



# Comparison of green turtle *Chelonia mydas* sex ratios at two time-points over 20 years at a foraging ground in Yaeyama Islands, Ryukyu Archipelago, Japan

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**ABSTRACT:** Sex ratio is an important factor in population dynamics as it influences the production of offspring; understanding the sex ratio of a given population is vital for the conservation of endangered species. In sea turtles, the sex of hatchlings is temperature dependent, with warmer incubation temperatures producing more female hatchlings. The rise in temperatures due to global warming may skew the sex ratios towards females. We studied the sex ratio of immature green turtles at a foraging ground in the Yaeyama Islands from 1997–1999 and from 2016–2017, using laparoscopy. The overall proportion of females was 68.2% (N = 314), and proportions were not significantly different between 1997–1999 (69.9%; N = 183) and 2016–2017 (65.6%; N = 131). Thus, sex ratios have not changed at this site over the last 20 years, suggesting minor or no effect of global warming/environmental influences on this population. The small size class (<55 cm straight carapace length [SCL]) was more female biased than the large size class (≥55 cm SCL) during both periods, which suggests the possibility of movement into or out of the foraging aggregation. Therefore, future research must clarify how the initial recruitment into the foraging aggregations, and the subsequent migrations affect sex ratios.

**KEY WORDS:** Green turtle · Sex ratio · Migration · Recruitment · Global warming

## 1. INTRODUCTION

The sex determination of sea turtles is temperature dependent. Previous studies have proposed 29–30°C as the pivotal temperatures for sex determination in sea turtles, within a general range of 27 to 31°C (review by Wibbels 2003). Exposure of embryos to higher temperatures within this range results in a higher ratio of female offspring, while exposure to lower temperatures within the range results in a higher ratio of males. An extreme bias towards one sex, e.g. complete feminization, could be a major

threat to the survival of species with temperature-dependent sex determination (Jensen et al. 2018). For example, one reason for the decline in the leatherback turtle population in Teregganu, Malaysia, was considered to be hatchery practices which led to high female hatchling production and reduced hatch success rates (Chan & Liew 1996). Furthermore, population structure, mortality rate, and sex ratio are fundamental demographic parameters. Sex ratios provide information about the reproductive potential of a population (Tarsi & Tuff 2012) and thus represent an important basis for estimating the size and pro-

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ductivity of populations of endangered species, including sea turtles (Casale et al. 2006, National Research Council 2010).

Green turtles *Chelonia mydas* have a circum-global distribution, but mainly occur in tropical and subtropical waters. They are listed as Endangered on the International Union for Conservation of Nature Red List (Seminoff 2004). Green turtle hatchlings inhabit pelagic waters for approximately 4 to 10 yr until they shift to occupy habitats in the neritic (shallow) zone. It has been estimated that green turtles require approximately 30 yr to reach maturity, with immature individuals spending about 20 yr in shallow foraging grounds (Zug et al. 2002, Balazs & Chaloupka 2004). Many previous studies have focused on the size distribution and survival rate of sea turtles inhabiting the neritic zone (Bjorn-dal et al. 2000, 2003, Koch et al. 2007, Colman et al. 2015, Kameda et al. 2017). However, those studies did not investigate the sex ratios of different size classes because the sex of immature sea turtles cannot be determined by their external morphology (Wibbels 1999). Consequently, our understanding of the sex ratio of immature sea turtles remains limited (Rees et al. 2016). The most recent review of meteorological data (IPCC 2014) has confirmed that there is a trend toward global warming, and global warming is expected to cause sex-ratio shifts in species with temperature-dependent sex determination (Hammam et al. 2007). Monitoring the sex ratio of immature green sea turtles at foraging grounds is important since it could be utilized to predict a population's future reproductive potential.

Previous genetic studies revealed that green turtles at certain foraging grounds are composed of aggregations of many individuals originating from different rookeries (Bass et al. 2006, Nishizawa et al. 2013). Therefore, the sex ratio of green turtles at foraging grounds represents a mixture of hatchling sex ratios produced over many years from the source rookeries. Previous studies of foraging grounds across the globe generally demonstrate female-biased sex ratios (e.g. Wibbels 2003, Allen et al. 2015, Braun McNeill et al. 2016). Limpus et al. (2009) reported that the sex ratio of small immature turtles (<65 cm curved carapace length, CCL) in Clack Reef, Australia, was more female biased than that of large immature turtles (>65 cm CCL) at the same site. Female bias in small-sized green turtles is considered an early sign of global warming (Limpus et al. 2009, Jensen et al. 2018) and the simultaneous warming of nesting beaches. Higher female sex ratios might also be due to warmer sand temperatures in unshaded areas of

beaches, resulting in part from a poorly managed relocation of eggs to open beach locations (e.g. Limpus 1993, Van de Merwe et al. 2006, Jensen et al. 2016).

The growth, or maturation, of sea turtles is a slow process. Long-term data is required to assess changes in sex ratio at given sites over time, due to recruitment, immigration, and emigration. However, the research periods in previous studies on sex ratios in immature sea turtles generally span less than 10 yr (Limpus et al. 2009, Delgado et al. 2010, Braun McNeill et al. 2016).

Our research group has been monitoring the coastal foraging areas of green turtles in the Yaeyama Islands of Ryukyu Archipelago, Japan, since 1995. A female-biased sex ratio of green turtle hatchlings has been reported in Taiwan, which is located near the Yaeyama Islands (King et al. 2013). However, there are no previous records of studies conducted on the juvenile green turtle population inhabiting this foraging ground. We recorded the sex ratios of turtles using laparoscopy and histological methods in 1997–1999 and 2016–2017. This study presents the first report to assess the sex ratios of immature green turtles inhabiting the marine area of Ryukyu Archipelago, in which the sex ratios of immature green turtles are compared at 2 time-points covering a long (20 yr) period.

## 2. MATERIALS AND METHODS

### 2.1. Study area

The Yaeyama Islands are located at the southern end of the Ryukyu Archipelago, northwestern Pacific (24° 00'–24° 40' N, 122° 45'–124° 30' E; Fig. 1), and have one of the largest coral reefs in Japan. Sea grass and sea algae flourish in the inner reefs, and immature green turtles inhabit these coastal areas for feeding (Kameda et al. 2017). Annual average air temperature is 24.9°C, ranging from 20.5 to 29.7°C across seasons in 2017 (Ishigakijima Local Meteorological Observatory; [www.jma-net.go.jp/ishigaki/menu/menuReport.html](http://www.jma-net.go.jp/ishigaki/menu/menuReport.html)). Annual water temperature is 26.1°C, ranging from 22.2°C to 29.7°C across seasons in 2016 (Ministry of Environment; [www.e-monitoring.jp/spot.html](http://www.e-monitoring.jp/spot.html)). The Yaeyama Islands consist of 8 islands. Many beaches on these islands are used as green turtle nesting sites, with Iriomote Island being one of the largest nesting sites in Japan (Kamezaki 1989).

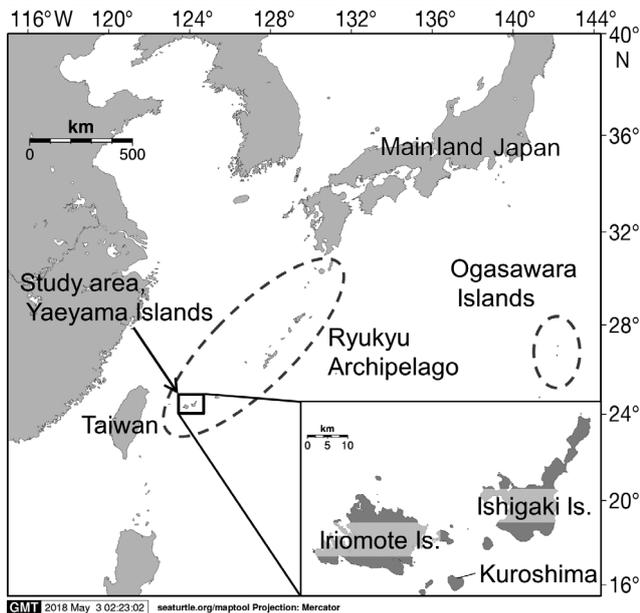


Fig. 1. Green sea turtle foraging grounds in Kuroshima, Yaeyama Islands. Dark grey dashed lines show the nesting sites of green turtles around the Yaeyama Islands and the Ogasawara Islands, Japan (Kamezaki 1989)

## 2.2. Sea turtle capture and measurement

Licensed sea turtle fishermen are allowed to legally catch green sea turtles, with a limit of 200 individuals per year, for local consumption in Yaeyama Islands. Throughout our study periods, from June 1997 to May 1999 and February 2016 to August 2017, licensed local fishermen captured turtles for our survey at intervals of 1 to 2 months. The local fishermen conducted snorkeling and dive surveys to search for and capture sea turtles, while the research team used entanglement nets (200 m long, 2 m high, 40 cm mesh size) for their capture surveys. Captured turtles were transported within 3 h to an outdoor flow-through saltwater tank at the Kuroshima Research Station, which is located on Kuroshima, south Yaeyama Islands. Straight carapace length (SCL) was measured to the nearest 0.1 cm using metal calipers (Kamezaki & Suganuma 1991). Turtles were tagged with an Inconel tag (Style 681; National Band and Tag Company) or a plastic tag (Jumbo tag; Dalton Tags) that was placed proximal to the first

large scale on both rear flippers for a capture-mark-recapture study. All turtles were released within 2 wk after the capture. All sampling procedures were permitted by the Marine Fisheries Coordinating Committee of Okinawa Prefecture, Japan (Approval #K28-4, K29-4).

## 2.3. Laparoscopy and sex determination

Sex determination by laparoscopy was performed by trained veterinarians and scientists as previously described by Owens (1999). The mean time between arrival at Kuroshima Research Station and laparoscopy was 7 d (range: 2–21 d). Specifically, 1 ml of anesthetic (Midazolam solution; Sandoz) per 5 kg body weight was injected into the pectoralis major muscle from the base of the front flipper. The sea turtle was restrained in an inverted position and a 1–2 cm incision was made on the peritoneal wall of the inguinal area. A laparoscope was inserted into the incision and the gonads were observed (Fig. 2). Sex was verified visually by the presence or absence of follicles, overall gonad coloration, and shape (Miller & Limpus 2003). The incision was sealed with 2 or 3 stitches of self-dissolving catgut. The turtles were kept in a dry room in the Kuroshima Research Station for 6 h and then returned to the water tank. Sea turtles were monitored for abnormal

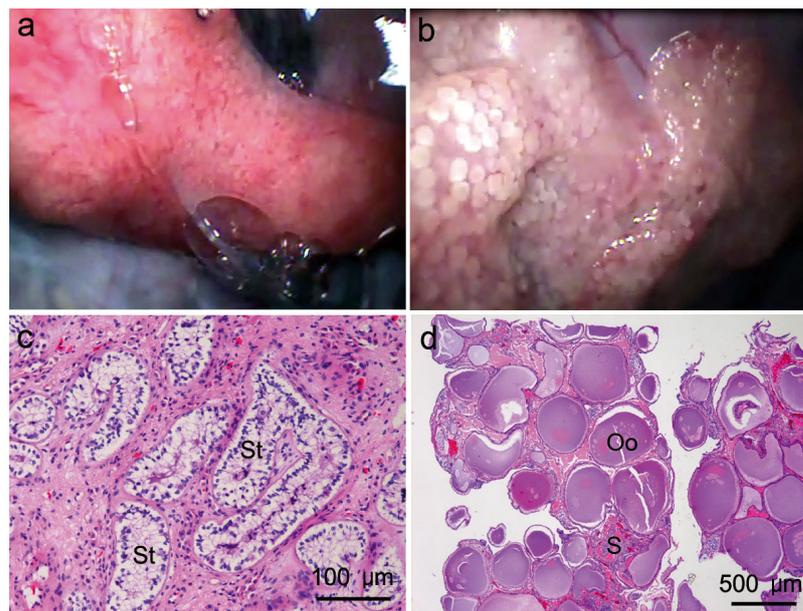


Fig. 2. (a,b) Photographs and (c,d) histology of (a,c) testis and (b,d) ovaries, obtained during the present study in 2016–2017. St: seminiferous tubules; Oo: oocytes; S: stroma. Staining in (c,d): hematoxylin and eosin

swimming behavior and were returned to the sea after recovery, which was within 14 d. The laparoscope used in 1997 to 1999 had a diameter of 3.5 mm and a length of 280 mm (NCS-3427D, Olympus). A different laparoscope with a diameter of 10 mm and length of 270 mm was used in 2016 to 2017 (A5252A, Olympus). Model A5252A allowed biopsies to be conducted, with 1 to 3 mm of gonads being collected from sea turtles to obtain histological evidence (validation) of sex. Biopsy samples were fixed in 10% formalin, and paraffin-embedded tissue sections were prepared routinely. Each section was stained with hematoxylin and eosin. Tissue slides were observed under a microscope (Nikon ECLIPSE 80i) to check for the presence of oocytes or seminiferous tubules (Fig. 2). Laparoscopy in 2016–2017 was based on guidelines for animal experiments from the School of Veterinary Medicine, Rakuno Gakuen University, Japan (Approval #VH16D2).

#### 2.4. Statistical analysis

A study from Ogasawara Islands reported the mean size of mature green turtles as 91 cm for males and 95 cm SCL for females (Tachikawa 1991). We based our analysis on this finding, and sea turtles with SCL >90 cm were thus considered to be mature and were removed from the analysis. The sex ratios in 1997–1999 and 2016–2017 and in each size class were compared using a chi-squared test. Confidence intervals (CI) of sex ratios were calculated using binomial distribution (Zar 1999).  $\chi^2$  was calculated using Excel (Microsoft).

### 3. RESULTS

A total of 314 immature turtles were captured from the foraging grounds in the Yaeyama Islands in 1997–1999 and 2016–2017 (Table 1). The overall proportion of females was 68.2% (95% CI: 62.7–73.3%,  $N = 314$ ), with a significant female bias ( $\chi^2 = 41.4$ ,  $df = 1$ ,  $p < 0.01$ ,  $N = 314$ ). Sex ratios in 1997–1999 were more female biased than in 2016–2017, but no statistical difference was observed ( $\chi^2 = 0.64$ ,  $df = 1$ ,  $p = 0.42$ ,  $N = 314$ ).

In 1997–1999, 56 males and 130 females were captured. The annual sex ratios in this period were not significantly different from each other ( $\chi^2 = 2.26$ ,  $df = 2$ ,  $p = 0.32$ ,  $N = 183$ ). In 2016–2017, 45 males and 86 females were captured and again there were no significant differences among years in the annual sex ratios ( $\chi^2 = 0.006$ ,  $df = 1$ ,  $p = 0.94$ ,  $N = 131$ ).

Overall, 56 gonad biopsy samples (17.6% of all turtles) were collected for histological validation in 2016–2017. Samples were distinguished as being 19 males and 37 females, all of which matched the laparoscopy sex determinations (i.e. validated our observations).

The mean SCL was 53.3 cm (SD = 8.7, range = 36.2–89.3,  $N = 183$ ) in 1997–1999 and 55.5 cm (SD = 8.9, range = 37.5–87.7,  $N = 131$ ) in 2016–2017. The size distribution of turtles was skewed toward smaller turtles (Fig. 3). Fig. 3 shows that sex ratios were female biased at <55.0 cm SCL (small size class), but were close to equal at  $\geq 55.0$  cm SCL (large size class) in both study periods (Fig. 3, Table 1b). Overall, across the study period, there were 48 males and 150 females in the small size class (75.8% female), and 52 males and 64 females (55.2% female) in the large size class (Table 1), with the sex ratio of smaller turtles more skewed toward females than that of the larger turtles ( $\chi^2 = 14.2$ ,  $df = 1$ ,  $p < 0.01$ ,  $N = 314$ ).

Table 1. Sex ratio (male:female) composition of foraging green turtles in 1997–1999 and 2016–2017 by year and size class (straight carapace length, SCL, in cm). 95% confidence limits (CI) for the proportion of females to males are also shown. Overall refers to the values for both study periods combined

	N	Male	Female	Sex ratio	Proportion of females (%)	95% CI
<b>Year</b>						
1997	118	31	87	1.0:2.8	73.7	64.8–81.4
1998	43	16	27	1.0:1.7	62.8	46.7–77.0
1999	22	8	14	1.0:1.8	63.6	40.7–82.8
Total 1997–1999	183	55	128	1.0:2.3	69.9	62.7–76.5
2016	46	16	30	1.0:1.9	65.2	49.8–78.6
2017	85	29	56	1.0:1.9	65.9	54.8–75.8
Total 2016–2017	131	45	86	1.0:1.9	65.6	56.9–73.7
Overall	314	100	214	1.0:2.1	68.2	62.7–73.3
<b>Size class (SCL, cm)</b>						
1997–1999						
<55	127	30	97	1.0:3.1	76.4	68.0–83.5
$\geq 55$	56	25	31	1.0:1.2	55.4	41.5–68.7
2016–2017						
<55	71	18	53	1.0:2.9	74.6	62.9–84.2
$\geq 55$	60	27	33	1.0:1.2	55.0	41.6–67.9
Overall						
<55	198	48	150	1.0:3.1	75.8	69.2–81.6
$\geq 55$	116	52	64	1.0:1.2	55.2	45.7–64.4

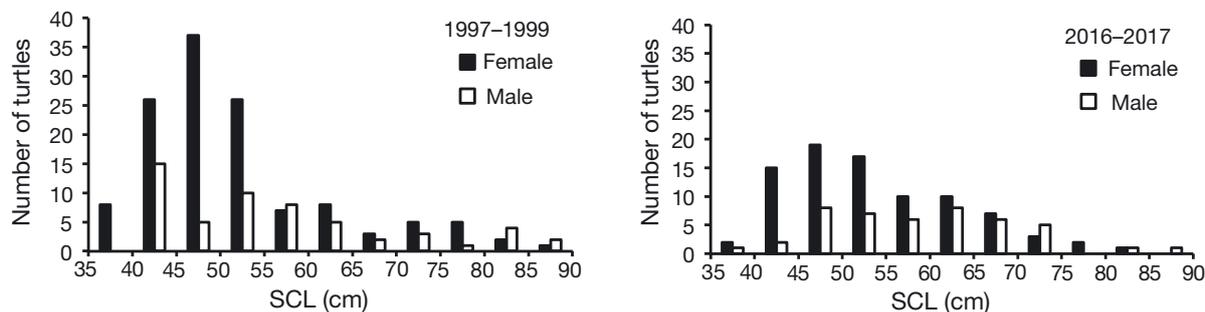


Fig. 3. Size distribution and sex of green turtles at foraging grounds in Yaeyama Islands, Japan (N = 183 in 1997–1999; N = 131 in 2016–2017)

#### 4. DISCUSSION

The overall proportion of females was 69.9% in 1997–1999 and 65.9% in 2016–2017; thus, the proportion of females did not increase. Our results show that the small size class had a greater female bias than the large size class in both 1997–1999 and 2016–2017. Annual growth rates of immature green turtles are 2.7 cm on Yaeyama Island (Kameda et al. 2017), so green turtles measuring 40 cm SCL in 1997–1999 are likely to measure about 90 cm SCL after 20 yr. If this difference in sex ratio between the size classes in 1997–1999 is the result of a greater number of smaller-sized females, then the sex ratio for the large size class should be more female biased in 2016–2017 than in 1997–1999, provided the smaller-sized turtles from 1997–1999 remained in this foraging ground. However, the sex ratios of the large size class in both periods were similar. Therefore, our results indicate that sex ratios at this foraging ground have remained unchanged over the past 20 yr.

Limpus et al. (2009) reported that the sex ratio of the small size class of immature green turtles was 1 male (M):4.2 females (F), while that of the large size class was 1M:2.2F at Clack reef, Australia. This difference in sex ratio might be an early sign of the effects of global warming, with higher temperatures in the nest skewing the bias of embryos and hatchlings towards females (Limpus et al. 2009). In addition, artificial incubation without shading results in greater female bias than some natural nesting beaches where cover from plant vegetation results in lower incubation temperatures (Limpus 1993, Van de Merwe et al. 2005). Thus, an increase in beach temperatures due to global warming might result in a greater female bias in small size classes of immature turtles in foraging grounds. Previous studies have proposed this possible effect of global warming. For example, Jensen et al. (2018) sug-

gested that the high female sex ratio (99% female) of juvenile green turtles in the rookeries of northern Australia is due to global warming. Additionally, predictions using air temperature and sand temperature indicate that the sex ratio of future hatchlings will be increasingly female biased for the same reason (Santidrián Tomillo et al. 2015, Laloë et al. 2016). However, our results demonstrated no change in sex ratio over different size classes and time. Therefore, it is unlikely that the different sex ratios in the immature size classes in this study were a result of increased temperatures due to global warming or artificial incubation.

A similar difference in the sex ratios of the immature size classes was also reported in the Bahamas (Bolten et al. 1992) and Shoalwater Bay, Australia (Limpus et al. 2005). Differences in sex-specific mortality and growth rate may explain the different sex ratios in immature size classes if mortality rates are higher in females than males or if females grow more slowly than males. However, differences in sex-specific growth rates and mortality rates were not observed among immature green turtles on foraging grounds in The Bahamas (Bolten et al. 1992) and the Great Barrier Reef (Limpus & Chaloupka 1997, Chaloupka & Limpus 2005). Like the Great Barrier Reef, the Yaeyama Islands are in the western Pacific, so it is possible that sex-specific mortality and growth rates of immature green turtles are similar in these 2 areas.

We propose 2 hypotheses to explain the different sex ratios of different class size demonstrated in this study. First, the migration of juvenile green turtles may differ with respect to sex. For instance, during the pelagic stage, Delgado et al. (2010) postulated that female loggerhead sea turtles migrate to neritic foraging grounds earlier than males in Madeira. In our study area, female green turtles of over 55 cm SCL might migrate to alternative foraging grounds, whereas males might remain in the Yaeyama Islands.

Size and sex-specific capture-mark-recapture data, satellite telemetry, and isotope analysis are required to identify these possible differing trends. Second, the migration of immature green turtles might differ with respect to size class. Green turtles in Japan nest in the Ryukyu Archipelago, which includes the Yaeyama Islands, and on the Ogasawara Islands (Kamezaki 1989). A previous study based on genetic analysis showed that green turtles in the Yaeyama Islands in the 40–50 cm SCL size class mainly originated from the Yaeyama Islands, whereas those in the 50–70 cm SCL size class mainly originated from the Ogasawara Islands (Hamabata 2014). Green turtles in the 40 cm SCL size class recruit from pelagic waters to neritic habitat in the Yaeyama region (Kameda et al. 2017). Isotope analysis has shown that many immature green turtles in the Yaeyama Islands are migrants from mainland Japan (Shimada et al. 2014). Additionally, the northern foraging aggregations of green turtles from mainland Japan mainly originated from the Ogasawara Islands (Nishizawa et al. 2013, 2014). Thus, the sex ratios of individuals in the 40–50 cm SCL class might be a result of recruitment from the Yaeyama Islands, while the sex ratios in the 50–70 cm SCL class might be due to the immigration of individuals from the Ogasawara Islands. Differences in the sex ratios for the immature size classes might be driven by the different sex ratios of hatchlings produced at each rookery.

The annual sex ratios of sea turtle hatchlings are not stable, due to sand temperature change caused by air temperature and other climate factors (Wibbels 2003). Therefore, long-term research is needed to analyze the effect of global warming on sex ratios (Fuller et al. 2013). The sex ratio of turtles at foraging sites is determined by sea turtles that hatched at various rookeries and migrated to the region over years to decades (Wibbels 2003). Differences in the sex ratios of immature size classes might thus be driven by the different sex ratios of hatchlings produced at the various source rookeries. An understanding of the sex ratio at the foraging grounds may enable an investigation of the effects of global warming on sea turtle sex ratios in the past few decades (e.g. Jensen et al. 2018). Our results propose that sex ratios at foraging grounds reflect not only primary recruitment but also immigration and emigration at later growth stages. Hatchling feminization may occur on a particular nesting beach, but this phenomenon will be neutralized at foraging sites if other nesting beaches produce more male hatchlings. The detection of change in sex ratio due to global warming may require the use

of multiple research techniques (e.g. genetics and endocrinology, Jensen et al. 2018). Future research should consist of long-term studies that focus on revealing the sex ratios of hatchlings produced from the Yaeyama Islands and Ogasawara Islands to understand how the sex ratio changes as sea turtles mature in the foraging grounds of the Yaeyama Islands. In addition, assessment of immature green turtle sex ratios at foraging grounds around the Yaeyama Islands, i.e. Taiwan and the Japanese mainland, should be incorporated to clarify sex-specific migrations from those regions.

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