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# Comparative Proteome and Phosphoproteome Analyses during Cyprid Development of the Barnacle *Balanus* (=*Amphibalanus*) *amphitrite*

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The barnacle *Balanus amphitrite* (=*Amphibalanus amphitrite*) is a major marine biofouling invertebrate worldwide. It has a complex life cycle during which the larva (called a nauplius) molts six times before transforming into the cyprid stage. The cyprid stage in *B. amphitrite* is the critical stage for the larval decision to attach and metamorphose. In this study, proteome and phosphoproteome alterations during cyprid development/aging and upon treatment with the antifouling agent butenolide were examined with a two-dimensional electrophoresis (2-DE) multiplexed fluorescent staining approach. Optimized protein separation strategies, including solution-phase isoelectric fractionation and narrow-pH-range 2-DE, were used in a proteomic analysis. Our results show that the differential regulation of the target proteins is highly dynamic on the levels of both protein expression and posttranslational modification. Two groups of proteins, stress-associated and energy metabolism-related proteins, are differentially expressed during cyprid development. Comparison of the control and treatment groups suggests that butenolide exerts its effects by sustaining the expression levels of these proteins. Altogether, our data suggest that proteins involved in stress regulation and energy metabolism play crucial roles in regulating larval attachment and metamorphosis of *B. amphitrite.*

**Keywords:** barnacle • IEF sample prefractionation • 2-DE • larval development • larval settlement • proteome • phosphoproteome

## 1. Introduction

Barnacles play an important role in the structure and function of intertidal ecosystems worldwide.[1] Barnacles are also the most dominant group of fouling organisms and thus the major target organism in antifouling research.[2] Like most benthic marine organisms, barnacles have a complex life cycle. After release by the adult into the plankton, the barnacle larvae go through six naupliar stages before transforming into the cyprid stage—the stage at which the larva becomes competent to attach and metamorphose. Once the larvae become competent cyprids, they must search the surrounding substratum and choose a suitable place for attachment and metamorphosis (both processes are often referred to as "settlement").[3−5] Upon encountering a suitable substratum, cyprids attach and metamorphose into juvenile barnacles. Searching behavior is often lengthy whereas attachment and metamorphosis are relatively quick processes. Therefore, it is reasonable to suggest that the "young" cyprids are not initially ready to attach or metamorphose and that biological changes have occurred in the "old" cyprids after the long search for a suitable substratum, making them truly competent to initiate attachment and metamorphosis. These biological changes may act as a "switch" to help cyprids avoid metamorphosis before encountering a suitable substratum.

Although barnacle attachment and metamorphosis are very important and have been studied for decades, we know surprisingly little about the molecular mechanisms involved in these processes. Almost entirely based on pharmacological assays, Clare et al. proposed a model of signal transduction pathways, involving G proteins, cAMP, protein kinases A and C, and other components, that is associated with larval settlement in barnacles; nevertheless, the overall picture of the molecular mechanisms associated with settlement remains blurry.[6−8] In the present study, we used proteomic tools to compare the protein profiles of "young" and "old" cyprids to identify, at the proteomic level, the changes that have occurred in the "old" cyprids to allow them to attach and metamorphose.

In addition, during the search for bioactive compounds that inhibit larval attachment and metamorphosis, we synthesized a potent antifouling butenolide that can inhibit larval attachment and metamorphosis based on the structure-antifouling activity-relationships of a group of butenolides that are naturally produced by two marine bacterial species.[9] High pharmaceutical ratio ($EC_{50}/LC_{50}$) of the synthesized butenolide suggests its low toxicity; a recovery assay showed that barnacle cyprids could attach and successfully metamorphose after the removal of the butenolide. Therefore, butenolide may specif-

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ically interfere with larval attachment and metamorphosis. In this study, we compared the protein profiles of cyprids treated with and without butenolide to determine whether butenolide interferes with larval settlement specifically or through some other intoxication process.

Furthermore, in three previous studies in our laboratory, we successfully used a two-dimensional electrophoresis (2-DE)-based proteomic approach to study the changes in the protein expression profiles in several developmental stages of the barnacle *Balanus* (=*Amphibalanus*) *amphitrite*, the marine polycheate *Pseudopolydora vesillosa*, and the bryozoan *Bugula neritina*.[10−12] In the present study, we used solution-phase isoelectric focusing (IEF) sample prefractionation coupled with narrow-pH-range 2-DE to improve the proteome coverage and to enrich the low-abundance proteins. We attempt to answer the following questions. (1) With improved techniques, can we identify more of the differentially expressed proteins and phosphoproteins that are important in the larval attachment and metamorphosis of *B. amphitrite*? (2) What proteins are involved in the larval attachment and metamorphosis of *B. amphitrite* and what major biological functions do they play? (3) Are phosphoproteins directly involved in regulating larval attachment and metamorphosis?

## 2. Experimental Section

**2.1. Sampling the Larvae.** Figure S1 of Supporting Information outlines the procedures for the *B. amphitrite* larval proteome preparation and analysis of differentially expressed proteins. Adult *B. amphitrite* were collected from the concrete columns of the pier at Pak Sha Wan in Hong Kong (22°21′45″N, 114°15′35″E) and the released naupliar larvae were reared to the cyprid stage, according to Thiyagarajan et al.[13,14] The nauplii developed into cyprids on day 4 when reared on a diet of *Chaetoceros gracilis* Schutt at 28 °C. Once the larvae had reached the cyprid stage, they were collected and divided into three experimental treatments (CON, CON-24, and INH). To collect the newly transformed cyprids (CON), the larval culture was examined every 2 h to ensure that the collected cyprids had just transformed from the naupliar VI stage. These larvae (CON) were immediately frozen in lysis buffer for protein extraction. To study the changes in protein expression during cyprid development and with butenolide treatment, the remaining larvae were divided into two groups: one group of larvae (CON-24) was cultured in filtered seawater for 24 h in the absence of butenolide, and the majority of swimming larvae completed attachment and metamorphosis after 24 h of cyprid development. The other group (INH) was incubated in filtered seawater with 10 $\mu$g mL$^{-1}$ butenolide for 24 h. During this treatment, the larvae did not attach and metamorphose and kept swimming like the 0 h control larvae. After 24 h, the swimming larvae from these two experimental groups were collected by filtration, washed, and frozen in lysis buffer for protein extraction.

**2.2. Protein Sample Preparation.** The larval samples were briefly rinsed several times with filtered seawater, and then placed in lysis buffer consisting of UTC (= 7 M urea, 2 M thiourea, 4% CHAPS) and 40 mM dithiothreitol (DTT). To inhibit any proteolytic or phosphorolytic activity that might occur during the protein extraction, a mixture of protease and phosphatase inhibitors (Roche Applied Science, Mannheim, Germany) was added to the lysis buffer. The samples were then frozen with lysis buffer in liquid nitrogen and stored at −80 °C until use. The larval proteins were solubilized with a sonicator (Branson Digital Sonicator 250, Danbury, CT) using six 1 min blasts of 20% duty cycle at output level 3, with 10 s pauses between blasts. After centrifugation for 15 min at 18 000× *g*, the pellet was discarded and the protein-contained supernatant was transferred to a clean tube. The proteins were reduced by dissolving them in 50 mM DTT, 25 mM Tris (pH 8.0). The reduced proteins were then alkylated by the addition of 100 mM iodoacetamide (IAA) and incubated in the dark for 20 min. To remove the sodium dodecyl sulfate (SDS), IAA, and other contaminants, the protein lysate was cleaned with a ReadyPrep 2-D Cleanup Kit (Bio-Rad, Hercules, CA). The protein concentration was estimated using an RC DC protein assay (Bio-Rad, Hercules, CA) which precludes interference by reducing agents and detergents.

**2.3. In-Solution IEF Fractionation.** The protein pellet (2 mg) was resuspended in 3.25 mL of lysis buffer plus 1% pH 3−10 ampholytes and prefractionated on a Zoom IEF Fractionator (Invitrogen, Carlsbad, CA) using the following five-step program: S1, 100 V for 30 min; S2, 200 V for 1 h; S3, 600 V for 1 h; S4, 800 V for 1 h; and S5, 1000 V for 2 h. Electrophoresis was carried out at constant power (10 W) and limiting current (10 mA) using a PowerPac HV Power Supply (Bio-Rad, Hercules, CA). Five fractions, F1−F5 (p*I* ranges: F1, 3.0−4.6; F2, 4.6−5.4; F3, 5.4−6.2; F4, 6.2−7.0; and F5, 7.0−10.0), were generated using this approach. After separation, a 16 $\mu$L aliquot of each fraction was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to check the fractionation efficiency. F1 and F2 were combined and F3 and F4 were combined, so that they could be isoelectrically focused on p*I* 3.0−6.0 and p*I* 5.0−8.0 first-dimension strips, respectively. These two combined fractions were precipitated with cold acetone and resuspended in rehydration buffer (UTC, 1% DTT, and 0.5% IPG buffer corresponding to the immobilized pH gradient [IPG] strip used). The solution was thoroughly mixed by vortexing, sonicated for 10 min, and incubated for 1 h at room temperature (24 °C). The solution was then centrifuged at 21 000× *g* for 15 min to remove any insoluble matter. The supernatants were stored in −80 °C until use. Three independently prepared samples were analyzed for each development stage or treatment to obtain statistically reasonable results.

**2.4. 2-DE.** First-dimensional electrophoresis was performed on a Protean IEF Cell (Bio-Rad, Hercules, CA). The two combined fractions were individually loaded onto IPG strips (Bio-Rad, Hercules, CA) corresponding to their p*I* values. The strips were rehydrated in the passive mode for 30 min, followed by 15 h of active rehydration (50 V, 20 °C). Isoelectric focusing was performed using the following program: 250 V for 20 min, 1000 V for 2 h, and with a gradient of 10 000 V for a total of 65 000 Vh. After focusing, the IPG strips were equilibrated for 15 min in equilibration buffer I (6 M urea, 2% SDS, 0.05 M Tris-HCl [pH 8.8], 50% glycerol, and 2% [w/v] 1,4-DTT) followed by 20 min in buffer II (same as equilibration buffer I except containing 2.5% IAA instead of DTT). The equilibrated IPG strips were loaded on the top of SDS-12.5% polyacrylamide gel (18 × 18 cm$^2$) and sealed with 0.5% (w/v) agarose. The second-dimensional gel electrophoresis (SDS-PAGE) was performed on a Protean II XL Multi-Cell electrophoresis apparatus (Bio-Rad, Hercules, CA). The running buffer system was the standard Laemmli buffer for SDS-PAGE. The gels were run at 16 mA gel$^{-1}$ and then at 24 mA gel$^{-1}$ once the bromophenol blue had passed through the agarose/acrylamide interface and stacked as a thin

layer. The electrophoresis usually lasted for approximately 8 h until the bromophenol blue reached the bottom of the gel.

**2.5. Multiplexed Gel Staining and Detection of Proteins.** All the gels were sequentially stained with a modification of the procedure of Thiyagarajan et al.[12] In brief, the gels were fixed in 50% methanol and 10% acetic acid. For phosphoprotein staining, the gels were incubated with Pro-Q Diamond fluorescent stain (Molecular Probes, Eugene, OR), followed by three rounds of destaining with 4% acetonitrile in 50 mM sodium acetate (pH 4.0). The gels were visualized with a 532 nm excitation laser and a 560 nm long-pass filter on a Typhoon Trio imager (GE Healthcare Life Sciences, Uppsala, Sweden). Following image acquisition, the gels were stained for total protein with Sypro Ruby fluorescent dye (Molecular Probes, Eugene, OR), destained in 10% methanol and 7% acetic acid, and scanned with a 582 nm excitation laser and a 610 nm 30 bandpass emission filter on a Typhoon Trio imager. For spot excision and subsequent mass spectrometry (MS) analysis, the gels were visualized with the modified G-250 Colloidal Coomassie Blue (CCB) staining method described by Schägger.[15] The resulting 2-DE protein patterns were obtained at 100 dpi resolution with a Molecular Imager GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA).

**2.6. Image Analysis.** The scanned gels were analyzed with the PDQuest Advanced software version 8.0 (Bio-Rad, Hercules, CA), according to the manufacturer's protocol. Automatic spot detection in each gel was verified by visual inspection. Landmarking was used to align the images, so that corresponding proteins were matched to each other. The spot intensities were normalized to equalize the total densities of each gel image. A multichannel viewer was used to merge the information from the Pro-Q-Diamond- and Sypro-Ruby-stained images, so that the types and levels of fluorescence in each individual gel could be distinguished. The analysis was performed in quantitative and qualitative modes. Changes in the normalized protein spot volumes in the proteomes and phosphoproteomes were compared between the experimental groups to determine the differential protein expression and phosphorylation during cyprid development and after butenolide treatment. Differentially expressed or phosphorylated protein spots with a change in protein abundance of at least 1.5-fold were detected by comparing the normalized spot volumes between each of the two experimental groups and were recorded as up- or down-regulated proteins. Student's $t$ test ($p < 0.01$, $n = 3$) was used in all of these comparisons.

**2.7. Trypsin Digestion and Mass Spectrometric Analysis.** We subjected 21 differentially regulated protein spots to identification using mass spectrometry. The spots were excised and digested according to the method described by Shevchenko et al.[16] Briefly, the gel plugs were washed for 4 h with 50% MeOH/50 mM $NH_4HCO_3$ and twice with $H_2O/CH_3CN$ (1:1, v/v), and were then dehydrated in 100% $CH_3CN$. The gel pieces were dried in a SpeedVac centrifuge (Thermo Electron, Waltham, MA) for several minutes before the addition of 20 $\mu$L of 10 ng/$\mu$L Trypsin Gold (Promega, Madison, WI) in 50 mM $NH_4HCO_3$ buffer. The gel pieces were covered with the digestion solution and allowed to stand on ice for 30 min. After rehydration with the enzyme solution, the excess digestion buffer was removed and the digestion was allowed to proceed at 37 °C for 16 h. The peptides were extracted using several volumes of $H_2O$/$CH_3CN$/trifluoroacetic acid (80:20:1, v/v/v). These fractions were pooled, dried in a vacuum centrifuge, and then redissolved in 20 $\mu$L of 5% (v/v) formic acid. The digests were cleaned with ZipTip (Millipore, MA) and subjected to analysis on a matrix-assisted laser desorption/ionization (MALDI) -time-of-flight/time-of-flight (TOF/TOF) tandem mass spectrometer (Autoflex III series, Bruker Daltonik, Bremen, Germany) and an electrospray ionization (ESI) -quadrupole time-of-flight (QqTOF) mass spectrometer (QSTAR XL, Applied Biosystems/Sciex, ON, Canada).

**2.8. Analysis of Peptide Sequences.** For the spots subjected to MALDI-TOF/TOF identification, PMF and MS/MS information obtained from the MS analysis was automatically submitted through MASCOT server (version 2.2.04, Matrix Science) to the NCBI nonredundant (NCBInr), MSDB, SwissProt databases and a customized *B. amphitrite* database (unpublished transcriptomic sequencing results from our laboratory). Mass searches were performed using mass tolerance settings of 75 ppm for the peptide precursor ions and 0.2 Da for the fragment ions. Oxidation at methionine residues and carbamidomethylation at cysteine residues were set as variable and fixed modifications, respectively, and one missed trypsin cleavage was allowed. For the spots subjected to ESI-QqTOF identification, the data were submitted through a locally installed MASCOT program to the MSDB database (3 239 079 sequences entries; updated September 2006) or the *B. amphitrite* database. The following parameters were used for the database search: mass tolerance settings of 0.2 Da for the peptide precursor ions and 0.5 Da for the fragment ions; fixed modification: carbamidomethyl (cysteine); variable modification: oxidation (methionine); and one tolerant missed cleavage. The Mowse score threshold for proteins was suggested by matching scores at $p < 0.05$.

**2.9. Western-Blot Analysis.** Patterns of regulation of HSP90 as suggested by the 2DE were confirmed with Western blotting. Different experimental samples (CON, 0 h cyprid; CON-12, 12 h cyprid; CON-24, 24 h cyprid and INH, 24 h treatment) were collected as described formerly and lysed in NP-40 lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 1% NP-40 in the presence of protease inhibitors). The total protein concentration was estimated using a Bradford protein assay (Bio-Rad, Hercules, CA). Equal amount of lysates (60 $\mu$g/lane) were separated on 12% SDS-PAGE and transferred onto a Hybond ECL nitrocellulose membrane (Amersham, Buckinghamshire, UK). After blocking, the membranes were incubated for 3 h at room temperature or overnight at 4 °C with antibodies of anti-Hsp 90 (Cell Signaling, Danvers, MA) and anti-Actin (Millipore, Temecula, CA) at a dilution of 1:1000. The membranes were incubated with horseradish peroxidase-conjugated secondary antibody at a dilution of 1:5000 for 1 h and developed using ECL Westernblotting analysis system (Millipore, Billerica, MA).

**2.10. Caspase-3 Activity Assay.** To examine whether endogenous stress level and the resulting apoptosis activity are changing during cyprid development/aging, the caspase-3 activity of different experimental groups (naupliar VI, 0 h cyprid, 12 h cyprid, 24 h cyprid, and 24 h treatment) were analyzed using a FRET (fluorescence resonance energy transfer)-based caspase-3 sensor provided by Prof. Donald C. Chang (HKUST).[17] This sensor C3 contains two fluorescent proteins (CFP and YFP) with the caspase-3 cleavage sequence DEVD in between. Once caspase-3 is activated, sensor C3 will be cleaved into separated CFP and YFP molecules. Therefore, the energy transfer will be abolished and the monomeric CFP or YFP can be detected. Due to the autofluorescence of the barnacle samples, we utilized Western-Blot analysis instead of fluorescence measurement to detect the cleavage of sensor C3. Sensor-

C3 stably expressed HeLa cells (S3) and different experimental groups of barnacle were harvested and lysed with NP40 lysis buffer. Five micrograms of S3 protein lysate was mixed with 60 $\mu$g of protein lysate of different experimental groups, and incubated at 37 °C for 1 h. After reaction, the lysates were subjected to Western-blot analysis and the intact and cleaved sensors were detected by anti-GFP primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and horseradish peroxidase-conjugated secondary antibody.

## 3. Results and Discussion

**3.1. Optimization and Improvement of Protein Separation Using Solution-Phase IEF Sample Prefractionation.** In this study, high-quality and reproducible phosphoproteome and proteome maps were obtained after evaluating different IEF methods before the second-dimension SDS-PAGE. Our results show that although reproducible reference proteome maps could be obtained when purified protein samples were directly focused on broad-range p*I* 3−10 first-dimension IPG strips (Figure S2A of Supporting Information), increased background noise was observed when the protein loading was higher than 500 $\mu$g strip$^{-1}$, thus hampering the detection and subsequent analysis of low-abundance protein spots. Two strategies were used to increase the sample loading capacity and reduce the sample complexity: (1) direct focusing of the unfractionated samples on narrow-range strips (p*I* 3−6 or p*I* 5−8); (2) sample prefractionation based on the p*I* value (p*I* 3.5−5.4 or p*I* 5.4−7.0), followed by sample focusing using the corresponding narrow-range strips (p*I* 3−6 or p*I* 5−8, respectively). Compared with the IEF prefractionation approach, there are some obvious drawbacks in focusing unfractionated samples on narrow-range strips. For example, sample cross contamination caused by incorrect focusing and the comigration of proteins with different p*I* values, limited sample loading, and limited dynamic range.[18,19] In contrast, the combination of IEF prefractionation, 2-DE, and multiplexed fluorescent staining produced protein maps with well-resolved spots. Furthermore, more low-abundance protein spots were detected using this approach. As shown in Figure S2B and C (Supporting Information), the numbers of protein spots detected and their separation and resolution were greatly improved over that shown by Thiyagarajan et al,[10,12] which detected just 400 spots in cyprid larvae of the barnacle *B. amphitrite* on a p*I* 3−10 gel. Similarly, using whole larvae and broad range techniques, approximately 480 spots have been detected in competent larvae of the polychaete *Hydroides elegans* (unpublished results) and about 450 spots in two-day-old coral larvae.[20] In this study, around 580 spots were reproducibly resolved in all of our cyprid samples using the prefractionation approach. This optimized 2-DE protocol has also been successfully used to separate the protein samples of another sessile marine invertebrate, the bryozoan *Bugula neritina* (unpublished results). Thus, our work demonstrates that solution-phase IEF sample prefractionation coupled to narrow-pH-range 2-DE is more successful in providing high-resolution proteome maps from complex, whole larvae samples than are traditional methods.

**3.2. Comparative Proteome and Phosphoproteome Analyses of "Young", "Old", and Butenolide-Treated Cyprids.** The optimized protocol was used to investigate developmentally- or aging-regulated proteins (comparison between the CON and CON-24) and proteins that are responsive to butenolide treatment (comparison between the CON, CON-24, and INH). Representative 2-DE gels of the three sample groups stained with Pro-Q Diamond and then with Sypro Ruby fluorescent dyes are shown in Figures 2 and 1, respectively. We reproducibly detected 582, 587, and 570 total protein spots in the CON, CON-24, and INH experimental groups, respectively (Figure 1D). Furthermore, only very few spots appeared or disappeared among the three experimental groups, indicating that there were no significant changes in the total protein expression of CON to CON-24 development or after butenolide treatment, supporting the hypothesis of Carpizo Ituarte and Hadfield[21] that larval metamorphosis in marine invertebrates may not require extensive *de novo* protein synthesis. Similar to the proteome gels, the number of phosphoprotein spots detected was similar between all experimental treatments: 203, 221, and 215 phosphoprotein spots were detected in the CON, CON-24, and INH experimental groups, respectively, accounting for 35−38% of the total protein in the corresponding groups (Figure 2D). Thus, the overall protein expression and phosphorylation are relatively constant during cyprid development and after butenolide treatment.

**3.3. Dynamic Changes in Protein Expression and Phosphorylation in Response to Cyprid Development and Butenolide Treatment.** To detect the numbers of differentially regulated proteins (including both qualitative and quantitative changes) during the cyprid development and upon the treatment of butenolide, the normalized spot volumes were compared between each of two experimental groups (CON/CON-24, CON-24/INH, and CON/INH). The numbers of proteins showing up/down-regulated expression (Figure 1E and F) or phosphorylation (Figure 2E and F) in these comparisons were calculated using PDQuest software. Both up- and down-regulation of proteins and phosphoproteins were detected in the "old" cyprids when compared to the "young" cyprids. Likewise, proteins and phosphproteins regulations were also observed in the butenolide-treated cyprids compared to the untreated cyprids (Figure 1 and 2).

To develop a more comprehensive understanding of how these differentially regulated proteins respond dynamically to cyprid development/aging or butenolide treatment, the differential expression and phosphorylation state of each target spot were analyzed by comparing their intensities among the three experimental groups. Twenty-one representative spots, which showed interesting patterns of differential regulation and were sufficiently abundant, were selected from different areas of the proteome images for further analysis (Figure 3). Their detailed differential expression patterns are shown in Figure 4. Except for spots 14 and 21, most of the proteins were down-regulated during development from CON to CON-24. This result was consistent with those of our previous studies, which also showed the down-regulation of protein expression during larval attachment and metamorphosis in two marine fouling invertebrates, the *Balanus amphitrite* and *Bugula neritina*.[10,12] Interestingly, after 24 h of butenolide treatment (INH), the expression levels of most of these proteins were not significantly down-regulated compared to the control. Therefore, we suspected that the down-regulation of these proteins is associated with the aging of the larvae and could be necessary for *B. amphitrite* cyprids to become fully competent and may be potential target proteins for antifouling compounds. In contrast, the expression levels of spots 14 and 21 were up-regulated during cyprid development. Once again, butenolide treatment prohibited the developmental regulation of these proteins; these two spots were not significantly up-regulated after butenolide treatment (INH). Since the expression levels of all

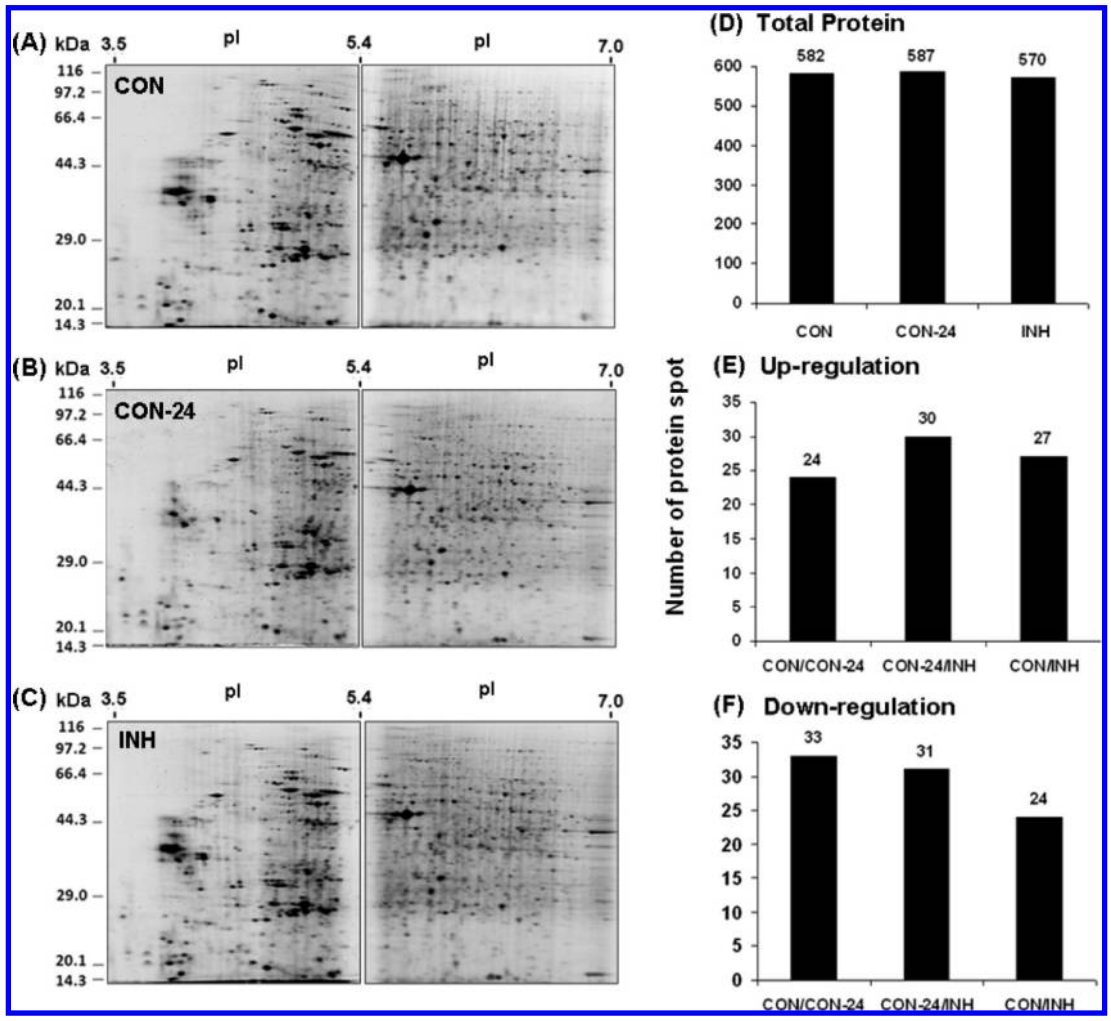


**Figure 1.** Representative total protein gels of the three experimental groups (A, B, and C) and the corresponding comparative analytical results (C, D, and E). Left panels: 2-DE gel stained with the total protein fluorescent dye, Sypro Ruby. Zero hour control swimming cyprid (A), 24 h control swimming cyprid (B), and 24 h inhibitor-treated swimming cyprid (C). Right panels: total numbers of protein spots reproducibly detected in the different experimental groups (D), numbers of up-regulated (E), and down-regulated (F) protein spots during development (CON/CON-24) or treatment with butenolide (CON/INH and CON-24/INH) of *B. amphitrite*. Student's *t* test ($p < 0.01$, $n = 3$).

the spots selected were more similar between the CON and INH groups than they were to the CON-24 group, we propose that butenolide may exert its effects by prohibiting the developmental regulation of expression level of these proteins and thus arrest development of the cyprid to the fully competent stage.

Of the 21 differentially regulated spots chosen for analysis, 6 were also differentially phosphorylated (spots 8−13, Figure 4 C). The two areas of the phosphoproteome images containing these 6 spots were magnified in Figure 5A and B and the phosphoprotein intensities and the phosphoprotein/protein intensity ratios of the spots are shown in Figure 5C and D. The down-regulation of phosphoprotein spots 10, 11, and 12 was observed in both the CON-24 and INH groups; this phosphorylation change is likely attributable to cyprid development or aging, rather than to compound treatment. Conversely, the phosphoprotein intensities of spots 8, 9, and 13 were down-regulated during cyprid development, but were up-regulated in the INH group compared to the levels in the CON-24 group, suggesting that the down-regulation of these phosphoproteins was inhibited by butenolide treatment. Although the mode of regulation of these phosphoproteins seems to be similar to that of the total proteins, it is possible that changes observed in the phosphoprotein intensities between treatments might be largely explained by the change in total protein expression. To determine whether the phosphorylation state of these proteins changed in response to cyprid development/aging or butenolide treatment, we examined the ratio of phosphoprotein to protein intensities for each spot. A change in the proportion of the phosphoprotein intensity in the treatments compared to the control suggests that the protein was de/phosphorylated during treatment. As shown in Figure 5D, the phosphoprotein/protein intensity ratios of spots 10 and 11 were relatively similar during cyprid development but were reduced in the INH group. This suggests that butenolide treatment leads to dephosphorylation of these two proteins and that maintenance of the phosphorylation level of these proteins may be important for larval attachment and metamorphosis. Both the protein and phosphoprotein intensities were down-regulated during cyprid development/aging and were sustained with butenolide treatment in protein spot 8, but the proportion of phosphorylation in CON-24 and INH increased compared to the CON group. This suggests that this protein was phosphorylated; moreover,

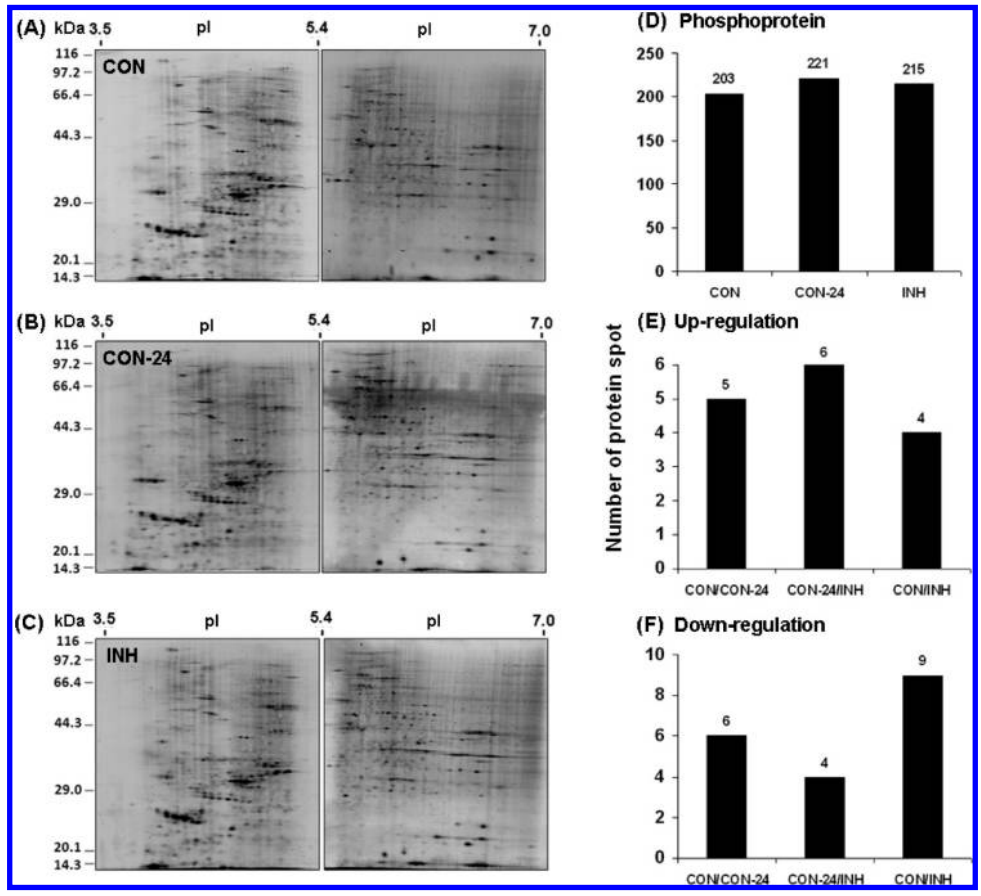


**Figure 2.** Representative phosphoprotein gels of the three experimental groups (A, B, and C) and the corresponding comparative analysis results (C, D, and E). Left panels: 2-DE gel stained with the phosphoprotein-specific fluorescent dye, Pro-Q Diamond. Zero hour control swimming cyprid (A), 24 h control swimming cyprid (B), and 24 h inhibitor-treated swimming cyprid (C). Right panels: numbers of phosphoprotein spots reproducibly detected in the different experimental groups of *B. amphitrite* (D), numbers of up-regulated (E) and down-regulated (F) phosphoprotein spots during development (CON/CON-24) or treatment with butenolide (CON/INH and CON-24/INH). Student's *t* test ($p < 0.01$, $n = 3$).

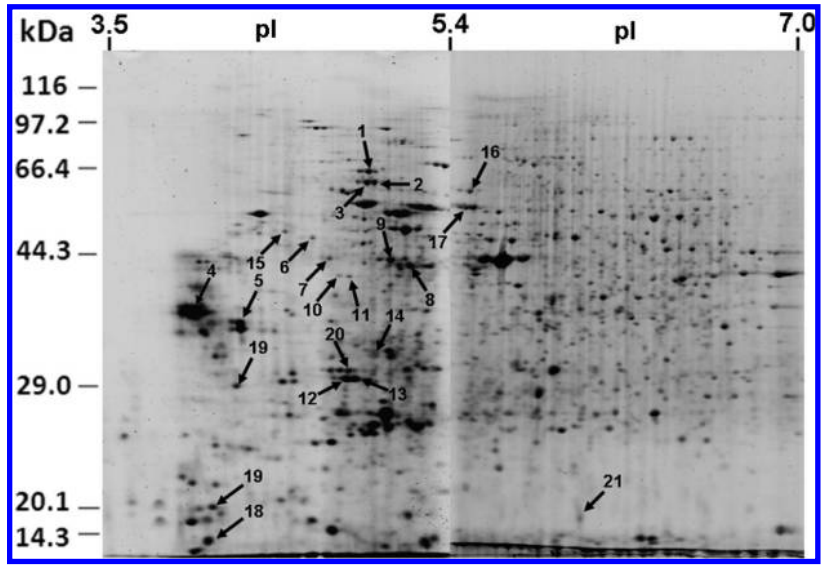


**Figure 3.** Combined (pH 3.5−5.4 and pH 5.4−7.0) image of a Sypro-Ruby-stained 2-DE gel of swimming *B. amphitrite* larvae. Protein spots marked with an arrow were selected for MALDI-TOF/TOF or ESI-QqTOF MS analysis. The spots selected for identification are numbered, but only the spots successfully identified with mass spectrometric analysis are listed in Table 1.

butenolide did not significantly interfere with phosphorylation of this spot (the proportion of phosphorylation was similar between the CON-24 and INH groups). There was no significant difference in the ratios of phosphoprotein/protein intensities between the CON, CON-24, and INH groups for any of the remaining spots, indicating that the phosphorylation state of these proteins remain constant during cyprid development and are not affected by butenolide treatment.

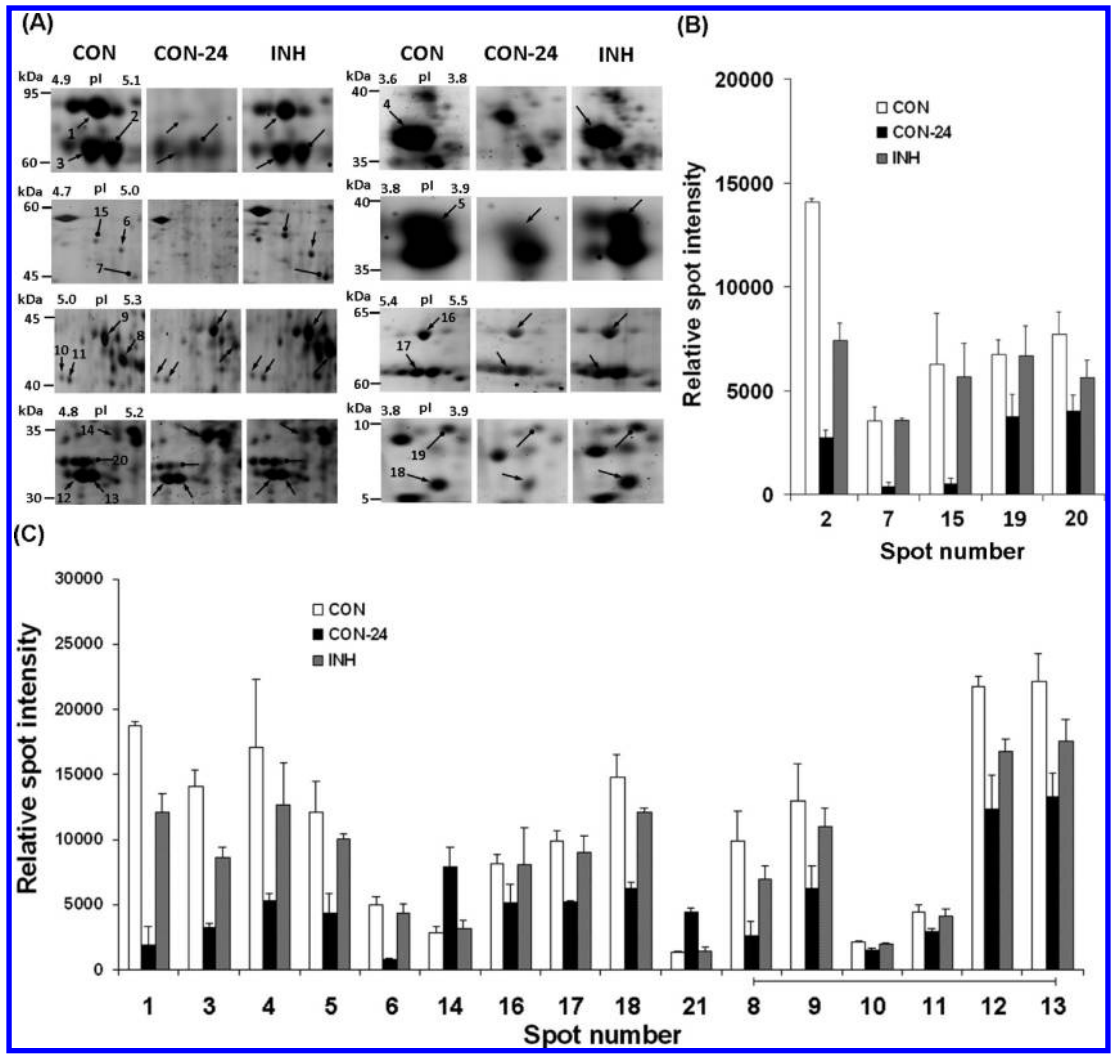


**Figure 4.** (A) Close view of the selected total proteins (indicated by arrows) regulated during cyprid development and affected by the inhibition of metamorphosis induced by butenolide treatment in *B. amphitrite*. Eight different areas in the high-resolution Sypro-Ruby-stained 2-DE gel were selected for each control stage (CON and CON-24) and inhibition treatment (INH). The identified spots are marked with a sharp arrow tip and the unidentified spots are marked with a round arrow tip. Graphical representation of the differentially expressed proteins that were (B) not identified and (C) identified by mass spectrometric analysis. Except for spot 10 (significantly altered at the phosphorylation level), all the spots were shown to be statistically different, with more than 1.5-fold variation (according to quantitative analysis with the PDQuest software). The spots underlined with the short line in C (spots 8−13) also showed differential phosphorylation.

The differential regulation patterns revealed in this study can be summarized into three categories. First, when the protein regulation (expression and/or phosphorylation) levels were similar in the CON and INH groups but different in the CON-24 group, we suggest that the differential regulation of this protein was associated with cyprid development/aging to a competent stage, and butenolide might sustain its expression/phosphorylation level. The expression regulation of most of the proteins in this study fell into this category. Second, if the protein expression/phosphorylation levels were similar in the CON and CON-24 groups but different in the INH group, we propose that the maintenance of the expression/phosphorylation level of this protein might be crucial for cyprid development and subsequent attachment and metamorphsis, and that butenolide might inhibit larval attachment and metamorphosis by changing this protein's regulation level. In this study, the phosphorylation regulation of spots 10 and 11 fell into this category. Finally, when a protein's expression/phosphorylation levels were similar in the CON-24 and INH groups but differ from those in the CON group, we consider that its regulation only responded to cyprid development and aging, and might not be affected by butenolide treatment. Using this comparative setup, we demonstrated that the differential regulation of the candidate target proteins was highly dynamic and may involve regulation at both the protein expression and phosphorylation levels. It is therefore important to study the differential expression and posttranslational modification of individual proteins. By comparing the expression levels of individual spots in different experimental groups, the target spots potentially involved in cyprid development/aging and the cyprid response to butenolide treatment were successfully screened for subsequent MS analysis.

**3.4. Identification of Differentially Regulated Proteins.** To better understand the differentially regulated proteins that might be crucial for *Balanus amphitrite* cyprids to develop to a stage capable of attachment and metamorphosis, representative spots whose expression or phosphorylation was reproducibly up- or down-regulated among the experimental groups

**Table 1.** Identification of Representative Proteins and Phosphoproteins That Are Differentially Expressed or Phosphorylated during Cyprid Development or during the Inhibition of Metamorphosis by Butenolide Treatment in *B. amphitrite*[a]

| spot no. | protein name | acc. no. | Mascot score (peptide matched) | sequence coverage (%) | $M_r$ (kDa)/p*I* | | expression fold change (phosphorylation fold change) | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | theoretical | experimental | CON/CON-24 | CON/INH | CON-24/INH |
| 1* | Chitin binding protein | Contig15426_12 | 117 (2) | 5 | 62.8/5.0 | 63.0/5.0 | –6.55 | –1.45 | 4.52 |
| 3# | BcDNA. GHO4637 | Q7KK51_DROME | 72 (9) | 15 | 74.3/7.9 | 64.5/5.0 | –4.33 | –1.64 | 2.65 |
| 4# | Vesicle-associated MP | gi/149053025 | 76 (5) | 58 | 13.0/8.0 | 39.5/4.1 | –3.23 | –1.35 | 2.38 |
| 5# | Chromosome 17 SCAF 15006 | Q4RQ97_TETNG | 87 (15) | 15 | 112.5/8.6 | 37.5/4.3 | –2.77 | –1.19 | 2.32 |
| 6# | Molybdenum cofactor sulfurase | gi/91077036 | 76 (11) | 18 | 88.1/6.4 | 48.3/4.7 | –6.20 | –1.17 | 5.32 |
| 8# | Oxidation resistance isoform | gi194306543 | 89 (15) | 23 | 94.3/5.1 | 42.3/5.2 | –3.79 (–2) | –1.41 (1.04) | 2.69 (2.07) |
| 9* | Hsp70 | T45473 | 52 (2) | 4 | 51.6/5.8 | 43.5/5.1 | –2.08 (–2.02) | –1.18 (1.04) | 1.76 (2.11) |
| 10* | Hsp90 | Q6TL18 DICLA | 189 (3) | 5 | 83.2/5.0 | 42.5/4.8 | –1.44 (–1.55) | –1.07 (–1.59) | 1.34 (–1.03) |
| 11* | Hsp90 | Q6TL18 DICLA | 125 (2) | 5 | 83.2/5.0 | 42.5/4.9 | –1.49 (–1.61) | –1.08 (–1.57) | 1.38 (1.02) |
| 12* | Tyrosine monooxygenase | Q2F637_BOMMO | 136 (4) | 7 | 28.2/4.9 | 29.0/4.9 | –1.76 (–1.55) | –1.30 (–1.40) | 1.36 (1.14) |
| 13* | Tyrosine monooxygenase | Q2F637_BOMMO | 144 (3) | 11 | 28.2/4.9 | 29.0/5.0 | –1.68 (–2.15) | –1.26 (–1.32) | 1.33 (1.63) |
| 14# | Actin | gi/157125380 | 280 (11) | 37 | 41.8/5.3 | 35.2/5.1 | 2.78 | 1.12 | –2.48 |
| 16* | ATP synthase beta subunit | Q2HZD8_PINFU | 67 (3) | 7 | 56.6/5.4 | 59.0/5.5 | –1.57 | –1.01 | 1.56 |
| 17* | mtHsp60 | Q6RFF9_9CNID | 138 (2) | 4 | 62.8/5.3 | 58.5/5.4 | –1.89 | –1.10 | 1.71 |
| 18# | Protein S100A10 | S10AA_BOVIN | 62 (4) | 68 | 11.2/6.8 | 11.2/4.0 | –2.37 | –1.23 | 1.93 |
| 21* | Arginine kinase | Q3HLS9_9HYME | 98 (2) | 14 | 18.7/4.6 | 18.6/5.9 | 3.42 | 1.12 | –3.06 |

[a] Spot number, protein name, MSDB, NCBInr, Swiss-Prot database accession number or Barnacle database contig number, MASCOT score (number of peptides matched), sequence coverage, experimental and theoretical molecular weight ($M_r$)/isoelectric point (p*I*), and the protein expression/phosporylation fold change between each comparison (CON/CON-24, CON/INH, and CON-24/INH) are indicated. Spots identified by MALDI-TOF/TOF and ESI-QqTOF are marked as # and *, respectively. Spot #1 was matched to one contig from our own unpublished cDNA library of *B. amphitrite*.

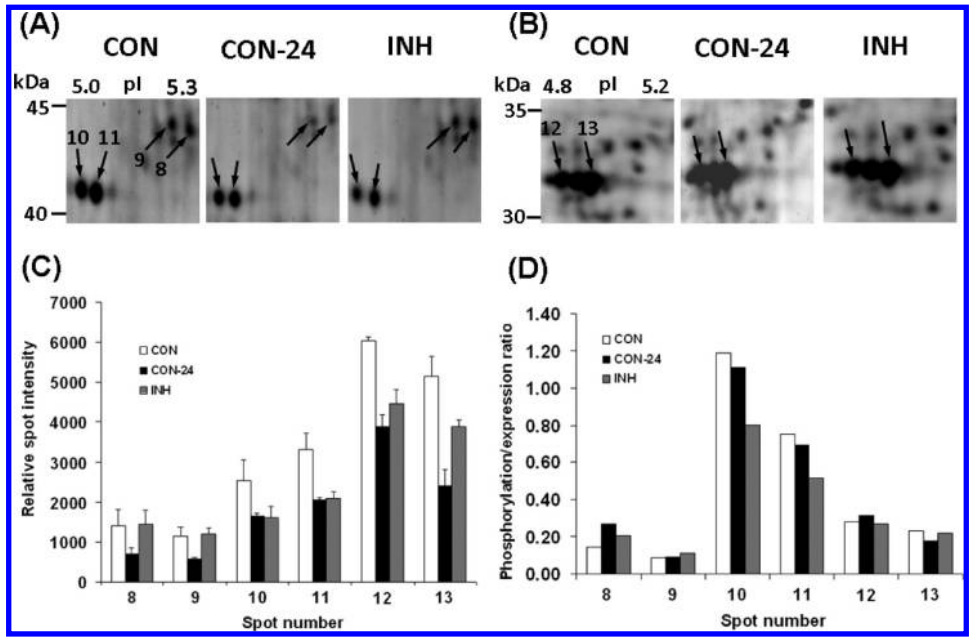


**Figure 5.** (A and B) Close view of selected phosphoproteins (indicated by arrows) regulated during cyprid development and affected by the inhibition of metamorphosis induced by butenolide treatment in *B. amphitrite*. Two different areas in the high-resolution Pro-Q-Diamond-stained 2-DE gel were selected for each control stage (CON and CON-24) and the inhibition treatment (INH). Graphical representation of (C) representative differentially regulated phosphoproteins and (D) the phosphoprotein-to-protein intensity ratios. All the spots were shown to be significantly different between treatments, with changes of more than 1.5-fold (according to quantitative analysis with the PDQuest software).

were analyzed by MALDI-TOF/TOF and ESI-QqTOF MS. Because the differentially regulated proteins detected by fluorescent staining cannot be completely visualized with CCB stain, we subjected only 21 differentially expressed spots (1.5-fold; $p < 0.01$) that were sufficiently abundant to mass spectrometric protein identification. Sixteen of these 21 differentially regulated spots were identified with confident matching scores ($p < 0.05$). The positions of the identified spots on the 2-DE map and their matching results are presented in Figure 3 and Table 1, respectively.

Because the larval development, attachment and metamorphosis of sessile marine invertebrates are dynamic and complex processes involving tissue remodeling and differentiation as well as a variety of biochemical and physiological changes mediated by differential gene and protein regulation,[22−25] a single target protein unlikely explains this complex process. Instead, it is more reasonable that a number of pathways will be turned on/off during cyprid development/aging and by antifouling treatments. Therefore, rather than interpreting the functions of each of the identified spots, the proteins were grouped by their biological functions to facilitate the discussion of their physiological significance. Although reversible protein phosphorylation is crucial for many biochemical and physiological processes in most organisms, the regulation of the phosphorylation of many proteins has only been studied in a few cases, and their biological and molecular functions might be comprehensible only in specific species or under certain conditions.[26,27] Therefore, the differential regulation of protein phosphorylation and its possible function in the *B. amphitrite* cyprid is not discussed in this study, but warrants in-depth study in future work.

**3.4.1. Stress-Associated Proteins.**

**3.4.1.1. Chaperones: Hsp90, Hsp70, and Hsp60 (Heat Shock Proteins).** As shown in Table 1, spots 10 and 11 were identified as Hsp90, and spots 9 and 17 as Hsp70 and mitochondrial 60-kDa heat shock protein, respectively. Many environmental factors influence reproduction, development, and larval settlement and metamorphosis of marine invertebrates. But, how these environmental changes are translated into cellular responses remains unclear in most cases. In addition to being known as stress-responsive proteins, heat shock proteins (HSPs) also act as the 'translator' to transduce the environmental changes in cell signaling pathways.[28−30] HSPs have multifunctional roles and are involved in many physiological processes, such as cell proliferation, development, and the regulation of cell death and survival. HSPs expression can be correlated with resistance to stress, which is largely achieved by a highly conserved and functionally interactive network of chaperone proteins that can rapidly respond to a variety of chemical, environmental, and physiological stresses.[28,31] Hsp60 is expressed in various species in the Porifera and Cnidaria and is considered to be generally important in marine invertebrates.[32] In the red flour beetle *Tribolium castaneum*, Mahroof et al. observed that the expression of the HSP 70 gene was greatly reduced in pupae just prior to metamorphosis.[33] Similarly, the expression of several HSP proteins was the lowest in the oyster larvae before they attach and metamorphose into spats.[34] In our study, we observed the same trend of lower expression level of HSP proteins in the "old" larvae that were just about to attach to a substratum than in the "young" larvae. HSP expression in the INH group was higher than that in the CON-24 group, suggesting that butenolide treatment might introduce some sort of stress to the developing larvae and consequently induces the up-regulation of these antistress proteins. The Western-Blot analysis using anti-Hsp90 antibody was conducted to verify the differential regulation results from 2-DE. As shown in Figure 6, the expression of Hsp90 beta was relatively constant during cyprid development/aging and butenolide treatment. In contrast, the Hsp90 alpha expression was down-regulated during cyprid development/aging and was sustained in butenolide treatment, which correlates well with our proteomic data.

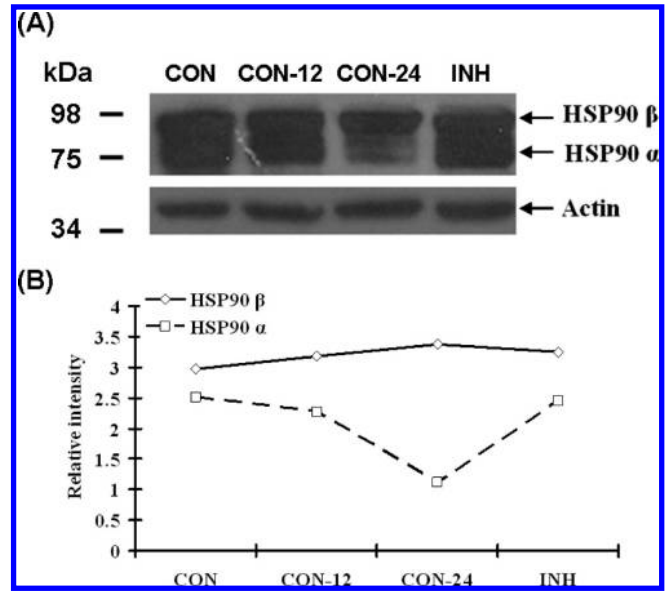


**Figure 6.** Western-Blot analysis of Hsp90 expression in different experimental groups of *B. amphitrite* (A). The positions of Hsp90 alpha and Hsp90 beta were indicated by arrows. Actin served as loading control of the analysis. The relative expression intensity of Hsp90 alpha and Hsp90 beta in different experimental groups (B). Open diamonds, Hsp90 beta; open boxes, Hsp90 alpha. The total protein concentration was determined by Bradford Protein Assay. Equal amount of protein (60 $\mu$g) was loaded into each sample lane and separated on 12% SDS-PAGE. CON, 0 h control swimming cyprid; CON-12, 12 h control swimming cyprid larvae; CON-24, control swimming cyprid larvae; INH, 24 h butenolide treatment.

**3.4.1.2. Transferase: Tyrosine 3-Monooxygenase Zeta Polypeptides (14-3-3 zeta).** Protein spots 12 and 13 were identified as isoforms of 14-3-3 protein that is involved in important cellular processes such as signal transduction, cell-cycle control, apoptosis, and stress response. The expression of certain isoforms of 14-3-3 protein is stress-regulated and it is well documented that this family of proteins has a major function in the control of apoptosis.[35,36] In this study, the expression of the zeta polypeptides of tyrosine 3-monooxygenase was down-regulated during the 24 h of cyprid development. This result is consistent with the expression changes in the HSPs during the same period of development and supports our argument that stress levels might be reduced during the cyprid development of *B. amphitrite*. Similar to the differential regulation of the HSPs by butenolide treatment, the expression levels of 14-3-3 protein in the INH group were up-regulated compared to those of the CON-24 group, indicating that 14-3-3 protein is probably involved in a stress-adaptive strategy.

**3.4.1.3. Antioxidant Proteins: Vesicle-Associated Membrane Protein (VAMP2) and Oxidation Resistance Isoform 1 (OXR1).** Spots 8 and 4 were identified as isoforms of OXR1 and VAMP2, respectively. The former is encoded by the *OXR1* gene in *Homo sapiens* and is considered a stress-response protein that is involved in protection against oxidative damage.[37] VAMP2 belongs to the synaptobrevin family and is one of the main components of a protein complex involved in the targeting and/or fusion of transport vesicles to their target membrane. Recently, it has been reported that the overexpression of the *Arabidopsis VAMP* gene can rescue plant and yeast cells from toxic concentrations of reactive oxygen species (ROS).[38] As indicated in Figure 4, the expression of both spots 8 and 4 was

down-regulated during the 24 h of cyprid development, which suggests a reduction in ROS and stress levels during development.

**3.4.1.4. Other Stress-Related Proteins: Molybdenum Cofactor Sulfurase 3 (MoCo Sulfurase 3) and S100 Calcium-Binding Protein A10 (S100A10).** Spots 18 and 6 were identified as the S100 calcium-binding protein A10 and molybdenum cofactor sulfurase 3, respectively. S100 protein belongs to the EF-hand $Ca^{2+}$-binding protein family and regulates a variety of cellular processes by interacting with different target proteins,[39] whereas MoCo sulfurase 3 sulfurates the molybdenum cofactor and is involved in the Mo-molybdopterin cofactor biosynthetic process.[40] Importantly, the expression levels of these two proteins increase in response to a variety of stresses. In our study, both the proteins were down-regulated during cyprid development, which might indicate a low level of stress experienced by larvae during this period. Interestingly, both the proteins were up-regulated in the INH group compared to the CON-24 group, suggesting that butenolide might sustain heir expression as an adaptive strategy to suppress stress levels. Therefore, our results indicate higher levels of stress in the INH group, which is consistent with the conclusions drawn from the differential expression of the HSPs, 14-3-3, and antioxidant proteins.

In summary, in the barnacle *B. amphitrite*, the expression of several groups of stress-associated proteins, including HSPs, 14-3-3 proteins, VAMP2, OXR1, MoCo sulfurase 3, and S100A10, was down-regulated during cyprid development. These results are somewhat surprising because it is generally believed that larvae experience higher stress when they are approaching metamorphosis competence and attachment. However, in the group treated with butenolide, the expression levels of all these proteins were similar to those in the 0 h control and were significantly higher than those in the 24 h control. To examine whether endogenous stress level and the resulting apoptosis activity are changing during cyprid development/aging, we employed a FRET-based caspase-3 sensor to detect the activity of caspase-3 in different experimental groups of barnacle. As shown in Figure S3 of Supporting Information, the caspase 3 activity decreased during cyprid development/aging, but was partially sustained by butenolide treatment. This result suggests the reduced apoptosis activity during development and is also in a good agreement with the differential expression of stress-regulated proteins. Although butenolide might exert its effects by maintaining the expression of these proteins, it is also probable that the expression of stress-related proteins is altered to overcome or balance the stress cause by the butenolide treatment. Either way, growth and development of the larvae is arrested by butenolide and their larval attachment behavior is inhibited.

**3.4.2. Proteins Related to Energy Production.**

**3.4.2.1. ATP Synthase/ATPase Beta Subunit.** Spot 16 was identified as the beta subunit of ATP synthase/ATPase, which is a ubiquitous membrane enzyme that plays a key role in biological energy metabolism. This enzyme is also called $F_0F_1$-ATP synthase or $F_0F_1$-ATPase and synthesizes cellular ATP from ADP and inorganic phosphate. However, this enzyme also catalyzes the reverse reaction and generates ADP by physically rotating its own subunit.[41] In our study, the expression of this subunit was down-regulated during cyprid development and was more strongly expressed in the INH group than in the CON-24 group. It has been reported that several energy metabolism pathways, including the creatine kinase pathway (CK system) and the arginine kinase pathway (ArgK system), act complementarily as a temporal energy buffer under specific conditions in a variety of biological systems.[42] Therefore, we suggest that the differential regulation of ATP synthase during cyprid development and during butenolide treatment is attributable to changes in the energy-utilization/production system in *B. amphitrite*.

**3.4.2.2. Arginine Kinase.** Arginine kinase (ArgK), a phosphagen kinase that plays an important role in the ArgK energy metabolism system, was also differentially expressed in the three groups of samples. This enzyme was first found in insects and more recently, it has been demonstrated that it acts as part of an important temporal energy buffer system in invertebrates.[42] The enzyme catalyzes the reversible phosphorylation of arginine by ATP, and the physiological importance of this reaction is in the provision of metabolic capacitance, because ArgK allows the reduction of the peak rates of ATP synthesis, which alternates between high- and low-energy consumption.[42,43] As shown in Figure 4, ArgK (spot 21) expression was up-regulated during cyprid development, indicating the corresponding up-regulation of phosphoarginine (PA) and the consequent greater energy release during the process. This result suggests that the 24 h of cyprid development immediately before larval attachment and metamorphosis was a high-energy-demanding period. Usually, *B. amphitrite* larvae will commence their metamorphosis immediately after attachment and the process is completed in a short time. To rapidly generate the large amounts of energy required during this active growth and development process, it is highly probable that the ArgK pathway is used as the dominant energy-metabolism system. It is interesting to note that the ArgK expression level is sustained (down-regulated relative to that of the CON-24 group) with butenolide treatment. This result agrees well with the regulation of ATP synthase during butenolide treatment and suggests that butenolide may exert its effect by preventing changes in the energy-metabolism pathway. Additionally, ArgK is highly expressed in the compound eye of the honeybee and a higher level of ArgK might be advantageous for the foraging behavior of this insect.[43] In the barnacle, the compound eye is a stage-specific organ that is only present in the naupliar VI and cyprid larval stages and disappears right after attachment and metamorphosis.[44] This may explain one of the probable uses of the energy released during this crucial developmental period. As in the insect system, high levels of ArgK might be related to the exploratory behavior of *B. amphitrite*, whereas in this case, it searches for a suitable habitat rather than for food.

## 4. Concluding Remarks

This study investigated the regulation of both protein expression and phosphorylation in response to the development of the *B. amphitrite* cyprid to a stage that is competent to settle and metamorphose, and in response to treatment with butenolid-a newly patented antifouling compound. The utilization of solution-phase IEF sample prefractionation and a 2-DE-based multiplexed proteomic approach significantly improved protein separation and allowed us to differentiate the responses of the target proteins to cyprid development and butenolide treatment. Our data show that the differential regulation of these target proteins is highly dynamic and acts at both the protein expression and posttranslational modification levels. Our results also indicate that different antifouling compounds may exert their effects by diverse mechanisms. Sixteen differentially expressed/phosphorylated proteins were successfully

identified by MALDI-TOF/TOF and ESI-QqTOF. Six kinds of stress-regulated proteins and two kinds of energy metabolism-related proteins are mainly involved in cyprid development and cyprid's response to antifouling treatment. The down-regulation of stress-regulated proteins and the up-regulation of ArgK in the CON-24 group indicate that cyprid development is a period characterized by reduced stress and high energy consumption. However, the expression of all these proteins was sustained during butenolide treatment, suggesting that butenolide exerts its antifouling activity by arresting the growth and development of the *B. amphitrite* cyprid. Because this compound is nontoxic to many marine species, some of these proteins deserve further study as potential candidates for the development of efficient antifouling treatments.

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**Supporting Information Available:** Supplementary figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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PR1000384