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Experiments with various Incubation Factors for Eggs of *Chelonia mydas* in China

Introduction

The Green Sea Turtle (*Chelonia mydas* Linnaeus, 1758) is an endangered species listed in Appendix I of CITES, the species has been afforded legal protection in most countries of the world. Many studies have been published on the incubation of *Chelonia mydas* eggs. However, most of them focused on the effects of nest depth (Chen 1993, Diong *et al.* 1999, Karavas *et al.* 2005) or temperature (Chen 1998, Booth *et al.* 2004) on hatching rates. Peters *et al.* (1994) suggested that decaying eggs were the result of microbial invasion after the embryo had perished, and Phillott *et al.* (2001) thought that the presence of fungi on sea turtle eggs might impede gas exchange. In mainland China, *China Sea Turtle Bay* (CSTB) in Huidong County, Guangdong Province, has been the only institution to carry out several studies on the relocation of clutches (Liang *et al.* 1990, Chen *et al.* 2007). As far as factors with an influence on embryonic development are concerned, few reports the world over have focused on the effects of egg surface cleanliness on hatching rates (e.g., Phillott *et al.* 2001).

At the CSTB (www.seaturtle.cn), vegetation on the sandy beach is largely limited to the seashore vine morning glory (*Ipomoea pes-caprae*) and the simpleleaf shrub chastetree fruit (*Vitex trifolia* L. var. *simplicifolia* Cham) that form a belt of 30-70 m

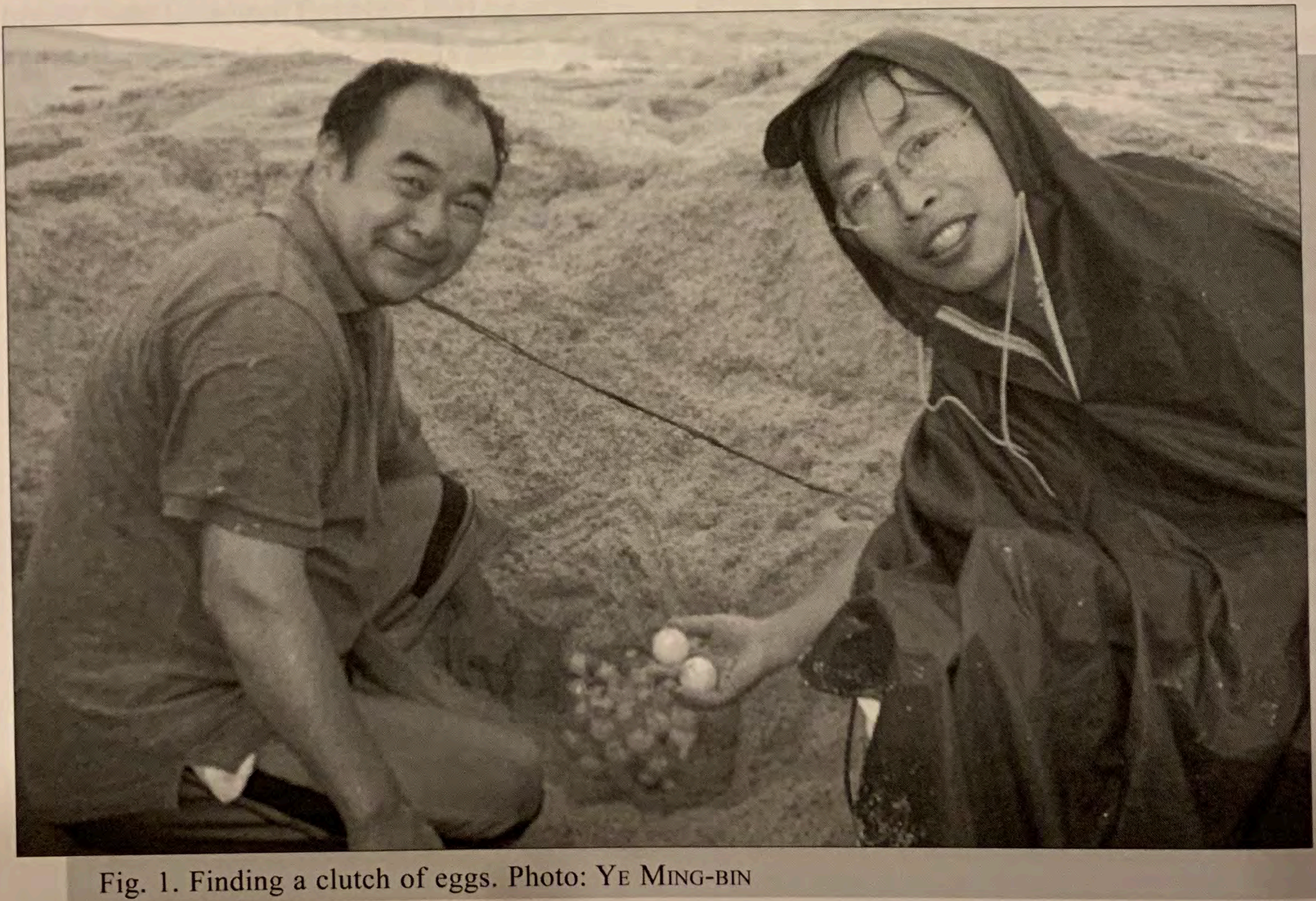


Fig. 1. Finding a clutch of eggs. Photo: Ye Ming-bin

Fig. 2.
The author (Xia Zhong-rong) relocating nests. Photo: Chen Hua-lin

in width. It was observed that, after having been relocated to a vegetated zone, hatching rates of eggs decreased (Chen 1998, Karavas *et al.* 2005). Under natural conditions, low hatching rates and low nesting success are believed to be associated with the vegetation zone in which sea turtles choose to nest (Karavas *et al.* 2005).

In recent years, we have investigated several factors that might be relevant to sea turtle hatching rates: nest depth, vegetation, egg surface cleanliness (mucus or albumen deposits), predators and pathogens. Generally, people tend to believe that oviductal mucus coating the egg surface has a positive effect on embryogenesis. However, hatching rates increased significantly when we removed coatings such as mucus, yolk and albumen from crushed eggs from the egg surface. We therefore hypothesized that mucus might not have any useful function other than to assist the laying process, and that contaminants on the egg surface will greatly reduce the hatching rate.

Our objective was to qualify the optimal combination of ecological factors on hatching rates in Green Sea Turtle eggs after relocation and compile practical guidelines for aiding protection efforts for this internationally endangered species.

Materials and methods

All of the 324 eggs studied in 2006 were laid by a three-footed turtle missing the left rear flipper (PIT: 4625523668, Inconel tag: A-0109) in the *National Gangkou Sea Turtle Reserve* (N 22°33', E 114°54'). A-0109 is approximately 80 years old and weighs about 150 kg. Her carapace curved length (CCL) was 104 cm and curved width (CCW) 93 cm. Because of her poor physical balance due to missing the left hind flipper, A-0109 often crushed some eggs while burying them, resulting in "surface contaminants" (yolk and albumen spilling from the crushed eggs) on some eggs. A total of 551 eggs from three sea turtles nesting at the CSTB were also collected for experiments in 2007 and 2008. The eggs were relocated to the experimental sites in the CSTB immediately after oviposition.

Experiment 1: The purpose of this experiment was to determine the possible influence of surface contamination on hatching rates. Twenty-one freshly laid eggs were randomly grouped in 7 eggs/nest in 2006. Surface contaminants (yolk and albumen from crushed eggs) were cleaned off with a 1‰ iodine solution using a brush. The control group consisted of 37 uncleaned eggs. In 2007, we cleaned 60 eggs of normal shape and colour in the same manner, with a control group of 60 eggs that

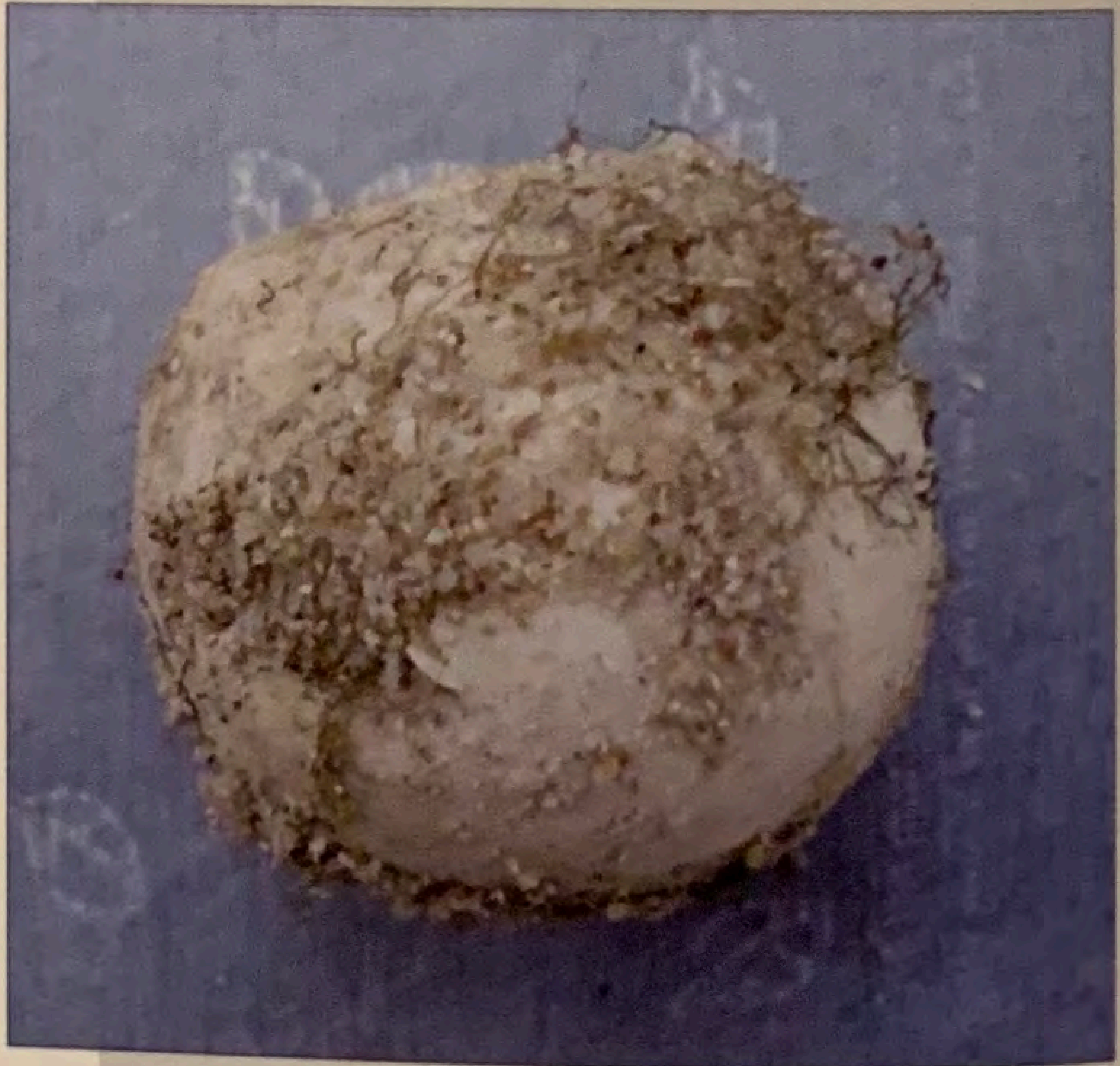
Fig. 3. Hatchlings for research. Photo: Xia Zhong-rong

were left stained with yolk and albumen. All the replacement nests in both 2006 and 2007 were 60 cm deep.

Experiment 2: The purpose of this experiment was to determine the possible influence of surface mucus on the hatching rate. Before A-0109 started closing her nest pit, her eggs were removed to prevent them from being crushed. Thirty eggs that were to form the manipulated group were soaked in 1‰ iodine solution for 90 seconds, after which the solution and surface mucus were wiped off with tissue paper. The control group (49 eggs) remained as it was. In 2007, the experiment was repeated three times with 120 eggs of normal shape and colour of which 20 each were treated thus. The depth of the replacement nests was set at 70 cm in both years.

Experiment 3: The purpose of this experiment was to determine the possible influence of nest depth on hatching rates. Five nest depths were studied: 40, 50, 60, 70 and 80 cm. The individual experimental nests were separated by about 30 cm from each other. A total of 198 eggs from two clutches were used (10-19 eggs in each nest; Tab. 3).

Experiment 4: The purpose of this experiment was to determine the possible influence of vegetation cover on the hatching rate. Eggs from one clutch were randomly split up into



Figs. 4 & 5. Eggs laid in the vegetation zone. Photos: Xia Zhong-rong two groups (30 eggs/group). Group 1 was buried in beach sand without vegetation cover; group 2 was buried in sand that was covered with beach vegetation. The nest depth for both groups was identical (50 cm), and the artificial nests were 30 cm apart. A total of 6 nests containing 10 eggs each were used in this experiment.

Experiment 5: The purpose of this experiment was to determine the possible influence of clutch size on the hatching rate. Eggs of normal shape and colour from two nests were selected at random in 2007. The eggs from each nest were split up into groups of 20, 40 and residual eggs, and separately reburied at a depth of 70 cm. The eggs were relocated to the experimental sites in the CSTB immediately after oviposition and buried in beach sand without vegetation cover.

Statistical analyses: Data from the samples were subjected to analyses of variance (f-tests, The Post Hoc Tests Multiple Comparisons) or were tested with t-tests using SPSS 11.0 Production Facility. For all analyses of variance, averages were considered significantly different at a p value of less than 0.05.

Results Influence of egg surface contaminants on the hatching rate (Experiment 1)

Table 1 shows that after brushing contaminants such as yolk and albumen of broken eggs off the eggs' surface, the average hatching rate was 86%, which is significantly higher than the 14% in the control group (t-test, $p < 0.01$). A similar result was found when the eggs were cleaned with a 1‰ iodine solution: the hatching rate increased to 88% as compared to the 17% of eggs left stained with yolk and albumen ($p < 0.01$). In spite of finding significant differences between manipulated and virgin groups, an equal extent of variance was noted within the two groups ($p > 0.05$) that suggests there was no difference in the egg characteristics in terms of maternal investment between A-0109 and the other female turtles.

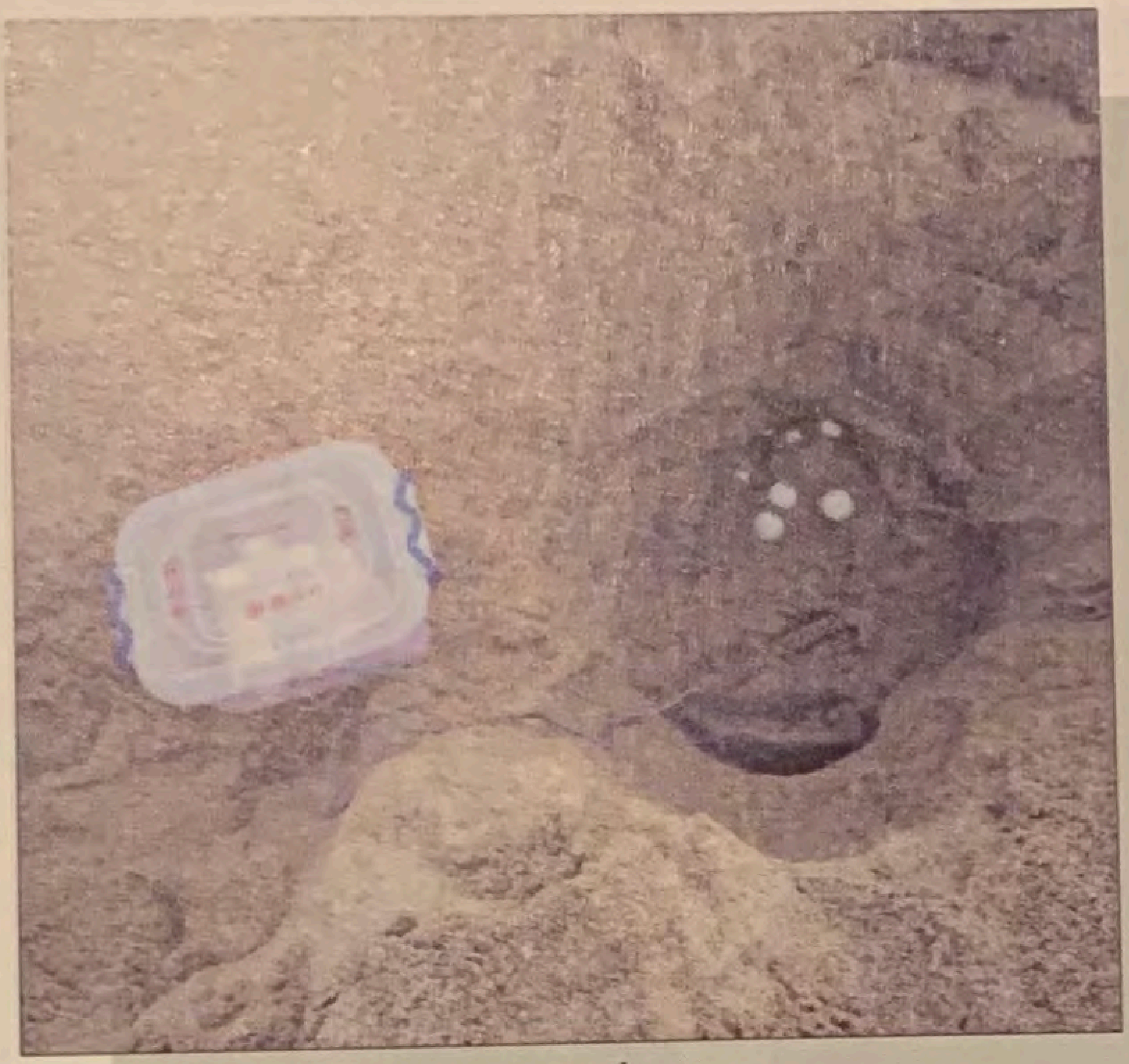


Fig. 6. Temperature logger.
Photo: Xia Zhong-rong

Fig. 7. Relocated nests in 2006.
Photo: Xia Zhong-rong

Fig. 8. Egg laying.
Photo: Xia Zhong-rong

	Manipulation	Nest	1	2	3	
Manipulated group	Experiment 1: Decontamination of egg surface with 1‰ Iodine solution	Number of eggs	7	7	7	Total: 21
		Hatching rate (%)	86	72	100	Average: 86
	Eggs cleaned with 1‰ iodine solution	Number of eggs	20	20	20	Total: 60
		Hatching rate (%)	95	90	80	Average: 88
Control group	Comparison	Nest	1	2	3	
	Eggs without treatments	Number of eggs	13	13	11	Total: 37
		Hatching rate (%)	15	0	27	Average: 14
	20 eggs from four-footed turtles stained with yolk and albumen	Number of eggs	20	20	20	Total: 60
		Hatching rate (%)	20	25	5	Average: 17

Tab. 1. Influence of egg surface contamination on hatching rates.

Influence of surface mucus on newly-laid eggs on the hatching rate (Experiment 2)

Results from both 2006 and 2007 are shown in Table 2. Levene's Test for Equality of Variances was F2006c = 0.389, P2006c = 0.566 (>0.05), F2006T = 0.235, and P2006T = 0.653 (>0.05); two samples of the control group and the manipulated group were assumed as equal variances. Due to the insignificant differences between the eggs of A-0109 and other turtles, the data from these two years were combined. The average hatching rate of the virgin control group was 75% as compared to the 93% in the manipulated group of eggs (1‰ iodine solution, soaking for 90 seconds). The t-test showed that soaking and removing surface mucus significantly increased the hatching rate (t = 8.034, p <0.001).

Group	Year	2006			2007			
Control group	Nest	1	2	3	4	5	6	
	Number of eggs	17	13	19	20	20	20	Total: 109
	Hatching rate (%)	88	69	74	80	75	65	Average: 75
Manipulated group	Nest	1	2	3	4	5	6	
	Number of eggs	10	10	10	20	20	20	Total: 90
	Hatching rate (%)	100	90	100	95	90	80	Average: 93

Tab. 2. Influence of mucus deposits on the hatching rate.

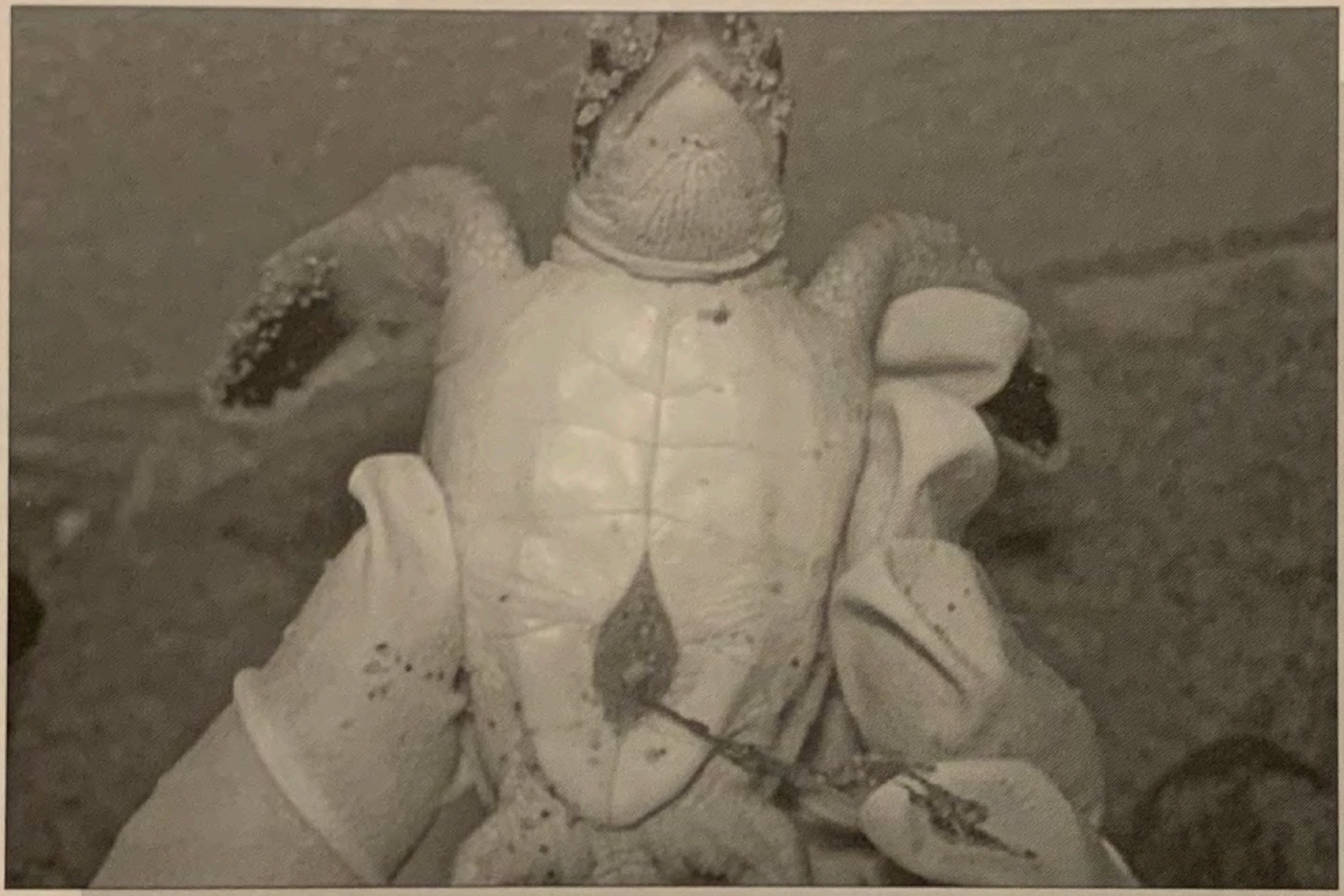


Fig. 9.
Navel scab.
Photo: XIA ZHONG-RONG

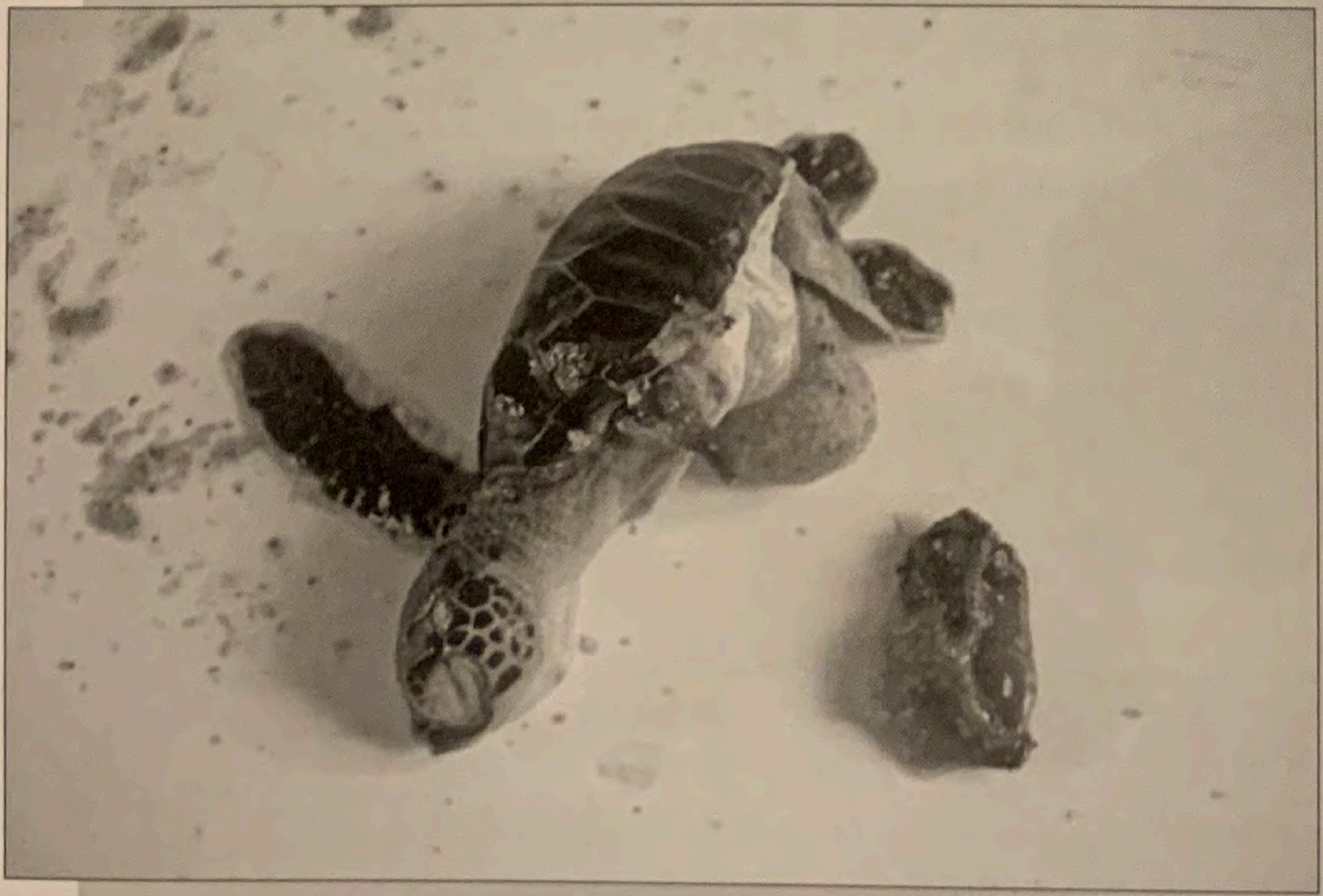


Fig. 10.
Turtle missing the left forelimb.
Photo: XIA ZHONG-RONG

Fig. 11. Egg and yellow mealworm (*Tenebrio molitor*). Photo: XIA ZHONG-RONG

Fig. 12. Insect larvae cause harm. Photo: XIA ZHONG-RONG

Fig. 13. Fungal growth and bacterial pathogens can cause egg failure. Photo: XIA ZHONG-RONG

Influence of nest depth on the hatching rate (Experiment 3)

Table 3 shows that a nest depth of 80 cm produced the highest hatching rate (average 90%), whereas 40 cm produced the lowest (average 20%). Analysis of variance indicated a significant difference among groups (f = 9.60, p = 0.001). Multiple comparisons using LSD likewise showed a significantly reduced hatching rate in the 40 cm group than in the other four groups (p <0.01).

Nest depth (cm)	Number of eggs	Hatching rate (%)	Average (%)
80	10	80.0	90.0
80	10	100.0	
70	14	86.0	87.3
70	17	88.0	
70	10	100.0	
70	10	100.0	
70	10	80.0	
70	10	70.0	
60	14	71.0	82.5
60	13	69.0	
60	11	72.7	
60	10	100.0	
60	10	100.0	
50	10	100.0	87.0
50	19	74.0	
40	10	10.0	20.0
40	10	30.0	

Tab. 3. Influence of nest depth on the hatching rate.

Influence of vegetation cover on the hatching rate (Experiment 4)

As is shown in Table 4, the average hatching rate was only 17% in the zone covered with vegetation, which is significantly lower than the 73% attained in the zone without vegetation cover (t-test, t = 3.001, p = 0.040). Levene's Test indicates an equality of variances between the two samples (f = 2.571, p = 0.184 [> 0.05]).

Group I	Nest	1	2	3	Average
Without vegetation cover	Hatching rate (%)	40	80	100	73
Group II	Nest	1	2	3	Average
With vegetation cover	Hatching rate (%)	10	30	10	17

Tab. 4. Influence of vegetable cover on the hatching rate.

Influence of clutch size on the hatching rate (Experiment 5)

Table 5 shows that the hatching rates of 20, 40 and remaining clutch sizes were 83, 70 and 49% on average, respectively. It would appear that a smaller clutch size produced a higher hatching rate.

Nest	1	2	3	4	5	6
Number of eggs	69	40	20	51	40	20
Number of hatchlings	34	26	17	25	30	16
Hatching rate (%)	49	65	85	49	75	80
Averages (%)	49 (nests 1+4)	70 (nests 2+5)	83 (nests 3+6)			

Tab. 5. Influence of clutch size on the hatching rate.

Multiple comparisons between the hatching rates in different clutch sizes show that the hatching rate was significantly lower in clutch sizes larger than 50 eggs than in the 20- and 40-egg groups ($p = 0.005$, or $p = 0.02$), while no significant difference was apparent between the two latter groups ($p = 0.071$).

Discussion and conclusions

The hatching rate of sea turtle eggs is apparently influenced by external factors such as nest depth (CHENG 1997, KARAVAS *et al.* 2005), temperature (DAVID *et al.* 2004), humidity (KARAVAS *et al.* 2005), vegetation cover (KARAVAS *et al.* 2005), deposits on the egg surface (e.g., mucus or spillage from broken eggs), predators (CHEN *et al.* 2007), pathogens (PHILLOTT *et al.* 2001), and probably other factors, too.

Influence of egg surface contaminants (such as yolk and albumen from broken eggs) on the hatching rate

Female A-0109 was missing a rear flipper, possibly as a result of a shark attack, which caused her to crush some eggs during and/or after oviposition. The spillage from these eggs contaminated the surface of intact eggs. Such surface contaminants can promote fungal growth and attract predators such as the pine root snake, dogs, crabs and insect larvae (KATILMIS *et al.* 2006), all of which have a potential of significantly reducing the hatching rate. Our results show that spilled albumen and yolk cause hatching rates to decrease when left on the shells of incubating eggs. Careful removal of these egg surface contaminants, on the other hand, greatly improved the eggs' chances of survival.

According to information obtained from some long-term citizens, local villagers used to visit the beach in numbers during the nesting season before the establishment of the reserve, locating turtle nests by pushing thin bamboo poles into the sand, then unearthed and took away the eggs for consumption. Some staff of the CSTB continued to use this method to locate nest sites, which resulted in a large number of destroyed turtle eggs and the contamination of entire clutches. Villagers furthermore stated that, in the 1940', more than 20 turtles nesting on this beach were missing a rear flipper. Supposing that these females were as unbalanced as A-0109, substantial contamination of their clutches and resulting losses are very likely.

Influence of surface mucus on the hatching rate

During oviposition, the birth canal secretes substantial amounts of mucus that drip into the egg chamber. As the results of Experiment 2 show, removing the mucus from the egg surface with 1‰ iodine solution improves

Fig. 14. "Three-footed turtle". Photo: Xia Zhong-rong

the hatching rate. Removal of surface mucus also eliminates the odour of the egg, reduces the risk of fungal infections, and so minimizes the chance of the eggs falling victim to predators and pathogens. It is likely that the oviductal fluids are already contaminated with pathogens from the sand surrounding the nest chamber (see also Phillott *et al.* 2001, Johnston *et al.* 2001). Lomholt (1976) furthermore suggested that liquid substances on the eggshell (of birds) must be removed from the pores and shell membrane to increase oxygen permeability.

It is likely that mucus on the egg surface might not have any positive effects on hatching rates and merely assists with the eggs' passing through the birth canal during oviposition. While one view is that oviductal fluids play a physiological role in that they confer some antibacterial protection to the eggs (Andrews *et al.* 2000, Al-Bahry *et al.* 2011), it may actually promote fungal growth and its strong odour could attract predators. On various occasions, we observed pine root snakes (*Oligodon formosanus*) above new marine turtle nests on Lanyu Island, Taiwan.

The proper depth for replacement nests

Diong *et al.* (1999) suggested that both hatching rates and incubation periods were similar between nests of 50 and 60 cm deep. Our results show that the hatching rate was similar among nest depths of 50, 60, 70 and 80 cm. However, it decreased significantly when the nest depth was set at only 40 cm. Temperature is known to influence the hatching rate and incubation period (Chen 1998, Diong *et al.* 1999). If the nest depth is less than 40 cm, sand temperature may fluctuate within a wider range than the embryo can withstand, whereas temperatures are more stable if the nest is deeper than 50 cm.

The depth of natural nests at the CSTB ranges from 65 to 80 cm (Cheng 1997). Our study shows that the average hatching rate is highest (90%) at a nest depth of 80 cm. Therefore, replacement nests should be set

Fig. 15.
A Green Sea Turtle nesting.
Photo: GU HOU-XIANG

up at a depth of more than 50 cm. A depth of 70 cm approximates the natural nest depth in the CSTB population, and sex ratios amongst hatchlings from nests at this depth are likely to approximate the population's natural sex ratio as well.

Vegetated zones may not be suitable for replacement nests

Vegetation has a negative impact on moisture levels at the depth of natural nests of 30 or even 50 cm deep (KARAVAS *et al.* 2005). Dry sand causes nests to collapse, rendering excavation attempts unsuccessful (KARAVAS *et al.* 2005). Moreover, we found that about 70% of the eggs in a nest set up in a vegetated zone were destroyed by sand-dwelling insect larvae like the yellow mealworm (*Tenebrio molitor*) during the incubation process. KATILMIS *et al.* (2006) found that *Pimelia* sp. (Tenebrionidae, Coleoptera) had the heaviest impact on loggerhead turtle nests on Dalaman beach, Turkey, and that fewer insects were found in nests farther away from vegetation.

On the sandy beaches of the CSTB, the vegetation consists mainly of *Canavalia maritima* (AUBL.), *Ipomoea pes-caprae*, *Vitex trifolia* LINN. var. *simplicifolia* CHAM, and *Panicum repens* LINN. The vegetated zone is about 70 m wide on the nesting beach, and the high tide water line can nearly reach it. Currently, we do not fully understand why the majority of Green and Loggerhead turtles avoid nesting in vegetated zones (KARAVAS *et al.* 2005). It is possible that it is because the plants may be competing with the eggs for available water. If the nest is located at a moist site, it will probably favour embryogenesis and produce larger hatchlings (CHEN 1992) with a higher demographic fitness (BOOTH *et al.* 2004). Temperature could be another major factor, though. The sand temperature at 50 cm deep was about 0.7°C lower in the vegetation zone than on the sun-exposed beach. Longer incubation periods caused by lower sand temperatures could also increase the risk of predation. In addition, at the CSTB, the majority of beach vegetation has strong rhizome and root systems that might discourage sea turtles from nest construction. KATILMIS *et al.* (2006) thought that decomposing roots might tend to promote worm infestation, which would then destroy the eggs. Plant roots may also cause hatchlings to be malformed in the nest (CHEN 1998). Our findings indicate that it might be sensible to remove or at least control beach vegetation as part of sea turtle conservation and the management program at the CSTB.

Influence of clutch size on the hatching rate

Monitoring data from the CSTB suggests that hatching rates were very high for several years after the establishment of the reserve, and then decreased (CHAN *et al.* 2007). We do not know the exact reason for this development, but action has to be taken to improve hatching rates.

Larger clutches tend to release more metabolic heat (CHEN 1998, ZBINDEN *et al.* 2006). This might speed up embryogenesis. Decaying embryos may infect neighbouring intact eggs, and the situation becomes worse in larger clutches where more decaying eggs and, as a consequence, higher levels of fungal and bacterial pollution are present (MILLER 1996). Our results suggest that a clutch size of more than 50 eggs could have a significant effect on the hatching rate as no such adverse effects on the hatching rate were found in the groups of 20- and 40-egg nests. Although sample sizes were relatively small in this experiment and most nests contained less than 40 eggs each, it appears that the effect of clutch size is not a significant one.

In summary, when relocating sea turtle eggs, it is recommended that oviductal mucus and other contaminants from the egg's surface be removed. Open, litter-free beach above the high tide line should be preferred for setting up replacement nesting sites. Nests should furthermore be installed away from the edge of the vegetation line at a depth of about 70 cm. If possible, large clutches should be split up into several small ones. Future research should focus on the influence of temperature, humidity, and nest size on the hatching rate in sea turtle nests.

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Fig. 16. Hatchlings leaving their nest. Photo: XIA ZHONG-RONG

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