



## Phenotypic plasticity of gut structure and function during periods of inactivity in *Apostichopus japonicus*

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### ABSTRACT

*Apostichopus japonicus* is a common sea cucumber that undergoes seasonal inactivity phases and ceases feeding during the summer months. We used this sea cucumber species as a model in which to examine phenotypic plasticity of the digestive tract in response to food deprivation. We measured the body mass, gross gut morphology and digestive enzyme activities of *A. japonicus* before, during, and after the period of inactivity to examine the effects of food deprivation on the gut structure and function of this animal. Individuals were sampled semi-monthly from June to November (10 sampling intervals over 178 days) across temperature changes of more than 18 °C. On 5 September, which represented the peak of inactivity and lack of feeding, *A. japonicus* decreased its body mass, gut mass and gut length by 50%, 85%, and 70%, respectively, in comparison to values for these parameters preceding the inactive period. The activities of amylase, cellulase and lipase decreased by 77%, 98%, and 35% respectively, in comparison to mean values for these enzymes in June, whereas pepsin activity increased two-fold during the inactive phase. Alginate and trypsin activities were variable and did not change significantly across the 178-day experiment. With the exception of amylase and cellulase, all body size indices and digestive enzyme activities recovered and even surpassed the mean values preceding the inactive phase during the latter part of the experiment (October–November). Principal Component Analysis (PCA) utilizing the digestive enzyme activity and body size index data divided the physiological state of this cucumber into four phases: an active stage, prophase of inactivity, peak inactivity, and a reversion phase. These phases are all consistent with previously suggested life stages for this species, but our data provide more defined characteristics of each phase. *A. japonicus* clearly exhibits phenotypic plasticity (or life-cycle staging) of the digestive tract during its annual inactive period.

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### 1. Introduction

Phenotypic plasticity of organ size and function can provide insight into the mechanisms an animal employs to accommodate a changing environment (Piersma and Drent, 2003). As the supply organ of nutrients to an animal, the digestive tract must be extremely plastic, responding regularly to changes in dietary composition, intake, and the metabolic state of the animal (Karasov and Martinez del Rio, 2007). Phenotypic plasticity of gut structure and function is predicted theoretically, and has been observed empirically (Karasov and Hume, 1997). Significant changes in the structure and the function of the digestive tract in response to food availability and season have been observed in a number of invertebrate taxa (Ebert, 1996; Honkoop et al., 2002), but none are more extreme than those seen in holothuroideans (Fankboner and Cameron, 1985; Liu et al., 1996; Li et al., 1996). Generally, holothuroideans (sea cucumbers) are deposit feeders that pass large amounts of sediment through their guts and assimilate

what organic material is available (Yingst, 1976; Moriarty, 1982; Mayer et al., 1997; Uthicke, 1999). They have simple, tubular digestive tracts that match the “plug-flow reactor” model of digestive physiology, under which food moves rapidly and in a unidirectional trajectory down the gut after ingestion (Penry and Jumars, 1987; Penry, 1989). Additionally, they have a suite of digestive enzymes that appear largely endogenous in origin (Féral, 1989; Mayer et al., 1997). Thus, sea cucumbers represent true detritivores that rely on endogenous digestive mechanisms to garner nutrients from their food.

Among holothuroideans, stichopodid sea cucumbers are the best studied, and consume detritus composed of macroalgae, molluscan shell fragments, crustaceans, echinoderm ossicles, pelagic and benthic foraminifera, and diatoms (Hauksson, 1979; Zhang et al., 1995). Because they feed on such a low-quality food, stichopodids feed continuously throughout the diel cycle (Yingst, 1976; Moriarty, 1982; Uthicke, 1999), and thus, have high levels of intake. However, many stichopodids experience seasonal food shortages (Fankboner and Cameron, 1985) or stop eating and become inactive in response to elevated water temperature (Mitsukuri, 1903; Sui and Liao, 1988; Liu et al., 1996; Li et al., 1996). During these times, food can be

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completely unavailable, or the animal is simply incapable of feeding altogether. Therefore, stichopodid sea cucumbers provide a perfect system in which to examine what happens to the gut structure and function of an animal in response to extreme ends of food availability and intake.

One of the best-studied stichopodid sea cucumbers is *Apostichopus japonicus* (Selenka), an epibenthic species with a geographical range spanning the northwest Pacific Ocean down to the Yellow Sea (Zhang et al., 2004). *A. japonicus* is utilized both as a “filter” in polyculture systems, including fish and shellfish (Yang et al., 2000; Zhou et al., 2006), and is captured directly from nature for human consumption in many parts of Asia (Sloan, 1984). As a temperate species with a life span of about four years (Michio et al., 2003), *A. japonicus* experiences a wide range of temperatures on an annual basis. Individuals of this species become less active as temperatures rise above 18 °C, and with the exception of very small individuals (<5 g), enter a stage of inactivity and cease feeding at temperatures ranging from 20–30.5 °C, depending on latitude, body size, and duration of the high temperature (Sui and Liao, 1988; Liu et al., 1996; Yang et al., 2005; Yuan et al., 2007). This period of inactivity usually lasts two–four months, and occurs during the summer (June–October; Li et al., 1996). The tell tale signs of this inactive phase in *A. japonicus* include gut atrophy, a cessation of feeding, a loss of 30–50% of body mass, and a lack of movement (Mitsukuri, 1903; Tanaka 1958a,b; Li et al., 1996; Liu et al., 1996).

Given that the digestive tract and associated organs can account for nearly 40% of an animal's metabolic rate (Wang et al., 2006), one would have the *a priori* expectation for *A. japonicus* to reduce the size and function of the digestive tract during this period of feeding cessation (Diamond and Hammond, 1992; Wang et al., 2006; Karasov and Martinez del Rio, 2007). In support of these expectations, *A. japonicus* decrease the width of their digestive tracts to approximately 1 mm in diameter, and shorten the length of the gut to about half of its length prior to the period of inactivity (Liu et al., 1996; Li et al., 1996). Changes occur on the level of the gut ultrastructure as well, as *A. japonicus* reduces the number of intestinal folds and villi, and the cells among the mucous membrane (indicated by nuclei) become sparse (Li et al., 2006). Whether this reduction represents hypotrophy (a shrinking of cells, but no change in cell number) or hypoplasia (an actual reduction in cell number) is unknown, but it is likely the former, because hypoplasia appears to be limited to mammals and birds (Starck, 2003).

Fu et al. (2006) and Choe (1963) found that *A. japonicus* expresses many types of digestive enzymes, including: metallo-protease, pepsin-like protease, serine protease, high-alkaline protease, dipeptidase, amylase, pectinase, cellulase, lichenase, and lipase. Furthermore, Fu et al. (2005) characterized the proteases in the guts of this species. These studies indicate that *A. japonicus* possess an array of digestive enzymes capable of hydrolyzing a variety of substrates. To our knowledge, however, there are no investigations into the changes in digestive enzyme activities (i.e., gut function) that occur during the inactive phase in *A. japonicus*, and we know of no studies that have examined the recovery of the gut following arousal from this period of inactivity. Exactly how do digestive enzyme activity levels change in response to a reduction in food intake, and an absence of nutrients in the lumen of the digestive tract? And, how quickly do enzymatic activity levels recover when the animal begins to feed?

In this study, we examined the variations in gross gut structure and digestive enzyme activities that occur before, during, and after the inactivity stage in *A. japonicus*. We measured the activity levels of the following six digestive enzymes that hydrolyze various substrates in the gut lumen: the carbohydrases amylase, cellulase, and alginase; the proteases pepsin and trypsin; and lipase. Because of the reduction in food intake that the animals experience during the inactive phase, we expected to observe lower activities of all six digestive enzymes and a reduction in gut size relative to body size, and that these parameters would return to normal levels shortly after arousal and re-feeding (Fankboner and Cameron, 1985; Cramp and Franklin, 2003).

## 2. Materials and methods

### 2.1. Animal collection and dissection

Individuals of *A. japonicus* ( $n=3-7$ ) were collected semi-monthly from June to November 2006 (across 178 day period) from a farm in Jaonan, Shandong province, P. R. China (35°44'N, 120°01'E). At the end of April every year, juvenile sea cucumbers (body mass ~30 g) were stocked in the farms' waters and were the source of animals for this study. Naturally occurring detritus is the only food source for the sea cucumbers in the farm, and the seawater on the farm is exchanged daily during high tides. Thus, the detrital material on the farm is representative of detritus in the surrounding coastal waters. However, we did not analyze the composition of the detritus in this study, so we do not know exactly what the cucumbers were consuming across the experiment. Because time of day can affect digestive function (Cleveland and Montgomery, 2003), animals were always obtained between 1100 and 1300 h. Upon collection, the animals were weighed (body mass [BM]  $\pm 0.01$  g), and dissected. The whole gut was removed by cutting at the esophagus and cloaca. The gut was uncoiled, without stretching, and then the whole gut mass (WGM  $\pm 0.01$  g), gut length (GL  $\pm 1$  mm) (German and Horn, 2006) and the remaining body wall (BWM  $\pm 0.01$  g) were measured. The guts were then cut longitudinally and washed thoroughly in ice-cold 0.1 M phosphate buffered saline (PBS, pH 7.4). On each sampling date, the guts were blotted dry with filter paper, weighed (GM  $\pm 0.01$  g), and frozen in liquid nitrogen. These gut samples were then stored at -80 °C until analyzed for digestive enzyme activities. Because the guts of the animals were smaller during the inactivity phase, it was necessary to pool the gut tissues of two to three individuals to have enough tissue in which to measure all of the digestive enzymes (Féral, 1989). Thus, the sample sizes for the analyses of digestive enzyme activities varied with sampling date, reaching a minimum of three during the inactivity period, and as high as seven in active phase.

From each of the individual cucumbers on each sampling date we calculated three digestive-somatic indices that allowed us to evaluate the size of the gut relative to the mass of the body. First, we calculated the mass of the gut contents (GCM) by subtracting the mass of the rinsed gut from the mass of the whole gut (WGM - GM). From this we calculated the relative gut content mass (RGCM = GCM / BM). Next, we determined the relative gut mass {RGM = gut mass (g) / body mass (g)} and Zihler's Index {ZI = gut length (mm) / 10[body mass (g)]<sup>1/3</sup>; Zihler, 1982}, as these indices have been used successfully in previous investigations of fish (German and Horn, 2006). The sea cucumbers varied in body mass during the experimental period, so the use of Zihler's Index (Zihler, 1982) allowed us to take into account these differences in body mass. Water temperature (°C) and salinity (‰) were also recorded at each sampling interval.

### 2.2. Assays of digestive enzyme activity

Following each sampling interval, guts were thawed, weighed, and homogenized in five volumes of ice-cold 0.1 M PBS, pH 7.4, using a manual glass homogenizer. The homogenates were then centrifuged at 10,000  $\times$ g for 20 min at 4 °C. The supernatant was then pipetted into clean centrifuge vials and stored at 4 °C until analyzed (usually less than 12 h).

The protein content of the homogenates was measured following Bradford (1976), using bovine serum albumin as the standard. All assays of digestive enzyme activities were carried out in duplicate and measured in a UV2102PC cuvette spectrophotometer (UNICO, Shanghai, China). Assays were run at saturating conditions as determined with gut tissues from *A. japonicus*, and all pH values were measured at room temperature.

Pepsin (E.C. 3.4.23.1) activity was assayed using casein as substrate following Pan and Wang (1997). Briefly, 2.0 ml of 0.5% casein in 0.2 M

citric acid–sodium citrate buffer (pH 3.0) was combined with 0.1 ml of 0.04 M EDTA–Na<sub>2</sub>, 0.4 ml gut homogenate, and 0.6 ml distilled water. The reaction mixture was allowed to incubate for 15 min at 37 °C, after which the reaction was stopped by the addition of 1 ml of 30% trichloroacetic acid. The arrested reaction mixture was then centrifuged at 825 ×g for 15 min. 1 ml of supernatant was then combined with 5 ml of 0.55 M Na<sub>2</sub>CO<sub>3</sub>, and 1 ml of Folin-Phenol reagent, and this mixture was incubated for 15 min at 37 °C. The absorbance of this solution was measured at 680 nm. Pepsin activity was determined with an L-tyrosine standard curve and expressed in U (1 µg of L-tyrosine liberated per minute) per mg protein. Blanks consisting of substrate and buffer were conducted simultaneously to which homogenate was added after the addition of trichloroacetic acid. Trypsin (E.C. 3.4.21.4) activity was assayed as described above for pepsin, with the exception that the buffer used was 0.05 M borax–sodium hydroxide, pH 9.8.

Cellulase (E.C. 3.2.1.4) activity was measured following Pan and Wang (1997). Briefly, 1 ml of homogenate was combined with 1 ml of 0.5% sodium carboxymethyl cellulose dissolved in distilled water, 4 ml of 0.1 M HAC–NaAC buffer (pH 4.5), and 1 ml of distilled water. The reaction mixture was allowed to incubate for 30 min at 40 °C, after which it was boiled for 15 min to stop the reaction. We then added 3 ml of 3, 5-dinitrosalicylic acid to the reaction mixture and boiled the solution for an additional 15 min. After cooling, 4 ml of distilled water was added and the absorbance was measured at 550 nm. Cellulase activity was determined with a glucose standard curve and expressed in U (1 µg of glucose liberated per minute) per mg protein. Blanks consisting of substrate and buffer were conducted simultaneously to which homogenate was added after the mixture was boiled. Alginase (E.C. 3.2.1.16) activity was assayed following Tang et al. (2005), and as described above for cellulase, with the exceptions that 0.5% sodium alginate dissolved in deionized water, and 0.1 M barbital sodium-hydrochloric acid, pH 7.6, were used as substrate and buffer, respectively.

Iodine-spectrophotometry was used to assay amylase (E.C. 3.2.1.1) activity using commercial kits of Nanjing Jiancheng Bioengineering Institute. The reaction temperature was 37 °C. The activities were expressed as U mg protein<sup>-1</sup>.

Lipase (E.C. 3.1.1.–) activity was determined by the simplified turbidimetric assay using commercial kits of Nanjing Jiancheng Bioengineering Institute. The reaction temperature was 37 °C. The activities were expressed as U mg protein<sup>-1</sup>.

### 2.3. Statistical analyses

Data were analyzed using SPSS 13.0, and Minitab (version 13) statistical software packages. Values are presented as means ± standard error of the mean (SEM). The values for the activity of each

digestive enzyme and for body mass were compared among time intervals with one-way ANOVA followed by a Tukey's HSD multiple comparison test. The digestive-somatic indices were compared among the sampling intervals with ANCOVA, using body mass as a covariate, and followed by a Tukey's HSD multiple comparison test (German and Horn, 2006). The variability and normality of the data were assessed prior to running ANOVA and ANCOVA with Levene's test and residual versus fits plots, respectively (German et al., 2004), and all data were suitable for analysis without any transformation.

The variation in digestive enzyme activities and digestive-somatic indices (RGM, RGCM and ZI), combined, were analyzed among time intervals using Principal Component Analysis (PCA) (Johnston et al., 2005; Falch et al., 2006; Brunborg et al., 2006). PCA allowed us to graphically visualize the overall changes in gut structure and function that occurred over the course of the experiment, and to observe how different these characteristics were before, during, and after the inactivity phase.

## 3. Results

### 3.1. Gross gut morphology, water temperature, and salinity

The body mass, digestive-somatic indices, water temperature, and salinity for each sampling interval are shown in Table 1. The body mass and each of the digestive-somatic indices varied significantly throughout the experiment. On 5 September, the body mass, RGM, RGCM, and ZI were 50%, 15%, 22%, and 28%, respectively, of the values for these parameters measured on 2 June, showing a sharp decline in the indices from the outset of the experiment. Each of these parameters on 5 September was significantly lower than the values on 2 June, with the exception of RGCM. Beginning on 10 October, the body mass and each of the digestive-somatic indices recovered to comparable values for these indices on 2 June, with BM and RGCM eventually exceeding the values at the outset of the experiment (Table 1). Body mass was not a significant covariate in the ANCOVA for any of the digestive-somatic indices. On 2 June and 21 June, the water temperature was 22.0 and 21.8 °C, respectively, and from that point, it increased, reaching a peak of 29.6 °C on 6 August. Salinity varied slightly, from 27.53 to 32.46‰, with the minimum in July due to rainfall.

When the animals were active, the digestive tract and respiratory trees of *A. japonicus* were fully functional, but once they entered the state of inactivity, the digestive tract and respiratory trees became considerably atrophied (Fig. 1a and b), and little food was observed in the gut throughout the inactive phase. On 6 August, 10% of sampled individuals had empty guts, but this proportion increased to 61.5% by 21 August, and to 77.8% on 5 September. Before and after these time intervals all individuals were found with food in their guts.

**Table 1**

Comparison of body mass (BM), body wall mass (BWM), relative gut mass (RGM), relative gut content mass (RGCM), and Zihler's index (ZI) in *Apostichopus japonicus* among the sampling dates

Date	Day	N	BM (g)	BWM (g)	RGM	RGCM	ZI	T (°C)	Salinity (‰)
2 Jun	1	3	117.73 ± 4.09 <sup>bc</sup>	77.10 ± 9.84 <sup>bc</sup>	0.021 ± 0.002 <sup>c</sup>	0.069 ± 0.008 <sup>abc</sup>	13.28 ± 0.71 <sup>c</sup>	22.0	31.52
21 Jun	19	4	118.65 ± 13.70 <sup>c</sup>	62.99 ± 10.56 <sup>ab</sup>	0.021 ± 0.002 <sup>c</sup>	0.069 ± 0.011 <sup>abc</sup>	13.27 ± 0.90 <sup>c</sup>	21.8	30.00
20 Jul	48	5	94.39 ± 7.83 <sup>abc</sup>	55.13 ± 4.57 <sup>ab</sup>	0.012 ± 0.001 <sup>b</sup>	0.076 ± 0.008 <sup>bc</sup>	9.69 ± 0.33 <sup>b</sup>	26.6	27.53
6 Aug	65	5	74.39 ± 7.83 <sup>ab</sup>	48.24 ± 6.53 <sup>a</sup>	0.011 ± 0.001 <sup>b</sup>	0.037 ± 0.006 <sup>ab</sup>	8.11 ± 0.36 <sup>b</sup>	29.6	29.73
21 Aug	80	7	58.60 ± 7.16 <sup>ab</sup>	41.50 ± 6.13 <sup>a</sup>	0.009 ± 0.002 <sup>ab</sup>	0.039 ± 0.013 <sup>ab</sup>	7.70 ± 1.06 <sup>b</sup>	27.8	31.80
5 Sept	95	5	58.56 ± 14.32 <sup>a</sup>	39.92 ± 2.85 <sup>a</sup>	0.003 ± 0.001 <sup>a</sup>	0.015 ± 0.013 <sup>a</sup>	3.77 ± 0.12 <sup>a</sup>	21.9	30.03
10 Oct	130	5	118.91 ± 10.99 <sup>c</sup>	77.63 ± 7.04 <sup>bc</sup>	0.013 ± 0.001 <sup>bc</sup>	0.084 ± 0.006 <sup>c</sup>	10.39 ± 0.52 <sup>bc</sup>	24.3	30.75
25 Oct	145	5	115.24 ± 13.89 <sup>c</sup>	64.50 ± 6.42 <sup>ab</sup>	0.015 ± 0.002 <sup>bc</sup>	0.089 ± 0.009 <sup>cd</sup>	9.96 ± 0.50 <sup>bc</sup>	18.7	30.38
9 Nov	160	5	133.21 ± 11.80 <sup>cd</sup>	78.69 ± 5.32 <sup>bc</sup>	0.015 ± 0.001 <sup>bc</sup>	0.091 ± 0.013 <sup>cd</sup>	9.70 ± 0.69 <sup>b</sup>	17.1	30.10
27 Nov	178	5	174.97 ± 9.97 <sup>d</sup>	95.79 ± 6.17 <sup>cd</sup>	0.018 ± 0.001 <sup>bc</sup>	0.136 ± 0.008 <sup>d</sup>	10.69 ± 0.47 <sup>bc</sup>	11.8	32.46
Date			F <sub>9,44</sub> = 12.61 P < 0.01	F <sub>9,44</sub> = 7.92 P < 0.01	F <sub>9,53</sub> = 5.29 P < 0.01	F <sub>9,52</sub> = 4.41 P < 0.01	F <sub>9,53</sub> = 9.27 P < 0.01	–	–
BM			–	–	F <sub>1,43</sub> = 0.01 P = 0.92	F <sub>1,42</sub> = 0.15 P = 0.70	F <sub>1,43</sub> = 0.44 P = 0.51	–	–

Values are mean ± SEM. Comparisons of BM and BWM were made with ANOVA followed by Tukey's HSD with a family error rate of P = 0.10. RGM, RGCM, and ZI were compared with ANCOVA (using body mass as a covariate) followed by a Tukey's HSD with a family error rate of P = 0.10. Values for BM, BWM, or a digestive-somatic index (RGM, RGCM, or ZI) with different superscript letters are significantly different (P < 0.004 for individual comparisons). Temperature and salinity were provided for reference and were not analyzed.

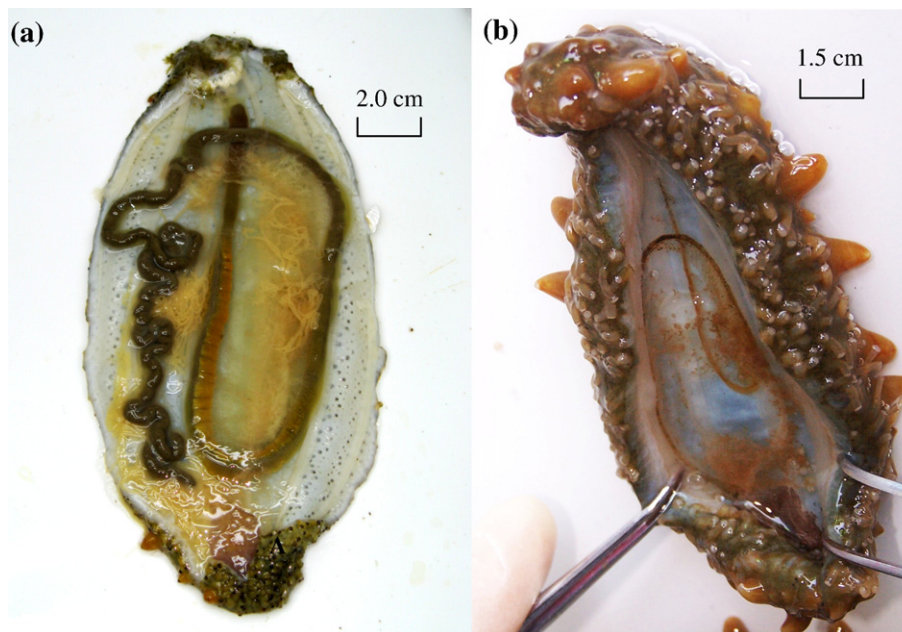


Fig. 1. The digestive tracts and respiratory trees in active phase (a) and in inactivity phase (b), showing the significant atrophy of the digestive tract in periods of inactivity.

### 3.2. Carbohydrase activities

Mean amylase activity varied significantly across the duration of the experiment ( $F_{9,36}=6.41$ ,  $P<0.01$ ; Fig. 2). It decreased after 20 July, where it reached a maximum of  $32.96 \pm 3.16$  U mg protein<sup>-1</sup>, towards a minimum activity ( $7.15 \pm 0.35$  U mg protein<sup>-1</sup>) on 5 September. Amylase activity generally increased after 5 September. The minimum amylase activity on 5 September was significantly lower than those observed on 2 June, 21 June, 20 July, 21 August and 10 October, whereas the activity on the other dates were not significantly different from those the minimum value on 5 September.

Mean cellulase activity changed significantly over the course of experiment ( $F_{9,35}=7.29$ ,  $P<0.01$ ; Fig. 2). It decreased from 2 June ( $1.39$  U mg protein<sup>-1</sup>) onwards to a minimum activity ( $0.02$  U mg protein<sup>-1</sup>) on 5 September. Then it slightly increased from October to November. The cellulase activities in *A. japonicus* on 5 September were significantly lower than those on 2 June, 21 June, and 21 August. Cellulase activities on all other dates were not significantly different from those observed on 5 September.

In contrast to the other carbohydrases, there was great variability in alginase activity within a given sampling date, and no significant differences were detected among sampling dates ( $F_{9,34}=0.56$ ,  $P=0.82$ ; Fig. 2). The mean activities remained relatively constant for the first 80 days of the experiment, but, like the other carbohydrases, reached a minimum activity ( $0.004 \pm 0.002$  U mg protein<sup>-1</sup>) on 5 September. The maximum mean alginase activity ( $1.12 \pm 0.54$  U mg protein<sup>-1</sup>) was observed on 25 October.

### 3.3. Protease activity

Mean pepsin activity changed significantly throughout the duration of the experiment ( $F_{9,36}=3.07$ ,  $P<0.01$ ; Fig. 3). Unlike the other enzymes, pepsin activity was relatively stable for the first 80 days of the experiment and the last 48 days, but the activity of this protease significantly increased to its highest levels on 5 September ( $2.67 \pm 0.99$  U mg protein<sup>-1</sup>). The pepsin activity on 5 September was significantly higher than the others except those observed on 2 June and 21 July.

Unlike pepsin, mean trypsin activity did not change significantly over the duration of the study ( $F_{9,36}=0.30$   $P=0.97$ ; Fig. 3), and was

extremely variable on a given sampling date. The highest trypsin activities were detected on 21 June ( $2.67 \pm 0.99$  U mg protein<sup>-1</sup>) and the lowest on 6 August ( $0.09 \pm 0.02$  U mg protein<sup>-1</sup>).

### 3.4. Lipase activity

Mean lipase activity changed significantly during the course of the experiment ( $F_{9,35}=4.37$ ,  $P<0.01$ ; Fig. 4). The activity of this lipolytic enzyme had a similar pattern to trypsin, peaking on 20 July ( $6.50 \pm 0.90$  U mg protein<sup>-1</sup>), and decreasing to a minimum activity of  $1.51 \pm 0.70$  U mg protein<sup>-1</sup> on 25 October. The lipase activities on 25 October and 9 November were significantly lower than those observed on 20 July and at the end of the experiment.

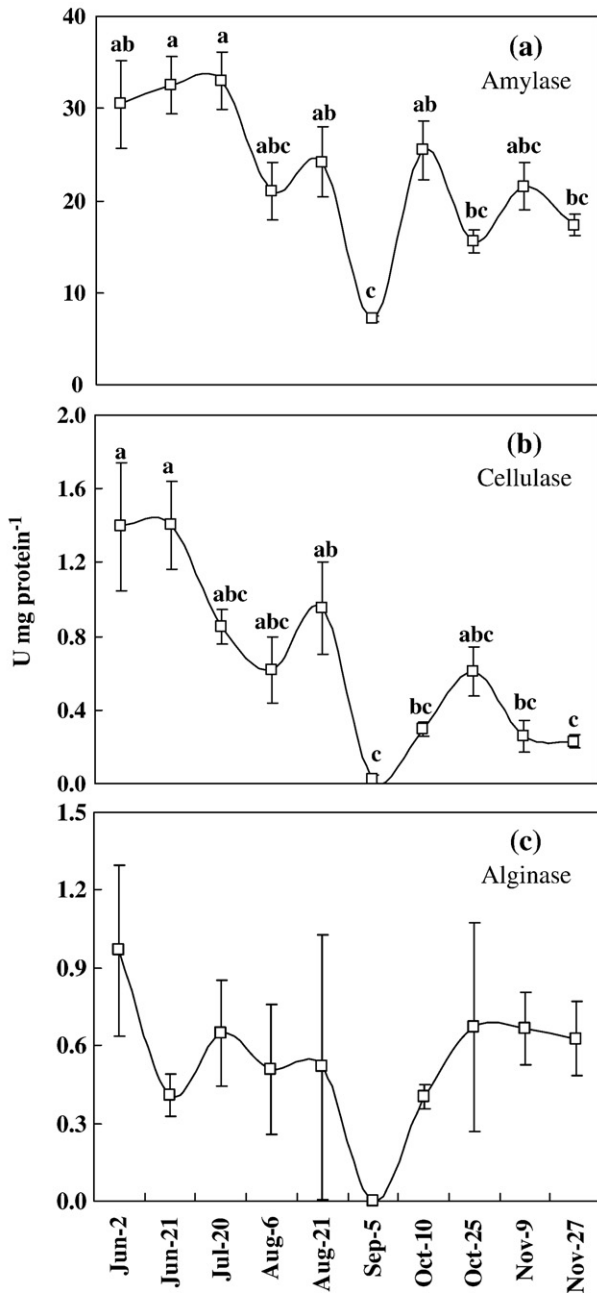
### 3.5. Principal component analysis

The PCA plots with scores and loadings of all samples based on the activities of amylase, lipase, pepsin, trypsin, cellulase, alginase and the digestive-somatic indices are shown in Figs. 5 and 6. These plots show that the data from a particular sampling interval cluster with dates near that interval. For example, all of the sampling dates in June and July are close to one another, as are the dates in August (Fig. 5). The data from 5 September stand alone from all of the other dates as being the most negative on both principle component axes. And, because the digestive enzyme activity and digestive-somatic index data were not the same at the end of the experiment as they were at the beginning, the dates from October and November tended to cluster away from those of June and July, primarily because of negative scores on the PC2 axis.

The loadings from the PCA analysis are shown in Fig. 6. With the exception of pepsin, all of the digestive enzyme activity and digestive-somatic index data fall in the positive for both principal component axes, whereas pepsin stands alone as being negative in both.

## 4. Discussion

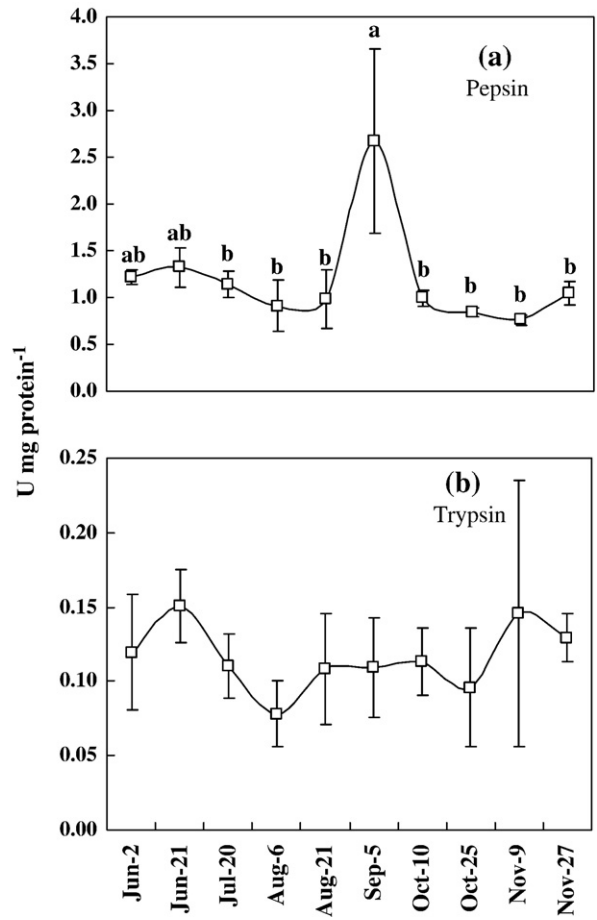
The results of this study support our hypothesis that *A. japonicus* significantly down regulates its gut structure and function during a period of inactivity that this animal enters during the summer months. Of the enzymes analyzed, pepsin was the only one to



**Fig. 2.** Amylase (a), cellulase (b) and alginase (c) activities as a function of date in *A. japonicus*. Values are means ( $\pm$ SEM,  $n=3-7$ ). Values were compared among sampling dates with ANOVA followed by a Tukey's HSD with a family error rate of  $P=0.05$ . Values that share a superscript are not significantly different.

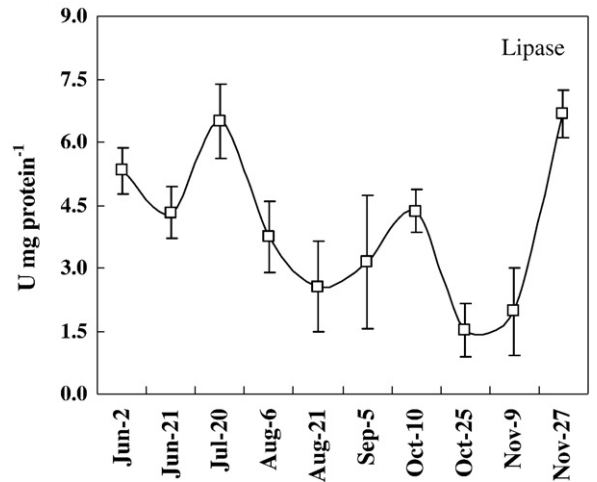
significantly increase in activity on 5 September, whereas the other five enzymes all decreased during this time. Furthermore, the body shrank and the gut was significantly reduced in mass and length during the inactive phase, and these parameters recovered following arousal and re-feeding. Thus, the gut of *A. japonicus* appears to be extremely plastic, undergoing significant changes in structure and function as it enters, endures, and recovers from a period of inactivity and a lack of food. In contrast to our predictions, *A. japonicus* does not restore its amylase and cellulase activities to the active phase levels upon re-feeding. These and the other findings of this study are discussed below.

The inactive phase is considered an indispensable state in the life history of *A. japonicus* (Li et al., 1996; Liu et al., 1996). Mitsukuri (1903) was the first to report that *A. japonicus* entered a period of inactivity and stopped feeding in late July when ocean temperatures warmed.



**Fig. 3.** Pepsin (a) and trypsin (b) activities as a function of date in *A. japonicus*. Values are means ( $\pm$ SEM,  $n=3-7$ ). Values were compared among sampling dates with ANOVA followed by a Tukey's HSD with a family error rate of  $P=0.05$ . Values that share a superscript are not significantly different.

The commencement of this inactive period has been denoted by a lack of sea cucumbers visible in the habitat (Mitsukuri, 1903; Tanaka 1958a,b), as well as by an absence of digesta in their guts (Li et al., 1996). Our results with farm-raised cucumbers largely corroborate these findings, as gut content mass decreases with increase in



**Fig. 4.** Lipase activity as a function of date in *A. japonicus*. Values are means ( $\pm$ SEM,  $n=3-7$ ). Values were compared among sampling dates with ANOVA followed by a Tukey's HSD with a family error rate of  $P=0.05$ . Values that share a superscript are not significantly different.

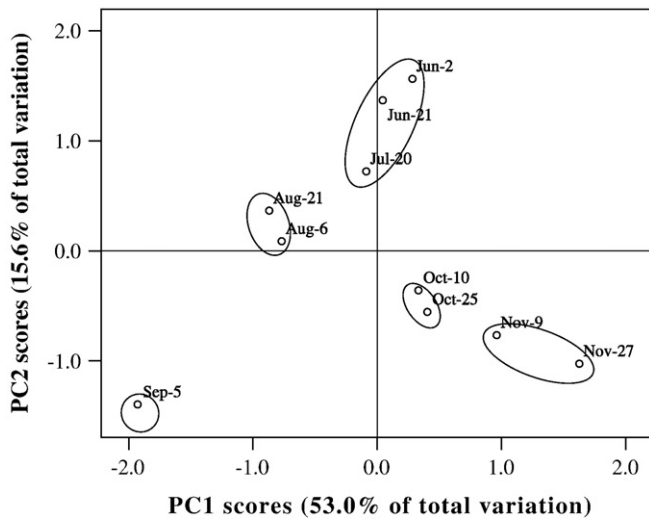


Fig. 5. Scores from a PCA model of digestive enzymes, indexes of digestive tract (RGM, RGCM and ZI). The clusters illustrated in the score plot points out the annual life cycle of *A. japonicus*.

temperature, and the animals become less active (FG, pers. obs.). Many authors have called the inactive phase in *A. japonicus* a period of “aestivation” (e.g., Sui and Liao, 1988; Liu et al., 1996; Yang et al., 2005). However, aestivation is defined as a period of hypometabolism during which animals cease feeding and movement, all in response to elevated temperatures and arid conditions (Storey, 2002). Thus, most animals that truly aestivate are terrestrial. Yang et al. (2006) provided some evidence that *A. japonicus* might enter a state of hypometabolism at higher water temperatures (25–30 °C), showing that cucumbers decrease oxygen consumption and nitrogen excretion rates by approximately 30% and 25%, respectively, compared to maximal values for these parameters observed at 20 °C. But, the animals were not held under these conditions for a long enough period of time to call it a true hypometabolism. And, Yuan et al. (2007) showed that *A. japonicus* decreased the amount of energy allocated towards respiration at 30 °C in comparison to lower water temperatures. Thus, although it is tempting to call the inactive phase in *A. japonicus* “aestivation”, more definitive evidence of hypometabolism is necessary to do so. What is clear is that the cucumbers cease feeding, in the wild and in the laboratory, in response to elevated water temperatures, even when food quality and quantity are held constant (Li et al., 1996; Yang et al., 2005; Yuan et al., 2007). If the cucumbers did not lower their metabolic rate in response to these elevated temperatures, as is suggested by Yang et al. (2006), then the cucumbers would have to increase food intake to meet their increasing metabolic demands. An increase in intake would cause an increase in gut size (Karasov and Martinez del Rio, 2007). However, we observed a decrease in gut size and intake, as evidenced by a decrease in gut content mass during the period of inactivity.

Choe (1963) divided the annual life cycle of *A. japonicus* into four phases: an active phase, prophase of inactivity, peak inactivity (which Choe called “aestivation”), and a reversion phase, during which the animals recover to active phase form. Our results support the presence of these four phases, as the principle components analysis produced five main clusters: June–July represent the tail end of the active phase and are represented by higher carbohydrase activities and ZI; August represents the prophase of inactivity during which the water temperatures were the highest (29.6 °C on 6 August) and there were decreases in carbohydrase activities, gut size, and gut content mass; September is the peak of inactivity, characterized by the highest pepsin and lowest carbohydrase activities, and the smallest gut size and gut content mass; and October–November (two clusters combined) represent the reversion phase, during which most of the

enzyme activities, and certainly food consumption (RGCM) and the gut size relative to body size, all increased. Clearly, each of these phases has its own characteristics based on gut structure and function. Piersma and Drent (2003) define phenotypic plasticity of this type as “life-cycle staging”, in which an animal undergoes predictable, reversible seasonal changes in some component of its physiology or morphology.

Water temperatures between 20 and 30.5 °C have been reported to elicit periods of inactivity in *A. japonicus* (Sui and Liao, 1988; Liu et al., 1996; Yang et al., 2005). We observed that cucumbers entered the inactive phase only after the water temperatures had been above 25 °C for over one month. Although the timing and duration of the periods of inactivity can vary with latitude, indicating that photoperiod may also be an important abiotic trigger, it appears that a lasting increase in water temperature is key in eliciting the inactive phase in *A. japonicus*. Additionally, the data we provide on the various annual phases in *A. japonicus* suggest that water temperature and animal movement may not be the best indicators of the onset of the inactive phase, but rather, a decrease in gut content mass in concert with drops in body mass and gut structure and function. In support of this, the carbohydrase activities (amylase, cellulase, and alginase) were all significantly (positively) correlated with ZI (amylase  $r=0.74$ ,  $P=0.015$ ; cellulase  $r=0.65$ ,  $P=0.04$ ; alginase  $r=0.70$ ,  $P=0.02$ ). Therefore, future analyses of the periods of inactivity in *A. japonicus* should take these characteristics into account.

Overall, the trends in digestive enzyme activity observed in *A. japonicus* during the inactive phase are consistent with data from other animals, either in aestivation, or under food deprivation. Amylase activity has been observed to decrease during starvation in a palinurid lobster (Johnston et al., 2004) and a panaeid shrimp (Comoglio et al., 2004), and a drop in  $\beta$ -glucosidase activity (which is always associated with cellulase) during aestivation has been observed in a terrestrial snail (Umezurike, 1976). Amylase (Sellos and Van Wormhoudt, 2002) and cellulase (Watanabe and Tokuda, 2001; Lo et al., 2003) are known to be of endogenous origin in invertebrate animals. Thus, the decrease in these endogenous enzymatic activities during a time of food deprivation makes sense in the absence of substrates for the enzymes because there would be no payoff from their production (Karasov, 1992; Caviedes-Vidal et al., 2000). On the other hand, alginase is known only from microbial sources in the digestive tracts of invertebrates (Monje and Viana, 1998) and fishes (Skea et al., 2005; Skea et al., 2007), which may help explain the variable, but continually detectable activity of this enzyme throughout the experiment. Although the animals began feeding and gaining mass during the reversion phase, their amylase and cellulase activities did not recover to levels observed in June and July (active phase), at least within the

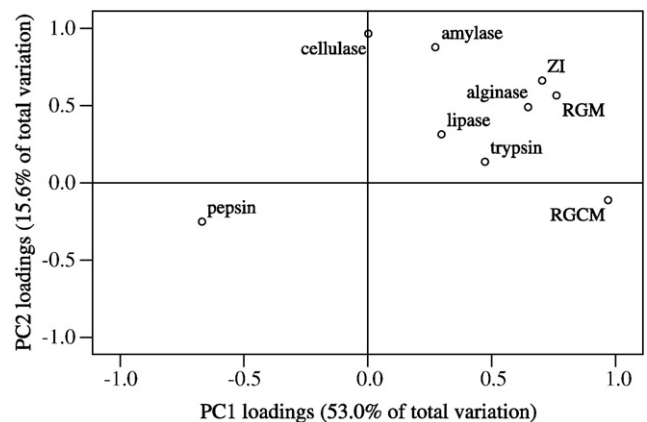


Fig. 6. Loadings from the corresponding PCA model in Fig. 5, showing the correlation between the digestive enzymes, RGM, RGCM and ZI.

time frame that we took measurements. It is unknown why the levels of these enzymes did not recover after the periods of inactivity, as did the activities of alginase and lipase. Amylase (Comoglio et al., 2004) and cellulase (Monje and Viana, 1998) activities are responsive to substrate level in the diet, and perhaps the food resources of the cucumbers were lower in starch and cellulose in October and November than they were in June and July. But, this is purely speculative, as we did not measure the starch and cellulose contents of the sediment on the farm over the course of the experiment.

Quite different from the other enzymes, pepsin activity suddenly spiked during the period of inactivity (September 5), to a much higher level than during the active phase. To our knowledge, a similar phenomenon has not been found in other animals, either in dormancy or under food deprivation, as this enzyme's activity typically decreased (Tanaka et al., 1999) or remained constant (Gildberg, 2004) under food deprivation. However, a significant increase in pepsin activity was observed when *A. japonicus* were cultured at 28 °C for 40 days, during which time the sea cucumbers gradually ceased feeding and entered the inactivity phase (Gao et al., unpublished). Our results are also consistent with Fu et al. (2006), who found that pepsin activity in *A. japonicus* increased noticeably from June to July, with the inactivity phase in their study occurring between July and August. The increase in pepsin activity during food deprivation may simply reflect an abundance of pepsinogen stored in gastric tissue. The overall decrease in gut mass, then, would result in an increase in pepsin activity per mg protein in our *in situ* assays of homogenized gastric tissue. We argue, however, that if this is the case, the patterns of all of the enzymes should have been the same. Instead, only pepsin activity clearly spikes during the peak period of inactivity, and the reasons for this remain unknown. Judd (2001) reported spikes in protease, esterase, and lipase activities in the early spring in a deposit-feeding polychaete (*Arenicola brasiliensis*), following a time of low food availability. But the increase observed by Judd (2001) was likely correlated with an increase in food availability in the early spring, unlike the spike in pepsin during an absence of food as we observed. The function of high pepsin activity during the period of inactivity warrants further investigation.

Interestingly, lipase activities were not lowest in September during the inactive phase, but later, during the reversion phase. This does not necessarily mean that lipids were not readily available in the diet, but may actually suggest that there were more lipids in the sediment following the inactive phase. Support for this comes from the observation that herbivorous fishes, which by nature consume low-lipid foods, have often been found to have higher lipase activities than carnivores, which consume a high-lipid diet (see German et al., 2004 for a discussion of high lipase activities in herbivorous fishes). The "limiting nutrient hypothesis" argues that when a resource is limiting, the activity for the enzymes that digest (or absorb) that resource will be high, but when the resource is readily available, the enzyme activities will be lower. This has been observed for epithelial mineral and vitamin transporters in vertebrates (Karasov and Hume, 1997) and may explain the patterns of lipase activities in fishes, and perhaps, in the cucumbers. This argument is being extended specifically for lipids in herbivorous and detritivorous animals, and not for other enzymes, which show more correlation with ingested substrates. We remain uncertain, though, as to why the cucumbers decreased, significantly so, their lipase activities during the reversion phase in October.

In conclusion, we found that *A. japonicus* undergoes significant changes in gut structure and function during periods of inactivity, including a cessation of feeding, elicited by elevated water temperature. This animal clearly exhibits phenotypic plasticity (or life-cycle staging; Piersma and Drent, 2003) in gross gut morphology and digestive enzyme activities in relation to food availability on a seasonal scale. Further work is necessary to evaluate why specific enzymes behaved in different fashions (e.g., why pepsin activity spikes in the feeding cessation period, and why lipase activity drops during reversion), and to further characterize the abiotic triggers of periods of inactivity in this

species. At any rate, this study suggests that during the summer months, *A. japonicus* will act poorly as a filter in polyculture systems, and should not be captured for human consumption during this period, as the cucumbers may be of the lowest nutritional quality at that time.

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