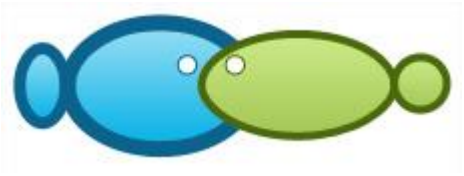

The effects of the amaranth extract (*Amaranthus* spp.) on the molting of orange mud crab (*Scylla olivacea*)

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Abstract. The aim of the present study was to investigate the effect of injected amaranth extract on hemolymph ecdysteroid concentration and molting of orange mud crab (*Scylla olivacea*). *S. olivacea* were injected with five different amaranth extract doses: 17, 29, 42, 54 and 67 μg mL^{-1} in order to quantify the treatment's effects on the molting percentage, molting duration, weight and width of carapace gains and survival rate. Those amaranth extract doses were referred to 20-Hydroxyecdyson hormone (20-HE) doses, in which 1 μg mL^{-1} of 20-HE is equivalent to 42 μg mL^{-1} of amaranth extract. Hemolymph molting hormones were analyzed using Ultra Fast Liquid Chromatography (UFLC). The initial and final concentrations of hemolymph ecdysteroid were analyzed by the t-student test. The results showed that: 1) the higher the injected concentration of amaranth extract, the higher the post injection hemolymph ecdysteroid concentration (from 1.15±0.17 μg mL^{-1} to 1.143-2.990 μg mL^{-1}); 2) the molting percentage ranged between 60.02-79.49%; 3) the molting duration was of 41.2-50.1 days; 4) the weight growth was of 18.92-25.41 g and the carapace width growth was of 7.62-10.53 mm; 5) the growth of biomass was of 395.33-486.33 g; 6) the survival rate ranged between 74.36-80.48%. A high dose of amaranth extract (phytoecdysteroid) injection leads to a high hemolymph ecdysteroid concentration during post-injection. An amaranth extract dose of 42 μg mL^{-1} was optimal for improving molting duration, molting percentage, weight gain and survival rate of *S. olivacea*.
Key Words: hemolymph ecdysteroid concentration, molting, productivity, survival rate.

Introduction. Soft shell crabs are cultivated and harvested shortly after changing the skin (molting) before the new skin or shell undergoes hardening. The soft shell crab products can be consumed with the shell. In addition, the nutritional value is also higher, due to the edible chitosan and carotenoids contained in the shell (Fujaya et al 2013). This product has a higher price than hard shell crabs of the same size, indicating an increasing demand.

The soft shell crab cultivation issues include: a low number of molting crabs, a long molting duration, low growth and survival rates. According to Fujaya et al (2009) and Fujaya (2011 a,b), the average number of crabs molting in ponds is still below 50% and the duration of molting is 60-90 days. To speed up the process of molting in crabs various efforts have been made, including: eye stalk ablation (Tamone et al 2005; Sudha & Anilkumar 2007), environmental stimuli (Azis 2008; Rangka & Sulaeman 2010), cutting claws and road legs (Widyastuti & Husni 2007), the addition of molting hormones (Tanaka & Naya 1995) and the use of phytoecdisteroids, namely the amaranth extract (Aslamyah & Fujaya 2011; Fujaya 2011b).

Ecdysteroid can also be found in insects, crustaceans and several plants, in which case it is called phytoecdysteroid. The phytoecdysteroid has an identical chemical structure with the growth hormone of insects and crustaceans (Feldman 2009). However, the functions of ecdysteroid in the plant are different from those in animals. Ecdysteroid in plants act as protective agents (poisons) against herbivorous insects (Schmelz et al 2002) and nematodes (Soriano et al 2004). The mulberry (*Morus* spp.) and amaranth (*Amaranthus* spp.) were identified as containing ecdysteroid. The mulberry plant has long

been used as feed in silkworm cultivation (Nguku et al 2007) and as a molting stimulant for crab larvae (Fujaya et al 2013) and for soft crab cultivation (Jompa & Suryanto 2014). Amaranth plants have been applied to stimulate molting for soft shell crab cultivation (Aslamyah & Fujaya 2011; Fujaya 2011a,b; Fujaya et al 2013). The ecdysteroid from Moraceae and Amaranthaceae plants are similar to the ecdysteroid found in the crab (*Callinectes sapidus*), their ecdybase being known as Inokosterone (or Callinecdysone A) (Fujaya et al 2013).

The phytoecdysteroid of amaranth and mulberry is abundant. These plants have been widely cultivated for consumption, for medicinal purposes and as feed for silkworms. Fujaya et al (2012) reported the production of 5-6 g of amaranth extract from 1 kg of dried amaranth which is used as a molting stimulant. The use of amaranth extract as a molting stimulant in crabs is more economical compared to the 20-HE molting hormone. 20-HE molting hormones are available in limited quantities and are expensive (Hasnidar 2014). The 20-HE molting hormone in a dose of 1.0 µg mL^{-1} is optimal for increasing the number of molting crabs, for speeding-up the molting time and for improving the growth and survival of *Scylla olivacea* (Hasnidar 2014; Tamsil & Hasnidar 2018). It was also the reference for determining the dose of amaranth extract which was tested in this study.

This study aimed to examine the effects of phytoecdysteroid on the increase of the hemolymph ecdysteroid concentration and molting of *S. olivacea*. Phytoecdysteroid was expected to be a replacement for 20-HE molting hormone, which is scarce and expensive, in order to achieve an enhanced productivity and efficiency in the soft shell crab cultivation.

Material and Method

Experimental study. A total of 225 *S. olivacea* with a carapace length of 61-62 mm and a weight of 50-51 g were obtained from crab fishermen in Siwa Kabupaten Wajo South Sulawesi, Indonesia, being selected based on the carapace weight and width criteria. The crabs were placed into a soft shell crab box, each box containing 1 crab. The crabs were reared in ponds by floating methods according to the soft shell crab farming procedure (Figure 1). During the rearing period, the crabs were fed with trash fish at a dosage of 10% of the crab body weight. Feeding was conducted once daily in the late afternoon (Fujaya et al 2012). The amaranth extract was identified as a phytoecdysteroid which has a lower ecdysteroid content than the 20-HE molting hormone. Thus, a higher dosage was needed in its application. A dosage of 1 µg 20-HE molting hormone is equivalent to 42 µg mL^{-1} of amaranth extract (Hasnidar 2014).

Figure 1. *Scylla olivacea* reared in ponds by the floating methods according to soft shell crab farming procedure (original).

Experimental design. A completely randomized design was applied, which consisted of 5 treatments and three replications, using 5 *S. olivacea* for each replication. The treatments were established as follows: Treatment A-amaranth dose of 17 µg mL^{-1}; Treatment B-amaranth extract dose of 29 µg mL^{-1}; Treatment C-amaranth dose of 42 µg mL^{-1}; Treatment D-amaranth dose of 54 µg mL^{-1}; Treatment E-amaranth dose of 67 µg mL^{-1}.

Hemolymph collection and extraction. The hemolymph collection and extraction were performed according to Fujaya & Trijuno (2007). The hemolymph collection was performed once (before the vitomolt injection), and three times (day 1, 3 and 5) post-injection. Hemolymph crabs were taken from the base of the fifth walking leg by using a syringe with needle, of 1 mL and 27-gauge. 1 mL of hemolymph was stored in eppendorf and mixed with anti-coagulants at a 1:1 ratio. The sample was stored in the freezer at a temperature of –20°C and then the sample was ready to be extracted. The hemolymph extraction procedure was: 1) 1 mL of hemolymph was added to 3 mL of diethyl ether, 2) the samples were homogenized with the vortex for 30 sec and then left for 2 min, 3) the upper layer is the ether phase containing the steroid, 4) the remaining residue was extracted 3 times, collected and the dry matter was infused at 40°C.

Measurement of ecdysteroid content. The dried residue of the extracted sample was dissolved with methanol pro UFLC, then inserted into UFLC auto-sampler vial. The sample was analyzed with a Shimadzu LC-20 AD Ultra-Fast Liquid Chromatograph (UFLC), with the characteristics: a) the column was a Shim Pack ODS C18 of 250 x 4.6 mm, b) with a reversed phase system, c) the mobile phase was: methanol-water (80:20 v/v), d) a flow rate of 1 $mL \cdot min^{-1}$, e) the temperature in the column was of 40°C, f) the detector was with photodiode array (UV) of 246 nm, g) the injection volume was of 10 µL. The quantification of the ecdysteroid used a standard series of 20-hydroxyecdyson (Sigma®).

Measurement of parameters. The hemolymph ecdysteroid concentration after injection was measured three times; the molting crabs percentage was calculated based on the number of molting crabs divided by the total number of crab multiplied by 100%; the molting time (duration) after injection was the time required before molting occurred; the growths of the absolute weight and width of the carapace were calculated according to Effendie (1979):

$$W = Wt - Wo$$
$$L = Lt - Lo$$

Where:
Wt - the final weight of the crab (g);
Wo - initial weight of the crab (g);
Lt - the final carapace width of the crab (mm);
Lo - the initial carapace width of the crab (mm).

The growth of biomass is the difference between the initial weight and final weight of crabs during the study. The survival rate was calculated by the initial number of crabs in the rearing pond divided by the final number of crabs after rearing. Water quality parameters were measured: temperature, pH, salinity, dissolved oxygen, nitrites and ammonia. Temperature, dissolved oxygen (DO) and pH were measured by a DO meter, salinity with a refractometer, ammonia and nitrite were analyzed by a spectrophotometer.

Data analys. The initial and final hemolymph ecdysteroid concentrations were analyzed by a t-student test, according to Sokal & Rohlf (1969). The analysis of variance (ANOVA test) at 5% significance level was performed, followed by a Duncan test, according to Steel & Torrie (1993). Excel 2010 and SPSS 22.0 version were used for statistic calculations. A descriptive and qualitative analysis was applied to the water quality parameters.

Results

Hemolymph ecdysteroid concentrations of amaranth extract post-injection. The mean initial concentration of hemolymph ecdysteroid (1.15±0.17 µg mL^{-1}) was not significantly different ($p>0.05$) from the amaranth extract concentration post-injection, between 17 and 29 µg mL^{-1}, observed on day 1, 3, and 5. The mean concentrations of

hemolymph ecdysteroid in day 1 and day 5 post-injection of 42, 54 and 67 µg mL^{-1} amaranth doses were significantly higher ($P<0.05$) than the mean initial concentration (1.15±0.17 µg mL^{-1}) before the injection. On day 3, however, the difference between the mean initial hemolymph ecdysteroid and the concentration after injection of all amaranth doses were not significant (Figure 2).

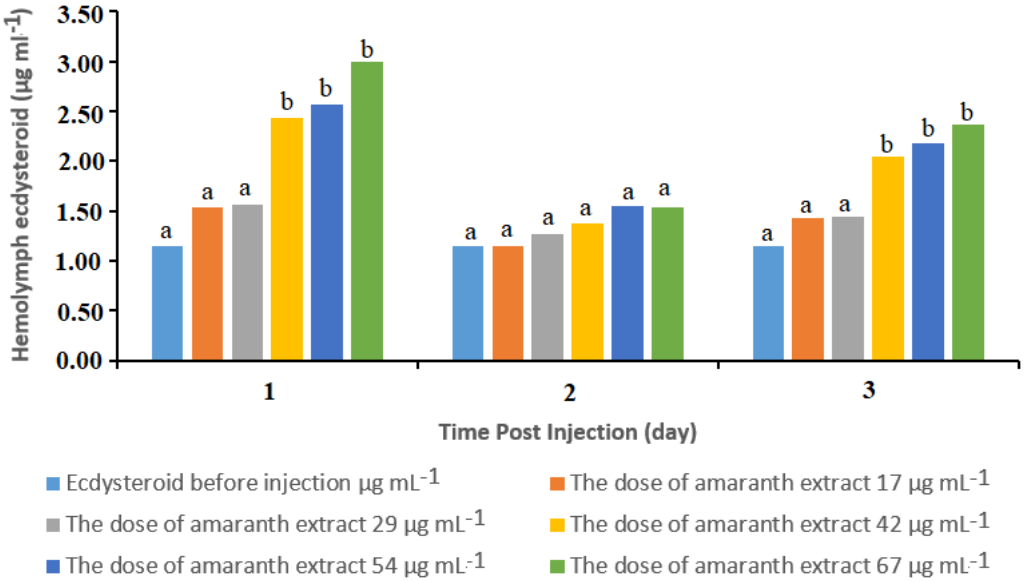

Figure 2. Concentration of ecdysteroid in hemolymph (µg mL^{-1}) of *Scylla olivacea* after amaranth extract injection on days 1, 3 and 5.

In Figure 2, the different letters on observations of days 1, 3 and 5 indicate the difference between the mean initial concentration (before injection) of hemolymph and the treatment concentration based on the Duncan test.

After the higher dose of amaranth extract was injected, a tendency to increase was observed in the concentration of ecdysteroid. Conversely, the control (treatment doses of 0 µg mL^{-1} or without the addition of amaranth extract) did not show any increase in the hemolymph ecdysteroid concentration. Post-injection, hemolymph ecdysteroid concentrations vary based on the time of observation. The hemolymph ecdysteroid highest concentration on the observation of the day1, decreased in the day 3, and rose again on the 5th day.

Molting percentage and molting duration. The highest molting percentage and the fastest molting time, of 79.49% and 41.6 days, respectively, were obtained from an amaranth extract dose at 42 µg mL^{-1} (Figure 3).

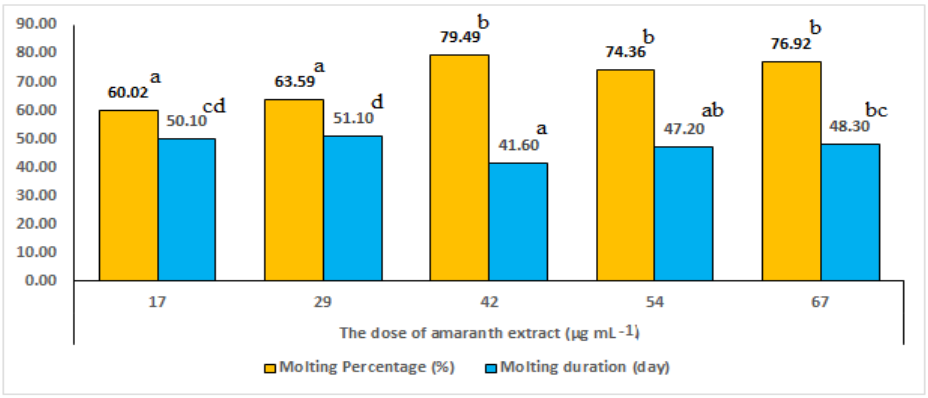

Figure 3. Mean molting percentage and molting duration of *Scylla olivacea* post injection of amaranth extract.

In Figure 3, the different letters above the value of molting percentage show that treatment 17 is not different with treatment 29, but is different with treatment 42, 54 and 67 µg mL^{-1}. The molting duration shows that treatment 17 is not different from treatments 29, 67 but is different from treatments 42, 54; treatment 42 is not different from 54, but different from 17, 29, 67 µg mL^{-1}, based on the Duncan test.

Growth of the absolute (weight and width of the carapace). The results growth weight and width carapace after injection of orange mud crab with amaranth extract (*S. olivacea*) showed that the dose of amaranth extract significantly affects ($p<0.01$) the rate of weight and carapace width increment of the experimental crabs. The growth of absolute weight and carapace width in *S. olivacea* at treatment doses of 42 µg mL^{-1} were of 25.4 g and 10.53 mm, respectively, higher than in other treatments (Figure 4).

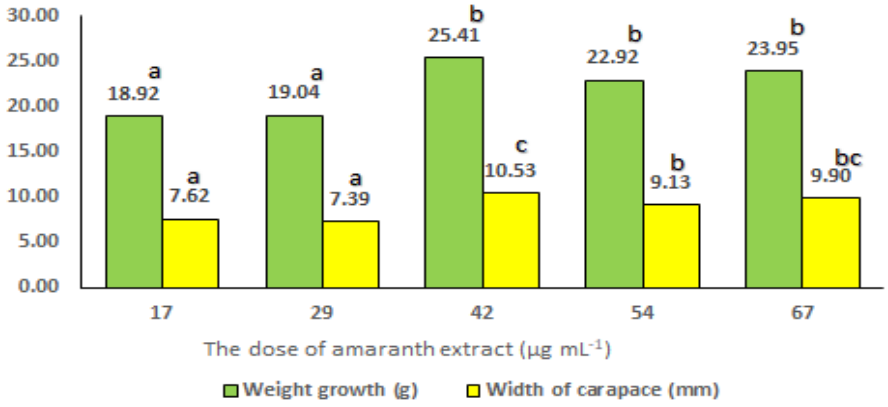

Figure 4. The changes in the carapace's weight and width of *Scylla olivacea* post injection of amaranth extract.

In Figure 4, the different letters above the value of the carapace's weight show that treatment 17 is not different from treatment 29, but it is different from treatments 42, 54 and 67 µg mL^{-1}. Treatment 42 is not different from treatments 54 and 67 µg mL^{-1}. Furthermore, the carapace's width shows that treatment 17 is not different from treatment 29, but it is different from treatments 42, 54 and 67; treatment 42 is not different from 67, but different from 17, 29 and 54 µg mL^{-1}, based on the Duncan test.

Weight gain and survival rate. The results show a significant ($p<0.05$) effect of different amaranth extract doses on the weight gain and survival rate of *S. olivacea.* The highest weight gain and survival rate were obtained from the treatment with an amaranth extract dose of 42 µg mL^{-1} (486.33 g and 80.48%, respectively) (Figure 5).

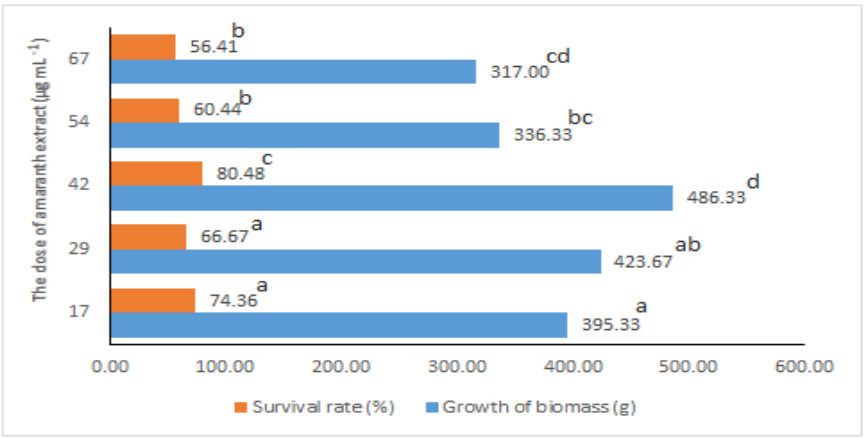

Figure 5. Survival rate and weight gain of *Scylla olivacea* after amaranth extract injection.

In Figure 5, the different letters above the value of the survival rate show that treatment 17 is not different from treatment 29, but it is different from treatments 42, 54 and 67 μg mL^{-1}. Treatment 42 is different from treatment 17, 29, 54 and 67 μg mL^{-1}. Furthermore, the weight gain shows that treatment 17 is not different from treatment 29, but it is different from treatment 42, 54 and 67; treatment 42 is not different from 67, but different from 17, 29, and 54 μg mL^{-1}, based on the Duncan test.

The results showed that the addition of an amaranth extract dose of 42 μg mL^{-1} produced a molting percentage, molting duration, weight growth, carapace width, growth of biomass and survival rate higher than at the doses of 17, 29, 54 and 67 μg mL^{-1}. An amaranth extract dose of 42 μg mL^{-1} is thought to be the right dose in inducing molting faster. Hormones are chemical messengers that are transported by blood to the target organs. The response of target organs to hormones can occur quickly or in several hours, days, months or even in several years, depending on the type of hormone. With the right dose of ecdystoid, the target organ, the cuticle, will respond to the chemical message (signal) for a precise and fast molting action. According to Meyer (2007), the molting process begins when the epidermal cells respond to hormonal changes through the rate of protein synthesis. Protein synthesis is the most basic growth process: without large-scale protein production, growth will not occur (Jobling et al 2001). Proteins play a role in the formation of new cells, including the formation of new cuticles or exoskeletons. An addition of amaranth extract doses of 54 and 67 μg mL^{-1} (>42 μg mL^{-1}) resulted in sub-optimal molting percentage, molting duration, weight growth, carapace width, growth of biomass production and survival rate, probably due to a higher hormone dose than required by the crab's organism, causing the hormone receptors' inhibition and a decrease of the ability of the target cell receptors to bind hormones (Dorrington 1979). A receptor is a biological device in the body that is tasked with recognizing the code carried by a hormone. If the performance of receptors decreases, the formation of new products such as proteins will be inhibited. Body cells have a certain limit in storing proteins: if this limit has been reached, each addition of amino acids to the body will be deaminated and used as energy or stored in adipose cells, as fat (Aslamyah & Fujaya 2011). The amaranth extract doses of 17 and 29 μg mL^{-1} (<42 μg mL^{-1}) also produce a sub-optimal molting percentage, molting duration, growth, growth of biomass and survival rate, this is likely due to a lower dose than required by the crab's organism for increasing the protein formation. According to Donaldson et al (1979), the most prominent metabolic action of the steroids is an activated protein metabolism.

Discussion. The dynamics of ecdisteroid increase are thought to be related to the half-life of the ecdysteoid hormone and to the molting cycle. Observations of the day 1 post-injection showed an increased ecdysteroid hemolymph concentration, probably due to the influence of the ecdysteroids contained in the injected amaranth extract, which increase the concentration of ecdysteroid in the crabs' hemolymph. The same phenomenon was observed in lobster treated with sea cucumber extract (containing a steroid compound, thetestosterone) by the immersion method: the effect on the hemolymph hormones depended on the administered testosterone levels (Riani et al 2010).

Observations of the day 3 post-injection showed a decrease in the concentration of the crab hemolymph ecdysteroid, likely due to the half-life ecdysteroid hormone. Ecdysteroid quickly undergoes a process of diffusion in the digestive organs and hepatopancreas. According to Fulierton (1980), ecdysteroid is metabolized rapidly in the gastrointestinal tract. This causes the hormones that enter through the gastrointestinal tract to partially accumulate in the hepatopancreas to subsequently experience metabolism and where they are partially absorbed by the intestine before being taken to the target organ. Observations of the day 5 post-injection showed that the concentration of ecdysteroid increased again, allegedly because the activated synthesis of ecdysteroid in the Y organ, for the preparation of molting. Increased ecdysteroid concentration in hemolymph is a signal for the body to begin the molting process. According to Chang & Bruce (1980); Suganthi & Anikumar (1999); Huberman (2000); Chung (2010), the low hemolymph ecdysteroid concentration, during the intermolting phase, increases significantly during premolting (preparation for molting) and drastically decreases after

molting. Hasnidar & Tamsil (2019) reported that mangrove crabs regulate their molting cycle based on the moon phase. The lunar phase influences the tides, the tidal phenomenon is suspected to be a signal to carry out the activities of molting in mangrove crabs. Furthermore, it is explained that in the relationship between the moon phase and the molting cycle, the new moon phase is the inter-molting stage (stage C_{1-4}), month ¼ I is early pre-molting (stage D_{0-1}), month ½ I is mid pre-molting (stage D_{1-2}), month ¾ I is late pre-molting (stage D_{3-4}) and ecdysis (stage E), full moon is post-molting (stage A and B). Molting is a physiological process controlled by the molting hormone (ecdysteroid) (Thompton 2015; Kuballa & Elizur 2007). The addition of ecdysteroid hormone influences the rate of molting. Fujaya et al (2012) reported that amaranth extracts contain hormone which are identical to the molting hormone, also containing protein, fat, ash and crude fiber, at concentrations of 28.94, 0.70, 8.09, and 17.58%, respectively. These components are thought to synergistically work together to increase the activity of protein synthesis and the cell carbohydrates, which effectively induce the molting process and growth rates. However, the response is largely determined by the right dose of amaranth extract.

Water quality parameters. The water quality parameters range of values and daily means were measured and compared to the optimum values for *S. olivacea* farming, according to the literature, as shown in Table 1.

Table 1

Range, daily mean and the optimum value of water quality parameters in *Scylla olivacea* rearing

Parameters	*Range of value*	*Daily mean*	*Optimum value*
Temperature (^{0}C)	20-35.7	31.04±2.535	25-35^{0}C[1,6]
pH	7-8.5	7.35±0.43	6.8-8.2[1,2]
Salinity (ppt)	15-35	29.25±3.390	25 ppt[2]
Dissolved oxygen (ppm)	2.1-6.43	4.82±1.338	>5 ppm[1,4]
Ammonia (ppm)	0.00-1.38	0.98±0.15.	<0.1[1,3,5]; <0.25[1,4]
Nitrite (ppm)	0.1-1.227	0.400±0.103	0.0-0.5[1,4]; 0.5-1.0[6]

[1]Fujaya et al (2012), [2]Karim (2008), [3]Wedemeyer & Mcleay (1981), [4]Gaude & Anderson (2011), [5]Turano (2007), [6]Shelley & Lovatelli (2011).

Water quality parameters such as temperature, pH, salinity, dissolved oxygen, ammonia and nitrite fluctuated but were still tolerated by the crabs. This can be seen in the high survival rate, above 50%, which ranged from 56.41 to 80.4%.

Conclusions. The addition of phytoecdysteroid (amaranth extract) increased the hemolymph ecdysteroid concentration. Injecting a high dose of amaranth extract leads to a high hemolymph ecdysteroid concentration in post-injection. An amaranth extract dose of 42 μg mL^{-1} was optimal for improving the molting duration, molting percentage, weight gain and survival rate of orange mud crabs.

Conflict of interests. The authors declare no conflict of interest.

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