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Isotopic trophic-step fractionation of the freshwater clam *Corbicula sandai*

Running title: Isotopic trophic-step fractionation of clam

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Abstract

Diet switch experiments with three different species of microalgae were conducted to estimate diet-tissue isotopic fractionation of the freshwater clam *Corbicula sandai*. In each experiment, *C. sandai* changed both the δ^{13}C and δ^{15}N values of soft tissues with little inter-individual deviations, reflecting the new diets. Isotope values of the clam reached the asymptotic value around 40 days after the switch. Equilibrium isotopic signatures, as well as turnovers of carbon and nitrogen in the whole soft tissues, were estimated by exponential decay models. Fractionations for *C. sandai* were from 0.1‰ to 0.7‰ for carbon, and from 2.1 to 3.6‰ for nitrogen, which fell within or close to the range of previously accepted fractionation values (0‰ to 1‰ for carbon and 3‰ to 4‰ for nitrogen). Half-life values in bivalves were about two times longer for carbon (12-22 days) than for nitrogen (7-9 days). The specific fractionation values estimated in this study provide important information for understanding inter-specific trophic relationships and aquatic food webs.

Keywords: bivalve, clam, diet, fractionation, stable isotope, laboratory experiment

Introduction

Corbicula sandai is an endemic bivalve, which inhabits Lake Biwa and the Yodo River. It had been dominant in the benthic community of Lake Biwa and played a major role in the aquatic ecosystem and water purification. It was also one of the most important fisheries resources in the lake, as the landing of *C. sandai* was at a high level of ~6,000 tons in the 1950s. However, it has started to decline since the 1960s and has become less than 100 tons (only 1 % of its peak) in recent years. It is thus highly required to clarify the cause of the decrease in its population to recover clam fisheries in Lake Biwa.

Generally the quality of food affects growth, survival and reproduction of animals. Clams obtain particles through filtration by holding its inhalant siphon above the sediment surface. There are several methods to investigate the diet of organisms. However, it is difficult to clarify the food source of filter feeding bivalves by the conventional methods. Direct observation of their feeding behavior provides little information on the food source in the field. Gut content analyses also are not suitable since they are often full of undistinguishable matter, which might be unassimilated.

In contrast to these conventional methods, stable isotope analysis has received increasing interest as it is based on assimilated food sources [1]. Early studies showed that the $^{13}C/^{12}C$ and $^{15}N/^{14}N$ ratios of an animal directly reflect the contribution of food sources assimilated and incorporated over time with slight enrichment of heavier isotopes (^{13}C and ^{15}N), compared to the lighter isotopes (^{12}C and ^{14}N) [2, 3]. Since the isotope composition in each primary source of organic matter has significantly different characteristics, this method has been used successfully in many studies on spatial and temporal variations in potential diets of bivalves [4-7]. In our previous paper, carbon and nitrogen isotope ratios of particulate organic matter in the water and the soft tissue of *Corbicula japonica*, which is closely related to *C. sandai*, were analyzed in the lower reaches of the Kushida River [8]. The results indicated that the contribution of terrestrial organic matter is significantly important for the diet of *C. japonica*, although the contribution gradually changes among sampling sites. In addition, Kasai et al. [9] investigated the diets of *C. japonica* in three brackish lakes by measuring stable isotope ratios. They showed that the diets of the clam are different among the lakes depending on the water residence time and consequent intensity of primary production.

The analysis in these papers was based on a fixed isotopic enrichment between animals and their diets, called trophic shift or fractionation (hereafter Δ). It has been commonly accepted in many studies that the average values for $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ are between 0 and 1‰ and between 3 and 4‰, respectively [2, 3, 10]. However, recent comprehensive investigations have pointed out that the actual degree of fractionation is more variable and this inconsistency depends on the species and/or tissue analyzed [11-13]. As for bivalves, Yokoyama et al. [14] conducted feeding experiments on *Mactra veneriformis* and *Ruditapes philippinarum*. They showed that $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ ranged from 0.6 to 0.9‰ and 3.4 to 3.6‰, respectively, which fell within the range of previously assumed fractionation values. On the other hand, it is reported that *Crassostrea gigas* and *Mytilus edulis* have $\Delta\delta^{13}C$ of 1.9‰ and 2.2‰, and $\Delta\delta^{15}N$ of 3.8‰ and 3.8‰, respectively [15]. Their $\Delta\delta^{13}C$ values are nearly twice as that commonly assumed, while the $\Delta\delta^{15}N$ values were comparable to the assumed fractionation value. These results indicate that Δ values are different among species.

There is still no information on the $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ values for *C. sandai*. Uncertainty in $\Delta\delta^{13}C$ or $\Delta\delta^{15}N$ could cause errors in estimates of the food source contributions to the diet of organisms in the field. The aim of this study was to determine $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ for the clam *C. sandai* based on laboratory feeding experiments. Early life stages of aquatic animals are generally important to determine the biomass because of the high mortality. In addition, *C. sandai* has unique early life ecology as it does not have larval stage, but settles to the bottom as a plantigrade just after the hatching. Therefore, we especially focused on the early life stages of *C. sandai*.

Materials and Methods

Small-sized juveniles of *C. sandai*, shortly after settlement were used for the experiments to attain sufficient growth in a relatively short time of the experiments. Firstly, mature adults of *C. sandai* were obtained from Lake Biwa, and eggs were taken from the adults. Then, newly hatched plantigrades of *C. sandai* were cultured in 600 l tanks equipped inside 25 l up-welling tanks (Tanaka Sanjiro Co., Ltd) filled with freshwater for 112 days before starting the experiments to acclimatize them to the new environment. Freshwater was pumped from Lake Biwa through a sand filter and cartridge filter (0.5μm mesh size, Advantec Toyo Kaisha, Ltd.) to reduce the concentration of particulate organic matter to close to zero. They were reared with green algae *Chlorella homosphaera*, which has been cultivated as a diet for *C. sandai* in Shiga Prefecture Fisheries Experimental Station [16]. Water temperature was maintained at 28 °C, which is close to the adequate temperature for optimal growth of plantigrade and juvenile stages of *C. sandai* in its seed production.

On 112 day (hereafter called the initial day), 25 clams were randomly sampled to determine the initial values of parameters, and then remaining clams were divided into three groups, each of which contained ~130 individuals. The first group (control group) was continuously fed *Chlorella homosphaera*. On the contrary, the diet was switched to the other algae in the second and third groups on the initial day. The second group was fed the diatom *Chaetoceros calcitrans* (YANMAR Co. Ltd), and the third group was fed the green algae *Chlorella vulgaris* (Chlorella Kogyo, Co. Ltd). These algae are relatively small in size and often used for seed production of bivalve aquaculture. All groups were fed 15 ml of the condensed diets once a day to get the concentration of the diets as ~100 thousand cells l^{-1} in the tanks. Each group was placed individually in a 15 l plastic vessel equipped inside a 5 l up-welling tank filled with freshwater and covered with a lid. The water temperature was maintained constant at 28 °C. The water in the vessels was kept still and replaced every two days.

Clams with initial shell lengths (SLs) of 2.5-3.2 mm were reared successfully. Feeding experiments were conducted for a total of 71 days for the all groups. The 10 reared clams were randomly sampled from each group on days 15, 29, 44, 57 and 71 for the determination of their isotopic changes. A small part of each diet algae was extracted on the same days to determine the isotope values of the diets.

Sampled clams were kept in filtered water for one day to remove intestine contents. After SL was measured to the nearest 0.1 mm, the whole soft tissue was removed and rinsed with distilled water under microscope. Then, the soft tissue of each individual was dried at 60°C for more than one day, and then dry weight (DW) was measured to the nearest 1 μg. The dried soft tissue was ground to a fine powder with a mortar and pestle, then put into a tin capsule. If the individuals were too small to measure the stable isotope values, especially at the initial stage of the experiments, each sample for the measurement was obtained by combining several individuals. Other samples were prepared by individual animals.

The stable isotope ratios are described by a per mil (‰) deviation from the respective international standards using the following equation:

$$\delta X = [(R_{sample}/R_{standard}) - 1] \times 1000,$$

where X represents ^{13}C or ^{15}N and R is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio. Peedee Belemnite and atmospheric N_2 are the standards for C and N, respectively. DL-Alanine was used as a secondary standard to verify the accuracy of stable isotope analysis. The standard deviations for the secondary standard were less than 0.10‰ for $\delta^{13}C$ and 0.12‰ for $\delta^{15}N$.

Negative experimental equations were fitted to the experimental isotope data as

$$y = a\exp(-bt) + c,$$

where y is the $\delta^{13}C$ or $\delta^{15}N$ value of the tissue in question, t is time, a and b are constants, and c is an asymptotic value of the tissue on the diet. The best-fit curves were optimized by the least squares method. The diet-tissue fractionation, $\Delta\delta^{13}C$ and $\Delta\delta^{15}N$ were calculated as the difference between the isotopic signatures of the diets and clams after equilibration. The half-life of each element (HL) was also calculated for each diet as

$$HL = \ln(0.5)/b\ .$$

HL corresponds to the time required to replace 50% of the initial tissue [17].

Results in the text are expressed as the mean ± SD with the number of samples analyzed (*n*).

Results

The isotopic compositions of the diets used in the experiments were fairly constant throughout the experiments. The overall $\delta^{13}C$ and $\delta^{15}N$ values to *Chlorella homosphaera*, *Chaetoceros calcitrans*, and *Chlorella vulgaris* were –15.0±0.2‰ and –6.3±0.3‰, –36.1±2.1‰ and 0.5±0.1‰, and –10.8±0.1‰ and –3.0±0.1‰, respectively (Table 1). The variation in $\delta^{13}C$ of *Chaetoceros calcitrans* was comparatively larger than the other diets, because three different batches were used during the experiment.

C. sandai were 2.8±0.2 mm SL and 0.111 ± 0.020 mg DW ($n = 25$) on the initial day. They increased in size and mass over the course of the experiments in all groups (Fig. 1). On the last sampling day, *C. sandai* were 3.8±0.4 mm SL and 0.241±0.102 g DW ($n = 10$) in the control group, 5.0±0.2 mm SL and 0.526±0.109 g DW ($n = 10$) in the second group, and 3.9±0.4 mm SL and 0.242±0.086 g DW ($n = 10$) in the third group. During the course of the experiment, *C. sandai* gained apparent averages of 37%, 99% and 26% in size and 131%, 656% and 58% in weight in the first, second and third group, respectively. DW of soft tissue increased in proportion to SL to the third power (Fig. 2).

In the control group, $\delta^{13}C$ values were almost unchanged during the experiment (Fig. 3). The mean value over the course of the experiment ($n = 21$) was -14.4±0.3‰. Taking into consideration the diet isotope values, the fractionation for soft tissue was calculated as $\Delta\delta^{13}C = 0.6‰$ (Table 2). $\delta^{15}N$ values of the clam decreased slightly, reflecting a slight decrease in $\delta^{15}N$ of the diet (Table 1). There was a significant negative relationship ($p < 0.001$, $r^2 = 0.57$, $n = 21$) between $\delta^{15}N$ and time elapsed. Since the final $\delta^{15}N$ value estimated from the regression line was -2.7‰, the fractionation value for soft tissue was calculated as $\Delta\delta^{15}N = 3.6‰$.

Both the $\delta^{13}C$ and $\delta^{15}N$ values of group 2 and group 3 changed following removal from their initial condition and converged on asymptotic values reflecting the switched diets during the experiments (Fig. 3). It is worth noting that deviations of isotope values for individuals sampled on the same dates were little, independent of their size. The exponential model provided a good fit for changes in both δ^{13}C and δ^{15}N for both diets ($r^2 > 0.95$, $p < 0.001$). The theoretical diet-tissue fractionations were calculated as $\Delta\delta^{13}C = 0.7‰$ and $\Delta\delta^{15}N = 2.1‰$ for *Chaetoceros calcitrans*, and $\Delta\delta^{13}C = 0.1‰$ and $\Delta\delta^{15}N = 3.3‰$ for *Chlorella vulgaris* (Table 2). HL values for the soft tissue were calculated as 22.2 days for δ^{13}C and 6.7 days for δ^{15}N for *Chaetoceros calcitrans*, and 12.3 days for δ^{13}C and 8.9 days for δ^{15}N for *Chlorella vulgaris*. The carbon HL values are longer than those of nitrogen. The relation between isotope values and DW of soft tissue shows that δ^{15}N values for both diets and δ^{13}C values for *Chlorella vulgaris* almost reached the asymptotic value when DW of soft tissue increased to 0.2 mg (nearly twofold increase, Fig. 4). It took a longer time for δ^{13}C changes to *Chaetoceros calcitrans*.

Discussion

C. sandai grew normally during the course of the experiments, because DW of soft tissue increased in proportion to SL to the third power with significant correlation ($r^2 = 0.92$, Fig. 2). The growths of *C. sandai* are enough to reflect the new diets in the isotope values of the clam body during the experiments (Figs. 1 and 4). There were some individuals showing smaller size and lighter weight on days 15 and 29 than the initial values (Figs. 1 and 2). It is not necessarily mean that they had poor growths, because the reared clams were randomly sampled and they should have been small on the initial day.

There was a tendency for the second group clams to gain more growth than the other groups. The dietary conditions could have influenced the growth, since all groups were cultured under the same conditions except for diets. *Chaetoceros calcitrans* is one of the most common species used to feed bivalves in recent cultivation techniques all over the world [18], as it has good nutritional properties such as high levels of polyunsaturated fatty acids [19]. This better condition would enhance the growth of the clams in the second group. On the other hand it is reported that *Chlorella homosphaera* and *Chlorella vulgaris* are the most suitable diets for newly hatched plantigrade stage of *C. sandai* [16]. This difference in growth rate could come from their size difference. Cells of Chlorella are several micrometers, which are considerably smaller than those of diatoms. This size would be suitable for plantigrade stage clams, but not be good enough for juveniles. Newly hatched clams could not ingest diatoms as they are too large to ingest. This indicates that *C. sandai* can change its diet depending on the stage.

In each group of the experiments, individuals sampled on the same dates had little deviations of isotope values and they converged on the asymptotic values reflecting each diet (Fig. 3). This indicates that individual differences in isotope ratios are negligible if they ingest a same diet. On the contrary, animals sampled from natural waters usually show variety of isotope values. Our results indicate that the variety is not caused by the inter-individual difference nor inter-species difference, but by the difference in ingested diets. The variation in isotope values of animals from natural waters should reflect that there are various food sources and animals consequently consume various diets in the natural fields.

Our experiments indicated that the diet-animal isotopic fractionations for *C. sandai* are almost within the range of the commonly accepted δ^{13}C and δ^{15}N fractionations [2, 3], although $\Delta\delta^{15}$N value for *Chaetoceros calcitrans* was slightly low. Previous studies reported a large variation in nitrogen fractionation for bivalves (Fig. 5) [14, 15, 20], so that $\Delta\delta^{15}$N value for *Chaetoceros calcitrans* in this study is not particularly out of the commonly accepted range. The fractionation in ^{15}N for benthic animals is influenced by the quality of diet such as protein contents and C:N ratios [21] and metabolic condition [14, 20]. However, such information cannot be obtained from the field, because there are generally various potential food sources in the field. This indicates that the usage of a specific value of isotope fractionation could lead errors in estimates of food source contributions in the diet of organisms in the field.

Recently, a comparison between δ^{15}N values of individual amino acids (phenylalanine and glutamic acids) has been applied for estimating the trophic level of organisms in food webs [24]. The advantage of this powerful method is that the δ^{15}N values of two amino acids from a single organism can show the trophic level of the organism [25]. However, δ^{15}N values of individual amino acids do not reflect any variation in environmental and/or physiological conditions of organisms, although they can provide accurate trophic levels without any information on primary producers. On the contrary, the bulk method, which is applied in this study, reveals predator-prey relationships directly, although it requires the analyses of isotope values of multiple organisms. A combination of amino acid method and bulk method would be the most suitable tool for understanding complicated ecosystems.

Our experiments showed that the HL value of the soft tissue of *C. sandai* for carbon in the second group was larger than those for nitrogen and those in the third group (Table 2 and Fig. 3), even though the growth in the second group was larger than the third group (Fig. 2). This difference could be caused by the large difference between the δ^{13}C values of the initial *C. sandai* and the diet (*Chaetoceros calcitrans*). As the δ^{13}C value of *Chaetoceros calcitrans* (-36.1‰ in average) is considerably lower than those of natural phytoplankton in freshwater lakes (-20‰ in usual) [22] and unrealistic, the HL value for carbon in the second group could not be applicable to field studies. The HL value for carbon in the third group was larger than that for nitrogen. This tendency was also reported for *Lateolabrax japonicus* from feeding experiments [23]. It is normal that the turnover rates are different between carbon and nitrogen to a varying degree [15]. It was suggested that the difference may correspond to a decoupling of the nitrogen and carbon metabolic pathways [17]. Overall, HL values depend on the turnover rate of tissues. Immature clams store a very low quantity of energy, with almost all assimilated food being used for growth. Therefore wild clams would have longer HL values than those estimated in this study.

In conclusion, as more information on the isotopic fractionation of bivalves has been required over the past decades, we provided Δ values for the freshwater clam *C. sandai* in order to contribute to the library of Δ values for invertebrate species. Stable isotope values for whole soft tissues of *C. sandai* satisfactorily converged on the asymptotic values with little inter-individual deviations reflecting the diets, and $\Delta\delta^{13}$C and $\Delta\delta^{15}$N values were 0.1-0.7‰ and 2.1-3.6‰, respectively. These values can promote better interpretation of food source of *C. sandai* in freshwater ecosystems. Phytoplankton, benthic microalgae and terrestrial organic matter show significantly different isotope values [5, 8, 22]. Comparing the isotope values of these potential diets and *C. sandai*, contribution of each organism to the clams' food source can be estimated. If the isotope analysis would demonstrate a preferred diet in the lake, increase in the diet could make a larger production of *C. sandai* in future.

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Figure captions

Fig. 1 Changes in shell length and dry weight of soft tissues of *Corbicula sandai* fed on three different algae during the experiments

Fig. 2 Allometry of the bivalve *Corbicula sandai* in the experiments. Open and closed circles indicate the data on and after the initial day, respectively

Fig. 3 Changes in δ^{13}C and δ^{15}N of diets (open circles) and soft tissue of *Corbicula sandai* fed on three different algae (solid circles) during the experiments. Solid lines and equations represent the best-fit model with coefficients of determination (r^2).

Fig. 4 Relation between dry weight of soft tissue and isotope values of *Corbicula sandai* fed on three different algae during the experiments. Dashed lines indicate isotope values of the diet

Fig. 5 Carbon and nitrogen fractionation values ($\Delta\delta^{13}$C and $\Delta\delta^{15}$N) for bivalves based on literature feeding experiments (open symbols) and estimated from the current experiments (closed symbols). Lipids were not removed from the samples of the all experiments. A shaded square indicates the range of the previously accepted δ^{13}C and δ^{15}N fractionations. Data source: 1) Yokoyama et al. (2005), 2) Yokoyama et al. (2008), 3) Dubois et al. (2007)

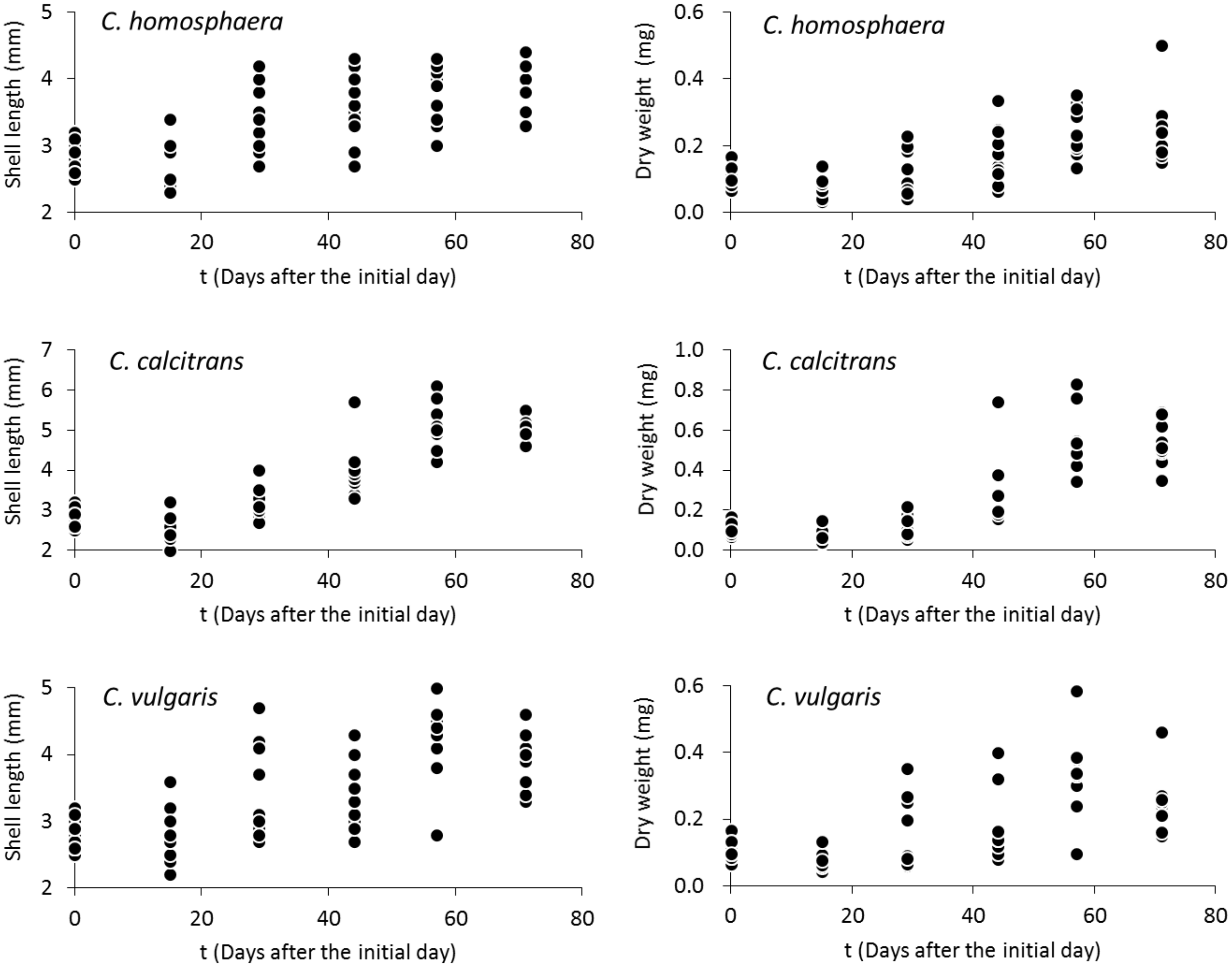
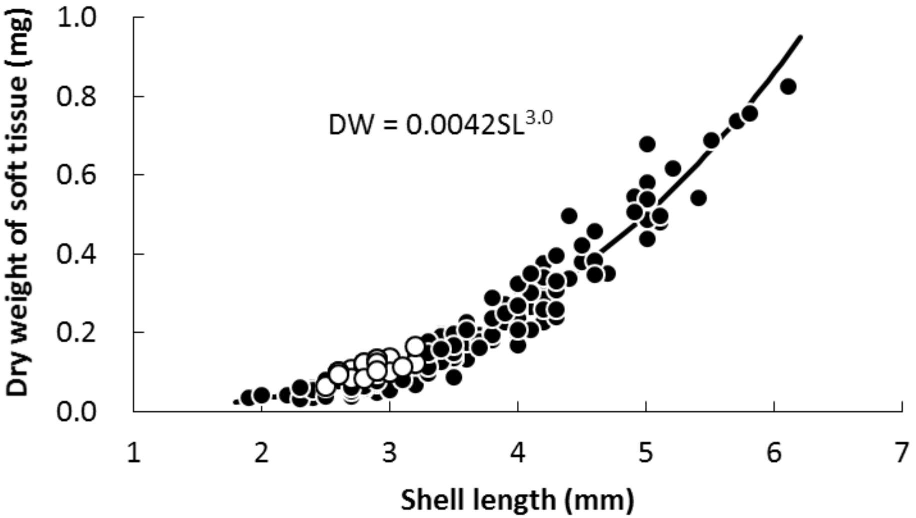


Fig. 1 Kasai et al.



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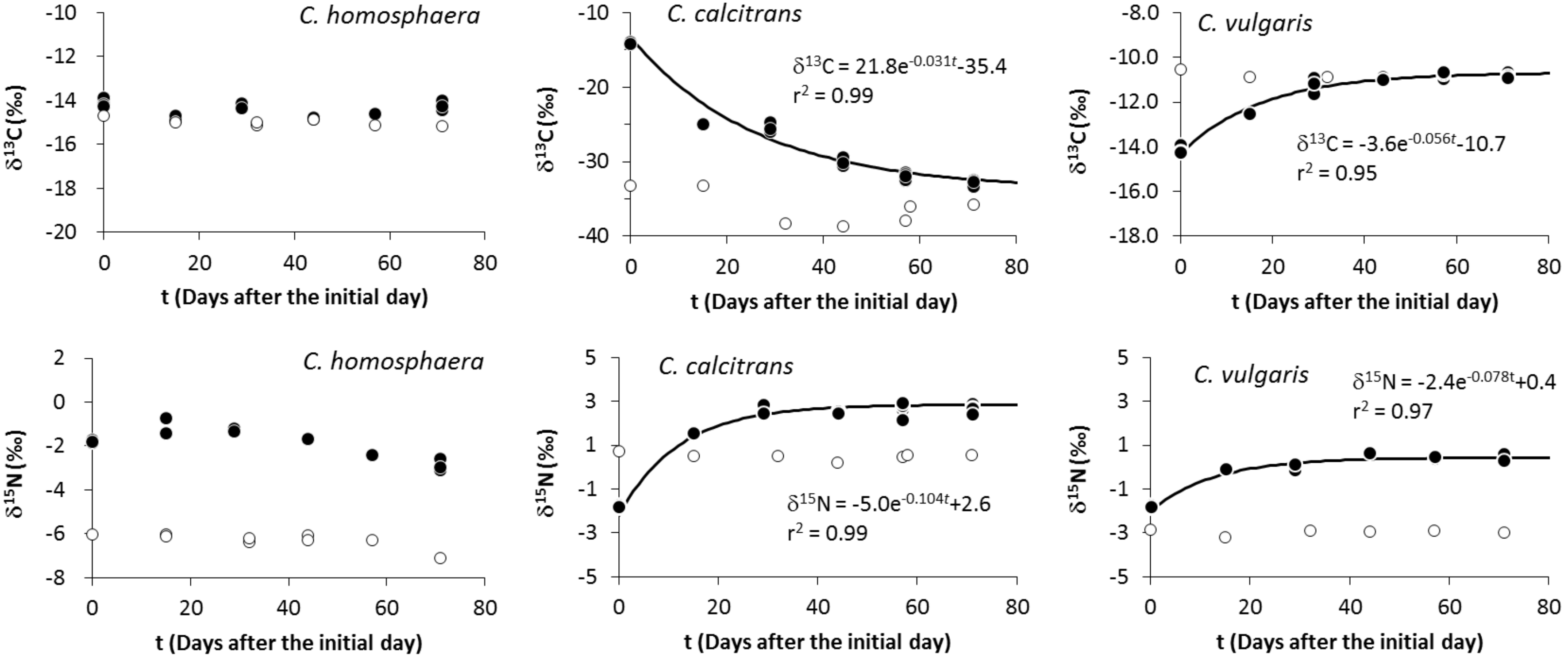

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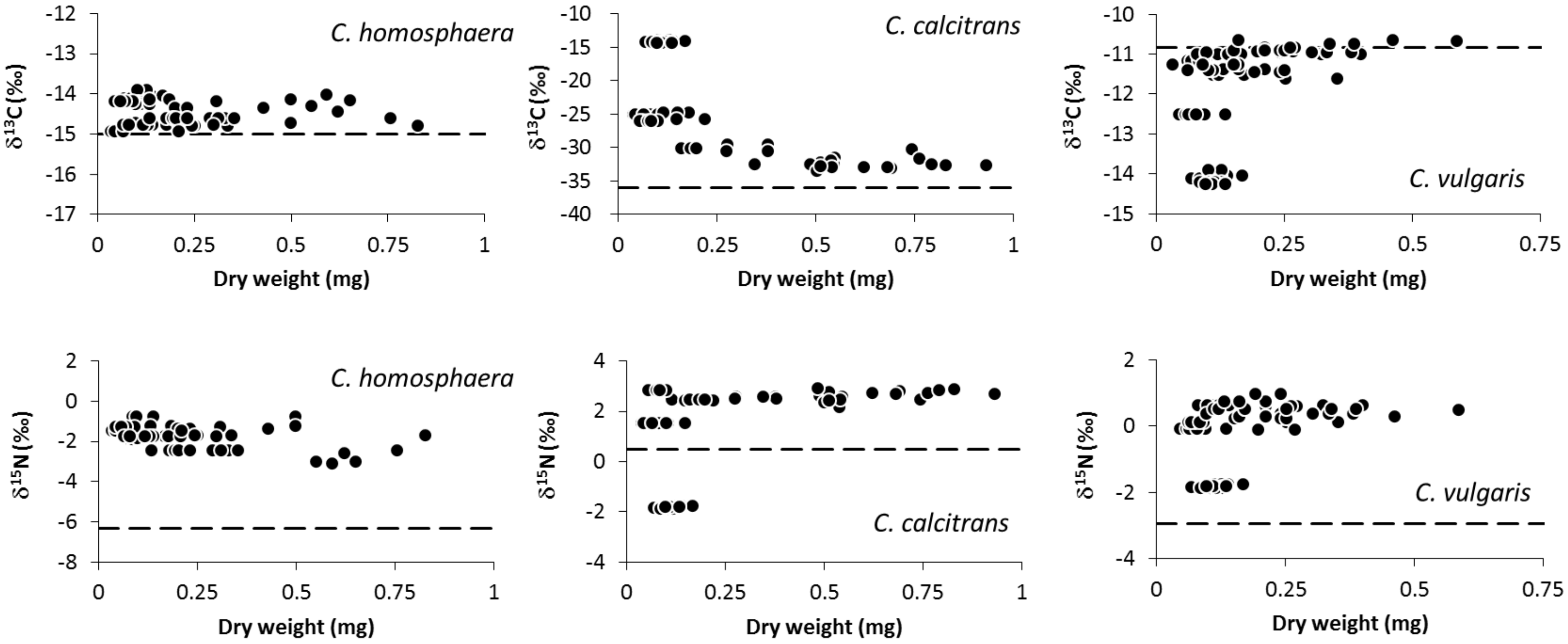

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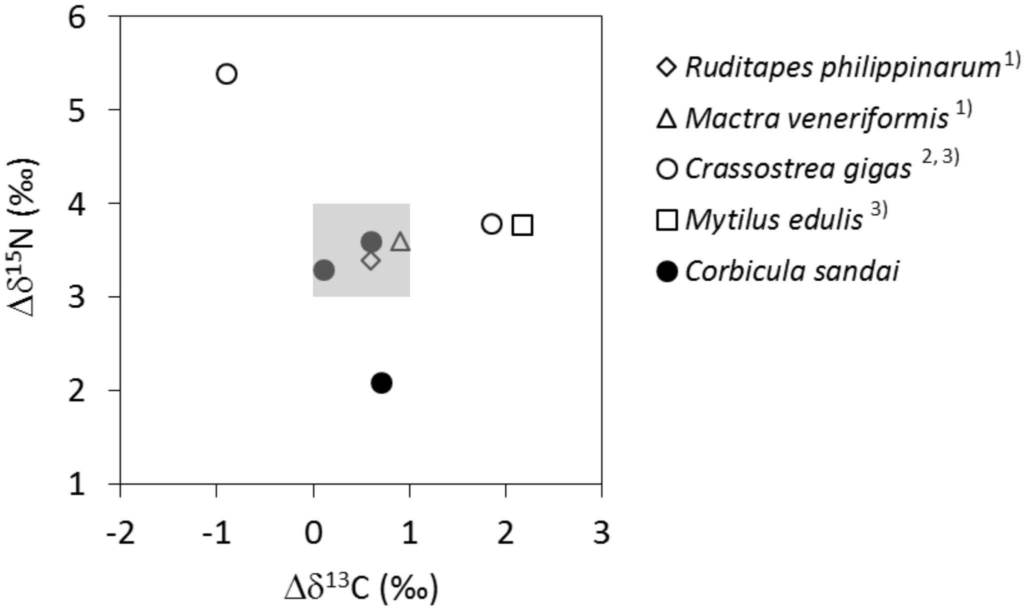

Fig. 5 Kasai et al.