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# Expressions of the Protein Phosphatases PP1 and PP4 during the Embryonic Diapause Process of the Silkworm *Bombyx mori*

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Reversible protein phosphorylation is accomplished by the opposing activities of kinases and phosphatases. We previously demonstrated the regulation of serine/threonine protein phosphatase (PP) type 2A (PP2A) and 2B (PP2B or calcineurin) during the embryonic diapause process of *Bombyx mori*. In the present study, we further examine the expressions of other PPs (PP1 and PP4) during embryonic stages. An immunoblot analysis showed that *Bombyx* eggs contained a 38-kDa PP1 catalytic subunit (PP1-C), a 38-kDa PP4 catalytic subunit (PP4-C), and a 120-kDa PP1 nuclear targeting subunit (PNUTS), each of which underwent differential changes between diapause and developing eggs during the embryonic process. In non-diapause eggs, eggs whose diapause initiation was prevented by HCl, and eggs in which diapause had been terminated by chilling diapausing eggs at 5°C for 70 days and then were transferred to 25°C, protein levels of PP1-C and PP4-C remained relatively high during the early embryonic stages and then decreased during middle (for PP1-C) or later (for PP4-C) embryonic stages. However, protein levels of PP1-C and PP4-C in diapause eggs remained at high levels during the first 8 days after oviposition. PNUTS protein levels showed inverse temporal changes, with increased levels being detected during the later embryonic stages of developing eggs. The direct determination of PP1 enzymatic activity showed higher activity in developing eggs than in diapause eggs. Examination of temporal changes in mRNA expression levels of *PP1-C* and *PP4-C* showed no difference between HCl-treated and diapause eggs. These results indicated that differential protein levels of PP1-C/PNUTS and PP4-C, and increased enzymatic activity of PP1 were likely related to the embryonic development of *B. mori*.

**Key words:** *Bombyx mori*, PP1, PP4, PNUTS, Phosphatase, Diapause, Gene expression.

## BACKGROUND

An ability to adapt physiology to changing environmental conditions is essential for all living beings. Insects respond to unfavorable conditions (such as freezing temperatures in winter and extreme heat and desiccation in summer) by altering their developmental trajectory and entering a developmental arrest called diapause (Xu et al. 1995; Hahn and Denlinger 2011; Shiomi et al. 2015; Hand et al. 2016). The silkworm, *Bombyx mori* enters diapause during the embryonic stage (Yamashita and Hasegawa 1985). Embryonic diapause occurs immediately after telson differentiation and mesoderm segmentation, when the embryo ceases cell division at the G2 phase (Nakagaki et al. 1991). If the eggs are incubated at 25°C, diapause can be maintained for about 1 year. However, diapause is terminated by exposure to 5°C for about 2 months

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(Yamashita and Yaginuma 1991). Diapause initiation can also be prevented by HCl treatment (with a specific gravity of 1.075 at 15°C) for 5 min at 46°C, 20 hours after oviposition. In addition, the *Bombyx* polyvoltine strain produces non-diapause eggs.

*Bombyx* eggs provide a good model system for investigating regulatory signaling during the diapause process. Numerous investigations were carried out on temperature-dependent changes in several key carbohydrate metabolism-related enzymes during the diapause process (Yamashita and Yaginuma 1991; Lin et al. 2009). It was also shown that numerous protein kinases are involved in the embryonic development of *B. mori*. The studied protein kinases include extracellular signal-regulated kinase (ERK), which is a member of mitogen-activated protein kinases (MAPKs) and glycogen synthase kinase (GSK-3β), a multifunctional protein kinase that plays important roles in regulating both glycogen synthesis and protein synthesis (Iwata et al. 2005; Fujiwara et al. 2006a b; Fijiwara and Shiomi 2006; Lin et al. 2009; Gu and Chen 2017). Phosphorylation of the translational repressor 4E-binding protein (4E-BP), which is generally accepted as a marker of target of rapamycin (TOR) activity (Bhaskar and Hay 2007), appears to be an early signaling in embryonic development in eggs in which diapause initiation was prevented by HCl treatment (Gu et al. 2011). More recently, we demonstrated differential regulations of insulin/Akt and protein kinase C-dependent phosphorylation between diapause and developing eggs (Gu et al. 2019 2020).

Protein phosphatases (PPs) antagonize the action of kinases and can no longer be viewed as passive housekeeping enzymes in signaling pathways (Tonks 2006 2013; Chen et al. 2007; Seshacharyulu et al. 2013). As all reversible phosphorylation events are dynamic and regulated by counteracting protein kinases and PPs, there is a clear rationale for the essential regulatory roles played by PPs during the diapause process. It has been reported that ecdysteroid-phosphate phosphatase (EPPase), which specifically catalyzes the conversion of ecdysteroid-phosphates to free ecdysteroids, is differentially regulated between diapause and developing eggs, and is thus involved in the *Bombyx* egg diapause process (Sonobe and Yamada 2004; Fujinaga et al. 2020; Gu et al. 2021). Recently, we demonstrated that the expressions of PP2A and PP2B (also known as calcineurin) are related to the embryonic diapause process of *B. mori* (Gu et al. 2017; Hsieh and Gu 2019). However, it is unclear whether other serine/threonine phosphatases are differentially regulated between diapause and developing eggs. Considering that an evolutionarily conserved role of PP1 in mitotic regulation was well established in yeast, *Drosophila*, *Xenopus*, and mammalian cells (Chen et al. 2007; Shi 2009) and that differential cell cycles exist between diapause and developing eggs in *B. mori* (Nakagaki et al. 1991), we hypothesized that, similar to PP2A and calcineurin, PP1 and other regulators may be differentially regulated between diapause and developing eggs. To test this hypothesis, we focused on the protein levels of the PP1 catalytic subunit (PP1-C) and PP4 catalytic subunit (PP4-C) in this present study.

PP1 is a major serine/threonine phosphatase and is ubiquitously expressed in all eukaryotic cells. PP1 regulates diverse cellular processes such as cell cycle progression, protein synthesis, carbohydrate metabolism, transcription, cytoskeletal reorganization, and signaling transduction (Chen et al. 2007; Shi 2009). Cellular PP1 rarely exists in free form; instead, PP1-C is associated with numerous regulatory subunits that control its phosphatase activity, substrate specificity, and cellular localization (Bollen et al. 2010). One of the key PP1 regulatory targeting proteins in the nucleus is the PP1 nuclear targeting subunit (PNUTS) (Allen et al. 1998). In mammalian cells, PNUTS is one of the two most abundant PP1-interacting proteins in the nucleus (Allen et al. 1998). A study of *Drosophila* showed that PNUTS is highly conserved between flies and humans and that it is essential for organismal growth, with mutant animals arrested in early larval developmental stages (Ciurciu et al. 2013). Although the involvement of PP1-C/PNUTS in regulating mitotic progression and development has been previously reported (Ciurciu et al. 2013; Wang et al. 2019), no study has been conducted on the temporal regulation of PP1-C/PNUTS during the *Bombyx* egg diapause process. In addition, PP4 is another ubiquitous serine/threonine phosphatase that is highly conserved between humans and invertebrates (Cohen 1991; Cohen et al. 2005). An investigation of *Drosophila* demonstrated that embryos with reduced levels of PP4 exhibited substantial loss of viability and that PP4 plays an important role in the organization of microtubules at centrosomes (Helps et al. 1998).

In the present study, we investigate expressions of PP1-C/PNUTS and PP4-C during the embryonic process of *B. mori*. Our results show that PP1-C and PP4-C undergo differential changes in protein levels during the embryonic stage between diapause and developing eggs. Protein levels of PNUTS showed inverse temporal changes compared to those of PP1-C and PP4-C, with increased levels being detected during later embryonic stages of developing eggs. Directly determining PP1 enzymatic activity further confirmed higher PP1 activity in developing eggs compared to diapause eggs. Based on these results, we suggested that expressions of PP1-C/PNUTS and PP4-C and PP1 activities are likely related to the embryonic development of *B. mori*.

## MATERIALS AND METHODS

### Experimental animals

A hybrid strain (Guofu × Nongfong) and a polyvoltine strain (P1) of the silkworm, *B. mori*, were respectively used to produce diapause and non-diapause eggs. The diapause status of eggs was manipulated by the following treatments as previously reported (Gu et al. 2019): (1) continuous exposure to 25°C to maintain a diapause state; (2) exposure to 5°C for 70 days after incubation at 25°C for 15 days after oviposition and then transfer to 25°C to allow embryonic development; (3) HCl treatment (with a specific gravity of 1.075 at 15°C) for 5 min at 46°C, 20 hours after oviposition to prevent diapause; and (4) allowing normal embryonic development in non-diapause eggs. When non-diapause eggs from the polyvoltine strain were incubated at 25°C, they hatched about nine days after oviposition. Eggs laid within 12 h were collected from each treatment and used for the experiments.

### PP1 enzymatic activity assay

PP1 activity was measured with a fluorescent-based RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay Kit R-33700 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. According to the manufacturer's description, tyrosine phosphatase activity cannot be detected with this assay kit. PP1 activity can be distinguished from PP2A, PP2B, and PP2C by adding different metal ions, such as $NiCl_2$, $MnCl_2$, and $Ca^{2+}$ to the assay buffer, which can enable differential phosphatase activities (Cohen 1991). Egg lysates were prepared using a low-detergent buffer (1% Nonidet P-40, 10 mM HEPES, 150 mM NaCl, 10% glycerol, 1 mM PMSF, and a complete protease inhibitor cocktail). In total, 50 µl of egg lysates was incubated with 1× PP1 phosphatase reaction buffer (pH 7.0) for 30 min at 37°C. The fluorescence intensity was measured using excitation at 355 nm and emission at 485 nm. According to the manufacturer's recommendations, data were presented as the percentage of PP1 enzymatic activity compared to eggs from the respective first day of each treatment.

### Antibodies

Anti-mouse PNUTS (sc-271681) and anti-mouse PP4-C (sc-374106) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rabbit PP1α (cat. no. 42582) (which detects PP1-C) and anti-rabbit heat shock protein (HSP) 90 (cat. no. 4874) antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase (HRP)-linked goat anti-rabbit and anti-mouse secondary antibodies were purchased from PerkinElmer Life Sciences (Boston, MA, USA).

### Western blot analysis

Eggs from different stages were homogenized in 10 µl of lysis buffer (10 mM Tris and 0.1% Triton X-100) at 4°C (Gu et al. 2020). Lysates were boiled in an equal volume of sodium dodecyl sulfate (SDS) sample buffer for 4 min, followed by centrifugation at 15,800 g for 3 min to remove any particulate matter. Each 5 µl of supernatant (approximately 12 µg of total protein) was loaded onto SDS gels. Following electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes using an Owl Bandit™ Tank Electroblotting System (Portsmouth, NH, USA), and the membranes were then washed with Tris-buffered saline (TBS) for 5 min at room temperature. Blots were blocked at room temperature for 1 h in TBS containing 0.1% Tween 20 (TBST) and 5% (w/v) nonfat powdered dry milk, followed by washing three times for 5 min each with TBST. Blots were incubated overnight at 4°C with the primary antibody in TBST with 5% bovine serum albumin (BSA). Blots were then washed three times in TBST for 10 min each and further incubated with the HRP-linked secondary antibody in TBST with 1% BSA. Following three additional washes, the immunoreactivity was visualized by chemiluminescence using Western Lightning Chemiluminescence Reagent Plus from PerkinElmer Life Sciences. Films exposed to the chemiluminescent reaction were scanned and quantified using an AlphaImager Imaging System and AlphaEaseFC software (Alpha Innotech, San Leandro, CA, USA). Quantified protein levels relative to HSP (loading control) were standardized to levels from the first day of embryonic stage.

### RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA from eggs (29 mg, about 50 eggs) was extracted using 600 µl of the TRI Reagent (Molecular Research Center, OH, USA) according to the manufacturer's protocol. The concentration was determined using a NanoPhotometer Pearl (Implen, Munich, Germany). First-strand complementary (c) DNA synthesis was performed using an iScript cDNA synthesis kit (Bio-Rad, CA, USA).

For the qRT-PCR analysis, total RNA was extracted from eggs on different days. A qRT-PCR was carried out in a 20-µl reaction volume containing 10 µl of SYBR1 Green Realtime PCR Master Mix

(Bio-Rad), 2 µl of a first-strand cDNA template, 2 µl of forward and reverse primers, and 4 µl of water. The iQ5 Real-Time PCR Detection System (Bio-Rad) was used according to the manufacturer's instructions. RT-PCR primers were designed according to parameters (no primer dimers and a product length of no more than 200 bp) outlined in the manual of SYBR1 Green Realtime PCR Master Mix. The annealing temperature for all reactions was 59.5°C. Because a previous study showed that *B. mori* ribosomal protein 49 (*rp49*) is the most stable gene during embryonic development (Kobayashi et al. 2014), it was chosen as a reference gene in the current study. The qRT-PCR was performed using the following primers: *PP1-C* forward, 5'-CCTTGAACGGGTTGATGTTTTAGT-3' and reverse, 5'-GGGAGGTTGCTGGGTTGT-3'; *PP4-C* forward, 5'-GTTCCTCACGACGGACCAAT-3' and reverse, 5'-GCCCTCCATGACAAGCTGA-3'; and *rp49* forward, 5'-CAGGCGGTTCAAGGGTCAATAC-3' and reverse, 5'-TGCTGGGCTCTTTCCACGA-3'.

### Statistical analysis

Data are shown as the mean ± standard error of the mean (SEM). Statistical comparisons between different groups were done using either Student's *t*-test (for comparison of two groups) or one-way analysis of variance (ANOVA) followed by Tukey's test (for more than two groups). A $p$ value of $< 0.05$ was considered significant. In addition, for the gene expression experiments, we set the significance value at $> 2.0$ for upregulated genes, and at $< 0.5$ for downregulated genes. Genes that exhibited more significant perturbations were then identified by ANOVA.

## RESULTS

### Expression of PP1/PNUTS and PP4 in silkworm eggs

As the first step to study the involvement of PP1-C/PNUTS in the *B. mori* embryonic diapause process, a Western blot analysis was used to examine whether differences exist in the levels of PP1-C, PNUTS, and PP4-C proteins between diapause eggs and eggs in which diapause initiation was prevented by HCl treatment. As shown in figure 1A, no significant differences were detected in the protein levels of either PP1-C, PNUTS, or PP4-C between eggs in which diapause initiation was prevented by HCl treatment and diapause eggs (treated with water) on day 4 after the treatment. Figure 1B shows a comparison between day 7 diapause eggs and HCl-treated eggs. As shown in the figure 1B, PP1-C and PP4-C protein levels of HCl-treated eggs were significantly lower than those of diapause eggs. Higher PNUTS protein levels were detected in HCl-treated eggs than in diapause eggs. These results imply the possibility that the expressions of PP1-C/PNUTS and PP4-C are related to the embryonic diapause process. In subsequent experiments, we studied temporal changes in each subunit during the embryonic diapause process in more detail.

### Changes in protein levels of PP1-C/PNUTS and PP4-C upon HCl treatment at 20 h after oviposition and in diapause eggs

To further clarify whether PP1 and PP4 were related to the embryonic development of eggs in which diapause initiation was prevented by HCl treatment, temporal changes in protein levels of PP1-C/PNUTS and PP4-C and PNUTS were examined in samples of diapause-destined eggs treated with HCl 20 h after oviposition to prevent the eggs from entering diapause. Results (Fig. 2) showed that levels of PP1-C and PP4-C remained relatively high during early embryonic stages. However, on day 5, levels of PP1-C sharply decreased and then remained low during later embryonic stages. Protein levels of PP4-C sharply decreased on day 7 and remained low on day 8. Protein levels of PNUTS remained low during early embryonic stages, and then gradually increased during middle and later embryonic stages. For diapause eggs, results (Fig. 3) showed that protein levels of PP1-C and PP4-C remained high during the first 8 days after oviposition. Protein levels of PNUTS remained low between days 0 and 8 after oviposition.

### Changes in protein levels of PP1-C/PNUTS and PP4-C in both non-diapause eggs and eggs in which diapause was terminated by chilling

To further study the correlation of temporal changes in protein levels of PP1-C/PNUTS and PP4-C with embryonic development, we used non-diapause eggs and eggs in which diapause was terminated by chilling during embryonic development. As shown in figure 4, in non-diapause eggs, levels of PP1-C and PP4-C decreased during middle and later embryonic stages. Increased protein levels of PNUTS were detected during the middle and later stages of embryonic development. Protein levels of PP1-C, PP4-C, and PNUTS in eggs in which diapause was terminated by chilling during the first 8 days after eggs were transferred to 25°C showed almost similar temporal changes as those in non-diapause eggs (Fig. 5). These results further confirmed that decreasing protein

levels of PP1-C and PP4-C and increasing protein levels of PNUTS are likely related to embryonic development.

### Changes in PP1 enzymatic activity

If PP1 is a potential candidate for regulating the *B. mori* embryonic diapause process, it would be expected that PP1 enzymatic activity would be differentially regulated in diapause and developing eggs. To test this hypothesis, PP1 enzymatic activity was determined in extracts of eggs using the RediPlate™ 96 EnzChek® Serine/Threonine Phosphatase Assay Kit. As suggested by the manufacturer's protocol, DTT and $MnCl_2$ were specifically used to optimize the PP1 assay. As shown in figure 6, PP1 activity remained at low levels in diapause eggs during the first 8 days after oviposition. However, in non-diapause eggs, eggs whose diapause initiation was prevented by HCl, and eggs in which diapause had been terminated by chilling, PP1 activity increased during the first several days, reached a peak during the middle embryonic stage, and then greatly decreased 5 or 6 days before hatching. These results showed that high

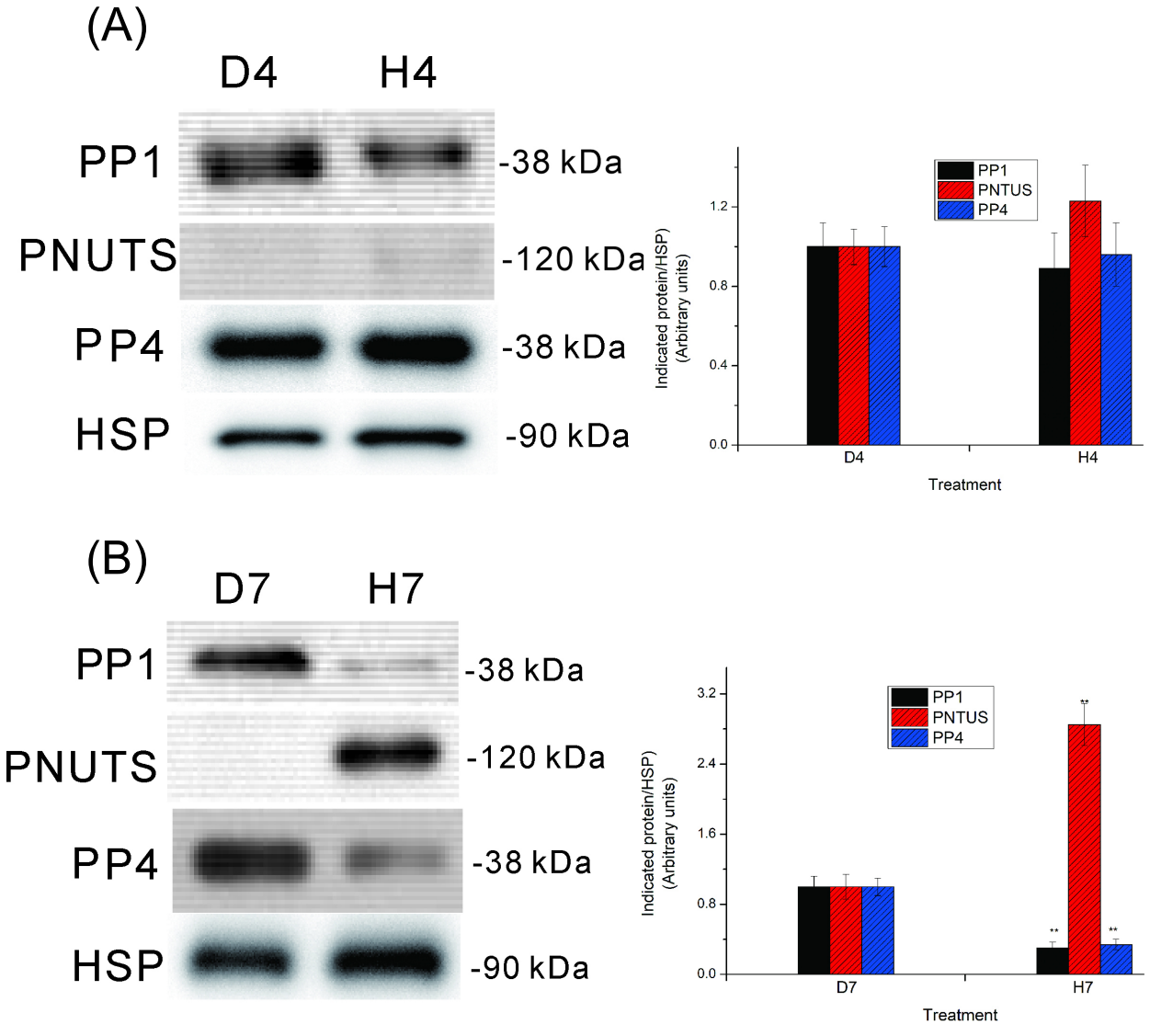


**Fig. 1.** Western blot analysis of PP1-C, PNUTS, and PP4-C in silkworm eggs. Following SDS-PAGE and immunoblotting, silkworm egg extracts (a quarter of one egg for each lane) were probed with anti-PP1α (PP1), anti-PNUTS (PNUTS), anti-PP4-C (PP4), and anti-HSP 90 (HSP) antibodies. (A) Results from day 4. D4, extracts from day 4 diapause eggs; H4, extracts from eggs whose diapause was prevented by HCl (4 days after HCl treatment). (B) Results from day 7. D7, extracts from day 7 diapause eggs; H7, extracts from eggs whose diapause was prevented by HCl (7 days after HCl treatment). Results shown in the left panel are representative of four independent experiments. Each protein band was quantified and normalized to the level of HSP in the right panel. Asterisks indicate significant differences compared to respective diapause eggs (by Student's *t*-test, ** $p < 0.01$).

PP1 activities are likely linked to *B. mori* embryonic development.

### Changes in mRNA expression levels of PP1-C and PP4-C

A further experiment was conducted to examine changes in mRNA expression levels of *PP1-C* and *PP4-C* in diapause eggs and diapause-destined eggs treated with HCl 20 h after oviposition to prevent the eggs from entering diapause. Results (Fig. 7) showed that on the first days after oviposition, higher levels of *PP1-C* and *PP4-C* were detected. Levels then decreased in both HCl-treated and diapause eggs. Between days 2 and 9, no significant differences were detected between HCl-treated and diapause eggs.

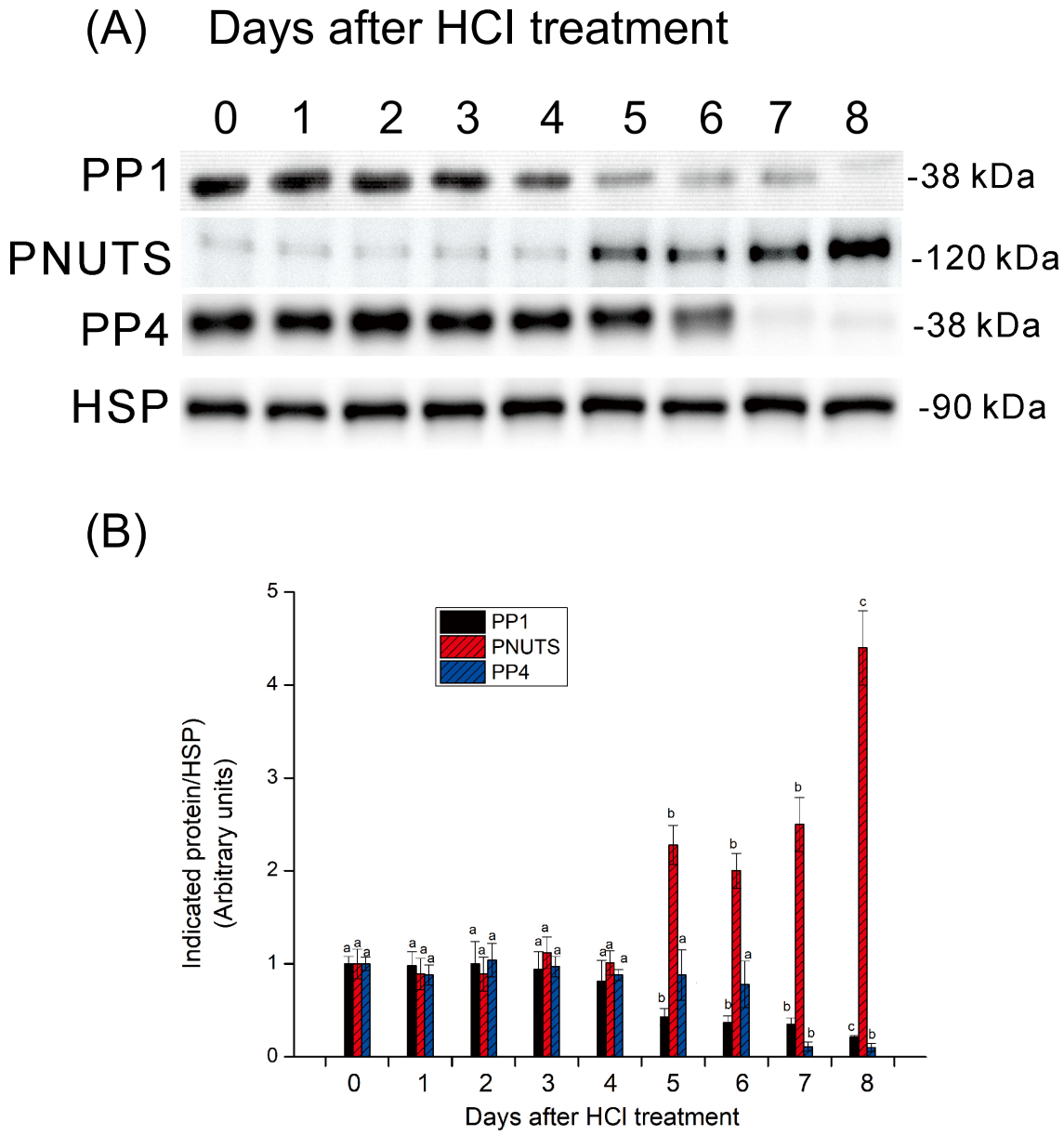


**Fig. 2.** Effect of HCl treatment on protein levels of PP1-C, PNUTS, and PP4-C during the first 8 days after treatment. Diapause-destined eggs that had been incubated at 25°C for 20 h after oviposition were treated with HCl and then incubated at 25°C. Egg lysates from each stage were prepared and subjected to an immunoblot analysis with anti-PP1α (PP1), anti-PNUTS (PNUTS), anti-PP4-C (PP4), and HSP 90 (HSP) antibodies. Lysates from a quarter of one egg were used for each lane. Molecular weight markers are shown on the right side of the gel (A). Results shown in the upper panel are representative of four independent experiments. Quantified protein levels relative to HSP were standardized to levels from the first day after HCl treatment. Different letters above the bars indicate significant differences (ANOVA followed by Tukey's test).

## DISCUSSION

Our previous studies demonstrated correlations between the differential expressions of two serine/threonine phosphatases (PP2A and calcineurin) and the insect egg diapause process of *B. mori* (Gu et al. 2017; Hsieh and Gu 2019). Results presented herein further clearly indicate that different changes occur in the expressions of two other serine/threonine phosphatases (PP1 and PP4) between diapause and developing eggs during embryonic stages. In diapause eggs, protein levels of PP1-C and PP4-C remained relatively high, and PNUTS protein levels remained very low. However, in HCl-treated developing eggs, decreased protein levels of PP1-C and PP4-C and increased PNUTS protein levels were detected during middle and later embryonic stages. Differences observed in the above protein levels were further confirmed using either non-diapause eggs or eggs in which diapause had been terminated by chilling, for which almost similar

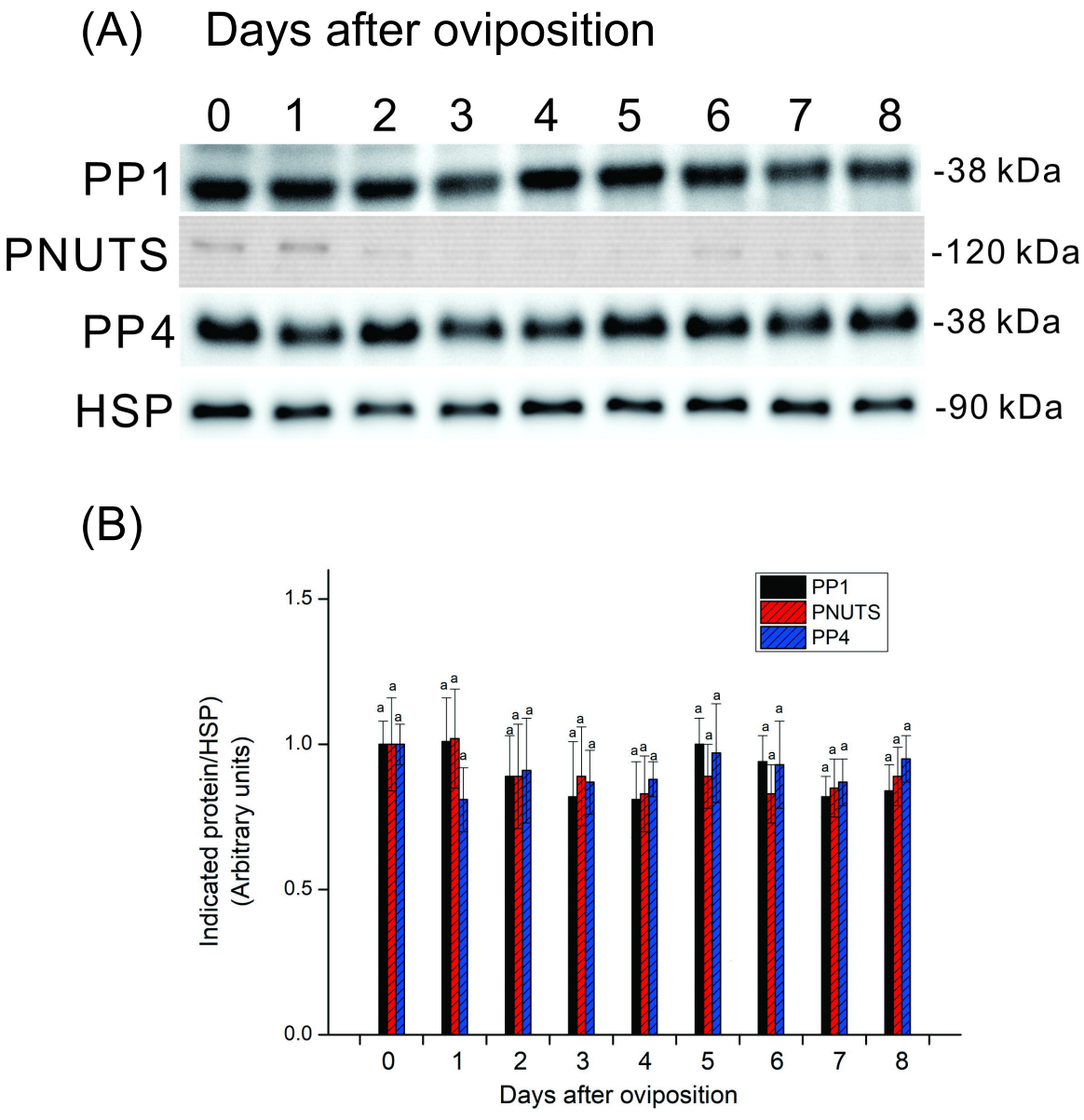


**Fig. 3.** Western blot analysis of protein levels of PP1-C, PNUTS, and PP4-C in diapause eggs. Egg lysates from each stage after oviposition were prepared and subjected to an immunoblot analysis with anti-PP1α (PP1), anti-PNUTS (PNUTS), anti-PP4-C (PP4), and anti-HSP 90 (HSP) antibodies. Lysates from a quarter of one egg were used for each lane. Molecular weight markers are shown on the right side of the gel (A). Results shown in the upper panel are representative of four independent experiments. Quantified protein levels relative to HSP were standardized to levels from the first day after oviposition. Different letters above the bars indicate significant differences (ANOVA followed by Tukey's test).

temporal changes were observed as those in HCl-treated eggs. Determination of PP1 enzymatic activity showed higher activities in developing eggs compared to those in diapause eggs. These results clearly implied that PP1 and PP4 are potentially linked to embryonic development during the silkworm diapause process, thus providing additional information for the complex regulatory mechanism underlying the embryonic diapause process (Fig. 8).

PP1 is one of the highly conserved serine/threonine phosphatases and dephosphorylates dozens of substrates, and thus plays essential regulatory roles in various cellular events including transcriptional regulation, cell cycle control, apoptosis, and telomere maintenance (Ceulemans and Bollen 2004). Mutations of PP1 in various fungi and *Drosophila* resulted in mitotic arrest or deficient cytokinesis (Axton et al. 1990; Baker et al. 1997). It was demonstrated that *in*

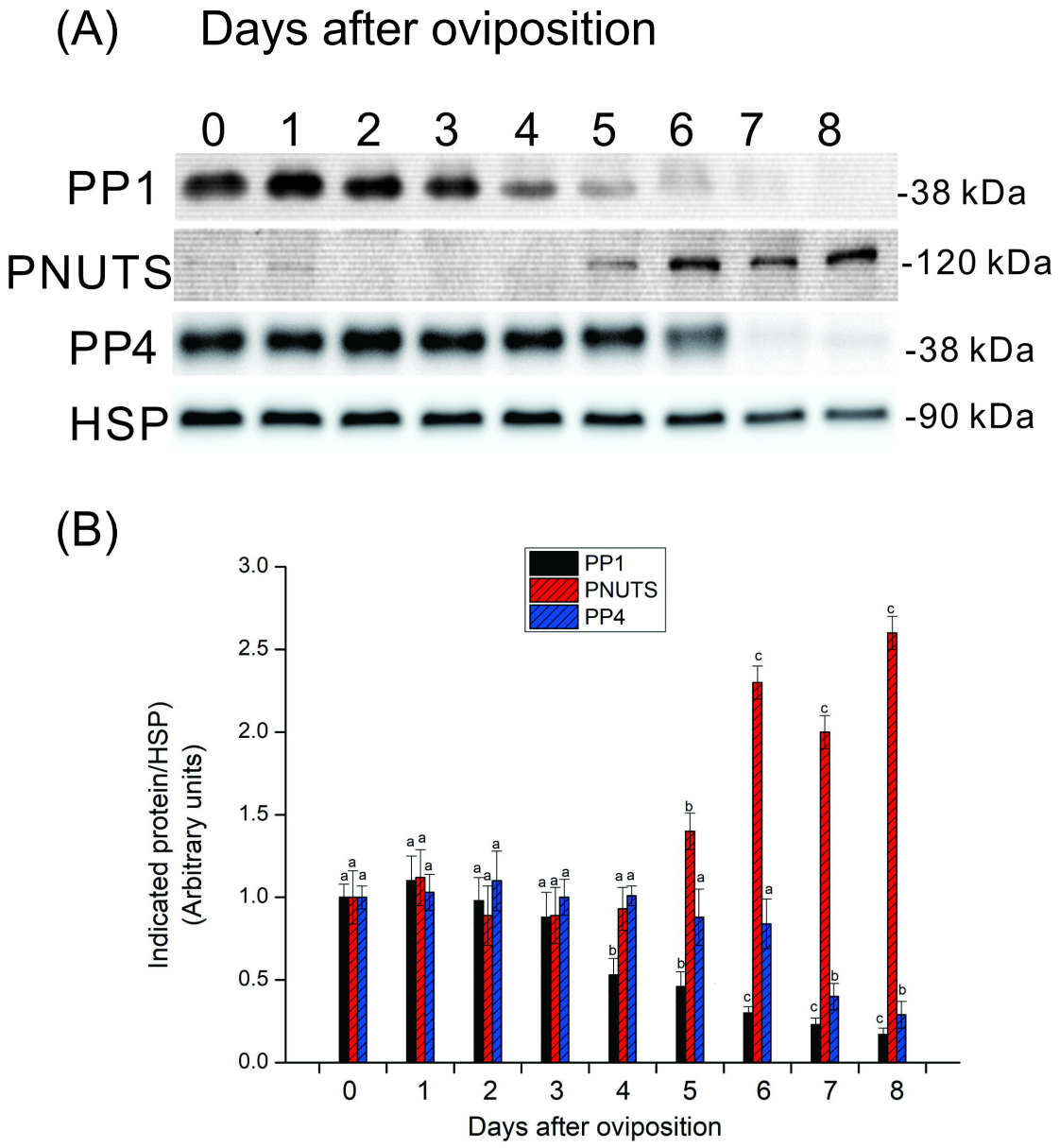


**Fig. 4.** Western blot analysis of protein levels of PP1-C, PNUTS, and PP4-C in non-diapause eggs. Egg lysates from each stage after oviposition were prepared and subjected to an immunoblot analysis with anti-PP1α (PP1), anti-PNUTS (PNUTS), anti-PP4-C (PP4), and anti-HSP 90 (HSP) antibodies. Lysates from a quarter of one egg were used for each lane. Molecular weight markers are shown on the right side of the gel (A). Results shown in the upper panel are representative of four independent experiments. Quantified protein levels relative to HSP were standardized to levels from the first day after oviposition. Different letters above the bars indicate significant differences (ANOVA followed by Tukey's test).

*vivo* PP1 is associated with a host of function-specific targeting and substrate-specifying polypeptides to form a variety of distinct multimeric holoenzymes. PNUTS is one of two most abundant PP1-interacting proteins in the nucleus. An analysis of *Drosophila* PNUTS showed that via its association with PP1, PNUTS regulates RNA polymerase II-mediated gene expression during larval development to support organismal growth (Ciurciu et al. 2013). Considering that in the diapause eggs of *Bombyx* the embryo stops cell division at the $G_2$ phase (Nakagaki et al. 1991), it is not surprising that there are very low levels of PP1 enzymatic activity. In developing eggs (non-diapause eggs, HCl-treated eggs, and chilled eggs), PP1 enzymatic activity increased during the early

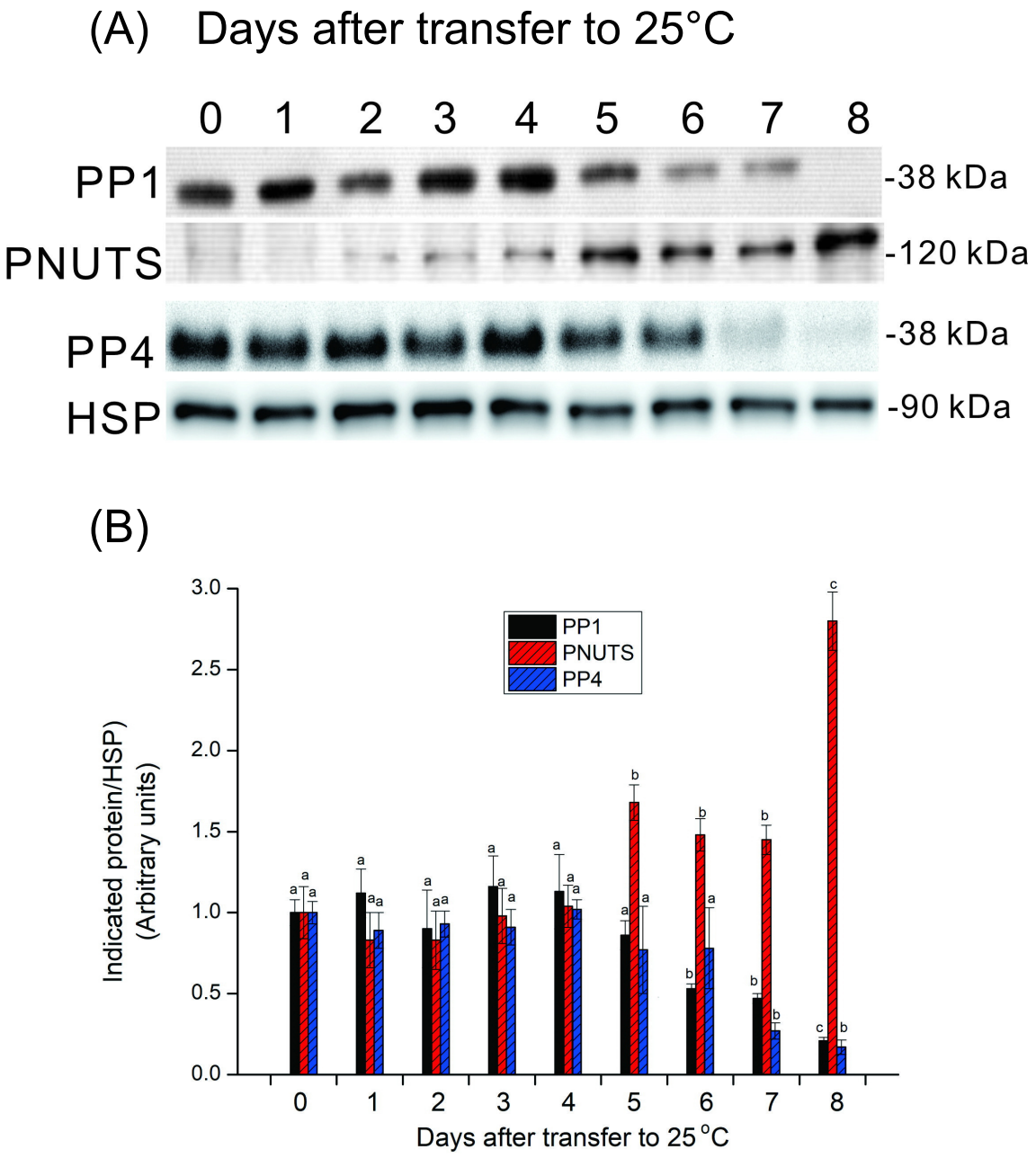


**Fig. 5.** Western blot analysis of protein levels of PP1-C, PNUTS, and PP4-C in eggs in which diapause had been terminated by chilling of diapausing eggs at 5°C for 70 days and then transferred to 25°C. Egg lysates from each stage after being transferred to 25°C were prepared and subjected to an immunoblot analysis with anti-PP1α (PP1), anti-PNUTS (PNUTS), anti-PP4-C (PP4), and anti-HSP 90 (HSP) antibodies. Lysates from a quarter of one egg were used for each lane. Molecular weight markers are shown on the right side of the gel (A). Results shown in the upper panel are representative of four independent experiments. Quantified protein levels relative to HSP were standardized to levels from the first day after transfer to 25°C. Different letters above the bars indicate significant differences (ANOVA followed by Tukey's test).

embryonic stages, reached a major peak during the middle embryonic development, and then decreased. In mammalian cells, it was demonstrated that PNUTS may act as both an inhibitor and a targeting subunit of PP1 and that a recombinant PNUTS (309–691 aa) fragment potently inhibited the catalytic activity of PP1 towards an exogenous substrate *in vitro* (Allen et al. 1998). In *Xenopus*, it appears that PNUTS plays a negative role in regulating PP1 activity (Fisher et al. 2014). Considering that PNUTS inhibits PP1, the increase in protein levels of PNUTS during the later embryonic stages in developing eggs may play a role in decreasing in PP1 activity after the major peak. In addition, low protein levels of PNUTS in diapause eggs may be related to the low levels of PP1 enzymatic activity.

Moreover, PP1 activity responded in the opposite direction to changes in protein levels of PP1-C. The protein levels of PP1-C remained relatively high in diapause eggs during the first 8 days of embryonic stages, but the levels decreased during the middle embryonic stages in developing eggs. Similar results were found in calcineurin of the *Bombyx* egg diapause process (Hsieh and Gu 2019). We suggested that changes in protein levels may be an attempt to compensate for changes in activity. Increased phosphatase activity but decreased protein levels were also demonstrated in the expression of calcineurin in the rat brain after the administration of antipsychotics (Rushlow et al. 2005). It was demonstrated that non-catalytic domains in the calcineurin A subunit negatively regulate enzyme activity and act as intramolecular inhibitors (Liu et al. 2005). The structure of PP1-C

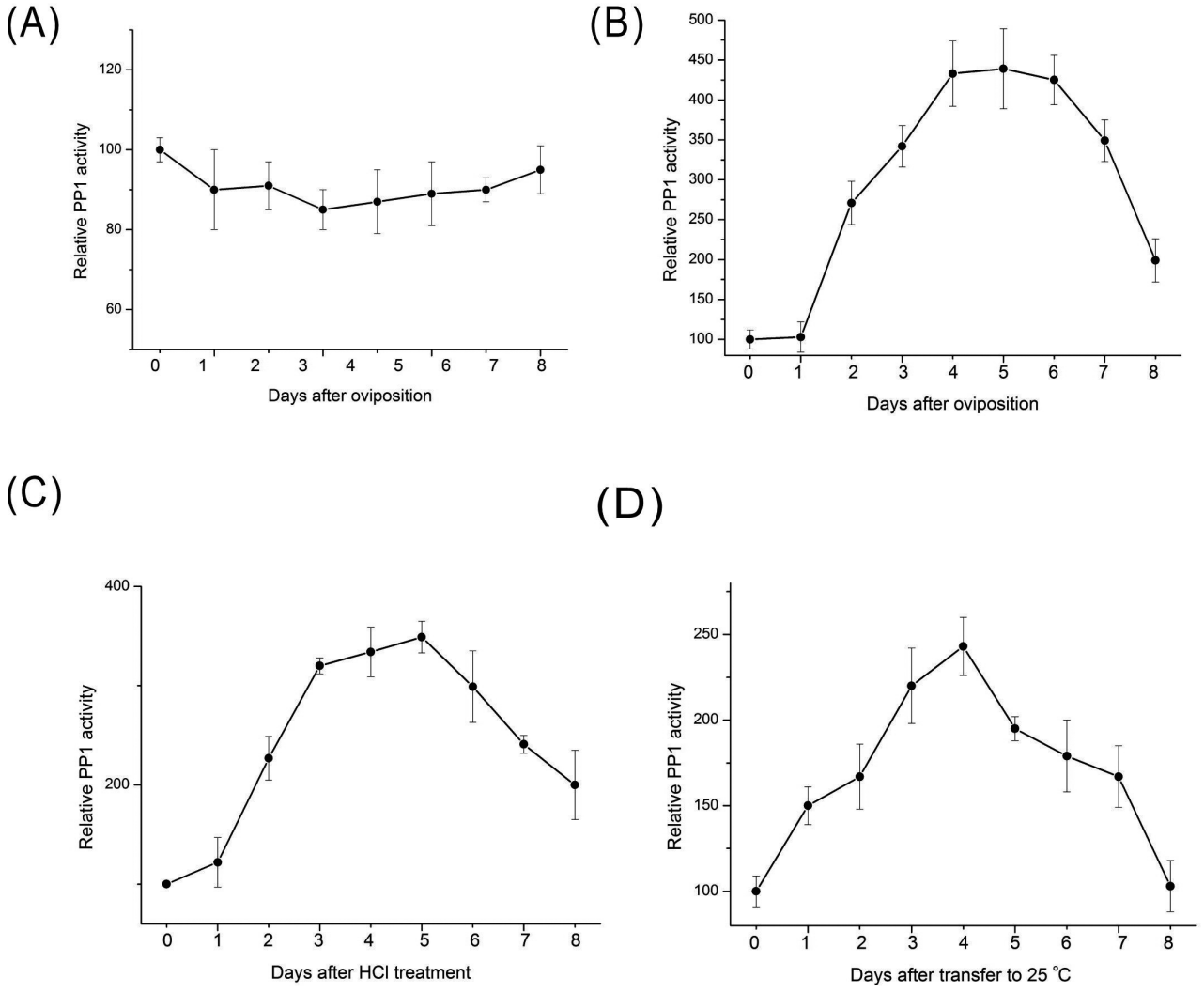


**Fig. 6.** Changes in PP1 enzymatic activity in *Bombyx* eggs. A, diapause eggs; B, non-diapause eggs; C, eggs whose diapause initiation was prevented by HCl; D, eggs in which diapause had been terminated by chilling. Egg extracts from each stage were prepared, and PP1 enzymatic activity was determined with a PP1 assay kit. The data represent mean ± SEM of four separate assays.

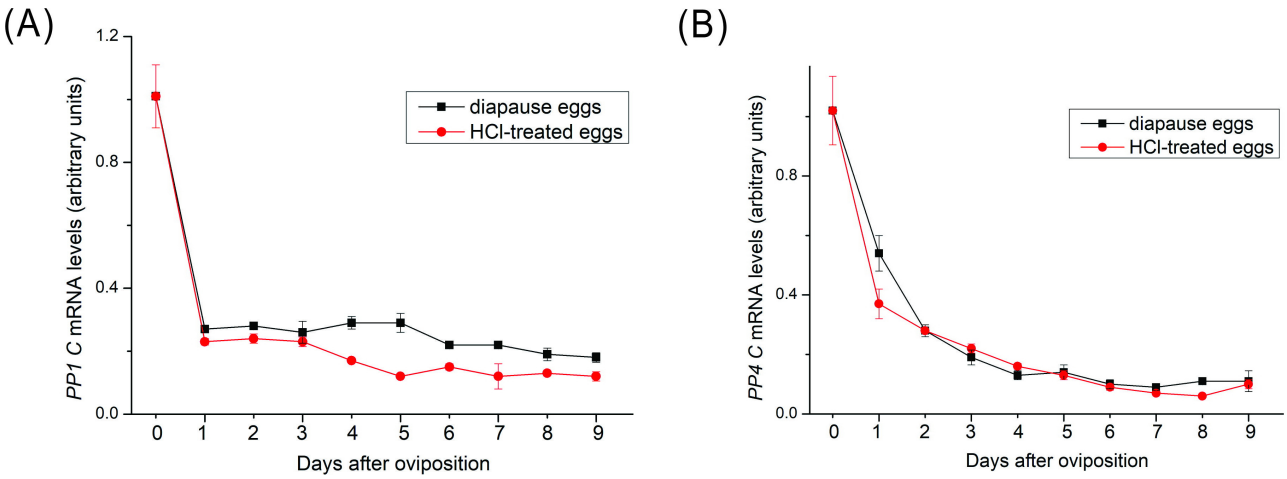


**Fig. 7.** Changes in mRNA expression levels of *PP1-C* (A) and *PP4-C* (B) in diapause eggs and diapause-destined eggs treated with HCl 20 h after oviposition to prevent the eggs from entering diapause. Egg extracts from each stage were then prepared, and mRNA expression levels were determined by qRT-PCR. The mRNA expression levels of *PP1-C* and *PP4-C* relative to *rp49* were standardized to the means of day 0 of diapause eggs and are represented as the relative amount ± SEM ($n$ = 4). Circles, HCl-treated eggs; squares, diapause eggs.

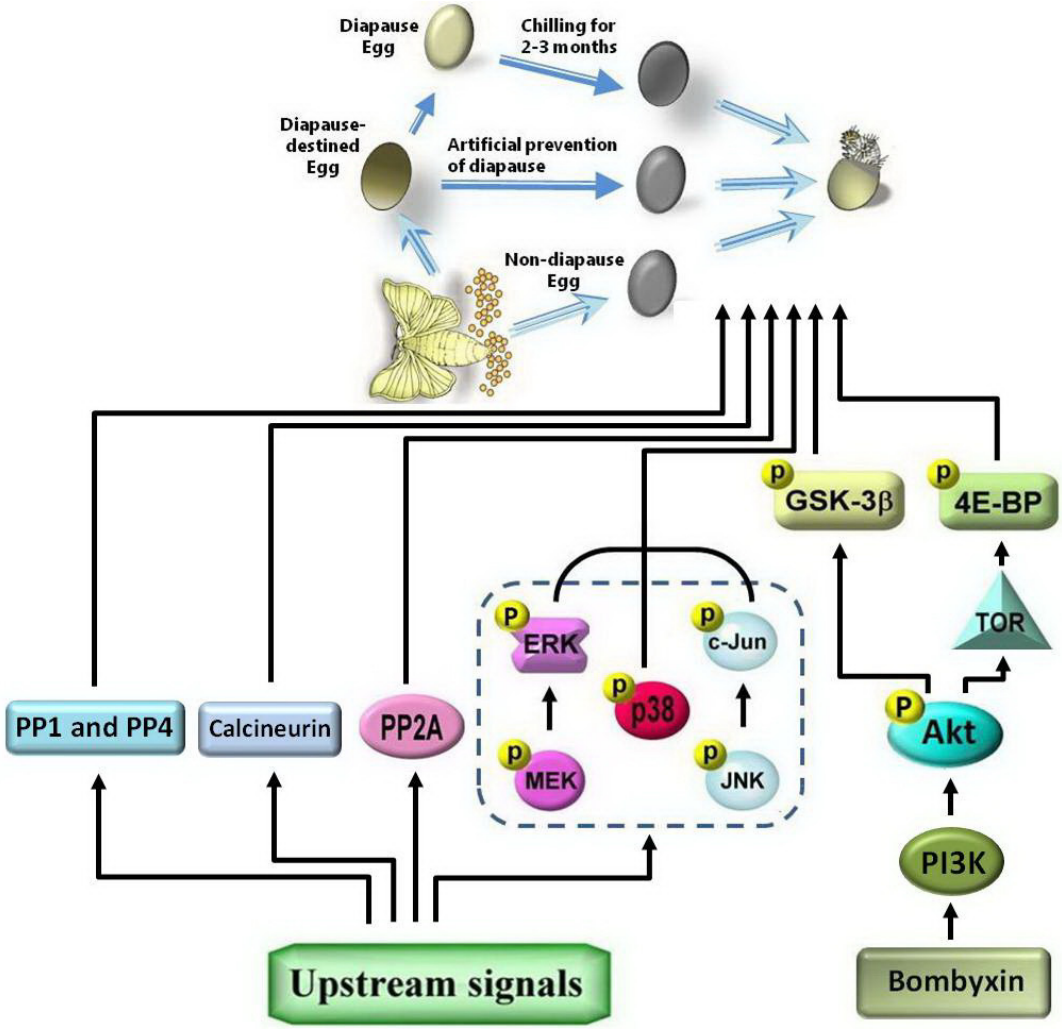


**Fig. 8.** Our current understanding of the signaling network involved in the *Bombyx* embryonic diapause process. See text for details.

is very similar to that of calcineurin A (Shi 2009). In addition, the phosphatase activity of PP1 is regulated by a number of endogenous inhibitory proteins such as inhibitor-1 and inhibitor-2 (Eto and Brautigan 2012). A *Drosophila* homologue of mammalian inhibitor-2 was previously reported (Helps and Cohen 1999). Although PNUTS potentially inhibited PP1 activity, identification of other PP1 inhibitory proteins in *Bombyx* remains to be investigated in future experiments. In addition, the present study showed that similar temporal changes in transcriptional levels of *PP1-C* were detected between diapause and developing eggs, indicating no correlation between the transcriptional expression of *PP1-C* and its enzyme activity.

## CONCLUSIONS

We demonstrated temporal expressions in PP1-C and PP4-C during the *Bombyx* embryonic diapause process. We found that inverse changes in protein levels of PP1-C and PNUTS exist between diapause and developing eggs. Higher PNUTS protein levels and PP1 enzyme activity in developing eggs compared to diapause eggs suggest a potential correlation between PP1 signaling and the *Bombyx* embryonic diapause process.

### List of abbreviations

PP, protein phosphatase.
PP2A, protein phosphatase 2A.
PP2B, protein phosphatase 2B.
PP1-C, PP1 catalytic subunit.
PP4-C, PP4 catalytic subunit.
PNUTS, PP1 nuclear targeting subunit.
ERK, extracellular signal-regulated kinase.
MAPKs, mitogen-activated protein kinases.
GSK, glycogen synthase kinase.
4E-BP, 4E-binding protein.
TOR, target of rapamycin.
EPPase, ecdysteroid-phosphate phosphatase.
HRP, Horseradish peroxidase.
SDS, sodium dodecyl sulfate.
PVDF, polyvinylidene difluoride.
TBS, Tris-buffered saline.
BSA, bovine serum albumin.
qRT-PCR, quantitative real-time polymerase chain reaction.

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**Availability of data and materials:** Raw data can be obtained upon reasonable request.

**Ethics approval consent to participate:** Not applicable.

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