or down-

regulate the expression of a gene. Gene-editing is of course just one of the tools available – but it is probably the most powerful available, especially given the absence of other technologies.

A persuasive example of the applied benefits that could come from the development of gene-editing and gene-drive methods is the possibility of engineering *Biomphalaria glabrata* to make the snail resistant to infection by schistosomes. The delivery vector could be engineered so that gene drive would push the resistance trait through the population. Similar methods might be used to control crop pests, by first screening for genes that define susceptibility to agrochemicals, or that induce avoidance behaviours (e.g. to crops). This knowledge could then be used for the development of novel chemicals that may be used as targeted molluscicides or to insert constructs which are then driven through the population. Likewise, for benefit of developmental biology, individual genes could be labelled with fluorescent markers and then used to trace molecules and cells through development, such as has been applied in other model organisms [e.g. 138]. In our own work on left-right asymmetry, such developments are necessary to trace the molecular dynamics of the cytoskeleton during chiral cleavage [139].

Cell culture and immortal cell lines

Molluscan cell lines are an essential complement for *in vitro* assays of gene function, proof-of-principle studies of viral transfection and electroporation, as well as testing of vaccines or molluscides. However, while primary culture cells have a role, there is only a single immortal molluscan cell line, *Bge*, derived in the 1970s with considerable effort from *B. glabrata* [67, 68]. By contrast, there over 500 lines derived from insects [67], but only the single *B. glabrata* line to represent the whole of the Lophotrochozoa.

The *Bge* line shows considerable karyotype variation from native snails [140], but still retains haemocyte-like morphology and behaviour, including encapsulation of schistosome sporocysts [141, 142]. To date, the *Bge* line has been mainly used to understand interactions between the snail-derived cells and schistosome larval stages, including immune resistance [143]. Most recently, the genome of the *Bge* cell line was sequenced, highlighting variation in genes that may have contributed to immortalization, especially genes involved in regulation of the cell cycle, including apoptosis and transcriptional regulation [143].

There is further considerable benefit that could be gained from deriving more lines from *B. glabrata*, as well as new lines from other species. Given the difficulties involved, one strategy could be to identify the genetic changes that make molluscan cells immortal. A starting point could be the application of genomics towards understanding the genetic changes that produce transmissible cancers in bivalves [53]. This knowledge could then be used construct new lines in a relatively straightforward manner – as well as also useful in bringing together a pan-metazoan view of cancer biology. It would also make any mollusc accessible to cell culture, and ultimately, enable e.g. organoid experiments that could be used to understand molluscan developmental pathways and cell signalling [144]. Unfortunately, it is not currently possible to derive long-term cultures directly from transmissible cancer cells (Stephen Goff, Michael Metzger pers. comm.).

Improved extraction of high-molecular weight DNA.

Extracting high-molecular weight and contaminant-free DNA is a continuing problem for molluscan research, recently brought into focus by new DNA sequencing methods that require long, contiguous, and break-free DNA, ideally from a single individual. Co-extracting contaminants such as mucopolysaccharides and polyphenols are often noted as a potential problem in that they may hinder library preparation and/or sequencing steps (although the reality is that the precise cause is rarely known or investigated). The usual mitigation strategy

is to try a variety of extraction methods [145-147], which frequently involve a reagent such as CTAB [148], and then selecting the most appropriate approach for the chosen organism.

It is a continuing problem that there is no high-molecular weight extraction method that routinely works for all molluscan taxa, exacerbated by the different but ill-defined demands of the various sequencing platforms. In our experience, a further problem is that there is considerable variation in DNA quality from individuals extracted at the same time, using the same method.

Improving both DNA preservation and extraction techniques will be important with respect to a suite of new directives [e.g. Earth Biogenome project; 149] that aim to sequence the genomes of all known eukaryotic taxa. For example, in the UK, the Sanger Tree of Life project has committed to sequencing the genomes of all native species within a few years [150]. In this case, the pinch-points are likely to be associated with technical issues such as DNA extraction or specimen collection and identification, rather than the sequencing itself.

There is also considerable potential in using the extraction of environmental DNA to monitor and/or identify molluscs [151-154]. The 'ancient' DNA that remains in molluscan shells and other material, including subfossil and museum species, also has the potential to transform our study of the past ecological and evolutionary dynamics. In this latter respect, several new studies have provided successful proof-of-principle [155-157]. While the methods for the extraction of ancient DNA have been available for some time, improved and cheaper sequencing technologies have enabled substantial recent progress in the insights that can be generated from this DNA. Access to a high-quality genome assembly for each species will provide another qualitative step forward, by enabling subtractive bioinformatic methods when sequencing degraded material.

Greater diversity in molluscan models

Despite our opening argument for transformative benefits associated with the availability of a diverse set of molluscan models, the reality is that the majority of molluscan research, including genomics, has mainly been restricted to the gastropods and bivalves, with a select group working on cephalopods. There are relatively few genomic studies and model species that represent the other classes, including the Scaphopoda (tusk shells), Monoplacophora, Polyplacophora (chitons), and the Aplacophora (Caudofoveata and Solenogastres). The notable exceptions are a growing resource of broad phylogenomic studies that have aimed to understand molluscan phylogeny and origins, mainly using transcriptome sequences [158, 159] but recently also genome data using strategically selected genomes [52, 160].

The lack of representative species from these other groups, mainly linked to difficulties in obtaining specimens, their sometimes small size and their (lack of) maintenance in a laboratory, is a continuing problem. The rapid improvement in technology and costs associated with methods for genomic resource development provide at least a partial solution. Even if a particular taxon is difficult to collect and study while alive, it is nevertheless now straightforward to develop genomic resources, which may then be used in comparative studies. Thus, in this respect the first chiton [97] and monoplacophoran [52] genome assemblies are important because they will advance the study of molluscan and animal evolution.

Concluding remarks

While molluscan genome assemblies are becoming commonplace, the absence of a fully 'mobilised' model mollusc means that there remain substantial challenges in devising methods to interrogate molluscan biology. However, as others have acknowledged [12] a single species is unlikely to ever meet all of the requirements. It remains the case that the biological question should dictate the species used.

We acknowledge that structural issues with respect to the study of molluscs (e.g. transgenesis, DNA extraction, cell culture, large and repetitive genomes) will be difficult to overcome. But by making the case that there are considerable benefits to studying molluscs, both from the perspective of general biology and for human health and well-being, we believe that major progress is still possible, by striving for a cohesive research community [48] that will ensure that this important phylum, and the wider grouping of Spiralia and Lophotrochozoa to which it belongs, is no longer neglected.

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Table 1. An overview of the some of the most well-developed molluscan model systems. All featured taxa have a completed genome assembly, albeit of varying assembly quality. Other resources (e.g. genome browser, tools for transgenesis) are comparatively rare, as are taxa that are suited to laboratory culture. The table is not comprehensive, instead featuring a set of diverse mollusc taxa with the potential for broader applicability as 'models'.

Class	Common name	Species[1]	Habitat	Genome browser	Laboratory life-cycle[2]	Inbred lines available	Immortal cell line	CRISPR-Cas9 transgenesis	Publications[3] 2010-2020	Publications[4] (%) genetics/genomics
Gastropoda	Apple snail	*Pomacea canaliculata*	Freshwater		Yes				553	12
	Bloodfluke planorb	*Biomphalaria glabrata*	Freshwater	Yes	Yes	Yes	Yes		1068	21
	Great pond snail	*Lymnaea stagnalis*	Freshwater	Yes[5]	Yes	Yes		Yes	1180	14
	New Zealand mud snail	*Potamopyrgus antipodarum*	Freshwater		Yes	Yes			339	14
	Periwinkle	*Littorina saxatilis*	Intertidal						652	19
	Abalone	*Haliotis spp.*	Marine	Yes					1743	28
	Cone snail	*Conus spp.*	Marine						364	26
	Eastern mudsnail	*Tritia (Ilyanassa) obsoleta*	Marine						175	15
	Owl limpet	*Lottia gigantea*	Marine	Yes					100	36
	Scaly-foot snail	*Chrysomallon squamiferum*	Marine						13	46
	Sea hare	*Aplysia californica*	Marine	Yes	Yes	Partial			1226	18
	Slipper snail / black-foot snail	*Crepidula spp.*	Marine		Yes			Yes	317	10
	Grove snail	*Cepaea nemoralis*	Terrestrial		Yes				171	20
	Giant African land snail	*Achatina fulica*	Terrestrial		Yes				289	14
	Garden snail	*Cornu aspersum*	Terrestrial						138	10
Bivalvia[6]	Manila clam	*Ruditapes phillipinarum*	Marine						1593	19
	Mussel	*Mytilus spp.*	Marine						8230	14
	Pacific oyster	*Crassostrea gigas*	Marine	Yes				Yes	6292	24
	Pearl oyster	*Pinctada fucata*	Marine						1008	36
	Scallop	*Argopecten*	Marine						577	29
	Scallop	*Patinopecten*	Marine						290	40
	Scallop	*Chlamys*	Marine						791	46
	Scallop	*Pecten*	Marine						695	11
	Softshell clam	*Mya arenaria*	Marine						410	16
Cephalopoda	Dwarf cuttlefish	*Sepia bandensis*	Marine						1171	9
	Longfin inshore squid	*Doryteuthis pealeii*	Marine						178	8
	Octopus	*Octopus spp.*	Marine	Yes					2874	10
	Squid	*Euprymna spp.*	Marine	Yes				Yes	243	50
Polyplachophora	West Indian fuzzy chiton	*Acanthopleura granulata*	Marine						34	3
Monoplacophora		*Laevipilina hyalina*	Marine						8	50

[1] Genus only is shown if several closely related species are in use

[2] Although many species can be induced to spawn in the lab, few are amenable to culture through the whole life-cycle and over repeated generations; excludes single reports

[3] Assessed by searching Web of Knowledge 2010-2020 using the genus name as a search term; for "*Conus*" it was also necessary to add "snail*" as a search term, for "*Mya*" and "Cornu" it was necessary to add the species name

[4] Assessed by searching Web of Knowledge 2010-2020 using the genus name as a search term AND "gene" OR "genes" OR "genomic" or "genomics"

[5] Not yet publically available

[6] Many bivalves can be grown in farms, sometimes over generations, but the conditions could not be described as "laboratory"; likewise, inbreeding is possible in many species (e.g. oyster), but they can not be described as "inbred lines" and are not generally available

Figure 1. Relationships among the major lineages of Mollusca, relative to other Lophotrochozoa, and Ecdysozoa and Deuterostomia outgroups. The structure of the phylogeny is based on that presented by Kocot at al. [52], using a phylogenomic dataset. Representative organismal images provided by Emily Jalinsky.

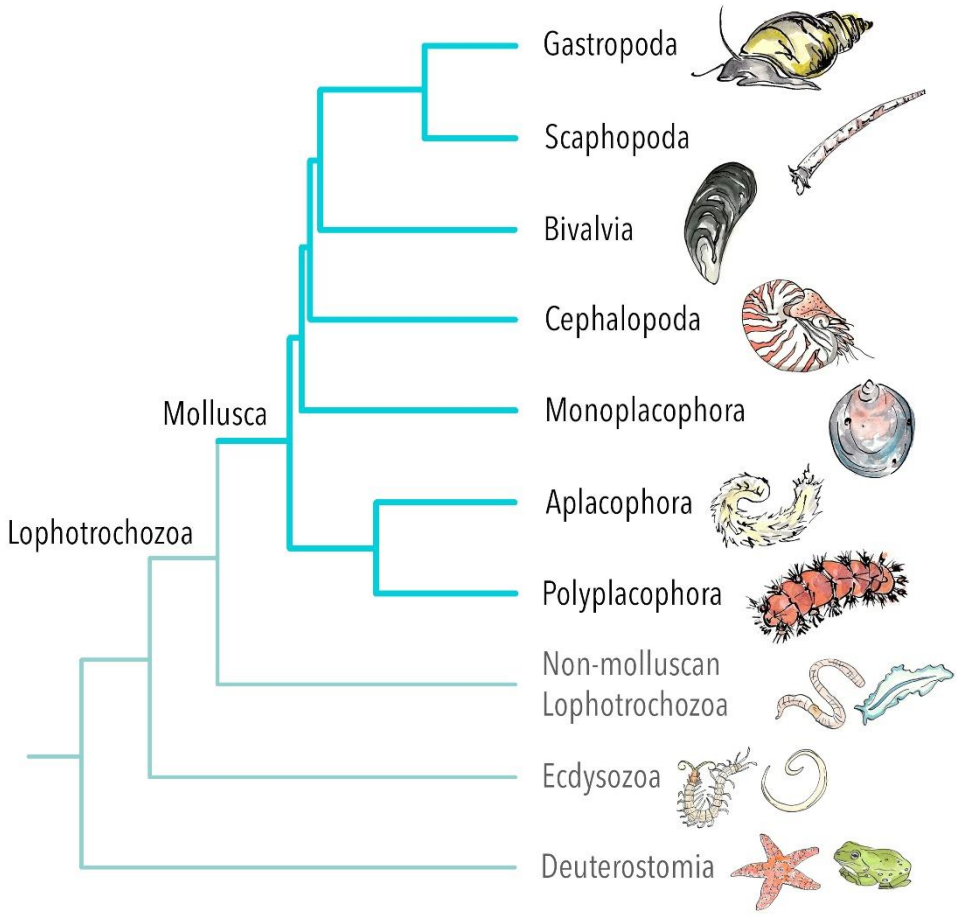

Figure 2. Idealised ‘ELCTR’ criteria for for the making of a model mollusc. Images from top: *Cepaea nemoralis* at the University of Nottingham (Daniel Ramos Gonzalez); *Potamopyrgus antipodarum* lab culture at the University of Iowa (Justin Torner); attendees at the Royal Society ‘Pearls of Wisdom’ molluscan genome meeting 2019 (Liam Helm); CRISPR-Cas9 cartoon (National Human Genome Research Institute, CC-BY-2.0); investigators using the MolluscDB [50] database (Chelsie Higgins).

Figure 3. Representative molluscs used in research and highlighted in this work. The classes Gastropoda (including pond snail *Lymnaea stagnalis*, bloodfluke planorb *Biomphalaria glabrata*, New Zealand mud snail *Potamopyrgus antipodarum*, slipper snail *Crepidula fornicata*, cone snails *Conus* spp), Bivalvia (including various clams, mussels, scallops) and Cephalopodia (including *Octopus*) all include several model species. In comparison, there no species that could be described as models in the other classes and groups, including the Scaphopoda, Monoplacophora, Aplacophora and Polyplacophora. Image credit: Emily Jalinsky.