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Proteomics insights: proteins related to larval attachment and metamorphosis of marine invertebrates

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The transition in an animal from a pelagic larval stage to a sessile benthic juvenile typically requires major morphological and behavioral changes. Larval competency, attachment and initiation of metamorphosis are thought to be regulated by intrinsic chemical signals and specific sets of proteins. However, the molecular mechanisms that regulate larval attachment and metamorphosis in marine invertebrates have yet to be fully elucidated. Despite the many challenges associated with analysis of the larvae proteome, recent proteomic technologies have been used to address specific questions in larval developmental biology. These and other molecular studies have generated substantial amount of information of the proteins and molecular pathways involved in larval attachment and metamorphosis. Furthermore, the results of these studies have shown that systematic changes in protein expression patterns and post-translational modifications (PTMs) are crucial for the transition from larva to juvenile. The degeneration of larval tissues is mediated by protein degradation, while the development of juvenile organs may require PTM. In terms of application, the identified proteins may serve as targets for antifouling compounds, and biomarkers for environmental stressors. In this review we highlight the strengths and limitations of proteomic tools in the context of the study of marine invertebrate larval biology.

Keywords: marine invertebrates, larvae metamorphosis, biofouling, proteomics, post-translational modifications

IMPORTANCE OF MARINE INVERTEBRATES: BARNACLES, BRYOZOANS AND POLYCHAETES

Marine invertebrates are important players in various marine ecosystems, including in biofouling, intertidal and sediment ecosystems. The barnacle *Balanus amphitrite*, the bryozoan *Bugula neritina* and the serpulid polychaete *Hydroides elegans* are the most dominant fouling species, are distributed worldwide, and are commonly used in larval settlement (including both attachment and metamorphosis) studies and antifouling research (Ten Hove, 1974; Khandeparker and Anil, 2007; Aguirre et al., 2008; Cohen, 2011). Marine polychaete worms, including *Capitella teleta*, *Pseudopolydora vexillosa*, and *Neanthes arenaceodentata*, occur in habitats ranging from subtropical to inshore waters, and are used as indicators of marine pollution and as toxicological test animals (Reish and Gerlinger, 1997; Greg and Kristian, 1998; Rouse and Fauchald, 1998; Glasby and Timm, 2008; Blake et al., 2009). Because of high sensitivity to marine contamination mussels have been used as indicators of marine pollution (Romeo et al., 2003). For instance, mussels and barnacles were used as biomonitors in coastal waters (Phillips and Rainbow, 1988) Abalone *Haliotis diversicolor supertexta* was used for investigating contamination of marine ecosystems by toxic chemicals (Zhou et al., 2010). Calcifying marine invertebrates including polychaetes and barnacles, are capable of physiological and behavioral adaptation to various environmental stressors and thus serve as model organisms in climate change research (Dupont and Thorndyke, 2009; Taylor et al., 2010). Abalone, gastropod mollusk, is used for food and their shells are often used for ornaments. Several invertebrates provide habitat to other species. For example, the mussel beds provide living space to the species living on them. Peracaridan species utilize *B. neritina* as living space (Conradi et al., 2000).

LARVAL DEVELOPMENT: COMPETENCY, ATTACHMENT, AND METAMORPHOSIS

The population dynamics of marine invertebrates are controlled by recruitment and post-settlement survival. The choice of attachment site is crucial because attachment to an unfavorable substrate can be fatal (Bishop et al., 2006). Factors that influence attachment and metamorphosis, including settlement cues, competency, and habitat, are selective for the larvae of a particular species. For instance, larval metamorphosis in barnacles is a dynamic and rapid process that involves substantial tissue remodeling and differentiation (Thiyagarajan et al., 2007; Maruzzo et al., 2012). Barnacle species have six nauplius stages and a cyprid larval stage, and larva–juvenile transitions may or may not require a metamorphosis inducer (Thiyagarajan et al., 2005). In the absence of cues, the larvae of *B. neritina* commonly

settle within several minutes (Woollacott and Zimmer, 2005). In *H. elegans,* the fertilized eggs hatch into young trochophore larvae, which develop into competent larvae and metamorphose into young juveniles. The larvae will not settle without suitable settlement cues, and the larval–juvenile transition requires substantial tissue reorganization and major morphological changes (Hadfield et al., 2001). In contrast, the young larvae of *P. vexillosa* can take 7–10 days to develop competency, and larval metamorphosis is a gradual process that does not require substantial development of juvenile organs (Mok et al., 2008). This type of metamorphosis in the absence of specific inducers is referred to as "spontaneous metamorphosis." Although past studies have contributed to our understanding of the basic underpinnings of the morphological, ecological and behavioral patterns of larval settlement, the molecular mechanisms that regulate larval attachment and metamorphosis are poorly understood. In recent years, it has been hypothesized that attachment and metamorphic events are regulated by changes in protein expression patterns and intrinsic signaling pathways that influence larval morphology and behavior. To test this hypothesis, proteomics tools are being used increasingly to identify proteins and pathways involved in larval development, despite technological limitations associated with the advancement of larval biology research in this area. Hence, the goal of this review is to describe the progress in marine larval proteomic research in relation to changes in protein expression patterns in different larval stages of marine invertebrates. Specifically, this review highlights: (i) technical challenges in larval proteomics research; (ii) proteome and phosphoproteome insights during larval attachment and metamorphosis; (iii) the involvement of signaling pathways during larval metamorphosis; and (iv) the usefulness of commonly expressed proteins in antifouling applications.

PROTEIN EXPRESSION PATTERNS DURING LARVAL DEVELOPMENT: TWO-DIMENSIONAL GEL ELECTROPHORESIS (2-DE) PROTEOMICS

Proteomics tools and pipelines used in the study of marine larval biology are outlined in **Figure 1**. A list of studies on larval proteomics is shown in **Table 1**. In the majority of these studies, the larval proteome was characterized by gel-based (2-DE) or gel-free proteomics, followed by mass spectrometry (MS) analysis. These approaches enabled documentation of changes in protein expression patterns and protein modification status at various stages in the larval lifespan. López et al. (2005) was the first group to use 2-DE proteomics approach to identify larval-specific proteins in the mussel *Mytilus galloprovincialis*. DeBoer et al. (2007) then conducted comparative proteome and transcriptome analyses to reveal symbiosis-related differences in coral larvae and dinoflagellates and found no significant changes in the protein profiles of the larvae. Thiyagarajan and Qian (2008) used 2-DE proteomics to document the global proteome expression pattern in the larvae of *B. amphitrite*. They optimized protein extraction and iso-electric focusing (IEF) conditions for a better resolution of protein spots on 2-DE gels and showed that the proteome pattern of cyprid larvae was very distinct from that of attached larvae and newly-metamorphosed juveniles. Another study (Mok et al., 2009) found that there are proteomic changes in the polychaete worm *P. vexillosa* during larval metamorphosis. They found that the proteome of competent larvae of *P. vexillosa* was similar to that of juveniles, whereas the proteomes of precompetent and

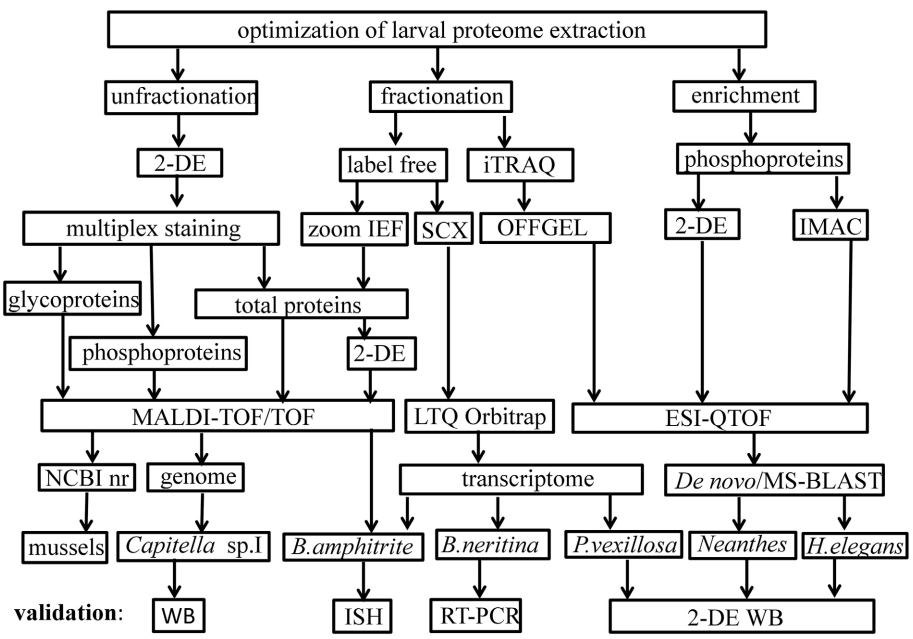

FIGURE 1 | Proteomics tools used to study marine larvae. Abbreviations: 2-DE, two-dimensional-gel electrophoresis; iTRAQ, isobaric tags for relative and absolute quantitation; IEF, isoelectric focusing; SCX, strong cation exchange; IMAC, immobilized metal ion affinity chromatography; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; ESI, electrospray ionization; NCBI nr, national center for biotechnological information non-redundant; WB, Western blot; ISH, *in situ* hybridization; RT-PCR, real-time polymerase chain reaction.

Table 1 | List of proteomics investigations focused on larval settlement and metamorphosis of marine invertebrates.

Marine animal	Objective(s)/hypothesis	Approach	Key findings/results	Source
LARVAL PROTEOME DYNAMICS DURING SETTLEMENT AND METAMORPHOSIS				
Mytilus galloprovincialis	Larval specific protein identification	Two-dimensional electrophoresis (2-DE) and matrix assisted laser desorption ionization with time-of-flight (MALDI-TOF)	Six species specific proteins were identified	López et al., 2005
Fungia scutaria	Investigation of biochemical or molecular events during the onset of symbiosis	2-DE, cDNA subtracted library, and real-time PCR	Age-related proteomics changes were detected. Changes related to symbiosis were not observed in the larval proteome	DeBoer et al., 2007
Balanus amphitrite	Documentation of overall protein expression patterns during larval development and metamorphosis	2-DE and MALDI-TOF/TOF	Proteome of the swimming cyprid was distinct whereas the attached and metamorphosed cyprid proteome were similar	Thiyagarajan and Qian, 2008
Pseudopolydora vexillosa	Hypothesis: metamorphosis related changes in the proteome are distinct in *P. vexillosa* compared with *B. amphitrite*	2-DE and MALDI-TOF/TOF	Proteomes of competent larvae were more similar to those of their juveniles	Mok et al., 2009
Bugula neritina	Hypothesis: first phase of metamorphosis is independent of *de novo* protein synthesis	2-DE, sequential staining and MALDI-TOF/TOF	Early morphogenetic changes were mediated by post-translational modifications (PTM) of available proteins	Wong et al., 2010
Hydroides elegans	To test the hypothesis that rapid larval settlement could be mediated by protein phosphorylation rather than synthesis of new proteins	2-DE, sequential staining and MALDI-TOF/TOF	During larval competency, the expression profiles of abundant proteins were similar, whereas protein phosphorylation drastically differed	Zhang et al., 2010b
Bugula neritina	To establish a comprehensive transcriptome and proteome dataset	454 RNA sequencing, APEX quantitative tool, and LC-MS/MS	882 proteins were identified and quantified during larval settlement. Transcript and protein reference dataset was constructed	Wang et al., 2010
Capitella teleta	Hypothesis: Specific mechanisms of larval metamorphosis vary between species	2-DE, sequential staining and MALDI-TOF/TOF	Cell division, cell migration, energy storage and oxidative stress proteins were abundantly expressed in competent larvae, whereas in juveniles, metabolism and transcriptional proteins were abundant	Chandramouli et al., 2011a
Pseudopolydora vexillosa	Documentation of protein phosphorylation dynamics during larval metamorphosis	Affinity chromatography, 2-DE, and MALDI-TOF/TOF	High degree of protein phosphorylation detected in competent larvae. Ten phosphoproteins were identified	Chandramouli et al., 2011b
Balanus amphitrite, Bugula neritina, Pseudopolydora vexillosa	Hypothesis: Inter-species differences in protein glycosylation account for differences in developmental processes	2-DE, sequential staining, MALDI-TOF/TOF and LC-MS/MS	Nineteen abundant glycosylated proteins were identified. The degree of glycosylation was high in *B. amphitrite* and *P. vexillosa,* but was decreased *in B. neritina*	Chandramouli et al., 2012b
Bugula neritina	Identification of molecular targets crucial for larval settlement and metamorphosis	ESI-Q-TOF, APEX quantitative tool, and inhibitor assay	1100 proteins were identified in swimming larvae and metamorphosing individuals. Energy metabolism and structural proteins were down-regulated, whereas transcription, a translation, and calcification proteins were up-regulated during metamorphosis	Zhang et al., 2011

(Continued)

Table 1 | Continued

Marine animal	Objective(s)/hypothesis	Approach	Key findings/results	Source
Neanthes arenaceodentata	Documentation of phosphorylation dynamics during early development	2-DE, Immobilized metal affinity chromatography (IMAC), and LC-MS/MS	Ten phosphopeptides and phosphorylation sites (Ser/Thr) were identified. Abundant isoforms of cytoskeletal proteins were identified in three early development	Chandramouli et al., 2012a
Pseudopolydora vexillosa	Specific questions: (i) using a quantitative proteomics approach, is it possible to quantify more differential proteins? (ii) Which molecular pathways are associated with metamorphosis?	454 RNA sequencing, iTRAQ, and ESI-Q-TOF	Mitogen-activated protein kinase (MAPK), calcium-, Wnt and Notch signaling pathways were enriched. One hundred and seven differentially expressed proteins were quantified during competency and metamorphosis	Chandramouli et al., 2013b
Neanthes arenaceodentata	Determination of proteins that influence unique reproduction pattern	2-DE and iTRAQ, and ESI-Q-TOF	Males worms showed a higher degree of similarity in protein expression patterns while females showed drastic changes in phosphoproteome before and after spawning	Chandramouli et al., 2013a
Balanus amphitrite	Identification of differentially expressed proteins during settlement and metamorphosis	Label-free quantitative proteomics	Energy metabolism, nervous system and signal transduction proteins were up-regulated in cyprids. Cytoskeletal, transcription and translation and biomineralization proteins were up-regulated in juveniles	Chen et al., 2014
Hydroides elegans	Comparative larval proteomes before and after competency	Gel-free high-throughput proteomics	Energy metabolism, redox homeostasis, and cytoskeleton development were involved in the development of larval competence.	Zhang et al., 2014
ROLE OF SIGNALING PATHWAYS DURING LARVAL METAMORPHOSIS				
Hydroides elegans	Identification of genes expressed in competent larvae	Suppressive subtractive hybridization (SSH), rapid amplification of cDNA ends (RACE), and *in situ* hybridization (ISH)	Transcripts are important for the reactive oxygen species signal transduction pathway and the p38 mitogen-activated protein kinase (p38 MAPK) pathway regulates larval metamorphosis	Wang and Qian, 2010
Hydroides elegans	Examination of the involvement of calmodulin (CaM) in larval settlement and metamorphosis	Gene cloning, *in situ* hybridization(ISH),and inhibitor assay	CaM was abundantly expressed in post-metamorphic juveniles and adults. CaM was continuously expressed in putative growth zones, branchial rudiments, and the collar region of competent larvae	Chen et al., 2012
Bugula neritina	Examination of the role of Wnt signaling in metamorphosis	*In situ* hybridization(ISH), DAVID analysis, gene orthology assignment and signal peptide prediction, and RT-PCR	Bnβ-catenin and BnFz5/8 were expressed in blastemas. BnWnt10 and BnsFRP were related to patterning of the polypide. BnWnt6 was expressed in the apical region of the pre-ancestrula epidermis	Wong et al., 2012
Balanus amphitrite	Hypothesis: p38 MAPK is involved in larval settlement	Gene cloning, antibody generation, confocal imaging, and settlement assay	Phosphorylated pp38 MAPK drastically decreased after settlement. Bar-p38 MAPK was highly activated in confronted extracts of adult barnacle	He et al., 2012

(Continued)

Table 1 | Continued

Marine animal	Objective(s)/hypothesis	Approach	Key findings/results	Source
Balanus amphitrite	Determination of the role of nitric oxide (NO)/cGMP signaling in larval attachment and metamorphosis	Quantitative PCR, co-incubation assay	NO regulates larval settlement through cGMP signaling	Zhang et al., 2012a
Balanus amphitrite	Identification of the upstream kinase of p38MAPK and its role in larval settlement.	Immunostaining and Western blot analysis	MKK3 activated the kinase function of p38MAPK, thereby influencing the larval settlement of *B. amphitrite*	Zhang et al., 2013
LARVAL PROTEOME RESPONSE TO SETTLEMENT INHIBITORS				
Balanus amphitrite *Bugula neritina*	Investigation of larval proteome and phosphoproteome responses to genistein, an inhibitor of metamorphosis	2-DE, sequential staining, and MALDI-TOF/TOF	Genistein reduced the expression of phosphoproteins in *B. neritina* but increased their expression in *B. amphitrite*	Thiyagarajan et al., 2009
Bugula neritina	Investigation of larval proteome and phosphoproteome responses to butenolide, an antifouling agent	Liquid IEF fractionation, 2-DE, sequential staining and MALDI-TOF/TOF	Settlement was mediated by changes in protein abundance and phosphorylation status. Butenolide affected the phosphorylation of proteins	Qian et al., 2010
Balanus amphitrite	Investigation of larval proteome and phosphoproteome responses to butenolide, an antifouling agent	Liquid IEF fractionation, 2-DE, sequential staining and MALDI-TOF/TOF	During cyprid development, stress and energy metabolism proteins were differentially expressed. Butenolide sustained the expression of proteins	Zhang et al., 2010a
Balanus amphitrite	Examination of the effects of poly-ether B on the larval proteome of barnacle	2-DE, sequential staining, and ESI-QTOF	Poly-ether interfered with proteins related to redox-regulatory mechanisms, thereby inhibiting larval settlement	Dash et al., 2012
Balanus amphitrite	Assessment of the effects of meleagrin on protein expression during cyprid development and aging	iTRAQ, 2D LC-MS/MS	Fifty proteins responded to treatment and aging, of which 26 were related to development and aging and 24 were specific to treatment	Han et al., 2013

competent larvae were more distinct. Availability of genome sequencing allowed a better characterization of the proteins involved in different biological processes during the development in ascidians (as reviewed in Inaba et al., 2007). Nomura et al. (2009) found the involvement of vitellogenin, elongation factors, actin-binding proteins and chaperones during embryonic development of *Ciana intestinalis*. In the abalone *Haliotis asinina*, the first proteomic study characterized protein domains from calcified shell, which were rich in glutamine, glycine, alanine, and aspartate (Marie et al., 2010). All the studies mentioned above have relied on low-resolution immobilized pH gradient (IPG) strips (13 cm in length; pH 3–10) for the first-dimension separation and Coomassie-brilliant blue (CBB) staining of the gels. Aggregation or overlapping of proteins spots limited the resolution of many spots on the 2-DE gels and could not really quantify actual changes in protein abundance. To overcome these shortcomings, Qian et al. (2010) and Zhang et al. (2010a) used combination of different methods to probe the larval proteomes of *B. neritina* and *B. amphitrite,* including pre-fractionation of the larval proteomes prior to 2-DE to reduce the sample complexity, sequential fluorescent dye gel staining to increase the detection limit (∼0.5–5 ng) and dynamic range (Panfoli et al., 2012) and implementation of narrow-range IPG strips (pH 4–7) to enhance resolution of the protein spots on 2-DE gels. In these studies, they used used a Zoom IEF fractionator (Invitrogen, Carlsbad, CA, USA) to fractionate the total proteome into two subproteomes of pH 3.0–5.4 and pH 5.4–7.0 and high-resolution narrow-range IPG (pH 4–7) strips followed by gel staining with SYPRO® Ruby (Molecular Probes, Eugene, OR, USA) to achieve better protein separation. The number of protein spots detected was more than that detected for unfractionated samples.

POST-TRANSLATIONAL MODIFICATIONS (PTMs) OF PROTEINS DURING LARVAL METAMORPHOSIS

The identification and characterization of protein modifications among marine invertebrates have received substantial attention in recent years. It has been hypothesized that the early phase of metamorphic events depends on PTMs of proteins. For example, in *B. neritina,* the rapid initial phase of metamorphosis was facilitated by PTMs of existing proteins (Wong et al., 2010). In fact, protein modification often plays a crucial role in many biological processes (Zhou et al., 2007). In particular,

protein phosphorylation is a rapid process that regulates many cellular processes and signal transduction pathways (Hunter, 1995). Thiyagarajan et al. (2009) demonstrated the usefulness of multiplex staining and 2-DE to probe the larval phosphoproteome of *B. amphitrite* and *B. neritina.* In their study, specific fluorescent dyes including Pro-Q Diamond and Sypro Ruby, were used to detect phosphoproteins and total proteins on a single 2-DE gel. This method enabled parallel analyses of total protein and phosphoprotein expression. The similar tools were used to study phosphoproteome patterns in other marine species. In *H. elegans,* the larval phosphoproteome pattern changed significantly during competency, whereas the proteome pattern was not affected (Zhang et al., 2010b). In *Capitella teleta* (this refers to *Capitella* sp. I in early publication) phosphorylated proteins were essential for the elongation of body segments, production of hooded hooks, and preparation of juvenile tissues during larval metamorphosis (Chandramouli et al., 2011a). Protein enrichment methods have enabled the separation of proteins according to their PTM (e.g., phosphorylation) (Gilchrist et al., 2006; Puente et al., 2006; Cantin et al., 2008). This approach was used successfully by Chandramouli et al. (2011b) to improve the detection and identification of phosphoproteins during larval metamorphosis of *P. vexillosa,* revealing that competent larvae exhibit a higher degree of phosphorylation than precompetent larvae and juveniles. However, MS analysis of phosphopeptides remains challenging (Leitner et al., 2011). Using immobilized metal affinity chromatography (IMAC) and liquid chromatography (LC)-MS/MS to enrich phosphorylated peptides, Chandramouli et al. (2012a) identified nine phosphopeptides and phosphorylation sites (Thr/Ser) that are crucial for the early development (ova and larval stages) of *N. arenaceodentata.* This enrichment strategy illustrated an effective method for the identification of phosphoproteins and phosphopeptides in a larval proteome. On the other hand, glycoproteomics is an emerging proteomic technology and can be used to detect specific glycosylation patterns and to identify glycoproteins in complex proteomes (Pan et al., 2011). This method has the advantage of sequential staining, whereby ProQ Emerald fluorescent dye is used to selectively detect glycoproteins on 2-D gels. Using thie method, Chandramouli et al. (2012b) identified 19 abundant glycoproteins involved in the stress response and metabolic processes during larval metamorphosis of three marine invertebrate species. They found that the degree of protein glycosylation in *B. amphitrite* and *P. vexillosa* was greater prior to metamorphosis, whereas glycosylation in *B. neritina* increased right after metamorphosis. Overall, these studies clearly demonstrated emerging proteomics technologies can be used to document both glycosylation and phosphorylation dynamics during larval metamorphosis.

GEL-FREE HIGH-THROUGHPUT PROTEOMICS AND COMPREHENSIVE LARVAL PROTEOME ANALYSIS

Although gel-based proteomics provided valuable information about larval protein expression patterns, limitations associated with the identification of low-abundance larval proteins prompted the development of high throughput gel-free approaches (López, 2007; Tomanek, 2011; Slattery et al., 2012). In addition, transcriptome sequence datasets for several marine invertebrates, including *B. neritina* (Wang et al., 2010), *B. amphitrite* (Chen et al., 2011; De Gregoris et al., 2011), the coral *Acropora millepora* (Moya et al., 2012) and *P. vexillosa* (Chandramouli et al., 2013b), are now available in the public domain, which allows larval biologists to conduct more gel-free proteome analysis. Label-free quantitative methods involving individual sample preparation steps followed by LC-MS/MS analysis. Quantification of the amount of proteins is based on mass spectrum counting of the identified peptides (Wong et al., 2010; Zhang et al., 2011; Chen et al., 2014). Wang et al. (2010) applied next generation RNA sequencing and high-throughput LC-MS-based proteomics to profile larval transcripts and proteins of *B. neritina.* They generated 131,450 high-quality transcripts which were used as a reference transcriptome for proteome analysis. About 882 proteins were identified and quantified for the swimming and attached larval stages of *B. neritina*. Zhang et al. (2011) identified >1000 proteins (among which 61 were differentially expressed) in swimming and in two post-attachment stages of *B. neritina*. These differentially expressed proteins were grouped in the several categories: energy metabolism, cytoskeleton, transcription and translation, and calcification. Chen et al. (2014) identified about 700 proteins in each stage of *B. amphitrite* (nauplius II, nauplius VI, cyprids and juveniles) using label-free quantitative proteomics and found that proteins related to energy metabolism, nervous system and signal transduction were up-regulated in cyprids, whereas those related to cytoskeleton, transcription and translation, cell differentiation, and biomineralization proteins were up-regulated in juveniles. Zhang et al. (2014) identified about 1500 proteins that are associated with energy metabolism, redox homeostasis, and cytoskeleton development during larval development of *H. elegans.*

Although label-free proteomic studies provide a robust platform for direct quantitative analysis of the proteome, it is very challenging and complicated to analyze many mass spectra derived from multiple MS runs (Haqqani et al., 2008). To address this issue, peptide labeling methods have been used for relative quantification of protein abundance in marine invertebrates (Chandramouli et al., 2013a,b; Han et al., 2013; Sun et al., 2013). In fact, isobaric tags for relative and absolute quantification (iTRAQ) have been established (Pütz et al., 2005) and used to multiplex 4–8 samples (Evans et al., 2012; Martyniuk et al., 2012). Using this method, the peptides are labeled and then the samples are pooled and desalted prior to LC-MS/MS. The protein or peptide abundance is calculated based on the chromatographic peptide peak intensity, or the height of the various labeled peptide samples. In addition, an improved fractionation technique involving the separation of proteins or peptides in a multi-well device according to their isoelectric points (pI) has been developed to reduce the proteome complexity (Agilent Technologies, Böblingen, Germany; Chenau et al., 2008). Chandramouli et al. (2013b) combined peptide OFFGEL fractionation with iTRAQ labeling to identify and quantify proteins involved in key molecular processes during larval metamorphosis of *P. vexillosa* and identified 107 differentially expressed proteins in precompetent, competent, and juvenile stages. Among these 107 proteins, 14 and 53 proteins were differentially expressed during competency and metamorphosis, respectively. Using the same approach,

Chandramouli et al. (2013a) identified a set of proteins that influence the unique pattern of reproduction in the polychaete *N. arenaceodentata* and demonstrated that male worms have consistent protein expression patterns, whereas female worms (particularly spent females) show marked changes in the phosphoproteome before and after spawning. These studies showed that labeling methods permit the relative and absolute quantification of proteins expressed during a specific developmental stage.

MOLECULAR PATHWAYS UNDERLYING LARVAL METAMORPHOSIS IN MARINE INVERTEBRATES

Signaling pathways that are possibly regulate larval metamorphosis of marine invertebratespecies have been a hot topic in recent years. It was reported that cyclic adenosine-monophosphate (cAMP) and calcium signaling in barnacles (Clare et al., 1995; Clare, 1996), inositol-1,4,5-tris-phosphate/diacylglycerol (IP_3/DAG) signaling in cnidarians (Fleck, 1997), nitric oxide/cyclic guanosine monophosphate (NO/cGMP), protein kinase C (PKC) in *Capitella* (Biggers and Laufer, 1999) and sea urchins (Bishop et al., 2001; Amador-Cano et al., 2006), and p38 mediated mitogen-activated protein kinase (MAPK) recently has been implicated in the regulation of larval attachment and metamorphosis in *H. elegans* and *B. amphitrite* (Wang and Qian, 2010; He et al., 2012) are possibly involved in larval settlement and metamorphosis of these animals. Furthermore, Zhang et al. (2013) demonstrated that MKK3 (the upstream kinase of p38MAPK) activates the function of p38MAPK, thereby influencing the attachment of *B. amphitrite* larvae. Nitric oxide (NO) and cyclic GMP (cGMP) signaling molecules have been shown to act as regulators of the initiation of metamorphosis in ascidians and in an echinoid (Bishop et al., 2001; Leise et al., 2001). Bishop and Brandhorst (2001) demonstrated that the NO/cGMP system acts as a negative regulator during metamorphosis of the sea urchin *Lytechinus pictus*. Zhang et al. (2012a) documented the role of NO and cGMP during larval attachment of *B. amphitrite*. Recently Ueda and Degnan (2014) reported that NO facilitates the induction of metamorphosis while inhibition of nitric oxide synthase (NOS) reduces rates of metamorphosis in the abalone *Haliotis asinine*. It appears that nitric oxide signaling can contribute to stress-related responses (up-regulation of HSP-90) and regulate the metamorphic transitions. The role of calcium in signal transduction pathways during larval metamorphosis has been well documented in the polychaete worm *Phragmatopoma californica* (Ilan et al., 1993). Chen et al. (2012) examined the role of calmodulin (CaM) in larval attachment and metamorphosis of *H. elegans* Wong et al. (2012) found that the Wnt signaling pathway appears to be important for morphogenesis in *B. neritina*. Based on the findings above, we anticipate that MAPK signaling is important for larval attachment in *B. amphitrite* and *H. elegans*. Wnt signaling typically influences larval metamorphosis of *B. neritina*. The importance of *Ca2+*/CaM signaling in the development of polychaetes is of significant interest.

COMMON PROTEIN EXPRESSION

Heyland and Moroz (2006) showed that larval attachment and metamorphosis in marine invertebrates is mediated by common metamorphic events. If this is true, one may expect that larval attachment and metamorphosis are regulated by a common set of proteins in multiple marine invertebrates. To identify common protein expression signatures (PESs) during larval attachment and metamorphosis of *B. amphitrite*, *P. vexillosa*, and *B. neritina*, Chandramouli et al. (2012b) found 11 commonly expressed proteins involved in cytoskeleton, metabolism, and stress responses. A similar set of common proteins were differentially regulated in multiple marine invertebrates. **Table 2** lists the commonly regulated proteins and their expression patterns. In general, changes in the expression patterns of cytoskeletal proteins (tubulin, actin, and myosin) were observed in all of the

Table 2 | Differentially expressed proteins commonly found in different marine invertebrates.

Protein	*B. amphitrite*		*B. neritina*		*P. vexillosa*		*C. teleta*
	Set	Meta	Set	Meta	Set	Meta	Meta
Tubulin	*	*	Down	Up	Up	Up	Down
Actin	Up	*	Down	Up	Up	Up	Down
Myosin	Down	*	Down	Up	Up	Down	Up
14-3-3	Down	Up	*	*	Up	Down	Down
HSP-90	Down	Down	Up	Up	Up	**	Down
HSP-70	Down	*	Down	Up	**	**	**
ATP synthase	Down	Up	*	Down	Up	Down	Up
Disulfide isomerase	Down	Down	*	*	Up	**	**
Malate dehydrogenase	Down	Down	Down	*	Up	Up	**
Calreticulin	Up	**	Up	**	Up	**	**
Vitellogenin	**	**	Down	Down	**	**	Down
Calmodulin	Up	**	Up	**	**	**	**
Enolase-phosphatase	**	**	**	**	Up	Down	Down

Abbreviations: set, settlement; meta, metamorphosis; up, up-regulated; down, down-regulated.

**No significant change in protein expression.*

***Proteins not identified.*

species studied so far, indicating the importance of cytoskeletal dynamics in maintaining the architecture and contractility of larvae. Similarly, proteins related to energy metabolism (ATP synthase, disulfide isomerase, vitellogenin, enolase-phosphatase, and malate dehydrogenase) and the stress response (HSP-90, HSP-70, 14-3-3 protein, and calreticulin) were often differentially expressed during larval attachment and metamorphosis, indicating that these proteins may be synergistically regulated and may control events in the metamorphosis of larvae to juveniles. To identify possible common proteins that are the molecular targets for antifouling compounds, Thiyagarajan et al. (2009) showed that genistein, which is an inhibitor of metamorphosis (Zhou et al., 2009), down-regulated the expression of phosphoproteins in settling larvae of *B. neritina.* In contrast, this compound induced the expression of phosphoproteins in *B. amphitrite.* Another potential antifoulant, butenolide (Xu et al., 2010) sustained the expression of stress and energy metabolism proteins in settling larvae of *B. neritina* and *B. amphitrite* (Qian et al., 2010; Zhang et al., 2010a). Dash et al. (2012) showed that polyethers affect proteins related to energy metabolism, oxidative stress and chaperones. Han et al. (2013) also found that meleagrin, a potential non-toxic antifoulant, affect the expression of cyprid major protein (CMP) and adhesive plaque matrix proteins (APMPs) and thereby interfere with the larval endocrine system and molting processes (Billinghurst et al., 2000). These studies indicated that proteins involved in oxidative stress, cytoskeletal dynamics, chaperones, apoptosis, and energy homeostasis are possible molecular targets of those antifouling compounds. In contrast, several other types of antifoulants act on ion channels, quorum sensing systems, neurotransmitters, genotoxic damage, and adhesive production (as reviewed in Qian et al., 2013), which indicates voltage dependent anion channels, acetylcholinesterase, phenoloxidase, chitinase, Ran GTPase activating protein, and NADH ubiquinone oxidoreductase are proposed as molecular targets. Because of housekeeping function and responsive to environmental changes, several PESs identified by proteomics studies may not be the direct targets but their differential expression may be an indirect physiological and biochemical response to various antifoulants.

CHALLENGES AND FUTURE DIRECTIONS

SAMPLE PREPARATION

The complexity of protein samples has always been a major obstacle of proteomic study (Hondermarck, 2003; Speicher, 2007; Shen et al., 2008). Larvae and adults of many marine invertebrates are protected by shell (e.g., *B. amphitrite*), calcareous tubes (*H. elegans*), and mucilaginous polysaccharide substances (*P. vexillosa* and *C. teleta*). Cell culture and tissue isolation methods have not yet been established for these species. The direct use of whole larvae or juveniles for proteomic studies results in an enormous increase in sample complexity, complicating downstream sample preparation for mass spectrometry (MS) analysis. Highly abundant proteins can mask those of a low abundance, potentially preventing the detection of signaling and receptor proteins that may be crucial for larval attachment and metamorphic transitions. To overcome these problems, we need to improve protein extraction protocols. In addition, larval proteins of polychaetes can rapidly degraded shortly after tissue lysis. To provent degradation, protease inhibitors are required during multiple sample preparation steps (Zhang et al., 2010b; Chandramouli et al., 2011b). In addition, to minimize the possible protein degradation, it is better to freeze the samples in liquid nitrogen or be used immediately. The combined use of strong detergents (8M urea), homogenization, and sonication methods can also significantly improve protein recovery (Sun et al., 2013). It is also highly recommended that prior to 2-DE, larval protein samples shall be purified from other contaminants through the use of acetone precipitation and 2-DE clean-up kits. Multistep strategies in sample preparation, fractionation, and MS separation of peptides may facilitate the detection of these proteins (Millioni et al., 2011). Among these methods, immunoaffinity columns seems to be more suitable for the depletion of high-abundant cytoskeletal proteins (Molnár et al., 2011), after which low-abundant proteins may bind to the column, be eluted with specific buffers, and identified by 2-DE and/or MS. This procedure facilitates the identification of potential protein candidates or biomarkers. Alternately, the enrichment of specific subset of phosphoproteins or peptides from a complex protein sample can be an effective approach (Batalha et al., 2012). Cost-effective specific immune-affinity reagents, such as antibodies and metal, are the subjects for targeted proteomic studies, particularly for elucidating the protein phosphorylation dynamics in signaling pathways. However, the development of protocols for protein enrichment is always challenged by the functional and structural diversity of phosphorylated proteins. Furthermore, we need to maximize the amount of proteins to begin with in order to have sufficient protein for down-stream analysis.

DE NOVO SEQUENCING AND MS BLAST

In general, mass spectra are searched against databases using dedicated softwares, such as Mascot and SEQUEST, and X! Tandem that compare peaks in MS spectra with precomputed peptide fragments (Liska and Shevchenko, 2003). However, this approach is not efficient for identifying proteins that are not included in the database. In *de novo* sequencing, peptide sequences are obtained from mass spectra without the assistance of databases either manually or via computer programs (Ma and Johnson, 2012). Sequence homology search engines, such as MS driven BLAST (MS BLAST) have been successfully applied in marine proteomics studies. For example, Shevchenko et al. (2005) identified 48 proteins in larval venom of the Brazilian moth *Cerodirphia speciosa*. Waridel et al. (2007) employed multiple layered integrated search strategy that included MASCOT, *de novo* sequencing and MS BLAST sequence-similarity search to identify proteins in green alga *Dunaliella salina*. Sun et al. (2010) identified 65 proteins from golden apple snail *P. canaliculata* using *de novo* cross-species method. We combined conventional and customized transcriptome databases search with MS-BLAST sequence similarity search and identified 21 proteins from *N. arenaceodentata* (Chandramouli et al., 2012a). The *de novo* sequencing and MS BLAST have shortcomings because they use partial peptide sequences for database search that are very short sequence tags that can be used to search a standard database, such as NCBI nr, SwissProt, Uniprot etc. to identify the homologous peptide.

SPECIES-SPECIFIC GENOME DATABASES

The major limitation in conducting molecular studies at either the genome or proteome level is the lack of genome sequences (López, 2007; Tomanek, 2011; Slattery et al., 2012). For example, in our study we only managed to identify about 200 proteins in *N. arenaceodentata* (Chandramouli et al., 2013a). Several studies have addressed this problem by constructing transcriptome databases of marine species under investigation, which has remarkably improved the quality of proteomics dataset. As more genome information becomes available, proteomics will provide a wealth of information about molecular processes that are associated with larval behavior and morphology.

VALIDATION OF PROTEOMIC DATA

The current proteomic tools were established mainly for model organisms, they need to be selectively optimized for investigating larval proteome. The lack of commercially available species-specific antibodies makes it challenging to experimentally validate proteomics data in functional studies. To overcome this problem, He et al. (2012) generated *B. amphitrite* species-specific p38 antibodies and overexpressed them in *E. coli*. The authors demonstrated activation of Bar-p38 MAPK during larval settlement through co-immunoprecipitating p38 antibodies with interacting partner proteins. He et al. (2013) used the same method to generate antibodies for cement proteins, cp20k, to localize those proteins in barnacle cyprids. Zhang et al. (2012b) also used co-immunoprecipitation and SDS-PAGE-LC-MS/MS to identify the binding proteins of icocyanide in three marine invertebrates. Furthermore, there has been no matured method for bridging the gap between gene/protein expression and larval development and behavior in marine species. RNAi or gene knockouts methods may offer the solution but require substantial effort to develop these methods for marine species.

CONCLUSION

Over the last 7 years, gel-based and gel-free proteomics have been used to study molecular mechanisms of larval attachment and metamorphosis in several species of marine invertebrates. Notably, proteomics studies have provided new tools to systematically document the plasticity and variability of the proteome in larval morphogenesis and behavior as well as the response of larvae to antifoulants and environmental stressors. These larval proteomic investigations have also provided substantial information of protein expression patterns, PTMs, and signaling pathways during larval attachment and metamorphosis, which improved our understanding of possible molecular mechanisms of larval attachment and metamorphosis.

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