Genes Genet. Syst. (2008) **83**, p. 423–426

Shorter telomere length with age in the loggerhead turtle: a new hope for live sea turtle age estimation

Hideo Hatase[1]*, Ryusuke Sudo[1], Kunihiro K. Watanabe[1], Takashi Kasugai[2], Tomomi Saito[2], Hitoshi Okamoto[2], Itaru Uchida[2] and Katsumi Tsukamoto[1]

[1]*Ocean Research Institute, University of Tokyo, 1-15-1 Minamidai, Nakano, Tokyo 164-8639, Japan*
[2]*Port of Nagoya Public Aquarium, 1-3 Minato-machi, Minato-ku, Nagoya, Aichi 455-0033, Japan*

(Received 4 July 2008, accepted 6 October 2008)

We verified whether telomere length shortens with age in the loggerhead sea turtle (*Caretta caretta*) by measuring telomere lengths (relative telomere to single copy gene [T/S] ratios) in whole blood and epidermis from 20 captive individuals with a real-time PCR method. There was no significant correlation between age and relative T/S ratios in blood. Although the correlation between age and relative T/S ratios in epidermis was not significant, older turtles had smaller relative T/S ratios in epidermis. It was thus demonstrated that telomere length in epidermis could be a useful age estimator for sea turtles. Relative age information obtained with this simple, rapid, non-invasive technique may help to advance our understanding of the ecology of endangered sea turtles. This is the first publication on age-related changes in telomere length among chelonians.

Key words: age, *Caretta caretta*, real-time PCR, reptile, telomere

Age information is crucial to better understand the enigmatic ecology of long-lived sea turtles that exhibit long-distance migrations throughout their lives. Although skeletochronology, i.e. counting growth marks in bony sections, has traditionally been used to estimate sea turtle ages (Avens and Goshe, 2007), this method cannot be applied to endangered live sea turtles. A non-invasive alternative has long been desired.

It has been reported that telomere length shortens with age in several organisms such as birds and humans, and potentially can be used as an age estimator for these groups (Monaghan and Haussmann, 2006). Telomeres are oligonucleotide repeats (TTAGGG for vertebrates) found at the end of eukaryotic chromosomes (Monaghan and Haussmann, 2006). Telomere shortening occurs during mitosis (Monaghan and Haussmann, 2006). Thus, mitotically active tissues such as blood and epidermis are expected to show more of a clear-cut tendency in telomere loss within individuals than other mitotically inactive tissues such as muscle (Takubo et al., 2002), so their telomere lengths may be a better age estimator.

If this age-related decrease in telomere length also exists in sea turtles, telomere length would be a useful estimator for their ages. In the present study, we verified whether telomere length shortens with age in the loggerhead sea turtle (*Caretta caretta*) by measuring telomere lengths in blood and epidermis from captive individuals whose ages and histories are known. We also examined whether there is some correlation between telomere lengths in different tissues within individuals, as has been shown in humans (Nakamura et al., 2002; Takubo et al., 2002).

Blood and epidermis samples were collected from 20 captive loggerhead turtles at the Port of Nagoya Public Aquarium (PNPA), Nagoya, Aichi, Japan, in late November 2007. Out of the 20 turtles, 16 turtles had been hatched and raised at PNPA. Their ages were thus known: five age-0 turtles, three age-1, three age-2, three age-10, one age-11, and one age-12. Because the remaining four turtles had been captured in the wild and transferred to PNPA in 1991 and 1992 (two turtles in each year), their ages were unknown. The body sizes of these four turtles have changed little since then. Because loggerhead turtles grow little after reaching sexual maturity (Dodd, 1988), the four turtles were likely to be mature at capture. Loggerhead turtles reach sexual maturity in about 20 years (Dodd, 1988). Thus, we roughly esti-

Edited by Chung-I. Wu
* Corresponding author. E-mail: hatase@ori.u-tokyo.ac.jp

mated their ages to be 35 and 36 by adding 15 and 16 years (i.e. 2007 minus 1991 or 1992) to the maturity age of 20.

Sample sites for blood and epidermis on the body of each turtle were cleaned with 100% ethanol. Blood samples were collected from the dorsal cervical venous sinus with an 18- or 20-gauge needle and syringe (Terumo Corporation, Tokyo, Japan) as a part of regular checks on health and well-being of the turtles (Kakizoe et al., 2007), and stored in a TNES buffer (10 mM Tris-HCl, pH 7.5; 125 mM NaCl; 10 mM EDTA; 1% SDS) at room temperature. Epidermis samples were sliced from the dorsal cervix with a razor (Beauty-M, Kai Razor Co., Ltd, Gifu, Japan) and stored in 100% ethanol at room temperature.

Total DNA was extracted from the samples with a QuickGene Mini80 (Fujifilm Corporation, Tokyo, Japan). Telomere length was measured from total DNA using a real-time PCR method (Cawthon, 2002; Callicott and Womack, 2006). Because this PCR-based technique requires only a minute faction of tissues (Nakagawa et al., 2004), we thought this is more suitable for the study of endangered species than others such as hybridization-based techniques. A specially designed oligonucleotide primer set hybridizes to the TTAGGG and CCCTAA repeats and selectively amplifies telomeric DNA: longer telomeres lead to quantifiable acceleration of amplification. We used a primer set developed by Callicott and Womack (2006). The amplification of telomeric DNA was compared to the amplification of a single-copy gene DNA of the same sample, and it was expressed as a T/S ratio. We used the 18S ribosomal RNA (rRNA) gene as the single-copy gene with the following primer sequences that had originally been designed for humans (Takara Bio Inc., Shiga, Japan): 18S-F: 5'-ACTCAACACGGGAAACCTCA-3' and 18S-R: 5'-AACCAGACAAATCGCTCCAC-3'. Because the 18S rRNA gene is highly shared between humans and turtles (Mallatt and Winchell, 2007), the above primer sequences were identical between them. To serve as a reference for standard curve calculation, five DNA concentrations over a 625-fold range were generated by serial dilution (dilution factor: five) of an individual turtle DNA sample for both telomere and 18S rRNA portions. The T/S ratio for each sample was compared to that of a reference standard, and it was expressed as a relative T/S ratio. Real-time PCR was performed three times for each sample, and the mean relative T/S ratio was used in data analysis (Table 1). The average standard deviations for relative T/S ratios in whole blood and epidermis samples were 14.1 and 12.7%, respectively (Table 1).

The procedure was carried out on a 7300 real-time PCR system (Applied Biosystems, CA, USA). Reaction conditions for both telomere and 18S rRNA portions were set at 50°C for 2 min and 95°C for 10 min followed by 40 cycles of data collection at 95°C for 15 s and a 60°C anneal-extend step for 1 min, followed by a dissociation stage consisting of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. Each reaction for both telomere and 18S rRNA portions included 10 µl of Syber Green PCR Master Mix (Applied Biosystems, CA, USA), 100 nM of each of the forward and reverse primers, template DNA, and enough distilled H_2O to yield a 20-µl reaction.

Table 1. Means and standard deviations (SD) of three measurements of relative T/S ratios in whole blood and epidermis for 20 loggerhead turtles (*Caretta caretta*)

Age	Blood		Epidermis	
	Mean	SD	Mean	SD
0	1.103	0.157	1.254	0.270
0	1.706	0.117	1.546	0.175
0	1.805	0.200	1.633	0.120
0	2.268	0.038	0.988	0.045
0	3.384	0.173	2.011	0.175
1	2.201	0.102	0.716	0.217
1	2.244	0.099	1.072	0.055
1	2.425	0.111	0.844	0.055
2	1.000	0.085	1.000	0.119
2	1.186	0.032	0.851*	0.095*
2	2.531	0.188	1.087	0.103
10	1.456	0.491	0.692*	0.200*
10	1.639	0.141	1.283*	0.081*
10	2.089	0.112	0.348	0.072
11	3.014	0.195	1.191	0.192
12	3.193	0.100	0.970	0.141
35**	2.323	0.061	0.754	0.076
35**	2.727	0.216	1.159	0.177
36**	1.299	0.045	0.642	0.048
36**	2.239	0.158	1.125	0.128

* One outlier was omitted from calculation.
** The ages of four old turtles (35 and 36) are estimated values (see text).

There was no significant correlation between age and relative T/S ratios in whole blood samples for loggerhead turtles (Spearman's rank correlation coefficient [r_s] = 0.12, n = 20, p = 0.60; Fig. 1A). And although the correlation between age and relative T/S ratios in epidermis samples for loggerheads was not significant (r_s = –0.41, n = 20, p = 0.07; Fig. 1B), older turtles did have smaller relative T/S ratios in epidermis samples. In addition, there was no significant correlation between relative T/S ratios in whole blood and epidermis samples within individual turtles (r_s = 0.17, n = 20, p = 0.45; Fig. 1C).

This study demonstrated that telomere length in epidermis may shorten with age in the loggerhead turtle. Although telomere length loss with age has been shown in several other taxa such as birds and humans

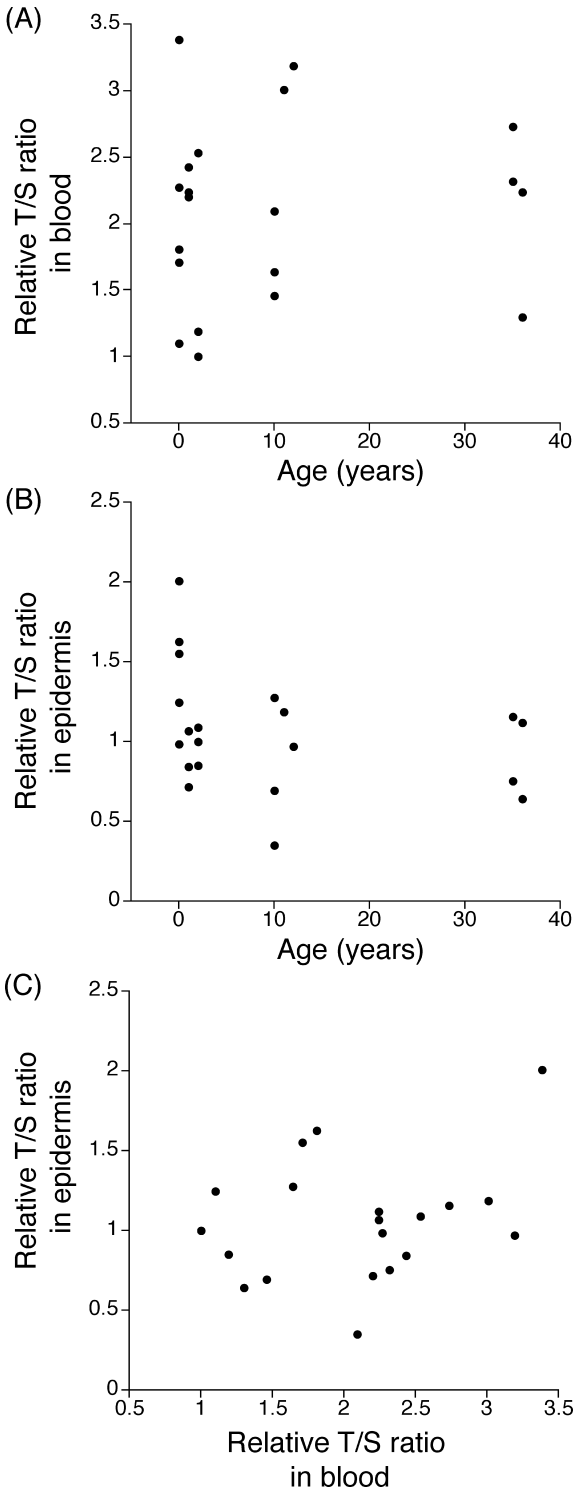

Fig. 1. Relationships between age and telomere length (relative telomere to single copy gene [T/S] ratio) in (A) whole blood and (B) epidermis for 20 loggerhead turtles (*Caretta caretta*). The ages of four old turtles (35 and 36) are estimated values (see text). (C) The relationship between telomere lengths (relative T/S ratios) in whole blood and epidermis within individual loggerhead turtles.

(Monaghan and Haussmann, 2006), this is the first publication that evaluated the trend in chelonians. In reptiles, age-related decrease in telomere length has been reported only in the American alligator (*Alligator mississippiensis*: Scott et al., 2006) and the garter snake (*Thamnophis elegans*: Bronikowski, 2008), but given the similarities in animal groups, we feel this method has potential in chelonians. For other taxa, telomere dynamics have generally not been investigated in epidermis samples, but in blood samples (Monaghan and Haussmann, 2006), with some exceptions in humans (e.g. Nakamura et al., 2002; Sugimoto et al., 2006). Significant correlations were found between telomere lengths in different tissues within individual humans (Nakamura et al., 2002; Takubo et al., 2002). The reason why we could not find significant correlations both between age and telomere length and between telomere lengths in different tissues within individuals may be due to a limited sample size (n = 20). Otherwise, the lack of significant correlations may be methodological, i.e., other telomere-measurement methods such as hybridization-based techniques might find strong patterns in chelonians like in other reptiles (Scott et al., 2006; Bronikowski, 2008; Haussmann and Mauck, 2008). In any case, telomere length in epidermis may be a useful estimator of not absolute but relative ages for sea turtles.

This simple, rapid, non-invasive technique has the potential to advance our understanding of the enigmatic ecology of sea turtles. For example, although size-related differences in the use of feeding habitats (neritic vs. oceanic) by adult female loggerhead turtles have been reported within several populations (Hatase et al., 2002, 2007; Hawkes et al., 2006), the mechanisms that produce and maintain this phenomenon are still unknown. Relative age information obtained with this method may become a key to help solve this phenomenon, which is closely related to facultative habitat shifts during ontogeny and alternative life histories in animals (Hatase et al., 2006; McClellan and Read, 2007).

We thank the staff (M. Ito, N. Toyamazaki, and M. Kobayashi) of PNPA for helping to collect samples. J. Aoyama taught us to use the real-time PCR system and M. J. Miller helped to improve the manuscript. This work was partly supported by a grant from the Research Fellowships of the Japan Society for the Promotion of Science (JSPS) for Young Scientists to H.H. The experiment was conducted in accordance with the Animal Care Authorization (No. P07-4) of the University of Tokyo.

REFERENCES

Avens, L., and Goshe, L. R. (2007) Comparative skeletochronological analysis of Kemp's ridley (*Lepidochelys kempii*) and loggerhead (*Caretta caretta*) humeri and scleral ossicles. Mar. Biol. **152**, 1309–1317.

Bronikowski, A. M. (2008) The evolution of aging phenotypes in snakes: a review and synthesis with new data. Age **30**,

169–176.
Callicott, R. J., and Womack, J. E. (2006) Real-time PCR assay for measurement of mouse telomeres. Comp. Med. **56**, 17–22.
Cawthon, R. M. (2002) Telomere measurement by quantitative PCR. Nucleic Acids Res. **30**, E47.
Dodd, C. K. Jr. (1988) Synopsis of the biological data on the loggerhead sea turtle *Caretta caretta* (Linnaeus 1758). US Fish Wildl. Serv. Biol. Rep. **88**, 1–110.
Hatase, H., Takai, N., Matsuzawa, Y., Sakamoto, W., Omuta, K., Goto, K., Arai, N., and Fujiwara, T. (2002) Size-related differences in feeding habitat use of adult female loggerhead turtles *Caretta caretta* around Japan determined by stable isotope analyses and satellite telemetry. Mar. Ecol. Prog. Ser. **233**, 273–281.
Hatase, H., Sato, K., Yamaguchi, M., Takahashi, K., and Tsukamoto, K. (2006) Individual variation in feeding habitat use by adult female green sea turtles (*Chelonia mydas*): are they obligately neritic herbivores? Oecologia **149**, 52–64.
Hatase, H., Omuta, K., and Tsukamoto, K. (2007) Bottom or midwater: alternative foraging behaviours in adult female loggerhead sea turtles. J. Zool. (Lond.). **273**, 46–55.
Haussmann, M. F., and Mauck, R. A. (2008) New strategies for telomere-based age estimation. Mol. Ecol. Resources **8**, 264–274.
Hawkes, L. A., Broderick, A. C., Coyne, M. S., Godfrey, M. H., Lopez-Jurado, L.-F., Lopez-Suarez, P., Merino, S. E., Varo-Cruz, N., and Godley, B. J. (2006) Phenotypically linked dichotomy in sea turtle foraging requires multiple conservation approaches. Curr. Biol. **16**, 990–995.
Kakizoe, Y., Sakaoka, K., Kakizoe, F., Yoshii, M., Nakamura, H., Kanou, Y., and Uchida, I. (2007) Successive changes of hematologic characteristics and plasma chemistry values of juvenile loggerhead turtles (*Caretta caretta*). J. Zoo Wildl. Med. **38**, 77–84.
Mallatt, J., and Winchell, C. J. (2007) Ribosomal RNA genes and deuterostome phylogeny revisited: More cyclostomes, elasmobranchs, reptiles, and a brittle star. Mol. Phylogenet. Evol. **43**, 1005–1022.
McClellan, C. M., and Read, A. J. (2007) Complexity and variation in loggerhead sea turtle life history. Biol. Lett. **3**, 592–594.
Monaghan, P., and Haussmann, M. F. (2006) Do telomere dynamics link lifestyle and lifespan? Trends Ecol. Evol. **21**, 47–53.
Nakagawa, S., Gemmell, N. J., and Burke, T. (2004) Measuring vertebrate telomeres: applications and limitations. Mol. Ecol. **13**, 2523–2533.
Nakamura, K., Izumiyama-Shimomura, N., Sawabe, M., Arai, T., Aoyagi, Y., Fujiwara, M., Tsuchiya, E., Kobayashi, Y., Kato, M., Oshimura, M., Sasajima, K., Nakachi, K., and Takubo, K. (2002) Comparative analysis of telomere lengths and erosion with age in human epidermis and lingual epithelium. J. Investigative Dermatol. **119**, 1014–1019.
Scott, N. M., Haussmann, M. F., Elsey, R. M., Trosclair, P. L. III, and Vleck, C. M. (2006) Telomere length shortens with body length in *Alligator mississippiensis*. Southeastern Naturalist **5**, 685–692.
Sugimoto, M., Yamashita, R., and Ueda, M. (2006) Telomere length of the skin in association with chronological aging and photoaging. J. Dermatol. Sci. **43**, 43–47.
Takubo, K., Izumiyama-Shimomura, N., Homma, N., Sawabe, M., Arai, T., Kato, M., Oshimura, M., and Nakamura, K. (2002) Telomere lengths are characteristic in each human individual. Exp. Gerontol. **37**, 523–531.