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EFFECTS OF PHYTOECDYSONE ON THE MOLTING PERIOD AND SURVIVAL RATE OF THE BLUE SWIMMING CRAB, *Portunus pelagicus*.

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ABSTRACT

To develop synchronized molting for soft-shell crab production, phytoecdysone, a plant steroid, is used as a stimulant instead of cutting eyestalks or limbs. Therefore, a study was performed to examine the effects of phytoecdysteroid extracts from *Vitex glabrata* on molting period and survival rate of the blue swimming crab, *Portunus pelagicus.* Post-molt (B) stage, inter-molt (C2) stage, pre-molts (D1 and D2) stages were, respectively, injected with a single dose of phytoecdysone at 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 μg/g body weight. The results showed that the effective doses significantly shortening molting periods were 0.4 μg/g BW, 0.5 μg/g BW, and 0.1, 0.4 μg/g BW when injections were performed on stages B, C2, and D1, respectively. However it could not induce molting by this method at stage D2, and molting was inhibited by the injection. An injection at stage B, survival rate of crab showed significantly higher than those of control at 0.1, 0.2, and 1.0 μg/g BW. As well, the survival rate of treatments showed significantly higher than those of control for injection at stage C2. In contrast, injection at stage D1, survival rates of 0.1, 0.2, and 1.0 μg/g BW were not significantly different from control, but were significantly lower than those of control at doses of 0.3, 0.4, and 0.5 μg/g BW. Survival rate was significantly different between control and treatments at injection of stage D2. The results indicated that, except for D2, the effective dosages for reducing the molting period injection at stage B, C2, and D1 were 0.4, 0.5, and 0.1 μg/g BW, respectively.

Keywords: Phytoecdysone, *Portunus pelagicus*, crab, molting.

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INTRODUCTION

The blue swimming crab (*Portunus pelagicus*) is an important commercial aquatic animal with high market demand, especially as soft-shell crabs. In Thailand, a report from The Department of Agricultural Economic Research, Ministry of Agriculture, Thailand indicated that its natural populations are declining (Department of Fisheries, 2010). Thus, the farmers want to produce crabs, including soft-shell crabs, to compensate for the declining natural resource. The soft-shell blue swimming crab is one agricultural product which offers both a good yield and benefit to farmers; however, the molting period of this species is quite long. It takes approximately 30-38 days for the next molt of adult crab when cultured in cement ponds, and 50-80 days in earth ponds, depending on sex, size, feeding, and salinity (Pratoomchat and Barnette, 2005). Currently, the yield of blue swimming crabs from the fishery is unreliable and varies seasonally.

The molt cycle of crustacean is controlled by the molting hormone, ecdysteroid is a hormone that can stimulate the molting cycle and induce intermolt crabs into a premolt stage (Skinner, 1985). It is known that ecdysteroid increases during the premolt (Soumoff and Skinner, 1983); thus, it might be possible to stimulate early molting stages into a premolt by using molting hormone (i.e., shorten and synchronize the molting period), which result in shortening the molting period. The important problem is an inadequate knowledge about appropriate doses of hormone and stage of molt that should be used. Overdosing could lead to hyperstimulation of the molting cycle and eventually kill the crab. In contrast, under dosing would have too little effect on the crab.

Phytoecdysone is a plant hormone which mimics ecdysone in crustaceans. Both hormones are classified as steroids but their side chains are different (Dinan, 2001). However, from previous studies, they have a similar effect in animals. Phytoecdysteroid used in this study has been found in *Vitex glabrata,* i.e., 20-hydroxyecdysone (99.3%), turkesterone (0.7%) and 20, 26-dihydroxyecdysone (0.006%) w/v (Werawattanametin et al., 1986; Suksamran et al., 1998). The structure of 20-hydroxyecdysone is similar to the molting hormone in arthropods and crustaceans. Several studies have shown that the molting period in some aquatic species, e.g., *Penaeus monodon*, can be shortened by either injecting or feeding with shrimp phytoecdysone prepared from crude plant (*V. glabrata*) extracts (Hutacharoen et al., 1989; Putchakarn, 1991). Many studies also reported that phytoecdysteroids could potentially play an important role in the aquaculture of many species (Hutacharoen et al., 1989; Putchakarn, 1991; Cho and Itami, 2004). However, the effects of exogenous ecdysteroids have been studied in many species, but they have not been assessed in *P. pelagicus*.

The blue swimming crab is an important commercial aquatic animal with high market demand, especially as soft-shell crabs. The soft-shell blue swimming crab is one agricultural product, which offers both a good yield and benefit to farmers; however, the molting period of this species is quite long in captive culture. It takes around 30-38 days for the next molt of adult crab cultured in cement pond, and 50-80 days in earth pond depending on sex, size, feeding, and salinity (Pratoomchat and Barnette, 2005).

The aim of this study was to evaluate the effect of phytoecdysteroid extracts from *V. glabrata* on molting period and survival in *P. pelagicus* via injection for improving synchronized production of soft-shell crab. This also included its potential to shorten the molting period in soft-shell crab production for supporting aquaculture production.

MATERIALS AND METHODS

Samples collection and preparation

Male *P. pelagicus* with carapace width of 80 - 90 mm (50 ± 5 g) were collected from the coast of Chon Buri Province, Thailand. After collection, they were immediately transported to an experimental aquarium facility at Burapha University where the crabs were placed in 0.5 x 1 m rectangular fiber-glass tanks filled with 20 ppt seawater which was changed twice daily. Crabs were held at stocking density of 20 crabs/m^2. Water condition such as pH, total ammonia, dissolved oxygen, and temperature,

within each rearing units, were monitored and maintained in order to maintain them within acceptable conditions (pH 7.9 - 8.2, total ammonia < 0.5 mg/ml, dissolved oxygen 4 - 6 mg/l, total nitrite < 0.20 mg/l, temperature 27 - 29˚C) throughout the experimental period.

The developmental stage of each crab was observed using a dissecting light microscope and classified into four molt stages; post-molt (B), inter-molt (C2), and pre-molt (D1 and D2). Ten crabs per replication for stages C2 and D1, and five crabs per replication for stages B and D2. Then they were injected with a single dose of 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 μg/g phytoecdysone (dissolved in sterile sea water) per body weight (BW) of crab at the base of chela, respectively. In each case, sterilized seawater was used as the vehicle for delivering the phytoecdysone. Crabs in the control groups were injected with an equivalent volume of sterile seawater. Three replicates were used. After that, the crabs were maintained and fed twice a day. The molt stage of each crab was examined every three days until they molted. The morphological changes during each molt stage were observed by light microscopy using the methods detailed by Pratoomchat (2007).

Statistical analysis

Differences in molting periods and survival rate were evaluated by one-way ANOVA and Duncan's new multiple regressions. Differences were considered significant at the level of $p \leq 0.05$.

Phytoecdysone extraction

Dried, powdered of barks *V. glabrata* were extracted with hexane and 95% ethyl alcohol (EtOH) in a Soxhlet apparatus. The EtOH concentration was concentrated to 300 ml, and EtOH (400 ml) and H_2O (2 L) were subsequently added. The filtered brownish solution was transferred to a continuous liquid-liquid extraction and further extracted with $CHCl_3$, Et_2O, and finally with EtOAc without 20-hydroxyecdysone. Re-crystallization from MeOH/EtOAc gave pure 20-hydroxyecdysone with a melting point of 240-242 ˚C. Spectroscopic analyses (UV, ir, ^1H nmr, ms) compared with the reported data confirmed the identity of this ecdysteroid (Werawattanametin et al., 1986).

RESULTS

Effect of phytoecdysone on molting period

For stage B, results showed that only the 0.4 μg/g BW dose exhibited a significant reduction ($p \leq 0.05$) for the molting period from stage B to D2. The dosages of 0.3 and 1.0 μg/g BW could also shorten the molting period, but this was not significantly different from the control (Figure 1). The molting period of crab at stage C2 injected with concentration of 0.5 μg/g BW was significantly shortened compared with the control, and significantly shorter than those of other treatments except for 0.4 and 1.0 μg/g BW, respectively (Figure 2). There was a dose dependent response across from low to high in dosage of phytoecdysone. Molting was effectively induced in the D1 stage by dosages of 0.1, 0.3, and 0.4 μg/g BW phytoecdysone. There were significant reductions in the molting period ($p \leq 0.05$) compared with the control (Figure 3). In contrast, an injection of phytoecdysone given to stage D2 crabs appeared to prolong molting period, as some crabs failed to molt (monitored over a period of 26 days post-injection; Figure 4). The experiment demonstrated that an injection of phytoecdysone was able to reduce the molting period in the remaining stages, except for D2 stage. Although different doses were effective for the different molt stages, each dose was able to reduce the molting period. At effective dosage in each molting stage, the molting period from B to D2 stage, from C2 to D1 stage, and from D1 to D2 stage were 7.8 ± 1.8, 7.4 ± 3.0, and 7.4 ± 3.6 days compared to those of control which were 13.2 ± 2.1, 9.5 ± 2.4, and 12.9 ± 2.7 days, which were shorter than control at 43%, 31%, and 45%, respectively .

Survival rates

The survival rate was presented as % survival fo each molting stage. The survival rate of the crabs in the control experiments of post-molt (B), inter-molt (C2), and early pre-molt (D1) were 83.3%, 85.7% and 88.9%, respectively. When compare with the phytoecdysone injection treatments in each stage, at stage B, the survival

rates of using phytoecdysone at 0.3, 0.4, and 0.5 μg/g BW were not significantly different from the controls, while injection at 0.1, 0.2, and 1.0 μg/g BW were significantly higher than those of the controls (Figure 5). At stage C2, the survival rates of all treatments were significantly higher than those of the controls. At stage D1 where the crabs were injected with phytoecdysone concentrations of 0.3, 0.4, and 0.5 μg/g BW, the survival rates were 50%, 55.2% and 63%, respectively (Figure 7), which were significantly lower than those of the controls. The survival rates of the crabs injected with phytoecdysone at 0.1, 0.2, and 1.0 μg/g BW did not differ significantly from those of the controls. Although, dosages of 0.3, 0.4, and 0.5 μg/g BW injected at stage D1 showed the lowest survival rates of all treatments and stages, there was no clear phytoecdysone concentration dependency. However, these results indicated that high concentrations of phytoecdysone were detrimental to the survival rates of pre-molt crabs. At stage D2, the survival rates were 100% in both treatment and control.

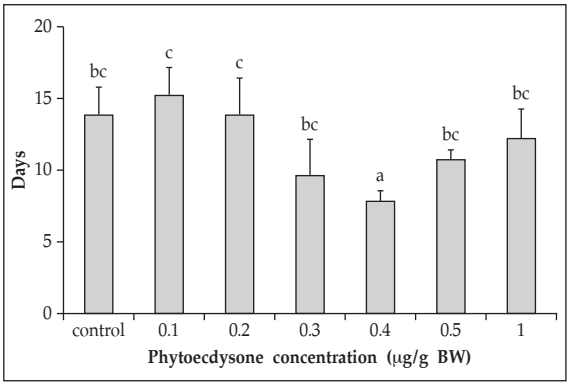

Figure 1. The molting period (in days presented as mean ± SE) from B to D2 stages following the injection at stage B with a series of concentrations of phytoecdysone w/w (μg/g). Means with different letters in each bar were significantly different ($p \leq 0.05$).

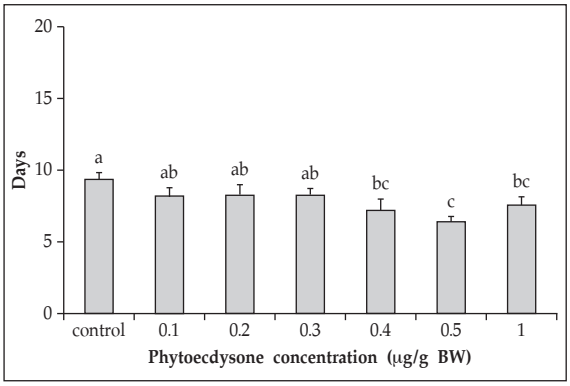

Figure 2. The molting period (in days presented as mean ± SE) from C2 to D1 stages following the injection at stage C2 with a series of concentrations of phytoecdysone w/w (μg/g). Means with different letters in each bar were significantly different ($p \leq 0.05$).

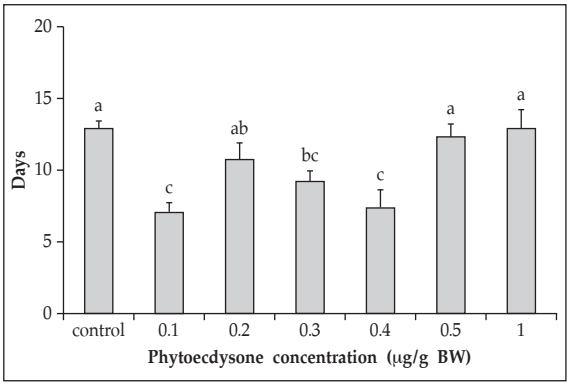

Figure 3. The molting period (in days presented as mean ± SE) from D1 to D2 stages following the injection at stage D1 with a series of concentrations of phytoecdysone w/w (μg/g). Means with different letters in each bar were significantly different ($p \leq 0.05$).

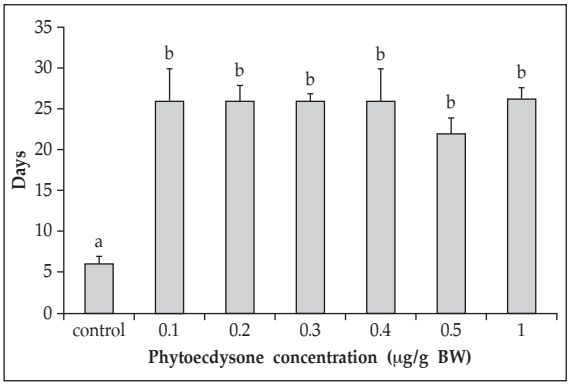

Figure 4. The molting period (in days presented as mean ± SE) from D2 to D3 stages following the injection at stage D2 with a series of concentrations of phytoecdysone w/w (μg/g). Means with different letters in each bar were significantly different ($p \leq 0.05$).

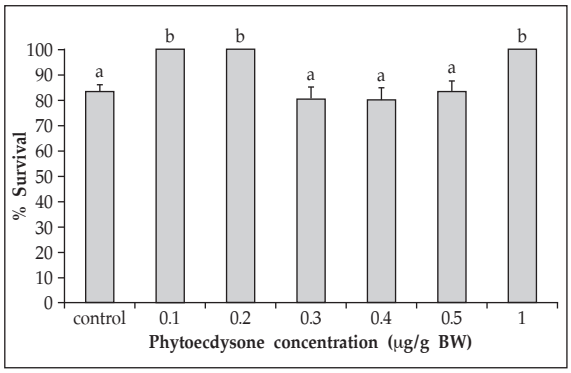

Figure 5. Survival rates (presented as percentage of survival ± SE) of *P. pelagicus* injected with a series of concentrations of phytoecdysone w/w (μg/g) from B to D2 stage. Means with different letters in each bar were significantly different ($p \leq 0.05$).

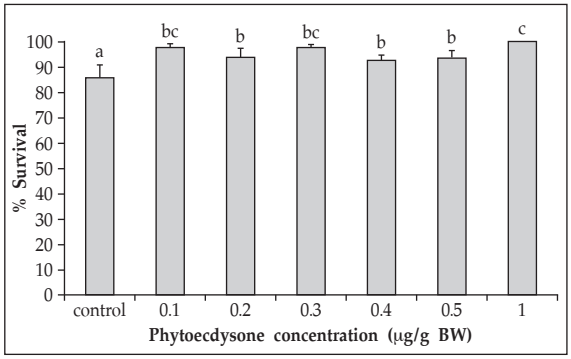

Figure 6. Survival rate (presented as percentage of survival ± SE) of *P. pelagicus* injected with a series of concentrations of phytoecdysone w/w (μg/g) from C2 to D1 stage. Means with different letters in each bar were significantly different ($p \leq 0.05$).

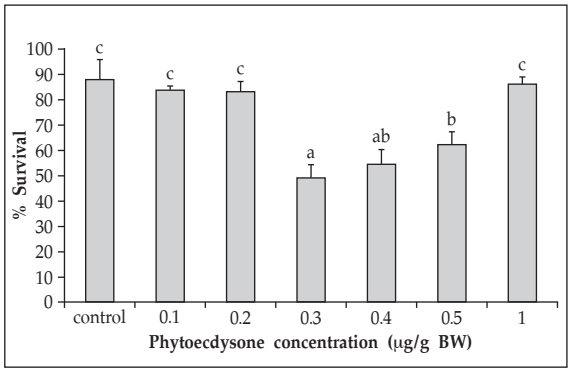

Figure 7. Survival rate (presented as percentage of survival ± SE) of *P. pelagicus* injected with a series of concentrations of phytoecdysone w/w (μg/g) from D1 to D2 stage. Means with different letters in each bar were significantly different ($p \leq 0.05$).

DISCUSSION

The hormone, ecdysteroid, has been shown to be a molt stimulating hormone in many crustacean species (Skinner, 1985). Basically, ecdysteroid is present at a low level (5 ng/ml) during the intermolt and increases markedly during premolt (44 ng/ml) where it stimulates and coordinates the integumental events leading to the next molt (Soumoff and Skinner, 1983). Phytoecdysone is actually less expensive but effective to induce molting of *P. pelagicus*. The findings in this study clearly demonstrate that a single injection of the phytoecdysone extracted from *V. glabrata* and given to crab, *P. pelagicus* at stage B, C2, and D1 can significantly reduce molting period. Different concentrations of phytoecdysone show different effects on specific molt stages. Firstly, the administration of a 0.4 μg/g BW of phytoecdysone applied to crabs at stage B can reduce the molting period of stage B to D2, with about 43% shortened when compare to control. Doses of 0.5 and 0.1μg/g BW for stage C2 and stage D1 could reduce the molting period by 31% and 45% when compare to control. The study concludes that phytoecdysone is able to reduce molting period in male *P. pelagicus* crabs at stage B, C2, and D1 *via* injection. This finding was in agreement with the observations of Hutacharoen et al. (1989) and Putchakarn (1991) who studied effect of phytoecdysone in *Penaeus monodon*. These authors reported that phytoecdysone, prepared from crude plant extracts of *V. glabrata*, reduced the molting period of the shrimp when incorporated into the feed or given as an injection. A study of Dall and Barclay (1977) indicated that when juvenile rock lobsters, *Panulirus longipes cygnus*, were injected at stage C4, were injected with 0.6 μg/g BW to 0.8 μg/g BW 20-HE, the molting period from D0 to D4 stages was shortened by about 40%. An earlier study using the crayfish, *Orconectes sanbornii sanbornii*, produced similar results (Stevenson and Tschantz, 1973). In intermolt male *Palaemonetes kadiakensis*, it was found that the administration of low doses of 20-HE increased the rate of molting (Hubschman and Armstrong, 1972). A 20-HE injection concentration of 50 μg/ crab at stages C2, C3, D0 of female crabs, *Emerita*

asiatica, enhanced molting which showed the percentage of reduction about 35%, 25%, and 20%, respectively (Gunamalai et al., 2004). The success of such low doses shows that this technique is an economically feasible means of initiating the premolt condition. On the contrary, treatment of male lobsters, *Homarus americanus*, during inter-molt and early premolt stages with either 0.25 or 0.75 μg/g 20-HE had no effect on the duration of their molt cycle. The lobsters subsequently underwent a normal molt, but when they were treated again with a dose of 0.75 μg/g 20-HE, the normal molt process was inhibited (Gilgan, 1980). However, it cannot utilize the phytoecdysone for crab at stage D2 because it shows the longer molting period. This indicated that overdosing of exogenous ecdysteroid might inhibit the molting of crustacean. With the injection of stage D3 juvenile, *H. americanus*, at a dose 1.0 or 5.0 μg/g of 20-HE delayed ecdysis (Cheng and Chang, 1991). It can be concluded that decreased ecdysteroid titers in the hemolymph of the lobster was a prerequisite to the initiation of ecdysis, and that the rate of development during stage D3 was regulated negatively by ecdysteroids. The results revealed that phytoecdysone must be applied with specific dosage for each molting stage. Longer molting period will occur with overdose treatment of phytoecdysone. The metabolism of phytoecdysone by the body follows a pathway close to that of 20-HE (McCarthy, 1980) which is processed quickly and removed from the body within a short period of time. If this is the case, the use of phytoecdysone would initiate the process of molting but as it is processed quickly it does not have any long lasting effects and may not leave any residues within the crab. The single administration of phytoecdysone when the natural level of ecdysone is low might stimulate the molt process in the crab. In contrast to this, administering a dose of phytoecdysone when the animal already has a high level of ecdysone might cause an imbalance in the hormone system, either delaying or preventing a normal molt event. Many studies have explored the use of phytoecdysone (Werawattanametin et al., 1986; Suksamran et al., 1998; Cho and Itami, 2004) or plant extracts which have a similar structure to molting hormone (Piyatiratitivorakul et al., 1996), as an alternative to eyestalk ablation, for the purposes of inducing molt events.

Administrations of even the lowest effective doses of exogenous 20-HE are usually lethal; several methods have been explored to administer lower, long lasting doses. The plant hormone, phytoecdysone, explored here which has a similar chemical structure to that of the crustacean molting hormone 20-HE (Werawattanametin et al., 1986; Suksamran et al., 1998), However, high dosage may cause high mortality. The safety doses for crab stages B, C2, and D1 should be 0.4, 0.5, and 0.1 μg/g BW, respectively. The effective doses which were applied to crabs at stages B, C2, and D1 did not cause mortality, but higher concentration of phytoecdysone injected at stage D1 might affect the survival rate within the experimental period. Because over dosage of molting hormone could result in hyperstimulation, and thus leading to death of crabs. In many crab species, the minimum effective dose of ecdysteroids was lethal to the fiddler crab, *Uca pugilator* (Rao et al., 1972), and *H. americanus* (Gilgan et al., 1977). In juvenile rock lobsters, *P. longipes cygnus*, at a concentration of 2.0 μg/g 20-HE could cause high mortality (Dall and Barclay, 1977). The cause of death may be the inability to uptake water which is crucial for the mechanism of molting as well as for the expansion of a new shell. However, endogenous ecdysteroid might be involved due to ecdysteroids level in most species started to increase in premolt stage (Skinner, 1985), and extra surge to high level of exogenous ecdysteroid in that time could be toxic.

This study demonstrated that the injection of different doses of phytoecdysone could significantly reduce the molting period, except for stage D2. As well, application of phytoecdysone at an effective dosage in each molting stage indicated that it did not affect the survival rate. Phytoecdysone can be used to produce soft-shelled crabs in the future. However, the application of phytoecdysone *via* injection should be investigated further for commercial soft-shelled crab culture.

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