Resposta ontogenética a temperaturas de incubação potencialmente impostas pelo aquecimento global no lagarto *Tropidurus torquatus* (Squamata: Tropiduridae)

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RESPOSTA ONTOGENÉTICA A TEMPERATURAS DE INCUBAÇÃO
POTENCIALMENTE IMPOSTAS PELO AQUECIMENTO GLOBAL NO LAGARTO
TROPIDURUS TORQUATUS (SQUAMATA: TROPIDURIDAE)

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"Mas, sob a rajada, ainda há barcos com velas

e há faróis, que ninguém sabe de que terras são."

MOURA, E. 1931. Interrogação.

Sumário

Resumo

A temperatura de incubação exerce grande influência sobre o desenvolvimento ontogenético dos seres vivos atuando, entre outros processos, sobre a expressão gênica. Assim esse é um dos principais fatores geradores de plasticidade fenotípica. Nos últimos anos, os efeitos das mudanças globais de temperatura têm instigado perguntas acerca das alterações nas trajetórias evolutivas das espécies e em especial das que apresentam maior suscetibilidade a fatores ambientais, como os lagartos ovíparos. No presente trabalho foi investigado o desenvolvimento de características morfológicas e fisiológicas da espécie de lagarto tropidurídeo *Tropidurus torquatus* em diferentes regimes de temperatura. No primeiro capítulo é explorada a morfologia genital de adultos de ambos os sexos de forma qualitativa e quantitativa, observando-se a alometria de três caracteres dos hemipenis nos machos e da região cloacal proctodeal-urodeal nas fêmeas e a relação morfológica desses caracteres entre machos e fêmeas. Nesse mesmo capítulo é estudado o desenvolvimento do hemipênis em embriões incubados em temperatura constante de 30°C, temperatura essa amplamente empregada na literatura como controle para a incubação de ovos de lagartos. O hemipênis de *T. torquatus* apresenta alometria positiva ontogenética e estática em seus lobos, enquanto a genitália feminina apresenta alometria negativa em todos os caracteres analisados. Apesar das diferenças registradas nos coeficientes alométricos, a Análise de Covariância indicou que não há diferença entre machos e fêmeas para os caracteres analisados. O padrão de alometria dos lobos do hemipenis de *T. torquatus* e o contraste com a morfologia genital da fêmea sugere que esta característica esteja sob processo de seleção sexual, cujo grau de interação morfológica pode impactar significantemente o processo de inseminação por meio de escolha críptica pelas fêmeas. No segundo capítulo é testada a influência de cinco diferentes regimes de incubação sobre traços de desenvolvimento dos ovos bem como da morfologia e fisiologia termal dos neonatos. Além disso é testada a influência do fator idade na fisiologia termal de neonatos e adultos. Os tratamentos empregados foram: a. em campo; b. 30°C; c. 32,5°C; d. 32,5°C com pico de 39°C durante duas horas por dia e e. 35°C. Com exceção de 35°C, com 100% de letalidade, todos os demais tratamentos registraram nascimentos, afetando negativamente a taxa de

sobrevivência. Além disso, a temperatura de incubação afetou tanto caracteres do desenvolvimento (massa e volume dos ovos, taxa de sobrevivência, razão sexual e tempo de desenvolvimento), quanto caracteres morfológicos (comprimento do corpo, comprimento da cauda, comprimento do tronco, comprimento da cabeça e altura da cabeça). Também houve influência da temperatura de incubação nos caracteres de fisiologia termal em dois dos cinco caracteres analisados, sendo eles, temperatura basal e temperatura crítica mínima. Todos os caracteres de fisiologia termal foram significativamente diferentes quando testados estatisticamente para o fator idade. Neonatos possuem menor temperatura preferencial e maior temperatura basal em relação aos adultos. Além disso, neonatos são mais suscetíveis as temperaturas críticas mínimas e mais tolerantes as temperaturas críticas máximas que os adultos. Contudo, a faixa de operação de temperatura (amplitude total) é menor em neonatos que em adultos. Entender a influência de temperaturas de incubação potencialmente impostas pelo aquecimento global sobre o processo de desenvolvimento e sobre a morfofisiologia de neonatos é de crucial importância uma vez que este pode ser considerado o estágio de vida mais crítico após o nascimento. Além disso, indivíduos adultos contam com uma adaptação ao ambiente onde vivem ainda não experimentada por neonatos. Assim, levando-se em conta o contexto das mudanças da temperatura média do planeta nas próximas décadas, o sucesso de estabelecimento e a manutenção das populações de lagartos ovíparos poderá ser afetada tanto com relação a influência desse fator sobre o desenvolvimento (taxa de sobrevivência, razão sexual) quando com relação a pressão sofrida nos estágios iniciais de vida (*fitness*).

Palavras-chave: desenvolvimento embrionário, hemipenis, alometria, plasticidade fenotípica, fisiologia termal

Abstract

Incubation temperature greatly influence the ontogenetic development of living beings, acting, among other processes, on gene expression. Thus, this is one of the main phenotypic plasticity generating factor. In recent years, the effects of global temperature changes have prompted questions about changes in the evolutionary trajectories of species and especially those that are more susceptible to environmental factors, such as oviparous lizards. In the present work, the development of morphological and physiological traits of the tropidurid lizard *Tropidurus torquatus* was investigated under different incubation temperature regimes. In the first chapter, the genital morphology of adults of both sexes is explored in both qualitative and quantitatively. We test for allometry on three hemipenial traits in males and three cloacal traits in females, and its morphological relationship between sexes. In this same chapter, we describe the hemipenial development of embryos incubated at a constant temperature of 30°C, a temperature widely used in the literature as a control for the incubation of lizard eggs. The hemipenis of *T. torquatus* shows positive ontogenetic allometry and static allometry in its lobes, while the female genitalia have negative allometry in all analyzed traits. Despite the differences recorded in the allometric coefficients, the Covariance Analysis indicated that there is no difference between males and females for the analyzed traits. The allometry pattern of the hemipenial lobes of *T. torquatus* and the contrast with the female's genital morphology suggests that this characteristic is under sexual selection, whose degree of morphological interaction can significantly impact the insemination process through cryptic choice by females. In the second chapter, is tested the influence of five different incubation regimes on egg development as well as on the thermal morphology and physiology of neonates. In addition, the influence of the age factor on the thermal physiology among neonates and adults is tested. Eggs were incubated under the following treatments: a. in the field; b. 30°C; c. 32.5°C; d. 32.5°C with a peak of 39°C for two hours a day, and e. 35°C. With the exception of 35°C, with 100% lethality, all other treatments registered births. Increase of temperature negatively affects the survival rate. In addition, the incubation temperature affected both developmental characters (egg mass and volume, sex ratio and duration of development), as well as

morphological traits (body length, tail length, trunk length, head length and head height). There was also an influence of the incubation temperature on the thermal physiology in two of the five analyzed traits (basal temperature and critical minimal temperature). All thermal physiology traits were significantly different when tested for the age factor. Neonates have a lower preferential temperature and a higher basal temperature when compared to adults. In addition, neonates are more susceptible to critical minimal temperatures and more tolerant of critical maximum temperatures than adults. However, the operating temperature range is smaller in neonates than adults. Understanding the influence of incubation temperatures potentially posed by global warming on the developmental process and on the morpho-physiology of neonates has a crucial importance, since it can be considered the most critical stage of life after birth. In addition, adult individuals have experimented adaptation to the environment not yet experienced by neonates. Thus, taking into account the context of changes in the average temperature of the planet in the coming decades both, the success of establishment and maintenance of oviparous lizard populations, may be affected by incubation temperature pressures on development (survival rate, sex ratio) and over early post-hatchling stages (fitness).

Introdução geral

Desenvolvimento ontogenético e plasticidade fenotípica

O estudo do desenvolvimento de um organismo e das transformações durante o processo de crescimento é conhecido como ontogenia (Gould, 1966). Esse campo da biologia do desenvolvimento tem sido amplamente desenvolvido nos dois últimos séculos e teve sua grande importância demonstrada desde o primórdio dos estudos na área (Gould, 1966, 1968; Alberch *et al.*, 1979). A ontogenia comparada, desde que se estabeleceu em meados do século XIX, tem contribuído fortemente para o entendimento das relações evolutivas e dos processos que geram variação nos organismos (Richman, Buchtová, & Boughner, 2006; Piekarski, Gross, & Hanken, 2014). Atualmente, a ontogenia é uma área multidisciplinar que utiliza diversas técnicas metodológicas para descrever e compreender desde o desenvolvimento morfofisiológico à ecologia funcional e evolução dos organismos nas micro e macro escalas (Werneburg, 2009).

Em conjunto com os processos de seleção natural, as mudanças ontogenéticas são responsáveis pela geração e manutenção da diversidade biológica, seja por meio de processos heterocrônicos, ou por plasticidade fenotípica (West-Eberhard, 1989; Klingenberg, 1998; Smith, 2001). Tendo em vista a possibilidade de estudo, sobretudo em espécies ou grupos modelo, onde é possível conduzir pesquisas de campo ou mesmo induzir experimentalmente gradientes fenotípicos e submetê-los a forças de pressão seletiva replicáveis, um dos objetivos da biologia contemporânea tem sido compreender como a variação ambiental age no processo de desenvolvimento e seleção de traços fenotípicos (West-Eberhard, 2005a,b).

Para a inferência e formulação de hipóteses dos processos responsáveis por gerar e manter a diversidade, é necessário investigar e descrever padrões (Klingenberg, 1998). As descrições dos fenótipos das espécies são de crucial importância, sobretudo quando feitas levando-se em conta os eventos do desenvolvimento. Estudos descritivos-comparativos conduzidos sob a perspectiva ontogenética fornecem pistas importantes, seja para a reconstrução de relações evolutivas (variação interespecífica), ou para a identificação de particularidades do

desenvolvimento das espécies (variação intraespecífica). Em ambos os casos a heterocronia – mudança na trajetória temporal de desenvolvimento de um traço fenotípico – atua como um dos principais mecanismos geradores de variação (Klingenberg, 1998; Smith, 2001).

A diversidade por variação intraespecífica é também gerada e mantida pelo processo de plasticidade fenotípica, definido como a capacidade de um genótipo gerar diferentes fenótipos quando sob ação de fatores ambientais que interfiram na sua expressão e trajetória ontogenética (Via *et al.*, 1995). O conjunto de características morfofisiológicas de um organismo pode ser mais conservado (fenótipo restritivo) ou apresentar um maior gradiente de variação (fenótipo plástico) sob efeito de um fator indutor de plasticidade como, por exemplo, a temperatura. Fenótipos plásticos são reconhecidamente mais vantajosos com relação a fenótipos fixos, uma vez que podem se ajustar a escalas de variação ambiental em diferentes magnitudes (West-Eberhard, 1989). Tal ajuste permite ao indivíduo um gradiente ambiental de sobrevivência e adaptação que não seria possível em casos de fenótipos restritivos. Não obstante, um fenótipo plástico também possui a capacidade de resposta não adaptativa, a qual pode ser neutra ou mesmo deletéria (Ghalambor *et al.*, 2007).

Nas últimas décadas, a integração dessas disciplinas tornou possível a emergência de novos campos como a biologia evolutiva do desenvolvimento (evo-devo) (Diogo, 2018) e sua integração com o estudo da evolução da forma em seu contexto ecológico (eco-evo-devo) (Abouheif *et al.*, 2014). Entre os estudos mais recentes têm se destacado, por exemplo, as pesquisas relacionadas as consequências das mudanças globais de temperatura sobre o risco de extinção e sobre os processos de evolução das espécies (Sinervo *et al.*, 2010; Pacifici *et al.*, 2015; Oostra *et al.*, 2018). A multidisciplinariedade da área é de fundamental importância uma vez que a vulnerabilidade de uma espécie ou população às mudanças ambientais é determinada por uma combinação de fatores tanto intrínsecos (fisiológicos, autoecológicos e genéticos) quanto extrínsecos (fatores ecológicos, mudanças ambientais regionais e micro-habitat) que possibilitam mensurar sua sensitividade, resiliência e capacidade de adaptação (Williams *et al.*, 2008).

A influência da temperatura na história de vida e no desenvolvimento dos ectotérmicos

A temperatura é uma condição determinante na história de vida dos organismos. Entre os metazoários, seu o efeito torna-se ainda mais determinante nos grupos ectotérmicos que, pela incapacidade de regulação da homeostase térmica de forma intrínseca, por meio de seus processos metabólicos, estão sujeitos a regulação extrínseca pela temperatura do ambiente onde se encontram, sendo então diretamente influenciados pela sua variação (Gunderson & Stillman, 2015).

Além disso, a temperatura também pode agir como um fator determinante nos processos biológicos que moldam a trajetória evolutiva de grandes grupos de organismos (Gunderson & Stillman, 2015). Enquanto a maioria dos clados dos amniotas são caracterizados por serem exclusivamente ovíparos (tartarugas, crocodilianos, aves e mamíferos monotremados) ou exclusivamente vivíparos (mamíferos metatérios e eutérios), os répteis da ordem Squamata (lagartos, serpentes e anfisbenas) apresentam uma complexa gama evolutiva desses dois modos reprodutivos, diversidade evolutiva essa que possui relação direta com a temperatura enquanto fator ambiental. Dos 141 eventos evolutivos de surgimento de viviparidade nos vertebrados, 115 deles ocorreram no clado Squamata, onde ambos os modos podem ser observados dentro da mesma família, do mesmo gênero e até mesmo em diferentes populações de uma mesma espécie (Sites, 2011). Os estudos iniciais acerca da evolução dos modos reprodutivos nos répteis apontaram uma ampla correlação entre a ocorrência da viviparidade e a distribuição geográfica dos clados onde tal modo reprodutivo está presente (Tinkle & Gibbons, 1977). Em síntese, as hipóteses iniciais correlacionam o padrão de viviparidade apresentado pelos Squamata à ambientes de temperaturas mais frias (ambientes de altitude ou de latitudes elevadas) (Revisado em Tinkle & Gibbons, 1977). Essas hipóteses foram recentemente revistas e confirmadas sob o ponto de vista filogenético, mostrando que os diferentes eventos de surgimento e reversão dos modos reprodutivos dos Squamata estão relacionados causalmente com condições ecológicas e distribuição filogeográfica dos clados (Ma *et al.*, 2014; Pyron & Burbrink, 2014).

Enquanto aproximadamente 20% das espécies de Squamata experimentam regimes de temperatura de incubação mais estáveis em decorrência da viviparidade, outros 80% (aproximadamente 8 mil espécies) são espécies ovíparas, cuja trajetória de desenvolvimento embrionário é diretamente influenciada por fatores do ambiente de postura dos ovos, em especial a temperatura e a umidade (Shine & Harlow, 1993; Shine, Elphick, & Harlow, 1997; Shine, 2001, 2004, 2006; Du, Lu, & Shen, 2005; Sites, 2011; Ma *et al.*, 2014). Buscando entender a magnitude dos efeitos dessas variáveis, diversos estudos têm se dedicado nas últimas décadas a investigar a plasticidade no desenvolvimento (plasticidade induzida durante os estágios de desenvolvimento intra-ovo), bem como a plasticidade no fenótipo dos indivíduos ao nascerem, em seu primeiro contato com o ambiente. Contudo, esse efeito pós-natal na plasticidade fenotípica em répteis tem sido menos estudado quando comparado ao volume de estudo com plasticidade no desenvolvimento (Goodman & Walguarnery, 2007; Bodensteiner *et al.*, 2020).

Nos últimos cem anos a temperatura global tem sofrido um aumento de 0.74°C e as previsões apontam para um cenário de aumento de 4.3±0.7°C nos próximos 80 anos (Kirtman *et al.*, 2013). Contudo, esse aumento pode ser refletido com maior intensidade em microescalas, afetando a biodiversidade em diversos níveis (Pacifici *et al.*, 2015). Um estudo de campo acompanhando ninhos de uma espécie europeia de lagarto (*Bassiana duperreyi*), por exemplo, demonstrou o aumento de 1.5°C na temperatura média dos ninhos em apenas dez anos e um aumento de 1.7°C na temperatura do ar envolta desses ninhos, durante o mesmo período de acompanhamento (Telemen *et al.*, 2009). Sendo a temperatura um fator crucial no processo de desenvolvimento e estando os ecossistemas sofrendo as constantes mudanças do aumento da temperatura global, diversas são as questões levantadas sobre os possíveis efeitos dessas alterações nos processos de evolução das espécies (Monasterio *et al.*, 2013).

De acordo com Noble *et al.* (2018), atualmente os répteis contam com três centenas de estudos de efeito da temperatura na plasticidade fenotípica em aproximadamente 140 espécies, sendo que os grupos melhor representados são os Squamata (75 espécies) e os Testudinata (69 espécies). Ainda de acordo com Noble *et al.* (2018), as características mais estudadas são: morfologia (164 estudos), seguida

por tempo de incubação (158 estudos), sobrevivência (139 estudos) e razão sexual (130 estudos); enquanto as características menos estudadas são: performance (72 estudos), fisiologia (59 estudos), comportamento (43 estudos) e crescimento (34 estudos).

A morfologia é o traço fenotípico mais abordado nos trabalhos de plasticidade fenotípica em répteis e seu estudo permite compreender quais mudanças corpóreas geradas a partir de gradientes de variação durante a embriogênese influenciarão o período inicial de vida e o estabelecimento do organismo no ambiente (Van Damme *et al.*, 1992; Elphick & Shine, 1998; Andrews, Mathies, & Warner, 2000; Rodríguez-Díaz *et al.*, 2010; Noble *et al.*, 2018). Neonatos de maior porte, por exemplo, possuem diversas vantagens que asseguram sua sobrevivência no início da vida como, sucesso na aquisição de alimento e escape de predadores (Ji, Qiu, & Diong, 2002). Nos estudos de plasticidade morfológica, duas das questões mais visitadas são a implicação ecológica de modificações plásticas na morfologia e os possíveis efeitos das alterações ambientais de temperatura nas espécies com determinação sexual temperatura-dependente durante o período embrionário (Revisado em Roush & Rhen, 2018). Mais recentemente, traços morfofisiológicos específicos como o desenvolvimento gonadal e a expressão hormonal, bem como o desenvolvimento de regiões cerebrais sensíveis a esses hormônios passaram a ser investigados de forma mais integrativa e aprofundada, buscando-se entender como fatores genéticos e bioquímicos modificados pela trajetória de incubação explicam variações em padrões metabólicos e mesmo comportamentais (Singh, Das, & Rhen, 2020).

A vasta maioria de estudos de fisiologia termal em lagartos concentram-se em organismos adultos (Sinervo *et al.*, 2010; Diele-Viegas *et al.*, 2019; Garcia-Porta *et al.*, 2019). Porém, não se pode desconsiderar que organismos adultos possuem uma trajetória de adaptação ao ambiente onde vivem e estudos que investiguem a fisiologia termal no nível de neonatos e juvenis são de grande importância e fazem-se necessários (Gvoždík, 2012). A plasticidade na termorregulação de neonatos incubados sob diferentes regimes de temperatura, bem como a persistência de efeitos da plasticidade no desenvolvimento no período pós-natal (resposta ontogenética) tem sido pouco estudada em répteis, contando com pouco mais de duas dezenas de estudos nesses dois tópicos (Revisado em Du, Elphick, & Shine, 2010 e Bodensteiner

et al., 2020). Enquanto aproximadamente metade dos estudos de efeito da temperatura de incubação na termorregulação de répteis não encontraram efeitos significativos, os resultados dos demais estudos demonstram que a resposta ontogenética pode variar especialmente com relação a resistência a temperaturas críticas, as quais podem ser maiores ou menores do que em adultos (Bodensteiner *et al.*, 2020). Uma possível explicação para isso seria que o fenótipo termal das espécies é resultante de uma adaptação evolutiva ao clima local: o estudo filogeográfico de Garcia-Porta *et al* (2019) com 262 espécies da família Lacertidae concluiu que nessa família de lagartos parâmetros da fisiologia termal foram selecionados de acordo com o clima do ambiente em que as espécies estão distribuídas. Além disso, modelos matemáticos construídos a partir de dados da literatura também indicam que a trajetória evolutiva de aclimatação de um organismo a um dado ambiente é determinada por uma relação tanto de fatores intrínsecos como a condição corpórea do mesmo, quanto por fatores extrínsecos como as condições do próprio ambiente (Rohr *et al.*, 2018).

Espécies de biomas com sazonalidade marcada como o Cerrado, possuem maior vulnerabilidade a variações que afetem o clima local (Nimer, 1979; Pinheiro *et al.*, 2002). Uma vez que as espécies estão adaptadas ao regime de temperatura e precipitação, os quais influenciam diretamente sua dinâmica reprodutiva e história de vida (Colli, 1991; Wiederhecker, Pinto, & Colli, 2002), estas estão fadadas a responder a mudanças nesses fatores com adaptação, caso sejam passíveis de plasticidade, ou a extinção (Thomas *et al.*, 2004; Bellard *et al.*, 2012). Contudo, não existem estudos de plasticidade fenotípica em espécies de lagartos do Cerrado, permanecendo desconhecidos os possíveis efeitos da alteração climática do bioma sobre a biologia das espécies.

Tropidurus torquatus: uma espécie modelo emergente para o neotrópico

Organismos ou espécies modelos são definidas como espécies não humanas amplamente estudadas frente a necessidade de entendimento dos fenômenos biológicos sobre os organismos viventes. Essas espécies são eleitas pela ciência de

acordo com critérios como a adaptação da mesma a condições de estudos de campo e de laboratório. Nos dias atuais, a rápida mudança dos ecossistemas tem culminado em altos níveis de extinção regional ou até mesmo total de várias espécies. Assim, há um alerta para a necessidade de estudos que explorem diversos grupos biológicos e não apenas aqueles consagrados como organismos modelo. Além disso, a utilização de poucos organismos modelo leva a generalizações cuja significância evolutiva e comparativa não são garantidas (Hedges, 2002; Alfred & Baldwin, 2015).

Os vertebrados contam com algumas das espécies mais utilizadas pela ciência como modelo para estudo de fenômenos ecológicos de interesse humano e seus efeitos fisiológicos, sendo elas o camundongo, o rato, a galinha, a rã africana e o peixe-zebra (Alfred & Baldwin, 2015). Dependendo do fenômeno específico a ser investigado, outras espécies têm se destacado como candidatas, como por exemplo a salamandra axolote, *Ambystoma mexicanum*, espécie modelo para o entendimento da capacidade de regeneração orgânica em vertebrados (Diogo & Tanaka, 2012). Os lagartos, por sua vez, têm se consolidado nos últimos anos como organismos modelo de previsão de extinção de espécies frente a mudanças ecossistêmicas, sobretudo com relação a perda de habitat e vulnerabilidade às mudanças climáticas (Sinervo *et al.*, 2010).

O gênero *Tropidurus* conta atualmente com 30 espécies descritas, amplamente distribuídas na América do Sul Cisandina em formações abertas de clima tropical e subtropical (Rodrigues, 1987; Carvalho, de Britto, & Fernandes, 2013; Carvalho *et al.*, 2018; Carvalho, 2013, 2021). São lagartos ovíparos e de médio porte que nas últimas décadas têm sido alvo de diversos estudos ecológicos, evolutivos e de desenvolvimento (Rapp Py-Daniel *et al.*, 2017).

Diversas espécies do gênero, em especial algumas representantes do grupo *Tropidurus torquatus* (*sensu* Frost *et al.*, 2001)*,* são extremamente abundantes em ambientes antropizados, sendo facilmente encontradas em áreas urbanas habitando geralmente jardins e muros residenciais (de Andrade, 2019). Dado esse fator, estudos morfológicos e de desenvolvimento recentes com a espécie *Tropidurus torquatus* têm sido conduzidos com indivíduos coletados em áreas urbanas (Silva *et al.*, 2016; Rapp Py-Daniel *et al.*, 2017; De-Lima *et al.*, 2019).

Entre os lagartos tropidurídeos, *Tropidurus torquatus* (*sensu* Rodrigues, 1987) tem sido a espécie do gênero mais amplamente estudada nas últimas décadas, sobretudo sob o ponto de vista ecológico, onde se encontram investigados aspectos como: morfologia e evolução (Kohlsdorf, Garland, & Navas, 2001; Kohlsdorf *et al.*, 2008; Pinto, Wiederhecker, & Colli, 2005; Kohlsdorf & Navas, 2007; Rocha-Barbosa *et al.*, 2008; Grizante *et al.*, 2010; Brandt *et al.*, 2016; De-Lima *et al.*, 2019; Miranda, Goulart, & Galdino, 2021), reprodução e gametogênese (Teixeira *et al.*, 1999; Vieira *et al.*, 2001; Wiederhecker *et al.*, 2002; Kiefer, Van Sluys, & Rocha, 2008; Van Sluys *et al.*, 2010; Ortiz *et al.*, 2014), desenvolvimento ontogenético (Rapp Py-Daniel *et al.*, 2017), fisiologia (Bergallo & Duarte Rocha, 1993; Kiefer, Van Sluys, & Rocha, 2005; Kohlsdorf & Navas, 2006; Beraldo & Garcia, 2007; Ribeiro *et al.*, 2008; Firmiano *et al.*, 2017; Longhini *et al.*, 2019), parasitologia (Ribas *et al.*, 1998; Pereira, Sousa, & De Souza Lima, 2012), dieta (Fialho, Rocha, & Vrcibradic, 2000; Carvalho *et al.*, 2007; Siqueira *et al.*, 2013), demografia e uso de habitat (Wiederhecker *et al.*, 2003; Kohlsdorf, Ribeiro, & Navas, 2006; Ribeiro *et al.*, 2008; Vieira *et al.*, 2011) e vulnerabilidade a mudanças climáticas (Piantoni, Navas, & Ibargüengoytía, 2016; Piantoni *et al.*, 2019a; Piantoni, Navas, & Ibargüengoytía, 2019b).

O potencial dessa espécie para estudos experimentais em laboratório foi destacado no estudo recente de Rapp Py-Daniel *et al.* (2017), estudo esse cuja metodologia traz as primeiras orientações de cuidados e manutenção em cativeiro para a obtenção de ovos a partir de fêmeas grávidas coletadas em áreas urbanas. Além disso, essa é uma espécie com o ciclo reprodutivo amplamente descrito em diferentes populações (Wiederhecker *et al.*, 2002; Ortiz *et al.*, 2014) além de contar com uma detalhada tabela de estagiamento embrionário, permitindo estudos de base comparativa no campo da biologia do desenvolvimento (Rapp Py-Daniel *et al.*, 2017). Contudo, um protocolo de manutenção bem estabelecido e que demonstre a possiblidade de reprodução da espécie em cativeiro ainda se faz necessário.

Além da presença em ambientes antropizados e da relativa facilidade de manutenção em cativeiro, os lagartos *Tropidurus* contam com um número de ovos por desova suficiente para garantir aleatorizações dos ovos de uma mesma ninhada em diferentes grupos de um mesmo experimento. Segundo Brandt & Navas (2011) o tamanho da desova é variável para os lagartos da subfamília Tropidurinae, variando

de 1 a 16 ovos e está filogeneticamente correlacionado ao tamanho corporal médio das fêmeas.

O estudo de Rapp Py-Daniel *et al.* (2017) registrou uma amplitude de 2 a 10 ovos por desova para a espécie *Tropidurus torquatus*, não mencionando a média. Um estudo de biologia reprodutiva conduzido por Wiederhecker *et al.* (2002) nessa mesma população de lagartos, registrou 6 ovos em média, por desova, com a ocorrência de até três desovas por fêmea em uma mesma estação reprodutiva. Além disso, segundo o mesmo estudo, há diferença entre as médias de ovos nas desovas de uma mesma estação com a diminuição no número médio de ovos de 7.5 na primeira desova, para 5.44 na segunda desova e finalmente 4.5 ovos na terceira desova. Uma segunda população de lagartos *T. torquatus* com seu ciclo reprodutivo bem descrito encontra-se na Argentina, onde as fêmeas apresentam 6.3 ovos por desova em média e amplitude de 4 a 9 ovos (Ortiz *et al.*, 2014). O estudo evolutivo de Brandt & Navas (2011) aponta que *T. torquatus* encontra-se na categoria com média de ovos por desova entre 5.85 e 6.41.

Objetivos

Objetivo geral

Descrever o a morfologia genital, a ontogenia hemipeniana e testar a alometria e coevolução de caracteres genitais da espécie de lagarto *Tropidurus torquatus*. Quantificar as variações plásticas causadas por cinco regimes de temperatura de incubação dos ovos, definidos de acordo com parâmetros da literatura e das projeções do Painel intergovernamental sobre Mudanças Climáticas (IPCC), em traços fenotípicos de história natural, morfologia e fisiologia termal de neonatos da mesma espécie.

Objetivos específicos do primeiro capítulo

- Descrever o desenvolvimento ontogenético da genitália masculina de *Tropidurus torquatus*;

- Testar alometria ontogenética no desenvolvimento dos caracteres hemipenianos;
- Descrever a genitália adulta masculina e feminina dessa espécie, testando a existência de alometria estática em caracteres genitais e;
- Verificar a coevolução das estruturas sexuais de machos e fêmeas.

Objetivos específicos do segundo capítulo

- Incubar ovos da espécie de lagarto *Tropidurus torquatus* em cinco diferentes regimes de temperatura, definidos de acordo com parâmetros da literatura e das projeções do Painel intergovernamental sobre Mudanças Climáticas (IPCC), obtendo dados de taxa de sobrevivência, razão sexual e tempo de desenvolvimento embrionário extra uterino;
- Quantificar as variações na morfologia dos ovos incubados sob diferentes regimes de temperatura com foco no volume, massa e morfologia da casca, nas duas espécies;
- Quantificar as variações na morfologia dos neonatos incubados a diferentes temperaturas, com foco em medidas corpóreas lineares;
- Quantificar as variações na fisiologia termal de neonatos incubados a diferentes temperaturas e compara-las com os padrões fisiológicos termais de adultos da mesma espécie;
- Testar as possíveis correlações das variações morfológicas e fisiológicas com os regimes de temperatura de incubação na espécie estudadas usando métodos estatísticos apropriados;
- Comparar os dados obtidos para a espécie com dados de outras espécies da literatura, buscando identificar as particularidades restritas a espécie, possivelmente explicadas pelos aspectos biológicos da mesma.

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Capítulo 1 – Morfologia, desenvolvimento, alometria e coevolução da genitália de *Tropidurus torquatus*

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Are hemipenial traits under sexual selection in *Tropidurus* lizards? Hemipenial development, male and female genital morphology, allometry and coevolution in *Tropidurus torquatus* (Squamata: Tropiduridae)

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Are hemipenial traits under sexual selection in *Tropidurus* lizards? Hemipenial development, male-female genital morphology, allometry and coevolution in *Tropidurus torquatus* genitalia (Squamata: Tropiduridae)

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Abstract

Male genitalia show a wide morphological variation among animals with internal fertilization and exhibit a high level of evolvability in lizards. Studies have suggested that sexual selection may be driving hemipenial evolution against natural selection and pleiotropy. Since the direct interaction of male and female genitals, coevolution of those is posited by several hypotheses of genital evolution. However, there are few studies on female genitalia morphology, leading to a lack of coevolution description and understanding. Studies of allometric pattern have filled some gaps in answering questions of male genital evolution and could be a powerful tool to clarify coevolution between male and

female genitals. Here, we studied *Tropidurus torquatus* genital morphology. The species of the *Tropidurus* lizards' group, an emerging Neotropical lizard model organism, are marked for having enlarged hemipenial lobes in contrast with other tropidurid species. In this study, we analyzed the hemipenial development in early and late stages, describing morphological variation and ontogenetic allometric pattern. We described male and female genital morphology, using quantitative traits, and examined their static allometric patterns and correspondence. Our study provides a quantitative discussion on lizard's genital evolution, suggesting sexual selection plays an important role in genital evolution on *Tropidurus* lizards.

Key Words

Ontogeny, female internal genitalia, ontogenetic allometry, static allometry, coevolution

Introduction

Snake and lizard species (Squamata) show a pair of intromittent male reproductive organs, called hemipenes. These organs are known to show considerable variation in shape, dimensions, and ornamentation, and are often more divergent than non-genital traits [1–4]. Usually, closely related species tend to differ in hemipenial morphology, leading to the use of hemipenis as a key taxonomic and phylogenetic character [5–8]. Along with this, hemipenial traits size have higher rates of evolution compared with other morphological traits [9,10]. Few studies investigating the ontogenetic development of hemipenial morphology exist, and the majority of them focus on the first stages of development [11–13]. As a consequence, little is known about how hemipenis morphological complexity and ornamentation are drive by developmental processes [11].

Several studies have suggested that sexual selection may be driving hemipenial evolution. However, a recent study suggests that hemipenial

morphological variation could be a result of sexual selection and pleiotropy acting simultaneously as antagonistic forces, where pleiotropy would constrain evolutionary speed, while sexual selection would accelerate evolutionary rates [3,14–16]. Studies of allometric patterns, static and ontogenetic, that have been extensively used to describe morphological variation in genitalia across species of insects and arthropods, still incipient among vertebrates, especially for squamates [17]. The allometric pattern is the description of the proportional size of a particular structure in relation to body size, calculated across multiple individuals in a population [18]. If the allometric pattern is measure across the developmental trajectory, it is called ontogenetic allometry [19]. Positive allometry often has been associated with traits under sexual selection, however, most of these conclusions were drawn exclusively from species with unusually expressed traits [e.g. 17,18]. Nevertheless, Bonduriansky [22] showed that positive allometry in specific traits is not always a predictor of sexual selection. Eberhard [18,23] described the allometric patterns for male genitalia across several species with internal fertilization. He showed that the vast majority of the analyzed species have negative allometric slopes in their genitalia. Based on these results he formulated the "one-size-fits-all" hypothesis, that is interpreted as evidence of stabilizing sexual selection, keeping male genital size constant, in a way to match the average female genital size in the population [17,18,23].

The direct interaction between male and female genitals in animals with internal fertilization implies that their morphology would be coevolving, either by females choosing for males are better stimulators or by an arms race between the sexes over the control of insemination and fertilization [24]. However, there is a lack of information about female genitalia morphology and consequently how coevolution plays in the role genital evolution [25]. Squamata´s female genitalia are an internal organ, soft and challenging object of study [24,26]. Few studies are available, however most of them focus on qualitative description of morphological variation, and fewer compare morphologies between sexes [24].

Tropidurus torquatus is an emerging Neotropical lizard model species, that shows a wide South American distribution and it is extremely abundant in open formations, especially in disturbed environments [27,28]. This species has been

extensively studied in ecology, phylogeny, physiology, and it is one of the few South American lizards species to have a complete staging table of post-ovipositional development [29]. Here we investigate the hemipenial development, morphology and examined the ontogenetic and static allometric pattern of this organ in *T. torquatus*. We described the female genital morphology for this species, and analyzed it static allometric pattern. Finally, we compared the allometric slopes of hemipenial and female genital traits.

Material and Methods

Embryo obtaining and processing

Ten gravid females of *Tropidurus torquatus* were captured by a noose in urban areas of Brasília (15°45'46.79"S 47°52'05.34"W), Federal District, Brazil, during the breeding season (Oct/2016 – Feb/2017). They were kept in terrariums with medium grain vermiculite as substrate until egg laying. To provide a stable maintenance and minimize distress, four to five females were placed in 60 X 40 X 50 cm terrariums equipped with heating plates at 35°C for thermoregulation. Lizards diet were composed by live cockroaches (*Nauphoeta cinerea*) floured with calcium and vitamin supplements for reptiles, and fresh water. The room were maintained ambient temperature (~25ºC) with a daylight cycle of 12 h.

Terrariums were inspected daily for the presence of eggs. Once spawning occurs, clutches were individually kept in 50 ml recipients containing 10 mg of vermiculite and 20 ml of water, conferring a humidity of 100%. The recipients were placed in an egg incubator with a constant temperature of 30°C. Eggs were dissected daily throughout the incubation period under an isotonic saline solution of 0.75% NaCl. In total were analyzed two to seven embryos per stage and summing a final sample of 57 embryos (Please refer to Supplementary Information S1 Table A, for a detailed specimen list). For immediate embryo euthanasia, 5 ml of 2% lidocaine hydrochloride was dissolved in saline solution during dissection. After *in vivo* photographic documentation, embryos were cold

fixed in Carson fixative during 24-48 h and stored in 70% ethanol. Captures were licensed by ICMBio/IBAMA under permit n° 55406-1. This study was approved by the Ethics Committee for Animal Use of the University of Brasília (UnBDOC n° 166980/2013).

In order to have a detailed visualization of the hemipenial morphological changes during embryonic development at different embryonic stages, 15 embryos were submitted to Scanning Electron Microscopy. Previously cold fixed in Karnovsky Fixative for 24 hours, samples were immersed in a solution of 2% osmium tetroxide, dehydrated in crescent solutions of acetone, critical-point dried with CO_2 in a Balzers CPD030 and coated with gold in a Leica EM SCD005 Sputter Coater. Analysis was performed in a JEOL JSM7000F Scanning Electron Microscope.

General developmental modifications of genitalia were described stage-by-stage, based on the embryonic staging table of *Tropidurus torquatus* [29] and vertebrate staging system [30].

Adult genitalia preparation and description

Genital morphological description and intraspecific comparison were made using both hemipenes, from 20 specimens of *Tropidurus torquatus*, thus totaling 40 hemipenes, and female internal genitalia from 20 females (detailed specimen list are provided at Supplementary Information, S1 Table C and D). All specimens used were collected in urban areas of Brasília, Distrito Federal, Brazil (ICMBio/IBAMA under permits n° 55406-1). The specimens were euthanized in accordance to methodological guides from the Ethics Committee of University of Brasilia (CEUA-UnB) with intraperitoneal administration of lidocaine hydrochloride (1.5 ml at 2% concentration).

In male specimens that have not its hemipenes everted during the fixative procedure, we prepare it following Pesantes method [31]. We made a small incision at the base of the tail, and both hemipenes were removed. They were kept in a potassium hydroxide 1.5% solution for up to three minutes and manually everted using forceps. After eversion, the hemipenes were washed in a solution of 70% ethanol for dilution of potassium hydroxide remnants. Finally, we injected

red colored petroleum jelly for better visualization of ornamentation and give a knot on the hemipenial base with surgical thread.

Female internal genitalia were obtained by dissection under stereomicroscope of previously formaldehyde-fixed female specimens deposited at LACV Scientific Collection. The cloaca was accessed by exposing the urogenital/digestive tract with a ventral incision in the abdominal region. Once exposed the urogenital/digestive tract, the cloaca was extracted by dissection surrounding the external face of cloacal lips. Finally, the cloaca was transferred for a Petri dish and dissected along the coronal section plan, thus resulting in the exhibition of the ventral and dorsal internal faces.

Hemipenial description were made using Klever & Bohme [32] and Dowling & Savage [2] terminology, and female cloacal terminology follows Sánchez-Martinez *et al.* [33].

The specimens and its genitals used on this study were deposited on Laboratory of Comparative Vertebrate Anatomy Scientific Collection, Department of Physiological Sciences, Institute of Biological Science, University of Brasilia, (LACV/ CFS/IB/UnB). Please refer to Supplementary Material for a complete list of analyzed specimens.

Morphological measurements and statistical analysis

Hemipenes of embryos (n=19) and adults (n=40), and internal female genitalia (n=20) were photographed under a Nikon SZM460 stereomicroscope coupled with a Cannon Power Shot digital camera. We perform morphometrical measurements for the following traits: hemipenial truncus length (TCL), hemipenial truncus width (TW), hemipenial lobes length (LL) and hemipenial total length (TTL). For female cloaca, we measured the following traits: proctodeal-urodeal region length (FPUR), urodeal corns length (FUC), and female cloacal total length (FIGL). All the measurements were obtained using ImageJ software. We also obtained data of snout-vent length (SVL) using a caliper rule, as a proxy for body size for embryo and adult specimens (raw data are provided at Supplementary Information, S1 Table B, C and D).

For adult male hemipenes, we tested asymmetry between left and right hemipenial traits using Paired *t*-tests. We also described the ontogenetic and static allometry for embryonic and adult hemipenial, and cloacal aforementioned traits. The coefficient of allometry (*b*) was estimated as the slope of a linear regression of trait values against SVL [18]. For each trait, we tested whether the coefficient of allometry was significantly different from isometry ($b = 1$) using slope test function on Smatr package of R software [34,35]. Coefficient values higher than one indicates positive allometry, while values smaller than one indicates negative allometry.

Finally, we tested the morphological correspondence of genitalia among sexes using ANCOVA tests for differences between male-female linear regressions. Were tested differences in allometric coefficients (slope) and male-female body size (intercept) for each male-female trait: TCL vs. FPUR, LL vs. FUC, and TTL vs. FIGL. All analysis and graphics were performed in R software [34,35].

Results

Early development of cloaca and genital primordium

Initial development of the genital-cloacal morphology occurs relatively fast during the early post ovipositional stages. The development of external genitalia begins two days post oviposition (DPO), at the ovipositional stage (stage 28). At this stage is distinguishable the presence of a genital swelling at the ventral-proximal portion of the hindlimb buds (Fig. 1, A and 2, A-B). Two pairs of bulbs located ventromedially on the further cloacal region arise from the genital swelling at the sixth DPO (Fig. 2, C). During 31 to 32 stages (8-11 and 11-14 DPO, respectively), remarkable modifications occur on the genital-cloacal morphology. At nine DPO a condensation of cells forms an epidermal depression along the region that further will develop into the cloacal opening (Fig. 2, D). By the tenth DPO, the posterior bulbs develop in the distinct genital primordia, whereas

anterior bulbs further develop into the anterior cloacal lip, by eleventh to the thirteenth DPO, being totally fused by fourteenth DPO (Fig. 2, E-F).
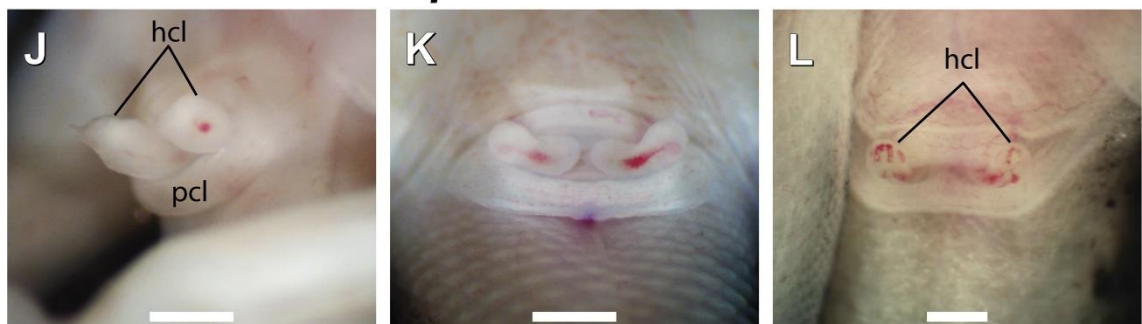
Early genital primordia
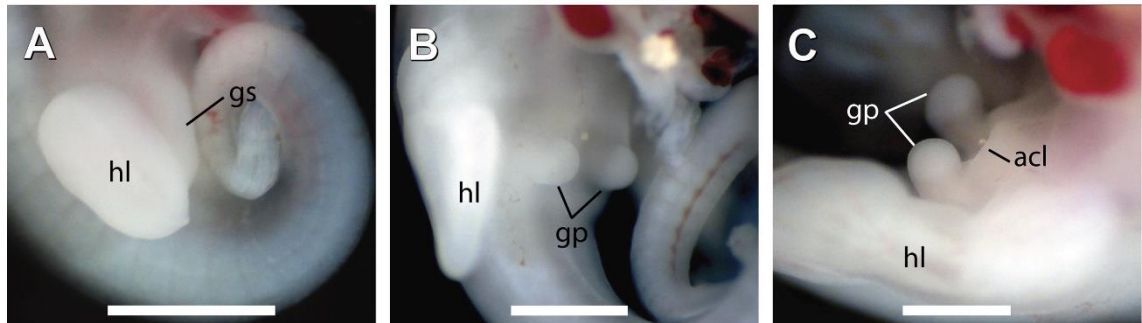
Hemipenis development
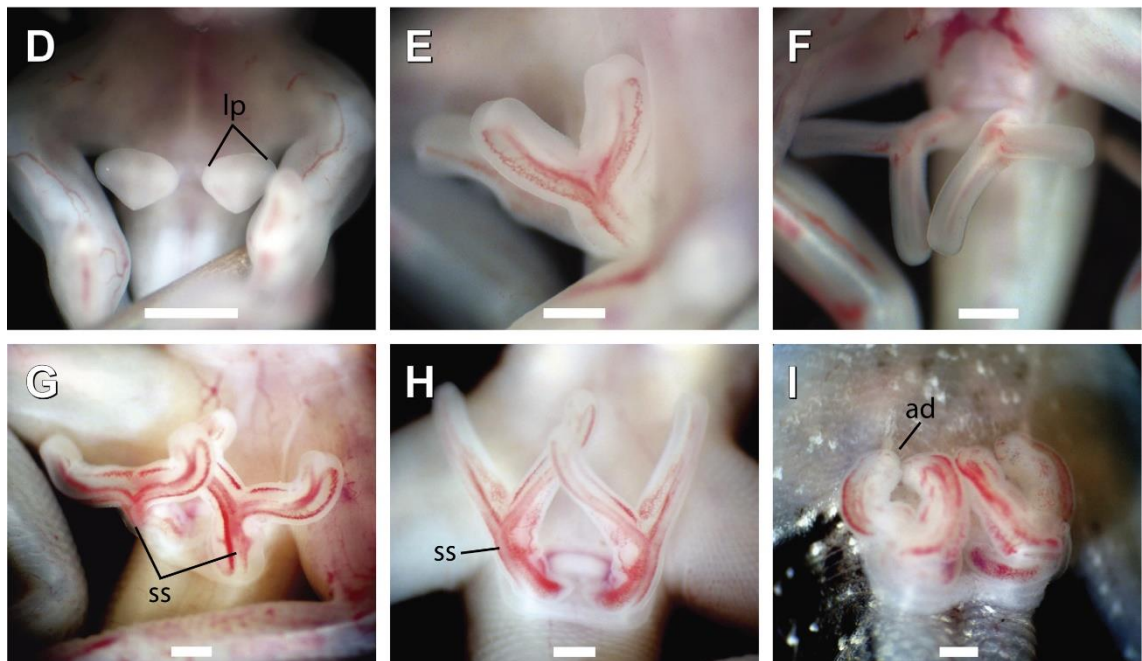

Hemiclitoris development

Figure 1. Genital development of *Tropidurus torquatus*. A, stage 29; B, stage 33; C, stage 34; D; stage 35; E, early stage 38; F, late stage 38; G, early stage 39; H, late stage 39; I, stage 40; J, late stage 37; K, stage 39; L, stage 40.

Legends: acl, anterior cloacal lip; ad, apical disc; gp, genital primordia; gs, genital swelling; hcl, hemiclitoris, hl, hindlimb; lp, lobes primordia; pcl, posterior cloacal lip; ss, spermatic sulcus. Scale bar: 0.5 mm.
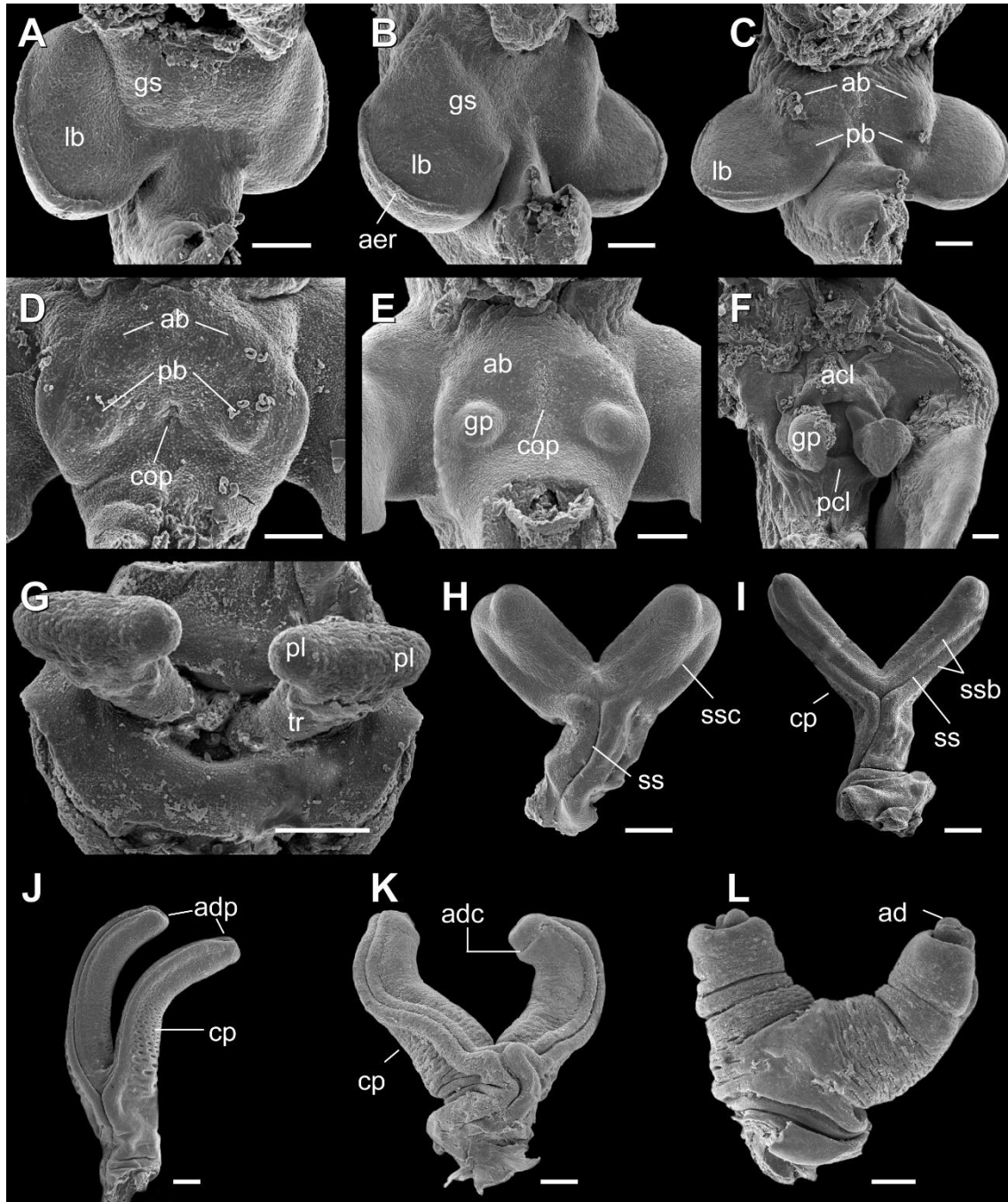
Figure 2. Hemipenial development of *Tropidurus torquatus* under SEM analysis. A, stage 28; B, stage 29; C; stage 30; D, early stage 31; E, late stage 31; F, late stage 32; G, stage 36; H, stage 37; I, stage 38; J, stage 40; K, stage 41; L, stage 42. Legends: ab, anterior bud; acl, anterior cloacal lip; ad, apical disc; adc, apical disc constriction; adp, apical disc primordia; aer, apical epidermal

ridge; tr, hemipenial truncus; cop, cloacal opening primordia; cp, calyces primordia; gp, genial primordia; gs, genital swelling; lb, hindlimb bud.; pb, posterior bud; pcl, posterior cloacal lip; pl, primordium of lobes; ss, spermatic sulcus; ssb, spermatic sulcus borders; ssc, spermatic sulcus constriction. Scale bar: A-F, 100 μm; G-L, 200 μm.

Late development of hemipenes

Late development of the hemipenial morphology is mainly characterized by the increase in the length of the hemipenial truncus and lobes and by further emergence of ornamentation and apical disc on the latter stages. By stage 35, the apical region of genital primordia shows a laterally expanded aspect in male genitalia which further develops in two lobes. From this stage it is possible to distinguish between male and female embryos by the presence of this distal expansion (Fig. 1, D).

The apical expansion of the hemipenial truncus protrudes laterally by stage 36 giving origin to the hemipenial lobes, which suffer a rapid increase in length until stage 37 (Fig. 2, G-H). The "Y" shaped hemipenes in stage 37 show a well-marked spermatic sulcus at the hemipenial truncus which is less marker along the lobes (Fig. 2, H). At stage 38 lobes length exceeds truncus length and the spermatic sulcus are now well developed along the lobes (Fig. 2, I). At apical region, each lobe showed a marked constriction, that will give place to the apical disc margins at the following stage. The spermatic sulcus is fully formed at stage 39 along the lobes, that ends on the formed apical disc (Fig. 1, G-H and Fig. 2, J-K). At the end of stage 40, a great number of pits give rise the primordium or ornamentation surrounding the lobes (Fig. 2, J). By the end of stage 41, the hemipenes start the inversion process to the inner side of the cloaca (Fig. 1, I and Fig. 2, L).

In females, modifications in hemiclitoris are restricted to little increase in size during embryonic development from the stage 35 to stage 41, whereas completely genital inversion occurs (Fig. 1, J-L).

Adult genital morphology

Tropidurus torquatus adult males show deeply bilobed hemipenes, with lobes length comprising a little more than truncus length ($t = 3.797$; $p = 0.001$) (Fig. 3, C; Table 1). The "Y" shaped spermatic sulcus arises proximally, forking at the level of lobes bifurcation and ending distally at the apical disc. The hemipenial truncus is nude and slightly thicker than the hemipenial truncus. Both sulcate and asulcate faces of the truncus are nude, although marked laterally by the presence of a flounce that enlarges at the base of the lobes and being continuous with lobes ornamentation. The lobes are fully ornamented with calyces with exception of the sulcate region. These calyces are deeply pronounced on the asulcate surface and decrease in size distally, being greatly reduced near the apical region (Fig. 3, A-B). Our SEM analysis shows the presence of a great number of reduced papilla that surrounds the margins of the calyces, distributed along the whole lobe. Additionally, the characterization of spermatic sulcus under SEM analysis shows it's continuously with the apical disc and the presence of a great number of parallel clefts that surrounds the apical disc (Fig. 4). Finally, we find no statistical significance between right and left hemipenial traits (Fig.3, D-G; Table 1).

Female internal genital morphology is also marked by its "Y" shape. The cloacal lumen shows a folded mucosa, with the proximal region, proctodeal-urodeal (FPUR) measuring 4.85±1.03 mm, and the distal region, the urodeal corns (FUC), showing a length of 5.23±0.89 mm, summing a total length (FIGL) of a10.08±1.72 mm. The urodeal corns are deeply bilobed, comprising approximately the same length that proctodeal-urodeal region ($t = 1.244$; $p = 0.2213$).
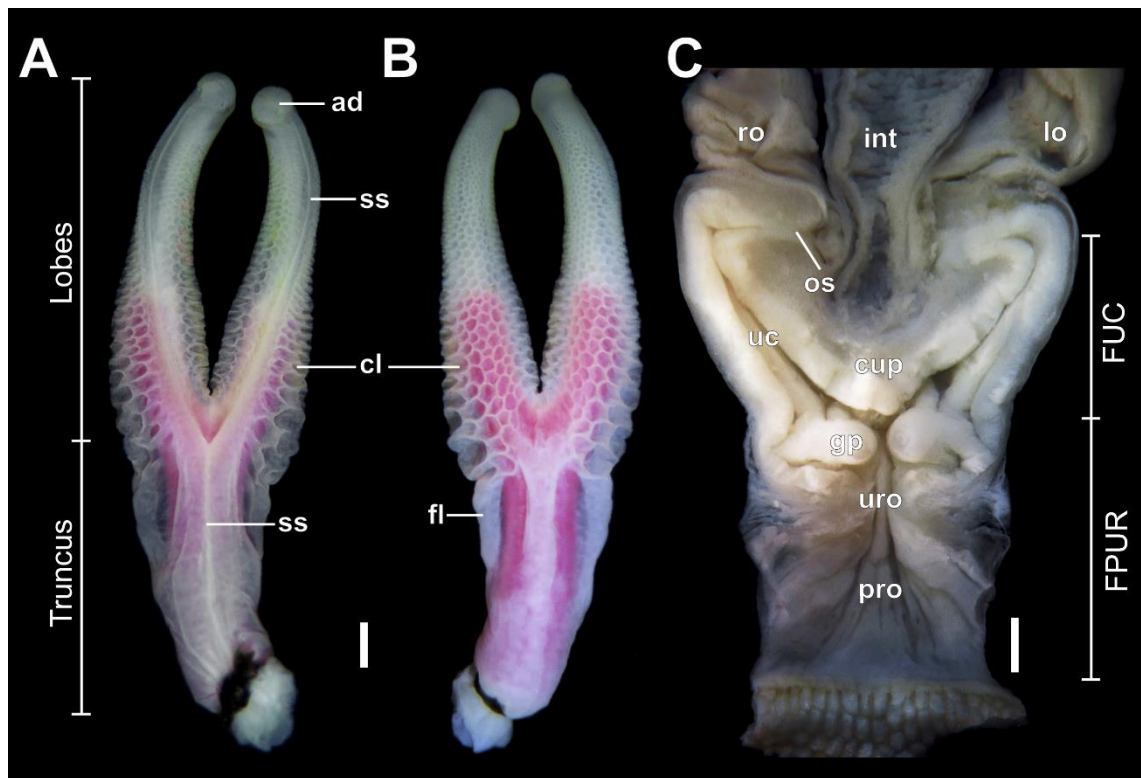
Figure 3. Male and female genital morphology of *Tropidurus torquatus*. **Measurements are indicated for hemipenial truncus, hemipenial lobes, female proctodeal-urodeal cloacal region (FPUR), and female urodeal corns (FUC).** Right hemipenis in (A) ventral and (B) dorsal view, and female internal genitalia (C). Figure legends: ad, apical disc; gp, genital papilla; int, intestine; lo, left oviduct; os, oviduct sphincter; ro, right oviduct; uc, urodeal corn; uro, urodeal region; pro, proctodeal region; ss, spermatic sulcus. Scale bars: 1 mm.
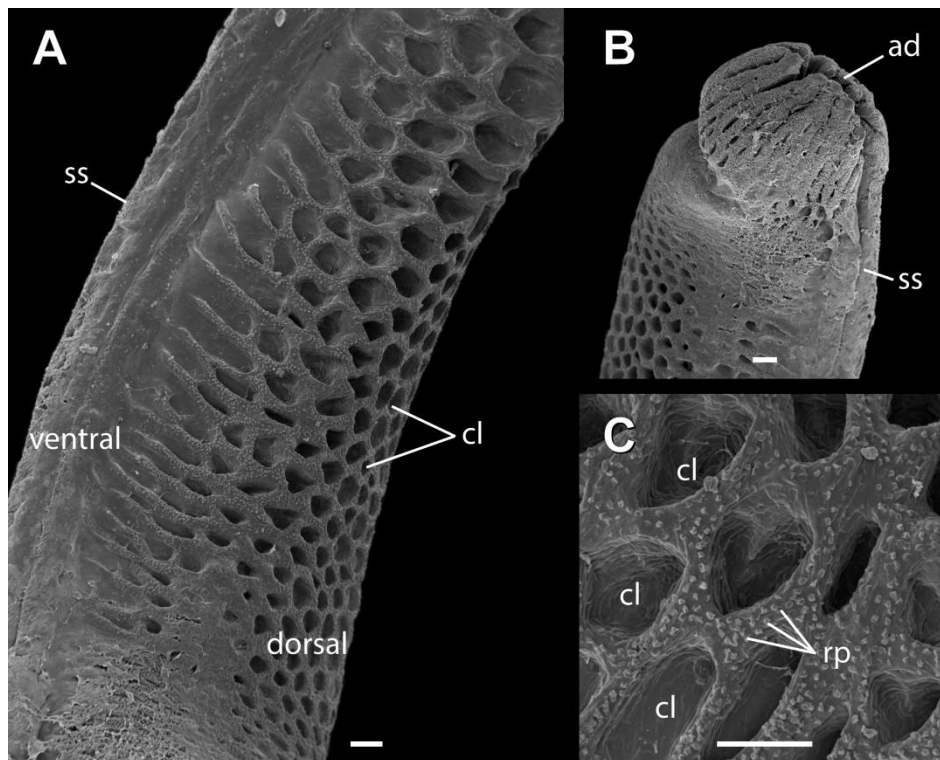
Figure 4. Adult hemipenis of *Tropidurus torquatus* under SEM analysis. (A) calyces, (B) apical disc and (C) reduced papilla. Figure legends: ad, apical disc; cl, calyces; rp, reduced papilla; ss, spermatic sulcus. Scale bar: 100 µm.

Table 1. A comparison between right and left hemipenial trait values of *Tropidurus torquatus*.

Trait	Right HP	Left HP	t	P
TCL	6.04 ± 0.86	5.89 ± 0.71	0.5434	0.591
TW	2.59 ± 0.41	2.36 ± 0.36	1.7297	0.094
LL	7.90 ± 1.76	8.10 ± 1.44	-0.35245	0.727
TTL	13.94 ± 2.32	13.99 ± 1.85	-0.065255	0.948

Legend: Mean and Standard Deviation for truncus length (TCL); truncus width (TW); lobes length (LL) and total length (TTL) in right and left hemipenes. Asymmetry results (t) and significance (P).

Ontogenetic and static allometry and male-female genital correspondence

For embryos, the allometric slope for truncus length was not significantly different from 1.0, indicating isometry, but slopes for lobes and total length were significantly over than 1.0, thus suggesting positive allometry during ontogenetic development of these traits (Table 2; Fig. 5, A). For adults, allometric slopes for truncus length and total length were not significantly different from 1.0, tending, however, to a negative allometry for the total length. On the other hand, the slope for lobes was significantly over than 1.0, thus indicating positive allometry for this trait (Table 2; Fig. 5, B). All analyzed female genital traits show negative allometry, with slopes significantly different from 1.0.

Males and females allometric patterns show intercepts values significantly different on all analyzed traits (Table 3). On the other hand, the slopes of the genital traits did not differ significantly (Table 3), although the hemipenial lobe length slopes were larger than urodeal corns length (Table 2).
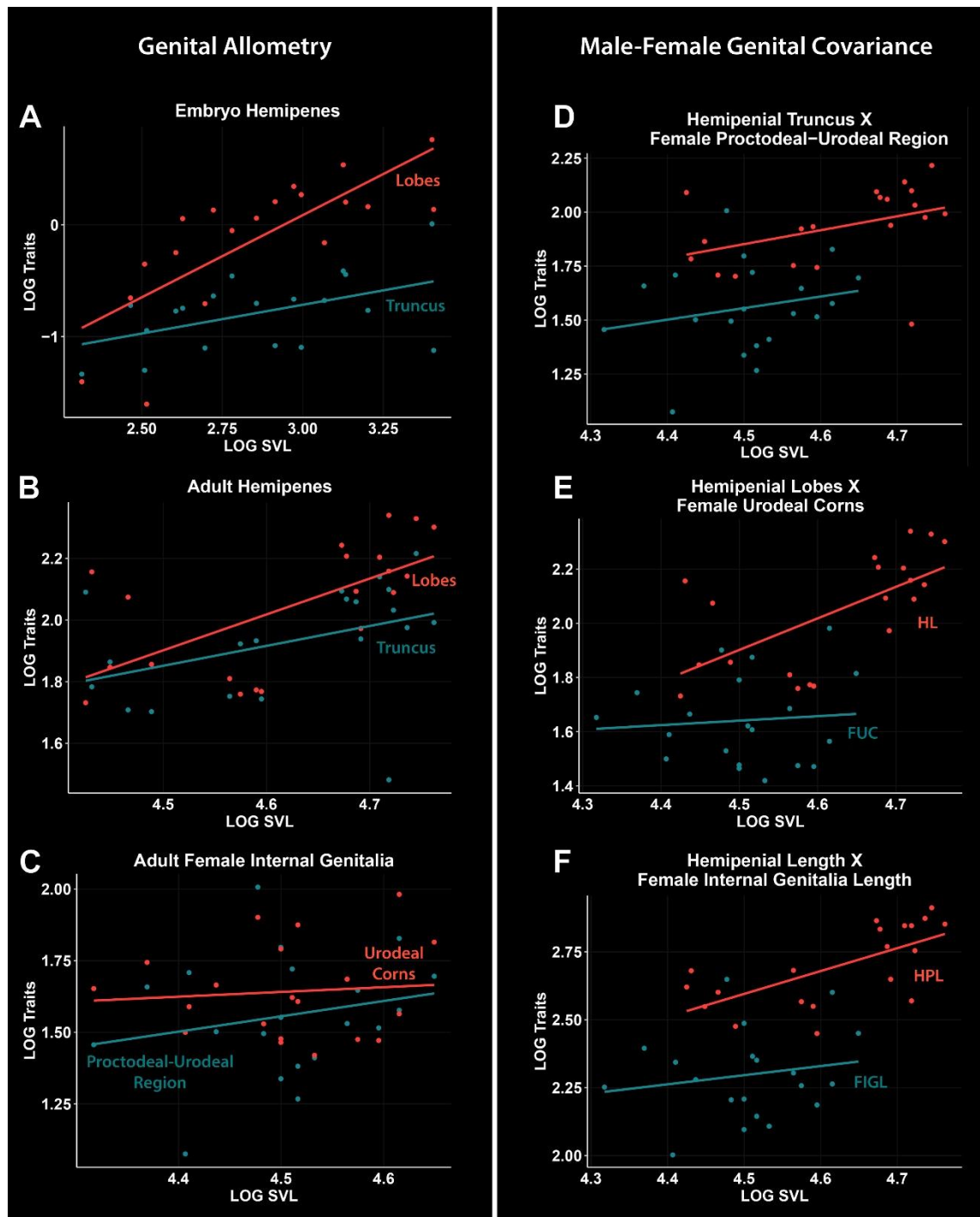
Figure 5. Regressions of log-transformed traits vs. log transformed body size (SVL) for genital traits of *Tropidurus torquatus*. A-C: regressions for hemipenial traits of embryos (A), hemipenial traits of adults (B), and female internal genitalia (C). D-F: comparison of male-female traits regressions for hemipenial truncus *vs* female proctodeal-urodeal region (D), hemipenial lobes *vs.* female urodeal corns (E), and hemipenial total length *vs.* female internal genitalia total length (F). Figure legends: FIGL, female internal genitalia total length; FUC,

female urodeal corns; FPUR, female proctodeal-urodeal cloacal region; HL, hemipenial lobes; HPL, hemipenes total length; HT, hemipenial truncus.

Table 2. Summary statistics for ordinary least square (OLS) regression of traits *vs.* body size (SVL).

Embryo hemipenes (n=19)	*b*	*r²*	*P*
Truncus length	0.514	0.232	0.7643
Lobes length	1.469	0.587	<0.001
Total length	1.093	0.605	0.0359
Adult male hemipenes (n=20)	*b*	*r²*	*P*
Truncus length	0.645	0.162	0.035
Lobes length	1.164	0.415	0.002
Total length	0.841	0.442	0.195
Female cloaca (n=20)	*b*	*r²*	*P*
Proctodeal-urodeal length	0.535	0.047	<0.001
Urodeal corns length	0.165	0.007	0.008
Total length	0.335	0.031	0.007

Legend: Allometric coefficient (*b*), coefficients of determination (*r²*) and significance deviation from a slope of one (*P*).

Table 3. ANCOVA results for difference in genitalia between sexes. *F*-statistics, and *P*-values for each analyzed trait.

Trait	Slope		Intercept	
	F	*P*	*F*	*P*
Hemipenial truncus *vs* female proctodeal-urodeal region	0.029	0.866	18.272	<0.001
Hemipenial lobes *vs* female urodeal corns	3.278	0.079	23.970	<0.001
Hemipenial length *vs* female internal genitalia length	1.148	0.291	38.231	<0.001

Discussion

Male genitalia shows a wide morphological variation along animals with internal fertilization and exhibit high levels of evolvability in lizards [9]. In the last decades, biologists world-wide have interested in explaining the developmental, genetical and evolutionary processes behind the morphological diversity [36]. Although megadiverse, the squamate clade is underrepresented in ontogenetic studies, especially for soft tissue organs, as the hemipenes. Most of the hemipenial studies have focused on morphological and genetical process exclusively during early developmental stages [13,37]. In this work, we investigate the male genital morphology of the Amazon lava lizard *Tropidurus torquatus* under a morphological and developmental approach, representing the first refined investigation for a tropidurid lizard. We have described quantitatively the morphology of male and female genitalia and demonstrate the correspondence between them. Finally, we showed that static and ontogenetical allometry along hemipenial traits in this species are a result of a rapid hemipenial growth during last embryonic stages. Ultimately, we discuss our results under phylogenetic, developmental and evolutionary perspectives below.

Among tropidurid lizards, morphological investigations of hemipenes are limited to its uses on phylogenetic analysis [38,39]. Moreover, *Tropidurus* genus hemipenial morphology descriptions are scarce [40]. Although, even in phylogenetic analyses, hemipenial characteristics has been underestimated. Frost *et al.*, [38,39] listed the general morphology of the genus *Tropidurus* with a combination of three characters: elongated hemipenial lobes, without apical disc and with calyces starting at the level of the lobes. Our data are in accord to this description in relation to lobes and ornamentation. However, we find that *T. torquatus* (and some other species not formally included in this work, i.e. *T. oreadicus* and *T. hispidus*) have a distinct disc on the apical region of each lobe. A possible explanation for these dissonant results could arise from the difficult eversibility of the apical region during hemipenial preparation from fixed specimens.

Development

Recent works have demonstrated that hind limb and hemipenial buds share the same morphogenetical basis [41]. This recent finding has mitigated questions among the degree of pleiotropy on early developmental stages driving evolutionary modifications related to hind limbs and hemipenes among lizards [3,15]. The development of the hemipenial buds and cloacal lips in *Tropidurus torquatus* is congruent with other Squamate species, showing an association between this structures on early developmental stages [13,29,37]. As well as in the other recent investigated species, *Anolis carolinensis* and *Python regius*, our SEM analysis shows a close association between hind limb buds and the genital swelling [13,37].

The independent primordia for genital and anterior cloacal lip buds, first reported on the general embryonic staging table of *Tropidurus torquatus* [29] is now confirmed. Our results show that the genital swelling further gives rise to genital and anterior cloacal buds, whose follows independent developmental trajectories. Moreover, the development of genital primordia posteriorly to the anterior external cloacal buds occurs as the other investigated squamates [13,37]. However, we did not exclude the possibility that the anterior cloacal buds may participate on the genital bud formation among other squamate lineages, since the particular aspects of genetical regulation of hemipenial-cloacal development [13,41], and especially the possibility of migration of genital bud on early embryogenesis [12]. New developmental studies for underrepresented squamate lineages may clarify the degree of the structural association during the development of the genital primordia and external cloacal structures.

Detailed information about late hemipenial development is even more scarce. Our SEM analysis reveals that all ornamental structures find on adult morphology begins its development during embryonic development, especially the primordia of the calyx and the apical disc structures. As so, post-embryonic development of hemipenes in *Tropidurus torquatus* may be restricted to its growth and remodeling of ornamental structures raised on late embryonic stages. A similar situation occurs on the gymnophthalmid lizard *Calyptommatus sinebrachiatus*, which hemipenes show ornamental structures by last

developmental stages, recognized even in the stereoscopic analysis [42]. Once ornamental structures have been recognized as crucial for differentiation between cryptic species with hemipenial variation [5,6,43], late embryological studies may explain this variation and should be considered in studies of hemipenial evolution driven by processes such as natural and sexual selection [15,44].

The allometric pattern of hemipenial lobes in *Tropidurus* lizards are driven by male-female genital correspondence?

The size and shape of hemipenial lobes play itself a source of general variation affecting the genital morphology among Squamates [1,4,5,45]. The enlarged-lobe condition is commonly found in different squamate groups [3]. Moreover, among tropidurid lizards, the hemipenial lobes are short in all *Stenocercus* and long in all *Tropidurus* and other related genera [38,39].

This work presents the first study of ontogenetic hemipenial allometry among squamates and the second of static allometry in hemipenial traits. Recently was studied the static allometric pattern of anole lizards subspecies of *Anolis grahami* [14]. Similar to *A. grahami grahami*, *Tropidurus torquatus* show an isometric pattern for hemipenial total length, with a tendency to negative allometry, and negative allometry for truncus length (Table 2). However, for *T. torquatus*, the allometric pattern for the hemipenial lobes is positive and has been driven by ontogenetic allometry, whereas the lobes show a rapid growth in late embryonic stages (Table 2).

Considering that allometric results over genital traits are a predictive of sexual selection [10,23,36,46,47], the allometric pattern found among *Tropidurus* hemipenial lobes could be explained by directional selection over this trait, caused as an action of two non-exclusive mechanisms: female cryptic choice and genital coevolution, as following discussed.

The direct interactions of genital traits during copula is directly related to reproductive success, especially when this process is leaded by female choice

[24]. The general "female cryptic choice" hypothesis predicts that male genitalia has a stimulatory function and females choose to copulate with males whose hemipenial morphology better fills that stimulating function [47]. Likewise, a crescent number of works have discussed the genital coevolution under this hypothesis [24,25,48–51]. Morphological characterizations of the vagina-cloacal region of *Tropidurus* lizards found an amount of large epithelial glands surrounding the anterior pars of the urodeal region [33]. So, if female cryptic choice is acting on *Tropidurus* genital evolution, the long hemipenial lobes may function as a stimulatory structure of females urodeal glands, being used as an indicator of good genes [52] or even serving as an efficient conductive system by which seminal fluid reaches the oviductual opening (Fig. 3-C).

Genital morphological interaction, been it congruent or antagonistic, is expected to reflect consequences on genitalia evolution, reproductive success, and speciation [24,51,53]. Moreover, the significance of studies of genital interaction during the copula can be recognized from morphological, physiological, ecological and evolutionary perspectives [24,54]. Using artificial hemipenial eversion inside female cloaca and micro CT scanning, Brennan [24] shows for garter snakes *Thamnophis radix*, that interaction of genital traits could occur, even when unexpected. In this species, the hemipenial "T" shape turns to a "Y" when inflated inside female cloaca, whereas the hemipenial lobes reach the vaginal pouch. As occurs in *Tropidurus torquatus*, the degree of this morphological interaction may have significant consequence for insemination success, under a stimulatory perspective. Nogueira *et al.*, [55] demonstrate for the gekkonid lizard *Hemidactylus mabouia*, that cloacal urodeal corns region play a phagocytic function of spermatozoa cells, representing an evidence of sperm selection. This physiologic condition is also found among mammals, birds, amphibians and reptiles (for a brief review see Nogueira *et al.*, [55]). Moreover, the occurrence of phagocytic cells specifically on the cloacal urodeal corns gives another source of support for the requirement of evolutionary strategies that better fit this post-copulatory selective mechanism. This becomes especially true for species which female oviductual opening are located distally from the urodeal corns, as occurs in *Tropidurus* studied species (this study, Fig. 3-C; Sánchez-Martínez [33]), thus representing an additional source of selective pressure for

hemipenial evolution [48]. More comparative cloacal studies further clarify this additional hypothesis of genital coevolution among lizards, as this study stands as the first quantitative evidence.

Following the demonstrated correspondence between male and female genital morphology of *Tropidurus* lizards, and assuming the possibility of hemipenial variation leaded by plasticity and ontogenetic effects [11,56,57], males with large lobes could be benefited in both aspects: the major stimulatory capability of urodeal glands and, therefore, by the amount of secretion produced by these glands, which can play as a mechanic and/or biochemical difficult for semen from future copulations. Evidences of female mate choice and sperm competition among lizards have been demonstrated in some study cases [10,58–60]. For the polymorphic phrynosomatid lizard *Uta stansburiana* is demonstrated that genital morphology is particularly associated with mating strategy, whereas "usurper male morphs" evolve a wider apical horn in contrast to other morphs [10]. For *T. torquatus*, by now, is demonstrated that male fitness plays a decisive point for the formation of harems [61]. However, experiments of female choice and male territorial dominance are still lacking.

Although the positive allometric pattern found in hemipenial lobes, the hemipenial total length in *Tropidurus torquatus* was recovered as isometric, tending to negative allometry, supporting the "one-size-fit-all" theory [18, 22]. This hypothesis predicts that males would be selected to have genitals that fit the average size of female genitals in the population, regardless of body size [18]. Thus, despite a possible directional selection in lobes size, the hemipenial overall length of *T. torquatus* could be under stabilizing sexual selection, corroborating the finding on *Anolis g. grahami* populations [14]. Moreover, it is plausible that different factors such as pleiotropy, natural and sexual selection, and male-female genital coevolution could be antagonistically driving the evolution of particular traits of *Tropidurus* male genitalia as argued in recent studies [15,41,48,51,62].

Acknowledgments

Author Contributions

Conceived and designed the experiments: JK, AKSL. Performed the experiments: AKSL, IPP, LP, PB. Analyzed the data: AKSL, IPP, JK. Wrote the paper: AKSL, JK.

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Capítulo 2 – Plasticidade fenotípica em traços morfológicos e fisiológicos de *Tropidurus torquatus*

Capítulo em fase de escrita a ser submetido ao periódico Scientific Reports sob o título:

Incubation temperature effects on morphology and thermal physiology of the emerging Neotropical lizard model organism *Tropidurus torquatus* (Squamata: Tropiduridae)

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Incubation temperature effects on morphology and thermal physiology of the emerging Neotropical lizard model organism *Tropidurus torquatus* (Squamata: Tropiduridae)

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Abstract

Incubation temperature is one of the most studied phenotypic plasticity inductors, since many animal lineages are oviparous with incubation trajectory affected by environmental temperature. In last decades, predictions of warming have aroused interest in this field in face of global climate changes. The aim of our study if test the influence of incubation temperature on developmental parameters, morphology and thermal physiology of the collared lizard (*Tropidurus torquatus*). We incubated eggs of the species at five temperature regimes ranging from field temperature (28.7°C) to 35.0°C. We found that increase in incubation temperature affects several investigated traits: egg mass gain is positively affected, without no influence in newborn mass; incubation duration is broadly reduced with temperature increase; survival rate is negatively affected by

temperature and 35.0°C is confirmed as a lethal incubation temperature; sex ratio is affected in the range of 30.0°C, prevailing the outbreak of females. Increase in incubation temperature also affects body and head size. Newborn thermoregulation and critic thermal maximum (CT_{max}) are not affected by incubation temperature. On the other hand, basal temperature (T_b) and critic thermal maximum (CT_{min}) were positively affected by this factor. Thermal traits were also affected by age, with newborns differing from adults in all analyzed thermal traits. Future modifications in incubation temperatures due to impacts of global warming on nest sites caused by warming may affect several features of the natural history, morphology and thermal physiology of the newborns of this species.

Keywords

Phenotypic plasticity, thermal developmental plasticity

Introduction

Developmental pathways are ruled by a wide range of factors that act over biological processes during embryogenesis, such as incubation temperature[1]. This factor, often recognized as a strong inductor of phenotypic plasticity under fluctuating environmental conditions, can modulate the phenotype in response to oscillations in metabolic rate and genetic expression[2,3]. Moreover, under conditions of extreme variation in incubation temperature, the collapse of ontogenetic processes leads to the phenotype being unfeasible, interrupting the developmental process[4].

Some groups of metazoans are especially more susceptible to temperature variations during development, such as oviparous reptiles[5]. In these heliothermic vertebrates, egg incubation is directly affected by fluctuating conditions in the laying environment, which are most strongly felt in species

whose embryonic development occurs inside the maternal body[6,7]. Although temperature-mediated phenotypic plasticity is a natural phenomenon, little is known about the possible effects of climate changes on biodiversity regarding the increase in average temperatures and extreme climate events potentialized by anthropogenic actions[8–10].

In the context of global climate change, especially concerning to the increase in the average temperature of the planet observed in the last decades and predicted for the next century, heliothermic vertebrates, mainly oviparous ones, have aroused relevant research interest given their vulnerability to such environmental changes[11–13]. Forecasting models have shown that one of the main forms of susceptibility of oviparous reptiles, such as lizards, to an increasing global temperatures, will occur through the reduction of energy gain due to changes in the foraging period, and changes in the potential distribution areas of these species[14]. In addition, the modification of incubation temperature in the nests caused by higher average temperatures of the surrounding environment offers direct risk to oviparous species in several ways, such as reducing birth rates, changing population sex ratio, and posing phenotypic restrictions with ecological implications (i.e. morphological malformations, reduced locomotor performance, altered thermal physiology, among others)[15,16].

Despite representing one of the largest vertebrate radiations, few species of squamate reptiles (lizards, snakes and amphisbaenians) had phenotypic traits investigated from the perspective of incubation temperature effects[17]. Besides, even fewer studies have investigated the effects of egg incubation temperature on thermal physiology features with ecological importance[16]. Moreover, none of these studies included Neotropical species, leaving a knowledge gap about the effects of drastic phenological changes can have on these organisms. Herein, we investigate the effect of incubation temperature on egg development and neonatal morphological and thermal physiological traits of the lizard species *Tropidurus torquatus*. This is a lizard species widely distributed throughout South America and extensively studied in the most diverse biological aspects[18–21]. In addition, this is a lizard species with a considerably simplified captive maintenance and one of the few South American lizard species to have a complete post-ovipositional embryonic staging table.

Methods

We obtained 144 eggs from 25 adult pregnant females of *Tropidurus torquatus* collected during the breading season (Oct/2018 – Jan/2019) from urban populations of Brasília (15°45'46.79" S 47°52'05.34" W), Distrito Federal, Brazil. The females were collected by noosing and kept in terrariums with medium grain vermiculite and washed sand in a proportion of 1:1 as a substrate, and with a light/dark ratio of 12h/12h, until oviposition. In order to minimize distress, four to five females were placed in 60X40X50cm terrariums supplied with food (live cockroaches *Nauphoeta cinerea* floured with calcium and vitamin supplements for reptiles), fresh water and heating plates at 35°C for thermoregulation. Animal capture was licensed by Brazilian Ministry of the Environment (ICMBio/SISBIO permit n° 63226-1). The experimental procedure described herein was fully approved by the University of Brasilia Ethics Committee (CEUA-UnB permit n° 116/2017).

Terrariums were daily inspected for the presence of eggs. Once oviposition occurred, the eggs were individually identified and their mass, length, and width were taken with aid of a precision digital balance and caliper, respectively. Each egg was transferred to a 50ml containers with 10g of vermiculite and 20ml of water, conferring humidity of 100%. Finally, the containers were randomly distributed in five treatments: tree egg incubators set at constant temperatures of (a) 30°C (n=33), (b) 32.5°C (n=28) and (c) 35°C (n=22), an egg incubator set at (d) 32.5°C with a variation to a peak temperature of 39°C during 2h per day (n=31) and an (e) artificial nest (n=30), built in the field.

For the construction of the artificial nest we observed the behavior of some females that usually spawn at wall corners under concrete slabs. Thus, the artificial nest consists of a wire cage placed in one of those wall corners covered with concrete slabs and equipped with a datalogger for temperature monitoring every ten minutes. Mean temperature of the artificial nest treatment (28.7°C) was calculated based on the mean of 20069 observations. Field temperature

fluctuations were more pronounced at its maximum temperatures, as the estimated mean temperature is 0.8°C above the median temperature registered (28.0°C). Mean daily maximum temperature was 39.1°C and mean daily minimum temperature was 22.7°C. Maximum and minimum temperature registered were 45.8°C and 20.9°C, respectively. A scheme of field temperature variation during incubation period is provided in Supplementary Figure SF1.

Each container was daily inspected for newborns. Egg measurements (mass, length and width) were repeated in intervals of 15 days in order to minimize egg handling and embryo distress. Once an egg hatched, the newborn was immediately weighed, placed in a plastic container and transferred to an experiment room for acclimation at room temperature (~25.5°C) for 24–48h, before thermal experiments. After experiments, newborns were euthanized with intraperitoneal administration of lidocaine hydrochloride (0.1ml at 2% concentration), fixed in a 10% formalin solution and preserved in 70% ethanol.

Developmental traits

Incubation and egg development were evaluated with respect to time of development (TOD), egg mass, and volume. Measurements of egg length, width and weight were regularly taken in a 7–10 day interval. Egg volume was calculated with the ellipsoid formula: $v = \frac{4}{3}\pi ab^2$, in which a corresponds to the major radius ($\frac{1}{2}$ egg length) and b corresponds to the minor radius ($\frac{1}{2}$ egg width). Final egg mass was estimated as the mean of the last measurement taken from eggs before hatching.

Morphological variables

Morphological dataset comprises data on sex, mass, and 15 body measurements from 82 newborns: SVL, snout-vent length; TLL, tail length; TTL, total length; BL, body length (pelvic girdle-neck fold length); INL, interbraquial-nasal length; HL, head length; HW, head width; HH, head height; HUM, humeral length; RAD, forearm length; HAL, hand length; FEM, femoral length; TIB, tibial length; and FTL, foot length. For sex determination the specimens were dissected under stereoscopic microscope for examination of gonads and presence of

oviductual primordia. Mass and morphological variables were measured with aid of digital scale and caliper, respectively.

Thermal physiology experiments

We measured five thermal traits from 52 newborns and 34 adults of *Tropidurus torquatus* (18 females, 73–104mm/SVL and 19 males, 88–112mm/SVL) during preferred temperature, minimum critic thermal tolerance and maximum critic thermal tolerance experiments: basal body temperature (T_b), preferred temperature (T_{pref}), critical thermal minimum (CT_{min}), critical thermal maximum (CT_{max}), and the thermal-tolerance range (Amp_{tol}). All measurements were based on cloacal temperature and token using a type "k" temperature probe.

Basal body temperature (T_b) was determined as the 24–48h post-acclimation temperature. Preferred temperature (T_{pref}) experiment consisted in the mean body temperature of the specimen placed in a thermal gradient with air temperature ranging from 10°C to 40°C. This temperature gradient was built using a halogen lamp (100W) and an ice pack placed in opposite sides of a 1m-length glass container wrapped with black self-adhesive contact paper to avoid visual distraction. The final preferred temperature was estimated as the mean of all body temperatures values taken every 10-20 minutes for 2 hours. Critical thermal minimum (CT_{min}) was evaluated by placing the specimen in a cold chamber with air temperature of ~4°C provided by ice packs disposed 4 cm above the substrate, avoiding direct contact with the ice pack. For critical thermal maximum (CT_{max}) evaluation, the specimen was put in a hot chamber with heating provided by constant input of hot water steam. In both cases, critical minimum and maximum body temperature corresponded to those temperatures in which the specimen lost its righting response. Thermal-tolerance range (Amp_{tol}) consists in the difference between the critical thermal maximum and minimum temperatures. Experiments were carried out with an interval of 24h between them, avoiding interference.

Data analysis

All analyses and graphics were performed using the R statistical platform version 3.6.2[22]. Variation in egg mass and volume during the developmental period were analyzed with ANCOVA, using the incubation period as covariate.

We also tested for mass differences between eggs and newborns using ANOVA. Egg final mass was estimated by the last egg measurement taken during the incubation period. General estimates of temperature effect on morphology and thermal physiology were tested with MANCOVA. Differences in mortality rate and sex ratio among treatments were tested with Pearson's Chi-squared test applying Yates correction for small sample size. Finally, morphological traits were Log transformed and analyzed by linear regressions and ANCOVAs, setting SVL and sex as covariates. For thermal physiological traits we also performed linear regressions and ANCOVAs using log-transformed data, with SVL and sex as covariates. Latent relationships in the data were identified by performing Principal Component Analysis (PCA) for incubation temperature and age effects. After performing the analysis, factor loadings were interpreted based in Tabachnik and Fidell[23] criteria for overlapping variance classification: excellent (>0.71), very good (0.7<0.63), good (0.62<0.55), fair (0.54<0.45), poor (0.44>0.32) or interpretable (<0.32). Normality and homoscedasticity were verified on log-transformed data prior to analysis.

Results

Influence of incubation temperature over egg mass and volume

Egg mass and volume were positively affected by incubation temperature. In both cases, mass and volume did not differ significantly between field (28.7°C) and control (30°C) treatments. Field treatment differed significantly from 32.5°C and 32.5°C (39°C/2h) treatments. In turn, control (30°C) treatment differed significantly from 32.5°C but not from 32.5°C (39°C/2h). Mean values of mass and volume did not differ statistically between eggs incubated at 32.5°C and 32.5°C (39°C/2h) did not differ for mass and volume means (Figure 1; Table 1). Egg mass increased approximately four times during incubation period; however, egg mass gain did not influence newborn mass (Figure 2.D; ANCOVA, F = 1468.01, P < 0.0001; egg final mass = 3.9g ± 0.9, egg initial mass = 1.1g ± 0.2, newborn mass = 0.9g ± 0.1).
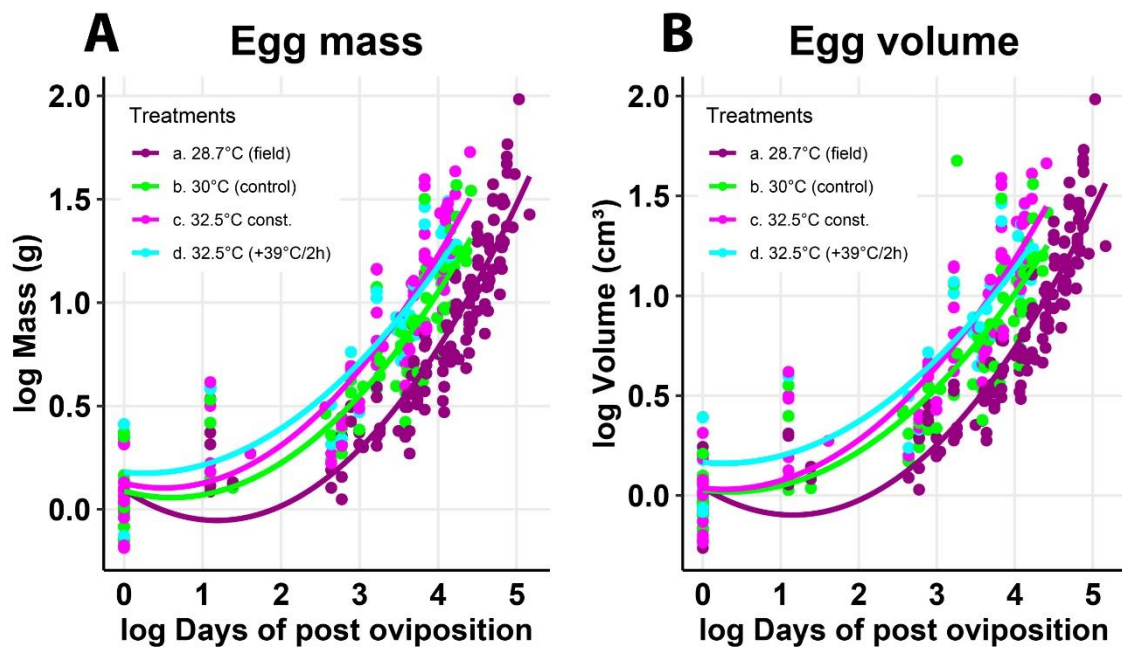
Figure 1. Egg mass (A) and volume (B) variation among temperatures during incubation period.

Table 1A. Egg mass gain rate during incubation period.

Temperature	Mean(sd)	Mass gain rate		Diference in mass gain curve among temperatures			Diference in mass among temperatures		
		b	r^2	F statistics		P	F statistics		P
28.7°C (field)	2.44(±1.2) *(a)	0.27	0.69						
30°C (control)	2.18(±0.9) *(ac)	0.25	0.75	0.4657	ns	0.70	11.892	*	>0.0001
32.5°C	2.36(±1.2) *(b)	0.28	0.77						
32.5°C(39°C/2h)	2.40(±1.0) *(bc)	0.25	0.72						

Table 1B. Egg volume gain rate during incubation period.

Temperature	Mean(sd)	Volume gain rate		Diference in volume gain curve among temperatures			Diference in volume among temperatures		
		b	r^2	F statistics		P	F statistics		P
28.7°C (field)	2.34(±1.2) *(a)	0.27	0.70						
30°C (control)	2.12(±1.0) *(ac)	0.25	0.71	0.7999	ns	0.49	11.996	*	>0.0001
32.5°C	2.26(±1.2) *(b)	0.29	0.78						
32.5°C(39°C/2h)	2.33(±1.0) *(bc)	0.24	0.68						

Influence of incubation temperature over survival rate, time of development, sex ratio, and morphology

Incubation temperature affected survival rate among treatments: field temperature, 30°C, and 32.5°C produced high rate of newborns, with 80%, 75.8%, and 71.9% survival, respectively. The 32.5°C (39°C/2h) treatment resulted in a 37% survival rate and the 35°C treatment was lethal, without hatches (Figure 2.A; X^2=28.19, P<0.0001). Sex ratio was affected only at 30°C treatment, resulting in 76% of females. Nevertheless, at 32.5°C treatment we registered a ~4:6 female:male proportion, thus differing from the 1:1 female:male proportion registered in the field and 32.5°C (±39°C/2h) treatments (Figure 2.B). Incubation temperature also affected time of development, with laboratorial treatments resulting in about half of the incubation period observed in the field treatment (Figure 2.C).

Morphological variables were affected by incubation temperature (MANCOVA, Pillai=1.05, F=2.46, P<0.0001), especially trunk, tail, and head traits (Table 2; Supplementary Figure SF2). Body length was the only trait affected by sex as covariate, males showing shorter bodies than females (14.2mm and 14.7mm, respectively; ANCOVA, F = 6.426, P = 0.01). Incubation temperature also influenced tail length, total length, body length, head length and head height traits. Mass, snout-vent length, interbrachial-nasal length, head width, and limb traits were not significantly affected (Table 2; Supplementary Figure SF2).
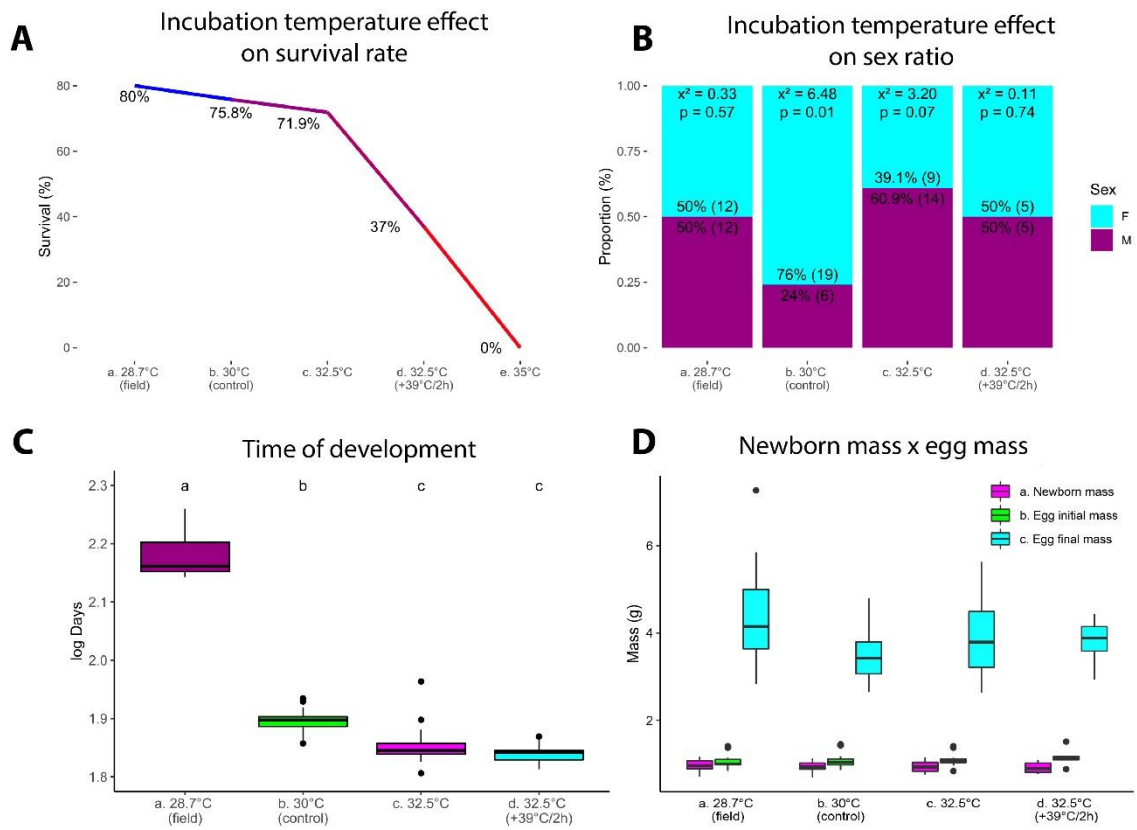
Figure 2. Incubation temperature effect on survival rate (A), sex ratio (B), time of development (C) and egg mass (D) of *Tropidurus torquatus*.

Table 2. Incubation temperature effects on morphological traits of *Tropidurus torquatus*.

Trait	Treatments				Effects					
	28.7°C (field, n=24)	30°C (control, n=25)	32.5°C (n=19)	32.5/C (39°C/2h) (n=14)	Incubation temperature			Sex		
					F statistics		p	F statistics		p
TOD	151.75 ±14.0 (*a)	78.8 ±3.2 (*b)	71.43 ±5.5 (*c)	69.3 ±2.8 (*c)	639.9	*	>0.0001	0.003	ns	0.96
MAS	0.96 ±0.1	0.94 ±0.1	0.93 ±0.1	0.9 ±0.1	0.391	ns	0.76	0.074	ns	0.79
SVL	30.2 ±1.2	30.5 ±1.4	30.8 ±1.6	30.4 ±1.6	0.533	ns	0.66	0.048	ns	0.83
TLL	41.5 ±6.1 (*ab)	42.0 ±5.5 (*ab)	44.6 ±4.1 (*a)	39.3 ±7.5 (*b)	3.497	*	0.02	0.108	ns	0.74
TTL	71.7 ±7.1 (*b)	72.5 ±6.5 (*ab)	75.3 ±5.3 (*a)	69.7 ±8.7 (*b)	4.265	*	0.008	0.161	ns	0.69
BL	14.0 ±1.0 (*b)	14.8 ±0.9 (*a)	14.7 ±1.1 (*a)	14.4 ±1.3 (*ab)	5.381	*	0.002	6.426	#	0.01
INL	12.2 ±0.6	12.0 ±0.6	12.0 ±0.7	12.0 ±0.5	0.617	ns	0.60	0.037	ns	0.85
HL	8.4 ±0.4 (*a)	8.2 ±0.3 (*b)	8.2 ±0.6 (*ab)	8.2 ±0.4 (*b)	3.096	*	0.03	2.316	ns	0.13
HW	6.3 ±0.4	6.3 ±0.3	6.2 ±0.5	6.3 ±0.4	0.266	ns	0.85	1.512	ns	0.22
HH	4.5 ±0.3 (*a)	4.5 ±0.2 (*ab)	4.5 ±0.3 (*a)	4.3 ±0.2 (*b)	3.416	*	0.02	0.13	ns	0.72
HUM	4.8 ±0.4	4.7 ±0.3	4.6 ±0.3	4.8 ±0.5	1.088	ns	0.36	1.627	ns	0.21
RAD	4.5 ±0.3	4.5 ±0.3	4.5 ±0.3	4.4 ±0.3	0.444	ns	0.72	0.118	ns	0.73
HAL	6.9 ±0.4	6.8 ±0.3	6.7 ±0.4	6.8 ±0.3	1.063	ns	0.37	0.475	ns	0.50
FEM	6.4 ±0.5	6.2 ±0.4	6.3 ±0.6	6.6 ±0.6	1.735	ns	0.17	0.242	ns	0.624
TIB	7.4 ±0.5	7.4 ±0.42	7.4 ±0.3	7.5 ±0.5	0.554	ns	0.65	0.19	ns	0.66
FTL	12.6 ±0.6	12.2 ±0.7	12.2 ±0.6	12.3 ±0.6	2.322	ns	0.08	0.008	ns	0.93

Influence of incubation temperature and age over thermal physiology

Incubation temperature affected thermal traits (MANCOVA, Pillai=0.602, F=3.1647, P<0.0001). ANCOVA tests show significant difference in Tb and CT_{min} traits with newborns incubated under field conditions showing smaller means (Table 3, Supplementary Figure SF3). Moreover, newborns from 32.5°C treatment also exhibit a significantly different mean. No significant differences were found in thermal preference, critical thermal maximum, and thermal tolerance range among incubation temperatures (Table 3). Our first PCA with TOD, SVL, and mass as proxies for development and size, respectively, resulted in three PCs explaining 65.52 of total variation (Table 5.A). PC1 explained 26.6% of the total variation and had very good positive loading from CT_{min} (0.59), a fair negative loading from TOD (-0.40), and a fair positive loading from T_b (0.44; Figure 3.A). This indicates a gradient from newborns with large developmental periods tending to small values of basal and critical thermal minimum temperatures. PC2 explained 20.4% of total variation and had good negative loading from mass (-0.48), a fair negative loading from TOD (-0.41) and good positive loading from CT_{max} (0.50). This indicates a gradient from newborns with large developmental periods and large mass values tending to small values of critical thermal maximum temperatures (Table 5.A; Figure 4.A). PC3 did not show interpretable overlapping variance for TOD loadings.

Table 3. Incubation temperature effects on thermal physiological traits of *Tropidurus torquatus*.

| | Treatments | | | | Effects | | | | | |
| | | | | | Incubation temperature | | | Sex | | |
Trait	28.7°C (field, n=22)	30°C (control, n=21)	32.5°C (n=22)	32.5/C (39°C/2h) (n=9)	F statistics		p	F statistics		p
T_{pref}	35.6 (±2.0)	35.9 (±2.3)	35.6 (±1.9)	36.5 (±1.4)	0.532	ns	0.6618	3.689	ns	0.06
T_b	26.3 (±2.0) (*b)	28.4 (±1.0) (*a)	28.5 (±1.4) (*a)	28.6 (±1.2) (*a)	12.331	*	>0.0001	0.314	ns	0.577
Ct_{min}	12.4 (±1.5) (*b)	14.4 (±2.3) (*a)	13.8 (±1.9) (*ab)	15.3 (±2.1) (*a)	5.766	*	0.0015	1.737	ns	0.19
CT_{max}	43.6 (±1.2)	43.9 (±1.6)	43.6 (±1.5)	44.5 (±2.0)	1.012	ns	0.393	0.04	ns	0.843
Amp_{tol}	30.0 (±3.5)	29.3 (±2.5)	29.6 (±2.6)	29.2 (±2.2)	0.26	ns	0.854	0.842	ns	0.36

Thermal traits also differed significantly between newborns and adults for all analyzed traits (Table 4). Although newborns showed smaller T_{pref} mean and larger T_{pref} variance significantly different from adults (Levene test, $F = 5.34$, $P = 0.02$), they proved to be less resistant to critical temperatures in total means and also exhibit a small variance for CT_{min} (Levene, $F = 9.63$, $P = 0.002$) and CT_{max} (Levene, $F = 7.81$, $P = 0.006$). No difference was found for T_b (Levene, $F < 0.001$, $P = 0.98$) and Amp_{tol} (Levene, $F = 2.53$, $P = 0.11$). Our second PCA with SVL and mass as proxies for body size/age resulted in three PCs explaining 75.18% of total variation (Table 5.B). PC1 explained 29.53% of total variance and had fair positive (0.40) and good positive (0.45) loadings from mass and SVL, respectively, a poor negative loading from CT_{max} (-0.35), and a fair positive loading from CT_{min} (0.41). This indicates a gradient from newborns (small values of SVL and mass) tending to large values of critic thermal maximum temperatures and small values of critic thermal minimum temperatures. PC2 did not show interpretable overlapping variance for critical thermal trait loadings and PC3 did not show interpretable overlapping variance for body size proxy loadings (Table 5.B; Figure 4.B).

Table 4. Age effect on thermal physiological traits of *Tropidurus torquatus*.

Character	Stage		Dimorphism in trait size among stages	
			F statistics	P
T_{pref}	Newborns	35.8 (±2.0)	12.412	>0.001
	Adults	37.1 (1.2)		
T_b	Newborns	27.8 (1.8)	29.019	>0.001
	Adults	26.0 (1.5)		
Ct_{min}	Newborns	13.8 (2.1)	71.695	>0.001
	Adults	10.0 (2.6)		
CT_{max}	Newborns	43.8 (1.5)	52.635	>0.001
	Adults	41.2 (2.3)		
Amp_{tol}	Newborns	29.6 (2.8)	5.3466	0.02
	Adults	31.2 (3.8)		

Table 5. Factor loadings of Principal Component Analysis ran on thermal and proxy-state related traits.

Table 5.A.

Trait	PC1 (26.6 exp.var.)	PC2 (20.4 exp. var.)	PC3 (18.5 exp. var.)
SVL	0.11	-0.28	-0.62
Mass	0.10	-0.48	-0.53
TOD	-0.41	-0.41	0.13
res_tb	0.44	0.23	0.04
res_tpref	0.12	0.20	-0.15
res_ctmin	0.59	0.01	0.02
res_ctmax	-0.04	0.50	-0.46
res_amptol	-0.49	0.42	-0.29

Table 5.B.

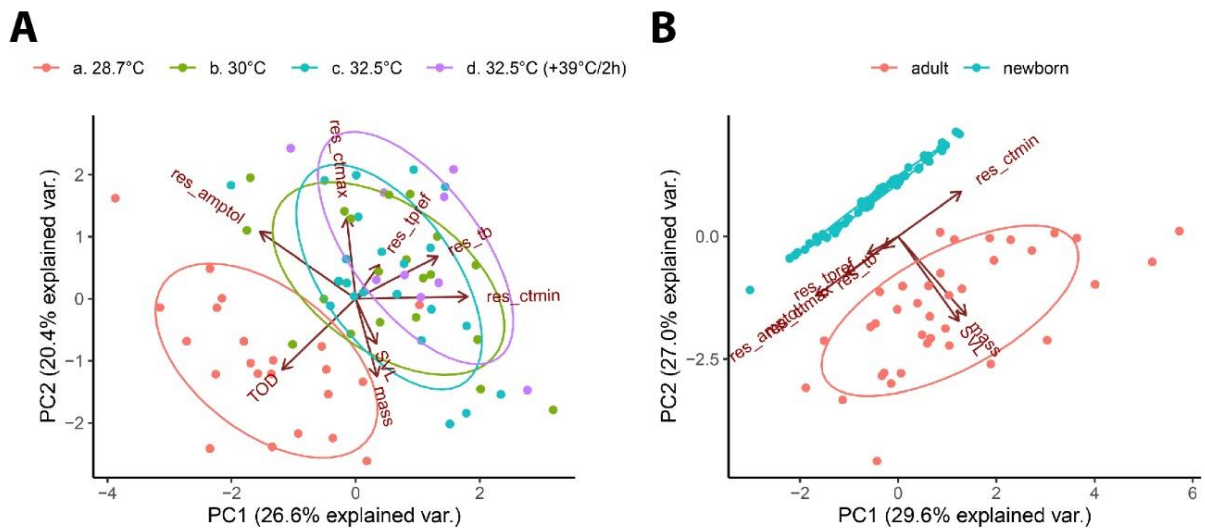| Trait	PC1 (29.5 exp.var.)	PC2 (27.0 exp. var.)	PC3 (18.7 exp. var.)
SVL	0.40	-0.58	0.002
Mass	0.45	-0.54	0.001
res_tb	-0.10	-0.08	0.59
res_tpref	-0.20	-0.12	0.41
res_ctmin	0.41	0.31	0.51
res_ctmax	-0.35	-0.28	0.45
res_amptol	-0.54	-0.41	-0.15

Figure 3. Principal component analysis biplot for incubation temperature (A) and age (B) effects over *Tropidurus torquatus* thermal physiological traits.

Discussion

The amount of studies on phenotypic plasticity in squamate reptiles is still insufficient vis-à-vis the diversity of this group, which represents one of the largest vertebrate radiations[4,17,24]. Moreover, there is a lack of in-depth studies on the effects of phenotypic plasticity inducers, such as temperature, during squamate development for Neotropical lizards. Our study investigates the effects of incubation temperature on egg development, and neonatal morphology and thermal physiology of the tropidurid lizard *Tropidurus torquatus*, an emerging Neotropical model organism. In addition, our comparison of natural incubation regime and different artificial temperature regimes aims to shed some light on the possible implications of climate change on the biology of this oviparous lizard.

The mass and volume of the eggs showed a great variation during development, being positively affected by incubation temperature. The mass gain reached an increase of up to four times the egg mass at the time of oviposition. However, this difference was not accompanied by the mass of hatched newborns, which was neither affected by incubation temperature nor by egg mass gain. Previous studies[15] argued that egg mass gain during incubation period does not influence on embryo development, even if the results show that egg mass gain by water absorption from the surrounding environment is influenced by temperature. This also occurs for *T. torquatus*, as we have no difference in neonate mass between incubation temperatures at the time of hatching, even though egg mass shows a positive temporal water gain. However, this is an issue that should be investigated in the future not only from the perspective of the direct influence of water absorption on mass gain, but also regarding the degree of thermoregulatory control the absorbed water plays inside the eggs[25,26].

In nature, oviparous species generally tend to choose locations that best mitigate the effect of environmental variables on egg development[27]. In addition of suitable nesting sites choice, most oviparous squamate species have a flexible egg shell that makes water gain from the laying environment possible, taking advantage of the combined action of shell porosity and yolk osmolarity[28]. This characteristic likely provides thermal and mechanical protection to the embryo due to the properties of the aqueous layer formed in the space between the embryo and the shell. It is reasonable to hypothesize that this characteristic doubly benefits the embryo: in addition to

promoting egg resistance against mechanical pressures, the thermal insulation of this layer also promotes an environment favorable to thermal equilibrium due to the thermodynamic properties of water, protecting the embryo from abrupt temperature variations. Temperature-induced egg mass and volume gains have been documented in other squamate species with flexible egg shells, and this is possibly a characteristic that contributed to their developmental success[29,30]. Nevertheless, not all squamates have flexible eggshells that can act as external incubators during post-ovipositional development, as is likely occurs in *T. torquatus* and other flexible egg shelled species. An open question remains regarding the influence of this phenomenon on the survival rates of embryos, also considering the species that have rigid calcareous egg shells.

As in several oviparous reptile species studied[15], the incubation temperature negatively affected the survival rate and incubation period of *T. torquatus*. This general pattern seen in *T. torquatus* and other lizard species is influenced by several factors such as environmental adaptation and the phylogenetic component itself[15,31–35]. It is interesting to note that *T. torquatus* exhibits a low tolerance to increased incubation temperature, with 100% lethality observed for eggs incubated at 35°C. This incubation temperature still generates viable neonates in other species, even with a birth rate considerably low.

The sex ratio of *Tropidurus torquatus* proved to be remarkably susceptible to genetic-environment interaction in a certain range of incubation temperature. While field and 32.5°C±39°C/2h treatments had a 1:1 sex ratio, 30°C treatment had a statistically significant higher proportion of female, and 32.5°C treatment had a not statistically higher proportion of males. However, the statistically significant mixed proportion with a greater number of females recorded in the 30°C treatment only allows us to hypothesize that in *T. torquatus* sexual determination can occur by an interaction between environmental and genetic factors and that this is not a species with temperature-dependent sexual determination, since there was no temperature range that registered an exclusivity of male or female neonates. In species where this situation occurs, the interaction between environmental and genetic factors has been demonstrated, where the duration and magnitude of the exposure of eggs to more masculinizing or more feminizing temperatures acts over hormonal expression processes that ultimately determine the development of either testes or ovaries[4].

A recent study revealed that changes in the morphology, growth and sexual maturity of *T. torquatus* populations have accompanied climatic changes in the last decades[36]. Our data show that the incubation temperature has an effect over body size traits. While the body and tail of *T. torquatus* are positively affected by increased incubation temperature, the size and height of the head are negatively affected. Not surprisingly, several studies have shown that the incubation temperature affects morphological traits; however, the ecological and physiological consequences of these changes remain unknown. In fact, there is a possibility that an increase in body size will benefit the species, since reproductive characteristics such as litter size are evolutionarily related to body size. On the other hand, a larger body demands greater energy gain. This goes against the prediction that lizard populations will be negatively affected by global warming, since there will be a decrease in potential daily foraging period, thus affecting energy gain[14].

Despite the great influence of thermal physiology on the survival of neonates in the initial periods of post-hatch life, this topic remains insufficiently explored[17,37]. Few studies have been conducted with the aim of understanding the effects of incubation temperature on the thermal physiology of newborn squamates; yet, comparisons across taxa show that the influence of incubation temperature on thermal traits exhibit conflicting patterns[38–40]. As in other lizard species studied, there was no change in the selected average temperatures of *T. torquatus* newborns hatched at different incubation regimes[25]. However, higher incubation temperatures significantly increased the metabolic rates of the embryos and neonates, which was reflected in the reduced incubation period and high T_b values positively affected by incubation temperature. Thermal physiological critics of *T. torquatus* may lead this species more vulnerable to future predict warming: resistance to critical cold temperature values by neonates decreases significantly with increment of incubation temperature. In fact, *T. torquatus* shows plasticity in CT_{min} values with negative implications since the neonatal cold critic values increase at higher incubation temperatures. This reduction in critic cold temperatures makes the species more vulnerable to abruptly colder climatic events, whose occurrence frequency is predicted to increase in the climate change scenarios. On the other hand, the absence of plasticity in CT_{max} values indicates that this is a trait possibly in a limit state of variation. The fact that incubation temperature does affect CT_{max} values of *T. torquatus* should make this species more vulnerable to temperature

changes in foraging environments induced by global warming. If not being able to adapt to the predict increase in maximum daily temperatures, the daily activity period of *T. torquatus* dedicated to foraging may be indeed circumstantially reduced, affecting important biological aspects such as energy gain and mate searching[39].

Few studies have investigated the effect of age on the thermal physiology of neonates of Squamate reptiles. The thermal critics variation in the few species investigated shows that there is no general rule about whether neonates are more or less susceptible to higher environmental temperatures than adults. While in the species *Eremias multiocellata* (Lacertidae), *Stenocercus guentheri* (Tropiduridae), and *Podarcis siculus* (Lacertidae) adults show higher CT_{max} values than juveniles, these are more tolerant than adults in the lizard *Sceloporus jarrovii* (Phrynosomatidae) and in the snake *Nerodia rhombifer* (Colubridae)[37]. Cases where there is no difference in CT_{max} between juveniles and adults include the lizards' species *Aspidoscelis sexlineatus* (Teiidae) and *Oligosoma maccannii* (Scincidae)[37]. Similar degree of variation across taxonomic groups has also been observed for the CT_{min}. The pattern observed from the thermal physiological traits of *T. torquatus* indicates that newborns thermoregulate in a lower temperature range when compared to adults. In addition, newborns have less resistance to colder temperatures and greater resistance to higher temperatures than adults. However, the temperature range of activity of the newborns is significantly smaller.

In conclusion, our study provides an in-depth investigation of the effects of incubation temperature on the plasticity of morphological traits and thermal physiology of the emerging Neotropical lizard model species *T. torquatus*. Further, it is the first study to investigate such effects in a lizard species from de Brazilian Cerrado. Our findings suggest that modifications in the incubation temperatures due to an increase in the temperature of nest sites caused by global warming may affect several features of the natural history, morphology, and thermal physiology of the newborns of this species. In addition, our results highlight the importance of observing such traits from the point of view of the global climate change paradigm and its implications for conservation of lizard diversity.

Acknowledgements

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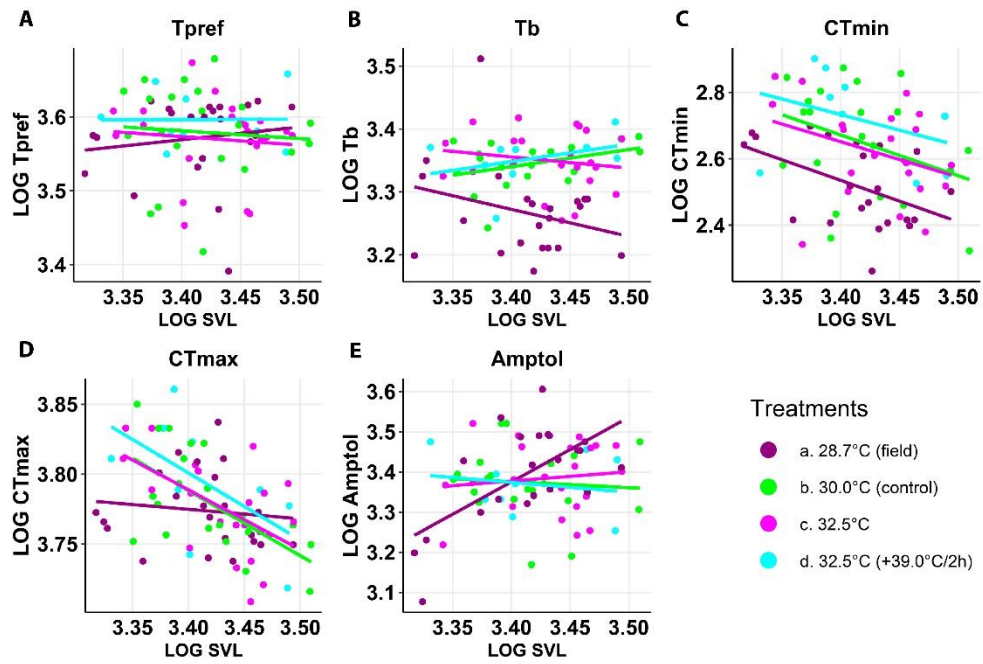
Supplementary Material
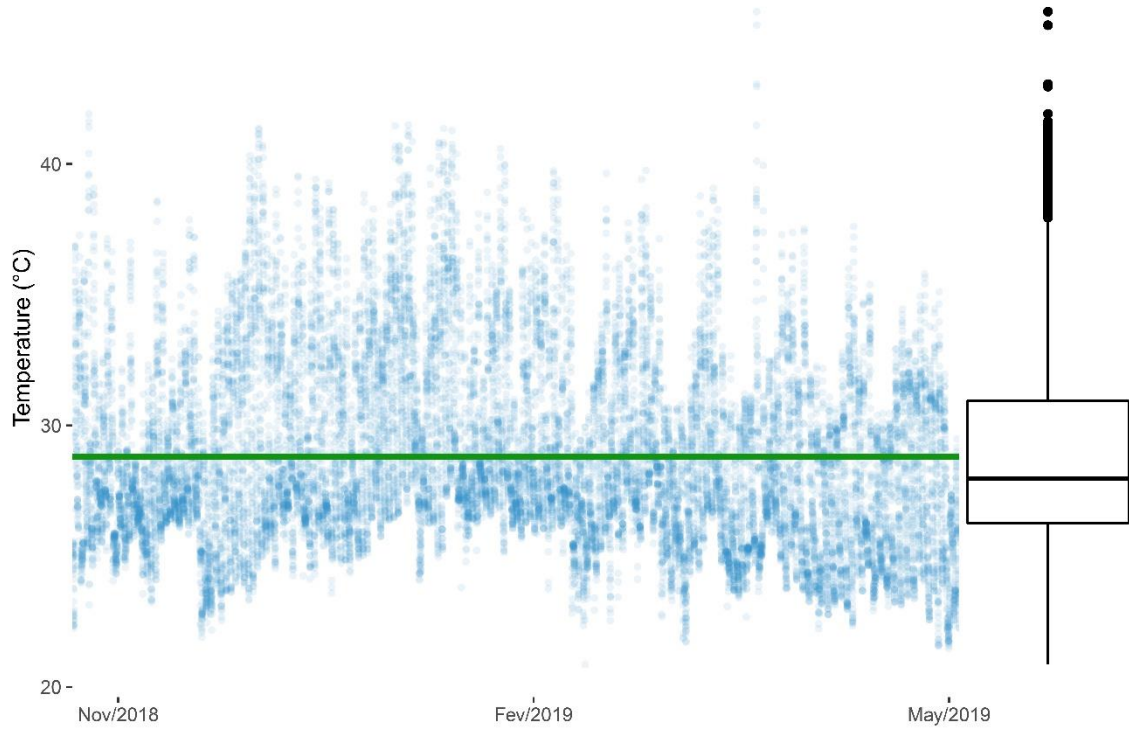
Supplementary figure SF1. Variation in artificial nest temperature during incubation period of *Tropidurus torquatus* eggs (Nov/2018 - May/2019)
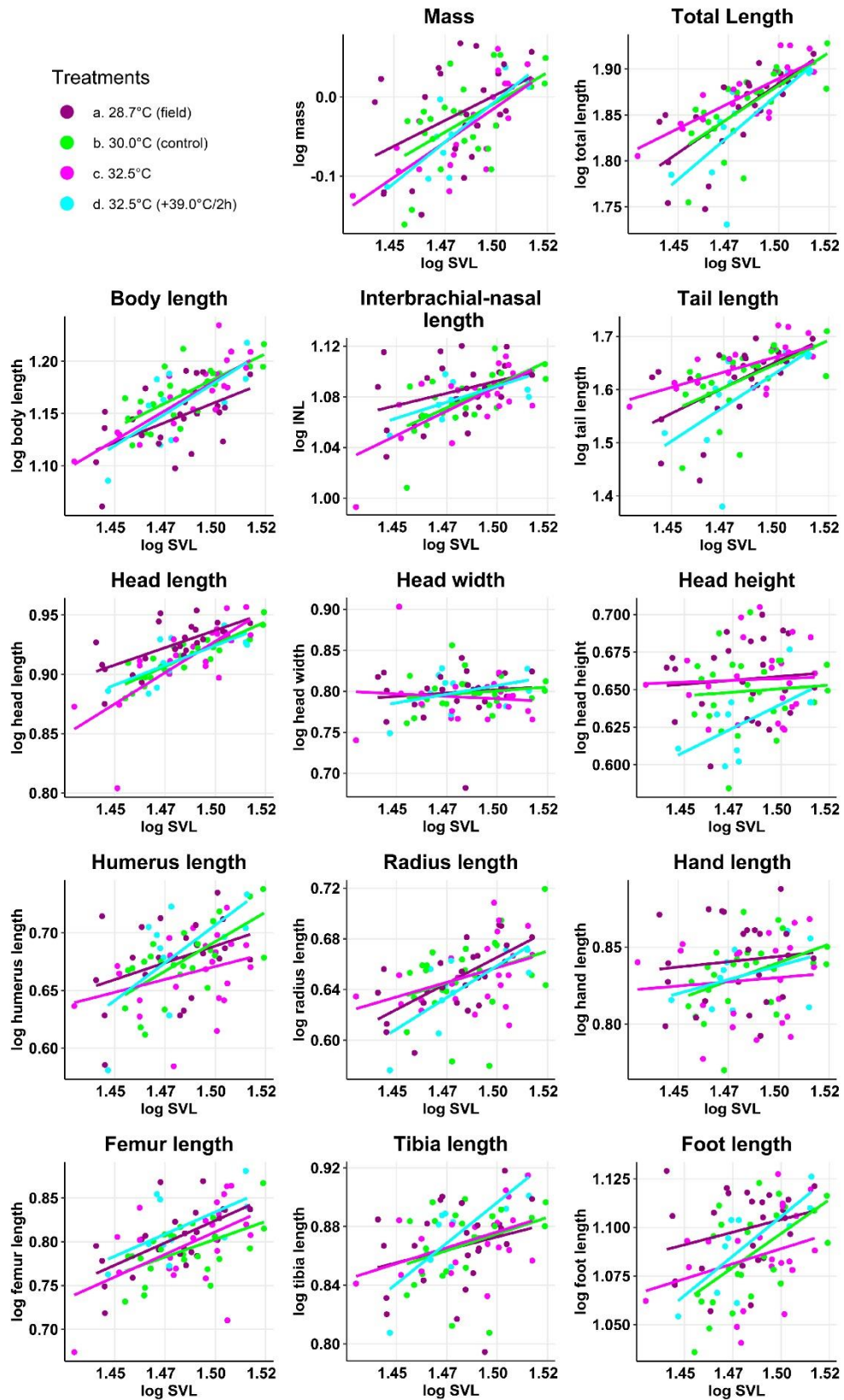
Supplementary figure SF2. Morphological traits of *Tropidurus torquatus* evaluated under four different incubation regimes.

Supplementary figure SF3. Morphological traits of *Tropidurus torquatus* evaluated under four different incubation regimes.

Apêndices

Apêndice 1 – Artigos publicados durante o mestrado (Referência bibliográfica)

- Anderson Kennedy Soares De-Lima, Fabrícius Maia Chaves Bicalho Domingos, Sacha Braun Chaves, Aline Pic-Taylor, Antonio Sebben, Julia Klaczko. A new scent organ for *Gymnodactylus* lizards (Squamata: Phyllodactylidae) and an updated evolutionary scenario for the origin of squamate epidermal glands. Biological Journal of the Linnean Society, Volume 125, Issue 3, November 2018, Pages 561–575, https://doi.org/10.1093/biolinnean/bly145

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- De-Lima AKS, Paschoaletto IP, Pinho LdO, Benmamman P, Klaczko J (2019) Are hemipenial traits under sexual selection in *Tropidurus* lizards? Hemipenial development, male and female genital morphology, allometry and coevolution in *Tropidurus torquatus* (Squamata: Tropiduridae). PLOS ONE 14(7): e0219053. https://doi.org/10.1371/journal.pone.0219053