Stable isotope signature and pigment biomarker evidence of the diet sources of *Gaetice depressus* (Crustacea: Eubrachyura: Varunidae) in a boulder shore ecosystem

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**Abstract:** *Gaetice depressus*, a varunid crab common on intertidal boulder shores, is a potential key organism for monitoring organic matter flow through the food web. In order to elucidate its biogeochemical role, the diet source and trophic position of this crab on the boulder shore of an island off the Izu Peninsula, Japan, were estimated using three approaches: foregut content examination, stable isotope signature (δ¹³C and δ¹⁵N) and pigment biomarkers. The results suggest that *G. depressus* utilizes green macroalgae (*Ulva* sp., *Ulvaria* sp.) as its main diet source together with red macroalgae (*Gloiopeltis complanata*, *G. furcata*). This crab also utilizes periphytic and planktonic microautotrophs (occasionally tissues of heterotrophs) when macroalgae prove insufficient due to seasonal change. Therefore, *G. depressus* can be considered to be an omnivore since it consumes both autotrophs and heterotrophs, although it obtains organic matter mostly from autotrophs.

**Key words:** diet source, foregut content, *Gaetice depressus*, pigment biomarker, stable isotope

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**Introduction**

Boulder shores comprise an ecosystem characterized by the presence of boulder layers forming a three-dimensional habitat (both surfaces and interstices). Boulders can 1) provide an increase in the surface for organism attachment, 2) slow down water currents, 3) accumulate detritus important as a temporal sink for fine organic particles, and 4) provide refuge for animals (Menge et al. 1985, Chapman & Underwood 1996, Takada 1999).

*Gaetice depressus* (De Haan, 1833), a common intertidal crab in East Asia (Ng et al. 2008), actively feeds during high tide and hides under boulders when they are uncovered at low tide (Depledge 1989, Davie & Ng 2007). It is very common on boulder shores along the Japanese coast (Fukui 1988, Sakamoto et al. 2006). Lohrer et al. (2000) stated that in the boulder shores of Japan, *G. depressus* is the most abundant of four common crab species, the other three being *Hemigrapsus sanguineus* (De Haan, 1835), *Leptodius exaratus* (H. Milne Edwards, 1834) and *Acmameopleura parvula* Stimpson, 1858. Its presence at high density suggests its importance in the biogeochemical cycle (including organic matter flow) in boulder shore areas (Nienhuis 1981).

Several ecological and behavioral studies on *G. depressus* have been conducted, investigating the molting process (Satoh and Suzuki 1985), mate choice behavior (Fukui 1995), feeding behavior (Depledge 1989), and anti-predator behavior (Sakamoto et al. 2006). However, little attention has been paid to its diet sources. On the one hand, De-
pledge (1989), who studied the feeding behavior of *G. depressus*, defined this species as omnivorous since it feeds on particulate organic matter (POM) in seawater and pieces of fish or mollusk flesh, according to behavioral observations in the laboratory. Davie & Ng (2007) also reported that this crab is a suspension feeder utilizing POM. It is also defined as herbivorous, utilizing phytoplankton as a diet source (Wolcott & O’Connor 1992). On the other hand, Mima et al. (2003) carried out an experimental study on prey-predator relationships and found that *G. depressus* is the main predator of a hermit crab, *Pagurus filholi* (De Man, 1887). Thus, the exact role of *G. depressus* in boulder shore food webs and the associated biogeochemical materials cycle is still uncertain.

To elucidate the diet source and trophic position of heterotrophic organisms, several approaches have been applied. Foregut content examinations can directly detect the actual diet consumed and has been widely employed (e.g. Cannici et al. 1996, Lawal-Are 2009) but the results sometimes reflect short-term scale sources and structurally stable diets. Stable isotope analysis of carbon and nitrogen ($\delta^{13}C$ and $\delta^{15}N$) is now used to estimate the prey-predator relationship (e.g. Nadon & Himmelman 2010, Bergamino et al. 2011) because stable isotope ratios reflect the long-term assimilated diet of an animal from its major dietary sources (Vander-Zanden & Rasmussen 1999, Post 2002).

High-performance liquid chromatography (HPLC) for plant pigment analysis has also been applied to determine the pigment composition of the contents of digestive organs as a possible tool to determine diet sources (e.g. Haug et al. 2003, Howell et al. 2004).

In the present study, samples including macroalgae, macrozoobenthos (including *G. depressus*), periphyton, and particulate organic matter (POM) were collected at a typical Japanese boulder shore. Stable isotope ratios and pigment composition were examined to estimate the major diet source of *G. depressus* in combination with foregut content analysis.

**Materials and Methods**

**Study site and sampling point**

This study was carried out on a boulder shore area of Ebisu Island, Suzaki, Shimoda, Shizuoka Prefecture, Japan (34°39′9″ N 138°57′54″ E; Fig. 1). The rocky shore of Ebisu Island can be divided into three regions, as defined by Raffaelli & Hawkins (1996): an exposed, flat rocky shore (southern and eastern, where cliff-rock dominates); a semi-exposed rocky shore (western); and a sheltered boulder-shore area (northern). Samples of benthic organisms were collected at the sheltered boulder-shore area on the north-

![Fig. 1. Map of Ebisu Island and the sampling site.](image)
ern side of Ebisu Island.

Three transect lines (each 23 m long) were set orthogonal to the shore line, 8 m apart. Two quadrats (50×50 cm) were set at the low and middle tidal area along each line (Fig. 2), for a total of 6 quadrats, where an abundant population of *Gaetice depressus* was confirmed by previous observations. The average time of submergence in seawater was 13.8±3.3 h and 12.2±0.3 h for the low and middle tidal areas, respectively.

**Sample collection**

Samples for stable isotope analysis were collected 10 times: in 2010 (April, May, June, August and September) and in 2011 (February, June, August, September and November). Samples for foregut content examination were collected 4 times: in May, June and September 2010, and in February, 2011. Samples for pigment analysis were collected twice for foregut content (May 2010 and June 2011) and 3 times for POM, periphyton and macroalgae (February, May and June 2011). Samples were stored at −80°C for pigment analysis and at −20°C for stable isotope analysis.

*Gaetice depressus* samples were collected at all 6 sampling points. Crab size (carapace width) ranged from 1.4 to 2.0 cm, mean 1.6 cm±0.2 SD. The crabs collected were cleaned with fresh seawater, placed on ice, transferred to the laboratory and stored for further analysis. For stable isotope analysis, one crab from each sampling point was used (total 6 individuals per sampling). The tissues within the abdominal and posterolateral parts of each individual were carefully dissected free, avoiding the digestive organs. Although it is possible that exoskeleton may affect the δ13C and δ15N values (DeNiro & Epstein, 1978; Carabel et al. 2006), only small amounts of exoskeleton from abdominal tissue was included. Tissues containing muscle and exoskeleton were dried to a constant weight at 60°C, then ground to a fine powder using a mortar and pestle and stored in a vacuum desiccator for further analysis.

Foregut content samples from *G. depressus* were removed, mixed with 5 mL filtered seawater and filtered onto glass fiber filters (Whatman GF/F, precombusted at 450°C for 6 h). POM was collected from surface sea water near the sampling points. Six aliquots of water sample (500 mL) were passed through glass fiber filters. Boulders or cobbles were collected to obtain periphyton (epiphytic microalgae) adhering as a biofilm, which was removed to 50 ml filtered sea water using a nylon brush. Aliquots of the suspension (15 mL) applied to glass fiber filters. Macroalgae (2 brown species; 7 red species; 6 green species) were collected by hand and transferred to the laboratory, then rinsed with fresh seawater to remove attached detritus and small organisms such as amphipods. Six species of macroalga were used, since they were the species observed to be abundant during 2010 and 2011: *Gloiopeltis complanata* (Harvey) Yamada, 1932; *Gl. furcata* (Postels & Ruprecht) J. Agardh, 1851; *Monostroma* sp.; *Ulva pertusa* Kjellman, 1897; *Ulva* sp.; and *Ulvaria* sp.

Macrozoobenthos other than *G. depressus* (crustacean, mollusk and polychaete species) were collected at the sampling point. Small-size crustaceans (*Pagurus filholi* and *Petrolithes* sp.) and polychaetes were kept for about three hours to empty the foregut (microscopic observation showed that a little foregut content remained after three hours). The whole body of each animal was then dried to a constant weight at 60°C. Two or three individuals were pooled together, then homogenized into a fine powder using a mortar and pestle. For large-sized crustaceans such as *Hemigrapsus sanguineus* and xanthoid species, tissues (including muscle and small pieces of exoskeleton) were collected from abdominal-posterolateral regions and from the chelipeds for determination of isotope ratios. The soft tissues of mollusks were removed from the shell, rinsed with fresh seawater, and muscle tissue was separated from the digestive organs for isotopic analysis. Further procedures for analysis were the same as for *G. depressus*.

![Fig. 2. Sampling points and quadrat design.](image-url)
Foregut contents examination

*Gaetice depressus* collected from the sampling point were dissected carefully under a binocular microscope to remove the foregut, which was subsequently opened, its contents diluted with filtered sea water (1 mL) in a petri dish for detailed examination under the microscope.

The relative degree of fullness (F), point scale of the content (PCT), and the number of content items (N) were examined. F was assessed visually and given one of the following five percentage classes (modified from Wear & Haddon 1987, Cannicci et al. 1996): 100% (full foregut), 75% (100%>F>65%), 50% (65%>F>35%), 25% (35%>F>5%) and 5% (only trace or empty). Then, 1 mL mixed foregut content was placed on a line-grid (50 lines) glass slide and observed under a binocular microscope to count the total number (N) of each category of foregut contents. The relative contribution (defined as PCT) of the foregut content categories to the total content (N) of each foregut was assessed on a five point scale: 0.05, 0.25, 0.50, 0.75 and 1.00 (following Wear & Haddon 1987, Cannicci et al. 1996). The total point value of each content (G) was weighted according to the actual fullness of the foregut in which it was found (\(G = \text{PCT} \times F\)).

Frequency of occurrence (FO) was assessed according to the presence of the foregut content category (Williams & Haddon 1987, Assis 1996). Geometric Importance Index (GII; Assis 1996) was determined to obtain the diet preference of *G. depressus* according to Amundsen et al. (1996). Geometric Importance Index (GII; Assis 1996) was determined to obtain the diet preference of *G. depressus* using three indices: total point value (G), frequency of occurrence (FO) and specific abundance (Pi). For instance, a foregut content category which had a G, FO and Pi value of 5%, 8% and 10%, respectively, will have a GII value of 17.3 (calculated based on the equation of Assis 1996 below).

\[
\text{GII}_j = \frac{\sum_{i=1}^{n} V_i}{\sqrt{n}}
\]

where \(GII_j\): index value for the \(i^{th}\) foregut content categories; \(V_i\): the vector for the \(i^{th}\) indices (G, FO, Pi) of the \(i^{th}\) foregut content categories; \(n\): the number of indices that will be used (\(n=3\) in case of three indexes).

Stable isotope ratio of organic carbon and nitrogen

Powdered samples of macrozoobenthos including *Gaetice depressus* were pretreated following Teece and Fogel (2004). Samples (POM, periphyton or foregut) dried on filter paper were acidified by exposure to the fumes from 11.25 M HCl for 1 minute. Acidification was not applied to powdered samples to avoid alterations in isotopic measurement (Jacob et al. 2005, Serrano et al. 2008, Bergamino et al. 2011). No treatment to remove the lipid materials was applied. The filter and powder samples were put into tin capsule (D=9 mm, h=10 mm, 0.63 mL, Ag99.99; Ludi Swiss AG). Stable isotope ratios of carbon and nitrogen were measured by an isotope ratio mass spectrometer (Delta Plus, Finnigan MAT) coupled with an elemental analyzer (EA1108, Carlo Elba).

Isotope data were expressed in the \(\delta\) notation as follows:

\[
\delta (\%o) = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1\right) \times 10000
\]

where \(R\) is the ratio of \(^{13}\text{C}/^{12}\text{C}\) or \(^{15}\text{N}/^{14}\text{N}\), \(R_{\text{sample}}\) is the count in the sample, and \(R_{\text{standard}}\) is that in the Vienna-Pee Dee Belemnite-limestone standard and atmospheric N\(_2\) (Bodin et al. 2007, Wan et al. 2010).

The data on the stable isotope ratio collected in 2010 and 2011 were pooled to seasonal groups in order to obtain the mean value which represent each season; spring 2010 (April and May), summer 2010 (June and August), autumn 2010 (September), winter 2011 (February), summer 2011 (June and August) and autumn 2011 (September and November).

The proportional contribution of potential diet sources was determined using a Bayesian analysis of stable isotope mixing models using sampling-importance-resampling (SIR), using MixSIR ver. 1.0.4 (Semmens and Moore 2008). The value of each contribution was obtained using a Dirichlet distribution as an uninformative prior \((A=1)\). The median value (50th percentile) was used to determine the contribution (over the range 0 to 1), assuming the median to be the highest posterior probability value (Moore and Semmens 2008: although when the posterior probability distribution is multimodal, this percentile may not adequately describe the posterior probability surface of the source contributions; see Semmens and Moore 2008).

Pigment analysis

For pigment composition analysis, Chlorophyceae (*Ulva* sp. \(n=2\) and *Monostroma* sp.; \(n=1\)), Rhodophyceae (*Gloiopeitlisc complanata*; \(n=1\), *Gl. furcata* \(n=2\) and *Gelidium elegans* Kützing, 1868; \(n=1\)), filter samples of POM (\(n=3\)), periphyton (\(n=6\)) and foregut content (\(n=10\)) were each placed in a vial to which was added 2 mL analytical canthaxanthin (internal standard). The vial contents were then homogenized using an ultrasonic homogenizer for three minutes and filtered using a syringe cartridge filter (Whatman PTFE filter, 0.45 \(\mu\)m). The filtrate was placed in a clean vial and mixed 1:1 (400 \(\mu\)L: 400 \(\mu\)L, \(v/v\)) with Tetrabutyl Ammonium Acetate (TBAA) solution ready for analysis by HPLC. Macroalgae from the groups Chlorophyceae (*Ulva* sp., \(n=2\) and *Monostroma* sp., \(n=1\)) and Rhodophyceae (*Gloiopeitlisc complanata*, \(n=1\); *Gl. furcata*, \(n=2\); *Gelidium elegans* Kützing, 1868; \(n=1\)) were analyzed in a similar manner, using samples of approx. 1 mg. These were each homogenized using an ultrasonic homogenizer, then prepared as for the filter samples.

High-performance liquid chromatography was per-
formed with an Agilent 1100 series HPLC system, using a ZORBAX Eclipse XDB-C8 4.6 mm ID X 15 cm column and HPLC Chemistation LC 3D system software (Agilent Technologies). The analytical conditions (including the solvent system and gradient times) were followed Van Heukellem & Thomas (2001). The standard pigments used as identification references were chlorophyll $a$, $b$, $c_3$, fucoxanthin, zeaxanthin, alloxanthin, 19-hexanoloxyfucoxanthin, peridinin, chlorophyllide $a$, pheophorbide $a$ and pheophorbide $a$ (Sigma Aldrich). Values for the concentration of the different pigments were calculated using factors from standard analyses of these authentic pigments.

The data on pigment concentration were pooled as a mean value for each category rather than for individual taxa (i.e. foregut content, POM, periphyton, Chlorophyceae and Rhodophyceae). Major peaks were identified for the samples except for foregut contents, but substantial chromatographic peaks for the pigments listed above were not identified, probably due to degradation of pigment structure in the foregut. In the present study, only the pigments identified are shown. Peaks for non-identified pigments were ignored.

Statistical analysis

The Shapiro-Wilk normality test was performed to estimate whether data were obtained from normal distributions. For data with apparently adequate normal distribution, one-way analysis of variance (ANOVA) or $t$-test was then performed. Tukey’s Honestly Significant Difference (HSD) test was performed as post hoc analysis used in conjunction with ANOVA to find which means are significantly different from one another. The Welch two sample $t$-test was used where the true ratio of variances differed from unity. An F-test was applied to obtain the ratio of variances. Where data were not obtained from a normal distribution or when the number of samples was small, the Kruskal–Wallis rank-sum test or Mann–Whitney–Wilcoxon test was used. Statistical analyses were conducted using R statistical software ver. 2.14.1 (R Development Core Team 2011).

Results

Foregut content examination

Twelve categories of foregut contents of *Gaetice depressus* were identified, as shown in Fig. 3. All categories were found in the foregut content samples taken in spring 2010, with fewer categories observed in other seasons. However, Chlorophyceae, animal tissues and unidentified tissues were found in all seasons.

The GII value of macroalgae is the highest compared to other foregut content categories for all seasons observed. High values around 100 were found in summer 2010 and winter 2011, but the values in spring and autumn 2010 were relatively low, at around 40. Other autotrophic organisms showed lower values (0 to around 20). Other autotrophic organisms including Centrales and Pennales were found as a secondary important group, although they were not found in the summer of 2010. Planktonic Chlorophyceae and Dinophyta also comprised foregut contents. Total values of autotrophs other than macroalgae varied over the GII range 7.9 (summer) to 79.8 (spring): high values were found in spring and autumn, when they were higher than those for macroalgae.

Only a small fraction of the samples was identified as a particular type of animal tissue, and animal tissues were

![Fig. 3. Geometric importance index (GII) value for each foregut content category for spring 2010 (n=4), summer 2010 (n=3), autumn 2010 (n=4) and winter 2011 (n=5).](image-url)
not identified by specific taxonomic groups for summer 2010, autumn 2010 or winter 2011. Arthropoda, Crustacea and Echinodermata were only found in spring 2010, but at low incidence (GII less than 10). The total value of heterotrophic organisms showed a mean GII of 27 throughout the observation period, much lower than that of autotrophic organisms (mean GII 118). The GII value of unidentified tissues, which were not distinguished as plants or animals, ranged from 20 to 45 throughout the study.

Stable isotope ratio of carbon and nitrogen

The data of the stable isotope ratio are presented in Table 1. Dual isotope plots of δ13C and δ15N (mean±SD) for Gaetice depressus and other organisms are shown in Fig. 4.

Gaetice depressus

The δ13C value of G. depressus ranged from −15.3±1.9 to −11.7±0.4‰ throughout the observations with an overall mean of −13.1±1.8‰. There were significant differences in the δ13C values at the p<0.05 level among the seasons. Post-hoc comparisons using Tukey’s HSD test (CI 95%) indicate that the mean value of δ13C in winter 2011 (−15.3±1.9‰) was significantly lower than the others. A slightly lower (−13.5±0.4‰) value than those in the other seasons was noticed in summer 2011. The δ15N value of G. depressus ranged from 7.4±0.5 to 9.1±2.4‰, with an overall mean of 7.8±1.4‰, and no significant seasonal differences were obtained at the p<0.05 level among the δ15N values.

Autotrophic organisms

The biomass of all macroalgae on the study site including the six dominant species (Gloiopeitlis complanata, Gl. furcata, Monostroma sp., Ulva pertusa, Ulva sp., and Ulvaria sp.) decreased during summer and autumn. The stable isotope ratio of macroalgae could not be measured in autumn 2010. Other macroalgal species were found during the study periods (e.g. Chondrus sp. Gelidium elegans, Grateloupi sp.; Sargassum sp.; data not shown), but only at a certain times during study, and with a low frequency of occurrence.

The value of δ13C for periphyton ranged from −16.6±1.3‰ (summer 2010) to −12.8±1.6‰ (winter 2011) with relatively high values in winter and summer in 2011, whereas POM ranged from −16.7±0.9‰ (spring 2010) to −14.9±0.5‰ (winter 2011) with a little seasonal change. Within the five most abundant species of macroalgae, the δ13C value of Ulva sp. was measured three times with a range of −13.0±0.6‰ (summer 2011) to −10.5±0.3‰ (spring 2010), and the seasonal difference was not significant (Kruskal-Wallis rank sum test, p=0.129; Table 1). The other two green algae showed a high variability: δ13C for Ulvaria sp. ranged from −16.7±0.9‰ (winter 2011) to −10.1±0.5‰ (autumn 2011); and for Monostroma sp. from −22.4±1.9‰ (winter 2011) to −14.4±0.2‰ (summer 2010). Two abundant species of red macroalgae (Gloiopeitlis complanata and Gl. furcata) exhibited lower δ13C values than those of Ulva sp. and Ulvaria sp. but were comparable with Monostroma sp. The red alga Gl. complanata showed δ13C values ranging from −18.1±1.5‰ (winter 2011) to −12.2±2.4‰ (autumn 2011) and Gl. furcata from −16.4±0.0‰ (summer 2011) to −13.9±2.2‰ (spring 2010).

The δ15N values for periphyton ranged from 2.4±0.8‰ (winter 2011) to 6.8±1.3‰ (summer 2011) and for POM from 1.1±0.8‰ (winter 2011) to 6.0±0.9‰ (autumn 2010). Both groups showed low values in winter 2011. The δ15N values of three green algae (excluding Ulva pertusa) varied from 3.9±0.8‰ to 7.1±2.3‰ and were similar to the two species of red algae, Gl. complanata and Gl. furcata (3.7±0.5–7.3±1.6‰).

Estimation of the proportional contribution of autotrophs as a potential diet source for G. depressus is shown in Fig. 5. The graph of likelihoods for SIR of all seasons shows that the mixing model calculation is reliable since the graph is not heavily right-skewed (Fig. 5). However, the likelihood graph for autumn 2010 shows the bars are relatively aligned. A similar pattern is also shown for autumn 2011.

It is estimated that Gl. complanata contributed a median of 0.16 (0.01–0.38; this and the following represent the 5 and 95% confidence percentiles) and Monostroma sp. contributed a median of 0.17 (0.01–0.60) to G. depressus in spring 2010. However, the posterior distributions of Gl. complanata and Monostroma sp. were bimodal, indicating low reliability. At the 75% confidence percentile, Monostroma sp. and Gl. complanata contributed 0.45 and 0.38, respectively.

During summer 2010, Ulva sp. was contributing the most at a median of 0.40 (0.12–0.56) followed by periphyton which contributed a median of 0.30 (0.24–0.36), while Ulvaria sp. contributed a median of 0.20 (0.02–0.52). During autumn 2010, periphyton showed a highest median value of only 0.99 (0.94–0.99). Ulvaria sp. contributed 0.68 (0.01–0.83) in winter 2011, and the two species of red algae contributed low posterior probabilities. In summer 2011, Ulva sp. showed a relatively high contribution with a median of 0.31, similar to the summer of 2010. Almost the same contribution (median 0.39) was calculated for other green algae, Monostoromia sp. and Ulva sp., in summer 2011. Other than the macroalgae, periphyton and POM showed a high contribution in autumn 2011, with median values of 0.52 (0.13–0.81) and 0.30 (0.04–0.57), respectively.

Macrozoothens

Stable isotope ratios of organisms with high biomass or high frequency occurrence (3 Crustacea and 6 Mollusca species) are shown in Table 1.

For the Crustacea Hemigrapsus sanguineus, Pagurus filholi and Petrolisthes sp., the ranges of δ13C values were com-
Table 1. Values of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ (mean ± SD%) of *G. depressus* and corresponding organisms (autotroph and macrozoobenthos) for different seasons.

| Code | Species | Spring 2010 | Summer 2010 | Autumn 2010 | Winter 2011 | Summer 2011 | Autumn 2011 | One Way ANOVA* | Kruskal-Wallis* | Welch T-test* |
|------|---------|-------------|-------------|-------------|-------------|-------------|-------------|----------------|----------------|--------------|
| 6d   | G. depressus | −12.3 ± 1.0 a | 7.9 ± 1.3 a | −11.8 ± 0.4 a | 7.6 ± 0.6 a | −11.7 ± 0.4 a | 7.4 ± 0.5 a | −15.3 ± 1.9 b | 7.6 ± 1.8 a | −13.5 ± 0.4 ab | 9.1 ± 2.4 a | −12.3 ± 0.4 a | 7.7 ± 1.2 a | 1.62e−13 | 18.473 |
| X    | Estimated diet | −0.3 4.5 | −12.8 4.2 | −12.7 4.0 | −16.3 4.2 | −14.5 5.7 | −13.3 4.3 | 0.592 | 0.775 |
| p1   | Periphyton | −15.6 ± 1.6 ab | 5.1 ± 0.7 a | −16.6 ± 1.3 b | 5.0 ± 1.2 a | −15.6 ± 1.0 ab | 5.1 ± 0.7 a | −12.8 ± 1.6 a | 2.4 ± 0.8 b | −16.4 ± 2.4 ab | 6.8 ± 1.3 a | −13.1 ± 4.1 a | 4.8 ± 1.5 a | 0.004 | 4.020 |
| p2   | Particulate Organic Matter | −16.7 ± 0.9 a | 5.4 ± 1.6 a | −16.1 ± 1.1 a | 5.9 ± 0.1 a | −15.6 ± 0.6 a | 6.0 ± 0.9 a | −14.9 ± 0.5 a | 1.1 ± 0.8 b | −15.3 ± 1.1 a | 2.0 ± 0.0 ab | −16.2 ± 5.7 a | 5.0 ± 2.4 a | 0.897 | 0.321 |
| p3   | Autotroph (macro) | −16.4 ± 1.6 a | 7.2 ± 2.1 ab | −16.3 ± 0.1 a | 4.6 ± 0.2 b | −18.1 ± 1.5 a | 4.1 ± 0.6 b | −18.2 ± 2.4 a | 7.3 ± 1.6 a | 0.050 | 4.752 |
| p4   | Gloiopeltis furcata | −13.9 ± 2.2 a | 6.8 ± 0.2 b | −14.4 ± 0.4 a | 3.7 ± 0.5 a | −16.3 ± 1.9 a | 4.5 ± 0.5 a | −16.4 ± 0.0 a | 4.6 ± 0.3 a | 0.008 | 13.602 |
| p5   | Monostroma sp. | −15.4 ± 1.0 a | 6.7 ± 1.8 a | −14.4 ± 0.2 a | 5.0 ± 0.0 a | −22.4 ± 1.9 b | 5.3 ± 0.7 a | −16.2 ± 0.2 a | 7.1 ± 2.3 a | 0.048 | 0.321 |
| p6   | Ussia portuensis | −10.5 ± 0.3 a | 5.1 ± 1.2 a | −10.5 ± 0.0 a | 4.4 ± 0.1 a | −13.0 ± 0.6 a | 4.7 ± 0.0 a | 0.129 | 0.019 | 0.069 |
| p7   | Ussia sp. | −11.4 ± 0.2 a | 4.0 ± 0.2 a | −16.7 ± 0.0 a | 3.9 ± 0.8 a | −10.1 ± 0.5 b | 6.3 ± 0.5 a | 0.082 |
| p8   | Ussia sp. | −11.7 ± 0.2 a | 4.0 ± 0.2 a | −16.7 ± 0.0 a | 3.9 ± 0.8 a | −10.1 ± 0.5 b | 6.3 ± 0.5 a | 0.001 | 6.148 |
| c1   | Batillaria multiformis | −11.5 ± 1.0 ab | 7.3 ± 1.0 a | −11.7 ± 0.2 ab | 7.6 ± 0.8 a | −11.6 ± 1.0 ab | 7.8 ± 0.3 a | −13.0 ± 0.3 b | 8.0 ± 0.7 a | −10.0 ± 0.8 a | 8.1 ± 0.8 a | 0.012 | 4.515 |
| c2   | Hapalopila radiata | −12.7 ± 0.0 ab | 7.6 ± 0.0 ab | −11.3 ± 0.4 ab | 8.6 ± 0.9 b | −14.4 ± 0.0 ab | 7.5 ± 0.0 ab | −15.4 ± 2.5 a | 4.8 ± 0.2 a | −10.6 ± 0.7 b | 8.6 ± 0.9 ab | 0.038 | 5.995 |
| c3   | Gloriopeltis complanata | −12.2 ± 0.6 a | 7.2 ± 0.6 a | −13.1 ± 0.4 a | 10.9 ± 0.4 a | −11.5 ± 0.3 a | 8.0 ± 0.0 ab | −12.5 ± 0.3 a | 10.9 ± 1.1 a | 10.7 ± 1.0 a | 11.4 ± 0.7 a | 0.097 | 2.246 |
| c4   | Lunella cinerea | −12.9 ± 0.6 a | 7.0 ± 0.7 a | −12.7 ± 0.2 a | 7.1 ± 0.2 a | −14.1 ± 0.0 a | 7.6 ± 0.1 a | −11.6 ± 0.0 a | 6.4 ± 0.2 a | −12.9 ± 0.2 a | 7.0 ± 0.1 a | −14.7 ± 3.9 | 7.3 ± 1.6 a | 0.010 | 0.441 |
| c5   | Monostroma latirostrum | −10.8 ± 1.2 a | 9.2 ± 1.0 a | −10.7 ± 0.0 ab | 8.7 ± 0.0 a | −14.2 ± 0.1 b | 8.7 ± 0.2 a | −7.5 ± 0.7 c | 8.2 ± 0.3 a | −10.5 ± 0.0 ab | 9.6 ± 0.9 a | 5.89e−3 | 11.284 |
| c7   | Nerita japonica | −12.5 ± 1.1 a | 7.4 ± 1.1 a | −12.0 ± 2.0 a | 8.3 ± 0.1 a | −14.3 ± 0.1 a | 8.8 ± 0.2 a | −11.4 ± 0.5 a | 9.2 ± 1.1 a | 0.605 | 0.726 |
| c8   | Pagurus filfolis | −11.4 ± 0.5 ab | 7.3 ± 1.3 a | −11.0 ± 0.5 b | 7.2 ± 0.7 a | −10.9 ± 0.6 ab | 7.8 ± 0.2 a | −11.5 ± 1.5 a | 6.3 ± 0.4 a | −17.3 ± 1.6 ab | 8.3 ± 0.8 a | −9.9 ± 0.2 a | 8.5 ± 1.0 a | 0.154 | 2.229 |
| c9   | Patelloida nana | −13.2 ± 0.0 a | 6.1 ± 0.0 ab | −11.2 ± 0.1 a | 6.4 ± 0.7 b | −13.0 ± 0.2 a | 9.5 ± 1.6 ab | −11.2 ± 1.4 a | 11.0 ± 0.5 a | 0.069 | 1.915 |

Note: Standard deviation calculated for two replicates or more. —, no sample, or missing data.

*Statistical tests were performed in order to compare differences among the seasons for each organism (see Materials and Methods).

Letters ‘a’, ‘b’, or ‘c’ at the end of $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ values represent the difference among values seasonally. The same letter indicates no significant difference. Values without these letters indicate no significant seasonal differences. Frequency (F) and probability (p) values are shown as follows: $\delta^{13}\text{C}$ (bold) and $\delta^{15}\text{N}$ (normal).

*Estimated diet of *G. depressus* (X) was calculated by subtracting stable isotope value of *G. depressus* with 1% and 3.4% for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, respectively. For instance in spring 2010, value of $\delta^{13}\text{C}$ is −13.3% which obtained from −12.3%−13.6%, and value of $\delta^{15}\text{N}$ is 4.5% which obtained from 7.9%−3.4% (see Discussion).

Higher classification: 1 Rhodophyceae (red macroalgae); 2 Chlorophyceae (green macroalgae); Mollusca; 5 Crustacea. (Species of Phaeophyceae, Polychaeta, and other organisms of low or rare abundance are not shown).
parable with those of *G. depressus*: *Hemigrapsus sanguineus* ranged from −15.4±2.5‰ to −10.6±0.7‰; *P. filholi* from −17.3±1.6‰ to −9.9±0.2‰; and *Petrolisthes* sp. from −13.2±0.0‰ to −11.2±1.4‰. The δ¹⁵N values of these Crustacea were also comparable (4.8±0.7‰ to 11.0±0.5‰).

For the four species of Mollusca *Japeuthria ferrea* (Reeve, 1847), *Batillaria multiformis* (Lischke, 1869), *Monodonta labio confusa* (Tapparone-Canefri, 1874) and *Lunella cinerea* (Born, 1778), the δ¹³C values varied from −14.8±0.0 to −10.0±0.8‰. Two species of *Nerita* had δ¹³C values between −14.3±0.1‰ to −7.5±0.7‰, and seasonal changes were found in *Nerita albicilla* Linnaeus, 1758. Species *B. multiformis*, *L. cinerea*, *M. labio confusa*, *Nerita albicilla*, and *N. japonica* Dunker, 1860, showed δ¹⁵N values comparable with *G. depressus* (Fig. 4, Table 1). However, values for *J. ferrea* (ranging from 11.0±1.1‰ to
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12.3±0.1% (mean value is 11.2±0.7%) were higher than for *G. depressus* and for those of the abovementioned macrozoobenthos species.

**Pigment analysis**

The concentration of pigments including chlorophyll *a*, -*b*, -*c*3, pyropheophorbide *a*, pheophorbide *a*, chlorophyllide *a*, fucoxanthin, alloxanthin, zeaxanthin and lutein were estimated from the samples (foregut contents, POM, periphyton, Chlorophyceae and Rhodophyceae) and are shown for each sample category in Fig. 6. The foregut content of *Gaetice depressus* contained 8 pigments, with pheophorbide *a* at the highest concentration followed by pyropheophorbide *a* and chlorophyllide *a*. Chlorophyll *a* and -*c*3 were not found in the foregut contents, and it is noteworthy that the former was abundant in potential diet sources.

Chlorophyll *a* was found in all other groups (POM, periphyton, Chlorophyceae and Rhodophyceae) at the highest concentration. In the Chlorophyceae, chlorophyll *b* was the most abundant pigment, while in POM, accessory pigments such as fucoxanthin and alloxanthin were at relatively high concentrations. Other pigments such as chlorophyll *c*3, chlorophyllide *a*, zeaxanthin and lutein were only found at low concentrations (Fig. 6).

**Discussion**

**Potential diet source of *Gaetice depressus***

It has been proposed that isotope fractionation occurs at an increase in trophic position: 3.4% for nitrogen (DeNiro and Epstein 1981, Minagawa & Wada 1984, Post 2002) and 1% for carbon (DeNiro and Epstein 1978, Schaal et al. 2008). Using these fractionation values, the isotope ratios of potential diet sources of *Gaetice depressus* were estimated and are shown as X in Fig. 4. The isotope ratios of X varied from −16.3 to −12.7% for δ13C and from 3.9 to 5.7% for δ15N throughout the present study. The narrower variation in δ15N than that in δ13C suggests that the trophic
position of *G. depressus* did not change drastically among seasons. The higher range of $\delta^{13}C$, on the other hand, indicates seasonal variability of diet sources.

The $\delta^{15}N$ value of the potential diet sources of *G. depressus* in summer (2010 and 2011) and winter (2011) agreed well with those of macroalgae. Although the $\delta^{13}C$ values of macroalgae varied depending on the species, the value of X was located at about the center of the range among the autotrophs in these three observations. This probably reflects the fact that *G. depressus* consumes several species of macroalgae and is not limited to a single species.

The MixSIR analysis indicates that *G. depressus* mainly uses two genera of macroalgal Chlorophyceae in summer: *Ulva* sp. and *Ulvaria* sp. in summer 2010, and *Ulva* sp. and *Monostroma* sp. in summer, 2011. However, in winter 2011, *Ulvaria* sp. was the main diet source, along with minor contributions from two red algae species, *Gloiopeilis compalanata* and *Gl. furcata*. Foregut content examination analysis in summer 2010 and winter 2011 also indicates a high contribution of macroalgae, with a high GII of about 100 in both seasons and a low GII for other autotrophs such as diatoms and Dinophyta. These findings agree well with the results of carbon and nitrogen stable isotope ratios.

Pigment composition of foregut content can provide information on the diet sources of *G. depressus* from another viewpoint, although the analysis was done only in the summer season. The amount of chlorophyll *a* in the foregut was small, though it was the most abundant pigment ex-
cept in the Chlorophyceae (in which chlorophyll $b$ was found to be the most important pigment). This is presumably due to degradation of chlorophyll $a$ during the digestion process (Howell et al. 2004, Yakobi & Ostrovsky 2008), since degradation products such as chlorophyllide $a$, pheophorbide $a$ and pyropheophorbide $a$, were abundant. Since chlorophyll $a$ is not unique to any particular algal group, it cannot be used as a biomarker to elucidate the particular diet source. Other pigments such as chlorophyll $b$, fucoxanthin, alloxanthin, zeaxanthin and lutein, however, are known as pigments specific to certain groups. The presence of chlorophyll $b$, zeaxanthin and lutein in the foregut content is probably due to ingestion of green (chlorophyll $b$) and red algae (zeaxanthin and lutein; Jeffrey and Vesk 1997, Howell et al. 2004). The results obtained from pigment analysis indicating that macroalgae are a diet source for $G. \text{depressus}$ are comparable with those from the stable isotope ratios and the foregut contents discussed above. However, the $\delta^{15}N$ of X was not comparable with those of macroalgae in spring 2010 and autumn 2011: the $\delta^{15}N$ of macroalgae was substantially higher than the estimated diet sources (X) of $G. \text{depressus}$, though the $\delta^{13}C$ values are within, or close to, those of macroalgae. In the spring season, macroalgal biomass was higher than that in autumn but similar to that in summer. The MixSIR results indicate that Monostroma sp. and Ulva sp. were possible diet sources in spring 2010, but their $\delta^{15}N$ values higher than X suggest that other diet sources with lower $\delta^{15}N$ than these macroalgae were also important. Considering that periphyton and POM exhibited $\delta^{15}N$ values comparable with X, it is conceivable that these microautotrophs are possible diet sources in this season as indicated by the MixSIR analysis. The lower contribution of macroalgae in summer was also found in the foregut content analysis. The GII value of macroalgae in spring 2010 was less than 50 while periphytic and planktonic microautotrophs, such as diatoms, Dinophyta and Nostocales, exhibited higher GII values. The total GII of microautotrophs (79.8) in spring 2010 was higher than that of macroalgae (41.9), indicating the relative importance of periphyton in this season.

Only a few species of macroalgae were observed in autumn 2011 and no sample was collected in 2010. The $\delta^{15}N$ value of $G. \text{depressus}$ in the autumn season, almost the same as in other seasons, suggests that $G. \text{depressus}$ mainly uses primary producers in its diet. Thus, it is conceivable that periphytic and planktonic microautotrophs are the major diet sources. This agrees with the high GII values of microautotrophs (63.6, including diatoms, Chlorophyceae and Dinophyta) in the foregut content analysis. However, the $\delta^{13}C$ values of periphyton and POM were generally lower than that of X, except in autumn 2011. The results of the MixSIR analysis also suggest that the estimation in autumn is less reliable, as shown in the pattern of Relative Likelihood (Moore and Semmens 2008; Semmens and Moore 2008). These findings indicate that other diet sources, whose samples were not collected for isotope analysis, were probably used by $G. \text{depressus}$ in autumn. Foregut content analysis indicates that $G. \text{depressus}$ used macroalgae even in autumn 2010, despite the low macroalgal biomass. In the autumn season, macroalgal fragments were found between and under boulders. Considering that macroalgae were found in the foregut contents, there is a possibility that $G. \text{depressus}$ uses detrital macroalgal fragments, with $\delta^{13}C$ comparable with or higher than X, in autumn.

Foregut content analysis indicates that $G. \text{depressus}$ also uses animal tissues (Fig. 3). Although little taxonomic information was available in most seasons, animal tissues were identified in every observation. It is likely that this crab consumes dead (detritus) instead of living macrozoobenthos for two reasons: first, the cheliped structure is not adapted for attacking hard shelled macrozoobenthos such as gastropods (Davie & Ng 2007); and second, the size of $G. \text{depressus}$ is relatively small in comparison with other available crab species, such as $H. \text{sanguineus}$ (according to Ng et al. 2008), or species of Holothuroidea (field observation). It is possible that $G. \text{depressus}$ consumes animal tissue detritus that is easily obtained by suspension feeding, or by scavenging when such detritus is present underneath boulders.

Summarizing the results obtained from the three methods, it seems conceivable that the diet sources of $G. \text{depressus}$ are based on the following preferences: 1) green macroalgae (Monostroma sp., Ulva sp., Ulvaria sp.) which can be obtained through grazing using the chelipeds, 2) periphyton or benthic autotrophs, 3) planktonic autotrophs (suspension feeding), 4) red macroalgae (two $Gloiopeplis$ species), and 5) heterotrophic detritus (scavenging).

**Trophic position of zoobenthoses**

In the present study, stable isotope ratios of carbon and nitrogen were measured for several macrobenthic species on Ebisu Island. Judging from the $\delta^{15}N$ values, most members of the macrozoobenthos except $Japeuthria \text{ferrea}$ (c3 in Table 1, Fig. 4) mainly use autotrophs for their diet. However, a significant variability of $\delta^{13}C$ values was found among the macrobenthic animals, implying that their feeding habits differ; some species mainly using benthic periphyton, whereas others usually feed on macroalgae.

According to its $\delta^{15}N$ values, the gastropod $Lunella \text{cirrata}$ was confirmed as a herbivore, in line with recent suggestions (Vermeij and Williams 2007), as well as other gastropod species such as $Batillaria \text{multiformis}$ and $Monodonta \text{labio}$ confusa, in line with Ishihi and Yokoyama (2010). $Hemigrapsus \text{sanguineus}$ is an omnivorous crab known to coexist with $Gaetice \text{depressus}$, possibly consuming a wide variety of species (e.g. macroalgae, grazers, filter-feeders, and carnivores; Lohrer et al. 2000). In the present study, $H. \text{sanguineus}$ exhibited a relatively stable $\delta^{15}N$ (around 8%) except for summer 2011 (c2 in Table 1, Fig. 4), indicating that primarily it uses autotrophs on Ebisu Island.
The δ15N value of *J. ferrea* was the highest (around 12‰) among the macrozoobenthos examined. This strongly suggests a carnivorous feeding habit for this gastropod, as also reported by Ota and Tokeshi (2000). Since *J. ferrea* has been widely observed along rocky shores from Kyushu to the Kanto region of Japan (Asakura and Suzuki 1987), it is likely to be a significant carnivore on the boulder shores of Japan. The stable isotope ratios of the estimated diet source of *J. ferrea*, which is calculated by subtracting 1‰ and 3.4‰ for δ13C and δ15N, respectively, are comparable to those of *G. depressus*. For instance, the estimated diet of *J. ferrea* in spring 2010 has δ13C and δ15N values of −12.2‰ and 7.6‰, respectively. These values are almost similar to δ13C and δ15N value of *G. depressus* (Table 1), indicating that *J. ferrea* is possible predator of *G. depressus*. However, further evidences are necessary to identify the predator(s), including observation of feeding behavior and analysis of the foregut contents. Considering that the *G. depressus* is one of the most abundant species in the boulder shore in Japan, the identification of the predator will afford the significant information both on the food web structure and organic matter flow in the boulder shore ecosystem.

δ15N has been used widely for analysis of trophic position, but it has been suggested that estimation solely by differences in δ15N sometimes leads to erroneous results (Cabana & Rasmussen 1996, Vander-Zanden & Rasmussen 2001, Caut et al. 2009). This more precise, multi-pronged evaluation of *G. depressus* in the present study, including measurements of foregut contents and of photopigments and their derivatives, has provided a more confident evaluation confirming the δ15N findings.

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