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Sources of otolith carbonate: experimental determination of carbon incorporation rates from water and metabolic CO_2, and their diel variations

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ABSTRACT: Although carbon isotopes in fish otoliths are widely utilized to obtain information on environmental records, uncertainty regarding the sources of otolith carbonate, either from fish diet or ambient water, limits detailed determinations. The present study experimentally determined absolute incorporation rates of carbon derived from 2 sources, metabolic CO_2 and ambient water, into otoliths using goldfish under controlled conditions. In addition, the proportions of the 2 sources in otolith daily increments were also determined by the diel variations in the rate of incorporation from the 2 sources. A group of fish was administered with D-[^{14}C-U]-glucose or was exposed to water containing $NaH^{14}CO_3$, and incubated for 1, 3, 6, 12 and 24 h. Another group was similarly treated with the radiocarbons at 06:00, 12:00, 18:00, 00:00 or again at 06:00 h, and were incubated for 6 h. After incubation, serum and otoliths were collected and were separated into organic and inorganic carbon fractions, and the incorporation rates from the 2 sources were determined. The rates of carbon incorporation from D-[^{14}C-U]-glucose injected and $NaH^{14}CO_3$ water were 0.27 (25.5%) and 0.79 (74.5%) nmol mg^{-1} otolith h^{-1}, respectively. During darkness (18:00 to 06:00 h), both the incorporation rate and proportion of metabolic-CO_2-derived carbon significantly decreased to ~50% of that during daylight. These results suggest that otolith carbonate is mainly derived from ambient water, and the ratio of carbon derived from metabolic CO_2 is lower in the D-zone than that in the L-zone of the otolith daily increments.

KEY WORDS: Otolith · Carbon source · Diel variation · Dissolved inorganic carbon · Metabolic CO_2 · Goldfish

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INTRODUCTION

Teleost otoliths (ear stones) are composed of calcium carbonate biominerals that contain a small amount (<5%) of organic matrix. Teleostean fishes have 3 pairs of otoliths, called sagitta, lapillus, and asteriscus, which are located in the membranous labyrinth of the inner ear end organs, sacculus, utriculus, and lagena, where they function as equilibrium and hearing organs. Since the finding of daily growth increments in otoliths (Pannella 1971), they have been widely utilized for assessment of the daily age and life histories of fish. It is thought that otoliths themselves are seldom reabsorbed after they have formed, and that the elemental composition of otoliths reflects external environments. Therefore, some elements in the otoliths, especially ratios of strontium/calcium and stable isotopes of carbon and oxygen, are widely used for reconstruction of various individual records, such as seawater/freshwater migrations (Tsukamoto et al. 1998), thermal history (Devereux 1967), and metabolic effects (Kalish 1991a). However, uncertainty in the proportion of otolith carbon derived from 2 sources, metabolic CO_2 (mCO_2) and dissolved inorganic carbon (DIC) in ambi-

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ent water, leads to difficulty in estimating individual histories by stable carbon isotopic ratio ($\delta^{13}C$) analyses (Weidman & Millner 2000). Therefore, it is important to determine the ratio of otolith carbonate derived from the 2 sources for any accurate estimation using $\delta^{13}C$.

Recently, Solomon et al. (2006) were the first to determine that the ratio of otolith carbon derived from mCO_2 and water is 0.17:0.83, using $\delta^{13}C$ in otoliths of rainbow trout *Oncorhynchus mykiss* maintained in a controlled environment. However, this study estimated only the average ratios of $\delta^{13}C$ through >10 d increments, because sample sizes were limited to >20 µm squares. In the near future, studies using otolith $\delta^{13}C$ will be able to elucidate sequential life histories using spot-to-spot analyses, such as those of strontium/calcium ratios, which are being conducted using electron probe microanalysis or laser ablation microprobe inductively coupled plasma mass spectrometry. In fact, some researchers have started the time-course predictions of $\delta^{13}C$ by spot-to-spot analyses (<10 µm, 1 to 5 d resolution) using nanoscale secondary ion mass spectrometry (Ushikubo et al. 2006). Therefore, the instantaneous rate of carbon incorporation is important to estimate the carbonate composition of otolith daily increments.

To understand otolith carbon sources at the daily increment level, carbon transport to otoliths and the rates of this transport must be studied at the diel level. Otoliths grow in the closed sac of the inner ear sacculus, which is filled with endolymph. Since the endolymph is an acellular environment and completely separated from the outer environments of blood and perilymph, otolith carbonate incorporation is directly regulated by the carbonate composition and supersaturation states in the endolymph (Takagi 2002). In the endolymph, pH and bicarbonate are maintained at higher levels (pH ~8.0, [HCO_3^-] ~30 mmol l^{-1}) compared with the blood (pH ~7.2, [HCO_3^-] ~10 mmol l^{-1}) (Mugiya & Takahashi 1985, Payan et al. 1997, 1999). Although Ca^{2+} levels are low (0.7 mmol l^{-1} in endolymph, 1.5 mmol l^{-1} in blood), the endolymph is totally supersaturated to the aragonite crystals (Takagi 2002). This carbonate environment is controlled by transportation of bicarbonate (Cl^-/HCO_3^- exchanger and carbonic anhydrase) from blood and proton elimination (Na^+/H^+ exchanger) to blood via the saccular epithelial cells (Payan et al. 1997, Tohse & Mugiya 2001).

In the present study, we experimentally determined the absolute instantaneous rates through 24 h and diel variations of carbon incorporation into otoliths from the 2 sources, mCO_2 and DIC in ambient water, using D-[^{14}C-U]-glucose and $NaH^{14}CO_3$. We also discuss in detail the incorporation pathways from the 2 sources and the effect of metabolic activity on the incorporation rates.

MATERIALS AND METHODS

Fish and maintenance. Goldfish *Carassius auratus*, weighing 8 to 10 g, were selected from our laboratory stocks and were used for the experiments. They were acclimated in aerated and dechlorinated tap water at 20°C for at least 2 wk before experiments, under a light regime of 12 light (06:00 to 18:00 h):12 dark (18:00 to 06:00 h). Fish were fed carp food pellets once a day, including the day of the experiments.

Expt 1: Incorporation of carbonates into otoliths. Twenty-five fish were anesthetized with 0.02% 2-phenoxyethanol, weighed, intraperitoneally administered with D-[^{14}C-U]-glucose (New England Nuclear) at a dose of 5 kBq mg^{-1} body weight in saline solution (135.0 mmol l^{-1} NaCl, 2.5 mmol l^{-1} KCl, 1.5 mmol l^{-1} $CaCl_2$, 2.0 mmol l^{-1} KH_2PO_4, 1.0 mmol l^{-1} $MgSO_4$, 10.0 mmol l^{-1} $NaHCO_3$, 10.0 mmol l^{-1} HEPES, 5 mmol l^{-1} glucose, pH 7.4), rinsed in water, and incubated at 20°C in a 60 l aquarium. These administrations were performed within 15 min. This time lag was adjusted at the time of sampling.

To determine the incorporation of inorganic carbon derived from ambient bicarbonate, another 25 fish were incubated in another 60 l aquarium filled with water containing 0.2 kBq ml^{-1} of $NaH^{14}CO_3$. To compare with the D-[^{14}C-U]-glucose experiment, these fish were intraperitoneally administered with the same amount of radiocarbon-free saline solution.

These 2 experiments were started at 06:00 h in the morning. In each of two 60 l aquaria, 25 fish were incubated and removed in groups of 5 each after 1, 3, 6, 12, and 24 h. During the incubations, the water was aerated to avoid increase in pCO_2 and decrease of pO_2. Ambient water (200 µl) was intermittently collected to determine variation in total CO_2 concentration and radioactivity. Total CO_2 concentration ranged from 0.75 to 0.84 mmol l^{-1} and varied little during incubation. After incubation, each group of 5 fish was quickly removed from the aquaria, immediately anesthetized, and sacrificed to collect blood and otoliths. After sampling the fish incubated for 12 h (at 18:00 h), remaining fish were incubated under dark conditions.

To monitor the differential otolith growth in the 2 experiments, inorganic carbon incorporation into otoliths from blood was also examined in the other group of 25 fish, which were intraperitoneally administered saline solution containing $NaH^{14}CO_3$ at 5 kBq mg^{-1} body weight. These fish were handled and sampled as above at 1, 3, 6, 12, and 24 h after administration. In addition, calcium deposition on otoliths over 24 h was determined using another 8 fish intraperitoneally administered saline solution containing $^{45}CaCl_2$ (5 kBq mg^{-1} body weight) (New England Nuclear). This group was incubated for 24 h and sampled for serum and otolith radioactivity.

Expt 2: Diel variations of carbonate incorporation rates. To determine diel variations in incorporation from the 2 different carbon sources, 2 additional experiments were conducted: one with D-[^{14}C-U]-glucose injection and the other with $NaH^{14}CO_3$ exposure. In the ^{14}C-glucose-injection experiment, groups of 3 fish each were acclimated in 10 aquaria (2 l of ambient water); 5 experimental groups (2 aquaria for each group) were started at 06:00, 12:00, 18:00, 00:00, and again at 06:00 h to examine diel variations. Within each of the 5 groups, one tank was incubated for 3 h and the other tank for 6 h. Handling (injection and sampling) of fish during the dark period (18:00 to 06:00 h) were conducted under dim light (<3 lux). For the $NaH^{14}CO_3$ exposure, 5 fish each were incubated in five 2 l aquaria, with incubations starting as above for ^{14}C-glucose. All 5 fish in each group were removed after 6 h. Other experimental conditions of this incubation are identical to those in Expt 1.

Sample collection and radioactivity. Blood was collected from the caudal aorta by amputating the caudal fin. The blood was blotted into a Petri dish filled with liquid paraffin to prevent diffusion of CO_2 into the air, recollected into a heparinized hematocrit tube with the liquid paraffin, and blood cells and serum were separated by centrifugation at 3000 × *g* for 10 min. The volume of serum was calculated from the length of the capillaries. Serum was then immediately tested for total CO_2 concentration or placed in the system for separation of inorganic and organic carbons and calcium, which is composed of 2 chambers containing perchloric acid for decalcification and collection of CO_2, respectively (Tohse & Mugiya 2001), to count the radioactivity. After collection of blood, otoliths (asterisci, largest otoliths in goldfish) of both sides of the inner ear were collected. They were washed 3 times in distilled water, dried overnight at 40°C for weighing, and ^{14}C of the organic and inorganic fractions and ^{45}Ca were separated by the system described above. Radioactivities of each fraction of serum and otoliths were separately counted with a liquid scintillation counter (Aloka, LSC-5100).

Determination of total CO_2 and glucose concentrations. Concentrations of total CO_2 in serum and water, and serum glucose were determined with the test kits, Infinity CO_2 (Sigma) and Glucose CII (Wako), respectively.

Calculation of carbonate incorporation rates. The rate of carbon incorporation into the otoliths (*y*, nmol mg^{-1}) was calculated by the equation:

$$y = S_c \times O_r / S_r \tag{1}$$

where S_c is the mean concentration (mmol l^{-1}) of carbon sources (glucose or total CO_2 in serum or in the water), O_r is the radioactivity of inorganic carbon in otoliths (dpm mg^{-1}) and S_r is the radioactivity of carbon sources (dpm µl^{-1}). Namely:

Carbon incorporation into otoliths from mCO_2 (nmol mg^{-1}) = serum glucose concentration (mmol l^{-1}) × radioactivity of inorganic carbon in the otoliths (dpm mg^{-1}) / radioactivity of organic carbon in serum (dpm µl^{-1}) (2)

Carbon incorporation into the otolith from DIC (nmol mg^{-1}) = total CO_2 concentration in the water (mmol l^{-1}) × radioactivity of inorganic carbon in the otoliths / radioactivity of inorganic carbon in the water (dpm µl^{-1}) (3)

Carbon incorporation into the otoliths from serum (nmol mg^{-1}) = total CO_2 concentration in serum (mmol l^{-1}) × radioactivity of inorganic carbon in the otolith (dpm mg^{-1}) / radioactivity of inorganic carbon in serum (dpm µl^{-1}) (4)

Since these concentrations and radioactivities were varied during the incubation (see 'Results'), S_c and S_r were standardized by obtaining the average values using the equation:

$$\int_{t0}^{t1} f(t)\,dt / (t_1 - t_0) \tag{5}$$

where t_0 and t_1 are the initial and final times of the experiments, and $f(t)$ is the concentration or radioactivity at time *t*.

Calcium deposition on the otoliths was calculated by the same equation using these parameters: S_c, calcium concentration in serum of 2.2 mmol l^{-1}, which was obtained in our previous experiment under identical conditions as the present study (Shinozaki & Mugiya 2000); O_r, otolith ^{45}Ca radioactivity; and S_r, radioactivity of serum.

Statistical analyses. One-way ANOVA was used for statistical evaluation. Differences among groups were assessed using the Tukey-Kramer post-hoc test and were accepted at $p < 0.05$.

RESULTS

Incorporation of carbon derived from metabolic CO_2

When fish were intraperitoneally administered ^{14}C-glucose, radioactivity in the ambient water was hardly detectable (Fig. 1A), indicating that inorganic ^{14}C in the otoliths is not incorporated from the water and is completely derived from mCO_2. Radioactivity of organic carbon in serum rapidly increased and peaked at 19.5 dpm µl^{-1} after 1 h (Fig. 1B), indicating that administered ^{14}C-glucose was rapidly circulated to the whole body. Radioactivity then decreased up to 24 h by metabolism of glucose to CO_2, and mCO_2 increased later, peaked at 3 h, and then decreased. This means that the mCO_2 was excreted to the water. On the other hand, inorganic ^{14}C was constantly being incorporated into the otoliths, and the radioactivity reached 36.8 dpm mg^{-1} (Fig. 1C). In contrast, no radioactivity

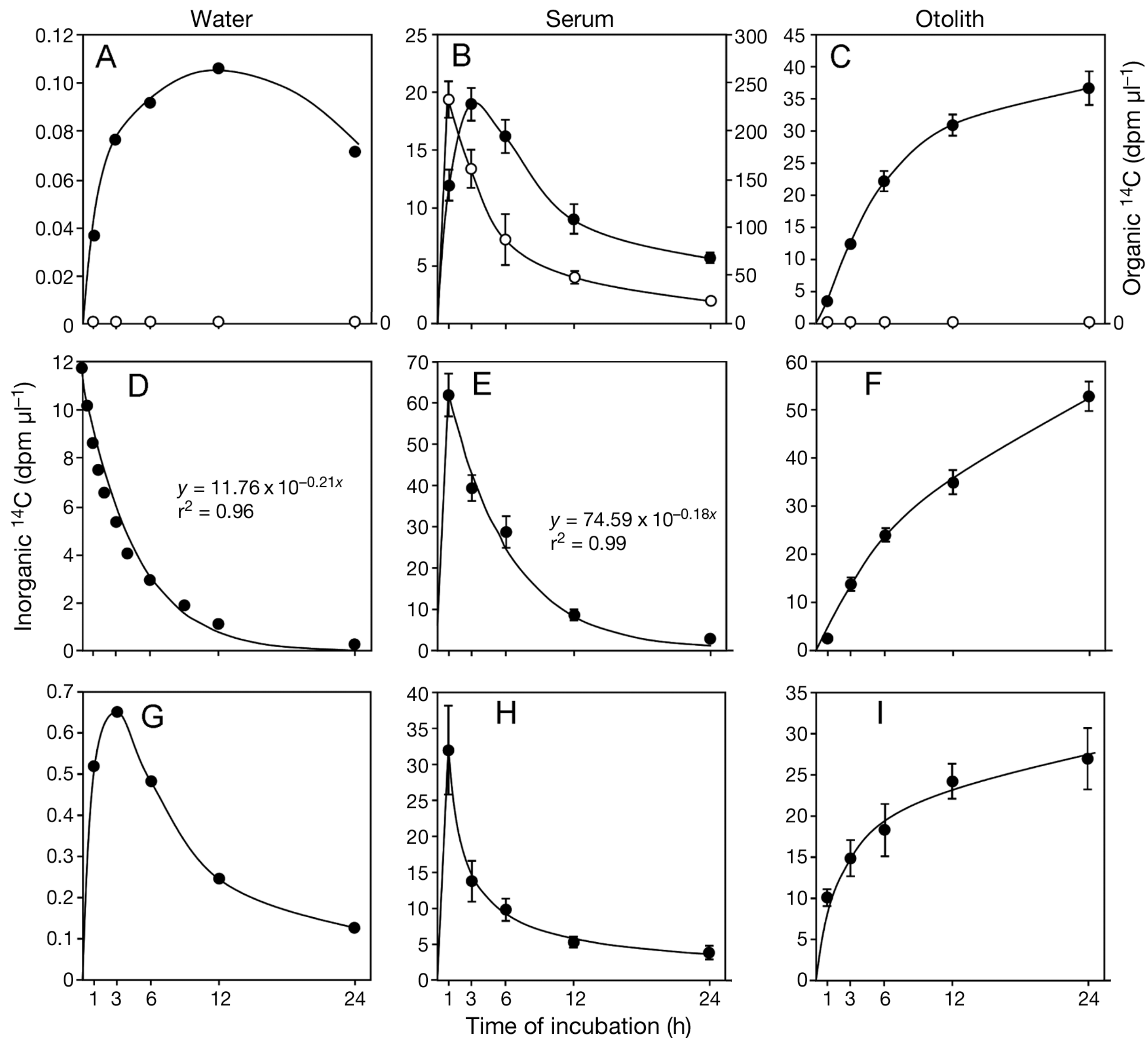

Fig. 1. *Carassius auratus*. Time-course variations of ^{14}C radioactivities in (A,D,G) ambient water, (B,E,H) serum, and (C,F,I) otoliths, when goldfish were administered (A–C) D-[^{14}C-U]-glucose or (G–I) NaH^{14}CO$_3$, or (D–F) were exposed to NaH^{14}CO$_3$. In Panels A to C, radioactivities of inorganic (●) and organic (○) carbon are indicated. Radioactivities in serum and otoliths are mean ± SE for 5 and 10 samples, respectively

was detected from the organic fraction of the otoliths. In this experiment, deposition of otolith carbonate derived from mCO_2 increased linearly up to 24 h incubation (Fig. 2A) and the incorporation rate (gradient of the regression line) was 0.27 nmol mg^{-1} h^{-1}.

Incorporation of carbonate derived from DIC in ambient water

When the fish were exposed to ^{14}C-containing water, radioactivity of the water decreased in inverse relation ($y = 11.76 \times 10^{-0.21x}$) to the incubation time (Fig. 1D), indicating that DIC is constantly exchanged for the CO_2 in the air and a part of this radiocarbon is incorporated into the fish. The time required to halve the radioactivity was ~3.3 h, which means that half the amount of DIC is exchanged for the CO_2 in the air during this period. On the other hand, total CO_2 concentration in the water was 0.75 to 0.84 mmol l^{-1} and changed little during incubation. Serum radioactivity increased rapidly to 62.0 dpm µl^{-1} and then decreased at the same rate ($y = 74.59 \times 10^{-0.18x}$) to that of the ambient water (Fig. 1E). In the otoliths, radioactivity increased throughout the incubation, finally terminating at 52.7 dpm mg^{-1} (Fig. 1F). The incorporation rate of the carbon derived from ambient DIC was 0.79 nmol mg^{-1} h^{-1} (Fig. 2B).

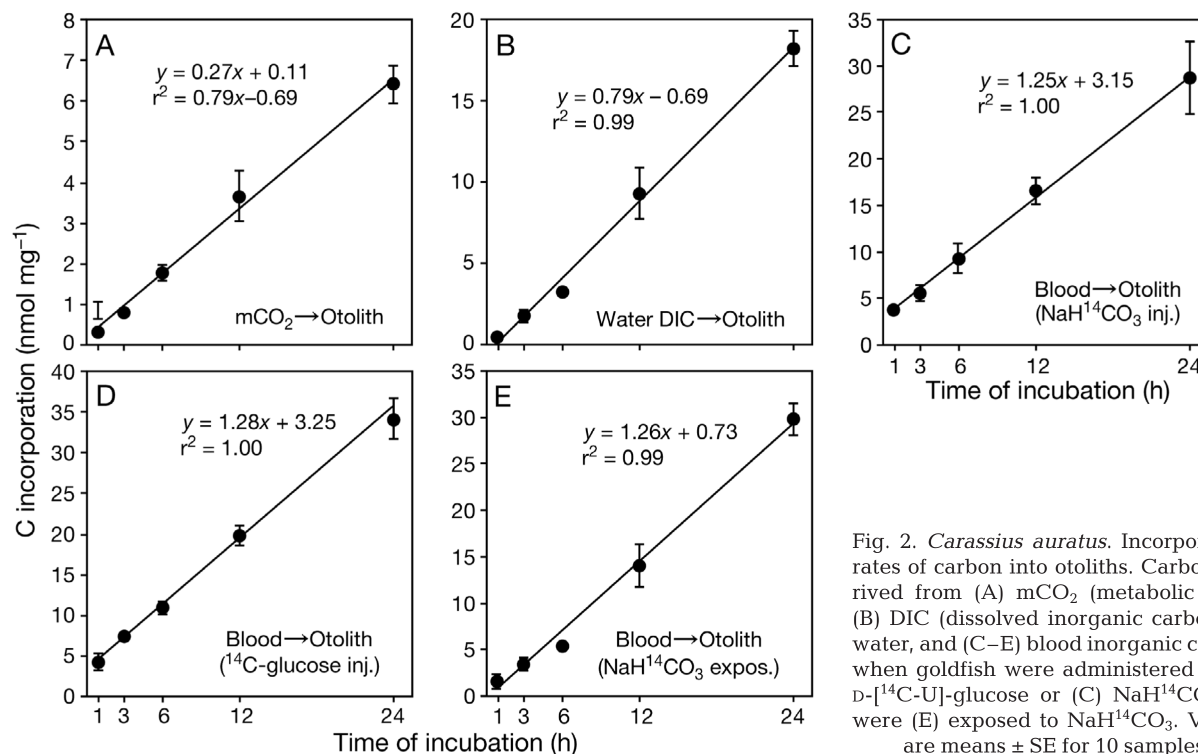

Fig. 2. *Carassius auratus*. Incorporation rates of carbon into otoliths. Carbon derived from (A) mCO_2 (metabolic CO_2) (B) DIC (dissolved inorganic carbon) in water, and (C–E) blood inorganic carbon when goldfish were administered (A,D) D-[^{14}C-U]-glucose or (C) $NaH^{14}CO_3$, or were (E) exposed to $NaH^{14}CO_3$. Values are means ± SE for 10 samples

Consistency of the incorporation rates and ratio of otolith carbon derived from the 2 sources

To confirm the consistency of carbon incorporation rates in the 2 experiments described above, incorporation of carbon from the blood was also examined by the administration of $NaH^{14}CO_3$. Administered ^{14}C radioactivity was barely detectable in the water (0.65 dpm µl^{-1} at the peak; Fig. 1G), suggesting that carbon in the blood was excreted to the water via gills. The injected ^{14}C was rapidly transferred to the blood within 1 h, with a peak of 32.0 dpm µl^{-1}, and the blood radioactivity decreased inversely with incubation time (Fig. 1H). Otolith radioactivity also rapidly increased, but saturated after 12 h and ended at 27.2 dpm mg^{-1} after 24 h of incubation (Fig. 1I). The incorporation rate of inorganic carbon in the blood as calculated from these radioactivities was 1.25 nmol h^{-1} (Fig. 2C). This rate can also be calculated from the radioactivities in the blood and otoliths, which were obtained from the ^{14}C-glucose injection and $NaH^{14}CO_3$ exposure experiments. The incorporation rates, 1.28 and 1.26 nmol mg^{-1} h^{-1} (Fig. 2D, E), were very similar to that obtained by $NaH^{14}CO_3$ injection. Therefore, it is certain that the incorporation rate of carbon in blood into otoliths ranges from 1.06 to 1.28 nmol mg^{-1} h^{-1} and that approximately 25% (0.27 nmol mg^{-1} h^{-1}) of carbon in otoliths is derived from mCO_2 and the remainder (0.79 nmol mg^{-1} h^{-1}) originates from DIC in water. This finding was also confirmed by the calcium incorporation rates. When fish were administered ^{45}Ca and incubated in the glucose-injection and $NaHCO_3$-exposure experiments, calcium incorporation rates were 24.37 ± 1.36 and 26.36 ± 2.18 nmol mg^{-1} 24 h^{-1}, respectively. This indicates that the difference in the activities of otolith growth in the 2 experiments is minor and that the calcium incorporation rate, approximately 1.04 nmol mg^{-1} h^{-1} (25 nmol mg^{-1} 24 h^{-1}), coincides with that of the total carbon incorporation, 1.06 nmol mg^{-1} h^{-1} (= 0.27 from mCO_2 + 0.79 from ambient water). Therefore, calcium and carbonates are incorporated into the otolith at a 1:1 ratio to form the calcium carbonate crystals.

Diel variations of glucose and total CO_2 concentrations in serum

Glucose concentration in serum ranged from 1.50 to 1.80 mmol l^{-1}, but there were no statistical differences among the groups (Fig. 3A). This level agrees with that found by Chavin & Young (1970), who examined serum glucose levels in goldfish incubated under various conditions and reported that serum glucose levels of well-acclimated goldfish are 25 to 35 mg 100 ml^{-1} (1.39 to 1.94 mmol l^{-1}) and do not vary diurnally. On the other hand, serum total CO_2 levels varied diurnally, with low levels during light (8.19 mmol l^{-1} at 15:00 h) and high levels during darkness (10.94 mmol l^{-1} at 00:00 h; Fig. 3B).

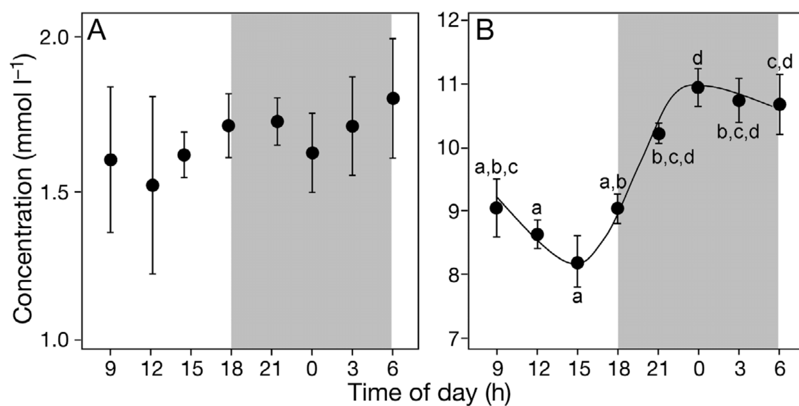

Fig. 3. *Carassius auratus*. Diel variations of (A) glucose and (B) total CO_2 concentrations in serum. Dark periods are indicated by gray background. Values are mean ± SE. (A) No significant difference was observed in the serum glucose concentrations. (B) Serum total CO_2 concentrations varied diurnally (n = 5 to 7, $F_{7,33}$ = 9.44, p < 0.0001). Significant differences (p < 0.05) between each of the groups, obtained by Tukey-Kramer post-hoc tests, are indicated by different letters (a to d)

glucose, fish incubated for 3 h were also collected to determine the correct variations in radioactivity for both organic and inorganic carbon in serum (Fig. 4A,B). However, radioactivity at 1 h could not be determined. Although radioactivity at 1 h was predicted from the results of Expt 1 (Fig. 1B), it is possible that considerable error occurred in the integral radioactivity from 0 to 3 h in serum, which could affect subsequent calculation of carbon incorporation into otoliths of fish incubated for 3 h (black bars in Fig. 6A). Accordingly, we considered these values to be less reliable than the results of the 6 h incubation, and the latter were used for determination of the incorporation rates. By the same reasoning, the carbon incorporation from blood in fish exposed to $NaH^{14}CO_3$, calculated from predicted sequential variations of serum radioactivity (Fig. 5B), were likewise considered less reliable.

In both experiments, the radioactivities in water and serum likely varied during the 0 to 6 h incubation period of Expt 1 (Figs. 4A,B & 5A). However, only mCO_2 radioactivity decreased during darkness (Fig. 4B). Indeed, the ratio of 6 h integral radioactivities of inorganic to organic carbon as an indicator of metabolic activity significantly decreased during darkness. This indicates that metabolic activity and subsequent production of mCO_2 in the fish is reduced in darkness. On the other hand, radioactivity of otoliths significantly decreased in darkness during both experiments (Figs. 4C & 5C).

Diel changes in the incorporation of carbon derived from the 2 sources

To determine the diel variations of carbon incorporation into otoliths from the 2 different sources, the incorporation rates of carbon derived from mCO_2 and ambient water were tested by 6 h incubations at various times (Figs. 4 & 5). The sequential variations in radioactivity of water containing $NaH^{14}CO_3$ could be determined by water collection during incubation, but serum radioactivity cannot be continuously monitored. Therefore, in the experimental administration of ^{14}C-

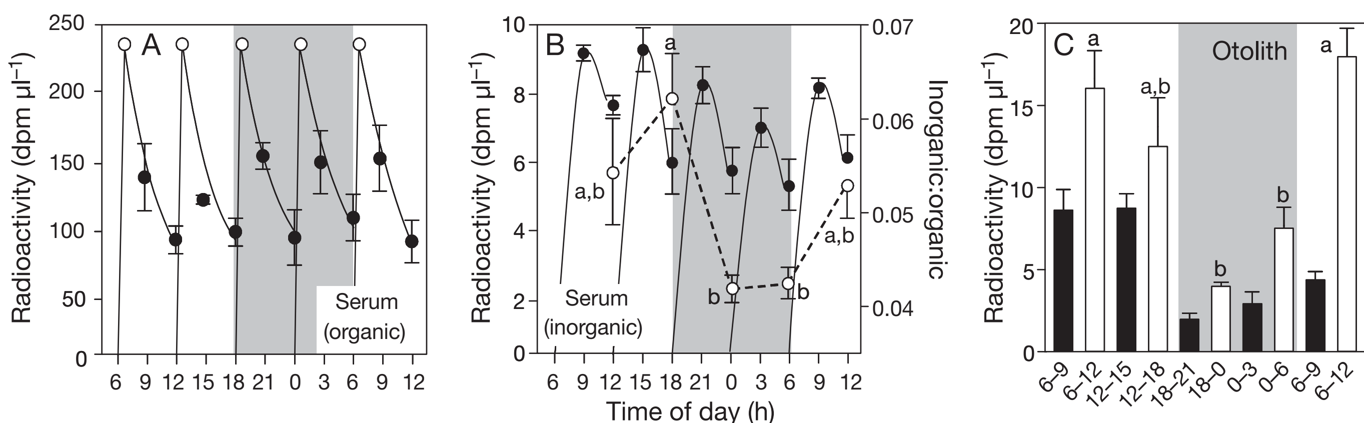

Fig. 4. *Carassius auratus*. Carbon radioactivities of serum and otoliths in fish injected with D-[^{14}C-U]-glucose and incubated for 3 or 6 h, at 6 different times of day (starting at 06:00, 12:00, 18:00, 00:00, and the next 06:00 h). Dark periods are indicated by gray background. Values are mean ± SE. (A) Radioactivity of organic carbon in serum (n = 3). Values at 3 and 6 h incubation (●) were actual values counted, whereas those at 1 h incubation (○) were predicted (see 'Results; Diel changes in the incorporation of carbon derived from the 2 sources' for details). (B) Radioactivity of inorganic carbon (left *y*-axis, ●) and integral radioactivity ratios of inorganic to organic carbon (right *y*-axis, ○) in serum. The ratio varied diurnally (n = 3, $F_{4,10}$ = 5.158, p = 0.0162). (C) Inorganic carbon radioactivity in otoliths of fish incubated for 3 (black bars) or 6 h (white bars). Significant differences in the Tukey-Kramer post-hoc test after ANOVA (6 h incubation: n = 6, $F_{4,25}$ = 8.176, p = 0.0002) are indicated by different letters (a, b)

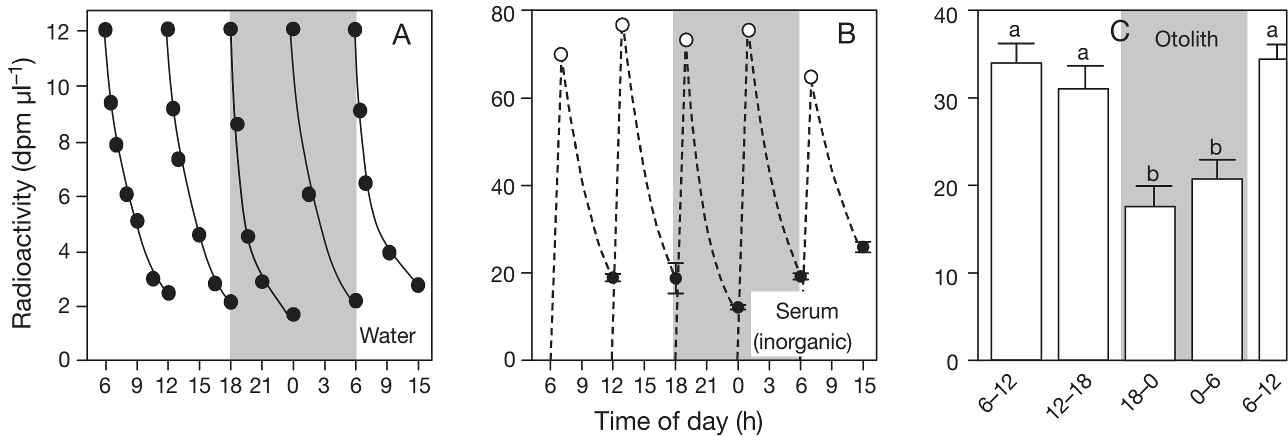

Fig. 5. *Carassius auratus*. Carbon radioactivities of water, serum, and otoliths in fish exposed to $NaH^{14}CO_3$-containing water and incubated for 6 h, at 6 different times of day (starting at 06:00, 12:00, 18:00, 00:00, and the next 06:00 h). Dark periods are indicated by gray background. Values are mean ± SE. (A) Radioactivity of water. (B) Inorganic carbon radioactivity in serum. Actual (●) and predicted (○) values are indicated. The decrement curves are also predicted. (C) Inorganic carbon radioactivity in otoliths. Significant differences in the Tukey-Kramer post-hoc test after ANOVA (n = 8 to 10, $F_{4,41}$ = 12.111, p < 0.0001) are indicated by different letters (a, b)

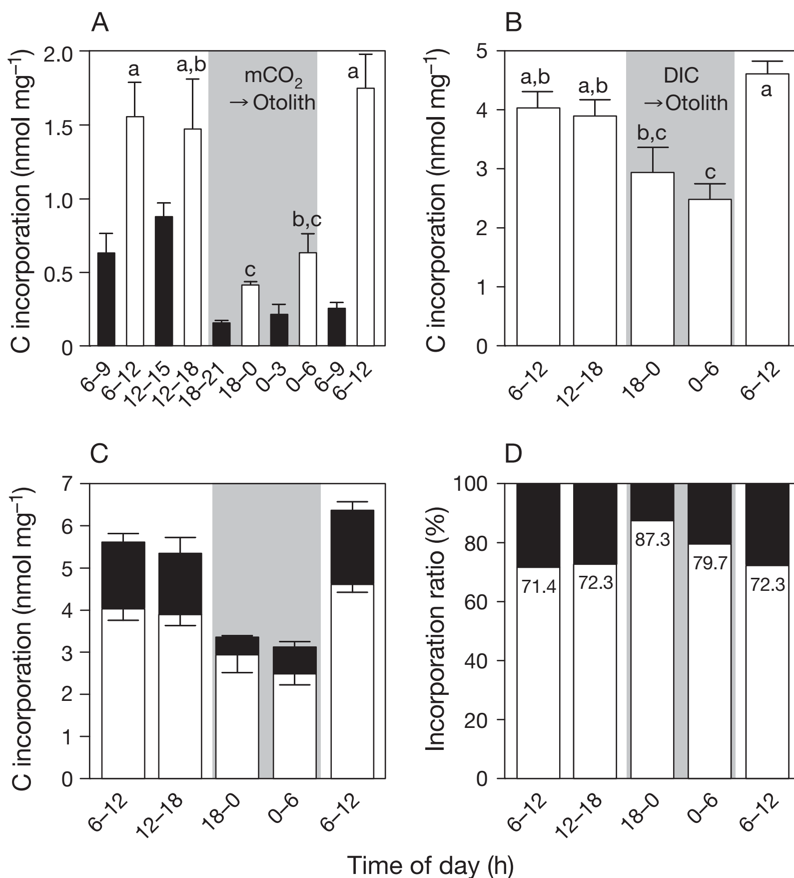

Fig. 6. *Carassius auratus*. Diel variations of carbon incorporation into the otoliths from (A) mCO_2 (n = 6, $F_{4,25}$ = 8.265, p = 0.0002) or (B) water DIC (n = 8 to 10, $F_{4,41}$ = 8.209, p < 0.0001). (C) Total carbon incorporation into the otoliths (black bars for mCO_2 and white bars for water DIC) for 6 h incubation, recalculated based on the results of Panels A and B. (D) Percentages of carbon incorporation from the different sources. Dark periods are indicated by grey background

During light periods in both experiments, carbon incorporation from both sources was similar to the 0 to 6 h incubation in Expt 1 (1.46 to 1.75 nmol mg^{-1} from mCO_2 [Fig. 6A]; 3.89 to 4.57 nmol mg^{-1} from water DIC [Fig. 6B]). However, these values significantly decreased in darkness (0.41 to 0.62 nmol mg^{-1} from mCO_2, 2.51 to 2.91 nmol mg^{-1} from ambient DIC). The total incorporation of carbon during light periods, 5.35 to 6.31 nmol mg^{-1} 6 h^{-1} (Fig. 6C), corresponded to the rate of total carbon incorporation estimated by Expt 1 (1.06 nmol mg^{-1} h^{-1}). On the other hand, in darkness, the incorporation decreased to 3.14 to 3.39 nmol mg^{-1}. The incorporation ratio of carbon derived from ambient DIC and mCO_2 varied diurnally between 71:29 and 87:13, with the higher ratio during light and the lower during darkness (Fig. 6D).

DISCUSSION

Although many studies have investigated carbon distribution in otoliths to assess the migration of fish (Iacumin et al. 1992, Schwarcz et al. 1998, Edmonds et al. 1999), little information on the sources of carbon in otoliths is available. To understand car-

bon composition in otoliths, it is necessary to consider the pathways of carbon transport, from sources to otolith incorporation. Endolymph, the fluid enclosed in the sacculi, has an elevated pH and high concentrations of CO_2-related ions (Mugiya & Takahashi 1985, Payan et al. 1997, 1999), which supersaturate to form aragonite crystals (Takagi 2002). These environmental features are formed by the anion transport system and carbonic anhydrase in the saccular cells, which play important roles in calcification of otoliths (Payan et al. 1997, Tohse & Mugiya 2001, Tohse et al. 2004). Specifically, CO_2-related ions in the blood are incorporated into the endolymph via the anion transport system and subsequently deposited on the otoliths.

Fish otoliths are primarily composed of calcium carbonate (>95%) and a small amount of organic matrix. The organic matrix has proteins (Degens et al. 1969), carbohydrates (Takagi et al. 2000), and their complexes (proteoglycans) as compartments. In the present study, we tested incorporation of organic carbon derived from glucose, but no radioactivity was detected in the organic fraction of otoliths, even though radioactivity of organic carbon in serum was detected 1 h after administration. This suggests that the study incubation time was too short to detect the organic carbon as synthesized proteins and glycosaminoglycans. Although we could not estimate the incorporation rate of organic carbon in this study, it is possible that carbon in the organic matrix may have a minor (<5%) effect on $\delta^{13}C$ in otoliths, especially in the D-zone, which is rich in organic substances compared with the L-zone.

When D-[^{14}C-U]-glucose was intraperitoneally administered, this radiochemical was rapidly incorporated into blood and metabolized. The maximum radioactivity of the metabolic ^{14}C was noted at 3 h after administration, suggesting that glucose is rapidly incorporated into the tissues and metabolized to CO_2 via glycolysis and the TCA cycle. This metabolic rate is comparable to a previous similar study using goldfish, and the mCO_2 production pathways are discussed therein (van den Thillart & Verbeek 1982). On the other hand, ^{14}C as water DIC was also rapidly incorporated into blood within 1 h of incubation. After the increase up to 1 h of incubation, blood radioactivity decreased at the same rate as the ambient water. This result suggests that the inorganic ^{14}C in blood was rapidly exchanged to water DIC via gills. However, DIC can be diffused into bodies and may account for most of the inorganic carbon incorporated into otoliths, since goldfish and other freshwater fish drink only a minimal amount of water (3.6 µl g^{-1} body weight h^{-1}; Kobayashi et al. 1983). Both the inorganic carbons derived from the 2 different sources are mixed in the blood and are incorporated into the otoliths via bicarbonate transporters (Tohse & Mugiya 2001). Therefore, the amount of carbon uptake from blood to otoliths is very similar among the 3 experiments: D-[^{14}C-U]-glucose administration (1.28 nmol mg^{-1} otolith h^{-1}), $NaH^{14}CO_3$ exposure (1.26 nmol mg^{-1} otolith h^{-1}), and $NaH^{14}CO_3$ administration (1.25 nmol mg^{-1} otolith h^{-1}). This incorporation, or otolith growth, compares favorably to our past studies using 2 freshwater fishes: the incorporation rate of calcium in goldfish (1.04 nmol mg^{-1} otolith h^{-1}; present study), calcium (~1.5 nmol mg^{-1} otolith h^{-1}; Mugiya 1984, Mugiya & Yoshida 1995), and inorganic carbon (~1.2 nmol mg^{-1} otolith h^{-1}; Tohse & Mugiya 2001) via the saccular epithelium in trout.

Since $\delta^{13}C$ in otoliths is thought to be affected by the 2 carbon sources, some studies have tried to estimate the source of otolith carbonate using $\delta^{13}C$ (summarized in Solomon et al. 2006). These studies reported that the proportion of metabolically derived carbon in otoliths ranges from 5 to 40%. However, most of these studies poorly control ambient water, diet, and other aspects of fish maintenance. In addition, none of these studies determined the instantaneous carbon incorporation rate, as described above. In our study, which controlled environmental conditions during fish incubation, absolute values of carbon incorporation rates could be determined, and the ratio of carbon derived from the 2 sources in otoliths could be calculated as 25% from mCO_2 and the reminder from water DIC. This value is within the mid-range of those estimated in the previously published studies. It is thought that the ratio of mCO_2-derived carbon in otoliths is affected by metabolic rate (Kalish 1991a,b, Iacumin et al. 1992, Gauldie 1996, Radtke et al. 1996, Schwarcz et al. 1998). However, no direct proof of this hypothesis has been reported, but Wright (1991) found that the increment width of otoliths is positively correlated with oxygen consumption (metabolic rate). The present study has proven this point: the ratio of inorganic/organic carbon in serum, which is an indicator of metabolic rate, and the incorporation rate of mCO_2-derived carbon into otoliths both simultaneously decrease during darkness. These results indicate that the proportion of carbon derived from mCO_2 decreases in the blood at night, and the carbon in blood is incorporated into the otoliths at that ratio. Therefore, the proportion of carbon derived from mCO_2 also decreases during darkness. Total carbon incorporation into the otoliths also decreases, indicating that activity of carbon transport in the saccular cells from blood to endolymph and the subsequent supersaturation state of the endolymph decreases.

The diel pattern of otolith growth has been studied morphologically and physiologically by several researchers. Morphological studies by Tanaka et al.

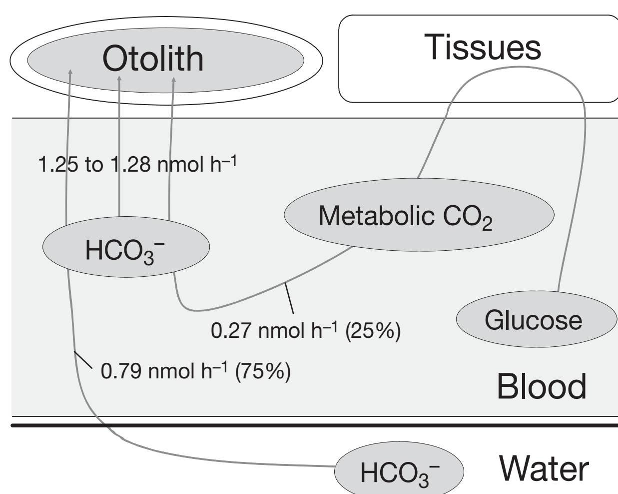

Fig. 7. Two different routes of carbon incorporation into otoliths from mCO_2 and DIC in water, and their incorporation rates

(1981) show that otolith formation slowed during darkness, and this phenomenon led to formation of the D-zone. A similar result was obtained using electron microscopy by Zhang & Runham (1992). Mugiya (1984, 1987) reported that calcium deposition on rainbow trout *Salmo gairdneri* otoliths decreased during darkness. Wright et al. (1992) reported that calcium deposition in Atlantic salmon parr *Salmo salar* otoliths declined during darkness under various photoperiods. Recently, it was revealed that the expression level of otolin-1 protein, a major component of the otolith matrix in saccular cells, is greater during darkness than daylight (Takagi et al. 2005). These results agree with those of the present study, suggesting that the carbon isotopic ratio in the D-zone is less than in the L-zone. The relationship between blood CO_2 concentration and otolith growth has also been evaluated in this study, but the results indicate little correlation between the two. Edeyer et al. (2000) reported diel variations in concentrations of calcium, total CO_2, and proteins in serum and endolymph, and suggested that the period of high CO_2 levels coincides with rapid otolith growth. In our study, however, metabolic rates, based on the ratio of inorganic serum ^{14}C-integral radioactivity, were high during periods of rapid inorganic carbon incorporation into otoliths, but total CO_2 levels in serum were low. These results suggest that inorganic carbon incorporation into otoliths was not related to total CO_2 levels in blood, but rather with metabolic rates. Indeed, Takagi (2002) and Takagi et al. (2005) revealed that the endolymph supersaturation state, which is directly involved in otolith calcification activity, is not affected by CO_2-related (bicarbonate and carbonate) ion concentrations in serum, but by the pH in the endolymph.

In conclusion, as summarized in Fig. 7, our study revealed that the otolith grows at a rate of ~25 nmol (2.5 µg) mg^{-1} otolith d^{-1}, but the growth rate is halved at night, suggesting that activity of carbonate transport in inner ear cells decreases. Carbon derived from mCO_2 was incorporated into otoliths as 28% of the carbonate during light, but decreased to 13 to 20% in the night. The remainder, about 75%, of otolith carbonate is derived from ambient DIC.

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