Reconstructing the life history of modern and fossil nautiloids based on the nitrogen isotopic composition of shell organic matter and amino acids

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Abstract. We determined the nitrogen isotopic compositions of organic matter in the shell material of modern nautilids (Nautilus pompilius) and a mid-Cretaceous cymatoceratid (Cymatoceras sakalavus from the Albian of Madagascar) to reconstruct the trophic ecology in natural habitats and its change over the animals’ lifetimes. The profiles of δ15N values along the growth direction exhibited a common pattern in modern Nautilus specimens, suggesting three ontogenetic stages in the trophic ecology of the animal. The Embryonic Stage, represented by the innermost shell formed during embryogenesis, is characterized by relatively elevated δ15N values (12–14‰). The δ15N values show a sharp decrease (by approximately 3‰) shortly after hatching of the shell, recorded between the 7th and 12th septa (the Post-hatching Stage). In the following Juvenile-mature Stage, δ15N values exhibit a relatively stable pattern in the range 10–12‰. The decrease in δ15N values during the transition from the Embryonic Stage to the Juvenile-mature Stage probably reflects a change in nutrient source from yolk to a diet. Analysis of shell material formed in an aquarium following capture from the ocean revealed a sudden drop in δ15N values, probably reflecting the change from natural to artificial diet. The compound-specific nitrogen isotopic compositions of amino acids from the shell of N. pompilius suggest that the above isotopic patterns obtained from the bulk material do indeed reflect changes in the apparent trophic level throughout its life. In contrast to modern Nautilus, δ15N values obtained for C. sakalavus show a gradual increase from 1.2 to 3.8‰ with increasing septum number, suggesting an increase of approximately one trophic step in the food web throughout its life.

Key words: Nautilus pompilius; fossil nautiloid; nitrogen isotopes; trophic level; amino acids trophic level; shell organic matter.

Introduction

The ecology of extinct animals has primarily been estimated based on fossil morphology and analogy to modern relatives. Even for extant species, the natural lives of organisms are poorly understood due to difficulties in undertaking direct observations of their life habits in nature. The composition of the skeletal remains of such organisms, however, potentially records their ecology as well as their living environment, thereby providing a geochemical approach to the indirect observation of remote and past lives. One of the most successful applications in this regard is stable oxygen isotopic analyses of carbonate mollusk shells, from which the ambient water temperature can be inferred. By determining the oxygen isotopic compositions of different stages of marginal-growing shell carbonate, for example, the life histories of both extant and extinct cephalopods can be reconstructed in terms of their living habitat (e.g., Oba et al., 1992; Landman et al., 1994; Moriya et al., 2003).

Analyses of the stable nitrogen isotopes of organic matter within calcified skeletal remains are potentially useful in estimating the nutritional sources during an animal’s lifetime. The stable nitrogen isotopic composition of animal organic tissue is known to reflect the animal’s diet, with a systematically increase in δ15N values toward the upper part of the hierarchy of the food web (e.g., Hobson and Welch, 1992; Keough et al., 1996; Yoshii et al., 1999; Ogawa et al., 2001; Post, 2002). The mean enrichment factor of δ15N in body tissue is ~3.4‰ per trophic step (e.g., DeNiro and Epstein, 1981; Minagawa and Wada, 1984). Calcified
skeletal tissue encapsulates organic material that is biosynthesized at the time of formation, resulting in the potential for preservation for much longer periods than body tissue, even in fossil specimens of the geologic past. Therefore, the nitrogen isotopic composition of skeletal tissue potentially preserves the pristine isotopic signature of organisms, directly reflecting their trophic ecology (e.g., Ostrom et al., 1993).

In this context, we have been exploring a new methodology to reconstruct the trophic ecology of modern and fossil nautiloids based on nitrogen isotopic analyses of shell material. As with other mollusks, nautiloids deposit a marginal-growing shell wall throughout their life, as well as periodically secreting aragonitic septa during the cycle of chamber formation. The organic matter of mollusk shells is largely conchiolin, a protein complex secreted from the mantle during shell growth, hence reflecting the nitrogen isotopic composition of the animal’s diet during the period of the shell formation. Therefore, the nautiloid shell material contains a continuous record of the nitrogen isotopic signature of its trophic state. Such data enable the reconstruction of dynamic changes in the trophic state of an individual throughout its life.

We determined the nitrogen isotopic composition of the bulk shell material of three specimens of a modern nautiloid, Nautilus pompilius of the family Nautilidae from the Philippines, sampled sequentially from the ventral shell wall along its growth direction, as well as samples of septa. We also determined the compound-specific nitrogen isotopic composition of amino acids from selected samples, which is a novel tool in estimating the trophic state of organisms. We also discuss the trophic history of N. pompilius and report the results of nitrogen isotopic analyses of aragonitic shell material from a fossil nautiloid, and consider the possible implications of these data for the animals’ trophic ecology.

Materials and methods

Materials

We analyzed one male (Tnb-53) and two female (Tnb-2, Tnb-88) specimens of Nautilus pompilius captured live in Balayan Bay, Batangas, Luzon, the Philippines, by K. Tanabe and T. Ubukata in 1993 (Figure 1). The male specimen was not kept alive, but the two female specimens were reared in an aquarium at the University of Tokyo for approximately 1 year, fed mainly on commercially available cultivated prawns. Besides, an additional adult specimen of N. pompilius (Rbt-1; shell only; source unknown) was used for analysis. We also analyzed a specimen of the fossil nautiloid Cymatoceras sakalavus (Cymatoceratidae) recovered from the Albian of Mahajanga Province, Madagascar, which preserves alagonitic shell materials and its original nacreous structure (Figure 2).

Isotopic analyses of bulk shell material

Aragonitic shell material corresponding to various growth stages was milled from septa and the nacreous layer of the ventral shell wall along its growth direction. Shells of N. pompilius were cut parallel to the median plane to obtain central slices of the shell (Figure 1). For specimens Tnb-53 and Rbt-1, each septum was carefully detached from the sliced specimens and pulverized after the surface layer had been planed off. For Tnb-2, Tnb-53, and Tnb-88, small amounts of powdered material (4–6 mg) were sampled from the ventral shell wall of the sliced specimens; here, only the core of the nacreous layer was sampled by planing parallel to the shell surface after the removal of the outer surface.

The fossil specimen was cut and polished along the median plane. Small amounts of powdered shell material (0.5–6 mg) were carefully sampled from the polished cross-section of each septum wall using an automated micromill sampler (GEOMILL326; Sakai, 2007). The precision of the sampler (<10 μm) enabled selective sampling from only the core layer of each septum. Sample material obtained by micro-milling of 2–3 adjacent septa were analyzed together because the amount of powder obtained from single septa was inadequate for accurate analyses.

Nitrogen isotopic analyses were performed using a ThermoFinnigan Delta plus XP isotope-ratio mass spectrometer coupled to a Flash EA1112 automatic elemental analyzer via a Conflor III interface (EA/IRMS; Ohkouchi et al., 2005). Nitrogen isotopic compositions are expressed as conventional δ-notation against atmospheric N₂ (AIR):

\[ \delta^{15}N \%o = 10^3[(^{15}N/^{14}N)_{sample}/(^{15}N/^{14}N)_{standard} - 1] \]

In practice, isotopic compositions were normalized using cross-calibrated in-house standards (Proline \[\delta^{15}N = 13.31 \pm 0.06 \%o\]) and Tyrosine \[\delta^{15}N = 8.44 \pm 0.05 \%o\]). The powdered shell material of N. pompilius was wrapped in precleaned tin capsules for EA/IRMS analysis. The powdered fossil shell was treated with 2 N HCl in precleaned silver capsules to remove excess carbonate and then wrapped in tin capsules (combustion improver) after dehydration. The estimated analytical precisions (1σ) of standards (analytical range: 2-35 μg nitrogen) were <0.5‰.
Nitrogen isotopic analyses of individual amino acids

Powdered samples were obtained from selected parts of the ventral shell wall that represent various ontogenic stages of the animal (Table 1). Amino acids were prepared by HCl hydrolysis and derivatized (N-pivaloyl/isopropyl derivatization) using a modified procedure described in Chikaraishi et al. (2007). The nitrogen isotopic composition of the individual amino acids was determined by gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) using a ThermoFinnigan Delta plus XP IRMS coupled to an Agilent Technologies 6890N GC via combustion and reduction furnaces. The analytical conditions for GC/C/IRMS analysis are described in Chikaraishi et al. (2007). In the present study, we focused on the δ¹⁵N values of glutamic acid and phenylalanine, from which information regarding the trophic ecology of the animal can be evaluated, as described below.

Recent studies have developed a novel methodology in estimating the trophic level of organisms based on the nitrogen isotopic composition of selected species of amino acids, such as glutamic acid and phenylalanine (McClelland and Montoya, 2002; Chikaraishi et al., 2007). Chikaraishi et al. (submitted to Limnology and Oceanography: Methods) suggested that glutamic acid is systematically enriched in ¹⁵N toward the upper levels of the food chain (8.0 ± 1.2 ‰ at each trophic step) as a result of metabolic processes; in contrast, phenylalanine shows little enrichment in ¹⁵N because of the absence of nitrogen-involving reactions in its dominant metabolic processes. Therefore, trophic level is estimated based on the δ¹⁵N values of glutamic acid and phenylalanine via the following equation, termed the “Amino acid Trophic Level (ATL):”

\[
\text{ATL} = \left( \delta^{15}\text{N}_{\text{Glu}} - \delta^{15}\text{N}_{\text{Phe}} + 4.2 \right) / 7.6
\]
where $\delta^{15}\text{N}_{\text{Glut}}$ and $\delta^{15}\text{N}_{\text{Phe}}$ are the nitrogen isotopic compositions of glutamic acid and phenylalanine, respectively. Unlike conventional estimates of trophic level based on isotopic analyses of bulk organic tissue, the ATL indicates the absolute trophic level without requiring knowledge of the isotopic composition of the primary producer at the base of the food web.

**Results**

The nitrogen isotopic compositions of septa in the two specimens of *N. pompilius* (Rbt-1 and Tnb-53) demonstrated similar ontogenetic variations with increasing septum number (Figure 3). From the 7th to 12th septa, the $\delta^{15}\text{N}$ values fell sharply, from 15.0 to 11.1 ‰ in Rbt-1 and from 14.0 to 10.0 ‰ in Tnb-53. After the 13th septum, however, the $\delta^{15}\text{N}$ values show only minor variations, increasing slightly (by about 1 ‰) to the last septum.

The nitrogen isotopic compositions of the ventral shell wall in the three live-caught specimens of *N. pompilius* (Tnb-2, Tnb-53, and Tnb-88) show similar relative isotopic variations along the growth direction (Figures 4 and 5). Elevated $\delta^{15}\text{N}$ values are observed in the innermost shell where located less than 60 mm in spiral length from the center of coiling. The values fall sharply (by 3–4 ‰) in the shell portion between approximately 60 and 100 mm in spiral length from the center of coiling, shifting from 13.4 to 9.7 ‰ for Tnb-2, 13.6 to 9.8 ‰ for Tnb-53, and 12.9 to 10.6 ‰ for Tnb-88. The $\delta^{15}\text{N}$ value varies only gradually, within a range of ~2‰, in the shell portion located more than 100 mm in spiral length from the center of coiling; however, Tnb-2 and Tnb-88 record a second sharp fall in $\delta^{15}\text{N}$ values (~2%) in the apertural shell portion that formed during the period in the aquarium.

The nitrogen isotopic values of septa of the *C. sakalavus* specimen are generally lower than those of *N. pompilius*, and gradually increase from 1.2 to 3.8 ‰ with increasing septum number (Figure 6).
Discussion

Modern nautiloid (Nautilus pompilius)

The $\delta^{15}$N values of shell material from live-caught specimens of *N. pompilius* suggest the occurrence of three ontogenetic stages in terms of trophic strategy: (1) the “embryonic stage,” when the yolk is the sole source of nutrients; (2) the “post-hatching stage,” during the early postembryonic stage; and (3) the “juvenile-mature stage” (Figure 4). An additional stage is identified for those specimens reared and fed on artificial diets (see below).

The embryonic stage is isotopically defined as an ontogenetic stage that occurs before the onset of a rapid drop in $\delta^{15}$N values within organic tissue. This stage is recorded in septa 1–7 and the early ventral shell portion before the primary constriction (less than 60 mm in spiral length from the center of coiling) (Figures 3–5). Previous observations of early postembryonic *Nautilus* specimens newly hatched in aquaria have shown that the first seven septa are secreted during the embryonic stage (e.g., Arnold, 1987), and that primary constriction developed at the time of hatching (Uchiyama and Tanabe, 1999; Figure 4). Therefore, this trophic stage corresponds to the period during which an embryo develops solely on nutrition derived from its yolk.

The $\delta^{15}$N values of the embryonic stage are $\sim$3‰ higher than those of the juvenile-mature stage, corresponding to approximately one trophic level (3.4 ‰; Minagawa and Wada, 1984). This difference in $\delta^{15}$N value probably arises because the yolk, the nutritional source of the animal during the embryonic stage, has a similar nitrogen isotopic composition to that of the mother (cf., Hobson, 1995). Therefore, the $\delta^{15}$N value of the embryo should indicate one trophic level higher than the adult animal in the juvenile-mature stage.

The post-hatching stage is isotopically defined as an ontogenetic stage that accompanies a rapid fall in the $\delta^{15}$N value of organic tissue. This stage is recorded in septa 7–12 and the ventral shell at 60 to 100 mm in spiral length from the center of coiling (Figures 3–5). The isotopic drop is probably related to a gradual change in nutrition source from yolk during the embryonic stage to its diet during the early postembryonic stage.
In fact, newly hatched *Nautilus* are known to start consuming diets while still possessing an external yolk sac (Uchiyama and Tanabe, 1999). The observed gradual shift in isotopic values therefore appears to be partially attributed to the delayed response to the diet change due to the gradual metabolic turnover of amino acids in body tissue.

The juvenile-mature stage is recorded in septum 13 and larger, as well as the ventral shell portion more than 100 mm in spiral length from the center of coiling (Figures 3–5). This stage is isotopically defined as the stage *after* the end of the rapid drop in the $\delta^{15}\text{N}$ value of organic tissue, representing the life period from the early postembryonic stage to the fully developed adult stage, during which time nutrients are exclusively supplied from the diet. The $\delta^{15}\text{N}$ values of shell material corresponding to this stage vary by only 2 ‰, less than the expected change for a single trophic step. The above results are therefore concordant with an ontogeny of *N. pompilius* that develops directly and presumably feeds on a similar diet throughout its life (Saisho and Tanabe, 1985). A broad trend of ~2 ‰ increase in $\delta^{15}\text{N}$ throughout the entire juvenile-mature stage of Tnb-2 may reflect a change in the size of the diet, as larger diets tend to occur at higher trophic levels.

An additional trophic stage is recorded by shell material formed after introduction to an aquarium environment (Figure 4), showing a rapid shift in nitrogen isotopic composition due to a change from natural to artificial diet. The observed drop in $\delta^{15}\text{N}$ values in Tnb-2 and Tnb-88 probably reflects the relatively low $\delta^{15}\text{N}$ value of the artificial diet (e.g., cultivated prawns) upon which they were fed while in captivity.

The nitrogen isotopic compositions of specific amino acids demonstrated that the isotopic stages identified based on analyses of bulk shell material do indeed reflect changes in the apparent trophic level rather than other factors such as environmental change (e.g., isotopic change of the primary producer) or physiological factors unique to the individual (Table 1). The ATL of Tnb-2 dropped from 4.5 during the
embryonic stage to 3.8 at the start of the juvenile-mature stage, presumably reflecting the change of nutritional source from yolk to diet. The ATL obtained at a later period of the juvenile-mature stage (3.7) is similar to that of the earlier period (3.8).

An ATL of 3.7–3.8 suggests that the trophic level of *N. pompilius* in its natural habitat in the Philippines was approximately 4, making it a tertiary consumer (a consumer placed three steps higher than a primary producer in the food-chain hierarchy). In the natural food web, however, the trophic level of organisms is best expressed with decimal fractions because their diets could consist of a mixture of organisms from variable trophic levels. For example, *N. pompilius* analyzed in the present study could have alternatively fed on a diet with a trophic level higher than 3 (e.g., crustaceans) and one with a trophic level lower than 3 (e.g., annelids and fishes that feed on planktons and/or algae). This interpretation is strongly supported by a previous analysis of the oesophagus and stomach contents of *N. pompilius* in Fiji (Saisho and Tanabe, 1985).

Fossil nautiloid (*Cymatoceras sakalavus*)

The δ¹⁵N values of shell samples of *Cymatoceras sakalavus* did not exhibit the three trophic stages recorded by modern *Nautilus* (Figure 6); instead, the values showed a gradual increase of 2.6 ‰ throughout its life, slightly less than the change thought to indicate a single trophic step. This finding may reflect a gradual change in diet during the ontogeny of this species.

The obtained δ¹⁵N values are generally low (1–4 ‰), indicating a low trophic level and perhaps a herbivorous diet during at least the animal’s early life stages. This finding challenges the common idea that all modern (and perhaps fossil) nautiloids are predators. The obtained δ¹⁵N values are unusually low, lower even than those of common primary consumers in the modern marine environment (e.g., Chikaraishi *et al.*, 2007). Moreover, the data do not indicate a distinct embryonic stage with elevated δ¹⁵N values, as observed in the modern nautiloid. This result may suggest that the egg of *C. sakalavus* hatched at a relatively early stage of
development, presumably prior to formation of the third septum.

We sampled and subsequently treated the analyzed shell material with special care to minimize the possibility of contamination in the laboratory; however, a potential pitfall of the current method is that we cannot exclude the possibility that organic matter was added to or formed within the samples during post-mortem degradation. Nonetheless, the non-random trends in the obtained profiles indicate that the isotopic signature represents at least in part a primary signal, and is not entirely overprinted by post-mortem events. Compound-specific isotopic analyses of amino acids of shell protein-derived peptides, may help to clarify the absolute trophic level of this fossil nautiloid, as such an analysis would be free of any post-mortem influences. Such analyses were not possible in the present study because of difficulties in analyzing the fossil specimen.

Conclusion and implications

The nitrogen isotopic composition of shell material of the modern nautiloid *Nautilus pompilius* records its trophic history through life. The apparently simple trophic history suggested by the present results, consisting of only three stages, is consistent with the predicted life history of a species that develops directly from an unusually large (ca. 3–4 cm in maximum size) and yolk-rich egg, without a larval stage. This reconstruction of the trophic history was further reinforced by compound-specific nitrogen isotopic analyses of amino acids. Thus, it is suggested that the present method is applicable in gaining an understanding of the trophic ecology of other mollusks and temporal changes in trophic ecology over the organism’s life, without the need for direct observations of feeding.

The method is also potentially applicable to studies of the trophic ecology of extinct mollusks. For example, Ammonoidea, an extinct ectocochilicate cephalopod group that flourished in Devonian–Cretaceous oceans, are known to possess much smaller embryonic shells (generally < 1 mm in diameter, excluding Early Devonian taxa) than nautiloids (Landman et al., 1996). This observation strongly suggests that newly hatched ammonoids fed on relatively small organisms (e.g., zooplankton) as a nutritional source, and that their trophic level at the early embryonic stage was lower than that of modern and fossil nautiloids. An understanding of the ecological roles of ammonoids is particularly important for analyses of

![Figure 6. Septal δ¹⁵N values obtained for the fossil nautiloid Cymatoceras sakalavus.](image-url)
Paleozoic and Mesozoic marine ecosystems, as the evolution and extinction of diverse ammonoid species are the most prominent aspects of nektonic/nectobenthic forms over this period. It will therefore be important in future studies to develop more reliable methods of measuring the primary nitrogen isotopic signature from fossil remains, including measurements from amino acids.

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