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Histological Evaluation of Hatchling Sex Ratios of Hawaiian Green Sea Turtles

Taylor Nelson, Jenny Estes, Thane Wibbels

The Hawaiian green turtle is a genetically distinct group of green sea turtle that is found throughout the Hawaiian Archipelago. Green turtles possess temperature-dependent sex determination (TSD) which can result in a variety of hatchling sex ratios. Thus, hatchling sex ratios are of conservation and ecological interest. French Frigate Shoals (FFS), part of the Hawaiian Islands National Wildlife Refuge, is an atoll where approximately 90% of Hawaiian green nesting occurs. The long term goal of this study was to produce a method for predicting hatchling sex ratios of clutches hatching on East and Tern Islands at FFS. The goal of the current study was to determine which temperatures produce males versus females in the Hawaiian green turtle. This was evaluated experimentally in three laboratory incubators. During the current study, the lower range of the TSD curve was evaluated (26.5, 27.5, and 28.5 °C). My primary responsibility during this project was to histologically evaluate the sex of hatchlings from the incubators that died of natural causes during the experiment. A total of 98 embryos, hatchlings, or post hatchlings were examined from three different temperatures (26.5, 27.5, and 28.5°C). The results indicate that those three temperatures produced primarily male hatchlings. These results were similar to those obtained through the laparoscopic examination of live turtles (Estes and Wibbels, unpublished data). The results also show a distinct clutch effect in which certain clutches have a greater tendency to produce a specific sex. We are using these results to design the experimental protocol for the 2007 nesting season during which these three incubators will be used to evaluate warmer female-producing temperatures in the Hawaiian green.

INTRODUCTION

The green sea turtle, *Chelonia mydas,* is distributed circumglobally in tropical and subtropical waters. The Hawaiian subpopulation of Green turtles represents a genetically distinct and isolated of green sea turtles inhabiting the Hawaiian Archipelago (Bowen, et al., 1992). Like most reptiles, the Hawaiian green sea turtle has temperature-dependent sex determination, or TSD (Wibbels, 2003). It has a Male:Female pattern of TSD (Figure 2), in which warmer temperatures produce females, and cooler temperatures produce males (Broderick et al., 2000; Mrosovsky, et al., 1984; Spotila et al., 1987; Standora and Spotila, 1985). TSD makes sex ratios of the Hawaiian green vulnerable to a number of environmental factors, and sex ratios can, therefore, vary with many factors including seasonal changes, rainfall, cloud coverage, and humidity (Wibbels 2003).

Over 90% of Hawaiian green turtles nest on French Frigate Shoals (FFS) (Balazs, 1980). FFS is an atoll located approximately 800 km northwest of Oahu in the Hawaiian Archipelago. The Hawaiian green turtle is an interesting candidate for sex determination studies for many reasons. Previous data collected over recent years on French Frigate Shoals indicated nesting beach temperatures to be cool (Estes et al., 2007), suggesting they might be indicative of male-biased sex ratios (based on pivotal temperatures published for green turtle in other areas of the world). However, necropsy data on stranded turtles (Koga and Balazs, 1996; Work et al, 2004; Chaloupka et al., in review) as well as data on immature turtles sexed via hormone analysis (Wibbels and Balazs, 1993) have indicated a balanced sex ratio in the Hawaiian green turtle population. Collectively these data support a hypothesis that the Hawaiian green turtle may have evolved a lower pivotal temperature in its sex determination, which allows it to produce balanced sex ratios at relatively cool incubation temperatures. Furthermore, a prerequisite for understanding the reproductive ecology of this population is knowledge of the naturally occurring hatchling sex ratios produced at French Frigate Shoals. Therefore, the collaborative study was initiated in 2006 in an effort to determine which temperatures produced each sex in the Hawaiian green, and in particular to determine if comparatively cool incubation temperatures produce a balanced sex ratio (Estes, et al., 2007). This study included the incubation of eggs at specific temperatures in incubators at Sea Life Park, Oahu. The purpose of the current study was to use histological techniques to determine

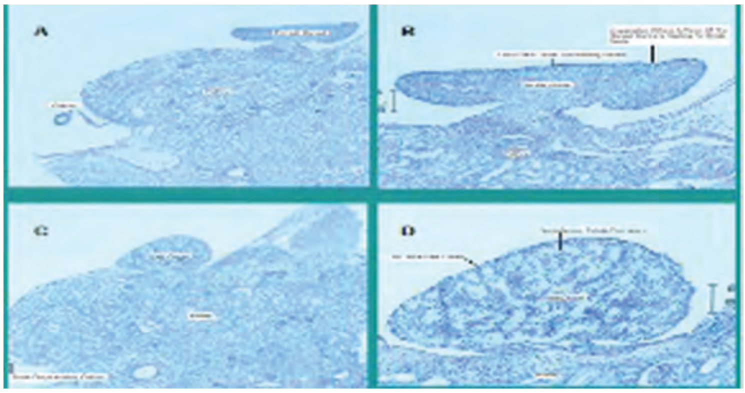


Figure 1: Photos of histology of turtle gonads. Photo (A) shows the characteristic of a female turtle. Note the well defined oviduct and in (B) the darkly stained cortex of the developing ovary. Photo (C) shows the characteristic of a male turtle. Note the regressed oviduct and the formation of tubules in the medullar region (D).

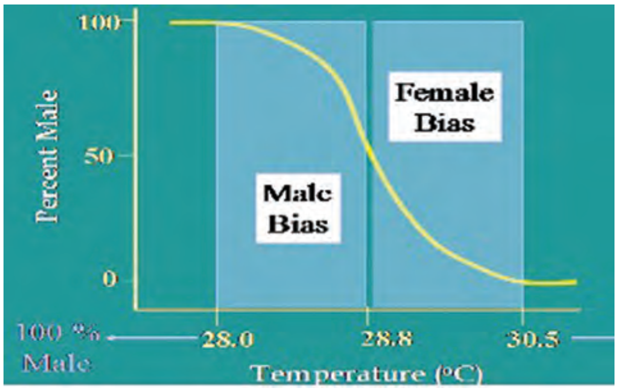


Figure 2: The M/F pattern of TSD in the green sea turtle.

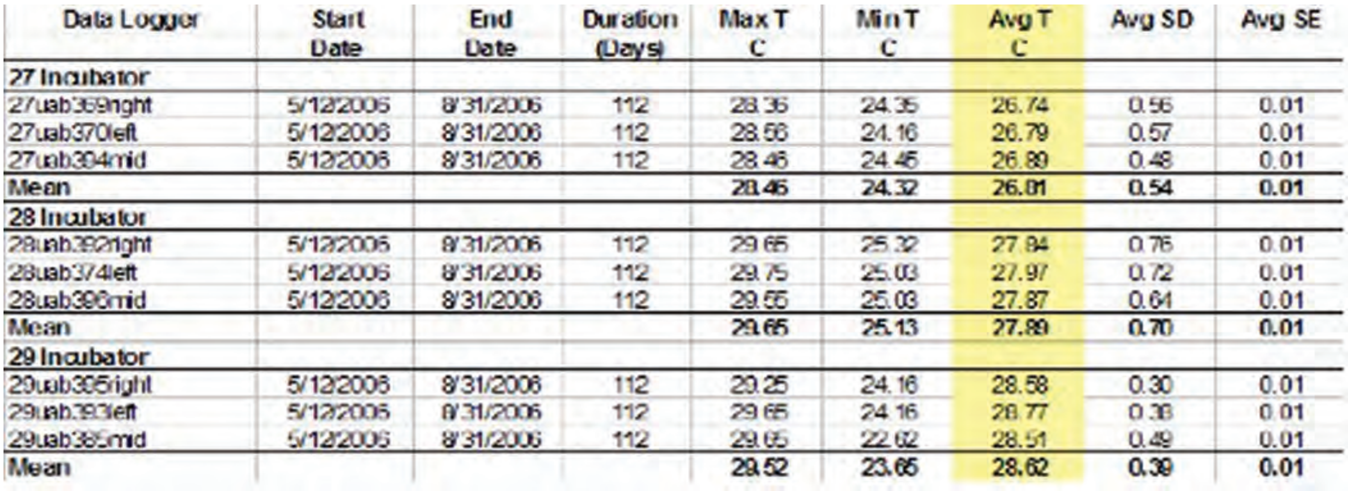
the sex of any embryos, hatchlings or post-hatchlings from the study that died of natural causes. These results were then compared and combined with sex ratio data from laparoscopic examination of live turtles from Estes et al., 2007.

MATERIALS AND METHODS

Three experimental incubators were installed at Sea Life Park, Oahu, in May of 2006. To address the cooler pivotal temperature hypothesis, the eggs were incubated at temperatures that were slightly cooler than the estimated pivotal temperature for the Costa Rican population of green turtles (i.e. incubator temperatures were set at 26.5, 27.5, and 28.5°C). These incubators were custom designed and built to maintain the desired temperatures within a few tenths of a degree (Table 3). Over an approximate two-month period, eggs were obtained from captive Hawaiian green turtles nesting on the artificial nesting beach at Sea Life Park. This work was conducted and supervised by biologists from Sea Life Park and the National Marine Fisheries Service. Eggs were obtained from multiple clutches and were distributed evenly to each of the three incubators. Egg incubation was monitored on a weekly basis and hatchlings were removed from the incubators and transferred to salt water tanks for rearing. The design of the study included the rearing of turtles for approximately four to five months, at which point they would be large enough for laparoscopic examination for sex determination. If any embryos, hatchlings or post-hatchlings died (of natural causes) during the experiment, they were preserved and the tissues were shipped to the University of Alabama at Birmingham (UAB) for histological verification of sex (Figure 1). The current study focuses on the results of the histological analysis. These results were used to verify those from a separate study in which the sex of live turtles from the same incubators was determined through laparoscopic examination (Estes et al., 2007).

During the current study, histological analysis of gonadal tissue was used to estimate the sex ratios produced in each of the three incubators (i.e. incubator temperatures were set at 26.5, 27.5, and 28.5°C). The embryos, hatchlings, or

Table 1: The incubators maintained mean temperatures at or very near the targeted temperatures of 27, 28, and 29°C.



Data Logger	Start Date	End Date	Duration (Days)	Max T C	Min T C	Avg T C	Avg SD	Avg SE
27 Incubator								
27uab369right	5/12/2006	8/31/2006	112	28.36	24.35	26.74	0.56	0.01
27uab370left	5/12/2006	8/31/2006	112	28.56	24.16	26.79	0.57	0.01
27uab394mid	5/12/2006	8/31/2006	112	28.46	24.45	26.89	0.48	0.01
Mean				28.46	24.32	26.81	0.54	0.01
28 Incubator								
28uab392right	5/12/2006	8/31/2006	112	29.65	25.32	27.84	0.76	0.01
28uab374left	5/12/2006	8/31/2006	112	29.75	25.03	27.97	0.72	0.01
28uab396mid	5/12/2006	8/31/2006	112	29.55	25.03	27.87	0.64	0.01
Mean				29.65	25.13	27.89	0.70	0.01
29 Incubator								
29uab395right	5/12/2006	8/31/2006	112	29.25	24.16	28.58	0.30	0.01
29uab393left	5/12/2006	8/31/2006	112	29.65	24.16	28.77	0.38	0.01
29uab385mid	5/12/2006	8/31/2006	112	29.65	22.62	28.51	0.49	0.01
Mean				29.52	23.65	28.62	0.39	0.01

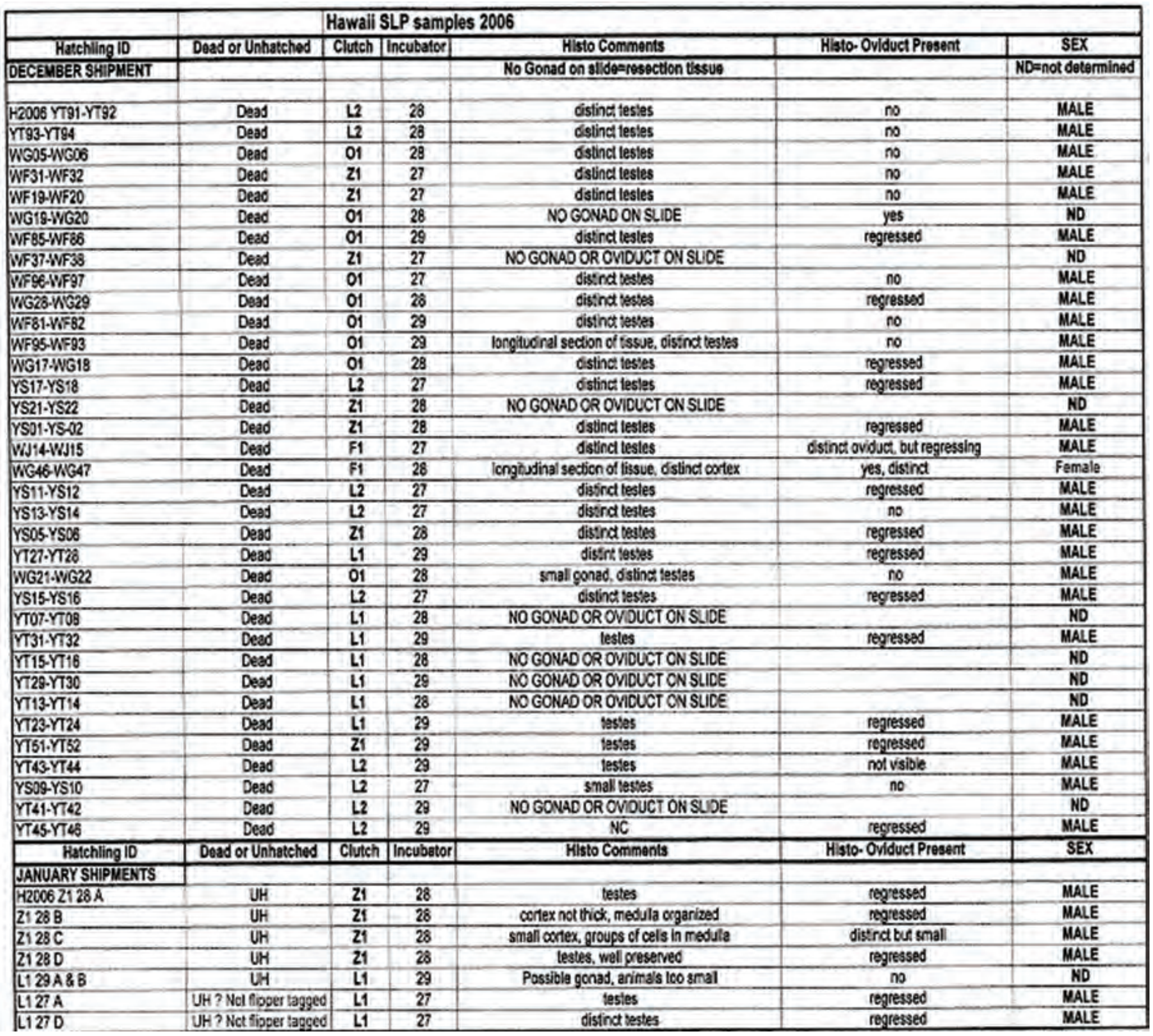
post-hatchlings that died of natural causes were preserved in formalin and the kidney/adrenal/gonad complex was then dissected from the turtle. These tissues were infiltrated and embedded with paraffin (Humason, 1972). Embedding the tissue with paraffin serves to firm the tissue so that sections can be cut using a microtome and placed on slides. After drying on a hot plate for at least 24 hours, the slides are stained through a staining procedure using hematoxylin and eosin (Humason, 1972). After staining is complete, the slides can be analyzed microscopically, examining the gonads for structural differences. A female gonad contains a dark cortex (Figure 1), pronounced oviduct, and a medulla comprised of degenerating seminiferous tubules (Wibbels, 2003). On the other hand, the male gonad has a medulla containing a developed system of seminiferous tubules (Figure 1) and lacks a dark stained cortex and well-developed oviduct (Wibbels, 2003).

RESULTS AND CONCLUSIONS

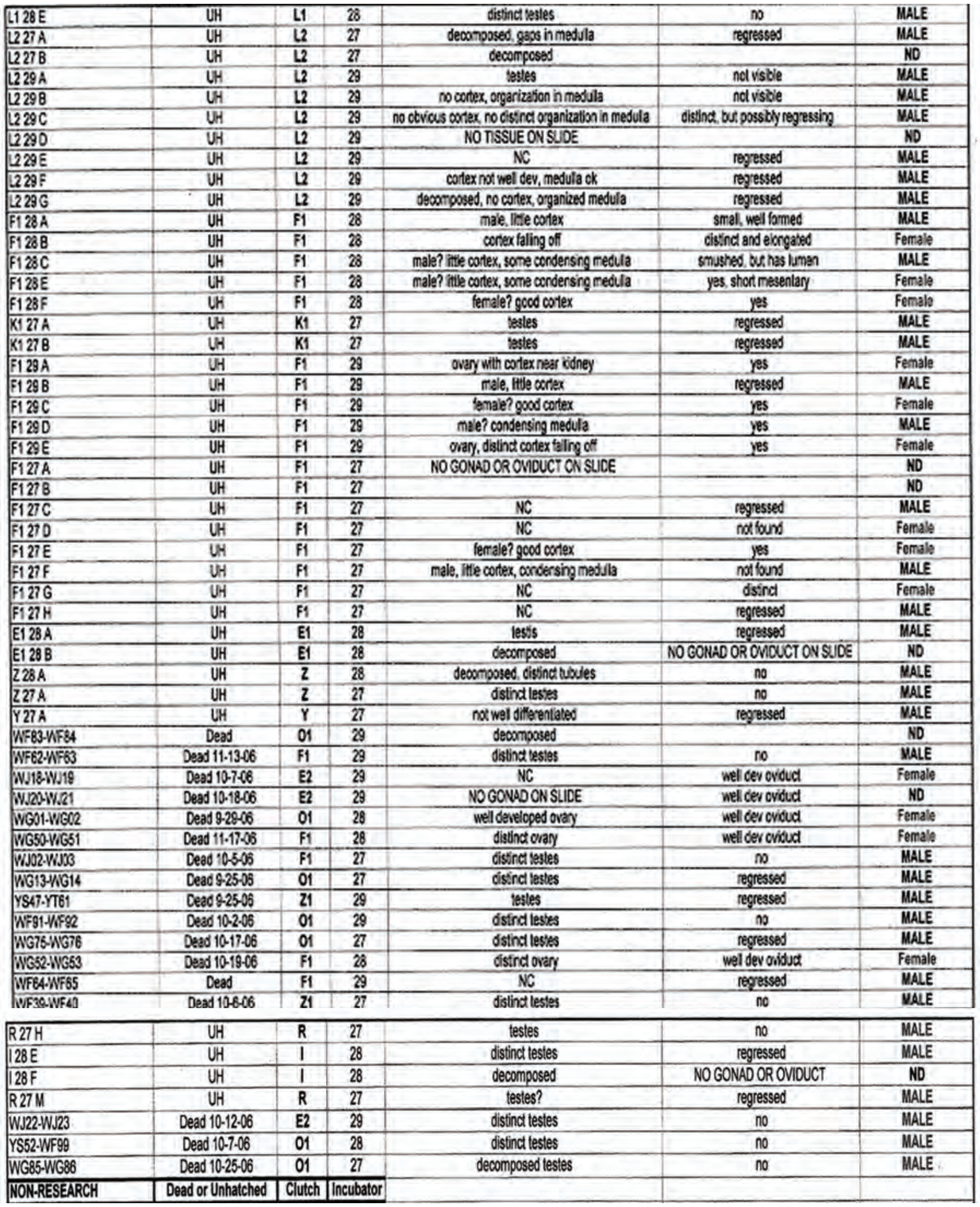
Tissues from a total of 98 turtles were examined in the current study (Table 2). Those tissues included turtles from all three incubators and from a variety of different clutches (Tables 2 and 3). Of those tissues, the majority were male (Tables 2 and 3). These data were similar to those from the laparoscopy study (Estes et al., 2007) and are summarized in Table 3. Collectively the results indicated that the three temperatures used in the incubators (26.5, 27.5, and 28.5° C) produced mostly males. This indicates that the pivotal temperature (temperature producing a 1:1 sex ratio) of the Hawaiian green turtle is higher thaN 28.5° C.

Collectively, the results do not support the hypothesis that the pivotal temperature in the Hawaiian green turtle is distinctly lower than those reported for green turtles in Suriname and Costa Rica. To the contrary, the results suggest that the Hawaiian green turtle has a pivotal temperature between approximately 28.5–29.0°C. The results have provided valuable data suggesting that the pivotal temperature in the Hawaiian green may be similar to those of other green turtles. Additionally, the results are consistent with previous studies of turtles with TSD (Dodd et al., 2006) suggesting that effects of temperature may vary between clutches as exemplified by clutch F1 in the current study. The results could also reflect potential

Table 2: Results from the histological evaluation of gonad tissue.



		Hawaii SLP samples 2006				
Hatchling ID	**Dead or Unhatched**	**Clutch**	**Incubator**	**Histo Comments**	**Histo- Oviduct Present**	**SEX**
DECEMBER SHIPMENT				**No Gonad on slide=resection tissue**		**ND=not determined**
H2006 YT91-YT92	Dead	L2	28	distinct testes	no	MALE
YT93-YT94	Dead	L2	28	distinct testes	no	MALE
WG05-WG06	Dead	O1	28	distinct testes	no	MALE
WF31-WF32	Dead	Z1	27	distinct testes	no	MALE
WF19-WF20	Dead	Z1	27	distinct testes	no	MALE
WG19-WG20	Dead	O1	28	NO GONAD ON SLIDE	yes	ND
WF85-WF86	Dead	O1	29	distinct testes	regressed	MALE
WF37-WF38	Dead	Z1	27	NO GONAD OR OVIDUCT ON SLIDE		ND
WF96-WF97	Dead	O1	27	distinct testes	no	MALE
WG28-WG29	Dead	O1	28	distinct testes	regressed	MALE
WF81-WF82	Dead	O1	29	distinct testes	no	MALE
WF95-WF93	Dead	O1	29	longitudinal section of tissue, distinct testes	no	MALE
WG17-WG18	Dead	O1	28	distinct testes	regressed	MALE
YS17-YS18	Dead	L2	27	distinct testes	regressed	MALE
YS21-YS22	Dead	Z1	28	NO GONAD OR OVIDUCT ON SLIDE		ND
YS01-YS-02	Dead	Z1	28	distinct testes	regressed	MALE
WJ14-WJ15	Dead	F1	27	distinct testes	distinct oviduct, but regressing	MALE
WG46-WG47	Dead	F1	28	longitudinal section of tissue, distinct cortex	yes, distinct	Female
YS11-YS12	Dead	L2	27	distinct testes	regressed	MALE
YS13-YS14	Dead	L2	27	distinct testes	no	MALE
YS05-YS06	Dead	Z1	28	distinct testes	regressed	MALE
YT27-YT28	Dead	L1	29	distint testes	regressed	MALE
WG21-WG22	Dead	O1	28	small gonad, distinct testes	no	MALE
YS15-YS16	Dead	L2	27	distinct testes	regressed	MALE
YT07-YT08	Dead	L1	28	NO GONAD OR OVIDUCT ON SLIDE		ND
YT31-YT32	Dead	L1	29	testes	regressed	MALE
YT15-YT16	Dead	L1	28	NO GONAD OR OVIDUCT ON SLIDE		ND
YT29-YT30	Dead	L1	29	NO GONAD OR OVIDUCT ON SLIDE		ND
YT13-YT14	Dead	L1	28	NO GONAD OR OVIDUCT ON SLIDE		ND
YT23-YT24	Dead	L1	29	testes	regressed	MALE
YT51-YT52	Dead	Z1	29	testes	regressed	MALE
YT43-YT44	Dead	L2	29	testes	not visible	MALE
YS09-YS10	Dead	L2	27	small testes	no	MALE
YT41-YT42	Dead	L2	29	NO GONAD OR OVIDUCT ON SLIDE		ND
YT45-YT46	Dead	L2	29	NC	regressed	MALE
Hatchling ID	**Dead or Unhatched**	**Clutch**	**Incubator**	**Histo Comments**	**Histo- Oviduct Present**	**SEX**
JANUARY SHIPMENTS						
H2006 Z1 28 A	UH	Z1	28	testes	regressed	MALE
Z1 28 B	UH	Z1	28	cortex not thick, medulla organized	regressed	MALE
Z1 28 C	UH	Z1	28	small cortex, groups of cells in medulla	distinct but small	MALE
Z1 28 D	UH	Z1	28	testes, well preserved	regressed	MALE
L1 29 A & B	UH	L1	29	Possible gonad, animals too small	no	ND
L1 27 A	UH ? Not flipper tagged	L1	27	testes	regressed	MALE
L1 27 D	UH ? Not flipper tagged	L1	27	distinct testes	regressed	MALE



L1 28 E	UH	L1	28	distinct testes	no	MALE
L2 27 A	UH	L2	27	decomposed, gaps in medulla	regressed	MALE
L2 27 B	UH	L2	27	decomposed		ND
L2 29 A	UH	L2	29	testes	not visible	MALE
L2 29 B	UH	L2	29	no cortex, organization in medulla	not visible	MALE
L2 29 C	UH	L2	29	no obvious cortex, no distinct organization in medulla	distinct, but possibly regressing	MALE
L2 29 D	UH	L2	29	NO TISSUE ON SLIDE		ND
L2 29 E	UH	L2	29	NC	regressed	MALE
L2 29 F	UH	L2	29	cortex not well dev, medulla ok	regressed	MALE
L2 29 G	UH	L2	29	decomposed, no cortex, organized medulla	regressed	MALE
F1 28 A	UH	F1	28	male, little cortex	small, well formed	MALE
F1 28 B	UH	F1	28	cortex falling off	distinct and elongated	Female
F1 28 C	UH	F1	28	male? little cortex, some condensing medulla	smushed, but has lumen	MALE
F1 28 E	UH	F1	28	male? little cortex, some condensing medulla	yes, short mesentary	Female
F1 28 F	UH	F1	28	female? good cortex	yes	Female
K1 27 A	UH	K1	27	testes	regressed	MALE
K1 27 B	UH	K1	27	testes	regressed	MALE
F1 29 A	UH	F1	29	ovary with cortex near kidney	yes	Female
F1 29 B	UH	F1	29	male, little cortex	regressed	MALE
F1 29 C	UH	F1	29	female? good cortex	yes	Female
F1 29 D	UH	F1	29	male? condensing medulla	yes	MALE
F1 29 E	UH	F1	29	ovary, distinct cortex falling off	yes	Female
F1 27 A	UH	F1	27	NO GONAD OR OVIDUCT ON SLIDE		ND
F1 27 B	UH	F1	27			ND
F1 27 C	UH	F1	27	NC	regressed	MALE
F1 27 D	UH	F1	27	NC	not found	Female
F1 27 E	UH	F1	27	female? good cortex	yes	Female
F1 27 F	UH	F1	27	male, little cortex, condensing medulla	not found	MALE
F1 27 G	UH	F1	27	NC	distinct	Female
F1 27 H	UH	F1	27	NC	regressed	MALE
E1 28 A	UH	E1	28	testis	regressed	MALE
E1 28 B	UH	E1	28	decomposed	NO GONAD OR OVIDUCT ON SLIDE	ND
Z 28 A	UH	Z	28	decomposed, distinct tubules	no	MALE
Z 27 A	UH	Z	27	distinct testes	no	MALE
Y 27 A	UH	Y	27	not well differentiated	regressed	MALE
WF83-WF84	Dead	O1	29	decomposed		ND
WF62-WF63	Dead 11-13-06	F1	29	distinct testes	no	MALE
WJ18-WJ19	Dead 10-7-06	E2	29	NC	well dev oviduct	Female
WJ20-WJ21	Dead 10-18-06	E2	29	NO GONAD ON SLIDE	well dev oviduct	ND
WG01-WG02	Dead 9-29-06	O1	28	well developed ovary	well dev oviduct	Female
WG50-WG51	Dead 11-17-06	F1	28	distinct ovary	well dev oviduct	Female
WJ02-WJ03	Dead 10-5-06	F1	27	distinct testes	no	MALE
WG13-WG14	Dead 9-25-06	O1	27	distinct testes	regressed	MALE
YS47-YT61	Dead 9-25-06	Z1	29	testes	regressed	MALE
WF91-WF92	Dead 10-2-06	O1	29	distinct testes	no	MALE
WG75-WG76	Dead 10-17-06	O1	27	distinct testes	regressed	MALE
WG52-WG53	Dead 10-19-06	F1	28	distinct ovary	well dev oviduct	Female
WF64-WF65	Dead	F1	29	NC	regressed	MALE
WF39-WF40	Dead 10-6-06	Z1	27	distinct testes	no	MALE
R 27 H	UH	R	27	testes	no	MALE
I 28 E	UH	I	28	distinct testes	regressed	MALE
I 28 F	UH	I	28	decomposed	NO GONAD OR OVIDUCT	ND
R 27 M	UH	R	27	testes?	regressed	MALE
WJ22-WJ23	Dead 10-12-06	E2	29	distinct testes	no	MALE
YS52-WF99	Dead 10-7-06	O1	28	distinct testes	no	MALE
WG85-WG86	Dead 10-25-06	O1	27	decomposed testes	no	MALE
NON-RESEARCH	**Dead or Unhatched**	**Clutch**	**Incubator**			

confounding factors associated with the experiment such as sampling bias. The turtles at Sea Life Park are of the same genetic stock as those that nest at French Frigate Shoals, but this breeding colony has been in captivity for over 30 years, and it is not clear if it is representative of the natural population. It would be of interest to compare the results from Sea Life Park to those of eggs from French Frigate Shoals. The results suggest a distinct clutch effect with clutch F1. The basis of clutch effects is currently unknown, but it is clear that such factors can affect the results. For example, had we only used one clutch of eggs (F1), the results would have been consistent with the low pivotal hypothesis. Alternatively, it could it be possible that other turtles at Sea Life Park have a propensity for the production of males. However, we have no basis for such assumptions, but the results from F1 exemplify the need for multiple clutches in such experiments.

The results generated a variety of questions regarding sex determination in the Hawaiian green turtle. For example, it is not clear why the previously recorded (cooler) beach temperatures at FFS are inconsistent with the previously recorded unbiased sex ratios in the Hawaiian archipelago. This inconsistency in data could be due to a variety of factors. For example, the sex ratios previously reported could have been due to beach temperatures in years prior to more recent beach temperature studies. There are other alternatives, including the possibility that metabolic heating within the nest could have a significant effect in the Hawaiian green's incubation temperature. Regardless, the data collected has proved to provide a firm platform for developing experimental design for the 2007 nesting season. During 2007 relatively warm temperatures (29 -31°C) will be evaluated in the three incubators in order to examine the upper portion (female-producing portion) of the sex determination curve in the Hawaiian green turtle.

Table 3: Sexes of turtles produced by each clutch relative to incubation temperatures. Most clutches produced males at all three temperatures. This table represents a compilation of data from laparoscopy, histology, and dissection.

CLUTCH	TEMPERATURE	SEX
E1	28	All Male
E2	27	All Male
E2	28	Predominantly Male with one female
E2	29	Predominantly Male with one female
K1	27	All Male
K1	29	All Male
F1	27	Mixed sea ratio, near 1:1 sex ratio
F1	28	Mixed sea ratio, female biased
F1	29	Mixed sea ratio, near 1:1 sex ratio
I	28	All Male
L1	27	All Male
L1	28	All Male
L1	29	All Male
L2	27	All Male
L2	28	All Male
L2	29	All Male
O1	27	Predominantly Male with one female
O1	28	Predominantly Male with one female
O1	29	All Male
R	27	All Male
Y	27	All Male
Z1	27	All Male
Z1	28	All Male
Z1	29	All Male

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