Science in China Series D: Earth Sciences

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Stable carbon isotope evidence for tracing the diet of the host *Hepialus* larva of *Cordyceps sinensis* in the Tibetan Plateau

CHEN Di[1], YUAN JianPing[1,2], XU ShiPing[3], ZHOU XiaoGang[1], ZHANG Yan[1], XU XiaoMing[4], ZOU ZhiWen[1], ZHANG GuRen[1] & WANG JiangHai[1,2†]

[1] State Key Laboratory of Bio-control, College of Life Sciences, Sun Yat-Sen University, Guangzhou 510275, China;
[2] School of Marine Sciences, Sun Yat-Sen University, Guangzhou 510006, China;
[3] State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China;
[4] R & D Center, Guangzhou Enenta Electrical Equipment Co., Ltd, Guangzhou 510635, China

Two types of *Hepialus* larvae with different diets were distinguished in the Sejila Mountain, Tibetan Plateau based on the stable carbon isotope data of the host *Hepialus* larva of *Cordyceps sinensis* and its closely adjacent tender plant roots and humus fractions. Type I is the larva chiefly fed by soil humus, and characterized by the δ^{13}C values of –22.6‰ to –23.4‰, and more than –23.4‰ in its heads. Type II is the larva chiefly fed by tender plant roots, and characterized by the δ^{13}C values of –24.6‰ to –27.6‰, and less than –24.6‰ in its heads. Our result has exceeded the traditional understanding that their food sources only come from the tender plant roots, and may provide evidence for choosing cheap and high-quality foods and further establishing artificial habitats in their large-scale reproduction.

stable carbon isotopes, host *Hepialus* larva of *Cordyceps sinensis*, humus substances, Tibetan Plateau

Cordyceps sinensis is a symbiote of *Hepialus* larva and fungus, formed through parasitization of the fungus *Hirsutella sinensis* on the larva of *Hepialus* (Lepidoptera, Hepialidae)[1,2], and is one of the well-known rare medicinal herbs in the Tibetan Plateau and its adjacent high-altitude areas[3]. *C. sinensis* is famous due to its lack of toxicity and specific medicinal efficacies. Modern pharmacological studies demonstrated that *C. sinensis* has various bioactivities, such as anti-tumor, immune regulation, lowering blood sugar and blood pressure, angiospasm and stimulating the biosynthesis of testosterone[4,5].

Many studies were presented by overseas specialists, but all of them were focused on the cultivation and fermentation of *Cordyceps* mycelium, and the cognition of active components[6–8]. However, the diet of *Hepialus* larva has not been well studied. Many domestic researchers systematically studied the diet of host *Hepialus* larva of *C. sinensis* by using the traditional diet analysis, and affirmed that this host *Hepialus* larva is an omnivorous insect, mainly feeding the tender roots of plants, such as Polygonaceae and Cuculidae, particularly *Polygonum macrophyllum* and *Polygonum viviparnm*[9–16]. Several scholars[17,18] discovered that it was preferred to eat carrot, yam and potato in the artificial feeding. Zhu et al.[15,16] speculated that the soil humus or rotten root might be one of food sources for the host *Hepialus* larva, but did not provide any authentic evidence.

Stable carbon isotope analysis has already been widely applied to study the circulation of material in the biosphere, and has a remarkable advantage that it may

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Received November 27, 2008; accepted February 24, 2009
doi: 10.1007/s11430-009-0068-1

[†] Corresponding author (email: wangjhai@mail.sysu.edu.cn)

Supported by the National Key Technology R & D Program (Grant No. 2007BAI32B05)

provide the long-term information of animals' diet and further trace the sources of material and energy with dispensable calibration[19−22]. Until now, no result on the diet of the host *Hepialus* larva of *C. sinensis* has been presented using this method. We studied its diet via adopting stable carbon isotope analysis, and discovered the humus-chiefly-fed *Hepialus* larva of in the Sejila Mountain, Tibetan Plateau. Here we report the evidence of stable carbon isotopes to corroborate the existence of two types of *Hepialus* larvae and trace their diet.

1 Materials and methods

1.1 Sampling

The host *Hepialus* larvae of *C. sinensis* (see photo in Figure 1) were sampled near the Experimental Base of Peculiar Bioresources in the Tibetan Plateau of Sun Yat-Sen University in the Sejila Mountain, Tibetan Plateau on June 20, 2007. The sampling location is at 94°36′16 E and 29°36′20 N, and its altitude is 4200 m. The sampling point is located at the shrubbery, and the main bush is *Rhododendron nivale,* whose root extended as deep as 45 cm. The soil at the sampling point might be divided into litter (7—9 cm), humus horizon (23 cm to 33 cm), and parent material horizon from the surface downwards. Thirteen host *Hepialus* larvae were randomly collected in a one-square-meter area at this sampling point, and all lived in the humus horizon soil. The humus horizon soils and tender roots of major plants in this area were simultaneously sampled.

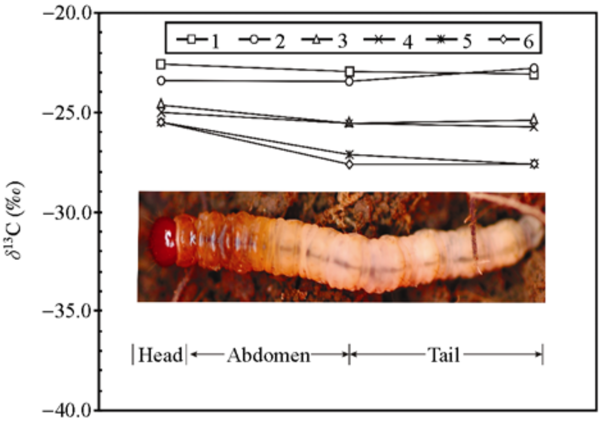

Figure 1 Morphological characteristics of the host *Hepialus* larva of *C. sinensis* in the Sejila Mountain, Tibetan Plateau and variation of the $\delta^{13}C$ ratios of different parts. 1—6 represent the sample numbers of *Hepialus* larvae.

1.2 Sample preparation

Hepialus larvae and tender plant roots were dried in the oven at 40°C. The dried roots were directly milled into 40 mesh powders by a glass mortar. The dried larva was first segmented into three parts of head, abdomen and tail, and then milled into 40 mesh powders, respectively.

The fractions of humic acid (HA), hydrophilic fulvic acid (Hil-FA), hydrophobic fulvic acid (Hob-FA), and humin (HM) were separated from the soil samples by using the following procedures:

(1) Weighed 20 g 100 mesh soil powder, then added 200 mL 0.1 mol/L NaOH and 5 mL 3% NaCl, input N_2, stirred and stood overnight at room temperature.

(2) Centrifuged the mixed solution (10000×*g*, 15 min) and collected the supernatant (HA + FA). The indissoluble part was humin (HM) after the removal of pyrite and silicates by thick HCl and HF.

(3) Acidified the supernatant (HA + FA) to pH = 1.2 with 4 mol/L HCl, stood overnight; centrifuged the supernatant (20000×*g*, 15 min), obtained the precipitate of HA; the filtrate was FA after passing the filter membrane with an aperture of 0.22 μm.

(4) Added 100 mL ethyl acetate into FA, and then separated the hydrophobic layer (containing hydrophobic fulvic acid, Hob-FA) and hydrophilic layer (containing hydrophilic fulvic acid, Hil-FA) by a separatory funnel; evaporated the ethyl acetate layer to get Hob-FA; added 0.1 mol/L $AlCl_3$ and 1 mol/L NaOH to the hydrophilic layer (Hil-FA), made the solution to pH = 5.0, stood overnight, and then obtained Hil-FA by centrifugation (10000×*g*, 15 min).

(5) Added the mixed solution of 0.3 mol/L HF and 0.1 mol $HClO_4$ into HA, continuously stirred for 5 h at room temperature to remove fine minerals, then obtained a precipitate (HA) by centrifugation (20000×*g*, 15 min); entirely redissolved HA with a 0.1 mol NaOH solution, then dialyzed until the electrical conductivity of the outer equilibrated solution less than 10 μS/cm, and finally obtained HA after the solution in the dialysis bag was evaporated.

(6) Milled the dried fractions of HA, HM, Hil-FA and Hob-FA into the powder samples for measuring their stable carbon isotope compositions.

1.3 Measurement of stable carbon isotope ratios

Based on the carbon contents in samples, the proper amount of each sample of *Hepialus* larvae, tender roots or fractions of HA, HM, Hil-FA or Hob-FA extracted from the soil in the humus horizon was weighted and loaded into a clean tin capsule, respectively. The prepared capsules were numbered and placed on a Sartorius 4503 MICRO balance. Their δ^{13}C values were measured by a CE EA1112 C/N elemental analyzer-DELTA $^{\text{plus}}$XL stable isotope ratio mass spectrometry. Our previously published paper[23] gave detailed experimental procedures and parameters. The corresponding standard deviation of analysis and the deviation between the measured data and pre-determined data were less than 0.3‰.

2 Results

2.1 Stable carbon isotope composition of *Hepialus* larvae and its variation

The data of stable carbon isotopes of *Hepialus* larvae are presented in Table 1. Table 1 shows that the variation of their δ^{13}C values is dramatic, and ranges from −22.6‰ to −27.6‰. Except for sample 2, the δ^{13}C values of the heads of the other *Hepialus* larva samples are all more than those of their abdomens and tails, whereas the δ^{13}C values of their abdomens and tails are nearly identical within an analytical error. The δ^{13}C value of the head of sample 2 is consistent with that of its abdomen, but slightly less than (0.4‰) that of its tail. There is an inconspicuous difference (0.2‰ to 0.6‰) among the δ^{13}C values of the heads, abdomens and tails of samples 1 and 2, while there is an evident difference (0.6‰ to 2.1‰) and a relatively wide range among the δ^{13}C values of the heads, abdomens and tails of samples 3 to 6.

Table 1 Stable carbon isotope ratios of the host *Hepialus* larva of *C. sinensis* in the Sejila Mountain, Tibetan Plateau[a)]

Sample	Type	δ^{13}C (‰)			Quantitative food structure (%)	
		Head	Abdomen	Tail	HA	TPR
1	Type I	−22.6	−23.0	−23.1	78.26	21.74
2	Type I	−23.4	−23.4	−22.8	66.67	33.33
3	Type II	−24.6	−25.5	−25.4	49.28	50.72
4	Type II	−25.0	−25.6	−25.7	43.48	56.52
5	Type II	−25.5	−27.2	−27.6	36.23	63.77
6	Type II	−25.5	−27.6	−27.6	36.23	63.77

a) HA, Humic acid; TPR, tender plant roots.

2.2 Stable carbon isotope composition of the tender roots of main plant species

Ten main plant species live near the sampling point, such as *Rhododendron nivale* and *Polygonum viviparum* (Table 2). The δ^{13}C values of their tender roots are also listed in Table 2. Table 2 shows that their δ^{13}C values range from −27.1‰ to −29.0‰, with a mean of −28.0‰, indicating that they all belong to C_3 plants[24].

Table 2 Stable carbon isotope ratios of the tender roots of main plant species in the habitat of the host *Hepialus* larva of *C. sinensis* in the Sejila Mountain, Tibetan Plateau

Sample	Plant species	δ^{13}C (‰)
1	*Rhododendron nivale*	−27.1
2	*Asrer souliei*	−27.3
3	*Aster barbellatus*	−27.6
4	*Saussurea sungpanensis*	−27.6
5	*Selaqinella vardei*	−27.8
6	*Polygonum viviparum*	−27.8
7	*Aconitum longilobum*	−28.5
8	*Fragaria nubicola*	−28.7
9	*Caltha scaposa*	−28.8
10	*Conringia planisiliqu*	−29.0

2.3 Stable carbon isotope composition of organic matter fractions from the humus horizon

The stable carbon isotope composition of HA, Hob-FA, Hil-FA and HM extracted from the soil in the humus horizon as well as the total organic matter (TOM) were presented in Table 3. Table 3 shows that the δ^{13}C value of TOM is −24.6‰; the δ^{13}C values of organic matter fractions widely range from −21.1‰ to −27.5‰; and HA and Hob-FA respectively possess the maximum and minimum δ^{13}C values. The δ^{13}C values of HA and HM (−21.1‰ and −23.5‰, respectively) are similar to those of type I (−22.6‰ to −23.4‰). The δ^{13}C values of Hil-FA and Hob-FA (−26.1‰ and −27.5‰, respectively) resemble those of type II (−24.6‰ to −27.6‰), but obviously different from those of Type I.

Table 3 Stable carbon isotope ratios of the organic matter fractions from the *Hepialus* larva's habitat soil at the humus horizon in the Sejila Mountain, Tibetan Plateau

Organic matter fractions [a)]	HA	Hob-FA	Hil-FA	HM	TOM
Fraction proportion (%)	1.07	0.17	42.03	56.73	100
δ^{13}C values (‰)	−21.1	−27.5	−26.1	−23.5	−24.6

a) HA, Humic acid; Hob-FA, hydrophobic fulvic acid; Hil-FA, hydrophilic fulvic acid; HM, humin; TOM, total organic matter.

3 Discussions and conclusions

3.1 Stable carbon isotope evidence to corroborate the existence of two types of *Hepialus* larvae

Based on the newly obtained data of stable carbon isotopes for *Hepialus* larvae, tender plant roots, and soil humus fractions (Tables 1—3), we conduct a simple digital calculation and further infer as below.

If a *Hepialus* larva eats the tender plant roots with the δ^{13}C values of −7.1‰ to −29.0‰ (Table 2), metabolism may enhance its δ^{13}C values up to 0.4‰ to 1.0‰[25,26]. If the maximum enrichment effect of +1.0‰ is considered, the larva fed wholly by these plant roots will have the δ^{13}C values of −26.1‰ to −28.0‰. If an analytical error of ±0.3‰ is further considered, the δ^{13}C values of the *Hepialus* larva fed by tender plant roots may be at the interval of −25.8‰ to −28.3‰.

Based on the result of the above digital simulation and the δ^{13}C values of *Hepialus* larvae (Table 1, Figure 1), the *Hepialus* larvae may be divided into two types. Type I (samples 1 and 2) is characterized by the δ^{13}C values of −22.6‰ to −23.4‰, more than −23.4‰ in the heads, and a small difference (less than 0.6‰) in the δ^{13}C values among heads, abdomens and tails (Table 1, Figure 1). Type II (samples 3 to 6) is characteristic of the δ^{13}C values of −24.6‰ to −27.6‰, less than −24.6‰ in the heads, and an evident difference (more than 0.6‰) in the δ^{13}C values among heads, abdomens and tails (Table 1, Figure 1). Obviously, type I has to ingest the food with a δ^{13}C value higher than that of these tender roots. Based on the data in Table 3, and combined with their living habits[1,11], we may affirm that type I of *Hepialus* larvae mainly ate the soil humus around them in habitats. The stable carbon isotope data of humus fractions (Table 3) further indicate that both HA and HM may be the foods of type I from point of view of single humus fraction. Given that the foods come from multiple humus fractions, it would become quite complicated, and need to be further confirmed by using the other methods, such as a feeding experiment.

The quantitative food structure of *Hepialus* larvae may also be further discussed on the basis of our newly obtained stable carbon isotope data. Because HA is the only humus fraction with the δ^{13}C value (−21.1‰) higher than that of *Hepialus* larva (−22.6‰), the δ^{13}C value of HA is the highest end member, while the tender plant root is the lowest end member for calculating the quantitative food structure. If the δ^{13}C values of *Hepialus* larvae are represented by those in their heads in Table 1 (the effects of their food residue and dejecta may be removed), and the δ^{13}C values of tender plant roots are assumed to be the mean of the δ^{13}C values of ten species of plants (−28.0‰). The *Hepialus* larvae also eat the HA fraction of soil humus with a δ^{13}C value of −21.1‰ except the tender plant roots as their food, then their quantitative food structures may be estimated as shown in Table 1. Obviously, the HA fraction in the soil humus at least accounts for the portions up to 66.67% to 78.26% for type I, and 36.23% to 49.28% for type II.

3.2 New discovery and sustainable utilization of *C. sinensis* resource

In recent years, with the elevation of Chinese living standard and health consciousness, the demand for *C. sinensis* has steadily increase and its price has subsequently increased quickly[1]. To alleviate the contradiction between the resource utilization of *C. sinensis* and entironment protection, many scientists have tried to enhance the yield of *C. sinensis* via an artificial intervention method of synchronously improving the density of *Hepialus* larvae and their infection rate by *H. sinensis* in *C. sinensis* habitats. Obviously, to realize this goal, it is necessary to solve the problem “how to breed *Hepialus* larvae on a large scale with a low cost”. Our new discovery of the humus-chiefly-fed *Hepialus* larva has broken through the previous understanding that the food source of larva only came from the tender plant roots[9−16], and may provide one novel idea to produce the low-cost and high-quality foods for *Hepialus* larvae without any new microcosmic ecological questions to be followed. This is to say that the humus fractions as the food of *Hepialus* larvae may rapidly be produced on a large scale in low-altitude regions, and then added into the soils of high-altitude *C. sinensis* habitats just like a fertilizer to feed *Hepialus* larvae. In the large-scale reproduction of *Hepialus* larvae, this new idea may provide evidence for choosing cheap and high-quality foods and establishing artificial habitats for them, and a technique support to relax the contradiction between the sustainable utilization of *C. sinensis* resource and the protection of vulnerable entironments in the Tibetan Plateau.

3.3 Conclusions

(1) Two types of *Hepialus* larvae were identified in the habitat in the Sejila Mountain, Tibetan Plateau. One is the *Hepialus* larva chiefly fed by soil humus and characteristic of δ^{13}C (−22.6‰ to −23.4‰), more than −23.4‰ in heads and less than 0.6‰ among heads, abdomens and tails. The other is the *Hepialus* larva chiefly fed by tender plant roots and characterized by δ^{13}C (−24.6‰ to −27.6‰), less than −24.6‰ in the heads, and more than 0.6‰ among heads, abdomens and tails.

(2) Based on the newly obtained data of stable carbon

isotopes, the digital simulation was conducted to elaborate the quantitative food structure of the host *Hepialus* larva of *C. sinensis* in the Sejila Mountain, Tibetan Plateau. The result demonstrated that the soil humus fraction HA was one food for types I and II of *Hepialus* larvae except the tender plant roots, and the portions of HA for Types I and II were 66.67% to 78.26% and 36.23% to 49.28%, respectively.

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