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Growth of the longline-cultured sea squirt *Halocynthia roretzi* in a temperate bay of Korea: Biochemical composition and physiological energetics

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ABSTRACT

The broad temperature range on the temperate coast of Korea has led fishermen to develop a unique and specialized procedure for the longline culture of ascidians. In Korea, warming of the coastal sea in winter has accelerated over the past few decades. After warmer winters, the rising temperatures of earlier springs have precluded the rearing of the ascidian spat in the culturing area and have imposed spring spat cultivation in colder-water nursery grounds. To examine the seasonal dynamics of energy reserves and the physiological strategies to optimize energy balance for the growth in the cultured sea squirt *Halocynthia roretzi*, its gross biochemical composition and physiological energetics were investigated monthly over two culturing periods from July 2005 to January 2006 and July 2013 to January 2014 in Geoje-Hansan Bay on the south coast of Korea. No indicators of the growth performance of sea squirts showed differences between the two culture practices (2005–2006 and 2013–2014) carried out using spat reared in different localities, i.e., the grow-out area and the colder nursery grounds. The seasonal patterns in accumulation and utilization of biochemical constituents in the sea squirt tissues were similar between the two periods. During the culturing period in the grow-out area, sea squirts retained physiological functions across the temperature range. Food energy acquisition and metabolic cost were positively related to water temperatures in the field conditions, probably due to the low and narrow range of suspended particulate matter (SPM) concentrations. A less-clear seasonal variability in food consumption rates yielded a seasonal discrepancy in scope for growth (SFG; i.e., negative during summer vs positive during autumn–winter). Consequently, the tissue weight and protein reserves of sea squirts varied concomitantly with the seasonal changes in SFG, supporting a fast growth during autumn–winter. Our results suggest that the spat reared in colder nursery grounds are suitable for cultivating sea squirts in the traditional grow-out area and support their sustainable culturing performance as an adaptation strategy to the winter–spring warming conditions that are unique to coastal seas.

1. Introduction

The sea pineapple *Halocynthia roretzi* (Drasche, 1884) is a hermaphroditic solitary ascidian occurring on shallow subtidal rocky bottom on the south and east coast of Korea and the northern Japan (Ikenoue and Kafuku, 1992). *H. roretzi* has become a popular aquaculture species in Korea and Japan because of their unique taste and high nutritional value as well as the bioactive compounds in the tissues (Inanami et al., 2001; Lambert et al., 2016). The aquaculture of *H. roretzi* in Korea started with an annual production of 39 tonnes in 1982, with production peaking at 42,800 tonnes in 1994 and 31,353 tonnes (which corresponds to approximately 60 million USD) recorded in 2016 (Statistics Korea, http://kostat.go.kr/). The longline aquaculture of sea squirts now guarantees a much higher commercial revenue (about 10 times more per line) than that of oysters (*Crassostrea gigas*) and mussels (*Mytilus edulis*). *H. roretzi* is native to northeast Asia, with an optimal growth temperature of 8–13 °C (Lambert et al., 2016). The southern coastal sea of Korea exhibits an annual temperature range of 5–25 °C (Kang, 2000; Sung et al., 2010). This broad range of temperatures has led the sea squirt aquaculture industry to develop a unique and

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specialized procedure (https://www.nifs.go.kr/). Seeds for the culture of sea squirts are collected after larval attachment to palm ropes in spawner-containing water tanks during December to January. Bundles of palm ropes to which spat at the early growing stage have adhered are transplanted to shallow coastal areas and deployed for more than 4 months (February–May/June), to allow them to adapt to the sea conditions and develop fitness. Subsequently, a coil of palm rope is bound on hanging ropes and suspended by longlines. These longline facilities are deployed in grow-out areas in small bays in early summer (June–-July) and the adults are harvested in the following winter–spring period as they reach 8–11 cm in body length (7–9 months after deployment).

Increase in the sea surface temperature (SST) has been projected to continue in the coastal waters of Korea. Over the past few decades, winter temperatures have elevated much faster than those of summer (Kang, 2000; Sung et al., 2010). One strategy that has been adopted in the aquaculture of tunicates is the development of alternative solutions to deal with the warming of the coastal seas. Warmer winters have caused an earlier rise in the spring sea temperatures. Since newly settled spat (length, < 3 mm) have a low resistance to warm temperatures (Jang, 1979), the spring warmth led the tunicate aquaculture industry to seek for alternative sites where physiological disturbances and mass mortality can be reduced or avoided. To overcome this vulnerability, the industry has selected the northeastern coast of Korea as a nursery ground for rearing spat before they are transplanted to the grow-out areas. Water temperature on the northeastern coast of Korea is comparatively lower, due to the North Korean Cold Current, which flows southward along the eastern coast of the Korean peninsula from the coast of Vladivostok (Lee et al., 2015). In this context, understanding the physiological adjustments of the tunicate culture under varying environmental conditions is crucial for the sustainable aquaculture practice in a warming environment.

The somatic growth, mortality, and reproduction of marine invertebrates are the result of time-integrated physiological responses in food intake and energy loss via feces production, ammonia excretion, and metabolism which are governed by variations in environmental conditions (Bernard, 1974; Deslous-Paoli and Héral, 1984; Bayne, 1998). In fact, the physiological processes of individual organisms are closely related to environmental conditions such as temperature and food availability, and the resultant energy budget is reflected by their scope for growth (SFG; Bayne and Newell, 1983). Such physiological activities determine the seasonal variations in the energy storage and utilization, as it can be inferred from changes in the tissue biochemical composition (Bayne, 1976; Newell and Bayne, 1980; Hawkins et al., 1985; Lee et al., 2017). Great efforts have been made to examine the physiological processes in relation to energy acquisition and expenditure, and the storage and allocation in marine bivalve mollusks (Bayne and Newell, 1983; Gosling, 2015), but relatively few studies have similarly examined physiological responses of tunicates to their environment.

While several studies have reported ecophysiology of *H. roretzi* (Numakunai and Hoshino, 1980; Ohkuma et al., 2000; Sekine et al., 2001; Kang et al., 2009; Lee et al., 2012; Donaghy et al., 2017), a limited number of studies the physiological responses to changes in salinity and temperature (Shin et al., 2007, 2011; Jeong and Cho, 2013). According to Shin et al. (2011), *H. roretzi* SFG at higher temperatures (20–25 °C) during spring and summer is negative, reflecting the increased metabolic costs and decreased feeding rates. Kang et al. (2015) also reported negative energy balance of *Styela clava,* another species of tunicate commonly cultured in a small bay on the south coast of Korea, during warmer seasons. In contrast, the SFG of *H. roretzi* and *S. clava* measured during fall and early winter when the SST ranged 8–15 °C, was reported to be positive, possibly due to the reduced metabolic rate and the increased feeding activity (Shin et al., 2011; Kang et al., 2015).

We examined physiological responses of *H. roretzi* in subtidal longline suspended culture to seasonal fluctuations in water temperature in a small temperate bay on the south coast of Korea. In this study, we aimed to test whether the spat developed in colder nursery grounds during a warmer spring, as an innovative culturing strategy, can support a sustainable aquaculture of the sea squirts in this warming water condition. Accordingly, we examined the seasonal variations in the reserve storage and expenditure in spats of *H. roretzi* raised in different nursery areas by measuring the gross weight of tissue biochemical constituents including proteins, carbohydrates, and lipids. We also determined rates of food consumption, feces production, ammonia excretion, and oxygen consumption of individuals to examine their integrated adaptive responses in the energy balance to the seasonal SST fluctuations. To our knowledge, this study is the first attempt to highlight the seasonal dynamics of the energy reserves in association with physiological strategies of *H. roretzi* in aquaculture to optimize the energy balance for the growth.

2. Material and methods

2.1. Site description and samplings

Geoje-Hansan Bay on the southern coast of Korea is a semi-closed bay with an area of about 55 km^2 (about 10 km long and 2–6 km wide) and 13.4% of the bay surface is being used for aquaculture (NIFS, 2014). The monthly mean sea surface temperatures vary from 9.0 to 25.6 °C, displaying a distinct seasonality, while the mean salinities stay within a narrow range of 29.5–34.2 PSU (Kim et al., 2019). The sea squirt spats to be used in aquaculture are harvested in winter from December to January, as SST stays around 10 °C. For the grow-out the sea squirt aquaculture industry uses 100–130 m long long-lines. The sea squirt string suspended on the long-line is a palm rope which includes approximately 100 sea squirt spats per 1 m. To avoid fouling organisms and cooler water temperature, the sea squirt strings are located at depth from 6 to 12 m below the surface. These hanging ropes are deployed mainly in June and the spats continue to grow during summer and winter.

Samplings were performed over two entire culturing periods, from April 2005 to January 2006 in Geoje-Hansan Bay (see Fig. 1). Since the sea squirt spats are transplanted to the colder nursery grounds located on the northeast coast of Korea during an early culture stage, we also sampled the sea squirt spats from the northeast nursery ground from May to June 2013. *H. roretzi* in the grow-out in Geoje-Hansan Bay from July 2013 to February 2014 were also sampled monthly, after the deployment of longline facilities.

A high prevalence of the soft tunic syndrome in the ascidian aquaculture has brought the disease symptoms (Nam et al., 2015). During our experimental period, the soft tunic syndrome and thereby the diseased individuals were undetectable probably due to younger individuals than 1.5 yr old (Hirose et al., 2014).

2.2. Environmental conditions

On each sampling occasion, water temperature and salinity were recorded using a conductivity, temperature, and depth meter (Sea-Bird Electronics Inc., Bellevue, WA). To determine food availability in the water column, 20 L of sea water samples were collected from 1 m below the water surface using a van Dorn water sampler. Water samples were pre-filtered through a 180 μm Nitex mesh to remove any large particles and zooplanktons on board. For measurements of total SPM, chlorophyll *a* (Chl*a*), and the biochemical composition of SPM, 1 l of seawater for each measurement was filtered using pre-weighed Whatman GF/F filters (47 mm, pore size = 0.7 μm). The filters containing the particles were kept in a cooler with dry ice and transported to the laboratory. Filters for SPM measurements were dried at 60 °C for 72 h and the mass was estimated from the weight difference of the filter before and after loading the samples. Chl*a* was extracted with 90% acetone for 24 h in the dark at –20 °C and its concentration was analyzed using a

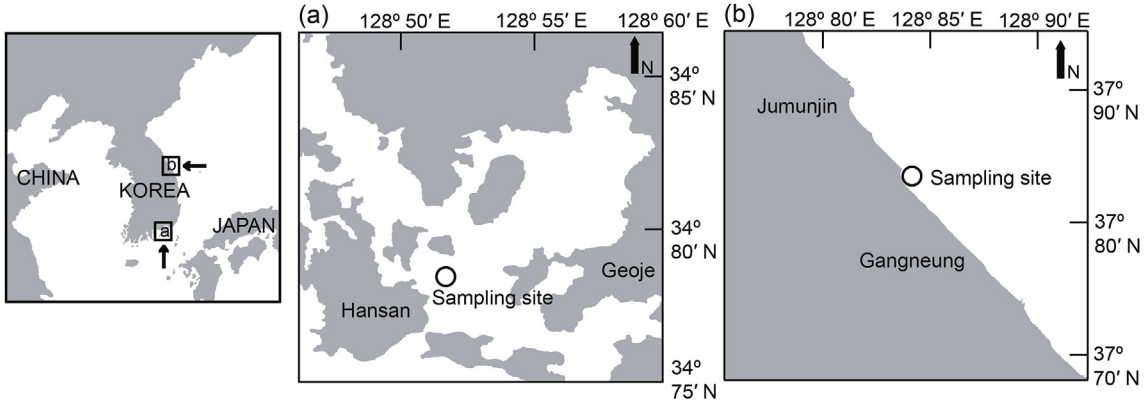

Fig. 1. Map showing the sampling sites: (a) the grow-out area for culturing sea squirts in Geoje-Hansan Bay on the southern coast of Korea and (b) the nursery ground for spat cultivation in the Gangnueng coast in the northeastern coast of Korea.

fluorometer (Turner Designs, Sunnyvale, CA) according to the procedure of Holm-Hassen et al. (1965). The levels of proteins, carbohydrates, and lipids of SPM were analyzed according to the same procedures used in the analysis of tissue biochemical composition as described below (Section 2.5). The food energy values in the water column were finally calculated by summing up the energy equivalents of the carbohydrate, protein and the lipids.

2.3. Biometric measurements

Twenty individuals of 0-yr old sea squirts were randomly collected monthly from the deployed longlines at each sampling site, kept in an icebox and transported to the laboratory within 12 h. The live tunicates were kept in filtered seawater overnight to depurate the gut contents. After removing the epifauna on the tunic, individual tunicates were dissected to separate the flesh tissue from the tunic. Flesh tissues were lyophilized at –80 °C and weighed to mg to determine the dry tissue weight (DW). The tunics were dried at room temperature for 3 days and weighed to determine the dry tunic weight. Whole dry weight (TW) was determined as the sum of dry tunic weight and DW. The condition index (CI) of sea squirt was calculated using the following equation, $CI = (DW/TW) \times 100$ (Petersen et al., 1997). The lyophilized tunicate fleshes were pulverized using a ball mill (Retsch MM200 Mixer Mill, Hyland Scientific, WA) and kept frozen at –70 °C until used.

2.4. Experimental design of physiological measurements

Measurements of physiological rates of *H. roretzi* were carried out monthly *in situ* on a 10 × 10 m barge floated on the culture ground, from July 2013 to February 2014. For the analysis, 30 specimens were selected randomly from the suspended rope and cleaned to remove the epi-bionts attached on the tunic. To determine the food consumption, feces production, and ammonium excretion rates, 9 specimens were placed individually in 1 L of flow-through open cylindrical chambers. Nine individuals were also placed in one linter flow-through closed chambers, to measure the oxygen consumption rate. As a control, one additional chamber was established without a sea squirt. Each series of chambers were partially or wholly immersed in large aquaria ($50 \times 150 \times 50\ cm^3$), which were filled with *in situ* seawater, to maintain the same thermal condition in the culture ground. Seawater supplied in each series of experiment was pumped from 1 m below the sea surface and distributed to individual experimental chambers using 10-channel peristaltic pumps (BT 100-1L, Longer Precision Pump Co., Baoding, China). The flow rates of the pumps were set to 50–100 ml min^{-1} depending on the size of individuals. These conditions ensured that the number of particles in the outflow of the feeding chamber and the concentrations of dissolved oxygen (DO) in the experimental chambers could be maintained at > 80% of *in situ* condition (Bougrier et al., 1995; Filgueira et al., 2006). Before measuring the physiological rates, all the specimens were acclimated to the experimental chamber conditions for 6 h. Subsequently, all the measurements were carried out for 24 h.

2.5. Gross biochemical composition

Five to 10 mg of the pulverized tissue samples used in each biochemical measurement. Total proteins were extracted with 1 N NaOH at 60 °C for 30 min and determined using the Folin–Ciocalteu phenol method with bovine serum albumin as the standard (Lowry et al., 1951). Total carbohydrates and glycogen were extracted overnight with 15% trichloroacetic acid at 4 °C, and glycogen was precipitated by centrifugation ($2000 \times g$) with 99% ethanol for 10 min. Carbohydrates and glycogen were measured using the phenol–sulfuric acid method with glucose as the standard (Dubois et al., 1956). Lipids were extracted using a mixture of chloroform and methanol (1:2) according to Blight and Dyer (1959), and the total lipids were determined using a spectrophotometer with glyceryl tripalmitate as the standard (Marsh and Weinstein, 1966). The energy equivalents of proteins, carbohydrates, and lipids were estimated using conversion factors of 24.0, 17.5, and 39.5 J mg^{-1}, respectively (Gnaiger, 1983).

2.6. Physiological energetics

Rates of food consumption, feces production, ammonium excretion, and respiration of individual sea squirt were measured for the energy balance equation. Food consumption rate (C) was determined by the weight difference of SPM between the outflows from the control chamber (i.e., without sea squirt) and the individual experimental chambers containing the sea squirt. On each experimental occasion, water samples were collected every 4 h from the outflows of the feeding chambers over the 24 h experimental period. Water was placed on. After lyophilizing the filters (pre-weighed Whatman GF/F filters 47 nm) containing the SPM at –80 °C, levels of proteins, carbohydrates, and lipids in the SPM were measured using the procedures as previously described. Subsequently, the rates were converted into energy equivalents (J d^{-1}) via the summation of the energy values of the biochemical constituents.

During the 24 h experimental period, the fecal materials were collected every 2 h directly from the feeding chambers using a Pasteur pipette, and kept in a 5 ml pre-combusted and weighed glass tube. The tubes were rinsed with distilled water to remove salt contents, lyophilized, and weighed to calculate the total mass of feces. Total protein, carbohydrate and lipids in the fecal materials were also determined using the methods previously described. Finally, the feces production rate (F) was converted into an energy equivalent (J d^{-1}) to estimate the energy loss by excretion.

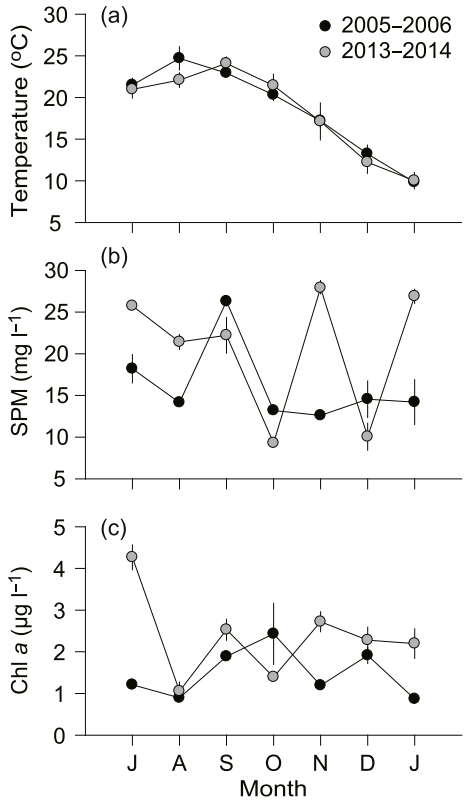

Fig. 2. Seasonal variations in (a) water temperature, (b) suspended particulate matter (SPM), and (c) chlorophyll *a* (Chl*a*) in the grow-out area of Geoje-Hansan Bay during the two culturing periods.

The ammonia excretion rate (U) was calculated based on the increase in ammonia concentration in the outflows from the experimental chambers compared to the control chamber. To determine the ammonia excretion rate, the outflowing waters were collected every 4 h over the 24 h experiment using a spectrophotometer according to Parsons et al. (1984). The excretion rates were finally converted into an energy equivalent (J d^{-1}) using a conversion factor of 24.83 J mg^{-1} NH_4-N (Elliot and Davison, 1975).

The respiration rate (R) was determined by measuring the DO level in the control and the experimental chambers. Oximetric probes (Oxy-10 micro, PreSens-Precision Sensing GmbH, Regensburg, Germany) were installed inside the closed flow-through experimental chambers and DO concentration was recorded every 30 s over the 24 h experimental period. The rates were converted into an energy equivalent (J d^{-1}) using a conversion factor of 14.0 J mg^{-1} O_2 (Gnaiger, 1983).

The monthly SFG of individual sea squirt was determined using the energy balance equation (Bayne and Newell, 1983), as $C = SFG + F + U + R$; $SFG = C–(F + U + R) = A–R$, where C = consumption; F = feces production; U = ammonia excretion; A = assimilation; and R = respiration. During the experiments none of the sea squirt used in the experiment exhibited pausing in their physiological activities.

2.7. Normalization of the biochemical and physiological measurements using standard animal

All the measured variables of the biochemical and physiological components were normalized to standard whole dry weight (TW) (2.586 g, the grand mean of all the specimens analyzed) and standard dry tissue weight (DW) (1 g) respectively, to eliminate the confounding effects of variation in different body size (Packard and Boardman, 1987). For this purpose, the TWs and DWs of sea squirts on each sampling occasion were fitted to the allometric equation $DW = aTW^b$, where a and b represent the fitted constants. Subsequently, least-squares regression analyses between DW and TW following logarithmic transformation (base 10) were performed to determine the fitted constants (i.e., the intercepts and slopes of the regression equation). Similarly, gross weights of the biochemical constituents (or physiological rates) were fitted against DW according to the allometric equation, $Y = aDW^b$, where Y represents the gross weights of the biochemical constituents (or physiological rates) of interest and a and b represent the fitted constants. Differences in the slopes between the regression equations were tested by analyses of covariance (ANCOVAs, Sokal and Rholf, 1995). Since the ANCOVA tests revealed no significant differences among the estimates of the slopes in the regressions of DW against TW, the common slopes ($\bar{b}$) and the adjusted intercepts ($\bar{a}$) were used to compute the monthly DWs of a standard animal by substituting the standard TW in the regression equations. The common slope and the adjusted intercepts in the regressions of biochemical constituents against DW were retained to calculate the gross weights of proteins, carbohydrates (glycogen), and lipids of a standard animal by substituting its computed monthly DWs. The monthly physiological rates were also standardized to 1 g DW using the procedure described above. Subsequently, a Bonferroni *post hoc* multiple-comparison test was performed to test differences between intercepts.

2.8. Statistics

First, the normality and homogeneity of variance of all the data were tested using the Shapiro–Wilk and Levene's tests, respectively. Differences in the environmental conditions and the biometric and biochemical measurements between the two cultivation periods were tested using a non-parametric Wilcoxon signed-ranks test (Sokal and Rholf, 1995). Pearson's product-moment correlation was carried out to determine the relationship between the abiotic and biotic components and between the biometric and the physiological variables. A multiple step-wise regression analysis was performed to identify major environmental factors that influenced the physiological parameters. The Wilcoxson test and Pearson's correlation analysis were carried out using SPSS (IBM SPSS Statistics, v. 22.0, IBM Corp., Armonk, NY) and the multiple step-wise regression analysis was performed using STATISTICA (STATISTICA 12, StatSoft Inc., Tulsa, OK).

3. Results

3.1. Environmental conditions

Water temperatures displayed a clear seasonal fluctuation, ranging from 9.8 to 24.7 °C, with a maximum in August and a minimum in February (Fig. 2a). Water temperatures were noticeably higher (18.7–19.8 °C) at the culturing site of Geoje-Hansan Bay in the spring (May–June) of 2013 than those (15.0–18.9 °C) in 2005 and at the spat-cultivation site on the Gangnueng coast (13.9–17.1 °C) in 2013. Salinity fell within a narrow range, between 32.5 PSU (September 2005) and 34.0 PSU (May 2005), with no apparent seasonal patterns in the two culturing periods (data not shown). Monthly SPM concentrations showed irregular seasonal variations, ranging from 12.6 to 26.3 mg l^{-1} during the culturing period of 2005–2006 and from 9.3 to 27.9 mg l^{-1} during the 2013–2014 period (Fig. 2b). No marked seasonal patterns in Chl*a* concentration were observed; it fluctuated from 0.87 to 2.43 μg l^{-1} during the culturing period of 2005–2006 and from 1.06 to 4.27 μg l^{-1} during the 2013–2014 period (Fig. 2c). No significant differences in SPM and Chl*a* concentration were observed between the 2013–2014 and 2005–2006 periods (Wilcoxon test, $P = 0.383$ and $P = 0.073$, respectively).

3.2. Condition index and dry tissue weight of a standard animal

A rapid increase in the TW of spat was detected at both nursery

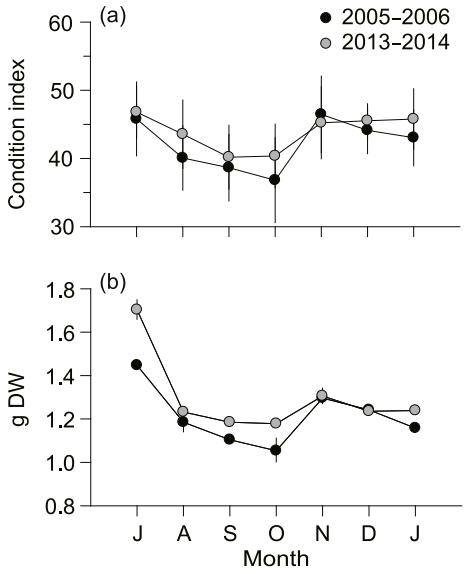

Fig. 3. Seasonal variations in (a) the condition index and (b) the dry tissue weight (DW) of a standard animal with a total dry weight (TW) of 2.586 g. The vertical bars represent (a) standard deviations and (b) 95% confidence intervals.

grounds (Geoje-Hansan Bay and the Gangnueng coast), which were exposed to similar environmental conditions during the cultivation (April to June) (Supplementary Table S1). After the deployment of longline facilities in the grow-out area in July, specimens with a broad size range (mean TW, 2.586 g over the entire sampling period) were collected for biochemical and physiological measurements on each sampling occasion (Supplementary Table S2).

The CI of sea squirts exhibited similar seasonal variations during the two cultivation periods (Wilcoxon test, $P = 0.306$), ranging from 36.8 to 45.8 in 2005–2006 and from 40.2 to 46.8 in 2013–2014 (Fig. 3a). The CI peaked in July, decreased slightly in summer, and was high during the late autumn–winter period of both cultivation periods.

Regressions of DW against TW in the sea squirt were highly significant (Table 1). ANCOVA revealed no significant differences in the estimate of the slope (b), with a common slope of 1.178 (± 0.017). The

Table 1
Parameters a (intercept) and b (slope) for allometric equation $DW = aTW^b$ between dry tissue weight (DW, g) and total (tissue+tunic) weight (TW, g) for *Halocynthia roretzi* during the cultivation after deployment in the grow-out area. Results of analysis of covariance (ANCOVA) to test significance of differences in slope are summarized at bottom of table. $\bar{a}$, recalculated using a common slope ($\bar{b}$) obtained from ANCOVA. CI, confidence interval.

Month	n	a	b	r	$\bar{b}$ ± 95% CI	$\bar{a}$
July 2005	20	0.429	1.181	0.882	1.178 ± 0.017	0.430
August	20	0.350	1.191	0.982		0.352
September	25	0.302	1.273	0.978		0.328
October	24	0.284	1.295	0.928		0.313
November	25	0.441	1.044	0.902		0.385
December	25	0.422	1.042	0.919		0.369
January 2006	25	0.370	1.119	0.898		0.344
July 2013	30	0.514	1.155	0.996		0.506
August	30	0.367	1.175	0.967		0.366
September	30	0.353	1.176	0.964		0.352
October	30	0.355	1.164	0.962		0.350
November	30	0.395	1.159	0.983		0.388
December	30	0.421	1.065	0.975		0.367
January 2014	30	0.360	1.197	0.979		0.368
		Fs	df	Significance		
ANCOVA		0.797	13, 373	$P = 0.664$		

Table 2
Parameters a (intercept) and b (slope) for allometric equation $Y = aDW^b$ between gross weights of biochemical components (Y, mg) and dry tissue weight (DW, g) for *Halocynthia roretzi* during the cultivation after deployment in the grow-out area. Y represents gross weights of proteins, lipids, carbohydrates, and glycogen. Results of analysis of covariance (ANCOVA) to test significance of differences in slope are summarized at the bottom. $\bar{a}$, recalculated using common slopes ($\bar{b}$) obtained from ANCOVA. CI, confidence interval.

Month	Proteins					
	n	a	b	r	$\bar{b}$ ± 95% CI	$\bar{a}$
July 2005	20	414.4	0.909	0.903	0.909 ± 0.017	408.4
August	20	318.5	1.003	0.938		366.7
September	25	378.8	0.842	0.949		428.7
October	24	451.8	0.820	0.927		393.6
November	25	483.5	0.948	0.892		517.7
December	25	451.0	0.938	0.900		449.9
January 2006	25	461.4	0.966	0.932		634.7
July 2013	30	465.9	0.983	0.989		587.6
August	30	432.0	0.949	0.969		431.6
September	30	407.0	0.973	0.946		409.4
October	30	437.6	0.945	0.893		436.6
November	30	475.3	1.103	0.960		605.5
December	30	512.4	0.924	0.949		622.4
January 2014	30	510.7	0.968	0.981		614.2
		Fs	df	Significance		
ANCOVA		1.030	13, 373	$P = 0.422$		
	Carbohydrates					
July 2005	20	329.2	0.909	0.903	1.024 ± 0.032	273.3
August	20	323.7	0.928	0.941		242.4
September	25	231.8	1.134	0.910		164.3
October	24	260.0	1.007	0.813		183.5
November	25	177.8	0.807	0.858		83.9
December	25	103.8	1.143	0.823		85.2
January 2006	25	16.9	1.182	0.841		9.7
July 2013	30	163.2	1.092	0.974		163.9
August	30	257.7	1.025	0.909		258.0
September	30	270.8	1.041	0.854		272.0
October	30	160.6	1.248	0.926		170.8
November	30	60.3	1.266	0.821		61.3
December	30	52.0	0.961	0.806		50.5
January 2014	30	62.7	0.916	0.806		119.8
		Fs	df	Significance		
ANCOVA		0.861	13, 373	$P = 0.595$		
Month	Glycogen					
	n	a	b	r	$\bar{b}$ ± 95% CI	ā
July 2005	20	219.9	1.048	0.938	0.965 ± 0.038	219.9
August	20	238.9	0.836	0.843		238.9
September	25	167.6	1.009	0.813		167.6
October	24	184.9	0.814	0.803		184.9
November	25	87.2	0.872	0.867		87.2
December	25	84.1	1.151	0.807		84.1
January 2006	25	10.1	0.908	0.826		10.1
July 2013	30	132.5	1.256	0.954		132.5
August	30	203.4	1.021	0.811		203.4
September	30	109.5	0.985	0.815		109.5
October	30	100.9	1.098	0.879		100.9
November	30	-	-	-		-
December	30	-	-	-		-
January 2014	30	22.5	0.805	0.805		22.5
		Fs	df	Significance		
ANCOVA		1.104	11, 313	$P = 0.147$		
	Lipids					

(*continued on next page*)

Table 2 (*continued*)

Month	Glycogen					
	n	a	b	r	$\bar{b}$ ± 95% CI	ā
July 2005	20	18.0	1.074	0.825	0.980 ± 0.024	19.4
August	20	17.1	0.980	0.895		17.1
September	25	16.1	1.053	0.945		25.3
October	24	14.1	0.982	0.930		14.1
November	25	6.4	1.019	0.888		4.1
December	25	5.0	1.130	0.822		5.1
January 2006	25	4.1	1.145	0.911		4.4
July 2013	30	5.9	0.901	0.952		5.8
August	30	13.0	0.888	0.949		12.9
September	30	15.1	1.061	0.954		15.4
October	30	13.7	1.021	0.922		13.9
November	30	8.8	1.288	0.934		9.0
December	30	8.9	1.037	0.899		11.6
January 2014	30	9.1	0.831	0.886		8.5
		Fs	df	Significance		
ANCOVA		1.697	13, 373	$P = 0.060$		

monthly DW values of a standard animal (TW, 2.586 g) were computed using the common slope, and intercepts were recalculated. The DW displayed similar seasonal fluctuations between the two cultivation periods (Wilcoxon test, $P = 0.371$); they were also similar to those of CI (Fig. 3b). Immediately after deployment in the grow-out area, the growth of sea squirts ceased and the DW of a standard animal exhibited a sudden decrease during the summer months (July and August). Subsequently, a weight increment occurred after a minimum was recorded in October in both cultivation periods.

3.3. Gross biochemical composition

Monthly regressions of the gross weights of biochemical components against DW in the sea squirt were also significant (Table 2), and ANCOVA revealed no significant differences in the estimates of the slope for individual components. The gross weight (mg) of each biochemical component of a standard animal were computed using common slopes (0.909 ± 0.017, 1.024 ± 0.032, 0.965 ± 0.038, and 0.980 ± 0.024 for proteins, carbohydrates, glycogen, and lipids, respectively), and intercepts were recalculated.

The seasonal patterns observed for the gross weights of the individual biochemical constituents of a standard animal paralleled each other in the two contrasting cultivation periods (Wilcoxon test, $P = 0.110$, 0.749, 0.337, and 0.949 for proteins, carbohydrates, glycogen, and lipids, respectively; Fig. 4). The protein weight of a standard animal accounted for about 44.9% (2005–2006) and 51.7% (2013–2014) of the variation in DW and followed a seasonal trend (i.e., a sharp decline between July and August and an increment after October) that was similar to that observed for the DW (Fig. 4a). Carbohydrate weight contributed less than 27.6% and 27.3%, respectively, to the DW of a standard animal during the two cultivation periods (Fig. 4b). The seasonal variations in glycogen weight paralleled those of carbohydrates, indicating that glycogen is a major component of carbohydrates (Fig. 4c). The carbohydrate maxima recorded in summer were followed by a gradual decrease during the entire cultivation period, with no weight increment observed (with the exception of a slight increase detected in January 2014). The contribution of lipid weight to the DW of a standard animal was negligible (< 2.5%) during the cultivation period.

Pearson's correlation coefficients showed that the CI, DW, and protein weight of a standard animal were significantly correlated with each other (Table 3). Water temperature exhibited a negative correlation with protein weight ($r = -0.534$, $P = 0.049$) and positive correlations with the carbohydrate (glycogen) and lipid weights of a standard animal ($r = 0.788$, 0.780, 0.671 and $P = 0.010$, 0.001, and 0.009, respectively).

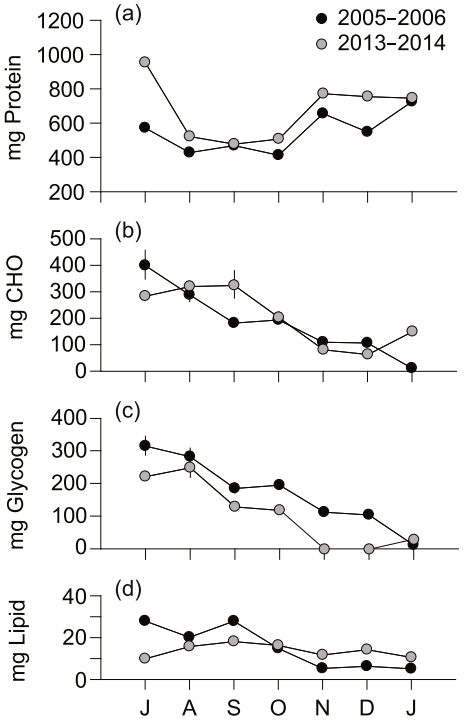

Fig. 4. Seasonal variations in the gross weights of proteins (a), carbohydrates (CHO) (b), glycogen (c), and lipids (d) of a standard animal (total dry weight, 2.586 g). The vertical bars represent 95% confidence intervals.

3.4. Physiological rates and scope for growth

The experimental conditions used for physiological measurements in *H. roretzi* reflected the monthly variations in water temperature and nutritional conditions that occur in the grow-out area of Geoje-Hansan Bay (Supplementary Table S3). Highly significant regressions of physiological rates against the DW of sea squirts were observed on each experimental occasion (Table 4), and analysis of variance revealed no significant differences among the estimates of the slope for individual physiological rates, with common slopes of 1.001 (± 0.034), 1.081 (± 0.059), 0.883 (± 0.064), and 0.945 (± 0.074) for food consumption, feces production, ammonia excretion, and respiration, respectively. Thus, these regression coefficients ($\bar{b}$) and recalculated intercepts ($\bar{a}$) were used to calculate the physiological rates of a standard animal with a DW of 1 g. All physiological rates were then converted to energy values, to estimate SFG.

The food consumption rates of a standard animal displayed a gradually decreasing trend, from 279.3 J d^{-1} in July 2013 (immediately after deployment in the grow-out area) to 248.5 J d^{-1} in January 2014 (harvest time) (Bonferroni *post hoc* test, $P < 0.05$; Table 4 and Fig. 5a). During the experimental period, a sudden decrease (230.6 J d^{-1}) in consumption rate was observed in October 2013. Energy loss by feces production varied between 12.5 and 40.5 J d^{-1}, with no seasonal trend (Fig. 5b). The ammonia excretion rates of a standard animal fluctuated from 1.9 to 5.4 J d^{-1}, with a unimodal peak detected in September–October (Fig. 5c). As a result, the assimilation rate [$A = C-(F + U)$] of a standard animal fluctuated from 206.1 to 257.6 J d^{-1}, displaying a seasonal pattern that was similar to that of the consumption rate (Fig. 5d). The respiration rate of a standard animal was considerably higher (272.4 J d^{-1}) in summer (September), followed by a decline through October and a lower level (186.5–201.3 J d^{-1}) in late autumn–winter (November to January) (Bonferroni *post hoc* test, $P < 0.05$; Fig. 5d). With the exception of October, there was a similar

Table 3
Pearson product-moment correlation coefficients between environmental (water temperature, salinity, suspended particulate matter, and chlorophyll *a*) and biometric (dry tissue weight, condition index, protein, carbohydrates, glycogen, and lipid) variables. *0.05 > P > 0.01; **0.01 > P > 0.001; ***P < 0.001. $n = 14$ for all cases. T, water temperature; S, salinity; SPM, suspended particulate matter; Chl. *a*, chlorophyll *a*; DW, tissue dry weight; CI, condition index; P, proteins; CHO, carbohydrates; G, Glycogen; L, lipids.

	S	SPM	Chl. *a*	CI	DW	P	CHO	G	L
T	−0.819**	0.121	0.044	−0.481	0.055	−0.534*	0.788**	0.780**	0.671**
S		0.115	−0.052	0.588	0.126	0.535	−0.477	−0.585	−0.546
SPM			0.431	0.189	0.330	0.320	0.217	0.027	0.176
Chl. *a*				0.225	0.557	0.592	0.090	−0.132	−0.164
CI					0.728**	0.796**	−0.148	−0.249	−0.423
DW						0.706**	0.313	0.235	−0.121
P							−0.354	−0.453	−0.520
C								0.863**	0.650*
									0.605*

seasonal trend between the respiration and consumption rates.

The SFG (expressed as the difference between the assimilation rate (A) and respiration rate (R)) of the physiological rates of a standard animal was negative during the weight-loss period of summer (Fig. 6). In contrast, a positive SFG was observed during autumn–winter, which was consistent with the protein accumulation observed in tissues of sea squirts during that period.

Multiple regressions showed that food energy was the only environmental variable that accounted for 59% of the variation in food consumption ($P = 0.045$, Table 5). Similarly, SPM concentrations were positively correlated with feces production in a standard animal, accounting for 65% of the variation ($P = 0.029$). Among the environmental variables, water temperature alone accounted for 89% and 70% of the total variances in the respiration rate and SFG, respectively, of a standard animal ($P = 0.001$ and 0.020, respectively).

4. Discussion

The difference in the growth performance between 2005–2006 and 2013–2014 that used the spat reared in different localities, i.e. the southern (grow-out area) and the northern nursery grounds is of major interest in the practice of longline culture in suspension of sea squirts. In this study, seasonal variation in the condition, DW, and gross weights of biochemical constituents of sea squirts (*H. roretzi*) was determined during the two contrasting culturing periods in a grow-out area of Geoje-Hansan Bay. No indicators of growth performance in sea squirts exhibited differences between culture practices that used spat grown in different localities.

The synchrony in the seasonal growth dynamics of sea squirts observed during the two culturing periods indicated that the spat reared at similar thermal conditions in spring should adapt to environmental conditions in the grow-out area. Interestingly, in terms of seasonal variations in DW, the culturing of sea squirts exhibited a single *b* value regarding the allometric relationship between dry flesh tissue weight and total dry weight. This result was inconsistent with previous findings obtained for the cockle *Cerastoderma edule* (Navarro et al., 1989) and *S. clava* (Kang et al., 2011). Those authors reported higher slopes during the slimming periods vs the growing phases and, therefore, more pronounced seasonal changes in flesh weight in young individuals. In contrast to this general pattern observed in marine mollusks (Bayne and Newell, 1983), the single *b* value obtained for *H. roretzi* in this study suggests that spat from different localities retain a uniform size-dependent growth performance under culturing conditions after deployment in the grow-out area.

The seasonal patterns in the accumulation and utilization of individual biochemical constituents in the tissues of *H. roretzi* were quite similar between the two culturing periods (2005–2006 and 2013–2014). The seasonal changes in CI and DW were paralleled by the

Table 4
Parameters *a* (intercept) and *b* (slope) for allometric equation $Y = aDW^b$ between physiological rate (Y, J d^{-1}) and dry tissue weight (DW, g) for *Halocynthia roretzi* during the cultivation after deployment in the grow-out area. Y represents rates of consumption, feces production, ammonia excretion, and respiration. Results of analysis of covariance (ANCOVA) to test significance of differences in slope are summarized at the bottom. $\bar{a}$, recalculated using common slopes ($\bar{b}$) obtained from ANCOVA. Superscripts indicate significant differences among elevations (P < 0.05). CI, confidence interval.

Month	Consumption					Feces production				
	a	*b*	*r*	$\bar{b}$ ± 95% CI	$\bar{a}$	*a*	*b*	*r*	$\bar{b}$ ± 95% CI	$\bar{a}$
Jul 2013	283.6	0.914	0.899	1.001 ± 0.034	279.3^{a}	19.4	1.117	0.950	1.081 ± 0.059	19.5^{g}
Aug	277.2	0.895	0.874		272.1^{a}	28.7	1.058	0.931		28.6^{f}
Sep	260.3	1.122	0.941		265.8^{ab}	22.3	1.217	0.974		22.8^{g}
Oct	228.4	1.057	0.866		230.6^{d}	12.7	0.985	0.917		12.5^{h}
Nov	264.3	0.915	0.799		260.4^{b}	28.6	1.141	0.804		28.9^{f}
Dec	246.9	1.005	0.952		247.1^{c}	16.2	1.047	0.838		16.1^{gh}
Jan 2014	243.7	1.114	0.978		248.5^{c}	40.6	1.068	0.853		40.5^{e}
	Fs	df	Significance			Fs	df	Significance		
ANCOVA	0.887	6, 62	$P = 0.274$			1.028	6, 62	$P = 0.473$		
	Ammonia excretion					Respiration				
Jul 2013	2.2	0.778	0.948	0.883 ± 0.064	2.2^{jk}	258.7	0.871	0.867	0.945 ± 0.074	255.4^{m}
Aug	2.8	0.851	0.951		2.8^{j}	253.2	0.888	0.953		250.7^{mn}
Sep	5.0	0.815	0.962		4.9^{i}	264.4	1.117	0.864		272.4^{l}
Oct	5.5	0.798	0.864		5.4^{i}	239.8	1.014	0.952		242.7^{n}
Nov	3.0	0.895	0.882		3.0^{j}	199.9	0.985	0.908		201.3^{o}
Dec	2.9	0.91	0.858		2.9^{j}	186.9	0.999	0.779		188.7^{o}
Jan 2014	1.9	0.887	0.952		1.9^{k}	189.9	0.841	0.900		186.5^{o}
	Fs	df	Significance			Fs	df	Significance		
ANCOVA	1.432	6, 62	$P = 0.455$			1.2715	6, 62	$P = 0.362$		

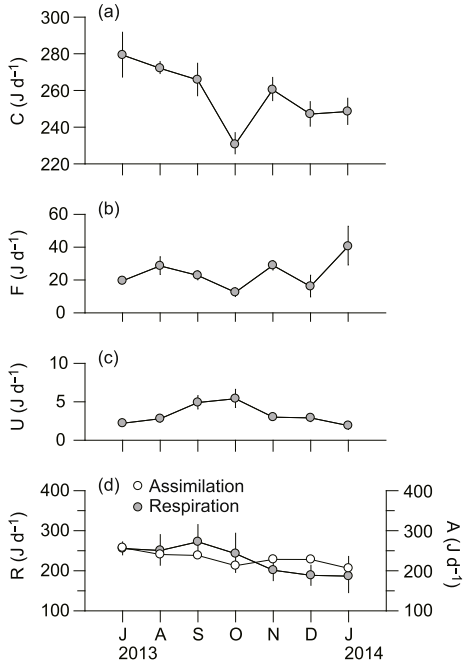

Fig. 5. Seasonal variations in the rates of physiological parameters in a standard animal with a dry tissue weight of 1 g. (a) Food consumption, C; (b) feces production, F; (c) ammonia excretion, U; and (d) respiration, R, and assimilation, A. The vertical bars represent 95% confidence intervals.

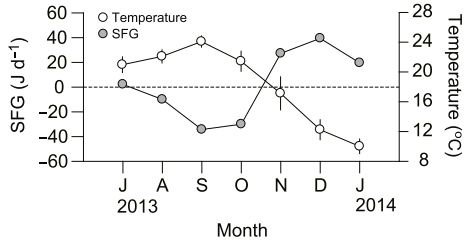

Fig. 6. Seasonal variations in seawater temperature and scope for growth (SFG) in a standard animal with a dry tissue weight of 1 g.

Table 5
Multiple regression analysis for several physiological (dependent) variables versus subsets of environmental (independent) variables. *0.05 > P > 0.01; **0.01 > P > 0.001. C, food consumption; F, feces production; R, respiration; SFG, scope for growth; FE, food energy; SPM, suspended particulate matter; T, water temperature.

Dependent variables	Independent variables	r^2	n	F
C	FE	0.587	7	7.10*
F	SPM	0.646	7	9.11*
R	T	0.890	7	40.35**
SFG	T	0.696	7	11.44*

levels of proteins during the two culturing practices. In fact, the gross weights of all biochemical components of a standard animal decreased concurrently with weight loss in its flesh tissues during the summer, after deployment in the grow-out area. The subsequent autumn–winter increase in DW was accompanied by a rapid replenishment of proteins. In contrast, carbohydrate (and glycogen) and lipid levels kept decreasing during the growing stage. As a result, the quantitative relevance of carbohydrates and lipids was negligible as sea squirts grew. Minor roles of carbohydrates and lipids in energy reserves in flesh tissues were also reported for *S. clava* (Kang et al., 2011). Moreover, given that gametogenesis and gonadal development occurred in *H. roretzi* 1 year after recruitment (Kim et al., 2001), the seasonal variations in DW and biochemical constituents observed in 0-age sea squirts in this study may reflect physiological responses to changes in environmental conditions, rather than to reproductive activity (including spawning events). Thus, the consistently low nutritional conditions caused by dense culturing stocks of suspension feeders underlie a lack of accumulation of carbohydrate and lipid reserves, even during a colder period with a positive energy balance resulting from physiological adjustments by sea squirts (Kang et al., 2000; Lee et al., 2018).

No significant relationships between the DW (and biochemical constituents) of a standard animal and the nutritional conditions (i.e., SPM and Chl*a* concentrations) of the water column were observed in this study. In contrast, the gross weights of biochemical constituents were closely related to changes in water temperature (Table 3). It should be noted that the SPM and Chl*a* concentrations in water columns of the Geoje-Hansan Bay fell within relatively narrow ranges and varied irregularly, with no seasonality, in the grow-out area during the culturing period. The results of monthly physiological measurements also revealed that water temperature had substantial effects on the physiological processes that control its energetic balance at the organism level (Table 5). A close relationship was found between the food consumption rate of a standard animal and water-column food energy concentration. However, the food consumption rates were positively correlated with water temperature ($r = 0.773$), with the exception of the extremely reduced rate recorded in October, which was caused by low food energy concentrations.

The upper critical thermal limit of sea squirts (*H. roretzi*) is considered to be around 26.5 °C; at this temperature, they close their inhalant and exhalant siphons and stop pumping water (Kim, 1980; Shin et al., 2011). The water temperature in the grow-out area was below this upper thermal limit, but fluctuated across a relatively wide range (9.8–24.7 °C) according to season, thus allowing sea squirts to retain physiological functions in proportion to temperature changes, as indicated by the monthly allometric models of physiological rates against DW (Bayne and Newell, 1983; Lee et al., 2018). In fact, together with the positive allometry observed between the rates of physiological components and DW, the identical estimates of exponents ($\bar{b}$) detected among the monthly equations support the conclusion mentioned above and allow us to make further comparisons of the rates across temperatures based on the intercepts ($\bar{a}$) of the equations. The weight exponent values of the individual physiological rates–DW regressions, 1.001, 1.081, 0.883, and 0.945 for food consumption, feces production, ammonia excretion, and respiration, respectively, overlapped with the upper limits of the values of suspension-feeding mollusks (Bayne and Newell, 1983; Gosling, 2015). This result indicates that the physiological responses of smaller sea squirt individuals to temperature fluctuations are more sensitive than those of other mollusks.

High concentrations of, and large fluctuations in, SPM (up to hundreds mg l^{-1}) may modify the feeding mechanisms of bivalves (Barillé et al., 1997; Navarro and Widdows, 1997) and other tunicates (Armsworthy et al., 2001; Montalto et al., 2017). No significant correlation between the feeding rates of the cultured sea squirts and SPM concentrations was found in this study. Instead, it seems likely that their food energy acquisition was dependent on thermal changes in our field conditions because of low SPM concentrations and their narrow range. The temperature-dependent trend observed for the feeding rates of *H. roretzi* agrees with those observed for the ascidian *S. clava*, which increase with increasing temperature over the range of 5–25 °C (Jiang et al., 2008; Kang et al., 2015). As described above, high water temperatures (exceeding 25 °C), which may inhibit the filtration rate of sea squirts, were not detected in this study.

In contrast to feeding rates, fecal production and ammonia excretion (as energy expenditure processes) did not exhibit significant correlations with water temperature, suggesting an inherent temperature independence for these components. In addition, while a significant correlation was found between feces production rate and SPM concentration, none of the two physiological components were related to

filtration (food consumption) and metabolic (respiration) rates. Energy losses by fecal material and ammonia excretion accounted for 5.4–16.3% and 0.8–2.3%, respectively, of the food energy that was consumed by a standard animal in this study. This result indicates that natural SPM diets are absorbed and assimilated with high efficiencies (83–92%) by the sea squirts in the grow-out area (Armsworthy et al., 2001). Although at present it is not clear which factors are associated with feces production and nitrogen excretion processes, it seems likely that the two processes are influenced by food availability (exogenous, SPM concentrations in this study), the feeding rate (endogenous) of the ascidian, and a combination of both (Jiang et al., 2008; Kang et al., 2015). Conversely, the O:N atomic ratio represents the relative proportion of protein, carbohydrate, and lipid substrates that are catabolized for maintenance metabolism by marine organisms and reflects well their nutritional status (Bayne and Newell, 1983). High values (70–180; data not shown) indicated that greater proportions of carbohydrates or lipids, compared with proteins, were catabolized during the entire culturing period, which supports the consistent declines in carbohydrate and lipid levels detected in flesh tissues of sea squirts from summer to winter.

In this study, the respiration rates (a proxy for metabolic rate) of *H. roretzi* were highly temperature dependent. Such a lack of metabolic adjustment to temperature changes has been reported for many marine ectotherms (e.g., *Ostrea edulis*, Newell et al., 1977; *Crassostrea virginica*, Shumway and Koehn, 1982; *S. clava*, Kang et al., 2015). In contrast, in other species (e.g., *Mytilus edulis*, Widdows and Bayne, 1971; *Cerastoderma edule*, Newell and Bayne, 1980), respiration rates are kept relatively constant in response to long-term changes in temperature. Respiration is one of the major energy-demanding processes through which ectothermic animals obtain energy for maintenance, growth, development, and performance (Kern et al., 2014). Another ascidian *Styela plicata* also showed temperature-dependent respiration rates but maintained low metabolic rates within a wide range of temperatures (Montalto et al., 2017). For the former group, including sea squirts, it appears that the high temperatures of summer give rise to a mismatch between metabolic requirements and available energy, resulting in a depletion of tissue energy and, thereby, a reduction in tissue weight. In that case, *H. roretzi* should adopt a compensatory strategy that is different from that of the species of the latter group, to compensate for the increased energy expenditure that occurs with the increase in temperature observed during summer. As the temperature increased, the sea squirt *H. roretzi* displayed a concomitant increase in filtration rate (food consumption) following its temperature-dependent regulation, to offset the increased metabolic costs, as shown in species of the former group (Bayne and Newell, 1983; see also the references mentioned above).

Interestingly, the clear seasonal pattern in SFG (i.e., negative during summer vs positive during autumn–winter) observed for *H. roretzi* challenges the synchronous adjustment of energy acquisition and expenditure processes (i.e., filtration rate and metabolic energy cost) to seasonal changes in water temperature. Despite the compensation resulting from the increased filtration rates regarding metabolic cost in sea squirts in warm conditions, a disruption of energy balance (i.e., negative SFG) led to tissue weight loss during summer. In contrast, despite concurrent reductions in both filtration rate and metabolic cost with a decrease in temperature, there was a corresponding positive SFG during autumn–winter. Such a seasonal discrepancy in SFG is more likely attributed to changes in metabolic cost than seasonal variability in food consumption rate, as shown by the coefficient of variation (SD/mean × 100) values (15.4 vs. 6.5; Fig. 5d). The seasonal trend observed for assimilation rate was paralleled by the trend in food consumption rate. The resulting seasonal pattern in the SFG of *H. roretzi* explained the seasonal dynamics in energy reserves and growth of its flesh tissues. As a result, while the negative SFG recorded during the warm period caused a decrease in nutrient reserves and weight loss in flesh tissues after deployment in the grow-out area, the positive SFG observed during the cold season supports the enhanced levels of protein reserves and DW during autumn–winter. However, despite the positive energy balance resulting from the thermal acclimation of the physiological processes of *H. roretzi*, the lack of recovery of carbohydrate (and lipid) levels in flesh tissues during the growing phase further suggest that the food that is available in the grow-out area is insufficient to meet the surplus of energy that is required for the accumulation of nutrient reserves (Navarro et al., 1989; Kang et al., 2000).

5. Conclusion

The development of nursery grounds in colder conditions to rear spat for the longline culture in the suspension of sea squirts on the coasts of Korea and employing a newly adopted procedure to provide spat that are suitable for this purpose are an inevitable consequence of recent demands to avoid failure in spat production. Spat that have been newly settled on palm ropes are placed in colder nursery grounds located on the northeastern coast of Korea because they can suffer severe mortality because of the increase in water temperature observed during spring on the southern coast of the country after a warm winter. The spat are kept there until they are deployed using longline facilities in grow-out areas because frequent and strong wind-generated waves and swells restrict the maintenance of the culturing facilities in the northeastern coastal sea. Sea squirts retained physiological function across the temperature range during the culturing period in the grow-out area. In addition to synchronous temperature-dependent adjustments in filtration rate and metabolic energy cost, a less-clear seasonal variability in food consumption rate yielded a seasonal discrepancy in SFG (negative during summer vs positive during autumn–winter). Despite consistent declines in carbohydrate and lipid levels, the tissue weight and protein reserves of sea squirts varied concomitantly with the seasonal trend in SFG. Finally, our results support the contention that sustainable longline culturing of sea squirts under the warming environmental conditions of shallow coastal seas may be achieved using spat reared in a newly developed cold nursery ground and that this alternative approach, which was adopted by fishermen, is acceptable as a common procedure to supply spat.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.734526.

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