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Metamorphosis Induction of the Dog Conch *Strombus canarium* (Gastropoda: Strombidae) Using Cues Associated with Conch Nursery Habitat

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Abstract: *Strombus canarium* is a commercially important gastropod that has great potential for advancement into aquaculture. In this study, the metamorphosis response of *Strombus canarium* larvae to various metamorphosis cues associated with conch nursery habitat and to KCl and GABA, were tested. Bioassays were run as static, no choice experiment and adopting a continuous exposure approach. *Strombus canarium* larvae showed strong metamorphosis responses when sediment (i.e., conch nursery habitat sediment/SD-NU) and detrital substrata (i.e., *Thalassia detritus* leachate/T-LC) from their nursery habitat were used ($p < 0.05$). There was no metamorphosis in treatments using sterilized conch nursery habitat sediment (SD-ST) and sediment taken from outside conch nursery habitat (SD-OT). Experiments using fresh macrophyte blades of *Enhalus acoroides* (EA), *Thalassia hemprichii* (TH), *Halophila ovalis* (HA) and *Ulva* (UL) and adult conditioned seawater (SD-SW) also showed negative respond. Conch larvae demonstrate active habitat selection during metamorphosis and no spontaneous metamorphosis was observed. Settlement in *S. canarium* is associative in nature where epibionts associated with conch nursery habitat could be the cue for the metamorphosis. However, the specific epibionts/inducers and mechanisms underlining the process were not studied and therefore are subjected to more detailed investigation. The use of KCl was comparable with treatments using natural inducers (SD-NU and T-LC), thus was suggested for application in hatchery spat production of the species.

Key words: Dog conch, gastropod, metamorphosis, settlement cues, veliger

INTRODUCTION

Metamorphosis in marine invertebrates involved significant morphological and physiological changes that facilitate the transformation of the larvae into young juvenile. Major ecological changes occurred, which include a shift from pelagic, encapsulated or brooded existence to that of an independent benthic individual (Gosselin, 1997; Bryan and Qian, 1998). The transformation process involved lots of energy due to significant increased in metabolic demand during the metamorphosis (Shilling *et al.*, 1996; Marsh *et al.*, 1999; Bryan, 2004). Successful metamorphosis is therefore, one of the most critical parts in supplying new recruits for the natural populations. It is also important for advancement of commercially important invertebrate species into aquaculture. The percentage of successful metamorphosis

is considered to be of primary importance and critical for aquaculture (Garcia-Lavandeira *et al.*, 2005) in order to maintain a steady supply and reliable source of seeds.

Metamorphosis in marine invertebrate is an active process and rarely occurred spontaneously. Upon reaching the metamorphosis competence stage invertebrate larvae will actively in search for these cues. In most cases the cues are very specific and generally reflects suitable habitat for the post-metamorphic life phase (Stoner *et al.*, 1996; Williams and Degnan, 2009). Abundant literature has shown that settling marine invertebrate larvae are influenced by specific physical, chemical and biological cues derived from a variety of sources in the marine environment (Manriquez *et al.*, 2004; Sawatpeera *et al.*, 2004; Holmes *et al.*, 2005; Xing *et al.*, 2008; Hayakawa *et al.*, 2009; Williams *et al.*, 2009).

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Strombus canarium Linnaeus, 1758 is a widely distributed conch species within the tropical waters and is highly associated with muddy bottom and seagrass bed areas (Purchon and Purchon, 1981; Poutiers, 1998; Cob *et al.*, 2009a). It is a commercially important conch species within the Southeast Asian region (Erlambang and Siregar, 1995; Cob *et al.*, 2008, 2009a, b). It has traditionally been fished and contributes to the economic of the local fishermen (Purchon and Purchon, 1981; Erlambang and Siregar, 1995).

Strombus canarium is a gonochoristic gastropod with separate sexes. Fertilization of egg is internal and large egg masses are laid by attaching them to seagrasses during spawning season from November to March. The egg mass is creamy-white in color and can easily be collected from the seagrass bed during low tides throughout the spawning season (Cob *et al.*, 2009d). The species showed high fecundity, which ranged from 48,000 to 71,000 eggs in a single egg mass (Cob *et al.*, 2009c). The larval stages of this conch have recently been described (Cob *et al.*, 2009c, 2009d) and it shared great similarity with other *Strombus* species (e.g., Davis *et al.*, 1993; De Navarrete *et al.*, 2007). The species certainly has great aquaculture potential. However, more information is needed, particularly regarding the settlement and metamorphosis of the species.

Recent study showed that there is specific nursery habitat for *S. canarium* population at Merambong Shoal, in the Straits of Johor, Malaysia (Cob *et al.*, 2009b). The purpose of the investigation was to determine parameters within the conch nursery habitat that might contribute as natural cues for conch larvae settlement and metamorphosis. Therefore, we tested and analyzed the effect of various natural cues associated with the nursery habitat. Apart from that the effect of K⁺ ion elevation and neurotransmitter GABA on *S. canarium* larvae were also conducted. The result is practically very important for management and conservation of the resources and hopefully lead to advancements in aquaculture of the species.

MATERIALS AND METHODS

Culture of *Strombus canarium* larvae: *Strombus canarium* egg masses were collected from Merambong Shoal, Johor Straits, Malaysia (01°19.778'N, 103°35.798'E). The egg masses were gently washed with 0.22 µm filtered seawater (FSW) to remove sand and debris and incubated in individual containers filled with FSW, at salinity 30±1 PSU and temperature 29±1°C until hatch. Larvae were held at concentrations of 1-2 larvae mL⁻¹ in 2 L round bottom glass container of 30±1 PSU and 29±1°C and fed daily with the algae *Isochrysis galbana* at 1000 cells mL⁻¹

(Cob *et al.*, 2009c). The culture medium was totally replaced every 2 days with freshly filtered FSW. After 16 days, larvae that exhibit exploration and swim-crawl behavior as well as other physical characteristics that indicates metamorphic competence such as the presence of dark-green pigmentation on shell, foot and mantle margin, development of proboscis etc. (Cob *et al.*, 2009c, d), were utilized for the settlement experiments.

Design of settlement experiments: Metamorphosis assays were run as static, no-choice experiments, with three replicates per treatment. Ten competent larvae were used in each replicate, which were taken from the same egg mass to reduce variability and all experiments were tested simultaneously. Larvae were introduced into a well of a 6-well plate (sterile Nunclon® 6-well polystyrene, 35 mm diameter, 15 mL volume), containing the respective settlement cues in 10 mL of FSW. The well plates (with lid on) were kept in an incubator at 29±1°C and salinity 30±1 PSU, under a 12L:12D light regimen. Larvae were subjected to continuous exposure to the metamorphosis inducers. A treatment with only FSW was used as a negative control, which also act as a test of spontaneous metamorphosis.

Larvae behavior was observed under a dissecting microscope. The larvae were considered have successfully metamorphosed when they lost their velar lobes and began to crawl using their foot (Cob *et al.*, 2009c). The conch juvenile showed active feeding activities immediately after metamorphosed (Cob *et al.*, 2009c). Percentage of metamorphosis was determined at 24, 48, 72 and 96 h after the initiation of the experiment. Abnormal behavior of the newly settled juvenile due to incomplete metamorphosis e.g., slow moving, inactive and/or not using foot to crawl (Boettcher and Targett, 1998; Cob *et al.*, 2009c) were also noted.

Metamorphosis assays

Settlement in response to fresh macrophyte blades: The larval settlement response to fresh macrophyte blades i.e., *Thalasia hemprichii* (TH), *Halophila ovalis* (HA), *Enhalus acoroides* (EA) and *Ulva* sp. (UL) were tested. The macrophytes were taken from conch nursery habitat during extreme low tide when the seagrass bed was exposed. The macrophytes were gently rinsed with FSW and their surfaces were carefully scraped to remove detritus, sediment and other epibionts. A small fragment (ca 1 cm²) of the macrophyte blades were introduced into the respective experimental well.

Settlement in response to seagrass detritus: Senescent and partially decomposed seagrass blades (i.e., macro-detritus) of *Thalassia hemprichii* (T-DET) and

Halophila ovalis (H-DET) were collected from the conch nursery habitat during extreme low tide. The macro detritus was gently rinsed with FSW to remove sediment, but the epibiota were kept intact (not scraped). Fragments of about 1 cm² of T-DET and H-DET were introduced into the respective experimental well.

Settlement in response to aqueous extract of seagrass detritus: Extract from partially decomposed leaf fragments (macro-detritus) of *Thalassia hemprichii* (T-LC) and *Halophila ovalis* (H-LC) were tested. The macro-detritus was gently rinsed in FSW to remove sand and unwanted debris and was further shredded and ground using pestle and mortar. FSW was added at a ratio of ~0.5 g detritus to 1 mL FSW. The solution was kept frozen for 1 week to lyse the cells. Prior the experiment the solution was allowed to thaw overnight and filtered using a qualitative whatman-1 filter paper. Assay concentrations of 10 mL aqueous extracts per 1 L FSW were used in the settlement experiment.

Settlement in response to surface sediment: The uppermost layer (5 mm) of top sediment was used for this experiment. The treatment for this experiment includes: sediment taken from conch nursery habitat (SD-NU), sediment taken from conch nursery habitat but further autoclaved (SD-ST) and sediment taken from outside conch nursery habitat (SD-OT) (i.e., from dense *Enhalus* bed of more than 80% coverage). The macro-detritus was manually removed from the sediment using forceps and smaller detritus was removed by decanting method using FSW. The sediments were transferred into the experimental wells to about 3-4 mm in thickness. Ten milliliter of FSW was added and the assays were conditioned for 24 h with mild aeration prior to the introduction of competent larvae.

Surface sediment conditioned seawater (SD-SW): Uppermost (5 mm) layer of sediment were taken from conch nursery habitat during extreme low tide. The macro-detritus was manually removed and FSW was added at a ratio of 0.5 g sediment per 2 mL FSW, with mild aeration. After 24 h the conditioned seawater was filtered using a 0.45 µm MF-millipore membrane filter and transferred to the experimental well.

Settlement in response to Adult Conditioned Seawater (AD-SW): Five adult specimens of *Strombus canarium* were taken from the field. The conchs were rinsed with FSW and their shells were scraped to remove epibionts and fouling and were kept in 2 L FSW with moderate aeration. After 24 h, the seawater was filtered using a

0.45 µm MF-Millipore membrane filter and introduced to the experimental well.

Settlement in response to elevated ion concentrations (K⁺) and neuroactive compound (GABA): There are many ions and neuroactive compounds that have been found to affect larva settlement in marine mollusks, however, only Potassium chloride (KCl) and γ-aminobutyric acid (GABA) were tested. KCl was selected as it is cheap and economical for commercial application (Yu *et al.*, 2008), whilst GABA has increasing been used by commercial hatcheries because of its higher efficiency compared with other settlement methods (Bernal and Beltran, 1998). The KCl stock solution of 1 M was prepared by dissolving the chemicals in distilled water. Serial dilutions with FSW were made immediately before commencing the experiments. Test concentrations were 5, 10 and 15 mM K⁺. Stock solution of GABA was prepared by dissolving the chemicals in FSW immediately prior to the experiments. The stock solutions of 10⁻² M were diluted to prepare solutions of 10⁻⁷, 10⁻⁶ and 10⁻⁵ M.

Data analysis: There were three replicates of each treatment. The percentage of metamorphosis in each experimental treatment was transformed (arcsine) before the statistical analysis was conducted. To improve the arcsine-transformation, those replicates with zero were given the value of 1/(4n), where n is the number of larvae in a single replicate (Yu *et al.*, 2008). Treatments in which percent metamorphosis was equal to zero for all replicates were not included in the statistical analyses. Normality and homogeneity of variance were tested using Anderson-Darling and Bartlett's test respectively. Data complied with the requirement for parametrical test were analyzed with a one-way ANOVA utilizing a post-hoc Tukey's test to compare difference between treatments and control (Yu *et al.*, 2008). Otherwise, the Kruskal-Wallis test followed by Dunn's method was applied (Gebauer *et al.*, 1998). All statistical analyses were conducted using the MINITAB® Statistical Software (Minitab Inc., State College, USA).

RESULTS

The cumulative percentage of metamorphosis after 24, 48, 72 and 96 h are presented in Table 1. Analysis of variance showed a highly significant different between treatments (oneway-ANOVA, F = 40.39, df = 35, p<0.05). Post-hoc analysis (Table 2) showed three different groups that significantly differed from one another. Higher percentages of metamorphosis were observed in treatments using sediment taken from nursery habitat

Table 1: Metamorphosis response of *Strombus canarium* larvae exposed to different inducers

Parameters	24 h	48 h	72 h	96 h
Sediment from nursery habitat (SD-NU)	93.33±3.34	100.00	-	-
Sediment conditioned seawater (SD-SW)	46.67±6.67	60.00±0.20	80.00±20.00	80.00±20.00
<i>Thalassia detritus</i> (T-DET)	43.33±8.82	50.00±10.00	50.00±10.00	50.00±10.00
<i>Thalassia detritus</i> leachate (T-LC)	80.00±5.77	100.00	-	-
<i>Halophila detritus</i> (H-DET)	20.00±5.77	20.00±5.77	20.00±5.77	-
<i>Halophila detritus</i> leachate (H-LC)	3.33±3.34	3.33±3.34	3.33±3.34	3.33±3.34
KCl 5 mM (K-5)	73.33±3.34	100.00	-	-
KCl 10 mM (K-10)	40.00±5.77	100.00	-	-
KCl 15 mM (K-15)	33.33±3.34	100.00	-	-
GABA 10 ⁻⁷ M (G-7)	6.67±3.34	16.67±6.67	-	-
GABA 10 ⁻⁶ M (G-6)	3.33±3.34	13.33±3.33	-	-
GABA 10 ⁻⁵ M (G-5)	3.33±3.34	33.33±13.33	-	-
Control (FSW)	0	0	0	0

Values are cumulative percentage of metamorphosis (Mean±SE, N = 3). FSW = 0.22 µm filtered seawater. Assays with negative metamorphosis response were not presented

Table 2: Tukey's pairwise comparisons between all treatments after 24 h exposure to the inducers

	SD-NU	SD-SW	T-DET	T-LC	H-DET	H-LC	K-5	K-10	K-15	G-7	G-6
SD-SW	*										
T-DET	*	O									
T-LC	O	*	*								
H-DET	*	*	O	*							
H-LC	*	*	*	*	*						
K-5	O	*	*	O	*	*					
K-10	*	O	O	*	O	*	*				
K-15	*	O	O	*	O	*	*	O			
G-7	*	*	*	*	O	O	*	*	*		
G-6	*	*	*	*	O	O	*	*	*		O
G-5	*	*	*	*	O	O	*	*	*		O

*Denotes significantly different and zero (o) denotes not significantly different, at 0.05 probability levels. SD-NU: Sediment from nursery habitat, SD-SW: Sediment conditioned seawater, T-DET : *Thalassia detritus*, T-LC: *Thalassia detritus* leachate, H-DET: *Halophila detritus*, H-LC: *Halophila detritus* leachate , K-5: KCl 5 mM, K-10: KCl 10 mM, G-7: GABA 10⁻⁷ M, G-6: GABA 10⁻⁶ M, G-5: GABA 10⁻⁵ M

(SD-NU), *Thalassia detritus* leachate (T-LC) and 5 mM KCl (p<0.05), followed by a slightly lower metamorphosis in treatments using sediment conditioned seawater (SD-SW), *Thalassia detritus* (T-DET), *Halophila detritus* (H-DET), 10 and 15 mM KCl (p<0.05). Lowest metamorphic responses were observed in treatments using GABA (10⁻⁷, 10⁻⁶ and 10⁻⁵ M) and *Halophila detritus* leachate (H-LC) (p<0.05). There was no metamorphosis occurred in treatments using fresh macrophyte blades (HA, TH, EA and UL), adult conditioned seawater (SD-SW), sterile nursery habitat sediment (SD-ST) and sediment taken from outside conch nursery habitat (SD-OT). In addition, there was no spontaneous metamorphosis as none of the larvae in the control treatments has metamorphosed.

Treatments using sediment from nursery habitat (SD-NU), KCl (5, 10 and 15 mM) and *Thalassia detritus* leachate (T-LC) showed the best result where all larvae in these treatments successfully metamorphosed after 48 h. Treatment using sediment conditioned seawater (SD-SW) also showed good metamorphic response where, 80% metamorphoses recorded after 96 h exposure. In treatment using *Thalassia detritus* (T-DET), up to 50% metamorphoses were recorded after 48 h, but no further metamorphosis recorded thereafter. For the treatments using *Halophila detritus* (H-DET) and *Halophila detritus*

leachate (H-LC), metamorphoses were only observed during the first 24 h (at 20 and 3.33%, respectively).

The neurotransmitter GABA was less efficient as inducers for the species as only 3 to 7% of metamorphoses were recorded. The juveniles from these treatments were relatively in poor shape compared to the other treatments. They were inactive and showed very slow respond when disturbed and the animal remained partially retracted into the shell with their columella opening facing upwards. All juveniles exposed to GABA were subsequently died by day 3 (72 h).

DISCUSSION

Recruitment is an important process that plays a critical role in the population dynamics and community structures of benthic marine organisms, especially those that develop from an initial planktonic larval phase. Successful recruitment can be influenced by both settlement success and early post-settlement mortality and how well they adapt to their new environment after metamorphosis (Stoner *et al.*, 1996; Pinedo *et al.*, 2000; Roberts, 2001). Thus, to some extent there might be some relationship between settlement and benthic invertebrate's habitat preferences. Previous studies

showed that *S. canarium* is highly associated with seagrass bed ecosystem and there is specific nursery habitat present where small juveniles can be found in large numbers (Cob *et al.*, 2008, 2009a). However, there was no metamorphosis response when the *S. canarium* larvae were exposed to fresh macrophyte blades (fragments of *Halophila ovalis*, *Thalassia hemprichii* and *Enhalus acoroides*), indicating that *S. canarium* settlement in seagrass beds may be related to features other than the seagrass itself. The seagrass ecosystem formed an important habitat and refuge for the species, but there might be other factors more important for survivorship of the newly settled juvenile.

Strombus canarium larvae on the other hand showed strong metamorphosis responses when sediment and detrital substrata from their nursery habitat were used, which is in agreement with previous studies on other *Strombus* species (Davis and Stoner, 1994; Boettcher and Targett, 1996; Stoner *et al.*, 1996). Many other invertebrate larvae also showed the same characteristic where positive responses to associative cues from their favorable feeding habitat were reported by Williams *et al.* (2007), Xing *et al.* (2008) and Hayakawa *et al.* (2009). This behavior might be important to ensure settlement in an environment most appropriate for their survival, as juvenile survivorship differed greatly among habitats with different physical and biological environments (Naylor and McShane, 1997; Herbert and Hawkins, 2006; Walker, 2007).

Highest percentage of metamorphosis was observed when, larvae were exposed to sediment taken from their natural nursery habitat. However, no metamorphosis was observed in treatment using the same sediment but sterilized, indicating that factors other than the physical characteristics of the substrate itself are more important for *S. canarium* larvae. Benthic sediment has in many instances been reported to emanate important metamorphic inducer for gastropods (Pederson and Page, 2000; Smith *et al.*, 2005; Walker, 2007). The upper layer of seagrass bed sediment is very rich in detritus and other associated epibionts (Kharlamenko *et al.*, 2001) that highly preferred by juvenile *Strombus*. The present study indicated that these associated epibionts within the nursery habitat might have emanated the most important cues for *S. canarium* larvae. However, the types of epibionts and their chemical properties however, need further investigation. According to Boettcher and Targett (1998), the types of chemical cues involved in *Strombus* metamorphosis and the mechanisms that controlling it share general features with chemoreception in adult aquatic invertebrates. They normally consists of chemicals with low molecular weight and soluble in water.

Autoclaved sterilization might have destroyed the epibionts as well as other important metabolites (cues) thus, resulted in negative metamorphic response by the larvae.

There was no metamorphosis response when cues associated with conspecific adult conch were used. The result of this experiment suggest that the important cues for *S. canarium* larvae settlement are not aggregative (cues associated with conspecifics), but rather associative (cues associated with non-conspecific-hosts, prey, or biofilm) in nature. Associative cues produced by marine algae, particularly the rhodophytes, were important in the settlement and metamorphosis of many invertebrate larvae (Morse, 1992; Huggett *et al.*, 2005; Hayakawa *et al.*, 2009). Although, juvenile *Strombus* lives in colony gregarious settlement has never been reported within the genus. This behavior might be an evolutionary selection to avoid being accidentally eaten up by the adults, which also engulf sediment and other particulate matter during feeding.

In KCl exposure trial, the recovery after 24 h exposure was relatively high and comparable with treatments using natural inducers (i.e., sediment from nursery habitat and *Thalassia detritus leachate*). A similar study on *S. gigas* reported between 52%-95% recoveries within 16 h exposure at 15 mM (Davis *et al.*, 1990). Another study by Boettcher and Targett (1998) reported KCl concentration between 20 and 22 mM induced levels of metamorphosis equivalent to those induced by the algal extract. Low concentrations of KCl (between 5 and 15 mM) are therefore suggested for large scale metamorphosis of the species studied, but test on commercial scale still yet to be conducted. Elevated K⁺ concentration induced metamorphosis in marine invertebrates presumably by depolarizing sensory cells (Boettcher and Targett, 1998). Potassium chloride has considerable potential for *S. canarium* aquaculture (spat production) mainly because it is readily available, inexpensive, effective and non-toxic (Yu *et al.*, 2008). It has been applied as metamorphosis inducer in other mollusks hatcheries with consistent dosage (Pechenik and Heyman, 1987; Davis *et al.*, 1990; Yu *et al.*, 2008).

The GABA is one of neurotransmitters that had the ability to inhibit velar cilia movement thus, induces the larvae metamorphosis behavior (Kaspar and Mountfort, 1995). GABA has been used by an increasing number of commercial hatcheries, because of it higher efficiency over other settlement methods (Bernal and Beltran, 1998). It has been reported to effectively induced settlement and metamorphosis in many mollusks larvae (Doroudi and Southgate, 2002; Zhao *et al.*, 2003; Garcia-Lavandeira *et al.*, 2005). In contrast, GABA was

less effective as metamorphic inducer for *S. canarium* larvae studied. The percentage of metamorphosis was very low in this treatment. Boettcher and Targett (1996) reported no significant effect on larval metamorphosis when exposed to 1 and 100 μ M GABA. The current study also showed evidence of toxic effects, which has been reported in other settlement studies using GABA (e.g., Bryan and Qian, 1998; Li *et al.*, 2006; Yu *et al.*, 2008). The toxicity might be due to long exposure to the chemical or through bacterial infestation as GABA provide a suitable substrate for bacterial growth.

CONCLUSION

The present study showed that *S. canarium* larvae demonstrate active habitat selection during metamorphosis. The metamorphosis responses were strongly influenced by the sediment and detrital substrata taken from their natural nursery habitat. Observations suggested that associated epibiont within the conch nursery habitat elicited the important cues for *S. canarium* larvae metamorphosis. As biological and chemical cues play great roles in the species metamorphosis, further investigations are therefore needed for extraction and characterization of the active compounds and the mechanism of the cues. The experiment also demonstrates that potassium chloride (KCl) has considerable potential in *S. canarium* aquaculture. It may provide a potentially effective and inexpensive method for enhancing large populations of synchronized spat production in *S. canarium* hatchery. As difficulties in the commercial culturing of mollusks mainly associated with larval metamorphosis, the establishment of metamorphosis cues for this species provide strong basis for advancement of the species into aquaculture.

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