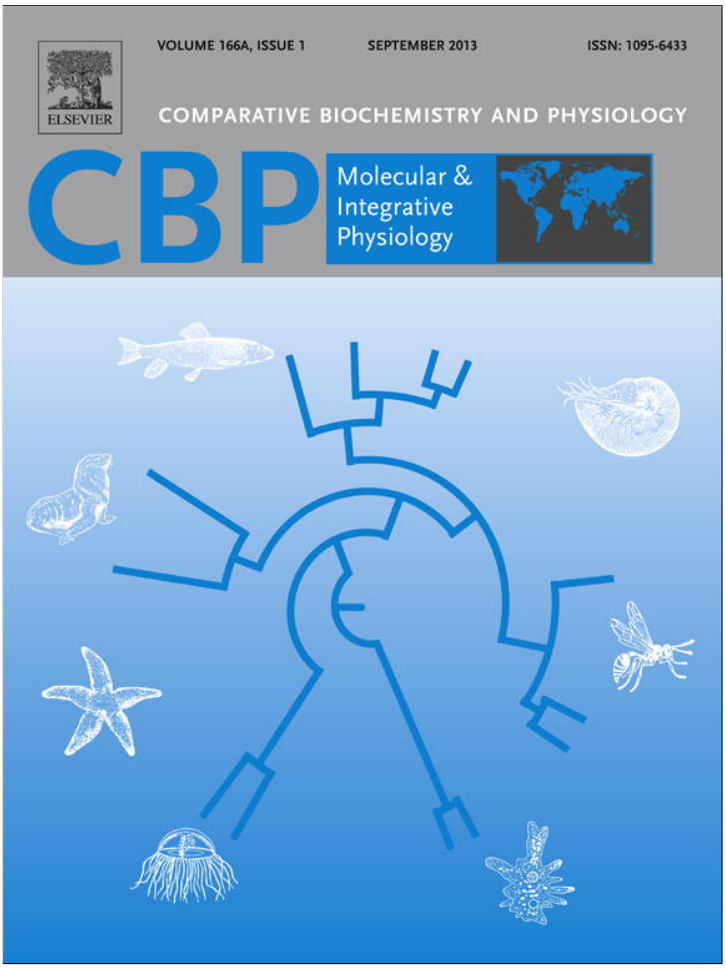

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Applying generalized linear models as an explanatory tool of sex steroids, thyroid hormones and their relationships with environmental and physiologic factors in immature East Pacific green sea turtles (*Chelonia mydas*)

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ABSTRACT

Generalized linear models were fitted to evaluate the relationship between 17β-estradiol (E_2), testosterone (T) and thyroxine (T_4) levels in immature East Pacific green sea turtles (*Chelonia mydas*) and their body condition, size, mass, blood biochemistry parameters, handling time, year, season and site of capture. According to external (tail size) and morphological (<77.3 straight carapace length) characteristics, 95% of the individuals were juveniles. Hormone levels, assessed on sea turtles subjected to a capture stress protocol, were <34.7 nmol T L^{-1}, <532.3 pmol E_2 L^{-1} and <43.8 nmol T_4 L^{-1}. The statistical model explained biologically plausible metabolic relationships between hormone concentrations and blood biochemistry parameters (e.g. glucose, cholesterol) and the potential effect of environmental variables (season and study site). The variables *handling time* and *year* did not contribute significantly to explain hormone levels. Differences in sex steroids between season and study sites found by the models coincided with specific nutritional, physiological and body condition differences related to the specific habitat conditions. The models correctly predicted the median levels of the measured hormones in green sea turtles, which confirms the fitted model's utility. It is suggested that quantitative predictions could be possible when the model is tested with additional data.

1. Introduction

One of the main goals of the ecologist working in the areas of toxicology and conservation medicine is to find out biomarkers (e.g. biochemical, physiological or histopathological) that are sufficiently sensitive and efficient to assess the health state of the wildlife populations before negative effects of the stress responses to the environmental change and habitat perturbation are evident along the population dynamic processes. Biomarkers are a tool for the integrative evaluation of the responses of an organism that have to cope with multiple factors (e.g. toxic or environmental) in its particular habitat (Mayer et al., 1992). The habitat of the organisms can also be characterized, in space and time, by evaluating the molecular and biochemical responses (biomarkers) associated to environmental factors, such as metal concentrations (Talavera-Saenz et al., 2007; Pereira et al., 2009; Labrada-Martagón et al., 2011). Nevertheless, the suitable interpretation of the biomarker responses depends on the understanding of their endogenous variability as well as which biological factors, such as age, gender, body condition, nutritional state, and exogenous factors, such as the temperature, seasonality and sampling, are associated to them (Mayer et al., 1992).

The complexity of the ecophysiological processes could be assessed by using multivariate statistics models, such as the multiple regression analysis, which deals with the description of the relationship between more than two variables and attempts to explain the simultaneous effect on the response variable. Multivariate data arise when several variables are recorded for a number of organisms, leading to a multidimensional observation for each individual. When the method of selection of the variables is by design, as in ecological and physiological studies, the variables of interest to be recorded are previously known to be descriptors of the system or phenomenon under investigation (e.g. cholesterol concentration and sex steroids) (Everitt and Hothorn, 2011). Multivariate statistics contribute to improving the information regarding key features and interesting patterns that could be missed or covered by using separate univariate analyses (Kleinbaum and Kupper, 1978; Everitt and Hothorn, 2011). When the individuals in a multivariate dataset have been sampled from a single population, the resulting model can be used for statistical inference. In this case, all the gathered information could be used

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to make inferences about the population of interest, rather than focus in the sample data per se (Everitt and Hothorn, 2011).

The choice of the adequate multivariate statistical analysis depends upon the general characteristics of the variables under investigation (Kleinbaum and Kupper, 1978). The generalized linear model (GLM) is an analysis that assumes the classic linear regression models and works under a variety of probability distributions (e.g. Poisson or binomial) in addition to the normal error. The non-normal biological data could be assessed in GLM by the transformation of the mean of the response variable through what is called a "link function", which is the union segment between the regression (explanatory variables) and the error distribution of the response variable of interest (Lindsey, 1997; Zuur et al., 2009).

Applying alternative statistical methods, such as GLMs, to assess complex ecological and physiological datasets is an effective approach to integrative studies, and is a particularly promising tool for research involving threatened or endangered wildlife. For instance, determinations of the circulating levels of specific hormones provides not only clues on the precise reproductive or behavioral status of sea turtles, but also data for understanding the population dynamics contributing to conservation biology (Balazs, 1995; Wingfield et al., 1997; Owens, 1999). Knowledge about the dynamics of growth and age at maturity is basic data for the development of sea turtle management strategies (Balazs, 1995). The nutritional and energetic status necessary for physiological functions including growth, maturity, vitellogenesis and reproduction could be understood by studying the factors involved in the variations of the hormone concentrations (Mayer et al., 1992; Rostal et al., 1998; Hamann et al., 2003). In addition, the effects of human disturbance and environmental endocrine disruptors on free living populations could be detected if the base sex steroids levels are previously determined (Wingfield et al., 1997; Valverde et al., 2008). Application of GLMs could contribute to analyze and evaluate the multiple environmental and physiological factors implicated in the reproductive hormone response in sea turtles.

There are numerous studies available on the reproductive physiology and hormonal variations (daily, seasonal, annual, with migration, reproductive and nutritional state) of green sea turtles (*Chelonia mydas*) in other regions of the world (e.g. Owens and Morris, 1985; Hamann et al., 2005), but information concerning those inhabiting the East Pacific ocean is scarce (Licht et al., 1980; Moon et al., 1998). Green sea turtle research has been focused on nesting areas, resulting in greater biological knowledge for the adult stage in comparison with the most recent advances concerning other age classes of the population structure, such as juveniles inhabiting feeding areas (Balazs, 1995; Bowen and Karl, 2007). Studies on variations of sexual steroids, including 17β-estradiol (E_2) and progesterone, have focused mainly on adult females during the reproductive cycles (e.g. Licht et al., 1979, 1980; Hamann et al., 2002; Al-Habsi et al., 2006). Studies on thyroid hormone levels in sea turtles are less common (Licht et al., 1985a; Owens and Morris, 1985; Moon et al., 1998, 1999), despite their physiological relevance for vertebrate growth, development and reproduction (Dickhoff and Darling, 1983).

Reproduction, growth and immunity are strongly influenced by the stress response, with physiological and behavioral consequences at the individual (Johnson et al., 1992) and population levels. Many factors, such as gender, season, ontogenetic shifts and reproductive status, appear to be associated to individual modulation of the adrenocortical responses to stressors in reptiles (Moore and Jessop, 2003). The capture-handling stress protocols can alter the testosterone (T) levels measured in sea turtles, especially in breeding males during the reproductive period (Jessop et al., 2002; reviewed by Moore and Jessop, 2003; Blanvillain et al., 2008). Nesting females, in contrast, appear to downregulate the corticosterone stress responses as a mechanism to optimize reproductive success (Jessop et al., 1999; reviewed by Moore and Jessop, 2003). The stress condition may not affect the sex steroid levels in immature sea turtles (Jessop et al., 2004), but information about this age class is scarce.

The goal of this study was to characterize the hormone levels between factors (age classes, season, year and handling time categories), and to evaluate the relationship and effect of the body condition, size, mass, nutritional state (total protein, glucose, cholesterol, and triglycerides), year and season, on the E_2, T and thyroxine (T_4) levels in immature East Pacific green sea turtles. The GLM was used as a statistical tool to fit the best explanatory model for each hormone and to identify the significant independent variables that contribute to the hormone levels measured in these sea turtles. It is imperative to understand the endocrine patterns of the East Pacific green sea turtle and the associated factors in order to answer questions regarding the relationship between those patterns and the habitat condition (e.g. food quality and availability), growth (sexual maturity), xenoestrogen compound levels, and in general, the health status of this sea turtle population.

2. Material and methods

2.1. Study area

The coastal lagoons of Baja California Sur (BCS) are feeding grounds and a favorable habitat for the growth of immature green sea turtles (Cliffton et al., 1995; Gardner and Nichols, 2001) in the northern distribution of the East Pacific population. For this study, organisms were captured alive during 2005 and 2007 in three coastal lagoons located in the occidental coast of BCS, Mexico, namely, Punta Abreojos (PAO, 26° 43′ 57" N and 113° 35′ 44" W), Laguna San Ignacio (LSI, 26° 43′–26° 58′ N and 113° 08′–113° 16′ W) and Bahía Magdalena (BMA, 25° 43′–24° 20′ N and 112° 15′–111° 20′ W) (Labrada-Martagón et al., 2010a). The green turtles inhabiting these lagoons have been reported as healthy, without severe lesions, anthropogenic damage or any sign of disease (Labrada-Martagón et al., 2010a). PAO, LSI and BMA are flooded depressions located inside the continental margin and protected from the Pacific Ocean by the presence of a sand barrier or island located parallel to the coast. They are characterized by a shallow bathymetry, with exception of the channels formed by erosion processes (De la Lanza, 1991). The geographical discontinuities and seasonal changes of the oceanographic processes in the area, such as the California Current and upwelling events, generate regional and temporal differences in habitat conditions (Dawson, 1951; Lynn and Simpson, 1987; Lluch-Belda, 2000). The biological value of the biodiversity in PAO, LSI and BMA is well recognized, as is their economical and cultural importance. These lagoons are important fisheries and tourism industry centers (Ávila and Saad, 1998; Guzmán, 1998; Martínez, 1998; Maya and Guzmán, 1998).

2.2. Animal capture and sample collection

The sea turtles were captured as previously described (Labrada-Martagón et al., 2010a, 2011) with monofilament fishing nets (100 m long; 20 cm mesh) monitored every 1–2 h in the three study sites. The handling time defined as the period from the time a turtle was recovered from the net to the time when the blood sample was obtained (Labrada-Martagón, 2011), considers the three stages of the capture stress protocol (capture, restraint and handling) (Wingfield et al., 1997). All animals were released immediately after sample collection; each individual was measured with calipers (±0.1 cm) to obtain the straight carapace length (SCL; from nuchal notch to tip of distal marginal scute) and weighed with a 150-kg spring scale (±0.1 kg). A blood sample was drawn from the venous cervical sinus (Owens and Ruiz, 1980) using double-ended Vacutainer© needles (1.5 in. length, 32 mm caliber) and Vacutainer© blood collection tubes without anticoagulant (7 mL, Becton Dickinson, Franklin Lakes, NJ, USA) as described elsewhere (Keller et al., 2006; Labrada-Martagón et al., 2010a, 2011). The collected blood was centrifuged (890 ×*g*) for 15 min in a field

centrifuge (Mobilespin, Vulcon Technologies, Grandview, MO, USA) to obtain the serum. Each sample was transferred into labeled microtubes (1.5 mL cryovials, Eppendorf©). Samples were immediately frozen and stored in a cryogenic shipper (5.4 L) (Taylor Wharton CP 100) for transportation to the laboratory where they were stored at −80 °C until analyzed.

2.3. Sex steroid (T, E_2) and thyroid (T_4) hormones

Sex steroid and thyroid hormone levels were determined in serum samples by direct competitive enzyme linked immunoassays (ELISA) using a polyclonal rabbit anti-T antibody, a monoclonal mouse anti-T_4 antibody or an anti-rabbit IgG, all of them coated onto the wells of 96 microwell plates (T and T_4, Diagnostics Biochem Canada Inc., ON, Canada E_2, Diagnostics Automation Inc., CA, USA). All the reagents and samples were brought to room temperature (18–25 °C) prior to use. All the samples were analyzed in duplicate following the supplier's instructions as previously reported for sea turtles (Ikonomopoulou et al., 2008). The standard curves, the sensitivity of the kits and the intra- and inter-assay coefficients of variability are shown in Table 1. The concentrations of the hormones are expressed in nmol T L^{-1}, nmol T_4 L^{-1} and pmol E_2 L^{-1}.

2.4. Data classification and statistical analyses

The average nesting size of the East Pacific green sea turtle (>77 cm SCL) was the criterion used to identify adult organisms (Seminoff et al., 2003; Koch et al., 2007). A relative body condition index (Krel) was estimated from the length (cm) and mass (kg) data of the sea turtles captured, using the parameters estimated from the mass–length regression model as described in Labrada-Martagón et al. (2010b). Data of individuals were grouped by season as defined by Koch et al. (2007): summer (May–October) and winter (November–April), and by handling time category defined as follows: (1) 20–35 min; (2) 36–60 min; (3) 61–90 min, and (4) >90 min.

Nonparametric tests were used on raw data since the assumptions of normality and homoscedasticity of variance were not accomplished when tested on the hormone levels. The Mann–Whitney U-test and Kruskal–Wallis test were used to assess differences in the hormone levels of sea turtles between factors (age classes, season, year and handling time categories) considering each study site and to evaluate differences between sites. Kruskal–Wallis test was used to evaluate differences in the handling time variable between study sites. The relationship between the handling time and the hormone levels were also evaluated by study site using the Spearman correlation analysis. All results with $p \leq 0.05$ were considered significant.

2.5. Data selection for modelling

From the total of 69 sea turtles captured, data obtained from the organisms captured in BMA ($n = 7$), LSI ($n = 5$) and PAO ($n = 31$) were considered to fulfill the creation of the GLMs of this study. The effect of the age class was included in the models by evaluating the *size* variable. The Krel was included as a biomarker of the nutritional state of the organisms (Labrada-Martagón et al., 2010a,b) and *mass* as indicator of internal (phenotypic) state of the organism (Clark and Mangel, 2000). The variable *handling time* was excluded from the analyses in order to keep the full database without the necessity of reducing the sample size (Katz, 2006), considering that (1) no differences in the hormone levels between the categories of handling time were found in the univariate analyses, the hormone levels of the individuals chosen for modelling were not correlated with the handling time in any study site ($p > 0.27$) and (2) *handling time* did not contribute significantly to explain the hormone levels of the sea turtles in a preliminary multivariate statistical model (data not shown).

Table 1
Standard curves, sensitivity and precision (CV%) of the enzyme linked immunoassays (ELISA).

Parameter	Standard curve	Sensitivity	Intra-assay variation (CV %)	Inter-assay variation (CV %)
Estradiol	0 - 3771 pmol L^{-1}	36.71 pmol L^{-1}	6.72	
			4.14	5.90
Testosterone	0 - 57.95 nmol L^{-1}	0.076 nmol L^{-1}	5.55	
			4.82	5.28
Thyroxine	0 - 411.84 nmol L^{-1}	7.72 nmol L^{-1}	4.25	
			3.55	4.08

2.6. Explanatory variables and predictive modelling procedure

The GLM was used in order to evaluate the simultaneous effect of the explanatory variables (independent variables) over the hormone levels measured in the green turtles, and to fit a predictive model for each hormone in terms of the explanatory variables with significant contribution (Kleinbaum and Kupper, 1978; Lindsey, 1997). Considering the error distribution of the hormone levels (dependent variables) GLM was performed using a *Poisson* error distribution with the *log* canonical link function when E_2 and T were evaluated and a *Normal* error with *identity* link for T_4 (Lindsey, 1997; Zuur et al., 2009). The *Poisson* GLM deals with the heterogeneous count data of the sex steroids and ensures non-negative outputs (Zuur et al., 2009). The fitted value of (y) is obtained by the using the inverse function of the canonical link function (e^x) (Crawley, 2007). The explanatory variables included in the analyses were size (cm), mass (kg), body condition (K*rel*), season (winter and summer), study site (PAO, LSI and BMA), and the concentration of biochemical parameters of the sea turtles (cholesterol, mmol L^{-1}; triglycerides, mmol L^{-1}; total proteins, g L^{-1}, and glucose, mmol L^{-1}) reported by Labrada-Martagón et al. (2010a) (Table 2). These serum metabolites were included considering their utility as biomarkers of body condition and nutritional state of green turtles (e.g. higher triglycerides and total proteins in organisms with higher body condition) (Hamann et al., 2005; Labrada-Martagón et al., 2010a,b).

The maximal model includes all the explanatory variables of interest (Crawley, 2007). In this study, the maximal models explored were those in which the relationship between the response variable and the explanatory variables had plausible biological explanations. When analyzing T and E_2 as dependent variables, T_4 data were included as one of the covariates to evaluate its relationship with the sex steroids, given its behavioral (e.g. to promote mating) and physiological functions associated with reproduction in reptiles (Dickhoff and Darling, 1983; Licht et al., 1985b; Moon et al., 1998). In the analyses for the creation of the E_2 model the concentration of T was incorporated as an independent variable due to its relationship with the former (Randall et al., 1997).

The random effect of the *year* to the hormone levels was evaluated using linear mixed effects models. The annual random effect was evaluated by estimating the variance around the intercept of the model, which is given by the different years of capture; a small variance ($d^2 = 0$) means that there is a lack of difference between the intercepts given by the years and the fitted line of the population model, and vice versa (Zuur et al., 2009). The best model (with or without the random effect of year) was chosen for each hormone by comparing the deviance residual (Bates et al., 2011). The software used was R v.2.14.0 and the models were fit by using the "stats" package (R Development Core Team, 2011) or the "lme4" package to fit the mixed effect models (Bates et al., 2011) accordingly. The simplification of the models was done by testing the significance of each variable in turn, at each step (Rodríguez-Estrella and Sánchez-Colón, 2004; Crawley, 2007). In this manner, all the alternative models were evaluated by adding variables (forward stepwise), extracting them (backward stepwise) or both simultaneously (forward/backward

Table 2
Concentration of sex steroids, thyroxine and biochemistry parameters in the East Pacific green sea turtle (*Chelonia mydas*) by study site.

Zone	Punta Abreojos				Laguna San Ignacio				Bahía Magdalena			
	Mean	SE	Range	n	Mean	SE	Range	n	Mean	SE	Range	n
SCL (cm)	65.16	1.95	43.9 – 92.4	37	49.74	2.36	39.7 – 62.2	10	58.23	1.64	46.3 – 80.3	22
Estradiol (pmol L^{-1})	195.19	30.06	21.25 – 1005.85	37	176.94	73.57	2.61 – 686.5	9	114.24	24.5	6.72 – 249.4	13
Testosterone (nmol L^{-1})	8.95	1.98	0.21 – 59.34	35	13.53	7.32	0.10 – 77.21	10	4.06	1.42	0.31 – 17.8	16
Thyroxin (nmol L^{-1})	27.67[a]	1.67	8.36 – 43.76	37	32.82[a,b]	1.29	27.03 – 39.38	10	33.98[b]	0.90	22.78 – 42.6	22
Cholesterol (mmol L^{-1})*	43.76[a]	2.3	20.98 – 84.43	37	36.32[a,b]	7.76	13.47 – 95.83	10	35.05[b]	5.04	14.50 – 57.62	10
Triglycerides (mmol L^{-1})*	1.83	0.21	0.27 – 5.47	36	1.67	0.22	0.89 – 2.94	10	1.26	0.25	0.45 – 2.97	9
Total Proteins (g L^{-1})*	56.3[a]	1.8	11.55 – 79.4	37	58.4[a,b]	6.7	28 – 96	10	47[b]	2	33 – 55.5	10
Glucose (mmol L^{-1})*	7.12	0.38	3.97 – 16.54	37	7.6	0.30	6.46 – 8.91	10	7.9	0.64	5.11 – 11.43	10

Abbreviations: SE, standard error; SCL, straight carapace length; Superscript letters denote statistically significant differences among groups, $p < 0.05$; * denote data reported by Labrada-Martagón et al. (2010a).

stepwise procedure). The selection of the minimal adequate model between stepwise procedures was done based on the residual deviance, a goodness of fit criterion (Zuur et al., 2009). The significant level ($p \leq 0.05$) was used to evaluate on each step time the contribution of the explanatory variables (Zuur et al., 2009). The parsimony criterion was also considered in the selection of the minimal adequate model. Finally, as a diagnostic method and model validation, the distribution of the residuals was evaluated by using the ordinary residuals for the model with *identity* link function and the deviance residuals for those models with *log* link function, to look for heteroscedasticity (trends in the degree of scatter of the residuals) (Zuur et al., 2009).

3. Results

From 2005 to 2007, a total of 69 sea turtles were captured, 37 in PAO, 10 in LSI and 22 in BMA. The predominant size classes in the three sites were juveniles (95.6% of the total captured). According to the external characteristics, seven organisms (10%) were classified as female adults due to their size (>77.3 cm LRC) and absence of secondary sexual characters such as tail size. From the total individuals classified as adults, 85.7% were captured in PAO ($n = 6$) and only 14.3% in BMA ($n = 1$). The SCL of the sea turtles captured in PAO was higher than those captured in LSI and BMA ($F_{(2,66)} = 9.96$, $p < 0.001$). In PAO the SCL ranged between 44 and 92.4 cm (mean ± SD, 65 ± 11.9 cm), in LSI the SCL ranged between 40 and 62.2 cm (49.7 ± 7.5 cm) and in BMA between 46.3 and 80.3 cm (58.2 ± 7.7 cm) (Fig. 1).

3.1. Handling time

The handling time was lower in PAO (20–183 min) in comparison with LSI (100–585 min) and BMA (25–840 min) ($H_{(2,51)} = 11.6$, $p = 0.003$). When data for all the sea turtles were plotted together, the variability in the hormone concentrations measured was evident in those animals in which the handling time exceeded 90 min (Fig. 2). Nevertheless, the hormone levels were not statistically different between the handling time categories created ($p > 0.05$) in the sea turtles from PAO and BMA. It was not possible to evaluate differences in hormone concentration for LSI between time categories. With exception of the E_2 levels of the sea turtles from BMA that were negatively correlated with handling time ($r_s = -0.51$, $p = 0.03$), the rest of the hormones levels measured were not correlated with the handling time employed ($p > 0.05$).

3.2. Sex steroids and thyroxine

The ranges and geometric mean of the hormone levels by study site are shown in Table 2. Only one sea turtle from LSI, classified as juvenile ($E_2 = 688.7$ pmol L^{-1}, $T = 76.34$ nmol L^{-1}), and one individual from PAO, classified as adult female ($E_2 = 1007.7$ pmol L^{-1}, $T = 59$ nmol L^{-1}), had sex steroid levels two times higher than the maximum value of the rest of the organisms. Without considering these 2 outliers, T levels in the three study sites were <34.7 nmol L^{-1}, E_2 levels <532.3 pmol L^{-1} and T_4 levels <43.75 nmol L^{-1}.

There were no differences in the hormone concentrations between age classes in any study site. There were no significant differences between seasons in the hormone concentrations of the individuals ($p > 0.40$). Inter-annual differences were found only in PAO, where the T_4 levels were higher in the organisms captured during 2006 in comparison with those of 2005 ($U_{(9,28)} = 28$, $p < 0.001$). Temporal differences could not be evaluated for individuals from LSI since they were only captured during the summer of 2006. When comparing study sites only T_4 levels were higher in sea turtles from BMA in comparison with PAO ($H_{(2,69)} = 6.54$, $p = 0.03$) (Table 2).

3.3. Fitted models

The equations for the prediction of the hormone levels of the East Pacific green turtles inhabiting the coast of BCS generated by using the parameters estimated by the minimal adequate models are presented by season and study site (Table 3).

3.3.1. Estradiol

The minimal adequate model to explain the E_2 concentration was the one fitted by using the *Poisson* error distribution (Table 4), according to the differences in the residual deviance between link functions (*Poisson*, 1429 residual deviance, 34 degrees of freedom; *Normal* 278140 residual deviance, 34 degrees of freedom). The fitted model explains 80% of the variance and suggests that the E_2 concentration was significantly related to an increase in cholesterol, glucose, T, and T_4 levels, was negatively correlated with the SCL, and that the hormone levels differ between seasons and study sites (Table 4).

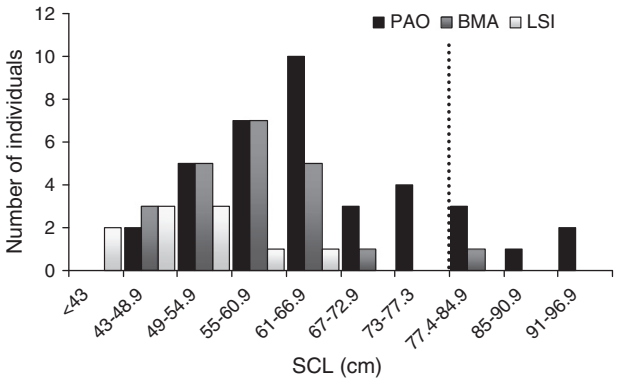

Fig. 1. Number of green sea turtles (*Chelonia mydas*) by size intervals. Dotted line represents the mean nesting size at Michoacán (77.3 cm SCL) according to Seminoff et al. (2003). (PAO = Punta Abreojos; LSI = Laguna San Ignacio; BMA = Bahía Magdalena.)

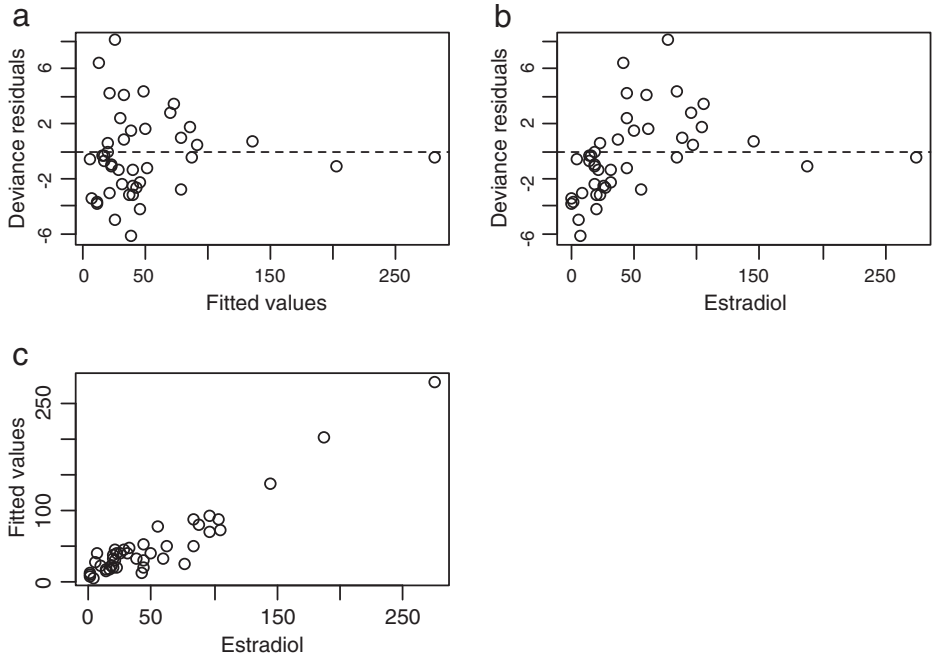

Fig. 2. Residuals plots of the minimal adequate model for the estradiol concentration of the East Pacific green sea turtles (*Chelonia mydas*) captured in the occidental coast of Baja California Sur.

No random effect of the variable *year* to the response variable (E_2 concentration) was found (variance $d^2 = 0.043$) and its inclusion did not improve the fitted model. The residuals diagnostic confirmed the adequacy of the fitted model (Fig. 2). A reasonably linear relationship between the fitted values and the E_2 data measured can be observed (Fig. 2).

3.3.2. Testosterone

The minimal adequate model to explain T concentration was the one fitted by using the *Poisson* error distribution (*Poisson*, 332 residual deviance, 36 degrees of freedom; *Normal* 6551 residual deviance, 36 degrees of freedom). The fitted model explained 49% of the T concentration by an increase in mass, negative relationships with SCL, cholesterol and glucose levels, and with a significant effect of the zone (Table 4). Annual random effect to the intercept of the fitted line in the model was not found (variance $d^2 = 0.0$). The residuals showed that the fitted values could be influenced by the higher values of T concentration (Fig. 3).

3.3.3. Thyroxine

The minimal adequate model for the T_4 concentration of the East Pacific green sea turtle was the one fitted by the link function *identity*. This model explained 23% of the variance of T_4 levels. The T_4 concentration was positively related to the glucose concentration and negatively to the SCL; nevertheless, the coefficient estimated for the *size* variable was not statistically significant ($b = 0$, $p < 0.07$) (Table 4). The inclusion of the *year* did not contribute to fit a better model (variance $d^2 = 0.27$), there was not a significant difference between models, with or without considering the annual effect ($p < 0.07$). The residuals of the model showed an evident constant variance and a normal distribution as expected for a fitted model (Fig. 4).

4. Discussion

Statistical models are simplified representations of reality that attempt to describe the relationship between independent and dependent variables with a probabilistic component, which involves the inclusion of variability due to unknown random factors (Hilborn and Mangel, 1997; Lindsey, 1997). These models smooth out the random irregularities in the data while attempting to detect patterns within (Lindsey, 1997). Regression models are the standard form of such descriptions and are the best single representation of the data (Hilborn and Mangel, 1997; Katz, 2006). The multiple parameters estimated from the data describe in various ways the variability or

Table 3
Minimal adequate models for the hormone concentrations of the East Pacific green sea turtle (*Chelonia mydas*) inhabiting in the occidental coast of Baja California peninsula.

Hormone	Study site	Season	Model	Median observed data	Median fitted data
Estradiol	PAO	Summer	$E_2 = e^{3.72+0.02Cholesterol+0.11Glucose+0.051T+0.012T_4-0.018SCL}$	150.14	145.85
		Winter	$E_2 = e^{4.49+0.02Cholesterol+0.11Glucose+0.051T+0.012T_4-0.018SCL}$	309.1	322.8
pmol L^{-1}	LSI	Summer	$E_2 = e^{1.95+0.02Cholesterol+0.11Glucose+0.051T+0.012T_4-0.018SCL}$	16.41	41.74
		Winter	$E_2 = e^{2.72+0.02Cholesterol+0.11Glucose+0.051T+0.012T_4-0.018SCL}$	nd	nd
	BMA	Summer	$E_2 = e^{2.52+0.02Cholesterol+0.11Glucose+0.051T+0.012T_4-0.018SCL}$	59.65	77.86
		Winter	$E_2 = e^{3.29+0.02Cholesterol+0.11Glucose+0.051T+0.012T_4-0.018SCL}$	60.5	63.4
Testosterone	PAO	Any	$T = e^{8.2 + 0.07Weight - 0.1SCL - 0.019Cholesterol - 0.244Glu\cos e}$	5.10	7.08
nmol L^{-1}	LSI	Any	$T = e^{9.32 + 0.07Weight - 0.1SCL - 0.019Cholesterol - 0.244Glu\cos e}$	14.89	19.74
	BMA	Any	$T = e^{6.97 + 0.07Weight - 0.1SCL - 0.019Cholesterol - 0.244Glu\cos e}$	0.55	1.53
Thyroxine nmol L^{-1}	Any	Any	$T_4 = 28.21 + 1.86Glu\cos e - 0.21SCL$	30.63	28.83

Abbreviations: E_2, estradiol; T, testosterone; T_4, thyroxine; SCL, straight carapace length; PAO, Punta Abreojos; LSI, Laguna San Ignacio; BMA, Bahía Magdalena; nd, no data available.

Table 4
Coefficients (*a*, *b*) fitted by the generalized linear models (GLM) with a *Poisson* error distribution for the hormone concentrations of the East Pacific green sea turtle (*Chelonia mydas*).

Model	Variable	Unstandardized coefficients		z	p	Res. dev. minimal model	95% Confidence interval for b	
		b	Std. error				Lower bound	Upper bound
Estradiol	Intercept	3.290	0.097	34.024	<0.001	1429	3.100	3.479
	SCL	−0.018	0.001	−13.888	<0.001		−0.020	−0.015
	Cholesterol	0.020	0.001	21.492	<0.001		0.018	0.022
	Glucose	0.110	0.008	13.755	<0.001		0.094	0.126
	Thyroxine	0.012	0.001	8.486	<0.001		0.010	0.015
	Testosterone	0.051	0.001	58.119	<0.001		0.049	0.052
	Season[Summer]	−0.773	0.039	−19.742	<0.001		−0.850	−0.696
	Site [LSI]	−0.565	0.078	−7.234	<0.001		−0.718	−0.412
	Site [PAO]	1.205	0.055	21.952	<0.001		1.098	1.313
Testosterone	Intercept	6.967	0.706	9.869	<0.001	332	5.573	8.343
	Weight	0.067	0.006	10.518	<0.001		0.054	0.079
	SCL	−0.100	0.013	−7.623	<0.001		−0.125	−0.074
	Cholesterol	−0.019	0.003	−5.566	<0.001		−0.026	−0.012
	Glucose	−0.244	0.029	−8.293	<0.001		−0.304	−0.189
	Site [LSI]	2.348	0.293	8.005	<0.001		1.808	2.967
	Site [PAO]	1.232	0.289	4.264	<0.001		0.702	1.843
Thyroxine	Intercept	28.208	7.909	3.567*	<0.001	2922	12.707	43.708
	SCL	−0.209	0.114	−1.839*	0.073		−0.432	0.014
	Glucose	1.857	0.601	3.089*	0.004		0.679	3.036

Abbreviations: Std. Error, standard error; Res. Dev., residual deviance; SCL, straight carapace length; LSI, Laguna San Ignacio; PAO, Punta Abreojos; *denotes t-Student test value obtained when using the link function *identity*.

dispersion of the responses (Lindsey, 1997). In other words, the mean value of the outcome changes linearly with multiple independent variables (Katz, 2006). In this study, significant linear models were built to explain the hormone levels of immature East Pacific green sea turtles inhabiting the coast of BCS. The information derived from the multivariate models created had not been elucidated before upon univariate analyses used in this or previous studies (Labrada-Martagón, 2011). The GLM successfully explained biologically plausible relationships between the hormone concentrations measured in the sea turtles and multiple explanatory variables, such as size, mass and blood biochemistry parameters of the individuals, and helped to clarify the effect of season, year and site of capture, as well.

The GLM are an extension of the linear regression analysis and could be used under many error distributions of the data without the necessity of assuming the normality of the response variable (Lindsey, 1997; Zuur et al., 2009). The GLM fitted a better model for the T_4 levels by using the link function *identity*, due to the normal distribution of this variable. In contrast, the GLM with the link function *log* for the *Poisson* error distribution was the best option for the sexual steroids data. GLM are excellent tool to deal with errors of the not

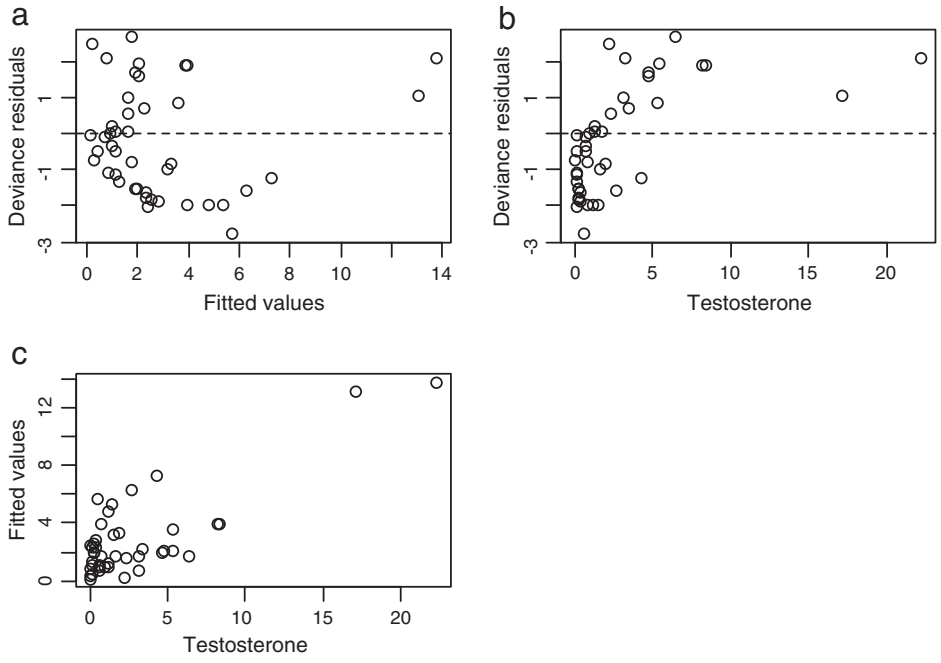

Fig. 3. Residuals plots of the minimal adequate model for the testosterone concentration of the East Pacific green sea turtles (*Chelonia mydas*) captured in the occidental coast of Baja California Sur.

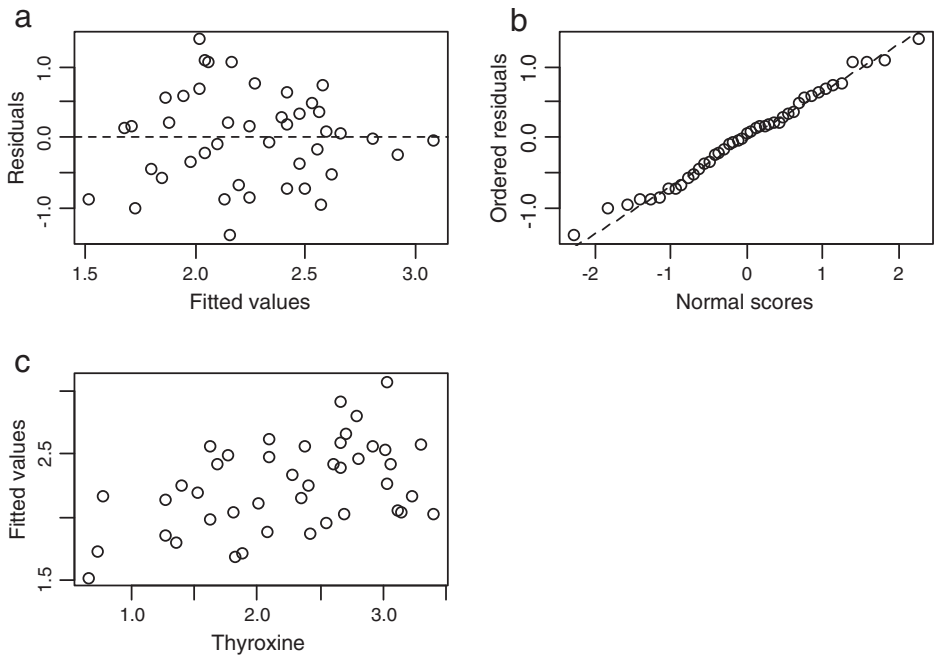

Fig. 4. Residuals plots of the minimal adequate model for the thyroxine concentration of the East Pacific green sea turtles (*Chelonia mydas*) captured in the occidental coast of Baja California Sur.

normally distributed response variable due to the link function, which relates the mean value of the response variable (y) to its linear predictors (Zuur et al., 2009). The performance of a variety of models can be compared when different link functions are used (*Normal vs Poisson* in this study) (Crawley, 2007).

When using the GLM with both, *identity* link or *log* link, to identify the explanatory variables related to the hormone levels, all the stepwise procedures generated the same minimal adequate model without discrepancies. These findings suggest consistency in the fitted models, independently of the analysis or simplification procedures used, and could be the result of the orthogonal data base employed. It is important, before modelling, to assess the correlation levels among explanatory variables, and to consider omitting highly related variables and missing treatment combinations, in order to avoid the effects of multicollinearity and the lack of constant variation during model simplification (Katz, 2006; Crawley, 2007).

In general, the models correctly predicted the median levels of the measured hormones in the green sea turtles (Table 3). For E_2 concentrations, the GLM generated a more complex model, which explains why it had significantly higher predictive potential than the other models, according to the lower residual deviance (variance not explained by the model), narrow interval coefficients of the estimated parameters and by the straight linear relationship between the fitted and the measured values. Even so, the predictive power of the models should be assessed with validation tests using new and independent data (Rodríguez-Estrella and Sánchez-Colón, 2004).

In this study, sex steroid concentrations of the sea turtles were explained by the combination of multiple covariates. The relationships between cholesterol, T and E_2 levels explained by the models are not metabolically surprising. Cholesterol is the precursor of the three major classes of hormones, including the reproductive ones; modification of the cholesterol structure yields a large number of related steroid hormones including T (Randall et al., 1997). E_2 is the product of the aromatization of T and, therefore, the presence and quantity of both hormones are closely related (Hernández-López and Cerda-Molina, 2012). The differences in the sex steroids levels, denoted by the models and related to environmental factors (such as season and study area), are in agreement with previous reports. The seasonal differences in the predicted E_2 levels of the sea turtles coincided with the seasonal changes in the composition of the diet and with the reduction of the activity of the animals, lipid content in blood (energy stores) and body condition during winter in the BCS population (Lopez-Mendilaharsu et al., 2003; Koch et al., 2007; Labrada-Martagón et al., 2010a,b). Specific nutritional (e.g. blood biochemistry parameters), physiological (e.g. antioxidant defenses) and morphological (body condition index) conditions and, therefore, differences in the growth rate of the green sea turtles between feeding grounds in BCS, have been attributed to differences in the quality and availability of food and to specific habitat and environmental conditions (Koch et al., 2007; Labrada-Martagón et al., 2010a,b, 2011; López-Castro et al., 2010). According to the models of the sex steroids fitted by study site, the organisms captured in PAO had higher concentrations of these hormones. The regional differences found in the sex steroids levels were also explained in the model by the differences in size and mass range of the individuals; these morphological variables contributed significantly to the measured hormone levels. Individuals inhabiting PAO had the widest size range, while the sea turtles from LSI were significantly smaller in comparison with other coastal lagoons (López-Castro et al., 2010). The lack of effect of year on hormone levels could be related to the prevailing environmental conditions of the region. Sea turtles inhabiting tropical or subtropical oceans, such as those from this study, could also have minor hormonal variations in an annual cycle and between years, due to the thermal stability of the environment (Licht et al., 1985a; Wibbels et al., 1990).

This study confirmed previous suggestions (Caldwell, 1962; Owens et al., 1978) that the size of the organisms and the absence of secondary sexual characters, such as tail size, are not good indicators of the reproductive stage of the East Pacific green sea turtles when used alone. No differences in the sex steroid hormone levels were found between age classes; only one (14%) sea turtle from those classified as adult females (>77.3 cm SCL, n = 7) had sex steroid concentrations within the ranges reported for adults in the nesting period (Wibbels et al., 1987, 1992). The energy stores for reproduction (stored lipid content) must be obtained prior to the breeding season and the migration to the nesting areas. Plasma triglyceride levels, protein concentration and body condition are the main factors involved in the regulation of the reproductive status during breeding and nesting (Hamann et al.,

2002, 2005). The lack of correlation between sex steroids and those variables during modelling aided in confirming the reproductive status (juveniles) of the population inhabiting the feeding grounds of BCS. The endocrine data of this study suggests that the green sea turtles inhabiting BCS may reach sexual maturity at sizes larger than the mean nesting size, criterion commonly used to establish the population structure for the East Pacific green sea turtle (Seminoff et al., 2002, 2003; Koch et al., 2007; López-Castro et al., 2010).

The glucose concentration, together with the SCL, contributed to explain the T_4 concentration of the green sea turtles. The activation of the thyroid system is the endocrine signal of the appropriate fitness and nutritional status of the organism to proceed with the physiological functions associated with an increased energetic cost (Dickhoff and Darling, 1983). Increased caloric intake, with carbohydrate-rich diets, results in an increase of peripheral T_4 levels, a relationship which was explained by the model in this study. Prolonged fasting or stress, under unfavorable environmental conditions, decreases the thyroid function and T_4 plasma levels (Dickhoff and Darling, 1983; Hadley, 1992). Captive sea turtles fed artificial food, enriched with elevated protein concentrations, had higher T_4 levels than wild sea turtles (Moon et al., 1998). The average T_4 (283 nmol L^{-1}) concentration of the sea turtles from this study was higher than that reported in free living immature green sea turtles, and was higher than the T_4 levels found in nesting green sea turtles in Michoacán and in Australia (Moon et al., 1998). In contrast, the ranges of the T_4 levels are within those reported for captive immature and male adult green sea turtles under constant feeding and water temperature (26 °C) (Licht et al., 1985a; Moon et al., 1999). This information corroborates that the green sea turtles from BCS were in an adequate nutritional and metabolic condition, as has been suggested before and denoted by the body condition of the individuals (Labrada et al., 2010a,b).

Studies on the T_4 levels in sea turtles are not common and its function in this group remains unknown (Licht et al., 1985b; Moon et al., 1998). In reptiles, such as those from the order squamata, the concentration of T_4 appears to be related to the behavior (e.g. to promote mating) and physiological functions associated with an increased energetic cost, such as that associated to growth, reproduction and nutrient assimilation (Dickhoff and Darling, 1983; Licht et al., 1985b; Moon et al., 1998). The results from this study confirm the relationship between T_4 levels and the sex steroid concentrations in green sea turtles, suggesting some physiological function of the thyroid hormone related probably to growth, maturity and reproduction of these turtles. However, a direct causal relationship between hormones should not be considered. The multivariate statistical models describe the variability of the responses of the dependent variables (sex steroids) related to multiple effects of the parameters involved (Lindsey, 1997; Katz, 2006). The regulation of hormone secretion and the endocrine action is achieved by coordination of a complex set of signals acting in the endocrine tissues, which responds directly to conditions of the extracellular (e.g. changes in ambient temperature) or intracellular (e.g. reduced glucose level) environment (Randall et al., 1997).

To the authors' knowledge, this is the first report concerning sex steroids and thyroid hormone levels of healthy, immature East Pacific green sea turtles; it provides novel information that can be used as reference for future studies. Lack of statistical differences in the hormone concentrations (T_4 or sex steroids) of the juveniles of this study between handling time categories coincides with previous reports where T levels of immature hawksbill turtles (*Eretmochelys imbricata*) and non-breeder male green turtles did not change after 5–8 h of capture stress protocol, even when the levels of corticosterone in those animals increased (Jessop et al., 2002, 2004). The increased variability of the hormone concentrations of those animals in which the handling time was >90 min and the relationship between the handling time and the E_2 levels of individuals from BMA (the site with the widest handling time range recorded) denotes the need to reduce the handling time and stress conditions and should not be ignored in future studies.

Models are constrained by assumptions and the available data; confirmation of the fitted models utility and precise quantitative predictions will be possible when tested against new additional data (Lindsey, 1997; Heppell et al., 2003). It is important to emphasize, for future comparisons and validations, that the measurements of the hormone levels of this study are not basal levels taken at time 0–15 min after capture, period which is reported to have no effect on the sex steroids levels due to the capture/restrain protocol (Jessop et al., 1999, 2002, 2004; Blanvillain et al., 2008). All the paired measures of the parameters considered in the models in this study came from the same samples, taken under similar handling time conditions. The capture stress protocol (capture, handling and restraint) assumes that all individuals are stressed in a comparable manner, across seasons, populations and even between species (Wingfield et al., 1997).

Due to the limited knowledge concerning the East Pacific green sea turtle population, modelling may be considered as exploratory and as hypothesis generators (Lindsey, 1997). The qualitative predictions presented in this study are generally reliable (e.g. regional and seasonal differences) and the information gathered regarding the relationships found between hormone levels with the morphology and biochemical parameters are biologically plausible and interesting. The use of statistical and mathematical modelling is a promising tool towards the understanding of the physiological processes and health status of the sea turtles. These tools have been successfully used in other areas of knowledge, such as population dynamics of sea turtles (e.g. Márquez et al., 1995; Heppell and Crowder, 1996; Bjorndal et al., 2000, 2003; Chaloupka and Balazs, 2007).

5. Conclusions

GLM analyses appear to be effective and practical statistical tools to predict sex steroids (T and E_2) and T_4 levels in immature East Pacific green sea turtles, and to evaluate the relationships between multiple blood biochemistry parameters, morphologic variables and temporal and regional factors over the hormone levels. The E_2 levels were related to an increase in cholesterol, glucose, T and T_4 levels in serum of the individuals with a significant effect of the season and study site. The T levels were related to decreased cholesterol and T_4 levels with regional differences in the hormone predictions. Independently of the season or study site, an increase in the T_4 concentration of the green turtles could be expected if an increase in the glucose level in serum of the individuals is measured. The SCL and mass of the organisms were morphological covariates with a significant contribution during modelling that has to be taken into account to explain the endocrine patterns of the green turtles. The generation of new, additional data and endocrine basal levels for the East Pacific green sea turtle population are recommended in order to validate the relationships found in this study and to continue evaluating population dynamics aspects in order to satisfactorily contribute to the conservation biology of the species.

Applying alternative statistical methods, such as GLMs, to complex ecological and physiological datasets can contribute to better understand the metabolic relationships between T_4, steroid hormones and other physiological and environmental variables in this and other wildlife species, particularly those inhabiting remote areas and those in which experimental manipulation is not possible (e.g., threatened or endangered species). In addition, GLMs can be used to understand the endogenous variability of biomarkers and which biological and exogenous factors are associated to them, as well as to evaluate which biomarkers are sufficiently sensitive and efficient to be used in health status assessment studies of wildlife populations. The latter could provide insight towards biologically and ecologically sound population management recommendations before the responses to environmental changes and habitat perturbation negatively affects population dynamic processes.

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