Factors controlling shell carbon isotopic composition of land snail *Acusta despecta sieboldiana* estimated from lab culturing experiment

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Abstract

The carbon isotopic composition ($\delta^{13}C$) of land snail shell carbonate derives from three potential sources: diet, atmospheric CO_2, and ingested carbonate (limestone). However, their relative contributions remain unclear. Under various environmental conditions, we cultured one land snail species, *Acusta despecta sieboldiana* collected from Yokohama, Japan, and confirmed that all of these sources affect shell carbonate $\delta^{13}C$ values. Herein, we consider the influences of metabolic rates and temperature on the carbon isotopic composition of the shell carbonate. Based on previous works and on results obtained in this study, a simple but credible framework is presented for discussion of how each source and environmental parameter can affect shell carbonate $\delta^{13}C$ values. According to this framework and some reasonable assumptions, we have estimated the contributions of different carbon sources for each snail individual: for cabbage (C_3 plant) fed groups, the contributions of diet, atmospheric CO_2 and ingested limestone respectively vary as 66–80%, 16–24%, and 0–13%. For corn (C_4 plant) fed groups, because of the possible food stress (lower consumption ability of C_4 plant), the values vary respectively as 56–64%, 18–20%, and 16–26%. Moreover, we present new evidence that snails have discrimination to choose different plant species as food. Therefore, we suggest that food preferences must be considered adequately when applying $\delta^{13}C$ in paleo-

environment studies. Finally, we inferred that, during egg laying and hatching of our cultured snails, carbon isotope fractionation is controlled only by the isotopic exchange of the calcite – HCO_3^- – aragonite equilibrium.

1 Introduction

Land snail shells are widely applied for studying paleo and present environment characteristics because most of those species which can be well preserved in Quaternary fossils are still extant today (e.g., Yapp, 1979; Lecolle, 1985; Goodfriend et al., 1989; Goodfriend, 1992; Zanchetta et al. 2005; Colonese et al., 2007, 2010 and 2011; Yanes et al., 2008a,b, 2009, 2011, 2012a,b, 2013; Kehrwald et al., 2010; Zaarur et al., 2011). Specifically, the carbon isotopic composition ($\delta^{13}C$ value) of land snail shell carbonate is regarded as useful to reconstruct the distribution of terrestrial C_3/C_4 vegetation, which itself is related to some environmental parameters such as rainfall amounts and temperature variations (Goodfriend and Magaritz, 1987; Goodfriend, 1992; Stott, 2002; Metref et al., 2003).

Land snail shell carbon has three sources: food, atmospheric CO_2, and ingested carbonate (limestone) (Goodfriend and Hood, 1983). Food sources such as plants, fungi, and other organic matter can be transformed into metabolic CO_2 in the body of a land snail by two pathways: direct digestion and breakdown of urea (Stott, 2002). The resulting CO_2 will dissolve into the bicarbonate pool in the hemolymph and then precipitate as shell carbonate. Atmospheric CO_2 can be introduced into the bicarbonate pool via respiration. Similarly, ingested carbonate can first dissolve into stomach acid to produce gaseous CO_2. Then it can be introduced into the bicarbonate pool, too. Some previous works have been undertaken to clarify their relative contributions both from observations and model calculations. Nevertheless, this topic remains poorly understood (Goodfriend and Hood, 1983; Goodfriend and Stipp, 1983; Stott, 2002; Metref et al., 2003; Balakrishnan and Yapp, 2004; Romaniello et al., 2008). Following are estimations of each carbon source based on published works: food source ratios vary as 25–40% (Goodfriend and Hood, 1983), 36–73% (Romainello et al., 2008), and 100% (Stott, 2002; Metref et al., 2003); atmospheric CO_2 has been estimated as negligible (Stott, 2002; Metref, 2003), 16–48% (Romaiello et al., 2008), and 30–60% (Goodfriend and Hood, 1983); ingested carbonate has been inferred as up to 30% (Goodfriend and Stipp, 1983), often negligible for small terrestrial gastropods of less than 10 mm, and as

always much less than 20–30% for larger species (Pigati et al., 2004, 2010), ~20% up to ~40% (Yanes et al., 2008a).

For solving problems of this kind, more studies including laboratory culturing experiments must be done. Stott (2002) and Meterf et al. (2003) reported two independent works related to land snail culture experiments. Both show a marked but discrepant correlation between land snail shell carbonate $\delta^{13}C$ and diet $\delta^{13}C$ with slopes less than one. Secondly, Stott pointed out that ingested carbonate does not contribute to shell carbonate $\delta^{13}C$ values based on results of snails fed with and without an added $CaCO_3$ source. Finally, according to their calculations and discussions, both papers reported that atmospheric CO_2 does not contribute to shell carbonate $\delta^{13}C$ values. Those reports notwithstanding, inconsistent observations and discussions emerged soon thereafter. Yanes et al. (2012a) reported that higher $\delta^{13}C$ values were observed during the younger growth stages of both living and fossil snails, which showed signs of a higher contribution of ingested limestone. Results of this research suggest strongly that environmental carbonate is incorporated as an important source for precipitating land snail shells (at least some snail species) and suggest that it can affect their shell $\delta^{13}C$ values. To the atmospheric CO_2, after summarizing the previous studies of relation between land snail shell carbonate $\delta^{13}C$ and shell organics/ body tissue organics/ diet (Stott, 2002; Goodfriend and Ellis, 2002; Balakrishnan and Yapp, 2004), McConnaughey and Gillikin (2008) pointed out that the offsets between $\delta^{13}C$ of shell carbonate and their diet (or body organics) are greater when the $\delta^{13}C$ of the latter diverge more from atmospheric CO_2, suggesting that the atmospheric CO_2 does contribute to shell carbonate.

These great discrepancies from published literatures are probably caused by: (1) the variability of land snail species studied with different ecological requirements, ethology, and other species-dependent-behaviors; (2) the variability of environmental conditions where these snails were living (e.g., $CaCO_3$-rich areas vs. $CaCO_3$-poor areas, wet areas vs. dry areas, hot/warm areas vs. cold/cool areas, etc.); (3) the limitation of calculations. Therefore, a better understanding of the contribution of each carbon source and related environmental controlling factors can promote this isotopic tool in the field of paleo environment reconstruction. In the present study, we cultured one land snail species (*Acusta despecta sieboldiana*) under different controlled conditions. A simple but credible framework was raised to discuss the mechanism of how each possible source and environmental parameter can affect shell carbonate $\delta^{13}C$ values based on previous works and results of this study. According to this

frame and some reasonable assumptions, we estimated the contribution of different carbon sources for each snail individual. This report is the first describing an attempt to estimate the contributing proportion of limestone ingested by snail individuals using stable isotope values compared with a previous method using calculations including radiocarbon dating (Goodfriend and Stipp, 1983; Pigati et al., 2010).

2 Materials and methods

2.1 Culturing of land snails

Land snail *Acusta despecta*, with a Japanese name 'Usukawa-maimai', is widely distributed around Japan except Hokkaido (Azuma, 1995), and Korea (Lee and Kwon, 1996). They mainly consume fresh plants (Suzuki and Yamashita, 1967; Takeuchi and Tamura, 1995) and live in the temperature ranging from 15 ℃ to 30 ℃ with the optimum from 25 ℃ to 30 ℃ (Kohno, 1976). Typically, the lifespan of *Acusta despecta* is around 1 year and the individual could become adult in 6 months from birth (Sumikawa, 1962; Okuma, 1982; Takahashi et al., 1992).

In this study, eight adult snails of *Acusta despecta sieboldiana* (a subspecies of *Acusta despecta*) were collected in Suzukakedai, Yokohama, Japan and were cultured at room temperature (ca. 25 ℃) from January, 2012. These snails began to lay eggs in March, 2012. Then the eggs were transferred into a stainless steel container and were covered with moist cloth in a 25 ℃ incubator. Most eggs hatched at around 3–4 weeks.

Larvae were distributed randomly into around 30 small transparent plastic boxes that had been perforated to allow air and vapor exchange: Each box contained 3–5 snails. Some later hatched larvae were added to May and June, 2012. Then four small boxes were put into semi-sealed big plastic boxes: two parallel groups were fed cabbage (*Brassica oleracea var. capitata*, green cabbage, C_3 plant, $\delta^{13}C$ = -28.4±1.2‰, n=12) that had been sprinkled with fine calcium carbonate powder ($CaCO_3$, $\delta^{13}C$ = 4.0‰). Another two were fed cabbage sprinkled with calcium phosphate ($Ca_3(PO_4)_2$). A NaCl saturated solution was used to produce high humidity conditions in each large box. The snails grew under natural light/dark cycles. The air and food were changed every two days. Then the big plastic boxes were put into three incubators with respective temperatures of 20 ℃, 25 ℃, and 30 ℃, until January, 2013; some snails from 20 ℃ group were cultured until May, 2013. At the end of culturing period, we recorded the length and height for each snail (see supplement, Table S2). Additionally, we

have checked the growth stage of one individual cultured at 25 ℃ (S43, Table S2), whose length is 10.1 mm. The result shows that this snail has already become an adult. Consequently, the lengths for most of other snails were larger than 10.0 mm, suggesting they probably reached adult stage. The growth phases of snails are presented in Fig. 1.

We also cultured some snails using corn (C_4 plant, $\delta^{13}C$ = -12.0±0.7‰, *n*=4; first we used *Miscanthus sinensis*, but the snails did not eat it; then we changed the food to corn) sprinkled with fine calcium carbonate powder in a 20 ℃ incubator.

Ultimately, snails were collected into labeled sampling bottles and were preserved at -40 ℃ for additional treatment.

2.2 Isotopic samples preparation and analysis

Frozen snails were dried using a cryogenic vacuum line. They were then washed with distilled water and kept in the water for 10 min. The soft body tissues of respective snails were separated from shells using a nipper without any damage. Then they were immersed into 3N HCl for 2–4 hr, rinsed with distilled water and then lyophilized. All dry tissues were ground to powder and wrapped in tinfoil (Sn) capsules. Each sample was introduced into a combustion tube from the auto-sampler and converted into gaseous CO_2 at 980 ℃ one by one. Then the resultant CO_2 was injected into a Cavity Ring-Down Spectroscopy (CRDS, L1121-I; Picarro, Inc., Santa Clara, CA, USA) for $\delta^{13}C$ measurement. More details describing the CRDS method and system are presented by Wahl et al. (2006) and Balslev-Clausen et al. (2013). All of the measured $\delta^{13}C$ values were normalized against two simultaneously measured standards, acetanilide ($\delta^{13}C$ = -33.62‰; Costech Analytical Technologies, Inc., Valencia, CA, USA) and sucrose ($\delta^{13}C$ = -13.55‰; Kanto Chemical Co., Inc., Tokyo, Japan) by two-point calibration method (Coplen et al., 2006). The analytical precision was better than 0.4‰ (*n*=3).

Shells were washed successively with distilled water, then with acetone under an ultrasonic bath for 60 min to eliminate the organic residues. After washing again using distilled water, they were put into labeled sampling bottles containing diluted hydrogen peroxide (10%) to remove the remaining organic matter. These bottles were kept overnight in a shaking machine with speed around 100 r/min. Finally, all the shells were dried using lyophilization and were crushed into a homogenous powder (the first 2 internal spirals were removed, which were inherited from their parents and accounted for less than 5% of the total shell mass) using an

agate mortar for isotopic analyses. To the collected adults and cultured snails, their shell powder (7–10 mg) was reacted with 103% phosphoric acid for more than 2 hr in a 25 ℃ water bath. Then the purified CO_2 was analyzed using isotope ratio mass spectrometry (MAT 253; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and calibrated against an synthetic calcium carbonate standard, Wako ($\delta^{13}C$ = -9.13‰; Wako Pure Chemical Industries, Ltd., Osaka, Japan). The precision was better than 0.1‰ (*n*=3).

To the larvae and eggshell, the powder (400±50 μg) was reacted with 103% phosphoric acid for more than 1 hr at 72 ℃. The resultant CO_2 was analyzed using an isotope ratio mass spectrometer (DELTA XL; ThermoFisher Scientific, Inc., Waltham, MA, USA) coupled with a PAL autosampler (GCPAL; CTC Analytics, Zwingen, Switzerland), and a GasBench II preparation device (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The $\delta^{13}C$ values were normalized against two simultaneously measured synthetic calcium carbonate standards, Wako ($\delta^{13}C$ = -9.13‰; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and Kanto ($\delta^{13}C$ = -20.62‰; Kanto Chemical Co., Inc., Tokyo, Japan) by two-point calibration method. The analytical precision was around 0.1‰ (*n*=3).

All of our in-house working standards mentioned here are calibrated with respect to the IAEA standards such as NBS-19 and the $\delta^{13}C$ values are reported relative to VPDB (Vienna Peedee belemnite).

In addition, the crystal structure of shell carbonate powder was checked using X-ray diffractometry (XRD, MXP3TA; Mac Science Ltd.). The results revealed that our snail shell carbonate is aragonite, although the eggshell carbonate is calcite.

3 Results

3.1 Summary of culturing experiment

Fig. 2a shows that snails that grew at low temperature (20 ℃) have a higher survival rate (72%) than those at 30 ℃ (16%). The reason probably is that, in high-temperature groups, the humidity is slightly higher so that the leaves are more easily putrified. Because the cultured environment is a sealed system with an air exchange per two days, the perishable leaves consumed oxygen and perhaps produced noxious gases which might have caused all the snails in one small box to have died. Therefore, these snails show higher mortality.

Egonmwan (2008) reported a positive correlation between calcium provision, snail body weight and shell length. High mortality was observed for snails that were deprived of a calcium source. Similarly, in our study, snails fed $Ca_3(PO_4)_2$ exhibited lower weight and shorter length. They were more translucent, more fragile, and had thinner shells, than snails fed $CaCO_3$ during our cultivation. Moreover, Fig. 2b shows a lower survival rate (30%) of snails fed $Ca_3(PO_4)_2$ than those fed $CaCO_3$ (50%), which is also consistent with results of the previous study, suggesting that calcium from the powder of $Ca_3(PO_4)_2$ was ingested only to a slight degree by land snails.

For the cabbage groups, shell weight proportions of the snails, which is equal to the mass of dry shell divide by their total body mass, cultured with $CaCO_3$ at different temperature are: 27.5±2.7% (20 ℃), 31.1±6.1% (25 ℃), 17.9±5.5% (30 ℃); those without $CaCO_3$ are: 7.8±2.9% (20 ℃), 5.7±1.8% (25 ℃), respectively. The discrepancy also reveals worse growth conditions at 30 ℃ among the temperatures discussed above. Those without $CaCO_3$ can get only a very small amount of calcium from either $Ca_3(PO_4)_2$, drinking water or diet. To the corn groups, preliminary results demonstrate that the snails probably have a higher shell weight proportion than those fed with cabbage (*t*-test, $p < 0.01$): 34.7±0.3% (corn, $CaCO_3$, 20 ℃) vs. 27.5±2.7% (cabbage, $CaCO_3$, 20 ℃).

3.2 Carbon isotope results

Body tissues of cultured snails yield a similar $\delta^{13}C$ value with same food source (Table 1), which are -27.9±0.6‰ (fed cabbage, *n*=38) and -10.8±0.7‰ (fed corn, *n*=3). No marked differences were found among temperatures or between those fed with and without $CaCO_3$.

Shell carbonate of cultured snails fed cabbage and $CaCO_3$ under 20 ℃, 25 ℃, and 30 ℃ respectively yielded $\delta^{13}C$ values of -9.7±0.5‰, -10.4±0.8‰ and -13.2±0.8‰ (Table 1., Fig. 6a). Those fed cabbage and $Ca_3(PO_4)_2$ respectively yielded values of -12.5±0.4‰, -13.7±0.7‰, -14.2‰. Snails fed corn and $CaCO_3$ at 20 ℃ yielded a value of 3.9±0.5‰.

We also measured the $\delta^{13}C$ values of collected adult snails, newly hatched snails, and eggshells, the results are, respectively, -11.5±1.2‰ (*n*=6), -11.0±0.1‰ (mixed samples, *n*>10), -13.1±0.9‰ (two groups of eggs, for each group, *n*>10).

4 Discussion

4.1 Land snail shell carbonate precipitation and carbon source estimation

The solid line of Fig. 3 shows a model of diet-controlled snail shell carbonate $\delta^{13}C$ (Francey, 1983; Stott, 2002), whereas the dashed line shows a flux balance model based on CO_2 diffusion when the input flux of CO_2 is equal to respired flux of CO_2 in the body fluid of land snails (Balakrishnan and Yapp, 2004). However, additional enrichments were observed in both the published results and this study (e.g. snails cultured at 20 ℃ and 25 ℃ with carbonate, this study), suggesting a contribution from ingested carbonate. In addition, because a common model seems unsuitable for all snails because of the different metabolic rates among snail individuals growing up at different conditions (Barnhart and McMahon, 1987), the estimation of shell carbon sources for each snail is expected to be useful and necessary.

The main sources of shell carbon are from diet, atmospheric CO_2 and ingested carbonate (Goodfriend and Hood, 1983). A simple framework of the possible mechanisms to precipitate shell carbonate is presented in Fig. 4 based on results of a previous study as well as results from the present study. Almost all of these three carbon sources are expected to transform into gaseous CO_2 and then dissolve into the hemolymph of snail (so-called bicarbonate pool) for reaching isotopic equilibrium with bicarbonate (HCO_3^-). The fractionation between gaseous CO_2 and HCO_3^- under equilibrium is controlled by temperature, shown as $\Delta^{13}C_{HCO3\text{--}g} = -(0.114 \pm 0.003)T$ (℃) + (10.78±0.05) ‰ (Mook et al., 1974; Zhang et al., 1995; Szaran, 1997). Therefore, the carbon isotope fractionations under our culturing temperatures might be 8.5‰, 7.9‰, and 7.4‰, respectively, at 20 ℃, 25 ℃, and 30 ℃. Finally, the shell aragonite will precipitate from bicarbonate pool in isotopic equilibrium (also designated as carbon isotope steady state, Balakrishnan and Yapp, 2004) and the carbon isotope ratio might be enriched to around 2.7‰ (Rubinson and Clayton, 1969; Romanek et al., 1991). Therefore, the total fractionation between each carbon source (diet, atmospheric CO_2 and ingested carbonate) and shell aragonite is expected to be an enrichment of 11.2‰, 10.6‰, and 10.1‰, respectively, at 20 ℃, 25 ℃, and 30 ℃. In addition, trace amounts of DIC dissolved in leaf water and drinking water might also introduce isotopic fractionations of shell carbonate. For instance, Pigati et al. (2004), based on radiocarbon dating technique, reported that aqueous carbon sources account for approximately 10% of the shell carbon for one species of *Catinella*, which is a semi-aquatic gastropod. However, no report to date describes a study of this source related to terrestrial gastropods.

For simplification of the model and calculations, we made two assumptions. First, no carbon isotopic fractionation takes place when the ingested carbonate dissolves into the water and reacts with stomach acid. Second, because of the variable sources and probably low contribution of DIC from directly ingested water, which are similar to all earlier published discussions, we will not involve such sources in our calculation. These assumptions can be evaluated for their propriety in future research. Results of those studies can be expected to improve the accuracy of this estimation method.

According to this framework, we will explain briefly how to calculate the contribution of each source to shell carbon based on the snail data we measured. First, from the mass balance model, we obtain the following.

$$\delta^{13}C_S = \mathrm{x}\delta^{13}C_t + y\delta^{13}C_a + z\delta^{13}C_c + b \quad (1)$$

$$\mathrm{x} + y + z = 1 \quad (2)$$

Therein, δ^{13}Cs = the isotopic composition of shell aragonite:

δ^{13}Ct = the isotopic composition of snail tissue. Based on our observations and discussions, see Sect. 4.2.2, we use the measurements for each snail, not an average value, because of the individual differences among snails.

δ^{13}Ca = the isotopic composition of atmospheric air (δ^{13}C of atmospheric CO_2 was observed from June 2010 to July 2011 in Suzukakedai, Yokohama, Japan. The average value is -9.5‰ (*n*=42, Zhang et al., unpublished data); the year decrease rate reported by Keeling et al. (2010) is approximately 0.02‰ per year. Therefore, we use -9.5‰ as the δ^{13}Ca value)

δ^{13}Cc = the isotopic composition of ingested carbonate (4.0‰)

x = proportion of metabolic CO_2; *y* = proportion of atmospheric CO_2; *z* = proportion of CO_2 from ingested carbonate

b = isotope fractionation value from gaseous CO_2 to aragonite at different temperatures (11.2‰, 10.6‰, and 10.1‰, at 20 ℃, 25 ℃, and 30 ℃, respectively)

Here we chose 20 ℃ group snails to demonstrate how we calculated the contribution of each source. When there is no $CaCO_3$ added, *z* = 0.

$$\delta^{13}C_S = \mathrm{x}\delta^{13}C_t + (1-x)\delta^{13}C_a + 11.2‰ \quad (3)$$

$$\mathrm{x} = (\delta^{13}C_S - \delta^{13}C_a - 11.2)/(\delta^{13}C_t - \delta^{13}C_a) * 100\% \quad (4)$$

$$x + y = 1 \quad (5)$$

From inputting the measured data, we obtain the *x* and *y* values shown in Table 1. For simplifying the calculation, to the snails fed $CaCO_3$, we assumed a similar *x*/*y* ratio (pCO_2) to those of all the snails, although individual differences might happen among snails attributable to different growth rates. Therefore we calculated the average *x*/*y* ratio in non-carbonate groups, and obtained

$$x / y = 3.2 \quad (6)$$

at 20 ℃, *b*=11.2‰. Therefore,

$$y = (\delta^{13}C_S - \delta^{13}C_c - 11.2)/(3.2\delta^{13}C_t + \delta^{13}C_a - 4.2\delta^{13}C_c) * 100\% \quad (7)$$

By combining Eqs. (2), (6), and (7), we calculated *x*, *y*, *z* values separately. Similar calculations were done at 25 ℃ and 30 ℃. Finally, for the C_4 plant groups, we assumed the same *x*/*y* ratio to the C_3 plant groups, which might not be accurate because the food consumption preference is different and C_3 plant groups have a higher growth rate than C_4 plant groups have. Then we calculated *x*, *y*, and *z* using the same method. All calculated results are presented in Table 1.

The calculated *x*, *y*, *z* values for snails fed C_3 plants reveal that the contributions of diet, atmospheric CO_2, and ingested limestone varied respectively as 66–80%, 16–24%, and 0–13%. Furthermore, for those fed C_4 plants, because of the potential food stress (lower consumption ability of C_4 plant), they vary respectively as 56–64%, 18–20%, and 16–26%. We observed a higher shell weight proportion of snails that had been fed corn compared with those that had been fed cabbage, suggesting a higher ingested limestone contribution, which is coincident with our calculations.

Fig. 5a presents a positive correlation between the calculated contribution of ingested carbonate and the shell weight proportion. Calcium carbonate can be transformed into Ca^{2+} in the stomach of land snails and can then be ingested into the hemolymph, and can finally be precipitated into the shell carbonate. Therefore, high calcium carbonate consumption is always correlated with a high shell weight proportion. The $\delta^{13}C$ of fed carbonate is more positive than $\delta^{13}C$ of food and atmospheric CO_2. For that reason, if snails consume more carbonate, then their shell $\delta^{13}C$ values are expected to be more positive, which is consistent to the relation presented in Fig. 5b.

As described earlier, the *x*/*y* ratio can vary among snail individuals. Therefore, the robustness of our estimation method was tested using the maximum and minimum *x*/*y* values observed from those snails fed without $CaCO_3$ at different temperatures. The results are presented in Table 2. For snails fed with cabbage and $CaCO_3$ at 20 ℃, the *x*/*y* values vary as 2.9–3.3. The maximum discrepancies of estimations by the maximum and minimum *x*/*y* value could be 1.3%, -2.9%, and 1.6% for *x*, *y*, and *z* values, respectively. For those fed with cabbage and $CaCO_3$ at 25 ℃, *x*/*y* values vary as 3.4–5.2. The maximum discrepancies could be 3.0%, -6.9%, and 4.1% for *x*, *y*, and *z* values, respectively. Considering individual differences among snails (calculated standard deviations of *x*, *y*, and *z* to the snails fed with cabbage and $CaCO_3$ are 3.9%, 1.6%, and 3.9%, respectively), these discrepancies are acceptable, especially for the estimated contribution of diet and ingested carbonate. Moreover, almost all of these re-estimated *x*, *y*, and *z* values are within in the scopes we have calculated based on an average *x*/*y* value, showing a satisfactory robustness of this estimation method.

At the same time, we have been aware of another possibility about the carbon isotope fractionation related to metabolic CO_2, which probably has already dissolved into snail body water when produced without any isotope fractionation generated from gaseous state to aquatic state. In this case, the total carbon isotope fractionation from metabolic CO_2 to shell aragonite would be 12.4‰, 11.8‰, and 11.2‰, at 20 °C, 25 °C, and 30 °C, respectively. We have calculated the contributions of each carbon source by this assumption and shown the results in the supplement (Table S1). In such circumstance, the contributions of diet, atmospheric CO_2, and ingested limestone for snails fed C_3 plants varied respectively as 70–85%, 13–19%, and 0–13%, while those fed C_4 plants varied respectively as 64–73%, 15–17%, and 11–22%. This estimation shows lower contribution of atmospheric CO_2 and higher contribution of diet than the previous one. Although we cannot eliminate either of these two possibilities with present knowledge, both of them implicate similar discussions and conclusions. Consequently, in the following discussions, we will only consider the data presented in Table 1.

4.2 Contribution of each carbon source to shell $\delta^{13}C$

4.2.1 Ingested carbonate (limestone in nature)

Stott (2002) reported no apparent differences in shell $\delta^{13}C$ of land snail *Helix aspersa* fed with and without $CaCO_3$. However, the deviation of ^{14}C ages estimation for some snail species in

nature shows an incorporation of limestone (Goodfriend and Stipp, 1983; Pigati et al., 2010). Consequently, ingested limestone is expected to play an important role in controlling shell carbonate $\delta^{13}C$, at least for some species. Heavier $\delta^{13}C$ values were observed in our cultivated land snail *Acusta despecta sieboldiana* fed $CaCO_3$ compared with those fed $Ca_3(PO_4)_2$, especially at 20 ℃ and 25 ℃ (Fig. 6a). These values are even heavier than the values predicted from a flux balance model (Balakrishnan and Yapp, 2004), which considers both metabolic CO_2 and atmospheric CO_2 (Fig. 3), strongly suggesting the involvement of ingested $CaCO_3$ powder (Fig. 6b). The estimated contribution for C_3 plant group is 0–13%; and for C4 plant, it varies: 16–26%. The different estimated proportions reveal different growth rates (metabolic rates) among snails. Apparently, snails fed C_4 plants might prefer involving higher proportions of limestone, which is probably caused by diet stress.

Fig. 7a (see Table 3) shows a decreasing trend of shell $\delta^{13}C$ along with the snail growth direction, which is consistent to the measurements of living land snail individuals reported by Yanes et al. (2012), who observed higher $\delta^{13}C$ values during the younger growth stages of land snails. This fact is explainable by the larger proportion of limestone ingested during the early period of land snails to enhance their growth rates. Egonmwan (2008) reported that the amount of food ingestion increases gradually, although calcium ingestion first increases (1–3 months, but increase rate is lower than diet consumption) and then decreases (4–6 months) during the first 6 months, suggesting that the contribution of ingested carbonate can be expected to decrease along with the snail growth. Our estimated contributions of ingested carbonate (Fig. 7b) support this supposition. To elucidate this phenomenon, similar studies should be done to observe snails fed with C_4 plants and those fed without $CaCO_3$.

4.2.2 Diet and food selectivity

DeNiro and Epstein (1978) reported a slight enrichment (approximately 1‰) of the snail body tissue $\delta^{13}C$ relative to their diet: romaine lettuce. Stott (2002) shown no significant isotopic offset between snail body tissue $\delta^{13}C$ fed lettuce and their diet, whereas about 2–3‰ depletion was observed for snails fed corn (Fig. 8a), which is regarded as attributable to the contribution of preculture carbon because they cultured snails from juveniles but not eggs. Our observations show slight enrichment among all snails relative to their diet: approximately 0.5‰ for cabbage and approximately 1.2‰ for corn. However, this enrichment is negligible when considering measurement precision among both snail individuals and vegetable samples. We suspect that the small discrepancies of the $\delta^{13}C$ values between snail body tissue and their diet

observed in the literature and this study may depend on the analytical precision and/or limitation of samples (e.g., sample size, growth condition, etc.). Therefore, we infer that the $\delta^{13}C$ values of snail body tissue are similar and that they should directly reflect the $\delta^{13}C$ values of their food.

Fig. 8b shows the observed relations between shell carbonate $\delta^{13}C$ of snails and their diet (This study; Stott, 2002; Metref et al., 2003). Evidently, all of these slopes are less than the expected slope 1, which is considered diet as a single source controlling shell carbon (Francey, 1993; Stott, 2002). Offsets suggest the influence of other sources such as limestone and atmospheric CO_2 causing these more positive values. The relative contributions of C_3 and C_4 type food might be different. Our estimation confirms this inference: the proportions of shell carbon from cabbage are 66–80% and from corn are 56–64%, which might reflect food stress among different plants. Metref et al. (2003) reported a C_3 vs. C_4 mixed experiment. Most snails show a preferential use of C_3 food. This study also found a preference of this kind in consuming C_3 but not C_4 plants, i.e., snails were growing faster when eating C_3 plants such as lettuce and cabbage than those fed a C_4 plant such as corn, which agrees with the observations reported by Metref et al. (2003). Moreover, almost all the snails fed *Miscanthus sinensis* (C_4 plant) died after 20–30 days, except one or two large individuals and their shell $\delta^{13}C$ values (-11.1±3.4‰) have no marked differences from newly hatched snail larvae (-11.0±0.1‰), suggesting that the snails were unable to consume *Miscanthus sinensis* at all. However, Stott (2002) reported a lower growth rate of snails cultured with dried sour orange tree leaves (C_3 plant) compared to those cultured by lettuce and corn, suggesting food quality such as water content or physical structure as the reason for food preference. To land snail *Acusta despecta*, food preference has been reported by Suzuki and Yamashita (1967), Takeuchi and Tamura (1995). For instance, this species could not eat *Oxalis corniculata* (C_3 plant), *Commelina communis* (C_3 plant), *Yoshinagella japonica* (Fungi) at all. Similar phenomenon also occurred in the nature observations. Hatziioannou et al. (1994) and Iglesias and Castillejo (1998) observed that land snails do not eat plant species at random. Baldini et al. (2007) reported that the feces of land snail *Cerion* collected from *Sporobolus domingensis* (C_4) plant exhibited $\delta^{13}C$ values more typical of a C_3 plant, suggesting a preference of C_3 plant as food of these snails. Anyhow, the food selectivity of land snails increases the difficulty of their application in the paleo-environment reconstruction, especially for the accurate study of C_3/C_4 vegetation distribution.

In addition, although some previous works reported that the fresh plants are the main diet for some land snail species (e.g. Suzuki and Yamashita, 1967; Colonese et al., 2014), it may not be common for all the species. According to the literature, land snails can eat decayed plant matter (Richardson, 1975), fungi, animal tissue (Mason, 1970), lichens (Baur, 1994) and other organic matter in nature. The consumed proportions of these food sources vary among land snail species (Mason, 1970).

Consequently, we suggest that the pre-investigation of food preference on living snails is important before applying shell carbon isotopic values in the plaeo-environment reconstruction of a certain species. In addition, future studies of food quality's influence on snail shell $\delta^{13}C$ values might be helpful, too.

4.2.3 Atmospheric CO_2

Although Stott (2002) and Metref et al. (2003) reported that atmospheric CO_2 does not contribute to shell carbonate $\delta^{13}C$ values based on culturing land snail *Helix aspersa*, some works clarify it as an important source of shell carbon. For instance, according to the combination of $\delta^{13}C$ and ^{14}C values observed from modern land snail shells, Romaniello (2008) calculated the contribution of atmospheric CO_2 varing from 16% to 48%. McConnaughey and Gillikin (2008) summarized the previous studies of relation between land snail shell carbonate $\delta^{13}C$ and shell organics (or body tissue organics / diet) and pointed out that the offsets are greater when the $\delta^{13}C$ of organics diverge more from atmospheric CO_2, i.e., when the slope of the shell vs. organic regression line is less than unity, suggesting that the latter does contribute to shell carbonate.

In this study, our calculation results revealed that atmospheric CO_2 can affect shell carbonate isotopic values, and our estimated contributions are, respectively, 16–24% (fed cabbage), 18–20% (fed corn). More clear evidence might be that, for snails growing up without $CaCO_3$ (Fig. 3), shell $\delta^{13}C$ values became heavier than the expected values controlled by one end member (diet). These estimated values can be attributed to two pathways: (1) atmospheric CO_2 directly being introduced into the bicarbonate pool via respiration; (2) by means of the imbibed water as dissolved atmospheric CO_2. The carbon isotope fractionation has no difference between these two pathways.

However, since the CO_2 concentration and its in-situ carbon isotopic value were not monitored in the semi-sealed system, the CO_2 gradually accumulated from the respiration of

snails and plant tissues might affect the accuracy of our estimations on atmospheric CO_2. Although Balakrishnan and Yapp (2004) inferred that the accumulated CO_2 (~240 ppm, vs. ambient background) produced by the respiration under forest canopy would contribute an insignificant variation of shell $\delta^{13}C$ values (~0.1‰), we suggest that additional studies based on experiments are needed. Besides, the limited accuracy of our assumption of similar diet/atmospheric CO_2 ratio at the same temperature must be acknowledged because the snails have individual differences of metabolic rates during their growth (Barnhart and McMahon, 1987). Therefore, to learn more accurate contributions of atmospheric CO_2, further incubation experiments are necessary, which are expected to include several parallel groups with labeled $\Delta^{14}C$ or $\delta^{13}C$-different CO_2 compositions, and also to record the concentration and isotopic composition variations of in-situ atmospheric CO_2 during cultivation.

4.3 Metabolic rate

Snail individuals have different metabolic rates in different environment conditions, or even in different growth phases of a single individual. Different metabolic rates reflect different partial pressures of CO_2 produced by diet, which consequently causes different flux ratios among CO_2 produced from metabolism, digestion of limestone, and atmosphere to approach a carbon isotope steady state in snail body fluids, and eventually produce different $\delta^{13}C$ values. Many discussions we presented above show influences of this kind, such as (1) snails cultured at 30 ℃ groups with $CaCO_3$ show more depleted $\delta^{13}C$ values than those at 20 ℃ and 25 ℃ groups (Fig. 6a). Correspondingly, they have a lower survival rate and lighter mass, suggesting stress from poor growth conditions (e.g., unsuitable temperature, perishable leaves, lack of oxygen, etc.); (2) for one snail individual, the trend of decrease in both $\delta^{13}C$ values of shell carbonate (Fig. 7a) and estimated that the contribution of ingested carbonate (Fig. 7b) reflects a decreasing metabolic rate along with snail growing up; (3) the different slopes presented in Fig. 8b reveal different metabolic rates among different snail species or different cultivation conditions.

Therefore, we suggest that the variations in metabolic rates attributable to the shift of environmental conditions, which can produce discrepancies of shell carbonate $\delta^{13}C$ values, should be taken into account in the paleo-environment studies. For example, Yanes et al. (2011) observed 3‰ higher moving average shell $\delta^{13}C$ values during the glacial interval (~15 to ~50 ka BP) than today, and inferred a larger proportion of C_4 plant during that period. However, in this study, we found that shell carbonate $\delta^{13}C$ values of land snails, for those fed

same diet (cabbage) and carbonate but growing up at different temperatures, can also vary as large as 3.5‰ (Fig. 6a).

Consequently, the carbon isotopic composition in land snail fossils can be considered as an auxiliary tool to understand changes of paleo-environment conditions, such as, a suddenly decreased temperature record during the Younger Dryas event.

4.4 Temperature

Theoretically, temperature can affect the fractionation factor between gaseous CO_2 and HCO_3^- in land snail body fluid from a relation presented as $\Delta^{13}C_{HCO3\text{--}g}$= - (0.114±0.003) T (℃) + (10.78±0.05) ‰ (Mook et al., 1974; Zhang et al., 1995; Szaran, 1997). Therefore, for shells precipitated at two different temperatures, the discrepancy between $\delta^{13}C$ values ($\Delta^{13}C$, ‰) is -0.11/ ℃ × T (Zhang et al., 1995; see also in Romanek et al., 1991, -0.13/ ℃ and Szaran, 1997, -0.10/ ℃). We observed a relation of -0.17±0.04/ ℃ among snails fed without $CaCO_3$ (Fig. 9), which is not significantly different from the theoretically expected relation. The small sharper trend might somehow reflect metabolic differences among the snails at different temperature conditions. The $\delta^{13}C$ discrepancy of 0.17‰ per degree is small compared with the contributions of different carbon sources. For that reason, no special consideration of environmental parameters of this kind is necessary on most occasions.

4.5 Carbon isotopic fractionations during snail laying and hatching

Metref et al. (2003) found that hatched and 1-day old snails showed a depletion of the $\delta^{13}C$ values of 2.5‰ compared to their parents. They inferred that shells of the hatching juvenile were built not only from eggshell calcite but also from isotopic depleted albumen. We have observed these $\delta^{13}C$ results for *Acusta despecta sieboldiana*: adult snails (parents), -11.5±1.2‰ (n=6); hatched snails: -11.0±0.1‰ (n>10, mixed samples); eggs: -13.1±0.9‰ (two groups of eggs, for each group, n>10). No obvious $\delta^{13}C$ depletion was found between hatched snails and adults.

Romanek et al. (1992) pointed out that fractionation factors among calcite, HCO_3^- and aragonite are temperature independent when they are in equilibrium conditions. Their values were reported as $\Delta^{13}C_{Calcite\text{-}HCO3\text{-}}$= 0.9±0.2‰, $\Delta^{13}C_{Aragonite\text{-}HCO3\text{-}}$= 2.7±0.2‰, $\Delta^{13}C_{Aragonite\text{-}Calcite}$= 1.8±0.2‰ (Rubinson and Clayton, 1969; see also in Romanek et al., 1992, $\Delta^{13}C_{Calcite\text{-}HCO3\text{-}}$= 1.0±0.2‰; $\Delta^{13}C_{Aragonite\text{-}HCO3\text{-}}$= 2.7±0.6‰; $\Delta^{13}C_{Aragonite\text{-}Calcite}$= 1.7±0.4‰). According to the

XRD results, all of our cultured snail shells are made of aragonite, whereas eggshells are made of calcite. We are striving to ascertain the isotopic fractionations based on calcite - HCO_3^- - aragonite equilibrium. At this isotopic equilibrium condition, (a) for the laying process: when the snail precipitate shell (aragonite) from bicarbonate pool, $\delta^{13}C$ will enrich 2.7‰; and when precipitate eggshell (calcite), $\delta^{13}C$ will enrich 0.9–1.0‰. Therefore, the difference between shell aragonite and eggshell calcite is expected to be 1.7–1.8‰, we obtain a similar value of 1.6‰, suggesting that snail eggshells are also precipitated from the bicarbonate pool and that they follow the equilibrium fractionation. (b) For the hatching process, when the larvae are hatching, we assume that the shell calcite will dissolve gradually into the egg water to form a bicarbonate pool to precipitate egg aragonite. Then the fractionation between egg calcite and hatched snail shell aragonite is expected to be around -1.8‰ to -1.7‰. Here we obtained a value of -2.1‰, which is also consistent with the assumed value if we consider the measurement error, suggesting that the shell aragonite is transferred from eggshell calcite at an isotopic equilibrium condition.

5 Conclusions

From culturing the land snail *Acusta despecta sieboldiana* under a controlled environment, we confirmed that diet, atmospheric CO_2, and ingested limestone are important sources controlling shell carbon isotopic composition. We have also discussed the influences of metabolic rates. Furthermore, temperature can affect shell carbonate $\delta^{13}C$ values by controlling the carbon isotopic fractionation of gaseous CO_2 - bicarbonate equilibrium. We presented a simple but credible framework to discuss the mechanisms of how each source and environmental parameter might affect shell carbonate $\delta^{13}C$ values based on this and earlier studies. According to this framework and some reasonable assumptions, we estimated the contribution of different carbon sources for each snail individual: to cabbage (C_3 plant) fed snails, the contributions of diet, atmospheric CO_2 and ingested limestone vary respectively as 66–80%, 16–24%, and 0–13%. Furthermore, to corn (C_4 plant) fed snails, because of the potential food stress (lower consumption ability of C_4 plant), they vary respectively in 56–64%, 18–20%, and 16–26%.

Secondly, we found that snails discriminate in their choices of different plant species as food. For instance, they can grow faster when eating C_3 plants such as cabbage compared with C4 plant such as corn than they can when they eat C_4 plants such as *Miscanthus sinensis*. This kind of food selectivity of land snails increases the difficulty of their application in the paleo-

environment reconstruction, especially for the accurate study of C_3/C_4 vegetation distribution. Finally, we inferred that during egg laying and hatching of our cultured snails, carbon isotope fractionation is controlled only by the isotopic exchange of calcite - HCO_3^- - aragonite equilibrium.

To prompt the application of carbon isotope in paleo-environment reconstruction, additional work should be undertaken in future culture experiments, especially in the following several aspects: (1) intra-shell measurements of snails fed with C_4 plants and those fed without $CaCO_3$ should be taken in future studies to verify the phenomenon of decreasing trend of shell $\delta^{13}C$ along with the snail growth direction; (2) influences of food quality such as water contents or physical structure on snail shell $\delta^{13}C$ values should be investigated; (3) snails can be cultured in air with labeled $\Delta^{14}C$ or $\delta^{13}C$-different CO_2 composition to ascertain the contribution of atmospheric CO_2 more accurately; (4) snails fed with C_4 plants under a non-carbonate condition should be evaluated; (5) more land snail species preferring varied environmental conditions should be studied to ascertain which inferences are common to all species and which are suitable for a given species.

Acknowledgements

This study was supported by a Grant-in-Aid for Scientific Research (#23224013) and a grant for the Global COE Program ‘From the Earth to Earths’ to Tokyo Institute of Technology. We thank Dr. Kazunori Hasegawa for helping us ascertain the species of cultured land snails, and Dr. Takuma Haga for helping us identify the growth stage of new generation. We thank Dr. Yingying Cheng for helping us run XRD measurements. Furthermore, our appreciation is extended to Dr. Xueqin Chi for her kind help during our cultivation of land snails. Finally, we are very grateful to Dr. Alexis Gilbert for his helpful comments.

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1 Table 1. Stable Isotope Results of Snails Cultured Under Different Conditions and the
2 Estimated Contributions of Their Shell Carbon Sources.

Snail No.	Temp. (℃)	$CaCO_3$[a]	Diet	Diet $\delta^{13}C$ (‰)	Shell $\delta^{13}C$ (‰)	Tissue $\delta^{13}C$ (‰)	*x** (%)	*y** (%)	*z** (%)	Shell weight proportion (%)
S1	20	+	Cabbage	-28.4	-9.6	-28.4	67.7	21.2	11.1	30.1
S2	20	+	Cabbage	-28.4	-9.1	-28.6	66.0	20.6	13.3	28.3
S9	20	+	Cabbage	-28.4	-9.1	-28.5	66.2	20.7	13.1	29.7
S10	20	+	Cabbage	-28.4	-9.7	-28.8	67.3	21.0	11.7	27.0
S11	20	+	Cabbage	-28.4	-9.7	-27.6	69.5	21.7	8.7	32.7
S12	20	+	Cabbage	-28.4	-9.5	-27.9	68.4	21.4	10.3	25.4
S5	20	+	Cabbage	-28.4	-9.5	-27.7	68.7	21.5	9.8	25.9
S6	20	+	Cabbage	-28.4	-9.8	-27.4	70.2	21.9	7.9	26.4
S13	20	+	Cabbage	-28.4	-9.4	-28.1	67.9	21.2	10.9	28.2
S14	20	+	Cabbage	-28.4	-10.2	-27.7	70.7	22.1	7.2	23.6
S15	20	+	Cabbage	-28.4	-11.0	-26.9	74.5	23.3	2.2	24.8
S3	20	-	Cabbage	-28.4	-13.1	-29.4	74.3	25.7	0.0	4.6
S7	20	-	Cabbage	-28.4	-12.3	-27.7	76.9	23.1	0.0	10.1
S8	20	-	Cabbage	-28.4	-12.3	-27.8	76.6	23.4	0.0	8.8
S16	20	+	Corn	-12.0	4.2	-10.3	59.1	18.5	22.4	34.4
S17	20	+	Corn	-12.0	4.0	-11.6	56.3	17.6	26.1	34.9
S18	20	+	Corn	-12.0	3.3	-10.5	63.7	19.9	16.4	-
S19	25	+	Cabbage	-28.4	-9.7	-27.5	69.6	17.4	12.9	33.1
S21	25	+	Cabbage	-28.4	-11.0	-27.7	72.8	18.2	9.0	31.0
S25	25	+	Cabbage	-28.4	-12.1	-28.7	74.1	18.5	7.4	19.9
S26	25	+	Cabbage	-28.4	-10.1	-27.7	70.5	17.6	11.9	34.5
S29	25	+	Cabbage	-28.4	-10.2	-27.3	71.6	17.9	10.5	37.8
S31	25	+	Cabbage	-28.4	-9.8	-27.0	71.0	17.8	11.2	30.2
S33[b]	25	+	Cabbage	-28.4	-9.9	-26.5	72.3	18.1	9.6	9.7
S22	25	-	Cabbage	-28.4	-14.9	-28.5	83.9	16.1	0.0	4.3
S23	25	-	Cabbage	-28.4	-13.7	-27.5	82.1	17.9	0.0	5.9
S27	25	-	Cabbage	-28.4	-13.6	-28.5	77.3	22.7	0.0	5.1
S28	25	-	Cabbage	-28.4	-13.4	-27.9	78.8	21.2	0.0	4.2
S32	25	-	Cabbage	-28.4	-13.0	-27.7	77.8	22.2	0.0	8.7
S34	30	+	Cabbage	-28.4	-13.7	-28.0	78.5	20.1	1.4	13.8
S35	30	+	Cabbage	-28.4	-13.5	-27.2	79.6	20.4	0.0	18.2
S36	30	+	Cabbage	-28.4	-13.1	-28.7	75.2	19.3	5.6	18.3
S37	30	+	Cabbage	-28.4	-13.3	-28.3	76.5	19.6	3.9	16.2
S38	30	+	Cabbage	-28.4	-13.4	-28.5	76.4	19.6	3.9	14.5
S39	30	+	Cabbage	-28.4	-11.4	-27.3	73.3	18.8	7.9	31.0
S40	30	+	Cabbage	-28.4	-13.8	-29.3	75.9	19.5	4.6	14.4

S41	30	+	Cabbage	-28.4	-13.5	-28.0	77.9	20.0	2.2	17.1
S42[c]	30	-	Cabbage	-28.4	-14.2	-28.2[d]	79.5	20.5	0.0	-

a.'-' means those fed $Ca_3(PO_4)_2$, which is thought to be hard and rarely ingested by snails;

b. snails regarded as a control group, cultured under an air-free-exchange system with a small amount of soil

c. mixture of four individuals cultured in the same condition (sizes are similar):

d. average value of snails from S34 to S41

* *x*, calculated proportion of metabolic CO_2; y, proportion of atmospheric CO_2; *z*, proportion of CO_2 produced from ingested carbonate

Table 2. Estimations of *x*, *y*, and *z* under Observed Maximum and Minimum *x*/*y* Values at 20 ℃ and 25 ℃.

Snail No.	x_{min} (%)	y_{min} (%)	z_{min} (%)	x_{max} (%)	y_{max} (%)	z_{max} (%)	Δx (%)	Δy (%)	Δz (%)
20 ℃, Cabbage	min: x/y = 2.9			max: x/y = 3.3					
S1	66.9	23.1	10.0	68.0	20.4	11.5	1.1	-2.6	1.5
S2	65.3	22.5	12.2	66.3	19.9	13.8	1.1	-2.6	1.5
S9	65.4	22.5	12.1	66.5	20.0	13.6	1.1	-2.6	1.5
S10	66.5	22.9	10.6	67.6	20.3	12.1	1.1	-2.6	1.5
S11	68.7	23.7	7.6	69.9	21.0	9.2	1.2	-2.7	1.6
S12	67.5	23.3	9.2	68.7	20.6	10.7	1.1	-2.7	1.5
S5	67.9	23.4	8.7	69.0	20.7	10.2	1.1	-2.7	1.5
S6	69.3	23.9	6.7	70.5	21.2	8.3	1.2	-2.7	1.6
S13	67.0	23.1	9.8	68.2	20.5	11.4	1.1	-2.7	1.5
S14	69.8	24.1	6.1	71.0	21.3	7.7	1.2	-2.8	1.6
S15	73.6	25.4	1.0	74.9	22.5	2.6	1.3	-2.9	1.6
20 ℃, Corn	min: x/y = 2.9			max: x/y = 3.3					
S16	57.7	19.9	22.3	59.6	17.9	22.5	1.9	-2.0	0.1
S17	55.1	19.0	25.9	56.8	17.0	26.2	1.7	-1.9	0.3
S18	62.2	21.5	16.3	64.2	19.3	16.5	2.0	-2.2	0.1
25 ℃, Cabbage	min: x/y = 3.4			max: x/y = 5.2					
S19	68.5	20.1	11.4	71.3	13.7	15.1	2.8	-6.5	3.7
S21	71.6	21.1	7.4	74.5	14.3	11.3	2.9	-6.8	3.9
S25	72.9	21.4	5.7	75.7	14.5	9.8	2.9	-6.9	4.1
S26	69.3	20.4	10.3	72.1	13.8	14.1	2.8	-6.6	3.8
S29	70.4	20.7	8.9	73.2	14.0	12.7	2.9	-6.7	3.8
S31	69.8	20.5	9.7	72.7	13.9	13.4	2.9	-6.6	3.7
S33	71.1	20.9	8.0	74.0	14.2	11.8	3.0	-6.7	3.7

'x_{min}', 'y_{min}', and 'z_{min}' are calculated by the minimum x/y values and 'x_{max}', 'y_{max}', and 'z_{max}' are calculated by the maximum *x*/*y* values; 'Δx', 'Δy', and 'Δz' show the discrepancies of estimations by maximum and minimum *x*/*y* values for each snail individual.

Table 3. Stable Isotope Results of Snail Individual Fractions and the Estimated Contributions of Their Shell Carbon Sources.

Sample	Spirals	Shell $\delta^{13}C$ (‰)	*x* (%)	*y* (%)	*z* (%)
S13_1	1–3	-8.2	64.6	20.2	15.2
S13_2	3–4	-9.2	67.2	21.0	11.9
S13_3	4–4.5	-9.3	67.5	21.1	11.4
S13_4	4.5–5	-10.0	69.5	21.7	8.8
S19_1	1–3	-8.1	65.1	16.3	18.6
S19_2	3–4	-9.0	67.8	16.9	15.3
S19_3	4–4.5	-9.1	67.9	17.0	15.1
S19_4	4.5–5	-9.2	68.1	17.0	14.9
S19_5	5–5.5	-12.6	77.9	19.5	2.6
S25_1	1–3	-9.2	66.0	16.5	17.5
S25_2	3–4	-10.8	70.6	17.6	11.8
S25_3	4–4.5	-12.0	73.9	18.5	7.6
S25_4	4.5–5	-12.4	75.0	18.8	6.2
S25_5	5–5.5	-13.1	76.9	19.2	3.9
S31_1	1–3	-8.1	66.1	16.5	17.4
S31_2	3–4	-9.2	69.4	17.3	13.3
S31_3	4–4.5	-8.8	68.3	17.1	14.7
S31_4	4.5–5	-10.8	74.0	18.5	7.5
S39_1	1–3	-9.1	66.7	17.1	16.2
S39_2	3–4	-10.4	70.5	18.1	11.4
S39_3	4–4.5	-11.4	73.5	18.9	7.6
S39_4	4.5–5	-12.8	77.3	19.8	2.8

Relative culturing conditions are shown in Table 1. Meanings of symbols ‘*x*’, ‘*y*’, and ‘*z*’ are the same as those shown in Table 1.

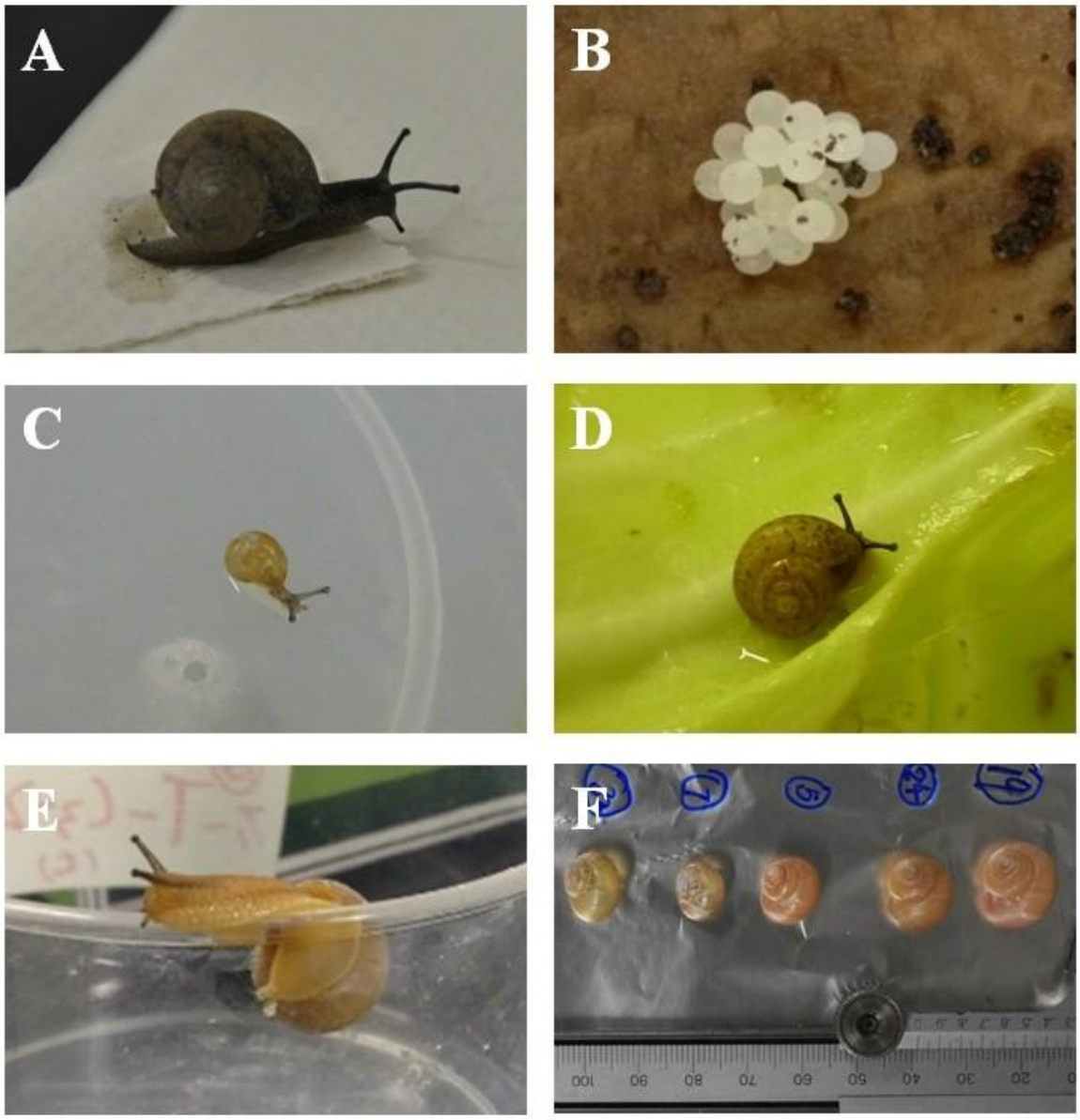


Figure 1. Growth phases of *Acusta despecta sieboldiana*: (A) adult snails collected from Suzukakedai, Yokohama, Japan (length: ca. 10–17 mm); (B) eggs (ca. 2 mm); (C) larva (1–3 days, ca. 2 mm); (D) juvenile (3–4 months, ca. 5–8 mm); (E) adult/juvenile (>6 months, >10 mm); (F) shells for isotopic measurement.

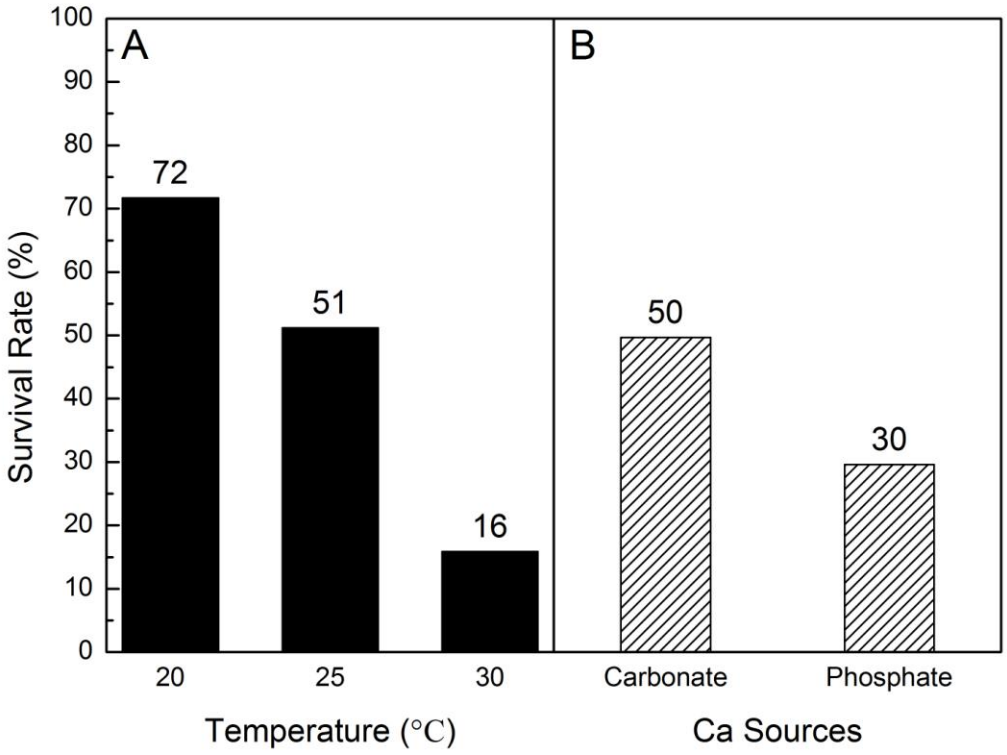
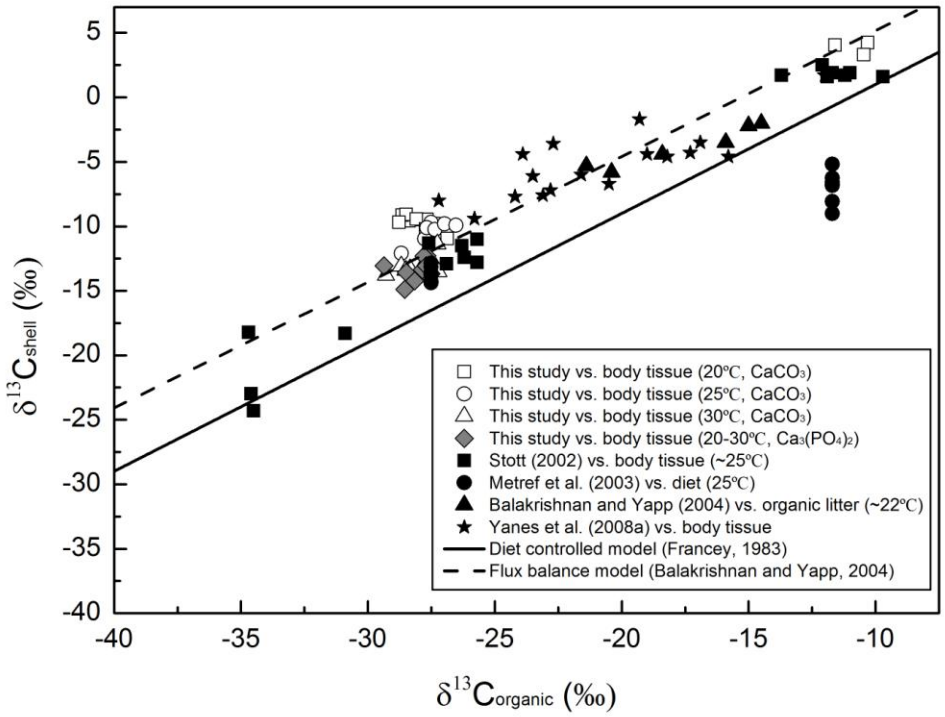


Figure 2. Snail survival rate: (A) at different temperatures; (B) with different calcium sources.



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3 Figure 3. Measured $\delta^{13}C$ of snail shell aragonite against $\delta^{13}C$ of associated organic matter

4 (This study; Stott, 2002; Metref et al., 2003; Balakrishnan and Yapp, 2004).

5

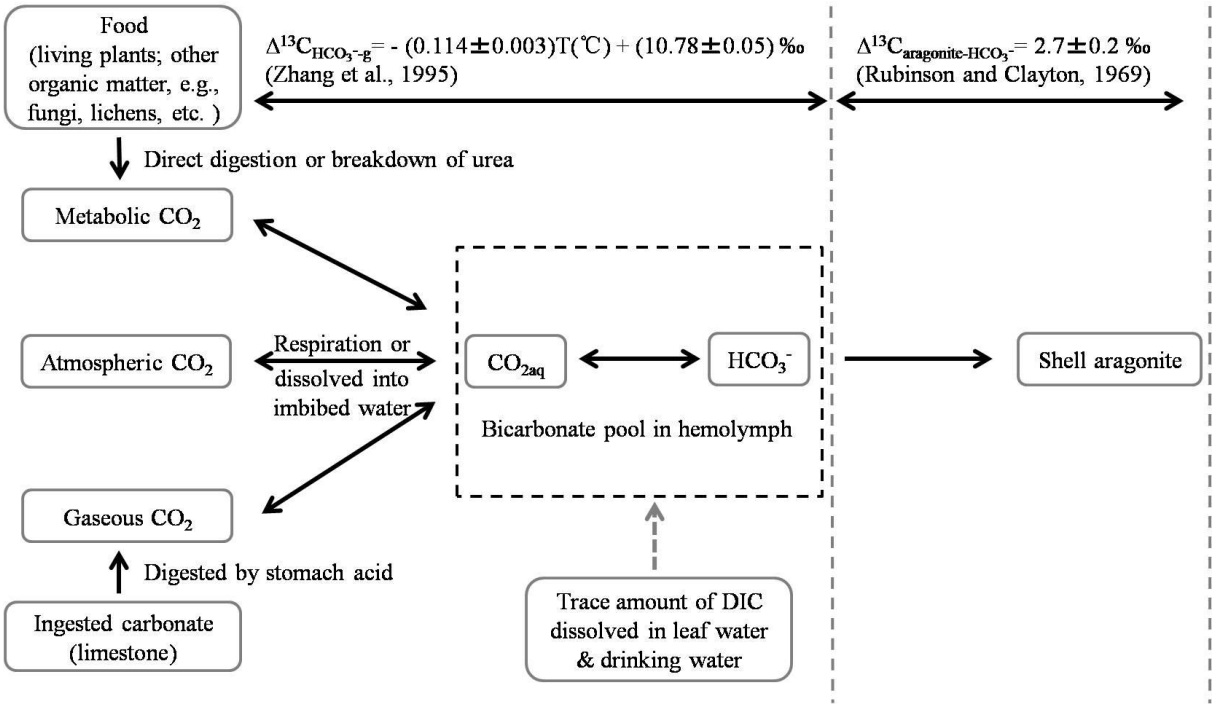
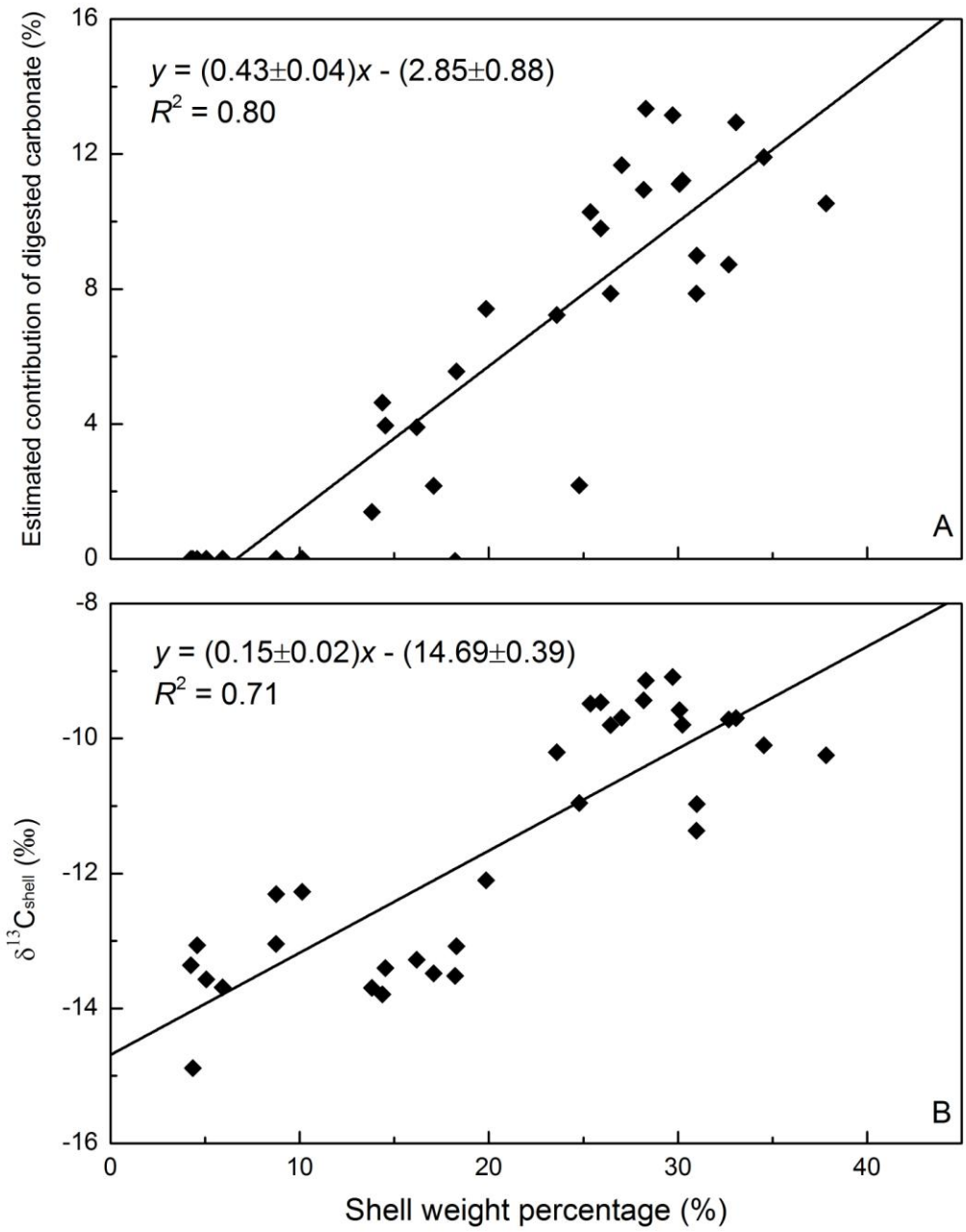


Figure 4. Simple framework of shell carbon sources and their relative isotope fractionation processes based on this and earlier studies.



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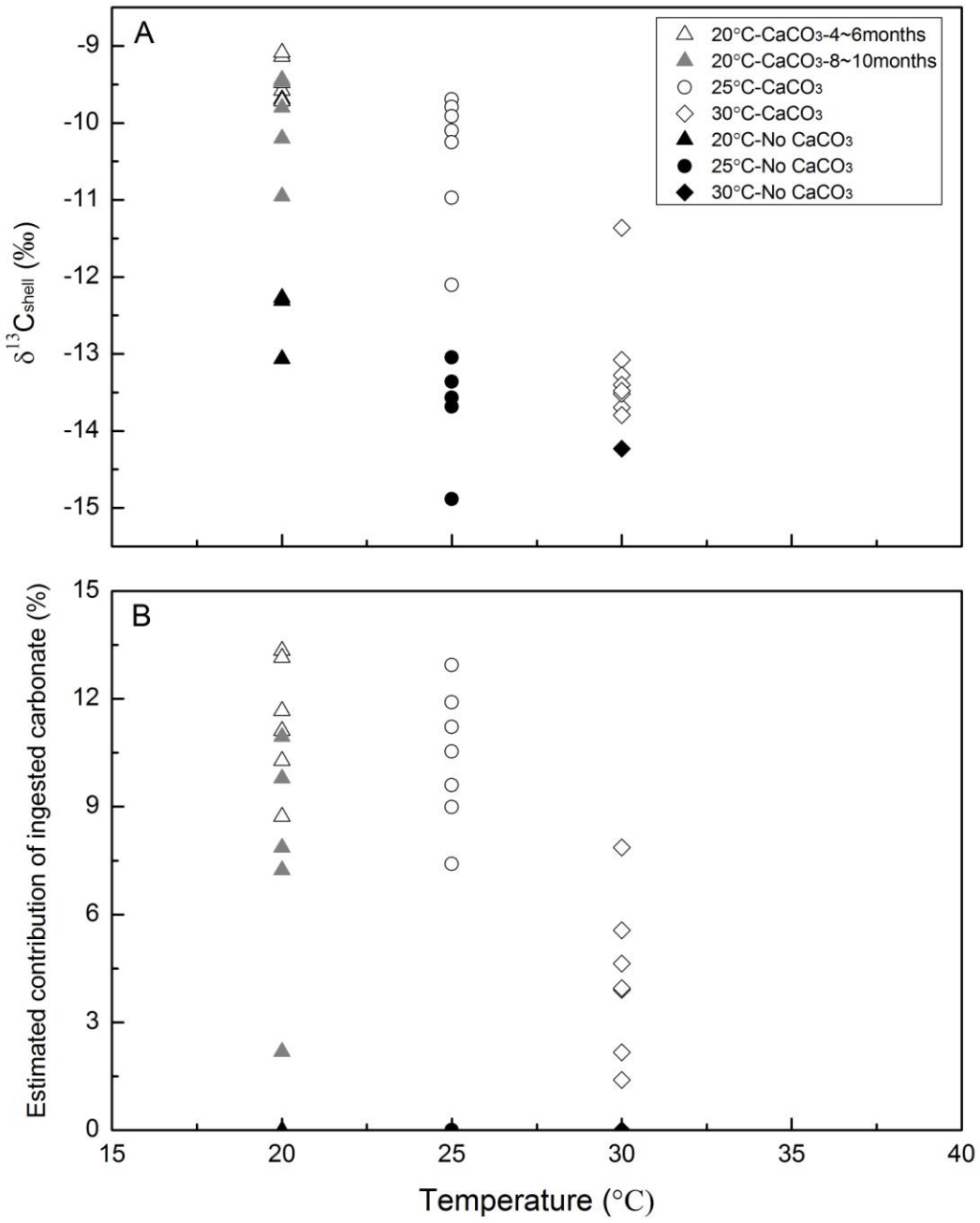
3 Figure 5. Relation between (A) estimated contribution of ingested carbonate and shell weight
4 percentage and (B) $\delta^{13}C$ of snail shell aragonite and shell weight percentage.



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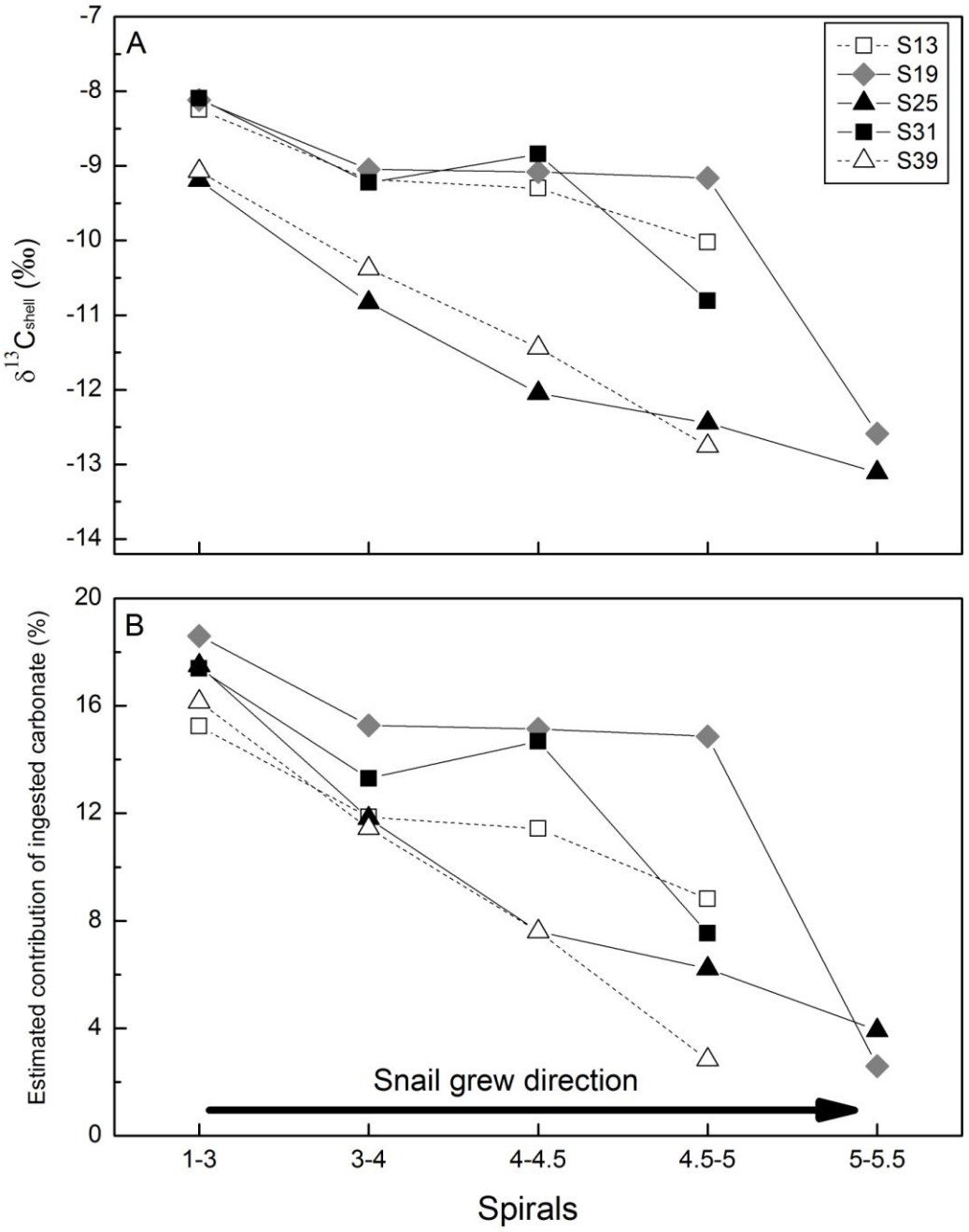
3 Figure 6. Bulk $\delta^{13}C$ of snail shell aragonite and estimated contribution of ingested carbonate
4 at different culturing conditions.



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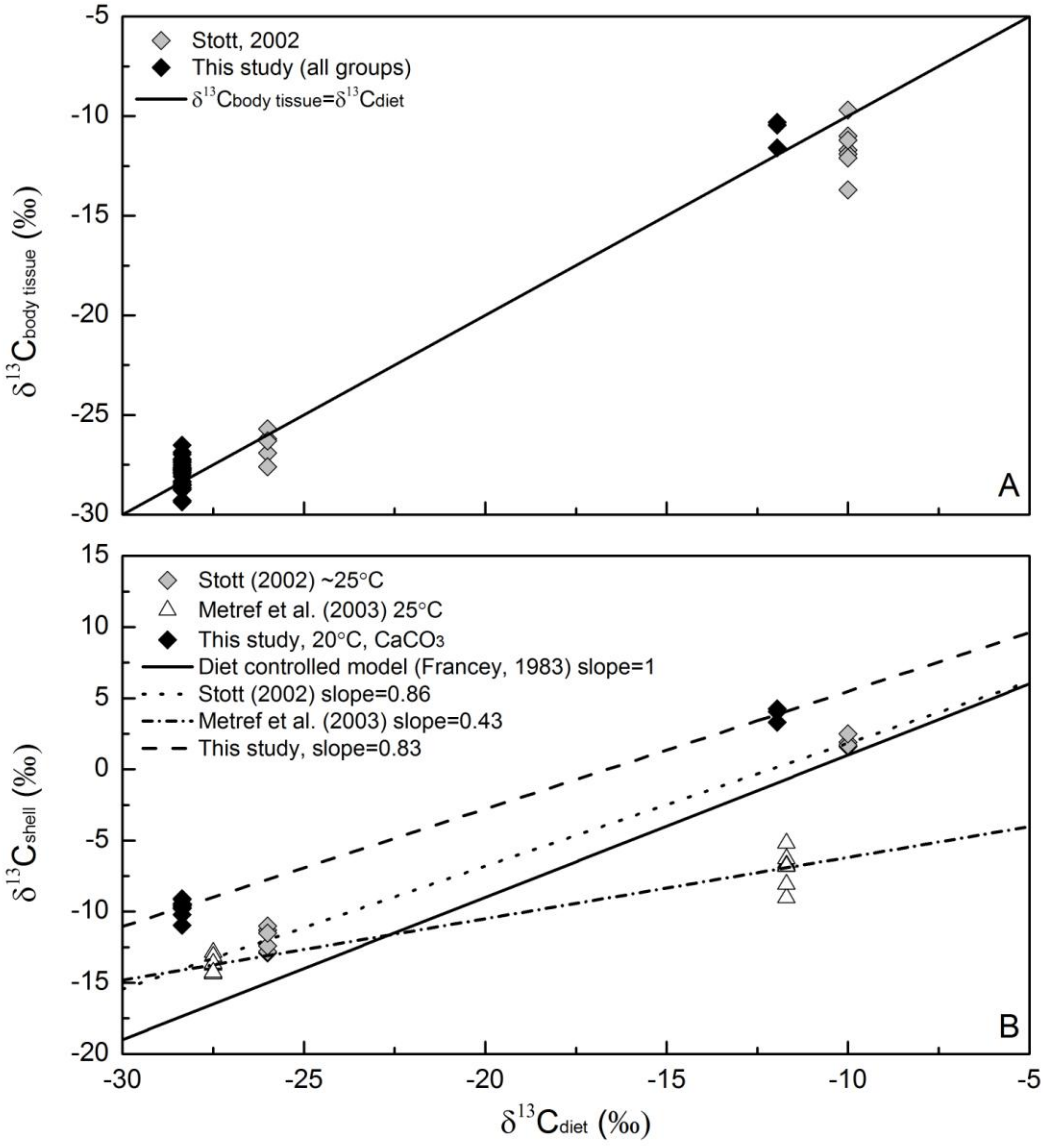
3 Figure 7. $\delta^{13}C$ of snail shell sections and their estimated contribution of ingested carbonate at
4 different growth phases.



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3 Figure 8. (A) $\delta^{13}C$ of snail body tissue against their diet. (B) $\delta^{13}C$ of snail shell aragonite

4 against their diet.

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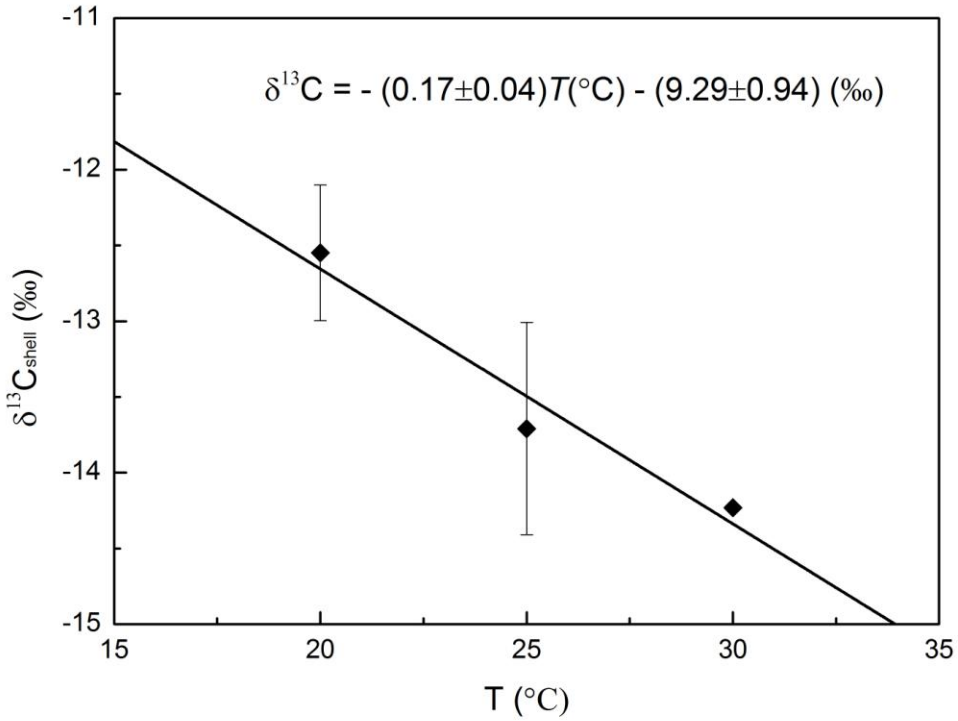

Figure 9. Relation between $\delta^{13}C$ of snail shell aragonite (fed without carbonate) and different culture temperatures.