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Biochemical indices as correlates of recent growth in juvenile green turtles (*Chelonia mydas*)

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ABSTRACT

Nucleic acid and protein concentrations and their ratios are increasingly used as correlates of nutritional condition and growth in marine species. However, their application in studies of reptile growth has not yet been validated. The green turtle (*Chelonia mydas*) is an endangered marine reptile for which assessing population health requires knowledge of demographic parameters such as individual growth rates. The purpose of this study was to evaluate a number of biochemical indices ([DNA], [RNA], RNA:DNA ratio, [protein], protein:DNA ratio, and RNA:protein ratio) in liver, heart, and blood as potential predictors of recent growth rate in juvenile green turtles under controlled feeding conditions. Intake of juvenile green turtles was manipulated over twelve weeks to obtain a range of growth rates. With the exception of $[RNA]_{blood}$, $[DNA]_{heart}$, and $[protein]:[DNA]_{liver}$, all biochemical indices demonstrated significant linear relationships with growth rate during the last 1.5 weeks of the study. The best single predictors of recent growth were hepatic [RNA] and [RNA]:[protein], which explained 66% and 49%, respectively, of the variance in growth. Contrary to expectations, these two indices were negatively correlated with growth rate. To investigate the possibility that hepatic [RNA] was higher in slow-growing turtles because of elevated expression of antioxidant genes, we quantified glutathione peroxidase activity and total antioxidant potential. Both measures of antioxidant function were affected by intake and growth histories, but these effects did not explain our results for hepatic RNA and protein concentrations. We developed a model that predicted 68% of the variance in specific growth rate (SGR) with the equation $SGR = -0.913(\ln[RNA]_{liver}) + 17.689(\text{Condition Index}) + 4.316$. In addition, our findings that [DNA] and [RNA]:[DNA] for blood were significantly correlated with SGR demonstrate the potential utility of minimally invasive tissue sampling that could facilitate instantaneous population monitoring.

1. Introduction

The green turtle, *Chelonia mydas*, is an endangered marine herbivore with a circumglobal distribution (Seminoff, 2002). Human exploitation of this species during the last several centuries has caused drastic population declines (Jackson et al., 2001). Assessing the current status and predicting the viability of wild populations of *C. mydas* requires the quantification of demographic parameters such as individual growth rates. However, measuring growth rates for long-lived and far-ranging green turtles typically entails either time-consuming mark and recapture programs in which recapture probabilities can be quite low (Limpus, 1992) or repeated sampling of a population for use in length-frequency analyses (Bjorndal et al., 1995). Typically, growth intervals of less than one year are considered unreliable for marine turtles because of the potential for errors in morphometric measurements (Chaloupka and Musick, 1997). Furthermore, growth rates calculated using morphometrics represent long-term, cumulative changes and often do not correlate well with biochemical indices of short-term growth (i.e., size changes that occur over the course of days to weeks) due to differences in the latency of these responses to environmental influences (Ferron and Leggett, 1994; Gilliers et al., 2004). Establishing techniques to supplement mark-recapture and length-frequency analyses for estimating recent growth rates of turtles upon first capture would substantially improve our ability to evaluate the instantaneous status of *C. mydas* populations.

Macromolecular indices (RNA concentrations, RNA:DNA ratios, RNA:protein ratios, and/or protein:DNA ratios) are frequently measured as indicators of protein synthesis potential and growth in marine fish and invertebrates (Bulow, 1970; Carter et al., 1998; Buckley et al., 1999; Dahlhoff, 2004; Caldarone, 2005; Mercaldo-Allen et al., 2006; Vidal et al., 2006). These indices are particularly useful for evaluating recent environmental conditions, as they reflect differences in growth rates over a period of several days (Rooker and Holt, 1996; Buckley et al., 1999; Vrede et al., 2002). The use of these indices depends on the assumption that total RNA content of a cell increases as the cellular demand for

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protein synthesis and growth increases (Buckley et al., 1999), while DNA content per cell should be relatively constant (Wallace, 1992). The RNA: DNA ratio is therefore an index of putative cellular protein synthesis capacity. Because nucleic acid concentrations and the ratios between them respond rapidly to fluctuations in food availability, they are considered reliable indicators of instantaneous condition and growth (Rooker et al., 1997; Okumura et al., 2002; Islam and Tanaka, 2005; Vidal et al., 2006). Despite widespread use as sensitive measures of recent growth rates in marine fish and invertebrates, nucleic acid ratios have not been validated for application to studies of reptile growth.

The purpose of this study was to evaluate the combined use of morphometric and biochemical indices as predictors of growth rates during the preceding 1.5 weeks in green turtles maintained under controlled feeding conditions. We also quantified glutathione peroxidase (GPX) activity and total antioxidant potential (AP) to investigate whether differences in biochemical indices could be explained by differences in antioxidant function, as intake has been shown to affect mRNA expression of antioxidant enzymes (Deruisseau et al., 2006). Liver and muscle (including the heart) are commonly sampled in studies of this kind, so our first objective was to evaluate the degree of concordance between our observed biochemical indices of growth in green turtles and published indices in other species. Because our ultimate goal is to develop new protocols for assessing growth rates of wild turtles, we also examined the potential for measuring nucleic acid concentrations in a tissue (whole blood) that can be obtained without destructive tissue sampling. Validating a blood-based physiological index is the first step toward development of minimally invasive growth measurement strategies.

2. Materials and methods

2.1. Animal care and morphometric measurements

A twelve-week feeding trial was conducted at the Cayman Turtle Farm in Grand Cayman, British West Indies, in accordance with the policies of the Institutional Animal Care and Use Committee at the University of Florida. *C. mydas* hatchlings were housed individually in sea water in 68-liter outdoor tanks and were fed turtle pellets (Melick Aquafeed, Catawissa, PA) twice daily. High and low water temperatures respectively averaged approximately 32.5 °C and 28.5 °C at the beginning of the study and 29.5 °C and 25.5 °C by the end of the study (Roark et al., in press). Prior to the beginning of the study, turtles were all fed ad libitum for one week to establish initial average daily intake (ADI). During the study, turtles in the ad libitum group (AL) were offered an excess of food each day for 12 weeks, turtles in the restricted group (R) were fed 50% of initial ADI each day for 12 weeks, and turtles in the restricted-ad libitum group (R-AL) were food-restricted for five weeks and then were fed ad libitum for seven weeks. The restricted ration was more than sufficient to fulfill basal metabolic requirements, as food-restricted turtles continued to gain weight throughout the trial. This treatment schedule elicited faster size-specific growth in R-AL turtles in weeks 7 through 10 (after the switch to ad libitum feeding) compared to the growth demonstrated by AL turtles during the same time interval (Roark et al., in press). Additional details about animal husbandry can be found in Roark et al. (in press).

Each turtle was weighed and measured weekly throughout the study. Data for body mass (BM) and straight carapace length (CL) were used to calculate specific growth rates (SGR) for each turtle during the final 10–11 days of the study according to the following equations:

$$SGR_{bm} = \left(\ln\left[BM_f\right] - \ln\left[BM_i\right]\right)*100 / t$$

$$SGR_{cl} = \left(\ln\left[CL_f\right] - \ln\left[CL_i\right]\right)*100 / t$$

where BM_i and CL_i represent body size 10 or 11 days prior to tissue sampling and BM_f and CL_f represent body size on the day of tissue sampling. Condition index (CI) was calculated as Fulton's K ($CI = BM_f / CL_f^3$; Ricker, 1975).

Analysis of variance (ANOVA) was used to compare BM_f, CL_f, CI, SGR_{bm}, and SGR_{cl} among treatment groups. If ANOVA revealed a significant difference, pairwise comparisons were evaluated using Tukey's Honestly Significant Different post hoc test (if variances were equal) or Tamhane's T2 post hoc test (if variances were unequal). All data were tested for normality (Shapiro–Wilk test) and homogeneity of variances (Levene's test) prior to parametric analysis and transformed, if necessary, to satisfy both assumptions. If transformation did not improve normality, data were tested using a Kruskal–Wallis test followed by pairwise Mann–Whitney tests with a Bonferroni adjustment for multiple comparisons.

2.2. Tissue collection

Turtles were euthanized after the twelve-week feeding trial as part of a larger study (Roark et al., in press). Tissues from these turtles were then analyzed for DNA and RNA concentrations (blood, heart, and liver) as well as protein concentration, glutathione peroxidase activity, and total antioxidant potential (liver). Seven AL turtles, ten R turtles, and ten R-AL turtles were weighed to the nearest 0.1 g, euthanized with an intramuscular overdose injection of ketamine (Ketaset, 100 mg/kg body mass) in the right pectoral muscle, and then decapitated.

A blood sample was collected from the decapitation site (as in Storey et al., 1993; Packard et al., 1997). The heart and a portion of the right lobe of the liver were excised, and blood, heart, and liver samples were snap-frozen in liquid nitrogen no more than 3 min after decapitation. Tissues were maintained at −80 °C until they were assayed as described below. Body composition (water, lipid, protein, and mineral contents) of ten additional turtles from each treatment group was also assessed, and these results are presented elsewhere (Roark et al., in press).

2.3. Nucleic acid and protein assays

Subsamples of frozen whole blood, heart, and liver were weighed, and DNA was isolated with DNeasy® kits (Qiagen Inc., Valencia, CA) using the manufacturer's protocol for animal tissues. To isolate RNA, subsamples of frozen heart and liver tissue (different from those used for DNA isolation) were weighed and then ground in liquid nitrogen using mortar and pestle. Frozen subsamples of blood and ground heart and liver tissue were homogenized using QIAshredder® spin columns (Qiagen Inc.). RNA was then isolated with RNeasy® Mini kits (Qiagen Inc.) using the manufacturer's protocol for isolation of total RNA from animal tissues. DNA and RNA were isolated separately from a minimum of three subsamples of each tissue (assuming a relatively homogeneous tissue composition) from each turtle for a total of at least 18 subsamples for each of 27 turtles, with the exception of a single turtle for which the heart was only large enough to permit extraction of DNA from two subsamples.

Nucleic acid concentrations in each subsample were determined using a PicoGreen® dsDNA quantitation kit (Invitrogen Corporation, Carlsbad, CA) and a RiboGreen® RNA-specific quantitation kit with DNase I (Invitrogen Corporation) by measuring fluorescence at standard fluorescein wavelength settings (485 nm excitation, 528 nm emission) using a fluorescent microplate reader. Data were collected using KCJunior™ data analysis software (Bio-Tek® Instruments, Inc., Winooski, VT).

To determine hepatic protein concentrations, subsamples of liver were homogenized in 1.0 ml of Sigma T-6789 buffer (0.05 M Tris, 0.138 M NaCl, 2.7 mM KCl, pH 8.0 with 1% bovine serum albumin) yielding a 10% weight:volume tissue solution for each sample. Liver homogenates were further diluted to 10%. Total protein concentration of each sample was evaluated using a Bradford assay with standard curves constructed using

dilutions of bovine serum albumin (BSA, 2 mg/ml undiluted). Concentrations of standards (in duplicate) and liver samples (in triplicate) were determined by absorbance at 595 nm with a 695 nm reference wavelength using a microplate reader.

Analysis of variance (ANOVA) was used to compare nucleic acid and protein concentrations and ratios among treatment groups as described above for morphometric indices. Ratios were calculated using concentrations averaged among replicates.

Spearman's rank correlation test was used to evaluate the strength of the relationships among the nucleic acid and protein indices measured. Regression models for predicting SGR_{bm} and SGR_{cl} were then developed using CI and all nucleic acid or protein indices as potential independent variables. Although body length has been correlated with RNA:DNA ratios in fish (Rooker et al., 1997), we did not include treatment group or any measure of total body size as independent variables in our linear regression models. We chose not to include BM or CL as variables because body size was strongly affected by diet treatment (Fig. 1), and the goal of this study was to assess the applicability of nucleic acid and protein measurements in estimating recent growth rates of wild turtles with unknown dietary histories.

Regression equations for SGR_{bm} and SGR_{cl} versus individual nucleic acid and protein indices were determined using least squares linear regression. Data were transformed, if necessary, to linearize them and to improve homoscedasticity. We verified the assumptions of linear regression by visually inspecting plots of studentized deleted residuals versus standardized predicted values.

To construct more comprehensive growth models for predicting SGR, data were analyzed using stepwise multiple linear regression. The same transformations used for linear regressions were used for stepwise multiple linear regressions. Condition index and all nucleic acid and protein indices measured for a single tissue type were included in separate models. A growth model incorporating condition index and all nucleic acid and protein indices for all tissues was also constructed. To enter a model, variables had to meet a 0.05 significance level.

2.4. Antioxidant assays

Glutathione peroxidase activity was evaluated using a total GPX assay modified from Nakamura et al. (1974). Diluted liver homogenate solutions (see above) were incubated for 3 min at 25 °C in a reaction cocktail containing 0.2974 U/ml glutathione reductase, 1.25 mM glutathione, and 0.1875 mM NADPH in a 100 mM potassium phosphate buffer with 10 mM EDTA (pH 7.4). T-butyl hydroperoxide (12 mM) was then added to the reaction mixture and the absorbance of the resulting solution at 340 nm was recorded every minute for 4 min using a microplate reader. A blank consisting of 100 mM potassium phosphate buffer with 10 mM EDTA (pH 7.4) was also assayed to evaluate glutathione-independent reaction rates. Samples and blanks were analyzed in triplicate.

Total GPX activity of each sample was calculated by determining the rate of change in absorbance of NADPH (ΔA_{340}/min, calculated using only linear data) and dividing this value by the extinction

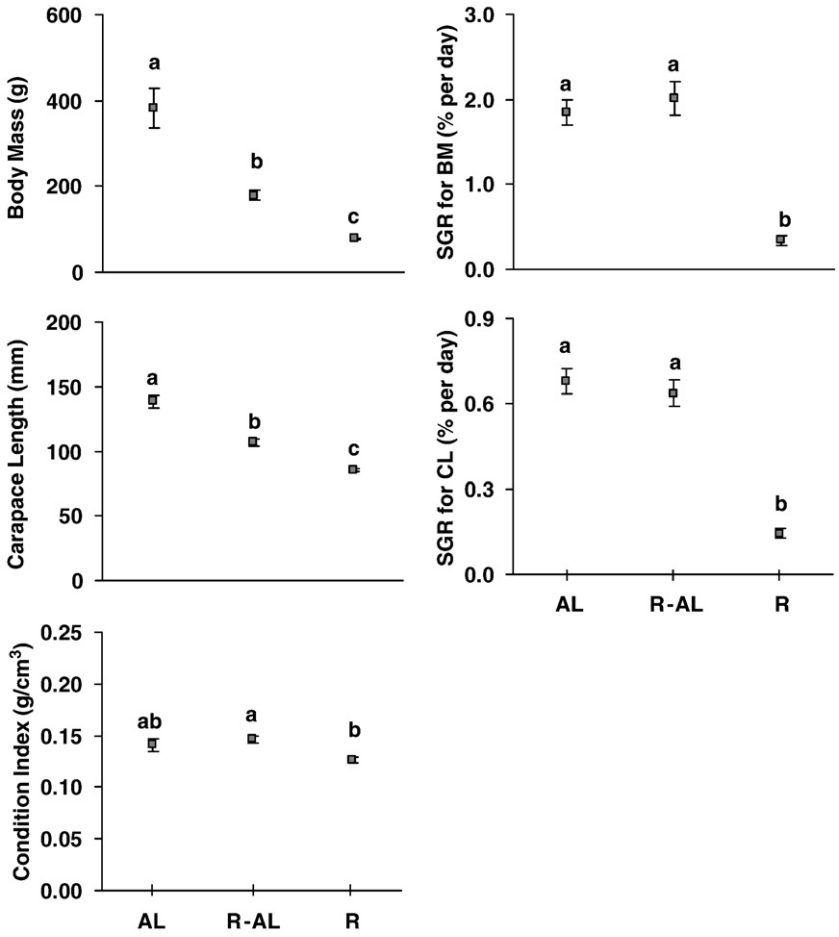

Fig. 1. Morphometric indices and growth rates (mean ± standard error) for turtles in each of three treatment groups at the end of a twelve-week feeding trial. Turtles in the AL group ($n = 7$) were fed ad libitum for 12 weeks. Turtles in the R-AL group ($n = 10$) were fed 50% of initial mass-specific AL intake for 5 weeks and then fed ad libitum for 7 weeks. Turtles in the R group ($n = 10$) were fed 50% of initial mass-specific AL intake for 12 weeks. Means were evaluated using analysis of variance with Tukey's Honestly Significant Difference or Tamhane's T2 post hoc tests (if appropriate) or using a Kruskal–Wallis test and pairwise Mann–Whitney *U* tests with a Bonferroni correction for multiple comparisons. Means that are significantly different at $p < 0.05$ are indicated by different letters. Abbreviations: SGR = specific growth rate, BM = body mass, CL = carapace length.

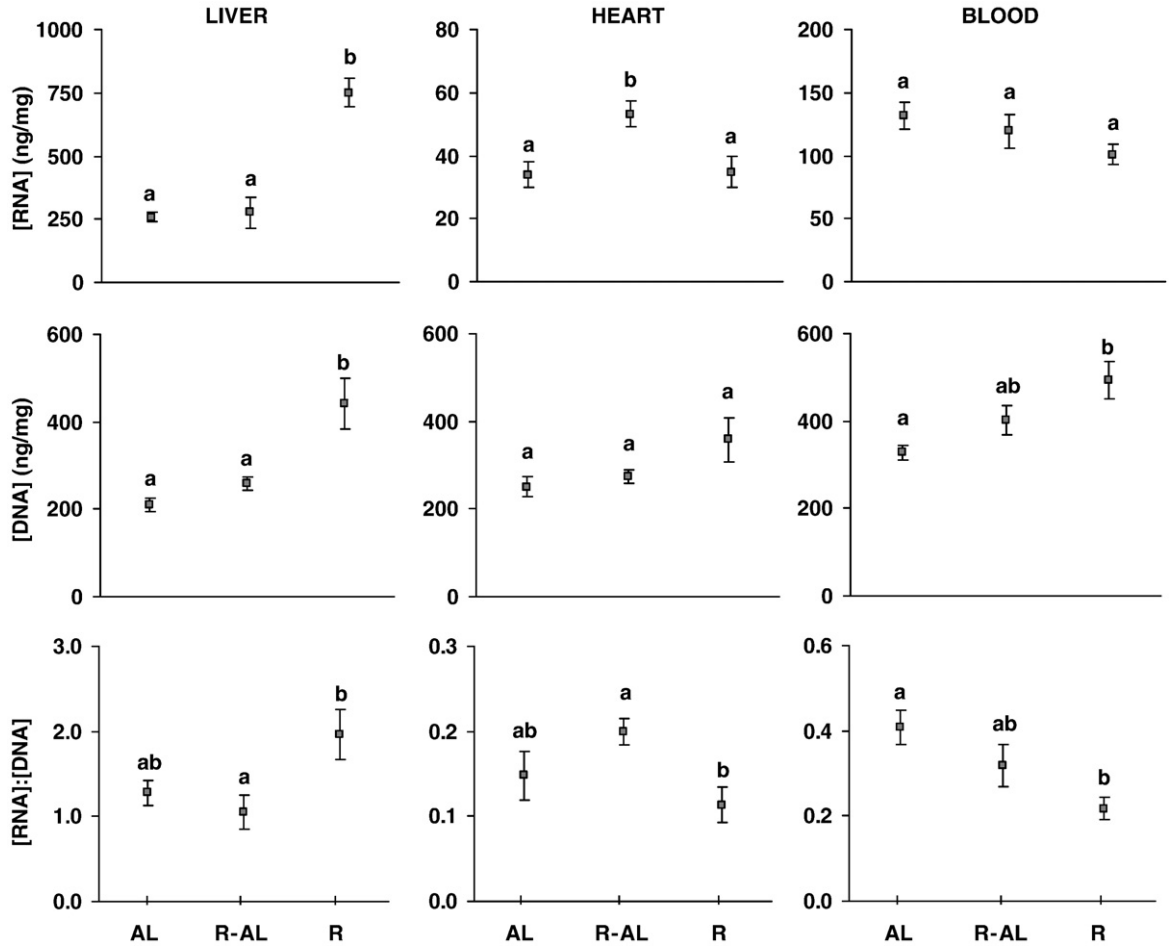

Fig. 2. Nucleic acid indices (mean ± standard error) for turtles in each of three treatment groups. Means that are significantly different at $p<0.05$ are indicated by different letters. Treatment groups, data analysis, and sample sizes are the same as in Fig. 1.

coefficient for NADPH (6.22). This quotient was doubled to account for stoichiometry and then multiplied by the final dilution factor. Activity of blanks was likewise calculated and subtracted from each sample's activity to yield total GPX activity. Total GPX activity was then normalized to DNA concentration of each sample.

Overall, non-specific antioxidant potential (AP) of homogenized liver samples was evaluated using the Bioxytech AOP-490 assay (OxisResearch, Portland, OR). This assay evaluates the total activity of cellular antioxidants including enzymes, small and large molecules, and hormones (OxisResearch Bioxytech Assay Systems, 2002). Samples from nine t_5 AL, nine t_5 R, seven t_{12} AL, eight t_{12} R, and eight t_{12} R-AL turtles and standards were analyzed in duplicate. A standard curve of uric acid was constructed and used to calculate AP of diluted liver homogenate samples as concentration (μM) of copper reducing equivalents (CRE). Total AP was then normalized to DNA concentration of each sample.

Analysis of variance (ANOVA) was used to compare GPX activity and total AP among treatment groups as described above for morphometric indices. All statistical tests were performed using SPSS for Windows (Release 11.0.0). Means are reported ± standard error with alpha set at 0.05.

3. Results

R-AL turtles grew more rapidly on a size-specific basis than AL turtles during weeks 7 through 10 (after the switch to ad libitum feeding), but this period of rapid growth ended prior to tissue sampling for the current study (Roark et al., in press). As a result, R-AL turtles were growing at comparable size-specific rates to AL turtles during the last 10–11 days of the 12-week study, and R turtles were growing significantly slower than both AL and R-AL turtles (Fig. 1).

Intake and growth patterns significantly affected all morphometric measurements of body size at the end of week 12 (Fig. 1; see also Roark et al., in press for morphometrics in each week of the study). At the time of tissue sampling, AL turtles were significantly larger than both R-AL and R turtles, and R turtles were significantly smaller than both AL and R-AL turtles. Despite differences in body size between AL and R-AL turtles, the condition indices (CI) for these two groups were comparable at the time of tissue sampling. Continuously food-restricted turtles were generally leaner than turtles feeding ad libitum, as CI differed significantly between R and R-AL turtles and approached significance ($p = 0.057$) between R and AL turtles.

Significant differences among treatment groups also existed for many of the biochemical indices we measured (Figs. 2 and 3). The patterns exhibited by the various biochemical indices varied depending on the tissue analyzed, particularly for [RNA] and [RNA]:[DNA] ratios.

Many of the morphometric and biochemical indices we measured demonstrated significant positive or negative correlations (Table 1). For most indices, significant correlations with growth rates were stronger when growth was expressed as SGR_{bm} rather than as SGR_{cl}. In some cases (e.g., $[protein]_{liver}$, $[RNA]_{heart}$, $[RNA]:[DNA]_{heart}$, and $[RNA]:[DNA]_{blood}$), the correlations between indices and growth were significant only for SGR_{bm}. Heart yielded the lowest and liver yielded the highest number of significant correlations between morphometric and biochemical indices and growth.

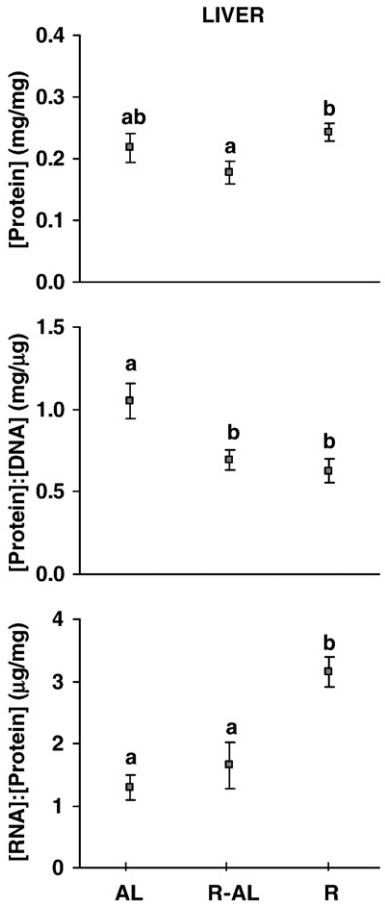

Fig. 3. Liver protein and protein-based indices (mean ± standard error) for turtles in each of three treatment groups. Means that are significantly different at $p<0.05$ are indicated by different letters. Treatment groups, data analysis, and sample sizes are the same as in Fig. 1.

When SGR_{bm} and SGR_{cl} were regressed against each index independently, all indices except $[RNA]_{blood}$, $[DNA]_{heart}$, and [protein]:$[DNA]_{liver}$ yielded significant relationships (Table 2). The R^2 values for significant relationships ranged from 0.161 to 0.659, with the strongest coefficients of determination achieved by regressing SGR_{bm} and SGR_{cl} against $[RNA]_{liver}$.

Stepwise multiple linear regression analyses for each individual tissue yielded a series of nine significant growth models (Table 3). SGR_{bm} was the dependent variable for five models, with two models (1–2) based on liver, one model (3) based on heart, and two models (4–5) based on blood. SGR_{cl} was the dependent variable for the final four models, with one model (6) based on liver, one model (7) based on heart, and two models (8–9) based on blood. The significant independent variables predicting growth rate in each of these equations are listed in the table in the order in which they were selected by the models.

When condition index and all biochemical indices for all tissues were combined and analyzed using stepwise multiple linear regression, the resulting models were identical to models 1 and 2 (for SGR_{bm}) and model 6 (for SGR_{cl}). The growth equation that best estimated recent growth rate was Model 2.

To investigate whether hepatic [RNA] was higher in slow-growing turtles because of elevated expression of antioxidant genes, we quantified glutathione peroxidase (GPX) activity and total antioxidant potential (AP). Instead of correlating with recent growth rates, both measures of antioxidant function were sensitive to intake and growth history (Fig. 4). Putative per cell GPX activity (nmol/min µg DNA) and total AP (nmol CRE/µg DNA) were significantly higher in AL turtles than in R and R-AL turtles, despite the fact that AL and R-AL turtles were growing at comparable size-specific rates by the end of the study.

4. Discussion

The purpose of this study was to evaluate the use of morphometric and biochemical indices for predicting recent growth rates in juvenile green turtles. Validation of assays with substantial predictive power for estimating growth would provide a less intensive alternative to tag and recapture programs and facilitate population monitoring in this endangered species. Nucleic acid concentrations and ratios hold promise as potential biomarkers of recent growth, as RNA content of tissues typically increases with feeding and growth in many marine organisms including krill (Shin et al., 2003), cephalopods (Melzner et al., 2005; Vidal et al., 2006), tuna (Carter et al., 1998), haddock (Caldarone, 2005), flounder, and tautog (Kuropat et al., 2002). Given the applicability of tissue nucleic acid content to growth studies in these organisms, we expected to find strong positive correlations between growth, RNA and/or protein concentrations, and ratios among nucleic acids and protein concentrations in green turtles. However, the observed relationships among growth rate and the various biochemical indices we measured were tissue-specific.

4.1. Nucleic acid and protein concentrations

Contrary to our expectations, the biochemical indices we measured were neither consistently, nor always positively, correlated with feeding treatment and growth rates. Perhaps most surprisingly, liver RNA concentration was inversely correlated with SGR. Turtles growing slowly had more total RNA (and thus presumably more protein synthesis capacity) in liver than turtles growing more rapidly. On the other hand, growth rate was not at all correlated with blood RNA content and was only mildly correlated with heart RNA content.

DNA content of blood and liver (but not heart) was negatively correlated with SGR, a trend that has also been observed in fish (Mercaldo-Allen et al., 2006). Because the amount of DNA in each cell should be constant (Wallace, 1992) except during cell division, this finding suggests that the density of cells in blood and liver increases in response to food restriction. In the blood, increased cell density could reflect elevated production of any of the six predominant types of nucleated blood cells found in green turtles, including erythrocytes, four types of leukocytes, and thrombocytes (Wood and Ebanks, 1984). The typical hematological response to caloric restriction in other species is either no change (Lochmiller et al., 1993) or a decrease (Maxwell et al., 1990b; Walford et al., 1992) in total leukocyte count, although the number of circulating basophils and thrombocytes has been shown to increase in food-restricted birds (Maxwell et al., 1990b; Maxwell et al., 1992).

Our DNA results could also reflect differences in the number of erythrocytes. Hematocrit may be correlated with body size in green turtles (Wood and Ebanks, 1984, but see also Bolten and Bjorndal, 1992), but this relationship (if it exists) should result in higher DNA concentrations in larger, rather than smaller, turtles. It is therefore unlikely that our results for DNA concentration reflect a body size dependence for erythrocyte number. Hematocrit does not normally increase during food restriction (e.g., Maxwell et al., 1990a; Lochmiller et al., 1993). However, enhanced erythropoiesis with concomitant microcytosis has been demonstrated in food-restricted birds (Maxwell et al., 1990a), suggesting that our DNA results may reflect differences in blood cell size between slow- and fast-growing turtles.

In the liver, our DNA results indicate that fast growth is achieved by hypertrophy more than hyperplasia. In snakes, feeding has been shown to increase the size of lipid droplets and glycogen deposits in hepatocytes, thus leading to hypertrophic growth of liver cells (Starck and Beese, 2002). Similarly, farm-raised marine turtles fed ad libitum are

Table 1
Spearman's rank correlations (ρ) for morphometric indices (a) and nucleic acid and protein indices for liver (b), heart (c), and blood (d) ($n = 27$ for each variable) in juvenile *Chelonia mydas*.

(a) Morphometrics

Variable	CL	CI	SGR_{bm}	SGR_{cl}
BM	**0.988****	**0.463***	**0.693****	**0.797****
CL		**0.391***	**0.649****	**0.785****
CI			**0.499****	**0.465***
SGR_{bm}				**0.839****

(b) Liver

Variable	$[RNA]_{liver}$	$[DNA]_{liver}$	$R:D_{liver}$	$[Protein]_{liver}$	$P:D_{liver}$	$R:P_{liver}$
BM	**−0.675****	**−0.673****	**−0.412***	−0.287	**0.428***	**−0.673****
CL	**−0.654****	**−0.635****	**−0.423***	−0.240	**0.426***	**−0.675****
CI	**−0.521****	**−0.534****	−0.168	−0.135	**0.427***	**−0.487***
SGR_{bm}	**−0.734****	**−0.519****	**−0.618****	**−0.561****	0.068	**−0.536****
SGR_{cl}	**−0.733****	**−0.442***	**−0.601****	−0.332	0.248	**−0.690****
$[RNA]_{liver}$		**0.609****	**0.778****	**0.433***	−0.292	**0.846****
$[DNA]_{liver}$			0.066	**0.423***	**−0.615****	**0.459***
$R:D_{liver}$				0.249	0.134	**0.703****
$[Protein]_{liver}$					0.332	−0.051
$P:D_{liver}$						**−0.545****

(c) Heart

Variable	$[RNA]_{heart}$	$[DNA]_{heart}$	$R:D_{heart}$
BM	0.031	**−0.394***	0.247
CL	−0.033	−0.369	0.195
CI	**0.429***	**−0.454***	**0.631****
SGR_{bm}	**0.435***	−0.292	**0.523****
SGR_{cl}	0.294	−0.217	0.374
$[RNA]_{heart}$		−0.062	**0.800****
$[DNA]_{heart}$			**−0.594****

(d) Blood

Variable	$[RNA]_{blood}$	$[DNA]_{blood}$	$R:D_{blood}$
BM	0.357	**−0.563****	**0.549****
CL	0.339	**−0.520****	**0.527****
CI	**0.543****	−0.237	**0.441***
SGR_{bm}	0.352	**−0.549****	**0.440***
SGR_{cl}	0.284	**−0.446***	0.352
$[RNA]_{blood}$		−0.253	**0.816****
$[DNA]_{blood}$			**−0.680****

Notes: Significant correlations are indicated in bold. Asterisks indicate level of significance (*$p<0.05$, **$p<0.01$). Abbreviations: BM = body mass, CL = carapace length, CI = condition index, SGR = specific growth rate, [ ] = concentration, R:D = RNA:DNA ratio, P:D = protein:DNA ratio, R:P = RNA:protein ratio.

known to have hepatocytes dominated by large lipid droplets (Solomon and Tippett, 1991). It is quite likely that our results for hepatic DNA content reflect more extensive lipid and glycogen deposition in turtles feeding ad libitum than in food-restricted turtles, therefore leading to more extensive hepatocyte hypertrophy in the former.

Increased lipid deposition in hepatocytes of fast-growing green turtles may also explain the negative correlation we observed between hepatic protein concentration and SGR. In other studies, however, overall protein content as well as protein content per cell was strongly and positively correlated with growth rate (Carter et al., 1998; Caldarone, 2005). To explore the incongruity between our nucleic acid and protein concentrations and those found in comparable studies in fish, we assessed cellular protein synthesis capacity by calculating ratios of RNA:protein (for liver only) and RNA:DNA (for all tissues). These ratios should both provide information about the protein synthesis capacity per cell, but the former is only valid as a measure of cellular RNA content if the protein:DNA ratio (a measure of protein content per cell) is unaffected by intake and growth. Because hepatic cellular protein content was influenced by treatment, only RNA:DNA ratio is an appropriate index of cellular RNA content for this study.

4.2. RNA:DNA ratios

Many authors have demonstrated significant positive relationships between RNA:DNA ratio (of muscle, liver, or whole body) and growth rate, particularly in fish (Westerman and Holt, 1994; Carter et al., 1998; Caldarone, 2005; Mercaldo-Allen et al., 2006). Given this common result, we expected to find similar trends in our turtle tissues. As predicted, heart and blood RNA:DNA ratios did correlate positively with growth, but they explained only a small percentage (16–28%) of the variance in SGR. On the contrary, hepatic RNA:DNA ratios were inversely correlated with SGR and explained 63–66% of the variance in SGR. We suggest several possible explanations for this discrepancy among tissues.

The liver is a mitotically active tissue, and elevated rates of cellular proliferation can lead to over-estimation of cell number (Darzynkiewicz et al., 1980). It is possible that the RNA:DNA ratios we calculated for liver of fast-growing turtles were under-estimates of the true cellular RNA content in fast-growing turtles. However, the difference in these ratios between fast-growing turtles in groups AL and R-AL and slow-growing turtles in group R is likely too substantial to result from differences in rates of DNA synthesis alone.

Instead, we suggest that our RNA:DNA ratios reflect real, tissue-specific differences in cellular ribosomal RNA content. Typically, RNA:DNA ratio declines as ribosomes are degraded during periods of food deprivation (Clemmesen, 1994). In our study, however, slow growth was induced by food restriction rather than starvation, and food-restricted turtles were never in negative energy balance. Studies in rodents have demonstrated that protein turnover rates and activity of gluconeogenic enzymes in the liver increase in response to food

Table 2
Growth equation parameters for juvenile *Chelonia mydas* as determined by least squares linear regression.

y	*x*	Intercept	Slope	Adjusted R^2	*F*	*p*
Body mass						
SGR_{bm}	$Ln[RNA]_{liver}$	8.040	−1.132	0.629	45.011	<**0.0001**
SGR_{bm}	$Ln[RNA]_{heart}$	−2.133	0.955	0.161	5.990	**0.022**
SGR_{bm}	$[RNA]_{blood}$	0.196	0.010	0.109	4.197	0.051
SGR_{bm}	$Ln[DNA]_{liver}$	8.574	−1.276	0.306	12.482	**0.002**
SGR_{bm}	$Ln[DNA]_{heart}$	6.808	−0.967	0.089	3.536	0.072
SGR_{bm}	$Ln[DNA]_{blood}$	12.305	−1.828	0.298	12.016	**0.002**
SGR_{bm}	$Ln(R:D)_{liver}$	1.610	−1.046	0.294	11.821	**0.002**
SGR_{bm}	$Ln(R:D)_{heart}$	3.054	0.852	0.253	9.804	**0.004**
SGR_{bm}	$Ln(R:D)_{blood}$	2.720	1.061	0.277	10.937	**0.003**
SGR_{bm}	$[Protein]_{liver}$	2.878	−7.203	0.196	7.353	**0.012**
SGR_{bm}	$Ln(P:D)_{liver}$	1.511	0.470	0.003	1.083	0.308
SGR_{bm}	$Ln(R:P)_{liver}$	1.934	−0.987	0.366	15.993	<**0.001**
SGR_{bm}	CI	−3.940	38.475	0.379	16.897	<**0.001**
Carapace length						
SGR_{cl}	$Ln[RNA]_{liver}$	2.549	−0.353	0.659	51.159	<**0.0001**
SGR_{cl}	$Ln[RNA]_{heart}$	−0.368	0.229	0.084	3.382	0.078
SGR_{cl}	$[RNA]_{blood}$	0.171	0.003	0.065	2.811	0.106
SGR_{cl}	$Ln[DNA]_{liver}$	2.610	−0.379	0.289	11.554	**0.002**
SGR_{cl}	$Ln[DNA]_{heart}$	1.984	−0.269	0.067	2.882	0.102
SGR_{cl}	$Ln[DNA]_{blood}$	3.486	−0.504	0.236	9.052	**0.006**
SGR_{cl}	$Ln(R:D)_{liver}$	0.550	−0.339	0.337	14.228	**0.001**
SGR_{cl}	$Ln(R:D)_{heart}$	0.899	0.217	0.164	6.107	**0.021**
SGR_{cl}	$Ln(R:D)_{blood}$	0.846	0.294	0.222	8.426	**0.008**
SGR_{cl}	$[Protein]_{liver}$	0.802	−1.584	0.083	3.357	0.079
SGR_{cl}	$Ln(P:D_{liver})$	0.535	0.203	0.047	2.283	0.143
SGR_{cl}	$Ln(R:P_{liver})$	0.669	−0.343	0.490	25.954	<**0.0001**
SGR_{cl}	CI	−0.903	9.951	0.262	10.245	**0.004**

Notes: Specific growth rate (SGR) for body mass (bm) or carapace length (cl) was regressed independently against each index ($n = 27$ for each variable). Significant *p*-values are indicated in bold. Abbreviations are the same as in Table 1.

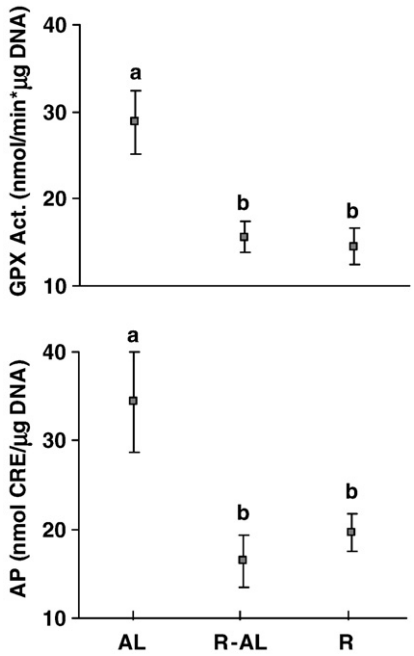

Fig. 4. Glutathione peroxidase (GPX) specific activity (act.) and antioxidant potential (AP; calculated as nmoles of copper reducing equivalents, CRE) in livers (mean ± standard error) of turtles in each of three treatment groups. GPX activity and total AP were normalized to tissue DNA concentration. Treatment groups and data analysis are the same as in Fig. 1. Sample sizes for GPX activities are the same as in Fig. 1. Sample sizes for AP are $n = 7$, 8, and 8 for AL, R, and R-AL turtles, respectively. Letters (a and b) indicate statistically significant differences ($p < 0.05$) among treatment groups within sampling periods.

restriction (Spindler, 2001; Hagopian et al., 2003). Thus, the effect of intake and growth rates on RNA:DNA ratios may differ depending on whether the individual is in positive or negative energy balance and the physiological role of the tissue studied.

It is also possible that ontogenetic or size-specific differences in the rate of growth of individual organs may complicate the interpretation of RNA:DNA ratios. Tissues that grow at different rates throughout development may demonstrate age- or size-specific protein synthesis potentials (and thus different RNA:DNA ratios) that do not correlate well with whole-animal growth rates. When we analyzed data on liver size in green turtles from another study (Roark et al., in press), we identified an allometric relationship between liver size and body size. In that study, the wet mass of livers from AL turtles in weeks 0, 5, and 12 scaled with $BM^{1.22}$ (95% confidence interval of the scaling coefficient = 1.17–1.28, data not shown) when data for liver and body size from all 3 sampling times were analyzed together. Despite the fact that liver size increases faster than total body size throughout the first three months of life, we found no evidence of any ontogenetic changes in liver growth rates during this time. To our knowledge, no further information about the ontogeny of organ growth in green turtles is currently available. However, as organ growth rates are likely age-dependent, we urge caution in the extrapolation of our results to green turtles beyond the age range we examined in this study.

4.3. Hepatic antioxidant function

To investigate whether our results for hepatic RNA content and RNA:DNA ratios could also be explained by differences in antioxidant function, we quantified glutathione peroxidase (GPX) activity and total antioxidant potential (AP). Glutathione peroxidase serves as one of several important enzymes in the antioxidant defense system. However, combating oxidative stress requires the concerted involvement of a variety of enzymatic and non-enzymatic molecules that scavenge or neutralize reactive oxygen species. Many of these molecules function synergistically (Niki et al., 1995; Böhm et al.,

Table 3
Growth equation parameters for juvenile *Chelonia mydas* as determined by stepwise multiple linear regression.

Tissue	Model #	*y*	x_1	x_2	Intercept	β_1	β_2	R^2	*F*	*p*-value
Liver	1	SGR_{bm}	Ln[RNA]		8.040	−1.132		0.629	45.011	<**0.0001**
Liver	2	SGR_{bm}	Ln[RNA]	CI	4.316	−0.913	17.689	0.680	28.567	<**0.0001**
Heart	3	SGR_{bm}	CI		−3.940	38.475		0.379	16.897	<**0.001**
Blood	4	SGR_{bm}	CI		−3.940	38.475		0.379	16.897	<**0.001**
Blood	5	SGR_{bm}	CI	Ln[DNA]	5.384	31.770	−1.402	0.547	16.705	<**0.0001**
Liver	6	SGR_{cl}	Ln[RNA]		2.549	−0.353		0.659	51.159	<**0.0001**
Heart	7	SGR_{cl}	CI		−0.903	9.951		0.262	10.245	**0.004**
Blood	8	SGR_{cl}	CI		−0.903	9.951		0.262	10.245	**0.004**
Blood	9	SGR_{cl}	CI	Ln[DNA]	1.730	8.058	−0.396	0.398	9.950	**0.001**

Notes: Specific growth rate (SGR) for body mass (bm) or carapace length (cl) was regressed against condition index (CI) and all biochemical indices for a particular tissue (liver, heart, or blood) ($n = 27$ for each variable). When specific growth rate was regressed against condition index and biochemical indices for all tissues together, the resulting models were identical to models 1–2 (for SGR_{bm}) and model 6 (for SGR_{cl}). Within tissues, variables are listed in the order in which they were selected by the models. Significant *p*-values are indicated in bold. Abbreviations are the same as in Table 1.

1997). For this reason, we also measured total, non-specific hepatic antioxidant potential. We focused specifically on liver because this organ plays an important role in metabolism and detoxification and is a major source of peroxides via oxidation reactions (Soto et al., 1993).

For both GPX activity and total AP, continuously ad libitum-fed turtles demonstrated elevated antioxidant function relative to continuously food-restricted turtles. However, GPX activity and total AP of R-AL turtles was comparable to those of R turtles despite the fact that R-AL turtles grew at rates comparable to those of AL turtles in the 10–11 days prior to the end of the study. These results suggest that green turtles typically grow sub-maximally to optimize their ability to prevent oxidative damage to lipids, nucleic acids, and proteins. Oxidative stress results from an imbalance between the rate of reactive oxygen species (ROS) production and the availability of antioxidants to scavenge these ROS within a cell (Agarwal et al., 2005). Given the assumption that ROS production increases with intake rate (López-Torres et al., 2002; Barja, 2004), our findings imply a cost of a period of rapid growth and suggest that sub-maximal growth protects individuals from the detrimental effects of impaired antioxidant defense. However, they do not explain our results for hepatic RNA content.

4.4. Predictive models of recent growth rates

To expand the predictive power of the various indices we measured, we incorporated condition index and all nucleic acid and protein indices measured for a particular tissue (liver, heart, or blood) into a series of models using stepwise multiple linear regression. We did not include either measure of antioxidant function in these models because of the significant effect of diet history on GPX activity and total antioxidant potential. The resulting predictive equations explained a maximum of 68.0% (range: 26.2–68.0%) of the variance in growth rate. This maximal predictive power was achieved by model 2, in which SGR_{bm} is estimated using liver RNA content and CI. The remaining indices, including nucleic acid concentrations and ratios for heart and blood, did not explain any additional variance in growth rate. Although a model for growth of juvenile green turtles (size range: 30–90 cm straight CL) in the Caribbean has previously been developed (Bjorndal et al., 2000), this model does not allow for discrimination of growth rates among individuals of similar size that may have experienced different nutritional conditions. Furthermore, the coefficient of determination for our Model 2 was greater than that of the earlier model and therefore indicates that the combined use of morphometric and biochemical indices holds promise for applications to studies of growth in wild populations.

In the various growth models we tested, CI was repeatedly selected as an independent variable with significant predictive power. Bjorndal et al. (2000) found a similar positive correlation between condition index and recent growth rates in wild green turtles. These findings are particularly interesting in light of criticisms of the use of ratio-based indices (Hayes and Shonkwiler, 2001) and suggest that, at least for green turtles, the use of "body condition" as measured using Fulton's *K* (Ricker, 1975) for predictive purposes is meaningful and appropriate. This conclusion is further supported by the fact that CI correlates with both short-term growth rates in small juveniles (8–15.5 cm; this study) and long-term growth rates in larger juveniles (25–82 cm; Bjorndal et al., 2000).

The growth model we developed fails to explain 32% of the variance in growth rates. This variance could potentially have been improved by measuring DNA and RNA concentrations from the same subsamples of tissue, but the protocols we used precluded us from doing so. The remaining unexplained variability in growth rate may result from a mismatch in the time scales over which the various indices in the model accurately detect changes in growth. As condition index relies on measurement of body mass (a result of tissue accretion) and body length (a result of bony growth), it most likely provides information about longer term growth processes than nucleic acid and protein concentrations, which fluctuate over shorter time scales (Ferron and Leggett, 1994).

Because sacrificing wild green turtles to collect liver samples for measuring nucleic acid concentrations is not an acceptable practice, the multivariate model that best predicted recent growth (model 2) has limited applicability in studies of wild turtle demography. However, the fact that several biochemical indices for blood (including DNA concentration and RNA:DNA ratio) were significantly correlated with growth suggests that further calibration of these assays for application to growth estimation is warranted. Indeed, 55% of the variance in body mass growth was predicted using only CI and concentration of DNA in the blood. Both of these parameters are easily measured with limited disturbance to the animal. Combining morphometric measurements with biochemical analyses of blood and other easily obtainable tissues (perhaps including dermis) could therefore be a minimally invasive technique for estimating recent growth rates in this endangered species. As biochemical indices of instantaneous growth are likely temperature-, size-, and age-dependent (Rooker and Holt, 1996; Caldarone, 2005), the utility of these indices in growth assessments of wild marine turtles in varying developmental stages and from different habitats deserves further study.

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