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Controle cardiovascular autonômico e metabolismo em embriões de lagartos (Reptilia; Lepidosauria)

MARINA RINCON SARTORI

Tese apresentada ao Instituto de Biociências do Câmpus de Rio Claro, Universidade Estadual Paulista, como parte dos requisitos para obtenção do título de Doutora em Ciências Biológicas (Área de Zoologia).

Setembro - 2016

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Olho o ovo na cozinha com atenção superficial para não quebrá-lo. Tomo o maior cuidado de não entendê-lo. Sendo impossível entendê-lo, sei que se eu o entender é porque estou errando. Entender é a prova do erro. Entendê-lo não é o modo de vê-lo – Jamais pensar no ovo é um modo de tê-lo visto. - Será que sei do ovo? É quase certo que sei. – Assim: existo, logo sei. O que eu não sei do ovo é o que realmente importa. O que eu não sei do ovo me dá o ovo propriamente dito."

Clarice Lispector – O ovo e a galinha

Resumo

Durante o desenvolvimento embrionário de répteis ocorrem mudanças em relação à demanda de oxigênio, devido à contínua formação de tecidos e crescimento do embrião. Além disso, os ovos de répteis estão sujeitos a variações ambientais, como mudanças de temperatura, que influenciam diretamente as taxas de processos fisiológicos do embrião. Estudos indicam que, em geral, sob temperatura constante, o consumo de oxigênio aumenta ao longo do período embrionário em três padrões distintos dentre os répteis: exponencial, sigmoidal e em pico. Desta forma, ajustes cardiovasculares devem acompanhar essas mudanças do padrão metabólico, compatibilizando a oferta de oxigênio à demanda necessária para cada estágio do desenvolvimento. Em adultos, uma das formas de se adequar o provimento de oxigênio pelo sistema circulatório ocorre através do controle neural do coração, via sistema autônomo simpático e parassimpático. O controle autonômico induz a aceleração ou diminuição da frequência cardíaca (f_H), através dos neurotransmissores, resultando no aumento ou diminuição da oferta de oxigênio aos tecidos. No entanto, no embrião, o sistema nervoso ainda não está completamente formado e os mecanismos de regulação utilizados são pouco conhecidos nos répteis. Dessa forma, neste projeto procuramos determinar o padrão de metabolismo (VO_2) e f_H em embriões do lagarto iguana, *Iguana iguana*. Foi também estudada a relação entre o VO_2 e a f_H durante a fase embrionária e os efeitos cronotrópicos da temperatura. E para finalizar, avaliamos os mecanismos de controle do coração pelo sistema nervoso autônomo em embriões de iguanas e em uma segunda espécie de lagarto, o teiú (*Salvator merianae*), que pertence a um clado distinto dentro da Ordem Squamata. Nossos resultados indicam que durante a fase embrionária de répteis: i. não há um acoplamento entre a taxa metabólica e a f_H, ii. a temperatura exerce um efeito direto na f_H e os ninhos escavados em profundidade minimizam as variações térmicas diárias, iii. há indícios de presença de receptores adrenérgicos e colinérgicos no coração do embrião logo após a oviposição, e iv. no entanto, o controle do coração é realizado principalmente via tônus adrenérgico, provavelmente através de catecolaminas circulantes enquanto o tônus colinérgico se inicia posteriormente, próximo à eclosão, devido a possível relação com o início do ritmo ventilatório e a necessidade de controle de interações cardiorrespiratórias.

Palavras-chave: frequência cardíaca, consumo de oxigênio, Sistema Cardiovascular, répteis, Squamata, ovo amniótico, Sistema Nervoso Autônomo, adrenérgico, colinérgico

Abstract

During the reptilian embryonic development a progressive change in oxygen demand occurs as the embryos differentiate new tissue and grow. Moreover, reptilian eggs are susceptible to environmental changes, especially in temperature, that directly influences metabolic processes in embryos. Studies have shown that under constant temperatures embryonic reptiles present increasing levels of oxygen uptake, in three distinct patterns: exponential, sigmoidal, and in peak. In order to match oxygen supply and demand there might be accompanying cardiovascular adjustments in each stage of the embryonic period. In adult organisms different levels of sympathetic and parasympathetic control of the heart, driven by neurotransmitters, promotes accelerations or reductions in heart rate (f_H), that ultimately alter the oxygen provision to embryonic tissues. However, the neural system is not completely formed during the embryonic development and cardiovascular regulatory mechanisms at this phase are not well understood. In this study we aimed to determine the patterns of metabolism (VO_2) and f_H in embryos of the green iguana, *Iguana iguana*. We also assessed the relationship between the VO_2 and f_H and chonotropic effects of temperature during the embryonic period. Finally, we investigated the autonomic control of the heart in embryos of iguanas and another lizard, the tegu (*Salvator merianae*), which belongs to another clade of the order Squamata. Our results show that in embryonic reptiles: i. a direct coupling between the metabolic rate and f_H is absent throughout the embryonic incubation, ii. there is a direct effect of temperature on the f_H and the depth in which nests are buried prevent major temperature changes, iii. there is an indication that cardiac receptors are present very early in development and iv. the control of the heart is mainly promoted by an adrenergic tonus via circulating cathecolamines, and the onset of cholinergic tonus is delayed, occurring only close to hatching which may be related to the ventilatory rhythm and the need to control cardiorespiratory interactions.

Key-words: heart rate, oxygen consumption, Cardiovascular system, reptiles, Squamata, amniotic egg, Autonomic Nervous System, adrenergic, cholinergic

Sumário

1. Introdução Geral

1.1 A fisiologia do desenvolvimento

Segundo Burggren e Warburton (2005), as perguntas que a fisiologia comparada do desenvolvimento busca responder, tratam de quatro temas principais:

1) Como ocorre a divisão regulatória dos sistemas fisiológicos em desenvolvimento em um organismo ainda não completamente formado?
2) Como as mudanças das condições ambientais afetam os sistemas fisiológicos em desenvolvimento?
3) Como as diferenças em capacidade fisiológica refletem em diferenças de valor adaptativo e, consequentemente, na evolução dos organismos?
4) Como o desenvolvimento pode constringir a evolução?

Estudos de fisiologia do desenvolvimento utilizam com frequência três modelos de espécies experimentais, o peixe-zebra (*Danio rerio*), o camundongo (*Mus musculus*) e a galinha (*Gallus gallus*). Os estudos se beneficiam da extensa informação da biologia dessas espécies, incluindo grande informação genética, fácil acessibilidade aos embriões, dentre outros fatores (BURGGREN, 2000). Porém, dependendo da questão a ser elucidada, novos modelos animais podem ser descobertos, beneficiando-se do princípio de August Krogh, que propõe que para cada problema fisiológico há um animal mais conveniente para ser estudado (e.g. KREBS, 1975). Em outros casos, estudos de desenvolvimento são motivados pelo interesse comercial, no intuito de estimular o crescimento e o aumento da produção, através da ampliação dos conhecimentos da fisiologia embrionária e dos efeitos benéficos ou deletérios de interferências ambientais. Neste caso, o ovo de galinha é novamente um organismo extensivamente estudado, assim como espécies de peixes comerciais (BROOK, 1975; WOOD et al., 2014).

Muitos estudos encontraram semelhanças em embriões e larvas de espécies pertencentes a grupos distintos de animais, o que pode ser relacionado como “características universais” do embrião de vertebrado, porém, ao se proceder o desenvolvimento embrionário, muitas características tornam-se peculiares de cada espécie, especialmente com relação ao sistema cardiovascular (BURGGREN, 2000). O coração de vertebrados primordialmente possui formato tubular, porém ao se diferenciar, revela diferentes formatos, que estão estritamente correlacionados com a função (MOORMAN et al, 2003). diferentes formatos gradualmente revelam diferenças fisiológicas específicas de cada grupo.

No caso da fisiologia cardiovascular em embriões, a maioria dos estudos se concentra

em fetos de mamíferos como ovelhas, camundongos e ratos (e.g. JI et al., 2003, RYCHIK, 2004), seguidos de vários estudos em aves, como galinhas de diferentes linhagens, e o emu (e.g. CROSSLEY; ALTIMIRAS, 2000; RUIJTENBEEK et al., 2002, CROSSLEY et al., 2003a), e de alguns poucos em peixes e anfíbios (e.g. BURGGREN; FRITSCHE, 1997, JACOBSSON; FRITSCHE, 1999, SCHEWERTE; FRITSCHE, 2003, MILLER et al., 2011). Os répteis são o grupo menos estudado, apesar de sua diversidade, destacando-se especialmente estudos com o crocodiliano *Alligator mississipiensis* e o quelônio *Chelydra serpentina* (e.g. EME et al., 2011; CROSSLEY et al., 2003b; CROSSLEY; ALTIMIRAS, 2005, ALVINE et al., 2013).

1.2 Os répteis

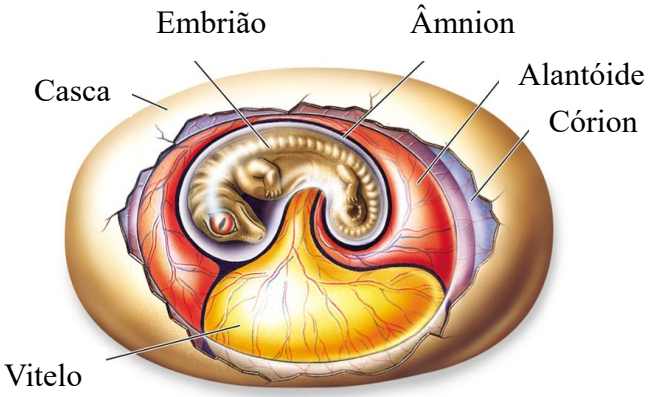
O grupo reptiliano é extremamente heterogêneo, tanto filogeneticamente quanto na ocupação de habitats, o que evidencia a importância de seu estudo. Os répteis tem origem polifilética a partir dos anfíbios, datada no período Triássico, em torno de 251 a 208 milhões de anos atrás (PORTER, 1972). Neste período pode-se traçar a origem dos quatro grupos, divididos com base na morfologia dos crânios: Anapsida, Sinapsida, Diapsida e Arcosauria. Anapsida é o grupo representado pelas atuais tartarugas viventes, que divergiu anteriormente ao estabelecimento das demais linhagens, sendo desta forma, o grupo mais isolado (CARROLL, 1969). Sinapsida é o grupo primitivo que deu origem aos precursores dos mamíferos. Diapsida engloba o grupo Lepidosauria, que se divergiu no grupo Ryncocephalia, que é representado por apenas um gênero vivente, o *Sphenodon*, e nos lagartos e serpentes (REIZS, 1997). Arcosauria, representado pelos jacarés e crocodilos viventes, tem sua ancestralidade compartilhada com os dinossauros e aves, através do grupo dos tecodontes (HUGHES, 1963).

De acordo com a base de dados mantida pelo pesquisador Peter Uetz (The Reptile Database), atualizado em agosto de 2015, o número de espécies de répteis viventes conhecidas é de 10.272, dentre as quais os lagartos são majoritários, com 6.145 espécies, seguidos de serpentes com 3.567, tartarugas com 341, crocodilianos com 25 e tuatara com apenas 1 espécie. Os répteis exploraram diversos habitats e desenvolveram especializações que permitiram que se distribuíssem por diversas zonas de extremos. Espécies de répteis são encontradas em ambientes de extremos de temperatura, desde desertos a ambientes abaixo de zero, extremos de salinidade, habitando zonas de água doce e salgada, extremos de altitude (POUGH et al., 2008) e extremos de disponibilidade de gases respiratórios, como ambientes anóxicos (MILTON; PRENTICE, 2007).

1.3 O ovo amniótico

Répteis representam a conquista definitiva do ambiente terrestre pelos vertebrados e, um dos principais motivos que possibilitou a total independência do meio aquático, foi o ovo amniótico, que propiciou que o desenvolvimento embrionário ocorresse independente do ambiente aquático (REISZ, 1997). O ovo amniótico, presente no grupo dos répteis e das aves, possui membranas extra-embrionárias que fornecem todos os nutrientes, água e permitem a passagem dos gases respiratórios necessários ao desenvolvimento do embrião. A casca confere proteção mecânica e possui poros que permitem a troca de água e de oxigênio e gás carbônico (RAHN et al., 1979). Logo abaixo da casca, encontra-se a membrana corioalantóica, que é vascularizada e realiza as trocas de gases respiratórios por meio de difusão com o ambiente externo através dos poros (PIIPER et al., 1980). O âmnion, envolve totalmente o embrião e, por ser uma membrana preenchida com líquido, o protege da dessecação que ocorreria caso estivesse em contato direto com o ar (ROMER, 1957). O alantóide também possui a função de armazenar água e excretas nitrogenados provenientes do metabolismo embrionário (e.g. SARTORI et al., 2012). O vitelo é uma reserva de proteínas e lipídeos, que garante o suprimento de combustível energético para a diferenciação e manutenção dos tecidos do embrião desde o período da oviposição até o período da eclosão dos filhotes (NOBLE, 1991). No entanto, ao contrário das aves, a maioria das espécies ovíparas de répteis depositam seus ovos em ninhos sem cuidado parental, estando dessa forma sujeitos à variabilidades ambientais de temperatura e umidade (GANS, 1996).

Fig. 1: Esquematização do ovo amniótico de répteis:



Fonte: http://www.sobiologia.com.br/conteudos/Reinos3/biorepteis.php. Acessado em 22 jul. 2016.

1.4 O desenvolvimento do sistema cardiovascular

Durante a embriogênese de vertebrados o primeiro sistema funcional e regulado é o cardiovascular. Em geral, o coração começa a bater precocemente no processo de desenvolvimento e em galinhas, por exemplo, inicia-se 3 dias após a oviposição, o que representa 14% do tempo de incubação de 21 dias (BURGGREN; WARBURTON, 1994). Nesta fase o coração não desempenha as funções de transporte, pois o processo de difusão é suficiente para suprir a demanda de oxigênio, porém possui a importante função de promover a angiogênese, dado a ligação entre morfogênese cardiovascular e fluxo sanguíneo (BURGGREN, 2004). A maturação do sistema cardiovascular ocorre através da junção entre o saco alantóico e o córion, formando a membrana corioalantóica irrigada com vasos sanguíneos e expansão dos vasos que irrigam o vitelo. A circulação extra-embrionária possui função análoga à placenta e ocorre em paralelo com a circulação intra-embrionária, que perfunde os órgãos internos em formação no embrião (BAUMANN; MEUER, 1992). Nos embriões mais tardios, após um considerável incremento de massa, o transporte através da convecção sanguínea é essencial para finalizar o desenvolvimento (BURGGREN; WARBURTON, 1994).

Para determinar o desenvolvimento do sistema cardiovascular, um dos parâmetros mais acessíveis é a medição da frequência cardíaca (f_H). Em algumas espécies de vertebrados o padrão da f_H ao longo do desenvolvimento foi determinado e uma grande variabilidade foi registrada. Em geral ocorre um aumento da f_H (HOLETON, 1971; WHITE, 1974; BURGGREN; DOYLE, 1986; PELSTER; BEMIS, 1991; PELSTER; BURGGREN, 1991) no estágio mais inicial do desenvolvimento. Em anfíbios pode-se observar uma relação marcante entre f_H e massa corpórea (Mb) como em *Lithobates catesbeianus*, e a ausência desta correlação em *Eleutherodactylus coquii* (BURGGREEN; DOYLE, 1986). Em répteis, o crocodiliano *Alligator mississipiensis* apresenta uma redução acentuada seguida de um decaimento gradual da f_H até o momento da eclosão (CROSSLEY; BURGGREN, 2009). Em aves o padrão parece estar associado com a divisão entre aves precoces, relativamente independentes logo após a eclosão, e altriciais, que requerem um cuidado parental extensivo após a eclosão. Nas espécies de aves precoces observa-se um aumento hiperbólico ou uma diminuição da f_H ao final da incubação ou logo após o "pipping interno" (momento em que o embrião perfura a membrana, tendo acesso à câmara de ar, em torno de 48h antes da eclosão). Em espécies altriciais observa-se um aumento progressivo durante a segunda metade da incubação, sem um decaimento no momento do "pipping interno" (TAZAWA et al., 1991, 1994). Em mamíferos o padrão humano apresenta um aumento acentuado da f_H até o 50° dia após a concepção, em seguida estabiliza e declina após o nascimento, um padrão oposto ao do cão doméstico (HOWE et al., 1991).

O padrão da f_H em embriões de vertebrados pode ser influenciado por mudanças de permeabilidade da membrana, mudanças na região do marca-passo ao longo da incubação e diferentes taxas e início do controle autonômico colinérgico, adrenérgico e hormonal (BURGGREN; PINDER, 1991; CROSSLEY et al., 2003b; NECHAEVA et al, 2007). Apesar do batimento cardíaco de vertebrados ser iniciado e seguir o ritmo ditado pelo marca-passo a frequência de batimentos é modulada constantemente. Uma das formas de regulação do sistema cardiovascular e da f_H se dá através do sistema neural, mais especificamente pelo o sistema nervoso periférico, através das ramificações simpática e parassimpática, e também através do sistema humoral. Os nervos autonômicos e os hormônios agem nos receptores presentes no coração e nos vasos sanguíneos através da transdução de sinais do sistema nervoso central (KLABUNDE, 2011). Através das inervações neurais, que liberam neurotransmissores, ou através dos hormônios circulantes, que se ligam a receptores específicos localizados nas membranas do miócito cardíaco ou na célula muscular lisa do vaso sanguíneo, ocorrem respostas que podem aumentar ou diminuir a força de contração, dilatar ou constringir os vasos, resultando em um fluxo sanguíneo suficiente para a perfusão dos tecidos de acordo com as demandas do organismo (LEVICK, 1991).

A temperatura de incubação interfere sensivelmente na f_H de aves e é muito importante para determinar taxas de desenvolvimento e crescimento e a duração da incubação (DEEMING; FERGUSON, 1991). Além disso, sabe-se que em aves a tolerância térmica de embriões também varia em função do desenvolvimento, por exemplo, embriões em estágios iniciais sobrevivem por muito mais tempo quando expostos a temperaturas baixas do que embriões mais avançados (TAZAWA; RAHN, 1986). No entanto, há poucos estudos sobre a influência da temperatura na f_H de répteis mesmo estando sujeitos a temperaturas de incubação muito mais variáveis. As fêmeas grávidas tem papel fundamental na escolha da temperatura corpórea e seleção de locais de oviposição (ANGILLETTA et al., 2000). Sabe-se que a temperatura pode influenciar a duração da incubação, o tamanho dos filhotes (CASTILLA; SWALLOW, 1996; SHINE et al., 1997) e na determinação do sexo em algumas espécies (FERGUSON; JOANEN, 1983). Nos embriões de répteis a f_H aumenta de acordo com a temperatura, e é capaz de se aclimatar, assim como a f_H de organismos adultos (DU et al., 2010a).

A f_H elevada de embriões é benéfica por estar associada a altas taxas de desenvolvimento, correlacionado com um maior sucesso reprodutivo. O nível de f_H elevado dos embriões quando comparados à de filhotes e adultos, são possíveis devido a existência de uma reserva energética garantida, o vitelo (DU et al., 2010b). Estudos mostraram que a f_H é mais altas em lagartos e quelônios quando comparados com serpentes e crocodilianos e que em

Squamata o número de batimentos cardíacos ao longo do desenvolvimento total é menor. Em algumas espécies as fêmeas são capazes de reter os ovos por um tempo maior antes da oviposição e essas diferenças de extensão da embriogênese, realizada antes e depois da oviposição, garantem diferentes períodos de incubação e diferenças de número total de batimentos cardíacos. As famílias de Iguanídeos e Gekkonídeos são as que possuem o menor número de batimentos, devido a uma provável divergência filogenética quanto à embriogênese realizada dentro e fora da fêmea (DU et al., 2011).

1.5 Regulação do sistema cardiovascular

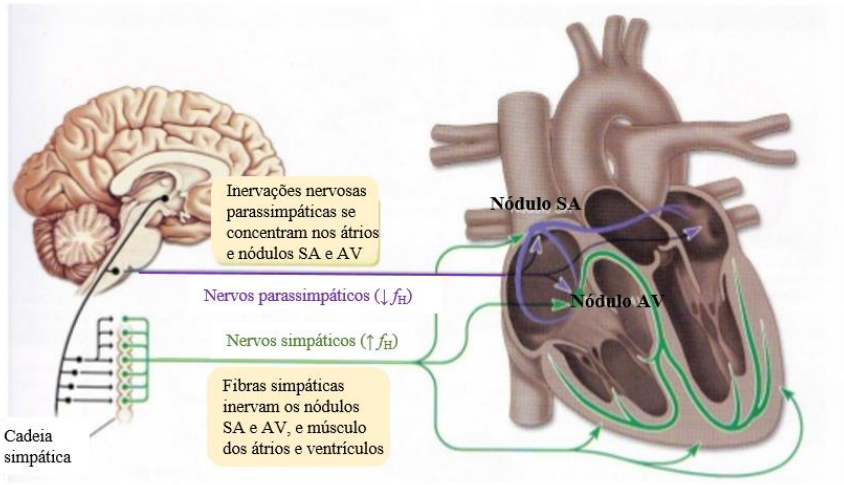
O sistema nervoso pode ser dividido em duas partes, o sistema nervoso central (SNC) e o sistema nervoso periférico (SNP). O SNC consiste do encéfalo e espinha vertebral enquanto o SNP consiste dos nervos cranianos, cervicais, espinhais, sacrais e nervos intrínsecos de cada sistema de órgãos. O SNP transporta informações do SNC para os órgãos efetores e levam informações sensoriais para serem processadas no SNC. O SNP é dividido em somático, que inerva músculos esqueléticos e os nervos e receptores sensoriais, e autônomo, que inerva músculos lisos, glândulas e vasos sanguíneos. Os nervos autonômicos controlam funções corporais, de forma involuntária (KRUK; PYCOCK, 1983).

O sistema autônomo é um dos principais efetores da regulação instantânea do sistema cardiovascular, e é um dos mecanismos que permite uma associação do débito cardíaco com o metabolismo. O sistema autônomo é dividido funcional e anatomicamente em simpático e parassimpático. O sistema parassimpático é responsável por processos que restauram as reservas de energia do organismo enquanto o sistema simpático é o responsável pelas respostas conhecidas como as de “fuga e luta”, promovendo a mobilização das reservas energéticas (HILL et al., 2012). No entanto, os dois sistemas estão sempre ativos, variando a dominância de cada um, dependendo da situação (KRUK; PYCOCK, 1983).

Em répteis adultos, durante o repouso, o coração é modulado predominantemente por influência do décimo nervo cranial, o vago, que libera o neurotransmissor acetilcolina (Ach). A acetilcolina, liberada pelo neurônio pós-ganglionar, se liga ao receptor muscarínico colinérgico presente no coração e promove redução da f_H e da força de contração (TAYLOR et al., 1999). O sistema simpático, por sua vez, libera catecolaminas, como a epinefrina ou norepinefrina, que se ligam aos receptores β-adrenérgicos do coração, promovendo aumento da taxa e da força de contração, que é geralmente associado, mas não restrito, ao exercício e condições de estresse do animal (OVERGAARD et al., 2002). A acetilcolina e a adrenalina são os responsáveis por iniciarem uma resposta no tecido efetor após se ligarem aos receptores e,

dessa forma, são chamados de agonistas. Substâncias que bloqueiam ou impedem os agonistas de iniciarem uma resposta são chamados de antagonistas ou bloqueadores. Como exemplo de antagonistas temos a substância atropina, que bloqueia os receptores colinérgicos muscarínicos e o propranolol, que bloqueia os receptores β-adrenérgicos (KRUK; PYCOCK, 1983).

Fig. 2: Esquematização da inervação autonômica sobre o coração em mamíferos.



Fonte: Adaptado de McARDLE, W.D.; KATCH, F.I.; KATCH, V.L. Fisiologia do exercício energia, nutrição e desempenho. Rio de Janeiro: Guanabara Koogan, 2003.

Em embriões de aves e répteis o sistema nervoso autônomo está se desenvolvendo e o momento em que se torna maduro para desenvolver a regulação cardiovascular é variável entre as espécies. Receptores adrenérgicos e colinérgicos foram encontrados desde o primeiro quarto da incubação em aves (BARRY, 1950), porém a inervação neural ocorre apenas tardiamente, evidenciado pelo início da presença de tônus colinérgico inibitório apenas em momentos próximos à eclosão (CROSSLEY; ALTIMIRAS, 2012; CROSSLEY, 1999). O tônus β-adrenérgico excitatório está presente pelo menos a partir da segunda metade da incubação em várias espécies de aves até o momento estudadas, a partir de 50-70%, resultando dos altos níveis de catecolaminas encontrados na circulação (CROSSLEY; ALTIMIRAS, 2000). Em répteis o tônus adrenérgico também foi encontrado presente nos períodos estudados no aligator americano *Alligator mississipiensis* (EME et al., 2011), na tartaruga *Chelydra serpentina* (ALVINE et al., 2013) e na serpente *Lamprophis fuliginosus* (CROSSLEY; BURGGREN, 2009). Porém, o início do tônus colinérgico varia, estando presente desde os 70% da incubação na tartaruga *C. serpentina* e 90% na serpente *L. fuliginosus*, enquanto é ausente no alligator durante o período de incubação embrionária.

Devido à escassez de estudos e a importância do grupo reptiliano, que representa o elo evolutivo entre anfíbios, aves e mamíferos, este estudo visou determinar ao longo do desenvolvimento embrionário de uma espécie de lagarto, o iguana (*Iguana iguana*), dados cardiovasculares básicos como padrão de f_H e efeitos cronotrópicos da temperatura assim como a determinação do início do controle autônomo. Também avaliamos o papel da f_H no transporte de oxigênio para manutenção do desenvolvimento embrionário, relacionando a f_H com taxas metabólicas e de crescimento. Para avaliar uma espécie de lagarto pertencente à suborderm Scleroglossa, um clado dicotômico à Iguania, selecionamos o teíú, *Salvator merianae*, em que determinarmos o padrão de frequência cardíaca e o papel do controle autonômico durante o período de desenvolvimento embrionário. Dessa forma, no escopo do trabalho, abordaremos dois dos principais temas da fisiologia comparada do desenvolvimento, no tocante às questões mecanísticas de processos de função e regulação cardiovascular e os efeitos da temperatura no sistema cardiovascular em desenvolvimento.

1.6 A espécie Iguana iguana

A espécie *Iguana iguana* é um lagarto herbívoro que faz parte da família Iguanidae, no clado monofilético Iguania. Na ordem Squamata distingue-se um segundo clado, Scleroglossa, baseado em características morfológicas (CONRAD, 2008). A distribuição da espécie ocorre desde o México, América Central e boa parte da América do Sul, em regiões tropicais e subtropicais. No Brasil, a espécie é encontrada na região Amazônica, parte da região Centro-Oeste, Pantanal, e na Caatinga. Um indivíduo adulto pode medir 180 cm de comprimento e pesar até 9 kg.

Fig. 3 Macho adulto do lagarto *Iguana iguana*, de aproximadamente 90 cm de comprimento total, pertencente ao criatório científico da UNESP Rio Claro (Jacarezário).

Fonte: arquivo pessoal.

Em cativeiro depositam aproximadamente entre 10 e 60 ovos em ninhos escavados no solo, nos períodos dos meses de setembro e outubro, que levam em torno de 70-74 dias para eclodir quando incubados artificialmente a 30°C (observações pessoais). A profundidade dos ninhos de iguana é de 25 a 50 cm (RAND, 1968). A profundidade do ninho tem direta influência no perfil térmico durante a incubação (HILLEL, 1980). Os ovos possuem cascas flexíveis, capazes de absorver água do meio externo, sendo que ao final da incubação apresentam o dobro do tamanho da oviposição (e.g. SARTORI et al., 2012).

1.7 A espécie Salvator merianae

O teiú (*Salvator merianae*) é um lagarto amplamente encontrado na América do Sul (AVILA-PIRES, 1995). É um dos maiores lagartos da família Teiidae, chegando até 5 kg e 1,6 m de comprimento (Fig. 4).

É uma espécie modelo para estudos fisiológicos, pois é um lagarto que possui atividade durante os meses quentes e chuvosos, e inatividade durante os meses mais frios e secos (ABE, 1983). Neste período de dormência, os teiús permanecem em seus abrigos, em jejum e com temperatura corpórea, metabolismo e função cardiorrespiratória reduzidos (ANDRADE et al., 2004). A reprodução ocorre logo após a emergência da dormência. As fêmeas depositam entre 20-60 ovos em ninhos construídos de folhas secas, cuja incubação dura em média 60 dias à temperatura de 30°C (observações pessoais).

Fig. 4 Fêmea adulta da espécie *Salvator merianae*, de aproximadamente 60 cm de comprimento total, pertencente ao criatório científico da UNESP, Rio Claro (Jacarezário).

Fonte: arquivo pessoal.

2. Objetivos

Neste trabalho buscamos determinar alguns parâmetros do desenvolvimento do sistema cardiovascular em embriões de lagartos, de duas sub-ordens distinstas Iguania e Scleroglossa. Dessa forma, ao longo do desenvolvimento embrionário da espécie de lagarto *Iguana iguana*, pertencente à sub-ordem Iguania, visamos determinar:

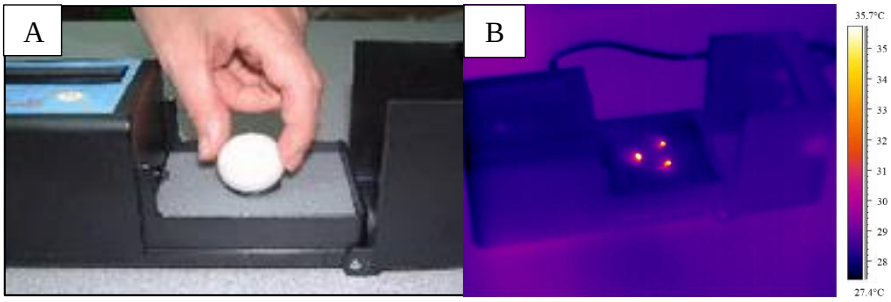
- padrão temporal de f_H exibido pelos embriões sob temperatura constante e a influência de alterações agudas de temperatura na f_H embrionária;
- a relação da f_H com taxa metabólica (VO_2), pulso de oxigênio (O_2 Pulse), tamanho corpóreo (M_b) e tamanho do coração (M_h);
- início e nível do controle autonômico da f_H..

E para verificar a existência de distinções entre as duas subordens visamos determinar o padrão temporal de f_H sob temperatura constante assim como o início e nível do controle autonômico na f_H. ao longo do desenvolvimento embrionário do teiú *Salvator merianae*, pertencente à sub-ordem Scleroglossa.

3. Metodologia

Para o monitoramento da f_H embrionária utilizamos o monitor cardíaco digital Buddy Systems (Avitronics, Truro, UK; Fig. 5A), que detecta os batimentos cardíacos do embrião no interior do ovo, de forma não invasiva, a partir de radiação infra-vermelha. No entanto, o equipamento é utilizado para medição da f_H instantânea para detectar viabilidade de ovos em granjas ou de forma pontual em estudos de aves e répteis (LIERZ et al., 2006; DU; SHINE, 2008). Para o estudo, solicitamos uma modificação do equipamento pelo fabricante, que providenciou uma saída digital, que acoplada a um sistema de aquisição de dados (PowerLab, ADInstruments, Sydney, Austrália) permitiu o monitoramento de longo prazo da f_H dos embriões. No entanto, a radiação infra-vermelha também é uma fonte emissora de calor (Fig 5B), o que causou o aquecimento dos ovos ao longo do tempo. Uma maneira de contabilizar o efeito do aquecimento, foi o monitoramento da temperatura interna dos ovos através de um termopar implantável (T-type, ADInstruments). Esta questão assim como os resultados do monitoramento da temperatura dos ovos quando medidos no monitor digital foram abordados no Capítulo I.

Fig. 5 A) Imagem do monitor digital cardíaco Buddy Systems, mostrando o local em que o ovo é colocado durante a medição da f_H; **B)** Imagem térmica do monitor digital cardíaco, mostrando o evidente aumento de temperatura dos sensores infra-vermelhos.



Fonte: A- https://www.avitronics.co.uk; B - arquivo pessoal.

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Capítulo I

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An appraisal of the use of an infrared digital monitoring system for long-term measurement of heart rate in reptilian embryos

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M.R.S is a postgraduate student in the laboratory of A.S.A. and performed the experiments and analyzed the data; D.A.C. initiated the study and supervised the work on turtles; E.W.T. inspected the data and amended the manuscript.

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Abstract

Measurement of heart rate (f_H) in embryonic reptiles has previously imposed some degree of invasive treatment on the developing embryo. Recently a non-invasive technique of f_H detection from intact eggs was developed for commercial avian breeders and has since been used in biological research. This device uses infrared light, enabling it to detect heartbeats in very early embryos. However, infrared light is a source of heat and extended enclosure of an egg in the device is likely to affect temperature with consequent effects on physiological processes, including f_H. We studied the effect of use of the monitor on the temperature of eggs and on f_H in two species of reptiles, the snapping turtle (*Chelydra serpentina*) and the green iguana (*Iguana iguana*). Egg temperature increased from a room temperature of 27-28ºC, by 26% in turtles and 14% in iguanas over one hour of enclosure, resulting in an increase in f_H of 76-81% in turtles and 35-50% iguanas. These effects on f_H can either be avoided by brief enclosure of each egg in the monitor or measured and accounted for during the design of long-term experiments.

Key words: reptiles; embryonic development; heart rate; Buddy®; infrared radiation; temperature

1. Introduction

Heart rate (f_H) during embryonic development has been the most commonly reported cardiovascular variable taken from a wide range of species of reptile, providing basic data regarding the maturation of cardiovascular function (Crossley et al., 2003; Crossley and Burggren, 2009; Eme et al., 2011; Sartori et al., 2015). Methods to acquire these data include direct measurements of arterial pressure (Crossley et al., 2003; Crossley and Altimiras, 2005; Eme et al., 2011; Alvine et al., 2013; Eme et al., 2013; Eme and Crossley, 2015), visual counting via a dissecting microscope (Nechaeva et al., 2007; Sartori et al., 2015) or impedance measurements (Bichard and Reiber, 1996, Crossley and Burggren, 2009). While these methods are useful for gathering information regarding maturation of the cardiovascular system they require some degree of invasive instrumentation, possibly disturbing and most often terminating embryogenesis for the individual embryo. Recent longitudinal studies of f_H prior to hatching in embryos of several species of lizards and turtles have utilized a noninvasive method for monitoring f_H using the transmittance or reflectance of infrared light from a digital egg monitoring system (Buddy®, Avitronics, Truro, UK). Publications using this system include: Lierz et al., 2006; Radder and Shine, 2006; Du and Shine, 2008; Du et al., 2009; Du and Shine,

2010; Du et al., 2010a; Du et al., 2010b; Du et al., 2010c; Du et al., 2010d; Du et al., 2011; McGlashan et al., 2012; Spencer, 2012; Angilletta et al., 2013; Aubret, 2013; Loudon et al., 2013; Zhao et al., 2013; Sartori et al, 2015. Infrared radiation (IRR) is an important source of heat (Herschel, 1801: Seigel et al., 2001) and devices emitting IRR are commonly used as a deliberate heating source. If the IRR emitted by the Buddy® system significantly alters the thermal environment of the egg it is likely to affect physiological processes, including f_H. Thus, there is clearly the potential for reporting unreliable data on progressive changes in f_H using this system. However, the potential heating effect of infrared light on the thermal status of reptilian eggs was not overtly considered in previous studies and has yet to be determined.

This investigation set out to characterize the changes in heart rate in the embryonic snapping turtle (*Chelydra serpentina*) and green iguana (*Iguana iguana*), when exposed to IRR. The snapping turtle represents one of the most extensively studied reptiles during embryonic development, allowing cross study and method comparisons within a species. We hypothesized that the infrared detection method would heat the turtle egg resulting in an elevation in heart rate. To test this hypothesis we studied embryonic snapping turtles at 70% and 90% of incubation and green iguanas from 30% of incubation until close to hatching. The eggs of green iguanas increase in mass during development (Sartori et al, 2015), possibly affecting their response to any heating effect from the infrared monitor.

2. Material and Methods

2.1 Experimental animals

Snapping turtle: On June 2013 eggs from snapping turtles, *Chelydra serpentina* were collected in northwestern Minnesota (Minnesota Department of Natural Resources Permit No. 18337 to DAC) and transported to the Biology Department at the University of North Texas, Denton, USA, where the experiments were performed. Upon arrival, eggs were numbered, weighed and placed in plastic boxes (volume approximately 3 litres) containing vermiculite mixed with water in a 1:1 ratio by mass. Water content of vermiculite was maintained by weighing boxes twice weekly and adding water as needed. The boxes were set in plastic Ziploc bags supplied with normoxic air (21% O_2) bubbled through water to maintain both oxygen and water saturation at adequate levels. The bags were maintained in incubators set to 30°C. Six eggs from different clutches were taken from incubators at each 70% and 90% of incubation time and weighed before assigned to the experiments.

Green iguana: Freshly laid eggs of green iguana, *Iguana iguana* were collected during the months of September and October of 2013 from captive gravid females that were part of the breeding program operating at the Jacarezário, Departamento de Zoologia, São Paulo State University (UNESP), Rio Claro, SP, Brazil. Eggs were weighed and immediately placed in trays (38 x 28.5 x 6.5 cm) containing water saturated vermiculite held at a constant temperature of 30±0.5ºC in incubators (Eletrolab, EL101/3, SP, Brazil). All eggs were examined daily for signs of mortality and the vermiculite sprayed with dechlorinated tap water to maintain humidity high. Six eggs were selected from different clutches at the developmental times of: 30%, 50%, 70%, 90% and just prior to hatching.

2.2 Instrumentation: Experiments were performed according to approved animal care protocols (UNT IACUC 11- 007 and CEUA-UNESP no. 6597 and no. 3680). The study utilized a digital egg monitor (Buddy® System, Avitronics, Truro, UK) that records f_H non-invasively by detecting movement via infrared sensors, and amplifies the resulting signal, enabling recordings to be obtained from early embryos. The digital egg monitors used in this study were customized by the manufacturers to provide an analog output signal via a BNC connector that was digitally transformed using a data acquisition system (PowerLab; ADInstruments, Bella Vista, NSW, Australia).

For temperature measurement, both snapping turtle and green iguana eggs were weighed and candled to detect a place for insertion of a thermocouple through the eggshell that avoided direct contact with the embryo or yolk. A patch of 1 cm^2 of latex glove was attached to the eggshell using cyanoacrylate glue (Loctite, USA). The eggshell was then punctured, through this patch, with a 26-gauge needle, and a flexible implantable thermocouple probe (BAT-4, Physitemp Instruments, NJ, USA or T-type, ADInstruments) was inserted approximately 5 mm into the egg. Eggs were then placed in the Buddy® chamber which was housed in a constant temperature chamber (EGC, OH, USA/Caltech EIP-010, PE, Brazil) held at 30±0.5°C and the lid of the instrument was closed following the manufacturers directions for use. Iguana eggs were surrounded by a ring of wet gauze in order to minimize evaporative water loss. The signal outputs from the egg monitors and from the thermocouples in the eggs and in the environmental chambers were relayed to the data acquisition system, (ADInstruments, PowerLab), and recorded simultaneously and continuously via LabChart software (ADInstruments, Bella Vista, NSW, Australia). Recordings were closely monitored and conducted until no major changes in temperature were detected, after a minimum of two hours. Egg temperature and f_H were collected every 10 minutes from the recordings for statistical

determination of the time elapsed until stabilization of egg temperature and relationships between temperature and f_H (Table 1). Temperature coefficients (Q_{10}) were calculated according to the following equation:

$$Q10 = \left(\frac{R2}{R1}\right)^{\frac{10}{T2-T1}}$$

2.3 Statistical analysis

Egg mass, initial and final temperatures (T_{min} and T_{max} respectively) and initial and final f_H (If_H and Ff_H, respectively) were tested within turtles with paired T-test and within iguanas with one-way ANOVA. A repeated measures ANOVA with time as the independent factor and temperature as the dependent factor was used to detect the point of stabilization of egg temperature. A post hoc Student-Newman-Keuls test was used to identify possible significant differences between the incubation groups. Linear regression analysis was performed with changes in egg temperature (independent variable) and f_H (dependent variable) at each point of incubation period. Tests for snapping turtle data were performed with the STATISTICA version 12 software package and for iguana data with SigmaPlot version 10.0. Significance was attributed at a level of 95% confidence. Data are presented as mean ± SEM.

3. Results

Eggs left inside the Buddy® warmed with time until they had reached a stable temperature, which was approximately 34°C in snapping turtle embryos (Fig. 1A) and to 32°C in green iguana (Fig. 1B). A summary of the data for each species and developmental group is detailed on Table 1.

In the turtle egg mass increased from 12.0±0.8g at 70% incubation to 12.9±0.3g at 90% incubation, an increase of 7.5%. Temperature stabilization occurred 40 minutes after the egg monitor was turned on at 70% incubation and 50 minutes at 90%. The mean temperature recorded after a period of 140 minutes was 34.0±0.1°C (n = 6) at 70% and 34.0±0.2°C (n = 6) at 90%. The increasing temperature had a direct effect upon f_H. At 70% mean f_H increased from 55±1 (beat · min^{-1}) to 96±3 (beat · min^{-1}), which represents a 76% increase. At 90% mean f_H increased from 45±3 (beat · min^{-1}) to 85±1 (beat · min^{-1}), representing 89% increase. Initial and final f_H of turtles were lower at 90% of incubation when compared to values at 70% incubation (Table I). Calculated temperature coefficients (Q_{10}) were 2.4 and 2.5 for 70% and 90%, respectively. A linear relationship between f_H and temperature of turtle eggs was strongly

supported by data analysis at both 70% incubation ($R = 0.90$; $R^2 = 0.82$; $P < 0.001$) and at 90% incubation ($R = 0.84$; $R^2 = 0.70$; $P < 0.001$) (Fig. 2A). f_H increased according to the following equations:

$$70\%: \ f_H = 6.4\ T - 123.7$$
$$90\%: f_H = 6.5\ T - 131.3$$

In the green iguana egg mass increased from 22.7±1.8g at 30% incubation to 33.8±0.1g immediately prior to hatching at 100% incubation, an increase of almost 50%. The increase in egg size was statistically different from initial values at 70% 90% and 100% of incubation (Table I). Data on egg temperatures and f_H for each of the embryonic periods tested are provided in Table 1. Temperature stabilized after 60 min at 30%, 50% and 90% of incubation, after 70 min at 70% and after 80 min at 100% incubation (Fig. 1B). As the resultant mean values of f_H with time were statistically similar we have reported the combined data. The combined mean temperature after stabilization for all periods of incubation was 31.9±0.1°C. Temperature affected f_H directly. The overall mean combined f_H increased from 73±1 to 105±2(beat · min^{-1}), representing an average increase of 44% (range of 35-50%). The combined temperature coefficient (Q_{10}) was calculated as 2.8. The linear regression of the combined data for all periods of incubation tested indicates a positive linear relationship between f_H and temperature ($R = 0.80$; $R^2 = 0.65$; $P < 0.001$) (Fig. 2B) that follows the equation:

$$f_H = 7.9\ T - 150.0$$

4. Discussion

The Buddy® monitor is a very effective non-invasive system for documenting embryonic viability, apparently delivering everything that the company (Avitronics, Truro, UK) describes on its web site. They state that it is the first digital egg monitor in the world. Using infrared transmitters and sensors it is capable of amplifying the "cardiovascular signal" of an embryo within the egg by as much as 20,000 times, allowing detection of the heartbeat of the embryo as early as 5 days after incubation has started. The monitor gives a digital read out of f_H onto a small screen. As such it gives the bird breeder "warming knowledge that everything is fine" with their valuable embryos. The company cautions that eggs may cool after removal from the nest and in a trial run they recorded f_H within the Buddy® as reducing from 260 to 190 (beat · min^{-1}) within 5 minutes. What they do not mention is the physical warming effect of the Buddy®

upon the eggs that we illustrate above. This will, of course, not be a problem for the bird breeder who merely wants to check the vitality of the egg by briefly placing it in the monitor. Neither is it a problem with the bulk of the experimental biologists that have checked embryo f_H from time-to-time by briefly placing them in the monitor. However, long-term measurements intended to establish how f_H changes during development in a given species must account for and document the heating effects of the system. In our studies these effects were observed during the first hour of enclosure within the Buddy® system. Turtle eggs warmed from 27ºC to 34ºC, causing an increase in f_H of about 80%. This relatively large warming effect may relate to the size of the eggs and the fact that they were placed directly in the monitor, with no protection against desiccation. Iguana eggs warmed from room temperature of 28°C with a f_H of around 73 (beats min^{-1}) up to a stabilised rate of about 105 (beat · min^{-1}) at a temperature of 32°C, taking between 60 and 80 min. This reduced warming effect may again relate to the relative size of the eggs and to the fact that they were protected against desiccation by being encircled by a wet gauze. Recorded differences in the stabilization times of iguana egg temperatures indicated an apparent trend for smaller eggs to warm faster. The 70% incubation turtle egg weighing around 12 g took half the time of a 100% incubation iguana egg at around 34 g. When we measured some eggs with the lid of the Buddy® held open the temperature of the eggs remained below 30°C, with the heart beating at around 75 (beats min^{-1}), despite being held in an incubator set at 30°C. Clearly, any investigation of long-term changes in f_H using enclosure of eggs within the Buddy® system must account for the warming effect. In most other studies using the Buddy® system (see Introduction) f_H was measured by brief enclosure of the egg in the monitor. For example, Du and Shine (2008) enclosed each egg for 2 min. However, McGlashan et al, (2012) when exploring the phenomenon of synchronous hatching in turtle embryos, subjected batches of eggs to different temperatures for 7 days then combined them at a set temperature or at a complex series of fluctuating temperatures and measuring outcome as hatching times and post-hatching development and growth. Metabolic compensation by embryos was measured as rate of carbon dioxide production and heart rate. To measure heart rate individual eggs were enclosed in a Buddy® egg monitor and a digital camera was used to record heart rate at 5 min intervals over 30 min with f_H taken as the average over this period. If the egg remained in the monitor throughout the 30 min period then it would have been subject to a similar heating effect to that recorded from turtle eggs in the present investigation. For eggs held at 26ºC the warming effect may have raised temperature to 31ºC. This implies that f_H was not measured under a steady-state regime and, assuming a Q_{10} of 2, it will have increased up to 37% during the time of measurement.

5. Conclusion

The Buddy® digital egg monitoring system provides a convenient and highly reliable technique for the non-invasive monitoring of f_H in very young bird embryos. For long-term measurements in reptiles it has to be noted that the infrared sensors cause a heating effect, which can have significant effects upon f_H. This effect may be a particular problem when studying relatively small eggs as they may heat more rapidly. The heating effect should always be measured and taken into account in the design of experiments and analysis of data or avoided by placing each egg in the monitor for brief periods, which is the intended primary use of the instrument.

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Table 1 Measured egg temperature and heart rate (f_H) profiles for snapping turtle (Tur) and green iguanas (Ig) within each percentage incubation period studied. T_{min} is the initial egg temperature recorded; T_{max} is the maximum egg temperature recorded; St is the time to temperature stabilization**,** initial and final f_H (I f_H and F f_H respectively) are the heart rates recorded at the T_{min} and T_{max}, respectively; Q_{10} is the calculated temperature coefficients; regression equations were provided by linear regression analysis with the respective R^2. Data presented as mean ± SEM. Statistical significance (P<0.001) is represented by lower case letters in turtles and capital letters for iguanas.

Group	*Egg mass (g)*	*T_{min} (°C)*	*T_{max} (°C)*	*St (min)*	*If_H (beat · min^{-1})*	*Ff_H (beat · min^{-1})*	*Q_{10}*	*Regression Equation*	*R^2*
Tur 70	12.0±0.8^{a}	27.6 ± 0.2^{a}	34.0 ± 0.1^{a}	40	54±1^{a}	96±3^{a}	2.4	f_H = 6.4T - 123.7	0.82
Tur 90	12.9±0.3^{a}	27.2 ± 0.4^{a}	34.0 ± 0.2^{a}	50	45±3^{b}	85±1^{b}	2.5	f_H = 6.5T – 131.3	0.70
Ig 30	22.7±1.8^{A}	27.4 ±0.4^{A}	32.0 ± 0.2^{A}	60	70±3^{A}	104±7^{A}	2.4	f_H = 10.1T - 217.7	0.73
Ig 50	25.0±0.9^{A}	27.7 ± 0.3^{A}	31.4 ± 0.2^{A}	60	69±4^{A}	104±3^{A}	3.0	f_H = 10.5T - 228.8	0.96
Ig 70	31.6±1.2^{B}	28.1 ± 0.4^{A}	32.0± 0.2^{A}	70	73±2^{A}	109±3^{A}	2.8	f_H = 8.1T - 155.5	0.78
Ig 90	33.2±0.9^{B}	28.2 ± 0.7^{A}	31.9 ± 0.2^{A}	60	79±2^{A}	107±4^{A}	2.3	f_H = 5.1T - 60.3	0.42
Ig100	33.8±0.1^{B}	29.3 ± 0.2^{A}	32.2 ± 0.2^{A}	80	72±4^{A}	100±3^{A}	3.1	f_H = 8.5T - 174.4	0.77

Figure captions

Figure 1 Profile of temperature change over time for A) snapping turtle egg measured at 70% (closed circle) and 90% (open circle) of incubation and B) green iguana eggs measured at 30% (closed square), 50% (open square), 70% (closed circle) 90% (open circle) and 100% (closed diamond) of incubation. The dashed line represents mean chamber temperature measured in all periods of incubation. Time zero represents the point of the first reliable measurement of heart rate. Snapping turtle egg temperature changed significantly after 40 min at 70% incubation (indicated by a single asterisk) and after 50 min at 90% (indicated by a double asterisk). Egg temperature from green iguanas changed significantly after 60 minutes (indicated by a single asterisk) at 30%, 50% and 90% of incubation. At 70% the temperature changed significantly after 70 minutes (indicated by a double asterisk) and at 100% after 80 minutes (indicated by a triple asterisk). Data are presented as mean ± SEM.

Figure 2 Pooled heart rate (f_H) responses for all embyros to increasing egg temperature (Temp) for A) snapping turtle eggs measured at 70% (closed circle) and 90% (open circle) of incubation and B) green iguana eggs measured at 30% (closed square), 50% (open square), 70% (closed circle) 90% (open circle) and 100% (closed diamond) of incubation. Data points represent f_H for all animals included in the regression for each age group. Linear regression lines for the 70% (dashed line) and the 90% (solid line) are presented for the turtles only. For clarity purposes statistical analysis results are presented in Table I.

Figure 1

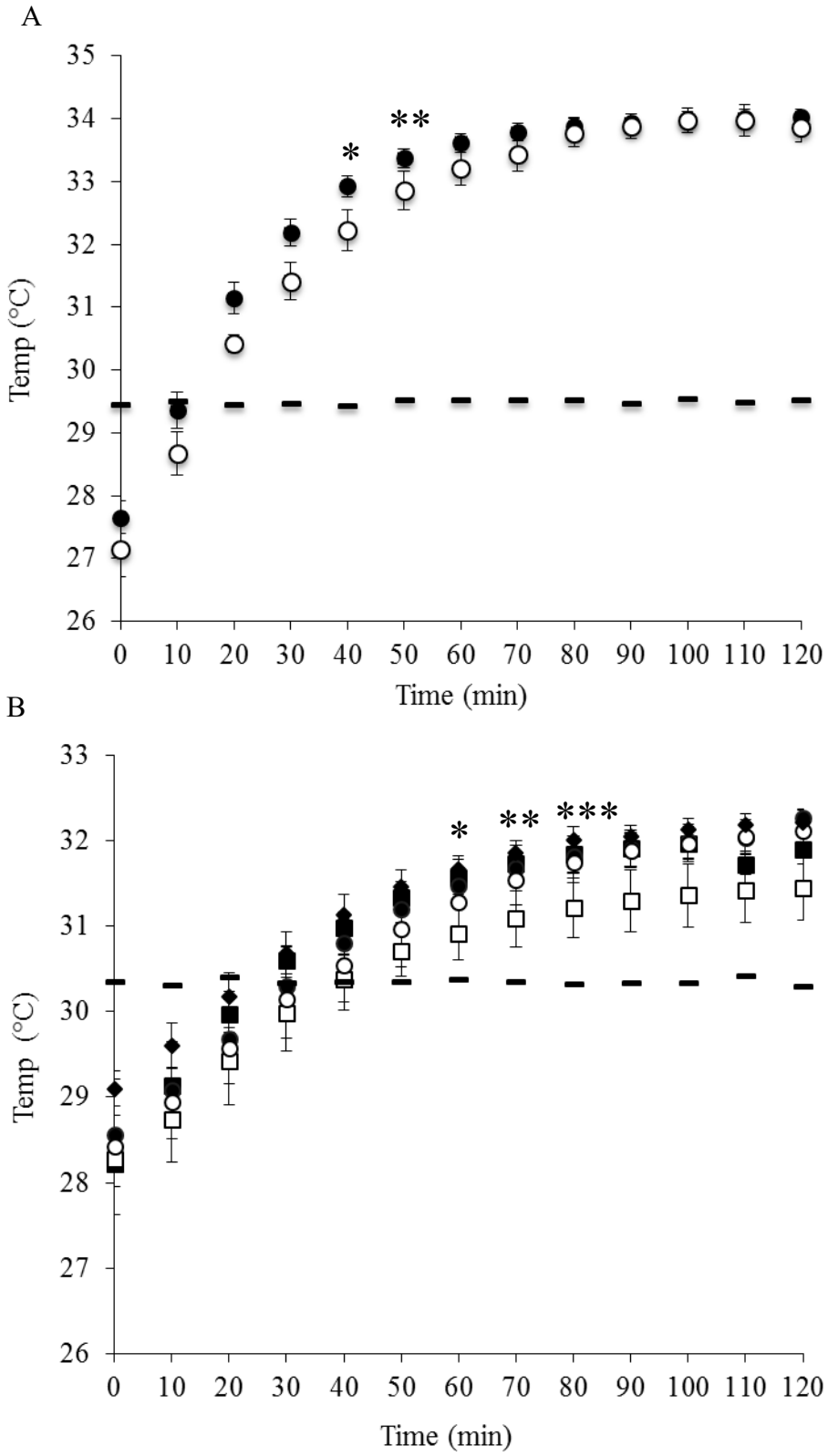
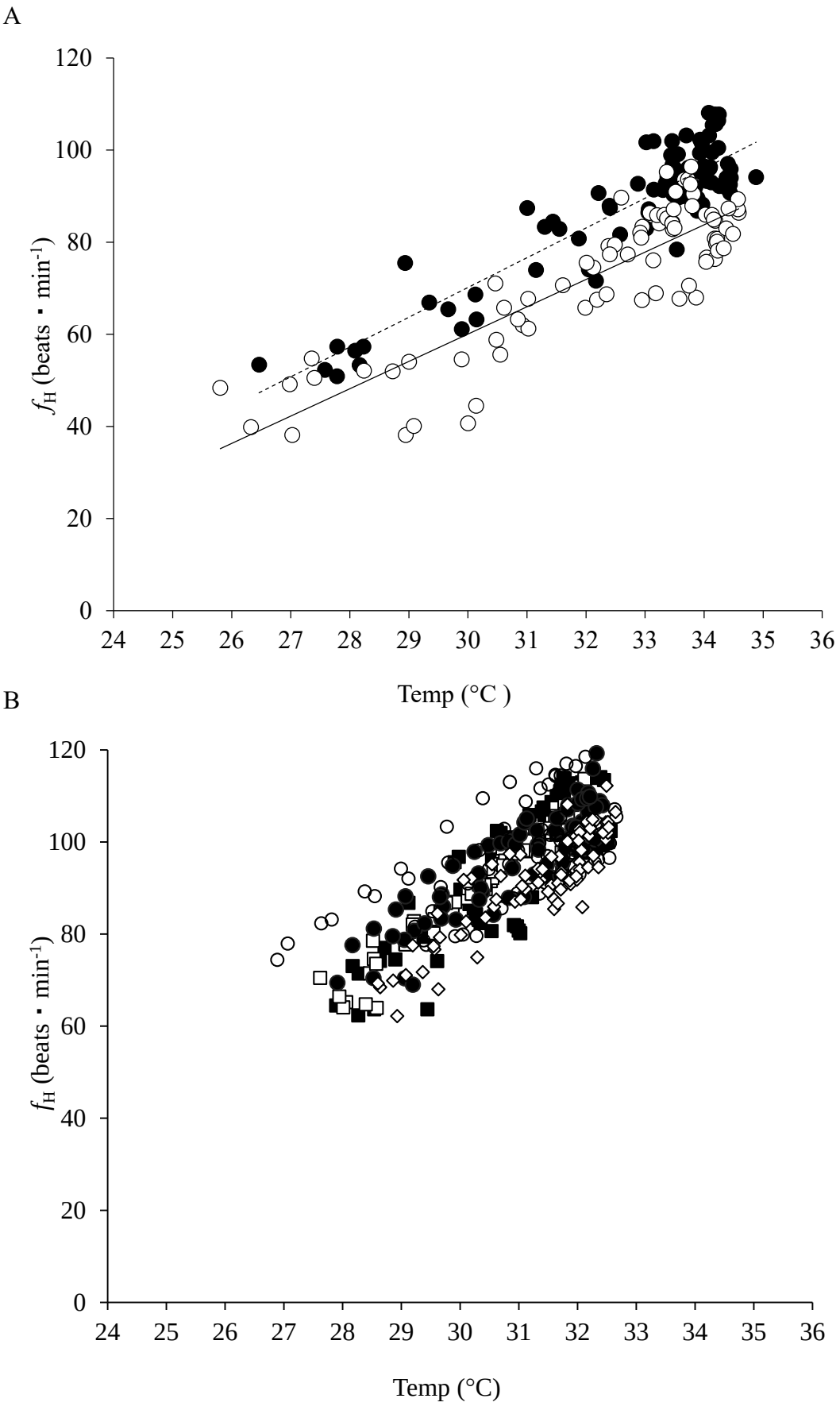


Figure 2


A
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Temp (°C)

Capítulo II

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Rates of O_2 consumption, heart rate and consequent O_2 pulse in embryos and hatchlings of the green iguana *Iguana iguana*

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Running title: O_2 pulse during ontogeny of the green iguana *Iguana iguana*

ms. has 23 pages, 5 figures, 2 tables

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List of abbreviations

A-V difference – arteriovenous oxygen content difference

CAM – chorioallantoic membrane

CaO_2 – oxygen content in oxygenated blood

CvO_2 - oxygen content in deoxygenated blood

f_H – heart rate

M_b – body mass

M_h – heart mass

O_2 pulse – Oxygen pulse, mean oxygen uptake per heart beat of routinely active animals

Q – cardiac output

VO_2 – rate of oxygen consumption (expressed for individual embryos and hatchlings)

Vs – calculated cardiac stroke volume

Abstract

Oxygen consumption (VO_2), heart rate (f_H), heart mass (M_h) and body mass (M_b) were measured during embryonic incubation and in hatchlings of green iguana (*Iguana iguana*). VO_2 increased in a sigmoidal pattern during embryonic development, doubling by the end of incubation, while f_H was constant, resulting in a 2.7-fold increase in oxygen pulse. The mean routine level of VO_2 in hatchlings, when circadian increases in VO_2 were ignored, was 1.7-fold higher, while f_H was reduced by half, compared to late stage embryos, resulting in a further 3.6 fold increase in oxygen pulse. No correlation between f_H and VO_2 was evident in embryos, indicating that predicting metabolic rate as VO_2 from measurements of heart rate is not possible in embryonic reptiles. Both body and heart mass increased in a non-linear fashion, however, both showed strong correlations with both f_H and VO_2. Our data suggest that convective transport supplying metabolism during late embryonic incubation is modulated by factors other than f_H, likely including changes in cardiac stroke volume, plus oxygen capacity and extraction (A-V diff), while in hatchlings the transition from chorioallantoic membrane (CAM) to pulmonary gas exchange may contribute to increased oxygen exchange capacity.

Key words: Iguana, reptile, embryo, growth, oxygen uptake, heart rate, oxygen pulse

1. Introduction

Early in reptilian embryonic development, oxygen is delivered to the embryonic tissues via diffusion (Andrews, 2004) but as the embryo grows and differentiates diffusion becomes insufficient to supply oxygen across the increased distances to the tissues. The processes of differentiation and growth require energetic cellular processes, increasing the oxygen demand (Vleck et al., 1980; Vleck and Hoyt, 1980) which is met by parallel development of and dependence on extra-embryonic tissue in the form of the chorioallantoic membrane (CAM) for gas exchange. While embryonic metabolism increases progressively during development, increases in oxygen consumption (VO_2) of reptilian embryos has been reported to be exponential, similar to altricial birds, sigmoidal, or to increase to a plateau or peak, that may relate to synchronous hatching (Dmi'el, 1970, Ackerman, 1981; Thompson, 1989; Whitehead and Seymour, 1990; Vleck and Hoyt, 1991; Aulie and Kanui, 1995; Booth, 1998). While these patterns have been documented, the cardiovascular adjustments that enable the adequate supply of oxygen to the embryonic tissues are not fully understood.

In adult animals heart rate (f_H) has often been used as an index of metabolic rate. Several studies have proposed calibration equations for the relationship between f_H and VO_2 at rest and in different states, in order to predict metabolic rate, often from free ranging animals (see Green, 2011). In many endothermic species (mammals and birds) f_H can be used to estimate VO_2 relatively reliably (Bevan et al., 1992; McPhee et al., 2003; Groscolas et al., 2010; Young et al., 2011; Currie et al., 2014). Nevertheless, in ectotherms that experience large fluctuations in body temperature, the influence of f_H on VO_2 can be confounded (Clark et al., 2006). For example, in adult reptiles f_H is a good predictor of VO_2 at different temperatures but not during rapid heating (Butler et al., 2002; Clark et al., 2006). However, the applicability of f_H as a predictor of VO_2 in embryonic reptiles remains in question.

In egg-laying embryonic animals f_H is a cardiovascular parameter that can be easily accessed during incubation (Taylor et al 2014; Sartori et al., 2015a). Measuring f_H requires minimally invasive techniques such as the cannulation of blood vessels perfusing the extra-embryonic, chorioallantoic membranes (CAM) (Crossley et al, 2003). Alternatively, it is possible to use a completely non-invasive method, with remote monitoring devices (e.g. Buddy System, Avitronics, Truro, UK) while taking appropriate precautions (Sartori et al., 2015b).

The goal of our study was to determine the relationship between $\dot{V}O_2$ and f_H during embryonic development and in post-hatching oviparous lizard *Iguana iguana.* In order to

understand how the changes in oxygen demand are met by the cardiovascular system we calculated the average oxygen uptake per heart beat, termed the 'oxygen pulse' and performed regression analysis between VO_2, f_H, body mass and mass of the heart of embryos at different points during ontogeny and subsequent growth.

2. Material and Methods

2.1 Egg collection and incubation

Iguana eggs were supplied from the breeding programme conducted at the Jacarezário, University of São Paulo State, Rio Claro, UNESP (IBAMA 673766). All experimental procedures were approved by the Ethics Committee on Animal Care at the São Paulo State University (CEUA-UNESP no. 6597). Fertile eggs from different clutches were collected soon after being laid and placed in plastic trays (38 x 28.5 x 6.5 cm) containing water-saturated vermiculite. The trays were held in constant temperature incubators at 30 ± 0.5ºC throughout total incubation time of approximately 74 days and water content of the vermiculite and viability of eggs were checked in a daily basis. After hatching, animals were maintained in plastic boxes (72 x 55 x 40 cm) provided with a light bulb for heating and a 12:12h light-dark phase. Animals were fed with dark green leafy vegetables three times per week and had free access to water and shelter. A total of 51 eggs and 21 hatchlings were used in the study.

2.2 Oxygen consumption ($\dot{V}O_2$)

For measurement of embryonic oxygen consumption (VO_2) a total of 19 eggs were selected from two different clutches. Every 72 hours, up to 6 eggs were put into cylindrical respirometry chambers of approximately 200 ml volume, attached to an intermittent closed system respirometer (Sable Systems, Las Vegas, USA).

The respirometry system was controlled via a computer interface (Datacan V, Sable Systems). A multiple flow controller (Multiplexer TR RM8, Sable Systems) operated an automatic switch between each of the six channels. Room air was pumped (SS-4 Sub-Sampler Pump, Sable Systems) through the system with airtight tubes. The multiplexer intercalated 50 minutes of an open phase, in which a continuous flow of air adjusted to 200 ml min^{-1} circulated through the chamber, with 10 minutes of a closed phase, in which the air of the selected chamber was dried with silica gel and re-circulated through the chamber and an

oxygen analyser (PA-10 O_2 Analyser, Sable Systems). Cycles of the open-closed phases were repeated from 4 to 7 times. The sampling frequency was 1 sec^{-1}.

In order to maintain a water saturated environment inside the chambers a piece of wet cotton wool containing approximately 1 ml of water was put at the bottom of the chamber. The chambers were maintained inside a climatic BOD (Eletrolab, EL101/3, SP, Brazil) at 30°C (± 0.5° C) during the measurements. For the measurement of hatchling oxygen consumption (N = 11, approx. 90 days old) we used the same protocol but used respirometry chambers made from PVC tubes with a volume of 110 ml. The hatchlings were fasted for four days and measured for a period up to 2 – 3 days, with occasional interruptions in the recordings for provision of water.

The values of oxygen percentages obtained were used to calculate the fractional decrease in oxygen of the chamber during the closed phase, and corrected with the residual air volume and time, to provide VO_2 values in µl O_2 $animal^{-1}$ h^{-1} (Klein et al, 2006). Data are shown as mean values ± SEM. The pattern of VO_2 in embryos was related to eventual hatching (eclosion) times, and for calculations of O_2 pulse and correlation analysis VO_2 was grouped in 10% increments of incubation time between 10% and 90%. For hatchlings, the 6 first hours of measurement were excluded and resting metabolic rates were calculated from stable values, excluding peaks related to sporadic bouts of activity.

Total volume of oxygen (VO_{2TOT}) consumed throughout the incubation period was calculated by the integral of the area under the VO_2 curve using SigmaPlot software. To calculate the energy required for total embryonic incubation we assumed that the main substrates used as fuel were lipids in which every ml of O_2 consumed corresponded to 19.64 J of energy (Thompson and Russel, 1999).

2.3 Heart rate (f_H)

Eggs were selected from eight clutches at the estimated percentage incubation times of 10%, 30%, 50%, 70% and 90%, based on oviposition date and the recorded mean of 74 ± 1 days of incubation at 30°C. Each egg was weighed and candled to select a region for the insertion of a thermocouple, in order to record egg temperature during the f_H measurements. A small piece of latex membrane was attached by cyanoacrylate glue to the eggshell over the selected region and the shell was then punctured beneath the latex patch using a 26G needle and an implantable tissue thermocouple (T-type thermocouple probe, AD Instruments) was inserted by 5 mm into the amniotic fluid. Despite the expertise of an author (e.g. Crossley et al, 2003) it proved challenging to cannulate CAM vessels in iguana embryos so that f_H could

not be monitored as blood pressure and blood samples could not be withdrawn for analysis. Accordingly, f_H was recorded using a digital egg monitor (Buddy System, Avitronics, UK), a non-invasive heart rate recorder that detects and amplifies, via infrared sensors, the signal of a heart beat within the egg, as described for birds and reptiles (Lierz et al., 2006; Du et al., 2009; Sartori et al 2015a). Each egg was enclosed in moistened gauze and placed in an egg monitor that was in turn placed within a portable incubator (Caltech EIP-010, PE, Brazil) set at 30 °C (± 0.5 °C). The signal outputs from the egg monitor and from the thermocouple (via a T-type Pod, AD Instruments, Sydney, Australia) were connected to a signal acquisition system (PowerLab, AD Instruments). f_H was determined when the egg had warmed to 30°C, which occurred at an average time of 32±6 minutes (Sartori et al 2015b).

For measurement of post embryonic f_H 10 three-month old hatchlings were instrumented with two lengths of Teflon insulated silver wire (A-M Systems, WA, USA), inserted subcutaneously either side of the mid-thoracic wall and secured to the skin with cyanocrilate glue. The hatchlings were enclosed in 50 ml Falcon tubes, with holes at both ends that allowed the anterior portion of the head and the tail to sit outside of the tube. These wire leads were externalized through a slot cut through the wall of the Falcon tube and connected to an ECG amplifier (Animal Bio Amp, ADInstruments). Signals of the ECG were recorded using LabChart data aquisition software (LabChart 7, ADInstruments) overnight, in order to obtain a resting value of f_H.

2.4 Masses and allometric relations

After completion of the f_H measurements embryos and hatchlings were killed by isoflurane vaporization and weighed. The heart was dissected out under a stereomicroscope (Zeiss Stemi 2000 C, Germany) and weighed on an electronic balance to the nearest 0.0001 g. Relations between heart and body mass, f_H, VO_2 and incubation time were determined using least-square or non-linear regressions.

2.5 Oxygen Pulse

Oxygen pulse is defined as the average volume of oxygen consumed by the animal (expressed as µl per animal) during each heart beat. This was calculated for each developmental stage (10-90% incubation and hatchlings) using the mean oxygen consumption values obtained for embryos in the whole egg, and for hatchlings as whole animals (µl O_2 min^{-1}) and dividing this value by the mean heart rate (beats min^{-1}) for each stage.

2.6 Statistical Analysis

We conducted a two-way ANOVA analysis to detect differences in embryonic VO_2 between the two clutches used. As they were statistically similar, data was combined and analysed with one-way ANOVA. A Student Newman Keuls *post hoc* test was used to detect differences between incubation periods.

Egg mass, M_b, M_h, f_H, and VO_2 for the selected percentages of the incubation period were analysed by One-way Anova with the *post hoc* test of Student Newman Keuls. When data failed assumptions of normality or homoscedasticity we used Anova on Ranks and the *post hoc* test of Dunn´s.

Least-squares linear regression analysis was used to calculate allometric equations to describe ontogenetic scaling relationships between masses, incubation time, VO_2 and f_H. When data failed the premisses of the test, we logarithmic transformed the data or used non-linear regressions, until normality or homoscedasticity were not violated and the best fit was found. All tests were performed with SigmaPlot v11 software. Significance level was attributed at 95% probability (P of 0.05).

3. Results

3.1 Growth and allometry

Table 1 shows mean data for egg mass, body mass (M_b); heart mass (M_h); f_H; VO_2 both for whole eggs and hatchlings (µl O_2 animal^{-1} h^{-1}), plus mass-specific values (µl O_2 g^{-1} h^{-1}) and O_2 pulse for all ontogenetic periods studied. The mass of embryos (M_b) increased markedly early in development with a 22 times increment between 10% and 70% incubation. Over the same period mass of the heart increased 6 times. However, in both cases these changes only became significant at 90% of incubation time, when both M_b and M_h had increased a further 2-3 times from the 70% level. 3-month old hatchling M_b and M_h both doubled from the 90% level. A linear plot of the relationship between individual values of M_h and the M_b of yolk-free embryos and of hatchlings revealed that the overall increase in M_h showed an apparent proportional relationship with M_b (Fig 1A). However, a double-log plot of these data (Fig 1B) to illustrate the separation of data obtained from early stage embryos, revealed a non-linear relationship with a relatively high degree of correlation (Fig. 1B; Table 2).

3.2 Oxygen uptake (VO_2)

Oxygen consumption of embryonic iguanas was characterized by a sigmoidal pattern that fitted a polynomial regression (Fig. 2; Table 2). VO_2 was approximately 312 ± 15 µl O_2 egg^{-1} h^{-1} throughout early and mid-incubation time. At the embryonic period close to hatching VO_2 increased, doubling to a mean value of 693 ± 49 µl O_2 egg^{-1} h^{-1} (Table 1), which was significantly above the value recorded at 10% incubation. However, mass-specific $\dot{V}O_2$ progressively decreased throughout the incubation period so that at 90% each gram of tissue consumed 26 times less oxygen than 10% embryos (Table 1). Based on the VO_2 data plot illustrated by Fig. 2, the total mean volume of oxygen (VO_{2TOT}) consumed throughout incubation, from the time close to oviposition to approximately 24h prior to hatching, was 654 ml, corresponding to a mean expenditure of 12.84 kJ of energy per egg. Dividing this value by the mean yolk-free body mass of close to hatching embryos it corresponds to 0.92 kJ per gram of embryo, which represents the total cost of producing a newly hatched animal.

In hatchlings, VO_2 was highly variable with peaks in a daily cycle that occurred between 09:00h and 22:00 h (Fig. 3), probably related to changes in activity levels with a circadian rhythm (Tosini and Menaker, 1995; Klein et al, 2006). During these peaks of activity VO_2 reached values from 2000-7500 µl O_2 $animal^{-1}$ h^{-1}. Excluding these peaks hatchling routine VO_2 was close to 1200 µl O_2 $animal^{-1}$ h^{-1} at 30ºC (Table 1; Fig. 3), which was significantly higher than in late stage embryos, being 4 times higher than the rate in 70% embryos and 2 times higher than in 90% embryos. Mass-specific VO_2 of hatchlings was reduced by 12% from that recorded from 90% embryos (Table 1).

3.3 Heart rate (f_H)

Mean f_H was unchanged in embryo iguanas, varying around 90 beats min^{-1} throughout development. The mean f_H of hatchlings of 42 ± 1 beats min^{-1} was calculated based on stable values recorded overnight. During the day it was possible to detect bouts of movement and a circadian effect in the f_H recordings of hatchlings that related to peaks in $\dot{V}O_2$. In these periods f_H reached values close to 80 beats min^{-1} that were discounted in the calculation of mean settled rates. Despite the stable rates recorded from hatchlings being half the rate in embryos of all stages, the mean values differed significantly only from the initial embryonic f_H at 10% incubation (Table 1; Fig. 4B).

3.4 Oxygen pulse

For calculation of O_2 pulse we divided the mean stable VO_2 (fig. 4A, transformed to µl O_2 animal^{-1} min^{-1}) by the mean stable f_H (fig. 4B), from the embryos at a range of % incubation times and from hatchlings. As mean heart rate was relatively constant during embryonic development the calculated values for O_2 pulse (Fig. 4C) resembled the pattern of variation in VO_2 plot, with O_2 pulse increasing 2.6-fold from 70% to 90% incubation. Hatchlings showed much higher values of O_2 pulse, which increased 10-fold from the value at 10% incubation. This increase was related to their reduced f_H and increased (4 times) $\dot{V}O_2$ (Table 1).

Throughout development, mean VO_2 and f_H were both closely correlated with mean M_b and, because of their proportional relationship (Fig. 1), with mean M_h. Table 2 shows the regression equations for the relationships between VO_2, f_H and mass. The relationship between mean VO_2 and mean f_H in the different periods of incubation and after hatching showed a barely significant, non-linear, negative correlation (Table 2; Fig 5) that underlies the values for oxygen pulse.

4. Discussion

The total energetic costs of producing a hatchling is influenced by the mass of the egg, so comparisons among amniotes are complex due to a large variation in egg sizes. Nevertheless, costs of producing a gram of iguana hatchling was within the range reported for other squamate embryos (D´miel, 1970). The final 30% of the embryonic VO_2 curve seems to be an important and critical part of incubation, as metabolism parallels the M_b exponential curve, indicating a rapid phase of growth at this period. According to the model proposed for avian embryonic metabolism (Hoyt, 1987; Vleck and Vleck, 1987) there is a link between the exponential increase in M_b and the exponential increase in metabolism due to a continuous increase in the costs of growth (incorporating new tissue) and costs of maintenance (proportional to the actual body mass). The result is that the final growth phase represents the most energetically expensive portion of the developmental process (Birchard and Reiber, 1995).

The pattern of VO_2 and the constancy of f_H during embryonic development in iguanas is similar to that found in some other species of lizards (Vleck and Hoyt, 1991; Birchard et al., 1995; Thompson and Stewart, 1997; Thompson and Russel, 1998, 1999; Booth et al., 2000; Taylor et al., 2014). The VO_2 pattern of embryonic iguanas fitted a polynomial curve and presents a sigmoidal format. The factors that contribute to both the VO_2 and f_H pattern in

reptiles, such a diverse and polyphyletic group, are not completely elucidated and may be phylogenetic, developmental or environmental (Thompson, 1989).

f_H seems not to increase in parallel to the increase in metabolic rate in embryonic reptiles (Nechaeva et al, 2007; Crossley and Burggren, 2009), and in some species, for example the turtle *Chelydra serpentina*, f_H can even decrease at the end of incubation (Alvine et al, 2013). The reduction in f_H is possibly due to developmental changes in the intrinsic cardiac rhythm (Nechaeva et al, 2007), or the onset of sympathetic and parasympathetic control of the cardiac tone (Burggren and Pinder, 1991; Crossley et al., 2003). The role of autonomic control of f_H in iguana embryos is important. An excitatory tone, probably due to high levels of circulating catecholamines, influences f_H throughout the entire incubation period, whereas inhibitory parasympathetic tone only becomes apparent immediately before hatching (Sartori et al., 2015a). After double autonomic blockade, which eliminates the excitatory tone, embryonic f_H was reduced to levels that ranged between 35-63 beats min^{-1}, close to values obtained for hatchlings that show an increased level of parasympathetic tone on the heart (Sartori et al., 2015a).

The inverse, non-linear relationship between VO_2 and f_H during embryonic development indicates that f_H is not a useful proxy of metabolism in this ontogenetic phase. The 2-fold increase in VO_2 at 90% incubation is accompanied by an unchanging heart rate, resulting in a 3-fold increase in O_2 pulse. Clearly, $\dot{V}O_2$ can vary independently of f_H. The relationship between the cardiovascular system and oxygen consumption (VO_2) is determined by the Fick Principle, in which the amount of oxygen consumed by an organism can be calculated from the equation: $VO_2 = (CaO_2 – CvO_2) \cdot f_H \cdot Vs$, where $CaO_2 – CvO_2$ is the difference in oxygen concentration between arterial and mixed venous blood (also known as tissue oxygen extraction and also termed A-V diff); f_H is the heart rate and Vs is the stroke volume, the volume of blood ejected from the heart during a single beat.

Based on the Fick Principle, O_2 pulse will be determined by the product of blood flow (the product of f_H and Vs) and the difference in oxygen content from oxygenated blood entering and deoxygenated blood that leaves the metabolizing tissue (A-V diff). Therefore changes in ventricular volume and blood oxygen carrying capacity, could together account for the higher rates of VO_2 in relation to f_H during the later periods of embryonic development (Tazawa and Whittow, 1994, Burggren and Pinder, 1991; Birchard and Reiber, 1996, Crossley and Altimiras, 2000). So far, embryonic Vs has not been measured directly but heart mass is often used as a correlated index for its prediction (Burggren and Pinder, 1991; Birchard and Reiber, 1996; Nechaeva et al., 2007). In the iguana embryonic and hatchlings´

heart mass represents 0.2 to 0.3% of the body mass, approximately half of the 0.6% proportion typically found in adult mammals (Schmidt-Nielsen, 1984). If the proportional increase in M_h reflects increases in Vs then this will be a factor contributing to the increased VO_2 seen during development. However, M_h increased 16 times and M_b 55 times during embryonic development and the logarithmic regression indicates a non-linear relationship. The apparent discrepancy in growth of heart mass relative to body mass may relate to the reduction in mass-specific rates of oxygen consumption as the embryo and hatchling grows, reducing the requirement for oxygen delivery per unit mass.

In iguana hatchlings O_2 pulse increased 4-fold as a result of a significantly 2-fold lower f_H and a $\dot{V}O_2$ that is 2-fold higher, in comparison with 90% embryos. Converting O_2 pulse for whole hatchling in µl to values of ml O_2 g^{-1} $beat^{-1}$ resulted in a value of 3.3×10^{-5}, which falls in the range of standard values obtained for other lizard species between 3×10^{-5} and 4×10^{-5} ml O_2 g^{-1} $beat^{-1}$ (Bennet, 1972). Hatching represents the transition from respiratory gas exchange over the vascularized extra-embryonic CAM to exchange over the lungs, which can increase the gas exchange capacity (Burgreen and Warburton, 1994, Aulie and Kanui, 1995).

In summary, based on the relationship we found between the f_H and VO_2 of green iguanas we can conclude that during the incubation period of reptiles f_H is not an appropriate predictor of VO_2 and should not be used as an index without further measurements to assess the relationship of these variables during the development of the species. One study (Aubret, 2013) used f_H data as an indication of resting metabolic rates in embryos of the snakes *Natrix maura* and *Natrix natrix*, which could have led to misinterpretations.

Mechanisms that match oxygen supply and demand during embryonic development in reptiles need to be explored. In addition to f_H and VO_2 measurements, studies should include: A-V difference, CAM blood flow, embryonic blood shunting and oxygen affinity of the embryonic hemoglobin. This broader approach was not possible in the present study due to the technical difficulty of cannulating blood vessels and future work will concentrate on species enabling this approach.

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Table 1 Egg mass, yolk-free body mass (M_b) of embryos and mass of hatchlings, heart mass (M_h), heart rate (f_H), rate of oxygen consumption ($\dot{V}O_2$) of embryos from 10 to 90% incubation and hatchling iguanas. Values are mean ± SEM, N is indicated in parenthesis. Mass-specific $\dot{V}O_2$ was calculated from mean $\dot{V}O_2$ and M_b, oxygen pulse (O_2 pulse) from mean data for f_H and $\dot{V}O_2$.

Age	*Egg mass (g)*	*M_b (g)*	*M_h (mg)*	*f_H (beats min^{-1})*	*$\dot{V}O_2$ (µl O_2 animal^{-1} h^{-1})*	*Mass-specific $\dot{V}O_2$ (µl O_2 g^{-1} h^{-1})*	*O_2 pulse (µl O_2 beat^{-1})*
10%	16 ± 0.6 (6) [a]	0.13 ± 0.01 (6) [a]	1.2 + 0.1 (5) [a]	111 ± 4 (6) [a]	321 ± 23 (13) [a]	2469	4.8 x 10^{-2}
30%	23 ± 1.6 (6) [b]	0.60 ± 0.05 (6) [ab]	2.1 ± 0.1 (6) [ab]	89 ± 2 (8) [ab]	330 ± 45 (8) [a]	550	6.1 x 10^{-2}
50%	25 ± 0.8 (6) [b]	1.31 ± 0.04 (6) [ab]	3.2 ± 0.6 (5) [ab]	88 ± 1 (6) [ab]	324 ± 39 (13) [a]	247	6.1 x 10^{-2}
70%	32 ± 1.1 (6) [c]	2.99 ± 0.13 (6) [ab]	6.8 ± 0.3 (6) [ab]	89 ± 2 (6) [ab]	272 ± 24 (14) [a]	91	5.0 x 10^{-2}
90%	33 ± 0.8 (6) [c]	7.20 ± 0.41 (6) [b]	19 ± 1 (6) [b]	90 ± 5 (6) [ab]	693 ± 49 (14) [b]	96	12.8 x 10^{-2}
Hatchl	-	13.9 ± 0.36 (8) [bc]	38 ± 2 (8) [bc]	42 ± 1(8) [b]	1166 ± 46 (11) [c]	84	46.3 x 10^{-2}

Table 2 Regression analysis of the relationships between body mass (M_b), heart mass (M_h), heart rate (f_H), oxygen consumption ($\dot{V}O_2$) during the different ontogenetic periods. When variables were not measured in the same animal mean values were used. Transformation of data were performed when necessary.

	R^2	*P*	***Regression equation***
log $\dot{V}O_2$ embryo vs log Inc time (t, in days) *	0.91	<0.001	$\log \dot{V}O_2 = 0.8 + 4.7 (\log t) – 4.3 (\log t)^2 + 1.3 (\log t)^3$
log $\dot{V}O_2$ vs log f_H **	0.67	0.05	$\log f_H = - 0.5 \log \dot{V}O_2 + 3.2$
log Mb vs log Mh **	0.93	<0.001	$\log M_h = - 0.8 \log M_b + 2.4$
$\dot{V}O_2$ vs M_b	0.94	0.001	$\dot{V}O_2 = - 64 M_b + 238$
log $\dot{V}O_2$ vs log M_h **	0.71	0.03	$\log \dot{V}O_2 = 0.36 \log M_h + 3.45$
f_H vs M_b	0.76	0.02	$f_H = - 3.9 M_b + 101.6$
f_H vs M_h	0.82	0.01	$f_H = - 1243.5 M_h + 100.6$

* indicates polynomial regression

** indicates a non-linear relationship

Figure captions

Fig. 1 A) Regression plot from body mass (M_b) vs. heart mass (M_h); **B)** A double logarithmic plot is included as an insert to illustrate the non-linear nature of the progressive change in heart mass with body mass during development. 10% is represented by filled triangles; 30% open circles, 50% closed circles, 70% open squares, 90% closed squares and hatchlings by asterisks.

Fig. 2 Profile of oxygen consumption (VO_2, mean rates ± SEM) recorded from iguana embryos throughout the incubation period at 30°C. Embryos of two different clutches were combined by plotting data against eclosion dates. Data are provided in µl O_2 egg^{-1} $h^{-1.}$

Fig. 3 Profile of oxygen consumption (VO_2) recorded from individual hatchling iguanas showing the marked circadian rhythm of metabolism. Data are provided as µl O_2 $hatchling^{-1}$ h^{-1}.

Fig. 4 Mean rates (± SEM) of **A)** heart rate (f_H, beats min^{-1}); **B)** oxygen consumption (VO_2, µl O_2 egg^{-1} $h^{-1.}$ and µl O_2 $hatchling^{-1}$ h^{-1}); and **C)** calculated oxygen pulse (O_2 pulse, µl O_2 $beat^{-1}$) of iguana eggs at 10, 30, 50, 70, 90% of incubation (closed bars) and after hatching (H; open bars). Dashed line represents eclosion time.

Fig. 5 Regression plot from embryos at 10-90% incubation and hatchling iguanas of mean VO_2 (µl $animal^{-1}$ h^{-1}) against mean f_H (beats min^{-1}). This reveals a negative, non-linear relationship. 10% is represented by filled triangles; 30% open circles, 50% closed circles, 70% open squares, 90% closed squares and hatchlings by asterisks.

Figures

Fig 1.

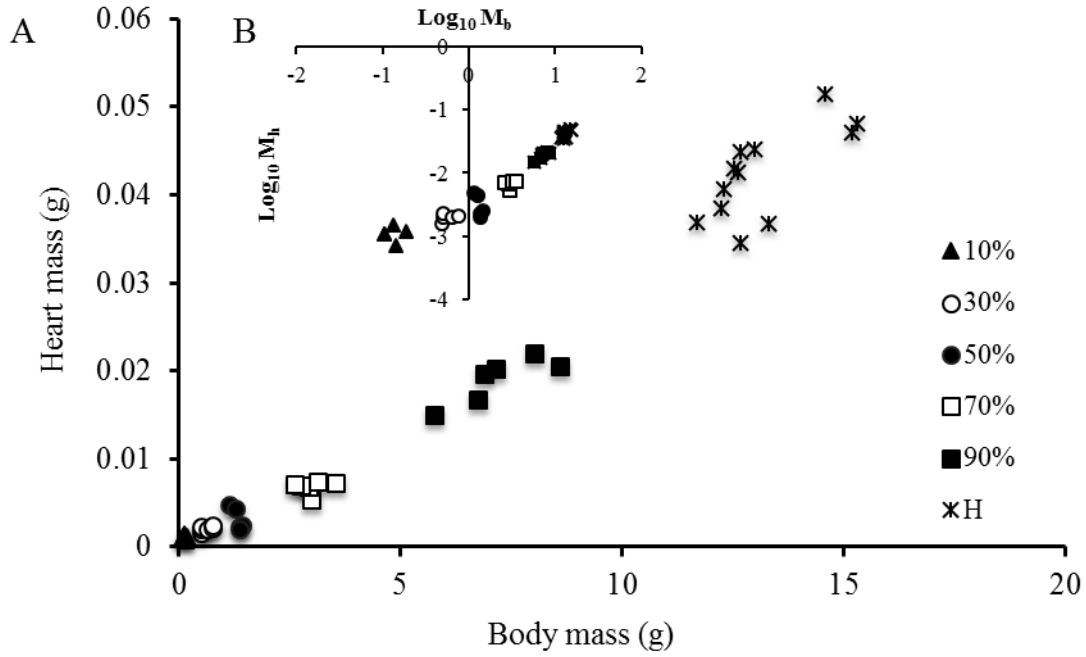


Fig. 2

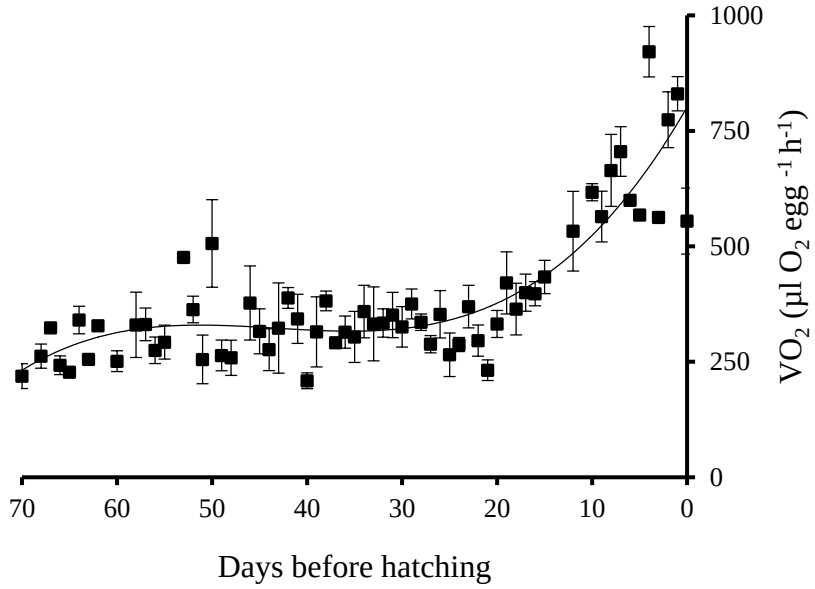

1000
750
500
250
0
70
60
50
40
30
20
10
0
VO2 (µl O2 egg -1 h-1)
Days before hatching

Fig. 3

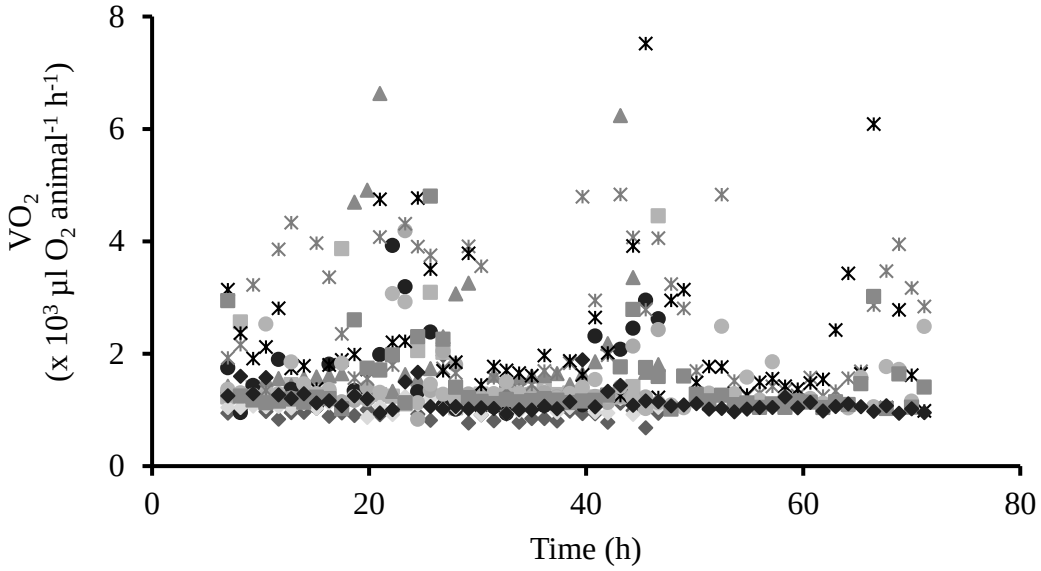

8
6
4
2
0
0
20
40
60
80
VO2
(x 10³ µl O2 animal-1 h-1)
Time (h)

Fig. 4

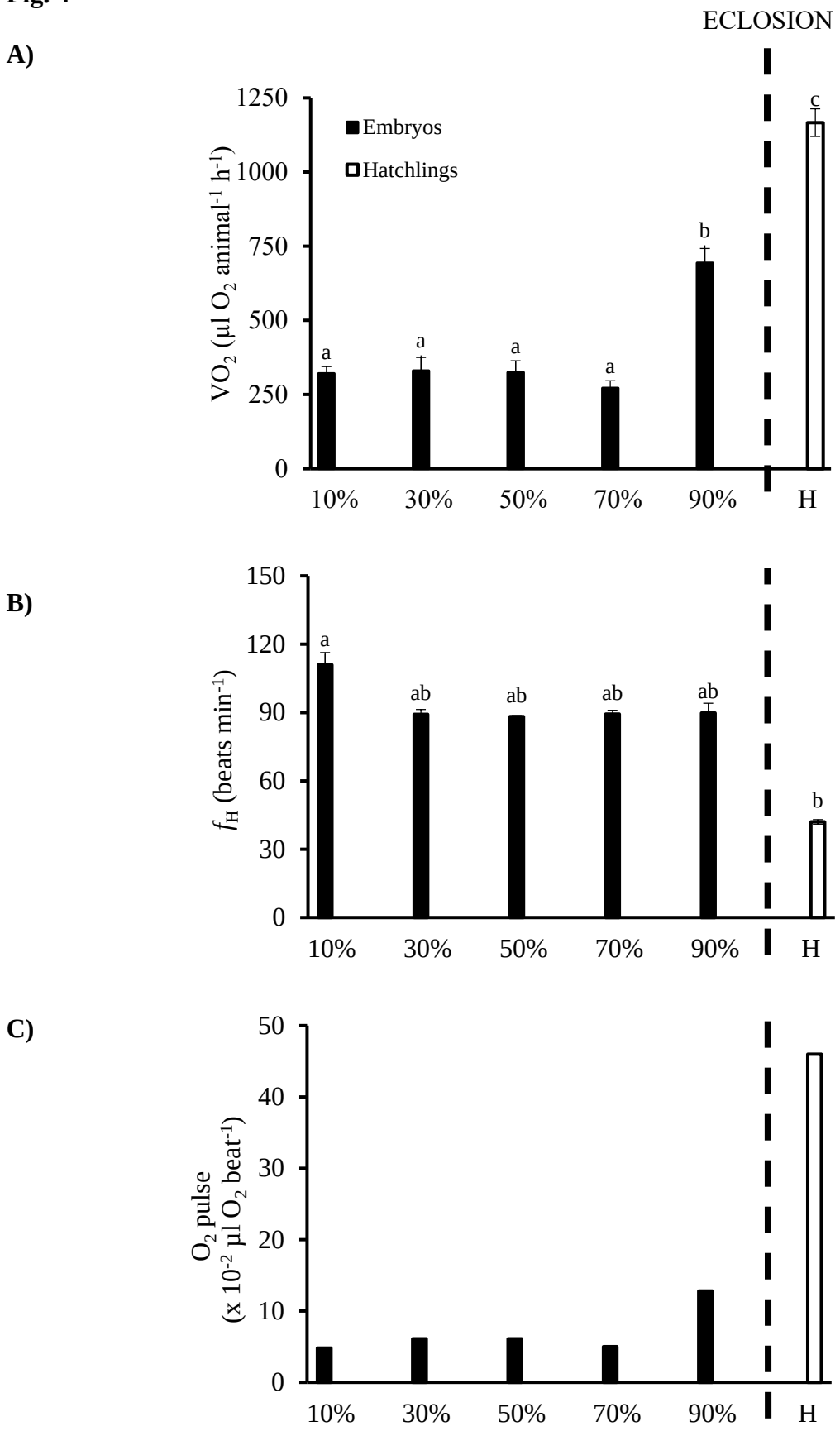
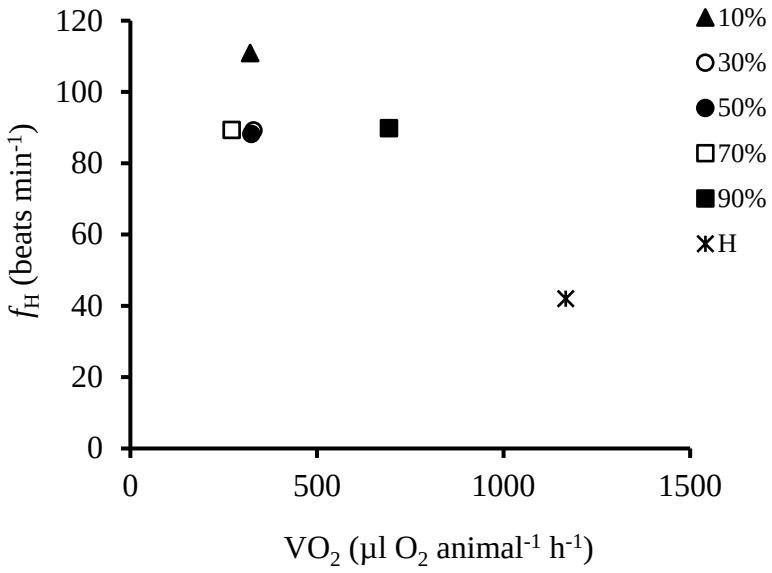

A)
ECLOSION
Embryos
Hatchlings
VO_2 (µl O_2 animal^{-1} h^{-1})
1250
1000
750
500
250
0
a
a
a
a
b
c
10%
30%
50%
70%
90%
H
B)
f_H (beats min^{-1})
150
120
90
60
30
0
a
ab
ab
ab
ab
b
10%
30%
50%
70%
90%
H
C)
O_2 pulse
(x 10^{-2} µl O_2 beat^{-1})
50
40
30
20
10
0
10%
30%
50%
70%
90%
H

Fig. 5


120
100
80
60
40
20
0
0
500
1000
1500
f_H (beats min^{-1})
VO_2 (µl O_2 animal^{-1} h^{-1})
10%
30%
50%
70%
90%
H

Capítulo III

**Manuscrito em preparação para publicação na revista Journal of Thermal Biology*

Pattern of heart rate and chronotropic effects of temperature in embryos of the lizard *Iguana iguana*

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Abstract

In lizards the pattern of heart rate (f_H) variation in developing embryos seems to be relatively uniform throughout development under constant temperature. However, reptilian embryos develop inside amniotic eggs that are deposited in nests without parental care and therefore are subjected to fluctuations environmental temperature. Temperature change has important effects in many biological processes and in developing embryos it can influence the duration of the incubation period and hatchling phenotype. We investigated the pattern of f_H at a constant temperature and the effect of acute changes of temperature on f_H at different periods in the development of embryos of the lizard *Iguana iguana*. We also measured nesting site temperaures from iguanas in captivity. We found that under constant temperature iguanas show a typical squamate pattern, in which f_H is maintaned at a relatively elevated level (~ 120 ± 1.3 beats min^{-1}) until mid-incubation period and thereafter maintained unchanged (108 ± 0.7 beats min^{-1}) until hatching. f_H increased with increasing temperature and both early and later embryos showed similar calculated temperature coeficients (Q_{10}). In our nesting site during

the period of reproduction, the range of temperature fluctuations in the depth where eggs are usually buried were narrower (8°C) if compared to the range found at a site closer to the surface (33°C) or the environmental range (23°C). So, temperature influenced directly the rate of heart beats in iguana embryos, and this effect was independent of the stage of the embryo and may be unrelated to metabolic rates. Temperature variation in the nest was dependent on the depth of burial, and the choice of nesting site by the females is predictive of the termal fluctuations that the eggs will experience.

1. Introduction

Interestingly, the heart is the first functional organ during embryonic differentiation in vertebrates, however, its main function is not necessarily the convective transport of respiratory gases, nutrients and wastes, typical of adultorganisms (Burggren and Crossley, 2009). Previous studies have shown that the heart starts to beat before transport function is required possibly to induce the proper formation of the heart and vessels during its morphogenetic processes (Burggren, 2004).

Heart rate (f_H) during embryonic development presents a large diversity of patterns in vertebrates. If the f_H reflected the time course of changes in metabolic requirements, it would increase progressively as embryos are cumulatively synthetizing new tissue and growing rapidly in size from the time of fertilization to the time of birth. Nevertheless, data gathered so far showed that the embryonic f_H may not be completely coupled to the oxygen requirements in embryonic reptiles (Barrionuevo and Burggren, 1999; Sartori et al., in press). However, the contributing factors of f_H patterns and its variations amongst different species are not completely understood. Some of these factors are thought to relate to changes in membrane permeability or in the pacemaker and different timing of onset and levels of neural autonomic and hormonal control (Burggren and Pinder, 1991; Crossley et al., 2003; Nechaeva et al, 2007).

There are few reptilian species studied so far, but a trend can be observed relatively to the different polyphyletic orders (Taylor et al., 2014). In Chelonia f_H seems to generally decrease during the last half of incubation (Spencer, 2012; Zhao et al., 2013; Nechaeva et al, 2007); in Squamata f_H seems to present a relative stable value throughout total incubation time (Radder and Shine, 2006; Crossley and Burggren, 2009; Du and Shine, 2008) and in Crocodilia f_H seems to increase at mid-incubation and then stay at a plateau until time of eclosion (Crossley and Burggren, 2009, Eme et al., 2013; Crossley et al., unpublished).

Most reptiles are oviparous and typically lay clutches of eggs in nests, buried (e.g. turtles, lizards) or covered by a mound of vegetation and soil (e.g. crocodilians) with no parental care or protection against predation and extremes of physical conditions, such as temperature,

humidity and concentration of respiratory gases (Ackerman and Lott, 2004). The range of behavioural and physiological responses to changes in ambient conditions shown by the freely mobile adult animal, breathing air are of course denied in some extent to the developing embryo enclosed inside its egg in the nest. So any of these physical factors are likely to affect the rate of embryological development. Specially temperature, is shown to fluctuate in natural nests, on a daily and seasonal basis (Shine and Harlow, 1996) and is likely to have an important effect on the rates and processes of development, including f_H (Deeming and Ferguson, 1991).

The present study focussed on the developing heart in embryos of lizards, determining the temperatures of captivity nesting sites, the pattern of f_H at a constant temperature, and the chronotropic effects of acute temperature changes in the lizard *Iguana iguana.*

2. Material and methods

2.1 Experimental animals

The eggs and adults for this study were supplied from the breeding programme (registered at IBAMA, n. 673766) operated by Prof Abe and his staff and students at the Jacarezário, Departamento de Zoologia, São Paulo State University (UNESP), Rio Claro, SP. Adult iguanas were maintained in large, purpose built outside vivaria with water *ad libitum* and fed daily with vegetables and fruits. Fertile eggs were laid by female green iguanas (*Iguana iguana*) at various dates in September/October 2010 and 2012. When freshly laid clutches of eggs were detected they were collected immediately and incubated under controlled conditions, enabling the factors determining successful development to be manipulated. In 2010, twenty-three clutches of eggs were collected comprising 923 eggs and in 2012, 24 clutches comprising 964 eggs. They were placed in trays of vermiculite that were held in constant temperature incubators at 30 ± 1.5 ^{o}C. The eggs were examined every day for any signs of mortality and sprayed with water to maintain humidity high. Eggs were selected for the physiological study from clutches at a range of development times.

2.2 Soil temperatures:

From October 27th through December 7th of 2011 data storage devices (Thermocron Data Logger, OnSolution Pty Ltd, Baulkham Hills, Australia) that continuously record temperatures were placed at nest collection sites in the enclosures that held the adult iguanas in order to record the temperatures to which eggs would normally be exposed. The devices were programmed to collect the temperature data every 20 minutes and then placed close to the soil

surface (5 cm deep) or buried in the depths of 25 cm which is the approximate depth at which females normally buried their eggs (Rand, 1972). The recorded temperatures were related to meteorological data collected on campus by the Metereological Station and Center of Environmental Analysis and Planning (CEAPLA/IGCE/UNESP, Rio Claro, SP).

2.3 Experimental protocol:

Developmental pattern of f_H: We selected eggs for f_H recording from 5 days post oviposition (dpo) and every 3 days thereafter, until the time to hatch, that occured near 73 dpo. Each egg was weighed, enclosed in moistened gauze to prevent desication and alocated in the egg digital monitor (Buddy System, Avitronics UK) inside a portable incubator chamber (Caltech EIP-010, PE, Brazil) at the temperature of 30ºC ± 1.5 ºC. This equipment record movements of the heart from outside of the egg (Buddy System, Avitronics UK), a non-invasive method used for birds (Lierz et al., 2006) and reptiles (Du et al., 2010). The egg monitors were proven to increase internal temperature of the egg so each egg allocated in the Buddy system was recorded for two hours, which would result in a stabilized mean egg temperature of around 32ºC (Sartori et al., 2015b).

Changes in acclimation temperature: Embryos ranging from different periods of incubation (20, 40, 60 and 90% of total incubation time) were selected for measurement of the acute effects in f_H to shifts of incubation temperatures. Temperature in the portable incubator was reduced from 30ºC to 25ºC and to 20ºC and each temperature was held for 2h to enable the embryos to acclimate to each new temperature. Internal temperature of the eggs after the 2h-acclimation period were checked by thermocouple in 14 eggs using a flexible implantable thermocouple probe (BAT-4, Physitemp Instruments, NJ, USA or T-type, ADInstruments). Temperature coefficients (Q_{10}) were calculated from the changes from the temperature achieved by the eggs subjected to changes in the incubator temperature from 20ºC to 25ºC ($Q_{10\text{-}1}$), 25 ºC to 30ºC ($Q_{10\text{-}2}$) and 20ºC to 30ºC ($Q_{10\text{-}3}$).

2.4 Statistical Analysis:

The pattern of f_H along incubation at constant temperature was tested with One-way ANOVA and the relationship between f_H and egg mass was acessed by linear regression. The effects of temperature and calculated Q_{10} were tested by Two-way ANOVA or ANOVA on ranks if normality failed, with age of embryos and temperature as variables. For *post-hoc* comparisons a Student-Newman-Keuls (SNK) test was used. All statistical tests were

conducted with the software package SigmaPlot. Significance was attributed to any changes at the 5% level of confidence (P = 0.05).

3. Results

3.1 Soil temperatures:

Air temperatures at the collection site varied between 11ºC and 34ºC on a diurnal cycle that was also affected by changing weather conditions (Table 1, Fig. 1). The larger variations we found were recorded during the passage of a weather front near the 20th day of measurement. Temperatures at the soil surface showed the larger variation range, rising to a daily maxima up to 48ºC. The plot of temperatures show that buried temperatures varied ranging from temperatures similar to the maximum temperatures recorded from air and to minimun temperatures recorded from the surface. At a depth of 25 cm, variations were over a smaller range of temperatures, of about 8 ºC, from 21°C to 29°C. Nevertheless, daily changes occured over a narrower range of 2.7°C.

3.2 Heart rate of intact eggs:

f_H from eggs maintained at constant incubator temperature of 30 ºC (representing an internal egg temperature of 32ºC) was related to age (days post oviposition), providing the curve of f_H pattern for iguanas illustrated in Fig. 2A. The plot showed a decreasing f_H value until mid-incubation time, with the mean for the period of 120 ± 1.3 beats min^{-1}. Thereafter f_H was maintained unchanged at 108 ± 0.7 beats min^{-1} until hatching. We found no clear relationship between f_H and egg mass (R^2 = 0.23; P < 0.001; Fig. 2B).

f_H measured at changing incubator temperatures of 20, 25 and 30ºC, represented internal egg temperatures of 23.5 ± 0.2ºC, 27.0 ± 0.2ºC and 31.9 ± 0.1ºC, respectively. Mean f_H in embryos from all periods of incubation increased significantly with temperature (P < 0.001, Fig. 3A) showing a strong correlation (R^2 = 0.82; P < 0.001; Fig. 3B). In each temperature f_H differed across the age groups (P < 0.05; Fig. 3A), and markedly at 32ºC f_H was higher in embryos from the first half of incubation (20 and 40%). Nevertheless, Q_{10} did not differ between the different incubation periods or temperature intervals and resulted in a mean value of 2.5.

4. Discussion

Vertebrate groups have distinct patterns of f_H during embryonic development. In mammals, the human fetus f_H markedly increase until 50 days after conception, stabilizes and decline soon after birth (Howe et al., 1991). The f_H pattern of birds can be divided into precocial and altricial. In the precocial species, in which the hatchling is relatively independent after eclosion, it is observed a hyperbolic increase, with a reduction in f_H at the end of incubation or soon after external pipping. In altricial species, that requires an extensive parental care after eclosion, it is observed a progressive increase in f_H during the last half of incubation, with no reduction related to pipping (Tazawa et al., 1991, 1994). In embryonic fish, the pattern is a progressive rise in f_H until it reaches a maximum, followed by a slower decline immediately prior to hatching (Pelster and Bemis, 1991; Rombough, 1997; Paine and Balon, 1984; Cunningham and Balon, 1986, Barrionuevo and Burggren, 1999). In amphibians, very early larvaes of the bullfrog *Lithobates catesbeianus* presented higher f_H that decreased and was maintained until hatching with a strong correlation between f_H and body mass (M_b). The pattern is the opposite in the frog *Eleutherodactylus coquii* and a body mass correlation is absent (Burggren and Doyle, 1986, Burggren et al., 1990).

In reptiles, a trend can be observed in the diferente orders (Taylor et al., 2014), but because of the lower amount of data avilable if it is unkown whether these variations are related to phylogenetis or to environmental conditions. The pattern found in iguanas, with a decrease in f_H during the first half of incubation and a constant level from mid-incubation until hatching seems to be a pattern shared by other squamates (Taylor et al., 2014). Interestingly, f_H seems not to be correlated to metabolic or growth rates during embryonic development (Sartori et al., in press) but seems related to the onset of autonomic cardiovascular control (Alvine et al., 2013). However, one study demonstrated in turtles f_H and metabolic rate (VO_2) strongly correlated at different temperatures (Du et al., 2010). Embryonic f_H in reptiles is influenced mainly by circulating cathecolamines throughout the entire incubation period (Crossley and Altimiras, 2000, Eme et al., 2011, Sartori et al., 2015a), but NANC factors like histamnine were shown to also have a role in the cardiovascular control (Crossley et al., 2013).

Differences in incubation temperatures can influence not only the embryonic incubation lenght, but have lasting effects on phenotypes of hatchlings (Shine and Harlow, 1996, Angilletta et al., 2000). Nevertheless, the range of temperatures measured at the site chosen

by female iguanas for deposition and burying of their eggs demonstrate that they are relatively protected against large fluctuations of environmental temperature during the incubation period (variation of 8°C compared to the air variation of 23°C, and surface of 33°C). Natural iguana nests in Panama were reported to be buried in depths from 20 to 70 cm (Rand, 1980) and the daily variation we found is similar to the reported value for natural nests (Booth, 1998). The increasing depth reduces the diurnal temperatures and variations but is dependent on the type of soil and its thermal properties. Temperatures are reported to be reduced at depths as shallow as 14 cm but more typically at 50 cm (Hillel, 1980).

Physiological processes typically results in Q_{10} close to 2 or 2.5, that is the temperature coefficient of most biochemical reactions. When processes are not sensitive to temperature Q_{10} values are close to 1 and when highly sensitive, values are higher than 2 (Randall et al., 1997). As f_H in embryonic resulted in a Q_{10} of 2.5, it reveals that f_H is only affected by temperature *per se* and no other mechanisms are involved. Our results are similar to zebrafish embryos that also did not differ considerably between the ages measured. In zebrafish embryos the Q_{10} for f_H yielded similar values to the iguana embryo (1.2-2.5). Nevertheless, in these embryos it was found a discrepancy between the Q_{10} of the VO_2 and the Q_{10} of f_H (Barrionuevo and Burggren, 1999), and we should measure how iguana embryos VO2 is affected by temperature changes in the future. In contrast, the Q_{10} for VO_2 measured in avian embryos was shown to decrease with the progression of incubation (Tazawa et al., 1989, Buttemer et al., 1988). These results indicate that the sensitivity of f_H to temperature in reptiles is based on a mechanism unrelated to the maturation of the embryo and probably unrelated to VO_2 reinforcing the independent relationship between f_H and metabolic demands of embryos, previously reported for fishes (Barrionuevo and Burggren, 1999; Sartori et al., in press).

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Table 1 Temperatures measured at the incubation site of captive iguanas. Air temperatures were registered by a metereological station, surface teperatures represent soil at 5 cm depth and buried temperatures represents soil at 25 cm depth. Means were calculated for a period of 41 days, at 20 min intervals.

T (°C)	Air	Surface	Buried
Min	11.00 ± 0.09	14.7 ± 0.1	21.17 ± 0.03
Max	33.70 ± 0.09	48.1 ± 0.1	29.17 ± 0.03
Amplitude	22.70 ± 0.09	33.4 ± 0.1	8.00 ± 0.03
Mean	21.97 ± 0.09	28.1 ± 0.1	25.21 ± 0.03

Table 2 Mean heart rates (± s.e.m.) of embryonic iguanas subjected to acute temperature changes, 23°C, 27°C and 32°C at 4 different points of the incubation period. Combined mean temperature coeficiente, Q_{10} (± s.e.m.) from the different ranges of temperature changes.

	f_H 23°C	f_H 27°C	f_H 32°C	Q_{10}
20%	55.1 ± 2.5 [a]	77.2 ± 7.0 [a]	110.2 ± 8.5 [a]	2.3 ± 0.1
40%	51.7 ± 5.1 [ab]	66.0 ± 2.6 [ab]	115.8 ± 7.4 [a]	2.6 ± 0.4
60%	43.1 ± 1.7 [b]	64.0 ± 1.9 [b]	102.9 ± 2.5 [b]	2.7 ± 0.1
90%	46.5 ± 3.1 [ab]	60.2 ± 3.1 [b]	100.7 ± 4.8 [b]	2.4 ± 0.2

Figure captions

Fig. 1 Soil and air temperatures from a holding pen in Jacarezário. The soil temperatures were measured from October 27th through December 7th of 2011 using data storage devices buried at 5 cm (surface, gray line) and 25 cm (buried, black line) depths. Air temperatures (red line) were provided by the meteorological station on UNESP campus.

Fig. 2 A) Pattern of f_H changes along incubation period (dpo, days post oviposition) of iguanas at a constant incubator temperature of 3°C that represents an internal egg temperature of 32°C. **B)** Linear regression between f_H and egg mass, showing no correlation.

Fig. 3 A) Mean heart rates of embryos at a range of developmental stages (20, 40, 60 and 90% of incubation) at egg internal temperatures of 23, 27 and 32ºC. **B)** Thermal dependence of f_H in iguana embryos. A total of 30 eggs at 23, 27 and 32ºC were measured at 20, 40, 60 and 90% incubation at 3 incubation temperatures.

Fig. 1

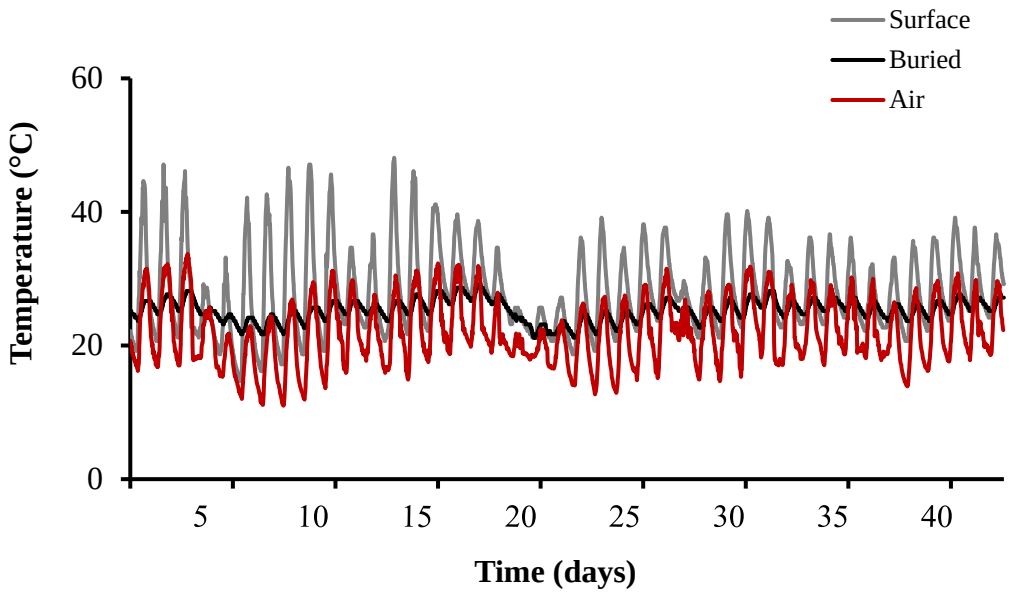

Surface
Buried
Air
60
40
20
0
Temperature (°C)
5
10
15
20
25
30
35
40
Time (days)

Fig. 2

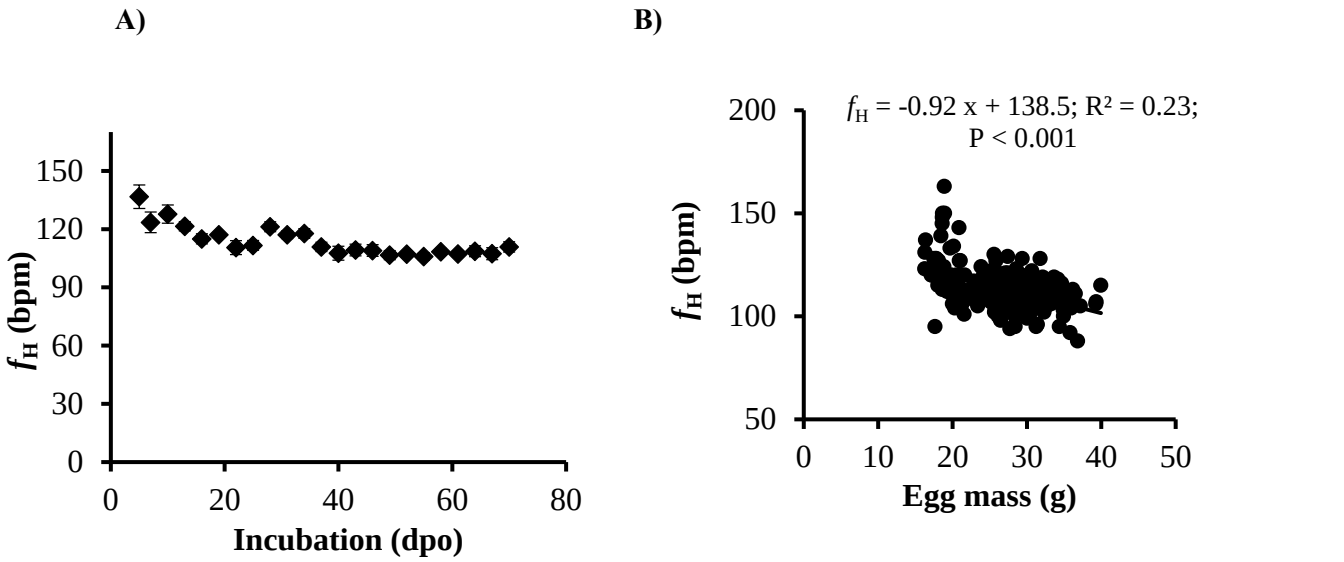
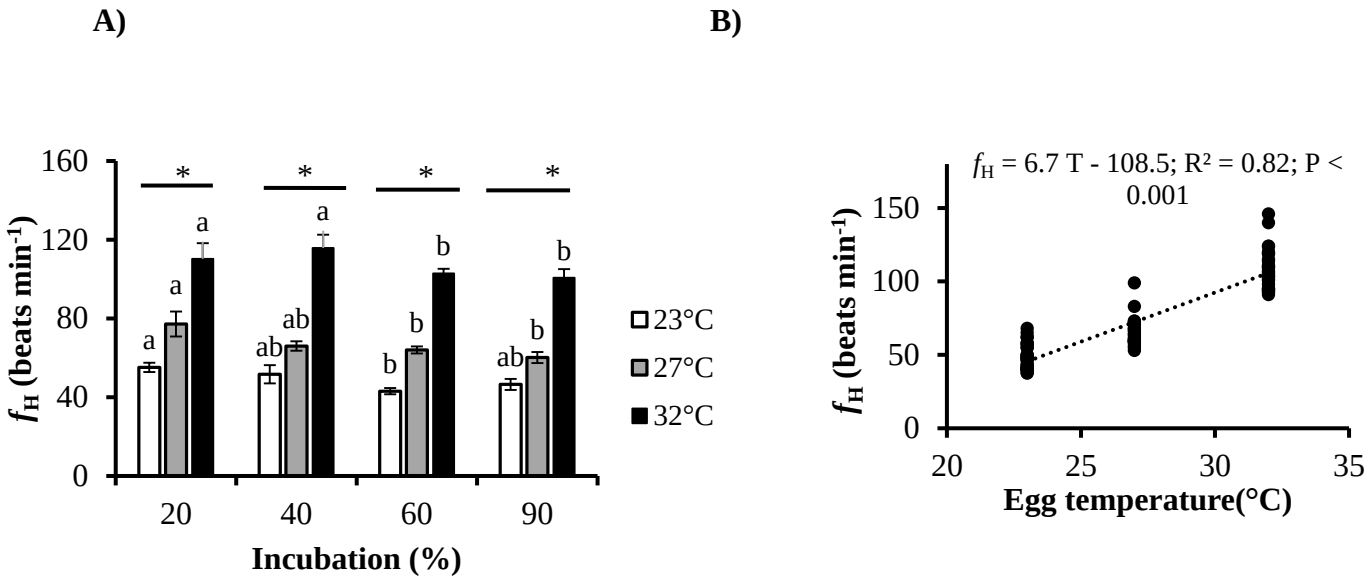

A)
B)
f_H (bpm)
Incubation (dpo)
f_H = -0.92 x + 138.5; R² = 0.23; P < 0.001
Egg mass (g)

Fig. 3


A)
B)
f_H (beats min^{-1})
Incubation (%)
23°C
27°C
32°C
f_H = 6.7 T - 108.5; R² = 0.82; P < 0.001
Egg temperature(°C)

Capítulo IV

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The progressive onset of cholinergic and adrenergic control of heart rate during development in the green iguana, *Iguana iguana.*

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List of abbreviations:

Ach - acetylcholine

Adr – adrenergic

Atr – atropine

Chol – cholinergic

f_H – heart rate

Hyp – hypoxia

Hyperc – hypercapnia

Prop – propranolol

Recov – recovery

Abstract

The autonomic control of heart rate was studied throughout development in embryos of the green iguana, *Iguana iguana* by applying receptor agonists and antagonists of the parasympathetic and sympathetic systems. In early embryos (< 10 days incubation) the chronotropic response to drugs topically applied to the heart was visually recorded while in later embryos drugs were injected into the amniotic fluid, with heart rate recorded remotely using an infrared monitor. Acetylcholine (Ach) slowed or stopped the heart and atropine antagonized the response to Ach indicating the presence of muscarinic cholinoceptors on the heart of early embryos. However, atropine injections had no impact on heart rate until immediately before hatching, when it increased heart rate by 15%. This cholinergic tonus increased to 34% in hatchlings and dropped to 24% in adult iguanas. Although epinephrine was without effect, injection of propranolol slowed the heart throughout development, indicating the presence of β-adrenergic receptors on the heart of early embryos, possibly stimulated by high levels of circulating catecholamines. The calculated excitatory tonus varied between 33-68% until immediately before hatching when it fell to 25-29%, a level retained in hatchlings and adults. Hypoxia caused a bradycardia in early embryos that was unaffected by injection of atropine indicating that hypoxia has a direct effect upon the heart. In later embryos and hatchlings hypoxia caused a tachycardia that was unaffected by injection of atropine. Subsequent injection of propranolol reduced heart rate both uncovering a hypoxic bradycardia in late embryos and abolishing tachycardia in hatchlings. Hypercapnia was without effect on heart rate in late stage embryos and in hatchlings.

Key words: Squamata, iguana, embryos, development, cardiovascular, hypoxia, hypercapnia, hatchlings

1. Introduction

The morphological and physiological changes accompanying embryonic development in reptiles are relatively poorly understood. This is in contrast to our knowledge of these processes in other oviparous vertebrates such as amphibians (Burggren, 1995; Burggren and Doyle, 1986) and birds (Crossley and Altimiras, 2000; Crossley et al., 2002; Crossley et al.,

2003a). The paucity of data for reptiles invites attention as they represent a key evolutionary link between the ancestral amphibians, birds and mammals. By far the most tractable cardiovascular parameter that can be measured in embryonic reptiles is heart rate (f_H), using non- or minimally invasive methods. These methods can be used to monitor the onset of central nervous control of heart rate via the autonomic nervous system, using appropriate pharmacological agents.

The ontogenetic development of f_H regulation in reptiles has been investigated primarily in American alligators, *Alligator mississippiensis* and common snapping turtles, *Chelydra serpentina*. Two main characteristics have emerged. Embryonic alligators maintain a constant and pronounced β-adrenergic tone throughout the final 40% of incubation that is attributable to circulating catecholamines and not mediated by an adrenergic innervation. They also lack cholinergic tone mediated by cholinoceptors until the time of hatching (Eme et al., 2011). Alligator embryos also lack central nervous control of f_H in response to hypoxia and exhibit a limited hypertensive baroreflex response (Crossley et al., 2003b; Crossley and Altimiras, 2005). We have recently obtained similar findings in the Paraguayan caiman (*Caiman yacare*) (Crossley et al., unpublished), possibly indicating this is a common feature of development in crocodilians. Like alligators, embryonic turtles possess a marked β-adrenergic tone on heart rate at 70% of incubation that was not attributable to sympathetic nervous outflow (Eme et al., 2013). Heart rate falls slightly before and markedly after hatching indicating the establishment of a vagal tone on the heart coincident with the onset of lung breathing (Birchard and Reiber, 1996). A number of turtles and tortoises lack cholinergic tone on f_H until the time of hatching (Crossley and Burggren, 2009; Taylor et al., 2014), although cholinergic tonus was verified during the final 30% of embryonic incubation in snapping turtles (Alvine et al., 2013). One preliminary study of the African brown housesnake *Boaedon fuliginosus* (Crossley and Burggren, 2009) suggested that cholinergic tone may be present prior to hatching. However, an extensive assessment of the onset of cholinergic tone on the heart has yet to be conducted in squamates.

The present study focused on measurement of f_H during embryonic development in the green iguana, *Iguana iguana.* The injection of appropriate agonists and antagonists, as well as exposure to changed levels of respiratory gases was used to reveal the onset and degree of autonomic control of the cardiovascular system in embryos and hatchlings. This is the first study of this aspect of development to provide data from a member of the superorder Lepidosauria that includes the extant lizards and snakes (the squamates) and is further distinguished by having extended the study to early embryos (< 10% incubation).

2. Materials and Methods

2.1 Experimental animals

The eggs and adults for this study were supplied from the breeding program operated by Prof Abe, his staff and students at the Jacarezário, Departamento de Zoologia, UNESP, Rio Claro, SP. Adult iguanas (*Iguana iguana*) were maintained in a large, outdoor vivarium with water *ad libitum* and fed daily with vegetables and fruits. Each year of the study clutches of eggs ranging in numbers from 15-50 eggs, were collected during the months of September and October. Eggs were weighed, placed in trays (38 x 28.5 x 6.5 cm) containing vermiculite and placed at a constant temperature of 30ºC ± 0.5ºC in incubators (Eletrolab, EL101/3, SP, Brazil). All eggs were examined daily for signs of mortality and the vermiculite sprayed with dechlorinated tap water to maintain humidity high. Eggs were selected for physiological study at 5-8 days, 18-20 days, 29-31 days, 43 days, 51-53 days and finally 67-71 days, listed as groups 1 to 6. For the present study we have identified our 6 groups according to the stages provided by Sanger et al. (2008) (Table 1). Group 6 animals had developed to a point immediately before hatching later confirmed by successful hatching of a subset of eggs. At the conclusion of experiments, embryos at all phases of development were killed by extended exposure to greatly increased levels of CO_2 (>20%) and were then preserved for separate morphological study. Hatchlings were held for experimentation for up to 90 days after hatching.

2.2 Experimental protocol

2.2.1 Studies on iguana embryos

A total of 107 embryos were used during this study. All experimental procedures were approved by the Local Ethics Committee on Animal Care at the São Paulo State University (CEUA-UNESP no. 6597). Prior to experimentation, eggs were weighed and heart rate was recorded to determine baseline values. For the earliest embryos (group 1) each egg was first "candled" over a bright, cold, fiber-optic light source to detect the position of the embryo. An approximately 1cm^2 window was then cut through the eggshell to view the heart, which at this early stage of development was not yet enclosed within the body wall. Heart rate was counted visually by means of a dissecting microscope (Stemi 2000 C, Zeiss, Germany), at room temperature (28±1ºC). In these experiments drugs (up to 0.2 ml) were applied topically to the heart surface via a fine 27-gauge hypodermic needle. In later stages the heart had increased in size and become enclosed within the body wall of the growing embryo so that it was no longer visible.

Attempts to measure an ECG or cannulate CAM (chorioallantoic membrane) vessels to measure blood pressure proved ineffective. Consequently, the heart's activity was recorded non-invasively using a digital egg monitoring system (Buddy System, Avitronics, Truro, UK), that recorded movements of the heart from outside of the egg, as described for birds by Tazawa et al (1994) and for reptiles by Du et al (2009). The Buddy system was customized by the supplier with an additional analog output at our request. This output was connected to a Powerlab system (ADInstruments, Sydney, Australia) with f_H recorded continuously, using data acquisition software (LabChart 7, ADInstruments). Each egg was placed in the monitoring device and surrounded by a ring of water saturated gauze. The device was then placed inside an incubator (Caltech EIP-010, PE, Brazil) set at 30±0.5ºC for at least 1 hour prior to recording. Baseline f_H was then recorded prior to each drug injection. In these experiments a portion of the egg shell was removed in order to assist selective injection of drugs close to the embryo and the lid of the monitor was opened during injection.

Drug injections: In early embryos (group 1) topical injection onto the heart was used for application of acetylcholine (Ach), the agonist for cholinergic receptors, and adrenaline (Adr), the agonist for adrenergic receptors, followed by the appropriate antagonists, atropine (Atr) or propranolol (Prop). All were applied drop-wise to the heart as solutions in physiological saline at a range of concentrations up to 10^{-3} molar. In later embryos (after 18 days of incubation) drugs were applied initially by opening a window in the shell and injecting the drugs in proximity to the developing embryo or through its body wall. Later, this relatively more invasive technique was replaced by injection through the eggshell. The egg was first "candled" as described above, to detect the position of the embryo and its yolk sac and an injection site close to the embryo that avoided injection into the yolk sac was marked on the outside of the egg. The drugs were then injected via this site into the amniotic fluid from whence they were absorbed into the embryonic circulation. After the baseline f_H was established a control injection of saline was given and responses recorded. This was followed by an injection of, 0.2 ml of the muscarinic cholinergic antagonist atropine (10^{-3} mol), and then the β-adrenergic antagonist propranolol (10^{-3} mol) (Sigma Aldrich) in close proximity to embryos at developmental stages from group 2 to 6. To ensure that the full effects of the drugs were recorded measurements were taken for up to one hour after the initial injection. The efficacy of each antagonist was verified by subsequent injection of the appropriate agonist or a second dose of the antagonist. Injection of drugs necessitated the opening of the lid of the egg monitoring system, causing brief changes in the local environment surrounding the egg.

Heart rate response to changes in respiratory gases: In order to uncover cardiac responses to changes in respiratory gas concentrations, early (group 2) and late stage (group 5-6) embryos were exposed to 5% O_2 (hypoxia) or 5% CO_2 (hypercapnia). Exposure to different gas mixtures was achieved by enclosing each egg monitoring system containing an egg in a sealed plastic bag (16 x 26.5 cm) supplied with gas mixtures produced by calibrated flowmeters (Cole Parmer, Vernon Hills, USA). The exit tubes were led to gas analyzing equipment (PA-10 and CA-10, Sable Systems, Las Vegas, USA) in order to ensure that the eggs were exposed to the correct experimental gas mixtures. Each gas mixture was applied to the eggs for 1 hr followed by a period of 30-60 min of recovery in air. During this series the lid of the monitor remained closed for the duration of each experiment. In a subset of studies egg temperature was recorded by inserting a thermocouple (T-type implantable thermocouple, ADInstruments) through the eggshell and membranes, to lie up to 5 mm into the amniotic fluid, clear of the embryo and yolk sac. Output from the egg monitoring systems and the thermocouple signals were connected to an interface (PowerLab plus Animal Bio Amp, ADInstruments, Sydney, Australia) and recorded with data acquisition software (LabChart 7, ADInstruments, Sydney, Australia). These experiments revealed that the infrared detection of f_H by the egg monitoring system causes a progressive increase in egg temperature. This warming effect was particularly intense in this second series of experiments, performed with the lid closed for prolonged periods, when egg temperature increased from a 28ºC to 32ºC over a period of 1 hour. However, if the lid was held open the temperature of the egg remained below 30ºC for 2 hours. These changes in temperature of up to 2ºC either side of the incubation temperature of 30ºC affected f_H (Sartori et al., 2015b). Their possible confounding effects on our study were mitigated by comparing all changes in f_H accompanying drug injection or changes in respiratory gas concentrations with baseline rates recorded immediately prior to and on recovery from treatment.

2.2.2 Studies on hatchlings

For measurement of f_H following drug injection or exposure to different gas mixtures iguana hatchlings (2-3 months) were instrumented with two lengths of Teflon insulated silver wire (A-M Systems, WA, USA), inserted subcutaneously either side of the mid-thoracic wall. The hatchlings were enclosed in 50 ml Falcon tubes (Cralplast, SP, Brazil) opened at each end to allow the anterior portion of the head and the tail to extend outside of the tube. The tubes containing the hatchlings were placed in an incubator set at 30°C (Caltech EIP-010, PE, Brazil). The wire leads were externalized via a slot cut through the wall of the Falcon tube and

connected to a preamplifier (Animal Bio Amp, ADInstruments). Raw data were recorded and selectively filtered, using data acquisition software (LabChart 7, ADInstruments) to separate the ECG and ventilatory signals. Data were collected overnight to determine the baseline f_H, then autonomic regulation of f_H was determined by injection of 0.25 ml of saline or the respective antagonists atropine (10^{-3} mol) or propranolol (10^{-3} mol) into the peritoneal cavity. In order to check complete blockade second doses of each drug were injected. In a separate series of experiments f_H responses to 10 min exposure to 10% O_2 or 5% CO_2 followed by a period of 30-60 min of recovery in air were accomplished as outlined for the embryonic studies.

2.2.3 Studies on juvenile and adult iguanas

Juvenile and adult iguanas (N= 7, mass= 902.6±60.3g) were first lightly anaesthetized in an atmosphere of CO_2 (see Wang et al, 1993; Taylor et al., 2009) then held under anesthesia on a respiratory pump (CWE, SAR-830/P Ventilator, USA) supplied initially with 5% isoflurane. This dose was reduced to 2% once anesthesia was established. The femoral vein was occlusively cannulated for injection of drugs as previously described (Busk et al., 2000). ECG electrodes were placed under the skin on opposing lateral sides of the thorax rostral or caudal to the heart and a reference electrode inserted dorsally, 1 cm lateral of the vertebral column. All leads were anchored to the back of the animal with a suture. The area around all operative lesions and suture points was injected with a local anesthetic (Lidocaine, Eurofarma, SP, Brazil). The animal was then placed in an incubator set at 30°C (Caltech EIP-010, PE, Brazil) for recovery and the catheter and ECG leads were led through a port in the wall. Animals were then left overnight in the incubator to recover from the effects of anesthesia and handling. The leads were connected to an ECG amplifier (Animal Bio Amp, ADInstruments) and raw signals were collected and processed as described for the hatchlings. Following measurements of baseline f_H 1.0 ml of atropine (10^{-2} mol) or propranolol (10^{-2} mol) was injected via the femoral vein and resultant changes in f_H recorded for up to one hour post-injection, for calculation of autonomic tones.

2.3 Statistical Analysis

The study of autonomic tones used a total of 74 eggs, 6 hatchlings and 7 adults. The responses to changes in respiratory gases used a total of 33 eggs and 12 hatchlings. All statistical tests were conducted with the software package SigmaPlot v. 11. The effects of drug injections

within each group, the comparison of adrenergic and cholinergic tonus between different groups and f_H changes in response to hypoxia and hypercapnia with or without blockade with drugs were all tested with one-way ANOVA. Student-Newman-Keuls (SNK) test was used for *post-hoc* comparisons, and each treatment was compared with the previous value. When the data failed a test of normality an ANOVA on Ranks and Dunn's test was used. Significance was attributed to any changes at the 5% level of confidence ($P < 0.05$).

3. Results

3.1 The embryos

A profile of the embryonic groups used in the study is detailed in Table 1. Egg mass from the first to the last group increased two-fold (from 15.4±0.5 g to 31.0±1.3 g) and embryo wet mass increased 60-fold (from 0.16±0.01 g to 9.76±0.13 g). The embryos were staged according to the staging table for *Anolis sagrei,* a lizard species closely related to the green iguana (Sanger et al., 2008).

Baseline embryonic heart rate: Baseline f_H in group 1 embryos were measured by direct visual observation at room temperature (28°C). They were significantly lower than baseline f_H in the succeeding groups 2-6 that were recorded using the egg monitoring system held inside the incubator at 30ºC. The mean rates were statistically similar within these latter groups (Table 2).

Effects of drug injection on embryo heart rate

Cholinergic blockade: Early embryos showed a clear, dose-dependent reduction in f_H to topic application of acetylcholine (Ach). This culminated in cardiac arrest at 10^{-2} molar (Figure 1). This effect was reversed by application of atropine, with f_H progressively recovering to the rate prior to injection of acetylcholine (Figures 1 and 2). Following atropine injection subsequent injection of acetylcholine had no effect (data not shown). This cholinergic inhibition was apparent in all embryos. However, injection of atropine alone into the eggs had no effect on all embryos from groups 1 to 5 (Table 2; Figures 2 and 3). Mean values of atropinized f_H are shown in Table 2 and also illustrated for groups 2-5 in Figure 3, as this shows the effect of prior injection of saline as a control for the injection of atropine. In contrast to groups 2-5, group 6 (67-71 days) embryos showed a significant increase of 17% in f_H following injection of atropine (Figure 4).

Adrenergic blockade: Topic application of adrenaline to early-stage embryos and injection into the amniotic fluid of later embryos had no effect on f_H (Fig. 2) but injection of Propranolol markedly reduced f_H at all stages of embryological development (Table 2; Figures 2, 3 and 4). Based on f_H values following atropine injection, the decrease in f_H induced by propranolol was approximately 45% in groups 1 to 5 and 32% in group 6.

3.2 Effects of drug injection on heart rate in hatchling iguanas:

Recordings of f_H in hatchlings revealed mean resting values of 43±1 bpm interrupted by brief bouts of tachycardia associated with spontaneous movements. Atropine injections increased f_H to 60±2 bpm representing an increase of 41% (Table 2). Propranolol injections reduced f_H to a mean of 43±5 bpm, representing a decrease of 28% from the atropinized value. The effectiveness of these treatments was verified by repeated injections of each antagonist into the peritoneal cavity.

3.3 Effects of drug injection on heart rate in adult iguanas:

Mean f_H after 24h of recovery from surgery was 28±1 bpm (Table 2). Intravenous injection of atropine (1ml of 10^{-2} molar) caused an increase of 38% in f_H (mean = 37±2 bpm). Subsequent injection of propranolol at a similar dose caused a decreased f_H from the elevated rate following atropine injection to a mean of 28±2 bpm, corresponding to a 24% decrease. Effectiveness of injection of both antagonists was verified by the intravenous injection of the appropriate agonist.

3.4 Calculation of autonomic tones:

Table 2 summarizes f_H before and after injection of atropine and propranolol in the 6 embryonic groups, hatchlings and adults. The adrenergic tonus on the hearts of all groups, plus the cholinergic tonus on the hearts of group 6 embryos, hatchlings and adult iguanas were calculated from the combined means of the mean cardiac intervals collected for individuals in each group (see Altimiras et al, 1997). Embryos in groups 1-6 had a clear adrenergic tone on the heart that varied between 33 and 68%, with the highest value found in group 3 that differed statistically from group 6 embryos, hatchlings and adults. Hatchlings had an adrenergic tone of 25% and adults of 29%. A cholinergic tone of 15% was first apparent in group 6 embryos, increasing to 34% in hatchlings and stabilising to 24% in inactive adults (Figure 5).

3.5 Effects of changes in the levels of respiratory gases

Exposure to 5% oxygen in early stage embryos (group 2) reduced mean f_H from 74±4 to 52±3 bpm, representing a 30% change that recovered in subsequent normoxia (data not shown). Initial f_H was unaffected by prior injection of atropine and hypoxia still elicited a decrease of 21% (Figure 6). In contrast, exposure of late embryos (groups 5-6) to hypoxia caused a small but significant initial tachycardia, increasing f_H from 115±1 to 125±1 bpm, representing a 8% change that was unaffected by injection of atropine (Figure 7A). Injection of propranolol caused an overall reduction in f_H (from 114±3 to 71±3 bpm) and abolished the initial tachycardia on hypoxic exposure, revealing a bradycardia of 30%, when f_H decreased to a value of 50±3 bpm (Figure 7B). Hatchlings showed a hypoxic tachycardia of 25% (from 44±1 to 55±4 bpm) that was unaffected by injection of atropine but abolished by propranolol (Figure 8). Exposure to 5% CO_2 of late (groups 5-6) embryos (data not shown) and hatchlings (Figure 8) was without effect on f_H despite a marked hyperventilation in the latter group (data not shown).

4. Discussion

4.1 Measurement of baseline heart rates

This is the first study of the ontogeny of cholinergic and adrenergic control of heart rate (f_H) throughout development in a squamate reptile, the green iguana, *Iguana iguana*. It differs from other studies as the responses to drug injection were measured in a wide range of developmental stages from very early embryos (< 10% incubation time) to immediately prior to hatching, as well as in hatchlings and adult lizards. Although we consider the data to be sufficiently reliable to enable us to describe the progressive development of autonomic control in this species, the data were complicated by varying temperature during experiments. The data on group 1 embryos was collected at room temperature (28ºC), while all subsequent embryonic groups were studied by enclosure of each egg in a monitoring device (the Buddy system, Avitronics, UK) that used low levels of infrared radiation to measure heart beats. Complete enclosure in the device resulted in progressive warming of the eggs from room temperature to a maximum of 32ºC over about 1 hour (Sartori et al., 2015b). Accordingly, initial f_H varied between experiments (Table 2; Figures 6 and 7). However, the potential for obscuring the effects of drug injection were avoided by a control injection of saline immediately prior to each drug injection, while exposure to changes in respiratory gases were compared to individual initial rates prior to exposure and expressed as % age changes. Reptilian eggs are routinely subjected to

temperature variations in their natural environment on a daily and seasonal basis (Ackerman and Lott, 2004).

4.2 Injection of drugs to reveal onset of autonomic control

The different routes of administration of drugs (i.e. topical application onto the hearts of very early embryos, injection in the amniotic fluid in later embryos, injection into the peritoneal cavity in hatchlings and injection through a cannula in the femoral vein in adults) could have resulted in different degrees of dilution of the drug. However, the doses were relatively high and subsequent injection of agonists or a second dose of the antagonist revealed that each antagonist had provided complete blockade of the receptors for the duration of the experiment.

Topic application of the cholinergic agonist acetylcholine markedly affected f_H in group 1 embryos, within the first 10% of incubation time, causing cardiac arrest at 10^{-2} molar concentration. This response was abolished by prior or subsequent injection of the antagonist atropine. These data show that the embryonic iguana heart possesses muscarinic cholinoceptors at the earliest stage in development, post-oviposition. Similar results were obtained from embryos at all stages of development, though the sensitivity to acetylcholine varied between clutches. Despite this clear demonstration of the existence of muscarinic cholinoceptors on the heart of early and mid-stage (1-4) iguana embryos, injection of atropine alone was without effect on f_H, revealing an absence of an inhibitory cholinergic tone on the heart. Only in group 6 embryos that had undergone more than 90% of development was there evidence of a cholinergic tone that increased in hatchlings and adults (Figure 5). These data were previously unavailable from squamate embryos, though a similar finding has been reported as unpublished data from the African brown house snake, *Boaedon fuliginosus* (Crossley and Burggren, 2009). The lack of a cholinergic tone on the heart, despite the recorded presence of receptors, implies lack of establishment of appropriate connections within the CNS. Alternatively, the vagal efferent supply to the heart may not yet have established functional connections with the heart or cardiac ganglion. It is also possible that the nerve fibers in early embryos lack the myelination required to confer a conduction velocity sufficient to affect beat-to-beat modulation of the heart (Foster et al., 1982; Crossley and Altimiras, 2000; Taylor et al., 2014). As cholinergic tone is often not detectable in late stage vertebrate embryos until close to or immediately after hatching or birth in a range of species (Taylor et al, 2014), we hypothesize that this may relate to the onset of respiratory rhythmicity or physical respiratory movements. In the red footed tortoise it appears during embryonic development (Crossley et al., 2013), as described here for the iguana while in the chicken cholinergic control appears post-hatching (Crossley and Altimiras, 2000).

A previous hypothesis suggested that these differences in timing of the appearance of cholinergic control of f_H between species relates to the varying provision of maternal care to the hatchlings of birds and reptiles. Chicks receiving a high level of maternal care immediately post-hatching show a delay in the full establishment of central control of f_H whereas reptiles lacking this care may require effective central control of f_H prior to hatching to ensure survival (Crossley and Altimiras, 2000).

Although the embryonic iguana heart was insensitive to injection of adrenaline β-adrenergic blockade by injection of propranolol markedly reduced heart rate at all stages of embryonic development (Table 2, Figs. 2, 3 and 4). This demonstrates that the heart possesses ß-adrenergic receptors from an early stage. There is evidence that many vertebrate embryos have both muscarinic cholinergic and β-adrenergic receptors on the heart at an early stage of development (Taylor et al., 2014). Cardiac muscarinic and adrenergic receptors were found to be present in embryonic chickens during the first quarter of incubation (Berry, 1950) and the enzymes responsible for the production or breakdown of the neurotransmitters acetylcholine and noradrenaline were also present during early development in chickens (Zacks, 1954; Ignarro and Shideman, 1968). The existence of an adrenergic tone on the embryonic heart may indicate high levels of circulating catecholamines as described for embryonic chickens (Crossley and Altimiras, 2000) and alligators (Eme et al., 2011). Lack of response to injection of adrenaline in iguana embryos may indicate these high levels of circulating catecholamines saturated the ß-adrenergic receptors on the heart. However, it is also possible that the injected adrenaline was oxidized during its passage from the amniotic fluid to the receptors on the heart.

4.3 Autonomic Tonus

Embryos prior to 90% of incubation lacked cholinergic tone but had a clear adrenergic tone on the heart that varied between 38 and 68%. Just prior to hatching this was 33% while a cholinergic tone of 15% then became apparent (Fig. 5). Hatchlings had an adrenergic tone of 25% and a cholinergic tone of 34% while in adults the adrenergic tone was 29% and cholinergic tone was 24% (Figure 5).

Thus, the onset of a cholinergic tonus, implying vagal control of the heart, in late stage iguana embryos was accompanied by the establishment of a level of adrenergic tone that closely resembled the level in hatchling and adult iguanas. This may indicate a switch from control exerted by high levels of circulating catecholamines to nervous control exerted by the CNS via the sympathetic innervation of the heart. While these data provide a glimpse of the ontogeny of cardiac regulation in a single species of squamate reptile the limited information available

constrains speculation regarding the commonalities within this order but provides a fertile ground for future studies.

Regulation of f_H in embryonic reptiles has been investigated primarily in American alligators and common snapping turtles. Two characteristics of regulation seem to emerge in the species investigated to date (see Taylor et al., 2014). In the American alligator tonic cholinergic receptor mediated regulation is absent until just prior to or after hatching (Eme et al., 2011). Alligator embryos also lack nervous control of the f_H response to hypoxia and exhibit a limited hypertensive baroreflex response (Crossley et al., 2003b; Crossley and Altimiras, 2005). They do, however, maintain a pronounced β-adrenergic cardiac tone of constant intensity, elevating f_H throughout at least the final 40% of incubation (Crossley et al, unpublished; Eme et al., 2011) but this is not mediated by sympathetic nervous system output, relying instead on hormonal regulation by circulating catecholamines (Eme et al., 2011). Unlike alligators, common snapping turtles (*Chelydra serpentina*) possess a clear cholinergic tone, depressing f_H over the final 30% of embryonic incubation. However, this is not a general Testudine characteristic as embryonic desert tortoise (*Gopherus agassizi*), Red-footed tortoise (*Chelonoidis carbonaria*), Red-eared slider turtles (*Trachemys scripta*) and D'Orbigny's slider (*Trachemys dorbigni*) lack cholinergic tone on embryonic f_H (Crossley and Burggren 2009; Crossley et al., 2013; Sartori, unpublished). However, the presence of tonic cholinergic depression of f_H has been identified in *C. carbonaria* just prior to hatching (the final 5% of incubation) and this may also be the case for the other 3 species studied (Crossley et al., 2013). Like the alligators, embryonic snapping turtles also possess a marked β-adrenergic tone on f_H that does not appear to originate from sympathetic nervous outflow (Eme et al., 2013). Similar β-adrenoceptor stimulation on f_H has been reported for *G. agassizi* (Crossley and Burggren, 2009), *C. carbonaria* (Crossley et al., 2013), *T. scripta* (Sartori, unpublished) and *T. dorbigni* (Sartori, unpublished). Thus while the onset of cholinergic receptor tone seems species-specific, the presence of β-adrenergic tone on f_H is a shared feature of the Testudine species studied. The possibility of control exerted by nonadrenergic and noncholinergic (NANC) factors has been considered and a role for histamine via H1 and H2 receptors was identified in the red-footed tortoise, *Chelonoidis carbonaria* (Crossley et al., 2013).

4.4 Responses to changes in respiratory gases

Exposure to hypoxia caused a significant bradycardia in early iguana embryos. However, this response was intact following injection of atropine suggesting that it was the result of a direct effect upon the heart rather than a reflex arising from stimulation of

chemoreceptors. Hypoxia also caused a marked bradycardia in embryos of the chicken. Early in development (day 12 of incubation) this hypoxic bradycardia appeared to arise from the direct effect of low oxygen on the cardiac muscle. Until day 18 of incubation there was no evidence for the change in f_H in hypoxia being a reflexive response involving the CNS. However, immediately pre-hatching, at day 21, injection of atropine limited the hypoxic bradycardia indicating that there was a component of the cardiac chronotropic response that was generated from the CNS via the parasympathetic nervous supply to the heart (Crossley et al., 2003c). This suggests that, contrary to the conclusion arrived at above, the chick does possess some cholinergic control of the heart established immediately before it hatches that is only revealed by hypoxic exposure. This was accompanied by a late onset of neural adrenergic control as is suggested by our data on iguana embryos. However, in late (group 5-6) iguana embryos hypoxia did not cause a bradycardia, indeed the response was a small but significant tachycardia, although a bradycardia was revealed after adrenergic blockade. Hatchlings also showed a tachycardia during hypoxia that was not affected by injection of atropine but was reduced by injection of propranolol. In neither stage was f_H affected by hypercapnia although hatchlings showed periods of marked hyperventilation. These data raise interesting questions regarding the onset of the secondary response to hypoxia in an air-breathing species. This comprises the masking of a reflex bradycardia during hypoxia by a secondary tachycardia in response to increased lung ventilation (Spyer, 1982; Daly, 1986). This explains the tachycardia in hatchlings, but not that displayed by group 6 embryos unless they had commenced initial lung ventilation. The absence of a cardiac response to hypercapnia in hatchlings, despite evident hyperventilation, also questions the development of the full spectrum of air-breathing responses in this species. These remain open questions.

5. Conclusion

A representation of the time course of recorded changes in the factors affecting f_H in embryonic iguanas is provided in Figure 9. These factors are plotted against %age of total incubation time to enable comparison with similar diagrams provided for chicken and emu (Burggren and Crossley, 2002). The figure reveals that an excitatory adrenergic tone is present on the heart throughout development in all three species. In addition, they all show evidence for muscarinic cholinoceptors on the heart from an early stage in development. However, the onset of an inhibitory tone varies between species with the iguana acquiring it prior to hatching while the normoxic chicken delays onset until post-hatching. Observation of the timelines for

the onset of autonomic control of the heart in vertebrate embryos provided by Taylor et al. (2014) reveals that all show a persistent presence of an excitatory adrenergic tonus throughout development. However, half the species studied, including birds, reptiles and a fish, showed delayed onset of an inhibitory, cholinergic tonus, to just before or even in some species beyond hatching. Human babies show the onset of respiratory sinus arrhythmia, indicative of cholinergic vagal control of f_H at 85% of gestation (Taylor et al., 2010) supporting the conclusion that late onset of this control immediately anticipates the need for control of cardiorespiratory interactions in the newly independent individual. A more thorough examination of the different developmental strategies must await investigation of the onset of chemoreceptive and baroreceptive control of the cardiovascular system in a range of species, including the iguana. This will require cannulation of CAM vessels, which is proving very difficult and is the reason why this investigation came to rely on remote monitoring of f_H.

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Table 1 Profile of the iguana embryonic groups (1-6) used in this study differentiated by incubation time, expressed in days from oviposition, % age incubation time and as stage by comparison with the sequence described by Sanger et al. (2008), plus mean egg mass± SEM. Number of observations is given in Table 2.

Group	Incubation time (days)	% of Incubation	Stage	Egg mass (g)
1	5 to 7	5-10 %	4	15.4 ± 0.5
2	18 to 21	25-30%	10	23.5 ± 0.5
3	29 to 31	35-40%	13	27.8 ± 1
4	43	60%	14	30.1 ± 0.8
5	51 to 53	65-70%	17	35.1 ± 1.1
6	67 to 71	95-100%	18	31 ± 1.3

Table 2 Effects of autonomic blockade on f_H in embryonic groups 1-6 (see Table 1), hatchlings (Hatchl) and adult iguanas. Values expressed as mean heart rates ± SEM, respective number of observations (N), and mean body mass. (*) indicates significant differences within f_H of each row, and (**) indicates significant differences within f_H of the embryonic groups.

Group	N	Body Mass (g)	f_H Settled (bpm)	f_H Post Atr (bpm)	f_H Post Double Block (bpm)
1	26	0.16±0.01	47.9±1.5 (**)	43.2±1.5	24.0±4.4 (*)
2	10	0.61±0.03	80.5±2.2	88.3±4.2	47.7±8.7 (*)
3	7	1.26±0.09	73.3±2.8	70.8±8.1	43.4±2.8 (*)
4	8	2.57±0.06	79.1±4.8	80.6±6.5	44.5±11.4 (*)
5	7	4.57±0.50	65.1±4.0	61.5±9.5	34.7±8 (*)
6	16	9.76±0.13	79.5±5.2	93.3±4.9 (*)	63.4±4.3 (*)
Hatchl	6	13.4±0.2	42.5±1.3	60.8±1.8 (*)	43.2±4.6 (*)
Adult	7	902.6±60.3	28.4±1.3	37.2±2.2 (*)	27.6±1.7 (*)

Figure captions

Figure 1. Recording of heart beats from a 19 day iguana embryo (group 2; egg mass 22.56 g) with the egg monitoring system (Buddy System; upper trace) and an instantaneous calculation of heart rate from the data acquisition interface (PowerLab; lower trace). Injection of acetylcholine (Ach) reduced the apparent force of recorded heart beats then caused cardiac arrest. Subsequent injection of atropine (Atr) resulted in a progressive recovery in the force and rate of heart beats back to the value measured before injection of Ach. The recording was interrupted for 3 min then 5 min as indicated respectively by the arrows.

Figure 2. Mean values of heart rate (f_H) for early iguana embryos (group 1; 5-8 days post oviposition; N=26). The first column represents the initial f_H, 10 minutes after opening the egg (Init); then following topic application of saline (Sal), acetylcholine (Ach); atropine (Atr); adrenaline (Epi) and propranolol (Prop). Ach caused a significant decrease in f_H that was abolished by the subsequent Atr injection. Although Epi was without effect, Prop caused a significant reduction in f_H.

Figure 3. Mean values (± SEM) of heart rate for iguana embryos of groups 2 (N=10), 3 (N=7), 4 (N=8) and 5 (N=7). The first column (Init) represents the stabilized f_H achieved one hour after placement in the egg monitoring system and following columns represents f_H values after injection of saline, atropine then propranolol (labelling as for figure 2). Propranolol caused a significant reduction in f_H in all groups, but atropine was without effect.

Figure 4. Mean values (± SEM) of heart rate (f_H) for late embryos of iguana (group 6; 67 – 71days after oviposition; N=16). Saline (Sal) injection was compared to initial (Init) values and there was no significant change in f_H. Atropine (Atr) injection was compared to a post-saline injection and a significant increase in f_H was detected. Propranolol (Prop) was compared to a post-atropine value and a significant reduction in f_H was detected.

Figure 5. Mean values (± SEM) of adrenergic and cholinergic tonus on iguana embryos (groups 1 to 6), hatchlings (H) and adults (A). There is an excitatory adrenergic tone on the heart of all embryos groups that is significantly higher in group 3 (asterisk symbol, P = 0.001) when compared to group 6, hatchlings and adult animals. An inhibitory cholinergic tone was

first apparent in group 6 embryos and this increased significantly in hatchlings but is not different in adult animals, as denoted by the different letters (P=0.003).

Figure 6. Mean values (± SEM) of heart rates (f_H) of iguana embryos from group 2 (18-20 days; N=8) exposed to hypoxia following injection of atropine. f_H was significantly reduced by hypoxia after atropine injection (p= <0.05).

Figure 7A. Effect of exposure to 5% oxygen (Hyp 5%) on heart rate in late stage iguana embryos (group 5-6, N=5). Hypoxia caused a tachycardia that was unaffected by injection of atropine (Atr).
Figure 7B Injection of propranolol (Prop) caused an overall reduction in heart rate and uncovered a hypoxic bradycardia (group 5-6, N=4).

Figure 8. Mean values (± SEM) of heart rate for hatchling iguanas subjected to hypoxia (10% O_2) and hypercapnia (5% CO_2), before (A, N=7) and after injections of the antagonists atropine (B, N=12) and propranolol (C, N=6). A) Hypoxic exposure elicited a tachycardia whereas hypercapnia exposure did not change f_H. B) Injection of atropine did not block the hypoxic tachycardia. C) Propranolol abolished the tachycardic response during hypoxia exposure.

Figure 9. Time course of the major changes in control of the heart of the green iguana throughout incubation and after hatching expressed as the percentage of incubation time based on days after oviposition, from laying of the eggs (0%) to hatching (100%). There is evidence of the presence of muscarinic cholinoceptors and of adrenergic tone on the heart from an early stage of development, but an inhibitory cholinergic tone is delayed until immediately prior to hatching. Hypoxia causes a bradycardia in early embryos that is unaffected by injection of atropine. In late embryos and hatchlings it causes a tachycardia that is abolished by propranolol.

Figure 1

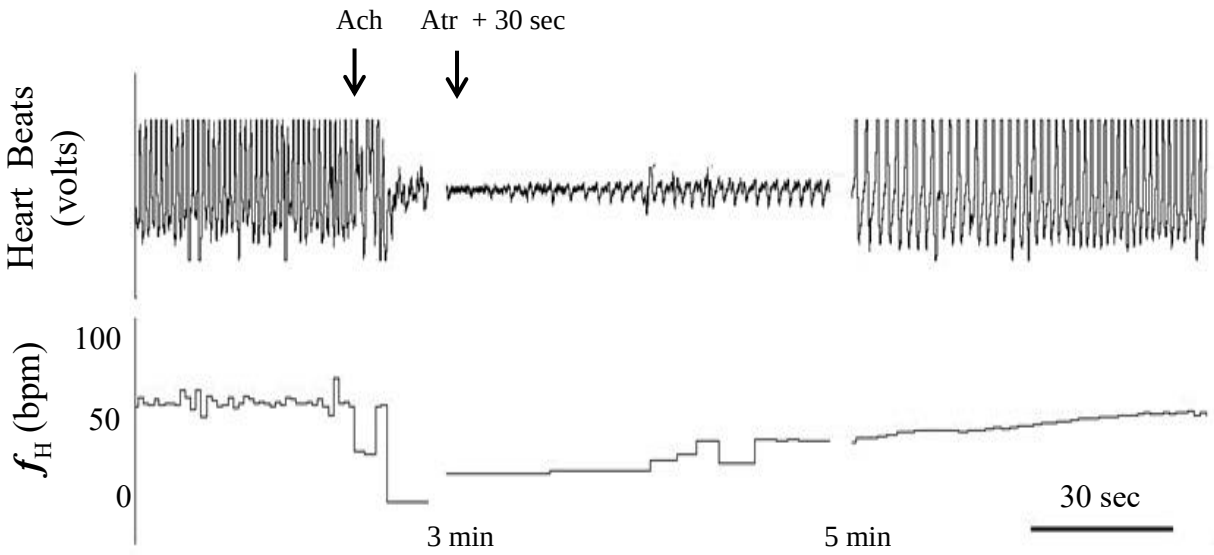


Figure 2

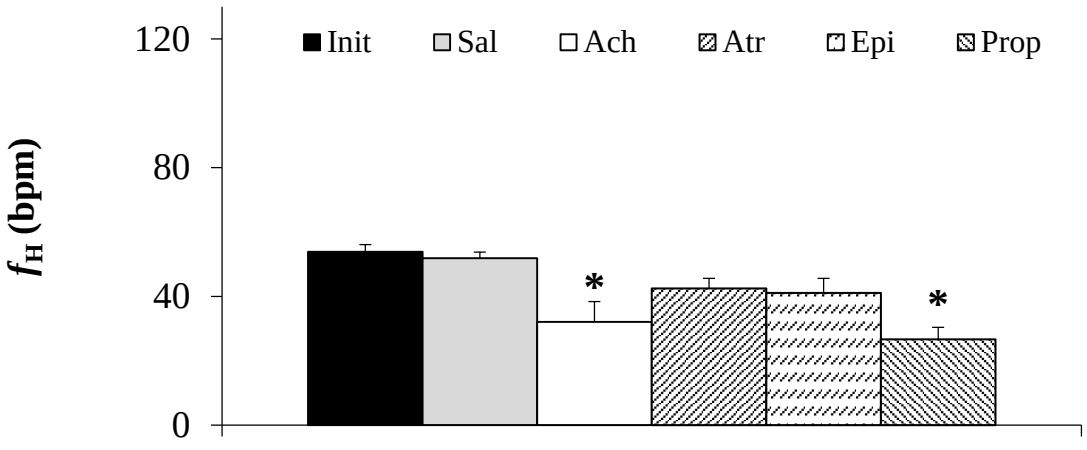


Figure 3

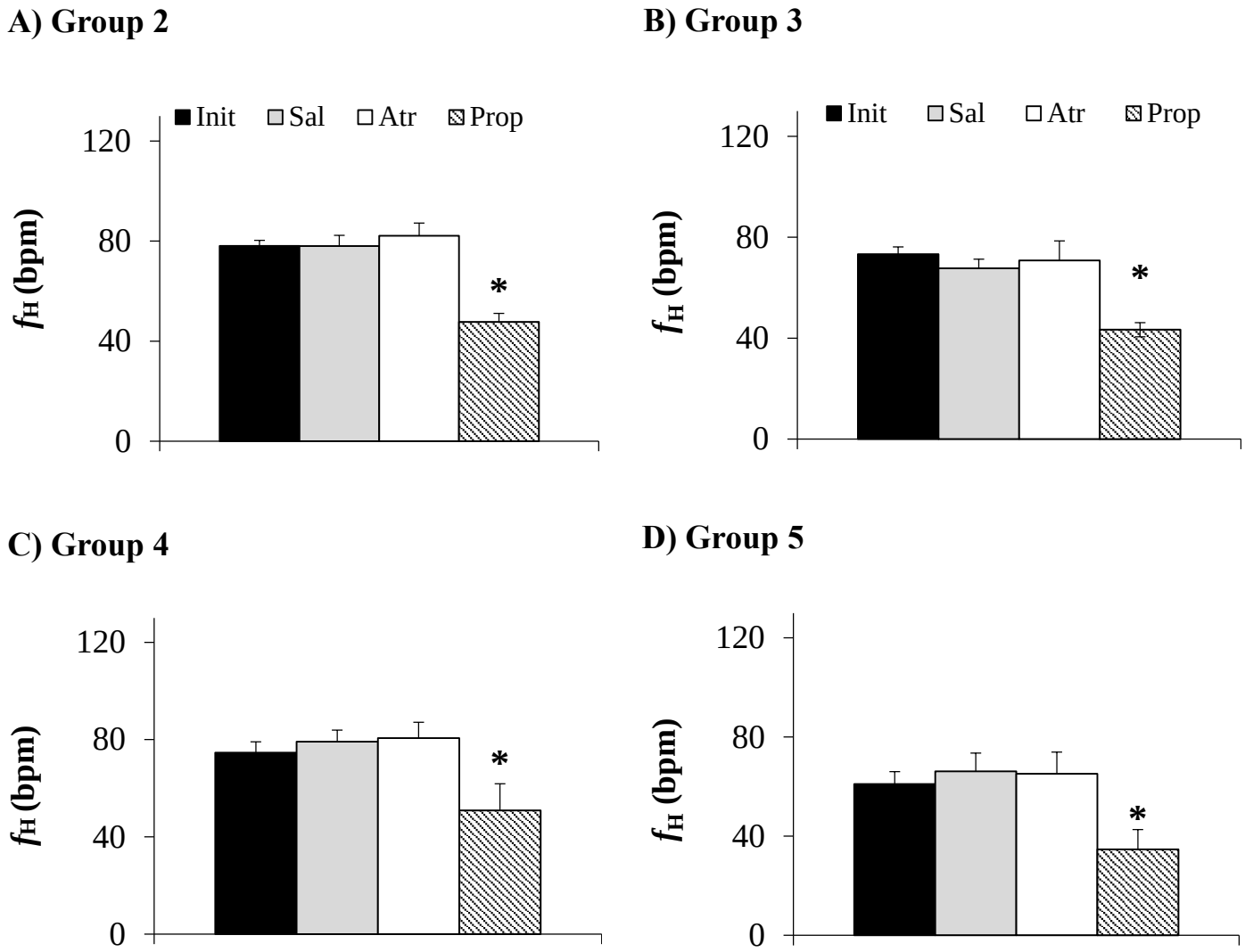

A) Group 2
B) Group 3
C) Group 4
D) Group 5
Init
Sal
Atr
Prop
f_H (bpm)
120
80
40
0
*

Figure 4

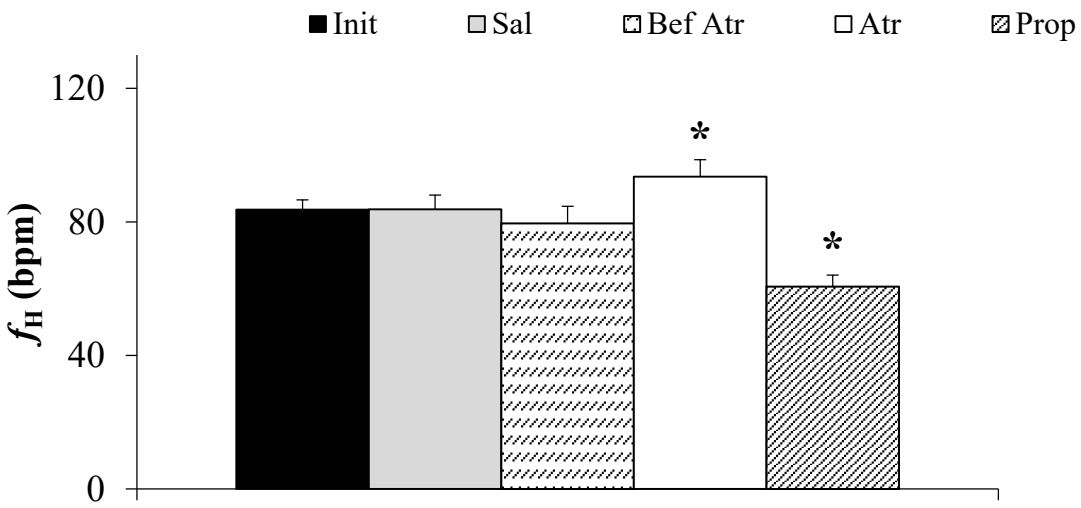


Figure 5

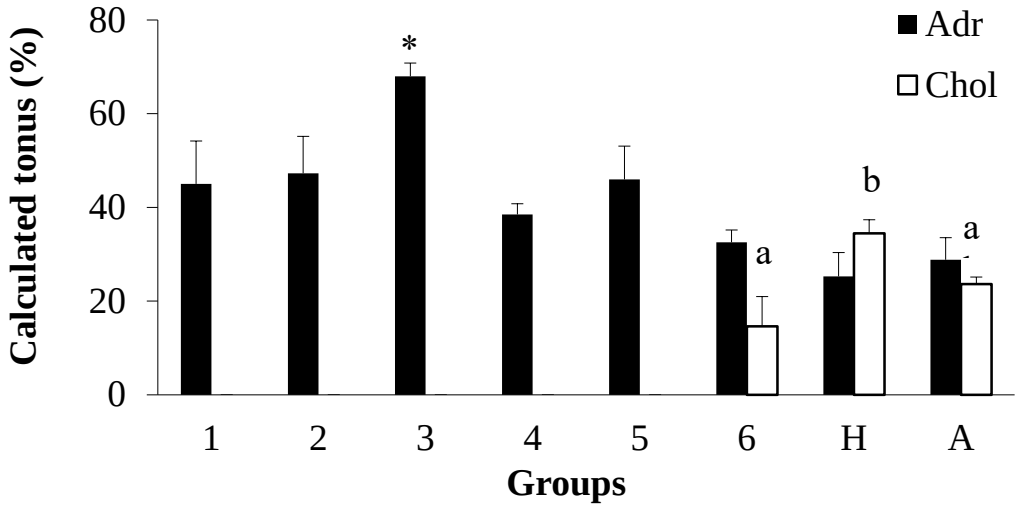


Figure 6

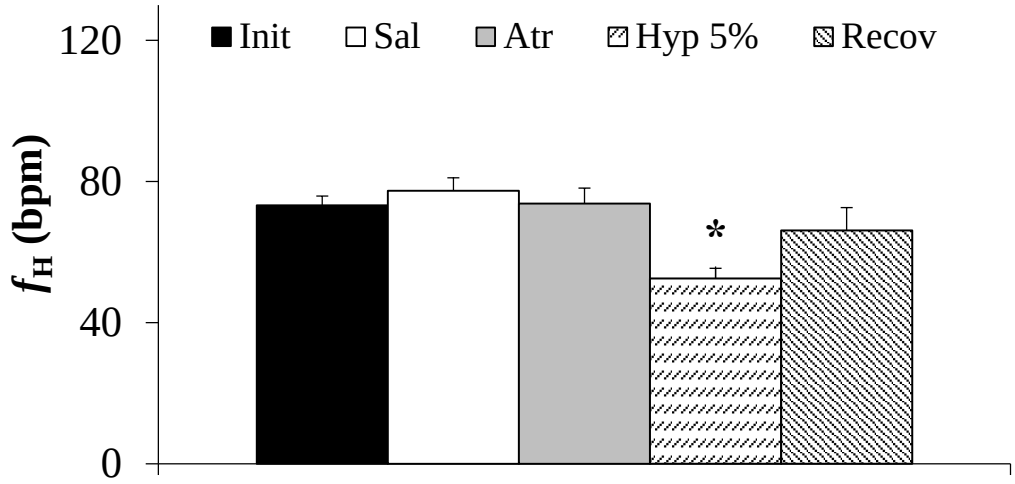
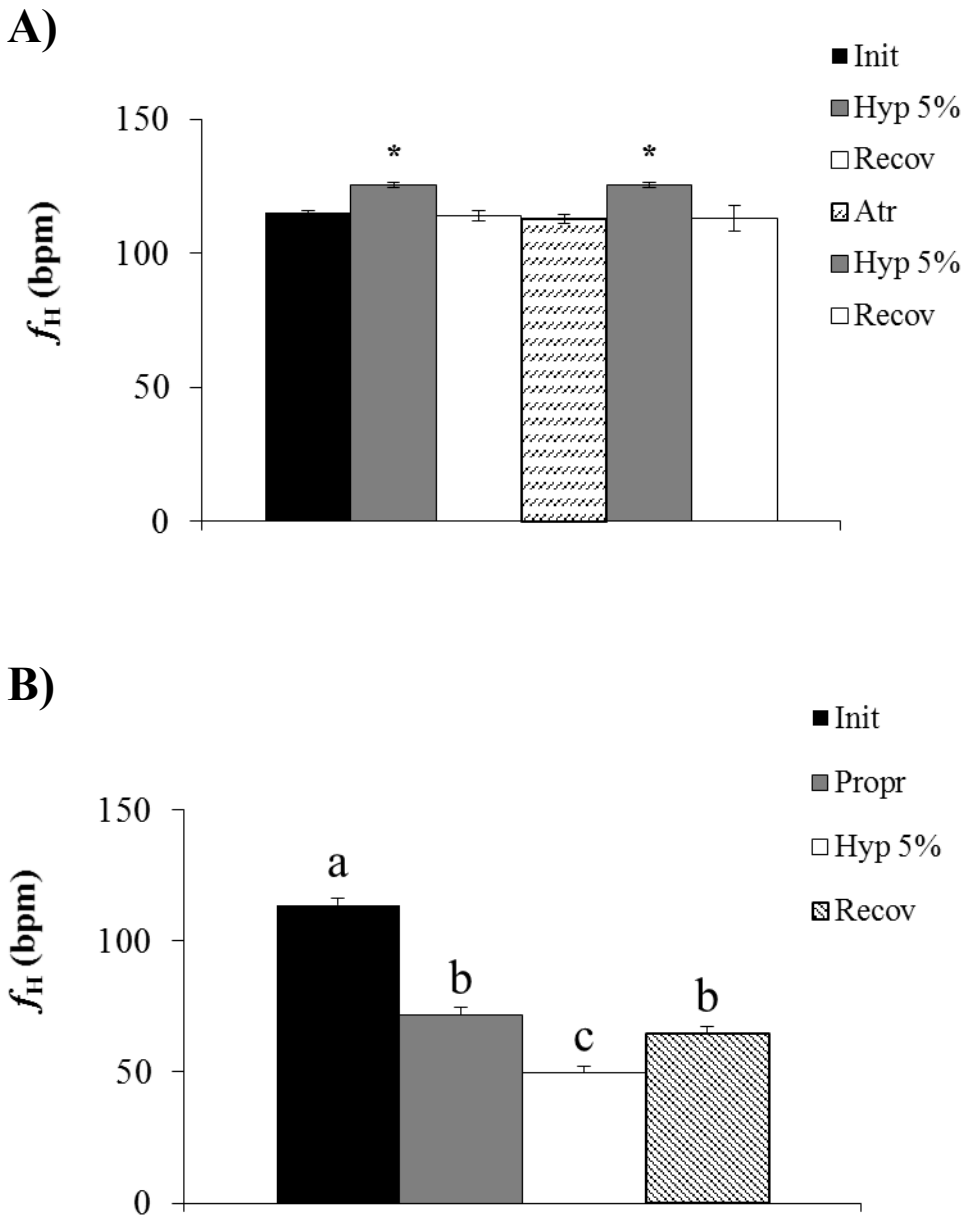


Figure 7


A)
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b

Figure 8

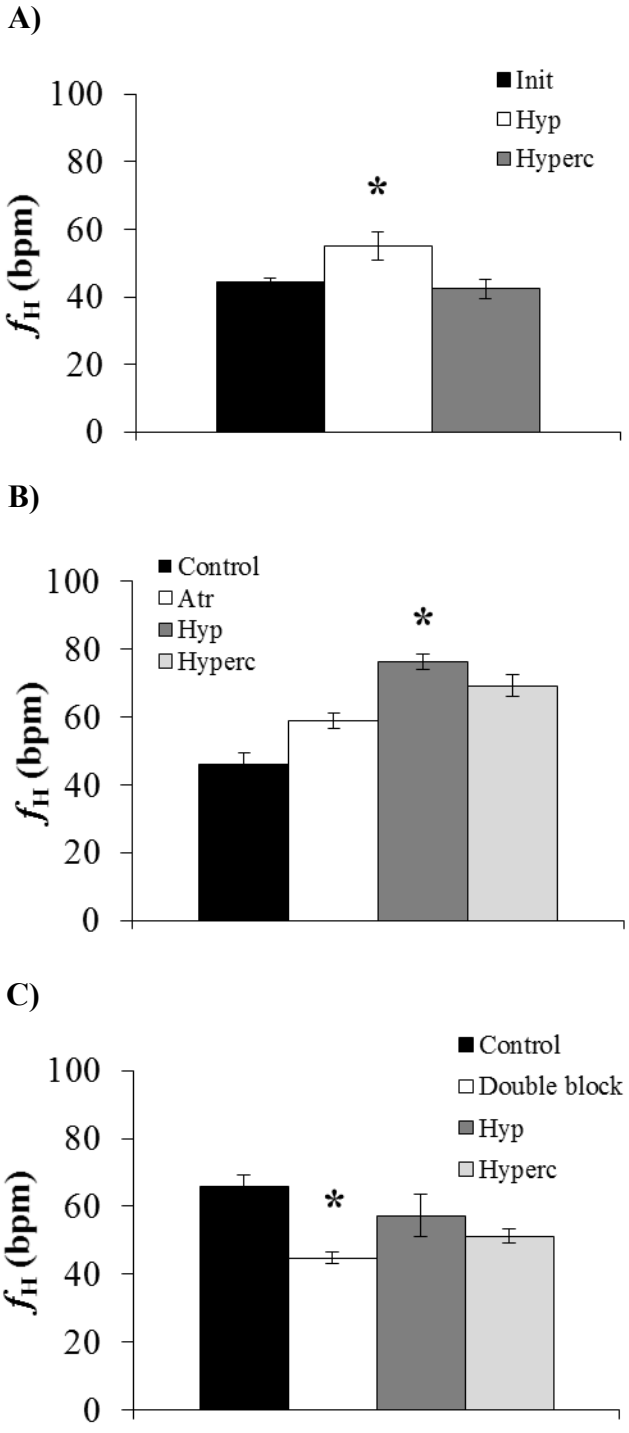
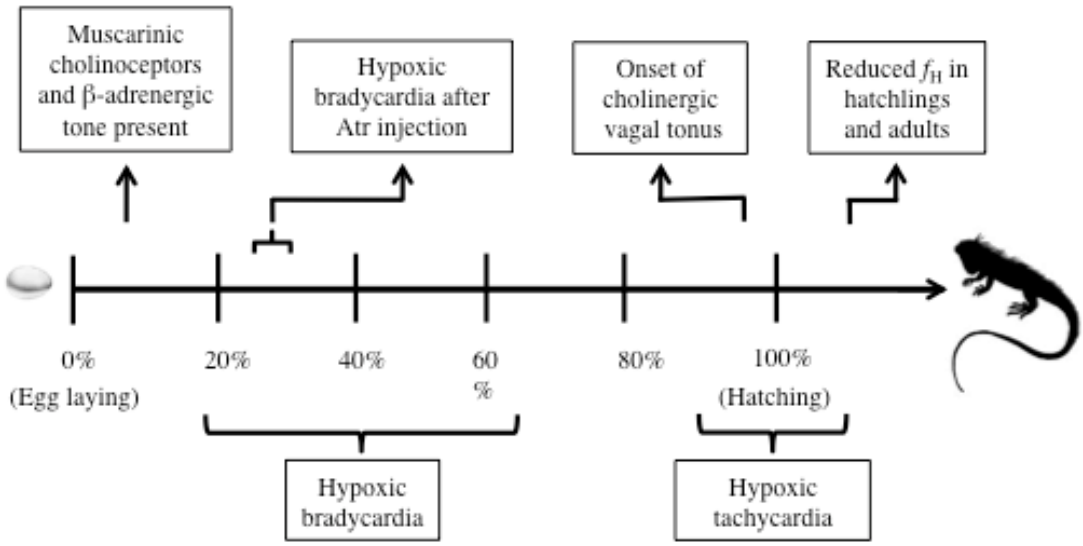

A)
Init
Hyp
Hyperc
f_H (bpm)
100
80
60
40
20
0
*
B)
Control
Atr
Hyp
Hyperc
f_H (bpm)
100
80
60
40
20
0
*
C)
Control
Double block
Hyp
Hyperc
f_H (bpm)
100
80
60
40
20
0
*

Figure 9


Muscarinic cholinoceptors and β-adrenergic tone present
Hypoxic bradycardia after Atr injection
Onset of cholinergic vagal tonus
Reduced f_H in hatchlings and adults
0% (Egg laying)
20%
40%
60 %
80%
100% (Hatching)
Hypoxic bradycardia
Hypoxic tachycardia

Capítulo V

**Manuscrito em preparação para publicação em revista a definir*

Controle autonômico em embriões e adultos do lagarto teiú *Salvator merianae*

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Resumo

Em geral, a frequência cardíaca (f_H) é controlada pela atividade do Sistema Nervoso Autônomo, que em embriões está em formação. Répteis adultos, quando em repouso, possuem tônus parassimpático predominante, o que mantém a f_H em níveis reduzidos. Durante a fase embrionária, a maior parte do período embrionário está sob influência do tonus simpático excitatório através de catecolaminas, mantendo f_H em níveis elevados, enquanto o tônus parassimpático inibitório tem início apenas ao final do período de incubação. Até o momento, a regulação da f_H durante o desenvolvimento embrionário de répteis foi estudada apenas em poucas espécies, e e entre os lagartos, em uma única espécie. Dessa forma, neste estudo avaliamos o padrão de f_H e sua regulação autonômica em uma espécie de lagarto da subordem Scleroglossa, o teiú, *Salvator merianae*. Os embriões apresentaram um aumento massivo em massa, porém, a f_H medida a 32°C, é decrescente na primeira metade da incubação, com média de 157 ± 2,2 bpm, e em seguida é mantida em um nível constante de 108 ± 0,9 bpm durante a metade final do desenvolvimento. O tônus adrenérgico excitatório exerceu um papel predominante ao longo de todo o desenvolvimento, variando de 21 a 48%, já que o tônus colinérgico em um nível de 11%, se tornou funcional apenas momentos antes da eclosão, o que

pode ter uma relação com a necessidade da interação entre o sistema cardiorrespiratórios no filhote. Já em adultos a 30°C, o tônus colinérgico aumentou para 70% e tornou-se predominante, comparado com o nível adrenérgico de 30%. O padrão de f_H e a regulação autonômica da frequência cardíaca foi similar ao encontrado no lagarto previamente estudado, pertencente ao clado Iguania.

Introdução

A ontogenia do desenvolvimento da regulação da frequência cardíaca (f_H) em répteis aos poucos tem sido mais explorada, porém continua inferior com relação à diversidade de estudos realizados com aves. Os primeiros estudos foram realizados com o jacaré americano *Alligator mississippiensis* (Crossley and Altimiras, 2005, Eme et al., 2011) e com a tartaruga *Chelydra serpentina* (Alvine et al., 2013), e um estudo recente acessou o desenvolvimento do lagarto *Iguana iguana* (Sartori et al., 2015a). No entanto, répteis são um grupo extremamente diverso, e suas ordens contemplam diferenças marcantes entre si e entre as diversas famílias que o compõem (Porter, 1972). O padrão de f_H embrionária em répteis parece refletir diferenças relacionadas com as ordens. Em Testudines, as espécies apresentam um declínio na frequência cardíaca durante a porção final do período incubatório, enquanto Squamata apresenta um valor constante e Crocodilianos um aumento da frequência durante a primeira metade da incubação (Taylor et al., 2014). Tal padrão de frequência cardíaca pode refletir o início da regulação autonômica, que por sua vez pode ter relações filogenéticas ou alguma influência ambiental.

Em comum os répteis e aves apresentam um constante tônus adrenérgico, presente ao longo de todo o período do desenvolvimento (Taylor et al., 2014), através da atuação de catecolaminas circulantes (Crossley and Altimiras, 2000; Eme et al., 2013). Porém diferenças quanto ao início e nível de controle colinérgico difere entre as espécies estudadas. No caso da tartaruga *C. serpentina*, foi verificado tônus colinérgico durante os 30% do período final de incubação (Alvine et al., 2013), enquanto no crocodiliano *A. mississipiensis* o tônus foi verificado apenas após a eclosão (Crossley and Altimiras, 2000) e em *I. iguana* o tônus foi verificado apenas no período antecedente à eclosão (Sartori et al., 2015a). No entanto, algumas tartarugas parecem não possuir controle autonômico durante o período de incubação (Crossley and Burggren, 2009; Crossley et al., unpublished) e estudos preliminares sugerem que a serpente *Boaedon fuliginosus* possui tônus colinérgico previamente à eclosão (Crossley and Burggren, 2009).

O presente estudo tem como foco elucidar o padrão de f_H sob temperatura constante e o controle autonômico do coração dos embriões do lagarto teiú, *Salvator merianae*. Os lagartos

fazem parte da Ordem Squamata, a mais diversa em número de espécies (Uetz, 2000). O teiú pertence à sub-ordem Scleroglossa, que engloba diversas famílias de lagartos e também serpentes, sendo considerado o clado irmão de Iguania (Conrad, 2008), cuja única espécie de lagarto já estudada quanto ao desenvolvimento do controle autonômico foi estudada (Sartori et al., 2015a).

Material e Métodos

Embriões de teiú

Os ovos de teiú (*Salvator merianae*) foram coletados em ninhos provenientes do Jacarezário, Departamento de Zoologia, UNESP, Rio Claro. O tempo de incubação destes ovos é em média de 60 dias. Os ovos foram incubados sob temperatura constante de 30ºC ± 1.5ºC, em caixas contendo vermiculita úmida, que eram checadas diariamente para saturação da umidade e viabilidade dos ovos.

Para determinar o padrão de f_H da fase embrionária ovos de diversas ninhadas foram selecionados a partir dos 5 dias pós oviposição (dpo) e medidos a cada 3 dias, até o momento da eclosão, próximo dos 61 dpo. Cada ovo foi pesado e em seguida a f_H monitorada com o monitor digital Buddy (Buddy System, Avitronics, Truro, UK) que utiliza luz infravermelha para detectar movimentos. Em nosso estudo anterior com ovos de iguana (Sartori et al., 2015b) detectamos um acréscimo de temperatura em consequência da propriedade de radiação de calor do equipamento. Em geral, a temperatura interna dos ovos estabiliza após 1,5 h e o acréscimo é de 2ºC acima da temperatura da incubadora. Dado que o tamanho dos ovos de teiús e iguanas são similares e o protocolo idêntico, assume-se que as f_H dos embriões de teiú refletem uma temperatura de 32ºC, após um período de estabilização de 2 h. O sinal de f_H adquirido foi acoplado ao software Powerlab (AD Instruments, Australia), que possibilita o registro e análise do sinal dos batimentos cardíacos e cálculo da f_H.

As idades selecionadas para o estudo de controle autonômico foram de 5, 15, 25, 35, 45, 55 dpo e próximo a eclosão, aos 59 dias, e foram denominados consecutivamente de grupo 1 a 7. Para os experimentos farmacológicos com embriões do grupo 1 (5 dias), os ovos foram abertos e o embrião removido em conjunto com a mebrana amniótica. Os batimentos cardíacos foram determinados sob estereomicroscópio, a temperatura ambiente, em torno de 28ºC. A frequência inicial foi determinada como a de repouso e em seguida uma série de injeções tópicas

foram realizadas na seguinte ordem: salina, acetilcolina (10^{-3} M), atropina (10^{-3} M) e propranolol (10^{-2} M).

Nos ovos a partir de 15 dias, a f_H de repouso foi determinada 2 h após os ovos serem colocados no monitor digital, no interior de uma incubadora a 30 ºC. Em seguida uma pequena janela foi realizada na casca dos ovos, expondo a localização do embrião e 0,2 ml dos antagonistas atropina (10^{-3} M) e propranolol (10^{-2} M) foram injetados próximos ao embrião. Nos estágios após 35 dias as injeções foram realizadas diretamente, após detecção do posicionamento do embrião, sem a abertura de janela na casca.

Teiús adultos

Teiús adultos durante a fase ativa após a estivação (n = 3, body mass = 3,12 kg ± 0,56) foram anestesiados com CO_2, em seguida entubados para serem mantidos anestesiados e mecanicamente ventilados (CWE, SAR-830/P Ventilator) inicialmente com 5% de isoflurano (BioChimico), diminuindo para 2% após a estabilização da anestesia. A veia femoral foi canulada para a injeção das drogas enquanto eletrodos de ECG foram colocados e suturados nas laterais (rostral e caudal ao coração) e o eletrodo referência no dorso. No local da cirurgia foi aplicado o anestésico local lidocaína (Pearson, SP, Brasil). Os animais foram colocados em caixas em uma sala mantida à temperatura de 30, durante as 24 horas de recuperação e durante a condução do experimento.

Análises estatísticas

Os efeitos dos testes farmacológicos para cada grupo de embriões e adultos, as massas dos ovos e embriões e a frequência cardíaca inicial ao longo do desenvolvimento foram testados pela ANOVA de uma via com o teste de Student-Newman-Keuls para comparações *post hoc*. Quando os dados não apresentavam normalidade ou variâncias homogêneas o teste utilizado foi o Kruskal Wallis com teste de Dunn´s. Todos os testes foram realizados utilizando-se o software SigmaPlot, com significância atribuída a 5% do nível de confiância (P=0,05).

Resultados

Os embriões de teiú de diferentes ninhadas se desenvolveram em um tempo médio total de 61,5 ± 2,1 dias. A massa dos ovos aumentou em duas vezes ao final da incubação devido a absorção de água. Os embriões aumentaram de 0,03 ± 0,01 g para 11,1 ± 0,1 g. A 32°C a f_H de embriões de teiú é elevada durante o primeiro quarto da incubação, em torno de 157 ± 2,2 bpm,

durante o segundo quarto é reduzida para um valor intermediário, e mantém-se constante durante a metade final da incubação, em torno de 108 ± 0,9 bpm (Fig 1A). A regressão entre a massa dos ovos e f_H não apresentou forte correlação, apresentando R^2 de 0,47 ($P < 0,001$; Fig. 1B).

Os resultados do ensaio farmacológico são apresentados na tabela 1. No grupo 1 (5 dpo) houve redução da f_H após a injeção de acetilcolina, e por demonstrar que logo no início do desenvolvimento já há indícios da presença de receptores no coração a acetilcolina não foi aplicada nos demais grupos. Em todos os grupos de embriões a f_H foi reduzida após a injeção de propranolol (10^{-2} M) e resultou em tônus adrenérgico calculados de 21 a 48%. Apenas o grupo 7, com embriões muito próximos à eclosão apresentou um aumento da f_H após a injeção de atropina, resultando em um tônus colinérgico calculado de 11%. Os tônus autonômicos adrenérgico e colinérgico foram calculados de acordo com Altimiras e colaboradores (1997).

Os adultos apresentaram à temperatura de 30ºC uma média de 11,6 ± 1,4 batimentos por minuto. Após injeção de atropina a f_H aumenta para 23,0 ± 2,6 bpm, representando um tônus colinérgico de 71% (Tabela 1, Fig. 5). Após a injeção de propranolol a f_H retorna para um valor de 16 ± 0,8 bpm, representando tônus adrenérgico de 29%. A figura 6 apresenta o resumo dos resultados obtidos.

Discussão

O padrão de frequência cardíaca em embriões de teiús apresentou um nível elevado no início da incubação, decaindo progressivamente até um valor que se manteve constante ao longo da metade final do período incubatório. Este padrão é similar ao encontrado na serpente *Lamprophis fuliginosus* (Crossley and Burggren, 2009) *e Iguana iguana* (Sartori, em preparação). A estabilização da f_H também ocorre durante a maior parte do período de incubação em outras espécies de Squamata como os lagartos *Pogona henrylawsoni* (Crossley and Burggren, 2009) e *Bassiana duperreyi* (Radder and Shine, 2006). Há um forte indício de que o padrão relativamente uniforme de f_H ao final do desenvolvimento seja um padrão típico de Squamata porém os fatores que influenciam nos diferentes padrões de f_H em répteis ainda não foram elucidados.

Os dados de regulação autonômica obtidos com os embriões de lagartos teiús corroboram os dados do lagarto *Iguana iguana* (Sartori et al., 2015a). Há indicação de que os embriões a partir de 5 dias já possuem receptores colinérgicos muscarínicos no coração assim como o início do tônus colinérgico ocorre apenas quando muito próximo ao momento de

eclosão. No caso dos adultos, os teiús apresentaram tônus adrenérgico similar ao de embriões, mesmo submetidos a uma temperatura inferior de 30°C. No entanto, teiús adultos possuem o fator adicional da sazonalidade e depressão metabólica nos meses de clima seco e frio (Andrade et al., 2004), o que pode ser um fator na variação do tônus colinérgico dependendo do período analisado. Considerações acerca das diferenças de temperatura entre o primeiro estágio embrionário, os estágios subsequentes e os adultos e também com relação às formas de aplicação dos fármacos se encontram em Sartori e colaboradores (2015a) já que o protocolo adotado foi similar ao utilizado em embriões de iguana.

De todos os répteis estudados até o momento, apenas a espécie de tartaruga *Chelydra serpentina* apresentou um evidente controle colinérgico antecipado, que ocorre a partir dos 30% final do período incubatório. As demais espécies apresentaram o início do tônus vagal apenas próximo à eclosão, como o iguana anteriormente mencionado, a tartaruga *Gopherus agassizi* (Crossley and Burggren, 2009) e o jabuti *Chelonoidis carbonaria* (Crossley et al., 2013). Esse pode ser o caso das demais espécies, os tigres d'água *Trachemys scripta* e *Trachemys dorbigni* (Sartori et al., não publicado, Taylor et al., 2014) que não foram analisadas nos períodos próximos à eclosão. No caso de Squamata, o padrão encontrado até o momento, nas espécies pertencentes ao clado Iguana e Scleroglossa foram similares.

Em adultos o tônus colinérgico vagal é relacionado com o controle das interações cardiorrespiratórias, tanto em mamíferos quanto em répteis (Taylor et al., 1999). Como em embriões de répteis o início do tônus colinérgico parece ocorrer justamente antes do pulmão se tornar funcional, no momento em que o filhote eclode. Esse adiamento da atuação do tônus inibitório pode ter relação com o início dos movimentos ventilatórios e com a necessidade do controle das interações entre os sistemas cardíaco e respiratório (Sartori et al., 2015a). Neste momento de troca da respiração através da membrana corioalantóica para a ventilação pulmonar é possível que se inicie uma interação entre as frequências cardíaca e respiratória, de forma a garantir uma troca efetiva de gases, de forma rápida e eficiente, no animal livre.

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Tabela 1. Dados dos grupos de embriões e adultos de lagartos teiú (Salvator merianae), indicando idade, n amostral, massa do ovo e corpórea e frequência cardíaca (f_H) de repouso e após tratamento farmacológico e consequente tônus autonômico calculado.

Grupo	dpo	N	Massa (g)		f_H (bpm)			% tonus	
			Ovo	Corpórea	Repouso	pós Atr	pós Bloq Duplo	Adr	Chol
1	5	8	12,7 ± 0,2	0,03 ± 0,003	74 ± 5	60 ± 5	47 ± 3	21 ± 2	-1,8 ± 2
2	15	7	21 ± 0,3	0,48 ± 0,009	135 ± 3	126 ± 4	85 ± 11	33 ± 7	-4 ± 1
3	25	8	21,4 ± 1,1	1,0 ± 0,2	128 ± 2	129 ± 2	79 ± 12	39 ± 8	-0,1 ± 1
4	35	8	27 ± 0,3	2,5 ± 0,2	112 ± 3	112 ± 3	59 ± 7	48 ± 6	0,7 ± 1
5	45	8	24,6 ± 0,4	6,6 ± 0,2	105 ± 4	105 ± 5	59 ± 7	44 ± 5	-0,4 ± 2
6	55	8	28,1 ± 0,5	13,3 ± 0,3	107 ± 3	110 ± 3	71 ± 6	35 ± 5	2,3 ± 2
7	60	6	23,5 ± 0,9	11,2 ± 0,05	106 ± 4	123 ± 5	83 ± 5	33 ± 3	11 ± 2
Adulto	-	3	-	3200 ± 560	11,6 ± 1,4	23,0 ± 2,6	15,8 ± 0,8	29 ± 7	71 ± 20

Legendas das figuras

Fig. 1: A) Média da frequência cardíaca de repouso (± erro padrão) ao longo do período de incubação, a partir de 5 dpo até 61 dpo, em intervalos de 3 dias, à temperatura de 32ºC; B) Regressão linear da f_H e massa dos ovos.

Fig. 2: Média (± erro padrão) dos valores de frequência cardíaca (f_H) de embriões do grupo 1 (5 dpo, N = 8), a temperatura ambiente. Inicial (Ini) representa o valor de f_H de repouso apresentado pelos embriões logo após sua remoção dos ovos, em seguida após aplicação de salina (Sal), acetilcolina (Ach), atropina (Atr) e propranolol (Propr). Ach e Prop causaram diminuição significativa na frequência cardíaca ($P < 0,001$). Atr anulou o efeito da Ach.

Fig. 3: Média (± erro padrão) dos valores de frequência cardíaca (f_H) de embriões dos grupos 2, 3, 4 e 5 (15, 25, 35 e 45 dpo, respectivamente). Inicial representa o valor de frequência cardíaca apresentado pelos embriões após 2 h de estabilização a uma temperatura interna de 32ºC. Os valores subsequentes representam as injeções de salina (Sal), atropina (Atr) e propranolol (Prop). Prop causou redução significativa da f_H em todos os grupos ($P < 0,001$).

Fig. 4: Média (± erro padrão) dos valores de frequência cardíaca (f_H) inicial e após a sequência de tratamento farmacológico de embriões nos grupos 6 e 7, estágios finais e próximos da eclosão (55 e 60 dpo, respectivamente). Propranolol causou reduções significativas da f_H nos dois grupos ($P < 0,02$) porém a atropina provocou o aumento da f_H apenas no grupo 7, de 60 ($P < 0,015$).

Fig. 5: Média (± erro padrão) de tônus adrenérgico (barras brancas) e colinérgico (barras pretas) nos diferentes grupos embrionários (32ºC) e em teiús adultos (30ºC).

Fig. 6: Linha do tempo de eventos do coração durante o período de incubação de embriões de teiú, desde o momento da desova (0%) até o momento da eclosão (100%). Há evidência da presença de receptores colinérgicos muscarínicos e de tônus adrenérgico em embriões no início do desenvolvimento. O tônus colinérgico inicia próximo ao período da eclosão e proporciona valores de f_H reduzidos em filhotes e adultos.

Fig. 1

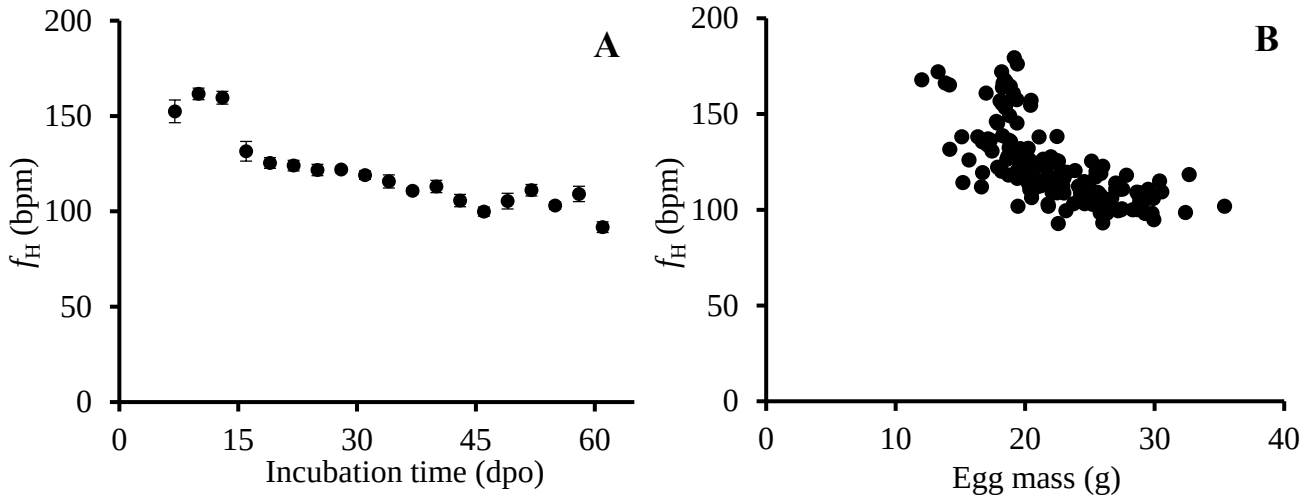

A
B
200
150
100
50
0
f_H (bpm)
0
15
30
45
60
Incubation time (dpo)
0
10
20
30
40
Egg mass (g)

Fig. 2

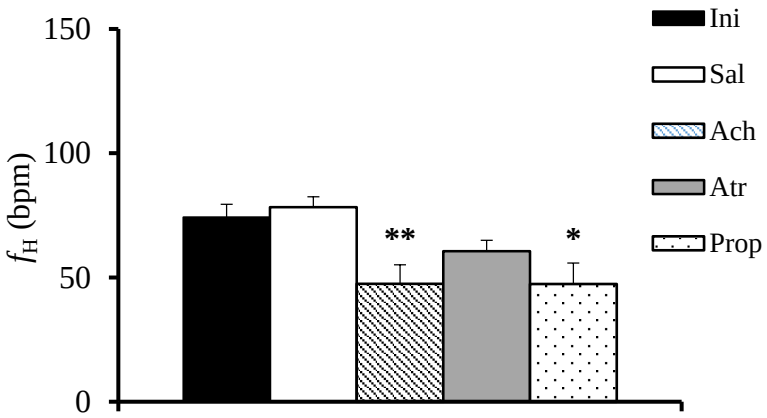

150
100
50
0
f_H (bpm)
**
*
Ini
Sal
Ach
Atr
Prop

Fig. 3

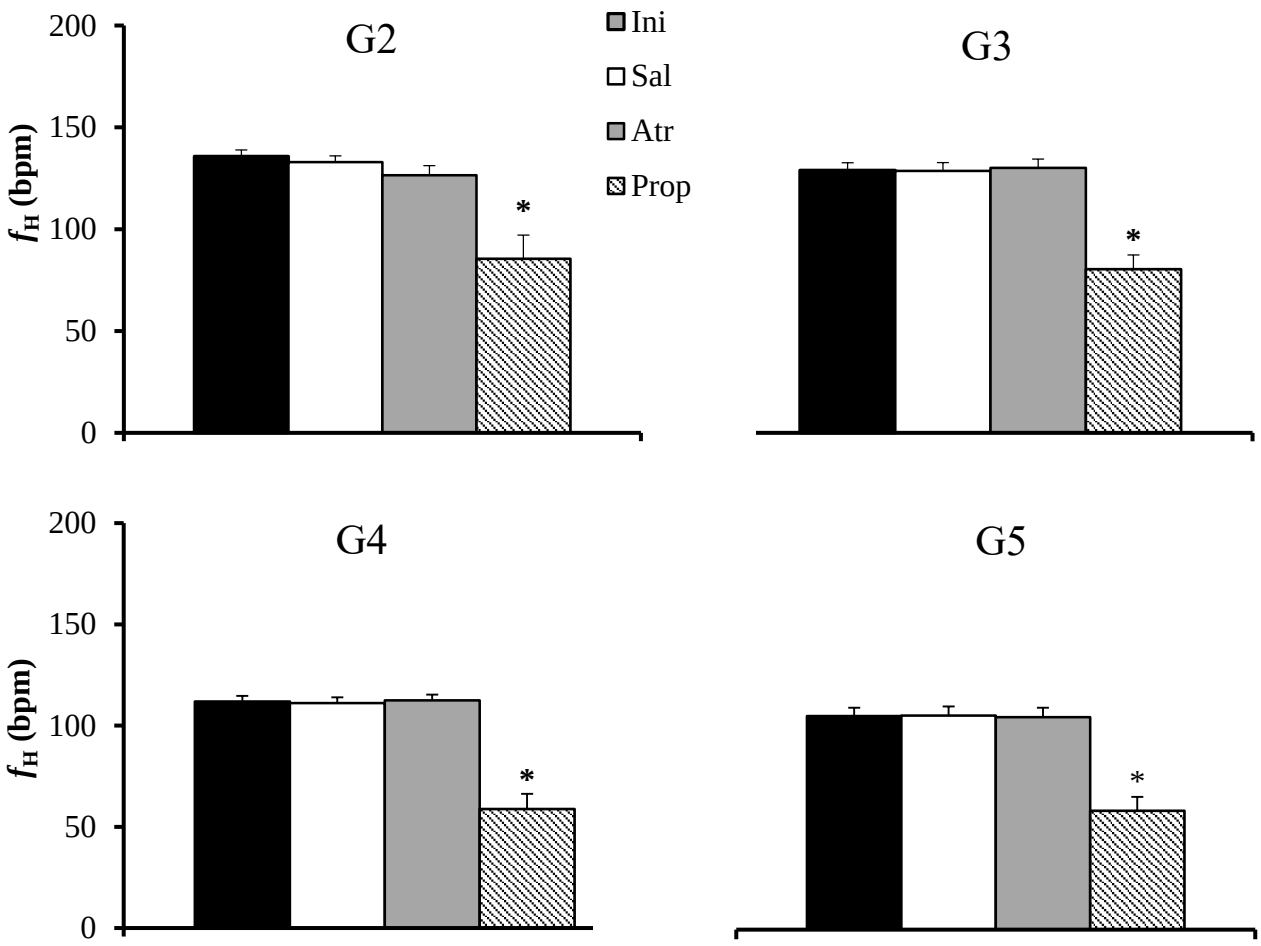


Fig. 4

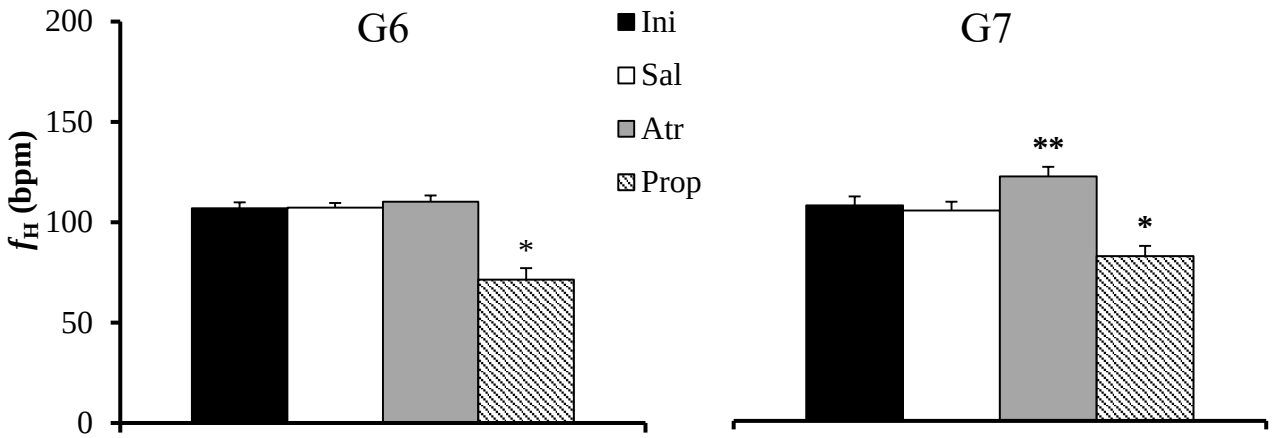

G6
G7
Ini
Sal
Atr
Prop
f_H (bpm)
200
150
100
50
0
*
**
*

Fig. 5

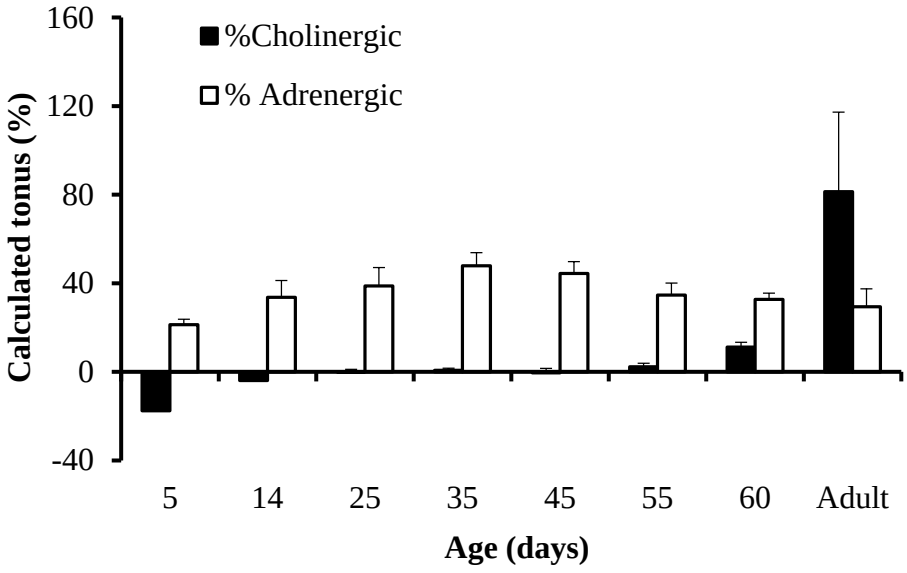
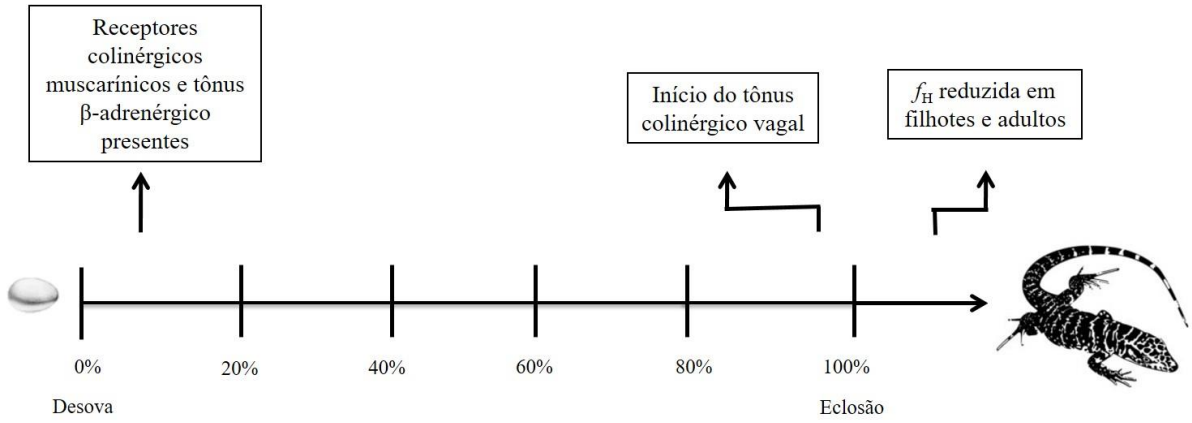

%Cholinergic
% Adrenergic
Calculated tonus (%)
160
120
80
40
0
-40
5
14
25
35
45
55
60
Adult
Age (days)

Fig. 6


Receptores colinérgicos muscarínicos e tônus β-adrenérgico presentes
Início do tônus colinérgico vagal
f_H reduzida em filhotes e adultos
0%
20%
40%
60%
80%
100%
Desova
Eclosão

Conclusões gerais

Ressaltamos que este trabalho foi o primeiro a determinar a ontogenia do controle autonômico de lagartos, com o diferencial de acessar embriões desde o início do período de incubação, a partir de aproximadamente 5 dias após a oviposição. Ademais, o conjunto de estudos relacionados com o desenvolvimento cardiovascular e o metabolismo de embriões de lagartos revelou que:

- O monitor cardíaco digital Buddy ao ser utilizado para medições de longa duração promove o aquecimento dos ovos, o que causa um efeito direto na f_H. Esse efeito deve ser determinado através da medição interna da temperatura dos ovos ou pela realização de leituras instantâneas.
- O padrão de f_H presente em embriões de iguanas apresenta um padrão constante ao longo do desenvolvimento, que aparenta ser compartilhado entre as espécies do grupo Squamata. O padrão de metabolismo embrionário apresenta uma curva sigmóide, semelhante ao de algumas espécies de lagartos, porém, distinto do padrão de serpentes, relatado como exponencial.
- A f_H embrionária não possui uma forte associação com taxas metabólicas ou de crescimento, não devendo ser utilizada como um índice de desenvolvimento embrionário. A taxa metabólica e o pulso de oxigênio são fortemente correlacionados entre si e com o crescimento, podendo ser utilizados como indicadores de desenvolvimento.
- A temperatura afeta diretamente a f_H e é independente do estágio de maturação embrionária. Os ninhos enterrados no solo fornecem um ambiente protegido contra grandes variações térmicas, minimizando as flutuações diárias.
- Apesar da presença de receptores muscarínicos colinérgicos e β-adrenérgicos no coração embrionário de iguanas desde estágios logo após a oviposição, a completa inervação parece ocorrer apenas ao final do período de incubação. Dessa forma a presença de tônus adrenérgico excitatório durante todo o período de incubação de iguanas, se deve à provável atuação de catecolaminas circulantes. O tônus colinérgico inibitório tem início um período muito próximo à eclosão, possivelmente relacionado com início do ritmo ventilatório.
- Embriões de iguanas submetidos a condições de hipóxia aguda apresentam bradicardia, quando no início do desenvolvimento, indicando um efeito direto no

coração e taquicardia, quando em estágios mais avançados do desenvolvimento, indicando a atuação de catecolaminas.

Perspectivas futuras

Os trabalhos realizados ampliaram a perspectiva sobre o desenvolvimento da fisiologia cardiovascular, sua regulação e sua relação com o metabolismo, possibilitando novas abordagens tais como:

- determinação do padrão de f_H, VO_2 e controle autonômico em um maior número de espécies de Squamata, para confirmar possíveis associações filogenéticas ou ambientais; por exemplo, embriões de serpentes apresentam padrão distinto de VO_2 porém não há dados sobre o padrão de f_H e controle autonômico no período embrionário.
- avaliação da atuação de outros fatores não colinérgicos não adrenérgicos (NANC), que atuam no controle cardiovascular ao longo do desenvolvimento embrionário de répteis;
- investigação de ajustes cardiovasculares durante o desenvolvimento embrionário, como volume sistólico, débito cardíaco e diferença arteriovenosa de conteúdo de oxigênio, assim como alterações em fatores que afetam a capacidade de transporte de oxigênio do sistema cardiovascular como concentração de hemoglobina, curvas de afinidade da hemoglobina, etc.
- avaliação dos efeitos de longo prazo de incubações a diferentes temperaturas, por exemplo sob temperaturas flutuantes, ou sob diferentes níveis de gases respiratórios como hipóxia e hipercapnia;
- determinação de janelas crítcas (“critical windows”), os períodos de maior vulnerabilidade à fatores ambientais, como mudanças de temperatura e níveis de gases respiratórios, durante o desenvolvimento embrionário, que influenciam a sobrevivência e os fenótipos dos filhotes.

Apêndice A

*Manuscrito em preparação referente ao estudo realizado durante o estágio no exterior (BEPE).

Oxygen provision to developing embryos of the snapping turtle *Chelydra serpentina*

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ABSTRACT

We have measured the variables determining the provision of oxygen to tissues of developing embryos of the snapping turtle, *Chelydra serpentina.* Measured variables included: heart rate (f_H); oxygen consumption (VO_2) and arterio-venous oxygen difference (A-V diff). Using the Fick equation we calculated cardiac output (Q). Relative blood flow (%Q) to the embryo and to the chorioallantoic membrane (CAM) was measured using microspheres. *In vitro* techniques on blood samples provided O_2 carrying capacity ($Ca_{Tot}O_2$); O_2 affinity curves to yield P_{50}; and hemoglobin content [Hb]. We measured these variables in embryos at 50, 70, and 90% of the incubation period. An exponential increase in body mass is paralleled by an increase in the mass of the heart and increased metabolic rate, measured as oxygen uptake. f_H did not change from 50% to 70% of incubation but was significantly reduced at 90%. [Hb] did not change but A-V diff doubled from 50 to 90% of incubation. P_{50} values revealed an increased affinity under 2% CO_2 and a decreased affinity under 6% CO_2 at 70 and 90% incubation. %Q to the embryo decreased from 50 to 70% incubation whereas it increased to the CAM that provides the surface for respiratory gas exchange across the eggshell. We conclude that embryos rely on an optimized binding of oxygen to Hb across the CAM and release of O_2 to the embryonic tissues during the latter stages of incubation, possibly ensured by different isoforms or allosteric effects on the hemoglobin.

INTRODUCTION

Most reptiles develop inside amniotic eggs, that are laid in nests dug in the soil, holes, or under molds, left with no parental care, thus, susceptible to changes in the environmental conditions (Ackerman and Lott, 2004). Several studies evaluated the effects of environmental hazards, i.e. temperature (Booth, 1998; Du and Ji, 2006, Du et al., 2010a), water restriction (Gettinger et al., 1984; Du and Shine, 2008) and respiratory gases levels (Booth, 2000; Crossley and Altimiras, 2005, Du et al., 2010; Marks et al., 2013; Eme and Crossley, 2015) on the physiology of the embryo. Yet, in contrast to the avian model, reptilian physiology under relatively "normal" conditions, are not completely understood.

Embryonic tissue growth and differentiation results in an increasing demand for oxygen during development, and in some species the metabolic rate of the pre hatch embryo is greater than those of the hatchling (Aubret, 2013; Du et al., 2010b), despite of embryos having no lung ventilation. Embryonic oxygen delivery within the developing cardiovascular systems need to match the metabolic demand of various tissues as they progress through development. At very early stages, matching of supply and demand is accomplished through diffusive steps alone (Burggren, 2004). With the increasing distances between metabolically active tissues and oxygen sources and the development of the chorioallantoic membrane (CAM) the provision of oxygen to developing embryos is replaced and dependent upon the PO_2 difference between the atmospheric air and blood vessels of the CAM in close contact to the eggshell plus convective transport in the embryonic circulation. By diffusion, oxygen moves from the surrounding air through the eggshell pores, traverse the inner shell membrane and reach the allantoic veins (Piiper et al., 1980; Corona and Warburton, 2000) combining reversibly with the hemoglobin contained in embryonic red blood cells. The blood embryonic heart pumps the blood to the metabolizing tissues, where the oxygen is unloaded from the hemoglobin (Burggren and Territo, 1995).

Possible mechanisms to supply increased oxygen demand include changes in heart rate or stroke volume in order to increase blood flow (Pearson et al., 1966), increased hematocrit and hemoglobin content, and changes in oxygen affinity of hemoglobin (Tazawa and Mochizuki, 1977). Evidences suggests that heart rate does not increase concomitantly with the increase in metabolic rate, and that the increase in heart mass may indicate an increase in stroke volume (Sartori et al., unpublished). Therefore, we investigated the properties of embryonic oxygen consumption and delivery in a reptilian species, the snapping turtle *Chelydra serpentina*. In order to understand the dynamics and adjustments of oxygen convective transport

in embryos, we measured oxygen consumption ($\dot{V}O_2$), heart rate (f_H), blood oxygen content (CaO_2 and CvO_2) and relative blood flow (%Qsys) at three different points of incubation. We predict that along development embryos will show increasing levels of metabolic rate, accompanied by increases in heart mass but not heart rate, supported by increasing levels of A-V difference and blood flow directed to the embryo.

MATERIAL AND METHODS

Eggs from snapping turtles were collected in northwestern Minnesota and transported to the Biology Department at the University of North Texas. Upon arrival, eggs were numbered, weighed and placed in plastic boxes (approximately 3 l of volume) containing vermiculite mixed with water in a 1:1 ratio by mass. Boxes were set in plastic ziploc bags ventilated with humidified atmospheric air to maintain the oxygen and water saturation at adequate levels. The bags were allocated in walk-in environmental chamber set to 30°C. Water content of vermiculite was maintained by weighing boxes twice a week and adding water as needed. Eggs were taken from incubators at 50%, 70% and 90% of a total incubation time of approximately 53 days, weighed and assigned for measurements of heart rate, $\dot{V}O_2$ and blood collection.

Embryonic heart rate (f_H)

Two wire electrodes were implanted through the shells of the eggs (N=6) to detect the beating signal of the heart via an impedance converter (UFI 2991, Morro Bay, CA). The signal output was recorded using LabChart Pro (V 7.2, AD Instruments, Colorado Springs, CO, USA), with a range of 1 to 5 V. The eggs were maintained in an environmental chamber at 30 ± 0.5°C. Measurements were recorded until stabilization, up to an hour, and the average of three values of f_H determined over the final 15 minutes was taken for each incubation period.

Embryonic oxygen consumption

Individual eggs (N = 6) were enclosed in hermetically sealed jars of 67 ml mean volume with two ports connected to three-way stopcock valves, which were closed for a period from one to four hours, depending of the stage of the embryo. We collected three samples from each egg using a 60 ml syringe that was connected to one side of the stopcock valve to withdraw a 20 ml gas sample. Between samples the jars were opened to restore gases concentration. Each sample was pumped through a Drierite column (W.A. Hammond Drierite Co. Ltd., Xenia, OH, USA) before reaching an O_2 (S-3A1) and CO_2 analyser (CD-3A, Thermox, Ametek, Berwyn, PA, USA) connected in series. The O_2 depletion and the CO_2 increase were recorded as

percentages with the Power Lab, and mean $\dot{V}O_2$ was calculated based on residual volume and time. Mass-specific $\dot{V}O_2$ was calculated based on embryo mass.

Oxygen Content

Eggs were candled to locate a tertiary CAM artery (that carry mixed oxygenated and deoxygenated blood) or vein (that carry oxygenated blood) for cannulation. Eggs were placed in Pyrex bottle caps insulated with cotton and set in temperature-controlled surgical chambers at 30 ± 0.5°C. A portion of the eggshell was removed and the targeted chorioallantoic artery or vein was occlusively cannulated under a dissection microscope (Leica MZ6, Leica Microsystems, Waukegan, IL, USA) using a heat-pulled polyethylene tube (PE-50) filled with 0.9% NaCl saline with 50 IU ml^{-1} heparin, as previously described (Crossley and Altimiras, 2000). The cannula was fixed to the eggshell with cyanoacrylate glue, and a blood sample (approximately 50-80 µl) taken with a 100 ul Hamilton syringe for analysis.

For the oxygen content analyses, a fraction of 8-20 µl of the blood sample was immediately inserted in a Tucker chamber (1.52 ml volume) filled with degassed ferrocyanite solution, maintained at 30ºC (± 0.5°C) via a circulating water bath. The oxygen sensor was connected to a gas analyser (Radiometer, Copenhagen, Denmark) and calibrated with the degassed Tucker solution according to the atmospheric pressure. Three oxygen partial pressure (PO_2, torr) values were recorded, one immediately before the injection of the sample in the chamber, and the others at the subsequent one and two minutes after injection and mixture of the blood sample with the degassed solution. The difference between the initial PO_2 value and the mean of the first and second minute values was used for the calculation of oxygen content (ml O_2 100 ml^{-1} blood) according to the equation proposed by Tucker (1967).

After all measurements and blood collection, embryos were euthanized in an atmosphere of vaporized isoflurane and embryo (M_b), heart (M_h), liver and yolk mass were measured to the nearest 0.01 g with an electronic balance. Embryonic stage was confirmed by comparison to the staging table of Yntema (1968) for *Chelydra serpentina* embryos.

Microspheres injection and processing

For the measurement of regional blood flow distribution we used colored polystyrene microspheres of 15 µm in diameter (Dye Track, Triton Technologies, San Diego, CA, USA), bigger in size than the red blood cells of the snapping turtle, in which minimum reported sizes are 21.6 µm in length and 12.5 µm in width (Frair, 1977). The basic concept of the technique consists of the injection of microspheres into the circulation where they were homogeneously

mixed in the blood and distributed throughout the vasculature. Upon reaching a capillary bed they become lodged in the tissue as they are not able to pass because of their larger diameter. The microspheres are trapped in the tissue beds in direct proportion to the volume of blood flowing to each tissue at the time of the central microsphere injection and are recovered and quantified according to the method outlined below.

For the injections, a tertiary vein of the CAM was catheterized. For 70 and 90% of incubation a tertiary artery of the CAM was also catheterized, allowing monitoring of arterial pressure. Microspheres were suspended in 0.9% saline containing 0.05% Tween 80 to prevent agglomeration of the microspheres at a final concentration of 1,500 spheres μl^{-1}. Due to embryonic volume load limitation a total of 52,500 yellow microspheres were injected at 50% incubation and 75,000 at 70% and 90% of incubation, after a period of stabilization from the microsurgery (aprox. 1 hour). The injections were performed with a 100 µl glass Hamilton syringe into the catheterized vein. After the end of the protocol a blood sample was withdrawn in order to detect if any microsphere remained into the circulation. Embryos were euthanized with an intravenous dose of 50 µg kg^{-1} pentobarbital. Whole embryo, CAM, and yolk membrane were immediately dissected, weighed and stored in either 15 or 50 ml falcon tubes.

We opted to use the sedimentation method, described in detail in materials provided by Triton, Inc., with microspheres. The first step was tissue digestion, that began immediately after dissection with the addition of 7 or 20 ml of 1 M KOH (Sigma Aldritch, St. Louis, MO) into the tissue tubes, depending on total volume. Before any further steps were attempted, 10,000 Blue microspheres were added to each tube as a process control, later used to calculate the % of microspheres lost during sample processing for recovery of microspheres. Three new empty centrifuge tubes were prepared with 10,000 Blue process control microspheres, subjected to the same steps as the samples tubes and used as a 100% recovery standard.

Tissues were sonicated to accelerate digestion using an ultrasonic homogenizer (Fisher Scientific, MA, USA), at 70% of full power for 30 s. The tubes were then filled with distilled water, vortex mixed and centrifuged at 1,500 G for 15 min (Marathon Centrifuge 3200R, Fisher Scientific, MA, USA). If, after centrifugation, there was remaining undigested tissue, the supernatant was aspirated to a level safely above each visible pellet and the alkaline digestion step repeated as many times as needed until no tissue remained visible. Tissues containing calcified tissue were set to an additional step, in which we added bone digesting reagent (0.12% EDTA, 5.38% HCl, 94% H_2O), also as many time as needed until no undigested tissue was visible.

When all tissue samples were digested satisfactorily, the samples moved to the second

step. The remaining pellet was re-suspended with 10% triton X-100 and vortex-mixed. The tubes were again centrifuged for 5 minutes at 1,500 G. The supernatant was aspirated and the tubes re-suspended with acidified ethanol (AE). The samples were strongly vortex mixed and then centrifuged at 1,500 G for 5 minutes. This step was repeated once again and the remaining supernatant was aspirated, leaving only a small amount of the AE over the pellet. Tube lids were left off overnight inside a fume hood to facilitate evaporation of remaining AE.

For tissues that did not digest after several repetitions of sonication, vortexing, and alkaline digestion, particularly yolk and whole embryo samples, we used the vacuum filtration method. In this method we used a 25 mm diameter 10 µm pore size filter (Triton Technology Part #31079) between an upper graduated cylinder and a filter screen. Using this method, insoluble tissue components are broken down into a very fine suspension of particles that can pass through the filter while microspheres, being 15 µm in diameter, are trapped. Trapped microspheres were washed with distilled water and ethanol and allowed to dry. The filter was then carefully transferred to a 15 ml falcon tube and cellosolve acetate was added. After soaking for at least one hour, with vortex-mixing every 15 minutes, the dye concentration was determined in the same manner as sedimented samples, described below.

After drying of sedimented pellets and filters, 250 µl of acidified cellosolve acetate (Sigma Aldrich, St. Louis, MO) was added to each tube and let stand for at least 15 min to allow the complete elution of the colored dye from the microspheres. The tubes were vortex-mixed again, then centrifuged for 5 min at 1,500 G to form a pellet of blanched microspheres and any remaining debris. 175 µl of the supernatant solution above the pellet was carefully drawn from the sample tube and transferred to a Crystal 96-well plate for dye analysis through a spectrophotometer (Synergy H1, Biotek, VT, USA) under the absorbance lengths of 650 nm for blue spheres and 440 nm for yellow.

To calculate total microspheres and recovery rates for each sample we entered the absorbance values at each wavelength from each tube into a calculation Excel file, provided by Triton. We summed the total microspheres of the individual embryos samples and calculated fractions of blood flow (%Qsys) to embryo, CAM and yolk.

Based on the regional blood flow distribution obtained from microspheres recovery, giving a value of %Q directed to the CAM ($\%Q_{CAM}$) and the A-V difference calculated from CAM oxygen content of arteries and veins, we can use the Fick equation to estimate blood flows/cardiac output ($\dot{V}O_2$ = blood flow x A-V diff). Then, from the proportional %Q we can also calculate the estimated blood flow directed to embryonic tissues ($\%Q_{EMB}$) and yolk membrane ($\%Q_{YOLK}$).

Statistical analysis

All data were tested for differences between the incubations periods using parametric one-way ANOVA but whenever data failed normality or homoscedasticity we used the non-parametric ANOVA on ranks. *Post hoc* tests of Student Newman Keuls or Dunn´s were used for detection of differences across groups. For oxygen content a T-test was used to account for differences between arterial and venous blood. Least square regressions were used to determine relationships between $\dot{V}O_2$, f_H, O_2 pulse, M_b and M_h. Data was transformed to logarithm when it failed to meet normal distribution and equal variances assumptions. The level of significance was established as $P < 0.05$ and data is presented as mean ± S.E.M. All statistical analyses were conducted with Sigma Plot V 11 software.

RESULTS

A total of 104 eggs were used in this study. Embryonic stages were defined according to Yntema (1968) staging table and at 50% of development, embryos corresponded to stages 19-20, at 70% to stages 23-24, and at 90% to stage 25 (Table 1). Egg masses were not significantly different between the incubation periods sampled (mean egg mass = 12.9 ± 0.2 g; Table 1). Embryonic yolk-free body mass (M_b) increased from 1.62 ± 0.08 g at 50% incubation to 7.16 ± 0.16 g at 90% ($P < 0.001$; Table 1), representing a 4.4-fold increase. Comparing the same periods heart mass (M_h) increased 2-fold and liver mass 4.5-fold.

f_H from 50 and 70% incubation did not differ and had respectively mean values of 56 ± 2 beats min^{-1} and 51 ± 1 beats min^{-1}. The rate significantly decreased at 90% to 43 ± 3 beats min^{-1} ($P < 0.001$; Fig. 1). Oxygen consumption ($\dot{V}O_2$) of whole eggs increased continuously from 510 ± 30 µl O_2 egg^{-1} h^{-1} at 50% to 993 ± 60 µl O_2 egg^{-1} h^{-1} at 70% and to 1204 ± 54 µl O_2 egg^{-1} h^{-1} at 90% ($P < 0.001$; Fig. 2). Mass-specific metabolic rates decreased progressively from 0.36 ± 0.02 ml O_2 h^{-1} g^{-1} at 50% to 0.16 ± 0.008 ml O_2 h^{-1} g^{-1} at 90% ($P < 0.001$). Oxygen pulse, the amount of oxygen consumed per heart beat increased significantly from 0.15 ± 0.01 µl O_2 $beat^{-1}$ at 50% to 0.48 ± 0.04 µl O_2 $beat^{-1}$ at 90% ($P < 0.001$; Fig. 3).

Oxygen content from blood samples withdrawn from the CAM artery did not differ between incubation periods and had a mean of 0.9 ± 0.1 mL O_2 100 ml $blood^{-1}$. Mean CAM mixed venous blood oxygen content was 2.7 ± 0.4 ml O_2 100 ml $blood^{-1}$ at 50%, 3.2 ± 0.3 ml O_2 100 ml $blood^{-1}$ at 70% and significantly increased to 5.0 ± 0.5 ml O_2 100 ml $blood^{-1}$ at 90% of

incubation ($P < 0.001$; Fig. 4). The resulting arteriovenous difference (A-V difference) represented an increase of 2-fold from 50% to 90% incubation.

Relative cardiac output directed to the embryo ($\%Q_{EMB}$) differed significantly between incubation times ($P < 0.001$; Fig.5). It presented the lower level at 70% of incubation, representing 66 ± 4% of the total cardiac output. At 50 and 90% of incubation the $\%Q_{EMB}$ were 84 ± 4% and 72 ± 8%, respectively. Relative cardiac output directed to the CAM ($\%Q_{CAM}$) was the lowest at 50% incubation, with a value of 9.2 ± 2.3%, increased to 31 ± 3.7% at 70% incubation, but at 90% decreased to 25 ± 6.2%. Relative cardiac output directed to the yolk membrane ($\%Q_{YOLK}$) did not differ statistically between incubation times, and represented an average of only 2.4 ± 0.6 % of the total cardiac output.

Based on the Fick Principle equation: $\dot{V}O_2$ = blood flow x A-V diff, it was possible to calculate the estimated blood flow to the CAM. The values obtained were the following: 0.33 ml g^{-1} min^{-1} at 50%, 0.17 ml g^{-1} min^{-1} at 70% and 0.07 ml g^{-1} min^{-1} at 90% of incubation, representing a total of 8-fold decrease. Based on the estimations of % Q, it is also possible to estimate blood flow to the embryo, that were 3.08 ml g^{-1} min^{-1} at 50%, 0.36 ml g^{-1} min^{-1} at 70% and 0.19 ml g^{-1} min^{-1} at 90% of incubation, representing a 16-fold increase (Table 1).

Embryonic M_b and M_h mass increased in a non-linear fashion. The same relationship occurred between O_2 pulse and M_h. Both regressions only attended homoscedasticity when the parameters were transformed in logarithms. f_H showed lower regression coefficients ($R^2 < 0.6$), when correlated with $\dot{V}O_2$, M_b and M_h, indicating a lack of correlation. $\dot{V}O_2$ and O_2 pulse presented high regression coefficients ($R^2 > 0.8$), indicating a strong correlation both with M_b and M_h.

DISCUSSION

The metabolic rates of the snapping turtle embryos measured in this study increased progressively towards the end of incubation. Previous reports (Gettinger et al, 1984; Miller and Packard, 1992; Birchard and Reiber, 1995) for snapping turtle embryos show that $\dot{V}O_2$ reached a maximum and rapid declined when close to hatching. This pattern in peak, is present in other turtles species and crocodilians (Thompson, 1989), and is suggested to be related to the synchronized hatching. The peaked pattern is thought to be related to an adaptive value as the synchrony of hatching lowers the individual predation risks (Spencer et al., 2001) but was also recently related to reduced costs when digging out of the nest after hatching (Rusli et al., 2016). Snapping turtles are know for synchronized nesting as a strategy to decrease predation

(Robinson and Bider, 1988), so it would be advantageous for the specie to also adopt the synchrony at hatching.

The heart rate of embryos decreased significantly at the end of incubation, which is in agreement with the previous report from Birchard (2000). It seems that this decrease is a pattern common in Testudines (Taylor et al., 2014) differing from the pattern found in Squamates, that shows a constant f_H throughout the incubation period (Birchard e Reiber, 1996; Nechaeva et al., 2007; Du et al., 2009; Sartori et al., in preparation). Embryonic f_H pattern differences in reptiles may be related to the polyphyletic origin of the group, but more studies are needed to elucidate if such difference can be related to ecological features or may have a phylogenetical noise. The drop in f_H seems to occur before those in the $\dot{V}O_2$, after the peak is reached, but no relationship seems to occur as we did not found a strong correlation between f_H and $\dot{V}O_2$, indicating that they are somewhat independent, as already reported for iguanas (Sartori et al., in press). However, there could be a similar trigger for the reduction in both $\dot{V}O_2$ and f_H.

The consequent oxygen pulse, calculated from $\dot{V}O_2$ and f_H data, increased progressively throughout incubation, corresponding to a higher oxygen consumption per heartbeat as the embryo grows. However, while $\dot{V}O_2$ doubled from 50% to 90%, heart rate decreased by 23% resulting in a 3-fold increase in O_2 pulse. O_2 pulse and $\dot{V}O_2$ correlates with M_b and M_h, being important for the provision of O_2 for the increasing maintenance costs of the growing process.

Oxygen content measured in CAM arteries (CaO_2) did not vary throughout development, but in CAM veins (CvO_2) it increased approximately 2-fold, consequently increasing the A-V difference by the same proportion. It is interesting to note that in embryos, veins of the CAM are the blood vessels that leave the gas exchanger, carrying oxygenated blood whereas arteries carry deoxygenated blood from the embryonic tissues to the gas exchanger. However, oxygenated venous blood mixes with deoxygenated blood due to the allantoic shunt, which results in a mixed oxygenated and deoxygenated blood (Tazawa and Takenaka, 1985). The mixture is estimated to be 10-15% of deoxygenated blood in the allantoic oxygenated vein of chicken embryos (Piiper et al., 1980), but it is not known in turtle embryos.

Several factors could affect the oxygen content of mixed venous blood: i) an increase in hematocrit or in hemoglobin concentration; ii) an increased saturation of the hemoglobin, related to differences in the hemoglobin oxygen binding curve; iii) changes in the level of allantoic shunting reducing the levels of deoxygenated blood mixed to oxygenated blood.

Turtles showed greater changes in blood flow when compared to chicken embryos. Blood flow to the CAM decreased 8-fold whereas flow to the embryonic tissues increased 16-fold from 50 to 90% turtle incubation. In chicks the changes are 2 and 3-fold, respectively for

Q_{CAM} and Q_{EMB}, from 15 to 90% of incubation (Mulder et al., 1997). Nevertheless, in one study in adults snapping turtles comparing regional blood flow measured with microspheres with values measured by a flow probe detected that the microspheres technique underestimated Qsys in 30% (Stecyk et al., 2004). Despite stroke volume was never directly measured in reptilian eggs (Nechaeva et al., 2007), it can be inferred from the heart mass index (Burggren and Pinder, 1991; Birchard and Reiber, 1996). Embryonic mass increased more than 4 times during the incubation period studied, while heart mass increased only by 2 times. This non-linear increase may not strongly affect the provision of oxygen because as the embryo grows it consumes a lower amount of oxygen per unit of body mass.

In summary, we found that in snapping turtles the embryonic f_H is relatively independent of the VO_2 and both follow a pattern present in other Testudine species. Lacking correlated changes in f_H, in order to match oxygen supply to the demand during embryonic development, the embryos apparently rely in changes on stroke volume, as heart grows and mass-specific VO_2 declines along incubation, and also in increases of oxygen content in oxygenated blood from the gas exchanger, both increasing the available O_2 to be delivered to tissues.

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Table I Mean (± s.e.m.) values of mass, collected and calculated cardiovascular data in embryos of Snapping turtles from 50, 70 and 90% of incubation. N for cardiovascular data is indicated in the parethesis.

Mass	***50%***	***70%***	***90%***
Egg (g)	12.4 ± 0.5	13.1 ± 0.4	13.4 ± 0.2
Yolk (g)	3.72 ± 0.15	2.71 ± 0.13	1.18 ± 0.07
Body (g)	1.62 ± 0.08	4.39 ± 0.13	7.16 ± 0.16
Heart (mg)	7.2 ± 0.2	16.5 ± 0.7	21.7 ± 0.8
Liver (mg)	47 ± 2	111 ±6	213 ± 8
N	21	24	21
Cardiovascular data			
f_H ***(beats min^{-1})***	55.7 ± 1.8 (7)	50.9 ± 0.6 (6)	42.8 ± 2.6 (6)
$\dot{V}O_2$ ***(µl anminal^{-1} h^{-1})***	510 ± 30 (6)	993 ± 60 (6)	1204 ± 54 (6)
O_2 pulse (µl O_2 beat^{-1})	0.15 ± 0.01 (6)	0.32 ± 0.02 (6)	0.48 ± 0.04 (6)
CaO_2	0.91 ± 0.3 (5)	0.93 ± 0.1 (5)	0.97 ± 0.2 (6)
CvO_2	2.74 ± 0.4 (5)	3.25 ± 0.3 (5)	4.97 ± 0.5 (6)
A-V diff	1.83	2.31	4
% Q_{CAM}	9.2 ± 2.6 (5)	31.6 ± 3.8 (18)	27.1 ± 6.3 (14)
% Q_{EMB}	83.7 ± 4.9 (5)	66.2 ± 3.7 (18)	71.5 ± 7.8 (15)
% Q_{YOLK}	6.4 ± 3.4 (4)	3.8 ± 0.9 (12)	0.14 ±0.1 (15)
Estimated data			
Q sys CAM (ml g^{-1} min^{-1})	0.33	0.17	0.07
CAM absolute flow (ml min^{-1})	0.32	0.16	0.04
Q sys EMB (ml g^{-1} min^{-1})	3.08	0.36	0.19
EMB absolute flow (ml min^{-1})	4.13	1.61	1.22

Table II Regression analysis results between VO_2 (µl O_2 egg^{-1} h^{-1}), f_H (beats min^{-1}), O_2 pulse (µl O_2 $beat^{-1}$), Mb (g) and Mh (g). Bold regression coefficients indicates a weak correlation.

	N	R^2	P	Equation
VO_2 vs f_H	18	**0.40**	0.005	f_H = -13.098 VO_2 + 61.339
log M_b vs log M_h	71	0.89	<0.001	log M_h = 0.7 log M_b - 2.27
f_H vs M_b	19	**0.56**	<0.001	f_H = -2.2 M_b + 59.1
f_H vs M_h	19	**0.60**	<0.001	f_H = -790.7 M_h + 61.4
VO_2 vs M_b	18	0.86	<0.001	VO_2 = 125 M_b + 374
VO_2 vs M_h	18	0.84	<0.001	VO_2 = 43121 M_h x + 267
log O_2 Pulse vs log M_b	18	0.89	<0.001	log O_2 Pulse = 0.688 log M_b - 0.915
O_2 Pulse vs M_h	18	0.91	<0.001	O_2 Pulse = 20.3 M_h + 0.02

Figure captions

Fig. 1 Heart rates presented by snapping turtles at 50, 70 and 90% incubation. Mean heart rates (beats min^{-1}) and SEM bars of embryonic turtles at different times of incubation period. At 90% the rate is lower than at 50% and 70% (One-way ANOVA + SNK; $P < 0.01$)

Fig. 2 Metabolic rates (VO_2) of embryonic snapping turtles at 50, 70 and 90% incubation. Total oxygen consumption (µl O_2 egg^{-1} h^{-1}) of *C. serpentina* eggs at 50%, 70% and 90% of the incubation period. The volume of oxygen consumed increased progressively throughout incubation period (One-way ANOVA + SNK; $P < 0.02$).

Fig. 3 Oxygen pulse of embryonic snapping turtles at 50, 70 and 90% incubation. Oxygen pulse (µl O_2 $beat^{-1}$) calculated from total VO_2 and f_H data. Oxygen pulse increased progressively at 70% and 90% of development (One-way ANOVA + SNK; $P <0.001$).

Fig. 4 Blood oxygen content of embryonic snapping turtles at 50, 70 and 90% incubation. Blood oxygen content in arteries and veins of the choriollantoic membrane of snapping turtle embryos. Arterial mixed blood oxygen content did not vary along development (ANOVA on Ranks; $P = 0.824$), but venous blood oxygen content increased at 90% of development (One-way ANOVA + SNK; $P<0.05$). Arterial mixed oxygen content was higher than venous content in every incubation period tested (T-test; $P < 0.02$).

Fig. 5 Relationship between body mass and heart mass in embryos of the snapping turtles at 50, 70 and 90% incubation Regression of log embryo body mass (M_b) against log heart mass (M_h) in the different periods of incubation. Data needed to be logarithmized, resulting in the regression which indicates a non-linear relationship ($R^2 = 0.89$; $P< 0.001$).

Fig. 6 Relationship between f_H and VO_2 in embryos of the snapping turtles at 50, 70 and 90% incubation. Regression f_H and VO_2 showed a lower coefficient, indicating lack of correlation.

Fig. 7 Relationship between f_H, VO_2 and O_2 Pulse with heart and body mass in embryos of the snapping turtles at 50, 70 and 90% incubation. Regression of A) f_H, B) VO_2 and C) O_2 Pulse, against heart (M_h) and body mass (M_b).

Fig. 8 Distribution of cardiac output in embryonic snapping turtles at 50, 70 and 90% incubation. Percentages of relative blood flow or systemic cardiac output ($\%Q_{sys}$) of the embryo, chorioallantoic membrane (CAM) and yolk sac at 50%, 70% and 90% of incubation, during routine normoxia at 30°C.

Fig. 1

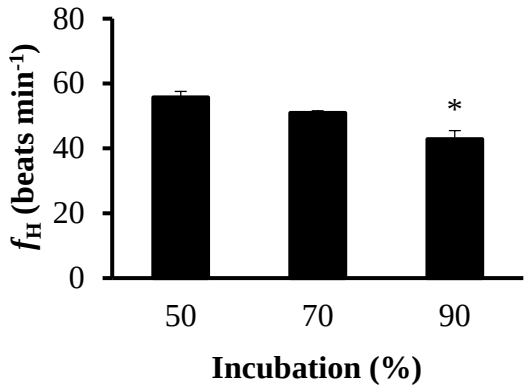


Fig. 2

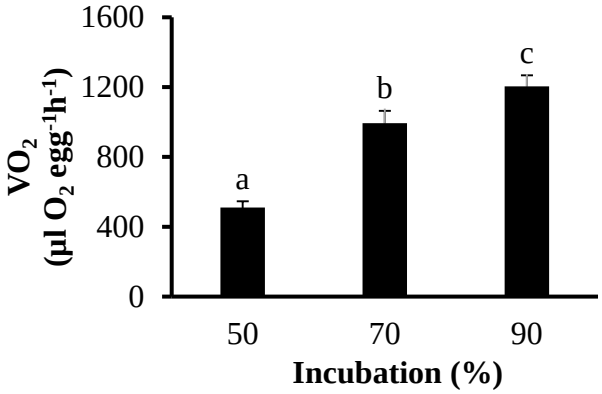


Fig. 3

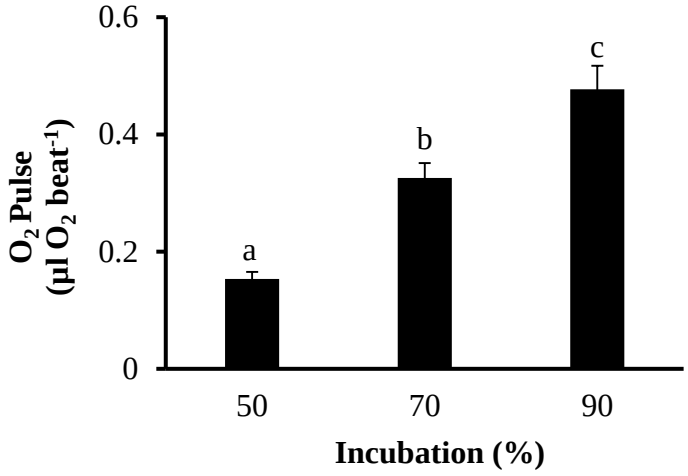


Fig. 4

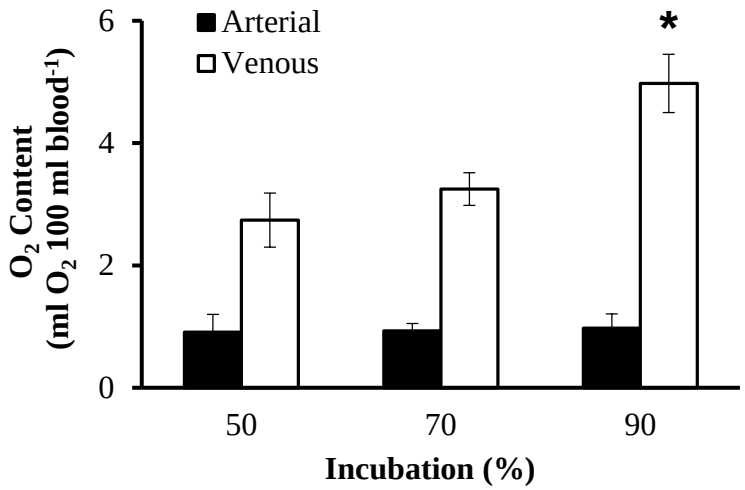

Arterial
Venous
*
6
4
2
0
50
70
90
Incubation (%)
O2 Content
(ml O2 100 ml blood-1)

Fig. 5

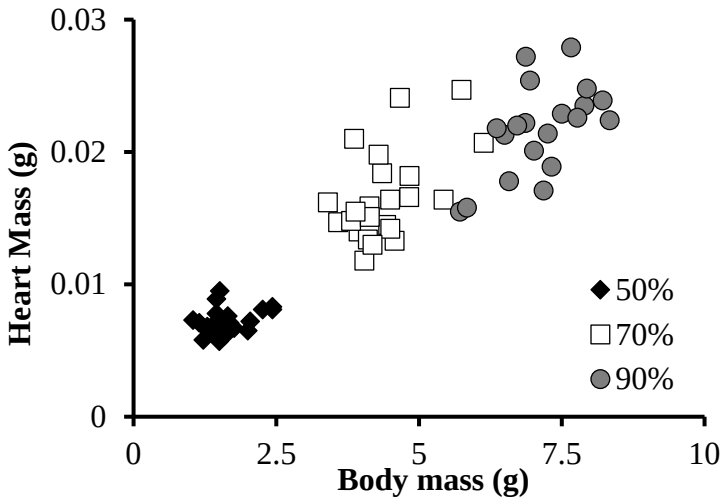

0.03
0.02
0.01
0
0
2.5
5
7.5
10
Body mass (g)
Heart Mass (g)
50%
70%
90%

Fig. 6

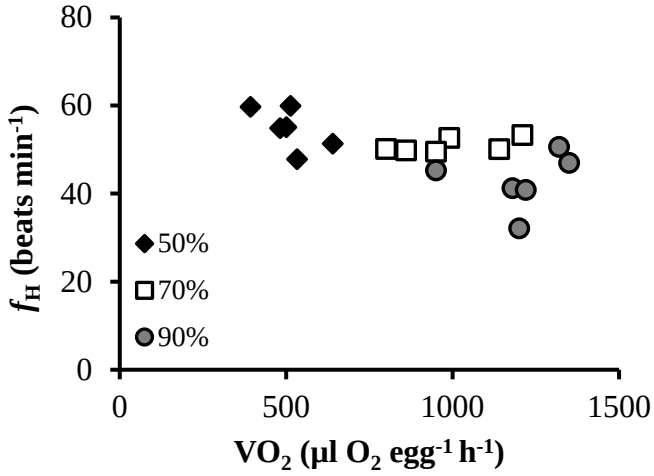

80
60
40
20
0
0
500
1000
1500
f_H (beats min^{-1})
VO_2 (µl O_2 egg^{-1} h^{-1})
50%
70%
90%

Fig. 7

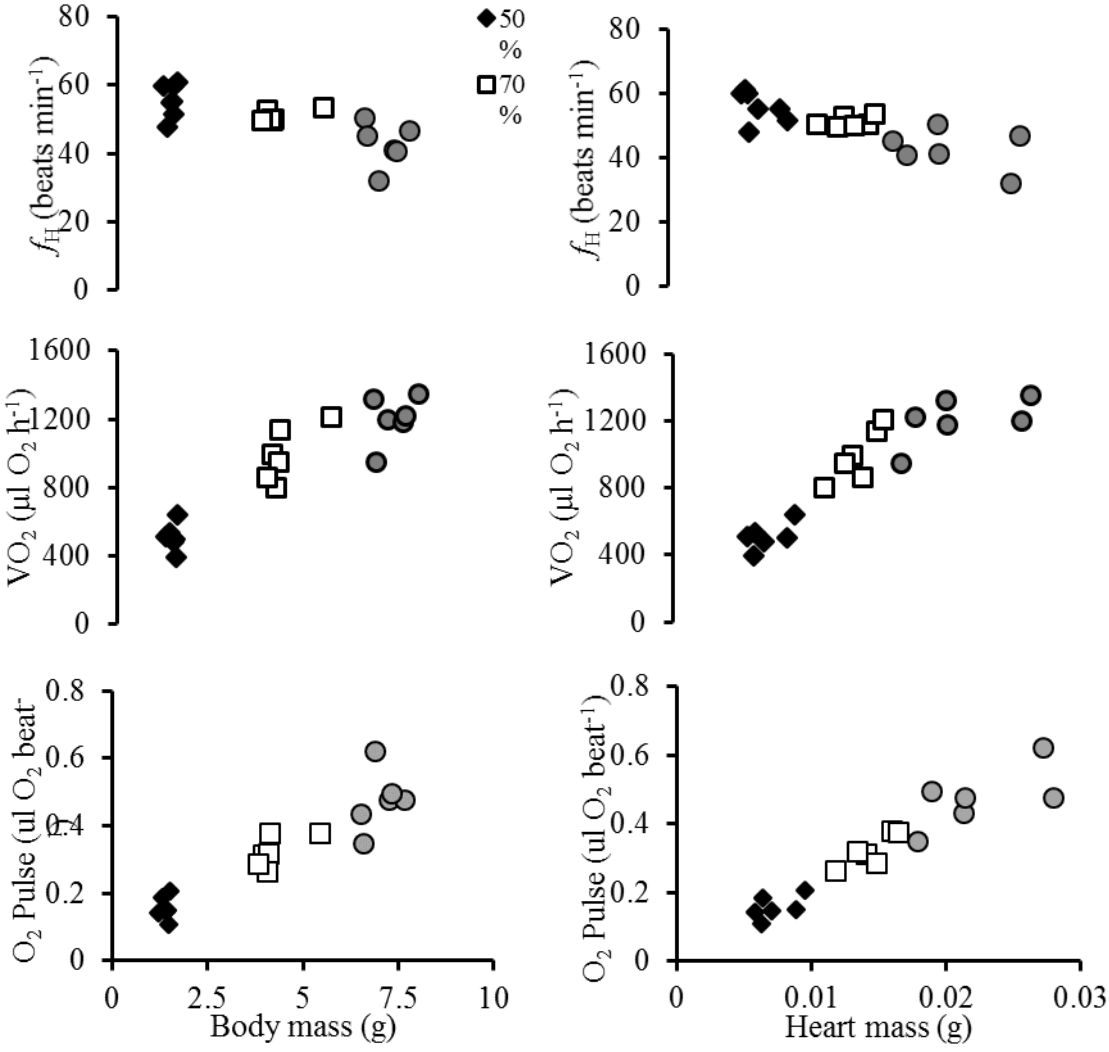
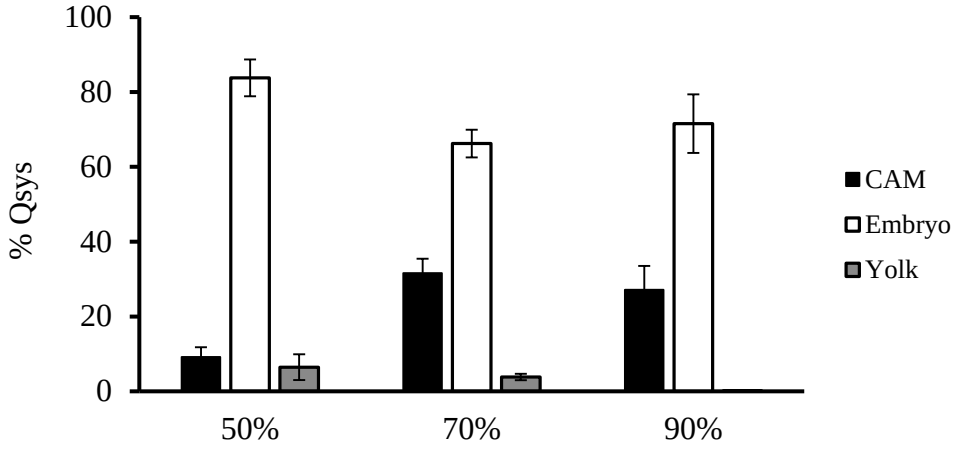

f_H (beats min^{-1})
VO_2 (µl O_2 h^{-1})
O_2 Pulse (ul O_2 beat^{-1})
50 %
70 %
Body mass (g)
Heart mass (g)

Fig 8



Apêndice B

**Artigo pertinente ao conjunto de trabalhos da tese*

J Comp Physiol B
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ORIGINAL PAPER

A role for histamine in cardiovascular regulation in late stage embryos of the red-footed tortoise, *Chelonoidis carbonaria* Spix, 1824

Dane A. Crossley II · Marina R. Sartori · Augusto S. Abe · Edwin W. Taylor

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Abstract A chorioallantoic membrane artery in embryos of the red-footed tortoise, *Chelonoidis carbonaria* was occlusively cannulated for measurement of blood pressure and injection of drugs. Two age groups of embryos in the final 10 % of incubation were categorized by the ratio of embryonic body to yolk mass. All embryos first received cholinergic and β-adrenergic blockade. This revealed that β-adrenergic control was established in both groups whereas cholinergic control was only established in the older group immediately prior to hatching. The study then progressed as two series. Series one was conducted in a subset of embryos treated with histamine before or after injection of ranitidine, the antagonist of H_2 receptors. Injection of histamine caused an initial phasic hypertension which recovered, followed by a longer lasting hypertensive response accompanied by a tachycardia. Injection of the H_2 receptor antagonist ranitidine itself caused a hypotensive tachycardia with subsequent recovery of heart rate. Ranitidine also abolished the cardiac effects of histamine injection while leaving the initial hypertensive response intact. In series, two embryos were injected with histamine after injection of diphenhydramine, the antagonist to H_1 receptors. This abolished the whole of the pressor response to histamine injection but left the tachycardic response intact. These data indicate that histamine acts as a non-adrenergic, non-cholinergic factor, regulating the cardiovascular system of developing reptilian embryos and that its overall effects are mediated via both H_1 and H_2 receptor types.

Keywords Cardiovascular · Reptilia · Histamine · Cholinergic · Adrenergic · Embryonic

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Introduction

Studies on control of the cardiovascular system of reptiles have explored changes in heart rate and blood flow, including the role of cardio-respiratory interactions associated with temperature change, activity levels, and feeding (Clark et al. 2005; Galli et al. 2004; Hicks et al. 2000; Wang and Hicks 1996a, b; Wang et al. 1997, 2001a, b). The role of the autonomic nervous system in instigating these changes has been investigated using pharmacological techniques. In the rattlesnake, *Crotalus durrisus,* vagal cholinergic tone on the heart predominates over adrenergic tone in inactive and active animals. Changes in heart rate in this species are largely due to withdrawal of a vagal tonus and sympathetic regulation of the systemic circulation (Wang et al. 2001b). During digestion, snakes such as pythons and boa constrictors can more than double their heart rate after ingestion (Secor and Diamond 1998; Secor et al. 2000). However, in the *Boa constrictor*, while the increase in heart rate during activity is predominantly due to withdrawal of vagal tone, this mechanism does not

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Apêndice C

**Artigo revisivo com dados pertinentes ao conjunto de trabalhos da tese*

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REVIEW

The phylogeny and ontogeny of autonomic control of the heart and cardiorespiratory interactions in vertebrates

Edwin W. Taylor[1,2], Cleo A. C. Leite[3,4,*], Marina R. Sartori[2,4], Tobias Wang[5], Augusto S. Abe[2,4] and Dane A. Crossley, II[6]

ABSTRACT

Heart rate in vertebrates is controlled by activity in the autonomic nervous system. In spontaneously active or experimentally prepared animals, inhibitory parasympathetic control is predominant and is responsible for instantaneous changes in heart rate, such as occur at the first air breath following a period of apnoea in discontinuous breathers like inactive reptiles or species that surface to air breathe after a period of submersion. Parasympathetic control, exerted via fast-conducting, myelinated efferent fibres in the vagus nerve, is also responsible for beat-to-beat changes in heart rate such as the high frequency components observed in spectral analysis of heart rate variability. These include respiratory modulation of the heartbeat that can generate cardiorespiratory synchrony in fish and respiratory sinus arrhythmia in mammals. Both may increase the effectiveness of respiratory gas exchange. Although the central interactions generating respiratory modulation of the heartbeat seem to be highly conserved through vertebrate phylogeny, they are different in kind and location, and in most species are as yet little understood. The heart in vertebrate embryos possesses both muscarinic cholinergic and β-adrenergic receptors very early in development. Adrenergic control by circulating catecholamines seems important throughout development. However, innervation of the cardiac receptors is delayed and first evidence of a functional cholinergic tonus on the heart, exerted via the vagus nerve, is often seen shortly before or immediately after hatching or birth, suggesting that it may be coordinated with the onset of central respiratory rhythmicity and subsequent breathing.

KEY WORDS: Autonomic nervous system, Parasympathetic tonus, Cardiorespiratory interaction, Heart rate variability, Respiratory sinus arrhythmia, Ontogeny, Vertebrate

Introduction

Each contraction of the vertebrate heart is initiated by a myogenic pacemaker, but the prevailing heart rate (f_H) is determined from within the central nervous system (CNS) via the autonomic nervous system (ANS), which exerts inhibitory parasympathetic and excitatory sympathetic influences on the heart. These mechanisms allow for gross matching of cardiac output to metabolism, but the effectiveness of gas exchange is also improved by tight beat-to-beat coordination of f_H to ventilation of the respiratory organs, whether they are gills or lungs. Animals with a discontinuous breathing pattern such as air-breathing fish or diving tetrapods typically display bradycardia during apnoea and a pronounced cardiac acceleration immediately upon the first air breath. In animals with regular and rhythmic ventilation, cardiorespiratory interactions are typically revealed as a rise in f_H during inspiration (Taylor et al., 1999). It has long been recognized that the overall rates of flow of air or water and of blood over the respiratory surfaces are matched according to their respective capacities for oxygen so that the ventilation-to-perfusion ratio varies from 1 in air breathers to 10 or more in water breathers, with the bimodal, air/water breathers among the lungfishes and amphibians showing variable ratios (Piiper and Scheid, 1977). As both ventilation and perfusion are typically pulsatile and sometimes intermittent, close co-ordination between the two rhythms would seem essential in order to optimize the effectiveness of respiratory gas exchange and its metabolic costs. Control of these cardiorespiratory interactions resides in the CNS. The cell bodies of the preganglionic neurones in both parts of the ANS are located within the brainstem or spinal cord where their activity can be influenced by afferent input from peripheral baroreceptors and mechanoreceptors as well as higher brain centres. These integrate afferent inputs from a range of central and peripheral receptors and coordinate central interactions between pools of neurones generating the respiratory rhythm and determining f_H variability (Taylor et al., 1999).

This review explores the central coordination of the cardiovascular and respiratory systems in vertebrates, and we will argue that, despite major differences in the construction and mode of operation of these systems, vertebrates share some fundamental similarities in the neural control of f_H and its coordination with the respiratory rhythm (Table 1). We introduce the review with a short description of the origin and nature of tonic nervous control to the heart and of cardiorespiratory interactions (CRI) in mammals and then describe and interpret our current knowledge of the other vertebrate groups.

While adrenergic influences on the heart are important in relation to activity levels, including fight or flight responses, the heart in routinely active or experimentally prepared vertebrates chiefly operates under a variable degree of inhibitory tonus, imposed by the parasympathetic arm of the ANS via the Xth cranial nerve, the vagus (Taylor et al., 1999). In a representative collection of mammals, while adrenergic tone varied from 4 to 30% (Fig. 1), inhibitory vagal tone varied between 15 and 102% (Fig. 2). The vagus is also responsible for instantaneous, beat-to-beat control of f_H (Bootsma et al., 1994; Taylor et al., 1999). The heart accelerates during inspiration in mammals and slows during exhalation. Power spectral analysis of f_H variability (HRV) (Grossman and Taylor, 2007) reveals a peak at a relatively high frequency that is respiration related and this relationship is termed respiratory sinus arrhythmia (RSA). In humans, RSA is normally well developed in juveniles

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