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RESEARCH ARTICLE

Environmental temperature alters the overall digestive energetics and differentially affects dietary protein and lipid use in a lizard

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ABSTRACT

Processing food (e.g. ingestion, digestion, assimilation) requires energy referred to as specific dynamic action (SDA) and is at least partially fuelled by oxidation of the nutrients (e.g. proteins and lipids) within the recently ingested meal. In ectotherms, environmental temperature can affect the magnitude and/or duration of the SDA, but is likely to also alter the mixture of nutrients that are oxidized to cover these costs. Here, we examined metabolic rate, gut passage time, assimilation efficiency and fuel use in the lizard *Agama atra* digesting cricket meals at three ecologically relevant temperatures (20, 25 and 32°C). Crickets were isotopically enriched with ^{13}C-leucine or ^{13}C-palmitic-acid tracers to distinguish between protein and lipid oxidation, respectively. Our results show that higher temperatures increased the magnitude of the SDA peak (by 318% between 32 and 20°C) and gut passage rate (63%), and decreased the duration of the SDA response (by 20% for males and 48% for females). Peak rate of dietary protein oxidation occurred sooner than peak lipid oxidation at all temperatures (70, 60 and 31 h earlier for 20, 25 and 32°C, respectively). Assimilation efficiency of proteins, but not lipids, was positively related to temperature. Interestingly, the SDA response exhibited a notable circadian rhythm. These results show that temperature has a pronounced effect on digestive energetics in *A. atra*, and that this effect differs between nutrient classes. Variation in environmental temperatures may thus alter the energy budget and nutrient reserves of these animals.

KEY WORDS: Gut passage time, Metabolic rate, Nutrient assimilation, Nutrient oxidation, Specific dynamic action, Stable isotopes

INTRODUCTION

An animal's diet provides it with mass and energy, but the processing of ingested food also requires energy – a phenomenon called specific dynamic action (SDA). SDA is defined as the increase in metabolic rate above the standard metabolic rate (the metabolic rate of a fasting, resting animal; SMR) in a postprandial animal (i.e. one that is digesting a meal). Generally, metabolic rate increases immediately after eating, reaches a peak and then gradually returns to the metabolic rate found prior to the meal (McCue, 2006; Secor, 2009). The timing and the magnitude of the peak and the total SDA energy costs differ between species and meal type, and are typically directly proportional to meal size (Andrade et al., 2005; McCue, 2006; Secor, 2009). The SDA accounts for approximately 10–30% of the energy content of the food in non-human animals (McCue, 2006; Andrade et al., 2005; Secor, 2009) and can be considered an important component of the energy budget.

In ectothermic vertebrates, temperature influences digestive energetics in diverse ways. Typically, SMR increases exponentially with temperature due to increased energetic costs of body maintenance (Bennett and Dawson, 1976; Niewiarowski and Waldschmidt, 1992; Clarke, 2003). The magnitude of the peak in postprandial metabolic rate increases with temperature while the duration of the SDA response tends to decrease (Andrade et al., 2005; McCue, 2006; Secor, 2009; Gavira and Andrade, 2013), but the total energy of the SDA response is usually not affected by temperature (McCue, 2006; Secor, 2009; Lei and Booth, 2014). Gut passage rates increase at higher temperatures (Jobling, 1981; McConnachie and Alexander, 2004) – although they may decrease above the preferred body temperature (Harwood, 1979; Van Damme et al., 1991; Xu and Ji, 2006). The increased gut passage rate and reduced SDA duration alleviates the impaired mobility of postprandial animals and thus may reduce predation risks (Pauly and Benard, 2002). Faster digestion may also allow the animal to eat more frequently (McConnachie and Alexander, 2004), which is especially useful when food is temporarily abundant. However, nutrient assimilation efficiency may decline when passage rate increases (McConnachie and Alexander, 2004; Karasov and Martínez del Río, 2007). Moreover, increased metabolic rates at higher temperatures may reduce the aerobic scope, which can limit energy allocation to other activities (Owen, 2001; Pörtner, 2001; Andrade et al., 2005). Hence, animals may have to compromise between digestion rate, energy expenditure and assimilation efficiency.

Assimilation efficiency depends on digestive enzyme activity and gut passage time (McConnachie and Alexander, 2004). As enzymatic activity increases and passage time decreases with temperature, often assimilation efficiency is temperature independent (Van Damme et al., 1991; Ji et al., 1996; Du et al., 2000; Chen et al., 2003; Zhang and Ji, 2004). Yet, in several lizard species, measures of assimilation efficiency are higher at intermediate temperatures and reduce at extreme temperatures (Angilletta, 2001; Zhang and Ji, 2004; Luo et al., 2006; Qu et al., 2011), and the thermal optima for assimilation of nutrients can differ from the temperature at which digestion rate is highest (Coggan et al., 2011; Lee et al., 2015). Further, the thermal optima of assimilation may vary between different dietary nutrients (e.g. lipids

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and proteins; Pafilis et al., 2007; Coggan et al., 2011; Clissold et al., 2013), but this has not been rigorously examined in ectothermic vertebrates.

The SDA is at least partly fuelled by the recently ingested meal (McCue et al., 2015a), but little is known about which nutrients are used to meet the energetic demands in processing that meal. Pythons primarily oxidize dietary proteins in the early stages of digestion and gradually increase their reliance on dietary lipid oxidation thereafter (McCue et al., 2015a). In the present study, we evaluated the influence of temperature on digestion energetics and nutrient oxidation in the lizard *Agama atra*. We measured the oxidation of dietary lipids and proteins during the SDA response across three ecologically relevant temperatures. We evaluated tracer oxidation and metabolic rate simultaneously, allowing us to determine whether proteins or lipids fuelled the energetic costs. Further, we determined the effect of temperature on gut passage rate, peak metabolic rate, SDA duration and total SDA cost.

MATERIALS AND METHODS
Lizards
The southern rock agama lizard, *Agama atra* Daudin 1802, is a widespread lizard in South Africa and encounters a wide range of environmental temperatures (Matthee and Flemming, 2002). It is a generalist insectivore and mostly uses the sit-and-wait strategy of foraging (Cooper et al., 1999).

Adult lizards [n=21 males, snout–vent length (SVL)=97±8 mm, body mass (M_b)=35.42±6.98 g; n=9 females, SVL=88±8 mm, M_b=26.53±3.57 g] were collected by hand or using standard nooses at Gifberg Mountain (South Africa), in May 2016. The lizards were transported to Stellenbosch University, South Africa, where they were individually housed in 45 l glass terraria, with sterilized coarse sand as substrate. A crevice-like refuge and a basking stone were provided for behavioural enrichment. Lizards received UV light (desert T8 lamps, ReptiZoo, China) from 8:00 to 18:00 h and additional light and heat source (Spot Lamp, 75W, ReptiZoo, China) from 10:00 to 14:00 h during the course of the experiment. Every other day, lizards were fed a meal of approximately 3% of their body weight consisting of a combination of crickets (*Acheta domestica*) and mealworms (*Tenebrio molitor*). Water was provided *ad libitum*.

Lizards were collected under the Cape Nature permit number 0056-AAA007-00206. All laboratory procedures were conducted with the approval of the Stellenbosch University Ethics Committee, reference number SU-ACUD14-00110.

Isotopically labelled crickets
To distinguish between the oxidation of dietary proteins and lipids, we labelled crickets with ^{13}C-leucine or ^{13}C-palmitic-acid tracers, respectively. We purchased a population of ~1000 adult house crickets (*Acheta domestica*) from Herpetology Africa (Everton, South Africa) and used them to create the populations (n≈200) that were raised on diets spiked with either of the two ^{13}C-tracers. Adult crickets were housed in glass terrariums kept at room temperature and provided with heat lamps for basking. They were fed with ground cat food (Catmor, Foodcorp, Randfontein, South Africa) and allowed to drink from moistened plastic sponges. The adult crickets laid eggs in dishes filled with moist soil (~2 cm deep) that were removed each week, and replaced with new soil-filled dishes. Soil containing the cricket eggs was kept moist in an incubator at 27°C, until 2 days after the first nymphs appeared. At this time, they were assigned to one of the two isotope treatment groups and were then relocated to larger glass terraria. Sets of nymphs were switched

to a diet of ground cat food supplemented with either ^{13}C-1-L-leucine (1 g tracer per kg of diet) or ^{13}C-1-palmitic acid (2 g of tracer per kg of diet; Cambridge Isotope Laboratories, Inc., Andover, MA, USA) to produce adult crickets isotopically enriched with ^{13}C in body proteins or lipids, respectively. These crickets were only used during experimental feeding trials (see below).

We measured energy and lipid content, and determined ^{13}C enrichment in lean and lipid fractions of crickets from the two treatments. Subsamples of 10 crickets per batch per tracer treatment were killed by freezing and dried at 60°C to determine body water content. The energy content of the crickets (based on dry mass) was determined using a bomb calorimeter (CAL2K-ECO, South Africa). Dry mass energy content was 22.86±0.14 kJ g^{-1} and did not differ between the two isotope treatments (unpaired t-test: t_{18}=−0.19, P=0.84). Water content of the crickets was 16.5% and did not differ between the isotope treatments (unpaired t-test: t_{18}=−1.32, P=0.20). Given that the energy content of live crickets was 27.38 kJ g^{-1}, we calculated the energy in the experimental meals (E_{meal}) by multiplying the mass of crickets consumed (M_{meal}, in g) by 27.38 kJ g^{-1}.

Lipids were separated from the lean mass by repeatedly washing the crickets in a chloroform:methanol (2:1) solution. Liquid fractions were collected and dried to recover the lipids. Lean tissues were then dried and ground. Subsamples of lean and lipid samples were analyzed in triplicate by the Stable Isotope Laboratory in the Department of Archaeology at the University of Cape Town, South Africa (Sealy et al., 2014). The δ^{13}C of lean tissue was −13.9±1.4‰ for untreated (control) crickets, 14.4±5.0‰ for crickets that received ^{13}C-leucine tracer and −4.8±2.2‰ for crickets that received ^{13}C-palmitic-acid tracer. The δ^{13}C of lipids were −18.4‰ for untreated crickets, −14.4±2.1‰ for crickets that received ^{13}C-leucine tracer and 23.4±6.0‰ for crickets that received ^{13}C-palmitic-acid tracer.

Experimental design
To evaluate the temperature effect on protein or lipid oxidation, individual lizards were randomly assigned to receive either crickets enriched with ^{13}C-leucine or ^{13}C-palmitic acid at the start of each trial. Each lizard was evaluated at each of three temperatures (20, 25 and 32°C) in a randomized order.

Before each trial, lizards were fasted for 5–6 days to become postabsorptive and enhance appetite. Then, lizards were offered a meal of ^{13}C-labelled crickets (10% of M_b). The exact amount consumed was determined by the difference in M_b (±0.0001 g; AX504, Mettler Toledo International) before and after feeding. Lizards that ate less than 3% of their M_b were excluded from the trials. Final sample sizes were 22 lizards for 20°C trials (12 for leucine and 10 for palmitic acid), 22 for 25°C trials (13 for leucine and 9 for palmitic acid) and 22 for 32°C trials (13 for leucine and 9 for palmitic acid).

Simultaneous evaluation of metabolic rate and tracer oxidation
Energetic costs and tracer oxidation were evaluated for each lizard by concatenating measures of rates of CO_2 production with δ^{13}C of the CO_2. Lizards were placed in individual metabolic chambers of 350 ml housed within an environmental chamber capable of heating or cooling (Sanyo Cooled Incubator, MIR-153, Sanyo Electric). The environmental chambers were darkened to minimize stress and activity.

To measure metabolic rate, a stream of CO_2-free dry air was generated using a purge-gas generator (PureGas, Broomfield, CO,

USA) and pumped through each metabolic chamber at 300 ml min^{-1} using a mass flow controller (Side-Track Model 840, Sierra Instruments, Monterey, CA, USA). We further ensured the absence of water and CO_2 by passing incurrent gas through columns of silica gel, soda lime and then drierite. Excurrent gas flowed into an 8-channel multiplexer (Sable Systems, RM8 Intelligent Multiplexer, V5, Las Vegas, NV, USA), programmed to sequentially switch between seven metabolic chambers and a baseline channel, allowing for 8 min recordings of the metabolic chambers and 4 min recordings of the baseline each hour. This cycle was repeated for the duration of the trial, except when gas flow was paused for ^{13}C-breath testing (see below). A calibrated LI-COR infrared CO_2/H_2O analyzer (Li-7000, LI-COR, Lincoln, NE, USA) measured ppm of CO_2 relative to the baseline in the active channel of the multiplexer. To avoid build-up of CO_2 during non-measurement periods, the inactive channels were flushed with dry CO_2-free air at ∼50 ml min^{-1}.

From each 8 min sampling period, the first and last 30 s were not used to avoid any small delays in airflow when switching between chambers. For the analysis, respirometry data collected over 6 h was averaged for each lizard. Many lizards showed high metabolic rate in the first hour of the trial, probably due to stress of handling, and most lizards required long periods (20 min) to equilibrate body temperature to the experimental temperature, causing a delay in adjustment of metabolic rate (see also Stinner and Wardle, 1988). Therefore, the first hour was excluded from the analyses. SMR was estimated as the lowest metabolic rate averaged during one sampling period (7 min) in postabsorptive animals at least 48 h after feeding. The SDA response was defined as rate of CO_2 production ($\dot{V}_{CO_2}$) in excess of the SMR. The highest measured $\dot{V}_{CO_2}$ was considered the peak in the SDA response. SDA duration was measured as the time between the start of the trial and the time at which the metabolic rate was equal to SMR. To estimate energy costs, the amount of O_2 associated with SDA was calculated from $\dot{V}_{CO_2}$, assuming a respiratory quotient (RQ) of 0.80 (Chappell and Ellis, 1987; Gessaman and Nagy, 1988; Wang et al., 1997; Hicks et al., 2000). Energy devoted to SDA (E_{SDA}) was then calculated, assuming 20.13 kJ l^{-1} O_2 (Jobling, 1981; McNab, 1999). The proportion of energy in the meal (E_{meal}; see above) that was devoted to SDA, named the SDA coefficient, was calculated as the $E_{SDA}/E_{meal}×100$.

Tracer oxidation
Breath samples were taken every 6 h during the first 3 days, and every 12 h thereafter, except for the trials at 20°C where breath samples were only taken once a day (because of the low metabolic rates). To allow accumulation of CO_2 in the chambers to levels required for isotope measurements (>0.3% of CO_2), airflow through the metabolic chambers was temporarily paused (2–4 h at 32°C, 4–6 h at 25°C, 20 h at 20°C). The CO_2 concentration within the metabolic chambers never exceeded 1%. Trials lasted 7 days at 20°C and 5 days at 25 and 32°C.

We collected 15 ml breath samples from metabolic chambers using a ground-glass syringe (Micro-Mate interchangeable, 20 ml; Cadence Science Inc., USA) and a resealable injection port. These volumes were injected into evacuated Exetainer vials (Labco Ltd, Lampeter, UK). The ^{13}C content was measured in each sample using a Helifan Plus (Fisher, ANalysen Instrumente, Germany) by non-dispersive infrared spectrometry interfaced with a FanAS autosampler as previously described in McCue et al. (2015a, 2017a). δ^{13}C values are reported in terms of the international standard, Vienna Pee Dee Belemnite (VPDB).

The δ^{13}C of the lizards' tissues (and breath) may progressively increase after consuming multiple meals of ^{13}C-labelled crickets; therefore, we measured δ^{13}C in the breath at the start of each trial, which served as a moving baseline from which we could calculate additional ^{13}C enrichment in the breath caused by the experimental meals. The rates of tracer oxidation (T in nmol min^{-1}) at any time point were calculated using the following equation (Welch et al., 2016):

$$T = \left(\frac{\dot{V}_{CO_2} \cdot AFE}{m \cdot k}\right), \tag{1}$$

where $\dot{V}_{CO_2}$ is the metabolic rate (in ml CO_2 min^{-1}), AFE is the atom fraction excess of ^{13}C in the breath (McCue and Welch, 2016), m is the molar mass of the tracer and k is the volume of CO_2 produced per gram of mixed substrate oxidized, using a value of 1.0 l g^{-1} (McCue et al., 2016, 2017b).

Nutrient assimilation and gut passage time
During the trial and the following 2 days, faeces and urates were collected from the chambers or terraria. Gut passage time was estimated by the period between the feeding and the appearance of the first faeces. Although in 30 out of 72 trials lizards produced more than one faecal pellet in the evaluated period, faeces were produced with at least an 18 h difference. As lizards had been fasted for at least 5 days before the trial, we are confident that these faeces are the product of the meal presented in the trial. Faeces and urates were dried in an oven at 50°C and then separated. Per trial, the first full-sized faecal pellet of each lizard was selected for isotope determination. A subsample of that pellet was taken and weighed (analytical balance, UMX2, ±0.0001 mg; Mettler Toledo International). Samples were sent to the Stable Isotope Laboratory in the Department of Archaeology at the University of Cape Town, South Africa, where isotope ratio of the faeces was determined by isotope ratio mass spectrometry (Sealy et al., 2014). Because the δ^{13}C within the meals of each treatment group did not differ, the temperature effect on apparent nutrient digestion efficiency was estimated by directly comparing the mean enrichment of the first post-feeding faeces across temperature treatments.

Statistical analyses
Data were analyzed with generalized linear mixed effects models (GLMM) in R (version 3.3.3; https://www.R-project.org), using the package lme4 (Bates et al., 2015). Initial models included temperature treatment, tracer, initial M_b, meal size and sex, and their second-level interactions, as fixed predictors, and individual as a random factor. Separate models were performed for the following response variables: SMR, SDA duration, magnitude of the SDA peak, timing of the SDA peak, total tracer oxidation, time to oxidation peak, magnitude of oxidation peak and δ^{13}C in the faeces for lizards receiving the ^{13}C-leucine or ^{13}C-palmitic acid labelled food. Parametric or gamma probability distributions were used depending on the nature of the data. Models were simplified by first dropping non-significant interactions, and likelihood ratio tests (LRTs) were used to compare different fixed-effect structures [using the anova function and specifying the method as maximum likelihood (ML); following Zuur et al., 2009; Crawley, 2013]. We provide P-values for fixed effects from the summary of glmer models but checked that interpretations did not differ from LRTs. When using gamma distributions, P-values are not presented, as they are not available from glmer outputs due to issues associated with obtaining P-values for generalized models (see Bates et al.,

2015). Diagnostics for GLMMs followed protocols in Zuur et al. (2009), and consisted of examining residuals and fitted values for violation of homogeneity and patterns in spread. *Post hoc* tests were performed using the glht function (multicomp package; Hothorn et al., 2008) for factor effects and lsmeans function (lsmeans package; Lenth, 2016) for interactions. Means and s.d. are reported throughout the manuscript.

RESULTS

Metabolic rate

Lizards displayed a circadian rhythm in metabolic rate ($\dot{V}_{CO_2}$), and the magnitude of daily changes was directly proportional to environmental temperature (Fig. 1, Fig. S1). At 25°C, lizards had the highest metabolic rate in the late morning and early afternoon (09:00–15:00 h), and $\dot{V}_{CO_2}$ was generally similar between late afternoon and night time. At 32°C, the highest metabolic rate was
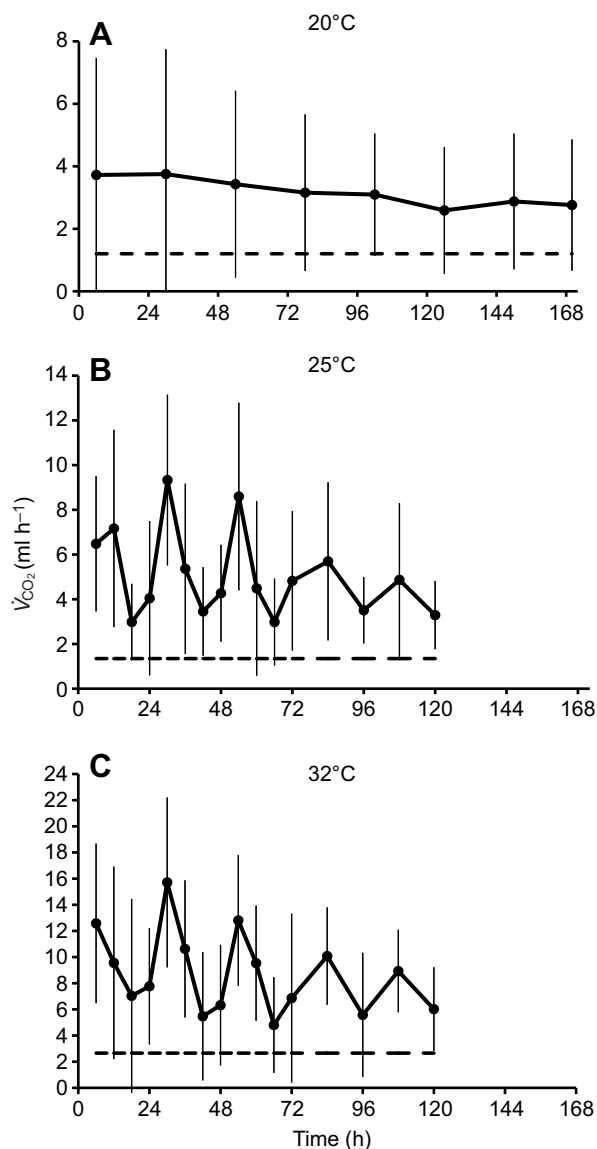

Fig. 1. Mean metabolic rates of *Agama atra* lizards across temperature treatments. (A-C) Temperature treatments of 20 (A), 25 (B) and 32°C (C) are shown. Vertical bars indicate s.d. Dashed lines indicate the average standard metabolic rate (SMR) for the given temperature. The specific dynamic action (SDA) response is the area between the dashed and solid lines.

also observed in the morning and early afternoon, but the metabolic rate in the evening (15:00–21:00 h) was generally higher than during night time. Because metabolic rate was only measured for 4 h each day at 20°C, we cannot quantify the magnitude of the changes in $\dot{V}_{CO_2}$ throughout the day. However, continuous metabolic rate measures from two lizards at 20°C showed a circadian rhythm in metabolic rate with very short yet high peaks in the morning (Fig. S1).

Thermal sensitivity

The best model for SMR showed an interaction between temperature treatment and sex (GLMM: $F_{2,63}$=2.19). In both sexes, SMR was affected by temperature (females: $F_{2,20}$=13.46; males: $F_{2,40}$=22.32; Table 1). SMR increased with temperature in females (Table 1). In males, SMR was significantly higher at 32°C, but did not differ between 20 and 25°C (Table 1). Although males were heavier than females (unpaired *t*-test: $t_{24.31}$=−5.31, $P<0.01$), body mass did not have a significant effect on SMR (GLMM: t=−1.46, P=0.14). The overall Q_{10} was 2.04±1.24, and did not differ between sexes (unpaired *t*-test: $t_{17.67}$=0.61, P=0.55).

Specific dynamic action

We found a significant interaction between sex and the duration of the SDA (GLMM: $F_{2,70}$=4.06, P=0.02). SDA duration in males was independent of temperature ($F_{2, 45}$=1.51, P=0.23). In females, SDA duration was longer at 20°C, but did not differ between 25 and 32°C (Table 1). Tracer type, M_b and meal size had no influence on the duration of the SDA response.

Time to SDA peak decreased at higher temperatures (GLMM: $F_{2,63}$=21.84), but differently in the sexes (interaction Temp×Sex: $F_{2,63}$=3.73). In males, the peak came significantly later at 20°C, but did not differ between 25 and 32°C (Table 1). In females, the peak came significantly earlier at 32°C, but did not differ between 20 and 25°C (Table 1). Tracer type, M_b and meal size did not affect time to the peak of the SDA response.

Temperature significantly increased the magnitude of the peak of the SDA response (GLMM: $F_{2,71}$=42.17, $P<0.01$; Fig. 1, Table 1). SDA peak increased with body mass (GLMM: $F_{1,71}$=9.70). Tracer type, sex and meal size did not affect the magnitude of the peak of the SDA response.

The SDA coefficient was higher at 32°C compared with those at 20°C and 25°C (GLMM: $F_{2,63}$=7.28; Table 1), suggesting that the relative cost of digestion was highest at 32°C. The SDA coefficient was negatively correlated with meal size (GLMM: $F_{1,63}$=26.64), suggesting that the relative cost of digestion was lower for larger meals.

Passage time

Two lizards (one at 20°C and one at 32°C) did not produce faeces within the observed period (time of trial and 2 days afterward) and were excluded from the analyses. Gut passage time decreased with temperature and differed significantly between all three temperature treatments (GLMM: $F_{2,65}$=130.73; Table 1). We found no effect of meal size, tracer type, M_b or sex for gut passage time.

Nutrient oxidation

Oxidation of dietary ^{13}C-leucine and ^{13}C-palmitic acid differed between tracer type and temperatures (Figs 2 and 3). Interestingly, however, temperature did not affect total protein oxidized (GLMM: $F_{2,37}$=0.16, P=0.85). Total lipid oxidized was lower at 20°C, but did not differ between 25 and 32°C (GLMM: $F_{2,28}$=25.10, $P<0.01$; Table 2). During the trials, heavier lizards oxidized more

Table 1. Metabolic and digestive responses across three temperature treatments in *Agama atra* lizards

Parameter	Sex	20°C	25°C	32°C
SMR ($\dot{V}_{CO_2}$ ml h^{-1})	Males	1.69±0.69[a]	1.58±0.44[a]	3.69±2.33[b]
	Females	1.08±0.23[a]	1.49±0.43[b]	2.07±0.5[c]
SDA duration (h)	Males	105.75±46.25[a]	85.60±22.16[a]	84.80±18.82[a]
	Females	135.33±36.72[a]	75.00±21.51[b]	69.75±11.08[b]
Time to SDA peak (h)	Males	100.75±59.14[a]	38.00±29.45[b]	24.00±11.10[b]
	Females	51.33±42.33[a]	45.00±19.24[a]	35.60±11.67[b]
Magnitude of SDA peak ($\dot{V}_{CO_2}$; ml h^{-1})	Both	5.00±3.43[a]	9.86±3.37[b]	15.90±6.20[c]
SDA coefficient (%)	Both	11.04±5.80[a]	7.10±9.20[a]	7.05±5.76[b]
Passage time (h)	Both	169.16±41.51[a]	113.39±35.39[b]	62.86±28.50[c]

Means (±s.d.) are reported for males and females separately when significant differences were found.
Different letters indicate significant differences between temperature treatments as indicated by *post hoc* tests ($P \leq 0.05$).

^{13}C-leucine (GLMM: $F_{1,38}$=10.37, P<0.01) and more ^{13}C-palmitic acid (GLMM: $F_{1,28}$=12.51, P<0.01). Meal size and sex did not influence total oxidation.

The peak in oxidation rate occurred later for lipids than for proteins at any given temperature (*post hoc* test: 20°C: $t_{61.29}$=−8.23; 25°C: $t_{61.70}$=−7.04; 32°C: $t_{61.70}$=−3.59; in all cases P<0.01; Figs 2 and 3). The timing of the peak in oxidation occurred earlier at higher temperatures for both proteins (GLMM: $F_{2,33}$=200.02) and lipids ($F_{2,23}$=39.41, P<0.01; Table 2).

The magnitude of the peak in protein oxidation was independent of temperature (GLMM: $F_{2,21}$=0.47, P=0.63). By contrast, the magnitude of the peak in lipid oxidation varied with temperature (GLMM: $F_{2,23}$=4.90, P=0.02); the peak in lipid oxidation was highest at 25°C, but did not differ between 20 and 32°C (Table 2). Interestingly, the magnitude of the peak in the metabolic rate was not related to the peak in either protein (GLM: $F_{1,33}$=0.26, P=0.11) or lipid ($F_{1,28}$=0.26, P=0.61) oxidation.

Apparent nutrient digestion efficiency
The ^{13}C recovered in the faeces can be used as a proxy for nutrients that were not assimilated. The δ^{13}C in the faeces of lizards that received the protein tracer decreased with temperature (GLMM: $F_{2,36}$=9.18; Table 2), suggesting improved assimilation at warmer temperatures. The δ^{13}C in the faeces of lizards that received the lipid tracer did not differ across temperature treatments (GLMM: $F_{2,23}$=2.11, P=0.14; Table 2). Sex, M_b and meal size did not affect δ^{13}C level in the faeces. The faeces may include waste material derived from previous ^{13}C-enhanced meals; however, we did not find an effect of number of previous trials of isotope level in

the faeces in lizards that received protein tracer (GLMM: F=2.23), nor in those that received lipid tracer (GLMM: F=1.85, P=0.16).

DISCUSSION
This study showed that temperature affected the digestion energetics, nutrient use and assimilation efficiency of the lizard *A. atra*. Dietary proteins were oxidized before lipids at all experimental temperatures. The peak in nutrient oxidation rate occurred earlier at higher temperatures for both proteins and lipids. Similarly, the SDA peak occurred earlier at higher temperatures, yet the timing of the SDA peak did not coincide or even correlate with the peak in tracer oxidation rate for either proteins or lipids. Further, whereas SDA peak increased with temperature, the peak in protein oxidation rate was independent of temperature and lipid oxidation peak was highest at the intermediate temperature (25°C). Hence, the costs of food processing did not dictate the oxidation of the nutrients of that particular meal. Rather the timing of oxidation of the nutrients may depend on the rate at which food assimilation makes the nutrients available for oxidation. Elevated temperatures increased the apparent digestion efficiencies of dietary proteins but not lipids. By contrast, the SDA coefficient was highest at 32°C. Thus, the increased costs of processing the meal at high temperatures may be partly compensated by the higher nutrient assimilation efficiency.

Temperature effects
The peak oxidation rate occurred earlier for both proteins and lipids at higher temperatures, and for lipids the magnitude of the peak also increased. At higher temperatures, food may be processed faster because gastrointestinal motility increases, presumably in response
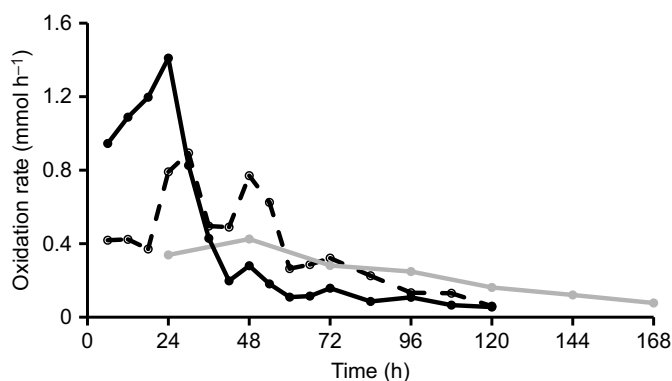

Fig. 2. Mean oxidation rate of leucine in *A. atra* across temperature treatments. Grey dots and line indicate ^{13}C-leucine oxidation rate at 20°C, white circles and dashed line at 25°C, and black dots and solid line at 32°C.
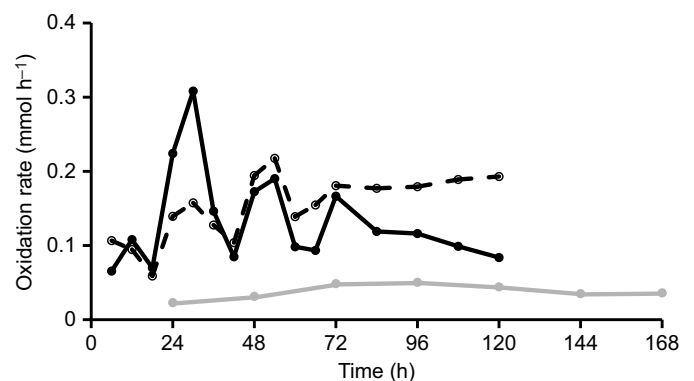

Fig. 3. Mean oxidation rates of palmitic acid in *A. atra* across temperatures. Grey dots and line indicate the rate of ^{13}C-palmitic-acid oxidation at 20°C, white circles and dashed line at 25°C, and black dots and solid line at 32°C.

Table 2. Tracer oxidation in *A. atra* lizards consuming ^{13}C-labelled cricket meals

Parameter	Nutrient	20°C	25°C	32°C
Tracer oxidation (mmol)	Proteins	65.10±41.41[a]	61.48±20.76[a]	61.11±28.13[a]
	Lipids	9.83±7.10[a]	27.04±8.51[b]	24.67±8.12[b]
Time to oxidation peak (h)	Proteins	72.00±14.47[a]	36.46±8.65[b]	12.46±1.66[c]
	Lipids	141.60±39.92[a]	96.00±25.46[b]	43.33±16.91[c]
Magnitude of oxidation peak (mmol h^{-1})	Proteins	25.01±6.11[a]	26.27±5.53[a]	26.23±4.94[a]
	Lipids	0.51±2.79[a]	7.93±4.84[b]	2.88±8.00[a,b]
δ^{13}C in faeces (‰)	Proteins	2.95±8.71[a]	−1.84±5.58[a,b]	−6.69±3.86[b]
	Lipids	−6.52±6.34[a]	−2.50±8.28[a]	−8.13±4.11[a]

Tracer oxidation specifies the total tracer oxidized during the trial (168 h at 20°C, 144 h at 25 and 32°C). Means±s.d. are given.
Different letters indicate significant differences between temperature treatments as indicated by *post hoc* tests ($P \leq 0.05$).

to increased rates of gastric acid secretion, enzyme production and nutrient absorption (reviewed in Andrade et al., 2005). Accordingly, at higher temperatures, we found a faster passage time, and an SDA response with higher and earlier peak, similar to other studies of different taxa (reviewed in Andrade et al., 2005; McCue, 2006; and Secor, 2009). The higher SDA peak, but the shorter duration of the SDA response at higher temperatures, predicts temperature-independent SDA coefficients (Wang et al., 2003; Secor et al., 2007; Lei and Booth, 2014). Yet we found that the duration of the SDA response was only reduced in females and not in males. Further, at 32°C the SDA coefficient was higher, indicating that costs of food processing also increased with temperature. In some earlier studies, the SDA coefficient also increased with temperature (Powell et al., 1999; Secor et al., 2007; but see Toledo et al., 2003). The increase in the SDA coefficient at higher temperatures may be caused by additional gut upregulation (e.g. increase intestine area); however, this is unlikely to occur in frequently feeding species such as lizards (Secor and Diamond, 2000) and these costs may be low (Overgaard et al., 2002). Other physiological responses, such as the increase in enzyme quantity and/or activity, may allow the higher digestion and passage rates (Secor et al., 2007).

Differences between nutrients
Dietary proteins were oxidized before lipids at all temperature treatments. The oxidation rate of proteins decreased during the last days of the trial, whereas lipid oxidation remained high at 20 and 25°C, but decreased at 32°C. In pythons, dietary proteins were oxidized before lipids and lipid oxidation remained elevated well into the postabsorptive period (McCue et al., 2015a). It is possible that a decrease in nutrient oxidation may reflect depletion of that nutrient. Total protein oxidation was similar between temperatures, suggesting that this may be indeed the case. However, protein assimilation was higher at higher temperatures, implying higher availability, although more proteins might have been used for other functions. After their assimilation, the amino acids of the proteins can be oxidized for energy, or can be incorporated into body tissue (growth) or used in reproduction. In fact, both growth rate and reproduction effort are known to increase at higher temperatures (Adolph and Porter, 1993). At lower temperatures, when growth is limited, proteins should be oxidized immediately, as the animal body cannot store them and would thus have to discard these nutrients.

Lipids contain more energy than proteins, yet can be stored and saved for later use (Allen, 1976; Jensen et al., 2012; Price, 2017). The delay in the peak of lipid oxidation compared with protein oxidation may suggest that lipids are conserved until an animal is fasting. Alternatively, the pathway of digestion, absorption into the blood stream and liberation for oxidation may be longer for lipids than proteins.

Nutrient assimilation
Although overall assimilation efficiency has often been found to be temperature independent (Van Damme et al., 1991; Ji et al., 1996; Du et al., 2000; Chen et al., 2003; Zhang and Ji, 2004; McConnachie and Alexander, 2004), nutrient-specific assimilation can either increase or decrease with temperature (Pafilis et al., 2007; Karameta et al., 2017). The recovery of ^{13}C in faeces of lizards that consumed crickets spiked with ^{13}C-leucine diminished with temperature, whereas it remained unaltered in lizards that received ^{13}C-palmitic-acid-spiked crickets. Our results indicate that, even though lipid assimilation was temperature independent, more proteins of the meal were assimilated at higher temperatures. Contrary to our findings, in Mediterranean lizards, lipid assimilation increased and protein assimilation decreased at higher temperatures (Pafilis et al., 2007).

Assimilation depends on the efficiency of physiological digestion processes and gut passage time, the latter of which decreases with temperature. Pafilis et al. (2007) suggest that the shorter gut passage time may be insufficient for optimal protein assimilation. In our study, gut passage time was also reduced at higher temperatures, but protein assimilation actually increased. The effects of reduced gut passage time may be countered by the increase in enzymatic activity at higher temperatures (McConnachie and Alexander, 2004). Contradictory results may have been caused by the confounding effects of waste in the faeces that was not derived from the recently ingested food. In the present study, isotope levels in the faeces indicate the presence of nutrients from the recent ingested meals. Although a portion may be waste material derived from ^{13}C-enriched meals from previous trials, the number of trials per animal was small (maximum three or four trials) and numbers of previous trials did not affect isotope level in the faeces. Further, slower digestion of lipids may result in later expulsion of lipid waste and tracer may have been present in later faeces. Still, the first faeces were notably enriched in ^{13}C, and second faeces were produced much later (>18 h).

At higher temperatures, ectotherms have higher maintenance costs (Bennett and Dawson, 1976; Clarke, 2003; McCue, 2004; Watson and Burggren, 2016). As expected, *A. atra* showed increased SMR with temperature. Yet, total protein oxidation did not differ between temperature treatments. Total oxidation of dietary lipids was lowest at 20°C, but the processing of the meal may have been incomplete, as suggested by the gut passage time (longer than the 7 days of the trial); no differences were found between 25 and 32°C. The temperature independence of total nutrient oxidation, but increased assimilation efficiency at higher temperatures, indicates that more nutrients must have been designated to growth or stored. It also implies that the portion of the SDA covered by the nutrients of the recently ingested meal would be temperature independent if SDA coefficients were similar. However, the SDA coefficient was higher at 32°C and, thus, a smaller part of these energy costs was

covered by the recently ingested meal, and lizards had to use more stored energy.

Our conclusions rely on the assumption that assimilation and oxidation of the ^{13}C-palmitic-acid and ^{13}C-leucine tracer was proportional to the rates of assimilation and oxidation of all lipids and proteins. Leucine is an essential amino acid that accounts for a significant portion of amino acids in proteins, and palmitic acid is likewise found in high quantities (Bukkens, 1997; Finke, 2002). Furthermore, the ^{13}C-tracer from dietary leucine and palmitic acid is readily included in body protein and fat tissues, respectively (McCue, 2011; McCue et al., 2015b; Levin et al., 2017). Therefore, the enhanced ^{13}C levels caused by palmitic acid and leucine tracers are likely to be a good proxy for total lipid and protein assimilation and oxidation. Still, different body tissues may vary in digestibility and it is currently unknown whether temperature has an effect on digestibility of tissues, especially of harder tissues like the exoskeleton. We did not distinguish between chitin and other protein tissues, and therefore we cannot exclude that the increase in apparent protein assimilation is caused by an increase in chitin digestion with temperature. Future studies should investigate this possibility.

Circadian rhythm

We found a strong circadian rhythm in metabolic rate. Higher $\dot{V}_{CO_2}$ was observed between 09:00 and 15:00 h, whereas $\dot{V}_{CO_2}$ was low in the late afternoon and at night. Many species display a circadian rhythm in their activity pattern, even in the absence of thermal and photo cues (e.g. Tosini et al., 2001). In the present study, however, metabolic chambers were very small and movement of the lizards was limited. Some reptile species show a circadian rhythm in postprandial metabolic rate even in constant dark at a constant temperature in small metabolic chambers (Hare et al., 2006), whereas others do not (Gavira and Andrade, 2013). Still, the circadian rhythm observed in this study is stronger at the beginning of the trial when animals are digesting than later when the SDA response has (almost) ended. This suggests that lizards display not only a circadian rhythm in SMR but also in the digestive response.

The circadian rhythm in the SDA may be caused by a circadian pattern in the activity of digestive enzymes, as observed in mammals (e.g. Saito et al., 1975; Konturek et al., 2011; Dibner and Schibler, 2015). Postprandial pit vipers showed that snakes exposed to temperatures fluctuating between 30°C during photophase and 20°C during scotophase exhibited large changes in metabolic rates over several days of digestion (Gavira and Andrade, 2013), but we are not aware of any studies that have evaluated possible circadian rhythms in activity of digestive enzyme in reptiles. If the capacity of processing food is reduced at the end of the day, prey items captured early in the day may give the animals a greater profit (faster and better assimilation of the nutrients) than a prey captured in the afternoon. However, the lack of zeitgebers may have altered the activity period of the lizards in this study, perhaps resulting in the reduction of digestive performance at the end of the day. The patterns should be evaluated under a normal day–night rhythm before any conclusions can be taken.

Ecological significance

We demonstrate a complex interaction among gut passage rate, assimilation efficiency, nutrient oxidation and digestive costs across temperatures. Low and high temperatures affect the digestion process and the energy budget differently, and probably result in distinct behavioural adjustments. At low temperature, many lizards display reduced appetite (e.g. Waldschmidt et al., 1986; Alexander et al., 2001; McConnachie and Alexander, 2004; Lei and Booth, 2014; Miller et al., 2014). *Agama atra* is known to only eat at temperatures above 19°C, and its selected body temperature in a laboratory thermal gradient varies between 32 and 36°C (Bruton, 1977; Van Berkel and Clusella-Trullas, 2018). The increase in gut passage time at low temperatures may cause decay of food in the stomach. A pilot trial showed that placing the lizards at 15°C after having eaten resulted in loss of liquids and regurgitation (results not shown), even though these lizards may be capable of locomotor activity at this temperature (Bruton, 1977). Lizards may manage the effect of reduced energy input by being inactive and maintaining lower body temperatures to reduce body maintenance costs (Lei and Booth, 2014). At high temperatures, the relative costs of food processing (SDA coefficient) increased; however, gut passage time decreased and nutrient assimilation increased. Hence, at high temperatures, animals may have higher food intake and nutrient assimilation, thus increase energy availability and potentially increase growth and reproduction (Andrade et al., 2005; Secor, 2009).

It is common for ectothermic vertebrates to select higher temperatures to improve digestive performance (e.g. Regal, 1966; Gatten, 1974; Lang, 1979; Sievert, 1989; Witters and Sievert, 2001). Climate change and habitat alteration predictions forecast changes in environmental temperatures, which are already perceptible in South Africa (IPCC, 2014; Ziervogel et al., 2014; Schreuder and Clusella-Trullas, 2016). This may reduce thermal heterogeneity and therefore thermoregulation opportunities (Sears et al., 2016; Basson et al., 2017). These constraints may affect how fast and efficiently ectotherms digest meals, and therefore could have substantial effects on the fitness of these animals. Our results indicate that oxidation of nutrients from recent meals drops after a few days. Protein oxidation drops at all temperatures, but also lipid oxidation drops at high temperatures, meaning that animals will have to use stored energy to cover the extra expenses of the SDA at high temperatures, as such depleting their energy reserves. Still, the increase in assimilation efficiency at these temperatures may cover part of the extra expenses. Overall, this study shows that temperature may alter nutrient assimilation and fuel use in ectotherms. Variation in temperature may alter the energy budget and nutrient reserves available to these lizards, and therefore affect other important fitness-related traits, such as growth and reproduction.

Data availability

Data are available from the Dryad Digital Repository (Plasman et al., 2019): dryad.tm38j84.

Supplementary information

Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.194480.supplemental

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