UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO" (*UNESP*)
CÂMPUS DE JABOTICABAL

EFFECT OF TEMPERATURE AND DIET (STARCH AND FIBER) ON EMBRYONIC DEVELOPMENT, ENERGY METABOLISM AND GROWTH OF RED-FOOTED TORTOISE (*Chelonoidis carbonaria*).

Pierina Jocelyn Mendoza Yengle
Zootecnista

JABOTICABAL-SP
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Discente: Pierina Jocelyn Mendoza Yengle
Orientador: Prof. Dr. Aulus Cavalieri Carciofi.
Coorientadora: Profa. Drª Kênia Cardoso Bícego.

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA DISSERTAÇÃO: EFFECT OF THE TEMPERATURE AND DIET (STARCH AND FIBER) ON THE EMBRYONIC DEVELOPMENT, ENERGY METABOLISM AND GROWTH OF RED-FOOTED TORTOISE (*Chelonoidis carbonaria*)

AUTORA: PIERINA JOCELYN MENDOZA YENGLE
ORIENTADOR: AULUS CAVALIERI CARCIOFI
COORIENTADORA: KÊNIA CARDOSO BÍCEGO

Aprovada como parte das exigências para obtenção do Título de Mestra em ZOOTECNIA, pela Comissão Examinadora:

Prof. Dr. AULUS CAVALIERI CARCIOFI (Participaçao Virtual)
Departamento de Clínica e Cirurgia Veterinária / FCAV / UNESP - Jaboticabal

Professor Adjunto CARLOS EDUARDO DO PRADO SAAD (Participaçao Virtual) p/
Departamento de Zootecnia / Universidade Federal de Lavras/MG

Dra. ELLEN DIERENFELD (Participaçao Virtual)
Nottingham Trent University / Nottingham/Inglaterra p/

Jaboticabal, 15 de abril de 2021

DADOS CURRICULARES DEL AUTOR

PIERINA JOCELYN MENDOZA YENGLE – Nascida em 29 de março de 1994, em Lima, Peru. Ingressou no curso de ‘Zootecnia da Faculdade de Zootecnia de Universidad Nacional Agraria la Molina (UNALM) em março de 2011, destacando-se as seguintes atividades: estágios na área de manejo de animais silvestres em diferentes zoológicos de Peru durante os anos 2011, 2013 e 2015, e desenvolvimento do trabalho de conclusão do curso em nutrição de filhotes de peixe-boi amazônico ‘Avaliação nutricional de filhotes de peixe-boi amazônico alimentados com quatro dietas não convencionais’. Obteve o título de Engenheira Zootecnista em abril de 2017. Em 2019, ingressou no curso de Mestrado em Zootecnia (Nutrição de Monogástricos) na Faculdade de Ciências Agrárias e Veterinárias de Jaboticabal. FCAV - UNESP como bolsista FAPESP, desenvolvendo vários trabalhos com ênfase em nutrição e fisiologia de animais silvestres. Participou de diversos eventos extracurriculares relacionados à área de animais silvestres, como 12th Biennial ZWNF/NAG Conference on Zoo and Wildlife Nutrition (2017), Frisco – Texas; 3rd Latin American Manatee Symposium - SILAMA 2018 and XII Congress of the Latin American Society of Specialists in Aquatic Mammals - SOLAMAC 2018 RT18; and Virtual Zoo Nutrition European Association of Zoos and Aquaria (EAZA), resultando em 4 publicações em anais de eventos e 2 publicações nas revistas científicas Natural Science and Aquatic Mammals.

DEDICATÓRIA

A mis padres Celso Mendoza y Rosella Yengle, y a mi hermano Jonathan, por acompañarme desde el primer momento que escogí esta carrera. En memoria de mis abuelos Manuel Yengle y Francisco Mendoza, de mi padrino Daniel Yengle y mi ángel Zasha, quienes desde el cielo cuidan mis pasos. A Dios por permitirme lograr cada meta, cuidarme y colocarme grandes personas en mi camino.

Aos meus pais Celso Mendoza e Rosella Yengle, e meu irmão Jonathan, pelo me acompanhar desde o primeiro momento em que escolhi esta professão. Em memória dos meus avós Manuel Yengle e Francisco Mendoza, do meu padrinho Daniel Yengle e de meu anjo Zasha, quem vigia os meus passos desde o céu. À Deus por me permitir alcançar cada objetivo, cuidando e colocando grandes pessoas no meu caminho.

À todos os animais silvestres vítimas do tráfico ilegal, por um trabalho em equipe pela conservação de nossa fauna

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- À minhas meninas do W.A.R.M.I., pelo aceitar e ser parte do projeto e começar esta aventura juntas, acreditar em mim e brindar suporte durante o tempo de redação.
- À todos meus amigos: Rony, Cecilia, Alvaro, Villegas, Kiara, Josué, Sarita, Anita, Lucas, Gabi, e David, e cada pessoa especial que ficaram enviando energias positivas para mim para continuar no caminho e não desistir, obrigada pelo apoio além da distância.
- À todas as estagiárias que ajudaram no cuidado e manejo dos animais, por cada recomendação, crítica e ideias novas que me ajudaram para melhorar o projeto cada vez mais, muito obrigada pelo amor e dedicação que amostraram com os animais.
- Às meninas que consegui coorientar, muito obrigada pela oportunidade de aprender juntas e poder fazer um trabalho em equipe, aprendi muito de vocês.
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- Finalmente, um grande agradecimento para cada um de meus jabutis, que permitiram que esta pesquisa seja feita: mis tortugas morrocoy.

SUMÁRIO

Página

CERTIFICADO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Efeito da temperatura ambiental no desenvolvimento embrionário e energia dietética (amido ou fibra) no metabolismo de filhotes Jabutis-piranga (*Chelonoidis carbonária*)"**, protocolo nº 006094/19, sob a responsabilidade do Prof. Dr. Aulus Cavalieri Carciofi, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 16 de maio de 2019.

Vigência do Projeto	01/06/2019 a 31/12/2020
Espécie / Linhagem	Jabuti piranga (*Chelonoidis carbonaria*)
Nº de animais	85
Peso / Idade	7.85 kg (2.68 – 13 kg) Adultos 265 g (30 – 500 g) Filhotes desde o nascimento até 1 ano e meio.
Sexo	Macho e Fêmeas
Origem	O Bosque Municipal Fabio Barreto, Ribeirão Preto e o Centro de Medicina e Pesquisa em Animais Selvagens (CEMPAS) da FMVZ/ UNESP, Campus Botucatu.

Jaboticabal, 16 de maio de 2019.

Fabiana Pilarski

Prof.ª Dr.ª Fabiana Pilarski
Coordenadora – CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n CEP 14884-900 - Jaboticabal/ SP - Brasil
tel 16 3209 7100 www.fcav.unesp.br

EFEITO DA TEMPERATURA E DIETA (AMIDO E FIBRA) NO DESENVOLVIMENTO EMBRIONÁRIO, METABOLISMO ENERGÉTICO E CRESCIMENTO DE JABUTIS PIRANGA (*Chelonoidis carbonaria*)

RESUMO

Existe pouca informação sobre as condições de incubação dos ovos, necessidades nutricionais e de manejo em cativeiro de jabutis piranga (*Chelonoidis carbonaria*) para a conservação. No primeiro estudo desta dissertação considerou-se que embriões de répteis respondem às mudanças da temperatura com ajustes metabólicos e fisiológicos que influenciam o sucesso de eclosão, fenótipo, e taxa de crescimento. As mudanças climáticas e o aquecimento global podem afetar as populações de répteis e a incubação artificial tem sido proposta como uma estratégia potencial para estudar e mitigar esses efeitos. Foram coletados ovos de jabuti piranga e incubados artificialmente às temperaturas constantes de 27,5°C e 29,5°C para determinar o efeito da temperatura no desenvolvimento embrionário mediante ovoscopia; a morfologia e a taxa de crescimento inicial do filhote. Os efeitos diretos da temperatura no período de incubação, massa perdida de ovos, índice de eclosão, tamanho e peso do filhote foram avaliados na eclosão e aos três meses de idade. Filhotes produzidos à 29,5°C apresentaram períodos de incubação mais curtos (141 dias) do que os de 27,5°C (201 dias; $p < 0,05$). A perda de massa de ovo, o peso e tamanho do filhote na eclosão não foram diferentes entre as temperaturas de incubação ($P > 0,05$). No entanto, o índice de incubação (taxa de sobrevivência) foi menor (64,5% versus 100%) em ovos incubados a 29,5°C, mas o peso e a largura do plastrão dos filhotes foram maiores aos 3 meses de idade do que para os filhotes dos ovos incubados a 27,5°C ($p < 0,05$). Esses resultados indicam claramente que a temperatura de incubação tem uma influência importante no sucesso eclosão e no tamanho e peso dos filhotes nos primeiros meses, influenciando a taxa de crescimento inicial.

O segundo objeto de estudo considerou que alterações da carapaça são um problema importante, observado em muitos zoológicos e criatórios ao redor do mundo. O objetivo do estudo foi determinar o efeito de dois alimentos, um com elevado teor de fibra e outro com elevado teor de amido, sobre o metabolismo energético, digestibilidade dos nutrientes e crescimento do jabuti piranga. Seguindo um delineamento inteiramente casualizado, com 20 filhotes recém eclodidos, aleatoriamente divididos em duas rações experimentais (10 filhotes por ração). O estudo teve uma duração de 18 meses, período no qual os filhotes receberam apenas sua respectiva dieta experimental, e água *ad libitum*. As avaliações realizadas nos animais incluíram: determinação dos coeficientes de digestibilidade aparente dos nutrientes e tempo de trânsito gastrointestinal aos 5 e 11 meses; determinação da taxa metabólica em repouso e pós-prandial, com cálculo do incremento calórico em câmaras de respirometría por calorimetria indireta aos 6 e 12 meses; avaliação do crescimento e características da carapaça, com especial atenção à formação de piramidismo; e determinação da composição corporal por absorciometria de raio-X de dupla energia (DXA) quando os animais atingiram 250 g de peso vivo. Valores de

P<0,05 foram considerados significativos. As análises foram conduzidas pelo R Studio Software (versão 3.2.3, AT). Os animais alimentados com a dieta alto amido apresentaram maiores consumos massa-específicos de matéria seca (12,75±3,11mg) em comparação aos animais da dieta alta fibra (10,20±3,37mg) (P<0,05). Apresentaram, ainda, menores tempos de trânsito e retenção gastrointestinal dos alimentos, de 2,95±1,01 e 8,09±2,05 dias, respectivamente (P<0,05), em comparação aos animais alimentados com dieta alta fibra (4,23±1,40 e 10,05±2,41 dias, respectivamente). Maiores eficiências digestivas dos nutrientes avaliados foram observadas em animais alimentados com a dieta com alto amido, com coeficiente de digestibilidade aparente da energia de 75,73±2,68% em comparação a 68,10±2,35% para a dieta alta fibra (P<0,05). A digestibilidade aparente da proteína bruta, no entanto, foi maior na dieta com alta fibra (P<0,05). Em relação à produção de calor em repouso e pós prandial na temperatura de preferência, estas não foram afetadas pela dieta (P>0,05). Foram observados incrementos calóricos de 43,85±14,92 e 41,33±14,66 kJ/kg/dia para as dietas alto amido e alta fibra, respectivamente (P>0,05). Os animais alimentados com a dieta de alto amido apresentaram aos 13 meses maiores larguras do plastrão e da carapaça, resultando em maiores taxas de crescimento da largura da carapaça do que os filhotes alimentados com a ração alta fibra (P<0,05). Sob as duas dietas animais desenvolveram carapaças com aparência piramidal, mas os alimentados com alto amido desenvolveram alterações de piramidismo com maior intensidade (P<0,05). Adicionalmente, os filhotes alimentados com alto amido apresentaram menor conteúdo mineral (1,88±0,15% versus 2,15±0,19%) e menor densidade óssea (0,13±0,01g/mm^2 versus 0,15±0,02g/mm^2) do que os alimentados com alta fibra (P<0,05), reforçando a piora na formação da carapaça.

No terceiro estudo, filhotes alimentados com a dieta alta em fibra foram mantidos à duas temperaturas (18°C e 28°C), para avaliar o efeito da temperatura no consumo de energia bruta e digestível, taxa metabólica em repouso e pós-prandial aos 6 e 12 meses, e a temperatura corporal superficial. Maior consumo massa-específico de energia bruta e ganho de peso foram obtidos na primavera e verão. A maior e menor taxa metabólica em repouso à 28°C foram obtidas na primavera e inverno, respectivamente. À 28°C, os animais apresentaram maior consumo diário de energia bruta e digestível, consumo de oxigênio, produção de CO_2, e taxa metabólica em repouso e pós-prandial, sendo a produção de calor em repouso de 30,56±4,07kJ/kg/dia à 28°C e de apenas 7,71±1,26kJ/kg/dia à 18°C (P<0,05). Coeficiente do incremento calórico específico foi também afetado pela temperatura, sendo a digestão menos dispendiosa energeticamente à 28°C do que a 18°C (P<0,05). Coeficientes respiratórios atípicos foram observados à 18°C (0,30-0,50). Adicionalmente uma forte influência da massa corporal foi descrita na taxa metabólica em repouso com exponente alométrico de 0,62 e 0,92 à 28°C e 18°C, respectivamente. E observou-se a prioridade de termorregular a temperatura superficial da cabeça sob temperatura baixa, e os animais apresentaram temperaturas corporais superficiais maiores pós alimentação.

Como conclusão, o presente trabalho indica a importância de se considerar o efeito da temperatura e da composição da dieta no metabolismo energético e crescimento de jabuti piranga. Alimentos com maior energia digestível, como elevado

amido pode induzir crescimento mais acelerado da carapaça com menor mineralização. Adicionalmente a importância de considerar a diferencia dos requerimentos de energia em diferentes regimes térmicos.
Palavras-chave: incubação, produção de calor, partição de energia, condições térmicas, nutrição

EFFECT OF TEMPERATURE AND DIET (STARCH AND FIBER) ON EMBRYONIC DEVELOPMENT, ENERGY METABOLISM AND GROWTH OF RED-FOOTED TORTOISE (*Chelonoidis carbonaria*)

ABSTRACT

There is very little information about egg incubation conditions, nutrient requirements, and captive management of the red-footed tortoise (*Chelonoidis carbonaria*) for conservation purposes. In the first study of this thesis, it was considered that reptile embryos respond to temperature changes with metabolic and physiological adjustments that influence hatchling success, phenotype, behaviour, and growth rate. Climate change and global warming can affect the reptile population by altering the frequencies of hatchling survival and phenotypes. Therefore, previous studies proposed artificial incubation as a potential strategy for mitigating these effects. Red-footed tortoise eggs were collected and incubated at constant temperatures of 27.5°C and 29.5°C to investigate the physiological effects of temperature on embryo development, hatchling morphology and early growth rate. The direct effects of temperature on the incubation period, lost egg mass, hatching index, hatchling size, and mass were evaluated at hatching and at three months of age. Hatchlings from 29.5°C presented shorter incubation times (141 says) than those from 27.5°C (201 days; $P<0.05$). Egg mass loss, hatchling mass, and size at hatching were not different between the incubation temperatures ($P>0.05$). However, the hatching index (survival rate) was lower (64.5% versus 100%) in eggs incubated at 29.5°C, but the hatchling mass and straight plastron width were higher at 3 months of age than those for eggs incubated at 27.5°C ($P<0.05$). These results clearly indicate that incubation temperature has an important influence on hatchling success and hatchling size and mass in the first months by influencing the early growth rate.

The second objective of the thesis considered that changes in the carapace are an important problem, observed in many zoos around the world. The objective was to determine the effect of two diets, one with a high fiber content and the other with a high starch content, on energy metabolism, nutrient digestibility, and growth of the red-footed tortoise. Following a completely randomized design, with 20 hatchlings, randomly divided into two experimental diets (10 hatchlings per feed). The study lasted 18 months, during which the animals received only their respective experimental diet, and water *ad libitum*. The evaluations performed on the animals included: determination of the apparent digestibility coefficients of the nutrients and gastrointestinal transit time at 5 and 11 months; determination of the metabolic rate at rest and postprandial, with calculation of the heat increment in respirometry chambers by indirect calorimetry at 6 and 12 months; evaluation of the growth and characteristics of the carapace, with special attention to the formation of pyramiding; and determination of body composition by dual energy X-ray absorptiometry (DXA) when the animals reached 250 g of body mass. Values of $P<0.05$ were considered significant. The analyzes were conducted by R Studio Software (version 3.2.3, AT). Animals fed with the high starch diet showed higher mass-specific dry matter intake (12.75±3.11mg) compared to animals fed with the high fiber diet (10.20±3.37mg) ($P<0.05$). They also presented shorter transit times and gastrointestinal retention of

food, of 2.95±1.01 and 8.09±2.05 days, respectively (P<0.05), compared to animals fed a high fiber diet. (4.23±1.40 and 10.05±2.41 days, respectively). Higher digestive efficiencies of the evaluated nutrients were observed in animals fed with the high starch diet, with an apparent energy digestibility coefficient of 75.73±2.68% compared to 68.10±2.35% for the high fiber diet. (P<0.05). The apparent digestibility of crude protein, however, was higher in the high fiber diet (P<0.05). Regarding the production of heat production at rest and post-prandial at the preferred temperature, these were not affected by the diet (P>0.05). Heat increments of 43.85 ± 14.92 and 41.33 ± 14.66 kJ/kg/day were observed for the high starch and high fiber diet, respectively (P>0.05). The animals fed with the high starch diet presented, at 13 months, wider plastrons and carapaces, resulting in higher growth rates of the carapace width than the hatchlings fed with the high fiber diet (P<0.05). Under the two animal diets, they developed pyramiding growth, but those fed with the high starch diet developed this doming with greater intensity (P<0.05). Additionally, hatchlings fed with the high starch diet had lower mineral content (1.88±0.15% versus 2.15±0.19%) and lower bone density (0.13±0.01g/mm^2 versus 0.15±0.02g/mm^2) than those fed with the high fiber diet (P<0.05).

In the third study, hatchlings fed with the high fiber diet were kept at two temperatures (18 ° C and 28 ° C), to assess the effect of temperature on the consumption of gross and digestible energy, resting and post-prandial metabolic rate at 6 and 12 months old, and surface body temperature. Higher mass-specific consumption of gross energy and body mass gain were obtained in the spring and summer. The highest and lowest resting metabolic rate at 28°C were obtained in spring and winter, respectively. At 28°C, the animals showed higher daily consumption of gross and digestible energy, oxygen consumption, CO_2 production, and rest and post-prandial metabolic rate, with a heat production at rest of 30.56±4.07kJ/kg/day at 28 °C and only 7.71±1.26 kJ/kg/day at 18°C (P<0.05). Specific heat increment coefficient was also affected by temperature, with digestion being less energy-intensive at 28°C than at 18°C (P<0.05). Atypical respiratory coefficients were observed at 18°C (0.30-0.50). In addition, a strong influence of body mass has been described on the resting metabolic rate with an allometric exponent of 0.62 and 0.92 at 28°C and 18°C, respectively. In addition, it was observed the priority of thermoregulating the surface temperature of the head under low temperature, and animals presented higher surface body temperatures after feeding.

In conclusion, the present work indicates the importance of considering the effect of temperature and the composition of the diet on energy metabolism and growth of red-footed tortoise. Feeds with higher digestible energy, such as high starch, can induce accelerated carapace growth with less mineralization. In addition, the importance of considering the difference in energy requirements in different thermal regimes.

Keywords: incubation, heat production, energy partition, thermal conditions, nutrition

LIST OF TABLES

CHAPTER II – THE INFLUENCE OF INCUBATION TEMPERATURE ON EMBRYO DEVELOPMENT, HATCHLING MORPHOLOGY AND EARLY GROWTH RATE IN RED-FOOTED TORTOISE (*Chelonoidis carbonaria*).

CHAPTER III - EFFECT OF HIGH DIETARY STARCH AND FIBER LEVEL ON ENERGY METABOLISM AND GROWTH OF HATCHLINGS OF RED-FOOTED TORTOISE (*Chelonoidis carbonaria*).

CHAPTER IV - TEMPERATURE EFFECT ON ENERGY METABOLISM IN HATCHLINGS OF RED-FOOTED TORTOISE (*Chelonoidis carbonaria*) IN CAPTIVITY.

LIST OF FIGURES

CHAPTER I - REVIEW OF LITERATURE

CHAPTER II – THE INFLUENCE OF INCUBATION TEMPERATURE ON EMBRYO DEVELOPMENT, HATCHLING MORPHOLOGY AND EARLY GROWTH RATE IN RED-FOOTED TORTOISE (*Chelonoidis carbonaria*)

Embryonal death cases at early development stage (1st, 3rd and 4th case) and at late development stage (2nd and 5th case). Embryo death characteristics are described: BV - broken vessels; AB - air cell; EB - embryo body; EA – forelegs; EH - embryo head; Vi - yolk sac.

Figure 4. Linear regression between *C. carbonaria* egg mass and size whit the hatchling mass and biometric parameters after incubation on two different temperatures …… 58

SCL: straight carapace length, HM_0: mass at hatching, SCW: straight carapace width, EHD: egg horizontal diameter. Circle represents eggs incubated at 29.5°C and diamond filled represents eggs incubated at 27.5°C. Showed equations represented the tendencies at each incubation temperature, obtained by simple linear regression.

Figure 5. Linear regression between *C. carbonaria* hatchling mass and biometric measurements after incubation at two different temperatures …….. 59

SCL: straight carapace length, SCW: straight carapace width, SPL: straight plastron length. Circle represents eggs incubated at 29.5°C and diamond filled represents eggs incubated at 27.5°C. Showed equations represented the tendencies at each incubation temperature, obtained by simple linear regression.

Figure 6. General and Accumulated Daily Growth Rate and Gompertz Growth Curve of *C. carbonaria* hatchlings from eggs incubated on two different temperatures……. 60

A-B. Gain rate of body mass ($g*day^{-1}$) and growth rate of straight plastron width (PW; $mm*day^{-1}$) at each week of age of hatchlings from two evaluated incubation temperatures. Showed equations represented the tendencies at each incubation temperature, obtained by quadratic regression.**C-D.** Growth rate of accumulated size index (SCL x SCW; mm^2*day^{-1}) along the first twelve weeks of age, and growth rate of size index (mm^2*day^{-1}) at each week at both evaluated temperatures. Showed equations represented the tendencies at each incubation temeprature, obtained by simple linear and quadratic regressions, respectively. **E-F.** Gompertz growth curves of body mass and plastron width of hatchlings during the first twelve weeks vs observed data at each evaluated temperature. Circle represents eggs incubated at 29.5°C and diamond filled represents eggs incubated at 27.5°C.

CHAPTER III - EFFECT OF HIGH DIETARY STARCH AND FIBER LEVEL ON ENERGY METABOLISM AND GROWTH OF HATCHLINGS OF RED-FOOTED TORTOISE (*Chelonoidis carbonaria*).

Specific-mass Intake of Gross Energy Intake J*gMBM^{-1}*day^{-1}: Mean Intake of gross energy per gram of metabolic body mass. *Metabolic mass= body mass^{0,86}* (Bennett and Dawson, 1979). **Body mass gain g*day^{-1}:** mean of body mass gain per day. Different letters in the same line color indicate significant difference (P<0.05) between months of age (age effect).

A: Mean Specific Dynamic Action of tortoises fed the high starch and the high fiber diet. Daily mean values of energy production per kilogram of body mass. Day 0 corresponds to fast state; on days 1, 2, and 3, tortoises were fed, and from day 4 until the end, animals were private of food again. Mean values of animals at 6 and 12 months old. **B:** Mean Specific Dynamic Action of tortoises at 6 and 12 months old. Daily mean values of energy production per kilogram of body mass. Day 0 corresponds to fast state; on days 1, 2, and 3, tortoises were fed, and from day 4 until the end, animals were private of food again. Mean values of animals fed the high starch and the high fiber diets. The shaded areas correspond to the range of resting metabolic rate (Mean±SD). Grey arrows indicate the first and last day of feeding. * Significant difference between ages (P<0.05).

A: Daily intake of gross and digestible energy, predicted metabolic energy (MEIp) less heat increment of the diet, and daily resting metabolic rate in hatchlings of 6 and 12 months old; related to body mass. Digestible energy was determined with the apparent digestibility coefficients obtained at 5 and 11 months old. Rest metabolic rate at 28°C. MEIp is the predicted intake of metabolic energy using a coefficient of 0.82 (Lickel, 2010). **B:** Linear regression Log10 of daily intake of gross and digestible energy and resting metabolic rate at 28°C with animals' body mass at 6 and 12 months old. The apparent digestibility coefficients obtained at 5 and 11 months old were also used in this graphic.

The red dotted line area indicates the wedge-shaped area of pyramiding growth. **A1:** Wedge-shaped area of a high degree of pyramiding in a tortoise fed with the high starch diet, **A2:** Wedge-shaped area of low degree of pyramiding in a tortoise fed with the high starch diet, **B1:** Wedge-shaped area of a high degree of pyramiding in a tortoise fed with the high fiber diet, **B2:** Wedge-shaped area of low degree of pyramiding in a tortoise fed with the high fiber diet.

CHAPER IV - TEMPERATURE EFFECT ON ENERGY METABOLISM IN HATCHLINGS OF RED-FOOTED TORTOISE (*Chelonoidis carbonaria*) IN CAPTIVITY.

Indirect calorimetry system of seven respirometry chambers: 10 minutes of measurement per chamber with three times baseline measurement about 5 minutes in the beginning, after the 2nd and 4th chamber. A thermometer was installed into one chamber that was placed in the center of the system. Blue arrows and lines (▬) refer to the transport of input samples of air to ventilate the chambers and baseline analysis. Red arrows and lines (▬) indicate the route of the output samples of chamber air to be analyzed until eliminated (exit). Black lines (▬) describe the data acquisition to Expedata Data Analysis Software by UI-3.

Evaluated components of the specific dynamic action: **RMR**-Resting metabolic rate after 10 days of fasting (--), **FMR_{MAX}** -maximum metabolic rate after feeding (--), **time to peak** (—), **duration of the peak** represented with a green shaded area, heat increment as a difference between FMR_{MAX} and RMR, and the **time to return to resting values** described as brown shaded area. Grey arrows indicate the first and last day of feeding.

A.Monthly Environmental Temperature: Monthly mean, maximum and minimum temperature from March 2019 to October 2020. **B. Specific-mass Intake of Gross Energy Intake $J*gMBM^{-1}*day^{-1}$ and Body mass gain $g*day^{-1}$:** Mean ±SD Intake of gross energy per gram of metabolic body mass. *Metabolic mass= body mass^{0,86}*

INTRODUCTION AND JUSTIFICATION

The Red-footed tortoise (*Chelonoidis carbonaria*) has been subjected to pressure due its use by indigenous communities for its meat, eggs, and illegal sale of specimens as pets (Valeris and Hernández, 2017). These treats have caused a decrease of populations becoming vulnerable to local extinction, being protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) in Appendix II (CITES, 1995). Most of the previously developed research on this species focused on reproduction, natural diet, seed dispersal function, behavior, and abundance.

The environmental influence on organism embryogenesis may be critical to understanding life-history strategies and distributions (Muth, 1980). Incubation temperature strongly influences the embryonic development of reptiles, including incubation duration (Gillooly et al., 2002), hatchling morphology, body shape, color, growth rate, locomotor performance, behavior, hatchling index, and sexual differentiation in many species (Brooks et al., 1991; Deeming and Ferguson, 1991; Gillooly et al., 2001; Du and Ji, 2003; Booth et al., 2004; Booth, 2006). In this context, a growing concern exists that global warming and climate changes may adversely affect reptile populations by altering nest temperatures. This may induce consequences for sex determination, frequency of phenotypes (Booth, 2006), and hatchling performance through their effects on locomotor ability and post-hatch growth (Booth et al., 2004).

An investigation of the effects of incubation temperature on embryos and hatchlings will provide information not only to understand the dynamics of embryonic development under different thermal conditions but also to determine which conditions are needed for husbandry and conservation red-footed tortoises. This knowledge is lacking for most South American reptile species.

Animals have energy costs that affect their physiological, anatomical, and behavioral strategies, and factors such as diet, environment, season, and hydric balance may influence the daily and annual bioenergetic needs (Paladino, 1985). However, few studies have been done on red-footed tortoise' physiology, metabolism, and nutrition.

In the wild, these animals have a varied diet with ripe fruit, flowers (at least 33 species), and large amounts of leaves and other fibrous items (Castaño-Mora & Lugo-Rugeles, 1979; Pritchard & Trebbau, 1984; Moskovits, 1985). Due this their natural diets have high content of fiber and low amounts of starch and sugars. However, diverse diets with high levels of very digestible carbohydrates are provided in captivity, mainly starch (dog extruded feed) or sugars (fruits) (Bartlett & Bartlett, 1996; Pizzuto and Mariana, 2001; Madera-Vergara et al., 2010).

Herbivorous tortoises are post-gastric fermenters that presented long duration of the digestive process (Stevens and Hume, 1998) which makes that different gastrointestinal tract regions contributes to the digestion energy cost, also called heat increment (Hailey, 1998). The digestion cost is essential in the energy balance perspective since this energy cannot be used for other activities such as maintenance, growth, and reproduction. The association of lower heat increment and greater metabolic energy of diets with a high starch content will result in energy excess being stored in the body as fatty tissue. Excessive digestible energy intake could affect the energy metabolism with acute adverse effects on animal health such as obesity, hepatic diseases (Carciofi and De-Oliveira, 2007), accelerated growth rates, and metabolic diseases in hatchlings (Pingleton, 2011), which have not been studied yet.

Ectothermic species can regulate its body temperature behaviorally. In this sense, the environmental temperature is an important factor to be taken into account to understand the energy partitioning and the digestive response at different thermal conditions. Increasing specific-species nutrition knowledge is a consequence of a better comprehension of diverse factors' effects on energy metabolism (Gienger et al., 2012), which allows the estimation of their energy requirements and avoids the development of metabolic disorders in captive conditions, for the conservation of this species.

CHAPTER I

REVIEW OF LITERATURE

1. Red-footed Tortoise: Distribution, conservation state, and ecological role.

The South American native species Red-footed Tortoise (*Chelonoidis carbonaria*) is found throughout the Caribbean; from Panama to Paraguay, including parts of Colombia, Bolivia, Peru, Ecuador, and Brazil (Davis, 1979; Vinke, 2008) in habitats such as savannas, forest-edges, forest clearings or along waterways (Davis, 1979).

Early studies failed to distinguish the red-footed from the yellow-footed tortoise (*Chelonoidis denticulata*), which confused the status of the northern South American tortoises (Ernst and Leuteritz, 1999) until Williams (1960) finally presented detailed evidence that they are different species. This species has only two predators: human beings and jaguars (*Panthera onca*) (Padarath, 2012). In fact, in other countries, the red-footed tortoises have been heavily hunted by rural and indigenous communities to consume their meat, eggs, and fat as part of their tradition (Pritchard and Trebbau 1984; Cuéllar, 2000; Soria and Noss, 2000; Rodríguez and Rojas-Suárez, 2008). This specie has been extirpated in some areas due to hunting and the conversion of their habitat into pastureland (Balée, 1985; Gorzula, 1989; Wang et al., 2011).

Another critical threat is the illegal pet market (Rodríguez and Rojas-Suárez, 2008). Tortoises are on the list of the most exported species to supply the exotic pet demand in the USA and Europe (Pritchard and Trebbau, 1984). In consequence of this significant impact on the tortoise populations, the *C.carbonaria* is protected by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) on Appendix II (CITES, 1995), and by the national legislation of different countries being classified as a vulnerable species (Noss, 2013; Valeris and Hernández, 2017). However, it has not been cataloged by IUCN yet.

In an analysis of fecal samples of red-footed tortoises, a total of 646 seeds of 11 different plant species were found; the most abundant species were *Ficus* sp. *Aechmea* sp. and *Genipa americana,* with high seed viability from 91 to 100% (Strong and Fragoso, 2006; Jerozolimski et al., 2009; Wang et al., 2011). Also, the great diversity of consumed viable seeds combined with a long retention time of seeds in the digestive tract and large traveled distances indicate this species as an effective dispersal agent, which contributes to the maintenance of species diversity and forest structure in Neotropical countries (Strong and Fragoso, 2006; Jerozolimski et al., 2009; Wang et al., 2011).

2. Environmental Temperature

2.1. *Preferred environmental temperature.*

Red-footed tortoises select habitats with temperatures close to 30°C, and they may estivate or brumate during too hot or cold season or when they cannot find available food (Moskovits, 1985; Paull, 1997). In a study of *C.carbonaria* in Bolivian Chaco, animals reduced their activity in the dry season and were inactive when air temperatures were below 20°C or above 37°C. Body temperature monthly varied between –2°C and +4°C in comparison with the environmental temperature (Noss et al., 2013). On Union Island, red-footed tortoises exhibited cloacal temperatures ranging from 26-34.6°C (28.1±1.3°C) at air temperatures from 25.3 to 30.8°C (28.9±1.8°C), with differences from -7.0°C to +1.5°C (-0.78 ± 1.44°C) between ambient and cloacal temperatures (Hedman et al., 2010).

The obtained data from wild conditions are considered for its management in captivity, and a mean temperature of 28°C has been usually recommended (Davis, 1979). Regarding newly hatched, higher, and stable temperatures are needed about 30-34°C (Valeris and Hernández, 2017). Also, San Diego Turtle and Tortoise Society (2011) indicate a range of preferred temperature between 80-90°F (26.7-32.2°C) and high air moisture levels for its ex-situ management.

Behavior thermoregulation in reptiles is a mechanism that allows them to meet their physiological thermal requirements in diverse habitat microenvironments (Avery, 1982; Vidal et al., 2010). Reptiles have various physiological mechanisms ranging from thermal hysteresis, heart rate, and peripheral circulatory adjustments (Seebacher and Franklin, 2005; Hutchison et al., 1966; Tattersall et

al., 2006). Another mechanism is respiratory cooling and the total water loss rates through evaporation (Tattersall et al., 2006; Withers, 1992; Scarpellini et al., 2015). For terrestrial species such as *C.carbonaria*, the daily regulation of body temperature is facilitated by the thermal environment's heterogeneity and relatively low heat transfer rates in the air (Seebacher and Fraklin, 2005).

2.2. *Temperature effect on embryo development*

Offspring phenotype is determined by genotype, maternal investment, and the environment (Ligon et al., 2009). There is growing evidence that environmental conditions substantially affect reptile embryogenesis (Braña and Ji, 2000). Influence on developmental rates, duration of incubation; hatching success; sex (for species with temperature-dependent sex determination); size; morphology; and physiology of hatchlings, has been reported (Bull, 1980; Packard and Packard, 1988; Booth and Thompson, 1991; Deeming and Ferguson, 1991). Furthermore, the long-term effect of incubation temperature on the physiology, behavior, locomotor performance, and post-hatching growth of hatchlings has also been described (Joanen et al., 1987; Braña and Ji, 2000; Morales-Mérida et al., 2017; Ryan et al. 1990; McKnight and Gutzke 1993; Janzen 1993; 1995).

Du and Ji (2003) reported a range of viable incubation temperatures, yielding living hatchlings for many species of oviparous reptiles. Thus, some aspects of how differences in embryos' thermal environment lead to morphological and performance differences remain poorly understood (Briggs, 2007). In this context, understanding reproductive biology and embryonic development are important to guide conservation programs for endangered species (Hernández-Montoya et al., 2017).

Usually, moderate temperatures are related to larger and locomotor well-performed reptilian hatchlings, and high temperatures with smaller, less developed, and poorly performed hatchlings with a higher proportion of deformed individuals (Gutzke and Packard, 1987; Packard and Packard, 1988; Van Damme et al., 1992; Phillips and Packard, 1994; Braña and Ji, 2000; Ji and Du, 2001a; Ji et al., 2002; Briggs, 2007). However, different responses have also been described when eggs are incubated at low temperatures. As it may induce

higher metabolic expenditure by embryos because of the long incubation length and, consequently, hatchlings were smaller in size or had lower energy reserves (Booth et al., 2000; Ji and Du, 2001b; Ji and Zhang, 2001; Ji et al., 2002; Du and Ji, 2003; Briggs, 2007). Also, eggs incubated at low temperatures usually do not result in deformed hatchlings but may increase embryonic mortality largely (Ji and Du, 2001a, b; Ji et al., 2002).

During the initial or the final stages of development, under adverse biotic or abiotic factors reptile embryos can arrest the growth by embryonic diapause or embryonic aestivation (Rafferty and Reina, 2012). The decrease of incubation duration as temperature increased and hatching success differed considerably among temperatures in some reptile' species, where eggs incubated at lowest and highest temperatures exhibited much lower hatching success than did those incubated at intermediate temperatures (Joanen et al., 1987; Du and Ji, 2003; Ligon et al., 2009).

In Venezuela, the laying season of wild red-footed tortoises has been reported between July and March; and it can change depending on the locality (Valeris and Hernández, 2017). In other countries such as United States, in Washington, the laying season of red-footed tortoise has been reported from June to December, with clutch sizes of four or five eggs in captivity. These eggs are laid into a hole depth, about 5 cm, where the nest area's soil temperature varies from 25-30°C and is usually about 1-2°C cooler than the air temperature (Davis, 1979).

Some studies about artificial incubation of *C.carbonaria* eggs reported incubation temperatures ranging from 26-27.5°C with an improved hatchling success with an incubation period of 185 days (Davis, 1979), and 29-31°C with incubation periods of 124-228 days (Hernández, 1997; Vinke, 2008). In this context, a unique study of incubation temperature effects on sex determination in red-footed tortoise by Hernández-Montoya et al. (2017) reported that from eggs incubated at three temperatures (29°C; 31°C and 33°C), the hatching success decreased with increasing temperature and all embryos died at the highest temperature. Also, embryo malformations were observed at these temperatures, indicating that further studies should focus on the developmental biology at temperatures below 29°C. In addition, it was suggested that this species exhibits a temperature-dependent sex determination mechanism with females produced

at or above 29°C. However, the production of male at temperatures below 29°C was not confirmed.

3. Reptile Nutrition in captivity

Moskovits and Bjorndal (1990) suggested that the foraging behavior, ecology, and population sizes of the red-footed and yellow-footed tortoises might be considerably affected by essential nutrients' availability. In general, herbivorous terrestrial chelonians eat a wide variety of plant species high in calcium and fiber and deficient in protein and fat (McArthur, 2004). In the wild, the food items that red-footed tortoises regularly eat have low energy amounts and digestibility, and high contents of the vegetable cell wall (especially cutin), are inferior in quality with high contents of the cell wall (especially cutin), low fermentability, low nitrogen, phosphorus, and total mineral concentrations; and relatively abundant calcium concentration with high Ca:P ratios. However, in the wild the dietary preferences include items with low Ca concentrations and Ca:P ratios, low cell wall content, higher fermentability, and higher nitrogen and phosphorus, being these items more palatable for this species (Fig. 1).

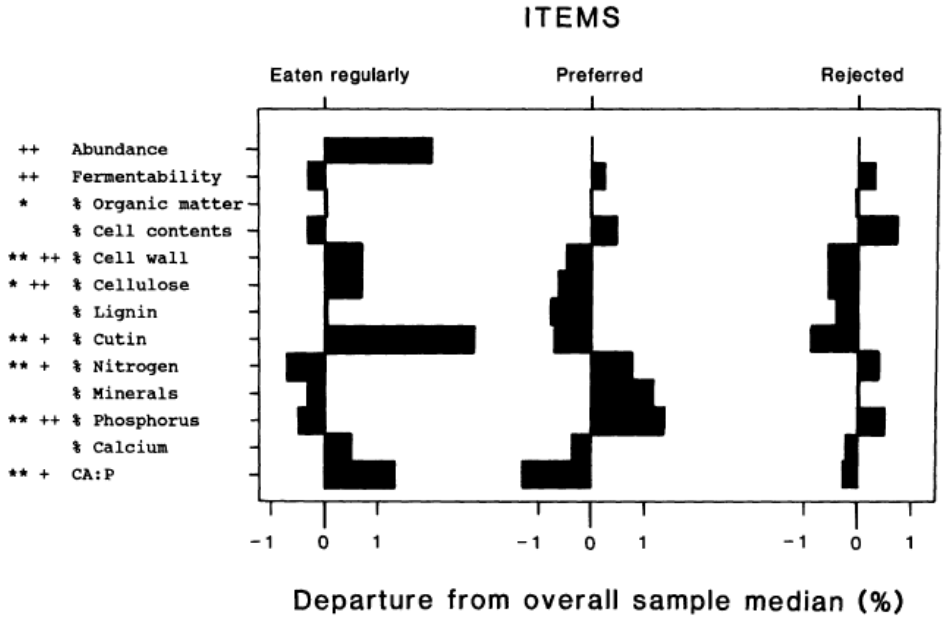

Figure 1. Nutrient composition profile of 12 rejected, 18 regularly eaten, and three preferred items by red-footed tortoises (*Chelonoidis carbonaria*) and yellow-footed tortoises (*Chelonoidis denticulata*). Levels of significance in comparison of medians: Wilcoxon (rank sums) * = P<0.05, ** P<0.01; median scores (number of points above the median) + = P<0.05, ++ = P<0.01. Source: Moskovits and Bjorndal (1990).

The primary source of energy in these diets are carbohydrates (Englyst et al., 1996). Understanding the digestive and absorption efficiency, and the rate and fermentation ability in the gastrointestinal tract is essential to study their nutritional effects. Short-chain fatty acids (SCFA) are products from carbohydrate fermentation in the hindgut and are largely absorbed before defecation. Dietary starches and endogenous carbohydrates are the principal substrates in omnivores, and the amount of starch that escaped digestion in the small intestine depends on the dietary source. Fibers include cellulose, hemicellulose, gums, mucilages, pectins, lignins, and various polysaccharides (Donoghue, 1996). In herbivorous species, dietary cellulose, hemicelluloses, and pectin are major substrates in the hindgut for microbial fermentation (Steven and Hume, 1998).

Captive tortoises are usually fed with various foodstuffs and feeding regimes from correct weed-based diets to the fruit, salad, and vegetable diets; specifically formulated commercial pelleted diets; to inappropriate diets including bread, milk, and cat or dog food (Pellett et al., 2015).

For red-footed tortoises, different diets have been described in captive conditions such as a mixture of chopped fruits and vegetables twice a week, and a feline diet added once a week (Davis, 1979); a mix of high calcium greens, fruits, vegetables, flowers, and a small amount of animal protein (San Diego Turtle and Tortoise Society, 2011); and a mixture of poultry, rabbit, or dog extruded food with vegetables (Hernández and Boede, 2000; Valeris and Hernández, 2017). The addition of extruded foods for domestic animals in omnivorous tortoise diets has the purpose of increasing the nitrogen content (Hernández and Boede, 2000) since these animals cover their nitrogen requirements with an opportunistic intake of carrion (Mourthe and Castro, 2017). These changes have had good results in adult females, where egg production was significantly increased (Hernández and Boede, 2000). However, these extruded foods have been made with ingredients containing high levels of starch, and fat, which can negatively affect the health of these animals. In this context, extruded foods for herbivorous animals (such as rabbits, cows, and horses) with low content of fat and starch and optimal fiber levels are more recommended (Highfield, 1990; Valeris and Hernández, 2017).

In concern to herbivorous reptiles, fiber is one of the most important dietary factors because it has a critical role in maintaining gut motility and the production

of volatile fatty acids. These species fed with low-fiber diets (less than 12%) have loose feces and are prompted to bloat or display lactate-induced diarrhea from fast fermentation of carbohydrates (Donoghue, 1996). However, there are no specific recommendations for omnivorous tortoises, and it is suggested a mixed protocol that follows carnivorous and herbivorous species' recommendations. Since these tortoises are opportunistic feeders of animal matter and there is no evidence of whether this can happens regularly, a proportion of 75:25 of plant and animal matter was suggested in captivity with an adequate fiber level of 10% (Donoghue, 1996), but this recommendation was not scientific evaluated yet.

Offered diet must provide the necessary digestible and absorbed energy to maintenance, activity, growth, and reproduction (Secor, 2009). Good nutrition is a fundamental basis for the welfare of captive-bred animal species. However, to provide an appropriate diet in terms of adequate nutrients amounts to meet the animal requirements is a big challenge in captivity conditions. These diets need to be as close as possible to what the same species eats in the wild with the adjustments of these animals' energy cost presented in captivity conditions (Pellett et al., 2015).

Metabolic disorders and nutritional deficiencies such as anorexia, metabolic bone disease, and hypovitaminosis A, have been constantly reported in reptiles (Paranzini et al., 2008). Reports of feeding and nutritional evaluations in tortoises are scarce. Long-term feeding with commercial pet foods that contain high protein levels (up to 45%) and high fat level have been associated with problems such as shell deformities of the skeletal system, pyramiding of the shell, and soft tissue calcification in growing tortoises (Donoghue, 1996; McArthur, 2004). High levels of energy and protein, low fiber and calcium diets have been related to abnormal growth in hatchlings leading to shell pyramiding, metabolic bone disease, vertebral collapse, and many other physiological problems (Chitty and Raftery, 2013). Thus, for forest-dwelling species such as Red-footed tortoises, a moderate offer of fruit is recommended (Pellett et al., 2015). Also, fiber provides energy to the animals through hindgut fermentation and aids gut motility. However, excessive fiber content limits calorie intake and may inhibits trace mineral absorption (Donoghue, 1996).

3.1. *Food habits and digestive physiology.*

Moskovits and Bjorndal (1990), in a study of the natural diets of red-footed and yellow-footed tortoises, reported a significant proportion of fruits throughout the year. Both species are categorized as primarily frugivorous, eating fruits of *Anacardiaceae, Annonaceae, Bromeliaceae, Lecythidaceae, Melastomataceae, Moraceae, Passifloraceae, Palmae, Rubiaceae*, and *Sapotaceae* families. Also, previous studies have described other dietary components such as live and dead foliage, stems, fungi, soil, sand, pebbles, insects, and animal matter throughout the year (Moskovits, 1985; Moskovits and Bjorndal 1990).

Previous studies have described this species as forest herbivorous tortoises (Bjorndal,1989; Donoghue, 1996). Various studies reported opportunistic necrophagy behavior by recovering samples of bones of an Agouti (*Dasyprocta leporina*) and a White-lipped Peccary (*Tayassu pecari*) from the scats of wild individuals (Moskovits, 1985) and by direct observations of scavenging (Mourthe and Castro, 2017). Consequently, the red-footed tortoise is now categorized as a diurnal, terrestrial, and opportunistic omnivore species (Moskovits, 1985) and in nutritional studies can be described as an opportunistic omnivore (Cheeke and Dierenfeld, 2010). Besides, its coprophagy behavior is a strategy to improve the absorption of nutrients (Valeris and Hernández, 2017) which enriches their intestinal flora (Rueda-Almonacid et al., 2007).

Enok et al. (2016) indicate that lizards, tortoises, and crocodilians, masticate their prey before ingestion. By breaking the food into smaller fragments, increasing the surface-to-volume area makes it more prone to enzymatic breakdown. Although knowledge of saliva lacks in reptiles, remarkably high concentrations of lysozymes in the python’s saliva were reported (Wang, unpublished). At the anatomical level, the size and compartments characteristics of the reptiles' gastrointestinal tract vary depending on dietary specialization (Karasov and Hume 2010; Karasov et al. 2011). The large intestine lies the caecum has a large diameter and, for herbivorous Chelonia, is the main site of fermentation as shown in Fig. 2 (Girling, 2013).

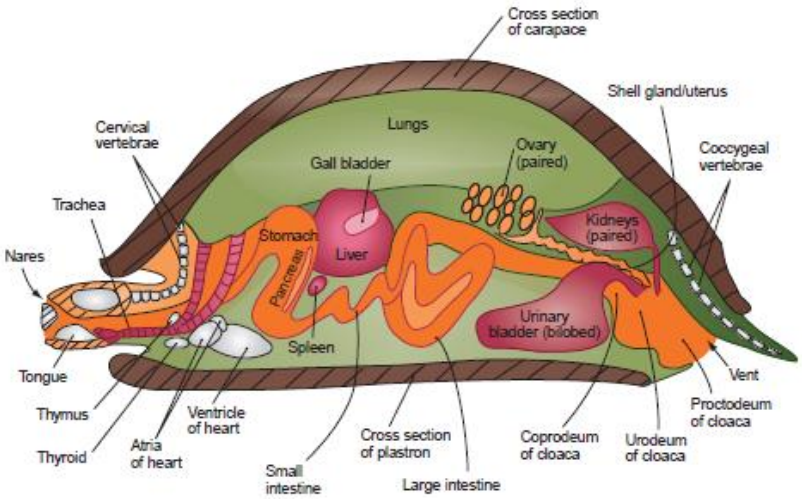

Figure 2. Section through the midline of a female tortoise. Source: Girling (2013).

In this context, herbivorous species tend to have a much longer digestive tract to increase gut retention time and to maximize nutrient absorption due to the significant amount of structural carbohydrates in their diet. Red-footed tortoises are described as an alloenzymatic digester and hindgut fermenter (Bjorndal, 1989; Stevens and Hume, 1998). An essential microbial population performs similar digestive functions in the rumen in ruminant species, but with lower efficiency in hindgut fermenters (Cheeke and Dierenfeld, 2010).

3.2. *Digestive Response of Herbivorous Reptiles.*

Generalist herbivores have flexible digestive responses that are influenced by diet, and physiological and morphological adaptations to a varied diet should be reflected in three interrelated animal responses: digestibility, food intake, and passage time (Bjorndal, 1989). This can be examined as the “digestive strategy” (Milton, 1981).

In animal nutrition, the terms “soluble fiber” and “insoluble fiber” represent specific parts of the plant cell wall and play a role in the gastrointestinal tract (Modica, 2016). Nonstructural carbohydrates make up the soluble fiber (resistant starch, pectins, gums, and β-glucans) and can be fermented, to a large extent, by the microbiota of the animal (Van Soest, 1994). On the other hand, the structural carbohydrates are more insoluble fiber (cellulose, hemicellulose, and

lignin), presenting reduced fermentation that require longer residence time on gastrointestinal tract (Van Soest, 1994).

These structural carbohydrates play nutritional and physical functions within the gastrointestinal tract (Van Soest, 1994). Their fermentation provides energy to the host in the form of short-chain fatty acids (Van Soest, 1994; Atlas and Bartha, 1998; Donoghue and McKeown, 1999; Stevens and Hume, 2004), vitamin B and vitamin K (Atlas and Bartha, 1998; Hooper et al., 2002). Also, in respect of physical function, the insoluble non-fermentable fiber adds bulk to the diet, resulting in a slower digesta passage rate and allowing more time for microbial fermentation to take place (NRC, 2007).

The relationship between feed intake and digestibility, with the physicochemical characteristics of the diet and the nutrient requirements, is influenced by the structure, morphological adaptations, and digestive tract morphology of some herbivorous reptiles (Bjorndal 1979; Troyer, 1984; Barboza, 1995). Fibrous components of herbivorous diets are resistant to degradation, and their digestion depends on disruption of the cell walls. This is limited by poor mastication in herbivorous reptiles (Throckmorton, 1973). Because the rate of digestion of structural carbohydrates is considerably slower than that of the cell contents, intake is limited by the need to retain these more slowly digesting residues.

Transit time in reptiles is dependent on the species (Vélez and Cobos, 1997), environmental factors (Zimmerman and Tracy, 1989; Torner, 2000), and diet quality and composition. In this sense, fiber digestion is related to the capacity and motility of the digestive tract to retain, mix and expose the ingesta to endogenous secretions and microbial symbionts (Barboza, 1995), where diets with high fiber content result in prolonged retention of digesta (Tracy et al., 2006; Madera-Vergara et al., 2010). This adaptation helps extract more available energy from the fibrous cell wall, as found in the foregut (Van Soest, 1982) and some hindgut fermenters (Foley, Hume, and Cork, 1989).

Tortoises are hindgut fermenters (Stevens and Hume, 1995), and hindgut fermentation may supply up to 30%-40% of the energy budget (McBee and McBee, 1982). The digestibility of cell-wall constituents may be as high as 54% (Troyer, 1984). Digestive efficiencies of fiber in herbivorous reptiles ranged from 37% to 86%, being similar to values in mammalian non-ruminants' herbivores

(30% to 65%) and ruminants (30% to 75%) (Van Soest, 1982), depending on diet quality.

The negative correlation between dietary cell wall content and dry matter digestibility had been reported in adults of red-footed and yellow-footed tortoises, juveniles of Galapagos tortoises (*Geochelone nigra*), and desert tortoises (Hatt et al., 2005; Bjorndal, 1989). Also, a negative correlation between dietary lignocellulose and organic matter digestibility was observed. These results evidence that the fiber content of the diet has a reducing effect on digestibility efficiencies, as observed all animal species.

In this context, the diet has been recognized as the critical factor for the discrepancy in digestive response, growth rate, and life expectation, between free-ranging and captive animals, in species such as Galapagos tortoises (*Geochelone nigra*) (Hatt et al., 2005). An increase in fiber levels has been proposed to reduce the accelerated growth rates that can reduce life expectations (Furrer et al., 2004). *Ad libitum* feeding, especially of a highly digestible feed, is not recommended for captive juvenile leopard tortoises (*Geochelone pardalis*), especially when food is daily offered. On this management animals freely eat amounts that can exceed 200% predicted herbivorous reptile field metabolic rate (Lickel, 2010). Insoluble non-fermentable fiber should be provided in the diet in sufficient quantities to fulfill the physical and nutritional function simultaneously. However, these amounts have yet objectively quantified for red-footed tortoises.

3.3. *Reptiles Energy Metabolism*

Studies focus on the population trend determination, preferred habitat use, and impact of human disturbance, are common topics in the research of threatened, endangered herpetofauna (Jodice et al., 2006). However, ecological energetics is an overlooked research area (McNab, 2002), and little information has been obtained about the energy metabolism of terrestrial chelonians (Auffenberg and Franz, 1982). Additionally, the major part of the studies about ventilation in chelonians was done in turtles (Jackson, 1973; Enstipp et al., 2011; Johnson et al., 2015; Cordeiro et al., 2016), or African and Asian tortoises' species (Burggren, 1977; Glass et al., 1978; Benchetrit and Dejours, 1980). This

indicates that a better characterization of tortoises' respiratory physiology and, in particular, about south American species is necessary (Trevizan, 2006).

One of the principal objectives of animals is to obtain sufficient food to meet their daily energy requirements. It is essential to understand which factors determine these needs and how they use that energy (Nagy, 2005). Additionally, how animals of different species allocate energy is still not very well understood (Hou et al., 2008; Kooijman, 2010). The lack of this knowledge has been a fundamental challenge in applied nutrition for managing animals in captivity. The determination of appropriate food (quantity and quality) to reach animal requirements is needed to prevent metabolic disorders and nutritional diseases (Higgins and Edward, 2009). Bennett and Nagy (1977) reported that the construction of time–energy budgets requires studies in the wild and the laboratory while simultaneously measuring energy expenditure through techniques such as indirect calorimetry (Speakman, 1997). Also, research about how temperature affects the metabolism helps to adjust and develop models to describe the energy partitioning (Lillywhite, 1987; Secor and Nagy, 1994; Beaupre, 1995, 1996).

The organisms can extract energy from their environment, process that energy, and allocate it among multiple competing functions such as maintenance and growth (Cox and Secor, 2007). In this sense, the daily energy expenditure and time-energy budgets help gain insight into an animal’s daily food requirements (Todd et al., 2009). Understanding population responses to environmental change requires detailed information on the influence of environmental factors on individuals' bioenergetic fluxes on producing energy precisely (Zaidan and Beaupre, 2003). Congdon et al. (1982) defined energy availability as total energy that comes from the energy intakes less the energy lost by respiration, feces, and nitrogenous waste.

Reptiles at rest use one-sixth to one-tenth of the energy that equal-sized resting endotherms used (Bennett and Dawson, 1976). In many species, essential functions such as reproduction, circadian rhythmicity, and thermoregulation are affected by diverse environmental factors (photoperiod, temperature, moisture, and others) (Krisch and Roles, 1984). Since the cost of maintenance is considered a conservative parameter in reptilian evolution, the study of environmental factors' influence on the resting metabolic rate has been

considered an important topic (Bennett and Dawson, 1976). One of these factors is the temperature, which directly influences body temperature affecting the metabolic rate, energy intake, and digestion rate, being increased on higher environmental temperatures (Blouin-Demers and Weatherhead, 2001; Tattersall et al., 2006; Cox and Secor, 2007).

Regarding the energy lost by respiration, the Specific Dynamic Action (SDA) is a critical and potentially significant component (Zaidan and Beaupre, 2003). Consumed food provides nutrients and calories, but processing a meal has costs, and it manifests itself as an increase in metabolic rate (heat production, O_2 consumption, or CO_2 production) that occurs after the food ingestion (Zaidan and Beaupre, 2003; Secor 2009; Sievert et al., 2013). The SDA is described as the cost of processing a meal, includes all the energy expended to ingest, digest, mechanical cost of transporting food through the gut, active transport and absorption, biochemical breakdown, use nutrients in anabolic reactions, muscle activity, secretion, and intermediary storage (Tandler and Beamish 1979; Blaxter, 1989; Andrade et al., 1997; Secor and Diamond, 1997; Secor, 2009).

The variability of energy utilization efficiency can reflect the differences in the meal (i.e., composition and size), the environment (i.e., temperature), and features of the organism (i.e., size, genetics, and metabolism) that exists both within and among species (Woods, 1982; Xu et al., 2006). About the meal influence, the increase of meal size causes an increase in the duration and magnitude of SDA (Andrade et al. 1997; Secor and Diamond 1997). Sievert et al. (2013) indicating that hard-bodied meals produce more significant increases in oxygen consumption than soft-bodied meals (Hailey, 1998; Secor and Boehm, 2006; Bessler et al., 2010). Also, larger meals yield higher SDA values than smaller meals of a given prey type (Andrade et al. 1997; Secor and Diamond, 1997; Toledo et al. 2003). Although the feed intake quantity is related to the body size, and larger animals eat larger meals or show higher feed intake (Jobling, 1981; Secor and Diamond, 1997).

On the other hand, it is relatively well known that SDA's magnitude is highly dependent on meal composition (Rubner, 1902; Lusk, 1915). Relatively low values of heat increment have been observed for fats and carbohydrates, and substantially higher values for protein, explained by the deamination of amino acids and nitrogenous excretion costs (Lusk, 1931; Hailey and Loveridge, 1997).

However, although this general concept, few studies in reptile species evaluated a specific nutrient effect on the specific dynamic action; most researchers were about meal size or comparing different diets, making it challenging to identify the impact of a unique nutrient on post-prandial metabolism.

McCue et al. (2005) reported that the food composition strongly affects the SDA of Burmese Python (*Python molurus*), where lipid meals did not result in measurable SDA and appeared to pass through the digestive tract unmodified. The protein produced a greater SDA than carbohydrate, and neither starch nor cellulose induced a measurable SDA. Besides, tortoises are hindgut fermenters (Stevens, 1988) and can digest foliage on over several days (Hamilton and Coe 1982; Bjorndal, 1987). As Hailey (1998) expresses, this long duration of digestion could make that different regions of the intestinal tract contribute to the SDA. However, in our knowledge, fiber or starch's effect has not been evaluated yet in herbivorous or omnivorous reptiles.

There are few studies about the effect of temperature on SDA in turtle and tortoise species. However, a pattern has been described in few species where the SDA shape was affected by temperature, as observed in slider turtle (*Trachemys scripta*), box turtle (*Terrapene ornata*) (Gatten, 1974), African tortoise (*Kinixys spekii*) (Hailey and Loveridge, 1997), and hatchlings snapping turtles (*Chelydra serpentina*) (Steyermark and Spotila, 2000). Increased oxygen consumption rates, shorter duration of the digestion process, faster oxygen consumption peak, and better digestive efficiency have been reported at a higher temperature (Sievert and Andreadis 1999; Toledo et al. 2003; Wang et al. 2003; Bessler et al. 2010). Nonetheless, positive, negative, and non-effects of temperature on assimilation efficiencies have been reported (Cox and Secor, 2007), being hypothesized that this may be a species-specific characteristic. Studies in different species, where animals were free to choose the environmental temperature during digestion, showed that many reptile species select a higher environmental temperature (Regal, 1966; Peterson et al., 1993; Dorcas and Peterson., 1997; Tattersall et al., 2006). Also, feeding provides the energy that increases the metabolic rate and leads to an elevated body temperature (Marcellini and Peters, 1982; Tattersall et al., 2004; Sievert et al., 2013).

The heat production during the digestion in terms of energy meal is described as the SDA coefficient. It correspond to an average of 17.6 ± 2.9% for crocodilians, 17.9 ± 1.3% for turtles, 17.9.1 ± 1.9% for lizards, 20.9 ± 0.7% for snakes, and 23%–35% for reptiles in general (Pierce and Wissing, 1974; Overgaard et al., 2002; Secor, 2009).

There are no studies about starch or fiber effect in tortoises in concern to diet effect on SDA coefficient. In comparison with another groups of species, such as fishes, Fu et al. (2007) indicated that catfish showed a higher SDA coefficient for protein than lipid (9.41% vs. 7.43%), and both were higher than the SDA coefficient for raw starch, which gave a coefficient of close to zero (0.84%). Also, mean values of Eurasian perch's oxygen consumption (*Perca fluviatilis*) decreased numerically when starch increased from 0% to 30% of the diet, and no significant change occurs when the starch level increases up to 30 % at the expense of lipid content (Keihani, 2013).

The influence of temperature on the SDA coefficient varies with different species (Zaidan and Beaupre, 2003). In *boas*, the digestion is less energetic costly at higher temperatures, which indicates an improvement of the energy return on the meal (Toledo et al., 2003; Tattersall et al., 2006). Other species presented constant SDA coefficients regardless of different body temperatures (Jobling and Davies, 1980; Secor and Faulkner, 2002; Wang et al., 2003).

About the effect of body mass, Chappell (1987) detailed that the relationship between body mass (BM) and metabolic rate (MR) adjusted in the general function $MR = a * BM^b$, had described two theoretical values of 0.67 and 0.75, from surface-volume considerations (Hemmingsen, 1960; Kleiber, 1961) and the principles of mechanical support and muscle biophysics, respectively (McMahon 1973; Heusner 1982, 1984). Contrarily, in squamate reptiles, the value of b showed greater variation (Andrews and Pough, 1985), being reported b values ranged between 0.8-1.0 in squamates and snakes (Greene et al., 2013; Nagy, 2005). Although a unique allometric exponent of 0.80 was described for reptiles in general (Naggy et al., 1999), little diversity in resting metabolic rate (RMR) and field metabolic rate had been reported within or among different groups of reptiles from a range of different habitats (Bennett and Dawson, 1976).

Few studies of body mass effect and equation of metabolism have been tested in juveniles, hatchlings and adults reptiles (Bennett and Dawson, 1976;

Bennett, 1982; Andrews and Pough, 1985). In this context, the application of generalized allometric equations developed in related species is common to predict the energy requirements in captivity (Higgins and Edward, 2009). However, to avoid over or under-estimated energy requirements, an accurate allometric exponent for a specific species should be obtained from measurements in the species in question (Andrews and Pough, 1985; Zari, 1993).

4. Growth and Estimated Body Composition

Some previous studies had indicated that tortoises in captivity often grow faster than their conspecifics in the wild. Better growth rates under captive conditions are most likely due to intense feeding or a balanced diet offered in high amounts (Furrer et al., 2004; Ritz et al., 2010). Also, fibrous material may not be incorporated in sufficient amounts on diets in captivity, increasing energy efficiency but exposing animals to undesirable high growth rates (Highfield, 1996; Senneke, 2003).

Faster growth rates positively influence to get considerably shorten generation times during the breeding stage of restocking programs relevant to endangered species. Because sexual maturity is a function of body size, an accelerated growth rate might lead to earlier sexual maturity and faster offspring production. However, greater growth rates are also linked to other pathological consequences such as obesity, high mortality, gastrointestinal illnesses, renal diseases, metabolic bone disease, or dystocia (Häfeli & Schildger, 1995; McArthur, 2004; McArthur and Barrows, 2004; Lapid et al., 2005; Donoghue, 2006; Hänse et al., 2010).

Schilliger (2000) considered reptile nutrition a complex task, because of the significant variability of metabolism and nutritional requirements among the almost 6400 species and their interaction with the environment. Then, inadequate diet and husbandry mismanagement indices the development of metabolic defects that may affect the bone morphology and function, which is the main medical disorder of chelonians in captivity conditions (Fledelius et al., 2005). Therefore, problems as pyramiding become apparent in growing juveniles (Frye, 1991). The scutes of the carapace of turtles become deformed and elevated, taking on a pyramid shape. Although pyramiding has been linked to nutritional

problems, its exact cause is still unknown (Frye, 1991). It has been hypothesized that it may be caused by an inadequate supply (excess or deficiency) of dietary vitamin D, calcium, phosphorus, or ratio Ca:P (Frye, 1991; Stancel et al., 1998), and accelerated growth with lack of proper bone mineralization. In this condition, the non-synchronic development of bone and scutes can result in a weak layer, which can make the bones of the shell porous and spongiform (Highfield, 1996; Redrobe 1996; Senneke, 2003).

Additionally, Highfield (2010) described an incorrect diet, specifically high protein and high energy, as a possible etiology of abnormal growth. Herbivorous reptiles are especially susceptible to nutritional deficiencies in captivity when the diet is deficient in mineral content -poor in calcium (Jacobson, 1989). Dietary associative effect may also occurs when one item of the diet can affect the digestive efficiency of another nutrient either positively or negatively (Bjorndal, 1991). This effect is more common in herbivorous and omnivorous species. Its occurrence may increase because they presented microbial fermentation to digest their diet (McWilliams, 2005). Phytates, oxalates, high-fat diets, and dietary protein can inhibit or affect calcium absorption (Jackson et al., 1976; Kirsch, 1984; Donoghe, 1996). However, scientific evidence that specific nutrients (other than calcium) such as starch or fiber may influence abnormal growth and pyramiding development in captive animals is limited.

Estimating body composition is an essential tool to detect nutritional and metabolic disorders (Elowsson et al. 1998), and the nutritional adequacy of a diet. The development and use of non-invasive techniques to get this information are necessary to study endangered species (Secor, 2003) without impact their populations, considering that more than 80 Chelonian species are listed as extinct, extinct in the wild, endangered, or critically endangered (IUCN, 2007). In this context, Stone et al. (2010) indicated dual-energy x-ray absorptiometry (DXA) as a technique that could be used to obtain this information. The use of this technique in reptiles has been reported in fewer species, including snakes (Secor, 2003), turtles (Fledelius et al., 2005; Stone et al., 2010), and lizards (Zotti et al., 2004). Besides being used for DXA validation, studies in green iguanas (*Iguana iguana*) and leopard tortoises (*Geochelone pardalis*) evaluate metabolic bone disease and bone density with calcium supplementation by DXA (Zotti et al., 2004; Fledelius et al., 2005).

Increasing dietary starch in fish has been influenced body composition, showing a positive relationship with lipid contents and negative effects on ash content (Wang et al., 2005; Wang et al., 2017; Sulaiman et al., 2020). However, no studies that evaluated the influence of the dietary starch and fiber levels on growth performance, body composition, and bone density in Chelonian and specifically red-footed tortoises has not been localized in the scientific literature.

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CHAPTER II

THE INFLUENCE OF INCUBATION TEMPERATURE ON EMBRYO DEVELOPMENT, HATCHLING MORPHOLOGY AND EARLY GROWTH RATE IN RED-FOOTED TORTOISE (*Chelonoidis carbonaria*)

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ABSTRACT

Reptile embryos respond to temperature changes with metabolic and physiological adjustments that influence hatchling success, phenotype, behavior, and growth rate. Climate change and global warming can affect the reptile population by altering the frequencies of hatchling survival and phenotypes. Therefore, previous studies proposed artificial incubation as a potential strategy for mitigating these effects. Red-footed tortoise (*Chelonoidis carbonaria*) eggs were collected and incubated at constant temperatures of 27.5°C and 29.5°C to investigate the physiological effects of temperature on embryo development, hatchling morphology, and early post-hatch growth rate. The direct impact of temperature on the incubation period, egg mass loss, hatching success, hatchling size, and mass was evaluated at hatching and three months of age. Hatchlings from 29.5°C presented shorter incubation times (141 days) than those from 27.5°C (201 days; $P<0.05$). Egg mass loss, hatchling mass, and size at hatching were not different between the incubation temperatures ($P>0.05$). However, the hatching success (survival rate) was lower (64.5% versus 100%) in eggs incubated at 29.5°C, but the hatchling mass and straight plastron width were higher at three months of age than those for eggs incubated at 27.5°C ($P<0.05$). These results indicate that incubation temperature influences hatching success and hatchling size and mass in the first months by influencing the early growth rate.

Key-words: Chelonian; Egg incubation; Hatchling growth; Ovoscopy; Thermal effect

INTRODUCTION

The egg stage is the most vulnerable life phase of many oviparous reptiles (Burger, 1976; Plummer, 1976; Tinkle et al., 1981). The environmental influence on organism embryogenesis may be critical to understanding life-history strategies and distributions (Muth, 1980). Incubation temperature strongly influences the embryonic development of reptiles, including incubation duration (Gillooly et al., 2002), hatchling morphology, body shape, color, growth rate, locomotor performance, behavior, hatchling index, and sexual differentiation in many species (Brooks et al., 1991; Deeming and Ferguson, 1991; Gillooly et al., 2001; Du and Ji, 2003; Booth et al., 2004; Booth, 2006). In this context, a growing concern exists that global warming and climate changes may adversely affect reptile populations by altering nest temperatures. This may induce consequences for sex determination, frequency of phenotypes (Booth, 2006), and hatchling performance through their effects on locomotor ability and post-hatch growth (Booth et al., 2004).

As incubation temperature increases (within a viable range), hatching occurs earlier because of embryonic development acceleration (Ewer 1979; Packard and Packard, 2001). Eggs incubated at moderate temperatures have high hatching success, whereas extremely high or low temperatures may be harmful or lethal for chelonian embryos (Gutzke and Packard, 1987). Hatchling size may be altered, as reported in loggerhead (*Caretta caretta*), green (*Chelonia mydas*), and olive ridley (*Lepidochelys olivacea*) sea turtles. Showed that hatchlings produced under warmer incubation conditions had smaller carapace dimensions (Glen et al., 2003; Booth et al., 2004; Booth, 2006; Maulany et al., 2012; Read et al., 2013). In contrast, prolonged incubation phases at lower temperatures seem to be related to longer times for yolk been converted into tissues, and these hatchlings tended to be larger (Booth et al., 2013). Long-term effects of incubation temperature on the physiology, growth, and behavior of reptile hatchlings were observed (Burger, 1989; Brooks et al. 1991; Etchberger, 1993; Roosenburg and Kelley, 1996), although the influence differed between species.

Red-footed tortoises (*Chelonoidis carbonaria*) are found throughout the Caribbean and South American countries (Vinke, 2008). The International Union has not evaluated this species for Conservation of Nature (IUCN). It has been included in Appendix II by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) since 1995. The diversity and high proportion of viable seeds after digestion (90%) consumed by *C. carbonaria*, combined with long seed retention times and daily movements, indicated that this species has an important ecological function as an effective dispersal agent of approximately 11 plant species (Fragoso, 2006; Wang et al., 2011). Many rural and indigenous communities in South American countries still hunt *C. carbonaria* for their meat (Pierret and Dourojeanni, 1966). This species has been extirpated in some areas (Pritchard and Trebbau, 1984; Balee, 1985; Gorzula, 1989; Walker, 1989). This species is also frequently kept in houses as a traditional pet. Despite this long history with humans, very few studies have been published about red-footed tortoises, and limited information about husbandry, feeding, and reproduction is available.

An investigation of the effects of incubation temperature on embryos and hatchlings will provide information not only to understand the dynamics of embryonic development under different thermal conditions but also to determine which conditions are needed for husbandry and conservation red-footed tortoises. This knowledge is lacking for most South American reptile species. The red-footed tortoise is a vulnerable species, and its determination mechanism of embryo development and the transitional temperature ranges are still unknown, and more information on incubation conditions is needed (Hernandez-Montoya et al., 2017). Therefore, the present study aimed to determine the effects of incubation temperature on embryonic development, hatchling morphology, and the early growth rate of *C. carbonaria* in captivity.

MATERIALS AND METHODS

All procedures, animal care, animal use, and experimental treatments were previously approved by the local Animal Care and Use Committee (CEUA, FCAV-UNESP, Jaboticabal protocol number 006094/19).

Egg sampling procedure

Adult *C. carbonaria* tortoises from different rescue centers were housed in three outdoor enclosures of 5.40 m × 4.00 m with a ground floor without vegetation covering and equipped with a group shelter and shaded areas. Tortoises were provided extruded feed and water *ad libitum*. From the detected nests, a total of 127 freshly laid eggs (0-4 days old) were collected during ten months. After collection, the eggs were cleaned and identified. The eggs were measured using a caliper (±0.01 mm) and weighed (±0.01 g). The following measurements were recorded: mass (EM), vertical diameter (straight length between opposite poles), horizontal diameter (horizontal length from side to side), and height (transversal diameter on the wide section).

Egg incubation conditions

Because natural incubation conditions for this species are unknown, we established our incubation conditions based on several considerations. First, red-footed tortoises live in areas with an average temperature between 21°C and 32°C (Wang et al., 2011), and they prefer habitats with temperatures close to 30°C (Moskovits, 1985). Second, captive management's general recommendations include air temperatures between 24°C and 30°C (Fowler and Cubas, 2001; Pingleton, 2009, Longley, 2010). Third, females lay their eggs in flask-shaped nests that are approximately 8-10 cm deep, and the ground temperature of the nest area, at a depth of 5 cm, varies from 25°C to 30°C and is approximately 1°C to 2°C cooler than the air temperature (Davis, 1979). Based on these considerations, the authors selected two incubation temperatures, 29.5°C and 27.5°C.

After biometry, eggs were randomly distributed to the two incubation temperatures. Two hatcheries were made using expanded polystyrene boxes with a 50-L capacity (58 cm x 38 cm x 38 cm; length, width, and height). Each box had an incandescent spotlight connected to a thermostat set at the established temperature±1.0°C (STC-1000, Diymore, SP, BR). A mix of vermiculite with water (1:2) was used as a bottom substrate to maintain moisture higher than 70% (Du et al., 2007; Fisher et al., 2014). Eggs were deposited and half-covered with vermiculite and periodically sprinkled with tap water. Each

hatchery's internal temperature and moisture data were registered twice daily using a digital hygrothermometer (Agetherm, AGT-TM10, Lima, Peru).

Embryo development was monitored using non-invasive methodologies, such as ovoscopy (candling eggs). Eggs were observed every two weeks beginning on the 15th day of incubation to determine fertility and check the embryo's growth and development. Each time the embryos were observed, their eggs were repositioned in new locations in the hatchery to mitigate the potential effect of local temperature variation among different positions in the incubator. Eggs with an initial darker area at the top were classified as possibly fertile, and eggs with a small reddish area with blood vessels extending away from it were confirmed as fertile. Every step of embryo development that could be identified using ovoscopy was recorded with pictures, and the incubation day was recorded. Embryo characteristics were described to identify not viable eggs (e.g., clear eggs, eggs showing blood rings or streaks, rotten smell, fungal or bacterial contamination on the shell, failed to develop or darker eggs) were removed from the incubator and discarded to avoid hatchery contamination.

At the end of the incubation period, the following parameters were recorded: fertility rate (%, the rate between fertile eggs and the total collected eggs); hatching success (%, the ratio of the number of hatchlings with the total fertile eggs); mortality index (%, the proportion of the total unsuccessfully developed embryos that were dead during the incubation with the total number of fertile eggs); incubation period (days); final egg mass (g; the last registered mass of the egg before hatching); mass loss of the egg (%); mass at hatching (g); egg embryo portion (%, egg mass fraction that represents the embryo mass); egg residuals portion (%, egg mass fraction that represents the residuals of yolk, white and shell). The following parameters were measured 4 to 7 days after hatching: hatchling mass; straight carapace length; straight carapace width (width measurement from site to site in the intersection of the 4th and 5th scute of the carapace); straight plastron length; and straight plastron width (width measurement from site to site of the 3rd line of the plastron).

Growth rate

The hatchlings' biometry and body mass were measured weekly during the first three months of life to measure growth rates and evaluate post-hatchling

development. The hatchling mass (±0.01 g), straight carapace length, straight carapace width, straight plastron length, and straight plastron width data were measured (±0.01 mm). The Gompertz function was used to determine the growth rates of the biometric measurements of interest (variables that presented temperature effect) when the hatchlings were three months old:

Eqn 1: $$BM = BMo + a_i * Exp\left(-Exp(b_i * c_i - b_i * Age)\right)$$

Where:

BM is the biometric measure of interest (g or mm)

BMo is the biometric measure value at hatching (g or mm)

Age in days

a_i is the biometric measure increment value (g or mm)

b_i is the growth rate of the biometric measure (g or mm day^{-1})

c_i is the inflection point or the time at the maximum growth rate

The asymptotic value comes from the sum of the increment (a_i) and the initial value (BMo)

The size index growth rate was calculated using the following equations (adapted from Kobayashi et al., 2018 and Booth et al., 2013):

Eqn 2: $$Daily\ growth\ rate\ at\ week\ n\ (mm^2 * day^{-1}) = (S_n - S_{n-1})/7$$

Eqn 3: $$Accumulated\ daily\ growth\ rate\ at\ week\ n\ (mm^2 * day^{-1}) = (S_n - S_0)/n°days$$

where *n* is the week number of rearing hatchling, S is the size index (Straight carapace length x Straight carapace width), and S_0 is the initial measurement.

Statistical analyses

Data normality and homoscedasticity were previously evaluated. Data were subjected to one-way ANOVA. The relationships between the initial egg mass and the biometric measurements of hatchlings were analyzed by Pearson correlation and general linear regression. The incubation temperature influence on mortality index and hatching index was tested by the Chi-square test. The incubation temperature effect on the incubation duration, egg mass loss, egg embryo portion, egg residuals portion, final egg mass, and accumulated and daily

growth rates were evaluated by adjusting the data to a general linear model (GLM). By considering the initial egg mass as a covariate (maternal effect). The influence of incubation temperature on hatchling size and mass was evaluated using a general linear model (GLM), with initial egg biometry (mass and width) as a covariate.

An ANOVA test with incubation temperature as an independent variable was performed to evaluate the temperature effect on the final biometric measurements at three months of age and growth rates. Hatchling biometric measurements were adjusted in a non-linear growth model of the Gompertz function using the PROC NLIN and the quasi-Newton method to determine the growth rate. All statistical analyses were performed using R Studio 3.6.3, and p values <0.05 were considered significant.

RESULTS

Embryo development and emergence at different temperatures

Of the 127 collected eggs, 79 and 48 eggs were incubated at 29.5°C and 27.5°C, respectively. The mean internal temperature and moisture of each incubator were 29.4±0.7°C and 78.9±14.1%, and 27.1±0.9°C and 84.3±15.4%, respectively. These were very close to the targets. The initial egg mass (IEM) was similar between treatments (P>0.05). The average initial egg mass and their Pearson correlation with biometric egg measurements are shown in Table 1.

Table 1. Egg biometry data at collection time and correlation coefficients between egg mass and the biometric measurements of eggs from *C. carbonaria*.

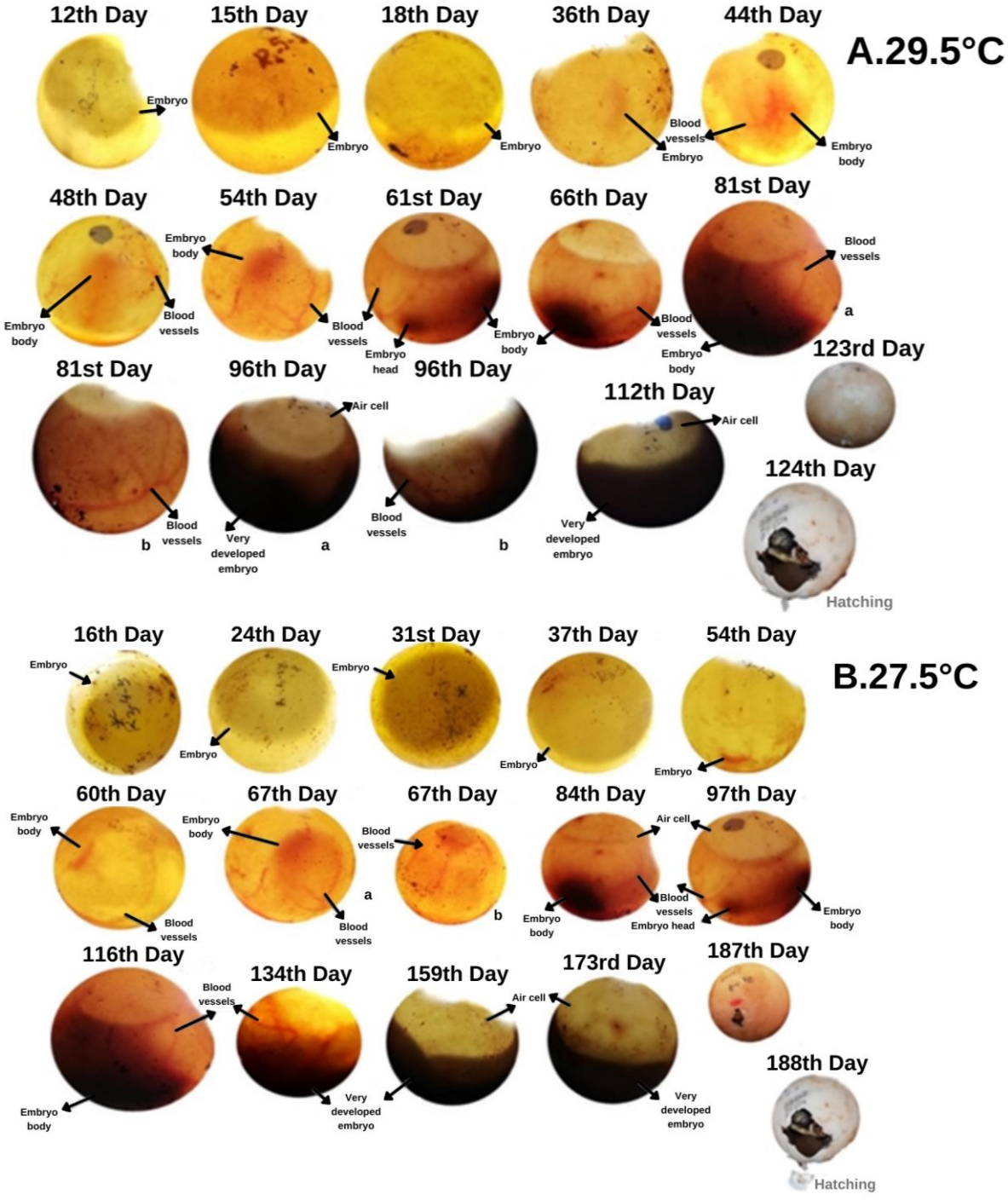
Parameters	Mean	Max	Min
Egg mass (g)	56.58	74.17	29.14
Egg vertical diameter (mm)	47.39	57.7	34.87
Egg horizontal diameter (mm)	45.08	68.34	33.51
Egg height (mm)	45.15	68.34	33.61
Pearson Correlation with egg mass			
Egg vertical diameter	Egg horizontal diameter	Egg height	Weight at hatching
0.60*	0.69*	0.69*	0.69*

*Significant Correlation

Schemes of embryo development from the 15th day until hatching were created for each incubation temperature (Fig. 1). Since the 12th day of incubation

at 29.5±1.0°C, it was possible to observe a dark area on the top of the egg, which expanded over several days to comprise most of the egg (15th-18th day). On the 36th day, a small reddish area and vessels could be identified, and the egg color returned to its original color. In the interval between the 44th and 48th days, blood vessels expanded and were more visible. From the 54th day, it was easier to observe and follow the embryo and blood vessel growth.

Figure 1. Scheme of embryo development of *C. carbonaria* during artificial incubation at two evaluated temperatures.



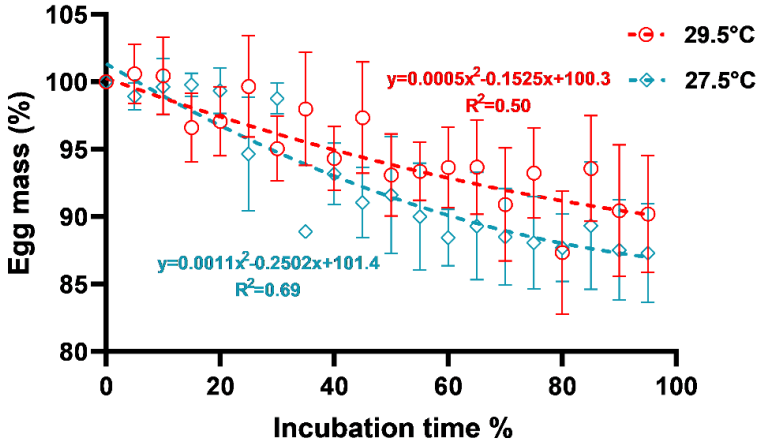
The tortoise's shape was visible in two more months, and robust blood vessels developed around the air cell. The embryo became larger in the subsequent two weeks and covered almost half of the egg. Its shape was not easily visible, but it was possible to observe some embryo movements. The embryo and vessels were more visible after the 81st day, as shown in Fig. 1A. The embryo was well developed on the 96th day, and it became more difficult to distinguish the embryo from the blood vessels. Only a large dark area with an air cell on the top was observed on the 112th day. That moment, hatching started, and almost all hatchlings started pipping the egg at night or in the early morning.

The initial stage of embryo development was observable beginning on the 16th day of incubation at 27.5±1.0°C, which was also described for eggs incubated at 29.5°C (Fig. 1B). A longer time was needed (24th - 31st day) for this area to cover all of the eggs. The egg color returned to its original color on the 54th day, and a small reddish area (EE) was observed. One week later, small vessels (VE) were observed (60th day). It became easier to observe the embryo and follow its growth on the 84th day. After three months of incubation (97th day), robust blood vessels developed around the air cell, and the shape of the tortoise was finally visible. Over the next two weeks, the embryo grew to fill most of the egg, and it was very active and possible to observe movements (116th day). The embryo was well developed (VDE) on the 134th day, but it was still possible to distinguish it from the blood vessels. Monitoring embryo development became difficult on the 159th day, and it was only possible to distinguish the air cells. After slightly more than five months of incubation, the hatching process started and occurred at night or in the early morning, similar to the 29.5°C IT.

The egg mass loss during incubation (10.53±4.43% and 12.69±3.66% of the starting weight), egg embryo portion (75.99±4.38% and 76.00±3.06%), residual egg portion (24.01±4.38% and 24.00±3.06%), final egg mass (FEM; 51.24±7.26 g and 45.83 g±5.49 g) and hatchling mass (HMo; 37.92±6.79 g and 34.81±4.27 g) at 29.5°C and 27.5°C, respectively, were similar between treatments ($P>0.05$). Apparently, all eggs lost mass at the same rate, regardless of IT, until 10% development. Between 15% and 75% embryo development, eggs incubated at 27.5°C apparently lost mass faster than eggs incubated at 29.5°C. During the interval between 80% and 95% development, eggs from both ITs

maintained or lost mass very slowly (Fig. 2). The hatching success was greater at 27.5°C (P<0.05; Table 2). The incubation temperature significantly reduced the incubation time (P<0.001). Tortoises incubated at 29.5°C required approximately 60 days less to start internal pipping (Table 2).

Figure 2. Egg mass reduction (%) during the incubation of *C. carbonaria* eggs at two temperatures



Mean and standard deviation of egg mass represented in percentage in relation with the initial mass, during the incubation process at each temperature. Showed equations represented the tendencies at each incubation temperature, obtained by quadratic regression.

Table 2. Number of eggs incubated, hatching index, and incubation period of eggs from *C. carbonaria* incubated at two temperatures.

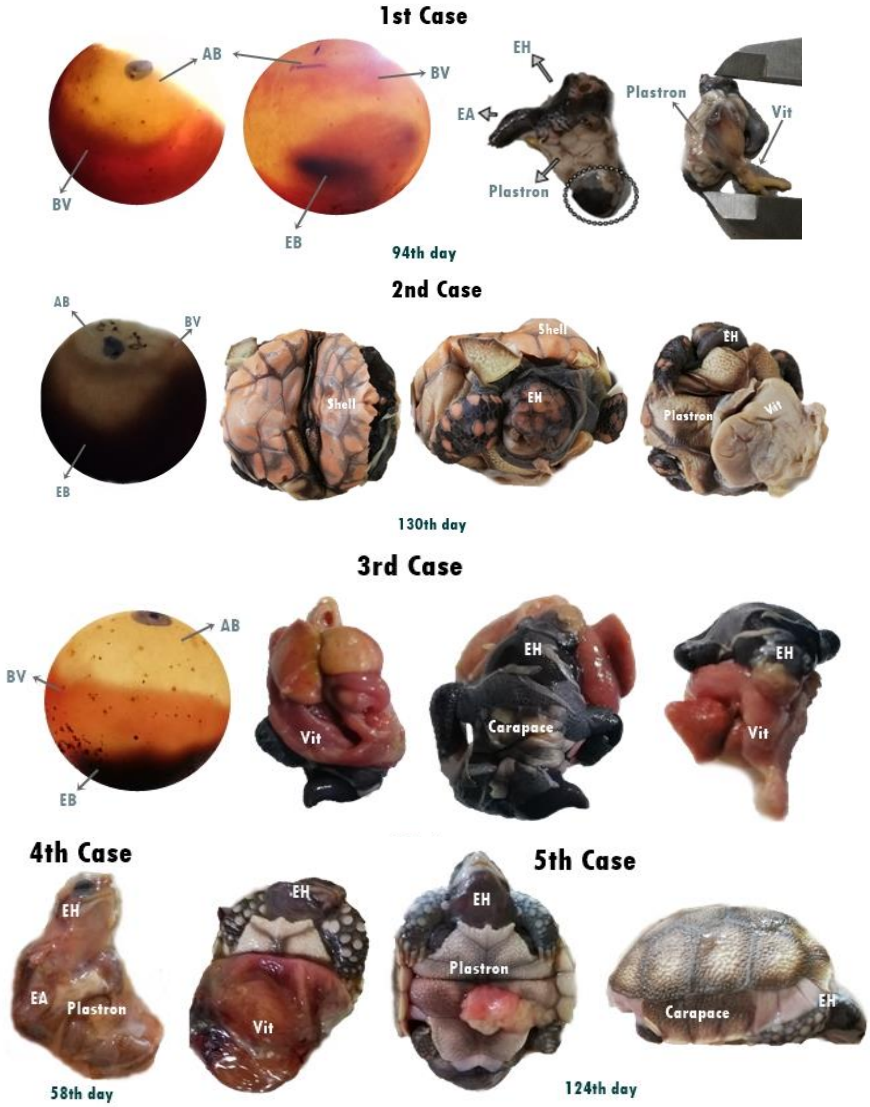
Parameters	Temperature on incubation	
	29.5°C	27.5°C
Incubated eggs (n)	79	48
Fertile eggs (n)	26	14
Hatched eggs (n)	17	14
Dead Embryos (n)	9	0
**Hatching success (%)	65.4	100*
**Mortality index (%)	34.6*	0
Incubation period to the point of pipping (days)	139.06±29.69	199.00±18.36*
Incubation period to total emergence (days)	141.06±29.85	201.00±18.25*

*statistically different (P<0.05).
**Chi-square test (X^2)

After pipping, tortoises took 1 to 2 days to emerge from the egg with no temperature effect (P>0.05). Cases of embryo death were observed only at 29.5°C and totaled 34.6% of the eggs. Unsuccessful embryo development was considered in two main stages: at the first two months (78% cases) and around the 130th day of incubation (22%). The embryo shape and abnormalities were

identifiable in five of the nine dead embryos. The other embryos were in the decomposition process, making it difficult to distinguish the embryo from the yolk and white. Embryonic death was suspected when the blood vessels were hard to observe, and blood was drawn away from the embryo to form a blood ring. Once this appearance was registered, these eggs were monitored for an additional week to confirm embryo death (five total cases). All of the embryos that died during the initial development presented normal development of the head and forelegs. However, some abnormalities were observed, such as organ exposure (broken yolk sac), absence or little development of the carapace and plastron, and hindlimb agenesis. For embryo death cases at the last stage of development, hatchling death was suspected when the shell color became dark and a rotten odor was detected. After egg opening, broken yolk sacs and vessels were observed in one case. The 2nd case showed abnormal development of the carapace with a creased and twisted appearance, but the head, forelegs, and hindlimbs were normal but in a mummified stage. In contrast, the 5th case did not present any abnormalities or deformation, and only a decomposition process was observed (Fig. 3).

Figure 3. Embryonic death cases of *C. carbonaria* eggs incubated at 29.5°C.



Embryonal death cases at early development stage (1st, 3rd and 4th case) and at late development stage (2nd and 5th case). Embryo death characteristics are described: BV - broken vessels; AB - air cell; EB - embryo body; EA – forelegs; EH - embryo head; Vi - yolk sac.

Hatchling development at different incubation temperatures

The initial straight carapace length, straight plastron width, and straight plastron length of hatchlings were similar between treatments (P>0.05). However, incubation temperature affected straight carapace width, and hatchlings from 29.5°C had a significantly wider carapace (P<0.05), as shown in Table 3. Accurate measurement of hatchling carapace at hatching was difficult because the shells were soft and flexible and curved downward along the caliper's edges.

Table 3. Biometric measurements of hatchlings of *C. carbonaria* on the first week and three months of age and the Gompertz Growth Curve parameters obtained for newborns incubated at two temperatures.

Age	Parameters	Temperature of incubation	
		29.5°C	27.5°C
1st week	Hatchling mass (g)	38.32 ± 6.75	35.07 ± 4.27
	Straight Carapace Length (mm)	50.08 ± 4.44	48.73 ± 2.55
	Straight Carapace Width (mm)	44.16 ± 4.07	41.53 ± 2.25*
	Straight Plastron Width (mm)	38.12 ± 4.84	36.90 ± 2.03
	Straight Plastron Length (mm)	44.05 ± 4.12	43.26 ± 2.34
3rd Month	Hatchling mass (g)	80.42 ± 15.35	66.44 ± 11.18*
	Straight Carapace Length (mm)	69.70 ± 4.01	68.33 ± 7.16
	Straight Carapace Width (mm)	58.51 ± 4.32	56.21 ± 3.87
	Straight Plastron Width (mm)	50.81 ± 4.45	46.62 ± 4.76*
	Straight Plastron Length (mm)	60.32 ± 3.95	59.64 ± 5.31

Gompertz model parameters

Temperature	Parameter	MSE	a_i	b_i	c_i
29.5°C	Hatchling mass	70.91	94.87 (24.78)	0.024 (0.02)	65.91 (18.94)
	Straight Plastron Width	10.63	20.03 (3.75)	0.029 (0.01)	45.67 (20.31)
27.5°C	Hatchling mass	96.48	74.13 (34.21)	0.023 (0.01)	65.37 (19.10)
	Straight Plastron Width	6.09	13.42 (2.32)	0.036 (0.01)	35.13 (21.32)

*statistically different (P<0.05).
(Estimation error)
MSE: mean square of error, a_i: Hatchling mass or straight plastron width increase at three months old, b_i: Hatchling mass and straight plastron width growth rate, and c_i: Age at the maximum growth rate of hatchling mass and straight plastron width during three months (days).

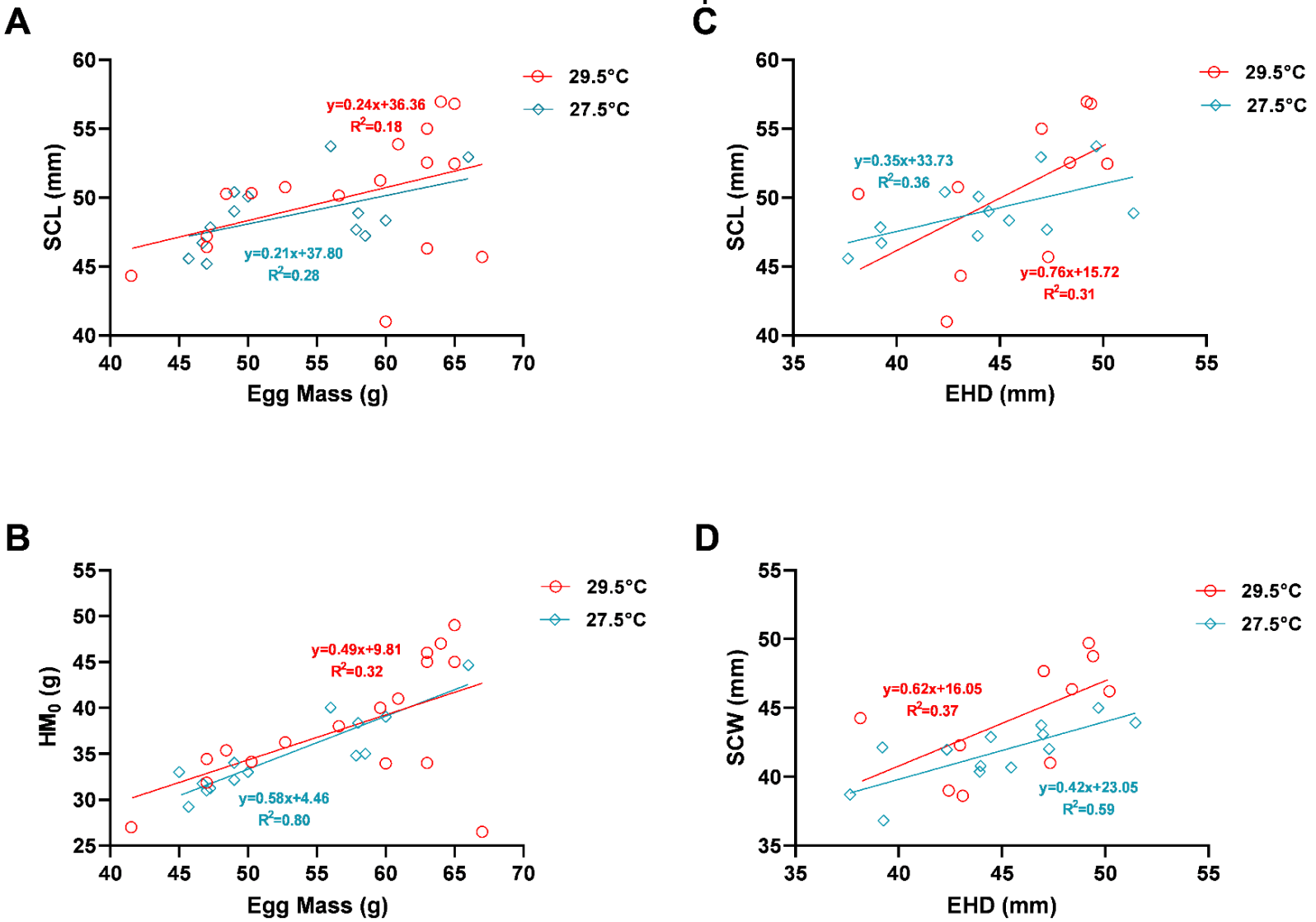
A maternal effect without incubation temperature influence was observed. Initial egg mass affected the hatchling mass at hatching (P<0.001) and straight carapace length (P<0.05), in which heavier and larger hatchlings were obtained from larger eggs (Fig. 4A-B). Egg horizontal diameter also influenced straight carapace length (P<0.05) and width (P<0.01) (Fig. 4C-D).

At three months old, the biometric measurements of hatchlings were similar between ITs, except for body mass and straight plastron width, which were higher for eggs incubated at 29.5°C (P<0.05). Hatchling mass strongly influenced the straight carapace length (P<0.001), straight carapace width (P<0.001), and straight plastron length (P<0.01) at hatching, with a direct relationship during the early growth phase (Fig. 5). The mean daily mass gain and straight plastron width

growth rate during the three months of observation were similar between ITs (P>0.05), with a mean gain of 0.62±0.13 g×day^{-1} and a mean increase in straight plastron width of 0.21±0.07 mm*day^{-1} for hatchlings from eggs incubated at 29.5ºC, and a mean gain of 0.55±0.08 g×day^{-1} with 0.21±0.09 mm×day^{-1} mean increase in straight plastron width on hatchings incubated at 27.5°C. However, the daily growth rate exhibited different patterns depending on IT. Hatchlings from incubation at 29.5°C showed a constant increase in growth speed every two weeks. In contrast, hatchlings from incubation at 27.5°C exhibited a reduced growth rate after the 6th week when their growth rate started to decrease (Fig. 6A). Due to these differences in the post-hatching growing pattern after the three months of observations (at the final week of observation), the daily growth rate was significantly greater (0.85±0.73 g×day^{-1}) for hatchlings incubated at 29.5°C than hatchlings incubated at 27.5°C (0.54±0.30 g×day^{-1}; P<0.05), which explained the higher final mass of hatchling incubated at 29.5°C. The incubation temperature did not influence the growth rate of straight plastron width (Fig. 6B).

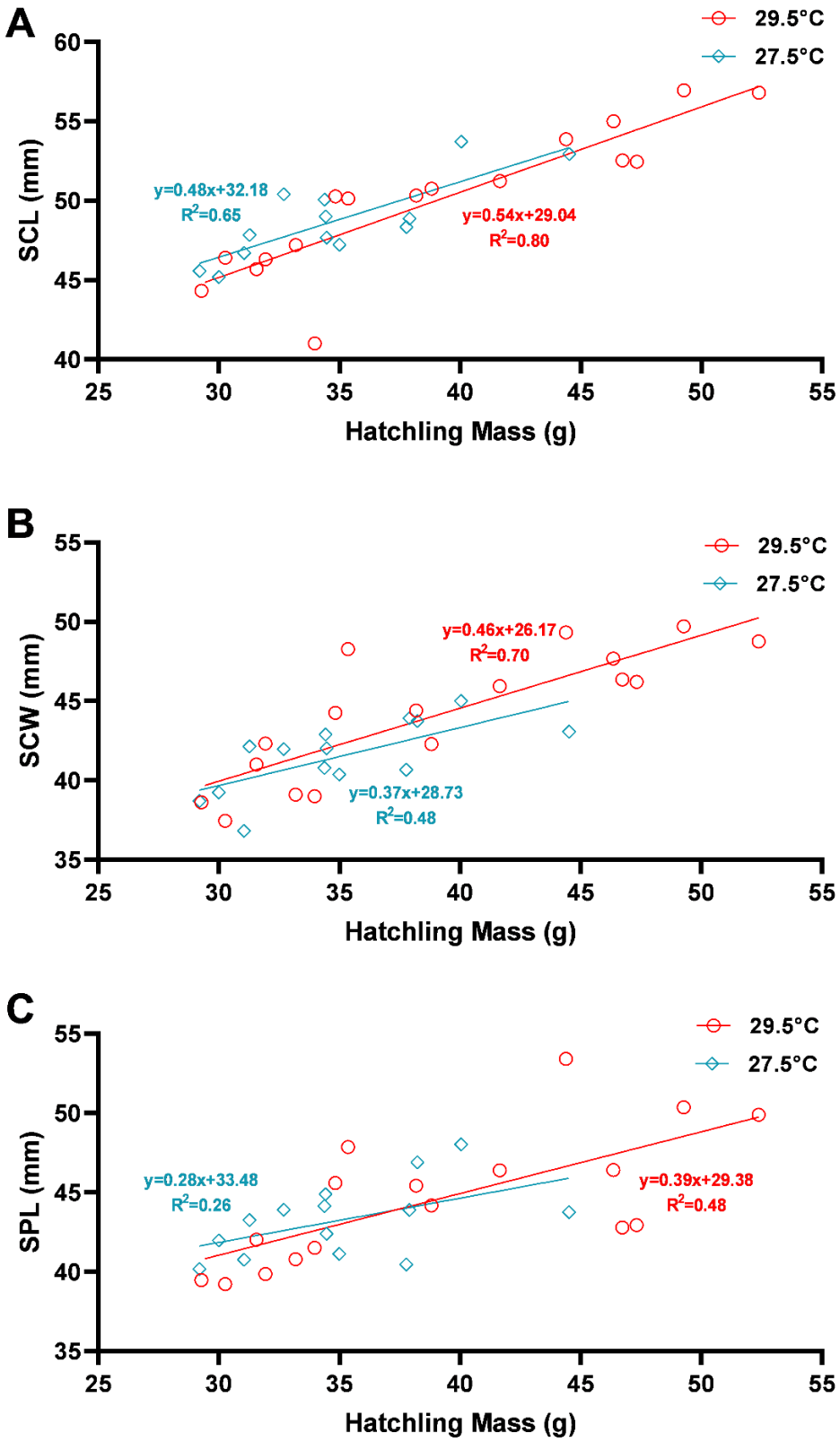
The size index (Straight carapace length x Straight carapace width; mm^2) of hatchlings was calculated every two weeks during 12 weeks. The accumulated daily growth rate of the size index at the 12th week was similar for the two incubation temperatures, 3.03±0.78 and 3.09±0.95 mm^{2*}day^{-1} for 29.5°C and 27.5°C, respectively (Fig. 6C). However, incubation temperature influenced the daily growth rates of the size index each week. Hatchlings incubated at lower temperatures grew at a higher rate (1.07±0.32 mm^2×day^{-1}) than the hatchlings incubated at higher temperatures (0.58±0.22 mm^2×day^{-1}). Nevertheless, at the 12th week, the daily size index growth rate of hatchlings incubated at 27.50°C (0.63±0.41 mm^2×day^{-1}) decreased significantly and was similar to the 29.5°C group (0.51±0.61 mm^2×day^{-1}) (Fig. 6D). The accumulated daily size index growth increased over the weeks, but the daily index size growth rate decreased with the advancement of weeks after hatching (Fig. 6C-D).

Figure 4. Linear regression between *C. carbonaria* egg mass and size with hatchling mass and biometric parameters after incubation at two different temperatures.



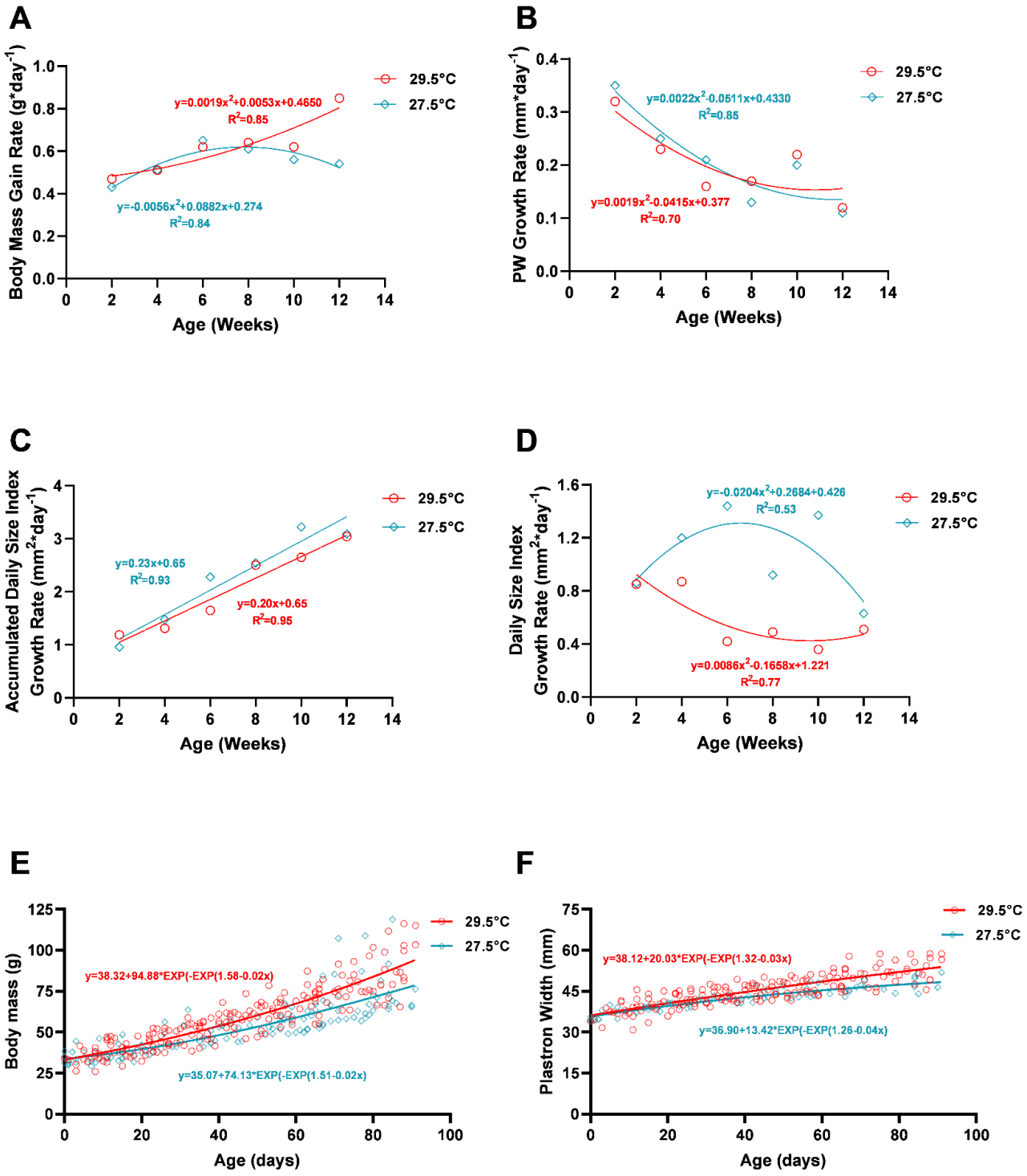
SCL: straight carapace length, HMo: mass at hatching, SCW: straight carapace width, EHD: egg horizontal diameter. Circle represents eggs incubated at 29.5°C and diamond filled represents eggs incubated at 27.5°C. Showed equations represented the tendencies at each incubation temperature, obtained by simple linear regression.

Figure 5. Linear regression between *C. carbonaria* hatchling mass and biometric measurements after incubation at two different temperatures.



SCL: straight carapace length, SCW: straight carapace width, SPL: straight plastron length. Circle represents eggs incubated at 29.5°C and diamond filled represents eggs incubated at 27.5°C. Showed equations represented the tendencies at each incubation temperature, obtained by simple linear regression.

Figure 6. General and Accumulated Daily Growth Rate and Gompertz Growth Curve of *C. carbonaria* hatchlings from eggs incubated at two different temperatures.



A-B. Gain rate of body mass (g*day^{-1}) and growth rate of straight plastron width (PW; mm*day^{-1}) at each week of age of hatchlings from two evaluated incubation temperatures. Showed equations represented the tendencies at each incubation temperature, obtained by quadratic regression.**C-D.** Growth rate of accumulated size index (SCL x SCW; mm^2*day^{-1}) along the first twelve weeks of age, and growth rate of size index (mm^2*day^{-1}) at each week at both evaluated temperatures. Showed equations represented the tendencies at each incubation temperature, obtained by simple linear and quadratic regressions, respectively. **E-F.** Gompertz growth curves of body mass and plastron width of hatchlings during the first twelve weeks vs observed data at each evaluated temperature. Circle represents eggs incubated at 29.5°C and diamond filled represents eggs incubated at 27.5°C.

Hatchling mass and straight plastron width data from both incubation temperatures were adjusted in a non-linear regression (Gompertz function) with age (Fig. 6E-F), and the following equations were obtained:

$$HM = 38.32 + 94.8755 * \exp\left(-\exp(1.58188 - 0.024 * \text{Age})\right)$$ [29.5°C];

$$HM = 35.07 + 74.1288 * \exp\left(-\exp(1.51046 - 0.023 * \text{Age})\right)$$ [27.5°C];

$$SPW = 38.12 + 20.0308 * \exp\left(-\exp(1.32443 - 0.029 * \text{Age})\right)$$ [29.5°C];

$$SPW = 36.90 + 13.4176 * \exp\left(-\exp(1.2646 - 0.036 * \text{Age})\right)$$ [27.5°C];

in which the estimation errors were 0.02, 0.03, 0.01, and 0.00, respectively.

The Gompertz model variables were used to describe the adjusted growth rate for hatchling mass/age and straight plastron width/age at both incubation temperatures. The model estimated a mass at three months old (Hatchling mass at hatching+a_i) of 133.19 g for hatchlings incubated at 29.5°C and 109.20 g for hatching incubated at 27.5°C. The adjusted straight plastron width/age estimated a straight plastron width at three months old (straight plastron width at hatching+a_i) of 58.15 mm and 50.32 mm for 29.5°C and 27.5°C, respectively. The estimated daily growth rates (b_i) were 0.024 g day^{-1} and 0.029 mm day^{-1} for hatchlings obtained from eggs incubated at 29.5°C, and animals from eggs incubated at 27.5°C resulted in estimated values of 0.023 g day^{-1} and 0.036 mm day^{-1} (Table 3).

DISCUSSION

Embryonic development at different temperatures

The mean egg mass was similar to the 50 g previously reported by Vokins (1977), and the minimum obtained mass was slightly lower than the 36.17 g reported for red-footed tortoises (Davis, 1979). Compared with chelonians in general, *C. carbonaria* eggs are lighter, although values as high as 74.17 g were also observed (Elgar and Heaphy, 1989). Egg horizontal diameter and vertical egg diameter have been widely used in morphological descriptions of eggs from different reptile species. The observed values of horizontal egg diameter and

vertical egg diameter in the current study ranged from 33.50 mm to 68.34 mm and 34.90 to 57.70 mm, respectively, which are similar to previously described values (Volkins, 1977; Davis, 1979; Moreira, 1991; Vanzolini, 1999; Hernandez and Boede, 2008). The EHe has not been used as part of the egg morphology description, possibly because it is similar to the egg horizontal diameter values. Hernández and Boede (2001) found that biometric measurements of eggs and clutch size were related to female size in *C. carbonaria* and observed that larger females produced more giant eggs. The female tortoises of the current study varied from 2.5 kg to 10 kg of body mass, which also must be considered when interpreting egg sizes.

A dark area on the top of the egg was observable on the 12th day of incubation at 29.5°C, which expanded over several days to comprise most of the egg from the 15th to 18th day. This characteristic indicates the early developmental stage of the embryo (Booth, 1998a), and it may be started on the axis of the egg in other species (Charles, 2008). During incubation, the egg mass loss was greater at higher incubation temperatures for snapping turtles (*Chelydra serpentina*) (Birchard and Reiber, 1995). In contrast, after incubation, the egg mass loss was similar for the two incubation temperatures in the present study, as was described for American alligators (*Alligator mississippiensis*) (Deeming and Ferguson, 1989). The observed mass loss of eggs in the current study ranged from 10% to 12%, which was greater than other species, such as soft-shelled turtles (*Trionyx triunguis*) (Leshem et al., 1991).

The incubation duration was substantially shorter at the higher temperature in the present study, a well-established condition for chelonians (Ewert, 1979; Du and Ji, 2003; Warner et al., 2011; Morales-Mérida et al., 2017). In *C. carbonaria,* a mean incubation duration of 176.90 days (124 to 228 days) in uncontrolled temperatures (Hernández, 1997) to values from 143.45 to 154.66 days have been reported (Hérnandez and Boede, 2008). At 29°C, Hernández et al. (2017) reported a slightly shorter incubation period (131±11.30 days) than the present study, and a longer incubation duration (185 days) was reported for incubations from 26.0°C to 27.5°C by Davis (1979).

The fertility rate observed in the current study was similar to the 42% previously reported for red-footed tortoises (Davis, 1979). This index is low

compared to other species, such as loggerhead sea turtles (*Caretta caretta*), hawksbill turtles (*Eretmochelys imbricate*), and soft-shelled turtles (*Trionyx triunguis*), with greater than 80% fertility (Leshem et al., 1991; Cañón and Orozco, 2004). Other factors, including nutrition, age (Pritchard, 1979), and captivity conditions (Werner, 1991), can affect fertility. The present study's hatching success was similar to the previously described indices for the species (Hérnandez, 1997). However, a slightly lower index of 45% and a higher value of 61% were also reported (Hérnandez and Boede, 2001). In this latter case, the value may have been underestimated because the fertility rate was not determined, and the hatching success was calculated with the total collected eggs. Hatching indices from 62.5% to 100% were described for different tortoises, turtles, and alligators (Joanen et al., 1987; Janzen, 1995; O'Steen, 1998; Briggs, 2007; Ligon et al., 2009; Carey, 2017). Species, such as loggerhead sea turtles (*Caretta caretta*) and hawksbill turtles (*Eretmochelys imbricate*), had significantly lower hatching success, between 12.68% and 14.84% (Cañón and Orozco, 2004). The higher mortality in the present study was associated with a high incubation temperature, which was similar to the mortality previously observed in red-footed tortoises by Hernández-Montoya et al. (2017) when the hatching success decreased from 52% at 29°C to 10% at 31°C and 0% (no hatchling) at 33°C of incubation. This negative effect has been reported in many tortoises, turtles, and lizards (Damme et al., 1992; Díaz-Paniagua, 2006; Fisher et al., 2014; Romito et al., 2015).

During embryonic development, the temperature may induce physiological adjustments, such as increased oxygen consumption to support energy expenditure for tissue synthesis and tissue maintenance (Birchard and Reiber 1995; Booth, 1998a; Ji et al., 2002; Briggs, 2007). At high incubation temperatures, turtle embryos show higher oxygen consumption in the second phase of incubation, with a more significant metabolic peak followed by an elevated metabolic decline before hatching. These changes reflect greater energy demand for tissue maintenance and biosynthesis, greater absolute growth rate, accelerated initial morphogenesis, and faster embryo development (Deeming and Ferguson, 1989; Congdon and Gibbons, 1990; Díaz-Paniagua et al., 2006). Incubation temperature also affects embryo phenotypic characteristics

(Yntema 1960; Telemeco et al. 2013; Ceballos et al. 2014; Gómez-Saldarriaga et al. 2016). Body malformations, including a twisted carapace and partial limb agenesis, have been described in *C. carbonaria* embryos during incubation at 29.0°C (Hernández-Montoya, 2017). High incubation temperatures also have adverse effects on organogenesis, producing malformations in the central nervous system, eyes, vertebral column, jaws, limb agenesis, and death (Yntema 1960; Webb and Messel 1977; Webb et al. 1983; Ferguson 1985; Burger et al. 1987; Deeming and Ferguson 1991). These potential alterations may explain the observed increase in mortality at high temperatures of incubation in the present study.

C. carbonaria eggs have rigid shells classified as endohydric eggs, with rigid and impermeable shells that are largely unaffected by environmental moisture. The water reserve in a rigid egg is not appreciably increased in a high moisture environment or depleted very much in a dry environment. The water supply to embryos is mainly dependent on the female parent at oviposition. (Packard, 1999). Internal water plays a crucial role in mobilizing yolk reserves by reptile embryos and consequently influences hatchling mass (Packard and Packard, 2001). An embryo with access to a relatively large reserve of water will consume more yolk and grow to a larger size before hatching than an embryo with limited water access (Packard, 1999). However, gas exchange is impaired when eggs are in a moisture-saturated environment. The presence of moisture levels for endohydric eggs that is much higher than the optimum may be more harmful to developing eggs than desiccation (Packard et al., 1977).

The hatchling masses from both ITs were slightly greater than the 17.80 g to 30.00 g interval described previously for red-footed tortoises (Davis, 1979). The straight carapace length, carapace width, and plastron length of the hatchlings in the present study were similar to the values reported by Davis (1979) and Hernández (1997). Straight plastron width has not been analyzed previously in hatchling morphometric descriptions of this species, but it was measured in other species (Gonçalves, 2010). The hatchling mass is dependent on incubation temperature in some, but not all, reptile species (Ji et al., 2002; Braña and Ji, 2000). Species may be divided into three main groups in this regard. In one group, hatchlings incubated at low temperatures are heavier and

have longer carapaces than hatchlings from intermediate and warmer conditions (Damme et al., 1992; Braña and Ji, 2000; Ji et al., 2002; Booth et al., 2004; Du et al., 2007; Warner et al., 2011; Booth et al., 2013). In another group, species with rigid-shelled eggs incubated at lower temperatures produce smaller and lighter hatchlings (Janzen, 1993; Remor de Souza & Vogt, 1994; Fisher et al., 2014). In the third group of species, little or no effect of incubation temperature is observed on hatchling mass and size (Joanen et al., 1987; Thompson, 1990; Leshem et al., 1991; Booth, 1998b; Du and Ji, 2003; Briggs, 2007; Delmas et al., 2008; Morales-Mérida et al., 2017). Apparently, according to the present study results, *C. carbonaria* belongs to the third group of species.

Maternal influence on hatchling phenotype may include genetic effects of maternal and paternal origin (Pearce and Avise, 2001; Booth et al., 2013), and non-genetic effects, primarily the initial egg mass (Gonçalves, 2010). Therefore, offspring phenotype was determined by incubation temperature and maternal investment. The process is highly variable between oviparous ectotherms and ranges from differential allocation of nutrients during egg formation to variation in the degree of post-laying care (Mcknight and Gutzke, 1993; Ligon et al., 2009). It has been demonstrated that the initial egg mass is more critical for hatchling size than incubation temperature (Roosenburg and Kelley, 1996; Booth et al., 2004; Booth et al., 2013). This relationship was confirmed for *C. carbonaria* in the present study because a significantly high positive correlation between hatchling size and egg mass has been observed (Díaz-Paniagua et al., 1997; O'Steen, 1998; Briggs, 2007; Ligon et al., 2009). The most direct maternal effect is related to egg size with the quality and quantity of the nutrients allocated to the egg (Booth et al., 2013), which have fundamental impacts on embryo development (Roosenburg and Kelley, 1996). Hatchling size is an essential parameter for survival, and larger hatchlings generally have a greater chance of survival because of their greater locomotor ability and ability to escape predation (Janzen et al., 2000; Hofmeyr et al., 2005; Booth, 2006).

The embryo mass constituted 76% of the initial egg mass in both ITs, and this proportion was slightly greater than the American alligator (*Alligator mississippiensis*) (66.76%) and soft-shelled turtle (*Trionyx triunguis*) (66%) (Deeming and Ferguson, 1989; Leshem et al., 1991). Also, mean egg residual

mass was not affected by temperature (10% to 12%), as previously reported for other reptile species (Deeming and Ferguson, 1989).

Hatchling development at different incubation temperatures

From 59 to 86 days post-hatching, the hatchlings obtained from both incubation temperatures in the present study were heavier with larger straight carapace widths and lengths than reported by Davis (1979) for *C. carbonaria*. Considering that the mass was only slightly greater at hatching, the biometric measurements were similar to previous reports, which indicates that the hatchlings in the current study exhibited a faster growth rate during the first three months. Possible explanations include differences in incubation temperature between studies, the quality of maternal nutrition and nutrient allocation to eggs, and post-hatching management, including environment and diet quality, which were likely better in the current study.

IT influences post-hatching growth (Brooks et al., 1991; Spotila et al., 1994; Demuth, 2001), as observed in the current study. At three months of age, hatchlings from 29.5°C exhibited higher mass and straight plastron width. The effects of incubation temperature on chelonian neonate growth are variable, even within individual species, and range from a positive correlation (Roosenburg and Johson, 1995; Roosenburg and Kelley, 1996), no observed temperature effect (Etchberger, 1993; Janzen 1995; Steyermark and Spotila, 2001; Briggs, 2007), or a negative correlation between incubation temperature and subsequent growth rate (Rhen and Lang, 1995; O'Steen, 1998; Braña and Ji, 2000; Demuth, 2001; Steyermark and Spotila, 2001; Kobayasshi et al., 2018). However, a general trend for faster post-hatch growth rates on higher incubation temperature is more prevalent (Deeming and Ferguson, 1989; Damme et al., 1992; Mcknight and Gutzke, 1993; Roosenburg and Kelley, 1996; Booth et al., 2004), as observed in the present study when the Gompertz growth curve was observed (Fig. 6E) and the daily growth rates at the 12th week were compared (0.85±0.73 g×day^{-1} at 29.5°C versus 0.54±0.30 g×day^{-1}).

Temperature also affects sex determination in many reptile species (Demuth, 2001; Steyermark and Spotila, 2001). In one group of species, eggs incubated at high or low temperatures produce males and females, respectively

(Brooks et al., 1991; McKnight and Gutzke 1993; Bobyn and Brooks, 1994). Red-footed tortoises have been described in another group, where males are produced at low temperature and females at a higher temperature and a mixed-sex group obtained from intermediate conditions (Gibbons and Lovich, 1990; Spotila et al., 1994; Hernández-Montoya et al., 2017). Sex may affect the growth of organisms that present sexual dimorphism, with faster growth rates on the sex that shows greater body size after sexual dimorphism is established (Gibbons and Lovich, 1990; McKnight and Gutzke, 1993; Spotila et al., 1994; Demuth, 2001). Considering *C. carbonaria* females are larger, it is possible to speculate females may grow faster than males, which may have affected the present study results because the hatchlings were not sexed. A faster growth rate is considered advantageous because hatchlings may spend relatively less time exposed to predators, handle larger prey items, and reach sexual maturity faster because sexual maturity in reptiles is determined by body size and not age (Booth, 2006).

In conclusion, the present study demonstrated that incubation temperature significantly affected embryo development, hatching success, incubation period, and early growth rate. Eggs incubated at 29.5°C exhibited a shorter incubation period, and the hatchlings had a faster growth rate. However, the eggs incubated at 27.5°C showed no embryo mortality with higher hatching success. Hatchling morphology was also more affected by egg size and mass than incubation temperature. These results suggest that the artificial manipulation of incubation temperature should be carefully considered to understand how current and future climate changes may affect the survival of the red-footed tortoise (*Chelonoidis carbonaria*), including its impact on male and female distribution, hatching rate, initial growth rate, and individual survival until adult fertile age.

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CHAPTER III

STARCH AND FIBER INTAKE EFFECTS ON ENERGY METABOLISM, GROWTH, AND CARAPACIAL SCUTE PYRAMIDING OF RED-FOOTED TORTOISE (*Chelonoidis carbonaria*).

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ABSTRACT

Carapace changes are a major problem, seen in many zoos around the world. The objective was to determine the effect of two diets, one with a high fiber content and the other with a high starch content, on energy metabolism, nutrient digestibility, and growth of the red-footed tortoise. Following a completely randomized design, with 20 hatchlings (10 hatchlings per feed). The evaluations performed on the animals included: determination of the apparent digestibility coefficients of the nutrients and gastrointestinal transit time at 5 and 11 months; determination of the rest and postprandial metabolic rate at 6 and 12 months; evaluation of the growth and carapacial scute pyramiding; and determination of body composition by dual energy X-ray absorptiometry (DXA). Animals fed with the high starch diet showed higher mass-specific dry matter intake (12.75±3.11mg versus 10.20±3.37mg), shorter transit times (2.95±1.01 days versus 4.23±1.40 days) and gastrointestinal retention of food (8.09±2.05 days versus 10.05±2.41 days), and higher digestive efficiencies. However, the apparent digestibility of crude protein was higher for the high fiber diet. Resting and post-prandial metabolic rate were not affected by the diet. At 13 months, the animals fed with the high starch diet presented wider plastrons and carapaces, and higher growth rates of the carapace width. Also, these animals developed pyramiding doming with greater intensity and had lower mineral content (1.88±0.15% versus 2.15±0.19%) and bone density (0.13±0.01g/mm^2 versus 0.15±0.02g/mm^2). In conclusion, foods with higher digestible energy, such as high starch content, can induce more accelerated carapace growth with less mineralization.

Keywords: *Chelonoidis, digestive response, heat increment, metabolic rate, body composition, pyramiding.*

INTRODUCTION

Structural carbohydrates play nutritional and physical functions within the gastrointestinal tract (Van Soest, 1994). At the nutritional level, their fermentation provides energy to the host in the form of short-chain fatty acids (Van Soest, 1994; Atlas and Bartha, 1998; Donoghue and McKeown, 1999; Stevens and Hume, 2004), vitamin B and vitamin K (Atlas and Bartha, 1998; Hooper et al., 2002). In respect of physical function, the neutral detergent fiber adds bulk to the diet, resulting in a slower digesta passage rate and allowing more time for microbial digestion and fermentation to occur (NRC, 2007).

Understanding population responses to environmental change requires detailed information about the influence of environmental factors on individuals' bioenergetic fluxes on producing energy specifically (Zaidan and Beaupre, 2003). Congdon et al. (1982) defined energy production as total energy from the energy intake less the energy lost by respiration, feces, and nitrogenous waste. In the energy lost by respiration, the Specific Dynamic Action (SDA) is a critical and potentially significant component (Zaidan and Beaupre, 2003), described as the cost of processing a meal (Secor, 2009). Tortoises are hindgut fermenters (Stevens, 1988) and can digest foliage low efficiently than ruminant mammals (Bjorndal, 1987), over several days (Hamilton and Coe, 1982). As Hailey (1998) expresses, this long duration of digestion could contribute to different regions of the gut to the SDA. However, to our knowledge, the effect of specific nutrients as dietary fiber or starch has not been evaluated yet in herbivorous or omnivorous tortoises.

A negative correlation has been described between the dietary cell wall (NDF) content and dry matter digestibility in adults of Red-footed, Yellow-footed tortoises (*Chelononoidis denticulata*), juveniles of Galapagos tortoises (*Geochelone nigra*), and Desert tortoises (*Gopherus agassizii*) (Bjorndal, 1989; Hatt et al., 2005). An adverse effect of dietary lignocellulose (ADF) and organic matter digestibility was observed in Galapagos tortoises. These results evidence that, in tortoises, the diet's fiber content has a reducing effect on digestibility efficiencies. The dietary associative effect occurs when one item of the diet can affect the digestive efficiency of another nutrient either positively or negatively (Bjorndal, 1991). This effect is more common in herbivorous and omnivorous

species. Its occurrence may increase because of its microbial fermentation (McWilliams, 2005).

Subsequently, inadequate diet and husbandry mismanagement may cause the development of metabolic defects that affect the bones' morphology and function, which is the most common medical disorder in captive chelonians (Fledelius et al., 2005). Pyramiding has been related to accelerated growth and lack of proper bone mineralization, in this condition, the synchronous growth of bone and scute attempts to continue can result in a layer of weakness which can make the bones of the shell porous and spongiform (Redrobe, 1996; Highfield, 1996; Senneke, 2003).

Greater growth rates under captive conditions are most likely due to intense feeding or high amounts of offered diet (Furrer et al., 2004; Ritz et al., 2010). As fibrous material is not easily or completely digested, it is common that captive tortoise diets present low fiber content and are rich in starch, which is a factor related to accelerated growth (Highfield, 1996; Senneke, 2003). Highfield (2010) described an incorrect diet, specifically high protein and high energy, as a possible cause of abnormal growth. On the other hand, increasing dietary starch in other species such as fish, influenced by body composition, showed a positive relationship with lipid content and adverse effects on ash content (Wang et al., 2005; Wang et al., 2017; Sulaiman et al., 2020). However, the effects of dietary starch and fiber levels on growth performance, pyramiding development, body composition, and bone density in captive Red-footed tortoises have not been evaluated yet.

In this concern, the diet has been recognized as the critical factor for the discrepancy in digestive response, growth rate, and life expectation between free-ranging and captive animals (Furrer et al., 2004; Hatt et al., 2005). Fiber should be provided in the diet in sufficient amounts to fulfill the physical and nutritional function simultaneously. However, these amounts have yet to be objectively quantified for Red-footed tortoises. Thus, in the present study, we evaluate the effect of high levels of dietary starch and fiber on energy metabolism in hatchlings of omnivorous Red-footed tortoise (*Chelonoidis carbonaria*). We hypothesize that animals fed with a high fiber diet will show lower feed intake and digestive efficiency due to their greater volume and higher content of structural carbohydrates. Also, this diet would be more challenging to digest, which can produce more significant energy costs during the post-prandial metabolism (SDA) and will result in a slower growth rate with smaller and lighter hatchlings. Contrarily, as starch is a sugar of easy assimilation, animals fed with a higher level of starch will present

higher content of body fat content, faster growth rates, and possible pyramiding doming with lower bone mass and density.

MATERIALS & METHODS

All procedures, animal care, animal use, and treatment were performed following the Animal Ethics and Use Committee of the Sao Paulo State University (UNESP, for its acronym in Portuguese). This committee approved the protocol of the study (n°006095/19).

1. Experimental animals and location

Twenty unsexed red-footed tortoises' hatchlings (*Chelonoidis carbonaria*) were used from hatching until 13 months old, with an average body mass at hatching of 39.60±5.38g. These hatchlings were obtained from the incubation process of 50 eggs, produced by adults' tortoises from three different captivity centers: Bosque Municipal Fabio Barreto, Ribeirão Preto; Centro de Medicina e Pesquisa em Animais Selvagens (CEMPAS) of FMVZ/UNESP, Campus Botucatu; and Zoológico de Ribeirão Preto. Their use was possible after a previously authorization of IBAMA (SISBIO número 60562-1). Animals were individually housed in cages of 0.31x0.57x0.77m on indoor-outdoor conditions, with access to natural photoperiod and environmental temperature. During the winter and autumn, an electric heater or infrared lamps were used to avoid low ambient temperatures (<20°C).

2. Treatments

Two formulations were developed to present similar crude protein and gross energy content. The same ingredients were used on both formulations, but to achieve a high starch content on the high starch diet purified corn starch was used, and to achieve high fiber content on the high fiber diet sugarcane fiber was used at similar inclusion amounts. The remaining ingredients were kept constant (Table 1). Ingredients were mixed and ground in a hammer mill with a 0.8mm screen sieve size, and then extruded in a single screw extruder with 250kg/h capacity (MEX 250, Manzoni Industrial, Campinas, SP) at the Extrusion Laboratory of the Faculdade de Ciências Agrárias e Veterinárias da UNESP, campus de Jaboticabal. After extrusion, the diets were dried on a double-step

drier at 110 °C for 15 min and coated with sunflower oil, poultry fat, and liquid palatant.

TABLE 1. Chemical composition in dry matter of experimental diets for growing tortoises with different starch and fiber contents.

Ingredients	High Fiber	High Starch
Proportion (%)		
Soybean meal 45%	16.49	16.49
Wheat bran	15.00	15.00
Sugarcane fiber	13.45	0.00
Corn Starch	0.00	13.45
Poultry meal	10.00	10.00
Wheat flour	10.00	10.00
Beet pulp	10.00	10.00
Alfalfa hay, grind	10.00	10.00
Corn Grain	4.00	4.00
Poultry fat	3.00	3.00
Liquid palatant	2.00	2.00
Cane yeast	2.00	2.00
Whole flaxseed	1.50	1.50
Dicalcium phosphate	0.79	0.79
Vitamin-mineral premix[1]	0.50	0.50
Calcium carbonate	0.50	0.50
Choline chloride	0.27	0.27
Common Salt	0.25	0.25
Mold inhibitor[2]	0.10	0.10
DL - Metionine	0.09	0.09
Antioxidant [3]	0.05	0.05
Chemical composition (% DM)		
Crude Protein	25.00	26.00
Crude Fiber	14.16	6.60
Neutral detergent fiber	39.20	24.85
Starch	16.08	27.71
Acid hydrolyzed fat	6.97	6.54
Calcium	1.10	1.10
Phosphorus	0.70	0.70
Gross energy (kJ*g DM^{-1})	19.52	18.85

1- Addition per kg of feed: Vitamin A – 18.750 UI, Vitamin D3 – 1.500 UI, Vitamin E – 125 UI, Vitamin K3 - 1.5 mg, Vitamin B1 – 5 mg, Vitamin B2 – 16.2 mg, Pantothenic acid– 37.5 mg, Vitamin B6 – 7.5 mg, Vitamin B12 – 45 mcg, Nicotinic acid – 0.625 mg, Folic acid– 0.75 mg, Biotin – 0.315 mg, Iron – 100 mg, Copper – 9.25 mg, Manganese – 6.25 mg, Zinc – 150 mg, Iodine – 1.87 mg, Selenium – 0.135 mg

2- Mold-zap Aquativa. Composition: ammonia diproprionate, acetic acid, sorbic acid and benzoic acid (Alltech of Brazil Agroindustrial Ltda.).

3- Banox. Composition: BHA, BHT, propyl gallate, and calcium carbonate (Alltech of Brazil Agroindustrial Ltda.)

Immediately after hatching, hatchlings were randomly distributed between the two treatments. They were then fed with their experimental diet since they have first eaten. The first month of life was considered an adaptation period to learn how to eat, and consume the egg yolk. The daily feed was offered at approximately 2.5% of body mass, and feeding was performed every day until six months old, and after that, tortoises were fed every other day (4 days a week). Water was offered *ad libitum*. The amount of offered and residual feed was weighed daily with a scale of 5kg capacity and ± 0.01 g sensitivity to obtain the daily intake of feed, that was recorded.

3. Response variables

Coefficient of total tract apparent digestibility of nutrients and gastrointestinal transit time

The coefficients of total tract apparent digestibility of nutrient and energy and the gastrointestinal transit time were determined two times, at 5 and 11 months old. For digestibility evaluation, chromium oxide (Cr_2O_3; Merck KGaA, Darmstadt, Germany) was used as an inert indigestible marker, added in 0.25% of dry matter of the offered feed. Ferric oxide (Fe_2O_3; 99.5% Êxodo cientifica, São Paulo-SP, Brazil) was used as an external marker in a proportion of 2.5% of dry matter to evaluate the diets gastrointestinal transit time. At each evaluation time (5 and 11 months), the gastrointestinal transit time was evaluated twice: one week after starting to offer the experimental diets added of Cr_2O_3; and at the end of the digestibility evaluation (Carciofi et al., 2007).

A single dose of ferric-labeled feed was given to study the digesta passage (transit and retention time), being registered as the time when animals started eating it. The transit time was described as the period between the food consumption and the moment when first red-marked feces were observed; and retention time as the interval until the elimination of first normal color feces (Van Weyenberg et al., 2006). To assure animals were in a constant Cr_2O_3 fecal elimination phase, hatchlings were fed the diet supplemented with Cr_2O_3 for one week, then dosed with ferric-labeled feed, and fecal collection for digestibility evaluation started after the first red-marked feces appeared. Feces collection lasted at least one month to have enough samples for chemical analysis, and

feces were stored at -20°C. During collection, feces were scored according to a 7 points system (Mendoza and Furuta, 2020).

At the end of the collection period, the feces were thawed, homogenized, and pooled to form a single sample per animal. Before performing the laboratory tests, the feces were dried in a forced-air oven at 55ºC for 72 hours and ground in a cutting mill with a 1mm sieve. Feed and feces were analyzed following the methods of the Association of Official Analytical Chemists (1995) to determine the dry matter by the oven-drying method (934.01), ash by muffle furnace incineration (942.05), crude protein applying the Kjeldahl method (954.01), ether extract in a Soxhlet system after acid hydrolysis (954.02), the neutral detergent fiber (NDF) analysis included a pretreatment of the sample with heat-stable alpha-amylase and was expressed exclusive of residual ash (aNDFom; Udén et al. 2005), and crude fiber by the Weende's method. The gross energy content of feces and diets were determined through bomb calorimetry on an automated Parr diabatic calorimeter (PARR Instrument-1281, Moline, EUA). Chromic oxide was quantified according to the method described by Fenton & Fenton (1979). The analytical calibration curve was built using standard solutions, prepared by digesting known amounts of Cr2O3 (1mg, 2mg, 4mg, 8mg, 10mg, 14mg, 20mg, and 26mg). Chromium was determined in a spectrophotometer (Labquest Bio 2000. Labtest Diagnóstica S.A., Lagoa Santa, Brazil) at 450 nm. All analysis was carried out duplicated and repeated when the coefficient of variation between replicates was higher them 5%.

Coefficient of total tract apparent digestibility of dry matter (DC_{DM}) and nutrients ($DC_{NUTRIENT}$) were calculated as described elsewhere (Harshaw, 2012):

$$DC\ (Dry\ Matter, \%) = 100 - (100 * \frac{\%Cr_2O_3\ food}{\%Cr_2O_3 feces})$$

$$DC\ (Nutrient, \%) = 100 - (100 * \frac{\%Nutrient\ in\ feces * \%Cr_2O_3\ food}{\%Nutrient\ in\ food * \%Cr_2O_3 feces})$$

Daily food intake and apparent digestibility coefficients were used to calculate and express the specific-mass intake per gram of metabolic mass of dry matter ($mg*day^{-1}$), digestible nutrients (DNut, $mg*day^{-1}$), gross energy (GEI, $J*g*day^{-1}$) and digestible energy (DEI, $J*g*day^{-1}$). The reptile allometric exponent of 0.86, described by Bennett and Dawson (1976), was used for the metabolic mass calculation.

Specific Dynamic Action (SDA) of diets

We quantified pre and postprandial metabolism via indirect calorimetry by measuring the oxygen consumption rate and the carbon dioxide production rate using open-flow system respirometry. Respirometry chambers were kept in a water bath to maintain the inside temperature constant and close to the preferred temperature (28°C), being monitored every 10 minutes. The chambers' internal temperature at each thermal regime was kept constant by using a water bath by using a 50W aquarium thermostat (AP-801, Ace Pet, Brazil). Moisture was maintained between 50-80%, and animals were kept under an artificial photoperiod of 12 hours of light and 12 hours of dark.

One week before measurements, hatchlings were habituated to the experimental chamber to reduce the stress and avoid the overestimation of heat production due to activity. Specific Dynamix Action's evaluations of the diets were performed at 6 and 12 months old in different seasons; depending on the animal age and size, they were randomly assigned to respirometry chambers of 900mL for evaluations at six months old and chambers of 2500mL for animals with 12 months of age. Inside of the chambers, animals were ventilated by positive pressure with a constant flux of air (250 ml/min) using an aquarium air pump (Jeneca AP-3500) coupled with a rotameter. A sub-sample of air (100ml) of the baseline and the output of the chamber was transported by the negative pressure of the SS4 pump (Sable System, Las Vegas, NV, USA), to be conducted to the desiccant (Drierite^{R} water-absorbent, W. A. Hammond DRIERITE, Xenia, OH, USA), before passing through oxygen and carbon dioxide analyzers (Sable Systems, Las Vegas, NV, USA). Certified gas mixture (21.1% O_2 and 1.03% CO_2) and pure nitrogen were used to calibrate the gas analyzers. Just data after the first hour (stabilization time) were considered for calculating metabolic rates. Also, these were measured for ten consecutive hours. In the morning, all animals were weighed before placing them into the chambers. At the end of the daily measurements, these animals were removed from the chambers and were housed into individual cages where water was offered *ad libitum*.

On the first day of evaluation (day 0), we measured the resting metabolic rate (RMR), with animals fasted for ten days before the evaluation (including an acclimatization period at 28°C during the two last days). Then, we *ad libitum* fed the animals with the experimental diets (7 animals per diet) for three days in the

early morning before starting the metabolism measuring in chambers (day 1 to 3). Offered and refused food was measured, and the intake was recorded. From day 4 onward, we kept the animals under prolongate fasting period for a maximum of 10 days to allow metabolism to return to RMR values. After 10d, if tortoises had not yet returned to resting levels, they were removed from the chambers, and the measurement was finished.

Data was collected and analyzed using ExpeData v.19 software (Sable System, Las Vegas, NV, USA). Values of oxygen consumption ($\dot{V}O_2$, L*kg^{-1}*min^{-1}) and carbon dioxide production ($\dot{V}CO_2$, L*kg^{-1}*min^{-1}) were used to calculate the resting metabolic rate (RMR, kJ*kg*day^{-1}), and maximum metabolic rate after feeding (FMR_{MAX}, kJ*kg*day^{-1}), corresponding to the peak heat production in the fed state. The heat increment (HI, kJ*kg*day^{-1}) was determined by the difference between FMR_{MAX} and RMR. The heat production by indirect calorimetry was estimated by Brouwer (1965) equation, which considered the $\dot{V}O_2$and $\dot{V}CO_2$:

$$HP\ (kJ)=16.17*\dot{V}O_2+5.02*\dot{V}CO_2$$

Where:

HP (kJ) is the total heat production.

$\dot{V}O_2$ (L) is the volume of consumed oxygen.

$\dot{V}CO_2$ (L) is the volume of production of carbon dioxide.

The specific dynamic action was described with the following parameters: the time to peak; the duration of the peak; time to return to resting values; the factorial scope of O_2 and CO_2 (O_2 Scope and CO_2 Scope) described as $\dot{V}O_{2\,Max}/\dot{V}O_{2Rest}$ and $\dot{V}CO_{2\,Max}/\dot{V}CO_{2Rest}$. Specific heat increments of the diets were expressed in three different terms: HI_{DM} (HI*g^{-1}), where the HI (kJ*kg*day^{-1}) is divided between the total intake of dry matter (g); HI_{DEI} (HI*kJ^{-1}) obtained by dividing HI (kJ*kg*day^{-1}) between the total intake of digestible energy (kJ); and SDA coefficient (%), defined as the SDA quantified as a percentage of digestible meal energy.

Growth and Estimated Body Composition

To measure growth rates, biometric measurements (±0.01mm) and body mass (±0.01g) of the hatchlings were recorded every two weeks. For each

tortoise, the following parameters were measured with a caliper ruler (ToolsWorld®, Brazil): straight carapace length (CL), measured on the midline of the shell from the anterior midpoint of the nuchal scute to the posterior tip of the longer of the pair of posterior marginal scutes; straight carapace width (CW) between 5th and 6th marginal scute; carapace height (CH) between 6th and 7th marginal scute; plastron straight length (PL), measured on the midline of the plastron from the jugular notch to anal notch; and plastron straight width (PW) at the 3rd line of the plastron. The pyramiding degree was evaluated by measuring the two sides and the base of the wedge-shaped indentation between the 2nd and 3rd vertebral scute to calculate its height and area.

For body composition evaluation, the lean body mass (%), fat content (%), bone mass content (%), and bone mass density (g/mm^2) were estimated by dual-energy x-ray absorptiometry when animals reached 250 g of body mass. Animals were immobilized by keeping them under low temperature (10°C) for 30-45 minutes to decrease their metabolism. The scanning was performed using a Discovery Wi™ DXA scanner equipment (Hologic, Bedford, MA) with QDR Software (for Windows) designed for the whole-body examination of laboratory animals (Hologic, Bedford, MA). Before scanning, the animals' weight was recorded, and the densitometer was quality-checked using Hologic® calibration models (anthropomorphic spine phantom and small-step phantom), as recommended by (Stone, 2010). Individuals were positioned in the dorsoventral projection and scanned twice without repositioning.

4. Statistical analysis

A factorial arrangement of treatments was used with two factors, a repeated measure mixed two-way ANOVA model was used. The factors varied among treatments and was diet with two levels (high starch or high fiber) and age with two levels (5 or 11 months old) in a 2 x 2 factorial arrangement totaling 4 treatments. The effects of both factors and their interaction were evaluated on the mass-specific daily intake of feed (mg*day^{-1}), digestible energy (J*day^{-1}), and digestible nutrients (mg*day^{-1}); daily production of feces (g*day^{-1}); transit and retention time (days); fecal score; and apparent digestibility coefficient of energy and nutrients (%). Also, the effect of age on the mass-specific intake of gross

energy in terms of body metabolic mass ($J*g*day^{-1}$) and body mass gain ($g*day^{-1}$) during thirteen months of the experiment was adjusted to a General Linear Model (GLM) and tested by Tukey-test.

To analyze both diets' specific dynamic action at two different ages, a factorial design was used with two factors repeated measures mixed model two-way ANOVA: age (6 and 12 months old) and diet (high starch and high fiber). Age and diet effects and their interaction were evaluated on the RMR ($kJ*kg*day^{-1}$), the FMR_{MAX} ($kJ*kg*day^{-1}$), O_2 Scope, CO_2 Scope, feed intake (%), heat increment ($kJ*kg*day^{-1}$), HI_{DM} ($HI*g^{-1}$), HI_{DEI} ($HI*kJ^{-1}$), and SDA coefficient (%). Data of daily intake of gross and digestible energy ($kJ*day^{-1}$), the estimated daily intake of the metabolic energy less heat increment MEIp-HI ($kJ*day^{-1}$), daily RMR ($kJ*day^{-1}$), and the respective body mass at both ages were log_{10} transformed. This data, except for MEIp-HI, was submitted under linear regression with body mass.

To evaluate the diet effect on the final body mass (g), final biometric measurements (mm); daily gain of body mass ($g*day^{-1}$); daily growth of carapace length, width, and height ($mm*day^{-1}$); daily growth of plastron length and width ($mm*day^{-1}$); wedge-shaped components; and estimated body composition at 250 g of body mass, a one-way ANOVA test with diet as a factor was conducted.
All analyses were conducted by R Studio Software (3.2.3, EUA version). A p-value less than 0.05 was considered the level of significance.

RESULTS

Coefficient of total tract apparent digestibility of nutrients and gastrointestinal transit time

Data on apparent digestibility and gastrointestinal transit time of both experimental diets at 5 and 11 months-old are given in Table 2. Age effect with higher digestibility at 11 months was observed for crude protein, acid-hydrolyzed fat, neutral detergent fiber, and gross energy ($P<0.05$). A diet effect was observed for all evaluated nutrients, with higher values for the starch diet ($P<0.05$), except for a similar digestibility for acid hydrolyzed fat ($P>0.05$). No diet x age interaction was observed ($P>0.05$). Age and diet also significantly influenced feces production, higher for the high fiber diet and animals at 11 months ($P<0.05$).

TABLE 2. Coefficient of total tract apparent digestibility and gastrointestinal transit time of experimental diets with high starch or high fiber contents for growing tortoises at 5 and 11 months old.

Parameter	Age	Diet		Mean	p-value		
		High Starch n=10	High Fiber n=10		Age	Diet	Interaction
Coefficient of total tract apparent digestibility (%)							
Dry matter	5 months-old	76.40±2.32	65.37±1.55	70.88±5.98	ns	<0.01	ns[1]
	11 months-old	76.48±2.15	67.55±4.36	71.68±5.63			
	Mean	76.44±2.18	65.83±1.95				
Crude protein	5 months-old	78.36±1.67	80.98±1.38	79.74±2.00	<0.01	<0.01	ns
	11 months-old	80.70±1.41	84.28±2.16	82.58±2.57			
	Mean	79.53±1.92	82.63±2.45				
Acid-hydrolyzed fat	5 months-old	47.16±11.76	50.51±10.72	48.59±11.03	0.04	ns	ns
	11 months-old	52.38±9.90	61.02±3.87	56.45±8.68			
	Mean	49.92±10.80	56.52±9.02				
Starch	5 months-old	99.59±0.04*	99.27±0.29*	99.34±0.29	<0.01	0.04	ns
	11 months-old	99.21±0.28*	98.83±0.38	99.00±0.38			
	Mean	99.29±0.30	99.03±0.40				
Neutral detergent fiber	5 months-old	54.93±20.20*	51.59±2.98*	53.26±9.47	0.03	0.03	ns
	11 months-old	69.99±2.32*	58.96±8.23*	64.48±8.47			
	Mean	62.46±12.04	55.28±7.42				
Gross energy	5 months-old	74.89±2.15	66.81±1.42	70.85±4.51	<0.01	<0.01	ns
	11 months-old	76.58±2.99	70.65±4.19	73.24±4.48			
	Mean	75.73±2.68	68.10±2.35				
Fecal Characteristics							
Production of feces, as-is (g*day^{-1})	5 months-old	0.88±0.17	1.23±0.41	1.06±0.36	ns	<0.001	ns
	11 months-old	0.96±0.25	1.19±0.33	1.08±0.31			
	Mean	0.92±0.21	1.21±0.36				
Production of dry feces (g*day^{-1})	5 months-old	0.28±0.07	0.41±0.13	0.34±0.12	0.04	<0.01	ns
	11 months-old	0.35±0.09	0.47±0.12	0.41±0.12			
	Mean	0.31±0.08	0.44±0.12				
Gastrointestinal transit time (days)	5 months-old	2.80±0.77	3.52±0.67	3.16±0.79	0.02	<0.01	ns
	11 months-old	3.11±1.22	4.95±1.60	4.03±1.68			
	Mean	2.95±1.01	4.23±1.40				
Gastrointestinal retention time (days)	5 months-old	7.01±1.26	8.76±1.74	7.93±1.74	<0.01	<0.01	ns
	11 months-old	9.07±2.18	11.35±2.34	10.21±2.49			
	Mean	8.09±2.05	10.05±2.41				
Fecal Score[2]	5 months-old	2.05±0.32	2.08±0.43	2.06±0.37	<0.01	ns	ns
	11 months-old	2.39±0.43	2.81±0.20	2.60±0.39			
	Mean	2.22±0.41	2.45±0.50				

Digestible Energy determined in vivo (kJ*g DM^{-1}) = High fiber 13.29 and High starch 14.27

[1] *ns*: not significant (P>0.05)

[2] Non parametric-Kruskal-Wallis test

*insufficient feces sample: Starch analysis [High Starch at 5 months old, n=2; High Fiber at 5 months old and High Starch at 11 months old, n=8] and NDF analysis [High Starch at 5 months old, n=2; High Fiber at 5 months old and High Starch at 11 months old, n=4; High Fiber at 11 months old, n=6].

The fecal score presented only an age effect, better for animals at 11 months (P<0.05). Difficulties related to obtaining enough fecal sample to conduct

all chemical analyses were observed, and starch and neutral detergent fiber digestibility was evaluated in less than 10 animals per feed and age. The gastrointestinal transit time and retention time were longer at 11 months and for animals fed the high fiber diet (P<0.05). No diet x age interaction was observed (P>0.05).

Feed, nutrients, and energy intake

The effects of age, diet, and their interaction on the daily intake of dry matter, digestible nutrients, and energy related to the metabolic body mass (body mass$^{0.86}$), are given in Table 3. All these variables were significantly affected by age, being lower at 11 months than at 5 months old (P<0.05). For digestible energy, for example, at 11 months, the intake was 30.1-33.25% lower, highlighting a marked reduction in metabolic rate at this age. Diet effect was observed only for digestible neutral detergent fiber intake, higher for animals fed the high fiber diet, and for digestible starch intake, higher for animals fed the high starch diet (P<0.05).

On the other hand, during the thirteen months of the experiment, a significant effect of age was observed on gross energy intake (P<0.00) and daily body mass gain (P<0.00), while the hatchlings were getting older, they presented lower specific-mass intakes of gross energy and greater growth rates (Fig. 1).

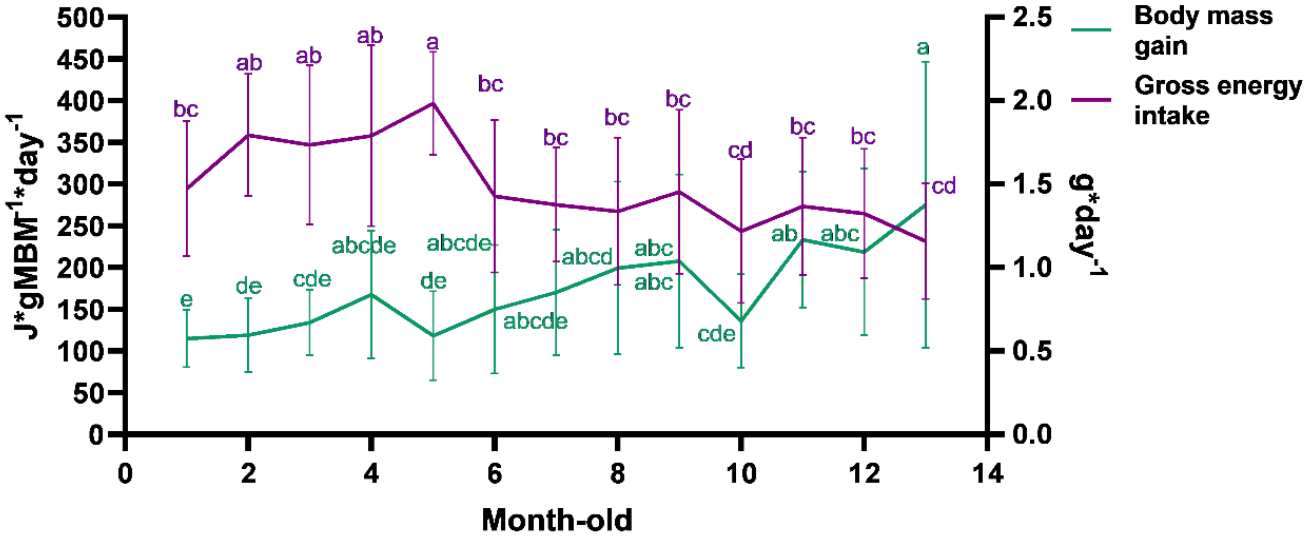
TABLE 3. Intake of dry matter and digestible nutrients and energy by growing tortoises at 5 and 11 months old fed with the high starch or high fiber diet.

Parameter	Age	Diet		Mean	p-value		
		High Starch n=10	High Fiber n=10		Age	Diet	Interaction
Dry matter (mg*day^{-1})	5 months-old	19.50±1.58	18.91±3.54	19.21±2.69	<0.001	ns	ns[1]
	11 months-old	13.81±3.60	11.73±3.42	12.77±3.58	<0.01	<0.01	ns
	Mean	16.66±3.98	15.32±5.00				
Digestible dry matter (mg*day^{-1})	5 months-old	14.90±1.20	12.35±2.29	13.62±2.21			
	11 months-old	10.61±2.96	8.04±2.90	9.33±3.14	<0.01	<0.01	ns
	Mean	12.75±3.11	10.20±3.37				
Digestible protein (mg*day^{-1})	5 months-old	4.20±0.33	4.17±0.75	4.18±0.57			
	11 months-old	3.11±0.83	2.71±0.84	2.91±0.84	<0.01	ns	ns
	Mean	3.65±0.83	3.44±1.08				
	5 months-old	0.65±0.15	0.78±0.14	0.70±0.15	<0.01	ns	ns

Digestible acid-hydrolyzed fat (mg*day^{-1})	11 months-old	0.52±0.16	0.54±0.14	0.53±0.15			
	Mean	0.58±0.17	0.64±1.0.18				
Digestible starch (mg*day^{-1})	5 months-old	6.11±0.11*	3.46±0.62*	3.99±1.24	<0.01	<0.01	ns
	11 months-old	4.14±1.07*	2.05±0.60	2.98±1.34			
	Mean	4.53±1.25	2.68±0.93				
Digestible neutral detergent fiber (mg*day^{-1})	5 months-old	0.88±0.18Ba	1.64±0.32Aa	1.33±0.47	<0.001	<0.001	<0.01
	11 months-old	0.76±0.18Aa	0.92±0.21Ab	0.85±0.21			
	Mean	0.81±0.18	1.28±0.45				
Digestible energy (J*day^{-1})	5 months-old	292.99±23.67	271.10±49.22	282.05±39.23	<0.01	ns	ns
	11 months-old	213.55±60.86	180.53±64.17	197.04±63.18			
	Mean	253.27±60.67	225.82±72.50				

Mean intake per gram of metabolic mass; *Metabolic mass= body mass^{0,86}* (Bennett and Dawson, 1979)
Digestible Energy determined in vivo (kJ*g DM^{-1}) = High fiber 13.29 and High starch 14.27
[1]*ns*: not significant (P>0.05)
* insufficient feces sample: High Starch at 5 months old, n=2; High Fiber at 5 months old and High Starch at 11 months old, n=8.
[a, b] Means in a column without a lowercase letter in common are different (P<0.05)
[A, B] Means in a row without a capital letter in common are different (P<0.05)

FIGURE 1. Gross Energy Intake and Body Mass Gain per Age of hatchlings from 0-13 months-old



Specific-mass Intake of Gross Energy Intake J*gMBM^{-1}*day^{-1}: Mean Intake of gross energy per gram of metabolic body mass. *Metabolic mass= body mass^{0,86}* (Bennett and Dawson, 1976). **Body mass gain g*day^{-1}:** mean of body mass gain per day. Different letters in the same color line indicate a significant difference (P<0.05) between months of age (age effect).

Specific Dynamic Action of experimental diets

No diet effect or diet x age interaction was observed (P>0.05), as shown in Table 4. Tortoise age, on the other hand, influenced most variables, with higher feed intake, resting and maximum metabolic rate, and specific heat increment in terms of dry matter and digestible energy intake for animals at 6 months than 12 months old (P<0.05).

TABLE 4. Feed intake and parameters of specific dynamic action observed for tortoises at 6 and 12 months, fed with the high fiber and high starch diet maintained on an environmental temperature of 28°C.

Parameter	Age	Diet		Mean	p-value		
		High Starch n=7	High Fiber n=7		Age	Diet	Interaction
Resting metabolic rate ($kJ*kg^{-1}*day^{-1}$)	6 months-old	34.35±4.93	32.30±2.62	33.33±3.94			
	12 months-old	24.91±6.53	23.18±5.74	24.05±6.01	<0.001	ns	ns[2]
	Mean	29.32±7.45	27.44±6.45				
Maximum metabolic rate in feeding state ($kJ*kg^{-1}*day^{-1}$)	6 months-old	82.08±5.92	77.61±17.72	79.67±13.30			
	12 months-old	65.77±17.72	61.05±8.47	63.41±13.64	<0.01	ns	ns
	Mean	72.76±15.90	68.78±15.61				
O_2 Scope	6 months-old	2.35±0.45	2.37±0.57	2.36±0.49			
	12 months-old	2.14±0.29	2.65±0.73	2.43±0.62	ns	ns	ns
	Mean	2.25±0.38	2.52±0.65				
CO_2 Scope	6 months-old	2.75±0.41	2.38±0.52	2.58±0.48			
	12 months-old	2.56±0.70	3.23±1.01	2.92±0.91	ns	ns	ns
	Mean	2.66±0.56	2.87±0.92				
Feed Intake (%)	6 months-old	3.09±0.96	2.75±0.87	2.92±0.90			
	12 months-old	1.89±0.74	1.86±0.42	1.87±0.57	<0.01	ns	ns
	Mean	2.49±1.03	2.27±0.79				
HI ($kJ*kg^{-1}*day^{-1}$)	6 months-old	47.28±8.50	45.32±18.29	46.30±13.74			
	12 months-old	40.85±19.00	37.85±10.65	39.35±14.96	ns	ns	ns
	Mean	43.85±14.92	41.33±14.66				
HI_{DM} ($HI*g^{-1}$)	6 months-old	11.46±4.50	14.56±9.60	13.01±7.38			
	12 months-old	6.22±2.47	6.91±1.73	6.57±2.09	<0.01	ns	ns
	Mean	8.67±4.37	10.48±7.52				
HI_{DEI} ($HI*kJ^{-1}$)	6 months-old	0.69±0.22	0.80±0.23	0.74±0.22			
	12 months-old	0.42±0.17	0.50±0.13	0.46±0.15	<0.001	ns	ns
	Mean	0.53±0.23	0.62±0.23				

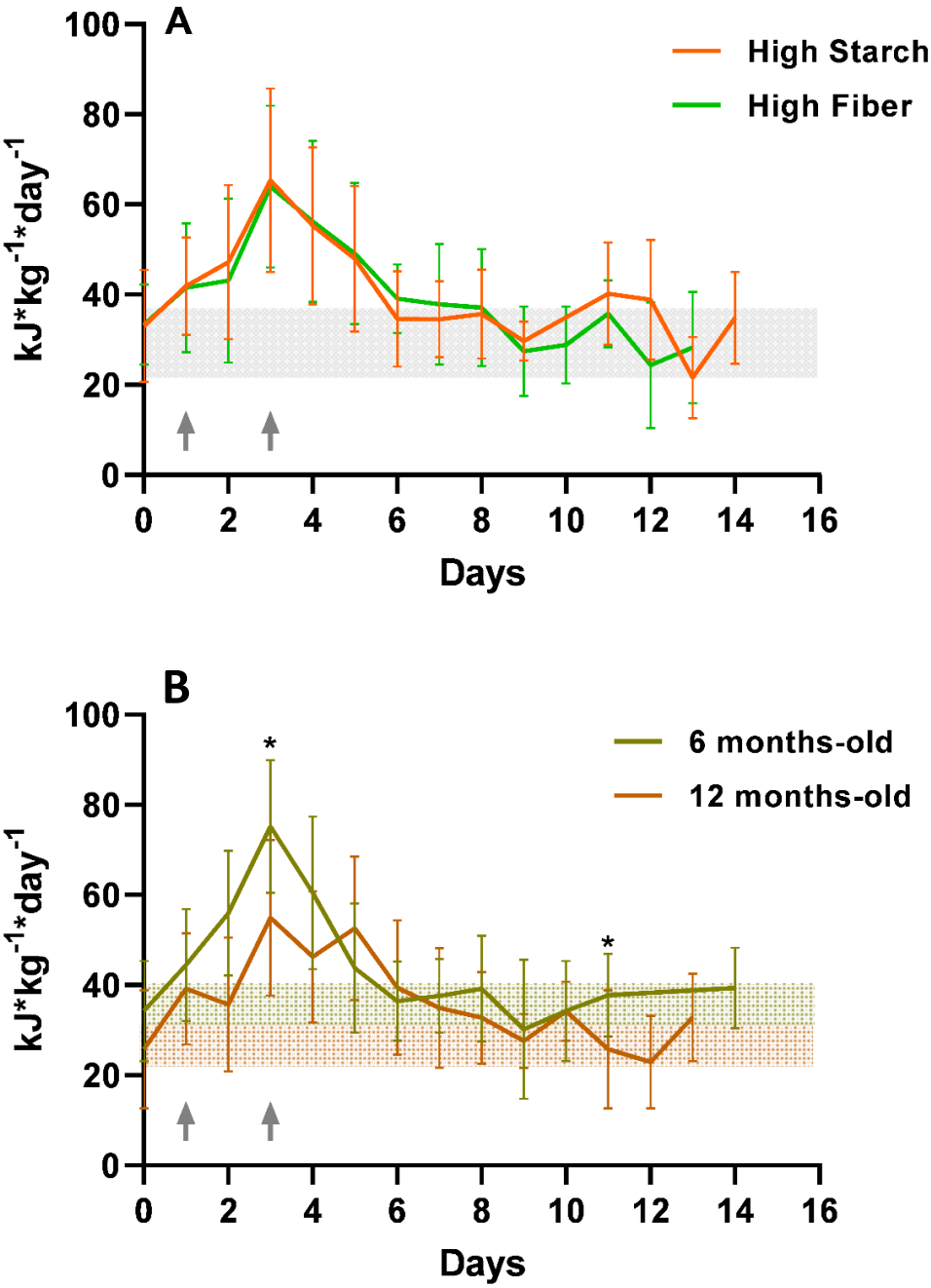
[1]Feed intake expresses in percentage related to the body mass.
[2]*ns*: not significant (P>0.05).
Digestible Energy determined in vivo ($kJ*g\ DM^{-1}$) = High fiber 13.29 and High starch 14.27.

The specific dynamic action responses of the tortoises fed with high starch and high fiber diet, maintained at 28°C, are illustrated in Fig. 2A., lasting no more than ten days. In Figure 2A is possible to observe that no diet effect was verified, with similar responses for high starch and high fiber diets ($P>0.05$). Differences were observed for tortoises at 6 and 12 months old (Figure 2B), with higher maximum heat production on day 3 and higher resting metabolic rate on day 11 for 6 months than 12 months old animals ($P<0.05$). The persistence of the peak was longer at 12 months old, about 72 hours approximately. Then, declining to the resting metabolic values appears to be slightly slower in older animals (12 months old). It is also possible to see in all curves that after feeding, heat production increased, reaching a peak on the third day of feeding, but it persisted less than 24 hours and declined to basal level on day 6, without diet or age effect ($P>0.05$).

Energy partitioning is explained in Fig. 3A., expressing the $\log_{10}$ transformed daily intake of gross energy, digestible energy, estimated metabolic energy less heat increment, and resting metabolic rate related to the body mass of Red-footed tortoises hatchlings at 6 and 12 months old. Also, this data is shown in linear regressions with the body mass, which resting metabolic rate equations presented a greater determination coefficient (Fig. 3B.).

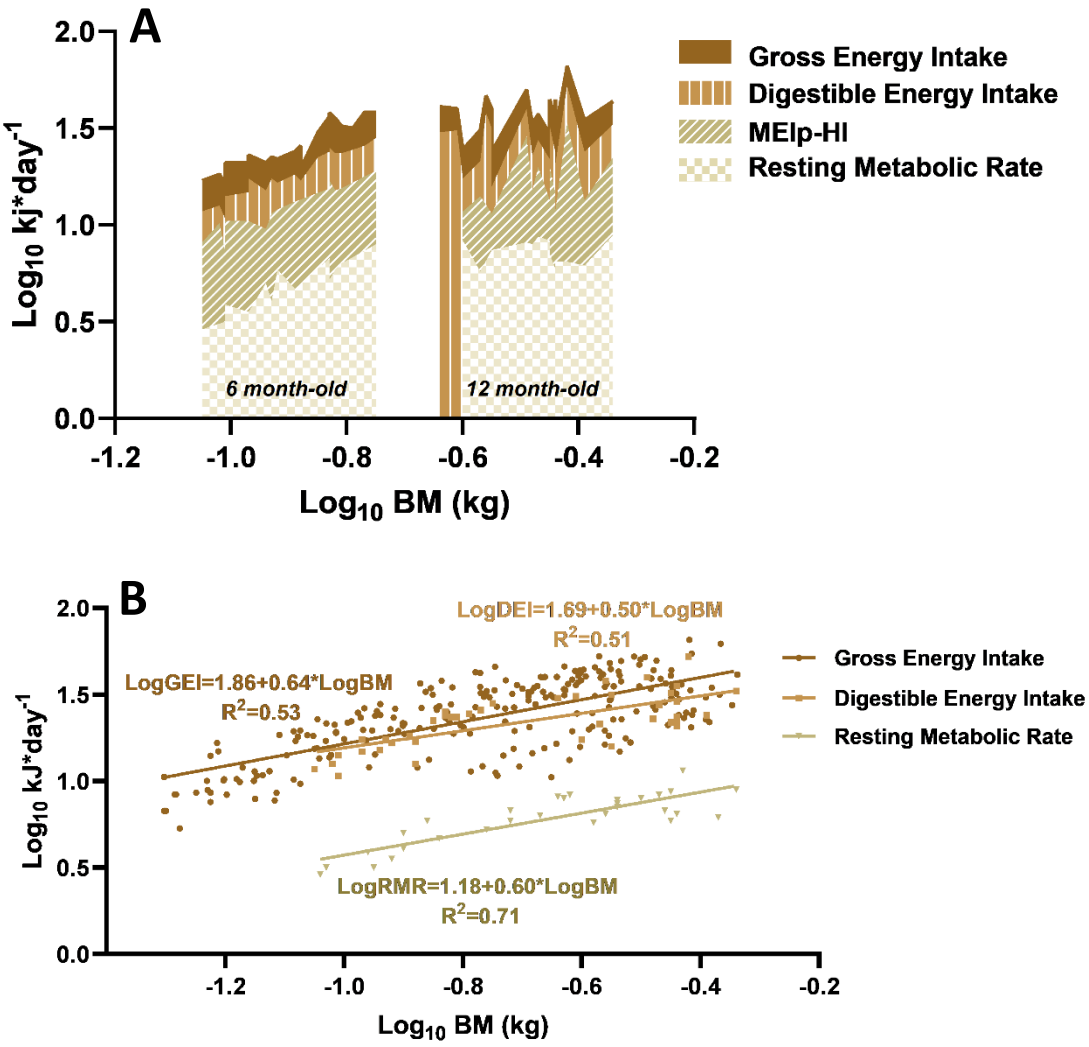
The maximum metabolic rate after feeding also increased with the feed intake. However, heat increment in terms of daily intake of digestible energy was qualitatively higher when animals were fed with a high fiber diet and/or at 6 months old. The SDA coefficient did not present effects of diet ($P=0.41$) or age ($P=0.18$), with mean values of 12.78±4.48% for high starch diet, 14.26±5.13% for high fiber diet, 12.22±4.46% and 14.65±4.93% at 6 and 12 months old, respectively. Finally, it was calculated that the 27.43±10.28% (at 6 months old) and 28.13±10.63% (at 12 months old) of digestible energy intake went to cover the resting metabolic rate.

FIGURE 2. Specific Dynamic Action of growing tortoises fed with high starch or high fiber diet, at 6 or 12 months old, maintained at an ambient temperature of 28°C.



A: Mean Specific Dynamic Action of tortoises fed the high starch and the high fiber diet. Daily mean values of energy production per kilogram of body mass. Day 0 corresponds to fast state; on days 1, 2, and 3, tortoises were fed, and from day 4 until the end, animals were private of food again. Mean values of animals at 6 and 12 months old. **B:** Mean Specific Dynamic Action of tortoises at 6 and 12 months old. Daily mean values of energy production per kilogram of body mass. Day 0 corresponds to fast state; on days 1, 2, and 3, tortoises were fed, and from day 4 until the end, animals were private of food again. Mean values of animals fed the high starch and the high fiber diets. The shaded areas correspond to the range of resting metabolic rate (Mean±SD). Grey arrows indicate the first and last day of feeding. * Significant difference between ages ($P<0.05$).

FIGURE 3. Energy Partitioning of hatchlings of red-footed tortoises at 6 and 12 months old related with body mass, transformed in Log_{10}.



A: Daily intake of gross and digestible energy, predicted metabolic energy (MEIp) less heat increment of the diet, and daily resting metabolic rate in hatchlings of 6 and 12 months old; related to body mass. Digestible energy was determined with the apparent digestibility coefficients obtained at 5 and 11 months old. Rest metabolic rate at 28°C. MEIp is the predicted intake of metabolic energy using a coefficient of 0.82 (Lickel, 2010). **B:** Linear regression Log10 of daily intake of gross and digestible energy and resting metabolic rate at 28°C with animals' body mass at 6 and 12 months old. The apparent digestibility coefficients obtained at 5 and 11 months old were also used in this graphic.

Growth and Estimated Body Composition

The initial biometric measurements of the hatchlings fed with both diets were similar (P>0.05; Table 5). At the end of the evaluated period of growth, carapace and plastron width and carapace width growth were lower for tortoises fed the high fiber diet (P<0.05). The final body mass was 12.5% lower, but this difference was not significant (P>0.05).

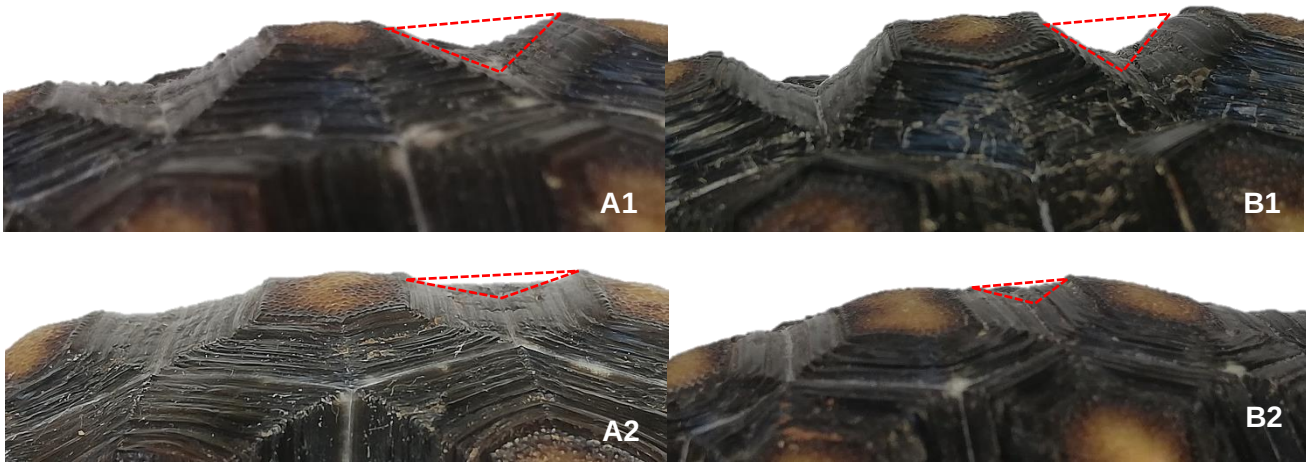
TABLE 5. Initial and final biometric parameters of growing tortoises fed with the high starch or a high fiber diet.

Parameter	Diet		P-value
	High Starch n=10	High Fiber n=10	
Initial body mass (g)	37.85±5.29	41.35±5.47	ns
Final body mass (g)	337.10±51.96	294.79±54.71	ns
Gain mass (g*day^{-1})	0.82±0.14	0.72±0.14	ns
Initial carapace length (mm)	49.91±5.13	53.81±4.06	ns
Final carapace length Growth (mm)	117.07±5.22	112.25±8.02	ns
Carapace length growth (mm*day^{-1})	0.18±0.01	0.17±0.02	ns
Initial carapace width (mm)	44.61±4.43	46.22±4.12	ns
Final carapace width (mm)	85.78±3.82	80.77±4.70	0.02
Carapace width growth (mm*day^{-1})	0.11±0.01	0.10±0.01	<0.01
Initial plastron length (mm)	45.75±4.62	46.65±4.53	ns
Final plastron length (mm)	100.06±4.88	95.87±6.74	ns
Plastron length growth (mm*day^{-1})	0.15±0.01	0.14±0.02	ns
Initial plastron width (mm)	40.36±5.14	41.46±7.58	ns
Final plastron width (mm)	72.75±2.24	70.86±3.27	0.04
Plastron width growth (mm*day^{-1})	0.09±0.01	0.08±0.02	ns
Wedge-shaped components			
Side 1 (mm)	7.03±0.76	6.75±0.90	ns
Side 2 (mm)	7.77±1.13	7.15±0.77	ns
Base (mm)	13.04±1.22	11.66±1.70	0.04
Height (mm)	3.69±0.69	3.77±0.39	ns
Area (mm^2)	22.66±8.01	22.09±4.84	ns

Pyramidal doming of scutes was observed in animals of both diets (Figure 4). However, no shell distortion or softening of shells was reported. Although the sides, height, and area of the triangle formed due to the pyramidal doming of the wedge-shaped scutes were statistically similar between tortoises fed both diets (P>0.05), its base was wider in animals fed with the high starch than the high fiber diet (P<0.05; Table 5). Considering the wedge-shaped area's geometry, as the base of the triangle formed was wider, the pyramidal formation was more intense in tortoises fed with the high starch diet.

The body composition of the hatchlings at 250g of body weight was similar in body fat and lean mass percentage (P>0.05), as shown in Table 10. However, the bone mass density and bone mass content were lower for growing tortoises fed with the high starch than the high fiber diet (P<0.05) (Table 6).

FIGURE 4. Degrees of pyramiding growth showed in animals fed with both experimental diets.



The red dotted line area indicates the wedge-shaped area of pyramiding growth**. A1**: Wedge-shaped area of a high degree of pyramiding in a tortoise fed with the high starch diet, **A2**: Wedge-shaped area of low degree of pyramiding in a tortoise fed with the high starch diet, **B1**: Wedge-shaped area of a high degree of pyramiding in a tortoise fed with the high fiber diet, **B2**: Wedge-shaped area of low degree of pyramiding in a tortoise fed with the high fiber diet.

TABLE 6. Estimated body composition of growing red-footed tortoises at 250g of body mass, fed with high starch or high fiber diets, determined by dual-energy X-ray absorptiometry.

Parameter	Diet		P-value
	High Starch n=8	High Fiber n=8	
Bone mass density (g/mm^2)	0.13±0.01*	0.15±0.02	0.02
Bone mass content (%)	1.88±0.15*	2.15±0.19	<0.01
Fat content (%)	7.49±2.35	6.49±1.68	ns
Lean mass content (%)	90.63±2.34	91.42±1.63	ns

DISCUSSION

Food intake, apparent digestibility, and passage time

The fiber content of both experimental diets used in this study is similar to other published tortoise zoo diets, such as for Galapagos giant tortoises (6.4%–15.3%) and Aldabra giant tortoises *Aldabrachelys gigantea* (15.3%) (Edwards,1991; Liesegang et al., 2001). Lower mass-specific intakes with high fiber content diets and less digestible energy have been continually reported in Desert tortoises (Meienberger et al., 1993; Barboza, 1995a; Tracy et al., 2006; Hazard et al., 2009). In herbivorous species, the fiber provides energy directly to

the intestinal microbes which fermentation products also provide energy and some vitamins to the host animal (Bondi, 1987; Dierick et al., 1989; Baer et al., 1997). However, relatively high fiber levels can be nutritionally disadvantageous because of their relative difficulty in digestibility (Swart et al., 1993; Baer et al., 1997; Souffrant, 2001). This can explain the lower mass-specific intake of animals fed with the high fiber diet.

About the observed age effect on energy intake, the declining of the mass-specific intake of gross and digestible energy during growth of the hatchlings is explained by the higher mass-specific metabolic rates of younger animals, in contrast with adults (Nagy et al. 1997), so these young tortoises need higher rates of nutrient assimilation (Tracy et al., 2006).

Bjorndal (1989), in a previous study of the digestive responses of the Red-footed tortoises and Yellow-footed tortoises, reported that the retention time of the particulate fraction of food varied as a function of diet quality, where the particulate fraction was retained longer for diets containing more cell wall. Fibrous components of the diet determine the digesta passage's speed because the microbiota digestion needs to retain the digesta into the cecum or colon. In this sense, Madera-Vergara et al. (2010) evaluated transit time of Red-footed adults fed with different diets, and animals fed with canine extrudate with low fiber content (3.5%) showed shorter transit time than those fed with a mix of vegetables. In the present study, this physiology was also observed, passage time of the digesta was longer in animals fed with a high fiber diet.

The age effect was described on digesta passage in just a difference of 6 months, being faster in younger animals, which is similar to what was described in other herbivorous reptiles (Troyer, 1984; Bjorndal et al., 1990; Tracy et al., 2006). When our data are compared with the transit time reported in adults of the same species, this difference is also observed about a mean of 3.60 days in hatchlings of 11 months-old and a mean of 5.21-9.50 days reported in adults (Bjorndal, 1989; Madera-Vergara et al., 2010). In contrast with adults of other species such as Galapagos tortoises, hatchlings of Red-footed tortoises of 11 months-old showed a lower mean retention time of 9.07 days versus 12 days at 26.7°C described for Galapagos tortoises (Sadeghayobi et al., 2011). Also, an increase in body capacity positively influences the digestive tract's capacity to store food (Parra, 1978), explaining the longer digesta passage in older animals.

In tortoises, digesta may be moved by peristaltic waves generated by the electrical rhythm of the muscular tunic cells in response to the animal's eating (Guard, 1980; Warner, 1981), in that sense, the food intake and transit time are often negatively correlated in Red-footed tortoises and other tortoises' species (Bjorndal, 1989; Meienberger et al., 1993; Lickel, 2010). This is similar to what we obtained from our results, where older hatchlings showed lower mass-specific intake and longer transit time. Also, in concern with diet, Barboza (1995b) described that retention times tended to reduce at higher intakes of low fiber and foliage diets. This agrees with the greatest mass-specific intake and the shortest transit time presented by animals fed with the high starch diet.

The same pattern was reported by Tracy et al. (2006) in Desert tortoises, and by that difference in passage rate, they explained that tortoises fed with a low-fiber diet acquire energy and other nutrients much faster than tortoises fed with a high-fiber diet. Also, if we link this with the high starch diet's greater digestibility efficiency, we can suggest that a low-fiber diet could yield much more energy per unit of time than a high-fiber diet.

Greater feces production in animals fed with high fiber diets was also described for desert tortoises (Barboza, 1995a). Previous studies of digestive efficiency in red-footed tortoise and reptiles, in general, used to evaluate diets with a different nutritional composition which makes it difficult to distinguish the real responsible nutrients for those results. However, if we focus only on the fiber effect, in a study of adults of red-footed and yellow-footed tortoises, Bjorndal (1989) reported a lower digestible efficiency of the energy of diets with high digestible cell wall content and high lignin content. A similar pattern has also been described in terms of digestive efficiency of dry matter and gross energy for desert tortoises and Galapagos tortoises fed with grasses with high neutral detergent fiber content (Meienberger et al., 1993; Barboza, 1995a; Barboza, 1995b; Hatt et al., 2005; Liesegang et al., 2001). Lower digestive efficiency of the fiber with a high fiber diet was similar to reports in desert tortoises, where diets with an increase of neutral detergent fiber (NDF) showed a decline of NDF digestibility (Barboza, 1995a). More easily digestible diets have lower amounts of plant structural materials, including hemicellulose, cellulose, and lignin (Tracy et al., 2006). In this context, Barboza (1995a) mentions that fiber digestion may therefore provide a major source of energy at low intakes of diets with high fiber

content. In contrast, high intakes of less dietary fiber may provide more energy from cell contents' digestion.

Less digestible efficiency of protein was observed in animals fed with the high starch diet in the present study, being similar to negative and low nitrogen digestibility previously reported in studies of adults of red-footed and yellow-footed tortoises fed with some fruits with low cell walls content or low digestible cell walls, and desert tortoises fed with grasses with lower content of neutral detergent fiber, respectively (Bjorndal, 1989; Meienberger et al., 1993). However, these fruits and grasses were poor in nitrogen content, which could also mainly influence the negative nitrogen balance.

In concert with the age effect, digestive efficiency can be better if food is ingested in smaller pieces. Although reptiles do not chew their food (Throckmorton, 1973), younger reptiles typically ingest smaller bites than adult reptiles do (Troyer, 1984; Bjorndal et al., 1990), where the reduction in dietary particle size would be mainly achieved through chemical action (Lobel, 1981; Bjorndal et al., 1990) and microbial action in the hindgut. This less efficient pattern in older hatchlings was observed in terms of starch digestibility in the present study. Previous studies with hatchlings of other herbivorous reptiles have also reported similar digestibilities as adults, indicating that their strategy to get a higher rate of digestion is by selecting high-quality food (Troyer, 1984; Mautz and Nagy, 1987; Bjorndal et al., 1990) and pass food through the gut more quickly (Tracy et al., 2006). All of this agrees with our results of better digestive efficiency of energy gross of 70.85% (at 5 months old) and 73.24% (at 11 months old) in comparison with adults of red-footed and yellow-footed tortoises (32%-65%) (Bjorndal, 1989). Also, our results of digestive efficiency of protein and gross energy were greater than reported values in juveniles and adults of other species such as Galapagos tortoises (63%) and desert tortoises (48-69%), respectively (Meienberger et al., 1993; Hatt et al., 2005).

On the other hand, a relationship has been described between passage time and apparent digestibility. Digestive efficiency of the refractory portions of an herbivorous diet can be better with an increase of the fermentation time in the large gut (Sibly, 1981; Allen and Mertens, 1988). However, in mammals' studies, the results have been inconclusive, a consequence of the difficulty in obtaining a wide range of transit times in endotherms (Meienberger et al., 1993), in concern

to our results, this relation was not observed where the high fiber diet presented longer passage time but lower digestive efficiency.

Considering all the obtained data, our results' diet effect evidenced again that this species does not have a fixed digestive strategy. As Bjorndal (1989) described, *C. carbonaria* presents a flexible digestive strategy where their digestive responses are determined by dietary choice.

Specific Dynamic Action of diets

Older hatchlings showed lower mass-specific metabolic rates at rest and after feeding, being similar to what was observed in Western fence lizards (*Sceloporus occidentalis*) and Tuatara (*Sphenodon punctatus*), where the mass-specific metabolic rates of the juveniles were greater than in adults (Bennett and Nagy, 1977; Cartland and Grimmond, 1994). This difference can be related to the basis of smaller body size of the immature animals as well as metabolic increments associated with rapidly growing tissue (Bennett and Nagy, 1977), which also explain the decrease of mass-specific feed intake, heat increment per gram of dry matter and digestible energy at 12 months old.

In the present study, with feed intakes of 1.87% - 2.92% of the tortoise body mass, the factorial scopes of oxygen and carbon dioxide were within previously reported ranges in different snakes' species such as Crossed pit viper (*Bothrops alternatus*) of 2.8-7.78 (Gavira and Andrade, 2013); Timber rattlesnakes (*Crotalus horridus*) of 2.8-8.9; and Cornsnakes (*Pantherophis guttatus*) of 2.3 (Sievert et al., 2013), with greater feed intakes of 5-50% of the body mass. On the other hand, our values were higher than what was described in an omnivorous tortoise, Speke's hinge-back tortoise (*Kinixys spekii*) of 0.98-2.03, fed with mushrooms and leaves (Hailey, 1998).

Evaluations in other species showed that the peak of heat production happened earlier, about 12-43 hours post-feeding in Gila monster (*Heloderma suspectum*), Common garter snakes (*Thamnophis sirtalis*), Burmese python (*Python molurus*), Timber rattlesnakes, and Crossed pit viper (Secor and Diamond 1997; Zaidan and Beaupre, 2003; Christel et al., 2007; Bessler et al. 2010; Gavira and Andrade, 2013; Sievert et al., 2013), in comparison with our maximum metabolic rates at 72 hours after feeding. Also, longer persistence of the heat production peak, which we observed at 12 months old, was described in

lizards that presented significantly elevated metabolic rates for 5 and 6 days when fed with rats and egg meals, respectively (Christel et al., 2007). Also, feed intake exhibited a positive relationship with the maximum metabolic rate after feeding, as reported in snakes, explained by the cost of processing more biomass (Andrade et al., 1997; Zaidan and Beaupre, 2003).

The ratio of the digestion cost with the meal energy, expressed as SDA coefficient, was within values described in juveniles of rattlesnake (*Crotalus durissus*) of 12-18% and Gila monster lizard of 13.8% of gross energy (Andrade et al., 1997; Christel et al., 2007). However, some species have reported lower values, such as Crossed pit vipers and Speke's hinge-back tortoise of 17-27% and 16-30% of digestible energy, respectively (Hailey, 1998; Gavira and Andrade, 2013).

In an evaluation of meal composition effect on specific dynamic action in pythons, lard, suet, cellulose, and starch were not digested and did not produce measurable SDA (McCue et al., 2005). However, as they are carnivorous species, their carbohydrate digestion is poor. Similar observations for carbohydrate digestion have been reported in caimans and turtles (*Pseudemys scripta*) (Coulson and Hernandez, 1983). When we compare to other species as Nile tilapia (*Oreochromis niloticus*), the heat produced during digestion was not affected by starch or cellulose-inclusion levels (Tran-Duy et al., 2008), similar to our results.

There are no specific studies about the starch or fiber effect on heat increment in herbivorous or omnivorous tortoises. Although the heat increment was not significantly affected by diet, we could observe that animals fed with the high fiber diet showed a similar maximum heat production during feeding, with a lower mass-specific intake of digestible dry matter and greater fiber intake than those from the high starch diet. In this sense, the HI_{DM} and HI_{DEI} of animals fed with the high fiber diet were qualitatively higher, as was observed in Speke's hinge-back tortoise feeding on leaves (Hailey, 1998). On the other hand, methane production in herbivore tortoises has been reported as an important energy loss. An increase in methane production was related to increased fiber digestibility at higher body sizes (Franz et al., 2011). In this sense, the non-significant effect of diet on heat increment, can be explained because the methane output was not

measured in the present study, being a critical energy loss that could evidence a greater energy cost of the high fiber diet.

Nagy (2001), by plotting information from different body mass studies and daily dry matter intake, described a mean allometric exponent of 0.92 for all reptiles and a range of 0.64-0.87 for herbivorous reptiles (Franz et a., 2011). In terms of gross energy, the allometric exponents of herbivorous tortoises were within 0.66-0.88 (Franz et a., 2011). These “b” values are higher than we obtained in terms of daily intake of gross energy (0.56-0.72) and digestible energy (0.34-0.66), which indicates a greater metabolic rate of red-footed tortoises. Also, in Nagy's (2001) study, just one Chelonian species was included: the desert tortoise. In concern to resting metabolic rate, a general allometric exponent of 0.80 was reported for reptiles (Nagy, 1987) and a range of 0.75-0.85 for reptiles and amphibians (Ultsch, 2013). However, some variation of the “b” value has been reported in a group of species as the case of pythons and boas, where the mean intraspecific allometric exponents for a standard metabolic rate and CO_2 production ranged between 0.57-0.86 (Chappell and Ellis, 1987) and 0.68-0.78, respectively (Secor and Dimond, 1997; Zaidan and Beaupre, 2003).

More studies in Chelonian species; mean “b” values from the resting metabolic rate scaled with the body mass of sea and non-sea turtles were 0.70 and 0.73 at fasting and rest state at 30°C (Ultsch, 2013). In this sense, we obtained allometric exponent values within 0.46 to 0.75, being slightly lower to a general slope range described in turtles from 0.69 to 0.79 (Ultsch, 2013). These reduced “b” values may be because our study was done with hatchlings, which have a lower percentage of ossified shell mass than do adults, as observed in other studies (Reese et al., 2004; Ultsch and Reese, 2008).

Also, the proportion of daily intake of digestive energy allocated to resting metabolic rate was lower than the 40-50% of the energy utilized by free-living Western fence lizards for maintenance costs (Bennett and Nagy, 1977).

Growth and Estimated Body Composition

Usually, in captivity conditions, diets with higher fiber content are recommended to produce a more natural growth rate in omnivorous and herbivorous tortoises and turtles. This is supported by comparisons of animals' growth performance in free-ranging and captivity conditions, where rapid growth

has been described in captive-bred animals of different tortoise species (Donoghue and McKeown, 1999; Furrer et al., 2004; Ritz et al., 2010). Although this difference can be explained by sufficient sunlight and over-supplementation of calcium and protein (Noegel and Moss, 1989), one crucial factor is the highly digestible food and the low fiber content of the zoo diets (Ritz et al., 2010). In this sense, captive Galapagos giant tortoises under a very digestible diet showed an accelerated growth related to negative consequences on life expectancy. An increase in dietary fiber level is recommended as an appropriate prophylactic measure to counteract this tendency (Hatt et al., 2005).

Continuous and significant increases in average body mass gain has been reported in juveniles of Soft-shelled turtle (*Pelodiscus sinensis*) with increasing dietary starch levels until 24-30%, but it was decreased at higher dietary starch content (>36%) (Kou et al., 2018). Although in the present study, animals fed with the high starch diet showed qualitative greater body mass gain and were 16.94% heavier than those animals fed with the high fiber diet, these differences were not significant. As to our knowledge, there are a few studies of this kind in reptile species, so if we compare with what was described for another group of species like fish, dietary starch has related to a positive influence on growth performance until an optimal level of dietary starch (Wang et al., 2005; Tan et al., 2009). This influence was observed in the present study in terms of final plastron width, final carapace width, and daily growth rate.

However, faster growth rates are linked to negative effects such as the development of obesity, high mortality, renal diseases, pyramiding, and metabolic bone diseases (Ritz et al., 2010). In concern to pyramiding growth, several environmental hypotheses have been described, linked to temperature and humidity, where juveniles of the African leopard (*Stigmochelys pardalis*) and spurred (*Centrochelys sulcata*) tortoises showed greater pyramid height because of increased heat exposure (Heinrich and Heinrich, 2016) and at dry environmental conditions (Wiesner and Iben, 2003). In the present study, the animals were exposed to the same environmental humidity and temperature during the whole experiment, modified just on the coldest days in winter and autumn; any environmental factor could influence the observed pyramidal growth, affecting all animals indistinct to the diet.

Dietary effects on pyramiding have also reported in Chelonia species, related to incorrect calcium and phosphorous content and low ratio Ca:P (Gerlach, 2004). Juvenile leopard tortoises that did not receive a calcium supplement showed a clear depletion of calcium in the body; also, tortoises that received three times the recommended calcium supplementation grew faster and metastatic calcifications were reported postmortem at the highest doses of calcium (Fledelius et al., 2005). However, in a study in Red-eared sliders turtles, pyramiding was not induced by dietary excess of calcium and/or phosphorus, suggesting that supplementation can be unnecessary if a balanced diet is fed and can result in lower growth rates (Stancel et al., 1998). Also, captive juvenile Galapagos giant tortoises fed with higher calcium concentrations in the diet were more efficient to digest the Ca, Mg and P, without pyramiding growth in any calcium concentration (Liesegang et al., 2001).

On the other hand, pyramidal growth was variably affected by dietary protein content in African spurred tortoises as a minor positive impact on the formation of humps (Wiesner and Iben, 2003). In the present study, the mass-specific intake of digestible protein was not different between diets. However, a significant effect of the diet was observed on the ratio between digestible energy and digestible protein ($P<0.01$). Animals fed with the higher starch diet showed a greater ratio of 69.16±1.93 J/mg compared to 65.41±2.24 J/mg of animals from high fiber diet and these were animals which presented greater levels of pyramiding with a greater base of the wedge-shaped. We could suggest a possible relation between greater ratios of digestible energy and protein intake and pyramiding growth.

Lower fiber dietary content influenced the pyramiding growth of species such as desert tortoises and red-eared slider turtles that presented pyramiding growth patterns at captivity conditions (Jackson et al., 1976; Stancel et al., 1998). The pyramiding pattern was not observed in leopard tortoises when their diet included more fiber content as natural vegetation of grasses, shrubs, and lucerne hays (Ritz et al., 2010). All of this agrees with the greater base of the wedge-shaped presented in animals fed with the high starch diet.

Even though an increase of calcium digestibility was reported in lower dietary fiber content and a greater ratio of dietary fat and fiber in juveniles of Galapagos Tortoises (Liesegang et al., 2001), as we mentioned before, a high

starch diet promoted accelerated growth rates, and this turn place much greater demands of calcium and vitamin D_3 and frequently resulted in relatively poor bone density (Highfield, 2010). Heinrich and Heinrich (2016) speculated that an unnatural growth rate might lead to the deposition of material between scutes faster than the shell can spread, leading to a conical uplift of carapacial scutes with more prominent pyramiding of the vertebral scutes. When the calcium balance is disrupted due to dietary imbalance, it affects processes such as renal function or intestinal absorption, and calcium is removed from the bone to maintain basic metabolic requirements (Moschilde, 1990). All of this could explain the less bone mass of tortoises fed with the high level of starch and their lower bone density, and linked with a wider bases of the wedge-shaped gives the idea of the effect on pyramiding development.

Increasing dietary starch has been related to higher body fat content and ash content decrease in another group of fish species (Wang et al., 2005; Wang et al., 2017; Sulaiman et al., 2020). However, in contrast to what we expected, non-diet influence on fat content was observed. Our results were similar to the only study in chelonians of dietary cornstarch's impact on body composition of juveniles of Soft-shelled turtle.

CONCLUSIONS

In conclusion, the present study shows that high starch and fiber levels have significant effects on mass-specific intake of dry matter, digestibility efficiency, passage time, final carapace width and its growth rate, pyramiding components, bone mass content, and density. Also, a surprisingly non-significant diet effect was observed on specific dynamic action. Hatchlings fed with the high fiber diet had a lower mass-specific intake of dry matter, apparent digestibility and growth rate of carapace width, shorter carapace width, and longer passage time. In contrast, animals from high starch diet presented a greater base of the wedge-shaped and low bone mass content and density. A significant effect of age was detected on digestibility efficiency, passage time, and mass-specific feed, digestive nutrients, and energy intake. This influence was also observed on resting metabolic rate and post-prandial metabolism; older animals showed a decrease in their metabolism. Our results indicate the importance of offering

enough fiber and limited starch contents in diets of omnivorous hatchlings tortoises as Red-footed tortoise (*Chelonoidis carbonaria*) in captivity, which can permit an optimal energy utilization by hatchlings reflecting a normal growth of these animals and avoid nutritional disorders as pyramiding development and lower bone density.

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CHAPTER IV

TEMPERATURE EFFECT ON ENERGY METABOLISM IN HATCHLINGS OF RED-FOOTED TORTOISE (*Chelonoidis carbonaria*) IN CAPTIVITY.

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ABSTRACT

Hatchlings of Red-footed tortoises (*Chelonoidis carbonaria*) in captivity, fed with a diet with high fiber level, were subjected to 28°C and 18°C, to evaluate the effect of temperature on the daily intake of gross energy, digestible energy, resting metabolic rate, post-prandial metabolism, and surface body temperature. During the experiment, greater mass-specific daily intake of gross energy and daily body mass gain was reported in spring and summer conditions. The highest and lowest resting metabolic rate of animals under 28°C were described in spring and winter, respectively. At 28°C, animals showed significantly greater daily intake of gross and digestible energy; O_2 consumption, CO_2 production and metabolic rate at rest (30.56±4.07kJ/kg/day at 28 °C versus 7.71±1.26 kJ/kg/day at 18°C) and after feeding; resting metabolic rate coefficient; and heat increment. Although the factorial oxygen and carbon dioxide scopes were not affected by temperature, a lower specific dynamic action coefficient was shown at a higher temperature; and untypical respiratory quotients were observed (0.30-0.50) at 18°C. A strong body mass influence was detected on resting metabolic rate, describing allometric exponents of 0.62 and 0.92 at 28°C and 18°C, respectively. On the other hand, the hatchlings under lower temperature prioritized to keep higher head surface temperature, and animals after feeding presented higher body surface temperature. Our results indicate an important effect of the environmental temperature on the energy requirement and its utilization in Red-footed tortoise' hatchlings in captivity, being proposed species-specific allometric exponents at different thermal conditions.

Keywords: *Chelonoidis, resting metabolic rate, post-prandial metabolism, temperature.*

INTRODUCTION

Ecological energetics aim to understand potential energetic constraints imposed on ecological patterns and processes by the metabolic requirements of interacting organisms (Irlich et al., 2009; Kearney and White, 2012) by studying the energy flow within an ecological system from how the energy enters to the living system, particularly by tracing the movement of energy through a food web until it is ultimately degraded to heat and irretrievably lost from the system (Humphreys, 2020). In this sense, the ecological processes crucial to an animal's growth, survival, and reproductive fitness have energetic costs (Tomlinson et al., 2014). However, this is an overlooked research area in herpetofauna (McNab, 2002), being rare in the energetics studies on terrestrial chelonians.

A total of four tortoises species are distributed in South America. Even when this is a significantly lower quantity of species in a smaller territory in comparison to Asia and Africa (32 species), studies on respiratory physiology and metabolism of South American tortoises are scarce (Trevizan, 2006). The major part of the respiratory physiology studies in chelonians have been done in African and Asian turtles species (Jackson, 1973; Burggren et al., 1977; Glass et al., 1978; Benchetrit and Dejours, 1980; Scantlebury and Minting, 2006; Enstipp et al., 2011; Johnson et al., 2015; Cordeiro et al., 2016).

Although Red-footed tortoises (*Chelonoidis carbonaria*), previously named *Geochelone carbonaria,* has a long history of human utilization for food by many South American tribes and the illegal pet market and are thought to be declining in some areas. However, it is not listed by International Union for Conservation of Nature and Natural Resources (IUCN) as a threatened species (Pritchard & Trebbau 1984; IUCN, 1989; Cuéllar, 2000; Soria and Noss, 2000; Rodríguez and Rojas-Suárez, 2008). Early studies failed to distinguish the red-footed tortoise from the yellow-footed tortoise (*Chelonoidis denticulata*), which resulted in confusion of the conservation status and the previously collected data until Williams (1960) finally presented detailed evidence that they are separate species. In concern to its ecological role importance, relatively recent studies described that the great diversity of consumed viable seeds combined with a longer retention time of seeds and great traveled distances indicate that this

species is an effective dispersal agent, which contributes to the maintenance of species diversity and forest structure in Neotropical countries (Strong and Fragoso, 2006; Jerozolimski et al., 2009; Wang et al., 2011). All these aspects highlighted the importance of developing diverse studies in the red-footed tortoise for conservation purposes.

Understanding how animals satisfy their daily energy requirements is fundamental to comprehend which factors determine these needs in energy and how animals can use that energy (Nagy, 2005). All of this is an important challenge in the applied nutrition for captive management of wild animals, where the offering of appropriate quantity and quality of food is required to satisfy the animal requirements and prevent metabolic disorders and nutritional diseases (Higgins and Edward, 2009). In this sense, the daily energetic expenditure and time-energy budgets are useful for gaining insight into an animal's daily food requirements (Todd et al., 2009), where energy production is defined as the total energy that comes from the energy consumption less the energy lost by respiration, feces, and nitrogenous waste (Congdon et al., 1982).

In the energy lost by respiration, the Specific Dynamic Action (SDA) is a critical and potentially large component (McCue and Lillywhite, 2002; Zaidan and Beaupre, 2003; Secor, 2009) because this energy component cannot be used for other functions such as maintenance, growth, and reproduction. The SDA is described as the cost of processing a meal, includes all the energy expended to ingest, digest, mechanical cost of transporting food through the gut, active transport, absorption, biochemical breakdown, use nutrients in anabolic reactions, muscle activity, and secretion, and intermediary storage (Tandler and Beamish 1979; Blaxter, 1989; Andrade et al., 1997; Secor and Diamond, 1997; Secor, 2009). Few studies have investigated how temperature affects the specific dynamic action (SDA) in chelonian species, where elevated oxygen consumption rates, shorter duration of the digestion process, faster oxygen consumption peak, and better digestive efficiency has been reported at higher temperatures (Sievert and Andreadis, 1999ab; Toledo et al., 2003; Wang et al., 2003; Bessler et al., 2010). Nevertheless, when the cost of digestion is expressed in percentage of the consumed energy (SDA coefficient), a variety of temperature influences between positive, negative, and non-effect have been described along with

different species (Jobling and Davies, 1980; Secor and Faulkner, 2002; Toledo et al., 2003; Zaidan and Beaupre, 2003; Tattersall et al., 2006).

In an era of unprecedented global environmental challenges, ecological energetics opens up the tantalizing prospect of a more predictive and mechanistic understanding of the drivers of threatened species decline (Tomlinson et al., 2014). Also, research about how temperature can influence the resting and postprandial metabolism is useful to adjust the development of models for energy partitioning in different thermal conditions (Lillywhite, 1987; Secor and Nagy, 1994; Beaupre, 1995, 1996).

Here, we aimed to test if i) the temperature affects the resting metabolic rate, daily intake of gross energy, daily body mass gain, and post-prandial metabolism, ii) if the cost of digestion related to the total intake of dry matter and digestible energy is affected by temperature, and iii) if there is a relationship between the resting metabolic rate and the body mass, to determine the allometric exponent in each evaluated temperature. Specifically, we tested this by measuring the metabolic rate of hatchlings of red-footed tortoises from 0 to 13 months old fed with a unique diet at 28°C and 18°C and recording the daily gross energy intake and body mass gain through the seasons.

MATERIALS & METHODS

1. Experimental animals and husbandry

We used a total of twenty unsexed hatchlings of Red-footed tortoises (*Chelonoidis carbonaria*) from 0 to 13 months-old, hatched in captivity, with an average of 39.60±5.38g to 315.95±29.92g of body mass, respectively. Animals were individually housed in cages of 0.31x0.57x0.77m in indoor conditions with access to the natural photoperiod. The environmental temperature was recorded twice daily (in the morning and evening) with a digital hygro-thermometer (HCT-2, Exbom, Brazil). During the austral autumn and winter, an electric heater and/or infrared lamps were used to prevent animals from experiencing very low temperatures that could limit the food intake. All procedures, animal care, animal use, and treatment were performed following the Animal Ethics and Use Committee of the Sao Paulo State University (UNESP, for its acronym in Portuguese). This committee approved the protocol of the study (n°006095/19).

2. Feeding trial and body weight monitoring

Hatchlings were fed with two isocaloric diets in gross energy with similar nutritional composition except for starch and crude fiber level (see Table 1), being distributed ten animals per diet. The feed was offered every day in 2.5% of body mass until 6 months-old, then feeding was performed every other day (4 days/week) in the same body mass relation. In this sense, mass-specific daily intake of food was determined by weighing the amount of offered and residual food with a scale of 5kg capacity and ± 0.01 g sensitivity (AD5002, Marte, Brazil). Also, water was offered *ad libitum*. Also, hatchlings were weighed every two weeks to measure the daily body mass gain per month ($g^* day^{-1}$).

The gross energy content of diets was determined through bomb calorimetry on an automated Parr diabatic calorimeter (PARR Instrument-1281, Moline, EUA) to determine the daily intake (GEI, $kJ^*animal^*day^{-1}$) and mass-specific intake (GEIm, $kJ^*kg^{-1}*day^{-1}$) of gross energy during the 18 months of the experiment.

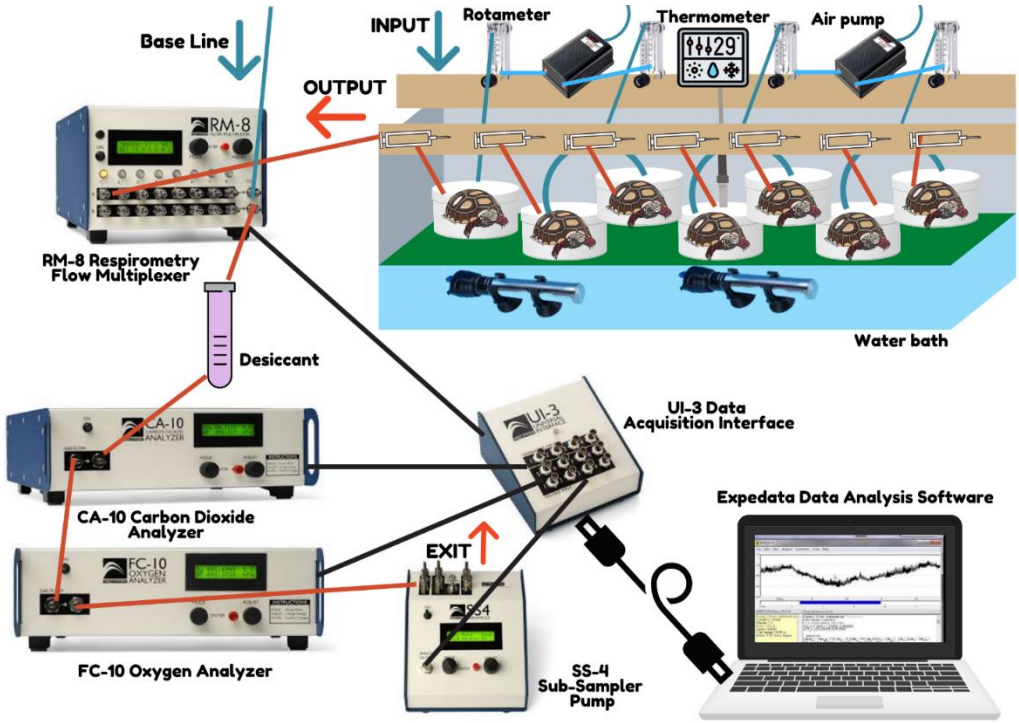
TABLE 1. Chemical composition of experimental diets for growing tortoises with different starch and fiber contents

Nutrients (%)	High Fiber (HF)	High Starch (HS)
Crude Protein	25.00	26.00
Crude Fiber	14.16	6.60
Neutral detergent fiber	39.20	24.85
Starch	16.08	27.71
Acid hydrolyzed fat	6.97	6.54
Calcium	1.10	1.10
Phosphorus	0.70	0.70
Gross energy ($kJ^*g\ DM^{-1}$)	19.52	18.85

3. Resting and Postprandial metabolism measurements

Previous studies reported a better adaptation of the Red-footed tortoise at 20-35°C with high moisture levels (Wang et al., 2011) and slow digestion rates and/or stopped peristalsis at 20°C has been observed in other reptile species (Skoczylas, 1978; Harwood, 1979; Pafilis et al., 2007); this information was considered to choose the evaluated temperatures: 18°C and 28°C.

FIGURE 1. Open-Flow Respirometry System for measuring metabolism.



Indirect calorimetry system of seven respirometry chambers: 10 minutes of measurement per chamber with three times baseline measurement about 5 minutes in the beginning, after the 2nd and 4th chamber. A thermometer was installed into one chamber that was placed in the center of the system. Blue arrows and lines (▬) refer to the transport of input samples of air to ventilate the chambers and baseline analysis. Red arrows and lines (▬) indicate the route of the output samples of chamber air to be analyzed until eliminated (exit). Black lines (▬) describe the data acquisition to Expedata Data Analysis Software by UI-3.

Resting metabolic rate and postprandial metabolism of hatchlings were measured via indirect calorimetry at two evaluated temperatures (18°C and 28°C) using an open-flow respirometry system (Fig. 1). Indirect calorimetry assesses the indirect calculation of the heat production in kJ by measuring the amount of oxygen consumed (VO_2) and carbon dioxide production (VCO_2) by the animal. The oxygen consumption rate ($\dot{V}O_2$) and carbon dioxide production rate ($\dot{V}CO_2$) were measured in individual respirometry chambers, made of translucent material (except for the lid), allowing the tortoise's visual observation without stressing the animals during oxygen consumption measurements. Data were discarded when the tortoise remained restless during the experimental trial by recording the period of activity. Additionally, to evaluate the Specific Dynamic Action, animals were randomly assigned to a respirometry chamber which consisted of two different sizes, depending on the animal age: chambers of 900mL for evaluations at 6 months old, and chambers of 2500mL for animals of 12 months old.

The chambers' internal temperature at each thermal regime was kept constant by a water bath using a 50W aquarium thermostat (AP-801, Ace Pet, Brazil). The chamber and environmental temperature were registered every 10 minutes, and the moisture was kept between 50-80%.

A Sable System eight-channel multiplexer-controlled subsampling by continuously cycling through all seven chambers available for simultaneous measurements. Each of the seven-individual flow-matched lines was then connected to one port of each respirometry chamber from the second port of the syringe barrel for subsampling. A separate line was connected on the 8^{th} channel for a baseline measurement. Inside of the chambers, animals were ventilated by positive pressure with a constant flux of air (250 mL/min at 28°C and 150ml/min at 18°C) by using an aquarium air pump (Jeneca AP-3500) coupled with a rotameter to control the airflow. A sub-sample (100ml/min at 28°C and 70ml/min at 18°C) of air of the baseline and output of the chamber was transported by the negative pressure of the SS4 pump (Sable System, Las Vegas, NV, USA) to be conducted to the desiccant (Drierite® water-absorbent, W. A. Hammond DRIERITE, Xenia, OH, USA) before to pass through oxygen and carbon dioxide analyzers (Sable Systems, Las Vegas, NV, USA). Certified mixture gases (21.1% O_2 and 1.03% CO_2) and pure nitrogen were used to calibrate the gas analyzers. Data of the first hour (stabilization time) were discarded because they were the time for gases to equilibrate and establish stable gas exchange values from the animal. All respirometry experiments lasted 11 hours per day (8:00 am – 7:00 pm).

4. Experimental protocol

Resting metabolic rate and postprandial metabolism were measured at 6 and 12 months old of hatchlings from the high fiber diet. One week before the measurements, we habituated hatchlings to the experimental chamber to reduce the stress and avoid the heat production super estimation due to the animal activity. Also, animals were weighed every day at the beginning and the end of the evaluations and kept under an artificial photoperiod of 12 hours of light and 12 hours of dark during the evaluations. At the end of the metabolism measurement, the animals were removed from the chambers and placed into their cages where they had access to *ad libitum* water.

At each evaluation, after 10 days of fasting (including an acclimatization to the evaluated temperature for two last days), the resting metabolic rate of the hatchlings was evaluated (Day 0). From day 1 to 3, we *ad libitum* fed the animals for three days in the earlier morning before starting the metabolism measuring in chambers. After feeding, animals were under prolongate fasting until to return to the resting values of $\dot{V}O_2$ and $\dot{V}CO_2$, a maximum of 10 days at 28°C and 12 days at 18°C. If after these periods the tortoises had not yet returned to baseline levels, they were removed from the chambers and the measurement was finished. Also, surface body temperature was daily measured from three body zones: head, front and rear legs at the beginning and final of the metabolic rate measurements using a digital infrared thermometer (Traceable ®, Texas, USA). This protocol was followed for evaluations in both temperatures (18°C and 28°C) and ages (6 and 12 months old), and all evaluations were first performed at 28°C, then at 18°C.

5. Data collection and calculations

Data was collected and analyzed using ExpeData v.19 software (Sable System, Las Vegas, NV, USA). Values of oxygen consumption ($\dot{V}O_2$, L*kg^{-1}*min^{-1}) and carbon dioxide production ($\dot{V}CO_2$, L*kg^{-1}*min^{-1}) was used to calculate the mass-specific resting metabolic rate (RMR, kJ*kg*day^{-1}), and maximum metabolic rate after feeding (FMR_{MAX}, kJ*kg*day^{-1}) observed at the peak of heat production. The heat production by indirect calorimetry was estimated by Brouwer (1965) equation, which considered $\dot{V}O_2$and $\dot{V}CO_2$:

$$HP\ (kJ) = 16.17 * \dot{V}O_2 + 5.02 * \dot{V}CO_2$$

Where:

HP (kJ) is the total heat production.

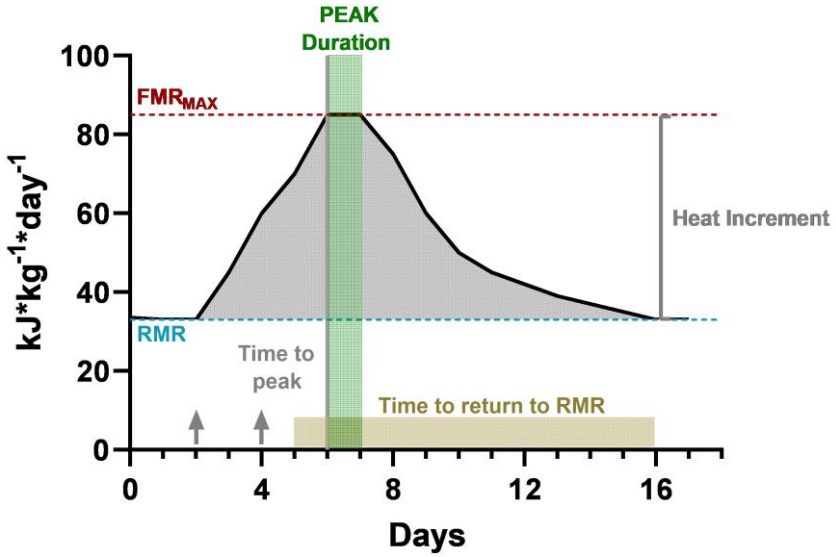
$\dot{V}O_2$ (L) is the volume of consumed oxygen.

$\dot{V}CO_2$(L) is the volume of production of carbon dioxide.

The specific dynamic action was described with the following parameters (Fig. 2): the heat increment (HI, kJ*kg*day^{-1}) as the difference of FMR_{MAX} and RMR; the time to peak; duration of the peak; time to return to initial values; the factorial scope of O_2 and CO_2 (O_2 Scope and CO_2 Scope) described as $\dot{V}O_{2\ Max}/\dot{V}O_{2Rest}$ and $\dot{V}CO_{2\ Max}/\dot{V}CO_{2Rest}$; respiratory quotient (RQ) at feeding and

fasting state, as the ratio of $\dot{V}CO_2$ to $\dot{V}O_2$ by the body allows inference about aerobic catabolism (Withers, 1992); RMR coefficient (%), quantified as a percentage of digestible meal energy; and temperature coefficient Q_{10} defined as the change of a metabolic rate as a consequence of increasing the temperature by 10°C.

FIGURE 2. Specific Dynamic Action Components



Evaluated components of the specific dynamic action: **RMR**-Resting metabolic rate after 10 days of fasting (- -), **FMR_{MAX}** -maximum metabolic rate after feeding (- -), **time to peak** (—), **duration of the peak** represented with a green shaded area, heat increment as a difference between FMR_{MAX} and RMR, and the **time to return to resting values** described as a brown shaded area. Grey arrows indicate the first and last day of feeding.

Specific heat increment of the diet at 28°C and 18°C were expressed in three different terms: HI_{DM} ($HI*g^{-1}$), where the HI ($kJ*kg*day^{-1}$) was divided between the total intake of dry matter (g); HI_{DEI} ($HI*kJ^{-1}$) was obtained by dividing HI ($kJ*kg*day^{-1}$) between the total intake of digestible energy (kJ); and SDA coefficient (%), defined as the SDA quantified as a percentage of meal energy. About the HI_{DEI} ($HI*kJ^{-1}$), apparent digestibility coefficients of the energy of a previous study were used to estimate the digestible energy intake (DEI), being 71.2% at 30°C and 70.7% at 20°C (Mendoza et al., unpublished data).

6. Statistical analysis

All analyses were conducted by R Studio Software (version 3.2.3, AT). Statistical significance was accepted when $P<0.05$. Data normality and homoscedasticity were previously evaluated, and when data were not normally distributed, a non-

parametric Kruskal-Wallis test was performed. The effect of environmental temperature on the mass-specific daily intake of gross energy in terms of body metabolic mass (J*g*day^{-1}) and body mass gain (g*day^{-1}) during eighteen months of the experiment was statistically tested by differences among means between months by Tukey post-hoc test. Also, as animals hatched at different times, metabolism evaluations at 6 and 12 months old were performed in different seasons, a possible seasonality effect on resting metabolic rate was determined by using General Linear Model (GLM) with the month as a factor and age as a covariate to eliminate the age effect, and a means comparison by Tukey post-hoc test.

Effect of temperature on specific dynamic action parameters: $\dot{V}O_2$ (ml*kg^{-1}*min^{-1}) and $\dot{V}CO_2$ (ml*kg^{-1}*min^{-1}) at fasting, $\dot{V}O_2$ (ml*kg^{-1}*min^{-1}) and $\dot{V}CO_2$ (ml*kg^{-1}*min^{-1}) at peak post-feeding, RMR (kJ*kg*day^{-1}), FMR_{MAX} (kJ*kg*day^{-1}), O_2 Scope, CO_2 Scope, feed intake (%), GEI (kJ*animal*day^{-1}), estimated DEI (kJ*animal*day^{-1}), heat increment (kJ*kg*day^{-1}), HI_{DM} (HI*g^{-1}), HI_{DEI} (HI*kJ^{-1}), SDA coefficient (%); RMR coefficient (%); and Q_{10} was statistically tested by one-way repeated measures ANOVA (temperature as independent variable).

Body mass (kg), daily intake of gross and digestible energy (kJ*day^{-1}), the estimated daily intake of metabolic energy less heat increment MEIp-HI (kJ*day^{-1}), and RMR (kJ*day^{-1}) at 18 and 28°C were $\log_{10}$ transformed to be plotted as kJ*day^{-1} vs body mass (kg). Besides, the influence of body mass on the before mentioned parameters except for MEIp-HI was evaluated to obtain the allometric exponent by using a General Linear Model (GLM) considering the thermal regimen as a factor and body mass as a covariate.

Surface body temperature was analyzed per body zone (head, front and rear legs) and state (feeding or fasting) by non-parametric Kruskal-Wallis test in each thermal regime.

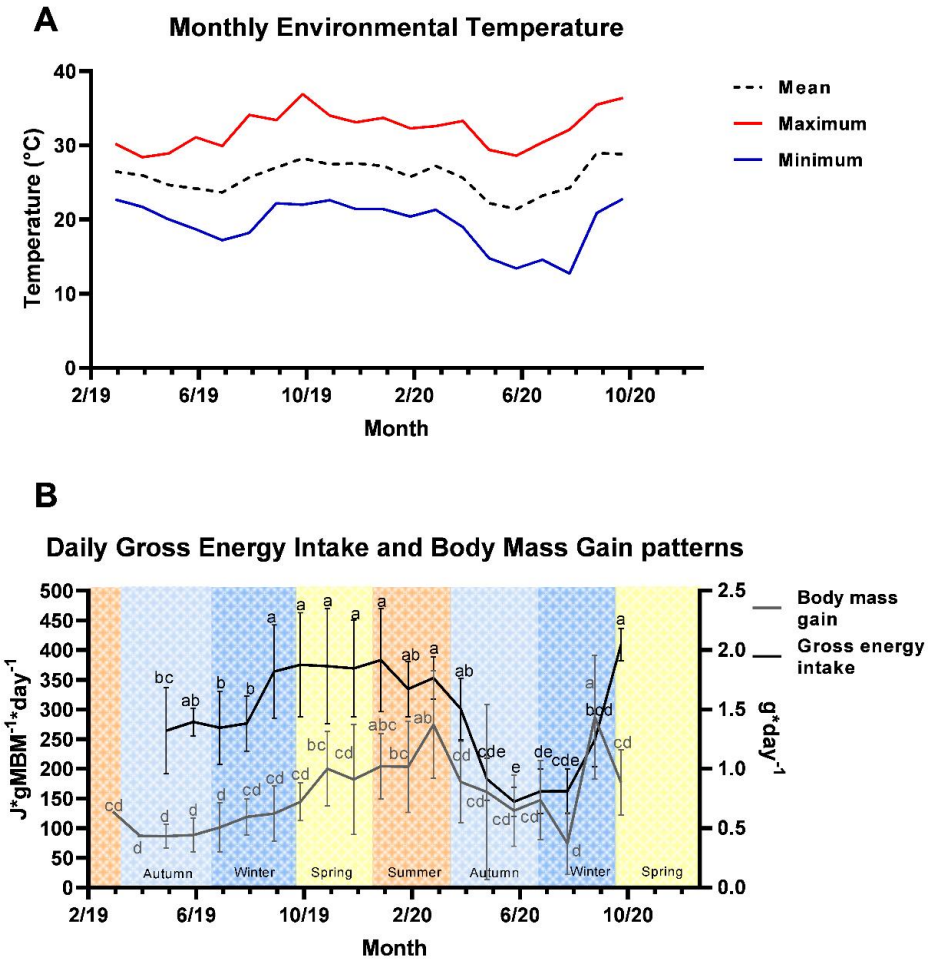
RESULTS

Environmental temperature and gross energy intake

Mean, maximum, and minimum monthly environmental temperature from March 2019 to October 2020 are shown in Fig 3A., where we can observe higher maximum temperatures between September and March 2020 coinciding with

spring and summer seasons in the southern hemisphere, respectively. We expect the lowest temperatures to be recorded in autumn and winter (April to August 2020).

FIGURE 3. Mass-specific Intake of Gross Energy and Body Mass Gain in relationship with environmental temperature (0-13 months-old).



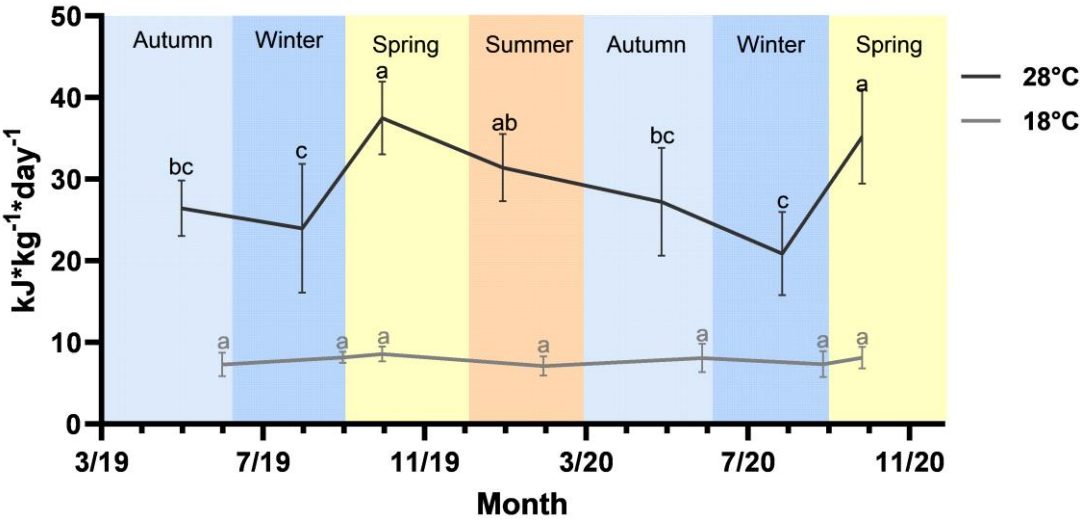
A. Monthly Environmental Temperature: Monthly mean, maximum and minimum temperature from March 2019 to October 2020. **B. Specific-mass Intake of Gross Energy Intake J*gMBM^{-1}*day^{-1} and Body mass gain g*day^{-1}:** Mean ±SD Intake of gross energy per gram of metabolic body mass. *Metabolic mass= body mass^{0,86}* (Bennett and Dawson, 1979), and mean ±SD of body mass gain per day per month from March 2019 to November 2020. Shaded areas color definitions: summer from December to March (Orange), autumn from March to Jun (Light blue), winter from Jun to September (Blue), and spring from September to December (Yellow). Different letters in the same line color indicate a significant difference (P<0.05) between months (environmental temperature effect).

A significant effect of time was observed in mass-specific daily intake of gross energy in terms of metabolic body mass (P<0.01) and daily body mass gain (P<0.01) during the experiment. Considering the environmental temperature as one of the main variables that monthly changes, greater GEIm, and daily body mass gain were reported in the warmer conditions of spring and summer. At the

same time, there was a decrease in lower temperatures during autumn and winter (Fig 3B).

As was before mentioned, experimental animals hatched at different times and could perform the metabolic rate measurement at both evaluated temperatures in each season. Time was used as an indirect indicator of temperature effect, or in this case as a possible seasonality effect, which significantly influenced the mass-specific resting metabolic rate at 28°C (P=0.031), being the highest and lowest values reached in spring and winter, respectively. However, a non-significant effect was identified when hatchlings were under 18°C (P=0.354) (Fig 4). The age effect was only reported at 28°C, where younger animals presented a greater mass-specific metabolic rate (P=0.013).

FIGURE 4. Mass-specific Resting Metabolic Rate in relationship with the season (0-13 months-old).



Rest Metabolic Rate kJ*kg^{-1}*day^{-1}: heat production after 10 days of fasting at 28°C and 18°C, measured in different seasons of the year. Shaded areas color definitions: summer from December to March (Orange), autumn from March to Jun (Light blue), winter from Jun to September (Blue), and spring from September to December (Yellow). Different letters in the same line color indicate a significant difference (P<0.05) between months (possible seasonality effect).

Specific Dynamic Action

During the respirometry evaluations, the mean temperature observed in the respirometry chambers was 27.7±0.7ºC and 18.6±0.7°C, close to established experimental temperatures. Except for oxygen and carbon dioxide scope, all the other evaluated parameters of the specific dynamic action were influenced by

ambient temperature. Also, the SDA coefficient was greater in animals kept at 18°C ($P<0.05$; Table 4). When maintained at 28°C, food and energy intake were approximately 2 times greater than at 18°C ($P<0.05$). These reflect the increase in metabolic rate and heat production verified, which can be observed by the temperature coefficient of oxygen Q_{10} (3.61±0.94) and carbon dioxide Q_{10} (3.94±1.19) values (Table 2).

TABLE 2. Feed intake and parameters of specific dynamic action observed at 28°C and 18°C. Mean values of tortoises fed with high fiber diet.

Parameter	Temperature	
	28°C	18°C
[1]Feed Intake (%)	1.86±0.29*	1.14±0.40
Gross Energy Intake ($kJ*day^{-1}$)	28.27±8.29*	14.16±5.40
Digestible Energy Intake ($kJ*day^{-1}$)	20.13±5.90*	10.01±3.82
Resting Metabolic Rate O_2 Consumption ($ml*kg^{-1}*min^{-1}$)	0.98±0.18*	0.26±0.04
Resting Metabolic Rate CO_2 Production ($ml*kg^{-1}*min^{-1}$)	0.75±0.15*	0.21±0.05
O_2 Consumption at peak ($ml*kg-1*min^{-1}$)	2.08±0.29*	0.61±0.17
CO_2 Production at peak ($ml*kg^{-1}*min^{-1}$)	2.18±0.51*	0.64±0.20
Resting Metabolic Rate ($kJ*kg^{-1}*day^{-1}$)	30.56±4.07*	7.71±1.26
RMR Coefficient (%)	28.51±8.63*	17.17±6.04
FMR_{MAX} ($kJ*kg^{-1}*day^{-1}$)	63.71±8.22*	19.03±5.42
Heat increment ($kJ*kg^{-1}*day^{-1}$)	36.84±9.16*	10.12±5.34
Oxygen Factorial Scope	2.52±0.65	2.32±0.47
Carbon dioxide Factorial Scope	2.97±0.97	3.15±0.56
HI_{DMI} ($HI*g^{-1}$)	7.66±1.90*	4.08±1.97
HI_{DEI} ($HI*kJ^{-1}$)	0.55±0.14*	0.30±0.14
SDA Coefficient (%)	14.26±5.13	20.57±8.72*

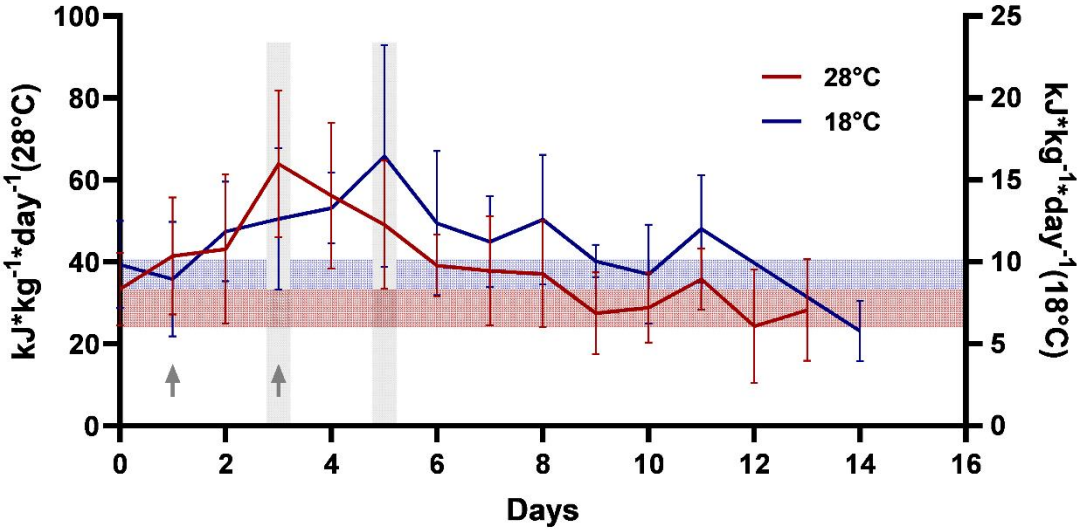
[1]Feed intake expressed in percentage related to the body mass (%).

*statistically different ($P<0.05$)

After the prolonged fasting period, hatchlings showed respiratory coefficient (RQ) values on the interval of 0.73 to 0.84 and 0.57 to 0.79 at 28°C and 18°C, respectively. Also, when hatchlings were placed at 18°C, low RQ values (0.30-0.50) were observed at the first hours after feeding, and only after 3 hours post-feeding, the values of RQ became to normal range. On the other hand, after feeding, although animals presented a mean RQ of 0.98 and 1.02 at 28°C and 18°C, respectively, high RQ values such as 1.11-1.22 at 28°C and 1.10-1.36 at 18°C were also observed for some individuals. The RQ of 1, that indicates carbohydrate metabolism, and postprandial state had a shorter duration of 3.83±1.90 days at 28°C than 5.13±1.69 days at 18°C ($P<0.05$).

Specific dynamic actions of hatchlings fed with the high fiber diet at 28°C and 18°C are described in Fig. 5; the heat production was expressed in different scales, as the values obtained at 28°C (left side y ax) was approximately 4 times greater than those obtained at 18°C (right side y ax). SDA lasted no more than eleven days, and the return to resting values happened faster and earlier at 28°C. At both temperatures was easy to identify oxygen increased after feeding and reached a peak of heat production earlier at 28°C than at 18°C; this occurred on the 3rd day of feeding and the 2nd-day post-feeding, respectively (day 3 and day 5). On the other hand, the peak's persistence was less than 24 hours at both temperatures, and the beginning of the decline of the heat production was slower at 18°C, where we could observe little peaks during the decrease.

FIGURE 5. Specific Dynamic Action of growing tortoises fed with high fiber diet maintained at an ambient temperature of 28°C or 18°C.



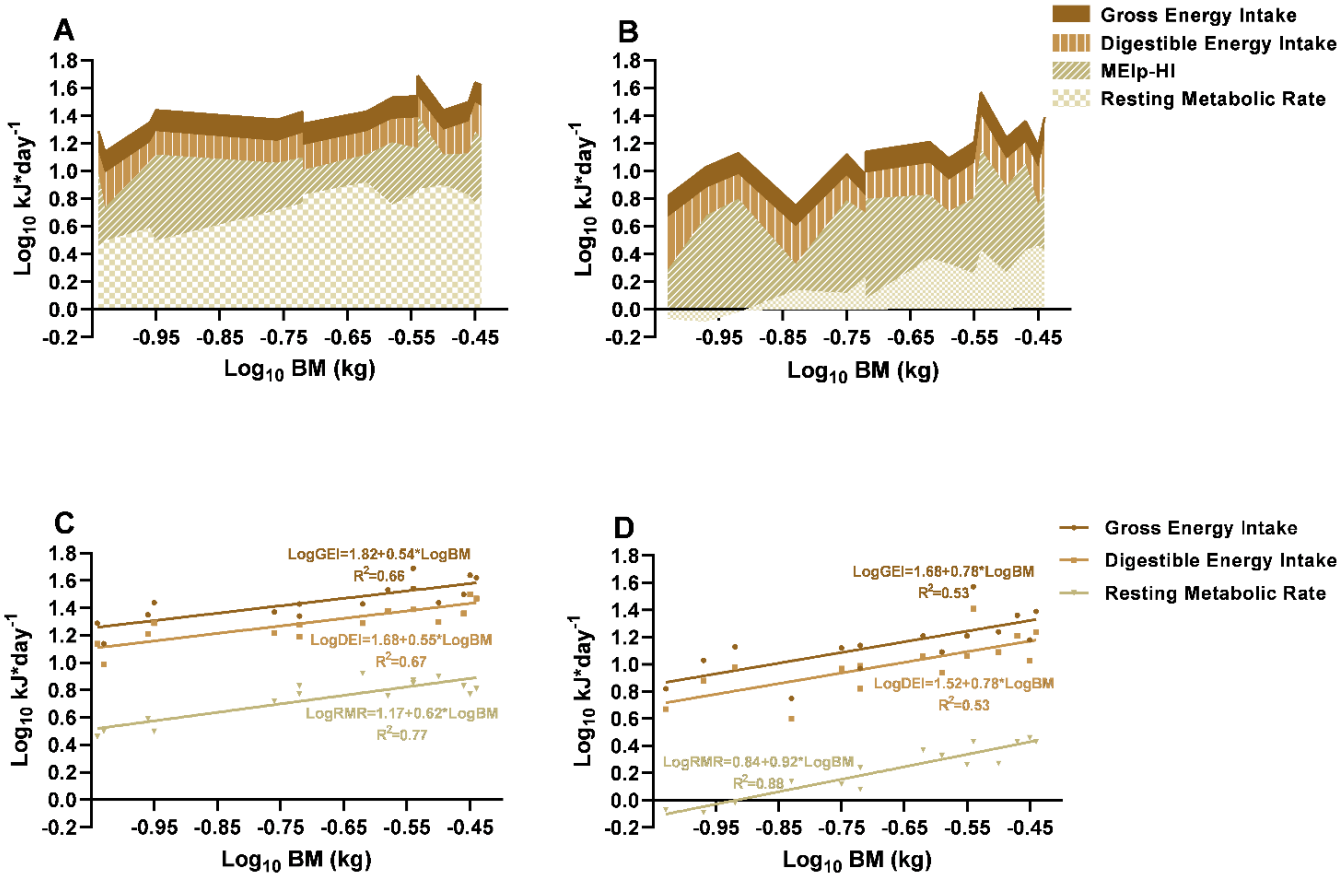
Mean Specific Dynamic Action of growing tortoises fed with a high fiber diet, maintained at two ambient temperatures (28°C and 18°C). Daily mean values of energy production per kilogram of body mass. Day 0 corresponds to fast state; on days 1, 2, and 3, tortoises were fed, and from day 4 until the end, animals were private of food again. The red (28ºC) and blue (18ºC) shaded area corresponds to the range of resting metabolic rate at each ambient temperature (Mean±SD). Results are the mean±SD value observed at 6 and 12 months old.

Significant effect of body mass was identified on GEI, DEI, MEIp-HI, and RMR at both temperatures ($P<0.01$). In this sense, energy partitioning at 28°C and 18°C is explained in the Fig. 6A and B, expressing in $\log_{10}$ the daily intake of gross energy, digestible energy, estimated metabolic energy less heat increment, and resting metabolic rate related to the body mass of red-footed tortoise'

hatchlings fed with the high fiber diet. Also, this data is presented in linear regressions with the body mass, where the rest metabolic rate equation at both temperatures showed a greater determination coefficient (Fig. 6C and D).

The daily intake of gross energy, digestible energy, and resting metabolic rate plotted against body mass (log_{10}-log_{10}) at each temperature scaled with respective mass exponents between 0.54, 0.55, and 0.62 at 28°C; and 0.78, 0.78, and 0.92 at 18°C, respectively.

FIGURE 6. Energy Partitioning of hatchlings of red-footed tortoises fed with high fiber diet related to body mass.



A and B: Daily intake of gross and digestible energy, metabolic energy intake less heat increment of the diet, and daily resting metabolic rate in hatchlings of 6 and 12 months-old related to body mass at 28°C (**A**) and 18°C (**B**). Digestible energy was determined with the following apparent digestibility coefficients of 71.2% (28°C) and 70.7% (18°C). MEIp is the predicted intake of metabolic energy using a coefficient of 0.82

(Lickel, 2010). **C and D:** Linear regression Log_{10} of daily intake of gross and digestible energy and resting metabolic rate with body mass at 28°C (**C**) and 18°C (**D**).

Surface body temperatures in the three evaluated body regions are shown in Table 3. When hatchlings were maintained at 18°C, the temperature of the head was higher than the legs (P<0.001), an effect not verified at 28°C (P=0.36). It was interesting to observe, also, that the mean body surface temperature (mean value of the three-body regions) was higher on the fed than the fasted state (P<0.01).

TABLE 3. Body surface temperature of tortoises maintained at ambient temperatures of 28°C or 18°C. Mean values of tortoises at 6 and 12 months, fed with the high fiber diet.

Parameter	Temperature	
	28°C	18°C
Front legs	28.25±0.59[a]	17.33±1.26[b]
Rear legs	28.16±0.67[a]	17.18±1.32[b]
Head	28.29±0.54[a]	17.90±1.06[a]
Fasting State[1]	28.16±0.61[b]	17.33±1.28[b]
Feeding State[1]	28.37±0.58[a]	17.76±1.15[a]

[a, b] Means in a column without a lowercase letter in common are different (P<0.05) Non parametric-Kruskal-Wallis test
[1]Mean value of the three body regions.

DISCUSSION

Environmental temperature and gross energy intake

Greater mean resting metabolic rate of animals kept under 28°C in all seasons compared to those from 18°C. The highest and lowest values at 28°C observed at spring and winter, respectively, agree with previous studies in European common lizard (*Lacerta vivipara*), Desert tortoises (*Gopherus agassizii*), and Andulate tortoise (*Chersina angulata*), where higher field metabolic rates have been detailed during warm seasons (spring and summer) (Al-Sadoon and Spellerberg, 1985; Henen et al., 1998; Setlalekgomo and Winter, 2012). Thus, the significant difference between months from different seasons suggests a possible seasonality pattern, which needs further research.

Additionally, in the present study, the depression of the metabolic rate during winter and autumn months happened with an increase in inactivity, which can be

explained by a reduced energy demand (Rocha and Branco, 1998). As lower energy is required, feed intake decreasing is usually observed in chelonians during winter or low temperatures (Moon et al., 1997; Hochscheid et al., 2004) with the purpose to conserve energy on cool conditions (Setlalekgomo and Winter, 2012), being similar to the reduced daily intake of gross and digestible energy observed at 18°C and months of colder seasons.

Specific Dynamic Action

The measured mass-specific consumption of oxygen at rest of Red-footed tortoise' hatchlings at both temperatures was greater than the mean of 0.18 ml O_2*kg^{-1}*min^{-1} before reported for this species by Trevizan (2016) in adults at normoxia condition, being approximately five greater at 28°C (0.98 ml O_2*kg^{-1}*min^{-1}). However, it was within the range of the resting mass-specific O_2 consumption values of 0.79-1.29 ml O_2*kg^{-1}*min^{-1} in other turtles and tortoise's species such as Green turtles (*Chelonia mydas*), Yellow-spotted Amazon River Turtle (*Podocnemis unifilis*), Hermann's Tortoise (*Testudo hermanni*), Red-eared slider turtle (*Trachemys scripta*), Andulate tortoise, and Mediterranean Spur-thighed Tortoises (*Testudo graeca*) (Crawford et al., 1976; Jackson et al., 1985; Hughes et al., 1971; Southwood et al., 2003; Cordeiro et al., 2016; Trevizan, 2016; Setlalekgomo and Winter, 2016). Also, under both experimental temperatures, hatchlings utilized less proportion of the daily intake of gross energy was used to resting metabolic rate if we compare with the 40-50% reported in species such as Western fence lizard (*Sceloporus occidentalis*) (Bennett and Nagy, 1977).

The increase of the resting O_2 consumption, CO_2 production, and in consequence, the metabolic rate, with an increase of temperature, has also been described in other reptile species such as Mud turtle (*Kinosternon subrubrum*), Australian crocodiles (*Crocodylus porosus*), Small-mouthed salamanders (*Ambystoma texanum*), Gopher tortoise (*Gopherus polyphemus*), Andulate tortoise, Boa constrictor (*Boa constrictor amarali*), Western desert iguana (*Dipsosaurus dorsalis*), and Three-toed skink (*Saiphos equalis*) (Dawson and Bartholomew, 1958; Grigg, 1978; Zari, 1993; Toledo et al., 2003; Litzgus and Hopkins, 2003; Finkler, 2006; Wu et al., 2009; Greene et al., 2013; Setlalekgomo and Winter, 2016; Goessling, et al., 2018). Likewise, other studies in ectotherms

that evaluated the relationship between temperature and metabolic rate during digestion reported a strong positive relationship, which agrees with the large increase of the maximum O_2 consumption, CO_2 production, and metabolic rate after feeding at 28°C (Secor and Diamond, 1995, 1997; Toledo et al., 2003; Wang et al., 2003; Greene et al., 2013).

In the present study, increased temperature was associated with a significant elevation of the heat increment, contrary to non or little effect of the temperature on total energy expended on SDA commonly described in snakes such as Timber rattlesnakes (*Crotalus horridus*) (Zaidan and Beaupre, 2003), Boa constrictor (Toledo et al., 2003), and Burmese pythons (*Python molurus*) (Wang et al., 2003), where was indicated that temperature could have more influence on the rate of postprandial response but little effect on the heat production. Nevertheless, it is important to highlight that forced feeding protocol is typically used in this kind of studies and animals were not available to control their food intake. On the other hand, the meal size had shown a strong influence on SDA with greater heat increment when animals ate large meals (Tattersall et al., 2004) because of the cost of processing more biomass and, considering that hatchlings showed higher feed intake at 28°C, this could explain the difference on heat increment between temperatures.

Reported constant SDA coefficients at different temperatures suggest that the energetic cost during digestion is temperature independent (Jobling and Davies, 1980; Powell et al., 1999; Wang et al., 2003). Nevertheless, Toledo et al. (2003) indicated that the ecological cost associated with feeding cost decreased by faster digestion rate at higher body temperature, which may vary among species. Thus, there are few cases where net energetic cost tended to be higher at lower temperatures in terms of SDA coefficient, independent of meal size (Zari, 1993; Toledo et al., 2003). This postprandial thermophilic response at higher temperatures could be beneficial not only by decreasing the SDA duration but also by improving the energetic yield associated with digestion (Toledo et al., 2003; Tattersall et al., 2006). On the other hand, the SDA coefficient is commonly expressed in terms of the meal's predicted gross energy. Some authors described a greater magnitude of this coefficient when it is calculated by considering the digestive efficiency since the evidence that the temperature can

influence the assimilation efficiency in some species (Greenwald and Kanter 1979; Lillywhite 1987). If information of efficiency at a digestive and metabolic level can be incorporated into the SDA coefficient at the experimental temperature, identifying significant differences could be easier than in the present study.

Previous studies have reported a temperature-dependency pattern in the duration and magnitude of the postprandial metabolic response. In this sense, snake species such as Timber rattlesnake adults, brown egg eater adults (*Dasypeltis inornate*), Common egg eater neonates (*Dasypeltis scabra*), Burmese python, and Boa constrictor presented shorter time to return to the fasting level completely, less time to peak and faster heat production peak after feeding, at increased temperature (Toledo et al., 2003; Wang et al., 2003; Zaidan and Beaupre, 2003; Greene et al., 2013), which is similar that we observed on specific dynamic action at the higher temperature. However, the mean factorial scope of oxygen and carbon dioxide at both temperatures were statistically similar to what was observed in Timber rattlesnakes and Burmese python (Zaidan and Beaupre, 2003; Wang et al., 2003).

In reptiles, higher body temperatures are required for digestion, and faster metabolic response after feeding (Cowles and Bogert, 1944) because the temperature influences the various gastrointestinal process by increasing gastrointestinal motility, secretion, and absorption (Mackay, 1968; Skoczylas, 1970a,b; Dandrifosse, 1974; Diefenbach, 1975ab; Skoczylas, 1978). Also, the maximal activity of several digestive enzymes of tortoises has been reported directly affected by temperature, which produced metabolic rate variation in different experimental temperatures (Setlalekgomo and Winter, 2016). In contrast, reduced appetite and digestion rate at lower temperatures can be explained by impaired ability to digest in these conditions (Wang et al., 2003). Its physiological performance declines at lower temperatures below the preferred temperature, reflected by decreasing its activity. Although, a non-significant effect of temperature was observed on apparent digestibility in red-footed tortoises at 30°C and 20°C (Mendoza et al., unpublished data) that cannot support the hypothesis of better enzymatic activity at high temperature in this species, and further research is needed in terms of metabolic efficiency.

The obtained temperature coefficient of oxygen and carbon dioxide at 28°C and 18°C, were similar to values described for other tortoises and turtle species such as Mud turtle (20-30°C), Speke's Hinged-back tortoise *Kinixys spekii* (10-25°C), Hermann's Tortoise (18-28°C), and Andulate tortoise (14-18°C) (Hailey and Loveridge, 1997; Kirsch and Roles, 1984; Litzgus and Hopkins, 2003; Setlalekgomo and Winter, 2016). However, lower values of Q_{10} between 1.5-2.9 have also been reported in other species of this group, as the cases of Common Snapping Turtle (*Chelydra serpentina*), Red-Eared Slider turtle, Ornate box turtle (*Terrapene ornata*), Green turtle, Loggerhead sea turtle (*Caretta* caretta), and Gopher tortoise (Lutcavage et al., 1987; Gatten, 1974; Steyermark and Spotila, 2000; Southwood et al., 2003; Goessling et al., 2018). On the other hand, when the Q_{10} had been determined in different intervals of temperatures, lower values were usually observed at higher temperatures (Snyder & Weathers, 1976; Zari, 1993; Hailey and Loveridge, 1997) and greater values at cooler temperatures (Gatten, 1974; Al-Sadoon and Spellerberg, 1985; Zari,1993), indicating that the rate of metabolic change is not isometric with temperature and an important transition from aerobic to anaerobic metabolism may occur at cooler conditions (Zari, 1993).

Resting RQs at both temperatures were closer to reported values in Common box turtle *Terrapene carolina* (0.57-0.70), Mud turtle (0.76±0.06 at 20°C), Western fence lizard (0.67±0.02), Lacerta lizards (0.80-0.88 at 10°C-30°C), and hatchling of Common snapping turtle (0.64-0.65 15-25°C) (Nielsen, 1961; Bennett and Nagy, 1977; Ultsch and Anderson, 1988; Steyermark and Spotila, 2000; Litzgus and Hopkins, 2003; Roe et al., 2005). In those studies, values that were around 0.70 have been considered as indicators of lipid catabolism (Bennett and Dawson, 1976; Litzgus and Hopkins, 2003); this means that our results of RQ during fasting indicates a combination of protein (0.80) and lipids (0.70) catabolism (Seidel, 1978; Withers, 1992).

Studies in juvenile Australian estuarine crocodiles and Green turtles had detailed low fasting RQs between 0.32-0.74 and 0.38-0.45, respectively (Prange and Jackson, 1976; Grigg, 1978; Jones et al., 2009), and in Australian estuarine crocodiles, this was observed at cooler conditions similar to our results at 18°C. This can be attributed to incorporating respiratory-derived CO_2 in the urine,

forming ammonium bicarbonate, buffering ammonia excretion (Grigg, 1978; Jones et al., 2009). Supporting this, Kleiber (1961) indicated that RQ less than 0.70 may be due to the production of uric acid in the excreta or gluconeogenesis from fat, and CO_2 excretion from the lungs is reducing (Coulson and Hernandez, 1964).

On the other hand, RQ >1, RQ =1 means a complete carbohydrate metabolism (Gessaman and Nagy, 1988), was reported after feeding at both temperatures in the presented study, and animals under low temperature showed higher values. In this sense, Gopher tortoises and Mud Turtles at colder temperature also showed a significant elevated RQ (Litzgus and Hopkins, 2003; Goessling et al., 2018), and these authors linked this with anaerobic digestive gases exiting the gastrointestinal tract, as significant anaerobic carbon dioxide production in fresh fecal samples. This explanation gets stronger if we consider that *C. carbonaria* is an omnivorous species but with heavy cellulose digestion by hindgut bacteria.

Body effect – Allometric scaling

Significant effect of body mass on resting metabolism, independent of the temperature, has been described in different reptile species such as the Western Fence lizard (Roe et al., 2005), Desert chameleon (Zari, 1993), Gopher tortoises (Jodice et al., 2006), Desert tortoise (Tracy et al. 2006), and Australian crocodiles (Gienger, 2012), by increasing whole-body metabolism and decreasing mass-specific metabolic rate, with the increment of body mass. This pattern agrees with our results, where higher mass-specific metabolic rates in smaller animals at both temperatures indicate greater oxygen consumption rates per unit of mass in smaller animals than larger ones, as before described by different authors (Benedict, 1938; Kleiber, 1961; Heusner, 1982; Cartland and Grimmond, 1994).

Previous studies in reptiles and amphibians to estimate “b” have usually been done at temperatures of 10-40°C, being described different mass exponents for squamate reptiles (Bennett and Dawson, 1976; Andrews and Pough, 1985) and amphibians (Gatten et al., 1992). At 28°C, the value of the mass exponent of resting metabolic rate was lower than 0.80, the mean allometric exponent reported for squamates and all reptilian taxa (Andrews and Pough, 1985; Chappell and Ellis, 1987). However, our results were similar to exponents

reported in Desert chameleon at 20-35°C (0.61-0.69) (Zari, 1993), Western fence lizard (0.61-0.82) (Roe et al., 2005), and Gopher tortoise (0.63) (Jodice et al., 2006). Also, this allometric exponent was lower than that previously calculated for herbivorous reptiles (0.80-0.813) (Naggy et al., 1999), supporting the suggestion of Jodice et al. (2006) that chelonians may expend energy at a slower rate per unit of body mass compared to other herbivorous reptiles, and that the daily energy expenditure chelonians may scale less strongly with body mass than in other reptiles. Also, the allometric model of herbivorous reptiles of Nagy et al. (1999) included only one Chelonian (Desert tortoise), and in a study of juvenile Leopard tortoises (*Geochelone pardalis*), the daily metabolic energy intake was approximately 30% overestimated by Nagy's equation (Higgins and Edwards, 2009).

In concern to turtle and tortoise species, diverse mass exponents have been reported at different temperatures for groups of species or a specific species: 0.86 at 20°C for turtles and tortoises (Bennett and Dawson, 1976), 0.82 at 22.9-28.4°C for Aldabra tortoises *Testudo gigantea* (Hughes et al., 1971), 0.79-0.83 a 22-29°C for sea turtles (Prange and Jackson, 1976; Wallace and Jones, 2008), and 0.81-0.94 at 15°C and 25°C respectively, for some freshwater turtles (Santos et al., 1990; Mathie and Franklin, 2006). An allometric exponent greater than 0.80 was observed in terms of resting metabolic rate at 18°C, being close to the before-mentioned studies. Higher allometric exponents have also been reported in other reptile species such as Australian crocodiles (0.85), Green turtle (1.0), Kalahari tent tortoises (1.03), and African egg-eating snakes (0.929–1.044) (Prange and Jackson, 1976; Scantlebury and Minting, 2006; Gienger, 2012; Greene et al., 2013).

Ultsch (2013), by a review of turtle studies, described a lower "b" value at higher temperatures (30°C) of 0.70 in non-sea turtles and sea turtles in comparing to 0.77 at 20°C. When turtles at rest and fasting were grouped, the same tendency of greater mass exponent at colder conditions was observed, 0.81 and 0.71 at 20°C and 30°C, respectively. This agrees with our results, with greater mass exponent shown in hatchlings kept under 18°C.

Another critical aspect to be considered is the age of the studied animals; reduced b value has been described when younger animals are included. The use of hatchlings can explain this by having a lower percentage of their total mass

as an ossified shell than do adults, which means they have a higher relative percentage of more active tissue (Ultsch, 2013). Understanding that the majority of previous studies have been done with adult animals, our lower mass exponents in comparison with other Chelonian species could be slightly influenced by age. Besides, growth requires extra food energy for the energy cost to synthesize new biomass (Nagy, 2000), this cost is about 33% - 40% for birds, mammals, fish, and garter snakes (Wieser, 1994; Rombough, 1994; Peterson et al., 1999).

In respect of the mass exponent of daily intake of gross and digestible energy at 28°C, these values were close to allometric exponents described for some herbivorous reptiles such as Desert lizard at 30°C (0.54) and Leopard tortoises (0.51) (Dawson and Bartholomew, 1958; Scantlebury and Minting, 2006). Although, at 18°C, we observed greater exponents being closer to the allometric exponent proposed range for herbivorous reptiles of 0.80-0.813 (Naggy et al. 1999).

Body temperature

Setlalekgomo and Winter (2016) express that some reptiles physiologically thermoregulate by changing their heart rate and conductance to control heating and cooling rates (Bartholomew and Lasiewski, 1965; Kour and Hutchison, 1970), jugular shunts to produce head–body temperature differences (Heath, 1970), and panting (Dawson and Templeton, 1963). Being suggested that head temperature may be primarily regulated than body temperature, where breathing patterns present thermoregulatory roles to control head and brain temperature in reptiles, particularly at higher temperatures to prevent the overheating of the brain (Tattersall et al., 2006).

Lower or similar head temperature than core body temperature at high environmental temperatures has been described in lizards (Webb et al., 1972), which the tendency of the initial rising of the brain temperature to reach a critical value and after that show similar or lower values than body temperature (Tattersall et al., 2006). It could happen in the present study in the animals kept under 28°C, where a non-significant difference was observed between the head and body temperature.

Contrarily, higher head and brain temperatures were described at lower and below preferred body temperatures (Tattersall et al., 2006). In this context,

Dorcas and Peterson (1997) indicated that reptiles use a combination of behavioral thermoregulation and physiological regulation to achieve regulation of head temperature, which appears to be more precisely regulated than body temperature (Webb et al., 1972; Gregory, 1990; Dorcas and Peterson, 1997). Also, this pattern was observed in our study in hatchlings kept under 18°C.

Finally, a significant increase in body surface temperature after feeding has also been detailed in snakes (Tattersall et al., 2004; Stuginski et al., 2011). The magnitude of the thermogenesis was positively related to the meal size. Although the feed intake was greater at 28°C, significantly higher body surface temperatures were presented at both temperatures after feeding.

CONCLUSIONS

We evaluated the resting and postprandial metabolic responses of Red-footed tortoise hatchlings (*C.carbonaria*) at different temperatures to understand the temperature effect on the energy metabolism and model the energy partitioning at different thermal conditions. We found that metabolism at rest and after feeding was significant influenced by temperature, where greater total and mass-specific daily intake of gross and digestible energy, shorter duration of the digestion process, the earlier and faster peak of heat production, greater heat increment, and low energy cost of digestion in terms of digestible energy was observed in hatchlings kept under higher temperature (28°C). However, a non-significant temperature effect was observed on the factorial scope of oxygen and carbon dioxide. Additionally, respiratory quotients lower than 0.70 and higher than 1.0 were possibly caused by increased CO_2 excretion in urine and gases from feces at 18°C, respectively. On the other hand, our results evidenced the priority of thermoregulating the head before body temperature in this species. The thermogenesis at both temperatures after feeding was enough for the surface body temperature to be different from when animals were in a fasting state. Finally, we found a strong effect of body mass on the daily intake of gross and digestible energy and resting metabolic rate, and at 28°C, animals showed allometric exponents lower than the general reptile' mass-exponent of 0.80. In conclusion, the present study indicates the difference in energy utilization by temperature, which is an important factor to consider to offer enough energy in

omnivorous tortoises diets in captivity as a Red-footed tortoise (*Chelonoidis carbonaria*). Also, a possible seasonality behavior was observed at 28°C that requires further in-depth research.

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FINAL CONSIDERATIONS

The present study demonstrated that incubation temperature significantly affected embryo development, hatching success, incubation period, and early growth rate. Higher temperature reduced the incubation period, and the hatchlings had a faster growth rate, and at a lower temperature, a higher hatching index with no embryo mortality was exhibited. Also, the egg size and mass significantly affected the hatchling morphology more than the incubation temperature.

High levels of starch and fiber in the diet significantly affect the mass-specific intake of dry matter, digestive response, final carapace width and its growth rate, pyramiding components, bone mass content, and density, without effect on specific dynamic action. It was demonstrated the importance of offering enough fiber and limited starch in diets of omnivorous hatchling tortoises such as the Red-footed tortoises (*Chelonoidis carbonaria*) was demostrated, which can permit optimal energy utilization and normal growth. Also, strong environmental temperature and age effects were evidenced on the resting metabolic rate and energy metabolism, where younger animals and/or at higher temperatures, animals have a greater mass-specific metabolic rate. On the other hand, an important relationship of the daily intake of gross and digestible energy and metabolic rate with the body mass was reported; described allometric exponents ranged from 0.54 to 0.62, which indicates a greater metabolic rate of red-footed tortoises' hatchlings in comparison to the predicted metabolic rate with the allometric exponent of 0.80 for reptiles in general.