Effects of Dew Grass Grinding Particle Size and Supplementation Level on Growth Performance, Digestive Enzyme Activity, and Non-Specific Immune Indicators of Macrobrachium nipponense (Post-print)

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Abstract

The present study was conducted to investigate the effects of ecdysterone-rich Cyanotis arachnoidea C.B.Clarke on growth performance, hepatopancreatic digestive enzyme activities, and non-specific immune indices of Macrobrachium nipponense. Experiment 1: C. arachnoidea with grinding particle sizes of 10, 30, 50, and 180 m was supplemented into the basal diet (without C. arachnoidea) to achieve an ecdysterone content of 10 mg/kg in each diet; Experiment 2: C. arachnoidea with the same grinding particle size (180 m) but different ecdysterone contents was added to the basal diet to achieve ecdysterone contents of 3.30, 6.60, 13.20, and 26.40 mg/kg, respectively. The eight experimental diets were fed to M. nipponense with an initial body weight of (0.08±0.01) g, with one control group fed the basal diet established in both Experiment 1 and Experiment 2. Each diet was assigned to three replicates with 70 shrimp per replicate, and the trial lasted for 60 days. After the feeding trial, shrimp in each group were challenged with Aeromonas hydrophila infection. The results showed: In Experiment 1, compared with the control group, the specific growth rate (SGR) and weight gain rate (WGR) of M. nipponense in groups supplemented with different grinding particle sizes of C. arachnoidea were significantly increased ($P<0.05$), while feed conversion ratio (FCR) was significantly decreased ($P<0.05$); no significant differences were observed among groups in survival rate (SR), hepatosomatic index (HSI), hepatopancreatic pepsin, trypsin-like protease, and amylase activities, total hemocyte count (THC), hemolymph phagocytic activity (HPA), and plasma superoxide dismutase (SOD) and alkaline phosphatase (AKP) activities ($P>0.05$); no significant difference was found in cumulative mortality after A. hydrophila challenge among all groups

(P>0.05). In Experiment 2, compared with the control group, the 6.60 mg/kg ecdysterone group and 13.20 mg/kg ecdysterone group showed significantly increased WGR and SGR (P<0.05), and significantly decreased FCR (P<0.05); the 26.40 mg/kg ecdysterone group exhibited significantly lower WGR, SGR, SR, and HPA than the control group (P<0.05), and its WGR and SGR were also significantly lower than those of other C. arachnoidea-supplemented groups (P<0.05); no significant differences in WGR and SGR were observed among the 3.30 mg/kg, 6.60 mg/kg, and 13.20 mg/kg ecdysterone groups (P>0.05); no significant differences were detected among groups in HSI, hepatopancreatic pepsin, trypsin-like protease, and amylase activities, THC, and plasma SOD and AKP activities (P>0.05); the cumulative mortality after A. hydrophila challenge in the 26.4 mg/kg ecdysterone group was significantly higher compared with other groups (P<0.05). These results indicate that dietary supplementation of C. arachnoidea to achieve ecdysterone content of 6.60–13.20 mg/kg exerts significant growth-promoting effects on M. nipponense, and there is no significant correlation between ecdysterone absorption in M. nipponense and the grinding particle size of C. arachnoidea. The main medicinal component of C. arachnoidea, ecdysterone, showed no immunoenhancing effect on M. nipponense, and excessive ecdysterone (ecdysterone content of 26.4 mg/kg in diet) also led to decreased survival rate and disease resistance in M. nipponense.

Full Text

Effects of Cyanotis arachnoidea C. B. Clarke Grinding Granularity and Supplemental Level on Growth Performance, Hepatopancreas Digestive Enzyme Activities and Nonspecific Immune Indices of Macrobrachium nipponensis

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Abstract

This study investigated the effects of *Cyanotis arachnoidea* C. B. Clarke, a plant rich in ecdysone, on the growth performance, hepatopancreas digestive enzyme activities, and nonspecific immune indices of the oriental river prawn *Macrobrachium nipponensis*. Two trials were conducted. In Trial 1, *C. arachnoidea* with different grinding granularities (10, 30, 50, and 180 m) was added to a basal diet to achieve an ecdysone content of 10 mg/kg in each experimental

diet. In Trial 2, *C. arachnoidea* with the same grinding granularity (180 m) but varying ecdysone contents was added to the basal diet to achieve ecdysone levels of 3.30, 6.60, 13.20, and 26.40 mg/kg.

Eight experimental diets were prepared and fed to *M. nipponensis* with an initial body weight of (0.08±0.01) g for 60 days. Both trials included a control group fed the basal diet without *C. arachnoidea*. Each diet had three replicates with 70 prawns per replicate. Following the feeding trial, prawns from each group were challenged with *Aeromonas hydrophila*.

The results showed that in Trial 1, compared with the control group, the specific growth rate (SGR) and weight gain rate (WGR) of prawns fed diets containing *C. arachnoidea* with different grinding granularities were significantly increased ($P<0.05$), while the feed conversion ratio (FCR) was significantly decreased ($P<0.05$). No significant differences were observed among groups in survival rate (SR), hepatopancreas index (HSI), activities of pepsin, trypsin-like protease, and amylase in the hepatopancreas, total hemocyte count (THC), haemolymph phagocytic activity (HPA), or plasma superoxide dismutase (SOD) and alkaline phosphatase (AKP) activities ($P>0.05$). Cumulative mortality after *A. hydrophila* challenge did not differ significantly among groups ($P>0.05$).

In Trial 2, compared with the control group, the 6.60 mg/kg and 13.20 mg/kg ecdysone groups exhibited significantly higher WGR and SGR ($P<0.05$) and significantly lower FCR ($P<0.05$). The 26.40 mg/kg ecdysone group showed significantly lower WGR, SGR, SR, and HPA compared with the control group ($P<0.05$), and its WGR and SGR were also significantly lower than those of other *C. arachnoidea* supplementation groups ($P<0.05$). No significant differences in WGR and SGR were found among the 3.30, 6.60, and 13.20 mg/kg ecdysone groups ($P>0.05$). HSI, hepatopancreas pepsin, trypsin-like protease and amylase activities, THC, and plasma SOD and AKP activities did not differ significantly among groups ($P>0.05$). The cumulative mortality after *A. hydrophila* challenge in the 26.40 mg/kg ecdysone group was significantly higher than that of other groups ($P<0.05$).

These results demonstrate that dietary supplementation of *C. arachnoidea* at ecdysone concentrations of 6.60–13.20 mg/kg significantly promotes growth in *M. nipponensis*, and that ecdysone absorption by the prawn shows no significant correlation with the grinding granularity of *C. arachnoidea*. The primary medicinal component of *C. arachnoidea*, ecdysone, does not enhance immunity in *M. nipponensis*, and excessive ecdysone (26.4 mg/kg diet) reduces survival and antiviral capacity.

Keywords: *Cyanotis arachnoidea* C. B. Clarke; *Macrobrachium nipponensis*; growth; digestive enzyme; nonspecific immunity

Introduction

Ecdysone exhibits typical steroid hormone properties and plays a crucial role in stimulating and regulating protein, carbohydrate, and mineral metabolism in crustaceans, promoting exoskeleton formation and facilitating molting. Crustacean molting steroids such as ecdysone (E), 20-hydroxyecdysone (20-HE), and 25-deoxyecdysone (25-DE) are synthesized and secreted by the Y-organ [1]. Since the 1970s, researchers have investigated crustacean molting and growth using ecdysone injection. Warner et al. [2] found that injecting an appropriate dose of ecdysone (2.14 μg/g) stimulated earlier molting and promoted growth in the crayfish *Orconectes obscurus*. Currently, exogenous ecdysone is commonly administered through feed to accelerate growth, increase molting frequency, and shorten the culture cycle in both commercial production and experimental research [3-6].

Cyanotis arachnoidea C. B. Clarke is an excellent source plant for ecdysone extraction and contains the highest known ecdysone content of any plant, reaching 1.2% of dry whole-plant weight and up to 2.9% in underground parts [7]. Compared with chemical drug additives, herbal medicines have low or no residues, do not induce drug resistance, and possess both nutritional and medicinal functions [8-10].

Ultrafine grinding technology processes herbs to micron-level particle sizes through mechanical grinding and impact. This technology enhances cell wall disruption rate, specific surface area, and active component dissolution, thereby reducing dosage, saving materials, and protecting resources [11-13].

Macrobrachium nipponensis, commonly known as the oriental river prawn or freshwater shrimp, is widely distributed in inland waters of China. Valued for its delicious taste and nutritional richness (16.9% protein, 1.3% fat, plus calcium, phosphorus, iron, and vitamins), it has become a prominent freshwater aquaculture species [14]. While ecdysone' s importance in crustacean growth, development, and reproduction has long been recognized [15], its effects on nonspecific immunity remain unclear. Only Wu et al. [16] have reported that ecdysone participates in immune response regulation in Pacific white shrimp (*Litopenaeus vannamei*), where 20-HE affects cholesterol metabolism and alters neural responses, with elevated exogenous 20-HE reducing immune response. Furthermore, whether grinding *C. arachnoidea* into powder enhances absorption of its active components in shrimp and crabs has not been reported. To investigate the relationship between *C. arachnoidea* grinding granularity and ecdysone absorption in *M. nipponensis*, and to determine whether ecdysone affects immune response, this study utilized ultrafine grinding technology to process dried *C. arachnoidea* into different particle sizes and added them at various levels to prawn diets to examine changes in growth performance, digestive enzyme activities, and nonspecific immune indices. The findings aim to provide technical support for developing new, efficient, and pollution-free feed additives that promote growth, reduce feed conversion ratio, and enhance disease resis-

tance in *M. nipponensis*.

Materials and Methods

1.1 Experimental Design and Diet Preparation

The experiment consisted of two trials. In Trial 1, *C. arachnoidea* with different grinding granularities (10, 30, 50, and 180 m) was added to a basal diet to achieve an ecdysone content of 10 mg/kg, producing four experimental diets (designated 1#, 2#, 3#, and 4#). A basal diet without *C. arachnoidea* served as the control (designated 0#1). In Trial 2, *C. arachnoidea* with the same grinding granularity (180 m, ordinary powder) was added at different levels to achieve ecdysone contents of 3.30, 6.60, 13.20, and 26.40 mg/kg, producing four experimental diets (designated 5#, 6#, 7#, and 8#). A basal diet without *C. arachnoidea* served as the control (designated 0#2).

The composition and nutrient levels of the basal diet are shown in Table 1 . *C. arachnoidea* with different grinding granularities was provided by Changxing Tsinghua Powder and New Materials Engineering Center Co., Ltd., with ecdysone contents of 0.74% for the 10, 30, and 50 m powders and 0.33% for the 180 m powder. Other feed ingredients were purchased from Zhejiang Jingbao Feed Co., Ltd. According to the experimental design, 0.13 g of 10, 30, or 50 m *C. arachnoidea* powder or 0.30 g of 180 m powder was added per 100 g basal diet to produce diets 1#-4#. For diets 5#-8#, 0.10, 0.20, 0.40, and 0.80 g of 180 m *C. arachnoidea* powder were added per 100 g basal diet, respectively. Control diets 0#1 and 0#2 were the basal diet without supplementation.

Experimental diets were prepared as follows: ingredients were ground and passed through a 60-mesh sieve, weighed accurately according to formulation, and mixed uniformly in stages. Fish oil and soybean oil were added and mixed again, followed by addition of appropriate water. The mixture was processed into 1.0 mm pellets using a small feed pelletizer, air-dried, and stored at -20 °C until use.

1.2 Experimental Animals and Culture Management

Juvenile *M. nipponensis* were obtained from Zhejiang Deqing Wuyue Aquaculture Co., Ltd. The juveniles had a body length of (1.71±0.07) cm and body weight of (0.08±0.01) g, and were healthy and active.

The experiment was conducted in glass aquaria (0.76 m × 0.36 m × 0.45 m) equipped with suspended mesh for shelter and climbing. Aerated tap water was used, with water temperature maintained at 26–28 °C, pH 7.6–8.1, dissolved oxygen >6.5 mg/L, and total ammonia nitrogen <0.01 mg/L.

After one week of acclimation, 2,100 uniformly sized juveniles were randomly stocked into 30 aquaria at 70 prawns per aquarium. During the trial, prawns in

three aquaria were fed one experimental diet twice daily (30% of daily ration in the morning and 70% in the evening) at 5% of wet body weight. Uneaten feed and feces were siphoned before feeding. Water was exchanged daily at one-third of the volume. The feeding trial lasted 60 days.

1.3 Sample Collection

On day 60, feeding was stopped for one day. Prawns in each group were counted and weighed for growth analysis and survival rate calculation. Hemolymph was collected from 20 prawns per replicate using a 1 mL sterile syringe and mixed with anticoagulant (30 mmol/L sodium citrate, 0.34 mol/L sodium chloride, 10 mmol/L EDTA, 0.115 mol/L glucose, pH 7.55) at a 1:2 ratio. One portion of anticoagulated hemolymph was used directly for total hemocyte count (THC) and haemolymph phagocytic activity (HPA) determination. Another portion was centrifuged at 700×g for 10 min at 4 °C, and the plasma was stored at -80 °C for SOD and AKP activity assays.

After hemolymph collection, prawns were dissected on ice. Hepatopancreas was excised, opened, washed with pre-cooled ultrapure water (4 °C, pH 7.0) to remove contents, blotted dry, and weighed for hepatopancreas index analysis. The weighed hepatopancreas was stored at -80 °C for pepsin, trypsin-like protease, and amylase activity measurements.

1.4 Measurement Indicators and Methods

1.4.1 Growth Performance Indices Specific growth rate (SGR, %/d) = 100 × (ln final mean weight - ln initial mean weight) / feeding days
Weight gain rate (WGR, %) = 100 × (final mean weight - initial mean weight) / initial mean weight
Hepatopancreas index (HSI, %) = 100 × mean hepatopancreas wet weight / mean prawn wet weight
Survival rate (SR, %) = 100 × number of surviving prawns at trial end / number of prawns stocked initially
Feed conversion ratio (FCR) = feed intake / (final mean weight - initial mean weight)

1.4.2 Nonspecific Immune Indices Total hemocyte count (THC) was determined directly using a hemocytometer under an optical microscope (200×). Haemolymph phagocytic activity (HPA) was measured using the neutral red phagocytosis method [17-18] and calculated as:

HPA = 100 × absorbance value / 10^1 hemocytes

SOD and AKP activities were determined using assay kits from Nanjing Jiancheng Bioengineering Institute. One unit (U) of SOD activity was defined as the enzyme amount causing 50% inhibition in the reaction system. One unit of AKP activity was defined as the amount producing 1 mg phenol per 100 mL plasma at 37 °C for 15 min.

1.4.3 Digestive Enzyme Activity Determination Hepatopancreas samples were homogenized in ice-cold ultrapure water at a 1:10 ratio (w/v) using a glass homogenizer in an ice bath. The homogenate was centrifuged at 3,000 r/min for 10 min, and protein content in the supernatant was determined using the Coomassie brilliant blue method. All digestive enzyme activities were measured using assay kits (Nanjing Jiancheng Bioengineering Institute) according to the manufacturer' s instructions.

1.5 Immune Protection Test

The *Aeromonas hydrophila* TPS-30 strain used for challenge was provided by Zhejiang Institute of Freshwater Fisheries. A preliminary experiment determined the 7-day median lethal concentration (LD) for *M. nipponensis* as 1×10 CFU/mL. On day 61, 20 prawns per replicate were randomly selected from remaining individuals and intramuscularly injected with 0.02 mL of 1×10 CFU/mL *A. hydrophila* TPS-30 strain between the second and third abdominal segments. Feeding was resumed 12 h post-challenge, and cumulative mortality was recorded over 7 days.

1.6 Data Analysis

All data are expressed as mean ± standard error (mean±SE) of three replicates. Statistical analysis was performed using SPSS 15.0 software. One-way ANOVA was used to test significance, and Duncan' s multiple range test was applied for inter-group comparisons. Significance level was set at $P<0.05$.

Results

2.1 Effects of Different Grinding Granularities at the Same Ecdysone Content on Growth Performance, Hepatopancreas Digestive Enzyme Activities, and Nonspecific Immune Indices

The effects of different grinding granularities at the same ecdysone content are presented in Table 2 and Table 3 . At a dietary ecdysone content of 10 mg/kg, prawns fed diets containing *C. arachnoidea* with different grinding granularities (10, 30, 50, and 180 m groups) showed significantly higher WGR and SGR ($P<0.05$) and significantly lower FCR ($P<0.05$) compared with the control group. No significant differences were observed among groups in HSI, SR, hepatopancreas pepsin, trypsin-like protease, and amylase activities, or nonspecific immune indices including THC, HPA, and plasma SOD and AKP activities ($P>0.05$). No significant differences were detected among the different grinding granularity groups (10, 30, 50, and 180 m) in any of these growth performance, digestive enzyme, or immune indices ($P>0.05$).

2.2 Effects of Different Ecdysone Contents at the Same Grinding Granularity on Growth Performance, Hepatopancreas Digestive Enzyme Activities, and Nonspecific Immune Indices

The effects of different ecdysone contents at the same grinding granularity are shown in Table 4 and Table 5 . At a constant grinding granularity of 180 m, the 6.60 mg/kg and 13.20 mg/kg ecdysone groups exhibited significantly higher WGR and SGR ($P<0.05$) and significantly lower FCR ($P<0.05$) compared with the control group. The 26.40 mg/kg ecdysone group showed significantly lower WGR, SGR, SR, and HPA than the control group ($P<0.05$), and its WGR and SGR were also significantly lower than those of other *C. arachnoidea* supplementation groups ($P<0.05$). No significant differences in WGR and SGR were found among the 3.30, 6.60, and 13.20 mg/kg ecdysone groups ($P>0.05$). HSI, hepatopancreas pepsin, trypsin-like protease and amylase activities, THC, and plasma SOD and AKP activities did not differ significantly among groups ($P>0.05$).

2.3 Immune Protection Effects of Different Grinding Granularities or Ecdysone Contents

As shown in Table 6 , no significant differences in cumulative mortality post-challenge were observed among groups in Trial 1 ($P>0.05$). In Trial 2 (Table 7), the 26.40 mg/kg ecdysone group exhibited significantly higher cumulative mortality compared with all other groups ($P<0.05$), while no significant differences were detected among the 3.30 mg/kg, 6.60 mg/kg, 13.20 mg/kg ecdysone groups and the control group ($P>0.05$).

Discussion

The *C. arachnoidea* powders with grinding granularities of 10, 30, and 50 m used in this study were produced by ultrafine grinding technology. Ultrafine grinding not only significantly increases the dissolution and extraction rates of active herbal components [11-13] but also improves absorption efficiency as particle size decreases [19-20]. Previous studies have shown that ultrafine-ground *C. arachnoidea* contains more than double the ecdysone content of ordinary powder (180 m), with no significant differences among the 10, 30, and 50 m powders [21]. Li et al. [20] found that ultrafine grinding of Huanglian Jiedu Powder increased utilization of geniposide by approximately 44% in chickens, though berberine absorption was not enhanced. In Trial 1 of the present study, no significant differences were observed among *C. arachnoidea* powder groups or between powder and ordinary powder groups in growth performance or hepatopancreas digestive enzyme activities of *M. nipponensis*, indicating that ecdysone absorption by the prawn is not correlated with grinding granularity. These findings suggest that smaller particle size does not necessarily improve absorption and that the optimal particle size for each herbal medicine should be determined

experimentally rather than assuming universal benefits from ultrafine grinding. Since ultrafine grinding of *C. arachnoidea* is more costly and yields less product compared with ordinary grinding, ordinary powder is recommended for use in *M. nipponensis* feeds.

Dietary supplementation with appropriate levels of ecdysone or molting promoters has been shown to promote molting and growth in Chinese shrimp (*Fenneropenaeus chinensis*) [3-4], giant river prawn (*M. rosenbergii*) [5], and red swamp crayfish (*Procambarus clarkii*) [6]. Significant molting and growth-promoting effects have been reported at dietary ecdysone levels of 5.33–10.67 mg/kg for Chinese shrimp (initial weight 1.95–3.30 g) [3], 2 mg/kg plant ecdysone for giant river prawn (initial weight 0.3 g) [5], and 0.50 mg/kg ecdysone for red swamp crayfish (initial weight 8.31 g) [6]. Chinese shrimp nauplii fed 300 mg/kg 17 -methyltestosterone reached the postlarval stage 2 days earlier, with 45.5% greater body length increase than controls [4]. In the present study, 10 mg/kg ecdysone in Trial 1 and 6.60–13.20 mg/kg ecdysone in Trial 2 significantly promoted growth in *M. nipponensis* without significantly altering hepatopancreas digestive enzyme activities, suggesting that the growth-promoting effect of appropriate ecdysone levels is achieved by shortening the molting cycle and increasing molting frequency rather than enhancing digestion. At 26.4 mg/kg ecdysone, growth performance and immune protection were significantly reduced, likely due to excessive ecdysone disrupting normal molting physiology. Hubschman and Armstrong [22] reported toxic effects of ecdysone in adult grass shrimp (*Palaemonetes* spp.) at injection doses of 0.25–5.00 µg/g. Luo and Wang [3] also found that 60 mg/kg dietary ecdysone inhibited molting and growth and caused toxicity in Chinese shrimp.

Many herbs contain immunomodulatory components such as organic acids, alkaloids, polysaccharides, volatile oils, waxes, glycosides, tannins, and unknown immune-active factors, often conferring immune-enhancing effects [16,23-24]. Deng et al. [25] reported that polysaccharides from *Cordyceps sinensis* significantly affected growth, immunity (phenoloxidase, alkaline phosphatase, acid phosphatase, lysozyme activities), and antioxidant indicators (SOD activity, reduced glutathione content, reactive oxygen species level, total antioxidant capacity) in Pacific white shrimp. *C. arachnoidea* contains steroidal ketones and sterols [26] that modulate immunity in mammals; ecdysterone inhibits anti-immunoglobulin E (IgE)-induced histamine release in rat mast cells and significantly reduces concanavalin A (ConA)-induced histamine release [27]. However, the present study found no significant enhancement of nonspecific immune indices (THC, HPA, plasma SOD and AKP activities) or disease resistance in the crustacean *M. nipponensis*, possibly due to low concentrations of steroidal components in *C. arachnoidea* [26].

Crustacean hemocytes (hemolymph cells) are critically involved in immune responses, including phagocytosis, agglutination, and encapsulation of pathogens. After engulfing microorganisms, hemocytes kill them through oxidative and

non-oxidative mechanisms [28]. In *Drosophila*, 20-HE promotes hemocyte differentiation, enhances hematopoiesis, affects hemocyte numbers, and regulates ecdysone-activated pathways [29]. In contrast, 20-HE significantly reduced hepatopancreas THC in Pacific white shrimp [16]. In this study, ecdysone from *C. arachnoidea* did not significantly enhance THC or HPA in *M. nipponensis*, and high ecdysone content (26.4 mg/kg) significantly reduced HPA.

Alkaline phosphatase is a major component of crustacean lysosomal enzymes [30]. Superoxide dismutase is a critical antioxidant enzyme that catalyzes dismutation of superoxide anion radicals (O ·) to H O , playing a major role in enhancing phagocytic defense and overall immune function [30]. Wu et al. [16] reported that 20-HE treatment significantly increased hepatopancreas SOD activity in Pacific white shrimp; high 20-HE concentrations inhibited O ·production while low concentrations promoted it, possibly by modulating NADPH oxidase activity and reactive oxygen species generation. The present results differ from these findings, as dietary ecdysone levels of 3.30–26.40 mg/kg did not significantly affect plasma AKP or SOD activities in *M. nipponensis*.

Conclusion

1. Dietary supplementation of *C. arachnoidea* at appropriate levels (providing 6.60–13.20 mg/kg ecdysone) significantly promotes growth in *M. nipponensis*. Ecdysone absorption by the prawn shows no significant correlation with grinding granularity. Given the higher cost and lower yield of ultrafine powder production, ordinary powder is recommended for use in *M. nipponensis* feeds.
2. The primary medicinal component of *C. arachnoidea*, ecdysone, does not enhance immunity in *M. nipponensis*. Excessive ecdysone (26.4 mg/kg diet) reduces survival and antiviral capacity.

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Note: Figure translations are in progress. See original paper for figures.