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Research article

Ecdysteroid hormones in molting cycle of mud crab (*Scylla paramamosain* Estampador, 1950)

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Article Info

Abstract

Importance of the work: Understanding ecdysteroid hormones and their role in the molting cycle of the mud crab (*Scylla paramamosain* Estampador, 1950) is vital for advancing commercial aquaculture, particularly for optimizing soft-shell crab production and ensuring sustainable farming practices.

Objectives: To investigate the ecdysteroid derivatives—20-hydroxyecdysone (20-HE) and ponasterone A (PoA)—in the molting cycle of the mud crab (*S. paramamosain*).

Materials and Methods: Crabs (*S. paramamosain*) were reared in a recirculating system. A hemolymph sample was collected from each crab. Ecdysteroid hormones—ecdysone (E), 20-HE, PoA—were measured using high-performance liquid chromatography (HPLC) and enzyme immunoassay (EIA) and analyzed based on analysis of variance and Student's t test.

Results: HPLC identified PoA as the primary ecdysteroid (33.59–675.06 ng/mL); however, E and 20-HE were undetectable using HPLC, while EIA detected 20-HE, (69.01–259.27 ng/mL). The PoA levels were significantly ($p < 0.001$) higher in the premolt stage than the intermolt stage. In addition, the 20-HE levels were significantly ($p < 0.05$) higher during premolt. The resulting PoA:20-HE ratios were approximately 1:1 in the intermolt stage and increased to approximately 2:1 in the premolt stage.

Main finding: PoA was identified and reported for the first time as the primary molting hormone in *S. paramamosain*, providing essential knowledge for optimizing aquaculture.

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Introduction

The mud crab, *Scylla paramamosain* Estampador, 1950)*, is a valuable product in the fishery industry, both in its hard and soft-shell forms in the Indo-Pacific region (Fazhan et al., 2025). Growth performance of hard-shell crab aquaculture and large-scale production of soft-shell crabs are constrained by molting—a periodic process in which the old exoskeleton is shed (Chung and Webster, 2003; Chen et al., 2020; Mykles and Chang, 2020; Mykles, 2021).

Molting is directly inhibited by a neuropeptide hormone named molt-inhibiting hormone (MIH; Chung and Webster, 2003; Mykles, 2021). MIH is known to suppress ecdysteroidogenesis by inhibiting cholesterol uptake and ecdysteroid secretion (Mattson and Spaziani, 1985; Saïdi et al., 1994). Consequently, bilateral eyestalk ablation is commonly used to induce molting by activating Y-organ (YO) to initiate ecdysteroidogenesis. The resulting increases in ecdysteroid titers in the hemolymph lead to the onset of the premolt stage, which subsequently culminates in ecdysis, where the newly molted soft-shell crab is in a stage where somatic growth and reproduction occur. Ultimately, the balance between these hormones dictates the timing and progression of the molting cycle.

It is well known that the crustacean YO synthesizes and secretes ecdysteroids as inactive precursor forms, including ecdysone (E), 25-deoxyecdysone (25dE), 3-dehydroecdysone (3DE) and 3-dehydro-25-deoxyecdysone (3D25dE); subsequently, these inactive forms (specifically E and 25dE) are converted into the biologically active forms, 20-hydroxyecdysone (20-HE) and ponasterone A (PoA), respectively, in peripheral tissues (Okumura et al., 2003; Mykles, 2021). This two-step process is crucial, allowing for tight regulation of molting by generating inactive precursors that can be activated rapidly only when required. In addition, several reports have shown that the nuclear ecdysone receptor (EcR) and its partner retinoid-X receptor (RXR), have several isoforms in crustaceans, particularly within the ligand-binding domain (LBD). This has been observed in several species, including *Callinectes sapidus*, *Crangon crangon*, *Eriocheir sinensis*, *Gecarcinus lateralis* and *Homarus americanus*, where these variations in the LBD of EcR are suggested to accommodate the different hydrophobicities of the active ecdysteroids (Tarrant et al., 2011; Verhaegen et al., 2011; Techa and Chung 2013; Chen et al., 2017).

Despite the economic importance of *S. paramamosain*, there remains a gap in the understanding of the specific types and concentrations of ecdysteroid hormones involved in its molting cycle. This knowledge is vital for developing strategies to control and accelerate molting in aquaculture settings. Therefore, the current study aimed to investigate the ecdysteroid hormone profile in the hemolymph of *S. paramamosain* throughout the molting cycle, identifying the primary hormones present and analyzing their concentration changes during different stages. The findings should provide a foundation for enhancing growth rate and improving the efficiency of soft-shell mud crab aquaculture.

Materials and Methods

Experimental site and rearing conditions

The research was conducted at the Ang Sila Marine Research Station, Chulalongkorn University, Thailand from 17 April to 17 June 2024, spanning 60 d and covering one complete molting cycle. Mixed male and female crabs were reared individually in plastic baskets (21 cm × 27 cm × 15 cm) floating within two 500 L closed recirculating water systems, with eight crabs per tank. Water quality was monitored and minerals were replenished weekly, as well as a 50% water change. The water parameters were maintained at: salinity, 27–34 parts per trillion; temperature, 28–30°C; pH, 7.5–8.5; alkalinity, 133–220 mg/L; calcium, 344.69–561.12 mg/L; magnesium, 1,269.85–1,602.45 mg/L; ammonia, 0.763–0.802 mg/L; and nitrite, 0.313–0.351 mg/L.

Animal preparation and sample collection

Animal ethics was conducted according to the Chulalongkorn University Protocol Review No. 2523013. Mud crabs (*S. paramamosain*) were obtained from MK AquaTech farm in Samut Prakan province, Thailand. Initial crab weights were in the range 22.65–45.01 g, with carapace widths in the range 53.1–66.8 mm. In total, 16 crabs were acclimated for 1 wk in the prepared rearing ponds prior to the start of the experiment to minimize transport stress and to allow acclimatization to the new environment. The crabs were fed commercial pellet feed once daily at 1800 hours, amounting to 10% of their body weight. Excess feed was removed immediately after feeding.

Hemolymph samples (100 µL) were collected every 2 d from each crab until the crab molted and then three times after molting, completing one full molting cycle. In total, there were 5–10, 4 and 3 times for samples in the intermolt, premolt and postmolt stages, respectively. Samples were collected using a 21G needle and a 1 mL syringe, inserted into the articular membrane between the body and the claw. A 100 µL hemolymph sample was immediately transferred to a sample tube containing 900 µL of methanol and stored at -20°C.

Chemicals and reagents

Various standards of ecdysteroids were purchased: 20-HE (purity ≥ 93%) from Sigma-Aldrich (Saint Louis, MO, USA); E (purity ≥ 95%) from Cayman Chemical Company (Ann Arbor, MI, USA); and PoA (purity ≥ 95%) from Glentham Life Sciences (Corsham, United Kingdom). Analytical grade acetonitrile, methanol, ethanol and formic acid were purchased from Fisher Chemical (Seoul, Korea). Hyper-grade water was prepared from Ultrapure water, Type I (Evoqua; Water Technologies LLC; Lennitech)). Octadecyl (C18) cartridges (200 mg, 3 mL) and the Agilent Poroshell 120 column (SB-C18 4.6 x 100 mm, 2.7 µm diameter) were purchased from (Fine Spec Co.Ltd, Bangkok, Thailand).

Preparation of standard substances and protocol for high performance liquid chromatography

The standard hormones E, 20-HE and PoA were prepared in range 50 pg/µL and 0.1 ng/µL, 0.5 ng/µL, 1 ng/µL, 5 ng/µL, 10 ng/µL and 20 ng/µL in methanol. The mobile phase consisted of A = water, B = acetonitrile, C = 0.1% formic acid and D = methanol. A running procedure was performed over 6 min as detailed in Table 1 using an HPLC system (1260 Infinity II; Agilent Technologies (Thailand) Co., Ltd.). Standard hormone substances were injected into the system at a volume of 10 µL.

Analysis of crab hemolymph samples using high-performance liquid chromatography

Each sample (100 µL) of crab hemolymph in 900 µL of methanol was vortexed thoroughly and then centrifuged (Okumura et al., 1989). Methanol was used as the solvent because it is a highly effective organic solvent for extracting lipophilic ecdysteroids (including PoA, 20-HE and E) from the hemolymph matrix. Its purpose is to precipitate proteins and solubilize the ecdysteroids in the supernatant for subsequent analysis. Furthermore, a recent report confirmed that methanol had superior extraction efficiency for ecdysteroids, such as 20-HE, compared to alternative solvents such as ethanol (Fang et al., 2022). The supernatant in the current study was collected, dried at 60°C and reconstituted with 120 µL of methanol (Hopkins, 1992) prior to the HPLC analysis. Then, an 80 µL aliquot sample of this reconstituted supernatant was passed through a 0.45 µm nylon filter and placed into a vial insert. For quantification, a standard curve was plotted and the resulting equation was used to determine the hormone concentrations. For the enzyme immunoassay (EIA) analysis, a separate portion of the initial hemolymph sample (40 µL) was dried and dissolved in 100 µL of phosphate buffered saline (PBS) solvent.

Analysis of crab hemolymph samples using enzyme immunoassay

A 20-HE EIA kit (Kerafast, Co. Ltd; Boston, MA, USA) was used according to the manufacturer's protocol and processed over 3 d. On day 1, a 96-well plate (fixed flat bottom, high binding) was coated with 20-HE conjugated to beta-lactoglobulin (BLG; 20-HE-BLG). The 20-HE-BLG conjugate was prepared by dissolving 7 µL of 20-HE-BLG in 10 mL of PBS. Next, 100 µL of this solution was added to each well and incubated at 4°C for 16 hr on a shaker. On day 2, the wells were washed twice with 100 µL of PBS buffered with Tween 0.05% (PBS-T). Then, either the 20-HE standard or hemolymph samples were added at 30 µL per well.

Table 1 Modified chromatographic conditions used for charactering ecdysteroid derivatives.

Time (min)	A (%)	B (%)	C (%)	D (%)	Flow (mL/min)	Max pressure limit (bar)
0.00	0.00	30.00	70.00	0.00	0.00	400.00
1.00	0.00	40.00	60.00	0.00	1.00	400.00
1.20	0.00	43.00	57.00	0.00	1.00	400.00
1.30	0.00	45.00	55.00	0.00	1.00	400.00
2.00	0.00	50.00	50.00	0.00	1.00	400.00
3.00	0.00	60.00	40.00	0.00	1.00	400.00
3.10	0.00	40.00	60.00	0.00	1.00	400.00
3.50	0.00	30.00	70.00	0.00	1.00	400.00
5.00	0.00	30.00	70.00	0.00	0.00	400.00

A = mobile phase; B=water; C=acetonitrile; C = 0.1% formic; D = methanol.

The 20-HE standard was prepared from an initial concentration of 1 ng/µL using 2-fold dilutions to achieve final concentrations ranging from 0.13 to 66.67 pg/µL. The primary antibody (anti-20-HE [EAB25] antibody) was prepared at a 1:40,000 ratio (e.g., 1 µL antibody in 10 mL of PBS supplemented with 0.2% milk block (PBS-MB). 50 µL of the primary antibody solution was added to each well. The plate was incubated at 4°C for 16 hours.

On day 3, the wells were washed twice with 100 µL of PBS-T. Then, 100 µL of the secondary antibody solution (peroxidase-labeled goat anti-rabbit Ab, prepared at a 1:20,000 ratio in PBS-MB) was added to each well and incubated at room temperature for 3 hr. Next, the wells were washed with 100 µL of PBS-T, after which 100 µL of 3,3′,5,5′-tetramethylbenzidine substrate (Merck; Saint Louis, MO, USA) were added to each well and incubated for 45 min at room temperature. The reaction was stopped by adding 100 µL of 1 M phosphoric acid. Absorbance was read at 450 nm using a Biotek Power Wave XS2 plate reader (BioTek Instruments, Inc, Vermont, USA).

Statistical analysis

The data on PoA hormone concentration were used to create a bar graph comparing PoA levels between the intermolt and premolt stages. These data were then statistically analyzed using a Student's t test in SPSS software (version 29.0.1.0; IBM Corp.; Armonk, NY, USA). The measured absorbance values were used to generate a graph of 20-HE hormone concentrations throughout the molting cycle. Data showing difference in 20-HE levels across molting stages were analyzed using one-way analysis of variance (ANOVA) in the same SPSS software, followed by Tukey's honestly significant difference (HSD) post-hoc test to identify specific pairs of means that differed significantly.

Results and Discussion

Analysis of ecdysteroid derivatives using high performance liquid chromatography

Optimizing conditions for separation of 20-hydroxyecdysone, ecdysone and ponasterone A

The study utilized HPLC techniques based on a method Fang et al. (2022) applied to plant extracts. As the current study involved crab hemolymph, it was necessary to adjust the conditions to suit the sample matrix. Optimization was required to ensure that the retention times of the standard

hormones were sufficiently separated from one another and from unwanted substances in the samples (Fig. 1). These observations suggested potential contamination or inappropriate machine settings, hindering accurate comparison between standard peaks and samples. Consequently, the analysis conditions were optimized and the three standard hormones (PoA, 20-HE and E) were reanalyzed to achieve clearer results and accurate peak confirmation.
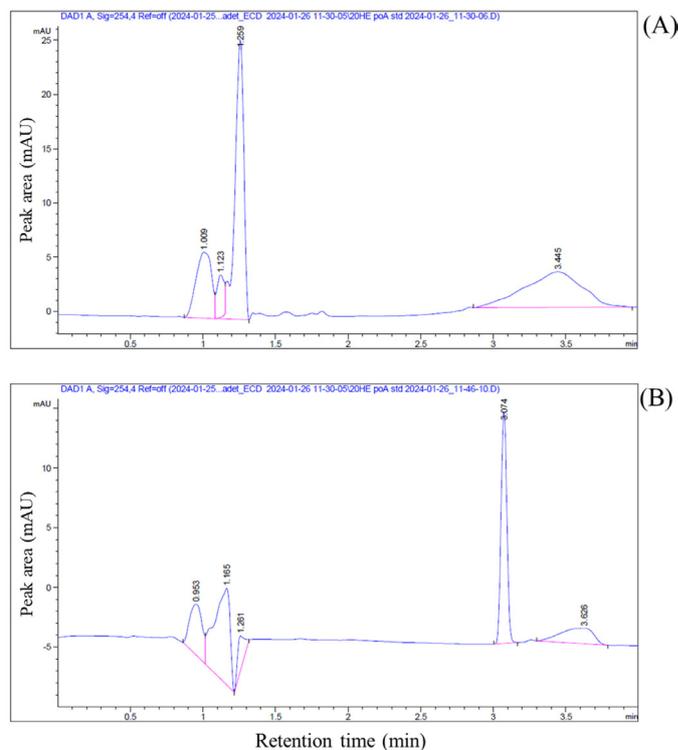
Fig. 1 Initial chromatogram profiles of standard hormones before condition optimization, resulting in retention times unsuitable for analyzing both hormone types: (A) 20-hydroxyecdysone; (B) ponasterone A.

The adjusted conditions for separation of three types of ecdysteroid derivatives were tuned and are presented in Table 1.

Based on the results, there was excellent separation of the three ecdysteroid forms, following re-analysis based on the conditions in Table 1. The retention times were 1.270 min for 20-HE, 1.660 min for E and 3.096 min for PoA (Fig. 2). This elution order was consistent with the hydrophobicity of the compounds, with the non-polar PoA binding most strongly to the C18 column and was therefore eluted last, while the most polar 20-HE was eluted first, followed by E. This pattern was similar to the findings in other reports (Wang et al., 2000; Ghosh and Laddha, 2006; Fang et al., 2022).
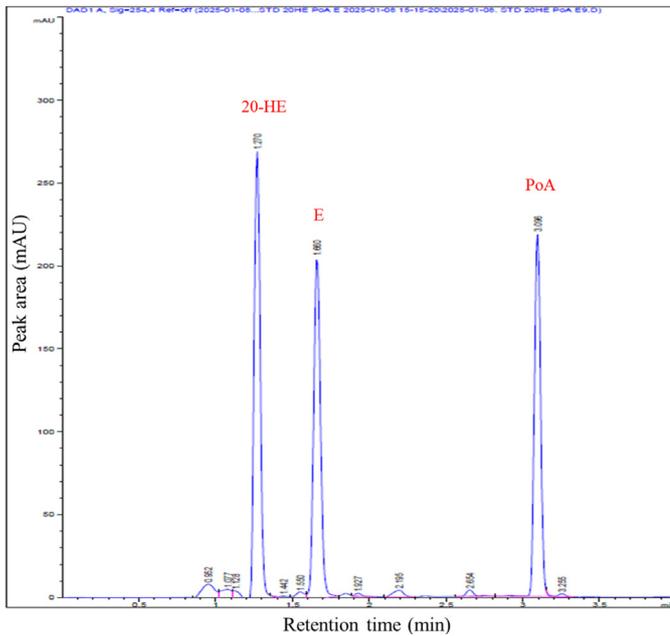
Fig. 2 Chromatograms of ecdysteroid standards (50 ng) identified based on retention times: 20-hydroxyecdysone (1.270 min), ecdysone (1.660 min) and ponasterone A (3.096 min).

*Characterization of ecdysteroid derivatives in hemolymph samples*

Prior to analysis, some hemolymph samples were tested to confirm that these HPLC conditions were suitable for the hemolymph samples. The hemolymph samples showed a peak at a retention time of 3.093 min, consistent with PoA, which confirmed the suitability of the HPLC analysis conditions for sample analysis (Fig. 3). Then, the calculated calibration curve and its corresponding linear equation were used to quantify each hormone in the crab hemolymph samples.

However, the HPLC results of the individual hemolymph samples collected during the molt cycle revealed that only PoA was detected consistently (Fig. 4), suggesting that PoA was the major form in *S. paramamosain* hemolymph, while 20-HE and E were minor ones. In addition, the amount of PoA in the intermolt samples was relatively low, often falling below the detection limit of the HPLC system. Consequently, all hemolymph samples collected during the intermolt period (10–20 d prior to ecdysis) were pooled as the intermolt sample, while those from 2–6 d prior to ecdysis were pooled as the premolt samples.

Concentration of ecdysteroids in hemolymph samples analyzed using high-performance liquid chromatography

The chromatograms of the hemolymph samples revealed only PoA, with 20-HE and E being below the limits of

detection for this specific HPLC technique and thus could not be quantified. This might be due to the relatively small volume of hemolymph collected from the individual samples (100 µL), a measure taken to avoid overstressing the animals. This suspicion was supported by Fujaya and Trijuno (2007), who reported that a 10-fold larger hemolymph sample (1 mL) collected from mature female *S. olivacea* was sufficient using an HPLC technique to successfully analyze 20-HE, detecting a range of 1,600−2,800 ng/mL.
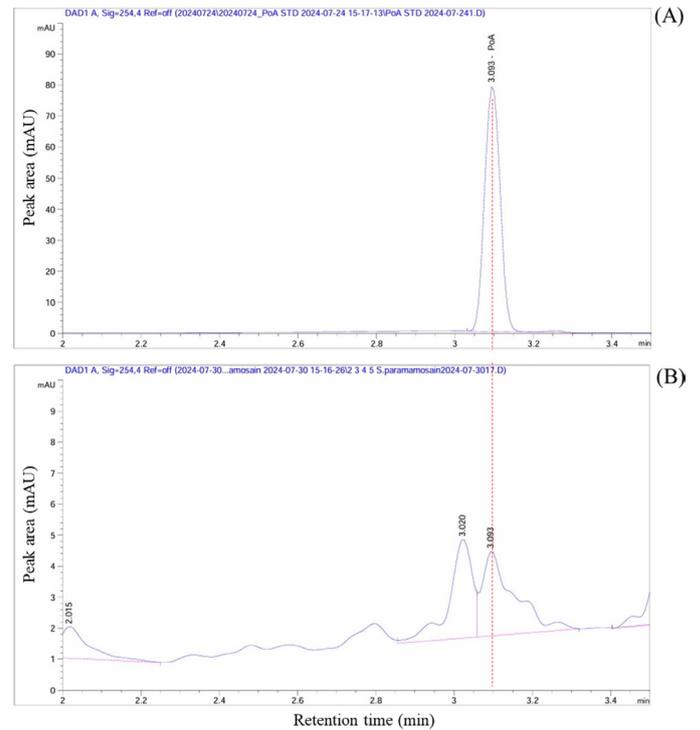
Fig. 3 Chromatograms of the mid-premolt stage showing the ponasterone A (PoA) standard (A) and the hemolymph sample (B). The dashed line indicates the PoA retention time (~3.09 min) used for peak identification.
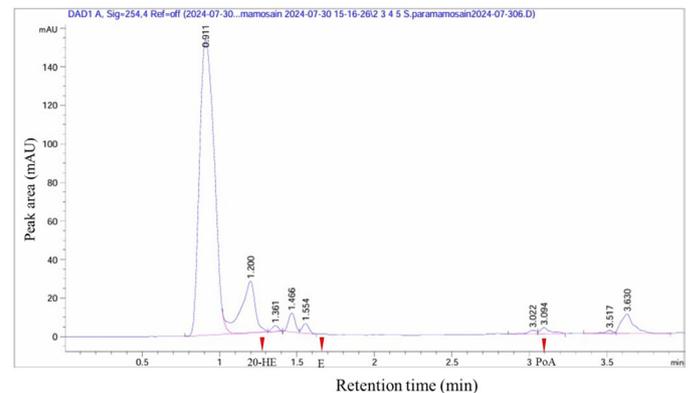
Fig. 4 Chromatogram of an individual hemolymph sample obtained from mid-premolt stage. Red arrowheads indicate the retention times of 20-hydroxyecdysone (20-HE; 1.270 min), ecdysone (E; 1.660 min) and ponasterone A (PoA; 3.094 min).

The HPLC technique was used to for the analysis of only PoA in the hemolymph samples, as the other ecdysteroids were undetectable. The quantity of PoA in the hemolymph samples was calculated using the calibration curve using Equation 1:

$$Y = 20.8885466X + 1.1351515 \qquad (1)$$

where X represents hormone quantity and Y represents the area under the peak (in milli atomic units seconds).

This equation was applied to calculate the PoA concentration in the pooled samples obtained from the intermolt and premolt stages.

Based on these results, it was clear that the PoA concentration was significantly (Student's t test analysis, $p < 0.001$) higher during the premolt stage in *S. paramamosain* in the pooled premolt sample (445.18 ± 54.64 ng/mL, n=11) than in the intermolt stage (78.46 ± 9.9 ng/mL, n=11), as depicted in Fig. 5. Crucially, the analysis of an individual, non-pooled sample from the mid-premolt stage yielded similar PoA levels (377.42 ± 63.6 ng/mL, data not shown), indicating that the sample pooling did not greatly alter the quantitative results, thereby confirming the accuracy of the pooling technique used for the low-volume hemolymph collection.
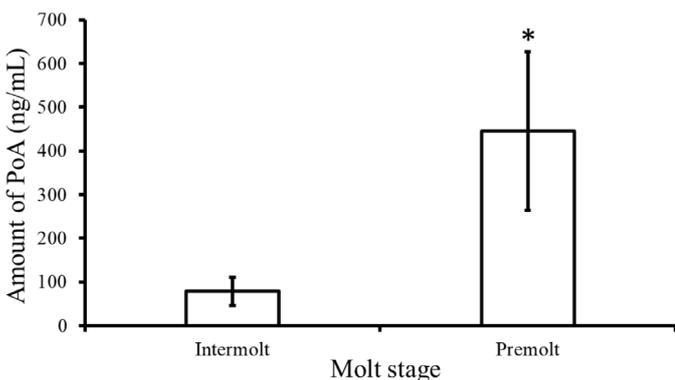
Fig. 5 Ponasterone A (PoA) levels in pooled hemolymph samples obtained from the intermolt and premolt hemolymph (n = 11 per stage), quantified by high-performance liquid chromatography. The asterisk indicates a highly significant difference ($p < 0.001$) between stages based on Student's t test. Error bars = ± SD.

Clearly, these findings identified PoA as the major ecdysteroid in the premolt hemolymph of the mud crab, *S. paramamosain*. To date, the current study is the first published report that has identified and documented the PoA levels in *Scylla* crabs; further investigation into other *Scylla* species is warranted.

Based on the current study, the finding that PoA was the major ecdysteroid aligned with other reports on other Brachyuran crabs, such as the blue crab (*C. sapidus*) and the European green crab (*Carcinus maenas*), which used similar reverse-phase HPLC methods (Styrishave et al., 2008; Chung, 2010). Notably, in *C. sapidus*, the PoA levels were detected in the range 210–330 ng/mL during the mid-premolt stage. In *C. maenas*, the PoA levels increased substantially from the intermolt stage (5−10 ng/mL) to a peak of 150−250 ng/mL during the mid-premolt stage (Styrishave et al., 2008).

The strong rise and peak in the PoA concentration in *S. paramamosain* at the premolt stage identified in the current study follows a pattern similar to that observed in the well-characterized species *C. sapidus* and *C. maenas* (Styrishave et al., 2008; Chung, 2010; Techa et al., 2013). In contrast, PoA was barely detectable in the swimming crab (*Portunus pelagicus*) (Sorach and Pratoomchat, 2017) and its levels in the Kuruma shrimp (*Penaeus japonicus*) were low, ranging between 2−7 ng/mL across a molt cycle (Okumura et al., 1989).

Analysis of 20-hydroxyecdysone using enzyme immunoassay

Since the 20-HE levels were undetectable using HPLC analysis, an available commercial EIA kit was applied. In total, 149 hemolymph samples were collected throughout the 60 day rearing period. The EIA analysis of these samples was divided into two batches to manage the high volume. Standard calibration curves were generated for each batch to ensure data accuracy. Equations 2 and 3 (for analyzing sample batches 1 and 2 , respectively) were used to determine the 20-HE concentration in each batch:

$$Y = -0.0551X + 0.6901 \text{ and } R^2 = 0.8953 \qquad (2)$$

$$Y = -0.0362X + 0.3262 \text{ and } R^2 = 0.9396 \qquad (3)$$

where X is the quantity of the sample substance, Y is the absorbance value at 450 nm and R^2 is the coefficient of determination.

The hemolymph samples from the crabs that had completed molting (n=7) were selected and aligned relative to ecdysis (day 0) as demonstrated by Chung (2010). Specifically, day -2 corresponded to the late-premolt (D_3) stage, day -4 to the mid-premolt (D_2) stage and day -6 to the early-premolt ($D_{0/1}$) stage. Days -20 to -8 were defined as the intermolt (C_4) stage.

Based on the current results, the levels of 20-HE in the mid-premolt stage (366.92 ± 92.22 ng/mL) and the late

premolt stage (265.66 ± 32.92 ng/mL) were significantly higher (based on one-way analysis of variance) than those in the intermolt stage and early premolt stage (58.97–149.53 ng/mL) and the postmolt (A/B) stage (98.66 ± 14.15 ng/mL), as shown in Fig. 6.
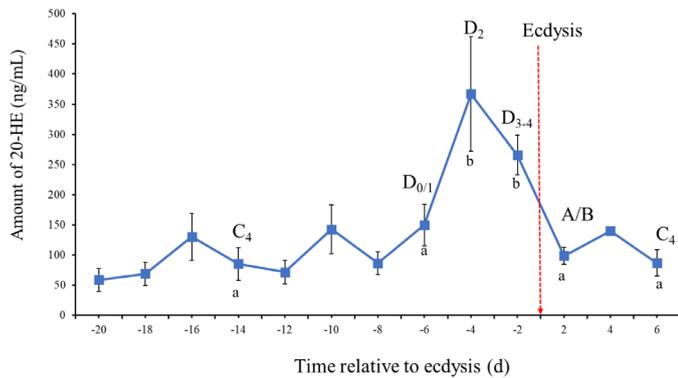
Fig. 6 Hemolymph 20-hydroxyecdysone (20-HE) profile of *Scylla paramamosain* across the molt cycle, quantified using a 20-HE enzyme immunoassay. Molt stages are C_4 (intermolt), $D_{0/1}$ (early-premolt), D_2 (mid-premolt), D_{3-4} (late-premolt) and A/B (post molt). Different lowercase letters indicate significant differences ($p < 0.05$) among stages (one-way ANOVA; $n = 7$).

Compared to the previous section on PoA, the understanding of 20-HE levels in crustaceans during a molt cycle appears clearer. For example, other researchers have reported that the 20-HE profiles consistently followed a pattern characterized by low levels during intermolt, a gradual increase in early premolt and a peak during the mid/late premolt stage, with a sudden drop before ecdysis (Okumura et al., 1989; Okumura and Aida., 2000; Thomton et al., 2006). The molt-related changes in the 20-HE levels of *S. paramamosain* in the current study followed a similar pattern to those observed in other species, including the closely related *Scylla* species. Specifically, in mature female *S. olivacea*, levels of 20-HE were low (~1,600 ng/mL) in the intermolt stage, increased in early-premolt stage (~2,000 ng/mL), peaked at the late-premolt stage (~2,800 ng/mL) and dropped to ~1,800 ng/mL in the postmolt stage (Fujaya and Trijuno, 2007). In other decapods, such as *C. sapidus* (Chung, 2010), *Cancer magister* (Thomton et al., 2006) and *G. lateralis* (Lee et al., 2007), intermolt hemolymph 20-HE was maintained at 10–45 ng/mL, 20 ng/mL and 11.45±0.86 ng/mL, respectively. Consistently, the 20-HE levels peaked at 83.35 ng/mL in *C. sapidus* and at 1,886 ± 186.2 ng/mL in *C. magister*.

However, in the current study, there was considerable variation in the measurement of the 20-HE levels in the *S. paramamosain* hemolymph using the 20-HE EIA kits from these other reports,

with the current detected levels (58.97–366.92 ng/mL) being notably lower than in the studies utilizing this technique in *Scylla* species. For example, in adult female *S. serrata*, the 20-HE levels were 1,573.12 ± 20.85 ng/mL (Nurcahyono et al., 2024). In contrast, in a phytoecdysteroid experiment involving juvenile *S. olivacea* (50–51 g), the initial 20-HE concentration was 1,150 ± 170 ng/mL and increased to 1,143–2,990 ng/mL at the post-injection stage (Hasnidar et al., 2021). This wide range across *Scylla* species raises concerns regarding the consistency and accuracy of 20-HE quantification using 20-HE EIA kits, potentially compounded by the limited hemolymph volume collected for the assay. For future analysis, a larger hemolymph volume, exceeding 500 µL, should be collected to ensure more reliable detection for hemolymph 20-HE.

Ratios of ecdysteroid derivative in hemolymph in intermolt and premolt stages

Since the PoA quantification was limited for individual intermolt samples, these samples were pooled for HPLC analysis. In addition, the premolt samples were pooled to facilitate a direct comparison. Consequently, the individual 20-HE results corresponding to the previously established period (intermolt: day -20 to -8; premolt: day -6 to day -2) were similarly pooled to ensure consistency for comparative ratio analysis

Notably, based on these results, the ecdysteroids were distributed differentially across the molt cycle—20-HE tended to be the major ecdysteroid during the intermolt stage, while PoA clearly dominated during the premolt stage. The quantitative analysis strongly supported this conclusion, with the ratio of PoA:20-HE being 1:1.17 in the intermolt stage and increasing to 1.71:1 in the premolt stage (Table 2).

Table 2 Proportion of ponasterone A (PoA) and 20-hydroxyecdysone (20-HE) in the intermolt and premolt stages.

Stage	Ecdysteroid (ng/mL)		Ratio
	PoA	20-HE	PoA:20-HE
Intermolt	78.50 ± 9.90	92.03 ± 12.08	1:1.17
Premolt	445.20 ± 54.60	260.71 ± 62.80	1.71:1

Furthermore, the increase in the PoA levels from the intermolt to the premolt stage was significantly greater (a 5.7-fold increase) than the 20-HE increase (a 2.8-fold increase). This premolt PoA:20-HE ratio was similar to the ratio observed in the blue crab, *C. sapidus*, where the PoA:20-HE ratio increased approximately three times during the early premolt stage (Chung, 2014; Techa and Chung, 2015). However, a unified analysis based on HPLC would be appropriate to obtain more precise and definitive ratios, since PoA and 20-HE

were measured by different assay methods (HPLC for PoA and EIA for 20-HE). To account for the detection limit of HPLC, future HPLC analysis should ideally use hemolymph samples of at least 1 mL so that other inactive and active ecdysteroids, as discussed earlier, could be definitively identified and quantified.

Based on the overall results, throughout the molting cycle of *S. paramamosain*, PoA was the major functional ecdysteroid in the critical premolt stage. This functional prominence aligned with findings in other arthropods. Several research studies involving insects, such as silkworm (*Bombyx mori*), fruit fry (*Drosophila melanogaster*) and dwarf wood scorpion (*Liocheles australasiae*), revealed that PoA was the most potent ecdysteroid for binding with both nuclear and membrane receptors in *B. mori*, *D. melanogaster*, and *L. australasiae* (Elmogy et al., 2004; Gonsalves et al., 2011; Miyashita et al., 2011; Petruccelli et al., 2020). Furthermore, genome-wide microarray analysis of *D. melanogaster* Kc167 cells revealed that PoA influenced far more responsive genes than 20-HE (Gonsalves et al., 2011), suggesting that *S. paramamosain* and other species where PoA is the major circulating ecdysteroid may utilize this enhanced potency to regulate a broader range of responsive genes critical for molting. Future studies should investigate this functional mechanism and gene regulation in the mud crab, specifically examining the effect of different titers of PoA or 20-HE and various PoA:20-HE ratios on ecdysteroid-responsive genes such as *spook* and *shadow* genes (Techa and Chung, 2015; Ittarat et al., 2025).

In conclusion, the current study has provided critical insights into the ecdysteroid dynamics during the molting cycle of the mud crab, *S. paramamosain*. A major finding was the consistent predominance of PoA as the major ecdysteroid, with its levels being substantially higher than 20-HE across all examined molt stages. This hormonal signature was strongly evidenced by the PoA:20-HE ratios, which increased from ~1:1 in the intermolt stage to a notable ~2:1 in the premolt stage. Furthermore, the increase in the PoA levels from intermolt to premolt was far more pronounced (5.7-fold) than the corresponding increase in 20-HE (2.8-fold). This pattern distinguishes our results from other reported studies in *Scylla* species and other decapods, where often, 20-HE has been reported as the primary circulating or most active ecdysteroid. These unique hormonal profiles underscore the species-specific regulation of molting. Future research should investigate the precise metabolic pathways of ecdysteroids in *S. paramamosain* and their functional implications. The detailed characterization of PoA as the predominant ecdysteroid should provide valuable knowledge for optimizing aquaculture practices, potentially leading to improved molting synchronization, growth rates and overall production efficiency in mud crab farming.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

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