PHYSIOLOGICAL AND LIFE-HISTORY RESPONSES TO PATTERNS OF FOOD AVAILABILITY

By

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To my family, without whom this work would not have been possible

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PHYSIOLOGICAL AND LIFE-HISTORY RESPONSES TO PATTERNS OF FOOD AVAILABILITY

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Green turtles (*Chelonia mydas*) experience nutritional stochasticity as oceanic-stage juveniles and should therefore be capable of compensatory growth (CG) following periods of nutritional stress. The purpose of the first phase of my research was to test for the occurrence of CG in green turtles and determine its mechanism(s). Food-restricted turtles (R) grew more slowly, differed in cell size and body composition, and had proportionally smaller digestive organs than turtles feeding *ad libitum* (AL). After food conditions improved, previously food-restricted turtles (R-AL) demonstrated CG. This growth pattern was elicited by enhanced food conversion efficiency rather than hyperphagia. The period of growth compensation may have ended when R-AL turtles attained a body composition similar to that of AL turtles. These findings indicate that growth rate, morphology, and body composition of juvenile green turtles are plastic in response to diet and that individuals can compensate for environmental variability to capitalize when conditions improve. However, CG was associated with altered antioxidant function. Activity of glutathione peroxidase and total antioxidant potential per liver cell were greater in AL turtles than in R and R-AL turtles, respectively, at the conclusion of the study. Therefore, impaired antioxidant capacity may be a cost of rapid growth in this species.

To elucidate long-term responses to food availability, I tested the effects of food restriction (FR) imposed during several life stages in Indian stick insects (*Carausius morosus*). Intake pattern affected age and size at each life-history transition, with size decreasing and age increasing in response to FR. Early-onset FR increased lifespan, but this increase was negated by detrimental effects on reproductive output. Late-onset FR negatively affected both longevity and reproductive output. *Carausius morosus* appears to allocate incoming adult-derived nutrients to egg provisioning and stored nutrients to adult somatic maintenance and survival. Thus, I found no evidence for a trade-off between fecundity and longevity because nutrients allocated to reproduction and maintenance do not appear to be derived from a common resource pool. These results demonstrate that fluctuations in food availability can significantly alter the expression of life-history traits and that the magnitude of these effects depends on the developmental stage during which food availability changes.

CHAPTER 1
GENERAL INTRODUCTION

Nearly every biological process depends to some extent on nutrition. Ingestion, digestion, and nutrient absorption determine an animal's capacity for maintenance, growth, reproduction, and survival. An individual's success thus depends on its capacity to extract and utilize nutrients from its food. However, many organisms experience variation in food availability throughout their lifetimes (Plötz *et al*. 1991, Boggs and Ross 1993, Carey *et al*. 2002a), and evolutionary adaptation to these fluctuations is critical to species survival. Fluctuations in food availability can result from climatic or seasonal dynamics of food sources and spatial or temporal heterogeneity of nutrient distribution (Dagg 1977, Smith and Ballinger 1994, Forman 1995, Arnekleiv *et al*. 2006, Schradin and Pillay 2006). Periods of "feast" and "famine" result in correspondingly fast and slow periods of growth, development, and reproduction (Ballinger 1977, Calbet and Alcaraz 1997, Kitaysky 1999, Morey and Reznick 2000). Such developmental plasticity is common to many species (Stearns 1982, Smith-Gill 1983, Schew and Ricklefs 1998), especially those in stochastic environments (Lochmiller *et al*. 2000).

One adaptation to cycles of low and high nutrient availability is compensatory growth (CG). This phenomenon manifests itself as a period of growth faster than that demonstrated by consistently well nourished conspecifics of the same age (Fig. 1-1) and can result in comparable body sizes for individuals with very different dietary histories (Broekhuizen *et al*. 1994, Metcalfe and Monaghan 2001). Compensatory growth presumably allows organisms to mitigate size-specific mortality risks and developmental time constraints induced by periodic nutritional stress (Arendt 1997). This growth pattern is typically effected by hyperphagia (increased feed intake), improved food conversion efficiency (defined as growth per unit of food consumed), or both (Miglavs and Jobling 1989, Ali *et al*. 2003). Compensatory growth has been documented in

many species (Wilson and Osbourn 1960, Ali *et al*. 2003, Jespersen and Toft 2003), particularly those that commonly experience environmental stochasticity. However, compensatory growth is certainly not a universal occurrence, especially in non-teleost species (e.g., Altwegg and Reyer 2003, Brzęk and Konarzewski 2004).

For my dissertation, I studied the physiological and life-history responses of animals to changes in food availability. The green turtle (*Chelonia mydas*) is an excellent model for pursuing questions about fluctuating food availability because of the nutritional stochasticity they are thought to encounter as juveniles. This stochasticity should select for growth patterns that allow individuals to maximize their growth rates when conditions are good. Green turtles consume a largely carnivorous diet during the juvenile oceanic stage (Reich *et al.* in review). Food availability during this time is thought to be spatially heterogeneous. At a size of 20-35 cm (carapace length), juvenile green turtles undergo an ontogenetic shift in habitat use and diet and recruit to neritic habitats, where they continue development while consuming a primarily herbivorous diet (Bjorndal 1997). Niche shifts are excellent opportunities for enhanced growth because such shifts often correspond to improved nutritional conditions (Ali *et al.* 2003). Although growth dynamics of oceanic-stage juveniles are unknown, post-recruitment growth rates vary temporally in response to oceanographic conditions (Limpus and Chaloupka 1997) and population density (Bjorndal *et al*. 2000). This variation in growth rates may have substantial life-history effects, because body size is correlated with juvenile survivorship (Chaloupka and Limpus 1998) and clutch size (Broderick *et al*. 2003) in this species. Because of the potential for nutritional stochasticity and the potential effects of body size on fitness in *C. mydas*, I predicted that green turtle juveniles should be capable of CG. Juvenile loggerheads with dietary

preferences similar to those of green turtles undergo CG in the wild (Bjorndal *et al*. 2003), although the mechanism for this growth pattern is unknown.

The purpose of the first phase of my research was to manipulate food availability in juvenile green turtles under controlled conditions to test whether previously food-restricted turtles can undergo CG and to evaluate hyperphagia and enhanced food conversion efficiency as possible mechanisms for this growth pattern. The results of Chapter 2 indicated that previously food-restricted green turtle juveniles are indeed capable of transient growth compensation after a return to *ad libitum* feeding. This finding suggests that growth rates of turtles under conditions of continuously high food availability are optimal rather than maximal (Metcalfe and Monaghan 2001) and that trade-offs between growth and fitness probably exist.

Rapid growth in many species may be associated with a variety of costs, many of which may not be paid until late in life (Einum and Fleming 2000 and references therein, Metcalfe and Monaghan 2001, Altmann and Alberts 2005, Nagy and Holmes 2005). To pursue this hypothesis, I examined antioxidant function of tissues from green turtles that had undergone CG. Antioxidants prevent free-radical induced oxidative damage to nucleic acids and proteins by converting reactive oxygen species (ROS) into less noxious compounds (Ji and Leichtweis 1997, Gredilla and Barja 2005). Caloric restriction depresses the rate of production of ROS (López-Torres *et al*. 2002, Barja 2004) and attenuates the accrual of irreparable damage to cellular macromolecules (Hyun *et al*. 2006). As a result, dietary restriction slows aging and thus extends lifespan relative to continuous *ad libitum* feeding in a diversity of species (Weindruch and Walford 1988, Austad 1989, Mair *et al*. 2003, Vaupel *et al*. 2003, Hatle *et al*. 2006b). Because *ad libitum* feeding is typically accompanied by accelerated aging, it is possible that periods of rapid growth induced by high food availability after a period of nutritional stress may

be costly in terms of survival. I hypothesized that the short-term benefits of CG may be countered by the rapid accumulation of oxidative damage, thereby imposing a cost to fast growth. My results confirmed that turtles with a history of CG suffered decreased cellular antioxidant potential relative to continuously *ad libitum*-fed turtles.

The diminished capacity for antioxidant defense exhibited by fast-growing turtles piqued my interest in the more long-term consequences of changes in food availability on life-history traits and trade-offs. Despite the tendency for caloric restriction to enhance longevity in the laboratory, nutritional stress experienced early in life can have profound life-history consequences (Metcalfe and Monaghan 2001). For example, growth rate, dominance status, and age at first parturition in spotted hyenas are strongly correlated with food availability during development (Hofer and East 2003). This "silver-spoon effect" (Grafen 1988, Madsen and Shine 2000) indicates that early nutritional conditions can have long-lasting effects. Resource limitation experienced later in life can also influence life history by decreasing reproductive output (Boggs and Ross 1993, Wheeler 1996, Olsson and Shine 1997, Carey *et al*. 2002b, Nagy and Holmes 2005). On the other hand, food restriction has been shown to extend an animal's reproductive lifespan (Hart and Turturro 1998), thereby partially mitigating the decline in reproductive rate caused by food scarcity.

Most of the work cited above, however, provides incomplete information about the effects of nutrition on fitness and life history because intake is not typically quantified throughout the entire lifespan in studies of this kind (Zera and Harshman 2001), particularly for long-lived species. As most animals experience fluctuations in food availability at some point in their lifetimes, a more complete understanding of the effects of diet on fitness requires intake

manipulation and quantification under controlled conditions during both juvenile and adult stages.

Furthermore, to evaluate the costs of reproduction and the effects of dietary restriction on fitness in sexual species, females must be allowed to mate. However, mating is often prevented (particularly in rodent studies) because experimental animals are maintained individually (Vaupel *et al*. 2003). In those studies where mating is permitted, co-housing individuals can complicate the quantification of individual intake and is known to influence longevity and fecundity due to the effects of crowding (Joshi *et al*. 1998). To avoid such problems, studies of food restriction and reproductive output are often undertaken using virgin or hermaphroditic females of invertebrate species. This approach suffers from limitations, including the fact that mating enhances fecundity (Chiang and Hodson 1950, Foster and Howard 1999, Chong and Oetting 2006) and that self-fertilization constrains reproductive output because of limits on self-sperm production in protandrous hermaphroditic species like *Caenorhabditis elegans* (Cutter 2004, Partridge *et al*. 2005). For these reasons, the choice of an appropriate model organism for the final phase of my dissertation research was of paramount importance.

Although the first phase of my research focused on green turtles, this species is not a suitable animal model for investigating correlations between intake and life-history traits. To evaluate the effects of food availability on development, longevity, and reproductive output, I adopted a novel approach to life-history experimentation by using a parthenogenetic species as my animal model. Using a parthenogen obviated the need for mating, and therefore allowed females to be housed individually, while still permitting natural reproductive processes. Given the paucity of parthenogenetic vertebrate species with a reasonable (i.e., less than two-year) lifespan that are amenable to laboratory culture, I chose to work with an insect species for the

final phase of my dissertation research. The Indian stick insect (*Carausius morosus* (Br.)) (Phasmatodea, Lonchodinae) is a relatively long-lived species that reproduces via apomictic parthenogenesis (Pijnacker 1966). This species is hemimetabolous and phytophagous, allowing for life-long, quantitative dietary manipulations using the same food source throughout the entire lifespan. My purpose was to determine the effects of differences in resource availability at several developmental stages on life-history traits that have substantial influences on population structure and dynamics, such as age and size at each developmental transition, longevity, and fecundity.

The overall goal of my dissertation research was to elucidate the physiological and life-history effects of variation in food availability by conducting feeding trials in a controlled laboratory setting. In Chapter 2, I explore the capacity for and mechanisms of CG in green turtles and evaluate the effects of intake and growth rates on body composition and digestive system morphology. In Chapter 3, I examine the effects of diet on cell size and protein synthesis capacity and assess the utility of a number of morphological and biochemical indices as potential predictors of recent growth in green turtles. In Chapter 4, I compare the antioxidant potentials of green turtles with known growth trajectories to establish a putative cost of rapid growth. In Chapter 5, I determine the life-history consequences of changes in food availability imposed either during development or after the attainment of sexual maturity in Indian stick insects. For this final chapter, I pursued questions about the effects of variation in intake on developmental transitions, longevity, and fecundity.

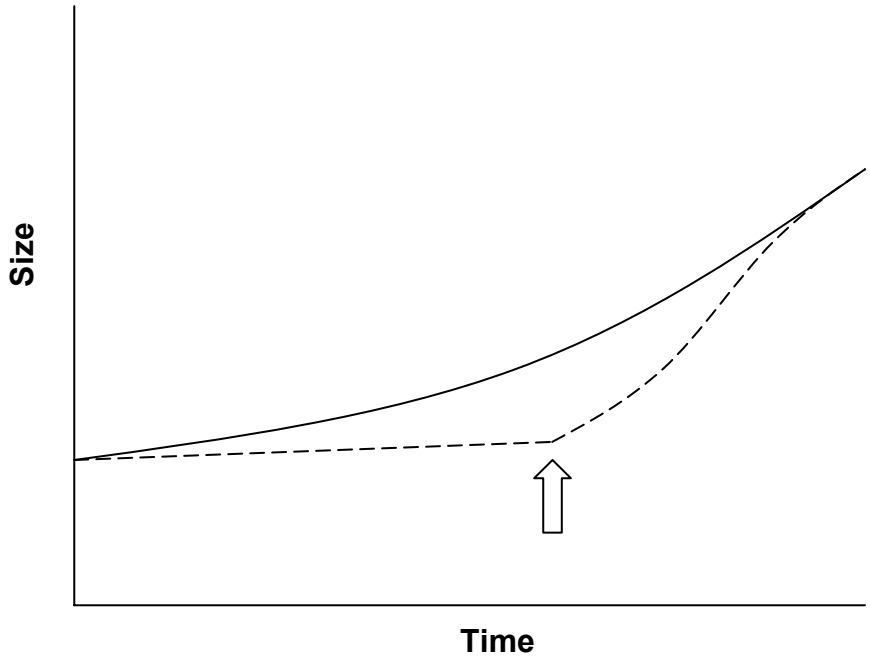


Figure 1-1. Hypothetical plot of size versus time for juvenile animals from the same cohort. The solid line represents individuals feeding *ad libitum*, and the dashed line represents individuals feeding at a restricted rate until the time indicated by the arrow, after which food was offered *ad libitum*. The rapid growth demonstrated by the previously-restricted individuals after the switch to *ad libitum* feeding represents compensatory growth.

CHAPTER 2
COMPENSATORY GROWTH IN RESPONSE TO A CHANGE IN FOOD AVAILABILITY IN JUVENILE GREEN TURTLES (*Chelonia mydas*)

Introduction

Many organisms experience varying levels of nutrient availability throughout their lifetimes as a result of spatial or temporal heterogeneity of food distribution. These periods of high and low food availability often lead to correspondingly fast and slow rates of growth, development, and/or reproduction (Ballinger 1977, Calbet and Alcaraz 1997, Kitaysky 1999, Morey and Reznick 2000). Given these life-history consequences, fluctuations in food availability should select for adaptations that allow a previously food-limited individual to maximize its ability to capitalize on better conditions when they are encountered.

One common response to alternating periods of high and low food availability is compensatory growth (CG). This phenomenon manifests itself as a period of accelerated growth during improved food conditions following a period of nutritional deprivation severe enough to restrict growth rates (Wilson and Osbourn 1960, Reid and White 1977). As a result, growth trajectories tend to converge, thereby minimizing the variance in body size among individuals of a cohort (Atchley 1984). Compensatory growth presumably allows organisms to mitigate the negative effects of slow growth on survival, development, and reproductive output. This growth pattern has been documented in invertebrates (Jespersen and Toft 2003, Dmitriew and Rowe 2005), fish (Skalski *et al*. 2005), reptiles (Bjorndal *et al*. 2003, Caley and Schwarzkopf 2004), birds (Kunz and Ekman 2000), and mammals (Lochmiller *et al*. 2000). However, compensatory growth is certainly not a universal occurrence, especially in non-teleost species (e.g., Altwegg and Reyer 2003, Brzęk and Konarzewski 2004).

Although the degree of growth compensation can vary depending on the species in question, the developmental stage of the organism at the times of restriction and improved food

availability, and the length and severity of the period of food restriction (Wilson and Osbourn 1960, Ali *et al*. 2003), the mechanisms for compensatory growth (when it is observed) are relatively conserved. Hyperphagia, or increased food intake, is the most common proximate cause of CG in a variety of animals (Ali *et al*. 2003). During hyperphagia, the rate of lipid accumulation may direct the duration of the compensatory response, with intake and growth rates returning to "normal" once adipose stores have been restored (Jobling and Johansen 1999, Johansen *et al*. 2001). Improvements in food conversion efficiency (FCE, defined as growth per unit of food consumed) can also allow for CG (Patterson *et al*. 1995, Boujard *et al*. 2000). Reductions in the mass of energetically expensive viscera (especially gut and/or liver; Hornick *et al*. 2000) during food restriction have been demonstrated in fish (Gaylord and Gatlin 2000), mammals (Weindruch and Sohal 1997), and birds (Hume and Biebach 1996, Karasov and Pinshow 1998). If decreased organ size persists for a period of time after a return to *ad libitum* feeding, the resulting combination of lowered metabolic expenditure and high intake might allow more nutrients to be allocated to growth. In this way, FCE would remain high, thus facilitating CG during this period.

In addition to affecting visceral organ size, variation in intake and growth rates can cause a variety of physiological changes. Body composition is one of the most plastic characteristics of organisms undergoing food restriction and subsequent realimentation. Sequential mobilization of reserves typically occurs when animals are food-limited, with lipid stores depleted preferentially compared to protein stores (e.g., Cherel *et al.* 1993, Tian and Qin 2004). During realimentation and CG, differential accretion of lipid and/or protein also occurs. Typically, the early phases of compensatory growth are characterized by lean tissue deposition while later phases are characterized by fat deposition. This differential accretion of lean tissue during the early stages

of realimentation may provide a mechanism for accelerated growth, as lean tissue deposition requires less energy than fat deposition (Hornick *et al*. 2000).

In this study, I examined the capacity for and mechanisms of CG in juvenile green turtles (*Chelonia mydas*). The green turtle leads an oceanic existence for the first several years of life (Carr 1987) and consumes a largely carnivorous diet during this time (Reich *et al.* in review). Intake of juvenile turtles in this stage most likely varies stochastically due to heterogeneous prey distribution. At a size of approximately 20-25 cm carapace length (for Atlantic *C. mydas*) or 35 cm carapace length (for Pacific *C. mydas*), green turtles undergo an ontogenetic shift in habitat use and diet by recruiting to neritic habitats, where they consume an herbivorous diet consisting of various species of algae and seagrass (Bjorndal 1997). Although growth dynamics of juveniles during the oceanic stage are unknown, post-recruitment growth rates are known to vary temporally as a result of variation in oceanographic conditions (Limpus and Chaloupka 1997) and population density (Bjorndal *et al*. 2000). This variation in juvenile growth rates may have substantial effects on fitness, as body size is correlated with juvenile survivorship (Chaloupka and Limpus 1998) and clutch size (Broderick *et al*. 2003) in *C. mydas*.

Because of the potential for stochasticity of the nutritional environment during the oceanic and neritic stages of development and the effect of body size on survival and reproductive output in *C. mydas*, I predicted that green turtle juveniles should be capable of CG. Loggerhead sea turtles, which are also largely carnivorous as young juveniles (Bjorndal 1997), have been shown to undergo CG in the wild (Bjorndal *et al*. 2003), although the proximate explanation for this growth pattern is not known. By manipulating food availability under controlled conditions, I tested whether previously food-restricted green turtles can undergo CG and evaluated hyperphagia and enhanced FCE as potential mechanisms for this growth pattern.

Materials and Methods

Animal Care

All animal care components of this study were performed at the Cayman Turtle Farm in Grand Cayman, British West Indies, in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Florida (permit #Z061). *Chelonia mydas* hatchlings (n = 115) were housed individually in sea water in 68-liter tanks arranged within a large outdoor concrete enclosure. Tanks were systematically arranged within the enclosure to minimize position effects. Fresh sea water was continuously circulated within the enclosure (at a depth of approximately 20-25 cm) to dampen the daily cycle of heating and cooling within each of the tanks. Water temperature was monitored daily using five min/max thermometers distributed throughout the array of tanks.

All turtles were maintained on an *ad libitum* diet for seven days prior to the beginning of the study to establish average daily *ad libitum* intake. Turtles were then randomly assigned to one of three treatment groups: *ad libitum* (AL), restricted (R), and restricted-*ad libitum* (R-AL). Turtles in the AL group were offered food *ad libitum* for twelve weeks. Turtles in the R group were offered approximately 50% of average initial mass-specific *ad libitum* daily intake for twelve weeks. Because AL turtles increased their mass-specific intake after week 0, the actual amount of food consumed by restricted turtles amounted to an average of 27% of the daily mass-specific intake of AL turtles. This ration was sufficient to maintain restricted turtles on a positive growth trajectory throughout the study. Turtles in the R-AL group were offered the restricted diet for five weeks and were then offered food *ad libitum* for the remaining seven weeks.

Turtles were provided 2.6-mm turtle pellets (Melick Aquafeed, Catawissa, PA) twice daily and were allowed to feed for a total of seven to ten hours each day. Pellets remaining in each

tank were counted every afternoon, and approximate intake was calculated based on the average mass per pellet (determined weekly), the known mass of pellets offered, and the number of pellets remaining. Intake was estimated in this way for each turtle on each of six days a week (weather permitting), with the seventh day reserved for tank cleaning. The water in each tank was replaced daily. A subset of pellets was counted and weighed each week to determine average pellet mass. Five food samples were weighed and dried every two weeks for nutrient analyses.

Body mass (BM, to the nearest 0.1 g) and minimum straight carapace length (CL, to the nearest 0.05 mm) of each turtle were measured weekly. Daily intake measurements and weekly body size measurements were used to calculate average daily intake (g/day), mass-specific daily intake (g/g*day), condition index (CI, g/cm^3) (Ricker 1975), food conversion efficiency (FCE, g/g and mm/g), and specific growth rate (SGR, %/day) for each turtle for each week of the study. FCE and SGR were calculated for both BM and CL using the following formulas:

$$FCE = (size_{t+1} - size_t)/(\text{average daily intake} * 7)$$
$$SGR = 100*[\ln(size_{t+1})-\ln(size_t)]/7$$

where $size_t$ is BM or CL in one week and $size_{t+1}$ is BM or CL in the following week. FCE and SGR were calculated for both BM and CL because gut fill accounted for up to 15.4% of total wet BM, and changes in CL are not affected by the mass of gut contents. Furthermore, straight CL is considered to be the most reliable measure of growth in green turtles (Balazs 1995).

Gut Morphology and Body Composition

Turtles were sacrificed prior to, during, and after the study for an analysis of gut morphology and body composition. Ten randomly chosen turtles (t_0 AL) were euthanized prior to the initiation of the study, at which time all turtles had been feeding *ad libitum* for at least one week. At the conclusion of the fifth week of the experiment (immediately prior to switching R-AL turtles to an *ad libitum* diet), ten AL (t_5 AL), five R, and five R-AL turtles were

euthanized. The R-AL turtles had, until the end of week five, been maintained on the restricted feeding treatment. Data for the five R and five R-AL turtles were therefore pooled into one group (t_5 R). Ten AL turtles (t_{12} AL), ten R turtles (t_{12} R), and ten R-AL turtles (t_{12} R-AL) were euthanized at the conclusion of the twelve-week trial. Turtles were weighed to the nearest 0.1 g and then euthanized using an intramuscular overdose injection of ketamine (Ketaset, 100 mg/kg body mass).

The liver and digestive tract (from the esophageal-gastric junction to the termination of the hindgut anterior to the cloaca) of each turtle were removed. The full gut was measured to the nearest 0.05 mm. Measurements included straight stomach length (SSL) and total intestine length (TIL). Small intestine and large intestine lengths could not be determined because the distinction between midgut and hindgut was not easily discernible. Gut contents from each turtle were gently removed from the excised gut using forceps. Wet masses of gut contents, liver, empty stomach, and empty intestine were determined for each turtle. Organ mass and length indices were calculated using the following formulas:

$$\text{Mass Index} = 100*M_{Or}/(BM - M_{GC})$$
$$\text{Length Index} = L_{Or}/CL$$

where M_{Or} is wet mass of each organ (liver, stomach, or intestine), M_{GC} is wet mass of gut contents, and L_{Or} is length of stomach or intestine.

All tissues and carcasses were dried at 60 °C for a minimum of seven days. Dried body tissues (liver, stomach, intestines, and carcass) were recombined for each turtle (n = 10 t_0, 20 t_5, and 30 t_{12} turtles) and ground for nutrient analyses. Each turtle was ground in a mill (C.W. Brabender Instruments, Inc., South Hackensack, NJ) with dry ice. Dried food samples (collected every two weeks during the study) were also ground in the mill (without dry ice). Dry matter (DM) content of each turtle and each food sample was determined by drying subsamples at

105 °C for 16 hours, and organic matter (OM) content was determined by combustion at 500 °C for three hours (AOAC 1960). Energy content of each turtle and food sample was determined by bomb calorimetry (Parr 1960; Parr Instrument Co., Moline, IL). Lipid content was determined by ether extraction using a soxhlet apparatus (AOAC 1984). Nitrogen content was determined using a modified Kjeldahl procedure. Samples were digested for at least four hours at 375 °C using a modification of the aluminum block digestion procedure of Gallaher *et al*. (1975). Nitrogen in the digestate was determined by semiautomated colorimetry using a Technicon Autoanalyzer (Hambleton 1977; Pulse Instrumentation, Ltd., Saskatoon, SK, Canada). All nutrient analyses were performed in duplicate unless relative error exceeded 2.0%, in which case additional analyses were performed. The ratio of lipid to lean contents was calculated by dividing DM lipid content by DM protein content (% nitrogen * 6.25; Hambleton 1977).

Statistical Analyses

Data for food samples collected at two-week intervals were analyzed using Kruskal-Wallis tests. Weekly data were analyzed using repeated measures ANOVA with Tukey's Honestly Significant Difference (HSD) post hoc tests. Data for each week of the study and for midpoint and endpoint samples were also tested for significance using one-way ANOVA with Tukey's HSD post hoc tests. Bonferroni corrections were not used to account for multiple comparisons among weeks with one-way ANOVA because of the risk of inflated Type II error (Perneger 1998). Data for t_5 and t_{12} samples were tested for normality (using Shapiro-Wilk test) and homogeneity of variances (using Levene's test) prior to parametric analysis. If both tests yielded a significant result ($p < 0.05$), data were transformed using a natural log, reciprocal, square root, square, reciprocal square, or reciprocal square root transformation. If transformation did not improve normality or homoscedasticity, or if no post hoc test could be performed (e.g., for t_5 samples), data were tested for statistical significance using a Kruskal-Wallis test and pairwise

Mann-Whitney U tests with α set at 0.017 (for t_{12} samples) to account for multiple comparisons. Analysis of covariance (ANCOVA) could not be used to evaluate midpoint and endpoint data for organ sizes because the assumption that covariate values have similar distributions and ranges for all treatment groups (Quinn and Keough 2002) was violated.

All data were analyzed using SPSS for Windows (Release 11.0.0). Only turtles that survived, displayed no external signs of illness, and continued to gain mass until the time of tissue sampling were included in the analyses. Data are expressed as means ± standard errors with alpha set at 0.05 unless otherwise noted.

Results

OM, energy, nitrogen, and lipid contents of food samples (n = 7, collected at biweekly intervals) were consistent throughout the experiment (Table 2-1). Although differences in energy content of pellets among weeks approached significance, the relative difference between the highest and lowest energy values measured was only 2.87% (for DM) and 2.73% (for OM).

R and R-AL turtles had comparable values for all repeated measures data collected during weeks 0 through 5. Data for these two groups were analyzed and are presented separately for this time period to demonstrate that there were no differences between groups prior to the switch to an *ad libitum* diet for R-AL turtles.

Intake in week 0 (during which all turtles were feeding *ad libitum*) was similar for all three treatment groups (ANOVA, $F_{2,112} = 0.946$, $p = 0.392$). Repeated measures ANOVA of intake during weeks 1 through 5 (n = 37 AL, 39 R-AL, and 39 R) and during weeks 6 through 12 (n = 17 AL, 29 R-AL, and 29 R) revealed significant time and treatment effects as well as significant interactions between time and treatment group (Table 2-2). The pattern of AL intake was significantly different from the patterns of R and R-AL intake during weeks 1 through 5 (Tukey's HSD post hoc test, $p < 0.0001$ for both comparisons), and intake patterns of all three

treatment groups were significantly different during weeks 6 through 12 (Tukey's HSD post hoc test, $p < 0.0001$ for all comparisons). Because body size of turtles in each group differed after week 0, intake was corrected for BM and re-analyzed as mass-specific intake.

The magnitude but not the overall pattern of mass-specific intake of R turtles differed from that of AL turtles for the duration of the experiment, and the magnitude and overall pattern of mass-specific intake of R-AL turtles was comparable to that of R turtles during weeks 1-5 and comparable to that of AL turtles during weeks 6-12. Repeated measures ANOVA of mass-specific intake (Table 2-2) revealed no significant linear interaction between time and treatment group ($p > 0.05$ for time * treatment interactions using tests of within-subjects contrasts in repeated measures ANOVA) during either weeks 1 through 5 or weeks 6 through 12. However, there was a significant linear effect of time on mass-specific intake during weeks 1 through 5 and during weeks 6 through 12, as demonstrated by the decrease in mass-specific intake for turtles in all treatment groups during these two time intervals (Fig. 2-1). The pattern of mass-specific intake of AL turtles was significantly different from those of R and R-AL turtles during weeks 1 through 5 (Tukey's HSD post hoc test, $p < 0.0001$ for both comparisons), and the pattern of mass-specific intake of R turtles was significantly different from those of AL and R-AL turtles during weeks 6 through 12 (Tukey's HSD post hoc test, $p < 0.0001$ for both comparisons).

Body size (BM and CL) in week 0 (during which all turtles were feeding *ad libitum*) was similar for all three treatment groups (ANOVA, $F_{2,112} = 0.992$, $p = 0.374$ for BM; $F_{2,112} = 1.109$, $p = 0.333$ for CL) . Repeated measures ANOVA of body size during weeks 0 through 5 and during weeks 6 through 12 revealed significant time and treatment effects as well as significant interactions between time and treatment group (Table 2-3). The pattern of body size (both BM and CL) of AL turtles was significantly different from those of R and R-AL turtles during weeks

0 through 5 (Tukey's HSD post hoc tests, $p < 0.0001$ for both comparisons for BM and CL), and the patterns of body size (both BM and CL) of all three treatment groups were significantly different during weeks 6 through 12 (Tukey's HSD post hoc tests, $p < 0.0001$ for all comparisons for BM and CL). Within individual weeks, body size of AL turtles was significantly greater than those of R and R-AL turtles in weeks 1 through 6 (for BM) and in weeks 1 through 7 (for CL), and body size of all three groups differed significantly in weeks 7 through 12 (for BM) and in weeks 8 through 12 (for CL) (Fig. 2-2).

There were significant effects of time and treatment on condition index (CI) as well as a significant time by treatment interaction during weeks 0 through 5. There was no significant overall effect of time on CI during weeks 6 through 12, although there was a significant interaction between time and treatment group (Table 2-3). The pattern of CI in AL turtles was significantly different from those of R and R-AL turtles during weeks 0 through 5 (Tukey's HSD post hoc test, $p < 0.0001$ for both comparisons), and the pattern of CI in R turtles was significantly different from those of AL and R-AL turtles during weeks 6 through 12 (Tukey's HSD post hoc test, $p < 0.0001$ for both comparisons). The difference between AL and R-AL turtles approached significance (Tukey's HSD post hoc test, $p = 0.076$). Within individual weeks, CI of AL turtles was significantly greater than CI of R and R-AL turtles during weeks 1 through 5, and CI of R-AL turtles was intermediate between those of R and AL turtles in week 6. In weeks 7 through 12, CI of R turtles was significantly lower than CI of R and R-AL turtles, and CI of R-AL turtles was consistently but not significantly lower than CI of AL turtles (Fig. 2-3).

Specific growth rates (SGR) for BM and CL also differed among treatment groups for all weeks of the experiment. Repeated measures ANOVA of SGR revealed significant time and treatment effects and interactions between time and treatment group during weeks 1 through 5

and during weeks 6 through 12 (Table 2-4). The SGR patterns (for both BM and CL) of AL turtles were significantly different from those of R and R-AL turtles during weeks 1 through 5 (Tukey's HSD post hoc tests, $p < 0.0001$ for both comparisons for BM and CL), and the SGR patterns (for both BM and CL) of R turtles were significantly different from those of AL and R-AL turtles during weeks 6 through 12 (Tukey's HSD post hoc tests, $p < 0.0001$ for both comparisons for BM and CL). Compensatory growth occurred during weeks 7 through 9, as demonstrated by significantly larger SGR in R-AL turtles relative to AL turtles in weeks 7 and 8 (for SGR_{bm}) and in weeks 8 and 9 (for SGR_{cl}) (Fig. 2-4).

Food conversion efficiencies (FCE) for BM and CL differed among treatment groups, but the patterns depended on whether FCE was calculated as mass gain per unit of food consumed or as carapace length gain per unit of food consumed. Repeated measures ANOVA of FCE revealed significant time and treatment effects during weeks 1 through 5 and 6 through 12 and significant interactions between time and treatment group during weeks 6 through 12 (Table 2-5). The pattern of FCE_{bm} of AL turtles was significantly different from those of R and R-AL turtles during weeks 1 through 5 and during weeks 6 through 12 (Tukey's HSD post hoc tests, $p < 0.01$ for all comparisons). The pattern of FCE_{cl} of AL turtles likewise differed from those of R and R-AL turtles during weeks 1 through 5, but the pattern of FCE_{cl} for all treatment groups differed significantly for weeks 6 through 12 (Tukey's HSD post hoc tests, $p < 0.0001$ for all significant comparisons). Specifically, the two groups feeding *ad libitum* after week 5 differed in conversion efficiencies, with R-AL turtles demonstrating enhanced FCE_{bm} in weeks 6 and 7 and enhanced FCE_{cl} between weeks 6 and 11 (Fig. 2-5). Trends in conversion efficiencies were more consistent among weeks when FCE was calculated as change in carapace length per unit of food consumed.

Visceral organ size expressed as a percentage of BM (minus M_{GC}) or CL was smaller in food-restricted turtles than in *ad libitum*-fed turtles, particularly at t_{12} (Tables 2-6 and 2-7). Liver was lighter and stomach and intestine were shorter in R turtles than in AL turtles at t_5. At the end of the feeding trial, liver and stomach were lighter and stomach and intestine were shorter in R turtles than in AL or R-AL turtles. Intestine mass was lighter in R turtles than in R-AL turtles at t_{12}, and the difference between R and AL turtles approached significance ($p = 0.052$). Turtles at 5 weeks and 12 weeks also differed significantly in body composition (Tables 2-6 and 2-8), with R turtles having higher N and lower OM, lipid, energy, and lipid:lean contents than AL turtles at both t_5 and t_{12}. Body composition of R-AL turtles at t_{12} was comparable to that of AL turtles.

Daily water temperatures dropped slightly as the experiment progressed into late autumn. Occasional variation in temperatures was the result of rainfall from tropical weather systems, including a hurricane that occurred during week 8 (Fig. 2-6).

Discussion

The data clearly demonstrate that green turtles are capable of CG within the first three months after hatching. This growth pattern was not simply the result of rapid gut filling after the diet switch, as both BM and CL increased proportionally faster in R-AL turtles than in AL turtles. However, turtles undergoing CG achieved only partial compensation, because body size of R-AL individuals did not catch up to that of AL individuals by the time the period of CG ended. The fact that smaller, previously food-limited turtles grew faster than larger turtles of the same age represents a strategy that decreases the phenotypic variance among individuals from the same cohort (Wilson and Réale 2006). It appears that green turtles are able to evaluate their growth patterns and adjust them, at least somewhat, toward a more "optimal" trajectory when nutritional conditions improve. Because the risk of mortality from predation decreases as size increases for juvenile sea turtles (Musick and Limpus 1997), this capacity for rapid growth when

food availability is high allows young green turtles to expedite progression through the vulnerable hatchling stage.

The mechanism for CG in juvenile green turtles is enhanced FCE rather than hyperphagia. Given that CG is often effected through increased intake, particularly in fish (e.g., Jobling and Johansen 1999, Johansen *et al*. 2001, Nikki *et al*. 2004, Tian and Qin 2004), I expected previously restricted green turtles to become hyperphagic when food was provided *ad libitum*. The fact that green turtles were not hyperphagic suggests that intake rates of AL turtles were already maximal and therefore could not be exceeded by R-AL turtles. McCauley and Bjorndal (1999) previously demonstrated that juvenile loggerhead sea turtles also do not increase intake beyond the maximal rate attained when feeding *ad libitum*. Animals that do not increase consumption rates even when food is abundant may be intrinsically limited by constraints on rates of digestion and/or passage (Speakman and Król 2005). In contrast, the hyperphagic response of fish undergoing CG indicates that continuously *ad libitum*-fed fish feed at sub-maximal rates.

It is possible that enhanced conversion efficiency was effected through improved digestibility via either behavioral or morphological changes. Although I did not quantify behavior throughout the study, the tanks of food-restricted turtles rarely contained feces because these individuals practiced coprophagy. Reingestion of feces when food is limited has been reported in rodents (Kenagy *et al*. 1999) and serves to increase digestive efficiency. The extent to which R-AL turtles relied on nutrient recycling is unknown, but continued reingestion of feces after the switch to an *ad libitum* diet could have allowed for the improved FCE and elevated growth rates I observed. Digestibility could also have been increased by upregulation of intestinal surface area. However, preliminary data on intestinal histology indicate that

food-restricted turtles had not only smaller guts than *ad libitum* turtles in terms of mass and length but also decreased intestinal surface area (Roark and Bjorndal, unpublished data). Because I did not evaluate density of epithelial transporters, I cannot rule out the possibility that uptake rates may have been enhanced in food-restricted turtles despite a significant reduction in intestinal mass, as has been shown in food-restricted birds (Brzęk and Konarzewski 2001).

Alternatively, the enhanced FCE of R-AL turtles relative to AL turtles, especially in the first few weeks after the switch to *ad libitum* feeding, may have resulted from decreased metabolic expenditure. I did not quantify metabolic rates, but I did find that several major visceral organs (e.g., liver, stomach, and intestine) in R turtles were smaller than those in AL turtles. By down-sizing organs that would otherwise require disproportionate metabolic expenditure to maintain them, R turtles may have been able to allocate less of their assimilated food into maintenance metabolism and more into growth. Similar results have been obtained in studies of fasted or food-restricted migratory birds (Lee *et al*. 2002, Karasov *et al*. 2004), which had significantly smaller digestive and assimilatory organs compared to birds fed *ad libitum*. In the intestines, this decrease in size was due largely to changes in the mucosal layer of the villi. The observed decrease in organ mass in these birds was reversed by several days of feeding *ad libitum*.

Because I sampled only at times t_0, t_5, and t_{12}, I was unable to determine the time course over which the organ sizes of R and R-AL turtles changed. Upon a return to *ad libitum* conditions, R-AL turtles may have experienced a delay in up-regulation of visceral organ size. The switch to *ad libitum* feeding at a time when maintenance expenditure was minimized would have allowed for the rapid growth I documented in R-AL turtles during weeks 7 through 9 (Ali *et al*. 2003).

Although both BM and CL increased proportionally faster in R-AL turtles than in AL turtles, these two morphometric measurements demonstrated different dynamics. During the first week of *ad libitum* feeding, the SGR_{bm} but not the SGR_{cl} of R-AL turtles was comparable to that of AL turtles. This decoupling of mass and length growth may result from differential allocation of assimilated nutrients in the first week of *ad libitum* feeding. Less energy is required to convert assimilated nutrients into reserve tissue than into more complex structural components such as skeletal tissue (Broekhuizen *et al*. 1994). Growth efficiency would therefore be enhanced in turtles that preferentially allocated nutrients to mobilizable tissue gain rather than to unmobilizable tissue gain, at least in the initial stages of elevated growth. The time lag in the increase of SGR_{cl} but not in SGR_{bm} may also result simply from a rapid increase in BM immediately after the diet switch due to filling of the gut. At t_{12}, gut contents accounted for an average of 9.9%, 12.7%, and 13.5% of total wet BM for R, AL, and R-AL turtles, respectively. Turtles on the restricted diet therefore carried proportionally less digesta than turtles feeding *ad libitum*, meaning that gut filling probably accounted for a small percentage of the initial increase in BM after the diet switch for R-AL turtles.

I used my measurements of BM and CL to assess body condition (as condition index, CI) of turtles in each treatment group for each week of the study. Not surprisingly, CI of R turtles decreased steadily until approximately the eighth week of the experiment, indicating that these animals were becoming leaner as the study progressed. My body composition results support this conclusion. Total body nitrogen content was higher and OM, lipid, and energy contents were lower in R turtles than in AL turtles at both t_5 and t_{12}. Somatic growth in food-restricted turtles therefore entailed either lower rates of lipogenesis and/or protein catabolism or higher rates of protein deposition and/or lipid catabolism than in AL turtles. In other studies, food deprivation

has been linked to down-regulation of the activity of lipogenic enzymes (Bastrop *et al*. 1991, Rosebrough and McMurtry 1993), and alterations in protein metabolism during food restriction are also common (Dhahbi *et al*. 2001, Hagopian *et al*. 2003).

Turtles that experienced a switch from restricted to *ad libitum* feeding demonstrated a rapid increase in CI after the diet switch, such that body condition of R-AL turtles was not significantly different from that of AL turtles by week 7. The rapid growth of R-AL turtles was accompanied by elevated lipid deposition between weeks 5 and 12. This increase in whole body adiposity and concomitant decrease in nitrogen content allowed R-AL turtles to achieve a body composition not significantly different from that of AL turtles by the end of the experiment.

Cessation of CG may have resulted from R-AL turtles attaining a tissue composition similar to that of AL turtles. In other words, repletion of body stores may have served as a signal regulating the duration of the compensatory response (Jobling and Johansen 1999, Ali *et al*. 2003). The fact that body composition was restored before full body size compensation was achieved provides further evidence that CG may have been regulated by condition rather than overall body size. In fish, several studies (e.g., Bull and Metcalfe 1997, Johansen *et al*. 2001) have shown that the rate of repletion of lipid stores rather than the attainment of a certain body size controlled the duration of the compensatory response. In contrast to my results, body composition in these fish studies exerted its effects by altering appetite rather than FCE. Because I found no evidence for hyperphagia, I conclude that juvenile green turtles, unlike many fish species, do not adjust their intake in response to adiposity.

My finding that turtles experiencing consistently high food availability grew more slowly than turtles undergoing compensatory growth implies that maximal growth rates may not always be advantageous for green turtles. Despite the potential for increased body size to provide a

benefit to the individual in terms of fitness (Roff 1992, Stearns 1992), fast growth may carry a variety of costs (Arendt 1997, Blanckenhorn 2000, Metcalfe and Monaghan 2001). In other animal models, these costs include delayed skeletal ossification (Arendt and Wilson 2000), weakened musculature (Christiansen *et al*. 1992), reduced locomotor performance (Billerbeck *et al*. 2001, Álvarez and Metcalfe 2005), accelerated telomere degradation (Jennings *et al*. 1999), and decreased longevity (Olsson and Shine 2002). The proximate determinant of such costs may be the accumulation of cellular damage during rapid growth, as modeled by Mangel and Munch (2005). These detrimental effects of rapid growth may explain the sub-maximal growth rates typically demonstrated by animals feeding *ad libitum* continuously (Mangel and Stamps 2001). I have demonstrated that cellular antioxidant potential of R-AL turtles is decreased compared to AL turtles, at least in mitotically active tissue (Chapter 4). If such costs place an upper limit on growth in green turtles, they may further explain the transient and incomplete nature of the compensatory response I observed.

This study is the first to document the existence of and mechanisms for CG in young green turtles. The capacity to grow quickly, albeit only transiently, provides juveniles an opportunity to mitigate some of the costs of being small. At the same time, however, the transitory nature of the CG response suggests that the benefits of accelerated growth are countered by costs potentially including decreased longevity and/or performance that may be mediated by altered antioxidant function. The extent to which CG is possible at different ages and during different life stages is unknown but deserves further study. For example, the ontogenetic shift in habitat use and diet that green turtles undergo as juveniles may provide an opportunity for CG, as such niche shifts often correspond to improved food availability (Ali *et al*. 2003). What is clear from this study is that differences in food availability can induce plasticity in growth, morphology, and body

composition in young green turtles. This plasticity could substantially affect life-history endpoints such as age and size at maturity, reproductive output, and longevity that directly influence the viability of green turtle populations.

Table 2-1. Kruskal-Wallis test results for nutrient content of biweekly food samples (n = 2 at each of 7 time points).

Source of variation	Content Mean ± SE	*df*	χ^2	*p*
Per DM				
OM (%)	91.95 ± 0.04	6	7.371	0.288
Energy (kJ/g)	20.92 ± 0.06	6	12.457	0.053
Nitrogen (%)	8.28 ± 0.05	6	1.943	0.925
Lipids (%)	16.78 ± 0.26	6	2.057	0.914
Per OM				
Energy (kJ/g)	22.75 ± 0.06	6	12.457	0.053
Nitrogen (%)	9.01 ± 0.05	6	2.114	0.909
Lipids (%)	18.25 ± 0.28	6	2.057	0.914

According to the Melick Aquafeed label, crude fiber was ≤ 5% and phosphorus was ≥ 1%.
Abbreviations: DM = dry matter, OM = organic matter.

Table 2-2. Repeated measures analyses of variance for weekly averages of daily intake and daily mass-specific intake.

Source of variation	*df*	*SS*	*F*	*p*
Intake (g/day), weeks 1-5				
Between subjects effects				
Group	2	451.274	168.22	< **0.0001**
Error	112	150.225		
Within subjects effects				
Time	4	22.823	57.65	< **0.0001**
Group * Time	8	44.743	56.51	< **0.0001**
Error (Time)	448	44.343		
Within subjects linear contrasts				
Time	1	22.108	71.99	< **0.0001**
Group * Time	2	43.336	70.56	< **0.0001**
Error (Time)	112	34.395		
Intake (g/day), weeks 6-12				
Between subjects effects				
Group	2	1.747×10^{3}	103.56	< **0.0001**
Error	72	607.367		
Within subjects effects				
Time	6	248.000	168.37	< **0.0001**
Group * Time	12	139.612	47.39	< **0.0001**
Error (Time)	432	106.055		
Within subjects linear contrasts				
Time	1	236.124	296.96	< **0.0001**
Group * Time	2	129.989	81.74	< **0.0001**
Error (Time)	72	57.250		
Mass-specific intake (g/g*day), weeks 1-5				
Between subjects effects				
Group	2	5.497×10^{-2}	359.41	< **0.0001**
Error	112	8.566×10^{-3}		
Within subjects effects				
Time	4	3.863×10^{-4}	17.02	< **0.0001**
Group * Time	8	1.834×10^{-4}	4.04	< **0.01**
Error (Time)	448	2.543×10^{-3}		
Within subjects linear contrasts				
Time	1	3.044×10^{-4}	19.64	< **0.0001**
Group * Time	2	7.915×10^{-6}	0.26	0.775
Error (Time)	112	1.736×10^{-3}		
Mass-specific intake (g/g*day), weeks 6-12				
Between subjects effects				
Group	2	4.330×10^{-2}	387.27	< **0.0001**
Error	72	4.025×10^{-3}		
Within subjects effects				
Time	6	7.750×10^{-4}	15.86	< **0.0001**
Group * Time	12	3.655×10^{-4}	3.74	< **0.001**
Error (Time)	432	3.518×10^{-3}		
Within subjects linear contrasts				
Time	1	3.465×10^{-4}	18.04	< **0.0001**
Group * Time	2	3.775×10^{-5}	0.98	0.379
Error (Time)	72	1.383×10^{-3}		

Three groups were tested: *ad libitum* for 12 weeks, food-restricted for 12 weeks, and food-restricted for 5 weeks followed by *ad libitum* for 7 weeks. When Mauchley's test indicated that the compound symmetry assumption was violated, Greenhouse-Geisser *p*-values are presented. Significant *p*-values are in bold.

Table 2-3. Repeated measures analyses of variance for weekly body mass, straight carapace length, and condition index.

Source of variation	*df*	*SS*	*F*	*p*
Body Mass (g), weeks 0-5				
Between subjects effects				
Group	2	7.591×10^{4}	75.97	< **0.0001**
Error	112	5.596×10^{4}		
Within subjects effects				
Time	5	1.022×10^{5}	600.23	< **0.0001**
Group * Time	10	4.433×10^{4}	130.23	< **0.0001**
Error (Time)	560	1.906×10^{4}		
Body Mass (g), weeks 6-12				
Between subjects effects				
Group	2	1.411×10^{6}	68.24	< **0.0001**
Error	72	7.442×10^{5}		
Within subjects effects				
Time	6	5.885×10^{5}	395.15	< **0.0001**
Group * Time	12	2.320×10^{5}	77.90	< **0.0001**
Error (Time)	432	1.072×10^{5}		
Carapace Length (mm), weeks 0-5				
Between subjects effects				
Group	2	6.012×10^{3}	50.02	< **0.0001**
Error	112	6.731×10^{3}		
Within subjects effects				
Time	5	2.599×10^{4}	2711.94	< **0.0001**
Group * Time	10	3.180×10^{3}	165.88	< **0.0001**
Error (Time)	560	1.073×10^{3}		
Carapace Length (mm), weeks 6-12				
Between subjects effects				
Group	2	5.701×10^{4}	80.43	< **0.0001**
Error	72	2.552×10^{4}		
Within subjects effects				
Time	6	2.891×10^{4}	1041.43	< **0.0001**
Group * Time	12	6.613×10^{3}	119.13	< **0.0001**
Error (Time)	432	1.998×10^{3}		
Condition Index (g/cm^3), weeks 0-5				
Between subjects effects				
Group	2	1.937×10^{-2}	22.75	< **0.0001**
Error	112	4.767×10^{-2}		
Within subjects effects				
Time	5	4.218×10^{-2}	657.58	< **0.0001**
Group * Time	10	5.556×10^{-3}	43.30	< **0.0001**
Error (Time)	560	7.185×10^{-3}		
Condition Index (g/cm^3), weeks 6-12				
Between subjects effects				
Group	2	3.872×10^{-2}	33.42	< **0.0001**
Error	72	4.171×10^{-2}		
Within subjects effects				
Time	6	1.351×10^{-4}	2.14	0.103
Group * Time	12	7.117×10^{-4}	5.63	< **0.0001**
Error (Time)	432	4.553×10^{-3}		

Three groups were tested: *ad libitum* for 12 weeks, food-restricted for 12 weeks, and food-restricted for 5 weeks followed by *ad libitum* for 7 weeks. When Mauchley's test indicated that the compound symmetry assumption was violated, Greenhouse-Geisser *p*-values are presented. Significant *p*-values are in bold.

Table 2-4. Repeated measures analyses of variance for weekly specific growth rates (SGR) for body mass (bm) and carapace length (cl).

Source of variation	*df*	*SS*	*F*	*p*
SGR_{bm} (%/day), weeks 1-5				
Between subjects effects				
Group	2	336.783	306.07	< **0.0001**
Error	112	61.620		
Within subjects effects				
Time	4	115.394	205.55	< **0.0001**
Group * Time	8	9.937	8.85	< **0.0001**
Error (Time)	448	62.875		
SGR_{bm} (%/day), weeks 6-12				
Between subjects effects				
Group	2	310.715	218.38	< **0.0001**
Error	72	51.222		
Within subjects effects				
Time	6	18.779	18.27	< **0.0001**
Group * Time	12	7.770	3.78	< **0.0001**
Error (Time)	432	74.002		
SGR_{cl} (%/day), weeks 1-5				
Between subjects effects				
Group	2	24.174	202.20	< **0.0001**
Error	112	6.695		
Within subjects effects				
Time	4	27.471	562.96	< **0.0001**
Group * Time	8	1.494	15.31	< **0.0001**
Error (Time)	448	5.465		
SGR_{cl} (%/day), weeks 6-12				
Between subjects effects				
Group	2	25.945	180.39	< **0.0001**
Error	72	5.178		
Within subjects effects				
Time	6	2.029	38.20	< **0.0001**
Group * Time	12	1.851	17.43	< **0.0001**
Error (Time)	432	3.824		

Three groups were tested: *ad libitum* for 12 weeks, food-restricted for 12 weeks, and food-restricted for 5 weeks followed by *ad libitum* for 7 weeks. When Mauchley's test indicated that the compound symmetry assumption was violated, Greenhouse-Geisser *p*-values are presented. Significant *p*-values are in bold.

Table 2-5. Repeated measures analyses of variance for food conversion efficiencies (FCE) for body mass (bm) and carapace length (cl).

Source of variation	df	SS	F	*p*
FCE_{bm} (g/g), weeks 1-5				
Between subjects effects				
Group	2	7.755	18.546	< **0.0001**
Error	112	23.418		
Within subjects effects				
Time	4	13.142	36.504	< **0.0001**
Group * Time	8	1.278	1.775	0.086
Error (Time)	448	40.323		
FCE_{bm} (g/g), weeks 6-12				
Between subjects effects				
Group	2	1.932	5.975	**0.004**
Error	72	11.640		
Within subjects effects				
Time	6	3.261	7.224	< **0.0001**
Group * Time	12	4.082	4.522	< **0.0001**
Error (Time)	432	32.498		
FCE_{cl} (mm/g), weeks 1-5				
Between subjects effects				
Group	2	24.260	198.288	< **0.0001**
Error	112	6.851		
Within subjects effects				
Time	4	18.711	232.633	< **0.0001**
Group * Time	8	0.123	0.767	0.574
Error (Time)	448	9.008		
FCE_{cl} (mm/g), weeks 6-12				
Between subjects effects				
Group	2	6.792	130.635	< **0.0001**
Error	72	1.872		
Within subjects effects				
Time	6	1.800	72.206	< **0.0001**
Group * Time	12	0.294	5.891	< **0.0001**
Error (Time)	432	1.795		

Three groups were tested: *ad libitum* for 12 weeks, food-restricted for 12 weeks, and food-restricted for 5 weeks followed by *ad libitum* for 7 weeks. When Mauchley's test indicated that the compound symmetry assumption was violated, Greenhouse-Geisser *p*-values are presented. Significant *p*-values are in bold.

Table 2-6. Omnibus F, χ^2, and p-values for analyses of variance of dissection data collected at weeks 5 and 12.

		Identity of Groups Tested in Pairwise Comparisons		
	Omnibus F and χ^2	AL and R-AL	AL and R	R-AL and R
Week 5				
BM (g)	$F_{1,18} = 30.06$, $\mathbf{p < 0.0001}$			
CL (mm)	$F_{1,18} = 21.98$, $\mathbf{p < 0.001}$			
LM Index (%)	$F_{1,18} = 80.05$, $\mathbf{p < 0.0001}$			
SM Index (%)	$F_{1,18} = 1.60$, $p = 0.222$			
TIM Index (%)	$F_{1,18} = 1.03$, $p = 0.324$			
SSL Index	$F_{1,18} = 19.28$, $\mathbf{p < 0.001}$			
TIL Index	$F_{1,18} = 8.03$, $\mathbf{p = 0.011}$			
% OM	$F_{1,18} = 20.22$, $\mathbf{p < 0.001}$			
N (% DM)	$\chi^2 = 12.10$, $\mathbf{p = 0.001}$			
N (% OM)	$\chi^2 = 14.35$, $\mathbf{p < 0.001}$			
Lipid (% DM)	$\chi^2 = 13.72$, $\mathbf{p < 0.001}$			
Lipid (% OM)	$\chi^2 = 11.84$, $\mathbf{p < 0.001}$			
Energy (kJ/g DM)	$F_{1,18} = 31.33$, $\mathbf{p < 0.0001}$			
Energy (kJ/g OM)	$\chi^2 = 14.29$, $\mathbf{p < 0.001}$			
Lipid:Lean	$\chi^2 = 14.29$, $\mathbf{p < 0.001}$			
Week 12				
BM (g)	$F_{2,27} = 227.3$, $\mathbf{p < 0.0001}$	**0.050**	**< 0.0001**	**< 0.0001**
CL (mm)	$F_{2,27} = 73.08$, $\mathbf{p < 0.0001}$	0.061	**< 0.0001**	**< 0.0001**
LM Index (%)	$F_{2,27} = 107.8$, $\mathbf{p < 0.0001}$	0.765	**< 0.0001**	**< 0.0001**
SM Index (%)	$F_{2,27} = 14.55$, $\mathbf{p < 0.0001}$	0.544	**< 0.0001**	**0.001**
TIM Index (%)	$F_{2,27} = 4.91$, $\mathbf{p = 0.015}$	0.892	0.052	**0.019**
SSL Index	$F_{2,27} = 17.86$, $\mathbf{p < 0.0001}$	0.150	**< 0.0001**	**0.001**
TIL Index	$F_{2,27} = 18.67$, $\mathbf{p < 0.0001}$	0.697	**0.0001**	**< 0.0001**
% OM	$F_{2,27} = 64.16$, $\mathbf{p < 0.0001}$	0.454	**< 0.0001**	**< 0.0001**
N (% DM)	$F_{2,27} = 11.05$, $\mathbf{p < 0.001}$	0.099	**< 0.001**	**0.043**
N (% OM)	$\chi^2 = 19.94$, $\mathbf{p < 0.0001}$	0.280	**< 0.0001**	**< 0.0001**
Lipid (% DM)	$F_{2,27} = 63.75$, $\mathbf{p < 0.0001}$	0.562	**< 0.0001**	**< 0.0001**
Lipid (% OM)	$F_{2,27} = 51.73$, $\mathbf{p < 0.0001}$	0.468	**< 0.0001**	**< 0.0001**
Energy (kJ/g DM)	$F_{2,27} = 69.97$, $\mathbf{p < 0.0001}$	0.467	**< 0.0001**	**< 0.0001**
Energy (kJ/g OM)	$F_{2,27} = 44.34$, $\mathbf{p < 0.0001}$	0.172	**< 0.0001**	**< 0.0001**
Lipid:Lean	$F_{2,27} = 73.56$, $\mathbf{p < 0.0001}$	0.696	**< 0.0001**	**< 0.0001**

When F values are reported, data were analyzed using analysis of variance with Tukey's HSD post hoc test. When χ^2 values are reported, data were analyzed using a Kruskal-Wallis test and pairwise Mann-Whitney U tests with α set at 0.017 to account for multiple comparisons among t_{12} groups. Statistically significant p-values are indicated in bold. Abbreviations: AL = *ad libitum* for 12 weeks, R = food-restricted for 12 weeks, R-AL = food-restricted for 5 weeks and *ad libitum* for 7 weeks, BM = body mass, CL = carapace length, LM = liver mass, SM = stomach mass, TIM = total intestine mass, SSL = stomach straight length, TIL = total intestine length, OM = organic matter, DM = dry matter.

Table 2-7. Organ masses (mean ± standard error) from turtles dissected at 0, 5, and 12 weeks reported as indices (organ mass or length as a proportion of body mass or carapace length, respectively).

Group	Week	*n*	BM (g)	CL (mm)	LM Index (%)	SM Index (%)	TIM Index (%)	SSL Index	TIL Index
AL	0	10	34.2 ± 0.6	61.7 ± 0.6	3.61 ± 0.15	0.92 ± 0.04	2.97 ± 0.12	0.67 ± 0.02	8.22 ± 0.18
AL	5	10	92.6 ± 9.6[a]	88.0 ± 2.6[a]	4.04 ± 0.17[a]	1.40 ± 0.09[a]	3.89 ± 0.21[a]	0.75 ± 0.03[a]	9.08 ± 0.22[a]
R	5	10	51.6 ± 1.9[b]	75.2 ± 1.0[b]	2.38 ± 0.07[b]	1.24 ± 0.09[a]	3.62 ± 0.16[a]	0.61 ± 0.02[b]	8.03 ± 0.29[b]
AL	12	10	227.5 ± 25.8[a]	117.4 ± 4.3[a] [1]	5.29 ± 0.27[a]	1.58 ± 0.11[a]	4.17 ± 0.15[ab] [2]	0.82 ± 0.01[a]	10.76 ± 0.26[a]
R-AL	12	10	150.0 ± 9.2[b]	105.7 ± 1.7[a] [1]	4.97 ± 0.20[a]	1.45 ± 0.07[a]	4.27 ± 0.17[a]	0.78 ± 0.02[a]	11.07 ± 0.34[a]
R	12	10	71.7 ± 1.0[c]	85.9 ± 0.9[b]	2.58 ± 0.10[b]	0.97 ± 0.05[b]	3.61 ± 0.16[b] [2]	0.69 ± 0.02[b]	8.91 ± 0.18[b]

[1]Difference between AL and R-AL approaches significance ($p = 0.061$).
[2]Difference between AL and R approaches significance ($p = 0.052$).
Within columns, values with different letter superscripts are significantly different within time periods (Kruskal-Wallis test or analysis of variance followed by pairwise Mann-Whitney U tests or Tukey's HSD post hoc test, $p < 0.05$). Abbreviations are the same as in Table 2-6.

Table 2-8. Body composition (mean ± standard error) of turtles dissected at 0, 5, and 12 weeks reported as percent of dry matter (% DM) and percent of organic matter (% OM).

Group	Week	*n*	% OM	Nitrogen Content		Lipid Content		Energy Content		Lipid:Lean
				% DM	% OM	% DM	% OM	kJ/g DM	kJ/g OM	
AL	0	10	85.5 ± 0.3	11.96 ± 0.09	13.99 ± 0.08	20.3 ± 0.4	23.8 ± 0.4	20.9 ± 0.1	24.4 ± 0.1	0.272 ± 0.006
AL	5	10	82.2 ± 0.3[a]	12.20 ± 0.21[a]	14.85 ± 0.28[a]	15.5 ± 1.4[a]	18.8 ± 1.7[a]	19.7 ± 0.3[a]	23.9 ± 0.4[a]	0.206 ± 0.023[a]
R	5	10	80.3 ± 0.3[b]	13.00 ± 0.06[b]	16.19 ± 0.04[b]	10.0 ± 0.1[b]	12.5 ± 0.2[b]	17.8 ± 0.1[b]	22.2 ± 0.1[b]	0.123 ± 0.002[b]
AL	12	10	81.4 ± 0.3[a]	11.68 ± 0.17[a]	14.35 ± 0.25[a]	18.6 ± 1.4[a]	22.8 ± 1.6[a]	20.5 ± 0.4[a]	25.1 ± 0.4[a]	0.258 ± 0.023[a]
R-AL	12	10	82.0 ± 0.3[a]	12.07 ± 0.12[a]	14.72 ± 0.15[a]	16.6 ± 0.8[a]	20.2 ± 1.0[a]	20.0 ± 0.3[a]	24.3 ± 0.4[a]	0.221 ± 0.013[a]
R	12	10	76.6 ± 0.5[b]	12.54 ± 0.10[b]	16.38 ± 0.06[b]	9.4 ± 0.3[b]	12.3 ± 0.4[b]	16.7 ± 0.1[b]	21.8 ± 0.1[b]	0.120 ± 0.004[b]

Within columns, values with different letter superscripts are significantly different within time periods. Treatment groups and data analysis are the same as in Table 2-7.

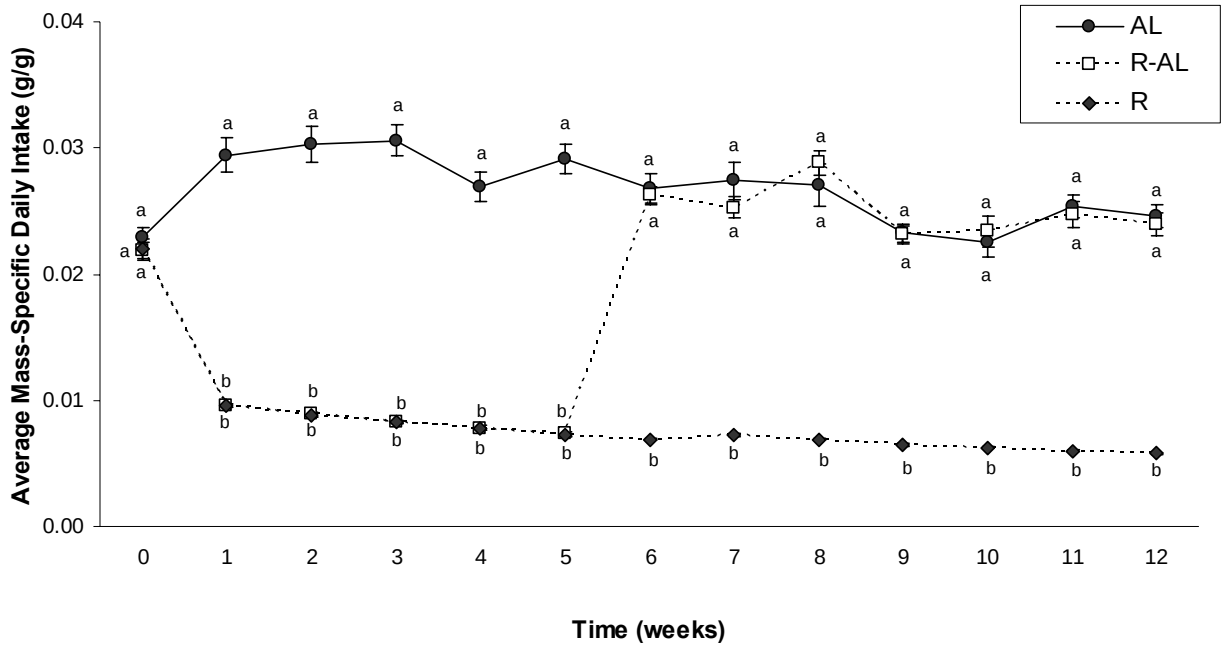


Figure 2-1. Average mass-specific daily intake (mean ± standard error) during each week of the feeding trial. Different letters indicate values that are significantly different within weeks (analysis of variance, Tukey's HSD post hoc test, $p < 0.05$). Turtles in the R-AL group were switched from a restricted diet to an *ad libitum* diet at the beginning of week 6. Sample sizes in weeks 0 through 5: $n = 37$ AL, 39 R-AL, and 39 R. Sample sizes in weeks 6 through 12: $n = 17$ AL, 29 R-AL, and 29 R. Abbreviations: AL = *ad libitum* for 12 weeks, R = food-restricted for 12 weeks, R-AL = food-restricted for 5 weeks and *ad libitum* for 7 weeks.

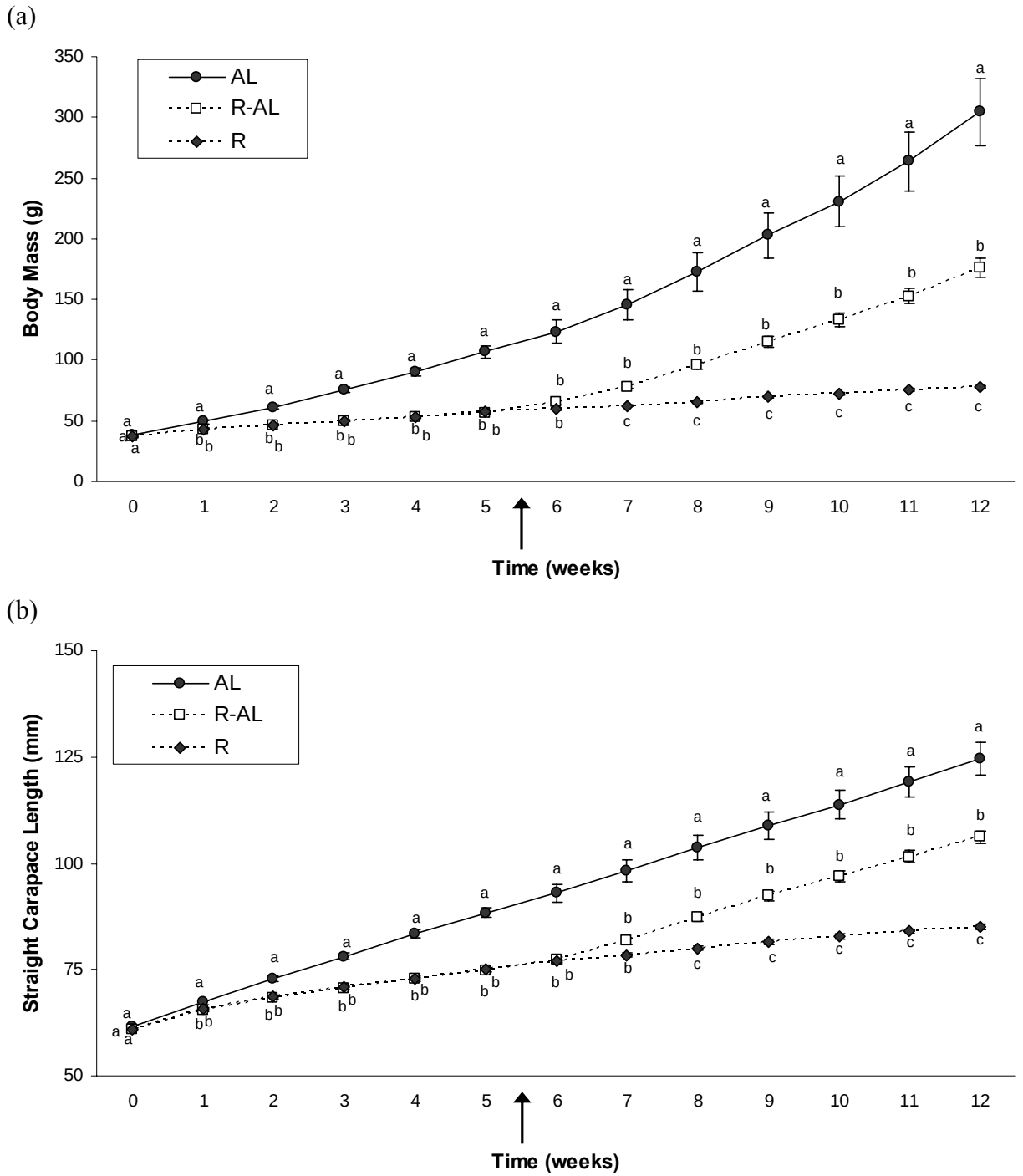


Figure 2-2. Body mass (a) and straight carapace length (b) (mean ± standard error) at the midpoint of each week. Different letters indicate values that are significantly different within weeks (analysis of variance, Tukey's HSD post hoc test, $p < 0.05$). The arrow indicates the time at which turtles in the R-AL group were switched from a restricted diet to an *ad libitum* diet. Sample sizes and abbreviations are the same as in Figure 2-1.

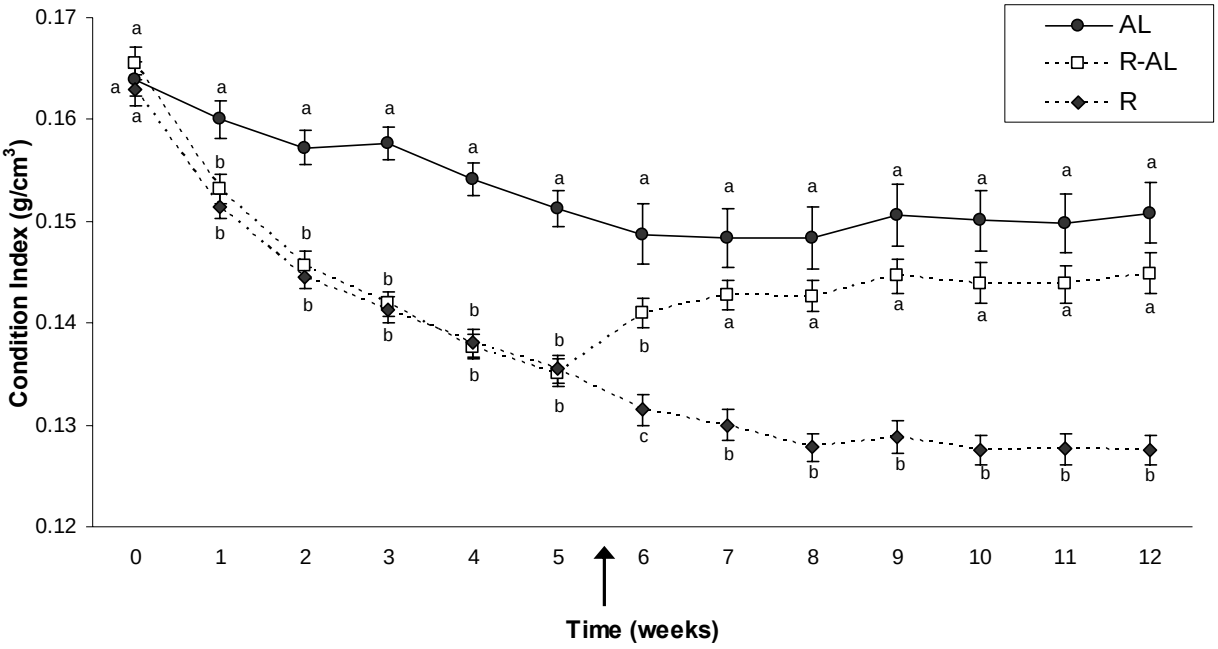
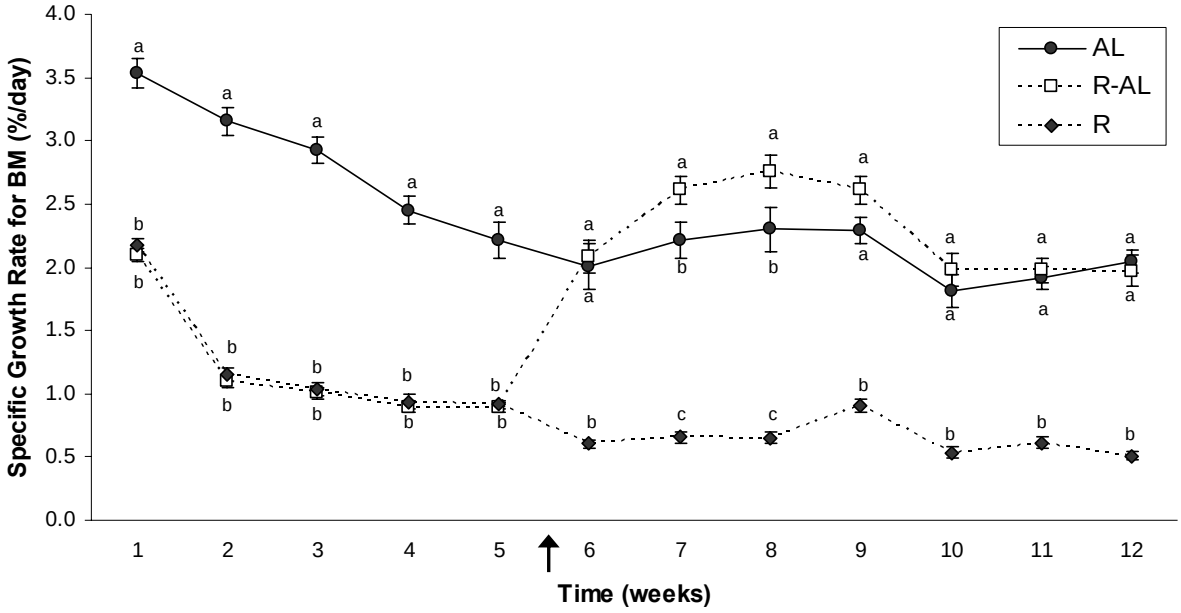


Figure 2-3. Condition index (mean ± standard error) in each week calculated as BM/CL^3, where BM = body mass (g) and CL = carapace length (cm). Different letters indicate values that are significantly different within weeks (analysis of variance, Tukey's HSD post hoc test, $p < 0.05$). The arrow indicates the time at which turtles in the R-AL group were switched from a restricted diet to an *ad libitum* diet. Sample sizes and abbreviations are the same as in Figure 2-1.

(a)



(b)

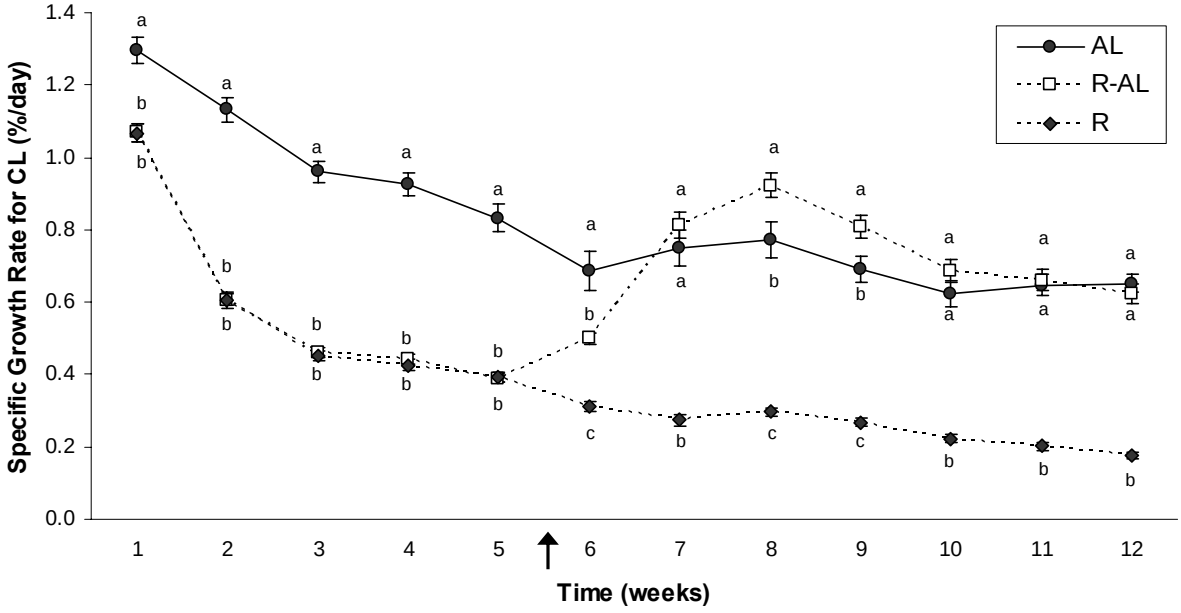
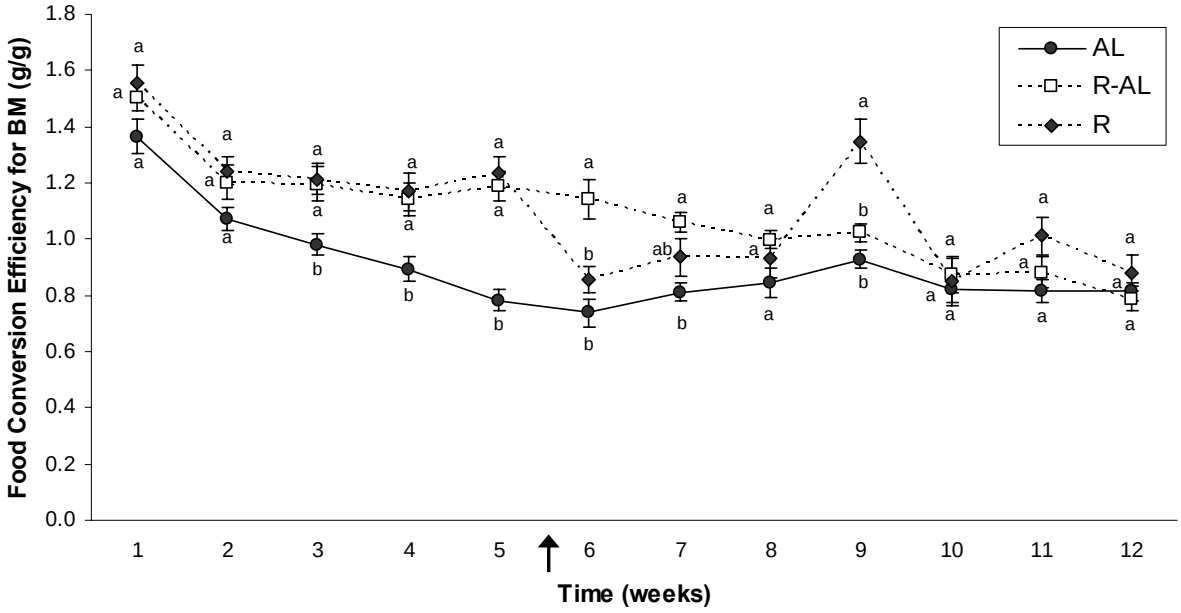


Figure 2-4. Specific growth rate (mean ± standard error) for body mass (BM, a) and straight carapace length (CL, b) during each week calculated as $100*(\ln[size_{t+1}]-\ln[size_t])/7$ where size = BM (a) or CL (b) and t = time (weeks). Different letters indicate values that are significantly different within weeks (analysis of variance, Tukey's HSD post hoc test, $p < 0.05$). The arrow indicates the time at which turtles in the R-AL group were switched from a restricted diet to an *ad libitum* diet. Sample sizes and abbreviations are the same as in Figure 2-1.

(a)



(b)

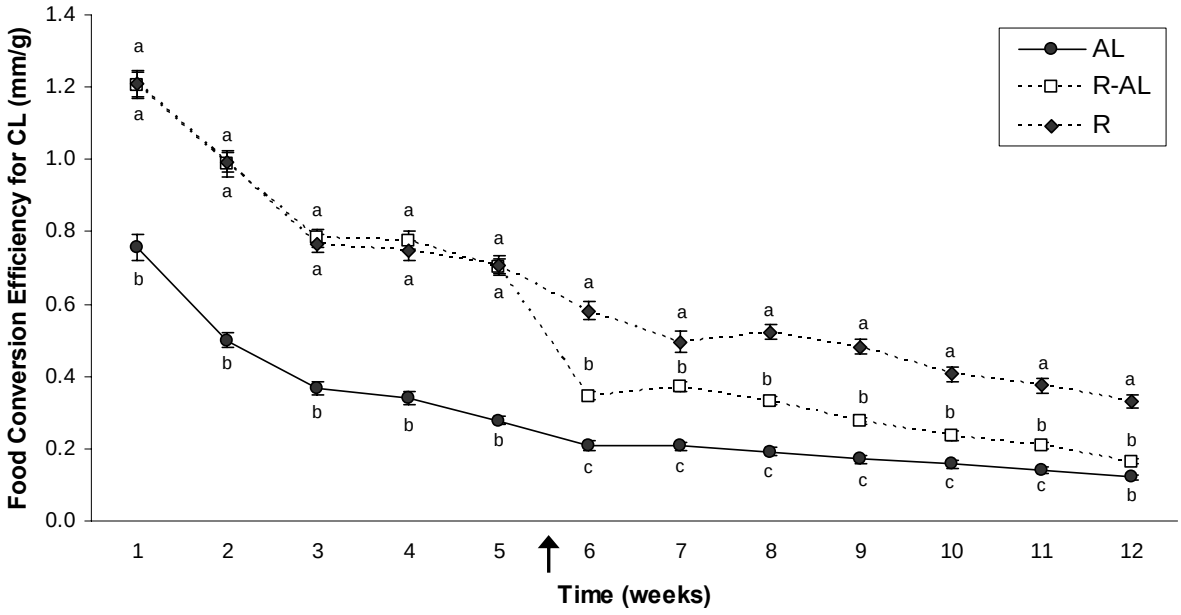


Figure 2-5. Food conversion efficiency (FCE, mean ± standard error) for body mass (BM, a) and straight carapace length (CL, b) during each week calculated as size change per unit of food consumed, where size = BM (a) or CL (b). Different letters indicate values that are significantly different within weeks (analysis of variance, Tukey's HSD post hoc test, $p < 0.05$). The arrow indicates the time at which turtles in the R-AL group were switched from a restricted diet to an *ad libitum* diet. Sample sizes and abbreviations are the same as in Figure 2-1.

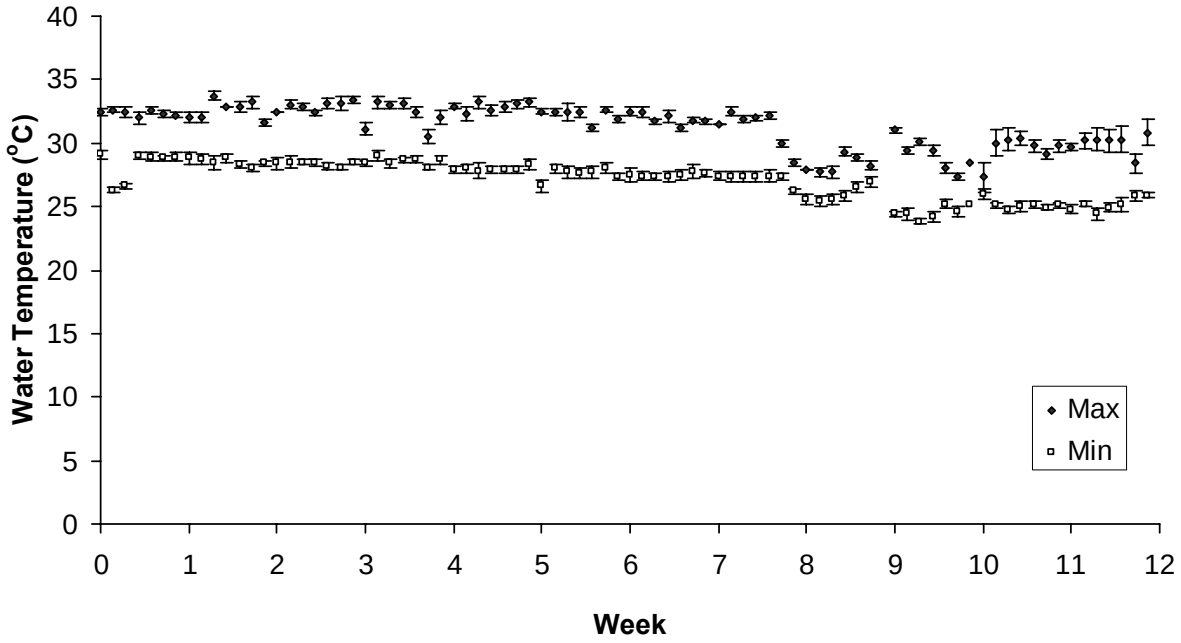


Figure 2-6. Daily water temperatures (mean ± standard deviation) throughout the feeding trial. Water temperature was monitored using five min/max thermometers in tanks without turtles. Water temperatures in weeks 8 and 9 fluctuated as a result of a hurricane.

CHAPTER 3
BIOCHEMICAL INDICES AS CORRELATES OF RECENT GROWTH IN JUVENILE GREEN TURTLES (*Chelonia mydas*)

Introduction

The green turtle, *Chelonia mydas*, is an endangered marine herbivore with a circumglobal distribution (Seminoff 2002). Overexploitation of this species by humans during the last several centuries has caused drastic population declines (Jackson *et al*. 2001). Development of effective management plans for this species requires knowledge of demographic parameters such as somatic growth rates. However, assessing growth rates for long-lived and far-ranging green turtles typically requires time-consuming mark and recapture programs in which recapture probabilities can be quite low (Limpus 1992). Furthermore, growth rates calculated using morphometrics represent long-term, cumulative changes and often do not correlate well with biochemical indices of short-term growth due to differences in the latency of these responses to environmental influences (Ferron and Leggett 1994, Gilliers *et al*. 2004). Establishment of alternative techniques for estimating recent growth rates of individual turtles upon first capture would substantially improve our ability to evaluate the instantaneous status of *C. mydas* populations and therefore to assess progress toward recovery goals for this endangered species.

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 (including messenger RNA, transfer RNA, and ribosomal RNA) should increase

as the cellular demand for 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 cellular protein synthesis capacity. Because nucleic acid concentrations and the ratios between them respond rapidly to fluctuations in food availability, they are considered reliable indices 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 use of morphometric and biochemical indices as predictors of recent growth rates in green turtles maintained under controlled feeding conditions. Because analyzing biochemical indices of growth typically requires homogenization of tissues extracted from euthanized individuals, I also examined the potential for measuring nucleic acid concentrations in whole blood, a tissue that is not typically tested in studies of this kind. Validation of a physiological growth index that can be assessed using minimally invasive sampling techniques and without sacrificing the animals would substantially enhance our ability to monitor short-term responses of green turtles to environmental perturbations.

Materials and Methods

Animal Care

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 (permit #Z061). Details of the animal care aspects of this study can be found in Chapter 2. Briefly, *Chelonia mydas* hatchlings were housed individually in sea water in 68-liter tanks. Turtles were fed turtle pellets (Melick Aquafeed, Catawissa, PA) twice daily.

Prior to the beginning of the study, turtles were all fed *ad libitum* to establish average daily intake. 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 average initial AL intake each day for 12 weeks, and turtles in the restricted-*ad libitum* group (R-AL) were fed the restricted amount of food for five weeks and then were fed *ad libitum* for seven weeks. The amount of food offered during food restriction was sufficient to maintain turtles on a positive growth trajectory. Turtles were weighed and measured (straight carapace length) each week.

Tissue Collection

At the conclusion of the twelve-week study, seven AL turtles, ten R turtles, and ten R-AL turtles were weighed to the nearest 0.1 g and euthanized with an intramuscular overdose injection of ketamine (Ketaset, 100 mg/kg body mass) in the right pectoral muscle. When each turtle failed to respond to a pain stimulus, it was decapitated.

A blood sample was collected from the decapitation site (as in Storey *et al*. 1993 and 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 three minutes after decapitation. Tissues were maintained at –80 °C until they were homogenized as described below.

Biochemical Assays

Tissues from each individual turtle were analyzed for DNA and RNA concentrations. 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. Subsamples of blood (different from those used for DNA isolation) were weighed but not ground in liquid

nitrogen because of their tendency to thaw quickly. 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 from each turtle for a total of at least 18 subsamples for each of 27 turtles unless tissue mass was insufficient.

The concentrations of DNA and RNA in each subsample of blood, heart, and liver tissue 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^{TM} data analysis software.

In addition, protein concentration of liver (but not heart or blood) was quantified for a separate project (Chapter 4), so I included those data in the analysis of this study. Hepatic protein concentrations were determined by Bradford assay (details, Chapter 4).

Statistical Analyses

Data for body mass (BM) and 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:

$$SGR_{bm} = (\ln[BM_f] - \ln[BM_i])*100/t$$
$$SGR_{cl} = (\ln[CL_f] - \ln[CL_i])*100/t$$

where BM_i and CL_i represent body size 10 or 11 days prior to tissue sampling, BM_f and CL_f represent body size on the day of tissue sampling, and t represents time (10 or 11 days). Condition index (CI) was calculated as Fulton's K ($CI = BM_f/CL_f^3$, Ricker 1975). Data for BM_f,

CL_f, CI, SGR_{bm}, SGR_{cl}, RNA concentration of each tissue, DNA concentration of each tissue, RNA:DNA ratio of each tissue, liver protein concentration, liver protein:DNA ratio, and liver RNA:protein ratio were compared for the three feeding treatments using analysis of variance (ANOVA). All ratios were calculated as the quotient of the average protein, DNA, and/or RNA concentration for a particular tissue. RNA:protein ratios should reflect RNA content per cell, but only if protein:DNA ratios (as a measure of cellular protein content) are consistent among treatment groups.

All data were tested for normality (using Shapiro-Wilk test) and homogeneity of variances (using Levene's test) prior to parametric analysis and transformed, if necessary, using a natural log, reciprocal, square root, square, reciprocal square, or reciprocal square root transformation. 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. If ANOVA revealed a significant difference, pairwise comparisons were evaluated using Tukey's Honestly Significant Difference post hoc test (if variances were equal) or Tamhane's T2 post hoc test (if variances were unequal). To evaluate repeatability of biochemical assays, coefficients of variation (C.V.) were determined for RNA and DNA concentrations in liver, heart, and blood and for protein concentration in liver.

Spearman's rank correlation test was used to test the strength of the relationships among BM_f, CL_f, CI, SGR_{bm}, SGR_{cl}, [RNA] in each tissue, [DNA] in each tissue, [RNA]:[DNA] in each tissue, liver [protein], liver [protein]:[DNA], and liver [RNA]:[protein]. Regression models for SGR_{bm} and SGR_{cl} were then developed using CI and biochemical indices as independent variables. Although body length has been correlated with RNA:DNA ratios in fish (e.g., Rooker *et al*. 1997), I did not include treatment group or any measure of total body size as independent

variables in my linear regression models. I chose not to include BM or CL as variables because body size was strongly affected by diet treatment (Fig. 3-1), and the goal of this study was to assess the applicability of RNA:DNA measurements in estimating recent growth rates of wild turtles with unknown dietary histories.

Regression equations for SGR_{bm} and SGR_{cl} versus each biochemical index were determined using least squares linear regression. Data were natural log-transformed, if necessary, to linearize them and to decrease heteroscedasticity. I verified the assumptions of linear regression by visually inspecting plots of Studentized deleted residuals versus standardized predicted values. To construct 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 biochemical indices measured for a particular tissue (liver, heart, or blood) were included in separate models. A growth model incorporating condition index and all biochemical indices measured for all tissues was also constructed. To enter a model, variables had to meet a 0.05 significance level. All statistical tests were performed using SPSS for Windows (Release 11.0.0). Means are reported ± standard errors with alpha set at 0.05.

Results

When fed to satiation, green turtle juveniles in the final 10-11 days of the 12-week trial grew at an average SGR_{bm} of 1.84% and 2.01% per day and an average SGR_{cl} of 0.68% and 0.64% per day for AL and R-AL individuals, respectively. Food-restricted turtles grew much more slowly at an average SGR_{bm} of 0.34% per day and an average SGR_{cl} of 0.15% per day (Fig. 3-1 and Table 3-1).

Intake and growth patterns significantly affected all morphometric measurements of body size (Fig. 3-1 and Table 3-1). At the time of tissue sampling, AL turtles were significantly

heavier and longer than both R-AL and R turtles, and R turtles were significantly lighter and shorter than both AL and R-AL turtles. Despite differences in body size between AL and R-AL turtles, condition indices for these two groups were comparable at the time of tissue sampling, and CI of R-AL turtles was significantly greater than CI of R turtles. The difference in CI between AL and R turtles approached significance ($p = 0.057$). Although R-AL turtles were food-restricted for the first five weeks of the study, they grew more rapidly than AL turtles during weeks seven through nine after the switch to *ad libitum* feeding, but this period of compensatory growth ended prior to tissue sampling (Chapter 2). As a result, R-AL turtles were growing at comparable rates to AL turtles during the last 10-11 days of the study, and R turtles were growing significantly slower than both AL and R-AL turtles. Significant differences among treatment groups also existed for many of the biochemical indices I measured (Figs. 3-2 and 3-3 and Table 3-1). 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 I measured demonstrated significant positive or negative correlations (Table 3-2). 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.

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

3-3). The R^2 values for significant relationships ranged from 0.161 to 0.659, with the best fits 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 3-4). 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. Despite the strong coefficient of determination for several SGR models, coefficients of variation for RNA and DNA concentrations in liver, heart, and blood and for protein concentration in liver (Table 3-5) were fairly substantial, indicating a high degree of interassay variation.

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 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, I expected to find strong positive correlations between growth, RNA and/or protein concentrations, and ratios among nucleic acids and protein concentrations in green turtles.

Contrary to my expectations, the biochemical indices I 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. I therefore infer that slow-growing R turtles had more total RNA, and consequently higher putative protein synthesis capacity, per unit of liver wet mass than fast-growing AL or R-AL turtles. Conversely, heart RNA concentrations in this study were positively correlated with SGR_{bm} (but not with SGR_{cl}) as expected, although this relationship was not strong. Growth rate had no apparent correlation with blood RNA content.

The pattern between DNA and growth rate was quite different from that between RNA and growth rate. Concentrations of DNA in blood and liver (but not in heart) were both negatively correlated with SGR, a trend that has also been noted in fish (Mercaldo-Allen *et al.* 2006). Because DNA concentration is a measure of the density of nuclei and therefore correlates with cell number, I conclude that total blood cell count increases in response to food restriction. It is unclear which of the six predominant types of nucleated blood cells in green turtles (Wood and Ebanks 1984) accounts for this increase in blood cell number. The typical hematological response to caloric restriction 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). My DNA results could also reflect differences in

red blood cell counts. 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 elevated DNA concentrations in larger, rather than smaller, turtles. It is therefore unlikely that my results for DNA concentration reflect a body size-dependence for this parameter. Hematocrit does not normally increase during food restriction (e.g., Maxwell *et al.* 1990a, Lochmiller *et al.* 1993). However, Maxwell *et al.* (1990a) demonstrated that enhanced erythropoiesis with concomitant microcytosis occurs in food-restricted birds, suggesting that my DNA results may reflect differences in blood cell size between slow- and fast-growing turtles.

My DNA results also indicate that liver growth results from hypertrophy more than hyperplasia in fast-growing green turtles. In reptiles, 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). Although I did not examine livers from my animals histologically, farm-raised marine turtles fed *ad libitum* are known to have hepatocytes dominated by large lipid droplets (Solomon and Tippett 1991). I surmise that a process of lipid and glycogen deposition similar to that observed by Starck and Beese (2002) occurs to a greater extent in green 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 I 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 mechanistic basis for the discrepancy between my nucleic acid and protein concentrations and those found in comparable studies using fish, I 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 index is only valid as a measure of cellular RNA content if the protein:DNA ratio (as 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.

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, I expected to find similar trends in my turtle tissues. Indeed, 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 29-34% of the variance in SGR. I 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, therefore, that the RNA:DNA ratios I 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, I suggest that my 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 my 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 increase in response to caloric restriction and that enzymes involved in gluconeogenesis are upregulated in the liver (Spindler 2001, Hagopian *et al*. 2003). A similar upregulation of metabolic enzyme production may have occurred in the livers of my food-restricted turtles. 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.

To expand the predictive power of the various indices I measured, I incorporated condition index and all biochemical indices measured for a particular tissue (liver, heart, or blood) into a series of models using stepwise multiple linear regression. The resulting predictive equations explained a maximum of 68% 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 juvenile green turtle growth in the Caribbean has previously been developed (Bjorndal *et al*. 2000), this model uses body size to predict recent growth and therefore 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 my 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. Specifically, using nucleic acid content to predict growth rates (for body mass) increased the coefficient of determination from 38% using CI alone to 55% using blood DNA content or to 68% using liver

RNA content. Predictive power is therefore substantially improved by incorporating biochemical indices into growth models.

In the various growth models I 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.

The growth model I developed fails to explain 32% of the variance in growth rates. A portion of this unexplained variability probably results from fairly large coefficients of variation for the biochemical assays I performed. This variation could potentially have been improved by measuring DNA and RNA concentrations from the same subsamples of tissue, but the nucleic acid isolation kits I used precluded me from doing so. Additionally, a number of nucleic acid quantification techniques are available (Caldarone *et al.* 2006), and it is possible that one of these techniques might have allowed for improved precision in measuring DNA and RNA content. 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 presumably 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. This coefficient of determination represents a loss of only 13% of the maximal predictive power achieved by the best model I developed. Both CI and blood DNA content are easily measured with limited disturbance to the animal. In combination with morphometric measurements, the blood cells of *C. mydas* may therefore allow for the development of minimally invasive techniques for estimating recent growth rates in this endangered species.

Table 3-1. Omnibus F, χ^2, and p-values for comparisons of means among treatment groups for the various morphometric and biochemical indices measured (n = 27 for each variable).

		Groups Tested in Pairwise Comparisons		
	Omnibus F and χ^2	AL and R-AL	AL and R	R and R-AL
Body Mass	$F_{2,24}$ = 99.476, **p < 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
Carapace Length	χ^2 = 22.013, **p < 0.0001**	**< 0.001**	**< 0.001**	**< 0.0001**
Condition Index	$F_{2,24}$ = 6.918, **p = 0.004**	0.684	0.057	**0.004**
SGR_{bm}	$F_{2,24}$ = 28.863, **p < 0.0001**	0.616	**0.002**	**< 0.001**
SGR_{cl}	$F_{2,24}$ = 62.995, **p < 0.0001**	0.737	**< 0.0001**	**< 0.0001**
$[RNA]_{liver}$	$F_{2,24}$ = 28.953, **p < 0.0001**	0.946	**< 0.0001**	**< 0.0001**
$[RNA]_{heart}$	$F_{2,24}$ = 6.076, **p = 0.007**	**0.021**	0.990	**0.015**
$[RNA]_{blood}$	$F_{2,24}$ = 1.946, p = 0.165	N/A	N/A	N/A
$[DNA]_{liver}$	$F_{2,24}$ = 12.349, **p < 0.001**	0.154	**< 0.001**	**0.010**
$[DNA]_{heart}$	$F_{2,24}$ = 2.111, p = 0.143	N/A	N/A	N/A
$[DNA]_{blood}$	$F_{2,24}$ = 5.278, **p = 0.013**	0.312	**0.010**	0.162
$[RNA]:[DNA]_{liver}$	$F_{2,24}$ = 6.546, **p = 0.005**	0.191	0.319	**0.004**
$[RNA]:[DNA]_{heart}$	$F_{2,24}$ = 4.678, **p = 0.019**	0.240	0.524	**0.015**
$[RNA]:[DNA]_{blood}$	$F_{2,24}$ = 5.224, **p = 0.013**	0.303	**0.010**	0.172
$[Protein]_{liver}$	$F_{2,24}$ = 3.545, **p = 0.045**	0.320	0.630	**0.036**
$[Protein]:[DNA]_{liver}$	$F_{2,24}$ = 7.655, **p = 0.003**	**0.012**	**0.003**	0.793
$[RNA]:[Protein]_{liver}$	$F_{2,24}$ = 13.438, **p < 0.001**	0.888	**0.003**	**0.003**

When F values are reported, data were analyzed using analysis of variance and pairwise comparisons were evaluated using Tukey's Honestly Significant Difference or Tamhane's T2 post hoc tests. When χ^2 values are reported, data were analyzed using a Kruskal-Wallis test with pairwise Mann-Whitney U tests. Statistically significant p-values are indicated in bold. Abbreviations: SGR = specific growth rate, bm = body mass, cl = carapace length, [ ] = concentration, AL = *ad libitum* for twelve weeks, R-AL = food-restricted for five weeks and *ad libitum* for seven weeks, R = food-restricted for twelve weeks.

Table 3-2. Spearman's rank correlations (ρ) for morphometric (a) and biochemical indices for liver (b), heart (c), and blood (d) (n = 27 for each variable).

(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****

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.

Table 3-3. 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**

Specific growth rate for body mass or carapace length 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 3-2.

Table 3-4. 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**

Specific growth rate for body mass or carapace length (the dependent variables) was regressed against condition index and 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 and 2 (for SGR_{bm}) and model 6 (for SGR_{cl}). 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 3-2.

Table 3-5. Coefficients of variation (C.V.) for RNA, DNA, and protein concentrations of *Chelonia mydas* tissues.

Tissue	Assay	C.V. (%)
Liver	[RNA]	33.2
Heart	[RNA]	32.2
Blood	[RNA]	20.0
Liver	[DNA]	32.4
Heart	[DNA]	35.2
Blood	[DNA]	38.9
Liver	[Protein]	11.0

Values represent averages of C.V.s for 27 individual turtles for each assay. A minimum of three replicates for each individual for each assay was performed unless sample mass was insufficient.

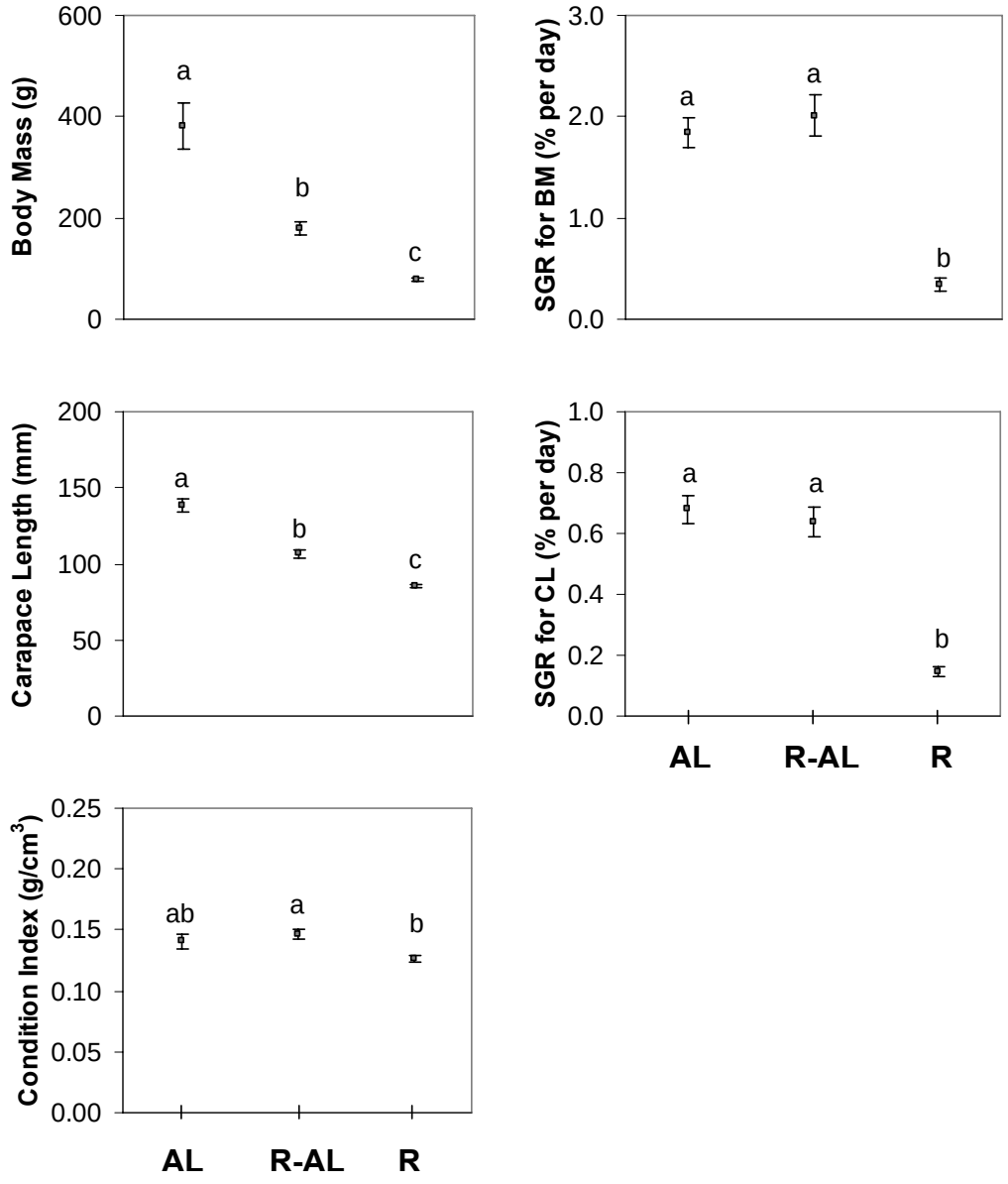
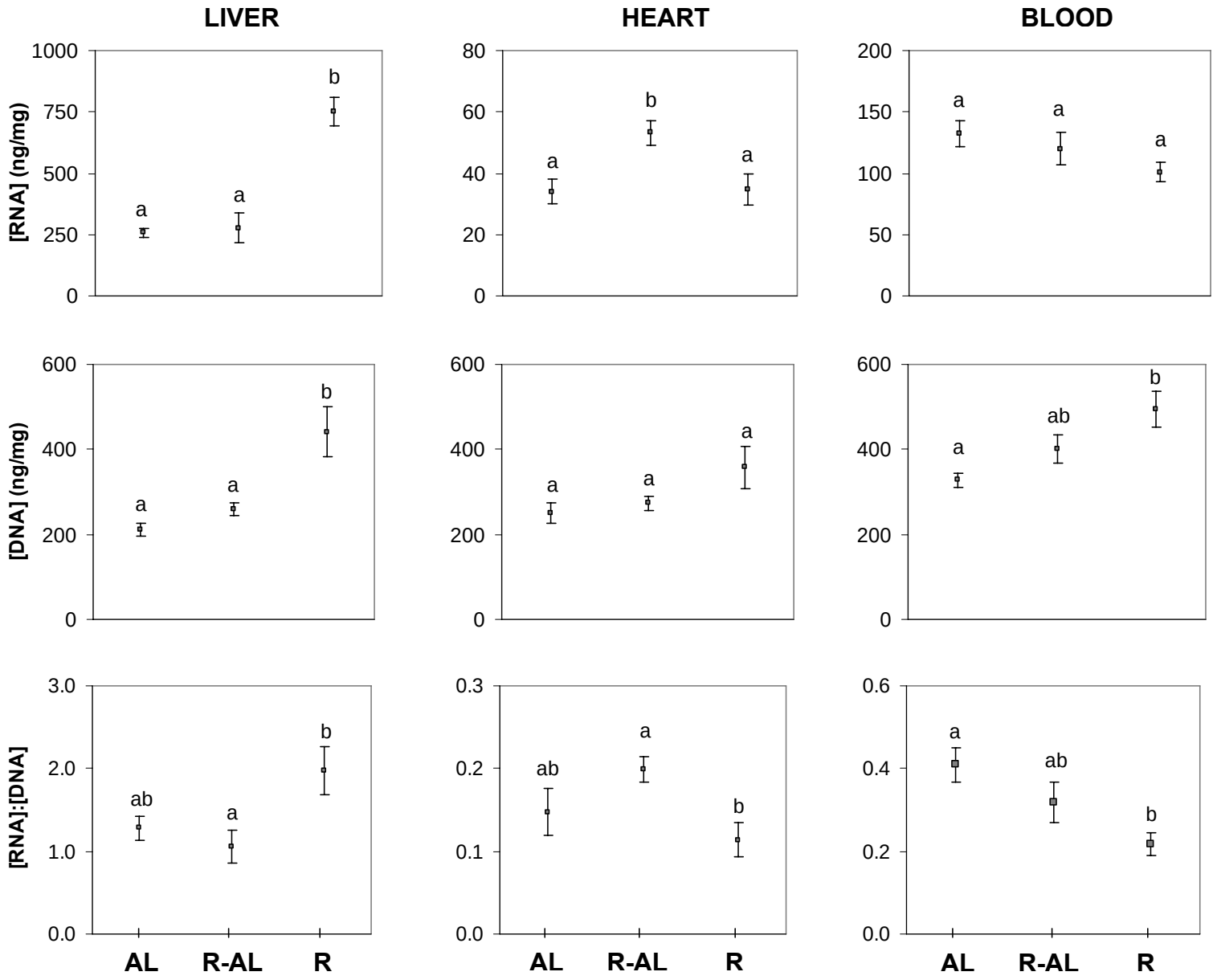


Figure 3-1. Morphometric indices and growth rates for turtles in each of three treatment groups. Turtles in the AL group (n = 7) were fed *ad libitum* for 12 weeks. Turtles in the R-AL group (n = 10) were fed 50% of initial mass-specific AL intake for 5 weeks and then fed *ad libitum* for 7 weeks. Turtles in the R group (n = 10) were fed 50% of initial mass-specific AL intake for 12 weeks. Each point represents mean ± standard error. Means were evaluated using analysis of variance with Tukey's Honestly Significant Difference or Tamhane's T2 post hoc tests 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.

Figure 3-2. Nucleic acid indices for turtles in each of three treatment groups. Each point represents mean ± standard error. Means that are significantly different at $p < 0.05$ are indicated by different letters. Treatments, data analysis, sample sizes, and abbreviations are the same as in Figure 3-1.


LIVER
HEART
BLOOD
[RNA] (ng/mg)
[DNA] (ng/mg)
[RNA]:[DNA]
1000
750
500
250
0
80
60
40
20
600
400
200
3.0
2.0
1.0
0.3
0.2
0.1
150
100
50
0.6
0.4
a
ab
b
AL
R-AL
R

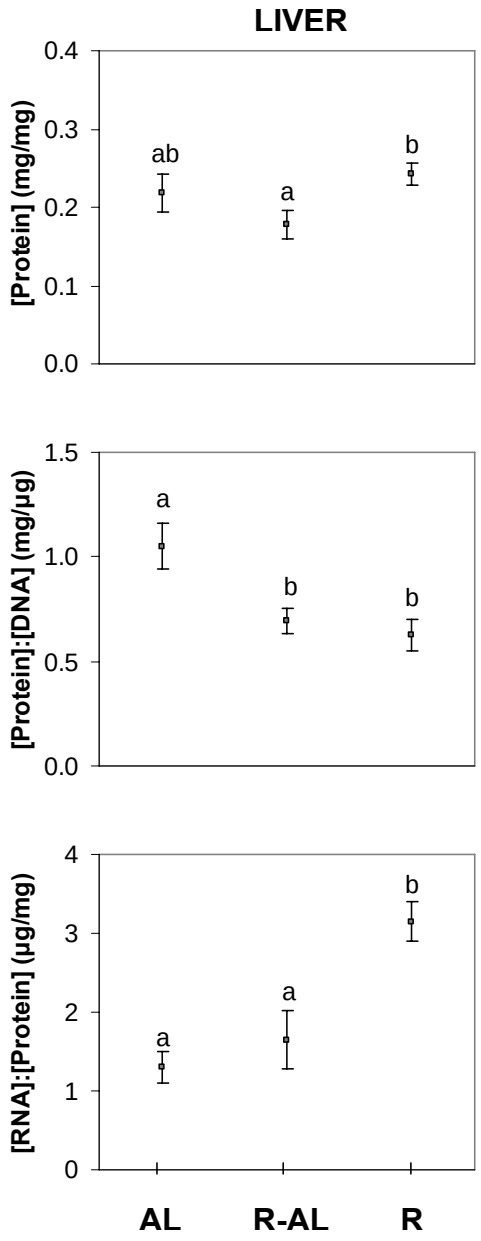


Figure 3-3. Liver protein and protein-based indices for turtles in each of three treatment groups. Each point represents mean ± standard error. Means that are significantly different at $p < 0.05$ are indicated by different letters. Treatments, data analysis, sample sizes, and abbreviations are the same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

CHAPTER 4
COMPENSATORY GROWTH AND ANTIOXIDANT STATUS IN JUVENILE GREEN TURTLES (*Chelonia mydas*)

Introduction

Poor early nutrition and resulting periods of depressed growth can have profound life-history consequences, some of which extend into subsequent generations (as reviewed by Metcalfe and Monaghan 2001). Slow growth that results in small size at a particular age or developmental stage can increase vulnerability to predation (Arendt 1997, Janzen *et al*. 2000), weaken dominance status (Richner *et al*. 1989), impede the establishment of feeding and/or breeding territories (Einum and Fleming 2000 and references therein), alter patterns of sexual dimorphism, or delay maturation and the onset of reproductive competence (Altmann and Alberts 2005). The nutritional environment an animal experiences early in its life can also adversely affect adult body size (Madsen and Shine 2000), survival (McDonald *et al*. 2005), fecundity (Nagy and Holmes 2005), and offspring size (Reznick *et al*. 1996).

Given the negative effects of slow growth and small size on performance, survival, and ultimately fitness, selection should favor compensatory strategies that allow individuals to grow rapidly when conditions improve (Metcalfe and Monaghan 2001). Such a period of 'catch up' or compensatory growth (CG) has been demonstrated in a number of organisms (Wilson and Osbourn 1960, Ali *et al*. 2003, Bjorndal *et al*. 2003, Jespersen and Toft 2003). Compensatory growth is characterized by growth rates greater than those of consistently well nourished conspecifics of the same age and can result in comparable body sizes for individuals with drastically different dietary histories (Metcalfe and Monaghan 2001). The benefits of a rapid increase in size include improved short-term survival due to decreased size-specific mortality (e.g., due to predation) (Arendt 1997, Metcalfe and Monaghan 2003) and enhanced reproductive

output, especially for organisms (e.g., ectotherms) in which fecundity is proportional to body size (Roff 1992).

Despite the potential fitness benefits of accelerated growth, the occurrence of CG suggests that maximal growth rates are not always optimal under conditions of ample food availability. Sub-maximal growth rates, even when food availability is high, presumably reflect a balance between the benefits and costs of rapid growth. Structures formed during periods of fast growth may be prone to weakness, as is the case for bird primary feathers (Dawson *et al*. 2000) and leg bones (Leterrier and Nys 1992) as well as fish scales (Arendt *et al*. 2001). Animals that have undergone a period of CG can incur costs including increased muscle protein degradation (Therkildsen 2005), decreased muscle mass (Bélanger *et al*. 2002), impaired locomotor performance (Álvarez and Metcalfe 2005), accelerated telomere shortening, and decreased longevity (Jennings *et al*. 1999). At the cellular level, animals feeding *ad libitum* (and therefore growing rapidly) typically produce more mitochondrial free radicals and thus experience more oxidative damage than calorie-restricted animals (Gredilla and Barja 2005). It has been suggested (although not tested, to my knowledge) that the detrimental effects of CG on performance and longevity may result from transient elevated rates of free radical-induced cellular damage (Mangel and Munch 2005).

In the present study, I manipulated growth trajectories of green turtle (*Chelonia mydas*) juveniles by controlling intake. Before and after a demonstrated period of CG, I evaluated muscle and liver glutathione peroxidase (GPX) activity and hepatic antioxidant potential (AP). Glutathione peroxidase is the major cytosolic and mitochondrial enzyme that catalyzes the reduction of hydroperoxides into water (Li *et al*. 2000), thereby protecting cells from oxidative damage to protein, lipids, and DNA (Barja 2004). Total AP reflects activity of antioxidant

enzymes such as GPX in addition to reducing capacity of non-enzymatic antioxidants (Sies 1997). Quantifying GPX activity and AP permits me to assess whether diminished capacity to combat cellular oxidative damage may be a cost of CG in this species. To my knowledge, this study is the first to test the effects of CG on these parameters.

Materials and Methods

Animal Care

All animal care components of this study were performed at the Cayman Turtle Farm in Grand Cayman, British West Indies. *Chelonia mydas* hatchlings were housed individually in 68-L bins of sea water. Turtles were fed turtle pellets (Melick Aquafeed, Catawissa, PA) twice daily.

Turtles in the *ad libitum* group (AL) were fed *ad libitum* for twelve weeks. Turtles in the restricted group (R) were fed approximately 50% of the average initial AL intake (on a mass-specific basis) for twelve weeks. Turtles in the restricted-*ad libitum* group (R-AL) were fed the restricted diet for five weeks and were then fed *ad libitum* for the remaining seven weeks. Turtles were weighed and measured weekly. Additional details regarding animal husbandry can be found in Chapter 2.

Tissue Collection and Homogenization

At the conclusion of the fifth week of the experiment (immediately prior to switching R-AL turtles to an *ad libitum* diet), ten AL (t_5 AL), five R, and five R-AL turtles were sacrificed. All R-AL turtles had, until the end of week five, been maintained on the restricted diet. Data for the five R and five R-AL turtles were therefore pooled into one group (t_5 R). The remaining turtles - seven AL turtles (t_{12} AL), ten R turtles (t_{12} R) , and ten R-AL turtles (t_{12} R-AL) - were sacrificed at the conclusion of the twelve-week trial. Turtles were euthanized with an intramuscular injection of ketamine (Ketaset, 100 mg/kg body mass).

After each turtle had been injected with ketamine and was no longer responsive to a pain stimulus, it was decapitated. Portions of the right lobe of the liver and the left pectoral muscle were removed with forceps and snap-frozen in liquid nitrogen no more than three minutes after decapitation. Tissues were maintained at –80 °C until they were homogenized as described below.

Subsamples of liver and pectoral muscle 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 and muscle homogenates were further diluted to 10% and 33%, respectively, to insure that enzyme activities would be within the range of the standard curves used. Total protein concentration of each tissue solution 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 tissue solutions (in triplicate) were determined by absorbance at 595 nm with a 695 nm reference wavelength using a microplate reader.

Glutathione Peroxidase Activity Assay

Glutathione peroxidase activity was evaluated using a total GPX assay modified from Nakamura *et al*. (1974). Diluted muscle and liver homogenate solutions (n = 46) were incubated for three minutes at 25 °C in a reaction cocktail containing 0.297 U/ml glutathione reductase, 1.25 mM glutathione, and 0.188 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 four minutes 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 duplicate (for muscle, because no significant relationships were found) or triplicate (for liver).

Total GPX activity of each sample was calculated by determining the rate of change in absorbance of NADPH (ΔA_{340}/min, calculating using only linear data) and dividing this value by the extinction 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 total protein concentration of each sample as determined by Bradford assay.

Total AP Assay

Overall, nonspecific 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 (e.g., superoxide dismutase, GPX, and catalase), small molecules (e.g., ascorbic acid), large molecules (e.g., albumin), and hormones (e.g., estrogen) (OxisResearch Bioxytech Assay Systems label, 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 (an antioxidant) 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 total protein concentration of each sample as determined by Bradford assay.

Statistical Analyses

Data for GPX activity and total AP were normalized to total protein content as described above. In addition, I also calculated these parameters per μg of DNA in t_{12} turtles using nucleic acid contents from Chapter 3 for liver only (DNA contents of muscle and t_{12} liver were not

evaluated for that study). Presumably, expressing GPX activity and AP per mg of protein reflects the proportion of total proteins functioning as antioxidants, whereas expressing these parameters per μg of DNA reflects antioxidant capacity per cell.

Data were tested for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) prior to parametric analysis. If either test yielded a significant result ($p < 0.05$), data were transformed using a natural log, reciprocal, square root, square, reciprocal square, or reciprocal square root transformation. If transformation did not improve homoscedasticity and Tamhane's T2 post hoc test could not be used, data were tested for statistical significance using a Kruskal-Wallis test. Otherwise, data within each sampling period were tested for statistical significance using analysis of variance (ANOVA). When statistically significant differences among t_{12} treatment groups were found, pairwise comparisons were evaluated using Tukey's Honestly Significant Difference (HSD) post hoc test (if variances were equal) or Tamhane's T2 post hoc test (if variances were not equal).

Data were analyzed using SPSS for Windows (Release 11.0.0). For all reported analyses, data are expressed as means ± standard errors (unless otherwise noted) with alpha set at 0.05.

Results

Turtles in the AL group grew significantly faster than those in the R group during each week of the study. After the switch from a restricted to an *ad libitum* diet (at the end of five weeks), R-AL turtles grew significantly faster than those in the AL group during weeks 7 through 9. This period of growth compensation ceased prior to the end of the study such that AL and R-AL turtles were growing at comparable rates by the time t_{12} samples were collected (as reported in Chapter 2). Different growth rates yielded significantly different body masses for turtles in each treatment sampled at the conclusion of week five and at the conclusion of week twelve (Fig. 4-1 and Table 4-1).

Diet treatments affected protein content of muscle (for t_5 turtles) and liver (for t_{12} turtles) expressed as concentration of protein per wet mass of tissue (Tables 4-1 and 4-2). After five weeks of food restriction, protein content of muscle was 59% greater in R turtles than in AL turtles, but this difference decreased after week 5 such that muscle protein content did not differ among treatment groups by week 12 of the study. Hepatic protein content only differed at t_{12}, with liver of R turtles containing 36% more protein than liver of R-AL turtles.

Specific activity of GPX in pectoral muscle did not differ significantly among treatment groups at t_5 or at t_{12} (Tables 4-1 and 4-3). Furthermore, interassay variation was quite high for measurements of muscle GPX activity (Table 4-4). However, differences in GPX activity of liver expressed per mg of protein approached significance ($p = 0.090$) for t_{12} turtles, with AL turtles demonstrating 20-24% greater hepatic GPX activity than R and R-AL turtles (Fig. 4-2 and Table 4-1). Because muscle GPX activity was not affected by intake and growth rates, I restricted my analysis of AP to liver.

Total hepatic AP per mg of protein differed significantly among treatment groups for both t_5 and t_{12} turtles (Fig. 4-2 and Table 4-1). After five weeks of food restriction, R turtles had a higher hepatic AP per mg of protein than AL turtles. After twelve weeks of food restriction, hepatic AP per mg of protein of R-AL turtles was significantly lower than that of R turtles, and the difference between AL and R-AL turtles approached significance ($p = 0.098$).

The aforementioned values for GPX activity and AP were calculated by correcting for total protein content of samples. Using DNA content per mg of liver from Chapter 3 as a correlate of cell number in t_{12} turtles, I also compared putative GPX activity and total AP per μg of DNA and found differences among treatment groups (Fig. 4-2 and Table 4-1). When normalized to tissue DNA content in this way, both GPX activity and total AP per cell were significantly higher in t_{12}

AL turtles than in R and R-AL turtles, despite the fact that AL and R-AL turtles were growing at comparable rates at this time.

Discussion

The objective of this study was to evaluate decreased antioxidant capacity as a possible cost of growth compensation in juvenile green turtles. By manipulating food intake during a controlled, twelve-week feeding trial, I elicited a CG response from previously food-restricted turtles after a switch to *ad libitum* feeding. Because compensating turtles grew faster than continuously *ad libitum* turtles, I conclude that growth rates in juveniles of this species are sub-maximal when individuals have continuous access to unlimited food. This finding suggests that the benefits of fast growth are countered by one or more costs, potentially including the accrual of cellular oxidative damage. Although Mangel and Munch (2005) incorporated oxidative stress into their CG model, the present study is the first to provide empirical evidence linking growth compensation with effects on antioxidant function.

To assess antioxidant capacity, I measured the activity of GPX in mitotic (liver) and post-mitotic (skeletal muscle) tissues. Specific GPX activity of liver but not muscle responded to diet, so I restricted my subsequent analysis of total, non-specific antioxidant potential to liver. Although the parameters I measured are typically expressed relative to protein concentration, I found differences in hepatocyte protein content among treatment groups (Chapter 3). As a result, differences in protein content per liver cell may have confounded my measurements of GPX activity and AP. In Chapter 3, hepatic DNA content of t_{12} turtles was measured for the same t_{12} turtles I examined here, allowing me to correct my measurements for total DNA content as a putative correlate of cell number. Doing so allowed me to compare the levels of antioxidant function per cell rather than assessing the proportion of total proteins functioning as antioxidants.

In this study, turtles with a dietary history of continuous *ad libitum* feeding for twelve weeks demonstrated higher hepatic GPX activity relative to turtles experiencing a continuous food restriction. Qualitatively, I observed the same pattern regardless of whether hepatic GPX activity was calculated relative to protein or DNA content. Glutathione peroxidase serves as one of several important enzymes in the cellular antioxidant defense system, and upregulation of its activity likely reflects increased endogenous production of organic hydroperoxides (Judge *et al.* 2005). My results therefore suggest that high intake and growth rates cause elevated oxidative stress in mitotically active tissues of juvenile green turtles.

The liver plays an important role in metabolism and detoxification and is a major source of peroxides via autoxidation reactions. It is therefore not surprising that I detected an upregulation of a component of the antioxidant defense system in liver of fast-growing AL animals. My GPX results parallel the finding that food restriction depressed hepatic GPX gene expression in young mice relative to *ad libitum* controls (Mura *et al*. 1996). Additionally, activity of antioxidant enzymes such as GPX typically increases with age due to elevated oxidative stress (Leeuwenburgh *et al*. 1994, Phaneuf and Leeuwenburgh 2002), but this increase is often attenuated by food restriction (Luhtala *et al*. 1994).

A counterintuitive result from the present study is the finding that R-AL turtles had GPX activities comparable to those of continuously food-restricted turtles at the conclusion of the study. Therefore, turtles in the R-AL group either produced fewer hydroperoxides and experienced less oxidative stress than continuously AL turtles during the final weeks of the study *or* experienced comparable levels of oxidative stress but demonstrated a reduced capacity to upregulate GPX activity. Because R-AL turtles underwent CG several weeks before the end of the experiment and had growth rates similar to AL turtles when tissues were sampled, I suggest

the latter scenario occurred. If my hypothesis is correct, then depressed antioxidant enzyme activity paired with elevated growth rates would exacerbate oxidative damage and therefore be a cost of CG.

Although my GPX results appear to support my initial predictions, specific components of the antioxidant defense system often respond differently to oxidative stress depending on the species and tissues measured. In addition, conflicting effects of dietary restriction on individual antioxidant enzyme activities are common. For example, GPX activities have been shown to increase (Agarwal *et al*. 2005), decrease (Grattagliano *et al*. 2004), or remain the same (Wu *et al*. 2003) in food-restricted animals relative to *ad libitum*-fed controls. Furthermore, individual enzymes in the antioxidant defense system do not function in isolation from each other. Instead, combating oxidative stress requires the concerted involvement of a variety of enzymatic and non-enzymatic molecules that scavenge or neutralize reactive oxygen species (ROS). Many of these molecules function synergistically (Niki *et al*. 1995, Böhm *et al*. 1997). For this reason, I measured total, non-specific hepatic antioxidant potential in t_5 and t_{12} turtles and again found differences among treatment groups.

When calculated relative to protein content, hepatic AP of R turtles was significantly higher than hepatic AP of AL turtles after five weeks of food restriction. Kalani *et al*. (2006) found similar results in *ad libitum*-fed versus calorie-restricted rats. After seven additional weeks, the difference between AL and R turtles was diminished, but R turtles had higher AP than R-AL turtles and AP of AL turtles was marginally higher than AP of R-AL turtles. These results indicate that diet history affects the proportion of total proteins that function as antioxidants within the liver.

However, when I corrected my values of t_{12} hepatic AP for DNA content, fast-growing AL turtles had higher AP per cell than both slow-growing R turtles and fast-growing R-AL turtles. As a result, turtles that underwent an earlier period of CG had decreased cellular antioxidant function compared to age-matched AL turtles feeding and growing at the same rate. Because I did not measure hepatic DNA concentration for t_5 turtles, I could not evaluate putative AP content per cell for these individuals.

The results of this study imply that individuals typically grow at rates that optimize their ability to prevent oxidative damage to lipids, nucleic acids, and proteins. Oxidative stress results from an imbalance between the rate of 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), my finding that *ad libitum*-fed turtles compensating for a prior food restriction also had diminished antioxidant function implies a cost of CG. It is unclear whether elevated oxidative stress during early development in this long-lived species would adversely affect longevity or performance. However, this study provides evidence of cellular stresses coincident with growth compensation and suggests that sub-maximal growth protects individuals from the detrimental effects of impaired antioxidant defense.

Table 4-1. Omnibus F, χ^2, and p-values for comparisons of means among treatment groups at five weeks (t_5) and twelve weeks (t_{12}).

	Omnibus F and χ^2	t_{12} Groups Tested in Pairwise Comparisons		
		AL and R	AL and R-AL	R and R-AL
Body Mass				
t_5	$F_{1,18}$ = 99.517, **p < 0.0001**			
t_{12}	$F_{2,24}$ = 99.476, **p < 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
Protein Content, Muscle				
t_5	$F_{1,18}$ = 5.952, **p = 0.025**			
t_{12}	$F_{2,24}$ = 0.698, p = 0.507	N/A	N/A	N/A
Protein Content, Liver				
t_5	χ^2 = 2.286, p = 0.131			
t_{12}	$F_{2,24}$ = 3.545, **p = 0.045**	0.630	0.320	**0.036**
GPX Specific Activity, Muscle				
t_5	$F_{1,18}$ = 0.507, p = 0.486			
t_{12}	$F_{2,24}$ = 0.226, p = 0.800	N/A	N/A	N/A
GPX Specific Activity, Liver				
t_5	$F_{1,18}$ = 0.811, p = 0.380			
t_{12}	$F_{2,24}$ = 2.660, p = 0.090	N/A	N/A	N/A
Total Antioxidant Potential, Liver				
t_5	$F_{1,16}$ = 10.443, **p = 0.005**			
t_{12}	$F_{2,20}$ = 5.135, **p = 0.016**	0.697	0.098	**0.015**
GPX Activity Per µg DNA, Liver				
t_{12}	$F_{2,24}$ = 9.617, **p < 0.001**	**0.001**	**0.003**	0.937
Antioxidant Potential Per µg DNA, Liver				
t_{12}	$F_{2,20}$ = 6.416, **p = 0.007**	**0.029**	**0.008**	0.808

When F values are reported, data were analyzed using analysis of variance, and pairwise comparisons of t_{12} samples were evaluated using Tukey's Honestly Significant Difference post hoc tests. When χ^2 values are reported, data were analyzed using a Kruskal-Wallis test. Statistically significant p-values are indicated in bold. Abbreviations: AL = *ad libitum* for twelve weeks, R = food-restricted for twelve weeks, R-AL = food-restricted for five weeks and *ad libitum* for seven weeks, GPX = glutathione peroxidase.

Table 4-2. Total protein concentrations of *Chelonia mydas* muscle and liver homogenates as determined by Bradford assay expressed relative to wet mass of homogenized tissue.

Treatment Group	Week	*n*	Protein Concentration, Muscle (mg/mg tissue)	Protein Concentration, Liver (mg/mg tissue)
AL	5	10	0.198 ± 0.023^{a}	0.178 ± 0.021^{a}
R	5	10	0.314 ± 0.040^{b}	0.241 ± 0.021^{a}
AL	12	7	0.173 ± 0.018^{x}	0.218 ± 0.024^{xy}
R	12	10	0.187 ± 0.026^{x}	0.242 ± 0.014^{x}
R-AL	12	10	0.211 ± 0.021^{x}	0.178 ± 0.018^{y}

Values represent means ± standard errors. Treatment groups and data analysis are the same as in Table 4-1. Letters (a and b for week 5, x and y for week 12) indicate statistically significant differences ($p < 0.05$) among treatment groups within sampling periods.

Table 4-3. Glutathione peroxidase (GPX) specific activity in *Chelonia mydas* muscle homogenate.

Treatment Group	Week	*n*	GPX Activity, Muscle (nmol/min*mg protein)
AL	5	10	2.78 ± 0.16
R	5	10	3.07 ± 0.37
AL	12	7	2.68 ± 0.12
R	12	10	2.66 ± 0.11
R-AL	12	10	2.78 ± 0.18

Values represent means ± standard errors. Treatment groups and data analysis are the same as in Table 4-1. No significant differences in GPX activity of muscle tissue were detected among treatment groups in either sampling period.

Table 4-4. Coefficients of variation (CV, %) for protein concentration, glutathione peroxidase (GPX) activity, and antioxidant potential (AP) assays.

Treatment Group	Week	Protein Content		GPX Activity		AP
		Liver	Muscle	Liver	Muscle	Liver
AL	5	8.0	9.2	6.7	26.9	19.1
R	5	10.8	9.1	6.3	38.2	19.2
AL	12	9.9	7.7	6.3	27.7	12.2
R	12	13.8	10.8	8.0	24.9	12.3
R-AL	12	9.0	7.7	6.4	24.0	24.3

Each value represents the average of individual CVs for each turtle and each assay. Treatment groups are the same as in Table 4-1. Protein content and hepatic GPX activity assays were performed in triplicate, whereas muscle GPX activity and hepatic AP assays were performed in duplicate.

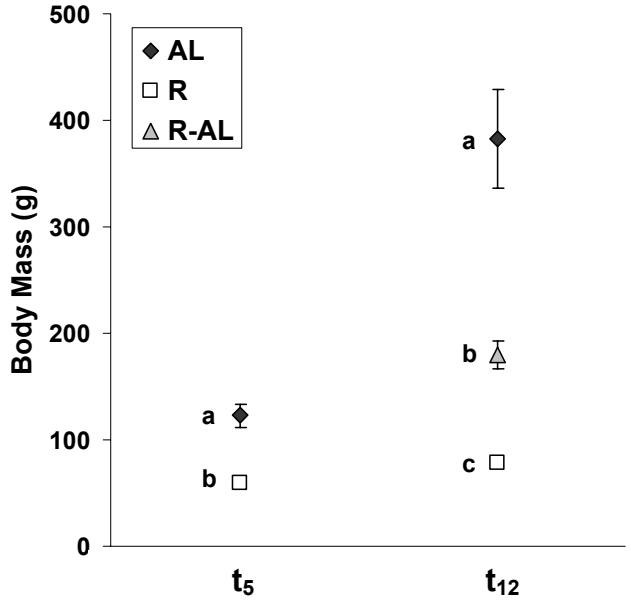


Figure 4-1. Body mass of turtles at five weeks (t_5) and twelve weeks (t_{12}), when tissues were sampled. Each point represents mean ± standard error. Sample sizes: n = 10 for all groups except t_{12} AL (n = 7). Treatment groups and data analysis are the same as in Table 4-1. Letters (a, b, and c) indicate statistically significant differences ($p < 0.05$) among treatment groups within sampling periods.

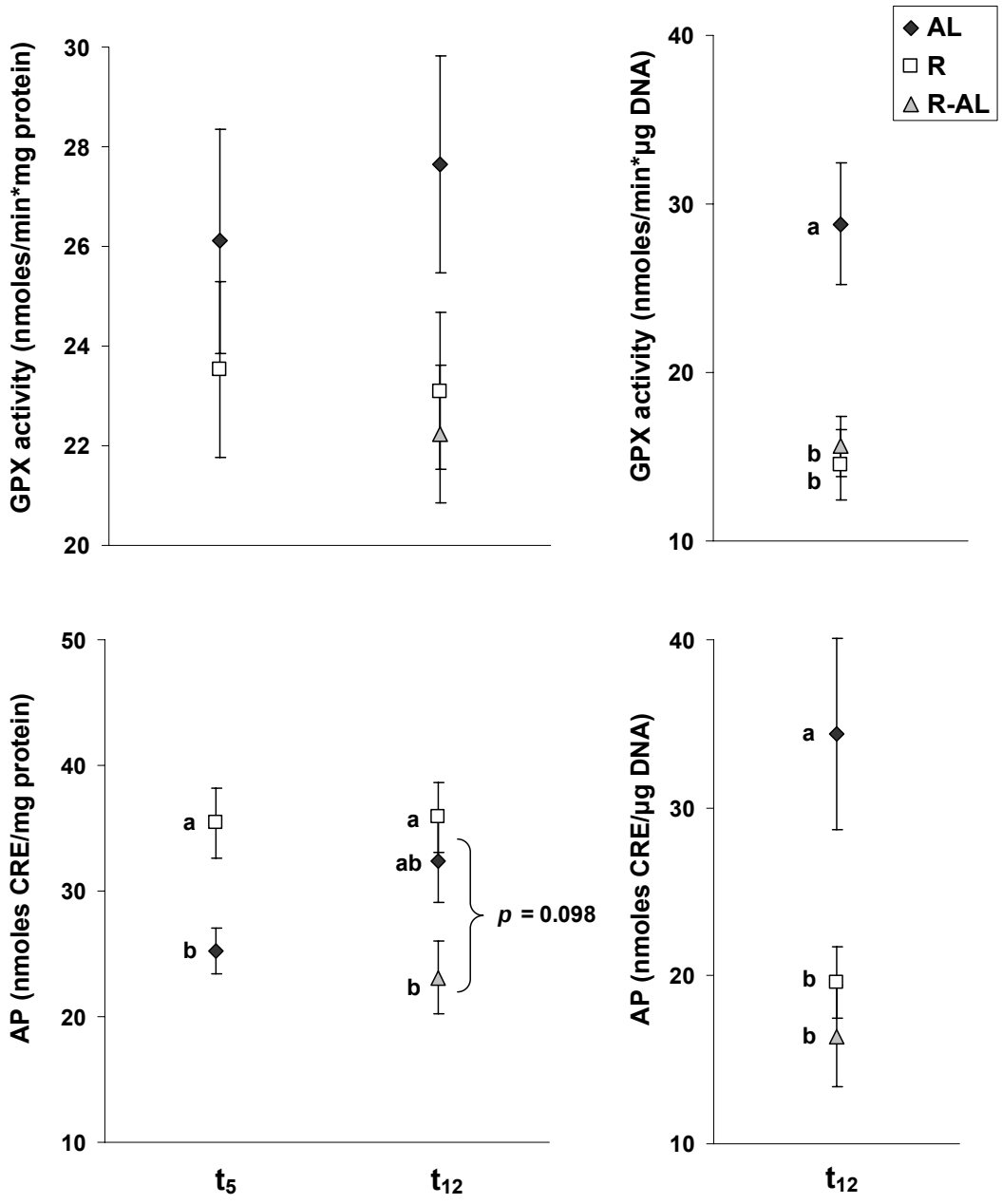


Figure 4-2. Glutathione peroxidase (GPX) specific activity and antioxidant potential (AP; calculated as nmoles of copper reducing equivalents, CRE) in Chelonia mydas liver homogenate at five weeks (t_5) and twelve weeks (t_{12}). For graphs on the left, GPX activity and total AP were normalized to total protein concentration as determined by Bradford assay. For graphs on the right, GPX activity and total AP were normalized to tissue DNA content as reported in Chapter 3 for t_{12} turtles. Each point represents mean ± standard error. Treatment groups and data analysis are the same as in Table 4-1. Sample sizes for GPX activities are the same as in Figure 4-1. Sample sizes for AP are n = 9 for t_5 turtles and n = 7, 8, and 8 for t_{12} AL, R, and R-AL turtles, respectively. Letters (a and b) indicate statistically significant differences ($p < 0.05$) among treatment groups within sampling periods.

CHAPTER 5
TIMING OF DIETARY RESTRICTION ALTERS THE EXPRESSION OF LIFE-HISTORY TRAITS IN A LONG-LIVED, PARTHENOGENETIC INSECT

Introduction

Life-history theory seeks to explain how natural selection optimizes life cycles to maximize fitness. Central to this body of theory is the assumption that developmental trajectories and life histories should demonstrate plasticity in response to environmental variation including food availability (Roff 1992, Stearns 1992). Because of extrinsic or intrinsic upper limits on rates of resource acquisition (Speakman and Król 2005), most animals cannot simultaneously maximize the allocation of nutrients to all traits that influence fitness. As a result, resources are differentially allocated to various reproductive and somatic functions according to priority rules (Boggs 1992, Zera and Harshman 2001).

These priority rules predict that survival should be favored over reproduction in times of resource limitation. One of the most pervasive findings in life-history studies is that food restriction (FR) leads to increased lifespan in a diversity of organisms including worms, spiders, insects, rodents, and primates (Weindruch and Walford 1988, Austad 1989, Turturro and Hart 1992, Mair *et al*. 2003, Hatle *et al*. 2006b). Coincident with lifespan extension, adult FR usually decreases or inhibits oogenesis and egg production (Chippindale *et al*. 1993, Wheeler 1996). Presumably, the suppression of reproductive activity during times of food scarcity allows food-restricted individuals to divert available resources into maintenance and storage, thereby increasing starvation resistance and the probability of survival until conditions more conducive to reproduction are encountered (Holliday 1989, Masoro and Austad 1996, Simmons and Bradley 1997).

This negative correlation between longevity and fecundity is interpreted as evidence for a cost of reproduction (Stearns 1992). A reproductive cost can also be expressed as a trade-off

between current and future reproduction (Calow 1979, Reznick 1985 and 1992). For such a cost to exist, the decreased survival demonstrated by very fecund individuals feeding at high rates as adults must be attributable to the process of egg production. However, preventing oogenesis or vitellogenesis in *ad libitum*-fed adult *Drosophila* did not decrease mortality rates (Mair *et al*. 2004). Additionally, Kaeberlein *et al*. (2006) found that FR in *C. elegans* extended lifespan even when the food restriction was imposed after the cessation of reproductive activity. Therefore, the enhanced longevity demonstrated by food-restricted adults may not result simply from a re-allocation of nutrients away from egg production and toward somatic maintenance and survival.

Although it is clear that the nutritional environment experienced during adulthood can directly alter reproductive output, juvenile food restriction can also influence fecundity indirectly through its effects on body size. High food availability during juvenile stages favors rapid growth and larger sizes at developmental transitions such that adult size is maximized. In insects, adult body mass is often strongly and directly correlated with fecundity (Honěk 1993, Tammaru *et al*. 1996; although see also Leather 1988), meaning that the conditions conducive to rapid juvenile growth and large adult size tend to maximize reproductive output. In contrast, when the nutritional environment during juvenile stages leads to slow growth rates, size at developmental transitions may shift downward to reduce the demographic costs of extended development time (Rowe and Ludwig 1991, Berrigan and Koella 1994, Leips and Travis 1994, Bradshaw and Johnson 1995, Day and Rowe 2002). The response to reduced intake in juvenile stages in insects is therefore a reduction in adult body size with a potential for concomitant decreased fecundity (Honěk 1993).

Whereas juvenile and adult food restriction can have similar effects on lifetime fecundity, their effects on longevity differ. In insects, the effects of juvenile FR on life-history traits and trade-offs are less clear than the effects of adult FR, primarily because few authors have assessed the responses of longevity to juvenile food availability. Those who have done so have reported that juvenile FR either has no effect (Tu and Tatar 2003) or a negative effect (Boggs and Freeman 2005) on lifespan. The pattern of resource acquisition early in an individual's lifetime therefore has the potential to alter later allocation patterns and the expression of life-history traits and trade-offs. Furthermore, allocation patterns may also change with age (models reviewed by Perrin and Sibly 1993), depending on the diets experienced in different life stages and the needs of the individual at the time of allocation. For example, the expression of a trade-off between current and future reproduction may depend on whether an organism relies on adult-derived nutrients or stored larval-derived nutrients for egg production (Boggs 1992).

It is clear, then, that resource acquisition patterns can profoundly affect the expression of life-history traits and trade-offs. Because fluctuations in food availability almost certainly occur for most animals at some point in their lifetimes (Boggs and Ross 1993, Carey *et al*. 2002a), a complete understanding of the effects of intake during different life stages requires data collection throughout an individual's entire lifespan. However, such experiments are rare, particularly for long-lived species. In addition, studies of FR and life-history trade-offs in insects often suffer from one or more fundamental limitations. Firstly, FR in insects typically entails *ad libitum* consumption of lower quality food rather than a quantitative reduction in the amount of food offered (Partridge *et al*. 2005). Furthermore, intake of these lower quality diets is typically not quantified, despite the need for such information when assessing trade-offs (Zera and Harshman 2001). In those studies that do impose a quantitative food restriction in which absolute

intake is limited, the restriction usually occurs only during adulthood and longevity is often not enhanced (Boggs and Ross 1993, Carey *et al*. 2002b, Cooper *et al*. 2004), contradicting the nearly universal finding of increased lifespan under quantitative FR in many taxa. In addition, to evaluate the costs of reproduction and the effects of dietary restriction on fitness in sexual species, females must be allowed to mate. However, co-housing individuals complicates the quantification of individual intake and can influence longevity due to the effects of crowding (Joshi *et al.* 1998). To avoid such problems, reproductive output of virgin female insects is often studied as a proxy for fitness, yet mating has been shown to enhance egg production in several species where unmated females would otherwise lay infertile eggs (De Clercq and Degheele 1997, Foster and Howard 1999). Furthermore, the males of many species can alter the physiology and behavior of females via sex peptides (Wolfner 1997, Gillott 2003, Carvalho *et al.* 2006) or nutritious nuptial gifts (Voigt *et al.* 2006). Lastly, sexual species incur a number of costs associated with reproductive behaviors including courtship, repulsion of unwanted mates, intrasexual competition, and locomotory costs of carrying mates during copulation (Watson *et al.* 1998).

To overcome these obstacles, I adopted a novel approach to life-history experimentation by using a parthenogenetic species as my animal model. *Carausius morosus* (Br.) (Phasmatodea, Lonchodinae) is a relatively long-lived species that reproduces via obligate apomictic parthenogenesis (Pijnacker 1966). Using a parthenogen as my animal model obviated the need for mating while still permitting natural reproductive processes. This species is hemimetabolous and phytophagous, allowing for life-long, quantitative dietary manipulations. Additionally, *C. morosus* consumes the same food throughout its lifetime, enabling me to test dietary treatments that spanned both juvenile and adult stages. My purpose was to determine the effects of

differences in resource availability at several developmental stages on life-history traits that have substantial influences on population structure and dynamics, such as age and size at each developmental transition, longevity, and fecundity.

Materials and Methods

Animal Husbandry and Feeding Treatments

This study was conducted in a USDA-approved quarantine facility within the Department of Zoology at the University of Florida (permit # PPQ 69292). Lights were maintained on a 12:12 light:dark cycle. Room temperature averaged 22.5-24.5 ^{o}C, and relative humidity averaged 45-55% throughout the trial. Twenty adult Indian stick insects (*Carausius morosus*) were obtained from the Exploratorium in San Francisco, California. Eggs laid by these females were individually incubated until hatching. The resulting offspring (n = 86) were systematically assigned to one of six treatment groups such that all experimental insects produced by a particular mother were evenly distributed among groups.

These insects were maintained individually for their entire lifetimes in plastic cages (29.5 cm x 19 cm x 19 cm) with locking vented lids lined with fine-mesh screening. Each cage was misted daily with deionized water to provide drinking water. Insects were fed discs cut from leaves of English ivy (*Hedera helix*) daily. Biopsy punches (Miltex Instrument Co., Inc.) were used to create discs of multiple diameters: 2 mm for first instar insects, 3 mm for second instar insects, 4 mm for third instar insects, 5 mm for fourth instar insects, 6 mm for fifth instar insects, and 8 mm for sixth instar insects and adults. When cutting leaf discs, care was taken to avoid major leaf veins such that discs contained as little vascular tissue as possible.

Insects were offered either more leaf discs than they could consume within 24 hours (*ad libitum*, AL) or a restricted number of discs (R) equal to 60% of the average daily mass-specific intake of AL-fed insects in the same life-history stage. Life-history stages were categorized as

each of six instars, the adult stage prior to first oviposition, and the adult stage after first oviposition. Because mass-specific intake of *AL* insects declined after first oviposition, the amount of food offered to food-restricted adults after first oviposition was decreased proportionally to match this decline.

Discs were offered according to five treatment schedules (Fig. 5-1). Insects in the *AL* group (n = 15) were offered food *ad libitum* for the duration of their lifetimes. This group served as the control group from which intake data were used to determine the appropriate number of discs to offer to restricted insects. Insects in the *R* group (n = 28) were offered the restricted amount of food for the duration of their lifetimes. Individuals in the *AL-R* groups were initially fed *ad libitum* and were switched to the restricted diet at the beginning of the fifth instar (*AL-R at* 5^{th}, n = 15) or at first oviposition (*AL-R at Ov*, n = 14). Insects in the *R-AL at* 5^{th} group (n = 14) were initially fed the restricted diet and were switched to an *ad libitum* diet at the beginning of the fifth instar. Food-restricted insects generally ate all of the food offered each day, although food-restricted adults occasionally failed to consume all discs. I initially planned a diet switch from restricted to *ad libitum* at first oviposition (*R-AL at Ov*) but was unable to test this treatment schedule because survival to oviposition was extremely low for insects maintained throughout the juvenile stages on the restricted diet. To ensure a sufficient sample size in the *R* group, all insects that were food-restricted throughout all six instars and successfully oviposited (n = 7) were maintained on the restricted diet throughout adulthood. The sample sizes indicated in each table and figure (except Figs. 5-5 and 5-6) include only those individuals that survived through the end of the sixth instar.

Physiological and Life-History Response Variables

Daily intake of each insect was estimated by determining the number of discs remaining each day and subtracting this quantity from the number of discs offered the previous day. Whole

discs were counted, and partial discs were pressed between microscope slides and scanned (Visioneer OneTouch scanner). Surface area of each disc fragment as a proportion of uneaten leaf disc surface area was determined using ImageJ (1.37v). A sample of each size of leaf discs was dried daily to constant mass at 60 °C and weighed. The approximate daily dry matter intake for each insect was then calculated as number of discs consumed * estimated dry mass per disc. Daily mass-specific intake was calculated using estimates of daily body mass computed from periodic body mass measurements, as described below.

Each insect was weighed weekly, at the end of each instar (defined as the day when no food was eaten in preparation for ecdysis), at first oviposition, and at death. Insects were also photographed at these times (Nikon Coolpix 3200), and body lengths at the end of each life-history stage were then determined using ImageJ. Body length was measured as the distance between the base of the antennal socket and the end of the tergum on the terminal abdominal segment. Measurements of body size at the end of each instar for *AL* insects were then fitted to the allometric equation $\ln(y) = \ln(a) + b\ln(x)$, where y = body mass and x = body length. Relative body mass (as an index of body condition) of insects in all treatment groups at the adult molt was assessed as the ratio between measured body mass and body mass predicted by the *AL* allometric equation (Perrin *et al*. 1990). Specific growth rate (SGR) of each insect in each life-history stage was calculated as:

$$SGR = 100*(\ln BM_f - \ln BM_i)/t$$

where BM_f is body mass at the end of a stage, BM_i is body mass at the beginning of a stage, and t is the time in that stage.

During each day of adulthood, all eggs laid by each female were collected and individually weighed. Average egg mass was calculated as the mean mass of individual eggs for each female.

Reproductive lifespan was calculated as the time between first and last eggs laid. Clutch size was not quantified because oviposition in *C. morosus* occurs continuously throughout the reproductive lifespan rather than in discrete clutches. After death, each female was dissected, and the number of ovarioles in both ovaries was determined. The numbers of fully chorionated eggs and non-chorionated eggs in terminal follicles and in the oviducts were counted. Although I intended to evaluate hatch success, egg viability was inexplicably low in this study, particularly compared to the expected hatchability of nearly 100% (Brock 2000).

Statistical Analyses

Analysis of variance (ANOVA) was used to test for differences among treatment groups. Data were first tested for normality (Shapiro-Wilk test) and homogeneity of variances (Levene's test) and transformed, if necessary, using a natural log, reciprocal, square root, square, reciprocal square, or reciprocal square root transformation. Pairwise comparisons were evaluated using Tukey's Honestly Significant Difference post hoc test (if variances were homogeneous) or Tamhane's T2 post hoc test (if variances were not homogeneous). If transformation did not normalize data, they were analyzed using a Kruskal-Wallis test, and pairwise comparisons were evaluated using Mann-Whitney U tests with α set at 0.005 to account for the number of comparisons tested (ten). Reproductive output was analyzed using ANOVA as described above and also using analysis of covariance with body mass at first oviposition as the covariate to test for size-independent differences in allocation to reproduction. Other covariates (including length at first oviposition, body mass at adult molt, and length at adult molt) could not be used because of significant interactions between these variables and treatment group.

Stepwise linear regression was used to determine the factors that best explained variance in reproductive output. For this analysis, the dependent variable was the cumulative fecundity of each female. The independent variables tested were mass-specific intake and growth rates during

the final instar; duration of the final instar; age, body mass, and relative body mass at the adult molt; duration of the pre-oviposition adult stage; mass-specific intake and growth rates during the pre-oviposition adult stage; age and body mass at first oviposition; cumulative intake during instars 1-4, 5-6, and 1-6; and cumulative intake during the pre-oviposition adult stage and during the reproductive lifespan. Cumulative intake and body condition were tested as potential determinants of reproductive output because insects have been shown to require a threshold level of food consumption or body stores to initiate reproductive processes (Juliano *et al.* 2004, Hatle *et al.* 2006a). Variables had to meet a 0.05 significance level to enter a model. Duration of the reproductive lifespan was dropped from this analysis because of collinearity with the first variable selected by the model.

Stepwise linear regression was also used to determine the factors that best explained variance in initial oviposition rate. For this analysis, the dependent variable was the cumulative number of eggs laid by each female on day 6 of the reproductive lifespan, and the independent variables tested were the same as those used above. Specific growth rate during adulthood prior to first oviposition was dropped from this analysis due to collinearity with other variables already selected by the models. To test for a longevity cost of reproduction in this species, least squares linear regression was used to examine the relationship between cumulative fecundity and lifespan (both total lifespan and adult lifespan). Least squares linear regression was also used to evaluate the strength of the relationship between fecundity and cumulative intake during the reproductive lifespan.

Kaplan-Meier survivorship curves were constructed for the entire lifespan ($n = 86$) and for adult lifespan ($n = 70$, including only those individuals that successfully molted to the adult stage). Pairwise comparisons among treatment groups were evaluated using log-rank tests with α

set at 0.005 to account for the number of comparisons tested (ten). Data were analyzed using SPSS for Windows (Release 11.0.0), and S-Plus (Version 7.0) was used for graphing smoothing functions and Kaplan-Meier curves.

Results

The diets I imposed yielded different mass-specific intake trajectories for insects in each treatment group (Fig. 5-2). Total dry mass of food consumed during each life-history stage except the first instar differed significantly among treatment groups (Table 5-1). In the second instar, food-restricted insects consumed more total food than insects feeding *ad libitum* despite being significantly smaller (Fig. 5-3 and Table 5-2) and receiving proportionally less food on a daily basis. This discrepancy is explained by the significantly longer instar duration in food-restricted groups relative to groups feeding *ad libitum* (Figs. 5-3 and 5-4a). The pattern of cumulative intake changed after the second instar and was dependent on diet history. Although cumulative intake prior to the adult molt differed among treatment groups ($F_{4,65} = 28.872$, $p < 0.0001$), the total amount of food consumed between hatching and first oviposition did not differ among treatment groups ($F_{4,53} = 1.321$, $p = 0.274$), hinting at a potential intake threshold for induction of reproductive activity (Juliano *et al*. 2004).

Size and age at each life-history transition differed significantly among treatment groups (Fig. 5-3 and Table 5-2). Insects did not differ in body mass among treatment groups at hatching ($F_{4,65} = 1.00$, $p = 0.414$). At the end of instars 1-4, body mass was greater and molting occurred at younger ages in initially *ad libitum* insects (groups *AL*, *AL-R at* 5^{th}, and *AL-R at Ov*) than in initially food-restricted insects (groups *R* and *R-AL at* 5^{th}). From the fifth instar until first oviposition, all treatment groups except *AL* and *AL-R at Ov* differed significantly in body mass and age at the end of each stage (Table 5-2). At death, body mass of *AL-R at Ov* insects was not significantly different from body mass of *AL* and *R-AL at* 5^{th} insects, but all other pairwise

comparisons of size were significantly different. Age at death differed significantly among all groups except *R* and *R-AL at 5th*.

Relative body mass also differed among treatment groups at the adult molt as determined by allometric analysis (Table 5-3). Least squares regression of body mass (y) and length (x) for *AL* insects at the end of each instar yielded the equation $\ln(y) = 2.7112*\ln(x) - 12.018$ ($F_{1,76} = 14077.66$, $p < 0.0001$, $R^2 = 0.995$). This equation was used to calculate predicted body masses at actual body lengths for each insect at the adult molt (Table 5-3). The ratio of actual to predicted body mass was lower for insects feeding at a restricted rate during the final two instars (groups *R* and *AL-R at 5th*), indicating that insects in these groups had proportionally lower body masses for a given body length than insects in the other three groups.

The duration of each life-history stage differed among treatment groups (Fig. 5-4a). Food-restricted insects generally progressed more slowly through each stage than insects feeding *ad libitum*. Previous diet history affected the duration of the fifth and sixth instars and the pre-oviposition adult stage for insects in groups *AL-R at 5th* and *R-AL at 5th*, as individuals in these groups progressed through these stages more rapidly than continuously food-restricted individuals (group *R*) but more slowly than continuously *ad libitum* individuals (group *AL*). Insects experiencing food restriction during adulthood prior to first oviposition laid their first eggs later in the adult stage than insects feeding *ad libitum* during this time. However, duration of adulthood after first oviposition was significantly shorter for insects that were food-restricted than for insects that were feeding *ad libitum* during this time, regardless of when the food restriction was imposed.

Food-restricted insects also grew more slowly than insects feeding *ad libitum* (Fig. 5-4b), although diet history affected the magnitude of this difference. After a switch from restricted to

ad libitum feeding at the beginning of the fifth instar (group *R-AL at 5th*), specific growth rates through the final two instars were comparable to those of continuously *ad libitum* insects (group *AL*). I therefore found no evidence of growth compensation in *R-AL at 5th* insects. However, insects that experienced a switch from *ad libitum* to restricted feeding grew significantly faster in both the fifth and sixth instars than insects that were continuously food restricted. All insects gained body mass between the adult molt and first oviposition, with growth of *R-AL at 5th* insects slower than that of *AL* and *AL-R at Ov* insects but greater than that of *R* and *AL-R at 5th* insects. All insects lost body mass between first oviposition and death. *AL-R at Ov* insects lost proportionally more body mass than *AL* insects during this time, but all other pairwise comparisons of adult growth rates after first oviposition were not significant.

An event history diagram depicting the lifespan of each insect in the study (Carey *et al*. 1998) demonstrates the variation in life histories and survivorship induced by diet treatments (Fig. 5-5). Pairwise log-rank tests of survival indicated that all groups except *R* and *R-AL at 5th* differed significantly in total lifespan (Fig. 5-6a). This result parallels the ANOVA results for age at death (Table 5-2). Pairwise log-rank tests of adult survivorship (Fig. 5-6b) indicated that longevity was greater for *AL* and *R-AL at 5th* insects than for all insects feeding at a restricted rate during adulthood, suggesting that food restriction experienced during reproductive activity negatively affected adult lifespan regardless of dietary history.

Treatment groups differed significantly in realized fecundity ($F_{4,53} = 50.31$, $p < 0.0001$, Fig. 5-7). These differences appear to result both from differences in reproductive lifespan ($F_{4,53} = 41.70$, $p < 0.0001$) and from differences in reproductive rate. The high initial slopes in Figure 5-7 for groups *AL*, *AL-R at Ov*, and *R-AL at 5th* corresponded to higher early oviposition rates (calculated as eggs laid per day in the first six days of the reproductive lifespan) in these groups

compared to groups *R* and *AL-R at 5th* ($F_{4,53}$ = 15.576, $p < 0.0001$). These differences suggest that egg output is enhanced early in the reproductive lifespan by *ad libitum* feeding during adulthood prior to first oviposition. The low reproductive output of *R* insects was compounded by low survival to first oviposition, such that egg production was severely diminished by lifelong FR. Differences in egg production did not simply result from differences in body size, as analysis of covariance revealed significant differences in adjusted mean fecundity when body mass at first oviposition was used as a covariate (Table 5-4). Adjusted mean fecundity also differed among groups when relative body mass at the adult molt was used as a covariate ($F_{4,53}$ = 42.872, $p <$ 0.0001; data not shown). Egg production was also altered by diet history through effects on average egg mass ($F_{4,53}$ = 8.195, $p < 0.0001$, Fig. 5-8), with insects experiencing a diet switch from *ad libitum* to restricted feeding producing significantly smaller eggs than continuously *ad libitum* insects. Differences in reproductive output do not appear to result from differences in ovarian morphology among groups (Table 5-5). However, although Tukey's HSD post hoc test did not reveal significant differences in ovariole number among treatment groups, a less conservative post hoc test (the Least Significant Difference test) indicated that initially restricted insects (groups *R* and *R-AL at 5th*) had significantly fewer ovarioles than initially *ad libitum* insects (groups *AL*, *AL-R at 5th*, and *AL-R at Ov*) ($p < 0.05$ for all significant comparisons).

Diet history did affect the number of eggs remaining in the ovaries at death (unfulfilled reproductive potential, $F_{4,53}$ = 5.286, $p = 0.001$), with *R* insects having more eggs remaining in the ovaries at death than *AL-R at 5th* insects. All other pairwise comparisons of unfulfilled reproductive potential were not significant. Groups also differed in potential fecundity ($F_{4,53}$ = 71.62, $p < 0.0001$, calculated as unfulfilled reproductive potential plus realized fecundity) and total reproductive investment ($F_{4,53}$ = 49.57, $p < 0.0001$, calculated as the summed mass of all

eggs laid by each female). The patterns for potential fecundity and total reproductive investment (data not shown) were identical to that demonstrated by realized fecundity.

I used stepwise multiple linear regression to identify the most significant determinants of realized fecundity (Table 5-6). Cumulative intake during the reproductive lifespan was the primary variable selected by the model, explaining 82.8% of the variance in fecundity (Fig. 5-9a). When potential fecundity (number of eggs laid + number of eggs remaining in the ovaries at death) was regressed against cumulative intake during the reproductive lifespan, the same relationship existed with an adjusted R^2 value of 0.847 (data not shown). In addition, growth rate during adulthood prior to first oviposition and cumulative intake during all juvenile stages were also selected as variables in a model that explained 92.8% of the variance in realized fecundity (model 3, $F_{3,54} = 245.46$, $p < 0.0001$). Stepwise multiple linear regression identified body mass at first oviposition, age at the adult molt, mass-specific intake during adulthood prior to first oviposition, and cumulative intake during adulthood prior to first oviposition as significant independent variables in a model that explained 70.6% of the variance in initial oviposition rate (model 7, $F_{4,53} = 35.20$, $p < 0.0001$).

The data do not support the contention that decreased longevity is a cost of reproduction, at least in *C. morosus*. On the contrary, fecundity was significantly and positively related to adult lifespan when data for all treatments were combined ($F_{1,56} = 25.67$, $p < 0.0001$, $R^2 = 0.302$, Fig. 5-9b). When potential fecundity (number of eggs laid + number of eggs remaining in the ovaries at death) was regressed against adult lifespan, the same relationship existed with an adjusted R^2 value of 0.343 (data not shown). However, analysis of covariance indicated that adult lifespan did not have a significant effect on reproductive output ($F_{1,52} = 3.284$, $p = 0.076$) whereas treatment did have a significant effect ($F_{4,52} = 32.463$, $p < 0.0001$). This result was confirmed by

individual regressions of fecundity versus adult lifespan for each treatment group, none of which was significant ($p > 0.2$ in all cases). Unlike adult lifespan, total lifespan was not significantly related to realized or potential fecundity ($p > 0.5$). I also found no evidence for a trade-off between number and size of eggs laid. There was neither a significant interaction between average egg mass and fecundity ($p > 0.2$) nor an effect of fecundity on average egg mass ($p > 0.3$). Individual regressions of average egg mass versus fecundity indicated that there were no significant relationships ($p > 0.3$) except in the case of *R-AL@5*th insects, for which there was a positive relationship between egg size and number ($F_{1,10} = 5.161$, $p = 0.046$, $R^2 = 0.274$).

Discussion

Developmental plasticity in response to food availability is nearly universal (Juliano *et al*. 2004 and references therein), and this study was no exception. In *C. morosus*, both size and age at each life-history transition depended on diet history. As is common in studies of this kind (e.g., Gebhardt and Stearns 1988 and 1993), insects that experienced FR prior to the onset of reproductive activity progressed through juvenile stages more slowly and were smaller at each molt than individuals feeding at a consistently high rate. Decreasing size and increasing age at developmental transitions represent a compromise between the need to maximize body size (because of its potential effects on fitness) and the need to minimize the demographic costs of extended development time (Rowe and Ludwig 1991).

This plasticity in development rate corresponded to substantial differences in survival trajectories among treatment groups. One of my most salient results was the finding that longevity enhancement is not a ubiquitous outcome of dietary restriction. Although individuals that experienced early-onset FR (*R* and *R-AL at 5*th) survived longer than initially *ad libitum*-fed individuals, this increased longevity was due solely to extended development time rather than to extended adult lifespan. Conversely, FR during adulthood decreased the duration of the adult

stage, such that *AL-R at Ov* insects had shortened lifespans compared to *AL* insects. A diet switch from *ad libitum* to restricted feeding during development extended the duration of the fifth and sixth instars relative to continuously *ad libitum* insects, but this difference was not sufficient to mitigate the negative effect of FR on adult lifespan.

As a result of decreased growth rates, insects that experienced FR at any point during development were smaller at the adult molt than *ad libitum* insects. Although subsequent reproductive output of food-restricted insects was significantly diminished, mean fecundities differed significantly among treatment groups even when corrected for body mass at first oviposition. Plasticity in adult size alone therefore does not explain the drastic differences in fecundity I observed among treatment groups.

Reproductive output may have been mildly constrained by ovarian morphology. Although I detected no significant differences in total ovariole number among treatment groups when a conservative post hoc test was used, I did find significant differences in ovariole number between initially restricted and initially *ad libitum* insects when a more liberal post hoc test was used. This result suggests that ovarian development in *C. morosus* is somewhat plastic in response to diet. In *Drosophila*, ovariole number responds strongly to larval diet (Tu and Tatar 2003) and is correlated with fecundity (David 1970). Although ovariole number in *C. morosus* appears to be much less plastic than in *D. melanogaster*, it is possible that decreased fecundity in food-restricted insects in this study is partially explained by differences in ovary size but only for insects that were food-restricted during early development.

The primary determinant of fecundity in this study was adult intake, with approximately 83% of the variance in reproductive output explained by the total amount of food consumed during the reproductive lifespan. Because of this strong, positive correlation between fecundity

and cumulative intake during the reproductive lifespan, I conclude that Indian stick insects use an "income" breeding strategy (sensu Stearns 1992, Jönsson 1997), in which the resources allocated to reproduction are acquired primarily during the reproductive period. Cumulative intake between the adult molt and the end of the reproductive lifespan was less strongly correlated with reproductive output, suggesting that the food acquired prior to first oviposition was allocated to some degree of pre-reproductive somatic growth rather than being allocated exclusively to egg production. The putative level of body stores accumulated by the time of the adult molt does not appear to dictate reproductive success, as demonstrated by significant differences among groups in realized fecundity when corrected for relative body mass.

An income breeding strategy is appropriate for an organism like *C. morosus*, in which oogenesis and vitellogenesis are non-cyclic and continuous (Bradley *et al*. 1995) throughout a comparatively long reproductive lifespan. Additionally, species that rely heavily on incoming resources for reproduction should have ovaries containing primarily immature oocytes immediately after the adult molt (Jervis *et al*. 2005), as is the case for *C. morosus* (Bradley *et al*. 1995). Although ovaries are present in juvenile stages, mature eggs are not present at the adult molt. Given this breeding strategy, it is not surprising that both mass-specific intake and age-specific fecundity in this study declined after first oviposition for adults feeding *ad libitum*. This decrease in consumption and production with time typifies insects that are income breeders (Kindlmann *et al*. 2001, Dixon and Agarwala 2002).

One might expect food-restricted insects that employ an income breeding tactic to extend the duration of reproductive activity and thereby to mitigate (at least partially) the effects of decreased daily intake on oviposition rate. Given that food-restricted flies respond in this way to FR (Carey *et al*. 2002a), I expected to see a similar pattern in this study. Surprisingly, females

could not simply compensate for adult FR by increasing the length of the reproductive lifespan. The very low reproductive output of *R* and *AL-R at 5th* females therefore resulted from the combined effects of decreased daily intake and shortened reproductive lifespan.

The proximate cause of shortened reproductive lifespan may relate to the level of body stores accumulated prior to adulthood. Insects feeding at a restricted rate late in development were lighter for their length than insects feeding *ad libitum* immediately prior to the adult molt. Although fat body mass and storage proteins were not quantified, these results imply that these individuals may have accumulated proportionally fewer body stores by the beginning of adulthood compared to insects feeding *ad libitum* during the final two instars. Therefore, food restriction late in development appears to have shifted allocation away from the accumulation of mobilizable reserves. I suggest that these reserves serve as the source of nutrients that are allocated to somatic maintenance after the onset of reproductive activity. Because *R* and *AL-R at* 5^{th} insects were smaller in both absolute and relative body mass at the adult molt, they probably depleted their limited stores more rapidly after the onset of reproductive activity than insects that were feeding *ad libitum* as young adults. Conversely, *R-AL at* 5^{th} insects were feeding *ad libitum* as pre-oviposition adults, although they were doing so at lower mass-specific rates than *AL* insects. Although *R-AL at* 5^{th} insects had relative body masses similar to those of *AL* and *AL-R at Ov* insects at the adult molt, they were nearly twice as old as *AL* and *AL-R at Ov* insects at first oviposition. It is therefore possible that reproductive lifespans of *R-AL at* 5^{th} insects were shorter than those of continuously *ad libitum*-fed adults simply because of age-specific declines in physiological function. It does not appear that shortened reproductive lifespans resulted from exhaustion of available oocytes, as almost all individuals in the study had chorionated eggs remaining in the ovaries at death.

In addition to sustaining the soma during adulthood, body stores present during early adulthood may also serve as a signal that coordinates rates of vitellogenesis and oviposition at the onset of reproductive activity (Moehrlin and Juliano 1998, Hatle *et al*. 2004, Juliano *et al*. 2004). In this way, body composition may function as an index of food availability that entrains subsequent reproductive function (Rowe *et al*. 1994). If this hypothesis is correct, *AL*, *AL-R at Ov*, and *R-AL at 5th* insects were committed to a high early oviposition rate in the first several days of the reproductive lifespan because these individuals had accumulated proportionally more body mass (with potentially higher levels of body stores) prior to first oviposition than *R* and *AL-R at 5th* insects. However, the mismatch between intake and pre-determined oviposition rate in *AL-R at Ov* insects after the switch to a restricted diet may have forced these individuals to supplement incoming resources by withdrawing nutrients from mobilizable stores that would otherwise have been allocated to somatic functions such as maintenance and survival. Data for all *AL-R at Ov* individuals were located above the regression line correlating fecundity with cumulative intake during the reproductive lifespan (Fig. 5-9a), suggesting that these individuals produced more eggs than would have been predicted by the amount of the food they consumed as reproductively active adults. If body stores were in fact used to supplement incoming nutrients for oogenesis, the exhaustion of these stores would then explain the shortened adult lifespan of *AL-R at Ov* insects relative to *AL* individuals.

The hypothesis that body stores determine adult lifespan and establish initial oviposition rate is supported by my data for insects that experienced a switch to the restricted diet late in development. Despite feeding *ad libitum* in the first four instars, *AL-R at 5th* insects had similar reproductive lifespans and fecundities as continuously food-restricted insects. Because growth is typically exponential in juvenile insects, body composition at the adult molt is largely

determined by food availability during the final instar(s) (Scriber and Slansky 1981). As a result, *ad libitum* feeding early in life does not appear to provide a substantial fecundity benefit for individuals that subsequently experience a decline in juvenile food availability. However, the marginal dependence of ovariole number on food availability during the first few instars does indicate that early nutritional conditions could potentially affect subsequent reproductive function.

Conversely, *ad libitum* feeding later in development and during adulthood provides nutrients necessary for somatic maintenance. However, mass-specific intake declined after first oviposition for both groups feeding *ad libitum* as reproductively active adults, suggesting either that oogenesis and vitellogenesis are less costly than somatic growth during juvenile stages or that digestive and reproductive functions decline with time due to senescence (Kindlmann *et al*. 2001, Carey *et al*. 2002a, Dixon and Agarwala 2002). Evidence for the latter hypothesis was demonstrated by the lower mass-specific intake of *R-AL at* 5^{th} insects, which matured at older ages, compared to *AL* insects after first oviposition. I therefore conclude that adult survival and reproductive decisions (such as age at first oviposition and initial oviposition rate) are based on the extent of reserves accumulated prior to maturity, whereas fecundity depends on food consumed during the reproductive lifespan in *C. morosus*. Perhaps not surprisingly, "capital" breeders (sensu Stearns 1992) tend to demonstrate the opposite strategy, allocating stored reserves to egg production and using adult-acquired nutrients to increase survival (e.g., Tammaru *et al*. 1996).

The apparent dependence of initial oviposition rate on accumulated body stores may result from differences in hormone signaling induced by diet. In insects, the fat body serves as the main depot for stored lipids and is responsible for synthesizing yolk proteins (e.g., vitellogenin) and

lipids for incorporation into developing follicles (Chapman 1998). Fat body mass and hemolymph vitellogenin titers decrease in response to adult FR in grasshoppers (Hatle *et al*. 2006a), suggesting that my early fecundity results may be mediated by differences in the size of this storage organ. Additionally, the synthesis of yolk compounds by the adult fat body is controlled by hormones including juvenile hormone (JH) and ecdysone (Klowden 2002), both of which have been shown to respond to feeding rates (Hatle *et al*. 2003, Tu and Tatar 2003). Specifically, JH stimulates vitellogenesis in most adult insects (Chapman 1998) but not in *C. morosus* (Bradley *et al*. 1995), so diet-induced differences in JH synthesis were probably not responsible for the decreased fecundity I observed in this study. However, JH does facilitate the uptake of vitellin by developing follicles in *C. morosus* (Bradley *et al*. 1995), suggesting that my results for egg size may reflect differences in JH signaling.

Unlike lipids, proteins are thought to be stored primarily in hemolymph (Chapman 1998). These hemolymph storage proteins are critical to egg production (Wheeler *et al.* 2000) and are responsive to diet (Hatle *et al*. 2004). Because egg production is a protein-limited process for phytophagous insects like *C. morosus* (Nijhout 1994, Chapman 1998), the quantity of hemolymph storage proteins present during adulthood may therefore serve as a nutrient sensor regulating reproductive output.

Contrary to my expectations, fecundity was significantly and positively correlated with adult lifespan and not correlated with total lifespan. My results therefore indicate that insects feeding *ad libitum* as adults did not incur mortality costs simply because they reproduced more than food-restricted insects. Consequently, decreased longevity is not a cost of reproduction in this species. This result contradicts the assumption that FR elicits a shift in allocation and therefore a trade-off between reproduction and survival (Stearns 1992). Furthermore, I detected

no evidence of a trade-off between current and future reproduction or between early fecundity and adult lifespan (Reznick 1985 and 1992). In fact, insects feeding *ad libitum* as pre-oviposition adults (groups *AL*, *AL-R at Ov*, and *R-AL at 5th*) exhibited correspondingly high initial oviposition rates and also higher cumulative fecundities than *R* and *AL-R at 5th* insects.

The longevity costs of reproduction may differ among species depending on the relative timing of resource acquisition and allocation to reproduction (Boggs 1992). The occurrence of a trade-off between longevity and fecundity requires that the resources allocated to reproduction or somatic maintenance are derived from a common resource pool and that the utilization of resources from this pool for egg production necessarily decreases the availability of resources for subsequent egg production or survival (van Noordwijk and de Jong 1986, Zera and Harshman 2001). The lack of a negative correlation between longevity and fecundity in *C. morosus* therefore implies that the processes of survival and reproduction may not compete for resources from a common pool. Instead, I suggest that females of this species allocate existing stores primarily to maintenance and divert incoming resources to egg production.

Although the diet treatments I imposed elicited clear and significant differences in egg production rates, these data alone do not establish that diet directly affects fitness. There is evidence that maternal nutritional environment can substantially alter offspring phenotypes (Wayne *et al*. 2006) and survival (Prasad *et al*. 2003) such that fitness depends on more than simply total egg output of a female. In addition, host plant quality has been shown to alter fertility of phytophagous insects with no effect on fecundity (Moreau *et al*. 2006). Furthermore, fertility can decline as age-specific intake declines over time in income breeders (Dixon and Agarwala 2002), thereby further uncoupling fecundity and fitness.

Consequently, my fecundity data in isolation are not sufficient to draw conclusions about the effects of diet history on fitness. Unfortunately, my ability to assess these effects was compromised by egg inviability. Compared to the expected hatch success of nearly 100% for captive *C. morosus* (Brock 2000), egg hatchability in this study was disappointingly low. I suggest two possibilities for this occurrence. The English ivy I fed to experimental animals may have been deficient in one or more limiting nutrients, thereby largely preventing embryogenesis. Alternatively, my incubation protocol may not have been appropriate for this species. However, my reproductive output results do indicate that food restriction imposed late in development and during reproductive activity has profound negative implications for fitness. Furthermore, high mortality prior to the onset of reproductive activity compounded the negative effects of lifelong FR on egg production and indicates that reproductive output is severely reduced by continuous, quantitative FR.

In summary, food availability strongly influences the expression of life-history traits and trade-offs in *C. morosus*. To my knowledge, this study was the first to evaluate the effects of quantitative dietary manipulations throughout life in a long-lived, hemimetabolous insect. My methodology allowed for accurate measurement of daily food consumption rates, a critical but often neglected component in studies of life history (Zera and Harshman 2001). In addition, using a parthenogenetic species as my animal model was a novel approach that obviated the need for mating while still permitting oviposition of fertile eggs. My data demonstrated that the life-history responses to differences in intake depended to a large extent on the timing of nutritional stress. Food restriction experienced at any point during life led to decreased fecundity. This decrease resulted primarily from differences in the quantity of reserves accumulated prior to the onset of reproductive activity and resulting differences in reproductive rate and adult survival. In

contrast, the effect of food restriction on overall lifespan depended on when the restriction was first imposed. As such, lifespan was maximized when food consumption was limited early in life, whereas reproductive output was maximized when food consumption throughout life was maximized. In effect, food restriction extended development but shortened adult lifespan, with negative consequences for final body size, reproductive lifespan, reproductive output, and, quite possibly, fitness. In *C. morosus*, it appears that storage reserves acquired early in life are essential for determining adult survival and for entraining the timing and rate of reproductive processes, but adult income is essential for egg provisioning. Putative fitness is therefore dictated both by past and current nutritional conditions.

Table 5-1. Cumulative intake expressed as the total dry mass consumed during each life-history stage in each of five treatment groups.

	Cumulative Intake (g dry matter)							
Treatment	First Instar	Second Instar	Third Instar	Fourth Instar	Fifth Instar	Sixth Instar	Pre-Ov Adult	Post-Ov Adult
AL	0.0082 ± 0.0002^{a}	0.0197 ± 0.0005^{a}	0.0411 ± 0.0009^{a}	0.0795 ± 0.0016^{a}	0.1558 ± 0.0025^{a}	0.3473 ± 0.0054^{ab}	0.5757 ± 0.3280^{a}	2.6454 ± 0.1859^{a}
AL-R at 5th	0.0080 ± 0.0002^{a}	0.0188 ± 0.0004^{a}	0.0403 ± 0.0008^{a}	0.0802 ± 0.0013^{a}	0.1438 ± 0.0018^{b}	0.3912 ± 0.0098^{c}	0.6500 ± 0.0293^{ab}	0.4289 ± 0.0478^{b}
AL-R at Ov	0.0079 ± 0.0002^{a}	0.0196 ± 0.0004^{a}	0.0410 ± 0.0007^{a}	0.0772 ± 0.0012^{a}	0.1550 ± 0.0016^{a}	0.3514 ± 0.0053^{b}	0.5747 ± 0.0135^{a}	0.9439 ± 0.0328^{c}
R	0.0088 ± 0.0003^{a}	0.0226 ± 0.0004^{b}	0.0358 ± 0.0010^{b}	0.0636 ± 0.0009^{b}	0.1289 ± 0.0031^{c}	0.3162 ± 0.0078^{ad}	0.7312 ± 0.0614^{b}	0.2287 ± 0.0242^{d}
R-AL at 5th	0.0084 ± 0.0004^{a}	0.0223 ± 0.0005^{b}	0.0352 ± 0.0011^{b}	0.0606 ± 0.0021^{b}	0.1061 ± 0.0035^{d}	0.2792 ± 0.0122^{d}	0.7529 ± 0.0491^{b}	1.5177 ± 0.1963^{c}

Life-history stages were categorized as each of six instars, the adult stage prior to first oviposition (pre-ov adult), and the adult stage between first oviposition and death (post-ov adult). Values represent means ± standard errors. Abbreviations: AL = *ad libitum*, R = restricted. See Figure 5-1 for a description of diet treatments. Sample sizes are the same as in Figure 5-2. Values with different superscripts are significantly different among treatment groups within life-history stages. See text for statistical analyses.

Table 5-2. Omnibus F, χ^2, and p-values for comparisons of body mass and age among five treatment groups within each life-history stage.

		Identity of Groups Tested in Pairwise Comparisons									
	Omnibus F and χ^2	1 & 2	1 & 3	1 & 4	1 & 5	2 & 3	2 & 4	2 & 5	3 & 4	3 & 5	4 & 5
Body Mass											
Hatch	$F_{4,65}$ = 1.00, p = 0.414	0.780	0.780	0.999	1.000	1.000	0.560	0.804	0.560	0.804	0.999
End of 1st Instar	$F_{4,65}$ = 21.67, **p < 0.0001**	1.000	0.999	**< 0.001**	**< 0.001**	1.000	**< 0.001**	**< 0.001**	**< 0.0001**	**< 0.0001**	0.998
End of 2nd Instar	$F_{4,65}$ = 108.34, **p < 0.0001**	0.827	0.999	**< 0.0001**	**< 0.0001**	0.926	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	1.000
End of 3rd Instar	$F_{4,65}$ = 115.37, **p < 0.0001**	0.998	1.000	**< 0.0001**	**< 0.0001**	0.997	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	1.000
End of 4th Instar	$F_{4,65}$ = 269.35, **p < 0.0001**	0.996	0.970	**< 0.0001**	**< 0.0001**	0.854	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	0.999
End of 5th Instar	$F_{4,65}$ = 260.84, **p < 0.0001**	**< 0.001**	0.998	**< 0.0001**	**< 0.0001**	**< 0.001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**0.002**
End of 6th Instar	χ^2 = 62.92, **p < 0.0001**	**< 0.0001**	0.801	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
First Oviposition	$F_{4,53}$ = 59.01, **p < 0.0001**	**< 0.0001**	0.993	**< 0.0001**	**0.010**	**< 0.0001**	**< 0.001**	**< 0.0001**	**< 0.0001**	**0.031**	**< 0.0001**
Death	$F_{4,53}$ = 33.26, **p < 0.0001**	**< 0.0001**	0.366	**< 0.0001**	**0.003**	**< 0.0001**	**0.001**	**0.019**	**< 0.0001**	0.243	**< 0.0001**
Age											
End of 1st Instar	χ^2 = 51.66, **p < 0.0001**	0.880	0.840	**< 0.001**	**< 0.001**	0.960	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	0.795
End of 2nd Instar	$F_{4,65}$ = 686.21, **p < 0.0001**	1.000	1.000	**< 0.0001**	**< 0.0001**	1.000	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	0.975
End of 3rd Instar	$F_{4,65}$ = 626.17, **p < 0.0001**	0.980	1.000	**< 0.0001**	**< 0.0001**	0.991	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	0.989
End of 4th Instar	$F_{4,65}$ = 518.30, **p < 0.0001**	1.000	0.994	**< 0.0001**	**< 0.0001**	0.995	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	0.997
End of 5th Instar	$F_{4,65}$ = 591.77, **p < 0.0001**	**< 0.0001**	0.942	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.001**
End of 6th Instar	$F_{4,65}$ = 888.90, **p < 0.0001**	**< 0.0001**	0.952	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
First Oviposition	χ^2 = 50.46, **p < 0.0001**	**< 0.0001**	0.840	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**
Death	$F_{4,53}$ = 95.65, **p < 0.0001**	**0.031**	**< 0.0001**	**< 0.0001**	**< 0.001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	**< 0.0001**	0.360

Identity of groups tested in pairwise comparisons: 1 = *AL*, 2 = *AL-R at 5th*, 3 = *AL-R at Ov*, 4 = *R*, and 5 = *R-AL at 5th* (where AL = *ad libitum* and R = restricted). See Figure 5-1 for a description of diet treatments. Sample sizes are the same as in Figure 5-2. When F values are reported, parametric tests were used. When χ^2 values are reported, nonparametric tests were used. See text for statistical analyses. Statistically significant p-values are indicated in bold.

Table 5-3. Relative mass (± standard error) at the adult molt of insects in five treatment groups, calculated as the ratio of actual to predicted body mass as determined by allometric analysis (see text for statistical analyses).

Treatment	Actual Mass/Predicted Mass
AL	1.087 ± 0.016^{a}
AL-R at 5th	0.977 ± 0.016^{b}
AL-R at Ov	1.093 ± 0.020^{a}
R	0.995 ± 0.014^{b}
R-AL at 5th	1.148 ± 0.013^{a}

Abbreviations: AL = *ad libitum*, R = restricted. See Figure 5-1 for a description of diet treatments. Sample sizes are the same as in Figure 5-2 for juveniles. Values with different superscripts are significantly different among treatment groups.

Table 5-4. Adjusted mean fecundity (± standard error) of insects in each of five treatment groups estimated using body mass at first oviposition as a covariate.

Treatment	Adjusted Mean Fecundity
AL	68.061 ± 4.386^{a}
AL-R at 5th	22.204 ± 4.524^{b}
AL-R at Ov	36.649 ± 4.190^{bc}
R	26.929 ± 6.815^{bc}
R-AL at 5th	42.719 ± 3.579^{c}

Abbreviations: AL = *ad libitum*, R = restricted. See Figure 5-1 for a description of diet treatments. Sample sizes are the same as in Figure 5-2 for adults. Values with different superscripts are significantly different among treatment groups according to analysis of covariance with a Bonferroni correction for multiple comparisons.

Table 5-5. Total number of ovarioles (mean ± standard error) in insects from each of five treatment groups upon post-mortem dissection.

Treatment	Number of Ovarioles
AL	53.38 ± 0.488
AL-R at 5th	53.00 ± 0.453
AL-R at Ov	53.23 ± 0.556
R	50.86 ± 1.262
R-AL at 5th	51.33 ± 0.449

Abbreviations: AL = *ad libitum*, R = restricted. See Figure 5-1 for a description of diet treatments. Sample sizes are the same as in Figure 5-2 for adults. Although the omnibus *F*-value was significant ($F_{4,53} = 3.387$, $p = 0.015$), Tukey's Honestly Significant Difference post hoc test found no significant differences among treatment groups. Results of a less conservative post hoc test (the Least Significant Difference test) indicated that initially restricted insects (groups *R* and *R-AL at 5th*) had significantly fewer ovarioles than initially *ad libitum* insects (groups *AL*, *AL-R at 5th*, and *AL-R at Ov*) ($p < 0.05$ for all significant comparisons).

Table 5-6. Parameters for equations predicting realized fecundity and initial oviposition rate as determined by stepwise multiple linear regression.

Model	y	x_1	x_2	x_3	x_4	intercept	β_1	β_2	β_3	B_4	R^2
1	Fecundity	Cum. Int. RL				9.038	28.053				0.828
2	Fecundity	Cum. Int. RL	Pre-Ov. SGR			-4.363	19.085	11.278			0.904
3	Fecundity	Cum. Int. RL	Pre-Ov. SGR	Cum. Int. Juv.		-40.833	18.140	11.973	57.654		0.928
4	In. Ov. Rate	BM at Ov.				-7.231	22.529				0.546
5	In. Ov. Rate	BM at Ov.	Age at Ad. Molt			-16.711	29.005	0.034			0.620
6	In. Ov. Rate	BM at Ov.	Age at Ad. Molt	MS Int. Pre-Ov.		-20.081	23.482	0.038	186.022		0.659
7	In. Ov. Rate	BM at Ov.	Age at Ad. Molt	MS Int. Pre-Ov.	Cum. Int. Pre-Ov.	-23.776	14.926	0.016	322.323	12.407	0.706

See methods for a list of independent variables tested. Significant independent variables are listed in the order in which they were selected. For all models, $n = 58$. All models are significant at $p < 0.0001$. Abbreviations: In. Ov. Rate = initial oviposition rate (total number of eggs laid during the first 6 days of the reproductive lifespan), Cum. Int. RL = cumulative intake during the reproductive lifespan, BM at Ov. = body mass at first oviposition, Pre-Ov. SGR = specific growth rate (per day) during adult stage prior to first oviposition, Age at Ad. Molt = age at the adult molt, Cum. Int. Juv. = cumulative intake during all juvenile stages, MS int. Pre-Ov. = average mass-specific intake during adult stage prior to first oviposition, Cum. Int. Pre-Ov. = cumulative intake during adult stage prior to first oviposition.

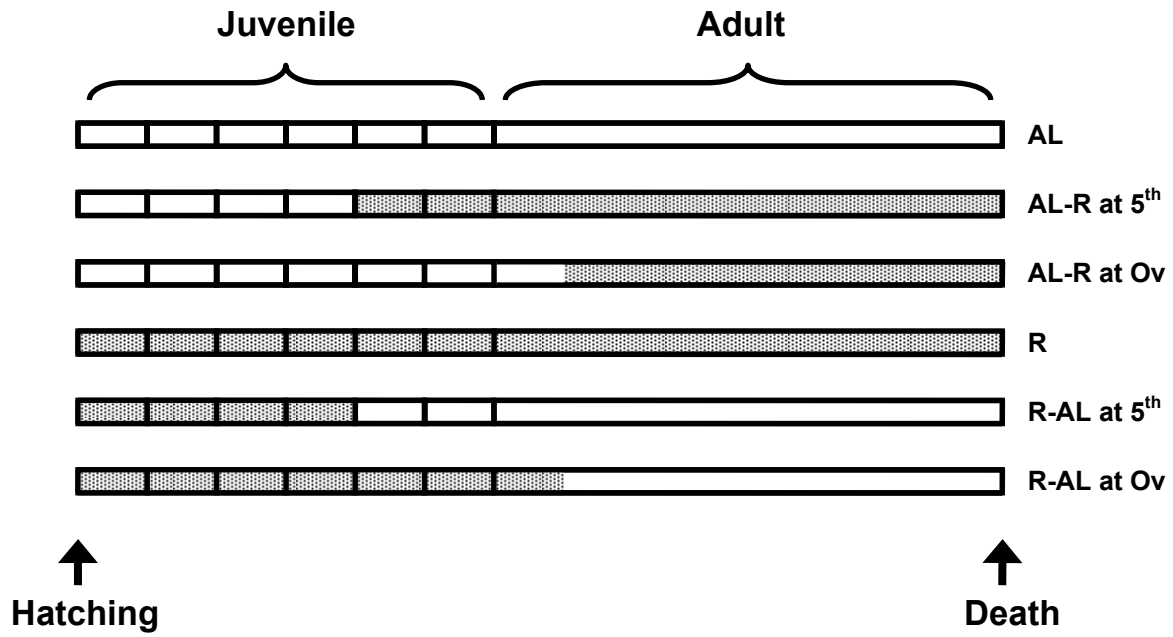


Figure 5-1. Experimental design for *Carausius morosus* feeding trial. Lifespans are represented by horizontal bars divided into six instars and an adult stage. Time is not to scale, and differences in timing of life-history transitions between groups are not graphically presented. Vertical lines in juvenile stages denote ecdyses. White bars represent life stages when food was offered *ad libitum* (AL); shaded bars represent life stages when food was restricted (R) to 60% of the amount of food consumed by insects in group *AL* on a percent body mass basis. Insects in groups *AL* and *R* were maintained for the duration of their lifespans on *ad libitum* and restricted diets, respectively. Insects in the *AL-R at 5th* and *R-AL at 5th* groups experienced a diet switch on the first day of the fifth instar. Insects in the *AL-R at Ov* group experienced a diet switch at first oviposition. Because survival to first oviposition was extremely low for insects that were food-restricted for the duration of juvenile development, I was unable to test the effects of a diet switch from R to AL at first oviposition.

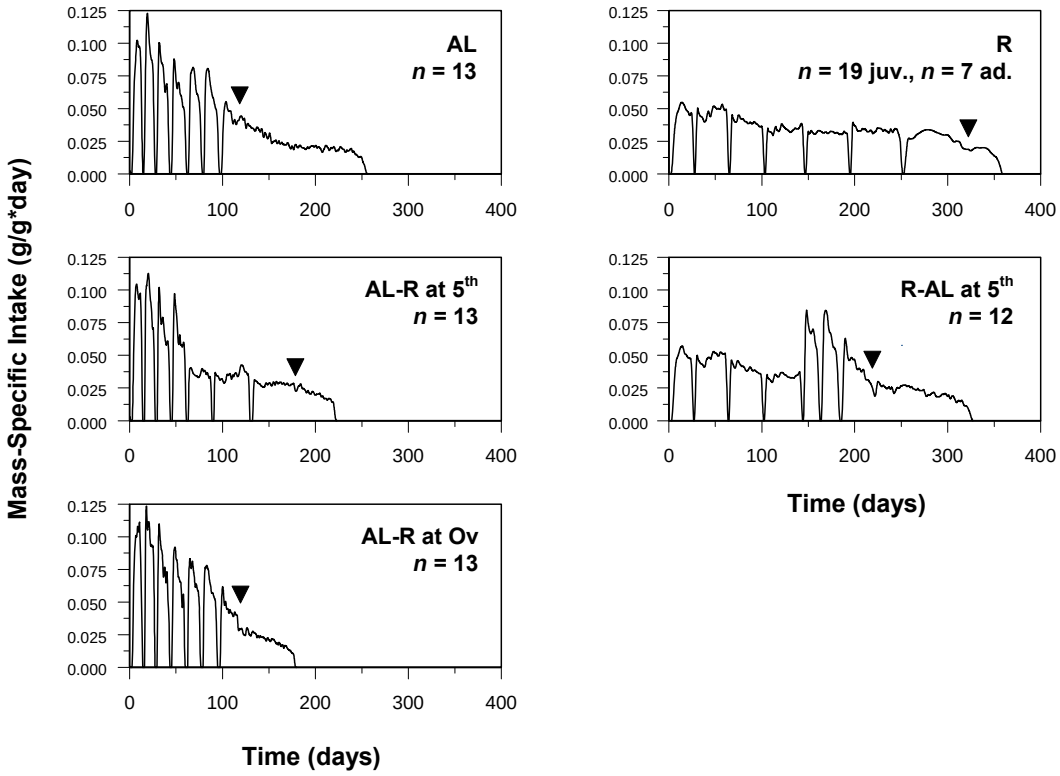


Figure 5-2. Mass-specific intake (g dry mass/g*day) consumed by insects in each of five treatment groups on each day of the study. Curves were constructed by scaling the duration of each stage for each insect to the average duration of that stage for each treatment group and fitting a loess smoothing function to these data. Points where mass-specific intake declined to zero correspond to ecdyses. The first six resulting time intervals for each group represent juvenile stages and the seventh time interval for each group represents the adult stage. Arrowheads denote the average age at first oviposition for each group. Abbreviations: AL = *ad libitum*, R = restricted, juv. = juveniles, ad. = adults. See Figure 5-1 for a description of diet treatments. Food restriction was imposed by offering restricted individuals approximately 60% of the mass-specific intake of insects in group AL on a stage-specific basis. Because mass-specific intake of AL insects declined after first oviposition, the amount of food offered to food-restricted adults after first oviposition was decreased proportionally to match this decline.

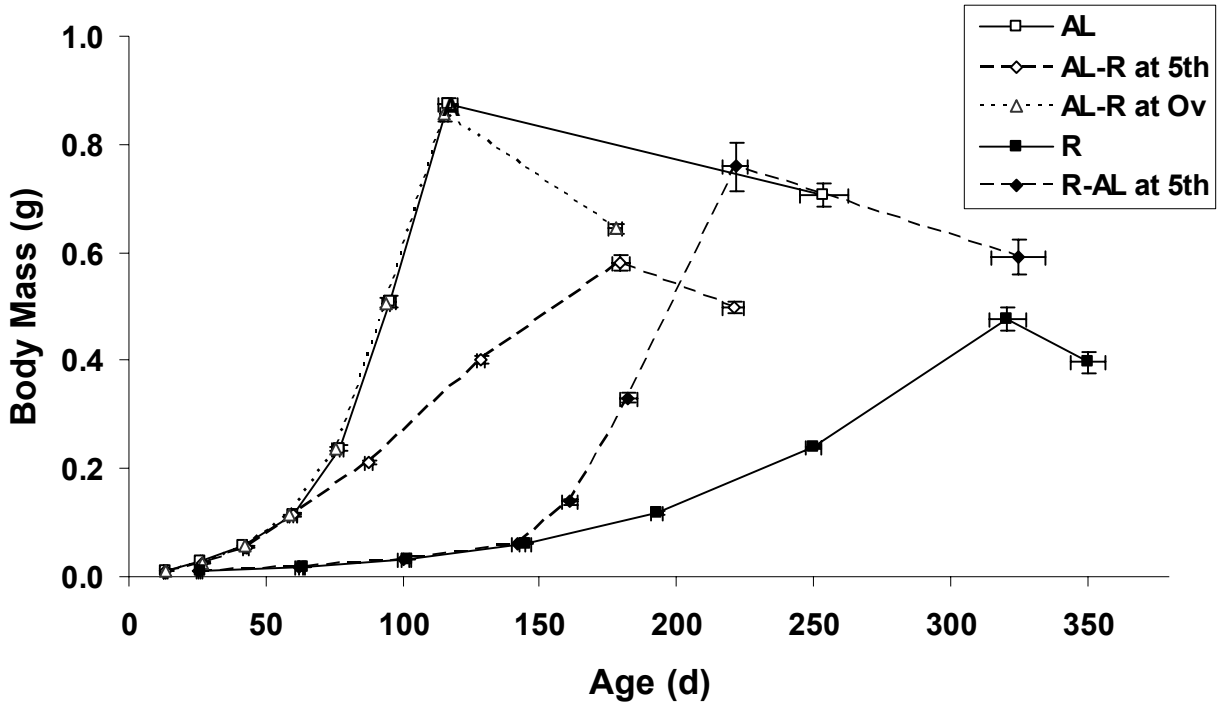
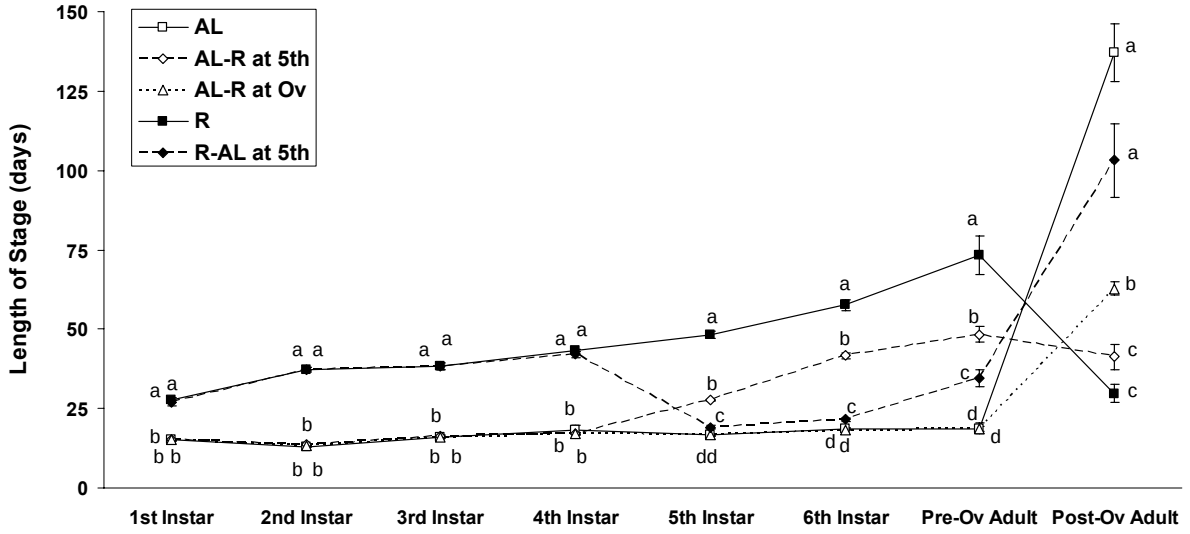


Figure 5-3. Age and size at each life-history transition for insects in each of five treatment groups. Each point represents mean ± standard error at the end of an instar (first six points for each line), at first oviposition (seventh point for each line), or at death (last point for each line). Abbreviations: AL = *ad libitum*, R = restricted. See Figure 5-1 for a description of diet treatments. Sample sizes are the same as in Figure 5-2. See Table 5-2 for *p*-values for size and age at each point.

(a)



(b)

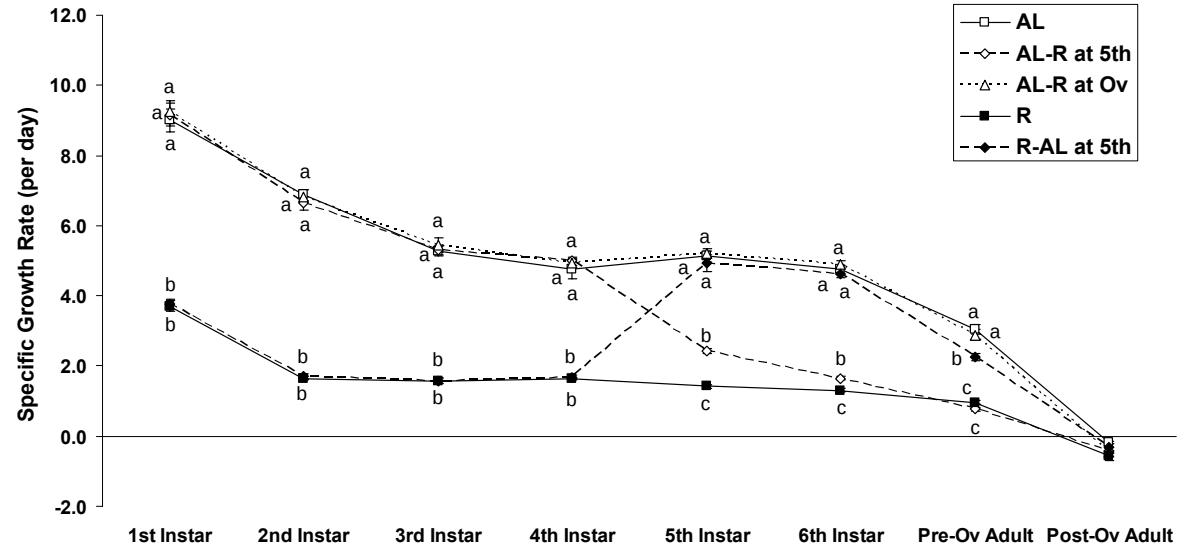


Figure 5-4. Duration of (a) and specific growth rate during (b) each life-history stage for insects in each of five treatment groups. Each point represents mean ± standard error. Stages were categorized as each of six instars, adult prior to first oviposition (pre-ov adult), and adult after first oviposition (post-ov adult). *AL-R at Ov* insects lost proportionally more body mass than *AL* insects after first oviposition, but all other pairwise comparisons of post-oviposition adult growth rates were not significant (Mann-Whitney U tests, $p > 0.005$). Abbreviations: AL = *ad libitum*, R = restricted. See Figure 5-1 for a description of diet treatments. Sample sizes are the same as in Figure 5-2. Means with different letters are significantly different among treatment groups within life-history stages. See text for data analysis.

Figure 5-5. Event history diagram depicting periods of *ad libitum* intake, restricted intake, and reproductive activity for individual stick insects maintained on five diet treatments. Each horizontal line represents the lifespan of one individual, with insects in each group arranged in order (top to bottom within a treatment group) from shortest to longest lifespan. Abbreviations: AL = *ad libitum*, R = restricted, Repr. Life. = reproductive lifespan. See Figure 5-1 for a description of diet treatments. Data for insects that died during the juvenile stages (i.e., insects represented by the shortest 1 or 2 bars for each treatment group) were not included in any analyses except for survivorship curves (Fig. 5-6).

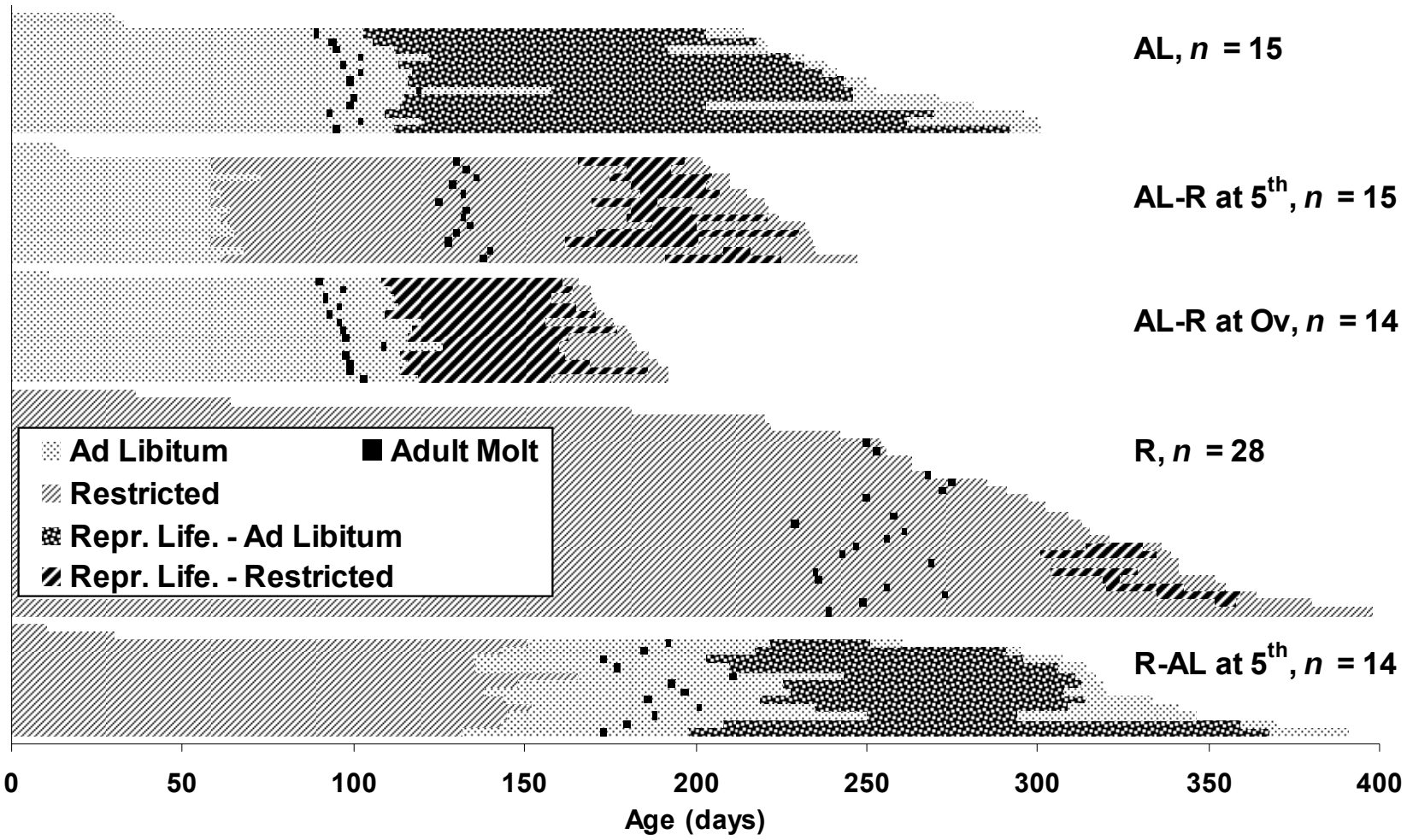
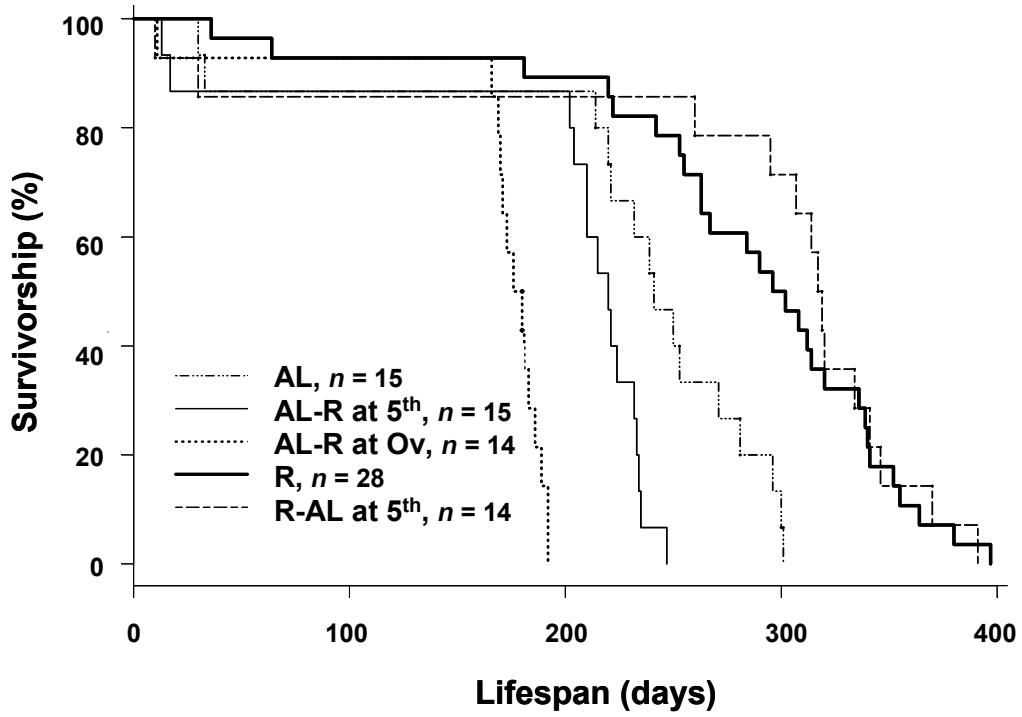
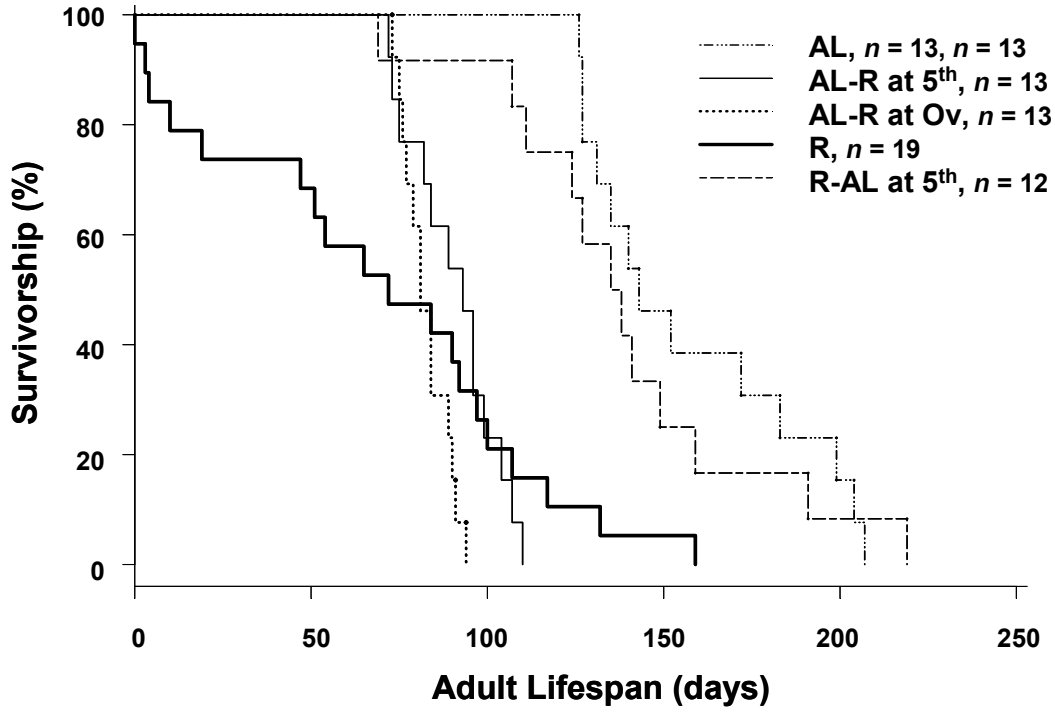

AL, n = 15
AL-R at 5th, n = 15
AL-R at Ov, n = 14
R, n = 28
R-AL at 5th, n = 14
Ad Libitum
Adult Molt
Restricted
Repr. Life. - Ad Libitum
Repr. Life. - Restricted
0
50
100
150
200
250
300
350
400
Age (days)

Figure 5-6. Kaplan-Meier survivorship curves for the entire lifespan (a) and for the adult lifespan (b) of insects maintained on five diet treatments, including insects that died prior to the adult molt. Abbreviations: AL = *ad libitum*, R = restricted. See Figure 5-1 for a description of diet treatments. For graph b, only insects that survived to adulthood are included. Only 7 of the insects in group *R* laid eggs, but all 19 individuals in this treatment group are included in the survivorship curve. For graph a, pairwise log-rank tests with α set at 0.005 to account for multiple comparisons indicated that all groups except *R* and *R-AL at 5th* differed significantly in longevity. For graph b, *AL* and *R-AL at 5th* insects had significantly enhanced adult longevity compared to *AL-R at 5th*, *AL-R at Ov*, and *R* insects.

(a)


Survivorship (%)
100
80
60
40
20
0
0
100
200
300
400
Lifespan (days)
AL, n = 15
AL-R at 5th, n = 15
AL-R at Ov, n = 14
R, n = 28
R-AL at 5th, n = 14

(b)


Survivorship (%)
100
80
60
40
20
0
0
50
100
150
200
250
Adult Lifespan (days)
AL, n = 13, n = 13
AL-R at 5th, n = 13
AL-R at Ov, n = 13
R, n = 19
R-AL at 5th, n = 12

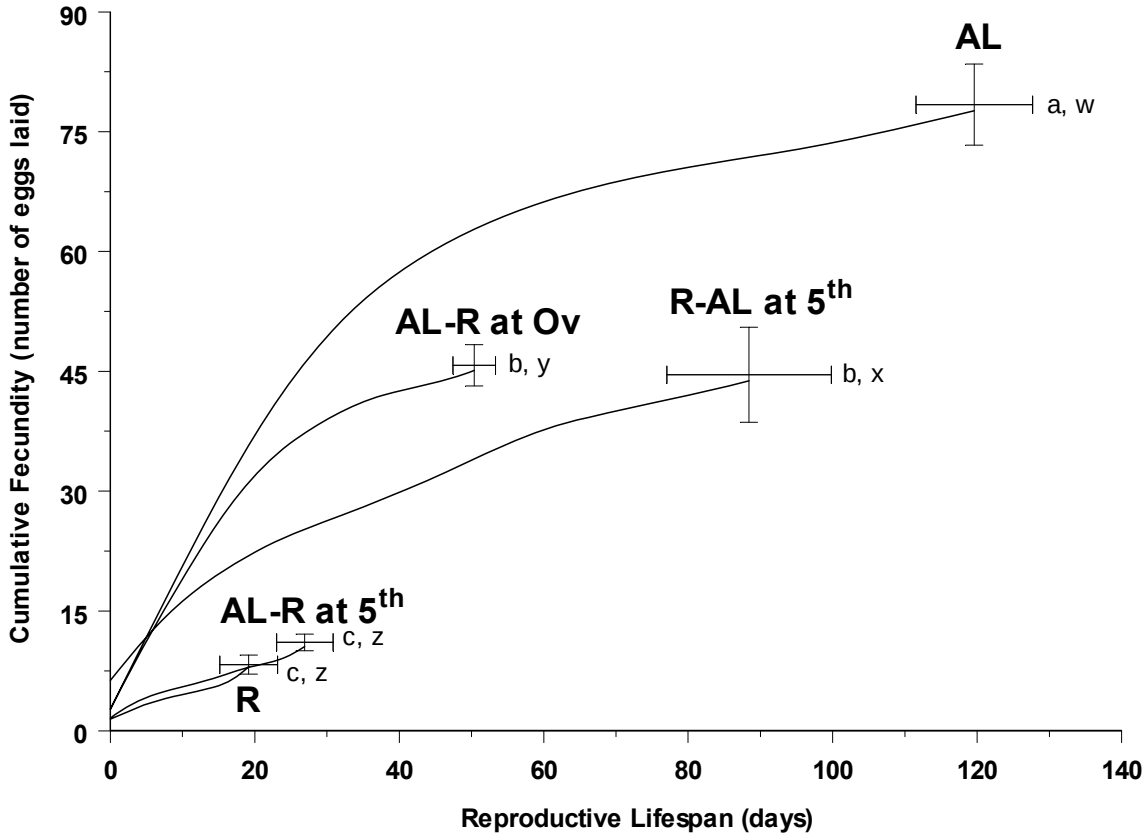


Figure 5-7. Cumulative fecundity of insects in each of five treatment groups. The x-axis represents days of the reproductive lifespan, with day 0 representing the first day of oviposition. Each curve terminates at a point corresponding to the mean duration (± standard error) of reproductive activity (x) and the mean fecundity (± standard error) for each group (y). Curves were constructed by scaling the reproductive lifespan of each insect to the mean reproductive lifespan for that group, averaging the cumulative fecundity of all insects in that group on each day of the reproductive lifespan, and fitting a smooth spline (*df* = 7) to the resulting averages. Abbreviations: AL = *ad libitum*, R = restricted. See Figure 5-1 for a description of diet treatments. Sample sizes are the same as in Figure 5-2 for adults. Different letters to the right of each endpoint indicate significantly different means for fecundity (a, b, and c) and reproductive lifespan (w, x, y, and z) among treatment groups. See text for data analysis.

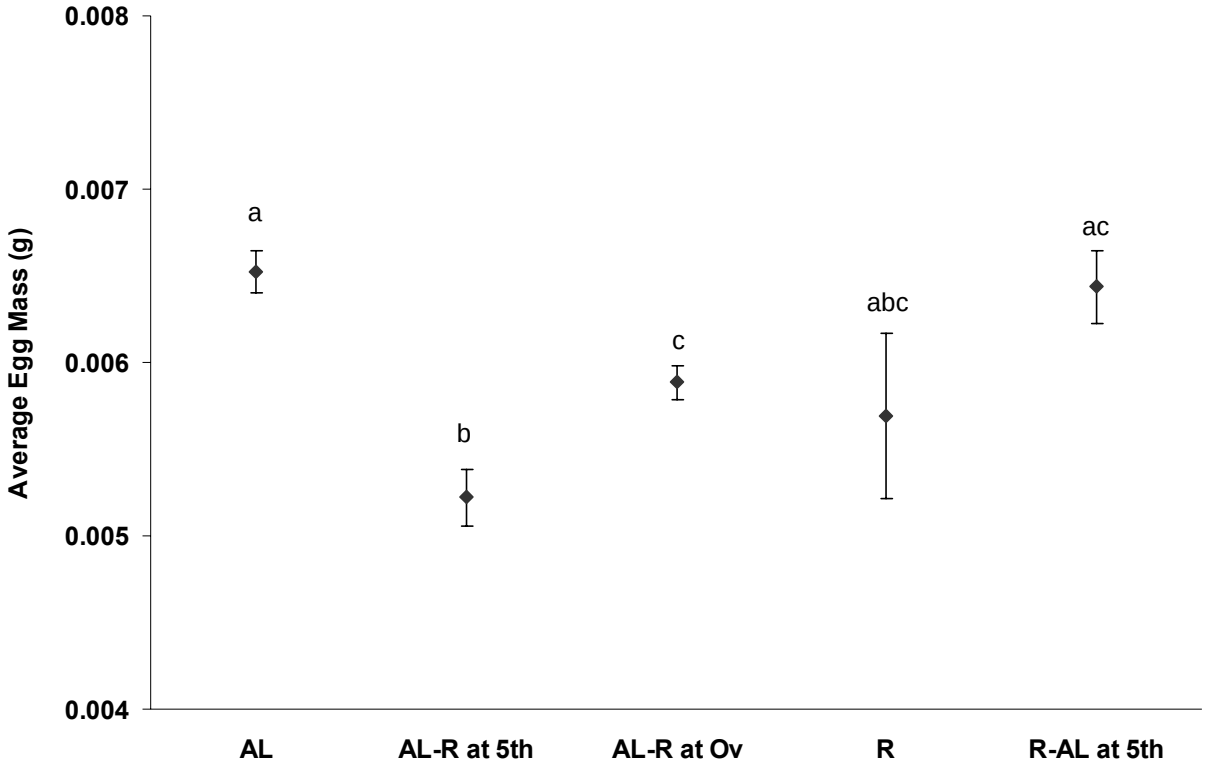
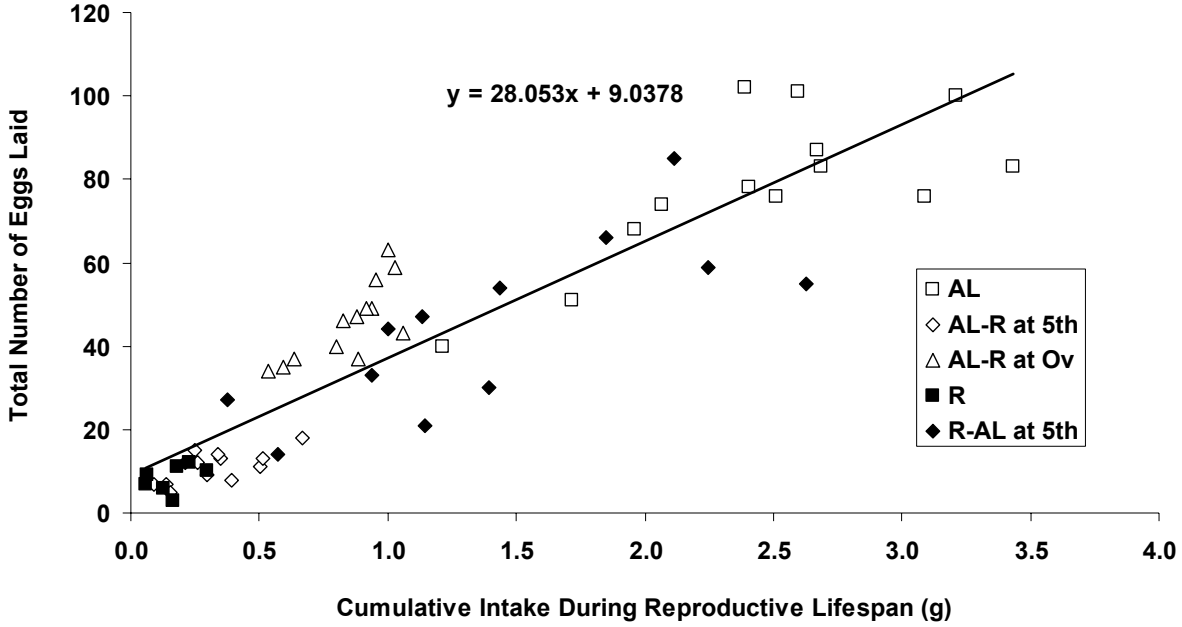


Figure 5-8. Average egg mass (mean ± standard error) for stick insects maintained on five diet treatments. Abbreviations: AL = *ad libitum*, R = restricted. See Figure 5-1 for a description of diet treatments. Sample sizes are the same as in Figure 5-2 for adults. Means with different letters are significantly different among treatment groups. See text for data analysis.

(a)



(b)

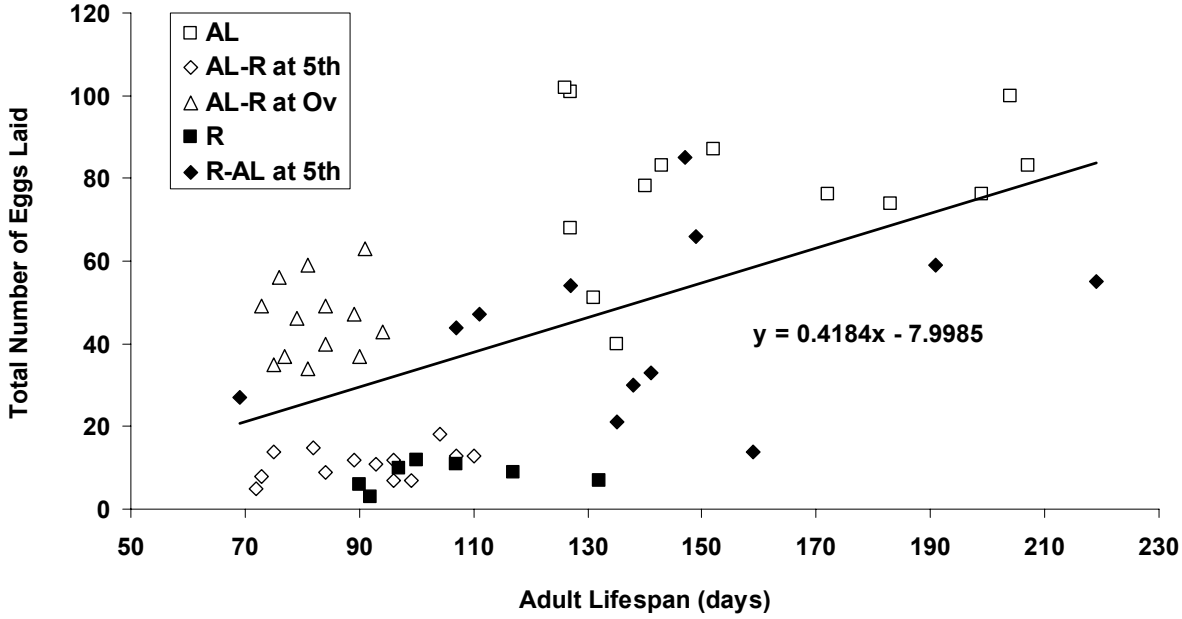


Figure 5-9. Relationships between realized fecundity and cumulative intake during the reproductive lifespan (a) and the duration of adult lifespan (b) for all insects that laid eggs (n = 58) as determined by least squares linear regression. The regression in a is significant ($F_{1,56}$ = 276.00, $p < 0.0001$) with an adjusted R^2 value of 0.828. The regression in b is also significant ($F_{1,56}$ = 25.67, $p < 0.0001$) with an adjusted R^2 value of 0.302. Abbreviations: AL = *ad libitum*, R = restricted. See Figure 5-1 for a description of diet treatments.

CHAPTER 6
SUMMARY AND CONCLUSIONS

Food availability is arguably one of the most fundamental and often-cited modulators of phenotypic and life-history plasticity. For my dissertation, I addressed questions about the effects of changes in food availability during different life stages in two taxa. In Chapters 2-4, I evaluated the physiological and morphological responses to short-term (e.g., 12-week) differences in food availability in a species (the green turtle, *Chelonia mydas*) that experiences nutritional stochasticity during the juvenile stage in the wild. To elucidate long-term responses to differences in food availability, I conducted a lifespan study using a more tractable animal model (the Indian stick insect, *Carausius morosus*). A summary of the major findings of my work can be found in Table 6-1.

Animals living in nutritionally stochastic environments demonstrate a variety of adaptations, including the capacity for compensatory growth (CG) (Wilson and Osbourn 1960, Reid and White 1977), that enable them to capitalize when conditions are favorable for growth and reproduction. Although CG has been documented in turtles and lizards (Bjorndal *et al*. 2003, Caley and Schwarzkopf 2004), my work is the first to assess the mechanistic basis for this growth pattern in reptiles. One of the most salient findings to emerge from my work on *C. mydas* was the fact that CG is effected via enhanced food conversion efficiency (FCE) rather than hyperphagia. This result stands in direct contrast to most CG studies in fish (reviewed by Ali *et al*. 2003).

Additionally, working at the Cayman Turtle Farm afforded me a unique opportunity to investigate growth dynamics in green turtles without sampling animals taken from the wild. As a result, I was able to elucidate the effects of intake and growth rates on a number of parameters (e.g., body composition, digestive tract morphology, nucleic acid content, and antioxidant

function) that cannot typically be studied in healthy individuals of this endangered species. Green turtles responded to food restriction by mobilizing lipid reserves, conserving protein reserves, and down-regulating the size of visceral organs. Assuming that digestive organs are energetically expensive to maintain (Hornick *et al*. 2000), a decrease in organ size and concomitant decreased metabolic rate may explain the improved FCE in food-restricted turtles. Turtles undergoing CG not only grew faster than continuously *ad libitum*-fed turtles but also adjusted their rates of tissue deposition such that body composition and organ morphology were restored after seven weeks of improved food conditions. Clearly, young green turtles have the capacity to adjust to fluctuations in food availability.

Given this flexibility in the response to changes in nutritional condition and the previously demonstrated effects of climate (Limpus and Chaloupka 1997) and population density (Bjorndal *et al*. 2000) on growth rates, a comprehensive assessment of green turtle population health requires a better understanding of short-term growth dynamics. I therefore explored the potential for measuring a number of biochemical indices as predictors of recent growth in juvenile green turtles. By analyzing RNA, DNA, and protein content of tissues from the same turtles I examined in Chapter 2, I was able to correlate these indices and ratios among them with known short-term growth rates (e.g., during the preceding 10-11 days). The models I developed predicted 55-68% of the variance in recent growth rates (Chapter 3). Specific growth rate for body mass was best explained (R^2 = 0.68) by RNA content of the liver and condition index (Fulton's K, Ricker 1975), and specific growth rate for carapace length was best explained (R^2 = 0.66) by only RNA content of the liver. Because these analyses rely on destructive tissue extraction, they are not widely applicable to studies of growth in wild turtles. However, specific growth rate for body mass was also explained moderately well (R^2 = 0.55) by condition index

and DNA content of blood. Both of these parameters are easily quantified with minimal disturbance to the animal, suggesting that biochemical indices hold promise as potential indicators of recent growth in wild turtles.

After demonstrating the substantial physiological and morphological plasticity of green turtles exposed to different nutritional environments over short time scales, I became interested in the long-term effects of food availability. A conspicuous feature of green turtle growth is the transient nature of CG after a switch from restricted to *ad libitum* feeding. In addition, the occurrence of CG indicates that "normal" growth rates in this species are sub-maximal, suggesting that rapid growth may be associated with one or more costs (Metcalfe and Monaghan 2001). Mangel and Munch (2005) posited that these costs could include elevated levels of oxidative damage incurred during CG. The results I presented in Chapter 4 provide the first empirical evidence supporting this hypothesis. Although antioxidant function of muscle (a post-mitotic tissue) was unaffected by diet, the activity of glutathione peroxidase (an antioxidant enzyme) and total antioxidant potential per cell in the liver (a mitotically active tissue) were approximately two-fold greater in continuously *ad libitum*-fed turtles than in continuously food-restricted turtles or fast-growing turtles that had undergone growth compensation. An impaired antioxidant defense system is therefore a cost of CG in green turtles. However, the duration of this impairment is unknown, as are its life-history consequences.

The long lifespan and large body size of green turtles were not conducive to evaluating long-term responses to fluctuations in food availability. Instead, I took a novel approach by using a parthenogenetic insect as my animal model for Chapter 5, and this tactic proved to be a fruitful one for investigating questions about life history. In contrast to my work on *C. mydas*, I did not find any evidence of CG in *C. morosus*. It is possible that an herbivorous diet precludes growth

compensation because digestive efficiency may be maximized in animals consuming a low-quality diet. Although I was unable to quantify the potential life-history costs of CG, my methodology allowed me to assess the effects of different diet treatments on traits such as development rate, longevity, and fecundity that could not be measured in *C. mydas*.

Not surprisingly, insects that experienced food restriction prior to the onset of reproductive activity progressed through juvenile stages more slowly and were smaller at each molt than individuals feeding at a consistently high rate. These results provide support for the model of Day and Rowe (2002) and suggest that development rate in response to food availability represents a compromise between selection for maximized body size (because of its fitness benefits) and selection against extended development time (because of its demographic costs) (Rowe and Ludwig 1991).

Although my results for age and size at developmental transitions are typical in studies of this kind, the quantification of lifespan and cumulative fecundity in individuals with drastically different developmental trajectories is a novel contribution of my research. My results indicate that quantitative food restriction experienced early in development extended lifespan, as is common in other animal models (e.g., Weindruch and Walford 1988, Austad 1989, Mair *et al*. 2003, Vaupel *et al*. 2003, Hatle *et al*. 2006b). However, this longevity enhancement resulted from extended development time rather than enhanced adult survival. Conversely, food restriction experienced later in development or at maturity significantly decreased total lifespan.

In contrast to my results for longevity, food restriction imposed at any point during the lifespan decreased fecundity. Putative fitness was therefore maximized when daily intake was also maximized throughout life. These findings indicate that the beneficial effects of early-onset food restriction on lifespan were negated by the detrimental effects on reproductive output.

Cumulative intake during the reproductive lifespan explained 83% of the variance in fecundity, indicating that *C. morosus* primarily allocates incoming adult-derived resources to egg provisioning. In contrast, body stores appear to be the source of nutrients allocated to somatic maintenance and survival during adulthood, with proportionally heavier females living longer as adults than smaller females. Given this breeding strategy, it is not surprising that I found no evidence for a trade-off between longevity and fecundity, as nutrients allocated to reproduction and maintenance do not appear to be derived from a common resource pool. These results indicate that fluctuations in food availability can significantly alter the expression of life-history traits and that the magnitude of these effects depends on the developmental stage during which food availability changes and on the timing of resource acquisition relative to allocation.

In conclusion, this dissertation provides new insights into the short- and long-term consequences of quantitative food restriction and has wide-reaching implications for studies of food availability in both vertebrates and invertebrates. Furthermore, the successful use of a parthenogenetic animal model underscores the importance of natural reproductive processes in studies of this kind, because the true fitness effects of diet can only be evaluated if reproductive potential is not constrained by methodological limitations. The results of my work highlight the need for further research into the proximate mechanisms underlying differences in life histories within and among taxa.

Table 6-1. Summary of traits measured for *Chelonia mydas* (top half of table) and *Carausius morosus* (bottom half of table) maintained on different schedules of *ad libitum* (ad lib.) and restricted (rest.) intake.

Trait Measured	Continuously Ad Lib.	Continuously Rest.	Rest. → Ad Lib. During Development	Ad Lib. → Rest. During Development	Ad Lib. → Rest. at First Oviposition
C. mydas					
Body Size	Large	Small	Intermediate		
Growth Rate	Fast	Slow	Fast, with CG		
Body Condition	High	Low	High		
Conversion Efficiency	Low	High	Intermediate		
Digestive Organ Size	Large	Small	Large		
OM, Lipid, and Energy Content	High	Low	High		
Nitrogen Content	Low	High	Low		
Cell Size	Large	Small	Intermediate/ Large		
RNA:DNA, Liver	Intermediate	High	Low		
RNA:DNA, Heart	Intermediate	Low	High		
RNA:DNA, Blood	High	Low	Intermediate		
Protein Content, Liver	Intermediate	High	Low		
Antioxidant Function	High	Low	Low		
C. morosus					
Body Size at First Oviposition	Highest	Lowest	High	Low	Highest
Age at First Oviposition	Youngest	Oldest	Old	Young	Youngest
Growth Rate (Juveniles)	Fast	Slow	Fast, with No CG	Intermediate	Fast
Body Condition at Adult Molt	High	Low	High	Low	High
Ovary Size	No Difference in Total Number of Ovarioles Among Treatment Groups				
Total Lifespan	Long	Longest	Longest	Short	Shortest
Reproductive Lifespan	Highest	Lowest	High	Lowest	Low
Adult Lifespan	Long	Short	Long	Short	Short
Lifetime Fecundity	High	Low	Intermediate	Low	Intermediate
Mass-Corrected Lifetime Fecundity	High	Intermediate/ Low	Intermediate	Low	Intermediate/ Low
Egg Success	High	Low	Intermediate/ High	Low	Intermediate

Abbreviations: CG = compensatory growth, OM = organic matter, Rest. = food-restricted, Ad Lib. = *ad libitum*-fed. Diet switches from *ad libitum* to restricted feeding were not tested for *C. mydas*.

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BIOGRAPHICAL SKETCH

Alison M. Roark was born in Norfolk, Virginia on October 26th, 1978. She attended Mills Godwin High School in Richmond, Virginia, where she was a leader in the marching and concert bands. After graduating in 1996, Alison attended the University of Virginia (Charlottesville) and obtained her Bachelor of Science in chemistry with specialization in biochemistry in 2000. She also fulfilled the requirements for the Distinguished Majors Program in biology with highest distinction and served as President of the Biology Society. Alison participated in undergraduate research in two different laboratories at the University of Virginia and also completed two Research Experience for Undergraduates programs, one at the University of Texas (Austin) and one at the Long-Term Ecological Research station in Oyster, Virginia. After her second year, she spent three weeks in San Salvador, The Bahamas, for a class in coral reef ecology and credits this course with steering her toward a career in academia.

In 2000, Alison joined the Department of Zoology at the University of Florida under the direction of Karen Bjorndal. In the spring of 2003, she completed her master's bypass. While at the University of Florida, Alison taught a number of classes, including functional vertebrate anatomy laboratory and the discussion sections and laboratories for both semesters of introductory biology. In her final year as a graduate student, she taught her own non-majors biology course (Cells, Organisms, and Genetics). In the summer of 2007, Alison begins a postdoctoral position in the laboratory of Lou Guillette through the Howard Hughes Medical Institute's Group Advantaged Training of Research (G.A.T.O.R.) program.