HHS Public Access

Author manuscript

Dev Comp Immunol. Author manuscript; available in PMC 2018 October 01.

Published in final edited form as:

Dev Comp Immunol. 2017 October ; 75: 3–15. doi:10.1016/j.dci.2017.03.013.

Comparative Immunogenomics of Molluscs

Jonathan H Schultz and **Coen M Adema**

Center for Evolutionary and Theoretical Immunology, department of Biology, University of New Mexico, Albuquerque, NM 87131, USA

Abstract

Comparative immunology, studying both vertebrates and invertebrates, provided the earliest descriptions of phagocytosis as a general immune mechanism. However, the large scale of animal diversity challenges all-inclusive investigations and the field of immunology has developed by mostly emphasizing study of a few vertebrate species. In addressing the lack of comprehensive understanding of animal immunity, especially that of invertebrates, comparative immunology helps toward management of invertebrates that are food sources, agricultural pests, pathogens, or transmit diseases, and helps interpret the evolution of animal immunity. Initial studies showed that the Mollusca (second largest animal phylum), and invertebrates in general, possess innate defenses but lack the lymphocytic immune system that characterizes vertebrate immunology. Recognizing the reality of both common and taxon-specific immune features, and applying up-to-date cell and molecular research capabilities, in-depth studies of a select number of bivalve and gastropod species continue to reveal novel aspects of molluscan immunity. The genomics era heralded a new stage of comparative immunology; large-scale efforts yielded an initial set of full molluscan genome sequences that is available for analyses of full complements of immune genes and regulatory sequences. Next-generation sequencing (NGS), due to lower cost and effort required, allows individual researchers to generate large sequence datasets for growing numbers of molluscs. RNAseq provides expression profiles that enable discovery of immune genes and genome sequences, reveal distribution and diversity of immune factors across molluscan phylogeny. Although computational *de novo* sequence assembly will benefit from continued development and automated annotation may require some experimental validation, NGS is a powerful tool for comparative immunology, especially increasing coverage of the extensive molluscan diversity. To date, immunogenomics revealed new levels of complexity of molluscan defense by indicating sequence heterogeneity in individual snails and bivalves, and members of expanded immune gene families are expressed differentially to generate pathogen-specific defense responses.

Keywords

Next-generation sequencing; Gastropoda; Bivalvia; Genome characterization; Transcriptomics

*Corresponding author: Coen M Adema, Tel.: +1 (505) 277 2743, coenadem@unm.edu.

1. Introduction

Historical observations of the association between exposure to disease and subsequent protection from future (human) illness eventually led to development of the smallpox vaccine by Jenner in the late 1700s (Owen et al., 2012), and of the Germ Theory, linking pathogens and disease (Walker et al., 2006). Studies of how immunity against pathogens is achieved culminated in our current understanding of immunology, mostly as it reflects the immune function of vertebrate animals. Some of the early studies of immunity, however, also benefitted from use of invertebrate organisms. Most famously, Metchnikoff discovered phagocytes and their role in immunity in starfish larvae (Metchnikoff, 1905). By using one (invertebrate) organism to make predictions of immune function in other (vertebrate) animals Metchnikoff gave rise to a new field of biology: comparative immunology. The power of comparative immunology begotten by investigating invertebrates is evident from landmark characterization of e.g. lectins (Prokop et al., 1968), antimicrobial peptides (Boman and Hultmark, 1987), Toll-like receptors (Lemaitre et al., 1996; Medzhitov et al., 1997), and RNA interference (Fire et al., 1998) that have expanded our understanding of immunity and revealed shared features among animals across phylogeny and evolution.

The study of immune function of molluscs (including snails, bivalves, cephalopods, others, see Fig. 1) is motivated importantly by notions that many molluscs are economically valuable food sources, especially in aquaculture (Carnegie et al., 2016), or may transmit infectious diseases of medical and veterinary relevance (Adema et al., 2012). Moreover, the highly diverse phylum Mollusca is second in size among animals only to Arthropoda and represents the generally understudied lophotrochozoan protostomes, one of three lineages of metazoan animals, along with ecdysozoan protostomes and deuterostomes (e.g. Erwin et al., 2011). As such, study of molluscs will continue to broaden understanding of the evolution of immune function across the range of metazoan phylogeny, especially because recent insights suggest that molluscs are capable of sophisticated and specific immune responses (Adema and Loker, 2015; Coustau et al., 2015; Guo et al., 2015).

This review briefly discusses the view of molluscan immune capabilities as it developed from investigations before the availability of immunogenomics. We then present the more specific characterization of molluscan immunity that was afforded by studies benefitting from PCR and Sanger sequencing. Following is a discussion of molluscan immune capabilities discovered from genome mining and transcriptome analyses, especially by relatively easily-applied large- scale next-generation sequencing techniques. Lastly, we consider current limitations of utilizing NGS data and discuss the future of molluscan comparative immunology now that large sequence datasets are increasingly available.

2. Molluscan Immunity

Historically, molluscan immunology has been studied in a small number of species represented within the diversity of the phylum Mollusca. Practical considerations that included ease of collection, animal size, reliable animal husbandry, selective rearing of genetic lineages, as well as relevance for disease transmission or economical (aquaculture) importance have focused consistent study toward a few species of the Gastropoda, and

increasingly so in recent times to some representatives of the classes Bivalvia and Cephalopoda, to the exclusion of other molluscan classes (Fig. 1). Experimental approaches for initial immunological studies included the monitoring of responses of bivalve and gastropod molluscs following exposure to inorganic material (e.g. Indian ink; Tripp and Kent, 1967), to pathogens, introduced either by bacterial injection or through infection with parasites, notably digenean flatworms like *Schistosoma mansoni* that causes significant infectious disease when transmitted to humans (Tebeje et al., 2016). Snails were observed to rapidly clear bacteria from circulation and survive the exposure, with indications of elevated immunity, a more rapid clearance, after an initial encounter (Bayne, 1980; van der Knaap et al., 1983a, 1981). Some individual snails among populations of otherwise parasite-susceptible *Biomphalaria glabrata* proved naturally resistant to digenetic trematodes, with more rapid responses toward a secondary exposure (Lie and Heyneman, 1979). Susceptibility to parasite infection was determined by the genetic background of snail and parasites (Richards et al., 1992). Professional phagocytic cells termed hemocytes, dwelling in the tissues or circulating with the blood fluid of gastropods and bivalves, phagocytose or encapsulate pathogens, eliminating these with cell-mediated cytotoxicity involving lysosomal enzymes and production of reactive oxygen species (Adema et al., 1991; Granath and Yoshino, 1983; La Peyre et al., 1995; McKerrow et al., 1985; Mohandas et al., 1985; van der Knaap and Loker, 1990). Depending on the species, molluscs may have either a single type or several functionally different categories of hemocytes, and these cells may originate from connective tissue or specialized organs, termed the amoebocyte producing organ (APO) in gastropods (Jeong et al., 1983), or from the white body organ in cephalopods (Claes, 1996; Cowden, 1972). Recognition of nonself and subsequent immune activation is mediated through lectins, initially referred to as agglutinins or cytophilic receptors for foreignness, present as humoral factors or on the surface of hemocytes (Cheng et al., 1984; Michelson and Dubois, 1977; Mullainadhan and Renwrantz, 1986; Renwrantz and Cheng, 1977; Rögener et al., 1985; van der Knaap et al., 1983b). Lectins are non-enzymatic, non-antibody proteins that function as pattern recognition receptors (PRRs) by binding to repetitive carbohydrate surface determinants that characterize groups of pathogens (pathogen associated molecular patterns, PAMPs) such as lipopolysaccharide (LPS) and peptidoglycans of bacteria (Vasta and Ahmed, 2009) and activate immune responses. Contrary to expectations regarding animal immunity drawn from a vertebrate perspective of immune function, and by the observation of some level of immunological memory in gastropods (Lie and Heyneman, 1979), no indications were found in molluscs, or invertebrates in general, of lymphocytic defenses, i.e. no T-cells, B- cells or the rearranging genes that drive generation of antigen-specific receptors (Warr, 1981). As a consequence, invertebrates were deemed to possess a rather unsophisticated innate-type immunity, with a reliance only on invariable, germline-encoded genes for general broad immune recognition of categories of pathogenic organisms. However, Klein (1989) championed the importance of investigating the immunity of invertebrates from new perspectives that are not myopically biased by norms of vertebrate immunology. While invertebrates may not possess all canonical features of the vertebrate immune system, as a result of a long independent evolutionary development they may bear homologs of immune mechanisms, as well as unique immune features that are specific to their lineage. Through analyses of such immune

features, comparative immunology can provide important insights into the evolution of immunity (Marchalonis and Schluter, 1990).

Continued study of molluscan immunology revealed several defense mechanisms, both analogous to aspects of vertebrate immunology and *de novo* from investigation of mollusc-pathogen encounters and the immune factors involved in these interactions. In the absence of specific reagents, comparative studies were often performed with heterologous techniques and reagents, borrowed from vertebrate immunology. The mechanism for production by molluscan hemocytes of toxic reactive oxygen species toward pathogens (Adema et al., 1994) was proposed to be homologous to that responsible for the cytotoxic respiratory burst of vertebrate phagocytes as both were sensitive to catechol-like phenol inhibitors of the vertebrate NADPH-oxidase enzyme complex that generates superoxide (Adema et al., 1993; Noël et al., 1993). Chemical inhibitors and immune-reagents raised against the components of vertebrate signaling pathways were applied to explore and characterize the role of MAPK and other signaling pathways in the regulation of molluscan immunity (Humphries et al., 2001; Walker et al., 2010; Zahoor et al., 2008; Zelck et al., 2007). A role for vertebrate-type cytokines in immune regulation was proposed, based on altered cell morphology and activity of hemocytes following exposure to (human) cytokines, cross-reactivity of molluscan hemocytes with immune- reagents specific for vertebrate cytokines such as Il-1, Il-6 and TNF, and reduced parasite success after treatment of snails with recombinant human cytokine (Connors et al., 1998, 1995a, 1995b; Granath et al., 1994; Hughes et al., 1992, 1991, 1990; Ottaviani et al., 2000, 1997, 1995a, 1995b, 1993; Ouwe-Missi-Oukem-Boyer et al., 1994; Stefano et al., 1991). Although the presence of functional cytokine-like factors in molluscs was not unanticipated and heterologous immune reagents may correctly reveal sequence homologs in molluscs (Williams and Gagnon, 1982), the actual identity of such factors remained to be confirmed due to the potential for false positive immune reactivity via the presence of aspecific antibody- binding proteins in some molluscs (Hahn et al., 1996) and the lack of evolutionary sequence conservation among animal cytokines (Beschin et al., 1999).

Study of mollusc-specific immune features, independent of concepts of vertebrate immunology, provided considerable understanding of how lectins function as non-self receptors. Immune activation resulted from a complex interaction between soluble and cell-bound lectin receptors, enabled by conformational changes after interaction with target epitopes (Cheng and Manzi, 1996; Dam, 1992; Renwrantz and Richards, 1992; Richards and Renwrantz, 1991). Along with lectins, hemocytes of *Mytilus edulis* also released cytotoxic factors with lytic activity toward target cells (Leippe and Renwrantz, 1988). The snail *Biomphalaria alexandrina* expresses multimeric hemolymph lectin molecules that are composed of different members of a family of glycan-binding lectins, potentially expanding the range of antigen recognition (Mansour, 1995). Levels of agglutinating activity (lectins) differed in individual Pacific oysters, *Crassostrea gigas* that were either susceptible or resistant to *Perkinsus marinus* parasites (Romestand et al., 2002). The gastropod *Biomphalaria glabrata* responded to *Echinostoma paraensei* (flatworm) infection with expression of increased levels of diverse hemolymph lectins distributed across several molecular weight ranges (Monroy et al., 1992), referred to as G1M, G2M, and 65 kd lectins (Adema et al., 1997a). These foundational research efforts provided a deeper understanding

of molluscan immune capabilities. Due to technical limitations, however, the factors or mechanisms involved could not yet be defined beyond general descriptors like “cross-reactivity with heterologous immune reagents”, “sensitivity to inhibitors”, “specific carbohydrate binding activity” or “specific molecular weight”. Regardless, molluscan immunology was well-positioned to benefit from the more detailed analysis of immune factors and mechanisms afforded by newly available molecular techniques and to develop the potential of comparative immunology for detailed tracking of the evolutionary development of immune function across animal clades.

3. Beyond “factorology”, immunogenomics phase 1

The first phase of molluscan immunogenomics began in the early 1990s, benefitting from several technological developments that facilitated routine access to molecular biology (PCR, cloning and automated dye-terminator Sanger sequencing) and to the internet (computational bioinformatics and rapidly growing public sequence databases) in a manner that made it possible to identify molluscan immune factors through characterization of coding sequences. A strategy that consisted of partial peptide sequencing (Edman degradation) of purified proteins to enable design of PCR primers and amplify cDNA for sequencing led to targeted characterization of the first snail lectins. The elucidation of the encoding sequences promoted these lectins from factors that were broadly defined by molecular weight and carbohydrate specificity to specific, named entities, with function and relationship to other defense factors defined by sequence. The parasite-reactive plasma lectins of *B. glabrata* described by Monroy et al. (1992) were found to include a diverse set of related fibrinogen-related proteins (FREPs), comprising N-terminal immunoglobulin superfamily domains juxtapositioned with a fibrinogen-like domain at the C-terminus (Adema et al., 1997b). The mucus of the slug *Incilaria burgsdorfii* yielded Incilarin A-C, three different C-type lectins that shared considerable sequence identity (44–55% identity of encoded amino acids; Yuasa et al., 1998). Screening of a cDNA library revealed sialic-acid binding activity derived from several lectins from *Limax flavus* (Gastropoda), also highly similar yet with different sequence identities, and each consisting of a single fibrinogen-like domain (Kurachi et al., 1998). Expression of related lectin sequences from multigene families may broaden the range of carbohydrate epitopes that can be bound, also considering that lectins may assemble as multimeric molecules under native conditions (e.g. Adema et al., 1997a; Mansour, 1995). Some of the newly characterized sequences were studied in greater detail. The full-length genome sequences were determined for several of these FREP genes (Léonard et al., 1999; Zhang et al., 2001). Moreover, it was observed that somatic mutation of FREP genes causes *B. glabrata* snails to generate individually unique repertoires of parasite-reactive FREP lectins (Zhang et al., 2004). Note that FREPs are discussed in detail by Portet et al. elsewhere in this issue. By revealing intronic sequence differences, Goodall et al. (2006) identified allelic variants of *B. glabrata* superoxide dismutase (SOD1) that differentially affected the production of reactive oxygen species in a manner that associated with parasite resistance/susceptibility phenotypes of individual snails.

The above targeted characterization of specific immune factors provided detailed sequence information that began to facilitate comparative immunology, i.e. considering the nature of molluscan defense factors relative to other organisms, and distribution of related factors

across phylogeny. The experimental work was laborious, however, and progress came at considerable effort and investment. Many more candidate immune factors were identified at a higher rate of discovery by transcriptomic studies that employed random gene discovery via high throughput sequencing of libraries of expressed sequence tags (ESTs). Short cDNA inserts, usually representing partial gene transcripts, generated by a number of strategies, including differential display reverse transcription PCR (DD-RTPCR), suppression subtraction hybridization (SSH), open reading frame EST (ORESTES), were cloned and sequenced to capture gene expression profiles from snails like *Lymnaea stagnalis* (Hoek et al., 1996) and prominently *B. glabrata*, untreated or exposed to pathogens. Aided by computational bioinformatics, EST projects recorded many transcribed sequences that helped reveal aspects of the immune system of gastropods (see Fig. 1). Based on sequence similarity with previously described defense factors, mostly from other animal taxa, *in silico* analyses identified gastropod genes for all components of an internal defense system ranging from non-self recognition (lectins); activation and regulation of immune responses; humoral defense factors (antibacterial proteins and peptides); cellular cytotoxicity (lysosomal enzymes, production and metabolism of reactive oxygen species), including antiviral responses as afforded by genes encoding the machinery for RNA interference. It is of note that up to 60% of EST data from molluscs cannot be annotated because they have no matches in (current) sequence databases. Designated as novel, unknown, a proportion of these sequences are likely unique to molluscan biology and await functional characterization. For an incomplete, yet representative listing of associated projects see: Bouchut et al., 2006; Hanelt et al., 2008; Ittiprasert et al., 2013; Knight et al., 1999; Lockyer et al., 2007a, 2007b; Miller et al., 2001; Mitta et al., 2005; Nowak et al., 2004; Raghavan et al., 2003; Schneider and Zelck, 2001.

Since, EST-based gene discovery has been applied widely to a range of additional species of gastropods, bivalves and cephalopods. This has provided a wealth of information regarding (partial) expressed gene sequences of molluscs (Fig. 1). To date, EST-library screening of this type continues to provide candidate immune factors for further characterization (e.g. Bai et al., 2009; Borisova and Gorbushin, 2014; Ding et al., 2011; Fleury et al., 2009; Goodson et al., 2005; Liao et al., 2013; Liu et al., 2011; Roberts and Goetz, 2003; Seo et al., 2016; Wang et al., 2009, 2007). For example, in 1999, Escoubas et al. recovered from the oyster *C. gigas* an EST sequence similar to mammalian IkappaB kinase (IKK) proteins that have central roles in cell (immune) signaling through activation of nuclear factor-kappaB (NF-kappaB). The recombinantly-expressed complete sequence effected expression of a NF-kappaB-controlled reporter gene. This provided a first indication of functional NFKb signaling in molluscs. The characterization of bivalve lectins and other defense factors has been driven mostly by initial identification of EST sequences (Kang et al., 2006; Korneev et al., 2002; Song et al., 2006). It is of particular interest that RNA interference is available for functional transcriptomics in gastropod and bivalve molluscs (Jiang et al., 2006; Owens and Malham, 2015; You et al., 2012). RNAi knockdown of gene transcripts, and thereby of protein expression, effected by either long double-stranded RNA or short interfering RNA has shown phenotypic changes in *B. glabrata* to demonstrate involvement of immune activities of FREP3 (Hanington et al., 2012, 2010), the cytokine MIF (Baeza-Garcia et al., 2010), and antibacterial LBP/BPI (Baron et al., 2013).

The analysis of EST datasets can reveal groups of genes that are functionally linked such that their expression provides evidence for active cellular, humoral and metabolic processes in an organism. However, EST-based expression profiles recorded from insert libraries best inform by presence of particular sequences. This technique provides modest information regarding relative expression levels of (constitutive) immune genes, that are perhaps increased in response to a pathogen or regarding the potential for pathogen-specific immune responses in molluscs. The lack of detection of a particular immune transcript sequence may be due to differential expression or incomplete sampling. Due to the great effort required for sequencing many individual EST clones, even in automated fashion, and the random nature of insert selection for sequencing, it is unlikely that EST projects yield full representation of the total diversity of expressed sequences.

Microarray approaches provide an alternative way to study transcriptomics that affords a more accurate comparison of expression levels of particular (immune) genes by differentially-treated molluscs. Several microarray platforms have been designed and applied to study immunity in gastropods and bivalves of medical or aquaculture relevance (De Zoysa et al., 2012, 2011; Dheilly et al., 2011; Fleury and Huvet, 2012; Jenny et al., 2007; Leite et al., 2013; Romero et al., 2015; Venier et al., 2011; Wang et al., 2016, 2010; Zhang et al., 2016). The number of gene targets included on initial microarray designs was limited by the modest extent of available sequence data but recent microarrays can harbor upward of 30,000 gene features such that immune sequences are likely included, even if these were not specifically selected as targets on the array. Microarrays that also contain unannotated gene targets have potential to identify unknown, novel candidate immune genes if these are differentially expressed following pathogen encounters. Microarray studies have shown novel aspects of immunity of particular species of molluscs. For example, different strains of the gastropod *B. glabrata* display distinct baseline expression profiles (Lockyer et al., 2012, 2008; Zahoor et al., 2014); this snail mounts pathogen-specific immune responses to Gram (−), Gram (+) bacteria and metazoan parasites (Adema et al., 2010), and FREP3 was identified as a common feature of successful defense responses (Hanington et al., 2012, 2010).

Such studies provided less insights to broaden comparative immunology. Microarrays are species-specific; they employ previously available sequences for a particular organism as hybridization targets to detect relative amounts of matching gene transcripts when comparing different RNA samples of the same organism. Comparison of results from expression studies for general features of molluscan immunity are difficult because microarrays are available only for a limited number of gastropods and bivalves and the various array platforms may differ considerably in number and representation of gene targets.

Nevertheless, the focus of molluscan immunogenomics on gene discovery through sequencing and characterization of (partial) cDNA sequences was highly effective in cataloguing and defining immune capabilities of bivalves and gastropods, even though the techniques available were unlikely to capture complete transcriptomes and thus did not reveal the full extent of gene diversity within molluscan species or the taxonomic distribution of particular molluscan defense genes. Furthermore, in this time of extensive

characterization of cDNA sequences, little information had accrued regarding intron/exon structures, distribution of genes in the genome, general genome architecture, and regulatory sequences for managing gene expression toward immune responses.

4. Genomes and next-generation sequencing, immunogenomics phase 2

Genome sequencing efforts, culminating in completion of the human genome in 2000, had led to development of scientific technology that was able to produce high quality genome sequence assemblies. However, the capacity for genome sequencing was limited. The complex and expensive hardware infrastructure that afforded genome sequencing was available only at a modest number of central facilities. Full genome characterization required a large, costly effort reliant on labor- intensive sequencing methods and challenging computational bioinformatics to organize massive sequence datasets into a genome assembly. Nevertheless, advocacy from (international) consortia of scientists led to inclusion of several molluscs with relevance for biomedical research or aquaculture among the exclusive group of organisms that enjoyed high quality, full genome characterization. Obviously, by providing insight into all aspects of organismal biology, genome sequences yield valuable resources, also for comparative immunology. To better qualify for full genome sequencing, some research communities developed complementary genomic resources. Bacterial artificial chromosome (BAC) libraries were developed for several molluscs like *B. glabrata* (Gastropda) and the oysters *Crassostrea virginica* and *C. gigas*, to gain initial access to genomic information (Adema et al., 2006; Cunningham et al., 2006; Raghavan et al., 2007). BAC libraries consist of considerable numbers of clones that represent the full genome with large (>100kb) inserts of molluscan genomic DNA. Selection and detailed analysis of BAC inserts is a somewhat protracted process, but allows for focusing investigative analysis towards genes of interest. Several BACs have been sequenced full-length in the case of *B. glabrata* (Tennessen et al., 2015a; Hanington et al., 2010). This further confirmed the presence of gene families of some immune genes in the genome of this gastropod: four FREP genes are present within a 115,524 base pair (bp) genomic region (Hanington et al., 2010) and *B. glabrata* BAC clone BG_BBa-10D22 (182,461 bp) contains five peptidoglycan recognition protein (PGRP) genes (GenBank: AC235813.3). Pending full genome assembly, this likely provides an incomplete view of these gene families in *Biomphalaria*. For instance, PGRP gene families comprising up to 12 different genes are present in other molluscs species such as *Bathymodiolus platifrons*, *C. gigas*, *Euprymna scolopes*, *Octopus vulgaris*, *and Solen grandis* (Castellanos-Martínez et al., 2014; Goodson et al., 2005; Itoh and Takahashi, 2008; Wei et al., 2012; Wong et al., 2015; Zhang and Yu, 2013). The use of BAC inserts containing defense/stress response sequences (actin and ferritin) as probes for fluorescent *in situ* hybridization (FISH) showed a repositioning of *B. glabrata* chromosomes in the nucleus following parasite exposure, likely in aid of active transcription of response genes (Knight et al., 2011). The utility of BAC libraries is underscored by continued use of this approach to characterize immune genes of additional molluscs in recent times, with continued observation of clustering of immune factors, e.g. two genes encoding lipopolysaccharide and beta- 1,3-glucan binding proteins in the Zhikong scallop *Chlamys farreri* (Kasthuri et al., 2013; Premachandra et al., 2012; Zhang et al., 2008; Zhao et al., 2012a). Large scale genome efforts, aimed at obtaining high genome coverage

and stringent computational genome assembly with availability of transcriptomic data for validation of predicted gene models have led to characterization of the full genomes of one bivalve *C. gigas*, the cephalopod *Octopus bimaculoides* and three gastropods *B. glabrata*, *Aplysia californica* and *Lottia gigantea* (see Table 1). Generally, molluscan genome assemblies consist of high numbers of genome scaffolds; concise assembly is challenged by large genome size and high repetitive content. The genome assemblies do provide, however, good representation of expressed protein-encoding genes and genome size, e.g. 97% and 83%, respectively, for *O. bimaculoides* (Albertin et al., 2015). As such, they give unprecedented access to the most complete gene complements ever for these molluscs, and this allows broad analyses, including those for immune relevant genes.

Accordingly, genome analyses can now identify gene sequences that validate and further develop previous inferences about immune function in molluscs. In the case of molluscan cytokines, previously indicated by time-appropriate, yet rather indirect methods, sequence and functional analyses have confirmed gene sequences for TNF, IL-17 and MIF among bivalves, gastropods and cephalopods (also see Gao et al., 2015; Rosani et al., 2015a). Sequence homologs for Il-1 or Il-6 were not recorded. It remains to be determined whether activities previously ascribed to these cytokines derive from functional analogs or yet other factors (Ottaviani et al., 1995a, 1993).

Molluscs have abundant genes encoding for a diversity of lectins, including many with C-type lectin domains, fibrinogen-related sequences or C1q domains (e.g Gorbushin and Borisova, 2015; Zhang et al., 2012). Molluscs also show expanded gene families of Toll-like receptors (TLR). Diversity of this PRR likely expands non-self recognition capabilities (Buckley and Rast, 2015). The availability of non-coding intergenic genomic sequences further enables exploration of (immune-relevant) regulatory mechanisms involving transcription factors and nuclear receptors (Humphries and Harter, 2016; Kaur et al., 2015). Moreover, molluscs possess the machinery to employ methylation for epigenetic regulation of gene expression (Fneich et al., 2013; Geyer et al., 2011) and genome-wide analysis of *C. gigas* suggests that a nucleotide sequence composition is biased towards GC content in families of inducible genes (like stress and environmental response genes) to facilitate methylation for epigenetic control of gene expression (Gavery and Roberts, 2010). The genome sequences also provide targets to develop markers for linkage studies. Such analysis of the association of particular SOD1 alleles with resistance to schistosome parasites indicated the presence of a linked cluster of redox genes and possibly other defense genes in the snail *B. glabrata* (Blouin et al., 2013). Additional analyses identified the so-called hyperdiverse Guadeloupe Resistance Cluster (HRC) and several other groups of genes as candidate genomic regions associated with different levels of susceptibility for parasite infection. Recent efforts have been made to identify the specific genes contributing to the differential susceptibility phenotype, but many remain to be characterized (Allan et al., 2017; Tennessen et al., 2015a, 2015b).

As indicated above, the availability of molluscan genomes facilitated comparative analyses of immune properties among diverse species representing different classes of molluscs. Mostly, however, the potential of genomic studies deepened comprehension of immunity unique to single molluscs. Rather than sparking comparative immunology across classes,

orders or even phyla, genome sequences tended to focus research inward, for a few model molluscs. This was of course because of the considerable challenge to complete genome characterization for any particular organism, dramatically limiting the number of species for which this was achieved. This situation changed significantly with the availability of next-generation sequencing (NGS) capabilities. Several novel high throughput sequencing technologies have been developed as alternatives to routine Sanger sequencing. Of these, some have already been relinquished (454, Solid) while Illumina technology has become a mainstay (Pettersson and Lundeberg, 2009). Compared to Sanger sequencing, NGS technology generates relatively short sequence reads (≤ 150 nt) that may challenge correct *de novo* assembly, but sequence is collected at massively increased fold-coverage and speed. Significantly, NGS carries a modest cost that now enables individual researchers to capture and characterize both genome and transcriptome profiles for their organism(s) of choice (Sohn and Nam, 2016). The third (next-next) generation of sequencing technology is already coming on-line, and by enabling significantly longer sequence reads, will improve sequence assembly capabilities (Rhoads and Au, 2015; Schadt et al., 2010). Of note, the amount of nucleic acid input material required for NGS is in the nanogram to microgram range, far less than required previously for Sanger technology. This enables complete sequencing from few or even individual organisms. With the current NGS capabilities, the number of sequenced genomes and transcriptomes, collected in the form of massive, comprehensive datasets is rapidly increasing (see Fig. 1). Without doubt, many more datasets are awaiting submission to public databases. These developments have rendered the unique status of traditional genome projects obsolete. In fact, some have mockingly declared the death of the genome paper (Smith, 2016), because such high-level prestigious publications are being replaced by reports that announce the availability of novel NGS data (e.g. Huang and Wu, 2015). The scrutiny and effort that yielded the initial set of genomes is unlikely to be paralleled and new genomes will not be assembled or annotated at the same level of quality. Clearly, however, NGS is of great benefit to comparative immunology. The capture of comprehensive (if not complete) genome and transcriptome sequence data, for any initial biological research question, provides an unprecedented data resource that can be mined with computational bioinformatics methods, and also for immune sequences. With NGS, indeed there are effectively no more non-model organisms for gene discovery (Dheilly et al., 2014).

The new sequencing capabilities have already shaped new understanding at different levels of molluscan immunogenomics. NGS characterization of expression profiles of a novel strain of *B. glabrata* increased the number of unique FREP gene sequences previously recorded from this gastropod, perhaps by also revealing novel alleles. Moreover, the non-selective manner of sequence sampling also identified FREP-related lectin sequences with similar domain structures (upstream IgSF sequences with a C- terminal lectin domain) but displaying either a galectin or a C-type lectin domain. These novel sequences, named galectin –related protein (GREP) and C-type lectin-related protein (CREP), respectively, are now grouped together with FREPs as Variable Immunoglobulin and Lectin domain containing molecules or VIgLs (Dheilly et al., 2015). Gorbushin and colleagues (Gorbushin et al., 2010; Gorbushin and Borisova, 2015) have tracked the distribution of defense factors like FREPs across gastropod phylogeny. Inspection of the genome of the euopisthobranch *A. californica*, and of NGS transcriptome data of hemocytes of the prosobranch *Littorina*

littorea, have revealed FREP genes outside of *Biomphalaria*. No FREPs were observed from the genome of the basal gastropod *Lottia gigantea*. Pending analyses of additional NGS datasets from other molluscs, this led to development of the hypothesis that FREPs are an evolutionary innovation that took place after the emergence of class Gastropoda within the Mollusca (Gorbushin et al., 2010). This is especially intriguing because *Mytilus galloprovincialis* (class Bivalvia) was reported to express diversified proteins that contain fibrinogen-related domains, also known as FReDs, thought to function in immunity (Romero et al., 2011). Numerous other FReD-containing proteins, e.g. those consisting of single FBG domains, have been recorded from a range of molluscs but these have not been reported to be diversified (Albertin et al., 2015; Gorbushin and Iakovleva, 2011; Zhang et al., 2012). A phylogenetic analysis of molluscan FReD sequences, including FREPs, does not reveal a clear evolutionary origin for canonical FREPs (Fig. 2a). The FREP sequences from *A. californica, B. glabrata*, and *L. littorea* cluster separately among other FReD-containing sequences. Moreover, the fibrinogen-related sequences of *Biomphalaria* and *Mytilus* shown to undergo extensive sequence diversification (Dheilly et al., 2015; Hanington et al., 2010; Romero et al., 2011; Zhang et al., 2004), fall out in different clades (Fig. 2b,c). This suggests that diversification of FReDs may have occurred independently at different stages during the evolution of molluscs. Of course, future investigations may uncover different instances of diversified FREPs and other FReD-containing proteins. Yet the fact remains that such systems for intra-individual sequence diversification greatly increase the overall FReD diversity that occurs in molluscs (Fig. 2b,c).

An apparently general concept of molluscan immunology was revealed by immunogenomic studies of NGS data involving gene complements and expression profiles from two classes: gastropods and bivalves. DeLeury et al. (2012) collected NGS transcription profiles of *B. glabrata* snails that were evoked by different pathogens (yeast, Gram (+) and Gram (−) bacteria). Zhang et al. (2015) independently exposed *C. gigas* oysters to multiple pathogens including (strains of) particular bacteria and a virus, to determine expression profiles using Illumina NGS. Similar results were observed from annotation and analysis of specific expressed sequences: both the gastropod and the bivalve molluscs studied possess expanded immune gene families, and in both molluscs, pathogens are met with generally similar immune processes or signaling pathways. However, individual sequences of particular gene families are differentially expressed to generate pathogen-specific immune responses. This indicates that molluscs possess complex immune systems with extensive discriminatory properties.

5. Limitations and potential, future immunogenomics

NGS has already shown itself to be a powerful tool for immunogenomics. It holds great promise to elevate molluscan immunology from detailed study of a few model species to a broad vantage point for true comparative immunology that can reveal commonly shared as well as unique immune features across the diversity of the phylum Mollusca. We must also consider carefully the limitations of NGS technology to maximize the future benefits. Current NGS methods generate massive datasets of relatively short read lengths. Computational assembly, especially *de novo* (i.e. without a reference genome or gene models), to reconstruct the genome or the transcript from which they derive, is very

challenging. Several software packages are available for assembly but these are each designed with different assumptions and assembly strategies in mind. Comparison of *de novo* genome assemblies with reference-based (human) genome assemblies of Han Chinese and Yourbal individuals showed that the *de novo* assemblies were 16.2% shorter than the reference genomes and were lacking over 2,000 protein coding exons (Alkan et al., 2011). When considered at the rate at which data is now being generated, these discrepancies can lead to erroneous conclusions about the absence of immune components for non-model, invertebrate organisms for which reference genomes are typically unavailable. Transcriptomes also succumb to assembly difficulties. The choice of assembly software can mean the difference between generating artificial, chimeric contigs, or producing many short, redundant contigs along with correctly assembled sequence contigs (Mundry et al., 2012).

Annotation of assembled sequence data to infer open reading frames, gene models and especially function, is another challenge for correct interpretation of molluscan immunology. For example, Blast2GO (Conesa et al., 2005) is one of the main tools for informative (automated) annotation of large sequence datasets from non-model organisms. The program relies on blast searches to identify transcripts with sequence similarity to a modest set of previously characterized sequences that have been associated with so-called Gene Ontology annotation to define their functional role in molecular, cellular or biochemical processes. Transcripts that do not share significant blast similarities with those included in the reference database are excluded from the annotation. Typically this affects a high proportion of molluscan sequences, and although GO annotation is available for non-model organisms through B2G-FAR (Götz et al., 2011), the resulting annotation may still not be suited for correct functional interpretation of molluscan immune factors. For instance, a FREP lectin transcript that contains two functionally distinct IgSF and fibrinogen-like domains may generate similarities to sequences with annotation that indicates antibody-based immune function and fibrinogen-mediated blood clotting, even though neither of these phenomena exist in snail biology. Thus it is of considerable importance to verify at least some results from *in silico* analysis of NGS sequence data, validating assembly of sequences of interest and applying knowledge of specific organismal biology.

Ultimately, immunogenomics can provide an essential mechanistic basis to evaluate the biological relevance of immune phenomena that may be inferred from observations at the organismal level(Hauton and Smith, 2007; Little et al., 2008). Surprisingly, we may uncover mechanistically supported immune phenomena in the lab that fit less with our perceptions of how immunity should benefit fitness. For instance, there is the unexpected observation of immune memory in the snail *B. glabrata* that prevents secondary infection by the parasite *Schistosoma mansoni* in snails that continue to sustain a primo infection by the same parasite strain, without obvious benefits to snail fitness (Pinaud et al., 2016).

For developing a representative overview of molluscan immunology, increasingly refined phylogenies are available to guide selection of species that will fill the current gaps in coverage by immunogenomics analysis of molluscan diversity (Kocot et al., 2011; Smith et al., 2011). As evident from figure 1, several classes of molluscs deserve particular attention for obtaining NGS data. Individual investigators are encouraged to release any available

relevant NGS datasets so that duplication of sequencing efforts can be avoided. The Global Invertebrate Genomics Alliance (GIGA community of Scientists, 2013; http://giga-cos.org/) aims to generate genomic sequences for 5000 (marine) invertebrates and may offer datasets for molluscan clades yet to be studied with regard to their immune function. Recent depositions of NGS data in public databases indicate that it is feasible to obtain and collect NGS data from molluscs of the classes Scaphopoda, Monoplacophora, Polyplacophora and Aplacophora. The currently available data of these lesser studied molluscs deserves analysis for immune genes. Encouragingly, genome size estimates have been made for several Aplacophora, Polyplacophora and Scaphopoda (Kocot et al., 2015) in light of interest to perform NGS-based genome characterization of representatives of these groups. Given the great diversity of the Mollusca, it is unlikely that full coverage by genome sequencing is ever achieved. However, strategic sampling will likely provide an initial overview that can help to identify taxa that may harbor unique aspects of immunbiology (gene gains or losses) and that are of particular interest for more detailed sampling.

A more comprehensive, inclusive understanding of immune function in molluscs will identify genes and immune mechanisms that can be targeted to bolster immune vigor in species with importance for aquaculture or conservation, or for control efforts aimed at molluscs that negatively impact humanity. Notably, it is worthwhile to consider the potential of the CRISPR/Cas9 system for genome editing (Singh et al., 2017) as a means for modifications of molluscan genomes in light of future functional immunogenomics studies.

In closing, recent studies that track distribution of IL-17 signaling components in over 30 bivalves (Rosani et al., 2015a), or that reveal that LBP/BPI proteins are not routinely conserved across animal phylogeny, yet are import immune factors in molluscs (Baron et al., 2016), show the great potential of modern immunogenomics to push molluscan immunology from detailed study of limited numbers of model species to true comparative immunology, revealing aspects of evolution of animal immune function across broad ranges of phylogeny.

Acknowledgments

The authors wish to thank E.S Loker and all other colleagues at the Center for Evolutionary and Theoretical Immunology at UNM for helpful discussions. This study was supported by NIH grant P30GM110907 from the National Institute of General Medical Sciences (NIGMS).

References

Adema CM, van der Knaap WPW, Sminia T. Molluscan hemocyte-mediated cytotoxicity: the role of reactive oxygen intermediates. Rev Aquat Sci. 1991; 4:201–223.

Adema CM, van Deutekom-Mulder EC, van der Knaap WP, Sminia T. NADPH-oxidase activity: the probable source of reactive oxygen intermediate generation in hemocytes of the gastropod *Lymnaea stagnalis*. J Leukoc Biol. 1993; 54:379–383. [PubMed: 8228616]

Adema CM, van Deutekom-Mulder EC, van der Knaap WP, Sminia T. Schistosomicidal activities of *Lymnaea stagnalis* haemocytes: the role of oxygen radicals. Parasitology. 1994; 109:479–485. [PubMed: 7800416]

Adema, CM., Hertel, LA., Loker, ES. Infection with *Echinostoma paraensei* (Digenea) induces parasite-reactive polypeptides in the hemolymph of the gastropod host *Biomphalaria glabrata*. In: Beckage, NE., editor. Parasites and Pathogens: effects on host hormones and behavior. Chapman & Hall; New York: 1997a. p. 76-98.

Adema CM, Hertel LA, Miller RD, Loker ES. A family of fibrinogen-related proteins that precipitates parasite-derived molecules is produced by an invertebrate after infection. Proc Natl Acad Sci U S A. 1997b; 94:8691–8696. [PubMed: 9238039]

Adema CM, Luo MZ, Hanelt B, Hertel LA, Marshall JJ, Zhang SM, DeJong RJ, Kim HR, Kudrna D, Wing RA, Soderlund C, Knight M, Lewis FA, Caldeira RL, Jannotti-Passos LK, Carvalho Odos S, Loker ES. A bacterial artificial chromosome library for *Biomphalaria glabrata*, intermediate snail host of *Schistosoma mansoni*. Mem Inst Oswaldo Cruz. 2006; 101:167–177. [PubMed: 17308766]

Adema CM, Hanington PC, Lun CM, Rosenberg GH, Aragon AD, Stout BA, Lennard Richard ML, Gross PS, Loker ES. Differential transcriptomic responses of *Biomphalaria glabrata* (Gastropoda, Mollusca) to bacteria and metazoan parasites, *Schistosoma mansoni* and *echinostoma paraensei* (Digenea, Platyhelminthes). Mol Immunol. 2010; 47:849–860. [PubMed: 19962194]

Adema CM, Bayne CJ, Bridger JM, Knight M, Loker ES, Yoshino TP, Zhang SM. Will all scientists working on snails and the diseases they transmit please stand up? PLoS Negl Trop Dis. 2012; 6:e1835. [PubMed: 23301104]

Adema CM, Loker ES. Digenean-gastropod host associations inform on aspects of specific immunity in snails. Dev Comp Immunol. 2015; 48:275–283. [PubMed: 25034871]

Albertin CB, Simakov O, Mitros T, Wang ZY, Pungor JR, Edsinger-Gonzales E, Brenner S, Ragsdale CW, Rokhsar DS. The octopus genome and the evolution of cephalopod neural and morphological novelties. Nature. 2015; 524:220–224. [PubMed: 26268193]

Alkan C, Sajjadian S, Eichler EE. Limitations of next-generation genome sequence assembly. Nat Methods. 2011; 8:61–65. [PubMed: 21102452]

Allan ER, Tennessen JA, Bollmann SR, Hanington PC, Bayne CJ, Blouin MS. Schistosome infectivity in the snail, *Biomphalaria glabrata*, is partially dependent on the expression of Grctm6, a Guadeloupe Resistance Complex protein. PLoS Negl Trop Dis. 2017; 11:e0005362. [PubMed: 28158185]

Ansorge WJ. Next-generation DNA sequencing techniques. N Biotechnol. 2009; 25:195–203. [PubMed: 19429539]

Baeza Garcia A, Pierce RJ, Gourbal B, Werkmeister E, Colinet D, Reichart JM, Dissous C, Coustau C. Involvement of the cytokine MIF in the snail host immune response to the parasite *Schistosoma mansoni*. PLoS Pathog. 2010; 6:e1001115. [PubMed: 20886098]

Bai Z, Yin Y, Hu S, Wang G, Xhang X, Li J. Identification of genes involved in immune response, microsatellite, and SNP markers from expressed sequence tags generated from hemocytes of freshwater pearl mussel (*Hyriopsis cumingii*). Mar Biotechnol (NY). 2009; 11:520–530. [PubMed: 19039623]

Barghi N, Concepcion GP, Olivera BM, Lluisma AO. Structural features of conopeptide genes inferred from partial sequences of the *Conus tribblei* genome. Mol Genet Genomics. 2016; 291:411–422. [PubMed: 26423067]

Baron OL, van West P, Industri B, Ponchet M, Dubreuil G, Gourbal B, Reichart JM, Coustau C. Parental transfer of the antimicrobial protein LBP/BPI protects *Biomphalaria glabrata* eggs against oomycete infections. PLoS Pathog. 2013; 9:e1003792. [PubMed: 24367257]

Baron OL, Deleury E, Reichart JM, Coustau C. The LBP/BPI multigenic family in invertebrates: Evolutionary history and evidences of specialization in mollusks. Dev Comp Immunol. 2016; 57:20–30. [PubMed: 26608112]

Bayne CJ. Molluscan immunity: induction of elevated immunity in the land snail (*Helix*) by injections of bacteria (*Pseudomonas aeruginosa*). Dev Comp Immunol. 1980; 4:43–54. [PubMed: 6768603]

Beschin A, Bilej M, Brys L, Torreele E, Lucas R, Magez S, De Baetselier P. Convergent evolution of cytokines. Nature. 1999; 400:627–628. [PubMed: 10458158]

Bettencourt R, Pinheiro M, Egas C, Gomes P, Afonso M, Shank T, Santos RS. High-throughput sequencing and analysis of the gill tissue transcriptome from the deep-sea hydrothermal vent mussel *Bathymodiolus azoricus*. BMC Genomics. 2010; 11:559. [PubMed: 20937131]

Blouin MS, Bonner KM, Cooper B, Amarasinghe V, O'Donnell RP, Bayne CJ. Three genes involved in the oxidative burst are closely linked in the genome of the snail, *Biomphalaria glabrata*. Int J Parasitol. 2013; 43:51–55. [PubMed: 23207063]

Boman HG, Hultmark D. Cell-free immunity in insects. Annu Rev Microbiol. 1987; 41:103–126. [PubMed: 3318666]

Borisova EA, Gorbushin AM. Molecular cloning of α-2-macroglobulin from hemocytes of common periwinkle *Littorina littorea*. Fish Shellfish Immunol. 2014; 39:136–137. [PubMed: 24830774]

Bouchut A, Roger E, Coustau C, Gourbal B, Mitta G. Compatibility in the Biomphalaria glabrata/Echinostoma caproni model: potential involvement of adhesion genes. Int J Parasitol. 2006; 36:175–184. [PubMed: 16310790]

Buckley KM, Rast JP. Diversity of animal immune receptors and the origins of recognition complexity in the deuterostomes. Dev Comp Immunol. 2015; 49:179–189. [PubMed: 25450907]

Bulat T, Smidak R, Sialana FJ, Jung G, Rattei T, Bilban M, Sattmann H, Lubec G, Aradska J. Transcriptomic and proteomic analysis of *Arion vulgaris*-Proteins for probably successful survival strategies? PLoS One. 2016; 11:e0150614. [PubMed: 26986963]

Carnegie RB, Arzul I, Bushek D. Managing marine mollusc diseases in the context of regional and international commerce: policy issues and emerging concerns. Philos Trans R Soc Lond B Biol Sci. 2016; 371

Castellanos-Martinez S, Arteta D, Catarino S, Gestal C. De novo transcriptome sequencing of the *Octopus vulgaris* hemocytes using Illumina RNA-Seq technology: response to the infection by the gastrointestinal parasite *Aggregata octopiana*. PLoS One. 2014; 9:e107873. [PubMed: 25329466]

Chen H, Wang L, Zhou Z, Hou Z, Liu Z, Wang W, Gao D, Gao Q, Wang M, Song L. The comprehensive immunomodulation of NeurimmiRs in haemocytes of oyster *Crassostrea gigas* after acetylcholine and norepinephrine stimulation. BMC Genomics. 2015; 16:942. [PubMed: 26576764]

Cheng TC, Marchalonis JJ, Vasta GR. Role of molluscan lectins in recognition processes. Prog Clin Biol Res. 1984; 157:1–15.

Cheng TC, Manzi JJ. Correlation between the presence of lathyrose with the absence of *Haplosporidium nelsoni* in *Crassostrea virginica* from two South Carolina tributaries where *Perkinsus marinus* also inhibits hemocyte agglutination by the *Lathyrus odoratus* lectin. J Shellfish Res. 1996; 15:391–394.

Claes MF. Functional morphology of the white bodies of the cephalopod mollusc *Sepia officinalis*. Acta Zool. 1996; 77:173–190.

Conesa A, Götz S, García-Gómez JM, Terol J, Taló M, Robles M. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics. Bioinformatics. 2005; 21:3674–3676. [PubMed: 16081474]

Connors VA, de Buron I, Granath WO. *Schistosoma mansoni*: interleukin-1 increases and decreases output of cercariae in schistosome-susceptible *Biomphalaria glabrata*. Exp Parasitol. 1995a; 80:139–148. [PubMed: 7821403]

Connors VA, de Buron I, Granath WO. *Schistosoma mansoni* interleukin-1 increases phagocytosis and superoxide production by hemocytes and decrease output of cercariae in schistosome-susceptible *Biomphalaria glabrata*. Ex Parasitol. 1995b; 80:139–148.

Connors VA, de Buron I, Jourdane J, Théron A, Agner A, Granath WO. Recombinant human interleukin-1-mediated killing of *Schistosoma mansoni* primary sporocysts in *Biomphalaria glabrata*. J Parasitol. 1998; 84:920–926. [PubMed: 9794631]

Coustau C, Gourbal B, Duval D, Yoshino TP, Adema CM, Mitta G. Advances in gastropod immunity from the study of the interaction between the snail *Biomphalaria glabrata* and its parasites: A review of research progress over the last decade. Fish Shellfish Immunol. 2015; 46:5–16. [PubMed: 25662712]

Cowden RR. Some cytological and cytochemical observations on the leucopoietic organs, the “white bodies” of *Octupus vulgaris*. J Invertebr Pathol. 1972; 19:113–119.

Cunningham C, Hikima J, Jenny MJ, Chapman RW, Fang GC, Saski C, Lundqvist ML, Wing RA, Cupit PM, Gross PS, Warr GW, Tomkins JP. New resources for marine genomics: bacterial artificial chromosome libraries for the Eastern and Pacific oysters (*Crassostrea virginica* and *C. gigas*). Mar Bitotechnol (NY). 2006; 8:521–533.

Dam TK, Sarkar M, Ghosal J, Choudhury A. A novel galactosyl-binding lectin from the plasma of the blood clam, *Anadara granosa* (L) and a study of its combining site. Mol Cell Biochem. 1992; 117:1–9. [PubMed: 1480160]

Davison A, McDowell GS, Holden JM, Johnson HF, Koutsovoulos GD, Liu MM, Hulpiau P, Van Roy F, Wade CM, Banerjee R, Yang F, Chiba S, Davey JW, Jackson DJ, Levin M, Blaxter ML. Formin is associated with left-right asymmetry in the pond snail and the frog. Curr Biol. 2016; 26:654–660. [PubMed: 26923788]

Deleury E, Dubreuil G, Elangovan N, Wajnberg E, Reichart JM, Gourbal B, Duval D, Baron OL, Gouzy J, Coustau C. Specific versus non-specific immune responses in an invertebrate species evidenced by a comparative de novo sequencing study. PLoS One. 2012; 7:e32512. [PubMed: 22427848]

Detree C, Nuñez-Acuña G, Roberts S, Gallardo-Escárate C. Uncovering the complex transcriptome response of *Mytilus chilensis* against saxitoxin: Implications of harmful algal blooms on mussel populations. PLoS One. 2016; 11:e0165231. [PubMed: 27764234]

De Zoysa M, Nikapitiya C, Oh C, Lee Y, Whang I, Lee JS, Choi CY, Lee J. Microarray analysis of gene expression in disk abalone *Haliotis discus discus* after bacterial challenge. Fish Shellfish Immunol. 2011; 30:661–673. [PubMed: 21215803]

De Zoysa M, Nikapitiya C, Oh C, Whang I, Shin HJ, Lee J. cDNA microarray analysis of disk abalone genes in gills and hemocytes after viral hemorrhagic septicemia virus (VHSV) challenge. Fish Shellfish Immunol. 2012; 32:1205–1215. [PubMed: 22465362]

Dheilly NM, Lelong C, Huvet A, Favrel P. Development of a Pacific oyster (Crassostrea gigas) 31,918-feature microarray: identification of reference genes and tissue-enriched expression patterns. BMC Genomics. 2011; 12:468. [PubMed: 21951653]

Dheilly NM, Adema C, Raftos DA, Goutbal B, Grunau C, Du Pasquier L. No more non-model species: The promise of next generation sequencing for comparative immunology. Dev Comp Immunol. 2014; 45:56–66. [PubMed: 24508980]

Dheilly NM, Duval D, Mouahid G, Emans R, Allienne JF, Galinier R, Genthin C, Dubois E, Du Pasquier L, Adema CM, Grunau C, Mitta G, Gourbal B. A family of variable immunoglobulin and lectin domain containing molecules in the snail *Biomphalaria glabrata*. Dev Comp Immunol. 2015; 48:234–243. [PubMed: 25451302]

Ding J, Li J, Bao Y, Li L, Wu F, Zhang G. Molecular characterization of a mollusk chicken-type lysozyme gene from *Haliotis discus hannai* Ino, and the antimicrobial activity of its recombinant protein. Fish Shellfish Immunol. 2011; 30:163–172. [PubMed: 20951809]

Ding J, Zhao L, Chang Y, Zhao W, Du Z, Hao Z. Transcriptome sequencing and characterization of Japanese scallop *Patinopecten yessoensis* from different shell color lines. PLoS One. 2015; 10:e0116406. [PubMed: 25680107]

Ertl NG, O'Connor WA, Papanicolaou A, Weigand AN, Elizur A. Transcriptome analysis of the Sydney rock oyster, *Saccostrea glomerata*: Insights into molluscan immunity. PLoS One. 2016; 11:e0156649. [PubMed: 27258386]

Erwin DH, Laflamme M, Tweedt SM, Sperling EA, Pisani D, Peterson KJ. The Cambrian conundrum: early divergence and later ecological success in the early history of animals. Science. 2011; 334:1091–1097. [PubMed: 22116879]

Escoubas JM, Briant L, Montagnani C, Hez S, Devaux C, Roch P. Oyster IKK-like protein shares structural and functional properties with its mammalian homologues. FEBS Lett. 1999; 453:293–298. [PubMed: 10405163]

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature. 1998; 391:806–811. [PubMed: 9486653]

Fleury E, Huvet A, Lelong C, de Lorgeril J, Boulo V, Gueguen Y, Bachère E, Tanguy A, Moraga D, Fabioux C, Lindeque P, Shaw J, Reinhardt R, Prunet P, Davey G, Lapègue S, Sauvage C, Corporeau C, Moal J, Gavory F, Wincker P, Moreews F, Klopp C, Mathieu M, Boudry P, Favrel P. Generation and analysis of a 29,745 unique expressed sequence tags from the Pacific oyster (Crassostrea gigas) assembled into a publicly accessible database: the GigasDatabase. BMC Genomics. 2009; 10:341. [PubMed: 19640306]

Fleury E, Huvet A. Microarray analysis highlights immune response of pacific oysters as a determinant of resistance to summer mortality. Mar Biotechnol (NY). 2012; 14:203–217. [PubMed: 21845383]

Fneich S, Dheilly N, Adema C, Rognon A, Reichelt M, Bulla J, Grunau C, Cosseau C. 5-methyl-cytosine and 5-hydroxy-methyl-cytosine in the genome of *Biomphalaria glabrata*, a snail intermediate host of *Schistosoma mansoni*. Parasit Vectors. 2013; 6:167. [PubMed: 23742053]

Gao D, Qiu L, Gao Q, Hou Z, Wang L, Song L. Repertoire and evolution of TNF superfamily in *Crassostrea gigas*: implications for expansion and diversification of this superfamily in Mollusca. Dev Comp Immunol. 2015; 51:251–260. [PubMed: 25910814]

Gavery MR, Roberts SB. DNA methylation patterns provide insight into epigenetic regulation in the Pacific oyster (*Crassostrea gigas*). BMC Genomics. 2010; 11:483. [PubMed: 20799955]

Gerdol M, De Moro G, Manfrin C, Milandri A, Riccardi E, Beran A, Venier P, Pallavicini A. RNA sequencing and de novo assembly of the digestive gland transcriptome in *Mytilus galloprovincialis* fed with toxinogenic and non-toxic strains of *Alexandrium minutum*. BMC Res Notes. 2014; 7:722. [PubMed: 25314922]

Geyer KK, Rodríguez López CM, Chalmers IW, Munshi SE, Truscott M, Heald J, Wilkinson MJ, Hoffmann KF. Cytosine methylation regulates oviposition in the pathogenic blood fluke *Schistosoma mansoni*. Nat Commun. 2011; 2:424. [PubMed: 21829186]

GIGA Community of Scientists. Bracken-Grissom H, Collins AG, Collins T, Crandall K, Distel D, Dunn C, Giribet G, Haddock S, Knowlton N, Martindale M, Medina M, Messing C, O'Brien SJ, Paulay G, Putnam N, Ravasi T, Rouse GW, Ryan JF, Schulze A, Wörheide G, Adamska M, Bailly X, Breinholt J, Browne WE, Diaz MC, Evans N, Flot JF, Fogarty N, Johnston M, Kamel B, Kawahara AY, Laberge T, Lavrov D, Michonneau F, Moroz LL, Oakley T, Osborne K, Pomponi SA, Rhodes A, Santos SR, Satch N, Thacker RW, Van de Peer Y, Voolstra CR, Welch DM, Winston J, Zhou X. The global invertebrate genomics alliance (GIGA): developing community resources to study diverse invertebrate genomes. J Hered. 2014; 105:1–18. [PubMed: 24336862]

Goodall CP, Bender RC, Brooks JK, Bayne CJ. *Biomphalaria glabrata* cytosolic copper/zinc superoxide dismutase (SOD1) gene: association of SOD1 alleles with resistance/susceptible to *Schistosoma mansoni*. Mol Biochem Parasitol. 2006; 147:207–210. [PubMed: 16564582]

Goodson MS, Kojadinovic M, Troll JV, Scheetz TE, Casavant TL, Soares MB, McFall-Ngai MJ. Identifying components of the NF-kappaB pathway in the beneficial *Euprymna scolopes*-*Vibrio fischeri* light organ symbiosis. Appl Environ Microbiol. 2005; 71:6934–6946. [PubMed: 16269728]

Gorbushin AM, Panchin YV, Iakovleva NV. In search of the origin of FREPs: characterization of *Aplysia californica* fibrinogen-related proteins. Dev Comp Immunol. 2010; 34:465–473. [PubMed: 20026348]

Gorbushin AM, Iakovleva NV. A new family of single fibrinogen domain lectins in *Mytilus*. Fish Shellfish Immunol. 2011; 30:434–438. [PubMed: 20951811]

Gorbushin AM, Borisova EA. Lectin-like molecules in transcriptome of *Littorina littorea* hemocytes. Dev Comp Immunol. 2015; 48:210–220. [PubMed: 25451301]

Götz S, Arnold R, Sebastián-León P, Martín-Rodríguez S, Tischler P, Jehl MA, Dopazo J, Rattei T, Conesa A. B2G-FAR, a species-centered GO annotation repository. Bioinformatics. 2011; 27:919–924. [PubMed: 21335611]

Granath WO, Yoshino TP. Lysosomal enzyme activities in susceptible and refractory strains of *Biomphalaria glabrata* during the course of infection with *Schistosoma mansoni*. J Parasitol. 1983; 69:1018–1026. [PubMed: 6674450]

Granath WO, Connors VA, Tarleton RL. Interleukin 1 activity in haemolymph from strains of the snail *Biomphalaria glabrata* varying in susceptibility to the human blood fluke, *Schistosoma mansoni*: presence, differential expression, and biological function. Cytokine. 1994; 6:21–27. [PubMed: 8003629]

Gregory, TR. Animal Genome Size Database. 2017. http://www.genomesize.com

Gueguen Y, Cadoret JP, Flament D, Barreau-Roumiguière C, Girardot AL, Garnier J, Hoareau A, Bachère E, Escoubas JM. Immune gene discovery by expressed sequence tags generated from hemocytes of the bacteria-challenged oyster, *Crassostrea gigas*. Gene. 2003; 303:139–145. [PubMed: 12559575]

Guo X, He Y, Zhang L, Lelong C, Jouaux A. Immune and stress responses in oysters with insights on adaptation. Fish Shellfish Immunol. 2015; 46:107–119. [PubMed: 25989624]

Hahn UK, Fryer SE, Bayne CJ. An invertebrate (Molluscan) plasma protein that binds to vertebrate immunoglobulins and its potential for yielding false-positives in antibody-based detection systems. Dev Comp Immunol. 1996; 20:39–50. [PubMed: 8738935]

Hanelt B, Lun CM, Adema CM. Comparative ORESTES-sampling of transcriptomes of immune-challenged *Biomphalaria glabrata* snails. J Invertebr Pathol. 2008; 99:192–203. [PubMed: 18590737]

Hanington PC, Forys MA, Dragoo JW, Zhang SM, Adema CM, Loker ES. Role for a somatically diversified lectin in resistance of an invertebrate to parasite infection. Proc Natl Acad Sci U S A. 2010; 107:21087–21092. [PubMed: 21084634]

Hanington PC, Forys MA, Loker ES. A somatically diversified defense factor, FREP3, is a determinant of snail resistance to schistosome infection. PLoS Negl Trop Dis. 2012; 6:e1591. [PubMed: 22479663]

Hauton C, Smith V. Adaptive immunity in invertebrates: a straw house without mechanistic foundation. Bioessays. 2007; 29:1138–1146. [PubMed: 17935208]

He Y, Jouaux A, Ford SE, Lelong C, Sourdaine P, Mathieu M, Guo X. Transcriptome analysis reveals strong and complex antiviral response in a mollusc. Fish Shellfish Immunol. 2015; 46:131–144. [PubMed: 26004318]

Hoek RM, Smit AB, Frings H, Vink JM, de Jong-Brink M, Geraerts WP. A new Ig-superfamily member, molluscan defence molecule (MDM) from *Lymnaea stagnalis*, is down-regulated during parasitosis. Eur J Immunol. 1996; 26:939–944. [PubMed: 8625992]

Hou R, Bao Z, Wang S, Su H, Li Y, Du H, Hu J, Wang S, Hu X. Transcriptome sequencing and de novo analysis for Yesso scallop (*Patinopecten yessoensis*) using 454 GS FLX. PLoS One. 2011; 6:e21560. [PubMed: 21720557]

Hu F, Pan L, Cai Y, Liu T, Jin Q. Deep sequencing of the scallop *Chlamys farreri* transcriptome response to tetrabromobisphenol A (TBBPA) stress. Mar Genomics. 2015:31–38.

Huang C, Wu W. Genomic resources of two landsnail, *Aegista diversifamilia* and *Dolicheulota formoensis*, generated by Illumina paired-end sequencing [version 1; referees: 1 approved]. F1000Research. 2015; 4:106.

Hughes TK, Smith EM, Chin R, Cadet P, Sinisterra J, Leung MK, Shipp MA, Scharrer B, Stefano GB. Interaction of immunoreactive monokines (interleukin 1 and tumor necrosis factor) in the bivalve mollusc *Mytilus edulis*. Proc Natl Acad Sci U S A. 1990; 87:4426–4429. [PubMed: 2352927]

Hughes TK, Smith EM, Barnett JA, Charles R, Stefano GB. LPS stimulated invertebrate hemocytes: a role for immunoreactive TNF and IL-1. Dev Comp Immunol. 1991; 15:117–122. [PubMed: 2060681]

Hughes TK, Smith EM, Leung MK, Stefano GB. Evidence for the conservation of an immunoreactive monokine network in invertebrates. Ann N Y Acad Sci. 1992; 650:74–80. [PubMed: 1605500]

Humphries JE, Elizondo L, Yoshino TP. Protein kinase C regulation of cell spreading in the molluscan *Biomphalaria glabrata* embryonic (Bge) cell line. Biochim Biophys Acta. 2001; 1540:243–252. [PubMed: 11583819]

Humphries J, Harter B. Identification of nuclear factor kappaB (NF-KB) binding motifs in *Biomphalaria glabrata*. Dev Comp Immunol. 2015; 53:366–370. [PubMed: 26277107]

Itoh N, Takahashi KG. Distribution of multiple peptidoglycan recognition proteins in the tissues of Pacific oyster, *Crassostrea gigas*. Comp Biochem Physiol B Miochem Mol Biol. 2008; 150:409–417.

Ittiprasert W, Miller A, Su XZ, Mu J, Bhusudsawang G, Ukoskit K, Knight M. Identification and characterisation of functional expressed sequence tags-derived simple sequence repeat (eSSR) markers for genetic linkage mapping of *Schistosoma mansoni* juvenile resistance and susceptibility loci in *Biomphalaria glabrata*. Int J Parasitol. 2013; 43:669–677. [PubMed: 23643514]

Jenny MJ, Chapman RW, Mancia A, Chen YA, McKillen DJ, Trent H, Lang P, Escoubas JM, Bachere E, Boulo V, Liu ZJ, Gross PS, Cunningham C, Cupit PM, Tanguy A, Guo X, Moraga D, Boutet I, Huvet A, De Guise S, Almeida JS, Warr GW. A cDNA microarray for *Crassostrea virginica* and *C. gigas*. Mar Biotechnol. 2007; 9:577–591. [PubMed: 17668266]

Jeong KH, Lie JK, Heyneman D. The ultrastructure of the amebocyte-producing organ in *Biomphalaria glabrata*. Dev Comp Immunol. 1983; 7:217–228. [PubMed: 6683673]

Jia X, Zou Z, Wang G, Wang S, Wang Y, Zhang Z. Gene expression profiling in respond to TBT exposure in small abalone *Haliotis diversicolor*. Fish Shellfish Immunol. 2011; 31:557–563. [PubMed: 21767652]

Jiang Y, Loker ES, Zhang SM. *In vivo* and *in vitro* knockdown of FREP2 gene expression in the snail *Biomphalaria glabrata* using RNA interference. Dev Comp Immunol. 2006; 30:855–866. [PubMed: 16442620]

Kang YS, Kim YM, Park KI, Kim Cho S, Choi KS, Cho M. Analysis of EST and lectin expressions in hemocytes of Manila clams (*Ruditapes philippinarum*) (Bivalvia: Mollusca) infected with *Perkinsus olseni*. Dev Comp Immunol. 2006; 30:1119–1131. [PubMed: 16690126]

Kasthuri SR, Premachandra HK, Umasuthan N, Whang I, Lee J. Structural characterization and expression analysis of a beta-thymosin homologue (Tβ) in disk abalone, *Haliotis, discus discus*. Gene. 2013; 527:376–383. [PubMed: 23680646]

Kaur S, Jobling S, Jones CS, NOble LR, Routledge EJ, Lockyer AE. The nuclear receptors of *Biomphalaria glabrata* and *Lottia gigantea*: implications for developing new model organisms. PLoS One. 2015; 10:e0121259. [PubMed: 25849443]

Kenny NJ, Truchado-García M, Grande C. Deep, multi-stage transcriptome of the schistosomiasis vector *Biomphalaria glabrata* provides platform for understanding molluscan disease-related pathways. BMC Infect Dis. 2016; 16:618. [PubMed: 27793108]

Klein J. Are invertebrate capable of anticipatory immune responses? Scand J Immunol. 1989; 29:499–505. [PubMed: 2658012]

Knight M, Miller AN, Patterson CN, Rowe CG, Michaels G, Carr D, Richards CS, Lewis FA. The identification of markers segregating with resistance to Schistosoma mansoni infection in the snail Biomphalaria glabrata. Proc Natl Acad Sci USA. 1999; 96:1510–1515. [PubMed: 9990054]

Knight M, Ittiprasert W, Odoemelam EC, Adema CM, Miller A, Raghavan N, Bridger JM. Non-random organization of the *Biomphalaria glabrata* genome in interphase Bge cells and the spatial repositioning of activated genes in cells co-cultured with *Schistosoma mansoni*. Int J Parasitol. 2011; 41:61–70. [PubMed: 20849859]

Kocot KM, Cannon JT, Todt C, Citarella MR, Kohn AB, Meyer A, Santos SR, Schandler C, Moroz LL, Lieb B, Halanych KM. Phylogenomics reveals deep molluscan relationships. Nature. 2011; 477:452–456. [PubMed: 21892190]

Kocot KM, Jeffrey NW, Mulligan K, Halanych KM, Gregory TR. Genome size estimates for Aplacophora, Polyplacophora, and Scaphopoda: small solengasters and sizeable scaphopods. J Mollus Stud. 2015; 82:216–219.

Korneev SA, Kemenes I, Straub V, Staras K, Korneeva EI, Kemenes G, Benjamin PR, O'Shea M. Suppression of nitric oxide (NO)-dependent behavior by double-stranded RNA-mediated silencing of a neuronal NO synthase gene. J Neurosci. 2002; 22:RC227. [PubMed: 12040086]

Kurachi S, Song Z, Takagaki M, Yang Q, Winter HC, Kurachi K, Goldstein IJ. Sialic-acid-binding lectin from the slug *Limax flavus*–cloning, expression of the polypeptide, and tissue localization. Eur J Biochem. 1998; 254:217–22. [PubMed: 9660173]

La Peyre JF, Chu FL, Vogelbein WK. *In vitro* interaction of *Perkinsus marinus* merozoites with eastern and Pacific oyster hemocytes. Dev Comp Immunol. 1995; 19:291–304. [PubMed: 8617400]

Leippe M, Renwrantz L. Release of cytotoxic and agglutinating molecules by *Mytilus* hemocytes. Dev Comp Immunol. 1988; 12:297–308. [PubMed: 3384159]

Leite RB, Milan M, Coppe A, Bortoluzzi S, dos Anjos A, Reinhart R, Saavedra C, Patarnello T, Cancela ML, Bargelloni L. mRNA-Seq and microarray development for the Grooved Carpet shell clam, *Ruditapes decussatus*: a functional approach to unravel host-parasite interaction. BMC Genomics. 2013; 14:741. [PubMed: 24168212]

Lemaitre B, Nicholas E, Michaut L, Reichart JM, Hoffmann JA. The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults. Cell. 1996; 86:973–983. [PubMed: 8808632]

Léonard PM, Adema CM, Zhang SM, Loker ES. Structure of two FREP genes that combine IgSF and fibronogen domains, with comments on diversity of the FREP gene family in the snail *Biomphalaria glabrata*. Gene. 2001; 269:155–165. [PubMed: 11376947]

Liao Z, Wang XC, Liu HH, Fan MH, Sun JJ, Shen W. Molecular characterization of a novel antimicrobial peptide from *Mytilus coruscus*. Fish Shellfish Immunol. 2013; 34:610–616. [PubMed: 23247103]

Lie KJ, Heyneman D. Acquired resistance to echinostomes in four *Biomphalaria glabrata* strains. Int J Parasitol. 1979; 9:533–537. [PubMed: 541167]

Little TJ, Colegrave N, Sadd BM, Schmid-Hempel P. Studying immunity at the whole organism level. Bioessays. 2008; 30:404–405. [PubMed: 18348252]

Liu L, Yang J, Qiu L, Wang L, Zhang H, Wang M, Vinu SS, Song L. A novel scavenger receptor-cysteine-rich (SRCR) domain containing scavenger receptor identified from mollusk mediated PAMP recognition and binding. Dev Comp Immunol. 2011; 35:227–239. [PubMed: 20888856]

Lockyer AE, Spinks J, Noble LR, Rollinson D, Jones CS. Identification of genes involved in interactions between Biomphalaria glabrata and Schistosoma mansoni by suppression subtractive hybridization. Mol Biochem Parasitol. 2007a; 151:18–27. [PubMed: 17081633]

Lockyer AE, Spinks JN, Walker AJ, Kane RA, Noble LR, Rollinson D, Dias-Neto E, Jones CS. Biomphalaria glabrata transcriptome: identification of cell-signalling, transcriptional control and immune-related genes from open reading frame expressed sequence tags (ORESTES). Dev Comp Immunol. 2007b; 31:763–782. [PubMed: 17208299]

Lockyer AE, Spinks J, Kane RA, Hoffmann KF, Fitzpatrick JM, Rollinson D, Noble LR, Jones CS. *Biomphalaria glabrata* transcriptome: cDNA microarray profiling identifies resistant- and susceptible-specific gene expression in haemocytes from snail strains exposed to *Schistosoma mansoni*. BMC Genomics. 2008; 9:634. [PubMed: 19114004]

Lockyer AE, Emery AM, Kane RA, Walker AJ, Mayer CD, Mitta G, Coustau C, Adema CM, Hanelt B, Rollinson D, Noble LR, Jones CS. Early differential gene expression in haemocytes from resistant and susceptible *Biomphalaria glabrata* strains in response to *Schistosoma mansoni*. PLoS One. 2012; 7:e51102. [PubMed: 23300533]

Mansour MH. Evidence for a family of schistosome glycan-binding lectins in *Biomphalaria alexandrina*. Dev Comp Immunol. 1995; 19:365–376. [PubMed: 8654664]

Marchalonis JJ, Schluter SF. Origins of immunoglobulins and immune recognition molecules. Bioscience. 1990; 40:758–768.

McDowell IC, Nikapitiya C, Aguiar D, Lane CE, Istrail S, Gomez-Chiarri M. Transcriptome of american oysters, *Crassostrea virginica*, in response to bacterial challenge: Insights into potential mechanisms of disease resistance. PLoS One. 2014; 9:e105097. [PubMed: 25122115]

McDowell IC, Modak TH, Lane CE, Gomez-Chiarri M. Multi-species protein similarity clustering reveals novel expanded immune gene families in the eastern oyster *Crassostrea virginica*. Fish Shellfish Immunol. 2016; 53:13–23. [PubMed: 27033806]

McKerrow JH, Jeong KH, Beckstead JH. Enzyme histochemical comparison of *Biomphalaria glabrata* amebocytes with human granuloma macrophages. J Leukoc Biol. 1985; 37:341–347. [PubMed: 2981947]

Medzhitov R, Preston-Hurlburt P, Janeway CA. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. Nature. 1997; 388:394–397. [PubMed: 9237759]

Metchnikoff, E. Immunity in infective disease. Cambridge University Press; Cambridge: 1905.

Michelson EH, Dubois L. Agglutinins and lysins in the molluscan family PLanorbidae: a survey of hemolymph, egg-masses, and albumen-gland extracts. Biol Bull. 1977; 153:219–227. [PubMed: 560878]

Miller AN, Raghavan N, FitzGerald PC, Lewis FA, Knight M. Differential gene expression in haemocytes of the snail Biomphalaria glabrata: effects of Schistosoma mansoni infection. Int J Parasitol. 2001; 31:687–696. [PubMed: 11336750]

Mitta G, Galinier R, Tisseyre P, Allienne JF, Girerd-Chambaz Y, Guillou F, Bouchut A, Coustau C. Gene discovery and expression analysis of immune-relevant genes from Biomphalaria glabrata hemocytes. Dev Comp Immunol. 2005; 29:393–407. [PubMed: 15707661]

Mohandas A, Cheng TC, Cheng JB. Mechanism of lysosomal enzyme release from *Mercenaria mercenaria* granulocytes: a scanning electron microscope study. J Invertebr Pathol. 1985; 46:189–197. [PubMed: 4045216]

Monroy F, Hertel LA, Loker ES. Carbohydrate-binding plasma proteins from the gastropod *Biomphalaria glabrata*: strain specificity and the effects of trematode infection. Dev Comp Immunol. 1992; 16:355–366. [PubMed: 1330758]

Mukherjee S, Stamatis D, Bertsch J, Ovchinnikova G, Verezemska O, Isbandi M, Thomas AD, Ali R, Sharma K, Kyrpides NC, Reddy TB. Genomes OnLine database (GOLD) v.6: data updates and feature enhancements. Nucleic Acids Res. 2017; 45:D446–D456. [PubMed: 27794040]

Mullainadhan P, Renwrantz L. Lectin-dependent recognition of foreign cells by hemocytes of the mussel, *Mytilus edulis*. Immunobiology. 1986; 171:263–273. [PubMed: 3754847]

Mundry M, Bornberg-Bauer E, Sammeth M, Feulner PG. Evaluating characteristics of de novo assembly software on 454 transcriptome data: a simulation approach. PLoS One. 2012; 7:e31410. [PubMed: 22384018]

Nam BH, Jung M, Subramaniyam S, Yoo SI, Markkandan K, Moon JY, Kim YO, Kim DG, An CM, Shin Y, Jung HJ, Park JH. Transcriptome analysis revealed changes of multiple genes involved in *Haliotis discus hannai* innate immunity during *Vibrio parahemolyticus* infection. PLoS One. 2016; 11:e0153474. [PubMed: 27088873]

Noël D, Bachère E, Mialhe E. Phagocytosis associated chemiluminescence of hemocytes in *Mytilus edulis* (Bivalvia). Dev Comp Immunol. 1993; 17:483–493. [PubMed: 8299847]

Nowak TS, Woodards AC, Jung Y, Adema CM, Loker ES. Identification of transcripts generated during the response of resistant Biomphalaria glabrata to Schistosoma mansoni infection using suppression subtractive hybridization. J Parasitol. 2004; 90:1034–1040. [PubMed: 15562603]

Nuñez-Acuña G, Gallardo-Ascárate C. Identification of immune-related SNPs in the transcriptome of *Mytilus chilensis* through high-throughput sequencing. Fish Shellfish Immunol. 2013; 35:1899–1905. [PubMed: 24080470]

Ottaviani E, Franchini A, Franceschi C. Presence of several cytokine-like molecules in molluscan hemocytes. Biochem Biophys Res Commun. 1993; 195:984–988. [PubMed: 8373430]

Ottaviani E, Caselgrandi E, Franceschi C. Cytokines and evolution: *In vitro* effects of IL-1, IL-1B, TNF-a and TNF-B on an ancestral type of stress response. Biochem Biophys Res Commun. 1995a; 207:288–292. [PubMed: 7857278]

Ottaviani E, Franchini A, Cassanelli S, Genedani S. Cytokines and molluscan immune responses. Biol Cell. 1995b; 85:87–91. [PubMed: 8882522]

Ottaviani E, Sassi D, Kletsas D. PDGF- and TGF-beta-induced changes in cell shape of invertebrate immunocytes: effect of calcium entry blockers. Eur J Cell Biol. 1997; 74:336–341. [PubMed: 9438129]

Ottaviani E, Franchini A, Malagoli D, Genedani S. Immunomodulation by recombinant human interleukin-8 and its signal transduction pathways in invertebrate hemocytes. Cell Mol Life Sci. 2000; 57:506–513. [PubMed: 10823250]

Ouwe-Missi-Oukem-Boyer O, Porchet E, Capron A, Dissous C. Characterization of immunoreactive TNF alpha molecules in the gastropod *Biomphalaria glabrata*. Dev Comp Immunol. 1994; 18:211–218. [PubMed: 8001700]

Owen, J., Punt, J., Stranford, S. Kuby Immunology. seventh. W.H. Freeman and Company; New York: 2013.

Owens L, Malham S. Review of the RNA interference pathway in molluscs including some possibilities for use in bivalves in aquaculture. J Mar Sci Eng. 2015; 3:87–99.

Patnaik BB, Wang TH, Kang SW, Hwang HJ, Park SY, Park EB, Chung JM, Song DK, Kim C, Kim S, Lee JS, Han YS, Park HS, Lee YS. Sequencing, de novo assembly, and annotation of the transcriptome of the endangered freshwater pearl bivalve, *Cristaria plicata*, provides novel insights into functional genes and marker discovery. PLoS One. 2016; 11:e0148622. [PubMed: 26872384]

Pauletto M, Milan M, Moreira R, Novoa B, Figueras A, Babbucci M, Patarnello T, Bargelloni L. Deep transcriptome sequencing of *Pecten maximus* hemocytes: a genomic resource for bivalve immunology. Fish Shellfish Immunol. 2014; 31:154–165.

Peñarrubia L, Sanz N, Pla C, Vidal O, Viñas J. Using massive parallel sequencing for the development, validation, and application of population genetics markers in the invasive bivalve zebra mussel (*Dreissena polymorpha*). PLoS One. 2015; 10:e0120732. [PubMed: 25780924]

Pettersson E, Lundeberg J, Ahmadian A. Generations of sequencing technologies. Genomics. 2009; 93:105–111. [PubMed: 18992322]

Philipp EE, Kraemer L, Melzner F, Poustka AJ, Thieme S, Findeisen U, Schreiber S, Rosenstiel P. Massively parallel RNA sequencing identifies a complex immune gene repertoire in the lophotrochozoan *Mytilus edulis*. PLoS One. 2012; 7:e33091. [PubMed: 22448234]

Pinaud S, Portela J, Duval D, Nowacki FC, Olive MA, Allienne JF, Galinier R, Dheilly NM, Kieffer-Jaquinod S, Mitta G, Théron A, Gourbal B. A shift from cellular to humoral responses contributes to innate immune memory in the vector snail *Biomphalaria glabrata*. PLoS Pathog. 2016; 12:e1005361. [PubMed: 26735307]

Premachandra HK, Wan Q, Elvitigala DA, De Zoysa M, Choi CY, Whang I, Lee J. Genomic characterization and expression profiles upon bacterial infection of a novel cystatin B homologue from disk abalone (*Haliotis discus discus*). Dev Comp Immunol. 2012; 38:495–504. [PubMed: 22878425]

Prokop O, Uhlenbruck G, Köhler W. A new source of antibody-like substances having anti-blood group specificity. A discussion on the specificity of *Helix* agglutinins. Vox Sang. 1968; 14:321–333. [PubMed: 5660844]

Raghavan N, Miller AN, Gardner M, FitzGerald PC, Kerlavage AR, Johnston DA, Lewis FA, Knight M. Comparative gene analysis of Biomphalaria glabrata hemocytes pre- and post-exposure to miracidia of Schistosoma mansoni. Mol Biochem Parasitol. 2003; 126:181–191. [PubMed: 12615317]

Raghavan N, Tettelin H, Miller A, Hostetler J, Tallon L, Knight M. Nimbus (BgI): an active non-LTR retrotransposon of the *Schistosoma mansoni* snail host *Biomphalaria glabrata*. Int J Parasitol. 2007; 37:1307–1318. [PubMed: 17521654]

Renwrantz LR, Cheng TC. Agglutinin-mediated attachment erythrocytes to hemocytes of *Helix pomatia*. J Invertebr Pathol. 1977; 29:97–100. [PubMed: 833437]

Renwrantz LR, Richards EH. Recognition of beta-glucuronidase by the calcium-independent phosphomannosyl surface receptor of haemocytes from the gastropod mollusc, *Helix pomatia*. Dev Comp Immunol. 1992; 16:251–256. [PubMed: 1323490]

Rhee JS, Kim BM, Jeong CB, Horiguchi T, Lee YM, Kim IC, Lee JS. Immune gene mining by pyrosequencing in the rockshell, *Thais clavigera*. Fish Shellfish Immunol. 2012; 32:700–710. [PubMed: 22289713]

Rhoads A, Au KF. PacBio sequencing and its applications. Genomics Proteomics Bioinformatics. 2015; 13:278–289. [PubMed: 26542840]

Richards EH, Renwrantz LR. Two lectins on the surface of *Helix pomatia* haemocytes: a Ca^{2+}-dependent, GalNac-specific lectin and a Ca^{2+}-independent, mannose 6-phosphate-specific lectin which recognises activated homologous opsonins. J Comp Physiol B. 1991; 161:43–54.

Richards CS, Knight M, Lewis FA. Genetics of *Biomphalaria glabrata* and its effect on the outcome of *Schistosoma mansoni* infection. Parasitol Today. 1992; 8:171–174. [PubMed: 15463608]

Roberts SB, Goetz FW. Expressed sequence tag analysis of genes expressed in the bay scallop, *Argopecten irradians*. Biol Bull. 2003; 205:227–228. [PubMed: 14583543]

Rögener W, Renwrantz L, Uhlenbruck G. Isolation and characterization of a lectin from the hemolymph of the cephalopod *Octopus vulgaris* (Lam) inhibited by alpha-D-lactose and N-acetyl-lactosamine. Dev Comp Immunol. 1985; 9:605–616. [PubMed: 4085673]

Romero A, Dios S, Poisa-Beiro L, Costa MM, Posada D, Figueras A, Novoa B. Individual sequence variability and functional activities of fibrinogen-related proteins (FREPs) in the Mediterranean mussel (*Mytilus galloprovincialis*) suggest ancient and complex immune recognition models in invertebrates. Dev comp Immunol. 2011; 35:334–344. [PubMed: 21034769]

Romero A, Forn-Cuní G, Moreira R, Milan M, Bargelloni L, Figueras A, Novoa B. An immune-enriched oligo-microarray analysis of gene expression in Manila clam (Venerupis philippinarum) haemocytes after a Perkinsus olseni challenge. 2015; 43:275–286.

Romestand B, Corbier F, Roch P. Protease inhibitors and haemagglutinins associated with resistance to the protozoan parasite, *Perkinsus marinus*, in the Pacific oyster, *Crassostrea gigas*. Parasitology. 2002; 125:323–329. [PubMed: 12403320]

Rosani U, Varotto L, Gerdol M, Pallavicini A, Venier P. IL-17 signaling components in bivalves: Comparative sequence analysis and involvement in the immune responses. Dev Comp Immunol. 2015a; 52:255–268. [PubMed: 26026244]

Rosani U, Varotto L, Domeneghetti S, Arcangeli G, Pallavicini A, Venier P. Dual analysis of host and pathogen transcriptomes in ostreid herpesvirus 1-positive *Crassostrea gigas*. Environ Microbiol. 2015b; 17:4200–4212. [PubMed: 25384719]

Safavi-Hemami H, Li Q, Jackson RL, Song AS, Boomsma W, Bandyopadhyay PK, Gruber CW, Purcell AW, Yandell M, Olivera BM, Ellgaard L. Rapid expansion of the protein disulfide isomerase gene family facilitates the folding of venom peptides. Proc Natl Acad Sci U S A. 2016; 113:3227–3232. [PubMed: 26957604]

Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evo. 1987; 4:406–425.

Salazar KA, Joffe NR, Dinguirard N, Houde P, Castillo MG. Transcriptome analysis of the white body of the squid *Euprymna tasmanica*. PLoS One. 2015; 10:e0119949. [PubMed: 25775132]

Schadt EE, Turner Kasarskis A. A window into third-generation sequencing. Hum Mol Genet. 2010; 19:R227–R240. [PubMed: 20858600]

Schneider O, Zelck UE. Differential display analysis of hemocytes from schistosome-resistant and schistosome-susceptible intermediate hosts. Parasitol Res. 2001; 87:489–491. [PubMed: 11411951]

Seo JK, Go HJ, Kim CH, Nam BH, Park NG. Antimicrobial peptide, hd Molluscidin, purified from the gill of the abalone, *Haliotis discus*. Fish Shellfish Immunol. 2016; 52:289–297. [PubMed: 27033467]

Simakov O, Marletaz F, Cho SJ, Edsinger-Gonzales E, Havlak P, Hellsten U, Kuo DH, Larsson T, Lv J, Arendt D, Savage R, Osoegawa K, de Jong P, Grimwood J, Chapman JA, Shapiro H, Aerts A, Otillar RP, Terry AY, Boore JL, Grigoriev IV, Lindberg DR, Seaver EC, Weisblat DA, Putnam NH, Rokhsar DS. Insights into bilaterian evolution from three spiralian genomes. Nature. 2013; 493:526–531. [PubMed: 23254933]

Singh V, Braddick D, Dhar PK. Exploring the potential of genome editing CRISPR-Cas9 technology. Gene. 2017; 599:1–18. [PubMed: 27836667]

Smith SA, Wilson NG, Goetz FE, Feehery C, Andrade SCS, Rouse GW, Giribet G, Dunn CW. Resolving the evolutionary relationships of molluscs with phylogenomic tools. Nature. 2011; 480:364–367. [PubMed: 22031330]

Smith DR. Goodbye genome paper, hello genome report: the increasing popularity of ‘genome announcements’ and their impact on science. Brief Funct Genomics. 2016

Sohn JI, Nam JW. The present and future of *de novo* whole-genome assembly. Brief Bioinform. 2016

Song L, Xu W, Li C, Li H, Wu L, Xiang J, Guo X. Development of expressed sequence tags from the bay scallop, *Argopecten irradians irradians*. Mar Biotechnol (NY). 2006; 8:161–169. [PubMed: 16625419]

Steelman BN, Connors VA. Chemokinetic effect of interleukin-1 beta on cultured *Biomphalaria glabrata* embryonic cells. J Parasitol. 2009; 95:772–774. [PubMed: 19090650]

Stefano GB, Smith EM, Hughes TK. Opioid induction of immunoreactive interleukin-1 in *Mytilus edulis* and human immunocytes: an interleukin-1-like substance in invertebrate neural tissue. J Neuroimmunol. 1991; 32:29–34. [PubMed: 2002089]

Tamura K, Stecher G, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evo. 2013; 30:2725–2729.

Tebeje BM, Harvie M, You H, Loukas A, McManus DP. Schistosomiasis vaccines: where do we stand? Parasit Vectors. 2016; 9:528. [PubMed: 27716365]

Tennessen JA, Théron A, Marine M, Yeh JY, Rognon A, Blouin MS. Hyperdiverse gene cluster in snail host conveys resistance to human schistosome parasites. PLoS Genet. 2015a; 11:e1005067. [PubMed: 25775214]

Tennessen JA, Bonner KM, Bollmann SR, Johnstun JA, Yeh JY, Marine M, Tavalire HF, Bayne CJ, Blouin MS. Genome-wide scan and test of candidate genes in the snail *Biomphalaria glabrata* reveal new locus influencing resistance to *Schistosoma mansoni*. PLoS Negl Trop Dis. 2015b; 9:e0004077. [PubMed: 26372103]

Tripp MR, Kent VE. Studies on oyster cellular immunity. In Vitro. 1967; 3:129–135.

van der Knaap WP, Sminia T, Kroese FG, Dikkeboom R. Elimination of bacteria from the circulation of the pond snail *Lymnaea stagnalis*. Dev Comp Immunol. 1981; 5:21–32. [PubMed: 7009245]

van der Knaap WP, Boots AM, van Asselt LA, Sminia T. Specificity and memory in increased defence reactions against bacteria in the pond snail *Lymnaea stagnalis*. Dev Comp Immunol. 1983a; 7:435–443. [PubMed: 6357878]

van der Knaap WPW, Sminia T, Schutte R, Boerrigter-Barendsen LH. Cytophilic receptors for foreignness and some factors which influence phagocytosis by invertebrate leucocytes: *in vitro* phagocytosis by amoebocytes of the snail *Lymnaea stagnalis*. Immunology. 1983b; 48:377–383. [PubMed: 6822406]

van der Knaap WP, Loker ES. Immune mechanisms in trematode-snail interactions. Parasitol Today. 1990; 6:175–182. [PubMed: 15463334]

Vasta, GR., Ahmed, H. Introduction to animal lectins. In: Vasta, GR., Ahmed, H., editors. Animal Lectins: a functional view. CRC Press; Boca Raton: 2009. p. 3-9.

Venier P, Varotto L, Rosani U, Millino C, Celegato B, Bernante F, Lanfranchi G, Novoa B, Roch P, Figueras A, Pallavicini A. Insights into the innate immunity of the Mediterranean mussel *Mytilus galloprovincialis*. BMC Genomics. 2011; 12:69. [PubMed: 21269501]

Walker L, Levine H, Jucker M. Koch's postulates and infectious proteins. Acta Neuropathol. 2006; 112:1–4. [PubMed: 16703338]

Walker AJ, Lacchini AH, Sealey KL, Mackintosh D, Davies AJ. Spreading by snail (*Lymnaea stagnalis*) defence cells is regulated through integrated PKC, FAK, and Src signaling. Cell Tissue Res. 2010; 34:131–145.

Wang H, Song L, Li C, Zhao J, Zhang H, Ni D, Xu W. Cloning and characterization of a novel C-type lectin from Zhikong scallop *Chlamys farreri*. Mol Immunol. 2007; 44:722–731. [PubMed: 16777225]

Wang L, Song L, Zhao J, Qiu L, Zhang H, Xu W, Li H, Li C, Wu L, Guo X. Expressed sequence tags from the zhikong scallop (*Chlamys farreri*): discovery and annotation of host-defense genes. Fish Shellfish Immunol. 2009; 26:744–750. [PubMed: 19328855]

Wang S, Peatman E, Liu H, Bushek D, Ford SE, Kucuktas H, Quilang J, Li P, Wallace R, Wang Y, Guo X, Liu Z. Microarray analysis of gene expression in eastern oyster (*Crassostrea virginica*) reveals a novel combination of antimicrobial and oxidative stress host responses after derm (*Perkinsus marinus*) challenge. Fish Shellfish Immunol. 2010; 29:921–929. [PubMed: 20708691]

Wang K, Pales Espinosa E, Tanguy A, Allam B. Alterations of the immune transcriptome in resistant and susceptible hard clams (*Mercenaria mercenaria*) in response to Quahog Parasite Unknown (QPX) and temperature. Fish Shellfish Immunol. 2016; 49:163–176. [PubMed: 26690665]

Wang K, del Castillo C, Corre E, Pales Espinosa E, Allam B. Clam focal and systemic immune responses to QPX infection revealed by RNA-seq technology. BMC Genomics. 2016b; 17:146. [PubMed: 26921237]

Wang Z, Wang B, Chen G, Jian J, Lu Y, Xu Y, Wu Z. Transcriptome analysis of the pearl oyster (*Pinctada fucata*) hemocytes in response to *Vibrio alginolyticus* infection. Gene. 2016c; 575:421–428. [PubMed: 26363408]

Warr GW. Immunity in invertebrates. J Invert Pathol. 1981; 38:311–314.

Wei X, Yang J, Yang D, Xu J, Liu X, Yang J, Fang J, Qiao H. Molecular cloning and mRNA expression of two peptidoglycan recognition protein (PGRP) genes from mollusk *Solen grandis*. Fish Shellfish Immunol. 2012; 32:178–185. [PubMed: 22119574]

Williams AF, Gagnon J. Neuronal cell Thy-1 glycoprotein: homology with immunoglobulin. Science. 1982; 216:696–703. [PubMed: 6177036]

Wong YH, Sun J, He LS, Chen LG, Qiu JW, Qian PY. High-throughput transcriptome sequencing of the cold seep mussel *Bathymodiolous platifrons*. Sci Rep. 2015; 5:16597. [PubMed: 26593439]

You Y, Huan P, Liu B. RNAi assay in primary cells: a new method for gene function analysis in marine bivalve. Mol Biol Rep. 2012; 39:8209–8216. [PubMed: 22544578]

Yuasa HJ, Furuta E, Nakamura A, Takagi T. Cloning and sequencing of three C-type lectins from body surface mucus of the land slug, *Incilaria fruhstorferi*. Comp Biochem Physiol B Biochem Mol Biol. 1998; 119:479–484. [PubMed: 9734332]

Zahoor Z, Davies AJ, Kirk RS, Rollinson D, Walker AJ. Disruption of ERK signalling in *Biomphalaria glabrata* defence cells by *Schistosoma mansoni*: implications for parasite survival in the snail host. Dev Comp Immunol. 2008; 32:1561–1571. [PubMed: 18619674]

Zahoor Z, Davies AJ, Kirk RS, Rollinson D, Walker AJ. Nitric oxide production by *Biomphalaria glabrata* haemocytes: effects of *Schistosoma mansoni* ESPs and regulation through the extracellular signal-regulated kinase pathway. Parasit Vectors. 2009; 2:18. [PubMed: 19386102]

Zahoor Z, Lockyer AE, Davies AJ, Kirk RS, Emery AM, Rollinson D, Jones CS, Noble LR, Walker AJ. Differences in the gene expression profiles of haemocytes from schistosome-susceptible and -resistant *Biomphalaria glabrata* exposed to *Schistosoma mansoni* excretory-secretory products. PLoS One. 2014; 9:e93215. [PubMed: 24663063]

Zelck UE, Gege BE, Schmid S. Specific inhibitors of mitogen-activated protein kinase and P13-K pathways impair immune responses by hemocytes of trematode intermediate host snails. Dev Comp Immunol. 2007; 31:321–331. [PubMed: 16926049]

Zhang SM, Léonard PM, Adema CM, Loker ES. Parasite-responsive IgSF members in the snail *Biomphalaria glabrata*: characterization of novel genes with tandemly arranged IgSF domains and a fibrinogen domain. Immunogenetics. 2001; 53:992.

Zhang SM, Adema CM, Kepler TB, Loker ES. Diversification of Ig superfamily genes in an invertebrate. Science. 2004; 305:251–254. [PubMed: 15247481]

Zhang Y, Zhang X, Scheuring CF, Zhang HB, Huan P, Li F, Xiang J. Construction and characterization of two bacterial artificial chromosome libraries of Zhikong scallop, *Chlamys farreri* Jones et Preston, and identification of BAC clones containing the genes involved in its innate immune system. Mar Biotechnol (NY). 2008; 10:358–365. [PubMed: 18283517]

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 PW, Paps J, Zhu Y, Wu F, Chen Y, Wang J, Peng C, Meng J, Yang L, Liu J, Wen B, Zhang N, Huang Z, Zhu Q, Feng Y, Mount A, Hedgecock D, Xu Z, Liu Y, Domazet-Lošo T, Du Y, Sun X, Zhang S, Liu B, Cheng P, Jiang X, Li J, Fan D, Huang R, Yang H, Du X, Chen L, Yang M, Gaffney PM, Wang S, Luo L, She Z, Ming Y, Huang W, Zhang S, Huang B, Zhang Y, Qu T, Ni P, Miao G, Wang J, Wang Q, Steinberg CE, Wang H, Li N, Qian L, Zhang G, Li Y, Yang H, Liu X, Wang J, Yin Y, Wang J. The oyster genome reveals stress adaptation and complexity of shell formation. Nature. 2012; 409:49–54.

Zhang Y, Yu Z. the first evidence of positive selection in peptidoglycan recognition protein (PGRP) genes of *Crassostrea gigas*. Fish Shellfish Immunol. 2013; 34:1352–1255. [PubMed: 23416224]

Zhang L, Li L, Zhu Y, Zhang G, Guo X. Transcriptome analysis reveals a rich gene set related to innate immunity in the eastern oyster. Mar Biotechnol. 2014; 16:17–33. [PubMed: 23907648]

Zhang L, Li L, Guo X, Litman GW, Dishaw LJ, Zhang G. Massive expansion and functional divergence of innate immune genes in a protostome. Sci Rep. 2015; 5:8693. [PubMed: 25732911]

Zhang SM, Loker ES, Sullivan JT. Pathogen-associated molecular patterns activate expression of genes involved in cell proliferation, immunity, and detoxification in the amebocyte-producing organ of the snail *Biomphalaria glabrata*. Dev Comp Immunol. 2016; 56:25–36. [PubMed: 26592964]

Zhao W, Fanning ML, Lane T. Efficient RNAi-based gene family knockdown via set cover optimization. Artif Intell Med. 2005; 35:61–73. [PubMed: 16084706]

Zhao C, Zhang T, Zhang X, Hu S, Xiang J. Sequencing and analysis of four BAC clones containing innate immune genes from the Zhikong scallop (*Chlamys farreri*). Gene. 2012a; 502:9–15. [PubMed: 22542508]

Zhao X, Wang Q, Jiao Y, Huang R, Deng Y, Wang H, Du X. Identification of genes potentially related to biomineralization and immunity by transcriptome analysis of pearl sac in pearl oyster *Pinctada martensii*. Mar Biotechnol. 2012b; 14:730–739. [PubMed: 22351046]

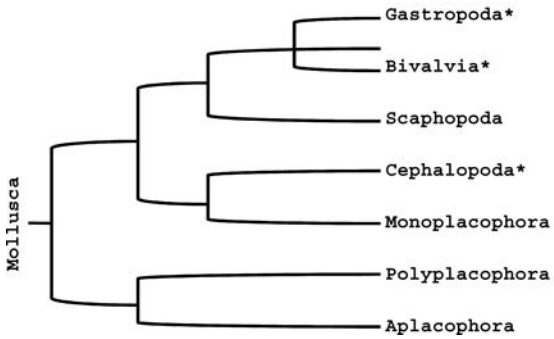
Zhao X, Yu H, Kong L, Liu S, Li Q. Comparative transcriptome analysis of two oysters, *Crassostrea gigas* and *Crassostrea hongkongensis* provides insights into adaptation to hypoosmotic conditions. PLoS One. 2014; 9:e111915. [PubMed: 25369077]

Zhao QP, Xiong T, Xu XJ, Jiang MS, Dong HF. *De novo* transcriptome analysis of *Oncomelania hupensis* after molluscicide treatment by next-generation sequencing: implications for biology and future snail interventions. PLoS One. 2015; 10:e0118673. [PubMed: 25775015]

Zuckerkandl, E., Pauling, L. Evolutionary divergence and convergence in proteins. In: Bryson, V., Vogel, HJ., editors. Evolving Genes and Proteins. Academic Press; New York: 1965. p. 97-166.

Highlights

- Historical review of molluscan immunity prior to development of modern techniques
- Coverage of species-specific gene complements for comparative immunology
- Identification of lineage-specific immune capabilities is achieved with NGS
- NGS informs on general features of molluscan immunity



	ESTs					SRA	
	1990	2000	2010	2016	# species	2016	# species
Gastropoda*	0	1232	630146	647770	23	1985	182
Bivalvia*	0	0	347393	382612	37	2829	127
Scaphopoda	0	0	0	0	-	7	6
Cephalopoda*	0	0	46081	114034	6	225	38
Monoplacophora	0	0	0	0	-	2	1
Polyplacophora	0	0	498	1548	2	5	5
Aplacophora	0	0	0	1907	2	13	11

Figure 1. Simplified phylogeny of the classes in the phylum Mollusca adapted from Kocot et al., 2011 and Smith et al., 2011

Asterisks indicate genome availability in public databases. For each class the number and increments of expressed sequence tag (EST) and short read archive (SRA) entries are indicated over time. Note that SRA entries were not available before 2008, coinciding with the advent of next-generation capabilities. Data were obtained by querying the SRA and EST databases of GenBank using name of class and modification dates as search terms.

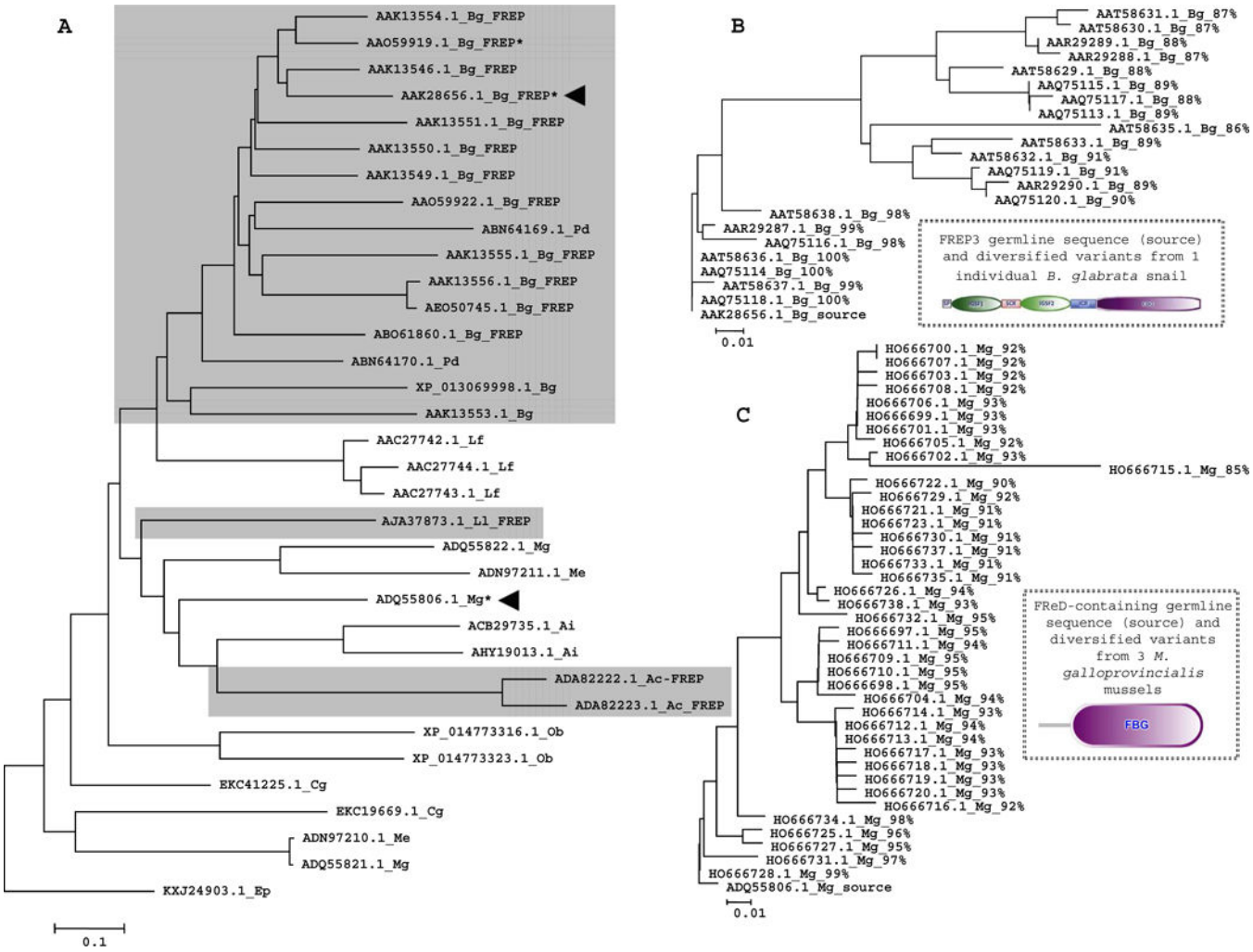


Figure 2. Phylogenetic reconstruction of molluscan fibrinogen-related domain (FReD) sequences

(A) Phylogenetic tree of FReD-containing sequences from three molluscan classes: Gastropoda (5 species), Bivalvia (4 species), Cephalopoda (1 species). A FReD sequence from the cnidarian (pre-bilaterian) *Exaiptaisa pallida* (Ep) is used as outgroup. The alignment of these FReD sequences included 103 amino acids (aa) Grey boxes highlight canonical fibrinogen-related proteins (FREPs: sequences containing 1 or 2 N-terminal IgSF domains and a C-terminal FBG domain). Asterisks indicate FReD-containing sequences reported to be extensively diversified (Dheilly et al., 2015; Hanington et al., 2010; Romero et al., 2011; Zhang et al., 2004). Arrowheads mark germ-line encoded sequences used in Fig. 2b,c.(B) Sequence diversity of a 127 aa region of the fibrinogen-related sequence of FREP3 (canonical structure indicated) recorded from the germline encoded (source) sequence and diversified variants from one individual *B. glabrata* snail. For each percentage value the number of different amino acids residues relative to the source sequence (127aa) are as follows: 100%=0aa, 99%=1aa, 98%=2aa, 91%=11aa, 90%=12aa, 89%=13aa, 88%=14–15aa, 87%=16aa, 86%=17aa.(C) Sequence diversity of a 129 aa region of the fibrinogen-related sequence of a single FReD-containing lectin (see structure) recorded from the germline encoded (source) sequence and diversified variants from three individual *M.*

galloprovincialis mussels. For each percentage value the number of different amino acids residues relative to the source sequence (129aa) are as follows: 100%=0aa, 99%=1aa, 98%=2aa, 97%=3aa, 96%=4–5aa, 95%=6aa, 94%=7aa, 93%=8–9aa, 92%=10aa, 91%=11aa, 90%=12aa, 85%=19aa. *Aplysia californica* (Ac), *Argopecten irradians* (Ai), *Biomphalaria glabrata* (Bg), *Crassostrea gigas* (Cg), *Limax flavus* (Lf), *Littorina littorea* (Ll), *Mytilus edulis* (Me), *Mytilus galloprovincialis* (Mg), *Octopus bimaculoides* (Ob), *Planorbella duryi* (Pd). All accession numbers are provided within the trees. Analyses were conducted in MEGA6 (Tamura et al., 2013) using the Neighbor-Joining (Saitou and Nei, 1987) and Poisson correction methods(Zuckerkandl and Pauling, 1965). Scale bars: units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated.

Table 1

Partial listing of publications reporting on NGS data collection from molluscs. The listing of transcriptomic data only includes immune-related studies. Information for genomic data was retrieved from the Genome database of NCBI. Publicly available genomes without a corresponding publication are indicated with an asterix.

Transcriptomic Data			
Class	**Organism**	**NGS Platform**	**Reference**
Gastropoda	*Arion vulgaris*	**Illumina HiSeq2000**	Bulat et al., 2016
Gastropoda	*Biomphalaria glabrata*	Illumina GAIIx	Pinaud et al., 2016
Gastropoda	*Biomphalaria glabrata*	Illumina HiSeq2000	Kenny et al., 2016
Gastropoda	*Conus geographus*	Illumina HiSeq2000	Safavi-Hemami et al., 2016
Gastropoda	*Littorina littorea*	Illumina HiSeq2000	Gorbushin and Borisova 2015
Gastropoda	*Oncomelania hupensis*	Illumina HiSeq2000	Zhao et al., 2015
Gastropoda	*Biomphalaria glabrata*	Illumina GAIIx	Deleury et al., 2012
Gastropoda	*Thais clavigera*	454 GS 20	Rhee et al., 2012
Bivalvia	*Crassostrea virginica*	Illumina GAIIx	McDowell et al., 2016
Bivalvia	*Cristaria plicata*	Illumina HiSeq2500	Patnaik et al., 2016
Bivalvia	*Mercenaria mercenaria*	Illumina HiSeq2000	Wang et al., 2016b
Bivalvia	*Mytilus chilensis*	Illumina HiSeq2000	Detree et al., 2016
Bivalvia	*Pinctada fucata*	Illumina HiSeq2000	Wang et al., 2016c
Bivalvia	*Saccostrea glomerata*	Illumina HiSeq2000	Ertl et al., 2016
Bivalvia	bivalvia		Rosani et al., 2015a
Bivalvia	*Bathymodiolus platifrons*	Illumina HiSeq2000	Wong et al., 2015
Bivalvia	*Chlamys farreri*	Illumina HiSeq2000	Hu et al., 2015
Bivalvia	*Crassostrea gigas*	Ion Torrent Proton	Chen et al., 2015
Bivalvia	*Crassostrea gigas*	Illumina GAIIx	He et al., 2015
Bivalvia	*Crassostrea gigas*	Illumina HiSeq2000	Rosani et al., 2015b
Bivalvia	*Crassostrea gigas*	Illumina GAIIx	Zhang et al., 2015
Bivalvia	*Patinopecten yessoensis*	Illumina HiSeq2000	Ding et al., 2015
Bivalvia	*Crassostrea gigas* *Crassostrea hongkongensis*	Illumina HiSeq2000	Zhao et al., 2014
Bivalvia	*Crassostrea virginica*	Illumina GAIIx	McDowell et al., 2014
Bivalvia	*Crassostrea virginica*	Illumina GAIIx	Zhang et al., 2014
Bivalvia	*Mytilus galloprovincials*	Illumina GAIIx	Gerdol et al., 2014
Bivalvia	*Pecten maximus*	Illumina HiSeq2000	Pauletto et al., 2014
Bivalvia	*Mytilus chilensis*	Illumina HiSeq2000	Nuñez-Acuña and Gallardo-Escárate 2013
Bivalvia	*Mytilus edulis*	454 GS FLX Titanium	Philipp et al., 2012
Bivalvia	*Pinctada martensii*	Illumina HiSeq2000	Zhao et al., 2012b
Bivalvia	*Patinopecten yessoensis*	454 GS FLX Titanium	Hou et al., 2011
Bivalvia	*Bathymodiolus azoricus*	454 GS FLX Titanium	Bettencourt et al., 2010
Cephalopoda	*Euprymna tasmanica*	454 GS FLX+	Salazar et al., 2015
Cephalopoda	*Octopus vulgaris*	Illumina GAIIx	Castellanos-Martinez et al., 2014

Genomic Data				
Class	**Organism**	**GenBank Assembly Accessi**	**Renference**	**Genome Size (bp)/database source**
Gastropoda	*Biomphalaria glabrata*	GCA_0 00457365.1	Adema et al., 2017: In prep*	9.16 × 10^8/GenBank
Gastropoda	*Conus tribblei*	GCA_0 01262575.1	Barghi et al., 2016	2.16 × 10^9/GenBank
Gastropoda	*Lymnaea stagnalis*	GCA_9 00036025.1	Davison et al. 2016	8.33 × 10^8/GenBank
Gastropoda	*Aegista diversifamilia Dolicheulota formosensis*	(SRA Accessions) SRR1918809 SRR1920140	Huang and Wu 2015	Data not available
Gastropoda	*Aplysia cali fornica*	GCA_0 00002075.2	Moroz and Kandel, 2006*	9.27 × 10^8/GenBank
Gastropoda	*Lottia gigantea*	GCA_0 00327385.1	Simakov et al., 2013	3.59 × 10^8/GenBank
Bivalvia	*Corbicula fluminea*	GCA_0 01632725.1	Peñarrubia et al., 2016*	Data not available
Bivalvia	*Dreissena polymorpha*	GCA_0 00806325.1	Peñarrubia et al., 2015	1.66 × 10^9/Animal Genome Size Database
Bivalvia	*Mytilus galloprovincial*	GCA_0 00715055.1	Nguyen et al., 2014*	1.62 × 10^9/GenBank
Bivalvia	*Crassostrea gigas*	GCA_0 00297895.1	Zhang et al., 2012	5.57 × 10^8/GenBank
Cephalopoda	*Octopus bimaculoides*	GCA_0 01194135.1	Albertin et al., 2015	2.33 × 10^9/GenBank