

RESEARCH ARTICLE

Beyond body size: muscle biochemistry and body shape explain ontogenetic variation of anti-predatory behaviour in the lizard *Salvator merianae*

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ABSTRACT

Anti-predatory behaviour evolves under the strong action of natural selection because the success of individuals avoiding predation essentially defines their fitness. Choice of anti-predatory strategies is defined by prey characteristics as well as environmental temperature. An additional dimension often relegated in this multilevel equation is the ontogenetic component. In the tegu *Salvator merianae*, adults run away from predators at high temperatures but prefer fighting when it is cold, whereas juveniles exhibit the same flight strategy within a wide thermal range. Here, we integrate physiology and morphology to understand ontogenetic variation in the temperature-dependent shift of anti-predatory behaviour in these lizards. We compiled data for body shape and size, and quantified enzyme activity in hindlimb and head muscles, testing the hypothesis that morphophysiological models explain ontogenetic variation in behavioural associations. Our prediction is that juveniles exhibit body shape and muscle biochemistry that enhance flight strategies. We identified biochemical differences between muscles mainly in the LDH:CS ratio, whereby hindlimb muscles were more glycolytic than the jaw musculature. Juveniles, which often use evasive strategies to avoid predation, have more glycolytic hindlimb muscles and are much smaller when compared with adults 1–2 years old. Ontogenetic differences in body shape were identified but marginally contributed to behavioural variation between juvenile and adult tegus, and variation in anti-predatory behaviour in these lizards resides mainly in associations between body size and muscle biochemistry. Our results are discussed in the ecological context of predator avoidance by individuals differing in body size living at temperature-variable environments, where restrictions imposed by the cold could be compensated by specific phenotypes.

KEY WORDS: Tegu lizards, Morphology, Muscle biochemistry, Behavioural shift, Temperature, Ontogeny, Predation

INTRODUCTION

Evolution of anti-predatory behaviour has been traditionally explained under the focus of natural selection because the success of organisms to avoid and/or chase predators probably affects their survival in natural environments (Lima and Dill, 1990; Lind and Cresswell, 2005; Magurran et al., 1993; Ydenberg and Dill, 1986).

When facing a predator and after choosing among possible anti-predatory strategies, animals are likely to adjust the characteristics and intensity of the elected behavioural response according to the perceived level of predation risk (Brown et al., 2006; Greene, 1988; Martín and López, 2003; Ydenberg and Dill, 1986). Many factors might influence the choice and intensity of the elected anti-predatory tactic, such as local conditions [i.e. environmental temperature, amount of light/period of day and vegetation cover/terrain characteristics (Savino and Stein, 1989; Christensen and Persson, 1993; Brodie and Russell, 1999; Shine et al., 2000, 2003; Schulte et al., 2004; Durso and Mullin, 2014)] or type and density of predators or their form of attack (Greene, 1988; Langkilde et al., 2004; Relyea, 2001; Seyfarth et al., 1980). The anti-predatory response is still dependent on the condition of the prey, for example, on its stage in the reproductive cycle, nutritional status (Brown and Shine, 2004; Burger et al., 1989; Shine et al., 2000) or its phenotypic characteristics. In the garter snake *Thamnophis ordinoides*, the degree of aggressiveness towards a predator is associated with differences in colour patterns (Brodie, 1989, 1992) and these characteristics may be established during embryo development, as observed by Hagman and colleagues (2015) in another snake, *Natrix natrix*.

From an eco-morphological perspective [based on Arnold's paradigm (Arnold, 1983; Garland and Losos, 1994)], the focus on anti-predatory behaviour is supported in morphological and physiological associations that directly influence performance and, in the latter instance, an individual's fitness. For fighters, predictions of biomechanical models derived for biting performance in squamates and fishes conclude that larger individuals and those with bigger heads will bite harder (Broekhoven and Mouton, 2014; Herrel et al., 2001a, 2002a,b; Verwajen et al., 2002). Individuals having such characteristics may therefore accept higher risks when facing a predator – a trend demonstrated even intra-specifically in males of the lizard *Lacerta monticola* (López et al., 2005). However, survival may also be achieved by running away from a given predator: how fast an animal can flee is frequently sustained by morphological associations, such as the variation in limb proportions observed in many lizard species (see Losos, 1990a,b; Bonine and Garland, 1999; Irschick, 2000; Melville and Swain, 2000; McElroy and Reilly, 2009, for some examples) that seem essential for success in escaping. Survival rates of hatchling lizards, for example, are positively correlated with sprint speeds and stride lengths, although this metric also benefits from increased body size (Miles, 2004; Warner and Andrews, 2002). Given that morphological features favouring combat against a predator do not necessarily enhance the performance of escape, a trade-off between fight and flight abilities might be expected (as observed in Anderson and Vitt, 1990; López and Martín, 2002; Cameron et al., 2013 in a sexual selection context). In fact, such

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List of symbols and abbreviations

AESM	adductor externus superfiliaris muscle
AICc	Akaike information criterion with a correction for finite sample sizes
CS	citrate synthase
ILIM	iliofibularis muscle
L_{fem}	femur length
L_{hum}	humerus length
L_{jaw}	upper jaw length
L_{rad}	radius length
L_{tib}	tibia length
LDH	lactate dehydrogenase
M_b	body mass
T_b	activity body temperature
TBS	total behaviour score

trade-off has been corroborated by data obtained in tegu lizards: individuals exhibiting higher bite forces are those having impaired escape responses because they run more slowly (Herrel et al., 2009).

The expected trade-off between fight and flight abilities probably also resides on physiological traits, such as those related to the metabolic and structural profiles of skeletal muscles. Increased proportions of slow-oxidative fibres – fuelled by aerobic metabolic pathways – might enhance endurance but would also require an improved oxygen delivery capacity and increased mitochondrial scope in skeletal muscles (Bennett, 1991; Gleeson and Harrison, 1986; Johnston, 1981). By contrast, muscle fibres having a glycolytic nature are fuelled by anaerobic metabolic pathways and contract quickly but also fatigue rapidly (Bennett, 1991; Gleeson and Harrison, 1986; Johnston, 1981). The relative contributions of aerobic and anaerobic profiles, established both by relative proportions of different compositions of fibre types and by the activity of specific enzymes, might be coupled with changes in muscle mass, contraction speeds, and elastic and force properties that determine muscle function and ultimately define organismal performance (Ashley-Ross and Barker, 2002; Baldwin, 1982; Bonine et al., 2001; Choi and Park, 1996; Gleeson et al., 1980; James et al., 2005; McBrayer and White, 2002; Pasi and Carrier, 2003; Putnam and Bennett, 1983; Seibel et al., 1998; Throckmorton and Saubert, 1982). As a consequence, we expect an association between the metabolic scope of different muscle groups and the performance exhibited in specific activities where these are involved, which in the context of anti-predatory behaviour, might encompass combat strategies, such as biting, and the escape response of running away.

The morphological and physiological relationships influencing the choice of a given anti-predatory strategy are not static and, especially in vertebrate ectotherms, these can vary according to the thermal conditions where predator–prey interactions occur. For example, locomotion and metabolism are, in general, reduced at low temperatures (Bennett, 1980, 1990; Huey, 1982; but see Angilletta, 2009; James, 2013 for reviews), and an animal's sensorial perception might also be restricted in such conditions (Van Damme et al., 1990). Accordingly, shifts between flight and fight behaviour in response to variation in body temperature have been demonstrated in several squamate lineages (Citadini and Navas, 2013; Crowley and Pietruszka, 1983; Hertz et al., 1982; Mautz et al., 1992; Polčák and Gvoždík, 2014; Schieffelin and De Queiroz, 1991). For example, the agamid lizard *Trapelus pallida* exhibits a temperature-dependent shift from evasive to aggressive behaviours that could be explained by increased thermal sensitivity of muscles used for sprinting in comparison to those involved in biting (Herrel

et al., 2007). Such a temperature-dependent shift in behaviour also has an ontogenetic dimension: adults of tegu lizards (*Salvator merianae*) run away from a predator at high temperatures but choose combat when it is cold, whereas juveniles exhibit the same flight strategy within a wide range of thermal conditions (de Barros et al., 2010). Given that ontogenetic development encompasses modifications at different levels of organization, it is plausible to predict that morpho-physiological parameters could at least partially explain the differences between adult and juvenile tegus in the anti-predatory responses exhibited at lower temperatures.

Ignoring ontogenetic components in studies focusing on temperature-dependent shifts of anti-predatory behaviour seems counterintuitive in face of the effects of a juvenile's survival for the perpetuation of populations in natural habitats. Here, we fill this gap by integrating physiological and morphological traits to test the hypothesis that multivariate models explain the ontogenetic variation in thermal relationships of anti-predatory behaviour reported in the Argentine black and white tegu *Salvator merianae* Duméril and Bibron 1839. Based on this hypothesis, we predict: (1) that enhanced glycolytic scopes in the limb musculature of juveniles compensate for the impairment imposed by small body sizes and allow these individuals to flee from predators even at limiting thermal conditions involving low temperatures; and (2) that modifications of shape, involving relative limb proportions or jaw length, also explain differences in anti-predatory behaviour between the ontogenetic classes of tegu lizards. To test this prediction, we combine behavioural results for recently hatched juveniles and adult tegus reported in de Barros et al. (2010) with new data for body morphometry and muscle biochemistry obtained in hindlimb and head muscles sampled from the same individuals. The integrative approach used here explores interactions among the multiple dimensions that are likely to determine whether a given prey will fight or flee when facing a predator.

MATERIALS AND METHODS

The dataset assembled to test our hypothesis is detailed here following three main categories: morphology, muscle biochemistry and behaviour. We focused on anti-predatory responses exhibited by juvenile and adult tegus at 22.5°C. From a previous study (de Barros et al., 2010) we know that, as body temperature decreases, most adults shift from running away to turning aggressive at 22°C, but juveniles remain evasive, and therefore at this temperature, the behavioural differences become prominent (see Table S1 to access behaviour frequencies in each experimental temperature and Table S2 and Fig. S3 for detailed analyses showing differences in anti-predatory behaviour between ontogenetic classes at lower temperatures).

Composed dataset

Animals and tissue samples

In this study, we combined results on anti-predatory behaviour previously published by de Barros et al. (2010) with new data on morphology and muscle biochemistry obtained for the same individuals studied before. All individuals of *Salvator merianae* used in this study were obtained from a captive population reared outdoors in large enclosures at Universidade Estadual Paulista in Rio Claro (São Paulo, Brazil; permit from IBAMA number 02001-000412/94-28). Tegu lizards are found in both rural and urban areas of Brazil, and animals are found in the wild at the University campus where the outdoor enclosures for captive colonies are located; we therefore assumed that animals used in our study were reared under equivalent conditions as natural populations – with both populations

even being exposed to the same occasional predators (i.e. birds and mammals). After obtaining behavioural data published by de Barros et al. (2010), we measured 17 recently-hatched juveniles (89.81 ± 19.13 g) and 10 adults at 1–2 years of age (871.05 ± 209.85 g) of *Salvator merianae* and sampled muscle tissues. Tegus were killed by overdose of anaesthetic (0.5 ml solution of xylazine and ketamine, 2:1 ratio) that was injected intraperitoneally in juveniles and in adults was inoculated directly into the tail vein in order to enhance anaesthetic effects. Animals were immediately measured (see below), and then tissue samples were removed for biochemical assays, which were performed in two muscles: the adductor externus superficialis (AESM) from the head and the iliofibularis (ILIM), adjacent to the femur. The AESM is involved in jaw movements, including bite (McBrayer and White, 2002; Schwenk, 2000), whereas the ILIM is recruited to push the body during locomotion (Bonine et al., 2005; Jayne et al., 1990). These two muscles are anatomically similar because they both have a central region mainly composed by slow-oxidative fibres that is surrounded by a region where most fibres are glycolytic or exhibit intermediate metabolic characteristics (Gleeson et al., 1980; Putnam et al., 1980; Throckmorton and Saubert, 1982). Samples used in the biochemical assays were dissected from the right side of individuals, immediately frozen in liquid nitrogen and then stored at -70°C . All the procedures were authorized by IBAMA permits (186/2006, number 14109-1/RAN IBAMA) and approved by the Ethics Committee for the Use of Animals in Research of the University of São Paulo (CEUA, protocol number 06.1.1390.53.5).

Morphology

Seven morphological traits of body size and shape were measured in all tegus before muscle dissection. Body mass was recorded in a digital scale and a digital caliper (Mitutoyo CD-15B; ± 0.01 mm) was used to measure snout to vent length (SVL), humerus and radius lengths (L_{hum} and L_{rad} , respectively), femur and tibia lengths (L_{fem} and L_{tib} , respectively) and upper jaw length (L_{jaw} , measured from tympanic aperture to the tip of the snout). For the statistical analyses all morphological variables were \log_{10} transformed, and scaling effects were explored through SMA regressions (see below).

Muscle biochemistry

Biochemical properties of hindlimb and head muscles were assessed based on maximum activity of two metabolic enzymes: lactate dehydrogenase (LDH) was used as an indicator of oxygen-independent pathways, whereas citrate synthase (CS) represented the muscle aerobic metabolism (Eme et al., 2009; Fields et al., 2008; Kirkton et al., 2011; Kohlsdorf et al., 2004; Norton et al., 2000; Rosa et al., 2009; Seibel et al., 1998; de Souza et al., 2004; Vetter and Lynn, 1997). Enzyme activities were measured using a Beckman DU-70 spectrophotometer under saturating and non-inhibitory substrate conditions, following de Souza et al. (2004), as further detailed. We performed all analyses with results obtained at 22.5°C because this is the temperature where ontogenetic differences in the anti-predatory responses exhibited by tegu lizards are prominent (de Barros et al., 2010). In order to minimize possible errors derived from enzymatic activity accessed solely at 22.5°C and with the aim of also evaluating effects of temperature variation on muscle biochemistry, we performed biochemical assays at three temperatures (18, 27 and 36°C) and constructed thermal-sensitivity curves for LDH and CS, which were then used to estimate activities of these two enzymes at 22.5°C . First, we pooled individual values obtained from biochemical assays into individual regression curves and inferred a linear

relationship between temperature and biochemical activity of LDH and CS for each ontogenetic category in both muscles at the thermal experimental interval accessed (see Tables S3, S4 and Figs S4, S5). Subsequently, fitted values at 22.5°C were estimated from each individual regression through a simple linear equation ($y=a+bx$). These procedures were implemented using TableCurve2D software (Systat). Enzyme activities of LDH and CS at 22.5°C were then used for calculation of the LDH:CS ratio in each muscle, used as an indirect measurement of glycolytic or oxidative potential of a tissue (Gleeson and Harrison, 1986).

In order to measure enzyme activities of LDH and CS in head and limb muscles, a cross-sectional fraction 1 mm thick of AESM and ILIM was extracted of each muscle sample previously obtained from tegus stored frozen at -70°C . These fractions included all fibres (white and red) composing the muscles, which are parallel-fibred tissues. Each sample from each individual was homogenized in nine volumes of buffer 20 mmol l^{-1} imidazole-HCl, pH 7.4 with 1 mmol l^{-1} PMSF (phenylmethylsulphonyl fluoride), 2 mmol l^{-1} EDTA and 0.1% Triton X-100. Cellular and mitochondrial membranes were lysed using a sonicator U-200Scontrol (IKA LaborTechnik) with 50% cycle and 0.5 amplitude settings for three periods of 10 s at 4°C . Samples were then centrifuged at 1500 rpm at 4°C for 2 min, and the supernatant was diluted according to each enzyme protocol. Specifically, activity of LDH was estimated as the absorbance of NADH at 340 nm. The assay consisted of 633 μl imidazole-HCl (100 mmol l^{-1}), pH 7.0, 35 μl dithiothreitol (100 mmol l^{-1}), 7 μl NADH (0.15 mmol l^{-1}), 7 μl homogenate (diluted 1:40) and 18 μl pyruvate (40 mmol l^{-1}). Pyruvate was omitted in the control. For CS assay, enzyme activity was measured as the reduction of DTNB (dithio-trinitrobenzoic acid) at 412 nm. The assay consisted of 522 μl Tris-HCl (100 mmol l^{-1}), pH 8.0, 70 μl DTNB (0.1 mmol l^{-1}), 50 μl acetyl-CoA (3 mmol l^{-1}), 28 μl homogenate (diluted 1:30) and 30 μl of oxaloacetic acid (20 mmol l^{-1}). Oxaloacetic acid was omitted in the control. All reactions were performed at a final volume of 0.7 ml and in duplicate. Maximum enzyme activity was set within a range of 90 s intervals, and mean values between duplicates were used in the analyses. Results were expressed in μmol of substrate converted to product per minute per gram of wet mass.

Behaviour: establishment of a total behaviour score (TBS)

We used the original dataset from de Barros et al. (2010) comprising 27 tegus (10 adults and 17 juveniles) to establish a ‘total behaviour score’ (TBS). As explained previously, we focused on anti-predatory responses exhibited at 22.5°C because at this temperature adult tegus shift from running away to confronting the predator, and therefore behavioural variation between juveniles and adults is more prominent. To construct the TBS, we assigned different weights to each of the five anti-predatory behaviours reported by de Barros et al. (2010), giving positive values to aggressive behaviours and negative values to escape behaviours as follows: bite=2, defensive posture=1, no response/immobility=0, walk=−1 and run=−2 (modified from Citadini and Navas, 2013). In order to differentiate the most aggressive individuals, we granted 5 extra points as a positive bonus to the TBS of individuals that bit or assumed a defensive posture with in the first 20 s of each duplicate test. By contrast, 5 points were subtracted from the TBS of individuals that exhibited escape strategies after all stimuli imposed at each duplicate test. As a result, the final TBSs varied from 50 positive points (i.e. 40 points in a hypothetical individual that bit in all trials added with 10 extra points from the positive bonus if the individual was aggressive within the first 20 s in both trials) to 50

negative points (i.e. –40 points in a hypothetical individual that ran away in both trials, and a further –10 points for being evasive in all stimuli). The normality of TBS was confirmed through a Shapiro–Wilk normality test ($W=0.9419$; $P=0.1357$). A detailed table including the total set of responses is provided in Table S1A–E.

Data analyses

All analyses were performed in R (version 3.1.0; <https://cran.r-project.org>) using RStudio (0.98.501). Statistical analyses were carried out in three complementary steps. First, we assessed the physiological disparity between juveniles and adults regarding muscle biochemistry. Then, we analysed allometric differences between juvenile and adult tegus. Finally, we combined morphological and physiological traits to test for associations with anti-predatory behavioural responses exhibited at 22.5°C, which were carried out using two complementary approaches: a canonical correspondence analysis (CCA) to test associations of each behavioural response separately and comparisons between complex linear models that combined morphological and physiological traits to explain the variation in TBSs. Implementation of the analyses performed in each step is further detailed in the three sections below.

Physiological disparity between juveniles and adults

Differences between juveniles and adults regarding enzyme activities in head and hindlimb muscles were tested using a two-factor analysis of variance (ANOVA). Specifically, we used as dependent variables either LDH activity, CS activity or the LDH:CS ratio, whereas muscle and age class were included as independent variables in the model, assuming interactions between terms. A *post hoc* test was performed, with ‘muscle-type’ as a fixed factor across age classes, using the R package ‘phia’ (<http://cran.r-project.org/package=phia>).

Morphological differences between age classes and construction of ‘shape’ matrix

We first tested for differences between juveniles and adult tegus using a Student’s *t*-test on SVL. Subsequently, we investigated allometric differences between age classes testing for significant associations between SVL and the remaining morphological measurements. We tested for significant differences in slope and elevation of regression curves using standard major axis (SMA) regressions implemented in the R package ‘smatr3’ (<http://www.bio.mq.edu.au/ecology/SMATR/>; Warton et al., 2012), where age was included as a covariate. After these allometric analyses, we performed between-group regressions because slopes were similar within ages for all traits; such an approach aimed to separate size

Table 1. Mean values with standard deviation for morphological and physiological traits examined in juvenile and adult tegu lizards

	Adults (N=10)	Juveniles (N=17)
Physiology		
ILIM LDH activity (units g ⁻¹)	790.89±114.34	863.25±139.30
ILIM CS activity (units g ⁻¹)	8.24±1.95	7.16±1.00
ILIM LDH:CS ratio	100.77±26.05	123.28±28.99
AESM LDH activity (units g ⁻¹)	386.06±45.36	319.14±39.23
AESM CS activity (units g ⁻¹)	8.77±1.56	9.49±1.36
AESM LDH:CS ratio	44.93±7.63	33.99±4.49
Morphology		
<i>M_b</i> (g)	871.05±209.85	89.81±19.13
SVL (mm)	285.40±17.73	141.36±10.06
<i>L_{jaw}</i> (mm)	68.60±3.31	37.39±1.70
<i>L_{fem}</i> (mm)	49.50±3.72	23.44±2.31
<i>L_{tib}</i> (mm)	65.20±4.98	28.71±2.44
<i>L_{hum}</i> (mm)	38.00±2.98	17.24±1.56
<i>L_{rad}</i> (mm)	43.70±2.21	21.19±1.66

effects from shape variation (Berner, 2011; Reist, 1985). From this analysis, we retained the regression residuals of the size-corrected humerus (RL_{hum}), radius (RL_{rad}), femur (RL_{fem}), tibia (RL_{tib}) (all measured on right-hand side of each animal) and upper jaw (RL_{jaw}) lengths, which were combined into the multivariate matrix ‘shape’.

Integrative analyses: canonical correspondence analysis and model comparisons

In order to identify relationships between morpho-physiological traits and anti-predatory behaviour in a multivariate framework, we first explored how studied traits were related by performing a CCA (Legendre and Legendre, 1998) and testing the axes significance using the R package *vegan* (<http://vegan.r-forge.r-project.org>). In this step, we used the frequencies of each behavioural category separately to consider the individual values (see detailed table in Table S1B), which totalled 27 observations. We used the ratio between LDH:CS activity of muscles and the shape components matrix as traits. In addition, we included log body mass (log M_b) to the model as a proxy of body size. We chose log M_b instead of SVL because muscle potential is also dependent on the cross-section area and the total number of muscle fibres, which relates to the total volume, rather than length, of the animal. As a complementary step of data analyses, we selected the morpho-physiological traits that most contributed to the variation observed in significant canonical axes and performed a multiple linear model using TBS as a dependent variable. We started by fitting a complex model [$\text{lm}(\text{TBS} \sim \text{ILIM LDH:CS ratio} + \text{AESM LDH:CS ratio} + \log M_b + RL_{jaw} + RL_{hum} + RL_{tib})$] and used a backward elimination to select the best models (Gotelli and Ellison, 2011). The

Table 2. Statistical results from the analyses of variance (ANOVA) performed with enzyme activity as a dependent factor and muscle and age as categorical predictors

	d.f.	LDH activity			CS activity			LDH:CS ratio		
		SS	F	P	SS	F	P	SS	F	P
Muscle	1	819,437	86.936	<0.001	1.430	0.700	0.407	15,590	38.209	<0.001
Age	1	28,202	2.992	0.090	3.230	1.585	0.214	754	1.848	0.180
Muscle:age	1	61,071	6.479	0.014	10.160	4.987	0.030	3524	8.636	0.005
Interactions										
Adductor (J versus A)	1	28,202	2.992	0.180	3.229	1.585	0.428	754	1.848	0.360
Iliofibularis (J versus A)	1	32,962	3.497	0.135	7.350	3.607	0.127	3191	7.821	0.015
Residuals	50									

P-values significant at the 0.05 level are indicated in bold.

SS, sums of squares for ANOVA; *F*, exact or approximate *F*-value; *P*, exact or approximate *P*-value (in case of interaction, *P*-adjusted value by Bonferroni); d.f., degrees of freedom; J, juveniles; A, adults.

Table 3. Results of standard major axis (SMA) regressions performed on morphological traits to examine between-age differences in tegu lizards

	Between-groups test				
	L_{jaw}	L_{fem}	L_{tib}	L_{hum}	L_{rad}
H_0 : slopes are equal	0.3825	0.7030	0.8935	0.9782	0.4821
Estimate (slope)	A=0.796 [0.501, 1.266]; J=0.634 [0.469, 0.855]	A=1.229 [0.789, 1.911]; J=1.372 [0.902, 2.085]	A=1.245 [0.823, 1.884]; J=1.193 [0.710, 1.981]	A=1.251 [0.675, 2.316]; J=1.239 [0.870, 1.765]	A=0.828 [0.434, 1.581]; J=1.073 [0.717, 1.608]
H_0 : no difference in elevation	0.0319	0.2241	0.7478	0.5277	0.8016
Estimate (elevation)	A=0.157 [−0.305, 0.618]; J=0.102 [−0.271, 0.476]	A=−1.481 [−2.519, −0.443]; J=−1.411 [−2.252, −0.571]	A=−1.200 [−2.230, −0.170]; J=−1.182 [−2.016, −0.348]	A=−1.471 [−2.495, −0.446]; J=−1.435 [−2.264, −0.606]	A=−0.789 [−1.708, 0.129]; J=−0.802 [−1.545, −0.059]
d.f.	1	1	1	1	1

P-values significant at the 0.05 level are indicated in bold. d.f., degrees of freedom.

significance level for retention of a given variable in the model was set to 0.05 and Akaike information criterion with a correction for finite sample sizes (AICc) was used to confirm the model with better fit, following Burnham and Anderson (2002).

RESULTS

This study integrates physiological and morphological traits for understanding variation in a temperature-dependent shift of anti-predatory behaviour that results in the differences among ontogenetic classes of tegus. The first step of data analyses here consisted of evaluating the physiological disparity between juveniles and adults. The main differences in muscle profiles were found in the LDH:CS ratio. Specifically, the ILIM is proportionally 3-fold more glycolytic than the AESM, and juveniles have hindlimb muscles proportionally 20% more glycolytic than adults, as detailed in *post hoc* tests (see Table 1 for absolute values and Table 2 for descriptive statistics). We also detected differences between muscles in maximum LDH activity (~2.5-fold higher in hindlimb

than head muscles) but ontogeny does not contribute to such differences. Maximum CS activity did not differ in relation to ontogeny or between muscles.

The second step of data analysis in this study consisted of testing for morphological differences between age classes. This analysis corroborated that juveniles are much smaller than adults in SVL ($F_{1,25}=659.6$, $P<0.001$, see Fig. S2 to access boxplot of size differences between juvenile and adult tegu lizards). Regarding body shape, we also identified differences in slope elevation between age classes in the size-corrected traits: juveniles have proportionally shorter upper jaws than adult tegus (Table 3).

The final step of data evaluation consisted of integrative analyses based on two complementary approaches. Results from the CCA indicated that increased $\log M_b$ and higher values of the LDH:CS ratio in the AESM are positively correlated to aggressive behaviour of tegus in the CCA1, whereas increased values of the LDH:CS ratio in ILIM and a relative shorter upper jaw are associated with evasive strategies on CCA2 (Fig. 1). Humerus and tibia lengths also slightly

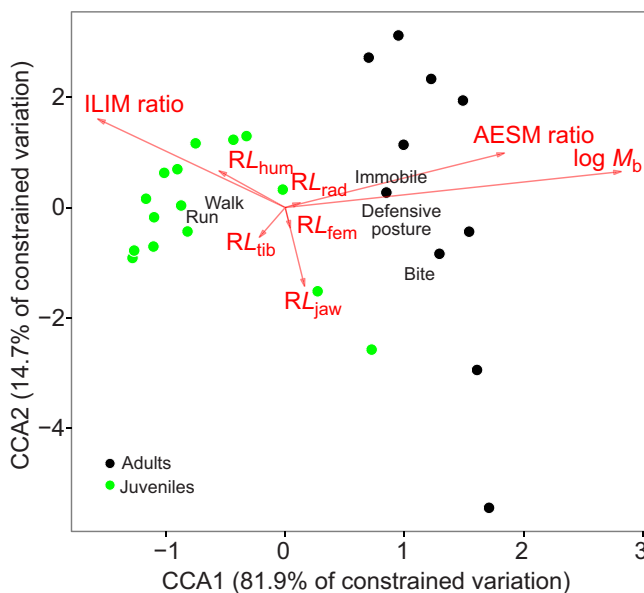


Fig. 1. Scatterplot of the first and second principal canonical axis for juvenile and adult *Salvator merianae*. Data were obtained from CCAs of morphophysiological traits (various bone lengths and muscle LDH:CS ratios, in red) and behavioural responses (walk, run, bite, etc., black text). $N=27$ observations. Red arrows indicate the direction and magnitude of each trait contribution for both representative CCA axes.

Table 4. Correlations between the morphophysiological traits and behavioural responses identified in the canonical correspondence analysis (CCA) with ANOVA results for maximum permutations (9999)

	Canonical variables			
	CCA1	CCA2	CCA3	CCA4
Behavioural response				
Bite	1.108	−1.296	0.624	−0.352
Defensive posture	0.860	−0.274	−0.052	0.067
Immobility	0.767	0.497	−0.012	−0.068
Walk	−0.576	0.107	0.128	0.049
Run	−0.729	−0.154	−0.139	−0.056
Morphophysiological trait				
AESM LDH:CS ratio	0.613	0.329	0.673	0.154
ILIM LDH:CS ratio	−0.524	0.536	−0.024	−0.010
$\log M_b$	0.939	0.217	0.106	0.176
RL_{jaw}	0.054	−0.476	−0.182	0.393
RL_{fem}	0.015	−0.124	−0.176	−0.311
RL_{tib}	−0.072	−0.180	−0.275	0.491
RL_{hum}	−0.185	0.222	−0.419	0.609
RL_{rad}	0.042	0.028	−0.302	0.012
Importance of constrained components (0.66 of variation)				
Eigenvalue	0.540	0.097	0.016	0.005
Proportion explained	0.819	0.148	0.025	0.008
Cumulative proportion	0.819	0.967	0.992	1.000
<i>F</i> -statistic	23.352	4.205	0.711	0.231
<i>P</i> -value	0.001	0.012	0.558	0.915

Numbers in bold indicate statistically significant differences for models.

Table 5. Results of tests of multiple linear model with TBS as dependent variable and body mass, shape components and LDH:CS ratio of muscles as independent variables

	Dependent variable: TBS				
	Model 1	Model 2	Model 3	Model 4	Model 5
AESM LDH:CS ratio	−0.339 (0.566) <i>P</i> =0.556	−0.339 (0.552) <i>P</i> =0.547			
ILIM LDH:CS ratio	−0.200 (0.114) <i>P</i> =0.094	−0.203 (0.106) <i>P</i> =0.070	−0.212 (0.104) <i>P</i> =0.054	−0.240 (0.099) <i>P</i>=0.024	−0.234 (0.098) <i>P</i>=0.026
log <i>M</i> _b	36.756 (9.088) <i>P</i>=0.001	36.716 (8.861) <i>P</i><0.001	32.728 (5.930) <i>P</i><0.001	32.416 (5.908) <i>P</i><0.001	32.542 (5.885) <i>P</i><0.001
<i>RL</i> _{jaw}	275.536 (275.278) <i>P</i> =0.329	262.537 (232.313) <i>P</i> =0.272	286.597 (225.703) <i>P</i> =0.218	173.753 (191.407) <i>P</i> =0.374	
<i>RL</i> _{hum}	−135.906 (135.713) <i>P</i> =0.329	−137.402 (131.558) <i>P</i> =0.309	−120.210 (126.698) <i>P</i> =0.354		
<i>RL</i> _{tib}	−9.799 (104.283) <i>P</i> =0.927				
Constant	−60.745 (22.923) <i>P</i> =0.016	−60.314 (21.924) <i>P</i> =0.012	−63.021 (21.168) <i>P</i> =0.007	−59.037 (20.702) <i>P</i> =0.010	−60.058 (20.596) <i>P</i> =0.008
Observations	27	27	27	27	27
<i>R</i> ²	0.709	0.708	0.703	0.691	0.680
AICc (dAICc)	237.3 (11.7)	233.2 (7.6)	230.0 (4.3)	227.7 (2.1)	225.6 (0.0)
Weight	0.002	0.015	0.076	0.236	0.671
<i>F</i> -statistic (d.f.)	8.104 (6,20)	10.204 (5,21)	13.031 (4,22)	17.149 (3,23)	25.498 (2,24)

The best model (M5) was selected from a complex model (M1) by backward step and AICc values. Standard errors are reported in parentheses. Numbers in bold indicate statistically significant associations for a given model.

contributed to the second axis (Table 4; Fig. 1). The second approach, based on comparisons of AICc, supports a best-fit model where log *M*_b explains anti-predatory behaviour together with the LDH:CS ratio in the ILIM (Table 5, Fig. 2A, B), whereas components of shape had no association with variation in TBS values. These results suggest that juvenile tegus, which more often escape from predators by adopting evasive strategies, exhibit higher glycolytic scopes on hindlimb muscles when compared with young adults.

DISCUSSION

The present study provides an integrative explanation for the ontogenetic variation in a temperature-dependent shift in the anti-predatory behaviour of tegu lizards. According to our results, the

ontogenetic differences in the temperature-dependent shift of anti-predatory behaviour previously reported in tegus (de Barros et al., 2010) resides in multidimensional associations that integrate body size with muscle biochemistry. In the ecological context of predator avoidance in natural habitats, adults are big and therefore might rely on strong bites during combat – a strategy that is inefficient in juveniles, which are comparatively smaller than adults. When tegus emerge from their shelters early in the morning to feed and/or thermoregulate, they experience body temperatures that can vary from 18 to 26°C depending on the season, reaching 33–37°C later in the day (Sanders et al., 2015) – the second range encompassing their preferential temperatures (Cecchetto and Naretto, 2015). Therefore, these animals may in fact face predators when their body temperatures are below their preferred range. Restrictions imposed by environmental thermal conditions may be compensated in juveniles by phenotypes that enhance escape, as further discussed.

Enzyme activities on hindlimb and head muscles

Results from this study confirm that muscles employed for different activities in tegus are metabolically distinct despite having similar fibre type architectures: ILIM, involved in locomotion, is proportionally more glycolytic than AESM, used to bite. Aerobic metabolism provides only a portion of the total energy used to sustain high levels of activity in lizards, and most of the available energy is derived from anaerobic metabolism (Bennett and Dawson, 1972; Bennett and Licht, 1972; but see Bennett, 1991 for a review). The relative proportion of aerobic and anaerobic pathways is however an important factor in these animals, and can be used for understating how the metabolic balance between two pathways influences muscle function. In tegus, AESM is activated during fast and high amplitudes that characterize crushing bites, as well as other head muscles, but is the only one active during slow closing bites (McBrayer and White, 2002). In this case, an equilibrated ratio between LDH and CS activities in the jaw muscle may reflect the important role of slow-oxidative fibres in this muscle for joint stabilization and prey manipulation (Throckmorton and Saubert, 1982), activities common to juveniles and adults.

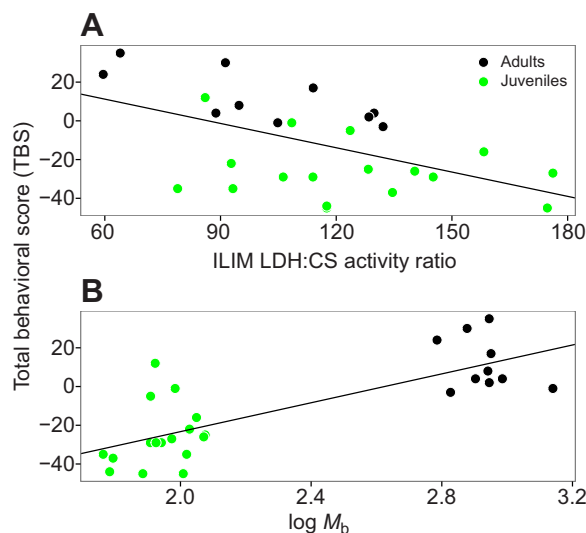


Fig. 2. Correlation between behavioural index and morphophysiological factors in tegu lizards. (A) TBS versus LDH:CS activity ratio in ILIM. (B) TBS versus log *M*_b. *N*=27 individuals. Solid lines indicate regression lines of the model corresponding to each factor.

The results for enzyme activity in the hindlimb muscle differ from those obtained for the jaw muscle: juvenile tegus have a hindlimb muscle that is proportionally more glycolytic than that of adults, a trend that contributes for understanding why recently-hatched tegus rely on running away from a predator regardless of thermal conditions (de Barros et al., 2010). Although energetic demands would favour during growth a reduction in the levels of aerobic metabolism coupled with increased anaerobic rates (for discussions on scaling-effects, see Somero and Childress, 1980; Childress and Somero, 1990; Norton et al., 2000; and Moyes and Genge, 2010 for a review), in juveniles locomotion may face increased selection pressures triggered by predation rates, summed to their developmental demands (Kirkton et al., 2011). In that sense, phenotypes that enhance escaping, such as hindlimb muscles that are comparatively more glycolytic, may compensate for restrictions imposed by environmental conditions involving low temperatures. Adults of *Salvator merianae*, in contrast, exhibit more equilibrated LDH:CS ratios in their hindlimb muscles, a balance that may grant suitable use of aerobic pathways during locomotion. Adult tegus are active foragers that course long distances in their natural habitats (Winck et al., 2011), and they depend on enlarged home ranges for feeding and maintaining large body sizes (Winck et al., 2011 and references therein). An equilibrium between aerobic and anaerobic pathways may not only sustain efficient locomotion of adults through their territories, but likely contributes for buffering muscle pH and enhancing lactate metabolism for glycogen re-synthesis after intense locomotion (Curtin et al., 1997; Gleeson, 1996; Passarella et al., 2008).

Ontogenetic variation of anti-predatory behaviour: integration between morphology, physiology and ecology

Recently-hatched tegus are small and rely on escape behaviours when facing a predator (de Barros et al., 2010). These young animals have enhanced glycolytic capacity in their hindlimb muscles, which probably contributes to success in escaping from an aggressor. The combination of an enhanced anaerobic metabolism in hindlimb muscles with the preference for strategies that involve escape is likely to be effective in animals that are too small to successfully injure a predator (de Barros et al., 2010), especially because access to energetic storages in glycolytic pathways is quick and produces powerful sprints (Bennett, 1991; Bennett and Licht, 1972; Childress and Somero, 1990; Somero and Childress, 1980). Increased LDH:CS ratios in hindlimb muscles of juvenile tegus seem particularly relevant at low temperatures, when enzyme activities may be impaired for sustaining energetic demands of continuous activity but combating the predator is not an efficacious choice.

However, the multilevel equation explaining evolution of anti-predatory behaviour in *Salvator merianae* is not composed solely of morphological proportions and biochemical scopes changing through ontogeny. Tegu lizards hatch in the summer (late December–January in South America) at ~15 g, and face an increase of ~5- to 7-fold in M_b in the subsequent 5 months, before experiencing their first dormancy period in the winter (de Souza et al., 2004). After dormancy, which involves partial loss of M_b , animals restart growing as soon as they resume feeding, and at the end of the first year, young adults can weigh 300 g (de Souza et al., 2004). This ontogenetic change in M_b is coupled with a remarkable differentiation in coloration (see supplementary material Fig. S1) and the cast of predators attacking juveniles and adults may be also variable (A.A., unpublished observations). The cryptic coloration of young tegus, together with the observation that they are rarely

observed moving around in their natural habitats (A.A., unpublished observations), corroborates the hypothesis of ontogenetic differences in the preferred strategy of tegus for avoiding predation: juveniles rely on camouflage and avoid direct interactions with predators, remaining restricted to small territories until reaching a body size that allows effective biting and expansion of home ranges (Winck et al., 2011; A.A., unpublished observations).

The behavioural shifts addressed here meet the ecology of tegu lizards regarding daily variation in body temperatures as well as seasonal changes involving dormancy periods. Early in the morning, the T_b of tegus is mainly influenced by temperature changes inside their shelters and, because this parameter is variable throughout the year (Sanders et al., 2015; Tattersall et al., 2016), in hotter seasons the lizards might avoid emerging from their shelters if T_b is too low. During the night, T_b decreases very slowly, especially in the summer, when nocturnal temperatures are higher – an effect that is more prominent in larger tegus because of thermal inertia (Tattersall et al., 2016). In colder months, however, the night-time minimum T_b in tegus remains low but maximum daily voluntary temperatures also decline, a pattern related to dormancy (Sanders et al., 2015). During this period, although tegus remain inside their burrows most of the time, they also emerge for briefly periods, and their T_b remains between 18 and 22°C (Andrade, 2016; Sanders et al., 2015). Such a condition represents a critical situation for survival, because animals may face predators when their T_b is below the preferred temperature and locomotion could be impaired. Therefore, the behavioural differences between newly hatched and young adult tegus at 22.5°C are ecologically relevant.

Body temperatures can change fast in small lizards because of their body size, but newborn tegus also rely on camouflage to escape from predators (see Fig. S1; A.A., unpublished observations). Consistently, our results indicate that recently hatched juveniles exhibit an enhanced metabolic capacity that is compatible with their propensity to run regardless of thermal conditions (de Barros et al., 2010). Adult tegus may also face predators when emerging from shelters during the winter, but warming might be slow as a result of their large body size. The preference of adult tegus for aggressive behaviour when facing predators presumably compensates for restrictions in escaping abilities imposed by specific environmental conditions that impair locomotion (de Barros et al., 2010; Brodie and Russell, 1999; Citadini and Navas, 2013; Crowley and Pietruszka, 1983; Hertz et al., 1982). Increased body size often enhances biting forces, probably reducing the threshold for the individual to engage an aggressive encounter because the animal will combat predators while minimizing its risk of injury (see Huyghe et al., 2005; Herrel et al., 2009; de Barros et al., 2010 for some examples). In addition to body size, bite performance is also affected by variation in body shape: wider and higher heads accommodate larger jaw muscles and increase jaw lever lengths, which improve bite performance (Herrel et al., 2001a,b; Vanhooydonck et al., 2007). Defensive postures potentially followed by effective bites also demand coordination between jaw, neck and forelimb, as jaw movements must be synchronized with body motion to grant suitable head velocity and jaw opening angles (Montuelle et al., 2009, 2012). For example, in teiids and cordyliforms, strikes on prey or during combat involve forelimb extension at the elbow joint to elevate the neck in the late phase of jaw opening (Montuelle et al., 2012). Because of these relationships, it is not surprising that some morphological measurements in *Salvator merianae*, such as relatively longer upper jaws and shorter humerus bones, have appeared as factors associated with fight behaviour in the CCA. However, associations

with body shape were less apparent in our dataset than those involving body size and muscle biochemistry – a trend that might be due to our sample being composed of young adults. The largest tegus used in our study are on average 900 g and 300 mm in terms of body size, representing a growth stage of 1–2 years of age, at which tegus are not completely sexually mature. Older animals that are capable of reproducing can reach 5–8 kg (A.A., unpublished observations). These animals can become more aggressive and they have enlarged head muscles and cranial structure, which enhances bite performance (see Herrel et al., 2009). Although we could not distinguish the sexes in our sample, in general, the males of *Salvator merianae* show a stronger degree of integration between the cranium, mandible and mandibular muscles, suggesting a sexual selection pressure linked to combat (Fabre et al., 2014). Our study provides evidence for identifiable differences between newly born animals and young adult tegus that have experienced their first cycle of dormancy, and the contrast between ontogenetic classes may be even more prominent when older animals are considered.

Extreme size disparity, in our study represented by recently hatched juveniles and young adults, imposes differences in prey–predator relationships between ontogenetic classes. As discussed here, absolute levels of locomotor output and variation in continuous activity and running bursts depend on morphology and physiology, and the influence of environmental thermal conditions in the preference for a given anti-predatory strategy is coupled with such phenotypic associations. In this study, we show that body size influences the anti-predatory strategy adopted by tegu lizards and we also promote the relative ratio between LDH and CS activity in hindlimb muscles as an important factor for understanding behavioural differences between ontogenetic classes. Other morphophysiological components, such as a longer upper jaw and increased LDH:CS ratios in head muscles, may also contribute to the shift favouring more aggressive behaviour when temperatures are low, although such an effect seems less apparent in our dataset. Organismal traits are often studied under compartmentalized approaches that dissect morphology from physiology in studies of behavioural variation that usually relegate ontogeny. However, we show that these dimensions interact in a complex equation that underlies ontogenetic variation in the thermal-dependent shift of anti-predatory behaviour in tegu lizards.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

F.C.d.B. performed all experiments and statistical analyses. T.K. designed the study and contributed to the statistical approach and data interpretation. T.K. and F.C.d.B. wrote the manuscript. J.E.d.C. provided support for biochemical assays and contributed to the discussion of results. A.S.A. provided the animals and contributed to the discussion, particularly regarding tegu ecology and behaviour.

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Supplementary information

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