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RESEARCH ARTICLE

Deformation Control and Mass Transfer in the Tunic of *Halocynthia roretzi*

Yoko Kato*

Faculty of Engineering, Tohoku Gakuin University, Chuo, Tagajo, Miyagi, 9858537, Japan

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Abstract:

Background:

It has been previously reported that the tunic of *Halocynthia roretzi*, mainly composed of cellulose, is actively deformed with mass transfer by the mechanical stimuli.

Objective:

In this study, how the tunic deforms in response to the mechanical environment was investigated.

Method:

The tunic specimen in the artificial seawater was still at 5˚C or underwent the mechanical stimuli at the temperature less than 10˚C. The mass and moisture content of the tunic, the concentrations of nitrate and dissolved organic matter in the artificial seawater used for the tunic, and the histological characteristics were evaluated.

Results:

The increase in mass of the tunic became lower as the region was closer to the bottom of *Halocynthia roretzi*. However, the decrease in mass caused by the mechanical stimuli was not different between the adjacent regions. Also, the tunic of the siphon, the tubular tissue for influx and efflux of the seawater, increased the mass more slowly after the stimuli. The size of the layer covering the outside of the tunic was inversely related to the increment in mass. The change in mass was corresponding to that in water content. The concentrations of nitrate and dissolved organic matter in the artificial seawater were enhanced 5 days after the stimuli while the concentration ratio of dissolved organic matter to nitrate was kept constant.

Conclusion:

The water content in the tunic was used for controlling the mass response to the mechanical environment.

Keywords: *Halocynthia roretzi*, Tunic, Cellulose, Chitin sulfate-like polysaccharide, Deformation, Mass, Dissolved organic matter, Nitrate.

1. INTRODUCTION

Cellulose, one of the most abundant substances in the world, can be found in various species, from higher plants to bacteria [1]. In Animalia (animal kingdom), the tunic of the ascidian, which covers the body of the ascidian entirely, is mainly composed of cellulose while the tunic shows various appearances from leathery to translucent one [2 - 4]. The tunic surface is constantly replaced by new one with continuous secretion so that the thickness is kept constant [2]. The tunic has blood vessels and various kinds of cells including hemocytes, receptor cells and neuro cells, which are

* Address correspondence to this author at the Faculty of Engineering, Tohoku Gakuin University, 1-13-1, Chuo, Tagajo, Miyagi, 9858537, Japan, Tel: +81-22-368-7837; Fax: +81-22-368-7070; E-mail: ykato@mail.tohoku-gakuin.ac.jp

dispersed in the tunic [2 - 4]. In the defense system of *Halocynthia roretzi*, one of the solitary ascidians, the substances secreted from the hemocytes play an important role [5 - 12]. As these characteristics show, the tunic is not passive tissue, but dynamic tissue [2, 3].

Halocynthia roretzi is entirely covered with its leathery tunic, where highly pure and crystalline cellulose Iβ [13, 14], water-soluble chitin sulfate-like polysaccharide, which is predominantly composed of sulfated chitin [15, 16], pseudokeratin [17], α-smooth muscle actin [18], F-actin [19], elastic fiber [18], nervous system [18], myocyte [18] and various types of hemocytes [12], have been found. While the mechanical stimuli on the tunic surface caused the contraction of the ascidian [4], the tunic of *Halocynthia roretzi* itself, where other organs were removed, was also actively deformed by the administration of acetylcholine [18] and α-chymotrypsin [19], mechanical [18, 20] and electrical [19] stimuli at room temperature. As the metallo-protease secreted by the hemocyte, whose substrate was the same as chymotrypsin, behaved more active at 20˚C than 4˚C [10], the deformation reported previously [18 - 20] would occur when the protease was active. If the tunic itself senses and responds to the mechanical stimuli, the tissue structure of the tunic would be suitable for the mechanical environments. The deformation by the mechanical stimuli was associated with a change in mass of the tunic [19], but a substance related to the change has not been identified. Considering that the tunic contains the water-soluble polysaccharide [15, 16], influx and efflux of water could involve this change.

The ultraviolet absorbance in the spectroscopic analysis has been used to evaluate the components of the seawater such as bromide, nitrate and dissolved organic matter [21 - 26]. The ultraviolet absorbance can be useful to evaluate substances discharged from the tunic when the tunic deforms.

In this study, how the tunic of *Halocynthia roretzi* deforms in response to the mechanical environment was investigated. Firstly, the response of the tunic to the mechanical environment with no mechanical stimulus was examined because the tunic could sense the mechanical environment and deform itself. The temperature was 5˚C in order to reduce the activity of the metallo-protease. Also, the mechanical stimuli were given to the tunic at the temperature lower than 10˚C. The mass and moisture content of the tunic specimen were measured in order to examine whether or not water is associated with a change in mass of the tunic. Substances discharged from the tunic were evaluated by spectroscopic analysis. Since the tissue structure could directly influence the deformation, the histological characteristics of the tunic were evaluated by observing the stained tissue with a light microscope.

2. MATERIALS AND METHODS

2.1. Tunic Specimens of *Halocynthia roretzi* and the Mechanical Environment with no Mechanical Stimulus

Figs. (**1A** and **B**) show one of the samples, *Halocynthia roretzi*, and the categories of the tunic specimens, respectively. The samples were obtained from Research Center for Marine Biology, Graduate School of Life Sciences, Tohoku University (Aomori, Japan) and Yamanaka Inc. (Miyagi, Japan). The tunic was removed from other organs with tweezers and trimming blades (Feather trimming blade; Feather Safety Razor, Co. Ltd., Osaka, Japan), and cut into appropriate sizes. The specimen was categorized into 4 groups as Figs. (**1A** and **B**) show: Siphon, tubular parts for influx and efflux of the seawater; M1, regions with spines; M2, regions with no spine; and Bottom, regions contacting the bottom of the muscle. The artificial seawater was made by Reef Crystals (Aquarium Systems, Sarrebourg, France) and deionized water.

All the samples were put into the artificial seawater at 5˚C in the refrigerator (MRP-215FS; Panasonic Healthcare Co., Ltd., Tokyo, Japan) up to 10 days. Each day of the period is named as Day 1 and so on. The increment in the mass of the tunic (Δm_r) and increment in the mass per day ($\Delta m_r/day$) were evaluated as the section 'Mass and Moisture Content of the Tunic' shows. The number of the specimens in each group was as follows: Siphon, 22; M1, 27; M2, 15; Bottom, 25; Inside (inner regions of M2 and Bottom), 7; Outside (outer regions of M2 and Bottom), 6. The measurement frequency was as follows: Siphon, every day (n = 14), every day or two days (n = 6) and every day or three days (n=2); M1, every day (n = 19), every day or two days (n = 4), every day or three days (n = 3) and every day or six days (n = 1); M2, every day (n = 7); every day or two days (n = 4), every day or three days (n = 3) and every day or six days (n = 1); Bottom, every day (n = 19), every day or two days (n = 3), every day or three days (n = 3) and every day or six days (n = 1); Inside, every day (n = 7); Outside, every day (n = 6).

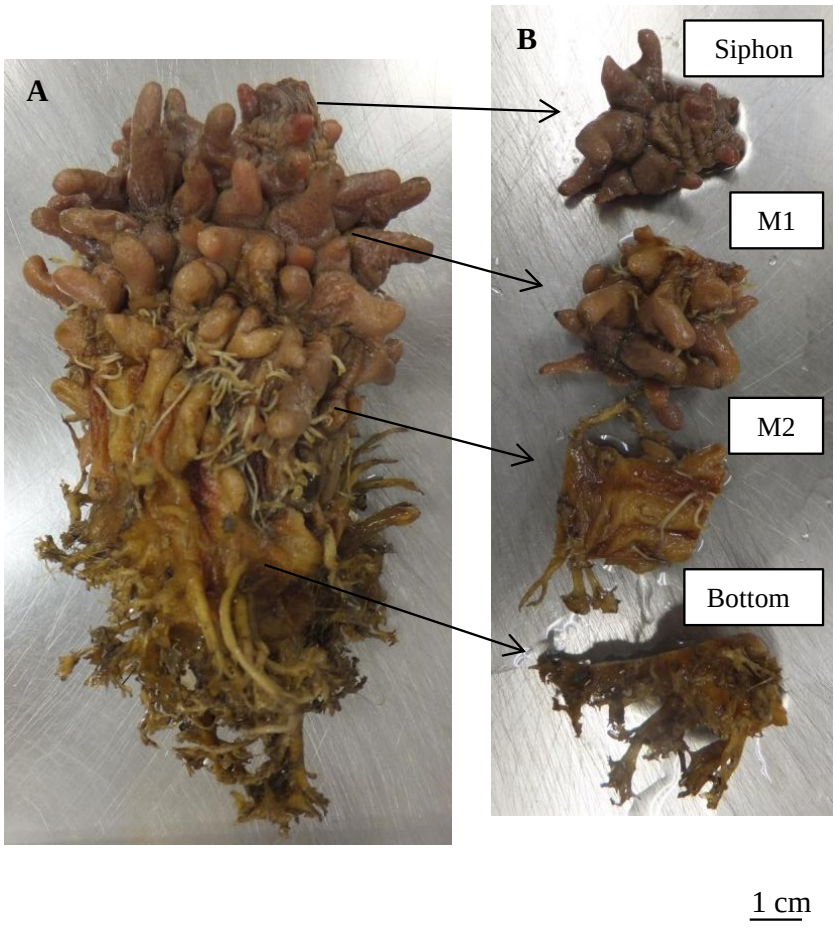

Fig. (1). The sample of *Halocynthia roretzi***(A)** and tunic specimens **(B)**. The tunic specimens were categorized into 4 groups: Siphon, tubular parts for influx and efflux of the seawater; M1, regions with spines; M2, regions without spines; Bottom, regions contacting the base of the muscle.

2.2. Mechanical Stimuli on the Tunic

The mechanical stimuli given to the tunic specimen at Day 1 were tapping the outer surface of the tunic specimen by tweezers for 3 minutes in the artificial seawater, at the temperature less than 10˚C, which was kept by ice packs. After the mechanical stimuli were given to the tunic specimen, the specimen and artificial seawater were at 5˚C in the refrigerator again, up to Day 7. The increment in the mass of the tunic (Δm_r) and increment in the mass per day ($\Delta m_r/day$) were evaluated as the section 'Mass and Moisture Content of the Tunic' shows. The number of the specimens was as follows: Siphon, 6; M1, 6; M2, 3; Bottom, 5. The frequency for measuring the mass was as follows: Siphon, every day (n = 4) and every day or five days (n = 2); M1, every day (n = 4) and every day or five days (n = 2); M2, every day (n = 2) and every day or five days (n = 1); Bottom, every day (n = 4) and every day or five days (n = 1).

2.3. Mass and Moisture Content of the Tunic

The mass of each specimen was measured by the balance (UW420S; Shimadzu Corporation, Kyoto, Japan) after removing the water at the surface of the specimen by wrapping it in paper wipers for 10 seconds (Kimwipe; Nippon Paper Crecia, Tokyo, Japan). The moisture content of the specimen was measured when the drying temperature was 200˚C by the moisture analyzer (MX-50; A&D, Tokyo, Japan) after removing the water at the sample surface by the aforementioned method and cutting the specimen into pieces by the trimming blade. The moisture content described by Equation (4) was measured when the change in the moisture content was less than 0.05%/min or 0.01%/min.

The mass of the specimen, m, was normalized by that just after making the specimen, m_o, as follows:

$$m_r = \frac{m}{m_o} \quad \textbf{(1)}$$

where m_r is the normalized mass. Hence, the increment in the mass of the tunic, Δm_r, is described as follows:

$$\Delta m_r = m_r - 1 \quad \textbf{(2)}$$

The increment in the mass of the tunic per day, $\Delta m_r/day$, is as follows:

$$\Delta m_r/day = \frac{m_{r_i} - m_{r_{i-1}}}{t_i - t_{i-1}} \quad \textbf{(3)}$$

where t_i is the time for the i^{th} measurement and m_{r_i} is the normalized mass at t_i. At giving the mechanical stimuli to the specimen, $\Delta m_r/day$ was calculated as follows, for convenience:

$$\Delta m_r/day = m_{r_{MS}} - m_{r_{pre}} \quad \textbf{(3')}$$

where $m_{r_{MS}}$ and $m_{r_{pre}}$ are the normalized mass at giving the mechanical stimuli and just before providing the stimuli at the same day, respectively. At the time of the measurement next to that at giving the stimuli, t_{post}, $\Delta m_r/day$ at t_{post} was calculated as follows:

$$\Delta m_r/day = \frac{m_{r_{post}} - m_{r_{MS}}}{t_{post} - t_{MS}} \quad \textbf{(3")}$$

where $m_{r_{post}}$ is the mass at t_{post}, and t_{MS} is the time at giving the stimuli.

The moisture content of the sample, C, is described as follows:

$$C = \frac{m - m_d}{m} \quad \textbf{(4)}$$

where m_d is the mass of the dried specimen when the moisture content was measured. m_{rw}, the mass of the water in the specimen normalized by m_o, is described as follows:

$$m_{rw} = \frac{mC}{m_o} = m_r C \quad \textbf{(5)}$$

Hence, the increment of m_{rw}, Δm_{rw}, is as follows:

$$\Delta m_{rw} = m_{rw} - 1 \quad \textbf{(6)}$$

The number of the specimens, which was used for evaluating Δm_{rw}, was 35, including 4 specimens just after receiving the mechanical stimuli at Day 2 (Tapping for 6 min at the temperature less than 10˚C), and 2 specimens 5 days after receiving the aforementioned mechanical stimuli.

2.4. Substances Discharged from the Tunic

The absorbance of the artificial seawater used for the tunic specimen was measured by the spectrophotometer (UV-1280; Shimadzu Corporation, Kyoto, Japan). The concentrations of nitrate and dissolved organic matter in the artificial seawater were evaluated by the absorbance at 220 nm [26] and integrated absorbance from 250 - 350 nm [25], respectively. The integrated absorbance, $\Sigma A_{250\text{-}350}$, was equal to the average of absorbance at 250 – 350 nm, $\bar{A}_{250\text{-}350}$, multiplied by the range of the wavelength, 100 nm. Hence, $\Sigma A_{250\text{-}350}$ is described as follows:

$$\Sigma A_{250-350} = 100\bar{A}_{250-350} \quad \textbf{(7)}$$

The absorbance at 250 – 350 nm was measured every 0.5 nm. Every absorbance was measured three times at least and averaged. Also, the artificial seawater without usage was evaluated in the same way. The number of the artificial seawater sample was as follows: without usage, n = 1; for the tunic specimen without the mechanical stimuli, n = 11; just after the mechanical stimuli were given to the tunic specimen at Day 2 (Tapping for 6 min at the temperature less than 10˚C), n = 2; 5 days after the aforementioned mechanical stimuli were given, n = 2. The tunic specimen in the artificial seawater was less than 0.2 g/ml while that was 0.1 – 0.35 g/ml when the mechanical stimuli were given.

2.5. Histological Characteristics of the Tunic

The tissue structure of the tunic was evaluated by observing the stained specimen. The specimen was fixed in 20% formalin, which was diluted by the artificial seawater. After the fixation, the specimen was embedded in paraffin, sliced and stained by the following staining methods: Hematoxylin-Eosin stain, distribution of cells; Elastica-Masson stain, distribution of fibers; Periodic acid/Shiff reaction (PAS reaction), simple stain and Gram stain, distribution of bacteria. All the processes after the fixation were carried out by Surgical Pathology Japan, Inc. (Miyagi, Japan). The stained sample was observed by the light microscope (BX51; Olympus Corporation, Tokyo, Japan).

2.6. Statistical Analysis

The difference in average between two groups and that between the periods in the same group were assessed by two sample t-test and paired t-test, respectively. In addition, the coefficient of determination, R^2, in the regression line was evaluated by F value which the following equation shows:

$$F = n\frac{R^2}{1-R^2} \quad \textbf{(8)}$$

where n is the number of samples. F shows the F-distribution, whose df_1 and df_2, degrees of freedom, is 1, and n-1, respectively. The level of significance was 0.05.

3. RESULTS

3.1. Tunic without the Mechanical Stimuli

Fig. (**2**) shows the increment in the mass, Δm_r, when the tunic was put into the artificial seawater at 5˚C, without the mechanical stimuli. While Fig. (**2A**) (Siphon) and Fig. (**2B**) (M1) show that Δm_r gradually increased as the period was longer, Fig. (**2D**) (Bottom) and Fig. (**2E**) (Inside) show the decrease in the mass. Fig. (**2C**) (M2) and Fig. (**2F**) (Outside) show that Δm_r increased as well as decreased. Fig. (**3**) shows the increment per day, $\Delta m_r/day$, in each group and division of the period, Day 1, Day 2-10 and Day 1-10, by mean ± SD (standard deviation). The period was divided into the three groups because the mechanical stimuli were given to the tunic at Day 1 in the other experiment. The result of the statistical analysis about $\Delta m_r/day$ is mentioned in Tables **1** and **2**. As Fig. (**3A**), Fig. (**3B**) and Table **1** show, $\Delta m_r/day$ was significantly different between all the adjacent groups at Day 1-10: Siphon > M1; M1 > M2; M2 > Bottom; Outside > Inside. The adjacent groups except for the combination, Inside and Outside, were significantly different at Day 2-10 while the two combinations, M1 and M2, and, Inside and Outside, were at Day 1. According to these results, M1 > M2 was maintained through the period although other combinations were not. Fig. (**3**) and Table **2** mention that the absolute values of $\Delta m_r/day$ in M1 and Inside were significantly decreased from Day 1 to Day 2-10. If the increment in the mass is caused by decreasing the stress in the tunic, compression of M1 and stretch of Inside at the initial state (Day 0) would be larger than those on other regions.

Table 1. Influence of the region on the increment in the mass per day ($\Delta m_r/day$).

No stimulus				Mechanical stimuli			
Comparison	Day 1	Day 2-10	Day 1-10	Comparison	Pre	MS	Post
Siphon and M1	N.S.	$p < 0.05$	$p < 0.05$	Siphon and M1	N.S.	N.S.	N.S,
M1 and M2	$p < 0.05$	$p < 0.05$	$p < 0.05$	M1 and M2 & Bottom	$p < 0.05$	N.S.	$p < 0.05$
M2 and Bottom	N.S.	$p < 0.05$	$p < 0.05$				
Inside and Outside	$p < 0.05$	N.S.	$p < 0.05$				

The average of the increment in the mass per day in each group was statistically compared with that in the adjacent region, by two sample t-test. No stimulus, the specimens without the mechanical stimuli; Day 1, Day 2-10, Day 1-10, the divisions of the period when the specimen put into the artificial seawater at 5˚C; Mechanical stimuli, the specimens with the mechanical stimuli; Pre, before giving the mechanical stimuli to the specimen (corresponding to Day 1); MS, at giving the mechanical stimuli; Post, after giving the mechanical stimuli (corresponding to Day 2-10).

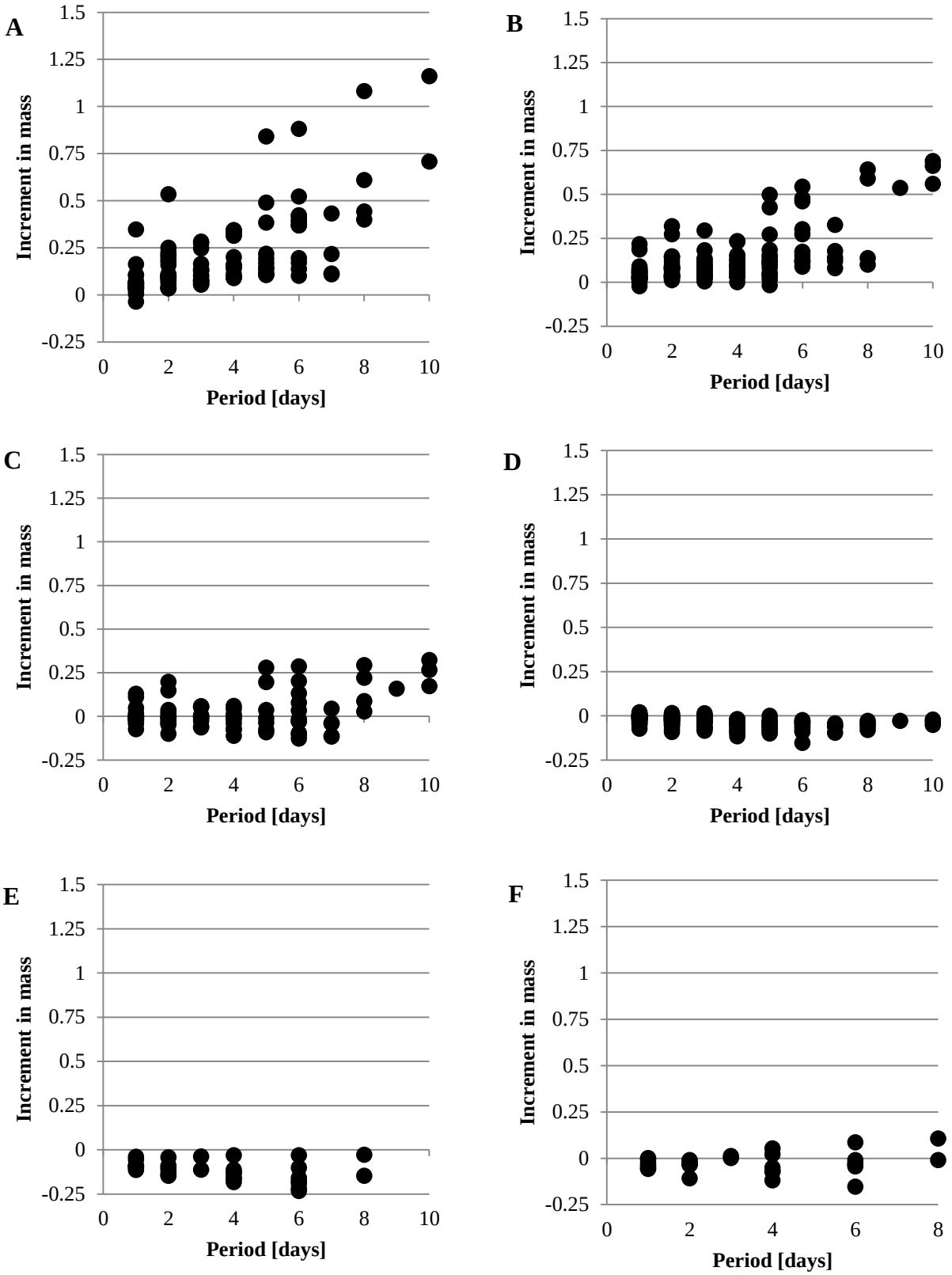

Fig. (2). Increment in the mass of the tunic (Δm_r) for the specimen without the mechanical stimuli. a, Siphon (n = 22); b, M1 (n = 27); c, M2 (n = 15); d, Bottom (n = 25); e, Inside (n = 7); f, Outside (n = 6).

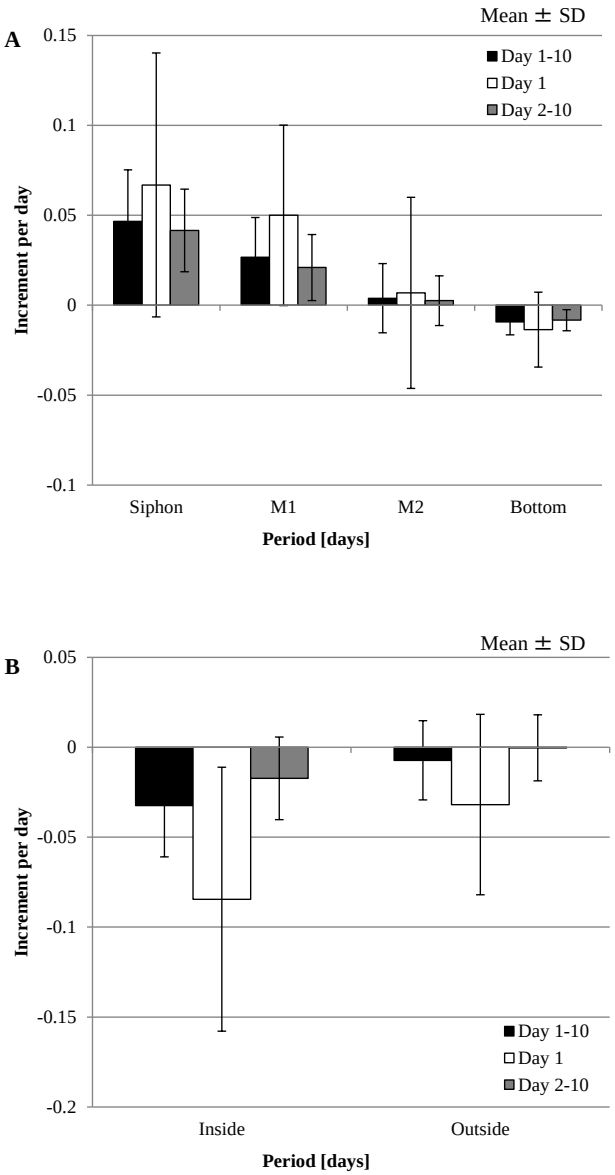

Fig. (3). Increment in the mass of the tunic per day ($\Delta m/day$) for the specimen without the mechanical stimuli. $\Delta m/day$ was evaluated at Day 1, Day 2–10, and Day 1–10 (mean ± standard deviation (SD)). A, Siphon (n = 22), M1 (n = 27), M2 (n = 15) and Bottom (n = 25); B, Inside (n = 7) and Outside (n = 6). The results of the statistical tests were described in Tables **1** and **2**.

Table 2. Influence of the period on the increment in the mass per day ($\Delta m/day$).

No Stimulus		Mechanical Stimuli			
Position	**Comparison**	**Position**	**Comparison**		
	Day 1 and Day 2-10		**Pre and MS**	**MS and Post**	**Pre and Post**
Siphon	N.S.	Siphon	$p < 0.05$	$p < 0.05$	$p < 0.05$
M1	$p < 0.05$	M1	$p < 0.05$	$p < 0.05$	$p < 0.05$
M2	N.S.	M2 & Bottom	$p < 0.05$	$p < 0.05$	N.S.
Bottom	N.S.				
Inside	$p < 0.05$				
Outside	N.S.				

The average of the increment in the mass per day at Day 1 was statistically compared with that at Day 2-10, by paired t-test. No stimulus, the specimens without the mechanical stimuli; Day 1, Day 2-10, the divisions of the period when the specimen put into the artificial seawater at 5˚C; Mechanical stimuli, the specimens with the mechanical stimuli; Pre, before giving the mechanical stimuli to the specimen (corresponding to Day 1); MS, at giving the mechanical stimuli; Post, after giving the mechanical stimuli (corresponding to Day 2-10).

3.2. Tunic with the Mechanical Stimuli

Fig. (**4**) shows the increment in the mass, Δm_r, when the mechanical stimuli were given to the tunic at Day 1. As Fig. (**4A**) (Siphon), Fig. (**4B**) (M1), Fig. (**4C**) (M2) and Fig. (**4D**) (Bottom) indicate, Δm_r was decreased by the mechanical stimuli in each group while Δm_r after the mechanical stimuli was diverse. The increment in the mass per day, $\Delta m_r/day$, of each group is indicated by mean ± SD in Fig. (**5**), where the number of the samples in M2 was small (n = 3) so that the group named as M2 & Bottom, including the samples in both the groups, was organized. In addition, the period was divided into 3 groups: Pre, before giving the mechanical stimuli to the tunic specimen (corresponding to Day 1); MS, at giving the mechanical stimuli; Post, after giving the mechanical stimuli (corresponding to Day 2-10). Tables **1** and **2** show the results of the statistical analysis about $\Delta m_r/day$. According to Fig. (**5**) and Table **1**, $\Delta m_r/day$ in M1 was significantly larger than that in M2 & Bottom at Pre and Post while $\Delta m_r/day$ in Spine and M1 did not show significant difference in all the divisions of the period. At MS, there was no significant difference between all the adjacent groups. As Fig. (**5**) and Table **2** show, all the combinations of the period divisions in Siphon and M1 indicated the significant differences in $\Delta m_r/day$: Pre > MS, Post > MS and Pre > Post. While $\Delta m_r/day$ in M2 & Bottom at MS was also significantly smaller than those of Pre and Post, the difference between $\Delta m_r/day$ in Pre and Post was not significant.

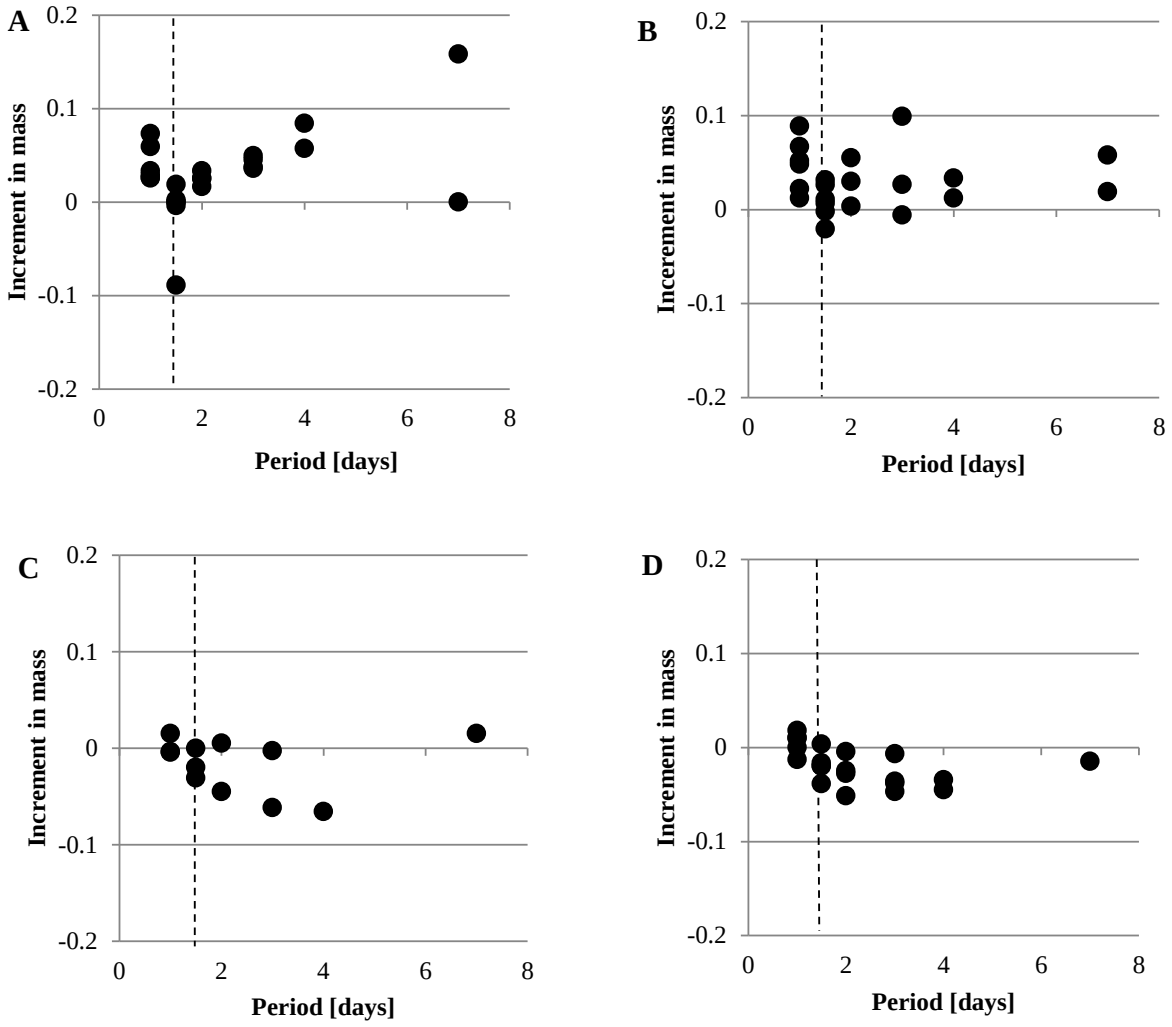

Fig. (4). Increment in the mass of the tunic which the mechanical stimuli were put on (Δm_r). A, Siphon (n = 6); B, M1 (n = 6); C, M2 (n = 3); D, Bottom (n = 5). The dotted lines indicate Δm_r when the mechanical stimuli were applied to the specimen just after the mass was measured at Day 1.

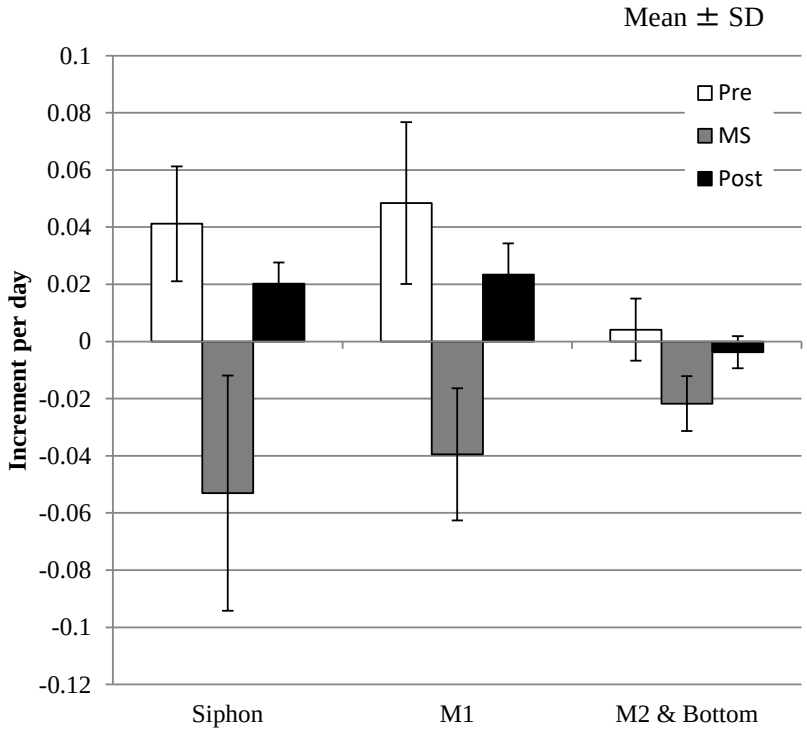

Fig. (5). Increment in the mass per day for the specimen with the mechanical stimuli ($\Delta m_r/day$). $\Delta m_r/day$ was calculated in each group for the following three divisions of the period: Pre, before giving the mechanical stimuli to the specimen; MS, at giving the mechanical stimuli; Post, after giving the mechanical stimuli. The results of the statistical tests were described in Tables **1** and **2**. Siphon, n = 6; M1, n = 6; M2 & Bottom, n = 8.

Because that Pre and Post are corresponding to Day 1 and Day 2-10 in the tunic specimens without the mechanical stimuli, respectively, $\Delta m_r/day$ in these corresponding periods was compared. As Tables **1** and **2** show, the results of the statistical tests related to $\Delta m_r/day$ of Siphons were changed by the mechanical stimuli. As Table **1** shows, $\Delta m_r/day$ of Siphon and M1 were significantly different at Day 2-10, but those at Post were not. Also, $\Delta m_r/day$ of Siphon at Day 1 was not significantly different from that at Day 2-10, but $\Delta m_r/day$ at Pre was significantly larger than that at Post. according to Table **2**. Fig. (**6**) shows the comparison between the corresponding divisions of the period in the same regions. $\Delta m_r/day$ of Siphon at Post was significantly smaller than that at Day 2-10. These results indicate that the influence of the mechanical stimuli on Siphon would be larger than those on other regions.

3.3. Mass and Water in the Tunic

The increment in the mass of water at the specimen, Δm_{rw}, and that in the mass of the specimen, Δm_r, are shown in Fig. (**7**). As the coefficient of determination, R^2, of the regression line in Fig. (**7**) show, Δm_{rw} was significantly proportional to Δm_r. Also, the gradient of the regression line was close to 1 so that Δm_r would approximate to Δm_{rw}. The results indicate that change in mass of the tunic would be caused by influx and efflux of water.

3.4. Substance Discharged from the Tunic

Fig. (**8**) shows the concentrations of nitrate and dissolved organic matter in the artificial seawater, which are corresponding to the absorbance at 220 nm, A_{220}, and the integrated absorbance from 250 nm to 350 nm, $\Sigma A_{250\text{-}350}$, respectively. The coefficient of determination, R^2, of the regression line for all the samples shows that the concentration of dissolved organic matter was significantly proportional to that of nitrate. Change in the concentrations of nitrate and dissolved organic matter was not clear just after the tunic was mechanically stimulated, but became clear 5 days after. This result indicates that the tunic would secrete the same substances whether or not the mechanical stimuli were given to it, but the mechanical stimuli could promote the secretion slowly.

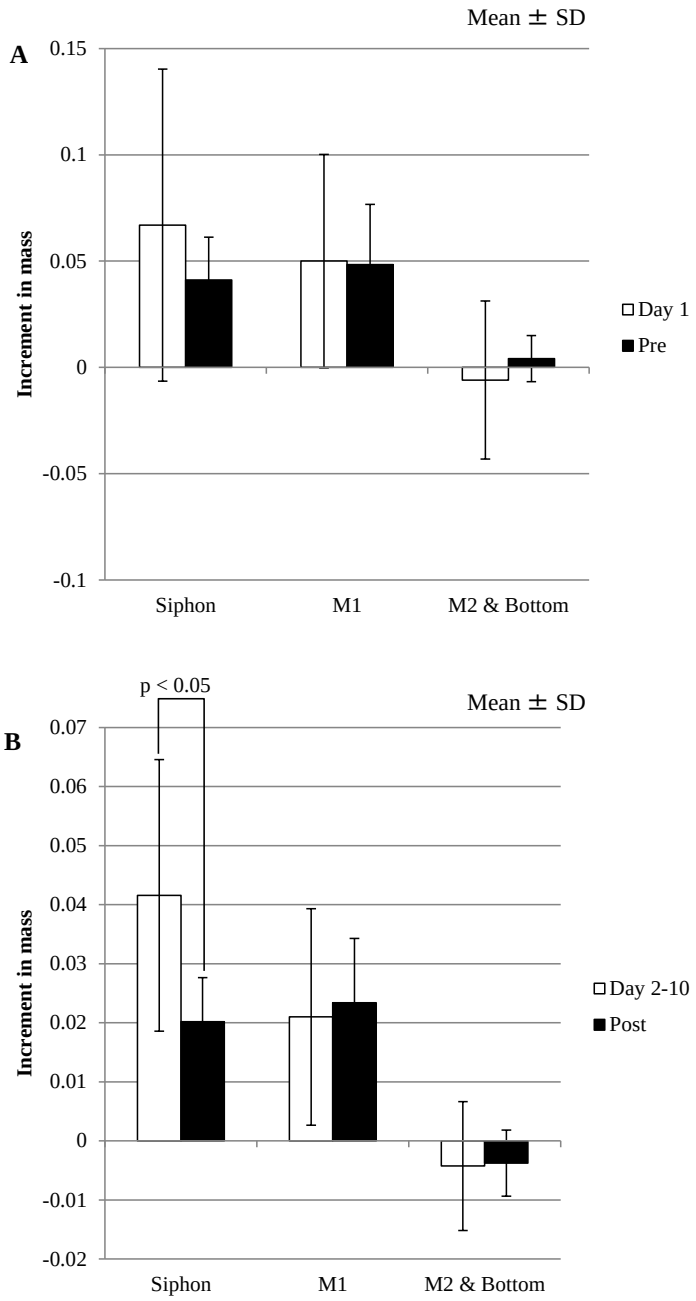

Fig. (6). Increment in the mass per day ($\Delta m_i/day$) before or after the mechanical stimuli were given. While $\Delta m_i/day$ in each group at Day 1 and Pre (A) did not show significant difference, those in Siphon at Day 2-10 and Post (B) were significantly different ($p < 0.05$).

3.5. Histological Characteristics of the Tunic

The distribution of bacteria in the tunic at Day 5 was examined by Hematoxylin-Eosin stain (Fig. (**9A**)), PAS reaction (Fig. (**9B**)), simple stain (Fig. (**9C**)) and Gram stain (Figs. (**9D** and **E**)). Fig. (**9A**) shows that the tunic specimen had cells because cytoplasm (pink) and nuclei (purple) were observed. The distribution of the region positive for PAS reaction in Fig. (**9B**) is similar to that for cytoplasm and nuclei in Fig. (**9A**). Figs. (**9C** and **D**) show that the region positive for simple stain and Gram stain was not observed. However, the positive regions, which would be corresponding to not bacteria, but phagocytes, were found in the area contacting the muscle as shown in Fig. (**9E**). These characteristics were the same as those of the control group (Day 0) so that the propagation of bacteria would be prevented in the tunic specimen.

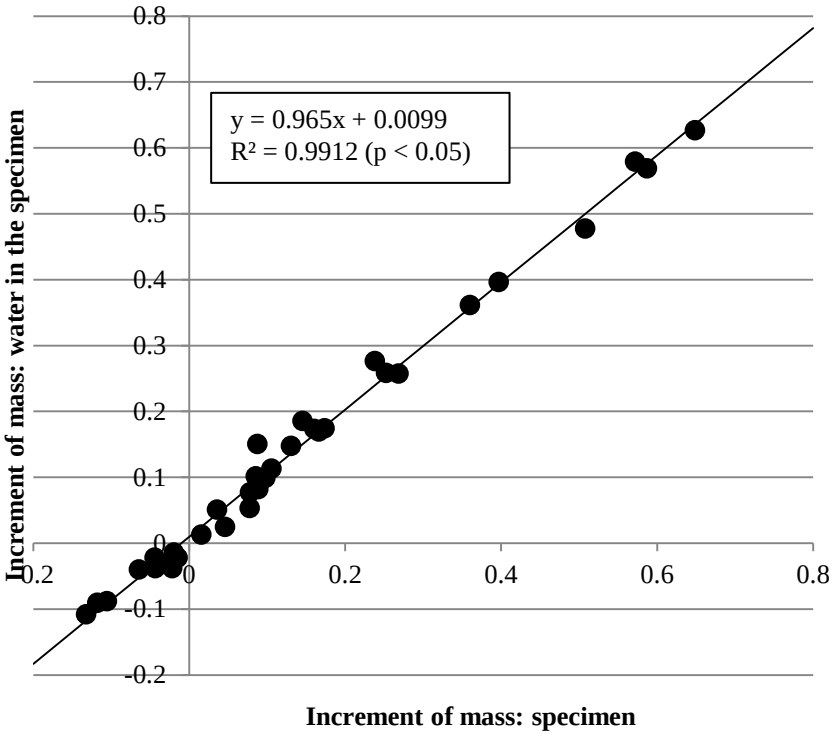

Fig. (7). Increments in the mass of the tunic (Δm_r) and mass of water in the tunic (Δm_{rw}). Δm_r was significantly proportional to Δm_{rw} (n = 35, p < 0.05). R^2, coefficient of determination.

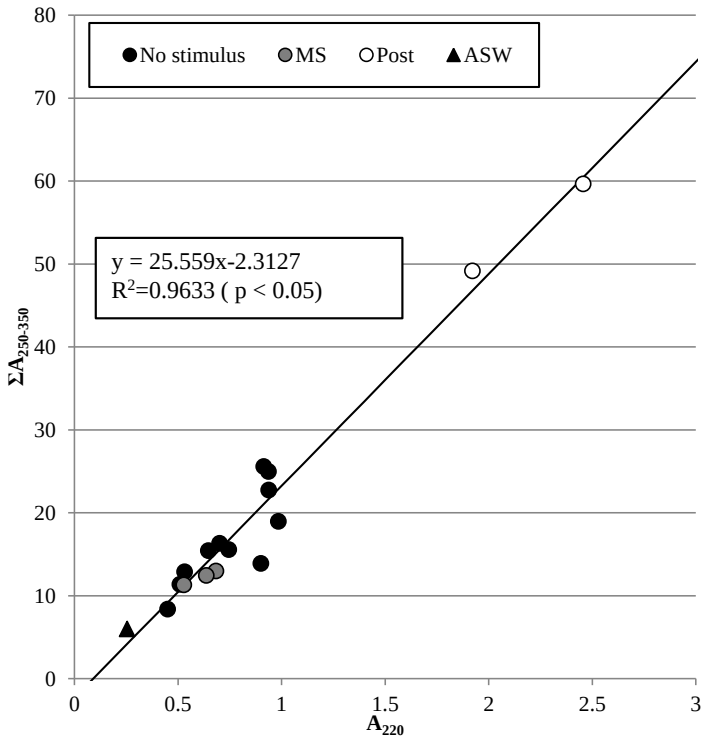

Fig. (8). Nitrate and dissolved organic matter in the artificial seawater used for the tunic specimen. The absorbance at 220 nm (A_{220}) and the integrated absorbance at 250 – 350 nm ($\Sigma A_{250\text{-}350}$) are corresponding to the concentrations of nitrate and dissolved organic matter, respectively. The following samples were evaluated: No stimulus, for the specimen without the mechanical stimuli (n = 11); MS, for the specimen when the mechanical stimuli were given to it (n = 2); Post, for the specimen after the mechanical stimuli were given (n = 2); ASW, not used for the tunic specimen (n = 1). The regression line for all the samples is indicated (p < 0.05).

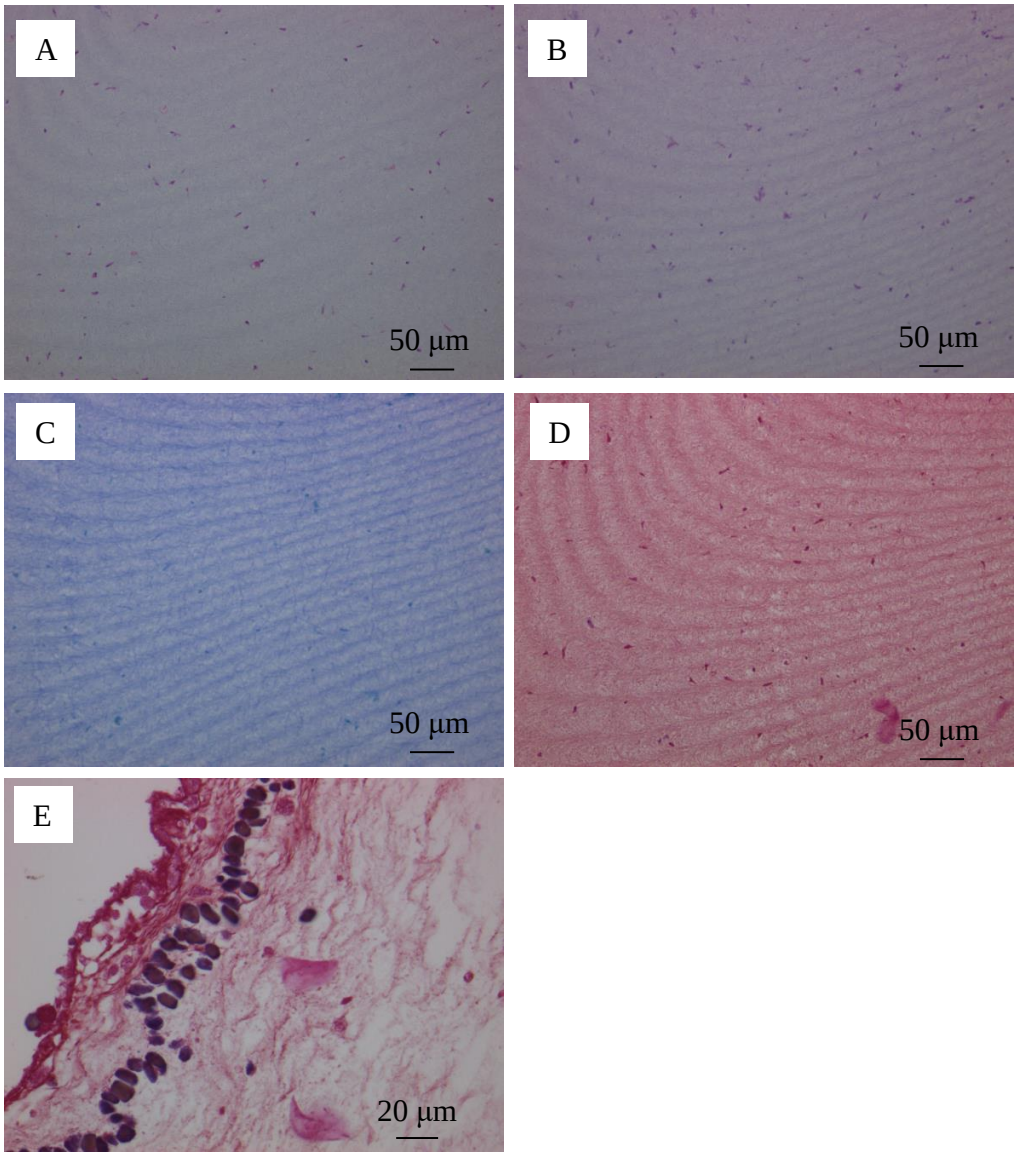

Fig. (9). Bacteria in the tunic of *Halocynthia roretzi* at Day 5. The specimen was stained by Hematoxylin-Eosin stain (A), Periodic acid/Schiff reaction (PAS reaction) (B), simple stain (C) and Gram stain (D and E).

Fig. (**10**) shows the outside of the tunic in each region at Day 0 (control) and Day 2-10 (Siphon, Day 5; other regions, Day 10), made by Hematoxylin-Eosin stain. Siphon was divided into two groups: Inner tunic and Outer tunic because the tunic covers the inner and outer surfaces of the tubular muscle. Uneven surfaces were observed in every region at both the divisions of the period (Day 0 and Day 2-10). The characteristics of the layer positive for Hematoxylin-Eosin stain at the outermost surface were diverse in each group but kept at both Day 0 and Day 2-10. The layer at Inner tunic of Siphon was scarcely observed while that at Outer tunic was distributed at the protrusion. At M1, the thin layer at the protrusion and the cells at the dent were observed. The layer at Bottom was relatively thick and distributed entirely. M2 also had the layer, whose characteristics were between those of M1 and Bottom. Fig. (**11**) shows the characteristic patterns on the inside of the tunic: layers, almost parallel to the outside and winding in some cases, and a blood vessel. The pattern of layers was observed in every region while blood vessels were observed mainly in Bottom, but also in M1 and M2. The distribution of blood vessels agreed with the previous reports [2 - 4]. These characteristics of the outside and inside tunic were also observed in the specimen receiving the mechanical stimuli at Day 2, whose mass was decreased just after the stimuli were given.

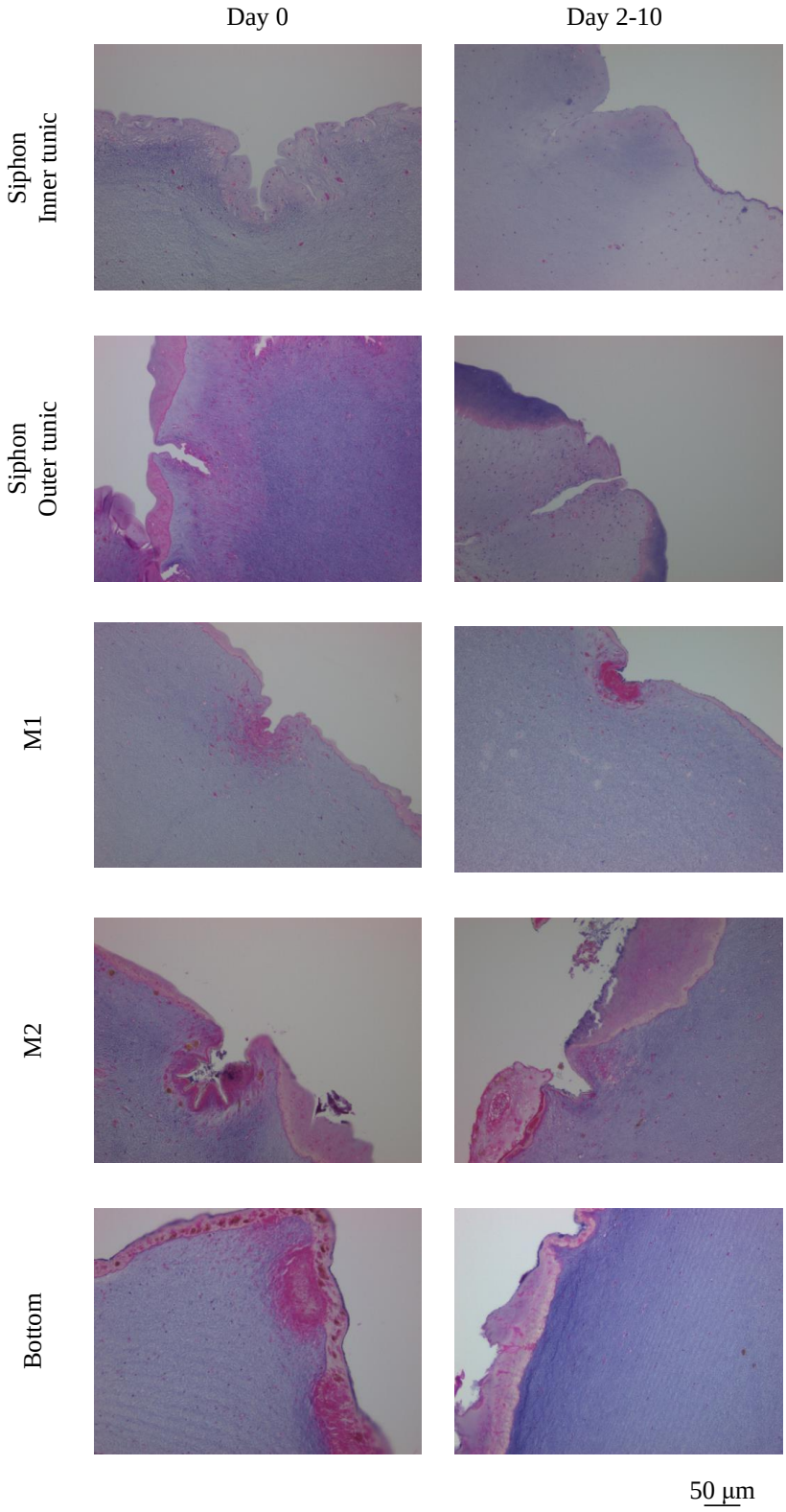

Fig. (10). Histological characteristics of the tunic outside at Day 0 and Day 2-10. All the specimens were made by Hematoxylin-Eosin stain. In Day 2-10, Siphon, including Inner tunic and Outer tunic, was at Day 5, and other regions were at Day 10.

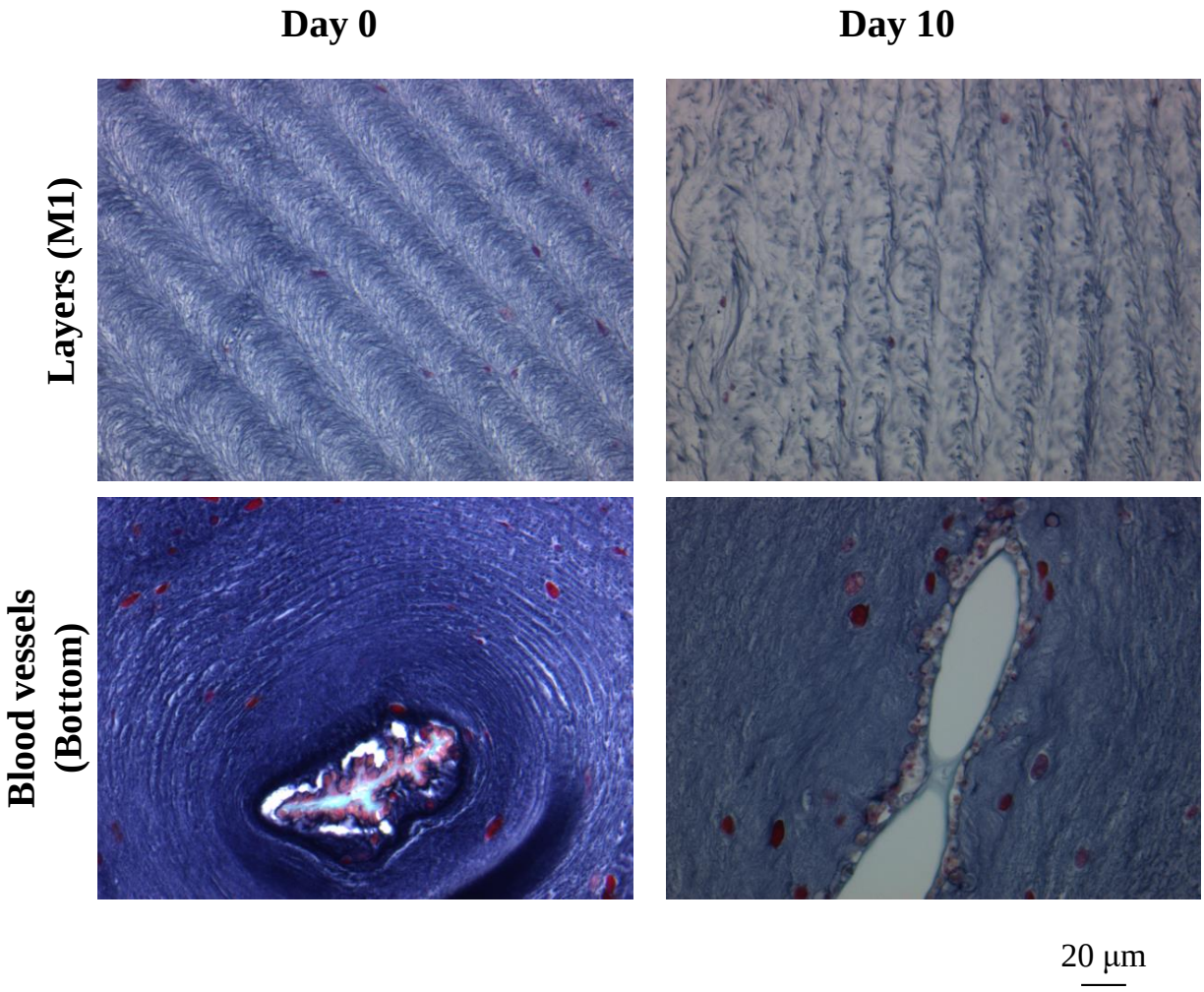

Fig. (11). Histological characteristics of the tunic inside. The characteristic patterns observed in the tunic inside, layers and a blood vessel, are indicated.

According to Fig. **3** and Table **1**, the increment in the mass of the tunic per day, $\Delta m_t/day$, was different in each region at Day 1-10 and Day 2-10: Siphon > M1; M1 > M2; M2 > Bottom. These characteristics would be reasonable if the layer positive for Hematoxylin-Eosin stain is hard so that it would limit the increase in mass of the inside, or if water hardly penetrates the layer so that influx and efflux of water in the tunic would be limited. Assuming that the blood vessels in the tunic could be collapsed by the change of the mechanical environment, it would be reasonable that $\Delta m_t/day$ at Bottom and Inside were negative.

4. DISCUSSION

In this study, how the mechanical environment causes the deformation of the tunic was investigated. In order to reduce the activity of the metallo-protease secreted from the hemocytes, the temperatures of the artificial seawater for the specimen in the refrigerator, and at giving the mechanical stimuli to the specimen were 5˚C and less than 10˚C, respectively. The following parameters, which would be associated with the deformation, were evaluated: change in the mass and water content of the tunic, and substances discharged from the tunic, and the histological characteristics of the tunic.

At the tunic specimen without the mechanical stimuli, the increment in the mass of the tunic per day, $\Delta m_t/day$, was larger as the region became closer to Siphon: Siphon > M1 > M2 > Bottom. In addition, $\Delta m_t/day$ at Inside was significantly lower than that at Outer. However, the difference in $\Delta m_t/day$ between the adjacent regions was not significant when the mechanical stimuli were given to the tunic specimen. Also, the mechanical stimuli reduced

$\Delta m_i/day$ at Siphon after the mechanical stimuli were given, but did not reduce those in other regions. The increment in the mass of the tunic was corresponding to that in the mass of water in the tunic whether or not the tunic was mechanically stimulated. As influx and efflux of water were corresponding to increment in the mass of the tunic, the distribution of water in the tunic would be helpful in investigating the movement of water. The method to evaluate the distribution will be developed in the future. The substances discharged from the tunic stimulated mechanically would be also the same as those without the stimuli because the concentration ratio of dissolved organic matter to nitrate was kept regardless of the mechanical stimuli, but the discharge was slowly increased after giving the stimuli to the tunic. This result indicates that the mechanical stimuli could enhance part of functions in the tunic related to the discharged substances slowly. In addition, according to the temperature in this study, the metallo-protease secreted by the hemocyte was less active than that in the previous studies [18 - 20], but the mechanical stimuli caused a decrease in the mass of the tunic. Substances except for the protease in the tunic would involve the response to the stimuli. The substances, discharged from the tunic regardless of the mechanical environment, will be analyzed in the future. In histological characteristics, the layer positive for Hematoxylin-Eosin stain at the outside was becoming thicker and spreading more widely as the region was closer to Bottom. While the layer pattern in the inside was observed at every region, the blood vessels were mainly observed at the inside of Bottom. Assuming that the layer positive for Hematoxylin-Eosin stain at the outside would hardly deform and indicate higher resistance for the penetration of water than the inside, it would be reasonable that $\Delta m_i/day$ became smaller as the position was closer to Bottom. In addition, the collapse of blood vessels could decrease $\Delta m_i/day$ at both Bottom and Inside. Mechanical characteristics of the layer and the blood vessels should be evaluated by an indentation test in the future. Cells in Inner tunic of Siphon would receive the mechanical stimuli more directly than those in other regions because the layer, positive for Hematoxylin-Eosin stain, scarcely covered the outside. That would make $\Delta m_i/day$ of Siphon at Post lower. The response of the cell to the mechanical environment will be investigated in the future. If $\Delta m_i/day$ at giving the mechanical stimuli is hardly influenced by the difference in the histological characteristics, the amount of substances related to contraction would play an important role in the deformation. Type of the substance related to contraction will be examined in the future study.

CONCLUSION

In this study, how the tunic of *Halocynthia roretzi* responds to the mechanical environment and deforms itself as result was investigated. In the specimen without the mechanical stimuli, the increment in the mass per day was higher as the region was closer to Siphon, whose outside was scarcely covered with the layer positive for Hematoxylin-Eosin stain. When the tunic received the mechanical stimuli, Siphon was mostly influenced in all the regions. However, the increment in the mass per day at giving the stimuli was not different from that in the adjacent region. Moreover, the increment in the mass was corresponding to influx and efflux of water in the tunic. Substances discharged from the tunic were slowly enhanced by the mechanical stimuli.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

Halocynthia roretzi, a solitary ascidian, is an invertebrate species, which legal controls are not imposed on in Japan.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

Declared none.

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