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Ontogenetic shifts in the digestive efficiency of an insectivorous lizard (Squamata: Agamidae)

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In most animals, juveniles are not merely miniature editions of adults. They differ in fact in numerous aspects, including morphology, anatomy, physiology, locomotor performance, and food preferences (Gould 1977). The specific requirements of a fully developed adult demand drastic, sometimes extraordinary, modifications in a suite of important features (Minelli 2006). Such dramatic changes result in distinct allometric rates in anatomy and higher physiological performances (Garland & Else 1987, Nagy et al. 1999). Energy input conforms to this general pattern (de Roos & Persson 2013).

Energy acquisition is one of the main problems animals have to overcome to survive (Karasov & del Rio 2007). Numerous factors affect the energy input, and digestion plays a crucial role. The effective digestive performance determines the amount of energy that can be allocated to growth, reproduction, and maintenance and thus is of pivotal importance (Karasov & Diamond 1988, Schmidt-Nielsen 1997). Most animals have the ability to adjust parameters of their digestion so as to overcome environmental or ecological restraints (Sibly 1981, Diamond 1991). Reptiles are definitely amongst the most successful taxa in this regard: by applying adequate shifts in their digestive performance, they may be able to maximize the extraction of nutrients and so ensure the optimal use of temporally and spatially limited resources (Tracy & Diamond 2005, Iglesias et al. 2009, Naya et al. 2011).

Reptilian digestion is characterized by high plasticity and may be adapted to the varying conditions of temperature and food quantity and quality (Durtsche 2004, McConnachie & Alexander 2004). Reptiles may thus increase or decrease the time food lingers in the digestive tract (van Damme et al. 1991, Sadeghayobi et al. 2011, Miller et al. 2013), improve digestive efficiency (McKinon & Alexander 1999, Pafilis et al. 2007, Moeller et al. 2015), alter the mass and the surface of intestine (Carretero 1997, Starck & Beese 2002, Naya et al. 2009), adapt the length of the digestive track (Vervust et al. 2010), expand fermentation in the small and large intestines (Bjorndal & Bolten 1990), intensify enzyme activity and accelerate brush-border transport rates (Buddington 1992), or even develop cecal valves to slow down food passage and hence increase energy absorbance (Herrel et al. 2008).

Two standardized indices are widely used to assess digestive performance: apparent digestive efficiency (ADE, the relative percentage of ingested energy absorbed through the gut) and gut passage time (GPT, the time food remains in the digestive tract). These two features offer a handy, yet reliable, description of the energy gains of organisms (Beaupre et al. 1993, McKinon & Alexander 1999). ADE in particular, through analysis of the major food components (lipids, proteins, and sugars), provides detailed qualitative information on digestive performance (Witz & Lawrence 1993, Pafilis et al. 2007, Vervust et al. 2010) and is complementary to the classical bomb calorimetry method (Johnson & Lillywhite 1979, McConnachie & Alexander 2004).

Ontogenetic changes in lizards have been reported in a wealth of contributions. Age has a clear impact on head shape and bite force (Herrel et al. 2006), thermoregulation (Tang et al. 2013), tail autotomy (Pafilis & Valakos 2008), antipredator behaviour (Martín & López 2003), habitat use (Stamps 1983, Keren-Rotem et al. 2006), diet (Rocha 1998, Durtsche 2000, Fialho et al. 2000, Durtsche 2004), home range, foraging and risk-taking (Bajer et al. 2015), and locomotor performance (Brecko et al. 2008). Here we focused on a largely insectivorous species (*Stellagama stellio*) and presumed that ontogeny would affect digestion.

Juveniles have relatively higher energy demands, associated with maturation and growth (Nagy 1982, Wikelski et al. 1993). On the other hand, mature individuals have to fuel the highly energy-consuming activities and physiological processes related to reproduction (e.g., yolk production in females and territoriality in males) (Khalil & Abdel-Messeih 1962, Derickson 1976). To assess the impact of age on the digestive performance, we measured the ADE for lipids, sugars, and proteins, GPT, and the length of the gastrointestinal tract.

Stellagama stellio (Linnaeus, 1758), formerly assigned to the genus *Laudakia* (Baig et al. 2012), is a diurnal, primarily rock-dwelling, robust lizard (snout–vent length up to 152 mm, mass up to 120 g) (Valakos et al. 2008). It feeds mostly on arthropods, but its consumption of vertebrate prey has also been reported (Düşen & Öz 2001, Lo Cascio et al. 2001, Baier et al. 2009, Karameta et al. 2015). Greece represents the westernmost point of its distribution and hosts the only natural European populations (the population on Malta is introduced) (Arnold & Ovenden 2003, Valakos et al. 2008). *Stellagama stellio* has a patchy range in this country, mainly limited to insular occurrences on the eastern Aegean islands, a few of the central Cyclades, on Corfu, and Paxoi in the Ionian Sea, and it has recently also been recorded from Crete (Spaneli & Lymberakis 2014). The only mainland population is found in the wider surroundings of Thessaloniki (Valakos et al. 2008).

A total of 25 individuals (13 adults and 12 juvenile) were captured in the area of Sagkri on Naxos Island in the Cyclades (37°1'44.57" N, 25°25'52.48" E) during July of 2012 and subsequently transferred to the laboratory facilities of the Biology Department at the University of Athens. Snout–vent length (SVL) was measured using a digital calliper (Silverline 380244, accuracy 0.01 mm). Lizards grow throughout their life, and thus body size typically serves as a proxy for age (Meiri 2007). Following the literature, we considered individuals with SVLs < 80 mm juveniles (Daan 1967, Loumbourdis 1981, Xyda 1983).

The captured lizards were housed in individual terraria (60 × 30 × 40 cm) that contained a tile to provide cover on a sand substrate. The animals were exposed to a natural photoperiod (sunlight entered through two 2.5 × 1.5-m windows). Temperature was regulated with 60-W incandescent heating bulbs (one over each terrarium) that were on for 8 h every day. An air-conditioning system operating non-stop ensured that the temperature would remain stable at 30°C. During the heated cycle, the temperature inside each terrarium varied from 28 to 36°C. The animals were fed with mealworms (*Tenebrio molitor*) dusted with a multivitamin powder (TerraVit Powder, JBL GmbH & Co. KG) every other day, and water was provided ad libitum. After an acclimatization period of three weeks, the animals were left to fast for five days after which no more faeces were found.

To estimate GPT, a mealworm incorporating a small indigestible plastic marker (3 × 2 × 0.1 mm) was force-fed to each lizard (van Damme et al. 1991). Faeces were collected and inspected for the presence of markers every hour day and night, and the time of collection was recorded. The time lapsed from feeding to the re-appearance of the marker was defined as GPT.

We measured the length of the gastrointestinal tract (GI tract) in six juvenile and twelve adult specimens deposited in the Herpetological Collection of the Natural History Museum of Crete (NHMC 80.3.93.237–240 and NHMC 80.3.93.255–268). Gastrointestinal tract length was measured using a digital calliper (Silverline 380244, accuracy 0.01 mm).

After the end of the GPT measuring experiment, the lizards were returned to the standard feeding schedule for a couple of weeks. We then started evaluating ADE-values by following a different feeding scheme (Pafilis et al. 2007, Vervust et al. 2010). Mealworms were weighed to the nearest 0.1 mg on a digital scale (i500 Backlit Display, My Weight, accurate to 0.01 g) and matched for mass to create groups of ten (for adult lizards) or six (for juveniles) similarly sized food items. Half of the mealworms (five for adults and three for juveniles) were force-fed to each lizard, while the other half was stored at -80°C for further biochemical analysis. Since digestive performance can be influenced by force-feeding (Harwood 1979), we followed the exactly same handling protocol to avoid biases. A sufficient amount of faeces (around 4.5 g) was collected within approximately two months.

Lipid extraction was performed from a homogenized sample (30–40 mg), using 1.5 ml of a 2:1 mixture of chloroform and absolute methanol. The homogenate was then centrifuged at 3,000 rpm for 10 min (at 4°C). The pellet that had formed was discarded and the supernatant used for quantifying the total lipid concentration with the aid of phospho-vanillin (Alexis & Papaparaskeva-Papoutsoglou 1986). We used a mixture of olive and corn oil (2:1 v/v) as standard.

Total sugar amounts were estimated with the classical Dubois et al. (1956) method. Faecal material or mealworms (150 mg) were homogenized with H_2O at a 1:10 w/v ratio and then boiled for 30 min. A subsample of 20 µl was diluted (1:500 v/v) in H_2O and incubated with 1 ml phenol (5% w/v) and 5 ml of 95% H_2SO_4. The sample was then incubated for 10 min at room temperature, and subsequently for 40 min at 30°C. Absorbance was read with a spectrophotometer at 490 nm (Novaspec II, Pharmacia Biotech). The glucose content was identified by comparison with a known glucose standard.

Total protein levels were measured with the Biuret method (Layne 1957). A sample of 0.1–1.2 g was homogenized using perchloric acid (PCA 10%, 1:5 w/v). The homogenate was centrifuged at 10,000 rpm for 5 min at 4°C and the supernatant discarded. The pellet was dissolved with 0.5 ml of 0.1 N NaOH and incubated at 37°C for 30 min. A sample of 50 μl was then diluted with 950 ml of ddH$_2$O; this was subsequently added to a volume of 4 ml of Biuret Reagent. We incubated this mixture for 30 min at room temperature and then read the absorbance at 550 nm. The standard used was bovine serum albumin (0.5–10 mg/ml).

Concentrations of lipids, sugars and proteins in both mealworms and faeces were used to calculate ADEs (denoted as ADE$_{L/S/P}$) for each individual, according to the following equation:

$$ADE_{L/S/P} = (I-E/I) \times 100,$$

where I = amount (lipids, sugars, or proteins) ingested and E = amount (lipids, sugars, or proteins) remaining in the faecal matter after digestion.

Differences in SVL, GPT, and GI tract length (and the ratio of GPT to SVL) between the two age groups were explored with the non-parametric Mann-Whitney U test. Pearson's correlation coefficient was used for calculating the relationship between SVL and GI tract length. To test for differences in ADE between adults and juveniles we used the Mann-Whitney U test. The use of the aforementioned non-parametric tests was based on the results of the Shapiro-Wilk normality test and the Levene's test for homogeneity of variances. Statistical analyses were conducted using SPSS version 22.0 software (SPSS Inc., Chicago, IL).

Snout–vent length and GI tract length were significantly greater in adults (SVL: Mann-Whitney U Test, U = 0, p < 0.001; GI tract: Mann-Whitney U Test, U = 5, p = 0.004) (Table 1). GPT did not differ between the two age groups (Mann-Whitney U Test, U = 11.5, p > 0.05), but the ratio of GPT to SVL was significantly higher in juveniles (Mann-Whitney U Test, U = 4, p = 0.018). Snout–vent length was highly correlated with GI tract length (r = 0.85, p < 0.001) so that we can consider SVL as a good proxy for GI tract length.

We found statistically significant differences in ADE-values for all three food compounds between the two age classes (Table 2). Adult lizards achieved higher ADE$_L$ (Mann-Whitney U Test, U =1, p < 0.001) and ADE$_S$ (Mann-Whitney U Test, U = 28, p = 0.007) than juveniles. However ADE$_P$ followed a reversed pattern and was higher in juveniles (Mann-Whitney U Test, U = 9, p < 0.001).

Our results suggest that age had indeed an effect on digestion. Nonetheless, we failed to detect a common pattern for the digestion of the main nutrients. Although adult lizards achieved higher digestive efficiencies for lipids and sugars, juveniles selected for higher ADE$_P$, GPT did not differ between the two age groups. The latter finding was rather unexpected, particularly as GI tract length was shorter in juveniles, advocating a shorter GPT.

Table 1. Morphological measurements and gut passage time in juvenile and adult individuals. SVL and GI tract length are given in mm and GPT in hours. Means ± standard deviation, sample size in parenthesis.

	Juveniles	Adults
SVL	52.83±7.13 (12)	110.30±7.02 (13)
GI tract length	147.72±13.74 (6)	236.66±54.47 (12)
GPT	67.33±20.18 (6)	86.00±32.04 (7)

Table 2. Apparent digestive efficiencies for lipids, sugars, and proteins. Means ± standard deviation; min.-max., sample size in parenthesis.

	ADE$_{Lipids}$	ADE$_{Sugars}$	ADE$_{Proteins}$
Adults (13)	76.9±9.2; 66.8–95.7	66.5±7.6; 55.5–80.0	74.4±10.9; 58.9–89.6
Juveniles (12)	44.5±12.1; 27.6–68.4	55.9±9.5; 44.9–72.7	93.0±5.2; 84.2–99.1

The time food remains in the GI tract affects the digestive performance (Scoczylas 1978, Hume 2005, but see Miller et al. 2014). According to what current theory predicts, two competing strategies might be adopted: animals can either evacuate their GI tract rapidly so as to maximize the rate of food intake, or retain digesta for longer periods in order to maximize digestive efficiency (Barton & Houston 1993, 1994). Previous research on the GPT of juveniles yielded contradictory results that depend on the feeding regime: herbivorous lizards tend to decrease GPT (Troyer 1984, Wikelski et al. 1993) whereas insectivorous species increase it (Xu & Ji 2006). Our results suggested that GPT-values did not differ between juveniles and adults in S. stellio. However, when food retention time was analysed taking into account body size, the ratio of GPT to SVL was significantly higher in juveniles than in adults. These results suggest that GPT in lizards is a species-specific feature and profoundly affected by life history and overall biology (Pafilis et al. 2007, Miller et al. 2013).

The length of the GI tract largely shapes the food passage rate (Lichtenbelt 1992, Vervust et al. 2010, Naya et al. 2011). Given the short GI tract of juvenile S. stellio, it would be reasonable to expect a low GPT. However, food retention time was similar in adults and juveniles (Table 1). GI tract length is not the only parameter that regulates GPT, though. Activity of luminal protein transport and gastrointestinal motility also impact on the duration of GPT (Da Diefenbach 1975, Hume 1989, Pafilis et al. 2007). These features might account for the high GPT in the young S. stellio and further specialized investigations are required.

The higher ADE$_L$ of adults should be attributed to the different energy needs between mature and juvenile individuals. Adults face higher lipid requirements (Loumbourdis 1987), both for fat storage (Simou et al. 2008) and fuelling of their everyday activities (Simandle et al.

2001), and hence have to achieve a higher ADE_L. Juveniles, in contrast, are free of energy-consuming biological processes such as reproduction.

Sugars represent a direct energy source that is immediately exploited by animals. In reptiles, larger body size implies higher energy requirements (Andrews & Pough 1985, Nagy 2005). Adult agamas were much larger than immature individuals (Table 1), and in order to support their overall metabolic needs, they increase food intake and energy turnover as they grow up (Loumbourdis & Hailey 1991). In this context, the higher ADE_S concurs with the increased energy needs of mature lizards.

Proteins serve as building blocks in the construction of tissues (Stevens & Hume 2004). Young animals that grow larger every day need a continuous flow of amino acids (Troyer 1984). In *S. stellio*, these demands were reflected in the significantly higher ADE_P that reached an impressive maximum of 93% in juveniles compared to 74.4% in adults (Table 2). By processing food at a slower pace, juvenile *S. stellio* offered extra time to digestive enzymes to process protein. Hence they seconded high ADEs for proteins and secured for themselves an optimised amino acid supply.

In conclusion, our study offers evidence for an ontogenetic shift in the digestive efficiency of the major food components. During the early stages of their life, *S. stellio* are more effective in absorbing and assimilating the building blocks that are required to support somatic growth. As they grow older and larger, they turn to be more efficient in the extraction of short- and long-term sources of energy (sugars and lipids, respectively) to fuel their higher energy demands. Further research on species with different feeding preferences and life history traits will shed more light on the fascinating adaptations of the reptilian digestive system.

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