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Earth and Planetary Science Letters 211 (2003) 381–393
EPSL
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Study of the diet effect on $\delta^{13}C$ of shell carbonate of the land snail *Helix aspersa* in experimental conditions

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Received 15 April 2002; received in revised form 17 April 2003; accepted 18 April 2003

Abstract

This study aims to demonstrate the influence of the metabolic CO_2 derived from the diet and of the atmospheric CO_2 on the shell carbonate $\delta^{13}C$ of the pulmonate snail *Helix aspersa maxima* raised under controlled conditions. Adult snails were analyzed and compared with three hatching and 1-day old young snails stemming from the same breeding. One day after, the 2-day old individuals were raised during 1 month. Three groups of gastropods were fed with fresh lettuce (C_3 plant, $\delta^{13}C = -27.49‰$), three groups with corn (C_4 plant, $\delta^{13}C = -11.7‰$), and three groups ate alternately both (C_3+C_4). The difference between the average $\delta^{13}C$ values of the adult snails on the one hand and the hatched and 1-day old snails on the other hand indicates a depletion of 2.47‰. Therefore, the isotopic parents–offspring signal is not preserved. The depleted ingested albumen by the snail embryo in the egg during the building of the shell could explain this depletion. The C_3 diet experiment gave the expected isotopic composition difference between the diet (lettuce) and the shells (average $\Delta^{13}C_{shell-lettuce} = 13.75‰ \pm 0.52$). This result shows a clear diet effect on the isotopic composition of the snail shells. For the C_4 experiment, the difference in carbon isotope composition between the corn and the shell ($\Delta^{13}C_{shell-corn}$) yielded an average value of $4.89‰ \pm 0.87$. The main result is that $\Delta^{13}C$ is not constant and appears to depend on the type of ingested food. Several hypotheses can arise from this study to explain the different fractionations: (a) differences in the quality of the two diets, (b) differences in turnover rate for C_3 and C_4 feeders. The groups regularly fed with mixed diet yielded $\delta^{13}C$ values showing a preferential use of C_3 food for most values. The C_3–C_4 mixed dietary alternation probably led snails to use mainly the lettuce instead of the corn powder.
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Keywords: *Helix aspersa*; $^{13}C/^{12}C$ ratio; diet; paleoclimatology

1. Introduction

Beside the soft body, terrestrial mollusk isotopic composition can be measured either on carbonate shell ($^{13}C/^{12}C$ and $^{18}O/^{16}O$) or on the organic matter present in the shell ($^{13}C/^{12}C$) [1].

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0012-821X/03/$ – see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0012-821X(03)00224-3

These measurements allow the reconstruction of paleoclimate parameters (temperature and humidity) and of the C_3/C_4 balance of the surrounding vegetation cover, respectively. Since an attempt by Bandel and Hoefs [2] and the work of Yapp [3], numerous stable isotope studies were realized on terrestrial mollusks, showing the sensitivity of their isotopic oxygen ratios to air–soil interface temperature and humidity [4–9]. More specifically, Lécolle [10] has shown that the oxygen isotopic ratios of snail species shell carbonate (i.e. Pulmonates, subclass of Gastropods, in which breath is made by means of a lung pocket and in which shell lacks operculum, and Prosobranchs, subclass of Gastropods, which breathes through gills, and in which the opening of the shell is closed by an operculum) collected in 125 localities in Western Europe and North Africa were related to the annual average oxygen isotopic composition of meteoric precipitation, and secondly to the average annual temperature.

Very few studies of $^{13}C/^{12}C$ in terrestrial snail shell organic matter were carried out to reconstruct the distribution of the C_3/C_4 plant ratios in relation to paleoclimatic parameters [11,12]. This method of studying terrestrial mollusks provides good results for modern and/or fossil shells [13,14]. Goodfriend [11,12], using snail shells from the Negev Desert, shows that the boundary between the C_3 and C_4 plants moved about 20 km southward from its current position during the middle Holocene. The snail shell isotopic composition changes were then interpreted as a change in the amounts of precipitation [11,12]. However, the recent results of Stott [15] show that paleoreconstructions of dietary plant communities based on the acid insoluble shell matrix cannot be used since a different $\delta^{13}C$ offset between C_3 and C_4 feeders is observed. The limitation of this method is that the terrestrial snail shell contains a very small amount of organic matter but the external organic matter of the periostracum (protective organic layer which surrounds the carbonate shell), which is present in the modern shells but quickly disappears after the death of the mollusk, is lacking in the fossil shells [1]. Thus, the use of stable isotopes of the organic matter preserved in snail shells requires relatively large samples due to the low concentration of organic matter in the shells. This implies working with species plentifully represented [1]. All these arguments justify the interest of studying snail shells whose carbonate amounts are more important than organic matter.

Recently, a study of shell carbonate isotopes of the large land snail *Achatina* sp. in South Africa allowed Abell and Plug [16] to link the $\delta^{18}O$ signal to paleoclimate changes. The analysis of the oxygen isotopic ratios of these shell fragments shows evidence of a sequence of climatic events, which includes coolings corresponding to the Older and Younger Dryas intervals, and an early Holocene abrupt warming. These authors reported $\delta^{13}C$ values they loosely interpreted in terms of C_4 plant occurrence, although they highlight the lack of studies correlating the $\delta^{13}C$ carbonate of snails with available diet. Other studies of $\delta^{13}C$ of the carbonates of some species of fossil snails sampled in Late Pliocene paleosols in central Italy [17] were interpreted after a simple comparison with literature data of different climatic regions (Israel, Europe and North Africa).

The contribution of diet to shell carbonate is complicated by essentially two factors. First, carbon from atmospheric CO_2 (by isotopic exchange across the body surface) may be incorporated in the carbonate shell. This implies that atmospheric CO_2 $\delta^{13}C$ may contribute substantially to shell $\delta^{13}C$ in addition to the metabolic CO_2 derived from the diet (released as respiratory CO_2, which dissolves in the body fluids) [1]. Second, fractionation and nutrient routing between diet and shell carbonate are poorly understood processes. Indeed, the assimilation of the food and its transformation into CO_2 and then into HCO_3^- in isotopic equilibrium can be perturbed by various factors. Besides, the contribution of foods with various nutritional values can put under stress the snail organisms and lead to disequilibrium in the diet fractionation. Soil carbonate is also a potential source to precipitate carbonate snail shell and, to a lower extent, carbonate may be provided through the ingested or diffused water [6]. Goodfriend and Hood [18] show that the contribution of soil carbonate input (which reacts with acid in the stomach to produce CO_2) to the shell of snail species of Jamaica can vary between

0 and 33%. Stott [15] shows that the contribution of CO_2 derived from limestone to adult *Helix aspersa* species did not influence the isotopic composition of the shell carbonate. However, if limestone $\delta^{13}C$ given to the snail in Stott's experiment is close to that of shell, an effect on $\delta^{13}C$ shell cannot be observed. Here, we aim to investigate the carbon isotopic composition of land snail shells *H. aspersa maxima* (Müller) under experimental conditions. We controled the isotopic composition of the food supplied to the snails of two generations (adult parents and offspring). We discuss these results by taking into account the impact of potential carbon sources and the length of the snails during the experiment. A variable temperature, which could influence the biochemical process of these ectotherm bodies [15], was avoided by performing this experimentation under constant temperature.

2. Materials and methods

2.1. Adult snails

Fifteen adult aragonite shells (land snail shell carbonate is almost invariably aragonite [19]) of *H. aspersa maxima* were randomly sampled in a breeding snail farm in Candillargues (Southern France), about 20 km from Montpellier (Fig. 1). These shells were fed during all their life with 'Alps Sanders' food (grains of wheat, oleaginous seeds, sugar, minerals). These snails received, in addition, calcium carbonate natural powder (Durance stone) to strengthen their shells, in order to increase their commercial value. Five samples of 'Alps Sanders' food (average $\delta^{13}C = -21.7‰ \pm 1.09$), and one sample of Durance stone ($\delta^{13}C = -6.07‰$) were analyzed, respectively, with an IRMS-EA at continuous flux Optima-Eurovector 3000 and a Micromass Multi Prep, IRMS Optima Ac 117. The values are expressed in δ notation (δ ‰) compared to the international standard, Pee Dee Belemnite (PDB), with an analytical precision for the $\delta^{13}C$ of $\pm 0.1‰$. We also expressed our results by calculating the difference in carbon isotopic composition between the shell and the diet ($\Delta^{13}C$). For the hatched and 1-day old snails, the $\Delta^{13}C$ is calculated using the Sanders diet isotopic composition ingested by their parents and their shell isotopic composition.

The largest diameter of the shells was measured as routinely done in malacology [20]. The shells were successively washed with distilled water and with acetone using an ultrasonic bath at 25°C during 30 min in order to eliminate the organic residues stuck on the surface of the periostracum. They were then dried in the oven at 90°C during 30 min before being crushed in a steel mortar. The resulting powder was sieved at 125 μm. To avoid contaminations between successive sample preparations, the sieve is washed after every crushed shell with diluted sodium hypochlorite and distilled water. 50 mg of aliquot powder was poured into a conical tube containing diluted hydrogen peroxide (H_2O_2 at 10%) in order to remove the remaining organic matter. After 2 h shaking, the samples were centrifuged at 4000 rpm during 15 min. The surnatant was removed and the powder rinsed out three times with distilled water. Ethanol (few ml, 70%) was added to the resulting powder poured in glass tubes to facilitate drying (at 90°C, 12 h). The powder is placed in vials in the multi-preparation autosampler of the mass spectrometer (Gilson XL 222). The CO_2 obtained by reaction (at 90°C) of the calcium carbonate with 103% anhydrous phosphoric acid [21] was analyzed with the Micromass Optima (Gilson XL 222) (Table 1a).

2.2. Hatching and 1-day old snails

Three hundred eggs stemming from the same breeding were brooded in a compost regularly moistened, in a dark chamber at ambient temperature (25° ± 0.5°C) (Fig. 1). Four $\delta^{13}C$ analyses of the compost provided an average value of $-25.88‰ \pm 0.12‰$.

Ten days later, most of the eggs hatched. Among the juveniles, three were randomly collected. The shell was immediately separated from the whole body by means of nippers; then the length was measured. Given the size and fragility of the shell of these juvenile gastropods, we could not prepare the shells as for the adults, the amount of powder being tiny and precious. Be-

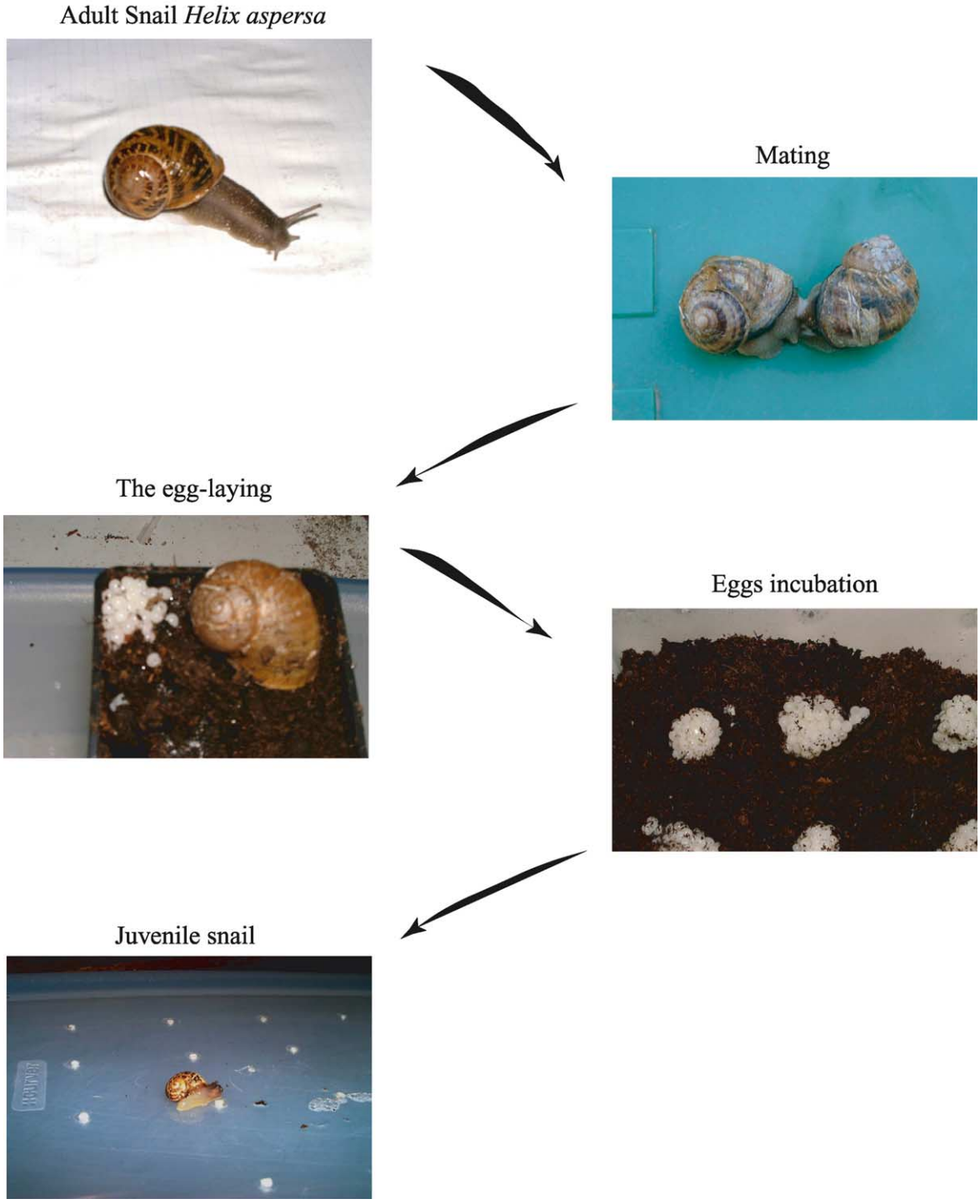

Fig. 1. Growth of *H. aspersa*. Reproductive cycle in the snail farm of Candillargues from an adult *H. aspersa* to a hatched juvenile individual (photos S. Metref).

Table 1
Stable carbon isotope results for adult, hatching and 1-day old individuals with indication of the $\delta^{13}C$ of the food for the adults

Snails	Length (mm)	Sanders diet $\delta^{13}C$ (‰)	Shell $\delta^{13}C$ (‰)	$\Delta^{13}C$ (‰)
(a) Adults				
1	40.0	−21.7	−8.47	13.23
2	39.8	−21.7	−9.34	12.36
3	40.3	−21.7	−9.09	12.61
4	42.0	−21.7	−8.57	13.13
5	42.2	−21.7	−9.03	12.67
6	40.5	−21.7	−8.79	12.91
7	44.8	−21.7	−9.49	12.21
8	40.4	−21.7	−9.29	12.41
9	36.5	−21.7	−9.27	12.44
10	40.0	−21.7	−9.25	12.46
11	38.3	−21.7	−8.98	12.72
12	39.4	−21.7	−8.64	13.06
13	41.3	−21.7	−9.17	12.53
14	37.0	−21.7	−9.12	12.58
15	37.5	−21.7	−9.17	12.53
Average	40 ± 2.16		−9.05 ± 0.3	12.66 ± 0.3
(b) Hatching				
1	4.9		−11.94	9.76
2	4.2		−11.89	9.81
3	4.7		−11.14	10.56
Average	4.6 ± 0.39		−11.66 ± 0.44	10.04 ± 0.44
(c) 1-day old				
1	4.9		−11.25	10.45
2	4.5		−11.43	10.27
3	4.6		−11.45	10.25
Average	4.7 ± 0.22		−11.38 ± 0.11	10.32 ± 0.11

fore crushing them in the agate mortar, shells were put in an ultrasonic bath of acetone at 25°C during 30 min, and dried in the oven during 15 min at 90°C. They were shook in hydrogen peroxide at 10% during 2 h, then washed in distilled water, and dried at 90°C. Afterward, they were analyzed the same way as the adults (Table 1b). The same procedure was repeated one day later (Table 1c).

2.3. One-month old snails

We used nine plastic boxes (30 cm × 18 cm × 12 cm), allowing air exchanges, placed in a room adjusted automatically at 25°C (± 0.5°C) during the whole experiment. Fifteen juveniles were randomly allocated to each box and raised. We chose plastic boxes in order to facilitate their cleaning, and the spraying and feeding of the snails twice a week. The photoperiod was adjusted mechanically to 18 h of light and 6 h of darkness, according to the period of growth and reproduction of the natural populations [22].

The snails were fed and sprayed according to a strict protocol. Three groups of young gastropods were fed with fresh lettuce (C_3 diet; Table 2), three groups with corn powder (C_4 diet; Table 2), and three groups ate both (mixed diet; Table 2), consisting of feeding them alternately 2 days with lettuce, then 2 days with corn until the end of the experiment. The mortality rate was about the same in all boxes (20%). For each type of food allocated, the experiment has been repeated three times in order to check the $\delta^{13}C$ variability in juvenile shells. Fragments of lettuce and corn powder provided to the mollusks were regularly

Table 2
Stable carbon isotope results for raised 1-month snails

Snails	Length (mm)	C_3 diet	C_4 diet	Mixed diet	Shell $\delta^{13}C$ (‰)	$\Delta^{13}C$ (‰)
1	11.6	+			−14.31	13.18
2	8.4	+			−13.76	13.73
3	7.0	+			−12.85	14.64
Average	9 ± 2.4				−13.64 ± 0.74	13.85 ± 0.74
4	8.6	+			−13.14	14.35
5	7.4	+			−13.64	13.85
6	7.9	+			−13.68	13.81
Average	8.0 ± 0.6				−13.49 ± 0.3	14.00 ± 0.3
7	7.9	+			−14.37	13.12
8	7.7	+			−13.62	13.87
9	7.5	+			−14.26	13.23
Average	7.7 ± 0.19				−14.08 ± 0.4	13.41 ± 0.4
10	5.8		+		−6.57	5.14
11	5.1		+		−6.27	5.4
12	5.1		+		−6.79	4.92
Average	5.3 ± 0.4				−6.54 ± 0.26	5.15 ± 0.26
13	5.7		+		−6.83	4.87
14	3.0		+		−8.01	3.69
15	5.0		+		−6.72	4.98
Average	4.6 ± 1.42				−7.19 ± 0.71	4.51 ± 0.71
16	5.3		+		−5.19	6.51
17	5.0		+		−8.08	3.62
18	3.0		+		−6.79	4.91
Average	4.4 ± 1.24				−6.69 ± 1.45	5.01 ± 1.45
19	10.5			+	−7.35	?
20	7.7			+	−7.54	?
21	8.0			+	−6.81	?
Average	8.73 ± 1.54				−7.23 ± 0.38	?
22	9.3			+	−6.32	?
23	6.9			+	−10.84	?
24	6.1			+	−9.97	?
Average	7.4 ± 1.7				−9.04 ± 2.4	?
25	7.8			+	−9.18	?
26	6.5			+	−9.05	?
27	7.2			+	−8.85	?
Average	7.2 ± 0.6				−9.03 ± 0.17	?

For each type of food allocated, the experiment has been repeated three times in order to check the $\delta^{13}C$ variability in juvenile shells. The question mark series represents the non-calculated $\Delta^{13}C$ (‰) values. Plus signs indicate the analyzed $\delta^{13}C$ of fed-lettuce diet (−27.49‰), fed-corn diet (−11.7‰) and fed-mixed diet snails.

sampled in order to check the variability of the isotopic signal of the food supply throughout the experiment. The $\delta^{13}C$ values remained constant (lettuce: $n = 20$, average = −27.49‰ ± 0.5; corn: $n = 4$, average = −11.7‰ ± 0.3). We analyzed more samples of lettuce than corn because the lettuce did not derive from the same set during the experiment and was renewed regularly, contrary to the corn. These tests are also justified as the isotopic composition of a determined food can vary in space and time, and would impact the feeders [23]. After 1 month of growth, three shells were randomly collected in each box. Then, for each diet experiment, nine individuals were analyzed for isotopic composition following the same procedure as for hatching and 1-day snails (Table 2).

3. Results and discussion

3.1. Adults–offspring diet relationships (Fig. 2)

The adult shells (Table 1a) yield a $\delta^{13}C$ varying from −9.49‰ to −8.47‰ (average = −9.05‰ ± 0.3) and the $\Delta^{13}C$ varies between 12.21 and 13.23‰ (average = 12.66‰ ± 0.3). The hatched individuals' $\delta^{13}C$ varies between −11.94‰ and −11.14‰ (average = −11.66‰ ± 0.44) (Table 1b). If we assume that the juvenile shells yield an isotopic signal related to the feeding of their parents, then the average $\Delta^{13}C$ is 10.04‰ ± 0.44. The 1-day old snails' values (Table 1c) range from −11.45‰ to −11.25‰ (average = −11.38‰ ± 0.11), and the estimated average $\Delta^{13}C$ is 10.32‰ ± 0.11. The difference between the average $\delta^{13}C$ values of the adult snails on the one hand and the hatched and 1-day old snails on the other hand indicates a depletion of 2.47‰ for the latter. Therefore, the isotopic signal parents–offspring is not preserved. The explanation for such a phenomenon may rely on physiological processes between parents and juveniles from the building of the eggs in the uterus, the egg-laying, the incubation in the ground, to the hatching of the juveniles seeking for food (Fig. 1) and on different $CaCO_3$ forms (aragonite, calcite).

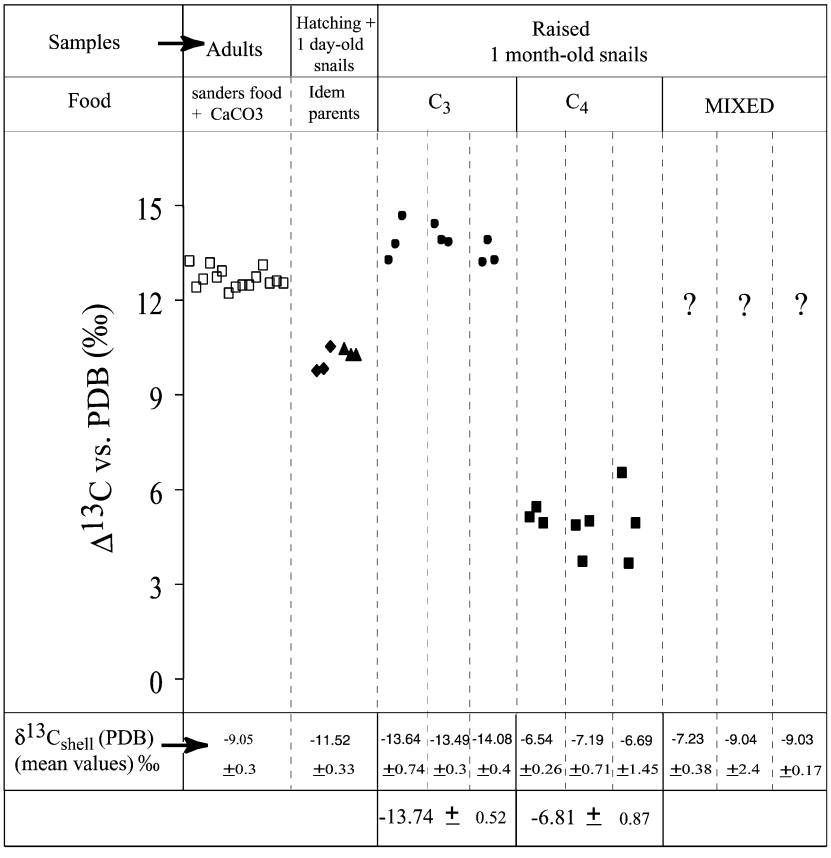

Fig. 2. Carbon isotopic composition of calcium carbonate shells in relation to the type of food supplied. The values of adult shells are more enriched than the hatching and 1-day old snails. The raised 1-month old snails fed with corn (C_4 plant) are more enriched than the snails fed with lettuce (C_3 plant). The $\Delta^{13}C$ ($\delta^{13}C_{shell} - \delta^{13}C_{food}$) for the individuals fed with lettuce is larger than the $\Delta^{13}C$ of snails fed with corn. The mixed fed snails seem to prefer C_3 food and the $\Delta^{13}C$ is not calculated and indicated by question marks. Open squares, adult snails; full diamonds, hatched individuals; full triangles, 1-day old juveniles; full circles and full squares, 1-month old juveniles.

The juvenile land snails inherit their shell from the calcium carbonate issued from the adults [24]. We tested the mineralogy of the juvenile shells. We randomly analyzed six shells from hatched individuals in our experiment in order to check the mineral composition of the protoconch using Fourier Transform InfraRed spectroscopy (FT-IR). The results indicate a band centered around 860 cm^{-1}, characteristic of aragonite [25]. There is thus no mineralogical difference between juvenile and adult shells which could have explained the depletion in the isotopic signal. Indeed, during the embryogenesis, the snail embryo dissolves and absorbs calcium from the calcite eggshell and redeposits it as aragonite for its larval body shell, i.e. protoconch [26], thus changing one crystal morph of $CaCO_3$ to another (Fig. 3).

The snail embryo, when growing in the egg, draws in an essentially proteinic food (albumen fluid). According to Kennedy and Krouse [23], isotopic composition differences are evidenced between the yolk and the white of the bird eggshells due to the different proportion of the major biochemical compounds (proteins, lipids and carbohydrates) and the inorganic nature of the shell. These components are ultimately derived from the diet of the parents [27]. The $\delta^{13}C$ of proteins is depleted compared to the $\delta^{13}C$ of the calcite, and is less depleted compared to lipids [28]. In the Stylommatophora, the most successful pulmonates order, calcium is known to originate from the digestive gland. It is carried through the blood as ions through the uterine epithelium specialized in the formation of the eggshells, where calcite is precipitated [24]. The eggs increase their calcium content as they move along the narrow walls of the uterus. The nucleation of dispersed calcite crystals starts within the jelly coating the egg which is strongly attached to the uterus wall [26,29]. This jelly also contains organic matter constituted by mucopolysaccharids, usually sulfated, and some proteins [26]. The complete egg of the pulmonate land snail *Strophocheilus oblongus* is, besides, constituted by 72% water, 18% albumen (dry weight) and 10% of shell proportions which can be generalized to the whole pulmonates [30]. We assume that the hatching juvenile snail of our study built its shell by reprecipitating free calcite within the jelly coat as aragonite as long as he ingested the depleted albumen. Such a mechanism could explain the depleted values measured between the adult and hatched individual of our experiment. This model is represented in Fig. 3.

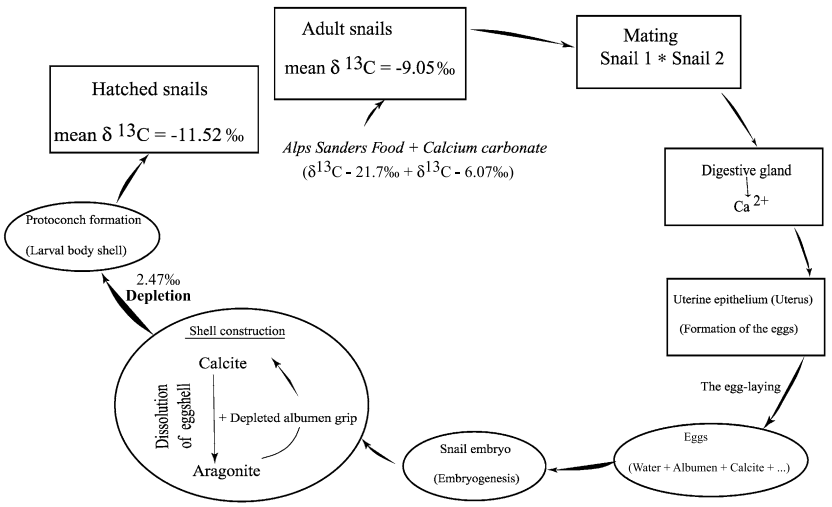

Fig. 3. Growth cycle indicating different phases. Indication of the hypothetic fractionation assumed to lead to depleted values in the hatched individuals.

3.2. Young snail shells after 1 month of growth (Fig. 2)

3.2.1. C_3 and C_4 experiments

After 1 month of growth, the individuals of the group fed with lettuce (Table 2) yielded $\delta^{13}C$ values varying from −14.37 to −12.85‰ (average = −13.74‰ ± 0.52). Their corresponding $\Delta^{13}C$ varies from 13.12 to 14.64‰ (average = 13.75‰ ± 0.52). This C_3 experiment shows a clear diet effect on the isotopic composition of the snail shells in agreement with the recent work of Stott [15]. The individuals fed with corn yielded enriched $\delta^{13}C$ values varying between −5.19 and −8.08‰ (average = −6.81‰ ± 0.87), showing a smaller $\Delta^{13}C$ value, ranging from 6.51 to 3.62‰ (average = 4.89‰ ± 0.87) compared with Stott's experiment [15] using also corn powder (average = 11.74‰ ± 0.37). The main result is that the difference between $\delta^{13}C$ shell and food ($\Delta^{13}C$) is not constant and appears to depend on the type of ingested food. This would be in disagreement with the general statement asserting that the isotopic relationships between the whole bodies of animals and their diets are similar for different species raised on the same diet and for the same species raised on different diets [13]. The food values of powder and lettuce are obviously different, and it is probable that what is metabolized by the snail (protein, sugar or lipid) is not present in equal amounts in the different diets which are characterized by different carbon isotopic compositions. This could partly explain the different $\Delta^{13}C$, but Stott, who fed his snails with the same types of diet, did not find any growth difference. In Fig. 4, we report $\Delta^{13}C$ versus the length of snail shells. The differences in quality of the two diets seem to have placed the animals in different growth states. This could also cause fractionation to differ. The snails fed with pure C_4 food are smaller than those fed with C_3 food and have more positive $\delta^{13}C$ values. According to Teeri and Schoeller [31], who performed an experiment on the beetle *Tribolium castaneum*, the enrichment in $\delta^{13}C$ of the whole body samples is independent of the animal growth rate. It appears that while the various flour mixtures could have a large influence on the growth of the beetles, the differences in growth did not affect the $\delta^{13}C$ value of the beetles. The comparison between insects and snails is not straightforward; however, we cannot avoid the hypothesis that the enrichment in the $\delta^{13}C$ of shell carbonate may be independent of both the snail size and the food quality. The other assumption that can be made, considering the short time of our experiment, is the turnover rate of the diet $\delta^{13}C$ signal. We suspect the young snails did not run out the stocks of C_3 nutrients that they would inherit from the parents. So, we assume that fractionation and the enrichment of the shell $\delta^{13}C$, with regard to the food $\delta^{13}C$, is probably processed gradually according to time and the age of the snails. Stott (personal communication) agrees with this interpretation, as the metabolic CO_2 is derived from the body. The body tissues did not

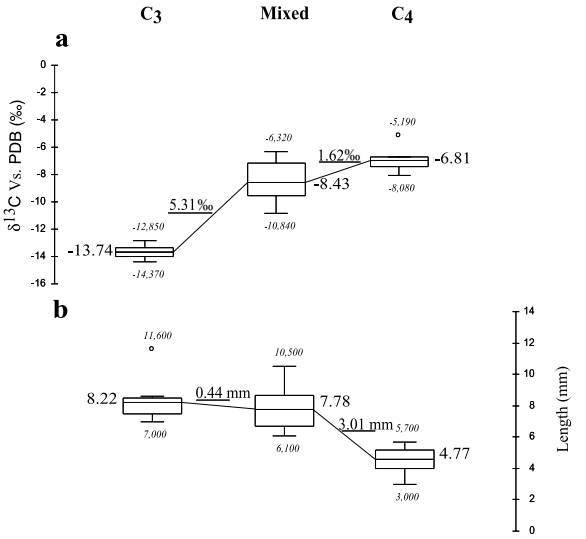

Fig. 4. $\delta^{13}C$ (‰) (a) and length (mm) (b) records reported as box plots versus type of food web. The boxes indicate 50% of the values and the upper and lower bars indicate 25% each. The horizontal strokes are the average values. In bold are the average values. Underlined values indicate the difference between C_3 and mixed experiments on the one hand, and between C_4 and mixed experiments on the other hand for both $\delta^{13}C$ (a) and length (b). Italic values indicate the higher and lower values except in the C_4 experiment because of the outlier $\delta^{13}C$ value of −5.19‰ and in the C_3 experiment of the outlier length of 11.6 mm.

acquire enough corn $\delta^{13}C$ over the course of the experiment to sufficiently influence the carbonate that was analyzed. Stott suggests, besides, another possibility of combining all of the shell aragonite formed during the experimental growth period, with the aragonite that formed early in the experiment, when the body tissue was still very depleted. This interpretation is plausible, but we believe this is not the case, as the C_4-fed snails still slightly grew compared to the C_3-fed snails. Then we assume the original C_3 signal has no significant influence on the values measured on the C_4-fed snail shells. However, another hypothesis, like weak air circulation in the breeding room, which could have created a disequilibrium between metabolic and atmospheric CO_2, could have led snails to badly assimilate the C_4 food compared with the more digestible C_3 food.

Goodfriend and Stipp [32] proposed that the ingested limestone was the main source for the shell carbonate, and Magaritz and Heller [5] proposed that the $\delta^{13}C$ from shells of the terrestrial *Theba pisana* in Israel was largely determined through air exchanges. Stott [15], after feeding individuals of *H. aspersa* with lettuce sprinkled with calcium carbonate powder ($\delta^{13}C$ value $\approx 2‰$; L. Stott, personal communication), indicates that the shells yielded $\delta^{13}C$ values slightly more depleted compared with individuals fed with only lettuce, in contrast to what one would expect if the $CaCO_3$ yielded were contributing carbon to the shell. The individuals fed with corn and $CaCO_3$ did not show any difference with those fed only with corn. The ingested $CaCO_3$ does not seem, therefore, to influence significantly the isotopic composition of the shell carbonate. Such results would then support the hypothesis that the main sources of carbon incorporated in the snail shell are mostly provided by diet and atmospheric CO_2, which disagrees with Goodfriend and Hood's [18] and Goodfriend and Stipp's [32] assumption. Using the mass-balance equation (Eq. 1), the proportion of the differential integration of metabolic and atmospheric carbon in the C_3- and C_4-raised snail groups can be estimated if we assume that the amount of CO_2 yielded by the limestone is null [15]:

$$\delta^{13}C_{\text{shell (aragonite)}} = \delta^{13}C_m(X) + \delta^{13}C_a(1-X) + 2.7‰ \quad (1)$$

where $\delta^{13}C_m$ is the isotopic composition of metabolic $CO_2 = \delta^{13}C$ of the consumed plants + $\approx 1‰$ $\delta^{13}C$ enrichment of the whole body tissue relative to the diet [13] $\Rightarrow -27.49‰\,(C_3)+1‰ = -26.49‰$; $-11.7‰\,(C_4)+1‰ = -10.7‰$; X is the fraction of metabolic CO_2 [(for $\delta^{13}C_{shell} = -13.74‰$ (100% C_3); for $\delta^{13}C_{shell} = -6.81‰$ (100% C_4))]; $\delta^{13}C_a$ is the isotopic composition of atmospheric CO_2 ($\approx -8‰$) [33] with $\alpha_{HCO3-CO2} = 1.008$ at 25°C (8‰) [34] $\Rightarrow$ the $\delta^{13}C$ of $HCO_3^- \approx 0‰$. 2.7‰ (± 0.2) is the isotopic offset between bicarbonate and aragonite precipitated in isotopic equilibrium [35].

Applying this mass balance equation to the results obtained for the groups fed with lettuce, we calculated that 62% of the ingested CO_2 has a metabolic origin (in our experiment, the $\delta^{13}C$ values of metabolic CO_2 were deduced from the $\delta^{13}C$ values of the analyzed plants, which probably is a rough approximation). Stott [15], who studied sub-adult to adult *H. aspersa* snails, has found almost the same value (57%), where the metabolic CO_2 derived from food slightly dominates the CO_2 derived from the atmosphere. Conversely, Stott obtained a small proportion of metabolic carbon for the snails fed with corn powder compared with our results (12% vs. 89%). The activity levels and environmental conditions in his experiment were similar and there were no significant differences in shell size or mass between the groups. In our study, the size of the shells of the corn-fed groups is half that of the lettuce-fed groups (Table 2). Goodfriend and Hood [18] indicated that the exchanges with the atmospheric CO_2 can be much more important in small individuals. This assumption would imply a strong dominance of the atmospheric CO_2. However, in our experiment, the atmospheric CO_2 is weakly ingested by the corn groups, which are small individuals, while it is much more important in the lettuce-fed groups, where it balances almost with the CO_2 derived from food metabolism. Then our results are not in agreement with Goodfriend and Hood [18]. The corn powder supplies may have

caused a stress to the snails, which would create a differential incorporation of atmospheric CO_2. But, according to Stott [15] and Stott (personal communication), this contrast in results supports that only metabolic CO_2 was involved. Therefore the atmospheric CO_2 is not contributing.

3.2.2. C_3 vs. C_4 mixed experiment

Table 2 shows that the three groups of individuals regularly fed with mixed diet yielded average $\delta^{13}C$ values of −7.23, −9.04 and −9.03‰. First, groups 2 and 3 show $\delta^{13}C$ values (−9.04 and −9.03‰) reflecting a predominant C_3 diet. Second, group 1 is typical of true mixed feeders (−7.23‰). Goodfriend and Magaritz [8], who studied the $\delta^{13}C$ relation of various desert snail taxa's carbonate with pure C_3 and with mixed C_3+C_4 plant communities, noticed a strong tendency for snails from plant communities with C_3 and C_4 species to be enriched in ^{13}C relative to snails from pure C_3 communities. The difference is highly significant and averages about 2–3‰. In our experiment, the $\delta^{13}C$ average value of the snails of group 1 is enriched by about 2‰ compared to snails of groups 2 and 3. This shows that the snails of group 1 incorporated more C_4 plants than the two other groups and thus reflects, probably like the snails studied in the southern Levant region [8], a mixed diet response. In Fig. 4, we can also notice that the length of the shells of these groups is conversely closer to that of the C_3 feeders rather than to the C_4 feeders. These results seem to support the hypothesis that the enrichment of the $\delta^{13}C$ of shell carbonate may be independent of the snail length and food quality, as shown by Teeri and Schoeller [31] on the beetle *Tribolium castaneum*. According to the protocol used for the mixed group experiments, we are not able to provide a $\delta^{13}C$ value for the food supply, since the food was alternatively given to the snails. Consequently, the difference in carbon isotope composition ($\Delta^{13}C$) between the diet and the snail shell carbonate is not calculated (see Table 2).

4. Conclusion

The most important result of our experiment is the direct response of the isotopic signal of terrestrial shell carbonate to the C_3 food supplied. The signature of C_3 plants is reflected without any ambiguity in the analyzed shells (average $\Delta^{13}C_{shell-lettuce} = 13.75‰ \pm 0.52$) but the answer to a C_4 food remains vague (average $\Delta^{13}C_{shell-corn} = 4.89‰ \pm 0.87$). Several hypotheses can arise from this study to explain the different fractionations, like the differences in quality of the two diets, which seem to place the snails in different growth states, and the differences in turnover rate for C_3 and C_4 feeders. Another hypothesis like food stress should be taken into account when doing paleodiet reconstruction. The variation in this parameter in sedimentary records could provide an indication of climatic or environmental crisis in the past. In addition, values yielded by fossil shells could be wrongly interpreted as related to the occurrence of C_4 plants in the studied area due to the influence of other environmental parameters. The groups regularly fed with mixed diet yielded $\delta^{13}C$ reflecting a predominant C_3 diet for two groups and probably a typical value of true mixed C_3–C_4 feeders for one group. This part of the study presently seems difficult to interpret and requires further investigations. We have shown that a fractionation between parents and juveniles exists which can reflect physiological processes followed during the building of the eggshells.

Acknowledgements

We acknowledge all the persons who contributed to the good progress of this work. To begin with Marc and Claire Diana ('l'Escargot amoureux' farm) for the adult and snail eggs supplied, Gilbert Pistre for the logistic support, Issam Moussa for the isotopic analyses, and M. Jean-Louis Bantignies, M. David Maurin from the GDPC (Groupe de Dynamique des Phases condensées, Université Montpellier II) for the infrared spectroscopy analysis. We are also grateful to Dr. M. Fontugne, Dr. L. Stott and two anonymous reviewers for their helpful comments, and to Dr. Philippe Jarne and Dr. Luc Madec for their scientific advice. This is ISEM Contribution

2003-004 and LDEO Contribution 6419. S.M. was funded by an Algerian Government Ph.D. grant.*[BARD]*

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