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Full Length Article

The Effects of Substrate Grain Size and Mud-Sand Ratio on the Burrowing Ability of *Urechis unicinctus* Juveniles

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Abstract

In order to find out the optimal substrate grain size and mud-sand ratio and improve the burrowing ability of *Urechis unicinctus* juveniles, we explored the effects of substrate grain size (fine sand: 125–250 μm; medium sand: 250–500 μm; coarse sand: 500–2000 μm) and mud-sand ratio (0:1; 3:7; 1:1; 7:3; 1:0) on the burrowing ability of juveniles with a body length of 1.50±0.14 cm and a weight of 0.50±0.06 g. Results showed that substrate grain size and mud-sand ratio significantly affected minimum burrowing time, burrowing time and burrowing rate respectively (P<0.01). In the fine sand, juveniles had the shortest mean minimum burrowing time and mean burrowing time, and the highest mean burrowing rate, as opposed to those of coarse sand. With the rise of mud content in substrate, the mean minimum burrowing time and mean burrowing time increased markedly (P < 0.01), while the mean burrowing rate decreased significantly (P<0.01). The interaction of substrate grain size and mud-sand ratio had a significant effect on minimum burrowing time and burrowing time (P<0.01), but showed no effect on burrowing rate (P>0.05). Thus, it can be concluded that smaller the substrate grain size and the lower the mud content, the shorter minimum burrowing time and burrowing time, the higher the burrowing rate and the burrowing ability. In the culture of *U. unicinctus*, farmers need to choose small grain size substrate with high sand content, and avoid large grain size substrate with high mud content.

Keywords: *Urechis unicinctus*; Substrate grain size; Mud-sand ratio; Burrowing ability

Introduction

Urechis unicinctus, a zoobenthos of Echiura, Xenopneusta, Urechidae and *Urechis,* inhabits in intertidal and subtidal zones of China, Japan and Korea (Ma *et al.*, 2016; Zhang *et al.*, 2016). *U. unicinctus* is delicious and nutrient-rich and also contains plenty of amino acids and many kinds of biologically active materials. Thus, it has a high medical and research value, hence the name, "Naked Sea Cucumber" (Kimura *et al.*, 1983; Wang *et al.*, 2007; Jo *et al.*, 2008; Sung *et al.*, 2008). At present, *U. unicinctus* is mostly obtained from natural fishing. However, due to overfishing and water pollution, *U. unicinctus* has been substantially depleted in the natural environment, thus the capture fisheries is unable to meet the needs of people. Thus, there is an urgent need to artificially culture *U. unicinctus* to protect its germplasm and meet the needs of market. At present, with the significant technological breakthrough in its artificial breeding, *U. unicinctus* can be the cultured artificially (Xu *et al.*, 2016).

In the process of culturing *U. unicinctus*, whether juveniles can burrow into substrate and their burrowing speed makes the difference between success and failure. If *U. unicinctus* juveniles can quickly burrow into substrate, it will be beneficial for lessening the chance of being preyed upon and washed out, thus improving the survival rate and reducing culture risks. It was found that zoobenthos' burrowing process was influenced by many elements, such as substrate types, water current, and contaminants, etc. (Shin *et al.*, 2002; Matozzo *et al.*, 2004; St-Onge *et al.*, 2007; Lai *et al.*, 2011). However, there is hardly any research on the burrowing behavior and burrowing ability of *U. unicinctus* in different circumstance.

Therefore, this research explored the burrowing behavior of *U. unicinctus*, and the burrowing ability on different substrates, which were formed by three kinds of substrate grain sizes (fine sand: 125–250 μm; medium sand: 250–500 μm; coarse sand: 500–2000 μm) and 5 kinds of mud-sand ratio (0:1; 3:7; 1:1; 7:3; 1:0). The burrowing ability was illustrated by quantitative analysis on different substrates, which provided optimum substrate conditions for its artificial aquaculture. This research can provide reference

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for understanding healthy culture and the behavioral science of *U. unicinctus*.

Materials and Methods

Experimental Material

Two-month-old *U. unicinctus* juveniles were purchased from Yantai Fisheries Research Institute (Yantai, China), and healthy juveniles with a similar size were chosen as experimental materials, with the mean length (at the state of contraction) of 1.50±0.14 cm and mean mass (at the state of draining water naturally from rectum and vesicula anale) of 0.50±0.06 g. Then juveniles were temporarily cultured in PVC receptacles (100 × 50 × 25 cm) for a week with an average density of 300/m^2. On the bottom of the receptacles was placed a 15 cm thick substrate, which was collected from the production intertidal zone of *U. unicinctus* and pumped with 5 cm aquaculture seawater. During the temporary culture period, the seawater temperature was 21±1℃, pH was 8.5±0.5 and salinity 30‰, with continuous oxygenating. The seawater was changed by siphoning each day. A 7.5×10^4 cells/mL microalgae (*Chlorella vulgaris* and *Mtzschiaclosterium*) was equally fed 3 times one day. The light intensity was controlled by fluorescent lamps and curtained under 50 lux to avoid the effect on the burrowing behavior of *U. unicinctus*, but make sure to observe the behavior clearly. Moreover, seawater was oxygenated for one hour before the formal experiment to ensure a sufficient dissolving of oxygen. During the formal experiment, the system had no feeding, oxygenating and water changing, and other conditions were consistent with the temporary culture period.

The experimental sand was obtained from the Yantai sea area (37.38°N, 121.59°E). It was dried and sorted with sieves (ISO 3310-1: 2016, Test sieves-Technical requirements and testing-Part 1: Test sieves of metal wire cloth) into three specific grades following the Wentworth scale (Buchanan, 1984): fine sand, 125–250 μm; medium sand, 250–500 μm; coarse sand, 500–2000 μm. The experimental mud (grain size<75 μm, GB/T 50145-2007, Standard for engineering classification of soil, China) was collected from the pond culture area in Dayao Town (Yantai, China). As shown in Table 1, the experimental substrate was made by fixing different grain size sand and different ratios of mud, with the mud-sand ratio of 0:1, 3:7, 1:1, 7:3 and 1:0, respectively.

Burrowing Behavior of *U. unicinctus*

The experiment was conducted in a PVC sink (30 × 25 × 25 cm). The sink bottom was laid with 15 cm thick fine sand substrate and 5 cm seawater was slowly pumped, after which it sat quietly for 1 h. 5 *U. unicinctus* were put in a sink for observing their burrowing behaviors when they scattered naturally on the substrate surface, and a photograph was taken every 5 sec using a camera.

Substrate Grain Size and Mud-sand Ratio to Affect Burrowing Ability of *U. unicinctus* Juveniles

The experiment was conducted in 65 PVC sinks (30 × 25 × 25 cm). A total of 13 treatments were established, and each had 5 replicates. On the bottom of each sink was placed a 15 cm thick substrate, which was prepared in the part of "Experimental Material", and 5 cm seawater was slowly pumped and then it was left for 1 h. Ten *U. unicinctus* juveniles were randomly placed in each sink. The preliminary experiment results show that the number of *U. unicinctus* was appropriate in each sink, which could ensure sufficient size of samples and clear observation and over half of the *U. unicinctus* could complete the burrowing process with most substrates within 1 h. When they scattered naturally on the surface of the substrate, we recorded the time immediately.

The standard for evaluating the burrowing behavior of *U. unicinctus* was that it could not be observed from substrate surface (Nel *et al.*, 2001; Huz *et al.*, 2002). Minimum burrowing time referred to the amount of time between their scattering naturally on substrate surface and the completion of burrowing by the first individual sample, while burrowing time was set between their scattering naturally on substrate surface and the completion of burrowing by 50% individual samples (Shin *et al.*, 2002; Zhou *et al.*, 2014). The computational formula of the burrowing rate (BR) is as follows:

$$BR = M/M_0 \times 100\%$$

Where M = the number that finished burrowing within 1 h and M_0 = the number introduced at the outset of the experiment.

Statistical Analysis

The experimental data were expressed as Mean±S.D. The difference between groups was compared based on a two-way analysis of variance (ANOVA), followed by a Duncan's post hoc test. The significant level was $P<0.05$, and the very significantly level was $P<0.01$. All statistical analyses were conducted utilizing SPSS statistics 17.0 software (IBM, Armonk, USA).

Results

Burrowing Behavior of *U. unicinctus*

The burrowing procedure of *U. unicinctus* could be divided into three stages: preparation, burrowing and terminal. The preparation stage was from scattering naturally on the substrate to the beginning of stretching rhynchodaenm. During this period, the body of *U. unicinctus* gradually changed from contraction to extension (Fig. 1A and B). The burrowing period mainly included stretching rhynchodaenm, rhynchodaenm burrowing into substrate, and the body erecting with transposing. Firstly, *U.*

unicinctus needed to stretch rhynchodaenm, which was the sign of burrowing (Fig. 1C). It gradually burrowed into the substrate through the friction between rhynchodaenm and substrate surface in the way of up-and-down peristalsis with the body wall contracting and extending (Fig. 1D). After the front end of body burrowing into substrate, the rear-end parts gradually erected slowly (Fig. 1E). *U. unicinctus* completed the burrowing process when the rear-end parts burrowed into substrate (Fig. 1F).

Substrate Grain Size and Mud-sand Ratio to Affect the Burrowing Ability of *U. unicinctus* Juveniles

Minimum burrowing time of *U. unicinctus* juveniles: As shown in Fig. 2, the minimum burrowing time increased with the rise of the substrate grain size. In Table 2, the analysis of variance showed that different substrate grain sizes considerably influence the minimum burrowing time of *U. unicinctus* juveniles (P<0.01). In Table 3, multiple comparisons suggested that juveniles had the shortest mean minimum burrowing time in fine sand, which was significantly smaller than that in medium sand (P<0.01), on the contrary of coarse sand. As shown in Table 4, the mean minimum burrowing time was the shortest in the substrate of total sand, but the longest in total mud. In Table 2, the analysis of variance showed mud-sand ratio had significant effects on the minimum burrowing time of juveniles (P<0.01). In Table 4, multiple comparisons suggested that the mean minimum burrowing time of total sand was significantly smaller than other groups (P<0.01), and the mean minimum burrowing time obviously increased with the rise of the mud content (P<0.01). The two-way ANOVA analysis showed that the interaction between substrate grain size and mud-sand ratio significantly (P<0.01) influenced the minimum burrowing time of *U. unicinctus* juveniles (Table 2).

Burrowing time of *U. unicinctus* juveniles: As shown in Fig. 3, the burrowing time of *U. unicinctus* juveniles increases in relation to the rise of substrate grain size and the mud content of substrate. It was shown in Table 2 that the substrate grain size had a considerable influence on the burrowing time of juveniles (P<0.01). In coarse sand, the mean burrowing time was the longest, on the contrary of fine sand, and the mean burrowing time in the substrate of fine sand, medium sand and coarse sand was 1514.92 s, 1666.44 s and 2098.52 s respectively (Table 3). In Table 2, the influence of mud-sand ratio on the burrowing time was of significance (P<0.01). In Table 4, the mean burrowing time of *U. unicinctus* juveniles was 237.80 s, 677.00 s, 1216.20 s, 1846.60 s, 4822.20 s, with the mud-sand ratio of 0:1, 3:7, 1:1, 7:3 and 1:0. The difference between each group was very significant (P<0.01). The two-way ANOVA analysis showed that the interaction between substrate grain size and mud-sand ratio significantly (P<0.01) influenced the burrowing time of *U. unicinctus* juveniles (Table 2).

Burrowing rate of *U. unicinctus* juveniles: The burrowing rates of *U. unicinctus* juveniles in different substrates were above 50%, except for total mud. In Table 3, the mean burrowing rate of juveniles in fine sand was the highest and reached 70.80%, and the difference between medium sand and coarse sand was not significant (P>0.05). As it was shown in Table 2, substrate grain size had very significant effects on the burrowing rate (P<0.01). The mean burrowing rate decreased as the mud content of substrate increased and the mean burrowing rate in total mud was just 32.00% (Fig. 4 and Table 4). The mean burrowing rate of juveniles on the

Table 1: Types of substrates used in this experiment

V_{mud} : V_{sand}	Substrate grain size			
	Mud	Fine sand	Medium sand	Coarse sand
0 : 1	×	√	√	√
3 : 7	×	√	√	√
1 : 1	×	√	√	√
7 : 3	×	√	√	√
1 : 0	√	×	×	×

The mean with "√" was the type of substrates set in this experiment; the mean with "×" was the type of substrates not set in this experiment

Table 2: Two factor variance analysis of substrate grain size and mud-sand ratio of *U. unicinctus* juveniles

Factors	Substrate grain size	Mud-sand ratio	Substrate grain size×Mud-sand ratio
Minimum burrowing time	*	*	*
Burrowing time	*	*	*
Burrowing rate	*	*	—

*means very significantly different (P<0.01), — means no significant differences (P>0.05)

Table 3: The mean minimum burrowing time, mean burrowing time and mean burrowing rate of *U. unicinctus* juveniles in different substrate grain sizes

Substrate grain sizes	Fine sand	Medium sand	Coarse sand
Mean minimum burrowing time (s)	382.56±293.24^{Cc}	423.12±278.92^{Bb}	546.24±295.84^{Aa}
Mean burrowing time(s)	1514.92±1687.79^{Cc}	1666.44±1652.59^{Bb}	2098.52±1606.37^{Aa}
Mean burrowing rate (%)	70.80±22.96^{Aa}	66.40±23.13^{ABb}	63.20±22.22^{Bb}

Different capital letters were very significantly different at the 0.01 probability level, with different minuscule letters being significantly different at the 0.05 probability level, and the means with the same letters did not have significant differences

Table 4: The mean minimum burrowing time, mean burrowing time and mean burrowing rate of *U. unicinctus* juveniles in different mud-sand ratio

V_{mud} : V_{sand}	0 : 1	3 : 7	1 : 1	7 : 3	1 : 0
Mean minimum burrowing time (s)	131.67±41.80^{Ee}	248.20±44.00^{Dd}	391.20±97.70^{Cc}	549.53±200.85^{Bb}	932.60±34.12^{Aa}
Mean burrowing time (s)	237.80±92.90^{Ee}	677.00±121.16^{Dd}	1216.20±391.85^{Cc}	1846.60±711.68^{Bb}	4822.20±229.01^{Aa}
Mean burrowing rate (%)	97.33±4.42^{Aa}	78.67±7.18^{Bb}	68.00±8.33^{Cc}	58.00±9.09^{Dd}	32.00±7.48^{Ee}

Different capital letters were very significantly different at the 0.01 probability level, with different minuscule letters being significantly different at the 0.05 probability level, and the means with the same letters did not have significant differences

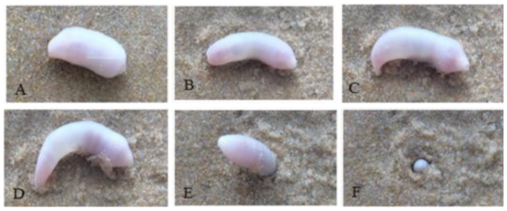

Fig. 1: The burrowing procedure of *U. unicinctus* juveniles. A, on the substrate; B, extending body; C, stretching out rhynchodaenm; D, burrowing into the substrate; E, erecting with around; F, finishing the burrowing procedure

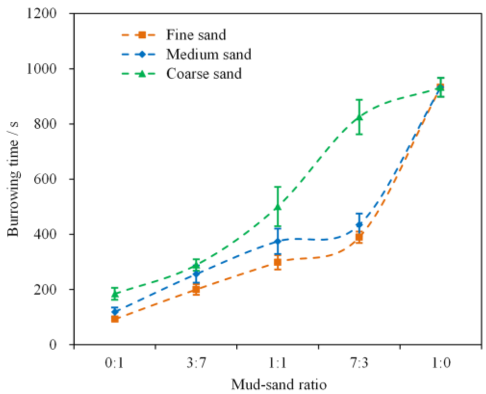

Fig. 2: The minimum burrowing time of *U. unicinctus* juveniles in different substrates

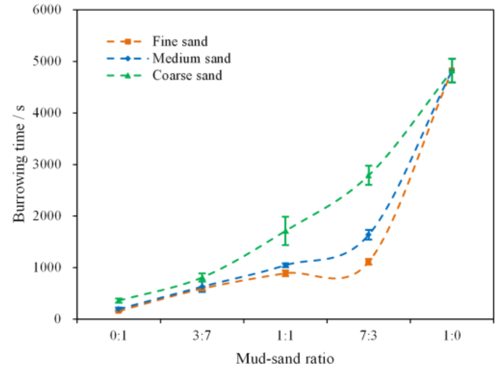

Fig. 3: The burrowing time of *U. unicinctus* juveniles in different substrates

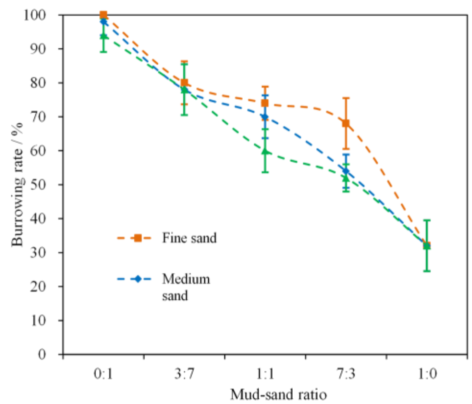

Fig. 4: The burrowing rate of *U. unicinctus* juveniles in different substrates

mud-sand ratio 0:1, 3:7, 1:1, 7:3 and 1:0 was 97.33, 78.67, 68.00, 58.00 and 32.00%, respectively. The analysis of variance showed that the mud-sand ratio had extremely significant (P<0.01) effects on the burrowing rate (Table 2). The two-way ANOVA analysis showed that the interaction of substrate grain size and mud-sand ratio had no significant (P>0.05) effect on the burrowing rate of *U. unicinctus* juveniles (Table 2).

Discussion

As one of the most important abiotic factors, the substrate condition affects growth, survival, behavioral response and geographical distribution of zoobenthos (Beisel *et al.*, 1998; Nel *et al.*, 2001; Bunn and Arthington, 2002). The measure of minimum burrowing time, burrowing time and burrowing rate can quantitatively reflect the ability of burrowing and the adaptability of substrate condition (Alexander *et al.*, 1993; Nel *et al.*, 2001; Huz *et al.*, 2002).

The study showed that the mean minimum burrowing time and mean burrowing time of *U. unicinctus* juveniles in fine sand was the shortest, followed by medium sand and lastly in coarse sand. The result indicated that juveniles had a better adaptability to small substrate grain size particles, and the burrowing ability decreased with the rise of substrate grain size. Based on the analysis above, the juveniles' body wall had a rich mucous gland (Liu, 2012), which could secrete mucus to lubricate substrate particles and body. With a relatively light weight of small grain size particles, juveniles could lubricate substrate by mucus to move particles easily, and burrow into the substrate. However, the quality of substrate particles increased with the rise of grain size and as the substrate surface became rough. The juveniles could not move substrate particles with a limited rhynchodaenm force and mucus secretion, which led to a drop in the burrowing ability.

In this study, *U. unicinctus* juveniles were specially explored for the mud-sand ratio of substrate. Greater the percentage of mud, longer the mean minimum burrowing time and mean burrowing time and the lower the mean burrowing rate of juveniles. Result showed that the burrowing ability decreased with the increase in the mud content in the substrate. It is believed that the substrate particles become smaller and texture denser after adding mud to the substrate, thus increasing the burrowing resistance (Sun *et al.*, 2017). Mud had a relatively higher content of organics than sand, and organics caused sand particles to be more adhesive to each other, thus resulting in the increase of burrowing resistance, and physiological stresses, which finally led to the reduction in the burrowing ability (Zwaan *et al.*, 1993; Laudien *et al.*, 2002; Zhou *et al.*, 2015).

When *U. unicinctus* juveniles burrowed into the substrate, it would be affected by more than one substrate condition (Shin *et al.*, 2002; Matozzo *et al.*, 2004; St-Onge *et al.*, 2007; Lai *et al.*, 2011). Therefore, it is necessary to consider the interaction of the substrate to choose a better environment for the breeding of juveniles. The interaction between substrate size and mud-sand ratio had a significant effect on the minimum burrowing time and burrowing time of juveniles. However, it had no significant effect on the burrowing rate. It was indicated that the interaction was influenced by time, which had an obvious influence on the short-term burrowing behavior of juveniles, but had little effect on the long-time burrowing behavior (Sun *et al.*, 2017). Analysis showed although under different substrates, the minimum burrowing time and burrowing time of juveniles differs, their burrowing ability was strong enough to adapt to different types of substrate. Except for total mud substrate, more than 50% of the juveniles could finish burrowing in other substrate conditions within 1 h. Other aquatic animal submergence studies found that some aquatic animals burrowing ability changed with water temperature, various sizes, flow rate and so on (John and Fernandez, 1989; Sakurai *et al.*, 1998; Huz *et al.*, 2002; St-Onge *et al.*, 2007). Whether or not a similar change will occur in *U. unicinctus* has to be further studied.

Conclusion

The smaller the substrate grain size and the lower the mud content, the shorter the minimum burrowing time and burrowing time, the higher burrowing rate and the greater the burrowing ability. The substrate grain sizes and mud-sand ratio are the essential factors affecting juveniles burrowing. In the culture of *U. unicinctus*, small grain size substrate with high sand content need to be chosen and large grain size substrate with high mud content must be avoided.

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