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 $\delta^{13}C$ and $\delta^{15}N$ 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*

63 in three brackish lakes by measuring stable isotope ratios. They showed that the diets of the clam are
64 different among the lakes depending on the water residence time and consequent intensity of primary
65 production.
66 The analysis in these papers was based on a fixed isotopic enrichment between animals and their diets,
67 called trophic shift or fractionation (hereafter Δ). It has been commonly accepted in many studies that the
68 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].
69 However, recent comprehensive investigations have pointed out that the actual degree of fractionation is
70 more variable and this inconsistency depends on the species and/or tissue analyzed [11-13]. As for
71 bivalves, Yokoyama et al. [14] conducted feeding experiments on *Mactra veneriformis* and *Ruditapes*
72 *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,
73 which fell within the range of previously assumed fractionation values. On the other hand, it is reported
74 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‰,
75 respectively [15]. Their $\Delta\delta^{13}C$ values are nearly twice as that commonly assumed, while the $\Delta\delta^{15}N$ values
76 were comparable to the assumed fractionation value. These results indicate that Δ values are different
77 among species.
78 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 $\Delta\delta^{15}N$
79 could cause errors in estimates of the food source contributions to the diet of organisms in the field. The
80 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
81 experiments. Early life stages of aquatic animals are generally important to determine the biomass
82 because of the high mortality. In addition, *C. sandai* has unique early life ecology as it does not have
83 larval stage, but settles to the bottom as a plantigrade just after the hatching. Therefore, we especially
84 focused on the early life stages of *C. sandai*.
85
86 **Materials and Methods**

87 Small-sized juveniles of *C. sandai*, shortly after settlement were used for the experiments to attain
88 sufficient growth in a relatively short time of the experiments. Firstly, mature adults of *C. sandai* were
89 obtained from Lake Biwa, and eggs were taken from the adults. Then, newly hatched plantigrades of *C.*
90 *sandai* were cultured in 600 l tanks equipped inside 25 l up-welling tanks (Tanaka Sanjiro Co., Ltd) filled
91 with freshwater for 112 days before starting the experiments to acclimatize them to the new environment.
92 Freshwater was pumped from Lake Biwa through a sand filter and cartridge filter (0.5μm mesh size,
93 Advantec Toyo Kaisha, Ltd.) to reduce the concentration of particulate organic matter to close to zero.
94 They were reared with green algae *Chlorella homosphaera*, which has been cultivated as a diet for *C.*
95 *sandai* in Shiga Prefecture Fisheries Experimental Station [16]. Water temperature was maintained at
96 28 °C, which is close to the adequate temperature for optimal growth of plantigrade and juvenile stages of
97 *C. sandai* in its seed production.
98 On 112 day (hereafter called the initial day), 25 clams were randomly sampled to determine the initial
99 values of parameters, and then remaining clams were divided into three groups, each of which contained
100 ~130 individuals. The first group (control group) was continuously fed *Chlorella homosphaera*. On the
101 contrary, the diet was switched to the other algae in the second and third groups on the initial day. The
102 second group was fed the diatom *Chaetoceros calcitrans* (YANMAR Co. Ltd), and the third group was
103 fed the green algae *Chlorella vulgaris* (Chlorella Kogyo, Co. Ltd). These algae are relatively small in size
104 and often used for seed production of bivalve aquaculture. All groups were fed 15 ml of the condensed
105 diets once a day to get the concentration of the diets as ~100 thousand cells l^{-1} in the tanks. Each group
106 was placed individually in a 15 l plastic vessel equipped inside a 5 l up-welling tank filled with freshwater
107 and covered with a lid. The water temperature was maintained constant at 28 °C. The water in the vessels
108 was kept still and replaced every two days.
109 Clams with initial shell lengths (SLs) of 2.5-3.2 mm were reared successfully. Feeding experiments were
110 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

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

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

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

137

138 **Results**

139 The isotopic compositions of the diets used in the experiments were fairly constant throughout the
140 experiments. The overall $\delta^{13}C$ and $\delta^{15}N$ values to *Chlorella homosphaera*, *Chaetoceros calcitrans*, and
141 *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
142 –3.0±0.1‰, respectively (Table 1). The variation in $\delta^{13}C$ of *Chaetoceros calcitrans* was comparatively
143 larger than the other diets, because three different batches were used during the experiment.

144 *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
145 size and mass over the course of the experiments in all groups (Fig. 1). On the last sampling day, *C.*
146 *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
147 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
148 the third group. During the course of the experiment, *C. sandai* gained apparent averages of 37%, 99%
149 and 26% in size and 131%, 656% and 58% in weight in the first, second and third group, respectively.
150 DW of soft tissue increased in proportion to SL to the third power (Fig. 2).

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

158 Both the $\delta^{13}C$ and $\delta^{15}N$ values of group 2 and group 3 changed following removal from their initial

159 condition and converged on asymptotic values reflecting the switched diets during the experiments (Fig.
160 3). It is worth noting that deviations of isotope values for individuals sampled on the same dates were
161 little, independent of their size. The exponential model provided a good fit for changes in both $\delta^{13}C$ and
162 $\delta^{15}N$ for both diets ($r^2 > 0.95$, $p < 0.001$). The theoretical diet-tissue fractionations were calculated as
163 $\Delta\delta^{13}C = 0.7‰$ 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
164 *Chlorella vulgaris* (Table 2). HL values for the soft tissue were calculated as 22.2 days for $\delta^{13}C$ and 6.7
165 days for $\delta^{15}N$ for *Chaetoceros calcitrans*, and 12.3 days for $\delta^{13}C$ and 8.9 days for $\delta^{15}N$ for *Chlorella*
166 *vulgaris*. The carbon HL values are longer than those of nitrogen. The relation between isotope values
167 and DW of soft tissue shows that $\delta^{15}N$ values for both diets and $\delta^{13}C$ values for *Chlorella vulgaris* almost
168 reached the asymptotic value when DW of soft tissue increased to 0.2 mg (nearly twofold increase, Fig.
169 4). It took a longer time for $\delta^{13}C$ changes to *Chaetoceros calcitrans*.
170
171 **Discussion**
172 *C. sandai* grew normally during the course of the experiments, because DW of soft tissue increased in
173 proportion to SL to the third power with significant correlation ($r^2 = 0.92$, Fig. 2). The growths of *C.*
174 *sandai* are enough to reflect the new diets in the isotope values of the clam body during the experiments
175 (Figs. 1 and 4). There were some individuals showing smaller size and lighter weight on days 15 and 29
176 than the initial values (Figs. 1 and 2). It is not necessarily mean that they had poor growths, because the
177 reared clams were randomly sampled and they should have been small on the initial day.
178 There was a tendency for the second group clams to gain more growth than the other groups. The dietary
179 conditions could have influenced the growth, since all groups were cultured under the same conditions
180 except for diets. *Chaetoceros calcitrans* is one of the most common species used to feed bivalves in
181 recent cultivation techniques all over the world [18], as it has good nutritional properties such as high
182 levels of polyunsaturated fatty acids [19]. This better condition would enhance the growth of the clams in

183 the second group. On the other hand it is reported that *Chlorella homosphaera* and *Chlorella vulgaris* are
184 the most suitable diets for newly hatched plantigrade stage of *C. sandai* [16]. This difference in growth
185 rate could come from their size difference. Cells of Chlorella are several micrometers, which are
186 considerably smaller than those of diatoms. This size would be suitable for plantigrade stage clams, but
187 not be good enough for juveniles. Newly hatched clams could not ingest diatoms as they are too large to
188 ingest. This indicates that *C. sandai* can change its diet depending on the stage.
189 In each group of the experiments, individuals sampled on the same dates had little deviations of isotope
190 values and they converged on the asymptotic values reflecting each diet (Fig. 3). This indicates that
191 individual differences in isotope ratios are negligible if they ingest a same diet. On the contrary, animals
192 sampled from natural waters usually show variety of isotope values. Our results indicate that the variety is
193 not caused by the inter-individual difference nor inter-species difference, but by the difference in ingested
194 diets. The variation in isotope values of animals from natural waters should reflect that there are various
195 food sources and animals consequently consume various diets in the natural fields.
196 Our experiments indicated that the diet-animal isotopic fractionations for *C. sandai* are almost within the
197 range of the commonly accepted $\delta^{13}C$ and $\delta^{15}N$ fractionations [2, 3], although $\Delta\delta^{15}N$ value for
198 *Chaetoceros calcitrans* was slightly low. Previous studies reported a large variation in nitrogen
199 fractionation for bivalves (Fig. 5) [14, 15, 20], so that $\Delta\delta^{15}N$ value for *Chaetoceros calcitrans* in this
200 study is not particularly out of the commonly accepted range. The fractionation in ^{15}N for benthic animals
201 is influenced by the quality of diet such as protein contents and C:N ratios [21] and metabolic condition
202 [14, 20]. However, such information cannot be obtained from the field, because there are generally
203 various potential food sources in the field. This indicates that the usage of a specific value of isotope
204 fractionation could lead errors in estimates of food source contributions in the diet of organisms in the
205 field.
206 Recently, a comparison between $\delta^{15}N$ values of individual amino acids (phenylalanine and glutamic

207 acids) has been applied for estimating the trophic level of organisms in food webs [24]. The advantage of
208 this powerful method is that the $\delta^{15}N$ values of two amino acids from a single organism can show the
209 trophic level of the organism [25]. However, $\delta^{15}N$ values of individual amino acids do not reflect any
210 variation in environmental and/or physiological conditions of organisms, although they can provide
211 accurate trophic levels without any information on primary producers. On the contrary, the bulk method,
212 which is applied in this study, reveals predator-prey relationships directly, although it requires the
213 analyses of isotope values of multiple organisms. A combination of amino acid method and bulk method
214 would be the most suitable tool for understanding complicated ecosystems.
215 Our experiments showed that the HL value of the soft tissue of *C. sandai* for carbon in the second group
216 was larger than those for nitrogen and those in the third group (Table 2 and Fig. 3), even though the
217 growth in the second group was larger than the third group (Fig. 2). This difference could be caused by
218 the large difference between the $\delta^{13}C$ values of the initial *C. sandai* and the diet (*Chaetoceros calcitrans*).
219 As the $\delta^{13}C$ value of *Chaetoceros calcitrans* (-36.1‰ in average) is considerably lower than those of
220 natural phytoplankton in freshwater lakes (-20‰ in usual) [22] and unrealistic, the HL value for carbon
221 in the second group could not be applicable to field studies. The HL value for carbon in the third group
222 was larger than that for nitrogen. This tendency was also reported for *Lateolabrax japonicus* from feeding
223 experiments [23]. It is normal that the turnover rates are different between carbon and nitrogen to a
224 varying degree [15]. It was suggested that the difference may correspond to a decoupling of the nitrogen
225 and carbon metabolic pathways [17]. Overall, HL values depend on the turnover rate of tissues. Immature
226 clams store a very low quantity of energy, with almost all assimilated food being used for growth.
227 Therefore wild clams would have longer HL values than those estimated in this study.
228 In conclusion, as more information on the isotopic fractionation of bivalves has been required over the
229 past decades, we provided Δ values for the freshwater clam *C. sandai* in order to contribute to the library
230 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

303 **Fig. 1** Changes in shell length and dry weight of soft tissues of *Corbicula sandai* fed on three different
304 algae during the experiments
305 **Fig. 2** Allometry of the bivalve *Corbicula sandai* in the experiments. Open and closed circles indicate
306 the data on and after the initial day, respectively
307 **Fig. 3** Changes in $\delta^{13}C$ and $\delta^{15}N$ of diets (open circles) and soft tissue of *Corbicula sandai* fed on three
308 different algae (solid circles) during the experiments. Solid lines and equations represent the
309 best-fit model with coefficients of determination (r^2).
310 **Fig. 4** Relation between dry weight of soft tissue and isotope values of *Corbicula sandai* fed on three
311 different algae during the experiments. Dashed lines indicate isotope values of the diet
312 **Fig. 5** Carbon and nitrogen fractionation values ($\Delta\delta^{13}C$ and $\Delta\delta^{15}N$) for bivalves based on literature
313 feeding experiments (open symbols) and estimated from the current experiments (closed symbols).
314 Lipids were not removed from the samples of the all experiments. A shaded square indicates the
315 range of the previously accepted $\delta^{13}C$ and $\delta^{15}N$ fractionations. Data source: 1) Yokoyama et al.
316 (2005), 2) Yokoyama et al. (2008), 3) Dubois et al. (2007)

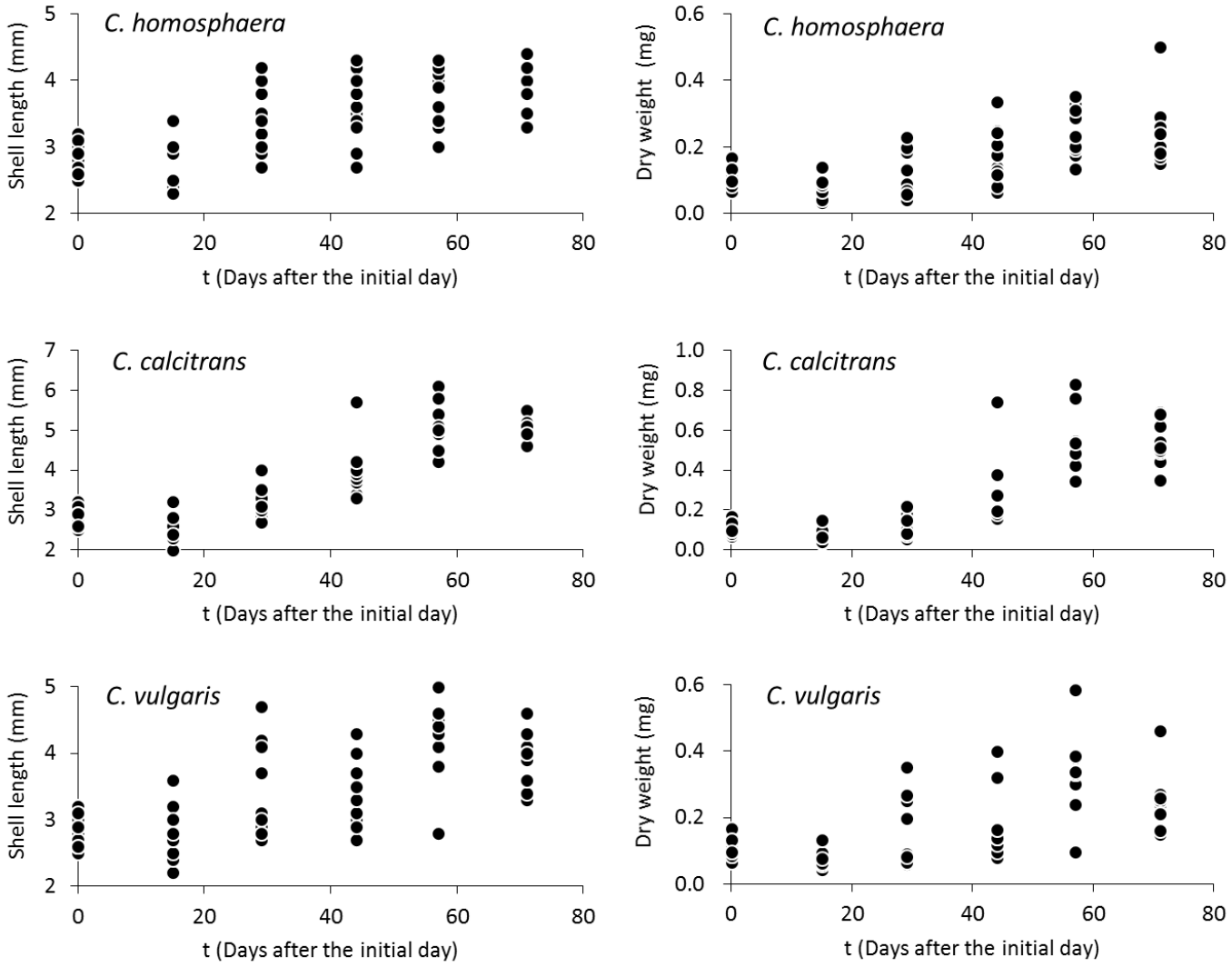

Fig. 1 Kasai et al.

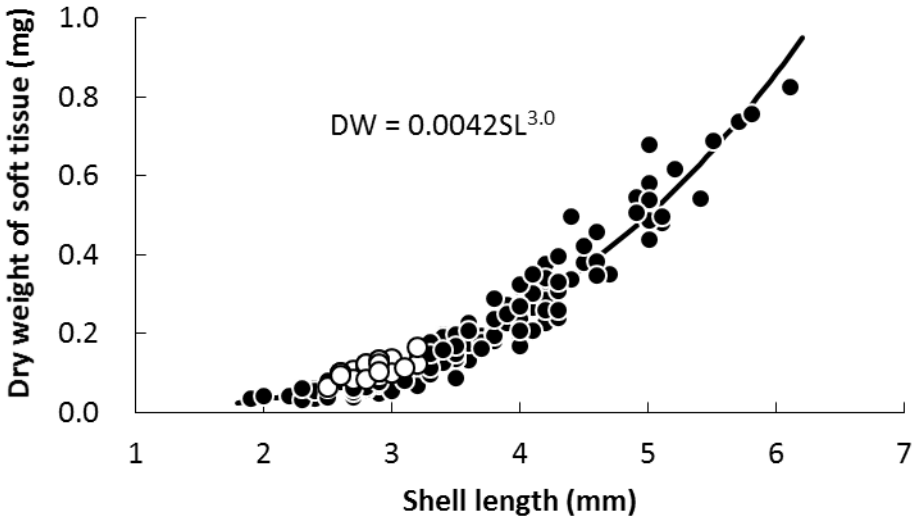

Fig. 2 Kasai et al.

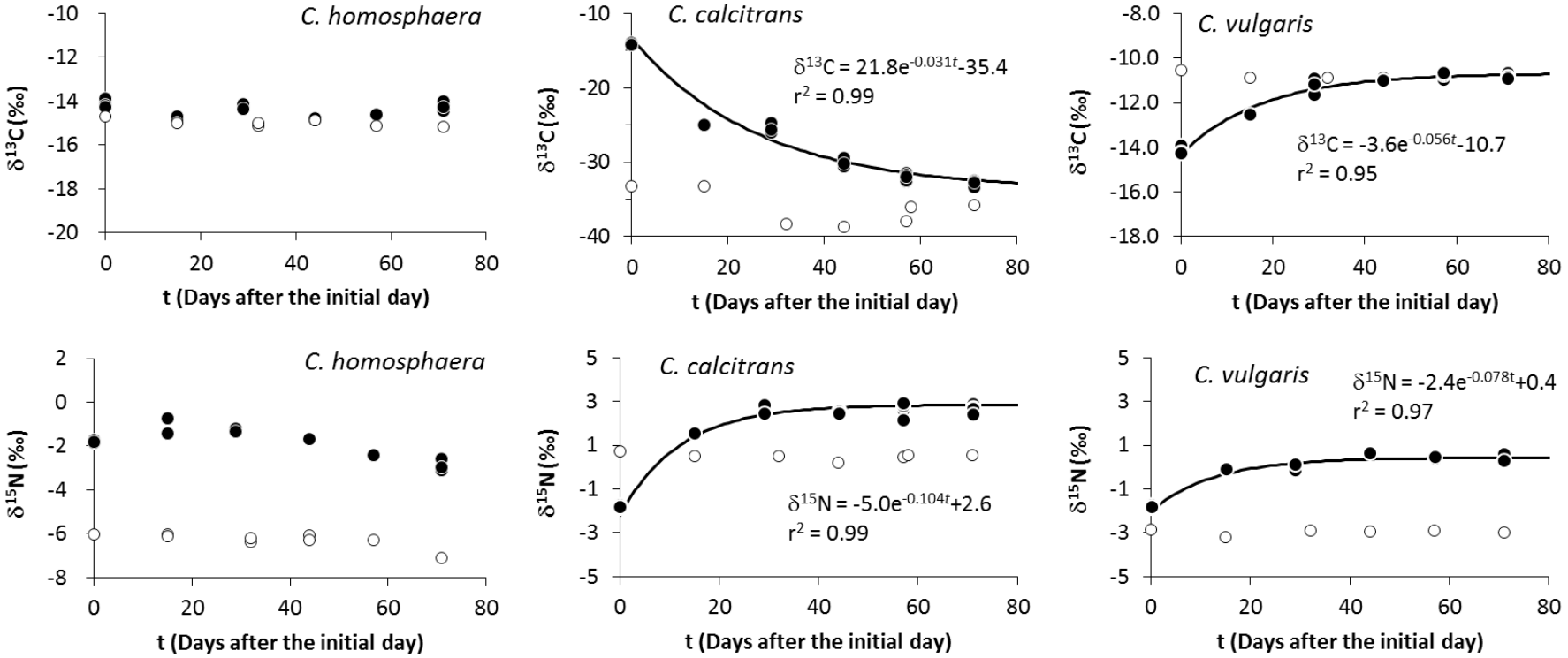

Fig. 3 Kasai et al.

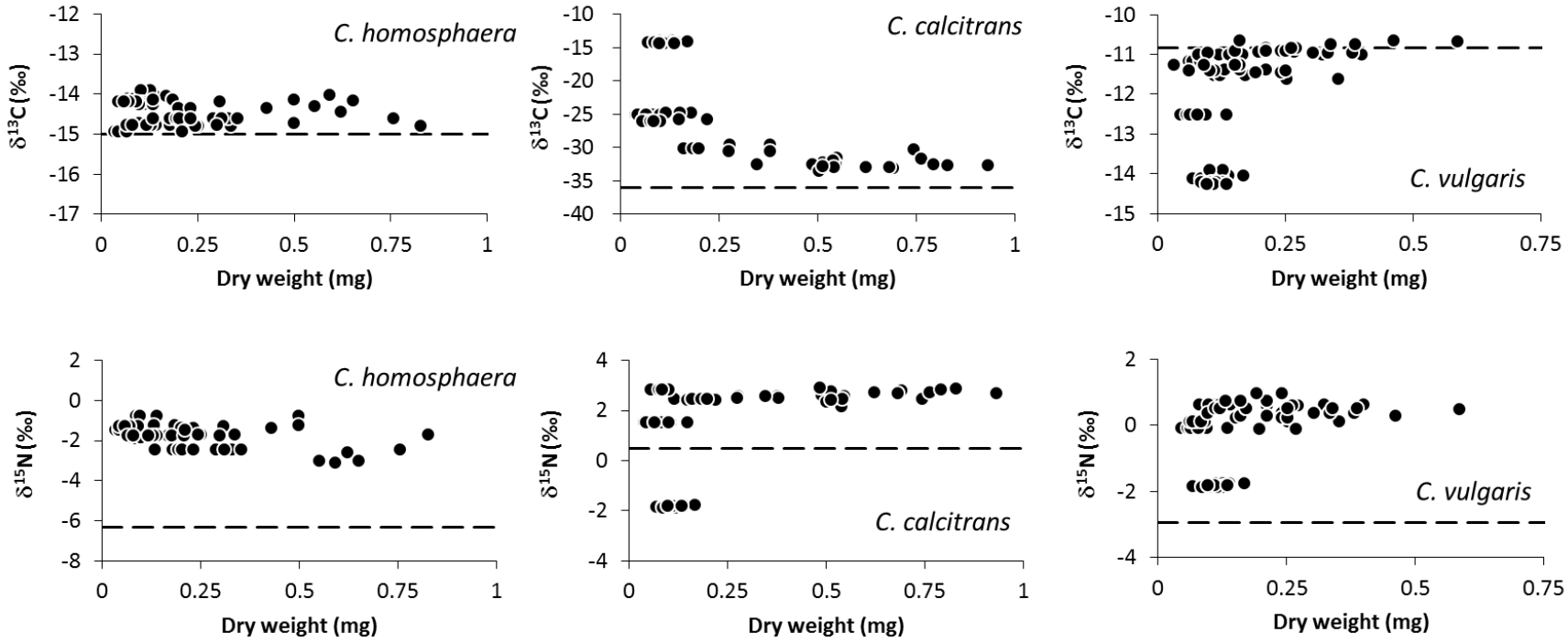

Fig. 4 Kasai et al.

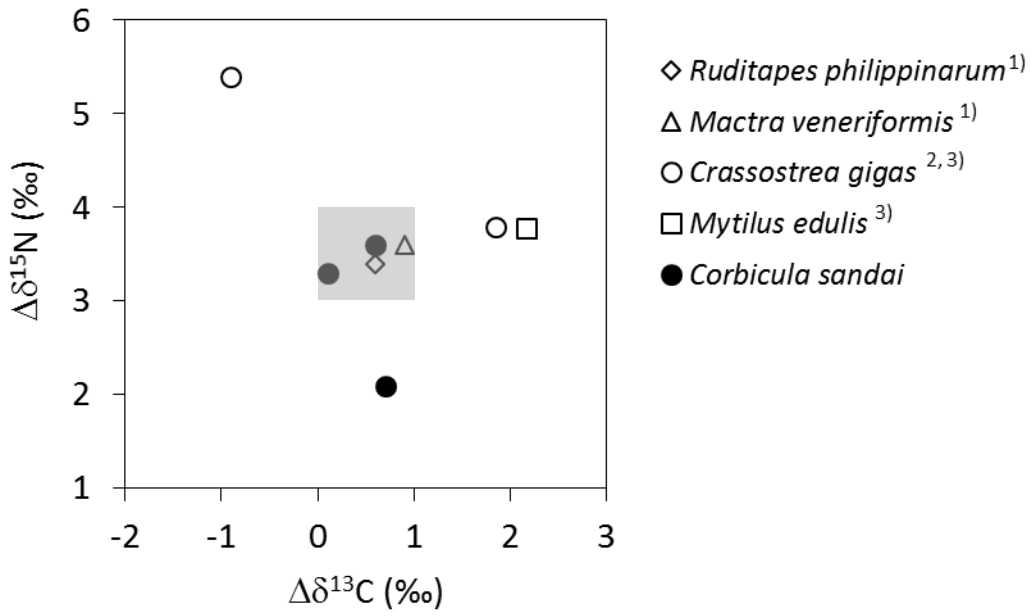

Fig. 5 Kasai et al.