1 iTRAQ-Based Proteomic Profiling of PLC-Mediated Expression 2 of 20E-Induced Protein in *Apolygus lucorum* (Meyer-Dür)

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Abstract: The polyphagous pest *Apolygus lucorum* has become the dominant insect in *Bacillus thuringiensis* (Bt) cotton fields. The hormone 20-hydroxyecdysone (20E) regulates multiple events in insect development and physiology. 20E responses are controlled by pathways triggered by phospholipase C (PLC)-associated proteins. However, 20E-modulated genes whose expression is affected by PLC remain unknown. Here, isobaric tag for relative and absolute quantitation (iTRAQ) and immunoblot were carried out for comparing differentially expressed proteins (DEPs) in *A. lucorum* in response to 20E and the PLC inhibitor U73122, respectively. Totally 1624 DEPs were, respectively, found in the 20E/control, U73122/control, and 20E+U73122/control groups. Venn diagram analysis further revealed 8 DEPs that were shared among the three groups. Immunoblot validated these findings, which corroborated and highlighted the reliability of proteomics. KEGG enrichment analysis showed that the DEPs were included in diverse signaling pathways. The largest portion of DEPs among the three groups were categorized in metabolic pathways. In addition, DEPs among the three groups were also found to regulate the Ras-MAPK and PI3K-AKT pathways. This is the first time that iTRAQ was carried out to assess proteome alteration in *A. lucorum* nymphs in response to 20E and a PLC inhibitor. These findings provide novel insights into protein expression in *A. lucorum* in response to 20E, and a more comprehensive understanding of the function of PLC in 20E signal transduction.

Keywords: *Apolygus lucorum*, 20-hydroxyecdysone, phospholipase C, iTRAQ, Proteomics, Western-blot

1 1. INTRODUCTION

2 *Apolygus lucorum* (Meyer-Dür), a worldwide agricultural pest, has become a key
 3 insect pest in cotton fields (Lu and Wu, 2008). With the rising plantation area of
 4 transgenic Bt cotton cultivars, *Helicoverpa armigera* (Hübner) has been controlled
 5 effectively (Wu *et al.*, 2005; Lu and Wu, 2011). However, *A. lucorum* is hard to
 6 control due to elevated mobility, broad host range and complex feeding properties (Lu
 7 *et al.*, 2010). At present, chemical control with different insecticides remains the most
 8 applied option for the management of *A. lucorum*. Extensive use of insecticides has
 9 resulted in *A. lucorum* resistance to these products, with inherent environmental risks
 10 (Lu *et al.*, 2007). Consequently, new tools, particularly those that target insect
 11 functions via molecular mechanisms for pest management, are urgently needed.

12 The insect steroid ecdysone and its active metabolite 20-hydroxyecdysone (20E)
 13 trigger and coordinate multiple events in insect development and physiology such as
 14 caste determination, diapause and longevity. Many effects of 20E are mediated by a
 15 heterodimer comprised of the ecdysone receptor (EcR) and ultraspiracle (USP)
 16 (Nakagawa and Henrich, 2009; Christiaens *et al.*, 2010). In detail, EcR contains a
 17 basic ligand-independent activation region near the N terminus, the main dimerization
 18 domain and a ligand-binding domain, which heterodimerizes with USP (Iwema *et al.*,
 19 2007; Xu *et al.*, 2020). This pocket with remarkable flexibility can functionally
 20 interact with the molting hormone 20E. The 20E-EcR-USP complex binds to a
 21 specific DNA response element (EcRE) to regulate the transcription of target genes
 22 (Boulanger and Dura, 2015).

23 As soluble compounds, steroid hormones easily diffuse into the cell nucleus via the
 24 cytoplasmic membrane, and interact with nuclear steroid hormone receptors to
 25 substantially modulate gene expression (Thompson *et al.*, 1995). Meanwhile, steroid
 26 hormones trigger non-genomic signaling via the cytoplasmic membrane (Meldrum,
 27 2007; Sandén *et al.*, 2011; Falkenstein *et al.*, 2000). Phospholipase C (PLC)
 28 constitutes a major class of regulatory enzymes contributing to non-genomic

1 membrane pathways controlling 20E signaling genes, mostly by hydrolyzing the polar
2 head groups in inositol-containing membrane phospholipids (Liu *et al.*, 2014; Cai *et*
3 *al.*, 2014). For instance, apoptosis in silkworm anterior silk glands is induced by 20E-
4 associated GPCR-PLC-IP₃-Ca²⁺-PKC signaling (Iga *et al.*, 2007; Manaboon *et al.*,
5 2009). In *H. armigera*, through GPCR-PLC-Ca²⁺ signaling, 20E induces a rapid
6 phosphorylation of cyclin-dependent kinase 10 (CDK10) to promote gene
7 transcription (Liu *et al.*, 2014). PLC substantially affects larva development and
8 pupation, and 20E could increase PLC amounts during molting and metamorphosis.
9 Via ErGPCR, Ga_q and Src kinases, 20E readily triggers tyrosine phosphorylation in
10 the SH2 domain of PLC and its migration toward the cytoplasmic membrane.
11 Through PLC/Ca²⁺ signaling, 20E induces EcRE's transcriptional activity by
12 modulating PKC phosphorylation at Ser 21, which is important for its interaction with
13 EcRE (Liu *et al.*, 2014; Jing *et al.*, 2015). In a previous study, we found 20E regulates
14 AlTre-1 through AlPLC γ and alters Vg expression and ovary development, thereby
15 facilitating female reproduction in *A. lucorum* (Tan *et al.*, 2020). These data indicated
16 that PLC participates in 20E signal transduction in a non-genomic pathway.

17 Currently, differential proteomics can help identify proteins with physiological
18 significance. Recently, isobaric tags for relative and absolute quantitation (iTRAQ)
19 were designed for the simultaneous assessment of 4 to 8 distinct specimens by
20 MS/MS, thereby increasing throughput and reducing assay-related errors (Ross *et al.*,
21 2004; Ye *et al.*, 2010). Additionally, this novel method is sensitive, automated and
22 multi-dimensional (Caubet *et al.*, 2010). Here, iTRAQ was utilized to investigate
23 proteome alterations in *A. lucorum* in response to 20E and the widely studied PLC
24 inhibitor U73122. These findings provide a basis for improved understanding of *A.*
25 *lucorum* nymph response to 20E, and a more comprehensive understanding of PLC's
26 function in 20E signal transduction.

27 2. MATERIALS AND METHODS

28 *Insect Culture*

1 *A. lucorum* specimens were obtained from *Vicia faba* grown in fields in Yancheng
2 (33.110N, 120.250E), Jiangsu Province, China, in July to August 2018. *A. lucorum*
3 nymph and adult individuals underwent rearing on *Phaseolus vulgaris* in a light
4 incubator at 25±1°C and 70±5% humidity under a 14 h-10 h light/dark cycle.

5 ***Sample Preparation***

6 About 24 h after molting, a total of 600 third instar stage nymphs were collected in
7 plastic cases with green beans. Midguts from early wandering third instar nymphs
8 underwent dissection in Ringer's solution, and were pre-incubated in 12-well tissue
9 culture plates with 500 µL of Grace's insect tissue culture medium for 30 min, with or
10 without 50 µM U73211 (Sigma, St. Louis, MO, USA). This was followed by
11 stimulation with 1.0 µM 20E (Sigma, St Louis, MO, USA) or distilled water for 12 h.
12 The assay consisted of four experimental treatments: (1) 20E and U73122; (2) 20E; (3)
13 U73122; (4) CK, negative control, treatment with distilled water. Upon treatment,
14 midguts were obtained, homogenized and submitted to centrifugation (16,000×g, 10
15 min).

16 ***Protein extraction and Digestion***

17 Specimens were pulverized in liquid nitrogen and treated with 0.5 ml lysis buffer (6
18 M urea, 2 M thiourea, 4% CHAPS, 40 mM Tris-HCl, 5 mM EDTA, 1 mM PMSF and
19 10 mM DTT; pH 8.0). After sonication (5 min at 4°C) and centrifugation (18,000×g,
20 4°C; 15 min), supernatants were collected. Protein amounts were assessed by the
21 Bradford assay. Protein (100 µg) digestion was performed by the FASP method (Tang
22 *et al.*, 2017) utilizing trypsin (Promega, Madison, WI) and 0.1M tetraethylammonium
23 bromide (TEAB) as buffer solution. Two biological replicates were pooled for iTRAQ
24 analysis.

25 ***iTRAQ labeling***

26 After trypsin digestion, lysate specimens (200 µg) were added to 200 µL 8 M urea in
27 150 mM Tris-HCl (pH 8.0) (buffered urea) and centrifuged (15 min, 14,000×g) in 10

1 kDa diafiltration tubes for concentration. The concentrated samples were treated as
2 described above for the lysates and incubated at ambient with 100 μ L 50 mM
3 iodoacetamide (Bio-Rad, 163-2109, Hercules, CA, USA) in buffered urea and
4 concentrated as described above. After two washes with 100 μ L buffered urea by
5 centrifugation (10 min, 14,000 $\times$ g), the specimens were further resuspended with 100
6 μ L of dissolution buffer (AB SCIEX, Foster City, CA, USA) and centrifuged as above.
7 Next, the pellets in 40 μ L dissolution buffer underwent digestion with 2 μ g trypsin
8 overnight at 37°C and vacuum drying. Further sample processing with the 8-plex
9 iTRAQ reagent (AB SCIEX) was carried out as directed by the manufacturer. Treated
10 and infected specimens underwent labeling (1 h, ambient) with iTRAQ reagents 113-
11 115 and 116-118, respectively. After vacuum drying, fractionation was carried out by
12 high pH Reversed phase chromatography (Hp-RPC).

13 ***Fractionation by Hp-RPC***

14 A Shimadzu LC-20AB HPLC Pump system was used for Hp-RPC. The iTRAQ-
15 labeled peptide mixture was added to 0.5 mL buffer A (20 mM NH_4HCO_2 in 2%
16 acetonitrile, pH 10) followed by injection into a Gemini-NX μ M C18 column (110 Å,
17 250 $\times$ 4.6 mm; Phenomenex, Guangzhou, China). Elution was carried out at 1
18 mL/min initially with buffer A for 10 min, followed by a 5-30% buffer B (20 mM
19 NH_4HCO_2 in 98% acetonitrile, pH 10; 15 min) and 30-80% buffer B (3 min) in buffer
20 A. Detection was performed at 214 nm, with fraction collection occurring at 1-min
21 intervals. Finally, the eluates were pooled in 10 fractions and submitted to vacuum
22 drying.

23 ***LC-ESI-MS/MS***

24 Various fractions underwent resuspension in a certain volume of buffer A (5%
25 acetonitrile and 0.1% formic acid in water) and centrifugation (18,000 $\times$ g, 10 min).
26 The final peptide content was adjusted to approximately 0.5 μ g/ μ L in all fractions.
27 After loading 8 μ L of each sample at 3 μ L/min on an Eksigent 425 2D HPLC system
28 (AB SCIEX), the peptides were eluted onto an in-housed packed analytical C18

1 column (internal diameter, 75 μ m). A 45-min gradient was initially applied at 0.3
2 μ L/min from 8 to 25% B (84% acetonitrile and 0.1% formic acid), and a 10-min linear
3 gradient to 80% B, which was maintained for 5 min.

4 A TripleTOF 5600 System was utilized to acquire data, with ion spray voltage at 2.3
5 kV, curtain and ion source gases at 30 and 6 PSI, respectively, and the chamber at
6 150°C. MS was carried out in the high-resolution mode ($>30,000$ fwhm) for TOF MS
7 scanning, with m/z of 350-1800 Da. For information dependent data acquisition (IDA),
8 scan was performed for 50 ms, collecting up to 30 product ions exceeding the cutoff
9 of 200 counts/s and showing a 2+ to 4+ charge.

10 ***iTRAQ protein assessment***

11 The raw data (*.wiff and *.wiff.scan) underwent analysis with ProteinPilot 5.0 (AB
12 SCIEX) against the *A. lucorum* transcriptome database. Search indexes were:
13 Specimen Type, iTRAQ 8plex (Peptide Labeled); Cysteine alkylation, iodoacetamide;
14 Digestion, Trypsin. FDR (false discovery rate) analysis was performed routinely for
15 identification. Differentially expressed proteins (DEPs) were considered with a 2-fold
16 change cutoff.

17 ***Bioinformatics analysis***

18 Two annotation databases, including Gene Ontology (GO) (Ashburner *et al.*, 2000)
19 and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata *et al.*, 1999), were
20 utilized for annotating and grouping DEPs. The GO numbers of DEPs were retrieved
21 with the Blast2GO software (<https://www.blast2go.com/>), and DEPs were further
22 classified with WEGO 2.0, a web tool for analyzing and plotting GO annotations (Jia
23 *et al.*, 2018). As for KEGG pathway analysis, the protein sequences of DEPs were
24 uploaded onto the KEGG Automatic Annotation Server (Moriya *et al.*, 2007) to
25 compute and group the DEP-integrated signaling pathways.

26 ***Protein analysis***

27 Immunoblot was performed for validating iTRAQ data. In brief, protein were
28 extracted from the midguts post-treatment with Tissue Protein Extraction Reagent Kit

(ZoonBio) as directed by the manufacturer. Protein quantitation utilized the bicinchoninic acid (BCA) assay (ZoonBio). Equal amounts of total protein underwent separation by 10% SDS-PAGE and electro-transfer on nitrocellulose membranes (Bio-Rad). After blocking (5% skim milk in Tris-buffered saline with 0.05% Tween 20; 1 h at 37°C), the membranes underwent incubation with primary antibodies targeting trans-sialidase, cuticle protein 19.8, pathogenesis-related protein 5, guanine nucleotide-binding protein and ras-related protein 1 for 1 h at 37°C. Then, horseradish peroxidase (HRP)-linked secondary antibody (ZoonBio) for 1 h at ambient. Development utilized Enhanced Chemiluminescence Advance Kit components (GE Healthcare, PA, USA). Quantitation was carried out by densitometry with ImageJ (<http://rsb.info.nih.gov/ij/index.html>). Relative protein amounts were obtained by dividing the normalized trans-sialidase, cuticle protein 19.8, pathogenesis-related protein 5, guanine nucleotide-binding protein and ras-related protein 1 densities by the normalized β -actin density based on mouse anti- β -actin antibody (ZoonBio).

3. RESULTS

Protein profiling and identification of differentially expressed proteins

To obtain a global view of proteome alteration in *A. lucorum* nymphs in response to different stresses (20E, U73122, and 20E+U73122), an iTRAQ-based quantitative proteomic approach was employed to perform comparative analysis among the three groups (20E/control, U73122/control and 20E+U73122/control). A total of 1624 non-redundant proteins with two or more unique peptides were identified (Table S1). Based on the regulation cutoff of 2.0-fold ($p < 0.05$), 98 (20 up-regulated and 78 down-regulated), 248 (42 up-regulated and 206 down-regulated), and 266 (57 up-regulated and 209 down-regulated) differentially-expressed proteins (DEPs) were, respectively, identified in the 20E/control, U73122/control and 20E+U73122/control groups (Table S2). For all DEPs among the three groups, the numbers of repressed proteins were higher than those of up-regulated ones, and DEPs were significantly less abundant in the 20E/control group compared with the other groups. Moreover, the numbers of

1 DEPs in the U73122/control and 20E+ U73122/control groups were similar.
 2 Venn diagram analysis (Figure 1, Table S3) further showed that only 8 DEPs were
 3 shared among all three groups. From pairwise comparisons of the 20E/control group
 4 with the U73122/control and 20E+U73122/control groups, a total of 20 and 18 DEPs
 5 were identified, respectively. A total of 155 DEPs were shared between the
 6 U73122/control and 20E+U73122/control groups. The numbers of shared proteins
 7 were over seven times greater than that of the 20E group. Furthermore, these 155
 8 DEPs exclusively showed the same trend in protein abundance changes (11 were up-
 9 regulated and 144 showed down-regulation).

10 ***Bioinformatics analysis and interpretation of DEPs***

11 Since no complete reference proteome database of *A. lucorum* has been published, we
 12 employed the Blast2GO suite (<https://www.blast2go.com/>), a commercial software, to
 13 annotate the DEPs found in each group, with the assigned GO numbers representing
 14 their functional relevance in the categories of cellular component (CC), molecular
 15 function (MF), and biological process (BP). The GO-assigned DEPs were then
 16 classified with WEGO 2.0, a web tool for analyzing and plotting GO annotations (Jia
 17 *et al.*, 2018). As shown in Figure 2, more DEPs in the 20E/control group were
 18 associated with intracellular localization compared with extracellular distribution.
 19 Multiple DEPs possessing catalytic and ion-binding activities were identified in the
 20 U73122/control and 20E+U73122/control groups, while many DEPs in the
 21 20E/control group contributed to the regulation of anatomical structure development
 22 and stress response.

23 “Zoomed-in” KEGG pathway analysis revealed that the identified DEPs were
 24 integrated into diverse signaling pathways (Figure 3, Table S4). The largest portion of
 25 DEPs among the three groups were associated with metabolic pathways. Comparative
 26 analysis showed no overlapping DEP among the three groups. All DEPs (n=24) in the
 27 20E/control group were down-regulated, while both up- and down-regulated DEPs
 28 were found in the U73122/control (8 up-regulated and 12 down-regulated) and

20E+U73122/control (17 up-regulated and 10 downregulated). Interestingly, multiple ribosomal proteins were identified as DEPs involved in the ribosome process/pathway. In the 20E/control group, all ribosomal proteins (n=11) were down-regulated, while almost all (11 out of totally 12) were up-regulated in the U73122/control group. In the 20E+U73122/control group, the numbers of up- and down-regulated ribosomal proteins were almost even (6:5), suggesting that 20E and U73122 treatments played opposite roles in the regulation of ribosome-driven protein translation. Compared with the 20E/control group, DEPs in the U73122/control and 20E+U73122/control groups were more enriched in the processes/pathways of lysosome, phagosome, endocytosis, phagosome (phagocytosis), endosome (endocytosis) and lysosome. A total of 20 non-redundant DEPs in the U73122/control and 20E+U73122/control groups, including 11 shared ones, were involved in these processes. Almost all these DEPs were down-regulated (16 out of 20). In contrast, only 4 DEPs (1 up-regulated and 3 down-regulated) in the 20E/control group were involved in these processes/pathways. Furthermore, several subunits (A, C, G, and E) composed of V-type proton ATPase (ATPeV) were identified as DEPs and classified into the phagosome group. Subunits A (catalytic unit) and G, identified in the 20E+U73122/control and U73122/control groups, respectively, were up-regulated, while subunits C and E (assembling the peripheral stalk to insert the complex into the membrane) were down-regulated in the 20E/control group.

21 **Validation of DEPs**

22 Western blot showed that trans-sialidase, cuticle protein, and pathogenesis-related
23 protein were clearly up-regulated by 20E, and down-regulated upon treatment with
24 U73122 and 20E+U73122, respectively. Ras-related protein 1 was obviously up-
25 regulated (Figure 4) in response to U73122 and 20E+U73122 treatments but clearly
26 down-regulated by 20E. Guanine nucleotide-binding protein was clearly up-regulated
27 by 20E+U73122, while treatment with 20E and U73122 showed no significant effects
28 on its expression. Results of Western blot confirmed and highlighted the above

1 proteomic findings.

2 **4. DISCUSSION**

3 Two-dimensional polyacrylamide gel electrophoresis (2-DE), combined with MS,
4 peptide sequencing and database search, represents a great tool for determining DEPs,
5 but is relatively complex and unsuitable for quantitating proteins in minute quantities.
6 iTRAQ, a novel stable isotope technique for protein measurement that employs mass
7 spectrometry (Zieske *et al.*, 2006; Pierce *et al.*, 2008), could comparatively assess
8 eight distinct specimens concurrently and examine proteome profiles upon treatment
9 with exogenous substances.

10 Ecdysteroids represent a group of steroid hormones regulating developmental,
11 reproductive and other critical biological events in insects. Meanwhile, 20E is
12 considered the major ecdysteroid in most insects (Spindler-Barth *et al.*, 2000; Lafont
13 *et al.*, 2005). Previous evidence indicates that 20E exerts effects through its receptor
14 (Thomas *et al.*, 1993; Hill *et al.*, 2013; Levin, 2015), a heterodimer comprising two
15 nuclear receptors, i.e., ecdysteroid receptor (EcR) and ultraspiracle (Usp), an ortholog
16 of the retinoid X receptor (RXR) in vertebrates (Yao *et al.*, 1992). In this study, after
17 treatment with 20E of *A. lucorum* nymphs, 98 (20 up-regulated and 78 down-
18 regulated) DEPs were identified, and 248 (42 up-regulated and 206 down-regulated)
19 and 266 (57 up-regulated and 209 down-regulated) were, respectively, identified in
20 the U73122/control and 20E+U73122/control groups. Venn diagram analysis further
21 showed that only 8 DEPs were shared in all three groups. Four proteins, including

1 pathogenesis-related protein 5-like, cuticle protein 19.8, trans-sialidase, and larval
2 cuticle protein A2B-like, were up-regulated after 20E treatment but down-regulated in
3 both the U73122/control and 20E+U73122/control groups. Meanwhile, cathepsin L1,
4 protein takeout (hemolymph juvenile hormone binding protein), ATP-dependent RNA
5 helicase p62-like, and myosin-9 isoform X1 were all down-regulated. Pairwise
6 comparisons of the 20E/control group with U73122/control and 20E+U73122/control
7 groups, respectively, revealed 20 and 18 DEPs, indicating that the panels of proteins
8 regulated by 20E and PLC were significantly different, in agreement with the nature
9 of these two reagents evoking distinct cellular responses (Liu *et al.*, 2014; Chen *et al.*,
10 2019; Tan *et al.*, 2020).

11 The cuticle of insects has multiple layers and three functional regions, including the
12 epicuticle, procuticle and endocuticle, differing in protein profile, structural properties
13 and physiological roles (Willis *et al.*, 1996). The properties of insect cuticle depend on
14 the construction of cuticular proteins, and various cuticle types show pronounced
15 differences in mechanical features in association with the characteristics of individual
16 proteins (Andersen , 2011). As shown above, all identified cuticular proteins were up-
17 regulated to varying degrees after 20E treatment (Table 1). Examples include larval
18 cuticle protein A2B-like, pupal cuticle protein C1B-like, cuticle protein 19.8 and
19 cuticle protein 19-like. This indicated that cuticular proteins are induced by ecdysone
20 addition in *A. lucorum* and 20E could evoke the up-regulation of these cuticular
21 proteins. Insects produce new cuticles each time the larva molts and various cuticle
22 types are required when the larva changes to the pupa, or when the latter

1 metamorphoses into the adult animal. Mounting evidence suggests that ecdysone
2 signaling is critical for cuticle biosynthesis and accumulation (Riddiford *et al.*, 2001;
3 Kozlova and Thummel, 2003). The production of cuticular proteins via the ecdysone
4 and juvenile hormones is well-known in multiple insects (Charles, 2010). In *Bombyx*
5 *mori*, several classes of cuticular genes with distinct profiles during development as
6 well as various ecdysone responses from wing disc have been reported (Zhong *et al.*,
7 2006; Futahashi *et al.*, 2008). Moreover, the temporal expression of CPR95 is
8 regulated by the ecdysone-responsive transcription factor E74A in the wing discs of
9 pre-pupa (Wang *et al.*, 2010). Indeed, most cuticular genes are induced by an
10 ecdysteroid pulse, with their expression requiring the existence and suppression of
11 20E (Nita *et al.*, 2009; Soares *et al.*, 2007; Wang *et al.*, 2009; Zhong *et al.*, 2006).
12 This is similar to what reported at the stage around ecdysis (Lemoine *et al.*, 2004;
13 Noji *et al.*, 2003; Wang *et al.*, 2009). Remarkably, 12 cuticular proteins and their
14 homologous molecules were down-regulated after U73122 treatment, while 7
15 cuticular proteins and their homologous molecules were down-regulated by
16 20E+U73122 (Table 2 and 3). Phospholipase C represents a group of enzymes
17 contributing to the cellular turnover of inositol-containing phospholipids (Dai *et al.*,
18 2015). The latter enzymes remove the polar head group from membrane lipids,
19 including phosphatidylinositol 4,5-bisphosphate (PIP₂), for generating inositol 1,4,5-
20 triphosphate and diacylglycerol, which are secondary messengers mobilizing calcium
21 in the cell and activating protein kinase C, separately (Klein *et al.*, 2011). It has been
22 reported that PLC is involved in 20E signal transduction. In this study, while PLC

1 activity was inhibited by the addition of exogenous U73122, it caused suppression of
2 cuticular proteins and their homologous molecules, consistent with Western blot
3 results. Cuticle protein 19.8 was clearly upregulated by 20E, but downregulated upon
4 treatment with U73122 and 20+U73122. This indicates that PLC may participate in
5 the cuticle protein biosynthesis via regulation by ecdysone.

6 Besides affecting development, molting, metamorphosis and reproduction, 20E is also
7 involved in the regulation of insect innate immunity. Previous reports demonstrated
8 that 20E induces apoptosis in insect metamorphosis and pathologies. In *B. mori* Bm-
9 12 cells, 20E induces cell cytotoxicity sequentially via autophagy and apoptosis (Xie
10 *et al.*, 2016). The induction and repression of immune reactions by 20E are species-
11 and developmental stage-specific. 20E upregulates prophenoloxidase 1 in *Anopheles*
12 *gambiae* (Ahmed *et al.*, 1999; Muller *et al.*, 1999) as well as hemolin in the fat bodies
13 of cecropia moth at the diapausing stage (Roxstrom *et al.*, 2005). As shown above, 2,
14 7 and 10 DEPs were found in the 20E/control, U73122/control and
15 20E+U73122/control groups, respectively, in the apoptosis pathway. Almost all DEPs
16 among the three groups were down-regulated except spectrin alpha (SPTA) and
17 tubulin alpha-1 in the 20E+U73122/control group. SPTA, a cytoskeleton with binding
18 sites for several proteins, is cleaved during apoptosis to alter membrane stability and
19 form apoptotic bodies. Up-regulation of SPTA by co-treatment of *A. lucorum* with
20 20E and U73122 may promote cell survival. Jointly, the above data indicate that 20E
21 and U73211 negatively regulate apoptosis, and 20E in combination with U73122 may
22 perform a double-edged role in this pathway. Besides positive effects, 20E also

1 suppresses immune response as reported in *Calliphora vicina*, *Bombyx mori* and
2 *Drosophila*, with downregulated AMPs under high ecdysone amounts at the pupal
3 stage, indicating ecdysone blocks innate immunity (Chernysh *et al.*, 1995; Beckstead
4 *et al.*, 2005; Tian *et al.*, 2010). In the larval molt and during larva to pupa
5 transformation, injected ecdysone induces the down-regulation of AMPs, including
6 lebocin-3, gloverin-like protein 2, and nuecin, in the fat bodies of *B. mori* (Tian *et al.*,
7 2010). The above discrepant findings indicate that complex mechanisms govern
8 ecdysone's function in innate immunity.

9 On the other hand, DEPs in the U73122/control and 20E+U73122/control groups
10 were more enriched in the processes/pathways of lysosome, phagosome, and
11 endocytosis. Phagosome (phagocytosis), endosome (endocytosis), and lysosome are
12 inter-crossed organelles for the degradation and recycling of macromolecules (Braun
13 and Niedergang, 2006). A total of 20 non-redundant DEPs in the U73122/control and
14 (20E+U73122)/control groups, including 11 shared ones, were involved in these
15 processes, and almost all these DEPs were down-regulated (16 out of 20). In contrast,
16 only 4 DEPs (1 up-regulated and 3 down-regulated) in the 20E/control group
17 participated in these processes/pathways. These data indicate that PLC predominantly
18 regulates the processes/pathways of phagosome, endosome, and lysosome in a
19 negative manner. PLC is known to contribute to the differentiation/activation of cells
20 controlling both innate and adaptive branches of the immune system. PLC also affects
21 tyrosine kinase-associated pathways for inhibiting Stat5 via recruitment of the
22 protein-tyrosine phosphatase SHP-1, with which PLC and Stat5 interact in the SPS

1 complex. This complex substantially regulates immune cell activation (Kawakami and
2 Xiao, 2014). Furthermore, several subunits (A, C, G and E) composed of V-type
3 proton ATPase (ATPeV) have been identified as DEPs classified into the phagosome
4 group. ATPeV is a multi-subunit protein of up to 14 different polypeptides located on
5 the organelle membrane. It is involved in the acidification of endocytic and
6 phagocytic organelles to regulate ligand release, macromolecule breakdown, and
7 cation uptake. Subunits A (catalytic unit) and G, identified in the 20E+U73122/control
8 and U73122/control groups, respectively, were up-regulated, while subunits C and E
9 (assembling the peripheral stalk to insert the complex into the membrane) were down-
10 regulated in the 20E/control group, indicating that 20E and U73122 have opposite
11 functions in the regulation of ATPeV activity and the subsequent phagosome
12 process/pathway.

13 The DEPs among the three groups were also found to regulate the Ras-MAPK and
14 PI3K-AKT pathways. In response to stimuli, activated Ras, a small G protein, triggers
15 the MAPK (RAF-MEK-ERK) and PI3K-AKT cascades to control critical cell events,
16 including growth, proliferation, differentiation, migration and apoptosis (Bonni *et al.*,
17 1999; Zhang *et al.*, 2014). Ras-related protein 1 (Rap1) was identified as a DEP in
18 both the U73122/control and 20E+U73122/control groups with increased expression,
19 consistent with Western blot results indicating that Rap1 was clearly up-regulated by
20 U73122. Rap1 was reported to interfere with mitogen-activated protein kinase
21 (MAPK) signaling by trapping the RAF protein. In insects, ecdysteroidogenesis by
22 prothoracic glands (PGs) is controlled by the brain peptide prothoracicotropic

1 hormone (PTTH) (Smith and Rybczynski, 2012; Johnson *et al.*, 2013). The mature
2 PTTH interacts with and induces its receptor Torso, a *D. melanogaster* receptor
3 tyrosine kinase (Grillo *et al.*, 2012; Johnson *et al.*, 2013). Induced Torso activates
4 MAPK signaling that encompasses Rap, RAF, MAPK kinase (MEK) and extracellular
5 signal-regulated kinase (ERK), stimulating ecdysteroidogenesis. External U73122
6 suppressed the expression of Rap1, suggesting that PLC negatively regulates the Ras-
7 RAF-MEK-ERK pathway, which is involved in the biosynthesis of 20E. Furthermore,
8 guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 (GNB1) was found
9 to be an up-regulated DEP in the 20E+U73122/control group, and Western blot
10 confirmed these results. GNB1 is a subunit of the heterotrimeric G protein. Its main
11 function is to form a G-beta dimer with the G protein γ subunit ($G\gamma$), and to generate a
12 heterotrimeric G protein with G protein α subunit ($G\alpha$). Heterotrimeric G protein
13 could receive the signal transferred by GPCR and pass it to downstream molecules. It
14 has been reported that G protein-coupled receptors (GPCRs) contribute to 20E as a
15 membrane receptor in signaling pathways and transfer the signals into the intracellular
16 space to open calcium channels, causing programmed cell death (Oldham and Hamm,
17 2007). Interestingly, PLC could be stimulated by GNB1 (Camps *et al.*, 1992). In the
18 lepidopteran insect *H. armigera*, 20E regulates calponin phosphorylation and
19 translocation into the nucleus (Liu *et al.*, 2011). 20E increases intracellular calcium
20 levels via the ecdysone-associated GPCR (Cai *et al.*, 2014), and 20E through G-
21 protein induction increases intracellular Ca^{2+} (Ren *et al.*, 2014). 20E controls gene
22 transcription via the GPCR, G-protein, PLC, calcium, and PKC non-genomic pathway

(Liu *et al.*, 2014). Another ecdysone responsible GPCR, termed ErGPCR2, also contributes to 20E-induced non-genomic biological events in *H. armigera* (Jing *et al.*, 2015), indicating that 20E cooperates with U73122 to positively regulate GPCR-mediated non-genomic pathway. In contrast, 14-3-3 ζ with down-regulated expression was identified as a DEP in the 20E/control group. In addition, 14-3-3 ζ , the adaptor protein, interacts directly with RAF to serve as a negative regulator of RAF function. Furthermore, 14-3-3 ζ also activates PI3K via binding of the p85 regulatory subunit, suggesting that 20E exerts different roles in the Ras-MAPK and PI3K-AKT pathways. Taken together, these data showed that 20E and U73122 exert opposite roles in Ras-MAPK and PI3K-AKT pathway regulation.

In summary, 98 (20 up-regulated and 78 down-regulated), 248 (42 up-regulated and 206 down-regulated) and 266 (57 up-regulated and 209 down-regulated) DEPs were differentially expressed in the 20E/control, U73122/control and (20E+U73122)/control groups. The functions of these proteins in 20E signaling as well as responses to 20E deserve further attention. For example, cuticular proteins were overtly involved. In addition, 20E was shown to modulate immune response in *A. lucorum*. Moreover, these findings provide a more comprehensive understanding of PLC's function in 20E signal transduction and protein interactions involving PLC under 20E regulation.

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Table 1 Cuticle proteins and homologies in 20E-treated *A. lucorum*

Protein	Blast Similarity Mean	Peptides (95%)	P-Value	Ratio(average)	Accession
larval cuticle protein A2B-like	95.12%	293	0.021508288	2.780555367	CL1466.Contig2
pupal cuticle protein C1B-like	94.02%	365	0.0361844	2.660523891	CL31.Contig4
cuticle protein 19.8	89.79%	55	0.01149462	2.155213952	Unigene44049
cuticle protein 19-like	77.81%	6	0.0120524	2.096478403	CL4340.Contig3

Table 2 Cuticle proteins and homologies in U73122-treated *A. lucorum*

rotein	Blast Similarity Mean	Peptides (95%)	P-Value	Ratio(average)	Accession
cuticle protein 7	83.01%	46	0.008437019	0.481294185	CL4657.Contig1
cuticle protein 10.9-like	77.66%	3	0.018015705	0.427869186	Unigene44908
cuticle protein 21-like	97.29%	569	1.97353E-05	0.405525729	Unigene12071
cuticle protein 21-like	92.38%	71	0.003097548	0.297643058	Unigene41367
larval cuticle protein A2B-like	95.32%	487	0.016687083	0.273016788	CL1002.Contig1
larval cuticle protein A2B-like	93.72%	548	0.00033265	0.272292286	Unigene44898
cuticle protein 16.5-like	68.54%	374	0.016726727	0.224266387	Unigene10138
cuticle protein 8-like	90.16%	57	0.010342484	0.213135041	Unigene19243
cuticle protein 3	83.07%	14	0.001466315	0.184845738	Unigene13702
cuticle protein 19.8	89.79%	55	0.009174642	0.184463348	Unigene44049
cuticle protein 70, isoforms A and B	100%	40	0.007508727	0.140011718	Unigene5243
larval cuticle protein A2B-like	94.89%	608	0.001391079	0.094018778	Unigene44897

1 Table 3 Cuticle proteins and homologies in 20+U73122-treated *A. lucorum*

2	Protein	Blast Similarity Mean	Peptides (95%)	P-Value	Ratio(average)	Accession
	cuticle protein 2-like	58.99%	2	8.47802E-06	0.472068041	Unigene2357
3	cuticle protein 16.5-like	69.61%	374	0.02345216	0.432273552	Unigene10138
	larval cuticle protein A2B-like	71.48%	487	0.004508908	0.41866003	CL1002.Contig1
4	cuticle protein 16.5	66.30%	57	0.037728864	0.385975547	Unigene23777
	larval cuticle protein A2B-like	71.65%	80	0.001128939	0.352907077	CL1002.Contig2_
5	larval cuticle protein A3A-like	67.08%	380	0.027402238	0.352296807	Unigene19994
	cuticle protein 16.5	67.72%	13	0.000400274	0.305423185	Unigene1414

Figure 1. DEPs with a change ≥ 1.2 -fold in *A. lucorum* after treatment with 20E, U73122 and 20E+U73122, respectively. Co-DEPs between two time points and among three time points were also determined.

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3 Figure 2. Gene ontology analysis of 612 proteins differentially expressed after
4 treatment with 20E, U73122 and 20E+U73122 of *A. lucorum*, respectively. Proteins
5 were annotated by biological process, cellular component, and molecular function

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2 Figure 3. KEGG pathway analysis of DEPs identified after treatment with 20E,
3 U73122 and 20E+U73122 of *A. lucorum*.

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2 Figure 4. (A) Heat map of Ras-related protein 1 (Rap1), cuticle protein 19.8, guanine
3 nucleotide-binding protein, trans-sialidase and pathogenesis-related protein in
4 different treatment groups of *A. lucorum* in ITRAQ analysis. (B) Western blot of Ras-
5 related protein 1 (Rap1), cuticle protein 19.8, guanine nucleotide-binding protein,
6 trans-sialidase and pathogenesis-related protein in different treatment groups of *A.*
7 *lucorum*. Actin was used for normalization.