UNIVERSIDADE ESTADUAL PAULISTA "JULIO DE MESQUITA FILHO"

FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS

CÂMPUS DE JABOTICABAL

ALTERAÇÕES METABÓLICAS E MORFOMÉTRICAS INDUZIDAS POR HIPÓXIA EM *Gallus gallus*

Paula Andrea Toro Velasquez

Zootecnista

JABOTICABAL – SÃO PAULO – BRASIL

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Paula Andrea Toro Velasquez

Orientadora: Profa. Dra. Kênia Bicego

Co-orientador: Prof. Dr.Marcos Macari

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutora em Zootecnia.

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DADOS CURRICULARES DA AUTORA

PAULA ANDREA TORO VELASQUEZ – natural de Medellín – Antioquia, Colombia, nasceu no dia 27 de julho de 1976. Em fevereiro de 1994 ingressou no curso de graduação em Zootecnia, na Faculdade de Ciências Agrárias, da Universidade De Antioquia da Colômbia em Medellín, formando-se em julho de 2000. Em agosto de 2004 iniciou o curso de Mestrado em Zootecnia (Área de concentração: Nutrição e Alimentação Animal, Linha de pesquisa: Avaliação de Alimentos para Animais) na Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista, Câmpus de Jaboticabal e concluiu em junho de 2006. Na mesma instituição, em março de 2010 iniciou o curso de Doutorado em Zootecnia (Área de concentração: Bioquímica e Fisiologia Animal, Linha de pesquisa: Biologia de Desenvolvimento Animal) concluindo em fevereiro de 2014. Realizou doutorado sanduiche no Laboratório do Professor Jacopo Mortola na Área de Fisiologia Respiratória, do Departamento de Fisiologia, da Universidade de McGill, em Montreal de outubro de 2012 a outubro de 2103, Quebec, Canada. Foi bolsista do Programa Convênio de Pós-Graduação (PEC-PEG) da CAPES durante março de 2010 até junho de 2011, Bolsista FAPESP de julho de 2011 a setembro de 2012 e bolsista BEPE durante o doutorado sanduiche.

“I have only made this letter longer because I have not had the time to make it shorter.”

“I would prefer an intelligent hell to a stupid paradise.”

“Nature is an infinite sphere of which the center is everywhere and the circumference nowhere.”

“We arrive at truth, not by reason only, but also by the heart.”

Blaise Pascal

“The true sign of intelligence is not knowledge but imagination.”

“Insanity: doing the same thing over and over again and expecting different results.”

“A person who never made a mistake, never tried anything new.”

Albert Einstein

“The greatest enemy of knowledge is not ignorance; it is the illusion of knowledge.”

Stephen Hawking

“Education is the most powerful weapon which you can use to change the world.”

“I learned that courage was not the absence of fear. But the triumph over it. The brave man is not he who does not feel afraid, but he who conquers that fear.”

“It always seems impossible until it’s done.”

Nelson Mandela

“In the depth of winter I finally learned that there was in me an invincible summer.”

Albert Camus

“What matters in life is not what happens to you but what you remember and how you remember it.”

“Nobody deserves your tears, but whoever deserves them will not make you cry.”

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“Friendship is the source of the greatest pleasures, and without friends even the most agreeable pursuits become tedious.”

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Certificamos que o Protocolo nº 024166/13 do trabalho de pesquisa intitulado **"Exposição à hipóxia durante a incubação e sua influência nos parâmetros morfofisiológicos de *Gallus gallus* pós-eclosão"**, sob a responsabilidade da Prof.ª Dr.ª Kênia Cardoso Bicego está de acordo com os Princípios Éticos na Experimentação Animal adotado pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 05 de novembro de 2013.

Jaboticabal, 05 de novembro de 2013.

Prof. Dr. Andrigo Barboza De Nardi
Coordenador - CEUA

Faculdade de Ciências Agrárias e Veterinárias
Via de Acesso Prof. Paulo Donato Castellane, s/n CEP 14884-900 - Jaboticabal - SP - Brasil
Tel. 16 3209 2600 - fax 16 3202 4275 www.fcav.unesp.br

ALTERAÇÕES METABÓLICAS E MORFOMÉTRICAS INDUZIDAS POR HIPÓXIA EM Gallus gallus

RESUMO- Baixa pressão parcial de O_2 durante o desenvolvimento embrionário e/ou fetal pode diminuir a taxa metabólica e a taxa de crescimento do embrião, podendo levar a alterações morfofisiológicas após o nascimento, além de atenuar a resposta ventilatória hipóxica no recém-eclodido de galinhas. No presente estudo, várias incubações foram realizadas para avaliar o efeito da hipóxia pre-natal sobre a taxa metabólica e a resposta à hipóxia aguda em diferentes linhagens e idades de *Gallus gallus*. Além disso, foi verificada a possibilidade de que a redução da taxa metabólica por si e não especificamente induzida por hipóxia prolongada durante desenvolvimento pré-natal seria um fator contribuinte sobre a diminuição da taxa metabólica em neonatos. Para isso embriões de galinha foram incubados a 35 ◦C (grupo de frio), que é conhecido por diminuir o consumo de oxigênio embrionário por ~30% ao longo de incubação, e aumenta o período de incubação em ~2 dias. Quanto a aves precoces, nenhum estudo havia abordado a influência da hipóxia em diferentes fases de incubação no desenvolvimento morfométrico do intestino, importante não somente para os processos digestivos e de absorção, mas também para a proteção contra patógenos; por isto avaliamos as características morfométricas e a quantidade de células caliciformes por vilo das regiões do intestino delgado em pintainhos recém-eclodidos de frango de corte.

Palavras-chave: incubação, taxa metabólica, intestino, células calciformes, respostas ventiltorias

METABOLIC AND MORPHOMETRIC RESPONSES INDUCED BY HYPOXIA IN *Gallus gallus*

ABSTRACT- Low O_2 partial pressure during embryonic and fetal development can decrease the growth and metabolic rate of the embryo, leading to morphophysiological changes after birth, and blunt the hypoxic ventilatory response in newly hatched chicken. In the present study, different conditions during incubation (cold and hypoxia) were performed to evaluate the effect of prenatal hypoxia on metabolic rate, and the response to acute hypoxia in different strains and ages of *Gallus gallus*. We also considered the possibility that prolonged metabolic depression per se, but not specifically induced by hypoxia, during incubation would be a contributing factor to decreased metabolic rate in neonate. Thus chicken embryos were incubated at 35◦C (cold group), which is known to decrease the embryonic oxygen consumption (VO_2) by ~ 30% throughout the incubation period and increases the incubation time in ~2 days. Finally, in precocial birds, no study has approached the influence of hypoxia at different stages of incubation in the morphometric development of the intestine, not only important for the digestive processes and absorption, but also for protection against pathogens, so we evaluated the morphometric characteristics and quantity of goblet cells per villi of the small intestine regions in newly hatched broiler chicks .

Keywords: incubation, metabolic rate, intestine, calciform cells, ventilatory response

CAPÍTULO 1 - Considerações Gerais

1. Introdução e revisão de literatura

Nos últimos anos, observa-se um crescente interesse da comunidade científica e dos produtores nas consequências pós-natais de mudanças ambientais (estresses nutricionais, térmicos, gasosos, dentre outros) durante o desenvolvimento pré e/ou perinatal, podendo ser resultado de adaptações epigenéticas, ou seja, de mudanças na expressão gênica (Hala et al., 2012; Jiménez-Chillarón et al., 2012) com possíveis alterações fenotípicas. Do ponto de vista zootécnico, a aquisição de tais conhecimentos visa melhorar o entendimento desses processos e os aspectos relativos à produção como, por exemplo, tolerância a estresses ambientais, prevenção de doenças, redução da janela de eclosão, dentre outros.

No presente estudo, o estímulo estressor abordado foi a hipóxia, queda da pressão parcial de oxigênio, durante a incubação de frangos de corte e/ou galinhas. Foram investigadas as influências da hipóxia pré-natal sobre o desenvolvimento morfométrico do intestino delgado e as características gerais dos neonatos de frangos de corte e as respostas metabólicas frente a uma nova exposição aguda à hipóxia na fase pós-natal (1 e 10 dias) de frangos de corte e galinhas. Também foi verificado se a redução metabólica induzida pelo frio durante a fase final da incubação teria o mesmo efeito que da hipóxia pré-natal sobre as respostas ventilatórias à hipóxia aguda de neonatos de galinhas.

1.1. Modelos Experimentais: Frangos de corte e galinhas poedeiras

A produção de frangos de corte e ovos de galinha em países de clima tropical e subtropical aumentou de forma marcante nos últimos 40 anos, sendo este um grande desafio para o setor devido à baixa tolerância ao frio na fase inicial e, principalmente para os frangos de corte, à baixa tolerância ao calor na fase final de criação. Nesse intervalo de tempo, o Brasil foi o país de maior destaque no cenário

avícola mundial, sendo o principal exportador e o terceiro produtor de carnes de frango (UBABEF, 2013). Segundo a USDA (United States Department of Agriculture) a tendência de crescimento do setor avícola do ano 2013 para 2014 é 2,7% para produção, 2,45% para o consumo, 3,46% para a exportação (todos maiores quando comparados aos das carnes bovina e suína) e 1,93% para importação (AVISITE, 2013).

Apenas cinco países produzem 55% do total de ovos no mundo, o equivalente a 64 milhões de toneladas do produto por ano. Hoje, no mundo, há cerca de 6,5 bilhões de poedeiras em produção, garantindo mais de 90% da produção global de ovos. A China, que é o maior produtor mundial de ovos, produziu em 2011 27,9 milhões de toneladas do produto. Atrás da China, seguem os Estados Unidos, a Índia, o Japão e o México no ranking dos maiores produtores de ovos. O Brasil aparece em sexto lugar (OVOSITE, 2013). Na década de 2002-2012 a produção brasileira de ovos aumentou perto de 35% (AVISITE, 2013). As pesquisas nacionais contribuíram sobremaneira para o avanço da produção de carne e ovo nas condições tropicais.

Aproximadamente desde a década de 1950 o melhoramento genético, praticado nas linhagens de frango de corte e galinhas de postura, levou a um grande progresso na produção de carne e ovos. As aves comerciais de hoje são híbridos produzidos por meio de cruzamentos entre linhagens intensivamente selecionadas para crescimento rápido, eficiência alimentar e rendimento de carne (frangos de corte) e produção de ovos (galinhas poedeiras), entre outras (Harvestein et al., 1994a, 2003). O direcionamento da seleção artificial de frangos de corte tem se baseado em uma alta taxa de ganho de massa corporal em um intervalo curto de tempo (Havenstein et al. 1994a,b); o melhoramento da composição corporal (alto rendimento de tecido muscular magro e baixo teor de gordura abdominal) também tem sido um importante critério de seleção (Rance et al., 2002).

1.2. Desenvolvimento pré-natal em *Gallus*

O desenvolvimento embrionário começa aproximadamente 3 horas após a fecundação, a qual ocorre na porção superior do infundíbulo do oviduto. Esse desenvolvimento continua progredindo paralelamente à formação do ovo no interior do oviduto da ave. Como a duração da formação do ovo é de ± 26 horas, o desenvolvimento embrionário no interior do organismo da ave tem aproximadamente 22 horas (Gilbert 2000).

Depois do inicio da incubação o evento mais importante para a clivagem do embrião é o estabelecimento de eixos de polaridade. Primeiro, os lados dorsal e ventral tornarão aparentes (o eixo dorso-ventral) e, em seguida, o ântero-posterior (o eixo crânio-caudal) (Bellairs & Osmond, 2005).

Pelas 22 horas de incubação, a maioria das células presumíveis da endoderme estão no interior do embrião, embora as células presumíveis mesodérmicas continuam a migrar para dentro de um tempo mais longo. No segundo dia de incubação a partir dos três folhetos embrionários (ectoderme, mesoderme e endorderme), começam a desenvolver os esboços primários dos órgãos: um sistema vascular primitivo surge para suprir as necessidades nutritivas do embrião e um coração tubular surge a partir deste sistema de vasos sanguíneos. Dois distintos sistemas circulatórios se estabelecem: uma circulação intra-embrionária e uma circulação extra-embrionária que se estende pelo saco vitelino. O Sistema Nervoso se diferencia a partir do ectoderme e pelo processo de Neurulação, um tubo cilíndrico se destaca das demais células ectodérmicas. A modelagem do corpo do embrião inicia-se mediante dobra cefálica, na região anterior, dobra caudal, na região posterior, e dobras laterais. Ao final do terceiro dia de incubação, as vesículas encefálicas e o aparelho ocular estão visíveis, o bico começa a se desenvolver e esboços das asas e pés estão presentes (Hamburger & Hamilton 1951).

No quarto dia de incubação uma torção e flexão do corpo do embrião estão visíveis e o embrião gira 90º para a direita e repousa com seu lado esquerdo sobre o vitelo. Com a flexão, a cabeça e a cauda se aproximam e o embrião tem a forma de

um “C”. A boca e a língua e as fendas nasais desenvolvem-se como partes dos sistemas digestório e respiratório, respectivamente. O coração tubular transforma-se em uma estrutura com quatro câmaras e pode ser visto batendo, se o ovo for aberto nesta etapa do desenvolvimento. No final do quarto dia de incubação, o embrião tem todos os órgãos necessários para sustentar a sua vida até a eclosão e inclusive para ser distinguido dos mamíferos. O embrião cresce e se desenvolve rapidamente. Pelo sétimo dia as papilas das penas aparecem nas asas e pés, o coração está completamente dentro da cavidade torácica e o embrião já tem muitas características de ave, assim ele passa a ser referido como feto. Após o décimo dia as plumas são visíveis e o bico endurece (Hambruger & Hamilton, 1951).

No décimo quarto dia as garras são formadas e o feto se move para a posição de eclosão. Após vinte dias, o pintinho está em posição de eclosão, o bico perfura a câmera de ar e a respiração pulmonar começa (*internal pipping*). Após 21 dias de incubação, o pintainho finalmente começa a sair da casca (*external pipping*) e eclode (Hambruger & Hamilton, 1951).

Em aves, a região das futuras divisões do intestino pode ser reconhecida pelo dia embrionário seis (E6). Uma pequena dobra do duodeno, que se encontra sob o lobo direito do fígado, é seguido pela curvatura do duodeno-jejuno e mais uma dobra que o liga ao saco vitelínico. O endoderme forma as camadas epiteliais do intestino e os ductos das glândulas mucosas, enquanto o mesoderme dá origem à parede muscular e a estruturas associadas (Bellairs & Osmond 2005). O peso do intestino, como peso relativo do embrião passa de 1% no dia 17 de incubação (E17) a 3,5% no momento de eclosão (Uni, 2006). Para ajustar-se à rápida transição de fontes de nutrientes internos para fontes externas, o intestino delgado da ave passa por alterações morfológicas, moleculares e celulares no final da incubação. Dois dias antes da eclosão, o intestino delgado de frangos tem a estrutura das vilosidades e um potencial de digestão e absorção de carbohidratos (Uni et al., 2003a). Embora a capacidade digestiva comesse a se desenvolver poucos dias antes de eclosão, a maior parte do desenvolvimento ocorre após o nascimento, quando o pintainho passa a consumir ração (Uni, 2006). Assim, a importância do desenvolvimento

precoce do intestino delgado (antes da eclosão e durante a primeira semana de vida) está relacionada ao próprio desenvolvimento geral do animal, tendo influências sobre o crescimento posterior e a sua saúde (Michell & Moretto, 2006). A superfície do intestino é coberta por uma camada de muco produzido pelas células caliciformes, o que é importante para a prevenção de patologias gastrointestinais e desempenha um papel na digestão e absorção de nutrientes (Forstner et al., 1995). A camada de muco atua tanto como um meio para proteção da borda em escova contra danos causados por produtos químicos ou microorganismos e ajuda no transporte entre o conteúdo luminal e a borda em escova (Forstner & Forstner, 1994). Recentemente também foi demonstrada uma importante função das células caliciformes na resposta imunológica inata, absorvendo as imunoglogulinas maternas e as secretando juntamente com o muco na superfície intestinal, mantendo essa primeira linha de defesa dos pintainhos até que eles produzam suas próprias imunoglobulinas (Bar-Shiba et al., 2014). O desenvolvimento das pequenas células secretoras de muco intestinal em frangos ocorre na fase tardia de incubação e na fase imediatamente após o nascimento (Uni et al., 2003b).

1.3. Hipóxia

A redução da pressão parcial de O_2, ou seja, a hipóxia pode ser encontrada em situações fisiológicas e patológicas. Situações fisiológicas resultam de ambientes hipóxicos como altas altitudes, tocas e habitats aquáticos privados de oxigênio, enquanto que condições patológicas podem ser apneia obstrutiva do sono, doença pulmonar obstrutiva crônica, hipoxemia associada com distúrbios circulatórios, especialmente os relacionados com a septicemia ou choque endotoxêmico (revisado por Bicego et al., 2007).

As respostas fisiológicas à hipóxia podem ser diferentes considerando se é uma exposição curta ou mais prolongada ou ainda dependendo da fase de desenvolvimento em que o animal se encontra, sendo críticas as fases pré e/ou perinatal para influenciar processos fisiológicos mais tarde na vida desse animal. Essas considerações das respostas à hipóxia no domínio do tempo são abordadas

abaixo.

1.3.1. Hipóxia aguda

Hipoxia gera respostas ventilatórias e metabólicas como aumento na ventilação, queda na taxa metabólica e queda na temperatura corporal (Tc) em recém-nascidos e adultos de diferentes espécies de aves e mamíferos (Gautier, 1996, Mortola 2009, Bicego et al., 2007) que tendem a reduzir a demanda por O_2 e facilitar a sua captação.

As respostas ventilatórias à hipóxia em aves e mamíferos são predominantemente ativadas por ação de quimiorreceptores chamados arteriais ou periféricos. Tais quimiorreceptores são sensíveis a mudanças das pressões parciais arteriais de oxigênio (PaO_2) e CO_2 ($PaCO_2$) e do pH arterial (pHa), estando, portanto envolvidos também nas respostas ventilatórias à hipercapnia e/ou à acidemia (Powell, 2000). Os mais importantes desses quimiorreceptores são os corpos carótideos (1-mm de diâmetro) localizados bilateralmente entre a artéria carótida e o gânglio nodoso do nervo vago (Adamson, 1958). Eles são ricamente perfundidos por um ramo da artéria carótida, e inervados por um ramo do vago. Os corpos carotídeos estão perto das glândulas paratireoide e ultimobranquial em aves e são envolvidos dentro da glândula paratireoide em algumas espécies (Yamatsu & Kameda, 1995), não sendo ainda bem conhecido o papel fisiológico dessas interações. A queda da PaO_2 promove ativação das células quimiosensíveis (células glomus) dos corpos carotídeos, sendo esta informação enviada ao sistema nervoso central via aferencias vagais.

É bem descrita a queda da Tc frente à hipóxia em recém-nascidos e adultos de muitas espécies (revisado por Bícego et al., 2007; Mortola 2009), bem como a redução da temperatura crítica inferior especialmente em mamíferos (Frappell et al, 1992; Mortola & Matsuoka, 1993; Tattersall et al, 2002). Essa queda da Tc frente à hipóxia não é uma simples hipotermia, constituindo um mecanismo regulado resultante da inibição da produção de calor e estimulação da perda de calor (Gautier

et al., 1987; Barros et al., 2001; Tattersall & Milson, 2003), além da seleção comportamental por ambientes mais frios (Hicks & Wood, 1985; Gordon & Fogelson, 1991; Malvin & Wood, 1992). Trata-se, portanto, de reduções da taxa metabólica e da Tc ativamente induzidas pelo organismo. Pelo menos em mamíferos, a hipóxia parece atuar nos neurônios sensíveis ao calor localizados na área pré-óptica do hipotálamo, principal região do encéfalo envolvida na termorregulação, para induzir essas alterações termoefetoras (Branco et al., 2006; Scarpellini et al., 2009; Tattersall & Milson, 2009). Para aves, o cenário do processamento central das informações térmicas é menos conhecido e parece apresentar variações quanto às regiões termosensíveis e termointegradoras no hipotálamo e fora dele (Bícego et al., 2007).

A sensibilidade do sistema nervoso central (SNC) frente à hipoxia tanto no que se refere às lesões deste e possíveis alterações no limiar termogênico e na termossensibilidade tem sido investigada em mamíferos (Vannucci & Hagberg, 2004; Tattersall & Milsom, 2009). Os trabalhos têm mostrado que o encéfalo "imaturo", isto é, durante o desenvolvimento embrionário ou fetal, parece ser resistente a condições de hipóxia ou hipóxia isquêmica. Fato este que não ocorre após a maturação do SNC. No feto, uma redução aguda da PaO_2 leva a respostas cardiovasculares envolvendo uma elevação da pressão arterial e redistribuição do débito cardíaco em favor de órgãos vitais (Ruijtenbeek et al., 2002). Em embriões de aves o aumento das concentrações de catecolaminas circulantes participa desta resposta (Mulder et al., 2000 e 2001).

1.3.2. Hipóxia crônica perinatal

Diversas alterações metabólicas e morfométricas foram demonstradas em aves submetidas à hipóxia durante o desenvolvimento pré-natal, tais como incremento na mortalidade, diminuição na massa corporal, anormalidades no desenvolvimento cardiovascular e de outros sistemas e até mesmo na embriogênese, como resultado da oferta restrita de oxigênio aos tecidos (Dzialowski et al., 2002; Chan & Burggren, 2005; Ghatpande et al., 2008). Em geral, o efeito

hipóxico depressor da taxa metabólica manifesta-se como redução da taxa de crescimento durante a embriogênese (Mortola & Cooney, 2008; Mortola & Awan, 2010) podendo levar à imaturidade de órgãos e sistemas no neonato. Além disso, também foi demonstrado que a exposição à hipóxia a partir do quinto dia de incubação de ovos de galinha altera a preferência térmica de pintainhos normóxicos recém-eclodidos, sendo que estes selecionam Tas menores do que os controles, diferença esta não observada naqueles com mais de 8 horas pós-eclosão (Azzan et al., 2007).

Existe uma interação multivariada entre o estágio de desenvolvimento, o ambiente, o tempo de exposição a um estímulo ambiental e o fenótipo do adulto, e quanto tempo vai levar para alcançar esse fenótipo. Além disso, durante o desenvolvimento pré e perinatal existem fases que apresentam maior sensibilidade a mudanças ambientais, sendo chamadas de “janelas críticas” (Burggren, 1998). Cada órgão, cada sistema orgânico e cada organismo tem suas próprias janelas críticas, ou seja, fases de maior suscetibilidade a apresentarem um fenótipo alterado devido à exposição a uma variação ambiental. Entender como processos fisiológicos derivam de estruturas anatômicas é particularmente importante na compreensão de como relações de forma e função surgem no embrião em desenvolvimento e, sobretudo, como essas relações mudam durante o desenvolvimento posterior do animal. Assim, o estresse hipóxico pode gerar diferentes efeitos no desenvolvimento (estruturais e fisiológicos), dependendo da fase e do intervalo de tempo de exposição à hipóxia (Burggren, 1998; Dzialowski et al., 2002; Chan & Burggren, 2005; Ghatpande et al., 2008).

Neonatos de galinha que foram expostos à hipóxia (crônica) durante a incubação apresentam reduzidas respostas metabólicas e ventilatórias a uma nova exposição hipóxica (aguda). Tal fato tem sido atribuído a possíveis alterações no SNC em regiões envolvidas no controle metabólico e ventilatório e a uma ação negativa sobre o desenvolvimento dos quimiorreceptores periféricos, os quais se tornam funcionais na última fase da incubação (Mortola, 2009).

Desta forma, tem sido sugerigo que a hipóxia crônica em recém-nascidos tem um efeito depressor a longo prazosobre a resposta ventilatória quando os animais são expostos a um novo episodio de hipóxia aguda na vida juvenil/adulta. Este fenômeno, tanto em mamíferos e aves, tem sido atribuído a um desarranjo do desenvolvimento normal dos quimiorreceptores (revisado em Carroll, 2003; Mortola, 2009). No entanto, em embriões de galinha, hipóxia crônica durante toda a incubação ou apenas no último terço, resultou em uma resposta ventilatória hipóxica diminuída (RVH) do recém-eclodido (Szdzuy & Mortola, 2007; Ferner & Mortola, 2009). Enquanto a hipóxia não carregava consequências nas RVH do recém-eclodido, se a hipóxia ocorre apenas em estágios anteriores embrionários (Ferner & Mortola, 2009). O fato de que a hipercapnia embrionária (Szdzuy & Mortola, 2008) e a hiperóxia (Bavis & Simons, 2008; Mortola, 2011a) pós-natal causou alguma diminuição das RVH foi considerado compatível com a ideia de que uma estimulação crônica dos quimiorreceptores pode interferir com o seu desenvolvimento pré-natal normal.

Hipóxia pré-natal reduz a taxa metabólica e impede o crescimento de muitos órgãos, incluindo os pulmões (Mortola, 2009). A possibilidade do que o baixo peso ao nascer, por si só e independentemente da hipóxia pré-natal, pode contribuir para a diminuição das RVH foi testado no recém-nascido experimentalmente e descartado (Mortola, 2010). Da mesma forma, a possibilidade de que a hipóxia pré-natal pode resultar em um incremento da impedância mecânica do sistema respiratório do recém-nascido também foi rejeitado (Mortola, 2011b). Ainda resta testar se a condição do hipometabolismo pré-natal, por si só independentemente da hipóxia, pode contribuir para alguma depressão das RVH neonatais.

É interessante notar que os trabalhos citados acima demonstrando as influências da hipóxia durante a incubação somente abordaram possíveis alterações morfofisiológicas durante a fase pré-natal (embrião e/ou feto) e/ou no primeiro dia pós-eclosão. Além disso, a grande maioria dos estudos foi realizada com linhagem de galinhas poedeiras, sendo que as alterações morfológicas avaliadas restringiram-se a possíveis mudanças de massa dos órgãos.

2. Objetivo

Diante do que foi apresentado acima, o presente estudo teve como objetivo investigar:

a) o efeito da hipóxia na primeira ou na última semana da incubação sobre características morfofisiológicas do intestino delgado (morfometria de vilos e quantidade de células caliciformes) e características gerais do recém-nascido de frango de corte (Capítulo 2).

b) as possíveis diferenças entre as respostas metabólicas à hipóxia aguda de pintinhos de frangos de corte (selecionado para crescimento rápido) e de galinha poedeira (crescimento lento) de 1 e 10 dias pós-eclosão e qual a interferência da hipóxia durante a fase final da incubação sobre essas respostas (Capítulo 3).

c) se a depressão metabólica fetal (dias 18-20 de incubação) pelo frio pode exercer a mesma influência que aquela pela hipóxia sobre a resposta ventilatória hipóxica no recém-eclodido de galinha poedeira (Capítulo 4).

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CAPÍTULO 2 - Effects of hypoxia during incubation on the development of the intestines in chicken hatchlings

Toro-Velasquez, Paula A.[1,2]; Souza, Lilian F.A.[1]; Gargaglioni, Luciane H. [1,2]; Macari, Marcos[1]; *Bícego, Kênia C.[1,2]

[1]Department of Animal Morphology and Physiology, College of Agricultural and Veterinarian Sciences, São Paulo State University, Jaboticabal, São Paulo, 14884-900, Brazil.

[2]National Institute of Science and Technology on Comparative Physiology (INCT- Fisiologia Comparada)

Running title: Late hypoxia incubation decreases goblet cell quantity in hatchlings

*Corresponding Author: Via de acesso Paulo Donato Castellane s/n, 14884-900, Departamento de Morfologia e Fisiologia Animal, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista Júlio de Mesquita Filho, Jaboticabal, SP, Brasil. Telephone: 55 16 32092656. Telefax: 55 16 32024275. E-mail: keniacb@yahoo.com.br; keniacb@fcav.unesp.br.

Abstract

Hypoxia during pre-natal development can affect body growth differently depending on the phase of incubation during which it is applied. Regarding precocious birds, no study assessed the influence of hypoxia during different phases of incubation on the morphometric development of the gut. The gut is important not only for digestion and absorption, but also for protection against exogenous pathogens. In this case, goblet cells play a significant role in gastrointestinal surface protection because of mucus secretion. We investigated the effect of 15% O_2 in air during the first (HxE) or the last (HxL) week of incubation on the morphometric characteristics and quantity of goblet cells per villus in regions of the small intestine in chicken hatchlings. Neither HxE nor HxL changed the villus height or surface in the duodenum, jejunum or ileum. The number of goblet cells per villus was lower in the duodenum of the HxL compared to the HxE and normoxia groups. Hypoxia at the beginning or the end phase of incubation did not affect body or intestinal mass. HxE, but not HxL, induced a higher quantity of immature neonates, characterized mainly by the presence of remaining membrane in the navel area, incomplete closure of the navel area and weak activity. The results indicate that hypoxia in the last week of incubation, but not in the first, seems to affect intestinal morphophysiological development, especially regarding the reduced quantity of goblet cells in the duodenum, which may lead to reduced protection of the brush border and poor immune defense and/or digestive function.

Key words: Goblet cells, villus, duodenum, jejunum, ileum

1. Introduction

Hypoxia is an important challenge during prenatal development and is reported to decrease body growth in avian embryos (Dzialowsky et al., 2002; Azzan and Mortola, 2007; Zhang and Burgreen, 2012) since primary energy saving during a low oxygen supply probably originates from the blunting of body growth (Mortola, 2009 for review). Thus, hatchlings may present anomalies that can influence performance or even survival, which may be related to the decrease in growth rate during embryonic and/or fetal development. Evidence exists that many of the abnormalities seen in late fetuses or hatchlings have their origins in mishaps at early stages, which are considered to be the most vulnerable phases to general disturbances (Bellairs and Osmond, 2005). However, chicken hatchlings subjected to hypoxia at the third, but not at the first or the second, week of incubation exhibit reduced body mass after 21 days of incubation (Dzialowsky et al., 2002). These results indicate that the embryo/fetus seems to be able to gain weight during the subsequent normoxia, compensating for the slower hypoxic growth rate (Dzialowsky et al., 2002).

Except for the chorioallantoic membrane, which develops out of proportion, there is a correlated reduction of body mass and the masses of several organs, such as the lungs, heart and intestine, in chicken hatchlings subjected to hypoxia during incubation (Azzan and Mortola, 2007). Regarding the intestine, its full development is associated with the maintenance of appropriate digestive and absorptive functions for the supply of nutrients, and also protection, since its enormous mucosal surface is associated with lymphoid tissue (Kasahara et al., 1993; Beal et al., 2006) and is covered by a mucus layer (Forstner et al., 1995). The mucus, produced by goblet cells, is important for preventing gastrointestinal pathologies, and also plays a role in nutrient digestion and absorption (Forstner et al., 1995).

The mucus layer acts as a medium for protection of the brush border against damage by chemicals and microorganisms, lubrication and also influences transport between the luminal contents and the brush border (Forstner and Forstner, 1994).

The region of future divisions of intestine in the chicken can be recognized by embryonic day 6 (E6) (Bellairs and Osmond, 2005). Intestinal mass, as a proportion of embryonic mass, increases from approximately 1% at 17 days of incubation (E17) to 3.5% at hatching (Uni, 2006). To accommodate the rapid transition from internal to external nutrient sources, the chicken small intestine goes through morphological, cellular and molecular changes toward the end of incubation. Two days prior to hatching, the small intestine has a villus structure and the potential for carbohydrate digestion and absorption (Uni et al., 2003a). Although the digestive capacity begins to develop a few days before hatching, its full development occurs post-hatch when the neonatal chick begins consuming food (Uni, 2006). Thus, the importance of early development of the small intestine is associated with influences on later growth, development and health of the animal (Michell and Moretto, 2006).

Even though there are many studies showing the effect of hypoxia during chicken embryo and fetus development on organ mass, including the intestines, (Dzialowsky et al., 2002; Azzan and Mortola, 2007; Zhang and Burgreen, 2012), no one has characterized this effect on the morphometric characteristics of the intestine, specifically the duodenum, jejunum and ileum, or the quantity of goblet cells. Therefore, in the present study we investigated the effect of hypoxia during the first or last week of incubation on the morphometric characteristics of segments of the small intestine and the quantity of goblet cells, as well as general physical characteristics, in chicken hatchlings.

2. Methods

Experiments were conducted on chicken hatchlings (broilers, Cobb-500® strain). Fertile eggs were obtained from a local supplier and placed in incubators (Premium Ecológica, Belo Horizonte, MG, Brazil) set at a temperature of 37.5°C and 60% relative humidity, with automatic egg rotation every hour. The eggs were distributed in three incubators as follows: 1) normoxia for the whole incubation (21% O_2; Nx); 2) hypoxia for the first week and normoxia during the rest of incubation (15% O_2 from day 0 to day 7; HxE); and 3) normoxia during the first two weeks and hypoxia for the third week of incubation (15% O_2 from day 12 to day 19; HxL). Hypoxia was induced by leaking a small stream of N_2 into the incubator under the control of a flowmeter (AFSG 165; 0.4-5 Lpm +-3%fe; White Martins, Brazil). The O_2 concentration in the incubator was continuously sampled by an O_2 analyzer (Sensepoint XCD, Honeywell, USA). The study wasconducted with the approval of the local Animal Care and Use Committee (protocol number 024166/13).

2.1. Internal pipping, external pipping and spread of hatching

At day 19 of incubation, all the eggs were candled, and those with evidence of a living fetus were transferred from turning trays to hatchery baskets and placed individually in a net sac (15 x 10 cm). Between 462 and 522 h of incubation, the transferred eggs were frequently checked (every 6 h), candled and verified for internal pipping, external pipping and hatching. The hatchlings were individually labeled and weighed. After 522 h of incubation (21.75 days), the hatching process was ended in all groups. For morphometric analyses of the intestine and the counting of gobletcells, chicks that hatched at around 504 h were used for all experimental groups.

2.2. General characteristics of hatchlings

All hatchlings were examined and scored for general characteristics as previously described by Tona et al., (2003). Briefly, physical parameters were scored according to their importance for the survival of the chick and the severity of possible anomalies. These included activity (good: 6; weak: 0), feathering (clean and dry: 10; wet: 8; dirty and wet: 0), eye condition (opened and bright: 16; opened and not bright: 8; closed eyes: 0), conformation of legs (normal legs and toes: 16; one infected leg: 8; two infected legs: 0), navel area condition (completely closed and clean: 12; not completely closed and not discolored: 6; not closed and discolored 0), yolk retraction (body with normal swallowed yolk: 12; body with swallowed large yolk and rather hard to the touch: 0), and status of the remaining membrane (no membrane: 12; small membrane: 8; large membrane: 4; very large membrane: 0) and remaining yolk (no yolk: 16; small yolk: 12; large yolk: 8; very large yolk: 0). Each chick was classified by its total score (maximum 100) resulting from the sum of the scores for all characteristics. For the morphometric analyses of the intestine and the counting of goblet cells, chicks with a score of 100 were used for all experimental groups (n= 6/group). The remaining hatchlings were used in other two studies.

2.3. Body and intestinal mass

Hatchlings were weighed and six were selected according to the average body mass of animals in each treatment (Nx, HxE or HxL) and euthanatized by lethal dose of ketamine (1.15 g/kg BW). The yolk sac and total intestine (small + large) were excised and weighed. The duodenum, jejunum and ileum were separated (see next section 2.4) and weighed. Body

and yolk sac masses were also determined in six other 19-day-old fetuses (E19; at this age, the yolk sac is not incorporated into the body yet).

2.4. Tissue sampling

The intestine samples consisted of pieces of about 2 cm: duodenum (from the pylorus to the distal duodenal loop); jejunum (from the distal duodenal loop to Meckel's diverticulum); and ileum (starting from Meckel's diverticulum to the ileocecal junction). The samples were washed in saline solution, fixed in Bouin, and then dehydrated in a series of increasing alcohol concentrations, diaphanized in xylene, and embedded in paraffin (Luna, 1968) for analysis with light microscopy.

2.5. Intestinal morphometry and goblet cell counting

Semi-serial 5-µm-thick cross-sections of the duodenum, jejunum and ileum were prepared and stained with hematoxylin-eosin for morphometry and periodic acid-Schiff reagent for the counting of goblet cells (Luna, 1968). Images were captured using a light microscope (Leica Microsystems Inc., USA) and were examined using the Image J® computerized analyzer (Rasband, 2004). Measurements of the villus height were taken from the basal region, which coincides with the upper portion of the crypts to the apex, and the crypts were measured from the base to the crypt-villus transition region. The villus surface area was calculated using the following formula: 2 x width / 2 x villus height (Sakamoto et al., 2000; Sohail et al., 2012), where width was measured at the middle part of the villus. The number of goblet cells per villus in the duodenum, jejunum and ileum was determined for a 200 m extension in the middle of the villus. A total of 30 villi and 30 crypts were considered per segment/bird (1 bird/replicate) for all the analyses.

2.6. Statistical analyses

Values are expressed as mean ± SEM. The data for incubation duration, body mass, yolk sac mass, organ mass, and intestinal parameters were processed with an analysis of variance using the General Linear Models procedure of SAS (SAS Institute Inc., 2002). Means were compared by the Tukey´s test and significance was based on $p < 0.05$. Data for chick scores and spread of hatching was analyzed with a two-tailed test for comparison of variance.

3. Results

3.1. Effect of early or late hypoxia on incubation duration and spread of hatching in broiler hatchlings

Hypoxia during the first or the last week of incubation did not affect internal pipping, external pipping or incubation duration (Table 1).

Figure 1 depicts the percentage of hatching during a 36-hour interval at the end of incubation (from 486 to 522 h). In normoxia, the peak of hatching occurred between 504 and 506 h corresponding to 40% of hatching. Late hypoxia induced a slightly lower peak of 30% hatching at the same time as normoxia. However, early hypoxia increased the spread of hatching, as demonstrated by a lower and larger peak (between 498 and 510 h) and a larger base of the hatching curve (Fig.1).

3.2. Effect of early or late hypoxia on general characteristics and body mass of hatchlings

The average scores of hatchlings were not different among groups (Table 2). For the normoxia group, 40.3% of animals had a score of 100 and 29% of them presented a score less than 88. These proportions were not different from those obtained in the late hypoxia group. In contrast, hypoxia during the first week of incubation induced a smaller proportion of hatchlings with a score of 100 (24.2%) and a higher proportion of chicks with a score less than 88 (46.8%) compared to normoxia and late hypoxia groups. The main problems observed in early hypoxia chicks were incomplete closure of the navel area, weak activity and the presence of remaining membrane in the navel area.

No differences among groups were observed in the body or yolk sac mass of 19-day-old fetuses or the body mass of hatchlings, while yolk sac mass of hatchlings was higher in the late hypoxia group compared with normoxia and early hypoxia (Table 3).

3.3. Effect of early or late hypoxia on intestinal development in broiler hatchlings

There was no effect of different incubation conditions on the absolute and relative masses of total intestine (small + large) or the duodenum, jejunum and ileum segments (Table 4).

In Table 5, it can be observed that there were no significant differences in villus height, crypt depth and villus area of the duodenum, jejunum and ileum among normoxia, early and late hypoxia groups.

The number of goblet cells in each villus in the duodenum was lower in late hypoxia, but not early hypoxia, compared to the normoxia group. For the other segments, jejunum and ileum, there were no significant differences among groups (Fig. 2).

4. Discussion

The main finding of the present study is that hypoxia during the last, but not the first, week of incubation interferes with the development of the small intestine, decreasing the number of goblet cells per villus in the duodenum of chicken hatchlings. As previously suggested (Dzialowsky et al, 2002; Azzan and Mortola, 2007; Zhang and Burggren, 2012), the present results reinforce the idea that critical windows for pre-natal development exist in chickens. Thus, hypoxia exerts general influences when applied during the first week of incubation (embryonic development) increasing the proportion of immature hatchlings, and affects maturation of systems, the digestive system in this case, when applied during the last week of incubation (fetus development).

Early hypoxia (HxE) affects the spread of hatching and general characteristics of hatchlings, but not the morphometry of the intestines

Despite the lack of treatment effects on duration of incubation, HxE increased the spread of hatching, inducing a larger base and lower peak of the curve (Fig. 1). This result seems to be related to the lower chick scores observed in the HxE hatchlings (Table 2). In fact, the main problems observed in the HxEchicks were the presence of remaining membrane in the navel area, incomplete closure of the navel area and weak activity, indicating hatchlings were in incomplete stages of development. In agreement with this assumption, acute hypoxia (2, 4 or 6 h) on embryonic day 2, 4 or 6 was reported to affect embryo survival and cause developmental abnormalities (Altimiras and Phu, 2000). In addition, incubation at an altitude of 2,000 m (corresponding to an approximate fraction of 17% O_2) during the first 10 days (Bahadoran et al., 2010) or during the entire incubation (Hassanzadeh et al., 2004) induced

hatching earlier than development at sea level. It is interesting to note that in both studies, the authors observed increased T4, T3 and corticosterone levels in embryos at days 10 (Bahadoran et al., 2010) and 19 (Hassanzadeh et al., 2004; Bahadoran et al., 2010), which may be a factor that induced early hatching in those studies and probably in our chicks as well. Thus, the imbalance in embryonic neurochemical regulations induced by hypoxia early in incubation may affect the proper time for total development before hatching, since a high percentage of our HxE hatchlings were immature.

None of the hypoxic conditions affected the body masses of fetuses (19 days) or hatchlings (Table 3). Bahadoran et al. (2010) also did not observe any difference in absolute or relative embryo body mass after incubation from 1-10 days at high altitude (1,800 m). Other studies also showed that hypoxia during incubation decreases growth rate (Azzan and Mortola 2007; Mortola, 2009), but after removal of hypoxia, chicken embryos are able to recover body mass (Dzialowski et al., 2002). On the other hand, Chan and Burggren (2005) found no differences in embryo and yolk mass at day 18 of incubation from early, late and continuous hypoxia, suggesting that embryos are able to recover from the deleterious effects of hypoxia on growth rate. In the present study, HxL hatchlings, but not HxE, presented bigger yolk sacs than the Nx group (Table 3). This result agrees with a hypoxia-induced reduction in metabolic rate (Mortola and Labbè, 2005) and, consequently, less yolk consumption during the late phase of incubation, while metabolic recovery possibly occurred in HxE hatchlings.

Late hypoxia (HxL) affects the number of goblet cells per villus in the duodenum, but not the spread of hatching and general characteristics of hatchlings

Both the absolute and relative masses of the intestine and its segments (duodenum, jejunum and ileum) were not affected in hatchlings by any incubation with hypoxia (Table 4). Azzam&Mortola (2007) demonstrated that at the same age, the intestines presented a lower specific weight in embryos incubated with hypoxia compared to a higher specific weigh of the brain and heart. However, the authors attributed this effect to the generalized blunted growth, as differences in organ weight between hypoxia and normoxia disappeared when organ weight was compared as a function of body mass. Thus, it appears that hypoxia has minimal selective effects on the growth of specific organs. Furthermore, we showed that neither HxE nor HxL caused changes in morphometric parameters of the duodenum, jejunum and ileum, such as villus height, crypt depth and villus surface area (Table 5). Pre-villus ridges appear in the chicken embryo duodenum on the 7^{th} or 8^{th} day and increase in numbers thereafter; at 18 or 19 days they elongate rapidly (Coulombre&Coulombre, 1958).

Despite the fact hypoxia did not change intestinal morphometry, the number of goblet cells per villus in the duodenum was reduced in the HxL group. It is known that the development of these cells occurs in the late embryonic and immediate post-hatch phase (Uni et al., 2003b). Goblet cells arise by mitosis from pluripotential stem cells at the base of the crypt (Cheng and Leblond, 1974) or from poorly differentiated cells in the lower crypt, referred to as oligomucous cells (Cheng, 1974). These cells migrate from the crypt toward the villus tip where they are sloughed into the lumen, a process that takes 2 to 3 days (Geyra et al., 2001). The goblet cells secret mucin, which is the main component of the mucus gel layer covering the surface of the gastrointestinal tract (Allen and Flemström, 2005; Uni, 2006). In

general, the mucus gel layer acts as a lubricant, improving the propulsion of chyme; protects the intestinal epithelium from enteric pathogens, modulating their adherence to the epithelium and acting as a physical barrier; acts as a stable and adherent layer where secreted bicarbonate creates a pH gradient that protects against luminal acid in the stomach and duodenum; protects the intestinal wall against proteolytic digestion by luminal enzymes (Allen and Flemström, 2005; Uni, 2006). At least in rats, acute intestinal ischemia induces mucin breakdown, which is accompanied by epithelial cell disruption and increased non-selective intestinal permeability (Chang et al., 2012). Interestingly, a new protective function of goblet cells was recently suggested, as they are thought to aid in the persistence of maternal IgA (important for immune defense at the perinatal period and first weeks post-hatch) in the chicken gut (Bar-Shira et al., 2014). These authors provided evidence that goblet cells serve as a reservoir for maternal IgA, as they seem to uptake this IgA (supplied by the egg) and release it along with mucin secretions, which are confined to the enterocyte surface, protecting this area and limiting IgA loss due to intestinal flushing. In our chicks, the hypoxia-induced ~20% reduction of goblet cells, especially in the duodenum (Fig. 2), may result in a poorly protected intestinal epithelium, affecting the integrity of this surface and, consequently, the general health of the animal, at least in the early phase post-hatch when hatchlings start to consume external food and water.

In summary, hypoxia during the late, but not the early, phase of incubation reduced the number of goblet cells per villus in the duodenum, which may affect protective and digestive/absorptive functions of the intestine in chicken hatchlings.

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Table 1 – Internal pipping, external pipping and incubation duration (ID) from the normoxia (n=45), early hypoxia (n=52) and late hypoxia (n=48) incubations of broiler chicken

	Normoxia	**Early hypoxia**	**Late hypoxia**	**p value**
Internal pipping (h)	476.10 ± 4.26	480.93 ± 4.35	476.73 ± 4.76	0.0683
External pipping (h)	488.85 ± 3.42	489.00 ± 3.16	490.79 ± 3.71	0.6183
ID (h)	506.44 ± 2.63	505.18 ± 3.33	505.09 ± 3.37	0.7285

Data are expressed as mean ± SEM.

Table 2 – Hatchling general scores from the normoxia (n=62), early hypoxia (n=62) and late hypoxia (n=61) incubations of broiler chicken

	Normoxia	**Early hypoxia**	**Late hypoxia**
% of chicks with score 100	40.32a	24.19b	40.98a
% of chicks with score < 88	29.03a	46.77b	29.51a
Average score of all chicks	91.60	87.56	91.90

Different letters (a, b) means significant difference among experimental groups ($p < 0.05$).

Table 3 – Body mass (without yolk sac) and yolk sac mass of broiler 19-day fetus and hatchling of the normoxia, early hypoxia and late hypoxia incubations

		Normoxia	**Early hypoxia**	**Late hypoxia**	**p value**
Fetus	Body mass (g)	33.45 ± 1.77	31.68 ± 0.76	29.56 ± 0.71	0.1006
	Yolk sac mass (g)	12.42 ± 0.79	14.65 ± 0.57	14.54 ± 0.77	0.0769
Hatchling	Body mass (g)	41.70 ± 0.56	41.22 ±0.55	40.41± 0.36	0.2117
	Yolk sac mass (g)	4.13 ± 0.46 b	4.93 ± 0.56 b	7.22 ± 0.40 a	0.0011

Data are expressed as mean ± SEM. N= 6. Different letters indicate statistical differences ($p < 0.05$)

Table 4 – Total intestine (small + large), duodenum, jejunum and ileum absolute and relative mass (% of yolk free hatchling mass) of hatchlings from the normoxia, early hypoxia and late hypoxia incubations

		Normoxia	**Early hypoxia**	**Late hypoxia**	**p value**
Absolut Mass (g)	Intestine	1.93 ± 0.14	1.87 ± 0.08	1.82 ± 0.07	0.7548
	Duodenum	0.40 ± 0.03	0.42 ± 0.02	0.40 ± 0.02	0.7929
	Jejunum	0.46 ± 0.05	0.44 ± 0.03	0.41 ± 0.02	0.6240
	Ileum	0.37 ± 0.04	0.33 ± 0.01	0.35 ± 0.02	0.4862
Relative mass (%)	Intestine	4.61 ± 0.29	4.54 ± 0.19	4.51 ± 0.18	0.9457
	Duodenum	0.95 ± 0.06	1.00 ± 0.06	0.96 ± 0.04	0.7786
	Jejunum	1.10 ± 0.11	1.07 ± 0.08	1.02 ± 0.05	0.7707
	Ileum	0.88 ± 0.08	0.79 ± 0.03	0.86 ± 0.05	0.5228

Data are expressed as mean ± SEM. N= 6.

Table 5 –Duodenum, jejunum and ileum villus height, crypt depth and villus surface area of hatchlings from the normoxia, early hypoxia and late hypoxia incubations

		Normoxia	**Early hypoxia**	**Late hypoxia**	**p value**
Duodenum	Villus height (µm)	825.4 ± 43.1	771.4 ± 41.6	790.6 ± 22.4	0.5887
	Crypt depth (µm)	78.3 ± 3.5	74.7 ± 5.2	76.0 ± 5.4	0.8707
	Villus surface area (mm^2)	0.261 ± 0.020	0.242 ± 0.024	0.222 ± 0.011	0.3768
Jejunum	Villus height (µm)	448.4 ±42	365.6 ±28	436.7 ±25	0.1871
	Crypt depth (µm)	55.8 ±5.1	61.2 ±4.0	58.5 ±2.7	0.6473
	Villus surface area (mm^2)	0.108 ± 0.014	0.096 ± 0.007	0.096 ± 0.012	0.7124
Ileum	Villus height (µm)	387.2 ±33	368.8 ±20	405.3 ±15	0.5627
	Crypt depth (µm)	64.3 ±2.3	66.3 ±2.9	61.4 ±2.6	0.2972
	Villus surface area (mm^2)	0.088 ± 0.009	0.098 ± 0.007	0.079 ± 0.005	0.1852

Data are expressed as mean ± SEM. N= 6.

Figure legends:

Fig. 1: Comparative percentages of hatched chickens from the normoxia (n = 45), early hypoxia (n = 52) and late hypoxia (n = 48) incubations. See Method section for details.

Fig. 2: Amount ofgoblet cells (number / 200 µm) per villus in the duodenum, jejunum and ileum of hatchlings from thenormoxia, early hypoxia and late hypoxia incubations. N = 6.

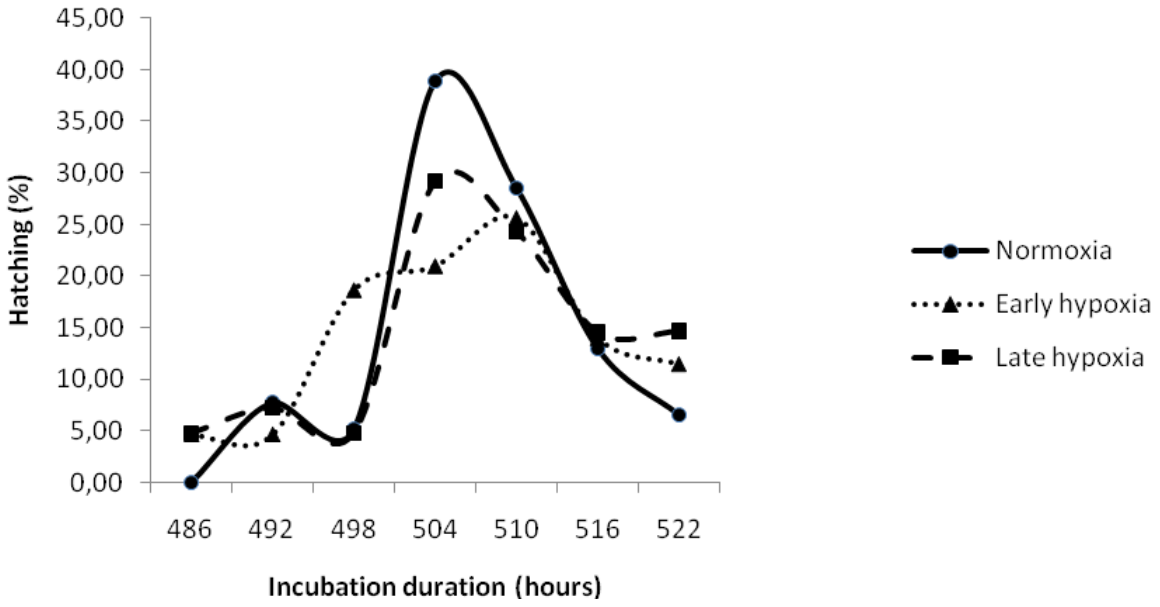
45,00
40,00
35,00
30,00
25,00
20,00
15,00
10,00
5,00
0,00
Hatching (%)
486
492
498
504
510
516
522
Incubation duration (hours)
Normoxia
Early hypoxia
Late hypoxia

Fig. 1

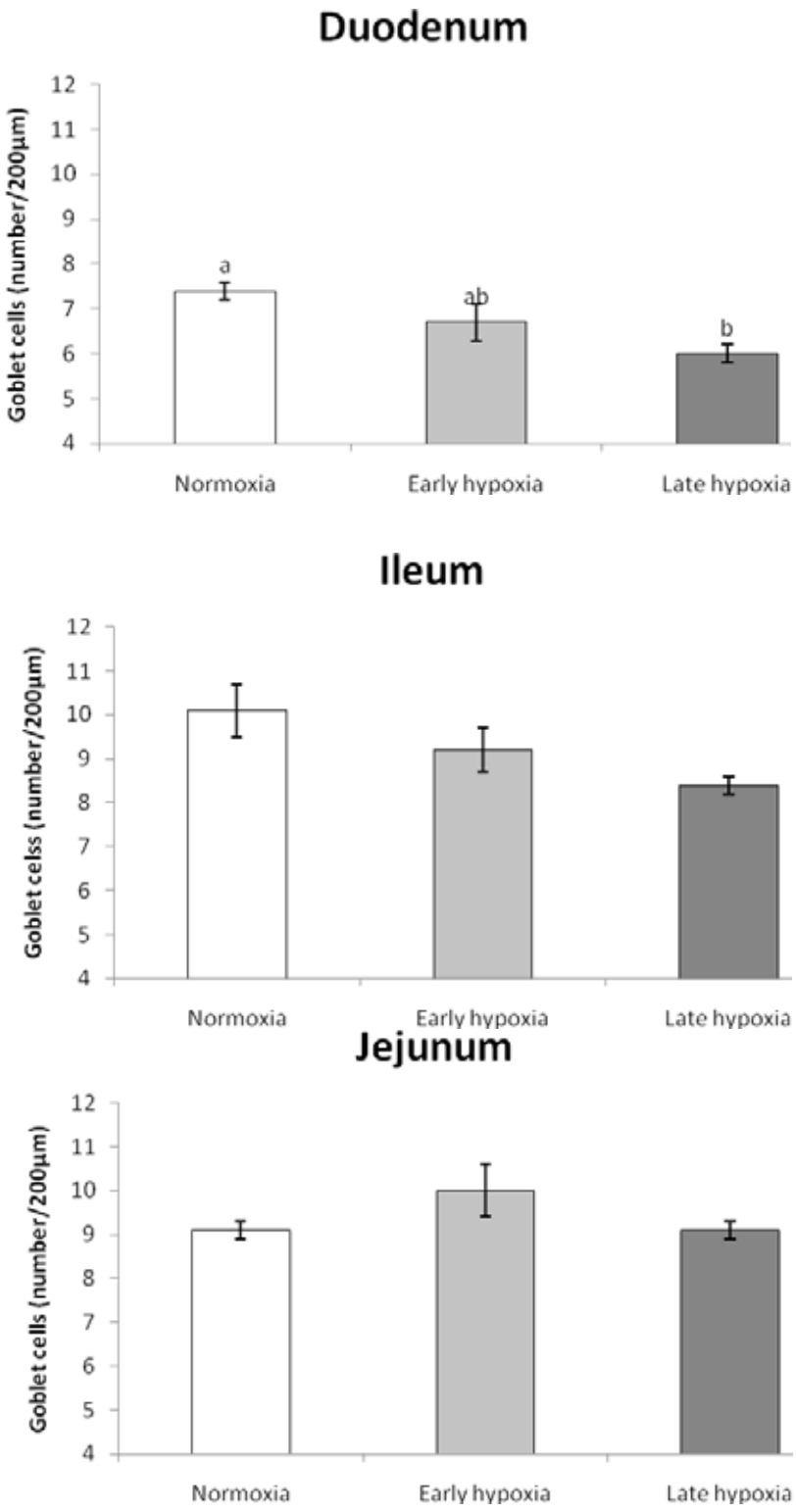
Duodenum
Goblet cells (number/200µm)
12
11
10
9
8
7
6
5
4
a
ab
b
Normoxia
Early hypoxia
Late hypoxia
Ileum
Goblet celss (number/200µm)
12
11
10
9
8
7
6
5
4
Normoxia
Early hypoxia
Late hypoxia
Jejunum
Goblet cells (number/200µm)
12
11
10
9
8
7
6
5
4
Normoxia
Early hypoxia
Late hypoxia

Fig. 2

CAPÍTULO 3 - Metabolic responses to hypoxia in chicks with different growth rates

Toro-Velasquez, Paula A.[1,2]; Mortola, Jacopo[3]; Amaral, Lara[1,2]; Fernandes, Marcia[1,2]; Gargaglioni, Luciane H. [1,2]; Macari, Marcos[1]; *Bícego, Kênia C.[1,2]

[1]Department of Animal Morphology and Physiology, College of Agricultural and Veterinarian Sciences, São Paulo State University, Jaboticabal, São Paulo, 14884-900, Brazil.

[2]National Institute of Science and Technology on Comparative Physiology (INCT- Fisiologia Comparada)

[3]Department of Physiology, McGill University, 3655 Promenade Sir William Osler, Montreal, Quebec, H3G 1Y6 Canada.

*Corresponding Author: Via de acesso Paulo Donato Castellane s/n, 14884-900, Departamento de Morfologia e Fisiologia Animal, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista Júlio de Mesquita Filho, Jaboticabal, SP, Brasil. Telephone: 55 16 32092656. Telefax: 55 16 32024275. E-mail: keniacb@yahoo.com.br; keniacb@fcav.unesp.br.

Abstract

For most endotherms, birds and mammals, the acute response to hypoxia includes decreases in metabolic rate and body temperature (Tb) and increase in ventilation, all together seems to minimize the imbalance between oxygen supply and demand. Therefore, in the present study we investigated what is/are the main factor(s) affecting metabolic depression by acute hypoxia in young chicks: age, rate of growth, body mass and/or previous exposure to hypoxia during incubation in the last week (late hypoxia: HxL). The resting metabolic rate (VO_2) normalized by body weight increased with age independent on the incubation condition for fast and slow growth. The exposure to acute hypoxia decreased VO_2 in all animals. Regardless incubation condition, the Fast H5 and Slow H10 chicks presented similar body weights but Fast H10 birds had higher resting metabolic rates. Fast H10 chicks presented the highest body weight and metabolic rate.

Key words: oxygen consumption, incubation, fast growth, slow growth,

1. Introduction

Energy metabolism in vertebrates is primarily based on aerobic processes. Thus, reduction of O_2 partial pressure (hypoxia) can affect all of these processes influencing cellular and organ functions including cell membrane transports, muscle tone, maintenance, tissue growth, locomotion, states of alertness and sleep and general behavior (Mortola and Maskrey, 2011). Hypoxia can be found in both physiological and pathological conditions. Physiological situations usually result from hypoxic environments such as high altitude, burrows and oxygen-deprived water habitats; as to pathologies, it can be listed obstructive sleep apnea, chronic obstructive pulmonary disease and hypoxaemia associated with circulatory disorders including those related to septicemic or endotoxemic shock (Tsioutou et al., 2005; Bicego et al., 2007, Tattersall and Milsom, 2009).

For most endotherms, birds and mammals, the acute response to hypoxia includes decreases in metabolic rate and body temperature (Tb) and increase in ventilation, all together seems to minimize the imbalance between oxygen supply and demand. These responses are observed in newborns and adults of several species (Gautier, 1996; Mortola, 2009; Bícego et al., 2007; Mortola and Maskrey, 2011) and the metabolic suppression is accompanied by increase in autonomic (Tattersall and Milsom, 2003) and behavioral (Mortola and Feher, 1998; Bícego et al., 2007; Mortola and Maskrey, 2011) heat loss responses. During prenatal stages, the hypoxic metabolic drop reflects mainly in depression of tissue growth because at this phase growth is the most energy-demanding function. In contrast, after birth, at least in mammals, thermogenesis becomes the principal source of energy expenditure, and its inhibition is the main factor involved in hypoxia-induced metabolic reduction (reviewed by Mortola and Maskrey, 2011). Moreover, there is a general consideration that the greater the

metabolic rate (thermogenesis), the larger the metabolic reduction during hypoxia. This reflects the fact that smaller, younger and exposed to cold mammals show a more pronounced reduction in metabolic rate during hypoxia (Mortola and Maskrey, 2011). To the best of our knowledge, no study compared metabolic effects of hypoxia in young birds of different ages and growth rates. In this context, chickens present interesting characteristics for this sort of investigation since they are precocial birds with detectible thermogenesis and independence from maternal care as early as at birth (Mortola, 2009, for review; Toro-Velasquez et al., unpublished).

Besides acute hypoxia, chronic exposure to low levels of oxygen can induce long lasting morphophysiological changes, especially if the challenge occurred at critical moments of pre and/or perinatal phases (Mortola, 2009, Dzialowski et al., 2002). Several metabolic and morphometric changes were demonstrated in laying hen chicks submitted to hypoxia during prenatal development as a result of tight supply of oxygen to tissues (Mortola, 2009, Dzialowski et al,. 2002; Chan and Burggren, 2005; Ghatpande et al., 2008). In general, the hypoxic reduction in the growth rate during embryogenesis can lead to immaturity of organ systems in the neonate (Mortola and Cooney, 2008; Mortola and Awan, 2010). Furthermore, it was shown that exposure to hypoxia from the fifth day of incubation reduces the thermal preference of newly hatched normoxic chicks; they select smaller ambient temperatures than the controls (Azzan et al., 2007). Neonates also show less pronounced metabolic reductions to acute hypoxia if they are incubated in low oxygen atmosphere (Szdzuy and Mortola, 2007).

Based on the considerations above, we investigated what is/are the main factor(s) affecting metabolic depression by acute hypoxia in young chicks: age, rate of growth, body mass and/or previous exposure to hypoxia during incubation. To this end, we used hatchlings

and 10 days old chicks of two strains of domestic chicken, a bred with a normal growth rate (Slow) and a bred which grows twice as quickly (Fast) (Gyles, 1989; Havenstein et al., 1994a,b).

2. Methods

Experiments were conducted on chicks (*Gallus gallus*) of fast-growing (Fast; Cobb 500®) and slow-growing (Slow; White Leghorn) strains at two different ages, 15-18 hrs (hatchlings; H0) and 10 days post-hatching (H10). Experiments were also performed using 5 days old Fast chicks (H5; see explanation in *Protocols* section). Freshly laid fertilized eggs were obtained from local supplier. After noting the weight, the eggs were placed in incubators set at the temperature of 37.5°C and 60% relative humidity, with a 45° egg rotation at least four times a day. The start of incubation was denoted embryonic day 0 (E0). Incubation temperature and relative humidity were monitored by sensors inside the incubator. Half of the eggs at E12 was transferred to a different incubator with hypoxia 15% until E19. At the end of incubation (E20, internal pipping phase) all eggs were moved to a hatchery-incubator with no rotation; hatching day and time were noted. Chicks were maintained in temperature-controlled and ventilated chambers under a light:dark cycle of 14h:10h up to the day of the experiment (5^{th} or 10^{th} day) with free access to water and commercial starter diet. Ambient temperature was about 32^{o}C (from day 1 to 6) and 30^{o}C (from day 7 to 10). The protocols were conducted with the approval of the local Animal Care and Use Committee (CEUA- number 024166/13).

2.1. Oxygen consumption (VO_2)

VO_2 was measured by an open-flow methodology (Szdzuy et al., 2008). The hatchlings (H0) of both strains were placed individually in a 200 mL container inside a temperature controlled chamber (BOD 347CDG, FANEM, Brazil) and a steady gas flow of 150 ml/min was continuously delivered through the respirometer. Regarding the H5 and H10 chicks, they were maintained in a 1000 ml container with a steady gas flow of 1500 ml/min continuously delivered through the respirometer. The outflow gas passed through a drying column (Drierite, Sigma) and the O2 and CO2 concentrations were recorded continuously by calibrated gas analyzers (Sable Systems International Fox, Henderson, NV). The inflow concentrations were monitored intermittently. After mathematical correction of the gas concentrations for a respiratory quotient different from unity (Depocas and Hart 1957; Mortola and Besterman 2007), VO2 was computed from the flow rate and the inflow-outflow gas concentration difference.

2.2. Protocols

The same protocol was conducted on chicks of all ages, strains and different incubation conditions (Normoxia: Nx and Hypoxia: HxL). Each bird was left undisturbed in the respirometer for at least half an hour at ~35ºC (H0) or 30 ºC (H5 and H10). Following this time for habituation, the chick was maintained for 15 min in normoxia, 15 min in hypoxia 15%, 15 min in hypoxia 10%, and 20 min in normoxia again (recovery). The same animal was used at H1, H5 and H10. We used Fast H5 chicks in order to isolate age and body mass as a factor of differences between strains, since Fast H5 and Slow H10 had a similar weight (Table 1).

2.3. *Data Analysis*

Data are presented as means ±1 SEM. Statistical comparisons among groups were done by one-way ANOVA (resting metabolic rates) and repeated measures two-way ANOVA (effect of acute hypoxia), with a Bonferroni's post hoc test. It was applied linear regression for testing the relation between metabolic depression during hypoxia and resting metabolic rate in normoxia. The statistical analysis was performed using the software GraphPad Prism 5.0. Differences were considered statistically significant at $P < 0.05$.

3. Results

The characteristics of the eggs and chicks (N=28) are in Table 1. The postnatal age was at least 10 hours at the time of the first measure of VO_2 consumption. The temperature at the respirometry chamber changed with age, for H0 it was 35-37 °C, for H5 it was 31-32°C, and for H10 it was 30-31°C for both strains and incubation conditions. The egg of various groups had statistically similar weight. Also the incubation age at hatching, the body temperature at H0 and H5 and the body weight at H5 were not different among strains and incubation condition. Fast H0 and H10 chicks were heavier than Slow ones ($p<0.0001$). Body temperature of Fast animals from hypoxic incubation was lower than the other groups ($p<0.01$).

3.1 Effect of hypoxia on metabolic rate of slow-growing (Slow) chicks incubated in normoxia (Nx) and late hypoxia (HxL)

The resting metabolic rate (VO_2) normalized by body weight increased with age independent on the incubation condition (Nx or HxL) ($p< 0.0001$) (Fig. 1a). The VO_2 of H0

were 18.77 ± 1.48and 23.02 ± 1.29 mL/kg.min, and of H10 were 49.2 ± 4.32 and 50.6 ± 3.18 mL/kg/min for the Nx and HxL incubations respectively. At the same age, no differences were observed in VO_2 between the two incubation conditions.

The exposure to acute hypoxia decreased VO_2 in all animals (Fig. 1b, c and d). The H0 and H10 chicks of the Nx incubation had a reduction in VO_2 at both hypoxic levels ($p<0.0001$), but this response was more pronounced in H10 submitted to 10% O_2 ($p< 0.001$; Fig. 1b). The VO_2 during recovery was higher in H10 at 10 min ($p<0.01$) compared to H0. For the HxL incubation group VO_2 reduced at 15%O_2 ($p< 0.05$) but did not decreased further at 10%O_2 in both ages (Fig. 1c). At 10 min after hypoxia exposure H10 had a higher VO_2 than H0 ($p< 0.05$). Comparing the two incubation conditions, it can be observed that the biggest difference among responses of the same age chicks was the much lower VO_2 of H10-Nx than H10-HxL during exposure to 10%O_2 ($p<0.001$; Fig. 1d).

The figure 2 depicts the comparison between the resting VO2 at the beginning of the experiment and during the recovery phase at 10 min (Fig. 2a) and 20 min (Fig. 2b) after the end of 15 and 10%O_2 exposure. At time 10 min values of H10 chicks of both Nx and HxL incubations were above the oblique line (line of identity: initial VO_2 = recovery VO_2), which means recovery VO_2 is higher than initial metabolic rate. In the fig 2.b all the data points are on the line of identity.

3.2. Effect of hypoxia on metabolic rate of fast-growing (Fast) chicks incubated in normoxia (Nx) and late hypoxia (HxL)

Regardless the incubation condition, the resting metabolic rate normalized by body weight increased with age ($p<0.0001$; Fig. 3a). The VO_2 of H0 were 22.65±0.51and

26.30±1.8 mL/kg.min, while those of H5 were 35.93±2.1 and 39.02±2.42mL/kg/min and of H10 were 40.85±1.4 and 39.25±1.1mL/kg/min for the Nx and HxL incubations respectively. H0 chicks had lower VO_2 than both H5 and H10 ($p<0.0001$) but no difference was observed between H5 and H10.

In the Nx incubation, metabolic rate of H5 and H10, but not H0, decreased during hypoxia 10%O_2. During the recovery time VO_2 of H0, but not H5 and H10, increased at time 10 min ($p< 0.0001$), decreasing at 20 min but remaining higher than in the other ages ($p< 0.05$; Fig 3b).

In the HxL incubation (Fig 3c) the hypoxic reduction of VO_2 was similar in H5 and H10 while it was less pronounced in H0 during 10%O_2 exposure ($p< 0.05$). After hypoxia, VO_2 of H0 increased at 10 min and remained high at 20 min compared with the other chicks ($p<0.001$).

Comparing Nx and HxL incubations, it can be observed that H5 as well as H10 chicks showed similar responses to hypoxia and recovery to normoxia, while H0-Nx presented a higher hypoxic VO_2 (10%O_2) than H0-HxL ($p<0.0001$; Fig. 3d).

The figure 4 depicts the comparison between the resting VO_2 at the beginning of the experiment and during the recovery phase at 10 min (Fig. 4a) and 20 min (Fig. 4b) after the end of 10%O_2 exposure. At the time 10 min data values of H0 chicks of both Nx and HxL incubations were above the oblique line (line of identity: initial VO_2 = recovery VO_2), which means recovery VO_2 is higher than initial metabolic rate. In the fig 4b all the data points are on the line of identity.

Comparisons of metabolic rates between fast- and slow-growing chicks incubated in normoxia (Nx) and late hypoxia (HxL)

The Fig. 5a depicts the relationship between body weight and resting absolute $V O_2$ of all animals in normoxia. The Fast and Slow H0 chicks of both Nx and HxL incubations showed similar weights and resting normoxic VO_2. Regardless incubation condition, the Fast H5 and Slow H10 chicks presented similar body weights but Fast H10 birds had higher resting metabolic rates. Fast H10 chicks presented the highest body weight and metabolic rate.

As can be seen in Fig. 5b hypoxic metabolic rates of Fast and Slow H0 chicks from both incubation conditions were close to the 0 line, which indicates no difference between the hypoxic and normoxic VO_2. Regardless incubation condition Slow H10 chicks had similar body weights as Fast H5; however the Slow H10 birds from Nx group showed further drop in hypoxic VO_2 while HxL incubation caused a great variability of the responses, with values similar to Fast H5. Fast H10 chicks, on the other hand, presented higher body weight but similar reduction in hypoxic VO_2 compared to Slow H10, independent on incubation condition.

Regardless both incubation condition and chicken strain, the higher the resting VO_2 (per unit weight) in normoxia the greater was its hypoxic drop (Fig. 6). The slopes of the linear regressions were not significantly different among groups.

Discussion

In the present study we demonstrate that the magnitude of drop in hypoxic VO_2 is

dependent on the level of normoxic resting metabolic rate in precocial chicks, which is similar to the pattern observed in several species of mammals (Mortola and Maskrey, 2011). This conclusion is based on the fact that regardless all conditions analyzed, i.e., age, body mass, growth rate, strain or hypoxia exposure during the last phase of incubation, the greater the normoxic resting VO_2 the larger the metabolic depression during acute hypoxia (Fig.6). However, differences are observed in the factors that contribute to the resting metabolic rate in these animals.

Although hypoxia during incubation is known to reduce growth rate, body weight of both slow and fast growing H0 chicks were not significantly affected by hypoxia during incubation (Table 1). In previous studies (Dzialowski et al., 2002; Azzam et al., 2007) it was suggested that the hatchling's body weight may be almost normal because in the process of hatching the abdomen incorporates the remaining yolk not consumed during embryogenesis (Mortola, 2009).

Slow H10 chicks had a higher metabolic rate normalized by body weight than Fast chicks. Konarzewski et al (2000) also studied two trains, Slow and Fast growing chicken and found that, despite growing six times faster, Fast chicks had lower resting VO_2, a difference that disappeared after one week of life. Those differences between strains in growth rate during the first week after hatching were not reflected in similar differences in the relative masses of the heart, liver, and small intestine. However, Fast animals had heavier intestines once they reached a body mass of 80 g, but had relatively smaller brain. If we consider the major tasks for energy in the animal are biosynthesis, maintenance and external work, but the most expensive budge for an organism is maintenance, gain muscle is less expensive than organ activities. Although Slow H10 chicks were almost 2 times smaller than Fast H10, they

showed similar hypoxic metabolic depressions (Fig. 5b), which may be attributed to the relatively higher resting metabolic rate of Slow H10, even higher than the Fast H5 of similar body weight.

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Table 1. Characteristics of the eggs and variables of the hatchlings

Incubation conditions	**Slow-Growing**		**Fast-Growing**		**P**
	Nx	HxL	Nx	HxL	
Number of hatchlings	8	8	6	6	-
Fresh egg weight, g	58.8±0.6	60.3±1.2	60.7±0.4	60.8±0.3	ns
Incubation age at hatching, days	20.2±0.2	20.7±0.2	20.2± 0.3	20.5 ±0.2	ns
Postnatal age, hours	> 10h	> 10h	> 10h	> 10h	-
Body weight (W), g H0	40.9±0.8b	38.9±0.6b	44.3±0.6a	42.7±0.3a	0.0001
Body weight (W), g H5	-	-	70.8±4.0	76.0±2.9	ns
Body weight (W), g H10	83.5±3.1b	75.4±4.3b	146.0±4.8a	163.0±3.3a	0.0001
Ta at study, °C H0	37- 37.5	37-37.5	35-36	35-36	-
Ta at study, °C H5	-	-	31-32	31-32	-
Ta at study, °C H10	30-31	30-31	30-31	30-31	-
Body temperature, °C, H0	40.2±0.2	39.7±0.2	39.7±0.2	39.5±0.1	ns
Body temperature, °C, H5	-	-	40.4±0.1	40.3±0.1	ns
Body temperature, °C, H10	40.9±0.1a	40.9±0.1a	40.8±0.1a	40.5±0.1b	0.01

Figure Legends

Figure 1- Slow growing strain-Resting metabolic rate ($\dot{V}O_2$) of neonates (H0) and ten days old chicks (H10) incubated in normoxia (Nx - Inc) and in hypoxia at the last week (HxL – Inc) (**a**). Percentage of change in $\dot{V}O_2$ (%$\dot{V}O_2$; normalizedby body weight) during hypoxia (Hx) 15 and 10% O_2 and after 10 (air 10') and 20 min (air 20') of recovery in H0 and H10 incubated in normoxia (Nx) (**b**), hypoxia (HxL) (**c**) and both Nx and HxL (**d**). Values indicated by different letters are significantly different from each other. * means significant differences between ages at the same time; # means significant differences between incubation conditions at the same age and time.

Figure 2- Slow growing strain-Air initial oxygen consumption ($\dot{V}O_2$)-air recovering $\dot{V}O_2$ diagram at 10 min (top panel) and 20 minutes (bottom panel) after hypoxia 10% O_2 in neonates (H0) and ten days old chicks (H10) incubated in normoxia (Nx) and in hypoxia at the last week (HxL). Each symbol refers to a different individual.

Figure. 3- Fast growing strain-Resting metabolic rate ($\dot{V}O_2$) of neonates (H0) and ten days old chicks (H10) incubated in normoxia (Nx - Inc) and in hypoxia at the last week (HxL – Inc) (**a**). Percentage of change in $\dot{V}O_2$ (%$\dot{V}O_2$; normalizedby body weight) during hypoxia (Hx) 15 and 10% O_2 and after 10 (air 10') and 20 min (air 20') of recovery in H0 and H10 incubated in normoxia (Nx) (**b**), hypoxia (HxL) (**c**) and both Nx and HxL (**d**). Values indicated by different letters are significantly different from each other. * means significant differences among ages at the same time; # means significant differences between incubation

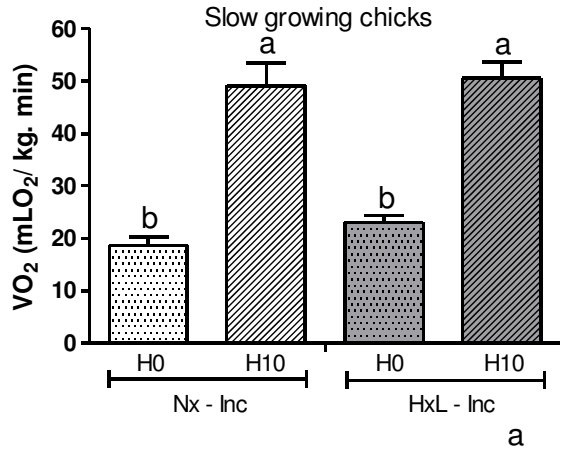
conditions at the same age and time.

Figure 4- Fast growing strain-Air initial oxygen consumption ($\dot{V}O_2$)-air recovering $\dot{V}O_2$ diagram at 10 min (top panel) and 20 minutes (bottom panel) after hypoxia 10% O_2 in neonates (H0) and ten days old chicks (H10) incubated in normoxia (Nx) and in hypoxia at the last week (HxL). Each symbol refers to a different individual.

Figure 5- a) Relationship between body weight and normoxic resting metabolic rate ($\dot{V}O_2$) in neonates (H0), five (H5) and ten days old chicks (H10) of slow growing (Slow; H0 and H10) and fast growing (Fast; H0, H5 and H10) chicken strains incubated in normoxia (Nx) and in hypoxia at the last week (HxL). The oblique dashed lines are isopleths of constant $\dot{V}O_2$ ml/kg.min (indicated by the numbers at top). **b)** relationship between body weight and drop in $\dot{V}O_2$ during hypoxia 10%O_2 in slow growing (Slow; H0 and H10) and fast growing (Fast; H0, H5 and H10) chicken strains incubated in Nx and in HxL.

Figure 6- Difference between hypoxic (10% inspired O_2) and normoxic oxygen consumption ($\dot{V}O_2$; normalized by body weight) in slow growing (Slow) and fast growing (Fast) chicken strains incubated in normoxia (Nx) and in hypoxia at the last week (HxL). The horizontal dashed

line indicates no difference between the hypoxic and normoxic $\dot{V}O2$. Each symbol refers to a different individual regardless age.



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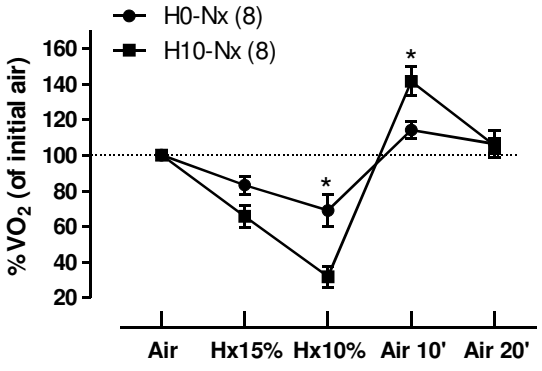

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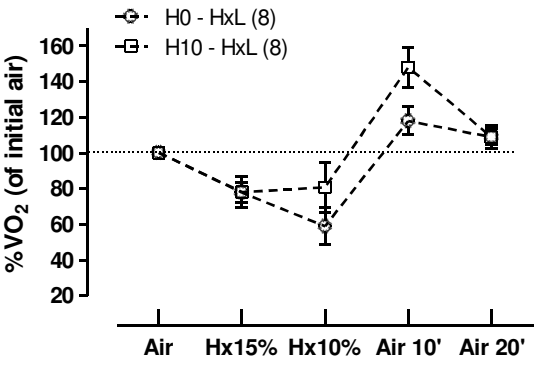

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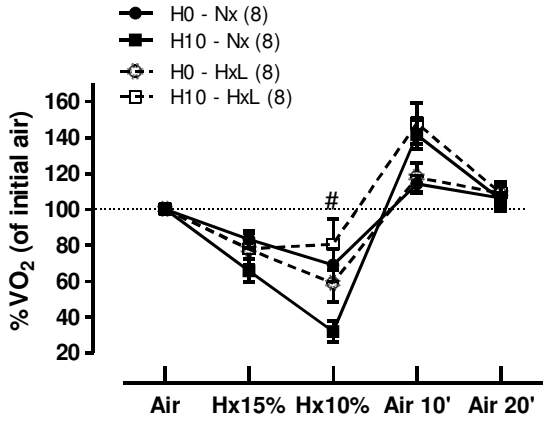


d

Figure 1

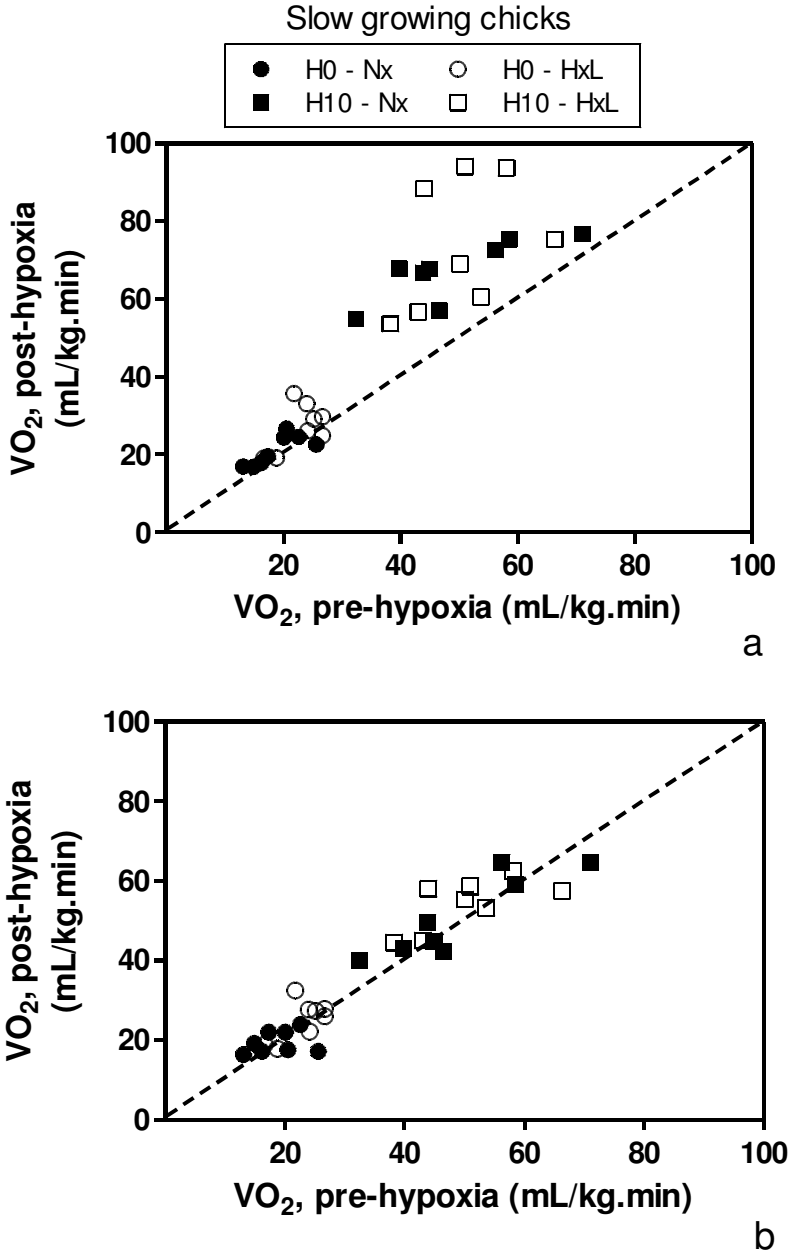
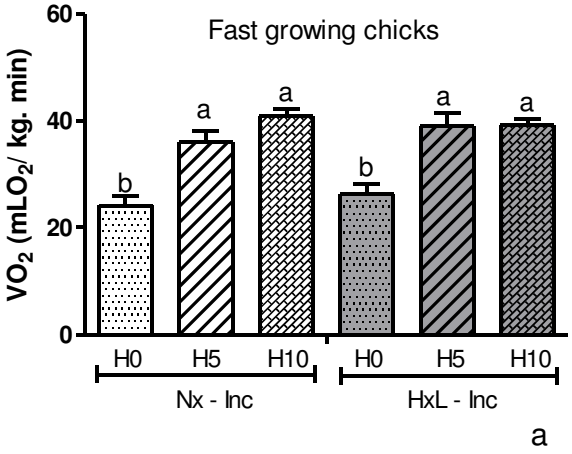

Slow growing chicks
H0 - Nx
H0 - HxL
H10 - Nx
H10 - HxL
VO2, post-hypoxia (mL/kg.min)
VO2, pre-hypoxia (mL/kg.min)
0
20
40
60
80
100
a
b

Figure 2



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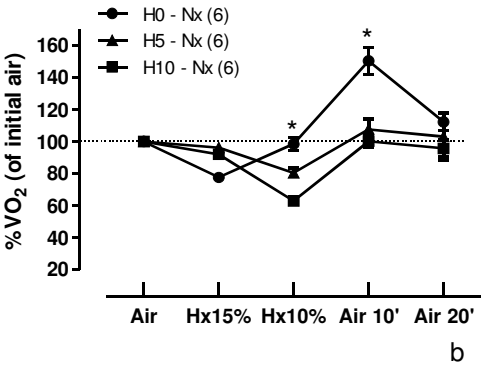

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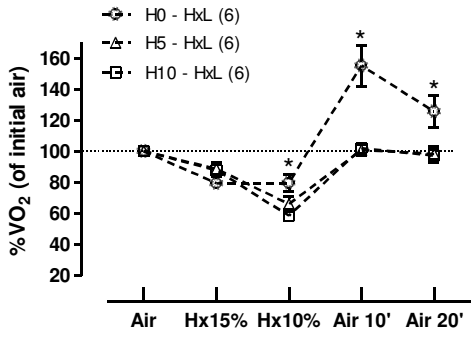

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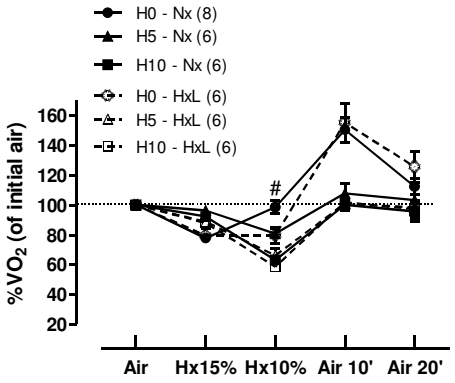

d

Figure 3

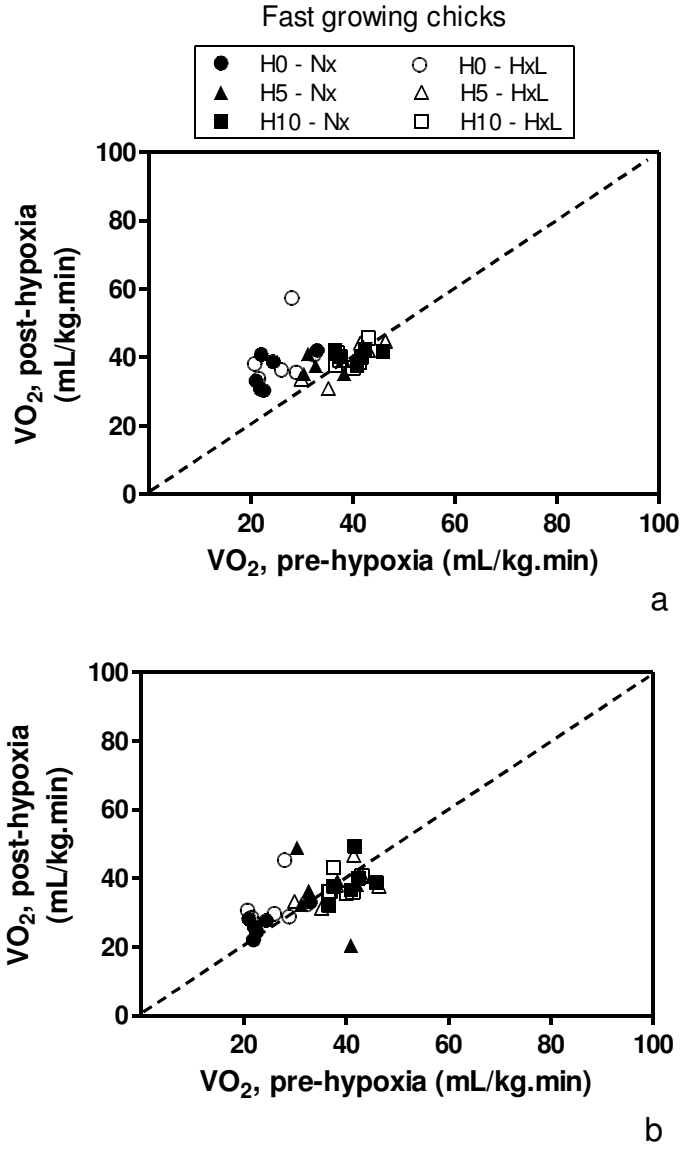
Fast growing chicks
H0 - Nx
H5 - Nx
H10 - Nx
H0 - HxL
H5 - HxL
H10 - HxL
VO2, post-hypoxia (mL/kg.min)
VO2, pre-hypoxia (mL/kg.min)
a
VO2, post-hypoxia (mL/kg.min)
VO2, pre-hypoxia (mL/kg.min)
b

Figure 4

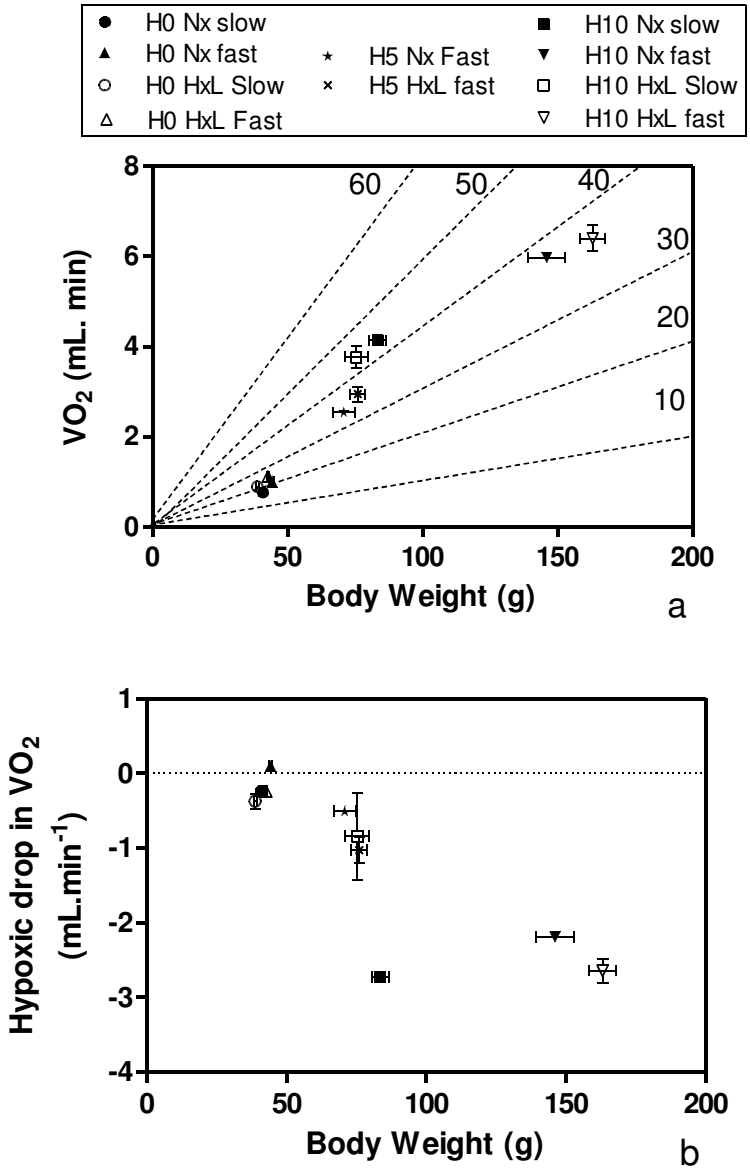
H0 Nx slow
H0 Nx fast
H0 HxL Slow
H0 HxL Fast
H5 Nx Fast
H5 HxL fast
H10 Nx slow
H10 Nx fast
H10 HxL Slow
H10 HxL fast
60
50
40
30
20
10
VO2 (mL. min)
Body Weight (g)
a
Hypoxic drop in VO2 (mL.min-1)
Body Weight (g)
b

Figure 5

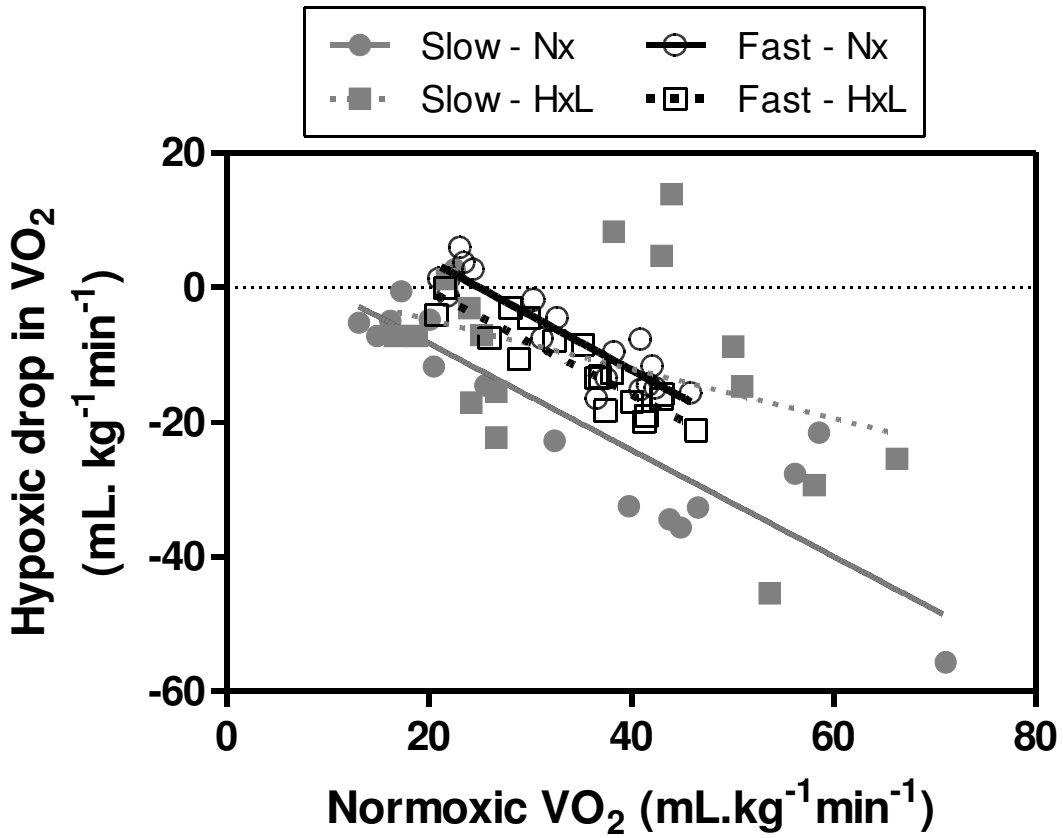
Slow - Nx
Fast - Nx
Slow - HxL
Fast - HxL
20
0
-20
-40
-60
0
20
40
60
80
Hypoxic drop in VO2
(mL. kg-1min-1)
Normoxic VO2 (mL.kg-1min-1)

Figure 6

CAPÍTULO 4-

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Short communication

Ventilatory response to hypoxia of the 1-day old chicken hatchling after prenatal cold-induced hypometabolism

Jacopo P. Mortola*, Paula Andrea Toro-Velasquez

Department of Physiology, McGill University, 3655 Promenade Sir William Osler, Montreal, Quebec H3G 1Y6, Canada

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ABSTRACT

Sustained prenatal hypoxia decreases the growth and metabolic rate of the embryo and causes a blunted hypoxic ventilatory response (HVR) in the newborn. The most likely interpretation is that the sustained hypoxic stimulation may interfere with the normal prenatal development of the chemoreceptors. However, we wanted to consider the possibility that the prolonged hypoxic hypometabolism may be a contributing factor. Chicken embryos were incubated at 35 °C (*Cold* group, $N = 14$), which is known to lower the embryonic oxygen consumption ($\dot{V}_{O_2}$) by ~30% throughout incubation, or at 37.5 °C (*Controls*, $N = 16$). Cold incubation delayed hatching by ~2 days. The 1-day old hatchlings had normal pulmonary ventilation ($\dot{V}_E$), measured by the barometric technique, and oxygen consumption ($\dot{V}_{O_2}$), simultaneously measured by an open flow methodology. During acute hypoxia (~15% or ~11% O_2) the hyperventilation (increase in $\dot{V}_E/\dot{V}_{O_2}$), the hyperpnea and the hypometabolism were almost identical between the two groups of hatchlings. We conclude that a sustained decrease in metabolic rate during the embryonic period by itself does not carry obvious consequences on the newborn's resting $\dot{V}_E$ and HVR.

1. Introduction

Sustained hypoxia in newborns has a long-term depressant effect on the ventilatory response to a new acute hypoxic episode later in life. This phenomenon, both in mammals and birds, has been attributed to a derangement of the normal postnatal development of the chemoreceptors (reviewed in Carroll, 2003; Mortola, 2009). Whether or not the same can happen with sustained prenatal hypoxia is difficult to establish in mammals, because maternal or placental responses become major confounding variables. However in chicken embryos, the development of which is free of extra-embryonic factors, hypoxia sustained for the whole duration or for only the last third of incubation resulted in a blunted hypoxic ventilatory response (HVR) of the hatchling (Szdzuy and Mortola, 2007b; Ferner and Mortola, 2009). Like postnatally, the effect of embryonic hypoxia on the hatchling's HVR has been attributed to a disturbance in the normal development of the chemoreceptors (Mortola, 2009). In support of this interpretation was the observation that prenatal hypoxia had to occur during the last portion of incubation, that is, at the time of the functional development of the chemoreceptors, while it carried no consequences on the newborn's HVR if it occurred only at earlier embryonic stages (Ferner and Mortola, 2009). The fact that also embryonic hypercapnia (Szdzuy and Mortola, 2008) or hyperoxia (Bavis and Simons, 2008; Mortola, 2011b) caused some postnatal blunting of the HVR were considered compatible with the idea that sustained stimulation of the chemoreceptors may interfere with their normal prenatal development.

Prenatal hypoxia lowers metabolic rate and stunts the growth of many organs including the lungs (Mortola, 2009). The possibility that the low birth weight by itself and independently of the prenatal hypoxia may contribute to the blunting of the newborn's HVR has been tested experimentally and dismissed (Mortola, 2010). Similarly, the possibility that prenatal hypoxia may result in an increase of the mechanical impedance of the newborn's respiratory system has been denied by specific measurements (Mortola, 2011a). What remains untested is the possibility that the prenatal condition of sustained hypometabolism by itself, and independently of hypoxia, may contribute to some depression of the neonatal HVR. To test this possibility we incubated the embryos in normoxia at the sub-optimal but viable temperature of 35 °C (instead of 37.5 °C), which lowers the embryonic oxygen consumption by about 30% throughout development (Mortola, 2006). The incubation of the chicken embryo in the cold offers an experimental opportunity to test the hypothesis that, even in absence of hypoxia, a sustained depression of embryonic metabolism may by itself cause some blunting of the newborn's HVR.

2. Methods

Freshly laid fertilized eggs of White Leghorn chickens (*Gallus gallus*) were obtained from a local supplier. At midday (embryonic

* Corresponding author. Fax: +1 514 398 7452.
E-mail address: jacopo.mortola@mcgill.ca (J.P. Mortola).

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Table 1
Characteristics of the eggs and resting ventilatory variables of the hatchlings.

Incubation conditions	Normothermia (*Controls*)	Hypothermia (*Cold*)	*P*
Number of hatchlings	16	14	
Fresh egg weight (g)	61.1 ± 0.8	62.6 ± 0.6	ns
Incubation age at hatching (days)	20.3 ± 0.2	22.2 ± 0.2	<0.001
Postnatal age (h)	20.0 ± 2.3	17 ± 1.2	ns
Body weight (W) (g)	41.6 ± 0.7	41.2 ± 0.6	ns
Ambient temperature at study (°C)	37.8 ± 0.1	37.8 ± 0.1	ns
Body temperature (°C)	40.1 ± 0.1	38.8 ± 0.3	<0.001
O_2 consumption ($\dot{V}_{O_2}$) (ml_{STPD}/min)	0.8 ± 0.03	0.8 ± 0.03	ns
$\dot{V}_{O_2}/W$ ((ml/kg)/min)	20.1 ± 0.8	20.0 ± 0.6	ns
Tidal volume (μl)	307 ± 22	303 ± 17	ns
Tidal volume/body weight (ml/kg)	7.4 ± 0.6	7.4 ± 0.5	ns
Breathing frequency (min^{-1})	64 ± 4	68 ± 4	ns
Ventilation ($\dot{V}_E$) (ml_{BTPS}/min)	18.7 ± 1.0	19.9 ± 0.6	ns
$\dot{V}_E$/W ((ml/kg)/min)	454 ± 26	483 ± 13	ns
Ventilatory equivalent ($\dot{V}_E/\dot{V}_{O_2}$)	22.8 ± 1.0	24.3 ± 0.6	ns

The *Cold* group was incubated at 35 °C for the whole incubation, hatching included, instead of the normal 37.5 °C (*Controls*). Values are means ± 1 SEM. *P*, level of the significant difference between the two groups (two-tailed *t*-test). ns, no significant difference ($P > 0.05$).

day 0, E0) the eggs were weighed and placed in two still air incubators set at 60% relative humidity and temperature of 37.5 °C (*Controls*, $N = 16$) or 35 °C (cold-incubated group, *Cold*, $N = 14$). From previous measurements we knew that incubation at 35 °C lowers the embryo's oxygen consumption by ~30% throughout development, delays hatching but is still compatible with survival (Mortola, 2006). The cold condition was maintained constant for the whole incubation, hatching included. The temperature and relative humidity inside the incubators were monitored by data loggers and directly by a mercury thermometer. Experiments were performed between 6 h and 24 h after hatching (usually ~17–20 h) to avoid the rapidly occurring physiological changes of the first postnatal hours (Mortola, 2009).

Measurements of breathing pattern at rest and of the $\dot{V}_E$ response to hypoxia were obtained with an 'ad hoc' adaptation of the barometric technique, which allowed for simultaneous measurements of the breathing pattern and oxygen consumption (Szdzuy and Mortola, 2007a). All aspects of data acquisition, analysis and experimental protocol were as previously employed in studies that required measurements of ventilatory chemosensitivity in hatchlings (Szdzuy and Mortola, 2007b; Ferner and Mortola, 2009; Mortola, 2010). Briefly, the animal chamber consisted of two sections in communication with each other; the hatchling was positioned in the smaller of the two ("nest" compartment). The temperature of the nest compartment, monitored by telemetry, was kept at ~37.5 °C by a water bath; it averaged about 9 °C higher than the temperature of the remaining portion of the animal chamber, to satisfy accuracy in the determination of tidal volume. The animal chamber had four leads; two were used for the recording of the breathing-related pressure oscillations and for the pressure–volume calibration of the chamber. The remaining two leads were for the passage of a steady flow of gas at the constant rate of 100 ml/min under the control of a needle-valve flowmeter. The gas consisted of air or of premade hypoxic mixtures. Calibrated gas analyzers monitored the O_2 and CO_2 concentrations of the inflowing and outflowing gases, after water vapor elimination through a drying column. The output of the analyzers, together with the breathing-related pressure oscillations, temperature and humidity values were continuously acquired at 100 Hz while displayed on a computer monitor. The gas fractional concentrations were mathematically corrected for the error introduced by a respiratory exchange ratio different from unity (Mortola and Besterman, 2007). Oxygen consumption ($\dot{V}_{O_2}$, ml/min at STPD, standard temperature, pressure and dry conditions) was calculated from the flow and the chamber in–out difference in O_2 concentration. Tidal volume (V_T, μl, at BTPS, body temperature, pressure and saturation conditions), breathing frequency (f, breaths/min) and minute ventilation ($\dot{V}_E = f \times V_T$, ml/min) were computed from the spirometric record (Szdzuy and Mortola, 2007a).

The hatchling was left undisturbed in the respirometer for at least half hour. Then, data collection started in air followed by hypoxia (~15% and ~11% O_2 balance N_2, 20 min each). Data of breathing pattern were the average of 150–200 breaths collected during the last 3–5 min of exposure, that is, at a time when gas composition in the respirometer has been stable for several minutes. Cloacal temperature was taken as representative of body temperature (T_b) and was measured with a thin thermocouple during air breathing and at the end of the hypoxic and hypercapnic runs.

Data are presented as means ± 1SEM. For each variable, statistical comparisons between *Controls* and *Cold* were done by two-tailed *t*-test. Differences were considered statistically significant at $P < 0.05$.

3. Results and discussion

Hatching in *Controls* occurred after 20.3 days of incubation; in *Cold*, incubation lasted about 2 additional days (Table 1). This longer incubation in *Cold* was the expected effect of the decreased metabolism and growth rate (Mortola, 2006). In fact, cold acts like a brake on embryonic growth, without compromising the maintenance component of metabolic rate (Vleck and Vleck, 1987; Hoyt, 1987); hence, throughout incubation $\dot{V}_{O_2}$ is less than same-age controls but appropriate for the weight of the embryo (Mortola, 2006). Like the embryo, the chorioallantoic membrane grows less in the cold keeping its functional adequacy to the embryo's gas exchange requirements (Tazawa, 1973). As it is the case following incubation in hypoxia (Szdzuy and Mortola, 2007b), after cold-incubation the hatchlings have body weights similar to controls (Mortola, 2006;Table 1). This is due, partly, to the longer incubation and mostly to the incorporation of any remaining yolk into the abdomen (Williams, 1994).

In normoxia, none of the metabolic and respiratory variables differed significantly between the two groups (Table 1). The only exception was T_b, which averaged 1.3 °C less in *Cold*, presumably because prolonged prenatal cold lowers the development of thermogenesis (reviewed in Mortola, 2009).

The hyperventilatory response to hypoxia (increase in $\dot{V}_E/\dot{V}_{O_2}$) and the response of its two components (the increase in $\dot{V}_E$ and

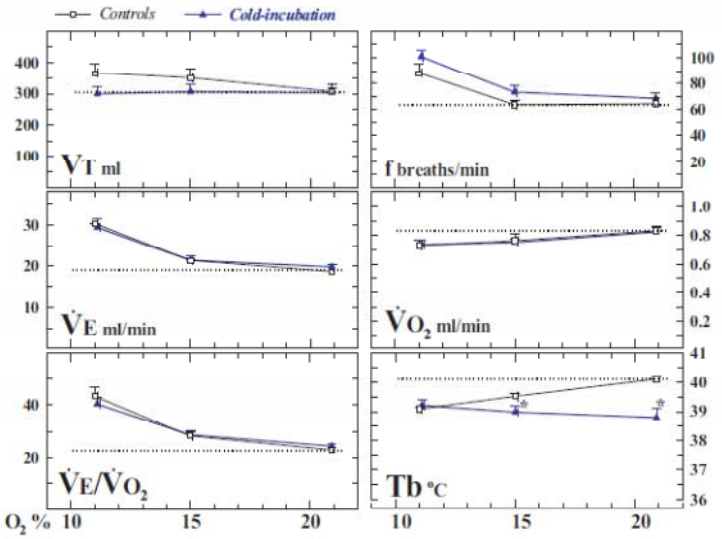

Fig. 1. Pulmonary ventilation ($\dot{V}_E$), breathing frequency (f) and tidal volume (V_T), oxygen consumption ($\dot{V}_{O_2}$) and ventilatory equivalent ($\dot{V}_E/\dot{V}_{O_2}$) during air breathing (20.9% O_2) and hypoxia (~15% and ~11% O_2) in 1-day old hatchlings. *Controls* and *Cold* refers to hatchlings incubated, respectively, at 37.5 °C (□, $N = 16$) or 35 °C (▲, $N = 14$). Symbols indicate mean values, bars are 1 SEM. *Statistically significant difference from controls ($P < 0.05$).

the drop in $\dot{V}_{O_2}$) were almost undistinguishable between *Cold* and *Controls* (Fig. 1). The only minor (and insignificant) differences were found in the response of the breathing pattern itself, which tended to be more rapid and shallower in *Cold*; it is interesting that this is the pattern most commonly adopted by adult mammals and birds during hypothermia, in an attempt to reduce respiratory heat loss (Mortola and Maskrey, 2011). Hence, the question that prompted the study, whether or not persistent prenatal hypometabolism may lower the HVR of the newborn, received a negative answer. The question was motivated by the general notion that the development of neural functions is contributed by the type of sensory information. With respect to respiratory control, many studies have shown that the HVR undergoes developmental plasticity following a prolonged modification of the gaseous stimuli, especially when this happens in the neonatal period (reviewed in Carroll, 2003; Mortola, 2009). As far as we know, this is the first experimental report to show that a major disturbance of metabolic rate sustained throughout the whole prenatal development by itself does not compromise the newborn's breathing pattern at rest or its response to a hypoxic stimulus. Previously, in neonatal rats, we found that sustained changes in metabolic rate caused by prolonged cold or by undernutrition carried no consequences on HVR (Sant'Anna and Mortola, 2002, 2003). The notion that changes in $\dot{V}_E$ occur promptly with changes in metabolic rate is a tenet of respiratory control, with innumerable experimental demonstrations even if the mechanistic basis of it remains poorly understood (Mortola and Maskrey, 2011). The current results indicate that the proper functionality of the $\dot{V}_{O_2} - \dot{V}_E$ coupling does not depend on the level of $\dot{V}_{O_2}$ attained throughout development. Presumably, this independence reflects the importance of having an appropriate gas exchange function irrespective of the individual's metabolic history. From the perspective that motivated this study, the current results exclude the possibility that the sustained hypometabolism, by itself, may contribute to the blunted HVR of the newborn following prenatal hypoxia, for which the altered development of the chemoreceptors remains the sole plausible interpretation.

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APÊNDICE 1

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The motility of the chicken embryo: Energetic cost and effects of hypoxia

Jacopo P. Mortola*, Alyssa S. Louis, Marina Simeonova, Paula A. Toro Velasquez

Department of Physiology, McGill University, Montreal, Canada

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ABSTRACT

We estimated the energetic cost of embryonic motility by relating the changes in embryo's motion to the changes in oxygen consumption ($\dot{V}_{O_2}$). Measurements were conducted on chicken embryos between day 10 and 18 of incubation. Embryonic gross body movement was quantified over ten continuous 3-min periods from the pressure oscillations inside the egg, measured through an implanted catheter, and was correlated to the synchronous changes in $\dot{V}_{O_2}$, measured by an open-flow methodology. Over the 30 min recording, movements could vary around the mean by up to four folds. The corresponding changes in $\dot{V}_{O_2}$ were minuscule (0.116 μl O_2/mmHg) or, for all age groups combined, only 2.3% of the mean $\dot{V}_{O_2}$, ranging from ~8% (day 10) to ~0.5% (day 16). At E18, hypercapnia and cold respectively increased and decreased motility. Differently, the effects of hypoxia on motility were variable among embryos. It is concluded that, in chicken embryos over the age period investigated, the cost of motility represents an almost negligible fraction of the total energy budget. Because of its low cost, motility can be maintained in hypoxia; conversely, reduction of motility in hypoxia does not provide an important energy saving.

1. Introduction

Embryos of terrestrial vertebrates develop in the aquatic environment of the amniotic sac. The amniotic liquid avoids dehydration and lowers the gravitational pull, with lesser risk of membrane and tissues adhesion. Whether in the womb or in eggs, motion is necessary to all vertebrate embryos for the homogeneous growth of the supporting tissues, skeleton and muscles (Pitsillides, 2006).

Embryonic motion can originate passively from external forces; in mammals, these can originate from postural changes of the pregnant female and myometrial contractions and, in birds, by the turning of the eggs and from contraction of the amnios. The smooth muscles cells of the amniotic sac have spontaneous contractions that stir the liquid (Nechaeva, 2011). As development progresses, eventually the embryo generates its own motion. In humans, fetal gross body movements are detectable by the seventh week (de Vries et al., 1986); in birds, embryonic movements can be recognized since the early days of incubation (Wu et al., 2001). The primary question of the present study has to do with the energetic cost required by the embryo to generate its own gross body movements.

Several studies have provided information regarding the cost of maintenance and growth in the developing embryo (Rombough, 2011); in these studies, the cost of motility was considered negligible, although direct experimental data are lacking. In fact, the only information currently available originates from the sheep fetus during the last part of gestation, when the fetal oxygen consumption ($\dot{V}_{O_2}$) was compared before and after complete muscle paralysis. In one experiment (Dalton et al., 1977) one hour of paralysis caused no changes in blood oxygenation, which should suggest that embryonic motion had no appreciable $\dot{V}_{O_2}$. Differently, in two subsequent studies paralysis of longer duration either increased oxygenation (Nathanielsz et al., 1982) or decreased fetal $\dot{V}_{O_2}$ (Rurak and Gruber, 1983). The drop in $\dot{V}_{O_2}$ was observed only in those fetuses that, before paralysis, presented breathing movements, suggesting that fetal breathing was the activity energetically costly (Rurak and Gruber, 1983). Because paralysis of skeletal muscles modifies the distribution of blood flow and oxygen delivery to various body districts, the possibility of hypoxic decrease in $\dot{V}_{O_2}$ complicates the estimate of the cost of motility. In the current study we calculated the cost of motility in behaving chicken embryos by correlating the changes of $\dot{V}_{O_2}$ to the changes in motion simultaneously recorded. Avian embryos permit to consider embryonic motility free from the influence of external factors (maternal, uterine and placental) characteristic of mammalian animal models. All embryos were studied before the internal pipping phase, to eliminate the possibility of breathing activity.

The motion of an avian embryo is easy to recognize visually, for example, by transillumination (video clip in Supplement 1) but

* Corresponding author at: Department of Physiology, McGill University, Basic Science Bldg, Room 1121, 3655 Sir William Osler Promenade, Montreal, Quebec, H3G 1Y6, Canada. Fax: +1 514 398 7452.
E-mail address: jacopo.mortola@mcgill.ca (J.P. Mortola).

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it can be difficult to quantify. Muscle electromyography (Bradley and Bekoff, 1990), kinematic analysis from videotapes (Bradley, 1999) and recording of muscle force (Nechaeva et al., 2010) have been useful for fine descriptions of muscle activations and motion of the body part under consideration, usually a limb. However, none of these techniques could provide a quantitative estimate of the total motility of the embryo. We have chosen to quantify motility by monitoring the pressure (P) changes inside the egg. P is the ratio between the force produced and the surface over which it is applied, which is the egg surface area, essentially constant among all eggs. Hence, the P recorded can be considered an index of the net force resulted from the motion of the embryo.

In addition to its energetic cost, we wanted to assess the effect of hypoxia on embryonic motility. Previously, in chicken embryos, brief (10 min) exposure to 10% O_2 did not modify the force generated by the motion of a limb at day 10 (E10) and caused only a transient depression of it at E14 (Nechaeva et al., 2010). In that study $\dot{V}_{O_2}$ was not measured. The fact that limb force persisted during hypoxia may suggest that in those embryos motility did not represent an energetically expensive function. With the present study we aimed, first, to quantify the energetic cost of embryonic movement in relation to the total $\dot{V}_{O_2}$ of the embryo and, second, the effect of hypoxia on $\dot{V}_{O_2}$ and motility. Finally, cold and hypercapnia were tested for a more complete interpretation of the results obtained in hypoxia.

2. Methods

Experiments were conducted on chicken embryos of the White Leghorn variety. Fertile eggs were obtained from a local supplier. After noting the fresh weight, the eggs were placed in incubators (Hova-Bator, Savannah, GA) set at the temperature of 37.5 °C and 60% relative humidity (both monitored by a data logger), with progressive 90° rotation at least 4 times per day. Incubation started at midday (embryonic day E0) and measurements were obtained at E10, E12, E14, E16 or E18, depending on the study protocol. The main measurements consisted in oxygen consumption ($\dot{V}_{O_2}$) and pressure (P) oscillations inside the egg, taken as indicative of embryo's motility.

2.1. Measurements of $\dot{V}_{O_2}$ and P

Oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production were measured by an open flow methodology adapted to the chicken embryo (Mortola and Labbè, 2005; Mortola and Besterman, 2007). The egg was placed inside a respirometer, which consisted of a 120-ml plastic container maintained at the desired temperature by a circulating water bath. A flow of 100 ml/min, under the control of a precision flow-meter, continuously passed through two openings in the lid of the respirometer. The inflow and outflow O_2 and CO_2 concentrations were monitored by a calibrated gas analyzer (CA-1B CO_2 analyzer, Sable Systems Int., Las Vegas, NV) arranged in series, after the gas had passed through a drying column. The gas fractional concentrations were mathematically corrected for the error introduced by a respiratory exchange ratio different from unity (Mortola and Besterman, 2007); then, $\dot{V}_{O_2}$ was computed from the flow rate and inflow–outflow O_2 concentration difference. The $\dot{V}_{O_2}$ values, calculated at standard temperature, pressure and dry conditions, are presented in μl/min.

A catheter was threaded through a third opening in the lid of the respirometer for monitoring the changes in pressure (P) inside the egg. To this end, on the day of the measurement, a hole of about 1 mm in diameter was manually drilled through the eggshell in the hemisphere opposite to the air cell, that is, far from the blunted end of the egg. The tip of a polyvinyl catheter (~55 mm long, 1.2 mm OD) was inserted 3–4 mm deep and kept in place by dental cement. In almost all experiments, a second catheter was similarly placed at approximately the opposite side of the egg as a precaution against accidental clotting or kinking. The two catheters joined together into the common catheter threaded through the lid of the respirometer, which was connected to a high-sensitivity differential P-transducer (DUXL30D, Data Instruments). Because the egg was positioned with the blunted end up, there was no backflow of egg liquid; hence, the air–liquid interface remained close to the catheter entrance at the egg surface. Although the catheter was air-filled, the fact that its tip rested within the egg liquid implied the presence of some hydrostatic pressure, which must have contributed to some inter-embryo variability in the absolute values of P. Hence, the results of each embryo were normalized by expressing P in percent of the mean (see Section 3). During acquisition the raw P signal was rectified, so that P values were always positive irrespective of whether the embryo's movement caused a P wave toward (positive) or away (negative) the catheter tip; then, the P signal was integrated every min, to yield mmHg/min. The baseline of the P signal was the average P recorded from several eggs after the embryo had been killed by 20–30 min exposure to CO_2 and N_2.

The temperature (T) inside the respirometer was monitored by telemetry. The relative humidity of the respirometer was not measured, but some condensation suggested that the humidity was close to the dew point. Gas flow rate, $[O_2]$, $[CO_2]$, T and P (raw, rectified and integrated) were acquired at 100 Hz, displayed on a computer monitor during on line acquisition and saved for later analysis.

The wash-out time of the whole set up (respirometer with the egg in it and connecting tubes) at the flow rate used in these experiments was computed by rapidly injecting a bolus of CO_2 and measuring the time of its detection by the analyzer; it was ~30 s. Because the recording of the P oscillation was virtually instantaneous, for the analysis the P signal was delayed by 30 s to achieve synchrony with $\dot{V}_{O_2}$.

2.2. Experimental protocols

The first goal of the study was to evaluate the energetic cost of motion, in the form of cost ($\dot{V}_{O_2}$) per unitary change in pressure (protocol 1). The second goal was to evaluate the effect of hypoxia on motility (protocol 2); this was done with three different approaches (protocols 2a,b,c). Finally, we wanted to investigate the effects on embryonic motion of non-hypoxic conditions that either did not influence $\dot{V}_{O_2}$ (hypercapnia, protocol 3) or decreased $\dot{V}_{O_2}$ (cold, protocol 4).

2.2.1. $P - \dot{V}_{O_2}$ relationship (protocol 1)

These measurements were performed on embryos at E10, E12, E14, E16 and E18. Data of $\dot{V}_{O_2}$ and P were collected for 30 min in normoxic–normothermic conditions and averaged over 3-min bins, so that each embryo produced 10 data points. In order to combine all embryos, the data of P and $\dot{V}_{O_2}$ were expressed as percentages of their 30-min average. Then, two analytical approaches (numerical and graphical) were used. By the first approach, the $\dot{V}_{O_2}$ values of the data points with $P > 105\%$ of the mean were compared statistically to the $\dot{V}_{O_2}$ of the data points with $P < 95\%$ of the mean. In all cases (see Section 3) the difference was statistically significant. Then, the difference in $\dot{V}_{O_2}$ (between $P > 105\%$ and $P < 95\%$) was divided by the corresponding difference in P, to yield the cost of motion (CM) per unitary change in P, CM/P (μl O_2/mmHg). The product between CM/P and the average P/min

gave the total CM (μl O_2/min); finally, the percent ratio of CM to the average $\dot{V}_{O_2}$ gave the fraction of resting $\dot{V}_{O_2}$ spent for motion.

Graphically, the percent values of $\dot{V}_{O_2}$ and P were grouped and averaged for 20% intervals of P (10–30% of the mean, 30–50%, 50–70%, and so on, up to 190–210%) until the last bin that grouped all the remaining data points (210–300%); they were all represented in a P(x-axis)–$\dot{V}_{O_2}$ (y-axis) plot and fit by linear regression to obtain the intercept on the y-axis (at $P = 0$), which represented the percent $\dot{V}_{O_2}$ when motility was nil. The difference from 100% gave the fraction of resting $\dot{V}_{O_2}$ spent for motion, similar to the numerical approach indicated above.

2.2.2. Effect of hypoxia (protocols 2a,b,c)

The effects of hypoxia were investigated with three different protocol variants, on different sets of eggs. The first (2a) was performed on embryos at E10, E12, E14, E16 and E18. First, data were recorded in normoxia (30 min). Then, the inflow port of the respirometer was connected to a gas-impermeable bag containing a pre-calibrated mixture of 10% O_2 in N_2. After a period of equilibration (10–15 min), recording resumed for 30 min. The average $\dot{V}_{O_2}$ and P in hypoxia were computed and represented in percent of the normoxic values.

By the second protocol (2b, at E16 and E18), embryos were exposed to normoxia, followed by moderate hypoxia (nominal 10% O_2), severe hypoxia (nominal 5% O_2) and recovery in air. Each exposure lasted 30 min and the average value of each condition was expressed in percent of the initial normoxic run.

The third protocol (2c, at E18) consisted in exposing embryos to 10% O_2 for two sequences of 30 min each. This protocol was an *a posteriori* decision, motivated by the results of protocol 2b (see Section 3.2).

2.2.3. Effect of hypercapnia (protocol 3)

Embryos (E18) were exposed to air, 2% CO_2, 4% CO_2 and recovery in air, in this order, 30 min each. The averages of $\dot{V}_{O_2}$ and P for each run were computed and represented in percent of the first air values.

2.2.4. Effect of cold (protocol 4)

Embryos (E18) were exposed to air, first in normothermia (nominal 37.5 °C, 30 min) followed by cold (nominal 32.0 °C, 30 min). The time interval between the two conditions was about one hour, which was the time required for the temperature within the respirometer to reach steady state. The average $\dot{V}_{O_2}$ and P values in cold were expressed in percent of those in normothermia.

2.3. Number of embryos and statistics

The number of embryos studied with the various protocols, and the characteristics of their eggs, are detailed in Table 1. Statistically significant differences in the comparisons between two groups of data (i.e., $\dot{V}_{O_2}$ at high and low P in protocol 1) and between two interventions (e.g., in protocols 2a and 4) were assessed by two-tailed t test, either paired or unpaired as appropriate. Comparisons among multiple interventions (e.g., in protocols 2b and 3) were tested by ANOVA for repeated measures, followed by post-hoc comparisons with Bonferroni's limitations. When normality test failed, comparisons were done on ranks, with the appropriate non-parametric post-hoc tests in the case of multiple comparisons (SigmaStat®, Jandel Scientific). In all cases a statistical difference was considered at $P < 0.05$.

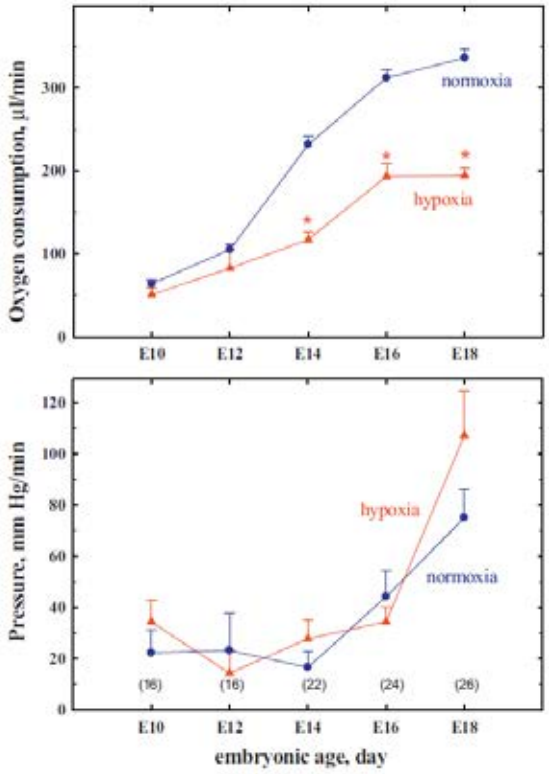

Fig. 1. Oxygen consumption (top) and motility-related egg pressure (bottom) at various embryonic ages. In blue are data collected during air exposure, in red during exposure to 10% oxygen. Symbols are group averages, bars are 1 SEM. *, statistically significant difference between normoxia and hypoxia. In brackets, number of embryos studied at each age.

3. Results

3.1. Cost of motility (E10–E18)

As the age of the embryos increased, so did $\dot{V}_{O_2}$, from 64 (±4) μl/min at E10–337 (±10) μl O_2/min at E18 (Fig. 1, top panel, blue); differently, the average P remained similar at ~20 mmHg/min until E16, and almost doubled at E18 (Fig. 1, bottom panel, blue). At any given age the variability of P among embryos was quite large, probably contributed by the hydrostatic effect on P, as mentioned in Section 2. For this reason, for each embryo the P and $\dot{V}_{O_2}$ values of each 3-min bin were expressed as percent of the average.

With all embryos combined, irrespective of their age (E10–E18), the $\dot{V}_{O_2}$ values at $P > 105\%$ were significantly higher than the $\dot{V}_{O_2}$ values at $P < 95\%$ ($P < 0.002$). The CM/$\dot{V}_{O_2}$, computed as indicated in Section 2.2.1, was 2.36%. The graphical representation of all these data points separated in P-bins of 25% (Fig. 2, bottom x- and left y-axes) was fit by the linear regression $\dot{V}_{O_2}$ (%) = 0.02 P (%) + 97.7 ($r^2 = 0.74$, $P < 0.01$). Therefore, CM derived from this graphical analysis was 2.3% of $\dot{V}_{O_2}$, virtually identical to the result of the numerical approach.

The same analytical approach was adopted for the embryos separated into age groups. As it was the case for the cumulative data, also for the individual age-groups the $\dot{V}_{O_2}$ values at $P > 105\%$ were statistically higher than the $\dot{V}_{O_2}$ values at $P < 95\%$. The CM/$\dot{V}_{O_2}$ (%) were 8.5, 2.9, 2.1, 0.4 and 1.0 at E10, E12, E14, E16 and E18, respectively. The graphical x–y representation of the P-binned data (Fig. 3) emphasized that, despite the large spreading of P, $\dot{V}_{O_2}$ changed very

Table 1
Embryos.

Protocol no. (experiment)	Embryonic age (day)	*N*	Fresh egg wt (g)	Temperature (°C)	$[O_2]$ or $[CO_2]$ (%)
1 (cost of motion)	E10	16	57.5 ± 0.7	37.3 ± 0.1	Air
	E12	16	57.7 ± 0.6	37.3 ± 0.1	Air
	E14	22	56.8 ± 0.5	37.5 ± 0.1	Air
	E16	24	58.2 ± 0.5	37.6 ± 0.1	Air
	E18	26	58.5 ± 0.6	37.5 ± 0.1	Air
2a (hypoxia: 10%O_2)	E10	6	56.5 ± 0.8	37.1 ± 0.1–37.4 ± 0.1	Air – 11.0 ± 0.2
	E12	6	56.4 ± 0.5	37.3 ± 0.2–37.5 ± 0.2	Air – 10.5 ± 0.2
	E14	12	56.7 ± 0.6	37.4 ± 0.1–37.4 ± 0.1	Air – 10.8 ± 0.2
	E16	15	58.4 ± 0.7	37.4 ± 0.1–37.5 ± 0.1	Air – 10.9 ± 0.2
	E18	17	57.8 ± 0.7	37.4 ± 0.1–37.4 ± 0.1	Air – 10.8 ± 0.1
2b (hypoxia: 10–5%O_2)	E16	8	57.4 ± 0.7	37.4 ± 0.1–37.5 ± 0.1 to –37.4 ± 0.1–37.4 ± 0.1	Air – 11.1 ± 0.2 to –6.3 ± 0.2 – Air
	E18	10	58.2 ± 0.8	37.3 ± 0.1–37.6 ± 0.2 to –37.4 ± 0.2–37.2 ± 0.2	Air – 10.9 ± 0.2 to –6.1 ± 0.2 – Air
2c (prolonged hypoxia)	E18	12	56.6 ± 0.8	37.4 ± 0.1–37.5 ± 0.1 to –37.4 ± 0.1–37.5 ± 0.1	Air – 10.5 ± 0.1 to –10.5 ± 0.1 – Air
3 (hypercapnia)	E18	5	59.4 ± 0.7	37.3 ± 0.1–37.7 ± 0.2 to –37.7 ± 0.2–37.8 ± 0.2	Air – 2.2 ± 0.1 to –4.0 ± 0.04 – Air
4 (cold)	E18	9	59.6 ± 1.4	37.7 ± 0.1–31.6 ± 0.1	Air

Values are group means ± 1 SEM. *N*, number of embryos. $[O_2]$, $[CO_2]$, oxygen or carbon dioxide concentration when different from air.

little. The fact that none of the correlation lines initiated precisely at $P = 0$ is the result of the binning over 3 min and group-averaging, and does not negate the fact that embryos, at times, could be motionless. The CM derived from the graphical analysis at E10, E12, E14, E16 and E18 were, respectively, 8.1, 3.2, 2.4, 0.6 and 0.9% of $\dot{V}_{O_2}$.

3.2. Hypoxia

In the older age-groups (E14-E16-E18) exposure to 10% O_2 significantly lowered $\dot{V}_{O_2}$, while it had variable and often insignificant effects on body motion (Fig. 1, red). One exception was the significant decrease observed at E16 with 10% O_2 (Fig. 5). Differently, severe hypoxia (5% O_2, Fig. 4) consistently reduced *P* (Fig. 5). Upon return to air, at E16 $\dot{V}_{O_2}$ and *P* rose toward the pre-hypoxic values, while in many E18 embryos *P* remained low.

Because 10% O_2 lowered motility at E16 but not at E18, and because additional severe hypoxia (5% O_2) eventually lowered motility at both ages, we questioned whether or not the difference between the two age groups with 10% O_2 may decrease had the hypoxia been maintained for a longer duration. The results of prolonged 10% O_2 exposure (30 min, repeated twice; Fig. 6) showed a trend in this direction, but confirmed that E18 embryos were more successful in maintaining motility close to the normoxic value than the younger group.

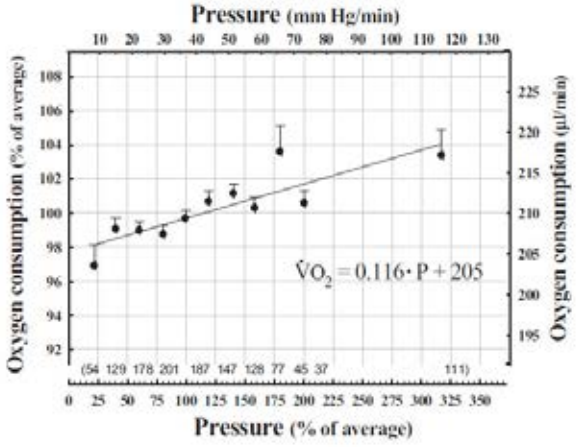

Fig. 2. Pressure (*P*) – oxygen consumption ($\dot{V}_{O_2}$) relationship for all embryos combined. First, the $P - \dot{V}_{O_2}$ data pairs of each embryo were computed in percent of the embryo's average. Then, all data were grouped by *P*-bins of 25% each (*N* of data points included in each bin is indicated in brackets) and plotted (*x*-axis at bottom and y-axis at left). Symbols are group means and bars are 1 SEM. The continuous line is the best fit linear regression. Extrapolation of the linear regression to $P = 0$ gave the percent $\dot{V}_{O_2}$ in absence of embryo's motion.
From the group means of $\dot{V}_{O_2}$ and *P* of all these embryos (respectively, 209.9 µl/min and 36.3 mmHg/min) the axes can be converted to absolute units (*x*-axis at top and y-axis at right); with these units, the slope of the linear regression was 0.116 µl O_2/mmHg, which represents the average cost of motion for the embryos during the second half of incubation. The y-intercept was 205.1 µl O_2/min, from which the average percent fraction of $\dot{V}_{O_2}$ required for motion was [(209.9–205.1)/209.9] × 100, or 2.3%.

3.3. Hypercapnia

These measurements were performed at E18 (Table 1). The $\dot{V}_{O_2}$ and *P* of these embryos in air averaged, respectively, 287 ± 42 µl/min and 43 ± 3 mmHg/min. Hypercapnia (2% and 4% CO_2) did not modify $\dot{V}_{O_2}$ while significantly increased embryo's motility, which returned to the control value upon restoration of normocapnia (Fig. 7).

3.4. Cold

These measurements were conducted at E18 (Table 1). In warm conditions (37.7 ± 0.1 °C) the $\dot{V}_{O_2}$ and *P* of these embryos averaged,

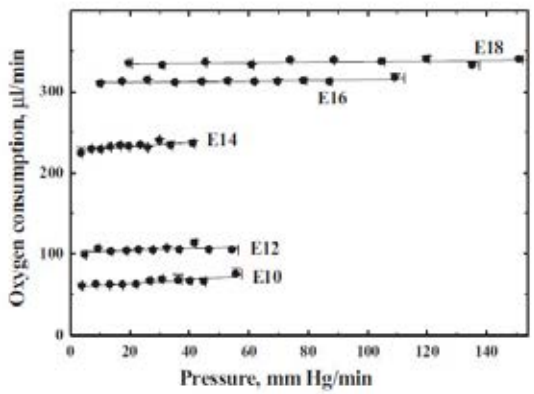

Fig. 3. Pressure – oxygen consumption ($\dot{V}_{O_2}$) data pairs for the five age groups studied. The curve of each age-group was constructed as for the cumulative curve of Fig. 2 (see Section 2).

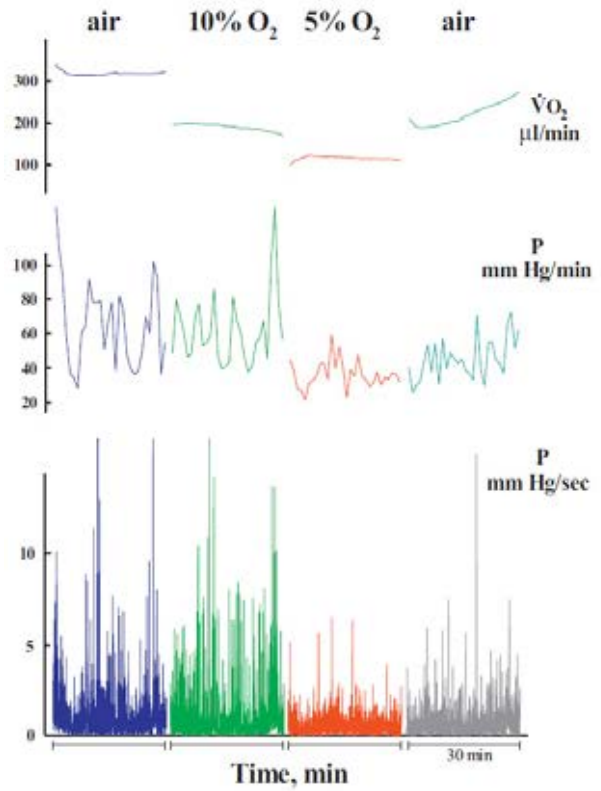

Fig. 4. An example of a tracing of motility-related pressure changes (bottom panel) in an embryo at E18, during air, hypoxia moderate (10% O_2) and severe (5% O_2) and recovery in air, each lasting 30 min. The bottom record is the sec by sec integral of the rectified pressure (P) signal. The middle panel shows the same signal averaged per min. At the top is oxygen consumption ($\dot{V}_{O_2}$), computed on a per min basis.

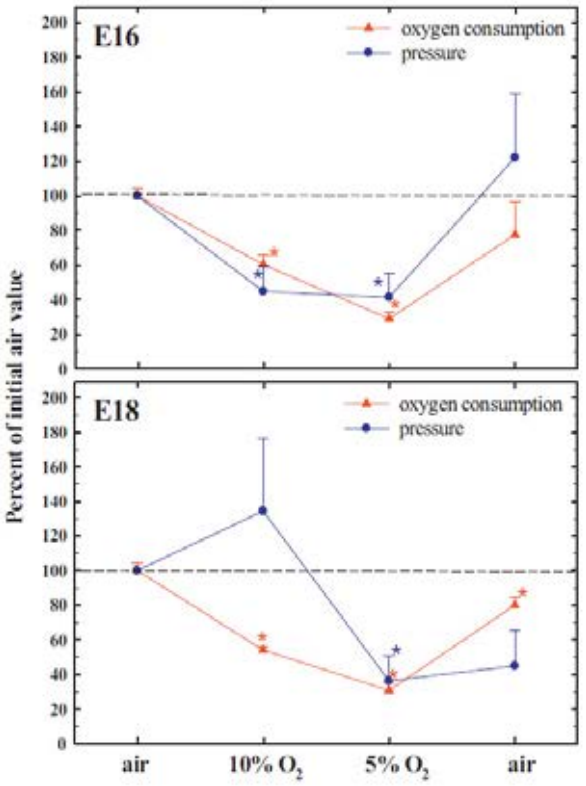

Fig. 5. Oxygen consumption (red triangles) and motion-related pressure (blue circles) during air, hypoxia with 10% or 5% O_2 and recovery in air, in embryos at day 16 (top panel) and at day 18 (bottom panel). Symbols are group averages, bars are 1 SEM. *, statistically significant difference from the initial air value.

respectively, 339 ± 23 µl/min and 36 ± 6 mmHg/min. Cold exposure (31.6 ± 0.1 °C) significantly lowered $\dot{V}_{O_2}$ to 77% (±3) ($P < 0.001$) and P to 46% (±16) ($P < 0.05$) of the warm values.

4. Discussion

The main results of this study with the chicken embryo as experimental model can be summarized as follows: (a) embryonic motility proved to be extremely economical; (b) severe hypoxia lowered motility, while moderate hypoxia had variable and different effects with age; (c) cold reduced, and hypercapnia increased, embryonic motility.

4.1. Methodological considerations

We have chosen to evaluate the motion of the embryo from the pressure (P) changes inside the egg. The signal was rectified, to give equal weight to positive and negative P waves, and integrated, to consider the cumulative P generated over time, rather than only its amplitude. The approach used to estimate the cost of motility was that of an iso-time correlation between the P and $\dot{V}_{O_2}$ averaged over 3 min-bins, for a total of 30 min. In addition to signal synchrony, achieved by introducing a delay of 30 s in the P signal to compensate for the delay intrinsic in the $\dot{V}_{O_2}$ recording, the approach relied on the occasional variations in embryonic motility throughout the 30-min recording period. In fact, either absence or a constant level of motility would have been of little avail. In practice, lack of motility was rarely the case, as previoulsy noted (Hamburger et al., 1965); rather, on average, motility changed by up to four folds on either side of its mean (Fig. 2). A totally different approach to estimate the cost of motility could have been that of comparing embryonic $\dot{V}_{O_2}$ before and after complete muscle paralysis. As mentioned in Introduction, this approach was applied to the lamb fetus (Dalton et al., 1977; Nathanielsz et al., 1982; Rurak and Gruber, 1983). In our model, paralysis would have been difficult to obtain without some degree of invasiveness, especially at the youngest ages when embryos weigh just a few grams (Mortola

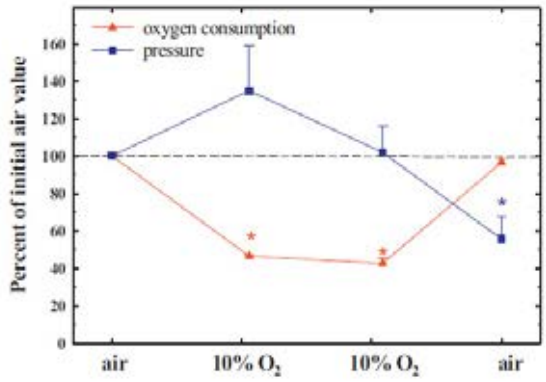

Fig. 6. Oxygen consumption (red triangles) and motility-related pressure (blue squares) during air, hypoxia (10% O_2) of 30 min repeated twice, and recovery in air, in embryos at day 18. Symbols are group averages, bars are 1 SEM. *, statistically significant difference from the initial air value.

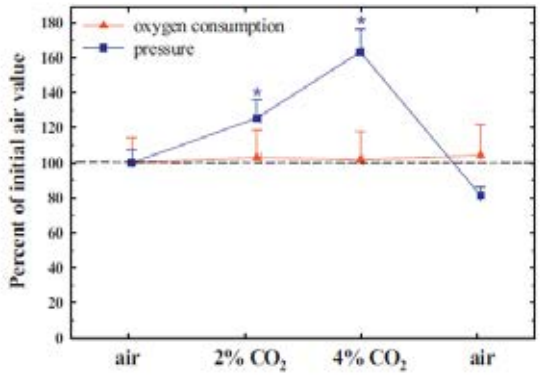

Fig. 7. Oxygen consumption (red triangles) and motion-related pressure (blue squares) during air, hypercapnia with 2% or 4% CO_2 and recovery in air, in embryos at day 18. Symbols are group averages, bars are 1 SEM. *, statistically significant difference from the initial air value.

and Al Awam, 2010). In chicken embryos mean artery pressure is low, less than 20 mmHg (Elfwing et al., 2011). Hence, another concern with total muscle paralysis was the possibility of hindering the distribution of blood flow and O_2 delivery to the various body districts. In such event, the hypoxic decrease in $\dot{V}_{O_2}$ (Mortola et al., 2010) would have complicated greatly any estimate of the cost of motility.

It is important to note that our approach quantifies embryo's motility *in toto*, with no possibility of distinguishing the source of it, active from the embryo's muscle contraction or passive from contraction of the amniotic sac. Occasional visual inspections in eggs windowed at the blunted end gave the strong impression that embryonic motion was almost exclusively of active origin and that the largest *P* fluctuations were produced by active muscle contraction. Because the largest values of *P* were the most influential in the analysis, the *P* oscillations due to passive movements could not have modified the general conclusion that the cost of embryonic motility was a minimal component of resting $\dot{V}_{O_2}$.

4.2. Cost of motility

The $\dot{V}_{O_2}$ values for $P > 105\%$ of the mean were consistently higher than those for $P < 95\%$ of the mean. Nevertheless, the extremely shallow slopes of the $P - \dot{V}_{O_2}$ relationships (Figs. 2 and 3) indicated that even large changes in motility did not entail major changes in $\dot{V}_{O_2}$. In fact, when embryos of all ages were combined, the cost of motion averaged only 2.3% of the mean $\dot{V}_{O_2}$; this represents a minimal portion of the embryo's budget, which is largely determined by the cost of tissue growth and maintenance (Mortola and Cooney, 2008). In the first part of incubation, the costs of growth and maintenance contribute approximately equally to the embryo's total energy budget; then, as incubation progresses, the cost of maintenance, which is proportional to embryo's size, continues to increase (Fig. 8) and progressively overshadows the cost of motility. Even at E18, when the embryo moved much more than at the younger ages, the motility-related $\dot{V}_{O_2}$ was ~1% of the total. The minimal energetic demands of motility help to understand some previous observations. For example, in the rainbow fish larvae during hatching an increase in body movements by several hundred folds caused less than 20% increase in $\dot{V}_{O_2}$ (Ninness et al., 2006). Also, some computations had indicated that the energy budget of embryos was close to balancing even if the cost of activity was completely ignored (Mortola and Cooney, 2008; Rombough, 2011), a conclusion that can be understood given the current results.

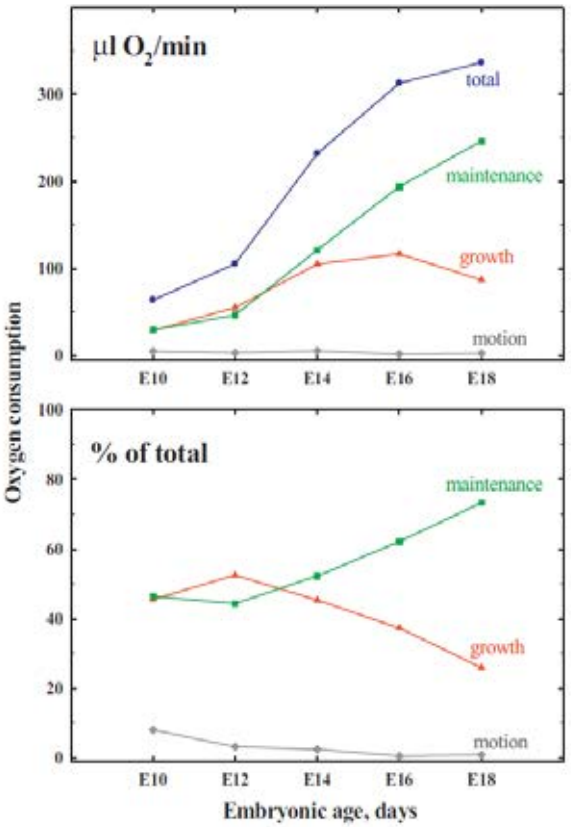

Fig. 8. *Top panel*: oxygen consumption used for tissue maintenance (green squares), body growth (red triangles) and motility (brown rhombi) in embryos of various ages; 'total' indicates the sum of all components (blue circles). The magnitudes of the maintenance and growth components were computed from previous data (Mortola and Cooney, 2008). *Bottom panel*: maintenance, growth and motility components in percent of the total oxygen consumption of embryos at different ages.

Our data were collected during the second half of incubation because it proved very difficult to detect movement-related *P* oscillations before E10. In addition, in the early part of incubation embryo's motility may result predominantly from the passive motion of the egg content, such as with contraction of the amnion, while active motility becomes dominant in the second part of incubation (Wu et al., 2001). Although muscle contractions can be observed in the cervical region since E3–E4 and extend downwards in the following days (Hamburger, 1963), they hardly generate any *P* because of the disproportion between embryonic mass and egg content. For example, at one week, the chicken embryo weighs only half a gram, and is surrounded by about 50 g of yolk and albumen, or by some 100 times its own weight (Mortola and Al Awam, 2010). Hence, at this age even massive activation of the embryo's muscles would not be able to overcome the mechanical load of the surrounding liquid, contrary to later stages when the embryo's body much outweighs the egg liquid. Even if not detectable as *P* swings, it is possible that the energetic cost of the primitive muscle contractions may represent a substantial component of the embryo's total $\dot{V}_{O_2}$. Indeed, it may not have been a coincidence that the highest fraction of the total $\dot{V}_{O_2}$ (~8%) was found in the youngest embryos investigated. Hence, one may argue that, had it been possible to collect measurements during the first half of incubation, the cost of motility may have turned out to be a larger percentage of the total $\dot{V}_{O_2}$ than apparent from the current data.

4.3. Effect of hypoxia

Hypometabolism in acute hypoxia is a well described response of many vertebrates, including the avian embryo (Bjønnes et al., 1987; Ar et al., 1991; Tazawa et al., 1992); hence, the reduction in $\dot{V}_{O_2}$ here observed in all hypoxic protocols was not a surprise. On the other hand, while severe hypoxia (5% O_2) lowered motility consistently in all embryos (Fig. 5), 10% O_2 had quite variable effects. In the age group that we studied the most, E18, 10% O_2 had no effects on the average motility of the whole group, but the individual responses could be large in either direction. Previously, it was noted that exposure to 10% O_2 did not modify the chicken embryo's spontaneous motion, quantified from limb movements, at E10 and caused only a transient but reversible depression at E14 (Nechaeva et al., 2010). The bioelectrical activity of the embryo's lumbosacral regions of the spinal cord, recorded in ovo, showed inhibition during 10% O_2 at E14 and E19, and no effects at E16 (Gonya-Magee and Stokes, 1980). Why in hypoxia the neuro-muscular activity responsible for the embryo's motility is variable and unpredictable is unclear. This is in sharp contrast to the other interventions tested in these experiments, cold and hypercapnia, which gave consistent decreases or increases in motility, respectively. Whatever the explanation for the variability in the effect of hypoxia may be, the fact that motility is often retained at the normoxic level can only be explained by the fact that the energetic cost of motility is minimal.

The possibility of retaining some motility in conditions of hypoxia is probably the manifestation at the embryonic level of a characteristic widespread in the animal kingdom. Vertebrates, invertebrates and unicellular organisms, when confronted by hypoxia, migrate to environments that provide the most adequate ambient conditions for that level of hypoxia (Malvin and Wood, 1992; Malvin, 1998); this survival strategy would not be possible if hypoxia curtailed motility. Similarly, many fish and marine organisms actively avoid hypoxia, in pursuit of better oxygenated environments (Wannamaker and Rice, 2000; Wu, 2002; Cook et al., 2011; Poulsen et al., 2011; Urbina et al., 2011).

Differently from hypoxia, acute hypercapnia did not modify embryonic $\dot{V}_{O_2}$, as previously noted (Menna and Mortola, 2003), even though motility increased by up to 60% (Fig. 7). This increase, according to the $P - \dot{V}_{O_2}$ curve (Fig. 2), should have caused less than a 2% rise in $\dot{V}_{O_2}$, an increase too small to be statistically detectable. Previously, neuronal bursts of activity in the spinal cord, presumably responsible for gross body movements, were found to decrease during the first minutes of hypercapnia followed by a return to the normal level, a pattern that was attributed to changes in the neuronal metabolic activity (Gonya and Stokes, 1978).

4.4. Conclusions

Motility permeates animal life at all levels of organization. Postnatally, body movements are primarily related to locomotion, for food gathering, escaping predators and finding mates, or to postural adjustments, as with the behavioral responses to ambient temperature. Prenatally, motility serves very different purposes and is both passive and active. The passive embryo's movements, like those caused by the periodic rotation of the egg by the incubating parent, are important in limiting the problem of adherence of the embryo to the surrounding membranes. Active movements can serve some heat control and communication with the incubating parent (Grāns and Altimiras, 2007; Du et al., 2011). However, the most essential role of active embryonic motility must be its contribution to muscle-skeletal development, which relies on the mechanical loads encountered by the spontaneous muscle contractions (Pitsillides, 2006; Nechaeva et al., 2010). Absence of active motility, as in experimental paralysis, unavoidably leads to poor cartilage formation and skeletal deformities. The current data have indicated that the embryo's active body movements are energetically very economical. This conclusion is in keeping with the general principle that life-indispensable functions cannot have evolved to be energetically expensive.

Because embryonic motility is rather inexpensive, it is possible for the embryo to continue to move in hypoxia, when body growth is curtailed. It also follows that cessation of motility during hypoxia would be of little benefit to the embryo, since it cannot be an important mechanism of energy saving.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.resp.2013.05.030.

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APÊNDICE 2

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The motility of the chicken embryo: Energetic cost and effects of hypoxia

Jacopo P. Mortola*, Alyssa S. Louis, Marina Simeonova, Paula A. Toro Velasquez

Department of Physiology, McGill University, Montreal, Canada

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ABSTRACT

We estimated the energetic cost of embryonic motility by relating the changes in embryo's motion to the changes in oxygen consumption ($\dot{V}_{O_2}$). Measurements were conducted on chicken embryos between day 10 and 18 of incubation. Embryonic gross body movement was quantified over ten continuous 3-min periods from the pressure oscillations inside the egg, measured through an implanted catheter, and was correlated to the synchronous changes in $\dot{V}_{O_2}$, measured by an open-flow methodology. Over the 30 min recording, movements could vary around the mean by up to four folds. The corresponding changes in $\dot{V}_{O_2}$ were minuscule (0.116 μl O_2/mmHg) or, for all age groups combined, only 2.3% of the mean $\dot{V}_{O_2}$, ranging from ~8% (day 10) to ~0.5% (day 16). At E18, hypercapnia and cold respectively increased and decreased motility. Differently, the effects of hypoxia on motility were variable among embryos. It is concluded that, in chicken embryos over the age period investigated, the cost of motility represents an almost negligible fraction of the total energy budget. Because of its low cost, motility can be maintained in hypoxia; conversely, reduction of motility in hypoxia does not provide an important energy saving.

1. Introduction

Embryos of terrestrial vertebrates develop in the aquatic environment of the amniotic sac. The amniotic liquid avoids dehydration and lowers the gravitational pull, with lesser risk of membrane and tissues adhesion. Whether in the womb or in eggs, motion is necessary to all vertebrate embryos for the homogeneous growth of the supporting tissues, skeleton and muscles (Pitsillides, 2006).

Embryonic motion can originate passively from external forces; in mammals, these can originate from postural changes of the pregnant female and myometrial contractions and, in birds, by the turning of the eggs and from contraction of the amnios. The smooth muscles cells of the amniotic sac have spontaneous contractions that stir the liquid (Nechaeva, 2011). As development progresses, eventually the embryo generates its own motion. In humans, fetal gross body movements are detectable by the seventh week (de Vries et al., 1986); in birds, embryonic movements can be recognized since the early days of incubation (Wu et al., 2001). The primary question of the present study has to do with the energetic cost required by the embryo to generate its own gross body movements.

Several studies have provided information regarding the cost of maintenance and growth in the developing embryo (Rombough, 2011); in these studies, the cost of motility was considered negligible, although direct experimental data are lacking. In fact, the only information currently available originates from the sheep fetus during the last part of gestation, when the fetal oxygen consumption ($\dot{V}_{O_2}$) was compared before and after complete muscle paralysis. In one experiment (Dalton et al., 1977) one hour of paralysis caused no changes in blood oxygenation, which should suggest that embryonic motion had no appreciable $\dot{V}_{O_2}$. Differently, in two subsequent studies paralysis of longer duration either increased oxygenation (Nathanielsz et al., 1982) or decreased fetal $\dot{V}_{O_2}$ (Rurak and Gruber, 1983). The drop in $\dot{V}_{O_2}$ was observed only in those fetuses that, before paralysis, presented breathing movements, suggesting that fetal breathing was the activity energetically costly (Rurak and Gruber, 1983). Because paralysis of skeletal muscles modifies the distribution of blood flow and oxygen delivery to various body districts, the possibility of hypoxic decrease in $\dot{V}_{O_2}$ complicates the estimate of the cost of motility. In the current study we calculated the cost of motility in behaving chicken embryos by correlating the changes of $\dot{V}_{O_2}$ to the changes in motion simultaneously recorded. Avian embryos permit to consider embryonic motility free from the influence of external factors (maternal, uterine and placental) characteristic of mammalian animal models. All embryos were studied before the internal pipping phase, to eliminate the possibility of breathing activity.

The motion of an avian embryo is easy to recognize visually, for example, by transillumination (video clip in Supplement 1) but

* Corresponding author at: Department of Physiology, McGill University, Basic Science Bldg, Room 1121, 3655 Sir William Osler Promenade, Montreal, Quebec, H3G 1Y6, Canada. Fax: +1 514 398 7452.
E-mail address: jacopo.mortola@mcgill.ca (J.P. Mortola).

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difference in *f* between aquatic and terrestrial species is present at birth, that is, before the exposure of the newborns to the aquatic experience (Mortola and Limoges, 2006). Similar data in birds are unavailable. Hence, the second goal of this study was to find out whether or not the difference in *f* between the adult chickens and ducks is already expressed at birth. In case ducklings had lower *f*, one may expect their tidal volume to be correspondingly higher to produce the necessary $\dot{V}E$, unless the ventilatory efficiency of their respiratory system was substantially greater than in chicks.

Finally, we investigated the response of the breathing pattern to chemical stimuli (hypoxia or hypercapnia) to examine whether or not similarities or differences in breathing pattern between the two species at rest were modified under increased ventilatory demands.

2. Methods

Freshly laid eggs of Muscovy duck (*C. moschata*) and White Leghorns chicken (*G. gallus*) were purchased from a local supplier. The eggs were stored at 15 °C for no more than 7 days. After noting the fresh egg weight, incubation started at midday (embryonic day zero, E0). The incubators were set at a temperature of 37.5 °C and relative humidity of 60%, monitored by a data logger, with at least four automatic rotations per day. At the end of the incubation, when embryos approached the internal pipping phase, eggs were transferred to a hatchery-incubator with no rotation. Measurements of oxygen consumption and breathing pattern during resting conditions and under chemostimulation were performed during the first postnatal day, on average at 16 h in both species, avoiding the first 6–8 h after birth when rapidly changing phenomena take place (Mortola, 2009).

The hatchling was weighed and placed in a Plexiglas respirometer, completely sealed and partially submerged in a circulating water bath. Oxygen consumption was measured by an open flow methodology and the breathing pattern was recorded by an application of the barometric technique. Cloacal temperature was taken as representative of body temperature (Tb) and measured by a fine tungsten–constantan thermocouple at the onset and end of the hypoxic and hypercapnic exposures.

2.1. Pulmonary ventilation

The barometric technique stands on the fact that, when an animal is breathing inside a sealed chamber, the air inspired is warmed and humidified from the ambient to the pulmonary values, raising the chamber pressure; the opposite occurs in expiration (Drorbaugh and Fenn, 1955). Its application to avian hatchlings has been described previously (Szdzuy and Mortola, 2007a). In essence, the respirometer consisted of two sections of ~100 mL and 200 mL, in ample communication; the hatchling was positioned in the smaller of the two ('nest' compartment), the temperature of which (monitored by telemetry) was maintained at 38 °C by circulating water from a servo-controlled water bath. The temperature (T) of the nest compartment was at least 9 °C above the remaining portion of the respirometer, so that the ΔT between animal and chamber was sufficiently large to satisfy accuracy in the computation of tidal volume (Mortola and Frappell, 1998). Temperature and humidity of the respirometer were monitored, respectively, by a thermistor and a humidity sensor. The animal chamber had four leads. Two leads were for the passage of a steady flow of gas at the constant rate of 150 mL/min, maintained by a negative pressure pump and under the control of a needle–valve flowmeter. The third lead was connected to a sensitive pressure-transducer for the recording of the breathing-related pressure (P) oscillations. The remaining lead was for the determination of the compliance K of the chamber, obtained by injecting volume in known amounts (ΔV) while recording the corresponding change in P (ΔP), so that $K = \Delta V / \Delta P$. For the recording of the breathing pattern, the flow through the chamber was momentarily interrupted by solenoid valves for the duration of about 1 min. This period of occlusion had negligible effects on the composition of the gases. Full details, possible problems and validation of the technique in its application to the hatchling have been given elsewhere (Szdzuy and Mortola, 2007a).

2.2. Oxygen consumption

Immediately before sealing the chamber for the measurements of $\dot{V}E$, gaseous metabolism (oxygen consumption $\dot{V}_{O_2}$ and carbon dioxide production $\dot{V}_{CO_2}$) were measured with an open-flow methodology (Frappell et al., 1992) adapted to the avian embryo and hatchling. A steady gas flow of 150 mL/min (either air or premade hypoxic or hypercapnic mixtures) was maintained through the respirometer. The outflow O_2 and CO_2 concentrations were continuously monitored by calibrated gas analysers (Sable Systems International Fox, Henderson, NV), while the inflow concentrations were checked intermittently, after either flow of gas had passed through a drying column (calcium sulphate).

2.3. Analysis and protocols

Data acquisition and analysis were as previously employed in studies that included measurements of ventilatory chemosensitivity in hatchlings (e.g., Szdzuy and Mortola, 2007b, 2008; Mortola, 2010; Mortola and Toro-Velasquez, 2013). The output of the O_2 and CO_2 analyzers, together with the breathing-related P oscillations, T and humidity values were continuously acquired at 100 Hz while displayed on a computer monitor. Breathing frequency (*f*, breaths/min) was computed from the breath-by-breath total cycle duration of the P recording. Tidal volume (VT, μL, BTPS) was calculated from the amplitude of the breathing related P-oscillations, K, Tb and the T and water vapour pressure of the respirometer (Szdzuy and Mortola, 2007a). For each condition, values of VT and *f* represented the averages of about 100 breaths. Pulmonary ventilation ($\dot{V}E$, mL, BTPS/min) equalled $f \times VT$.

For the computation of oxygen consumption ($\dot{V}_{O_2}$, mL/min, STPD), first, the gas fractional concentrations were mathematically corrected for the error introduced by a respiratory exchange ratio different from unity (Depocas and Hart, 1957; Mortola and Besterman, 2007). Then, $\dot{V}_{O_2}$ was calculated as the product between the gas flow and the chamber in–out difference in O_2 concentration.

The hatchling was left undisturbed for at least 30 min, after which time data were collected in air. Then, the inflow line was switched to a gas impermeable 15-litre bag for the delivery of hypoxic (15% or 10% O_2, in this order), or hypercapnic (2 or 4% CO_2, in this order) gases, with a period of air (30 min) between the two exposures. These gas mixtures were prepared by blending the pure gases from pressurised tanks and checking their final concentration with calibrated gas analysers. Each exposure lasted 20–30 min and data were collected during the last 2–3 min. All measurements were conducted at the ambient T of 37.5–38 °C (37.9 ± 0.1 and 37.8 ± 0.1 °C in chickens and ducklings, respectively).

2.4. Number of animals and statistics

Experiments were conducted on 21 hatchlings per species, of which, after the measurements in air, 7 had exposure to hypoxia, 7 to hypercapnia and 7 to both hypoxia and hypercapnia; in this latter case, hypoxia and hypercapnia had a 30 min period of air breathing in between and were presented in alternate order among animals. Hence, for each species, 21 hatchlings produced data during air breathing; of these, 14 had measurements taken during hypoxia and 14 during hypercapnia.

Data were presented as means ± 1 SEM. A difference between chicken and duck hatchlings during air-breathing (N = 21 per group) was evaluated statistically by two-tailed *t* test. The responses to hypoxia and hypercapnia were compared statistically between the two species

by one-way ANOVA (N = 14 per group), followed by the post-hoc Bonferroni's limitations for the two comparisons of interest between the two species (at 15 and 10% O_2 for the hypoxic response and at 2 and 4% CO_2 for the hypercapnic response). In all cases, a difference was considered statistically significant at $P < 0.05$.

3. Results

The ducklings were about 10 g (or 23%) heavier than the chicks; hence, data between the two species were compared both in absolute terms and after normalisation by body weight (Table 1). Body temperature averaged ~40 °C in both groups of hatchlings.

3.1. Breathing pattern during resting conditions

The main differences in the resting breathing between the two sets of hatchlings are given in Table 1. Breathing frequency averaged 46 ± 3 in ducklings and 63 ± 3 in chicks, because of significant differences in both inspiratory and expiratory times. Tidal volume was higher in ducklings than in chicks, in absolute values (565 μL ± 37 and 330 μL ± 18, respectively) and after normalisation by body weight (11.3 mL/kg ± 0.7 and 8.1 ± 0.4, respectively). The values in chicks are in agreement with those collected previously on chicken hatchlings of similar age (Szdzuy and Mortola, 2007b, 2008; Ferner and Mortola, 2009; Mortola, 2011b; Mortola and Toro-Velasquez, 2013), which, all combined, averaged 70 breaths/min and 6.4 mL/kg for f and tidal volume, respectively.

In separate sets of hatchlings (N = 6 per species) we measured f visually, simply by counting the breathing-related movements of the air sacs over several minutes through a plastic window of the hatchery-incubator, while the hatchling was resting undisturbed. This was done to consider the possibility that the confinement of the plethysmograph required by the barometric technique may have caused qualitative changes in the breathing pattern. In reality, this did not seem to have been the case; in fact, f averaged 66 ± 4 and 51 ± 4 breaths/min in chicks and ducklings respectively ($P < 0.02$), close, although not quite identical, to the average values measured by barometric plethysmography.

In ducklings, resting $\dot{V}_{O_2}$ was higher than in chicks, even after taking into account the difference in body weight, while the differences in $\dot{V}E$ were not as large (Table 1); therefore, the ventilatory equivalent $\dot{V}E/\dot{V}_{O_2}$ (20.8 ± 1.5) averaged slightly, yet significantly, less than in chicks (25.3 ± 1; $P < 0.02$). In both species the newborns' values exceeded those of the adults.

Table 1
Resting values in chicken and Muscovy duck hatchlings.

	Chickens (N = 21)	Muscovy ducks (N = 21)	P	Adult chickens, ducks[a]
Fresh egg mass, g	59.8 ± 0.5	80.7 ± 0.9	<0.0001	
Post-hatching age, hours	16.4 ± 1.1	16.4 ± 2.4	ns	(adults)
Body mass, g	40.8 ± 0.5	50.3 ± 0.9	<0.0001	1900, 2160
Body temperature, °C	40.0 ± 0.1	40.1 ± 0.2	ns	41.6, 40
Oxygen consumption, mL · min^{-1}	0.79 ± 0.03	1.26 ± 0.07	<0.0001	27, 50
mL · kg^{-1} · min^{-1}	19.6 ± 0.7	25.1 ± 1.4	<0.001	14.2, 23.1
Tidal volume, μL	330 ± 18	565 ± 37	<0.0001	29,300, 69,000
mL/kg	8.1 ± 0.4	11.3 ± 0.7	<0.001	15.4, 31.9
Breathing frequency, breaths/min	63 ± 3	46 ± 3	<0.0001	20.5, 10.5
Inspiratory time, ms	221 ± 10	297 ± 19	<0.001	
Expiratory time, ms	334 ± 18	499 ± 31	<0.0001	
Ventilation, mL · min^{-1}	20.2 ± 1.2	25.1 ± 1.5	<0.02	590, 700
mL · kg^{-1} · min^{-1}	496 ± 28	499 ± 30	ns	311, 324
Ventilatory equivalent ($\dot{V}E/\dot{V}_{O_2}$)	25.3 ± 1.0	20.8 ± 1.5	<0.02	22, 14

[a] From the data compiled by Frappell et al. (2001).

3.2. Responses to hypoxia and hypercapnia

Both species responded to hypoxia and hypercapnia with an increase in $\dot{V}E$, but differed in their breathing pattern (Fig. 1). In hypoxia, ducklings increased f more than the chicks did, so as to abolish the difference in f present at rest. At the same time, the ducklings retained a higher tidal volume at all levels of hypoxia; hence, the hypoxic hyperventilation was similar in both species (Fig. 2), and the ventilatory equivalent in 10% O_2 approximately doubled the value during air-breathing. Hypoxia lowered Tb by about 0.4–0.5 °C with 15% O_2 and by 0.8–1.0 °C with 10% O_2 in ducklings and chicks, respectively; these drops did not differ significantly between species ($P > 0.05$).

In hypercapnia, both f and tidal volume increased. However, ducklings retained their tendency to breathe with a f lower than chicks and their increase in tidal volume was moderate (with 2% CO_2: 120 ± 7% and 147 ± 7% of the air value in ducks and chicks, respectively, $P < 0.02$; with 4% CO_2: 131 ± 9% and 168 ± 8%, respectively, $P < 0.005$). Hence, during hypercapnia $\dot{V}E$ increased less in ducklings than in chicks (Fig. 1). Because metabolic rate changed little in either species, the degree of hypercapnic hyperventilation was significantly lower in the ducklings ($P < 0.001$; Fig. 2).

4. Discussion

The data indicated that ducklings, by comparison to chicks, had a) slightly higher ventilatory efficiency, b) lower resting f, and c) a blunted hypercapnic hyperventilation.

4.1. The ventilatory equivalent of avian hatchlings

The functional advantages of the cross-current gas exchange mechanism in birds have long been recognised (Scheid and Piiper, 1972, 1986). The air sacs are the bellows that provide the unidirectional flow through the secondary bronchi, from which air capillaries originate, surrounded by the blood capillaries, within the rather rigid lungs (Maina et al., 2010). It is this arrangement that permits birds to gas exchange with a $\dot{V}E$ lower than mammals. From the allometric analysis of many species of adult birds (Frappell et al., 2001) $\dot{V}E$ and $\dot{V}_{O_2}$ at 1 kg were, respectively, 386 mL BTPS/min and 16 mL STPD/min; these values yield an average size-independent ventilatory equivalent of ~24 mL BTPS · mL STPD^{-1} (Frappell et al., 2001), about 70% of adult mammals (Mortola, 2001). The experimental values pertinent to adult chickens and ducks are, respectively, 22 (Kassim and Sykes, 1982; Gleeson, 1985) and 14 mL BTPS · mL STPD^{-1} (Jones and Holeton, 1972). Here we found that in the hatchlings of these two species the ventilatory equivalent averaged 25 (chicks) and 21 (ducklings) (Table 1). Hence, the current results in hatchlings confirmed the adult trend of a greater ventilatory efficiency in the duck than in the chicken, even though both hatchlings had ventilatory equivalents slightly higher than the corresponding adults. In the semi-precocial Wedge-tailed shearwater (*Puffinus pacificus*), the ratio between the average $\dot{V}E$ provided by Pettit and Whittow (1982) and the $\dot{V}_{O_2}$ by Ackerman et al. (1980) yields a ventilatory equivalent of 25; this is close to those of the chicken and duck hatchlings and is slightly higher than the value (22) computed from the allometric functions for a 400 g adult shearwater (Frappell et al., 2001). In summary, from the data currently available it seems that in hatchlings the ventilatory efficiency is slightly lower than in adult birds. It would be interesting to extend the analysis to other species, especially to hatchlings of altricial species; but data on $\dot{V}E$ are totally lacking.

In neonatal mammals, the incomplete formation of alveoli and the mass-specific larger dead space probably explain why in some species the ventilatory equivalent exceeds the adult values (Mortola, 2001). Similarly, it is possible that incomplete expansion of the air sacs or some remaining fluid in the bronchi could increase the mechanical impedance of the hatchling's respiratory apparatus, diminishing its

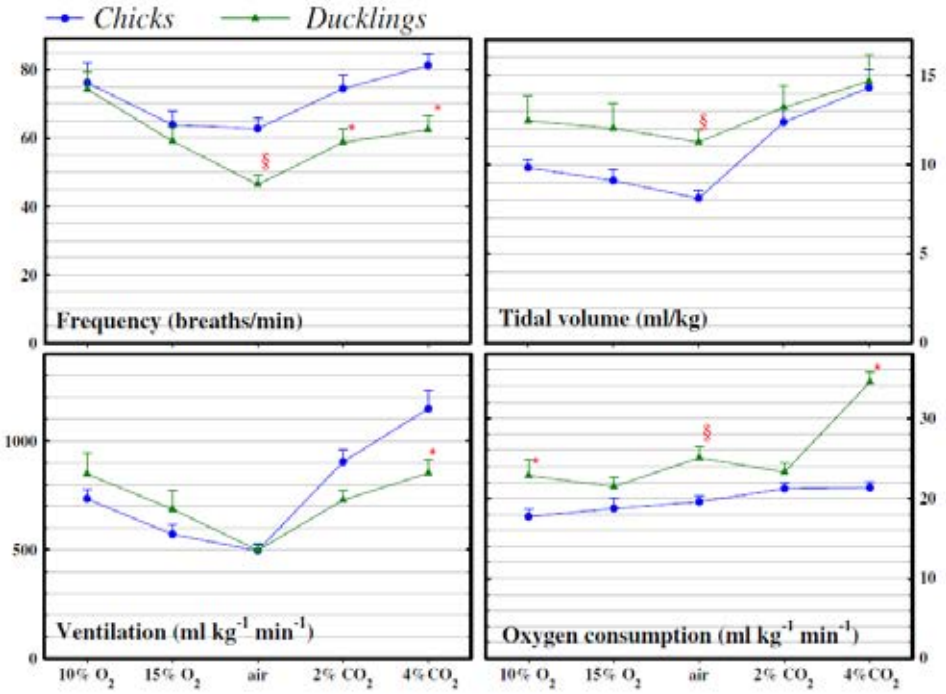

Fig. 1. Breathing pattern during air breathing, hypoxia (15 or 10% O_2) and hypercapnia (2% or 4% CO_2) in chicken and duck hatchlings (blue circles and green triangles, respectively). Values are group means, bars 1 SEM. *Significant difference between the two groups ($P < 0.05$) during air breathing (§, $N = 21$) or during the hypoxia or hypercapnia (*, $N = 14$). (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this article.)

ventilatory efficiency. It is of interest to compare the values of the hatchlings to those of a typical 1.9 kg adult chicken and 2.2 kg adult duck (Frappell et al., 2001), to examine in detail the variables responsible for the differences in ventilatory equivalent between newborns and adults (Table 1, rightmost column). From these comparisons it emerges that the hatchlings' $\dot{V}_{O_2}$, normalised by body weight, exceeds the adult's value by 38% (chicken) and 10% (duck), while $\dot{V}E/kg$ exceeds it by 50–60%. The disproportionately higher $\dot{V}E$ in hatchlings is entirely determined by f, which is 3 (chicks) to 4.5 times (ducklings) higher than in adults (Jones and Holeton, 1972; Powell et al., 1978; Kassim and Sykes, 1982; Gleeson, 1985), while mass-specific tidal volume is no more than half that of the adults. Hence, the higher ventilatory equivalent of the hatchling, by comparison to the adult, is due to the higher $\dot{V}E$ and this is accompanied by a more rapid and shallow breathing pattern. It is possible that the mechanical properties of the respiratory system contribute to the newborn–adult difference in breathing pattern. In fact, during resting breathing the hatchling's respiratory system is overinflated above its passive volume, which reduces greatly the compliance of the respiratory system (Mortola, 2011a). In addition, at birth any yolk residue, some 20% of the embryo's weight (Mortola and Al Awam, 2010), gets incorporated into the abdomen (Williams, 1994), a process that must create an elastic load to the respiratory system. Whenever respiratory compliance is lower than normal, it is energetically favourable to adopt a faster and shallower breathing pattern. In birds, despite the unidirectional gas flow, ventilation–perfusion inhomogeneity introduces an important physiological dead space (Hastings and Powell, 1986). Hence, like in mammals, the fast and shallow breathing requires a disproportionate increase in $\dot{V}E$ to maintain adequate ventilation of the gas exchange area. In conclusion, the more rapid and shallow breathing pattern of the hatchling, possibly determined by a lower respiratory compliance, decreases the ventilatory efficiency and requires ventilatory equivalents higher than in adults.

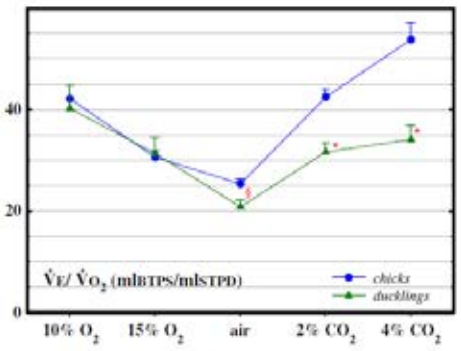

Fig. 2. Ventilatory equivalent ($\dot{V}E/\dot{V}_{O_2}$) of chicken hatchlings (blue circles) and ducklings (green triangles) during air, hypoxia (15 or 10% O_2) or hypercapnia (2% or 4% CO_2). Symbols indicate group means ($N = 14$), bars 1 SEM. *Significant difference between the two groups ($P < 0.05$) during air breathing (§, $N = 21$) or during the hypoxia or hypercapnia (*, $N = 14$). (For interpretation of the references to colours in this figure legend, the reader is referred to the web version of this article.)

4.2. Breathing frequency

At rest, the chicken and duck hatchlings were breathing, respectively, 63 and 46 breaths/min, an interspecies difference qualitatively confirmed by the visual measurements of f collected outside the body plethysmograph.

In both mammals and birds the resting f of aquatic species, when examined allometrically over a wide range of body size, is lower than in terrestrial species (Mortola and Limoges, 2006; Mortola and Seguin, 2009). A satisfactory explanation for this difference remains elusive. One possibility is that lung inflation maintained by breath-holding improves buoyancy at the water surface; presumably, this has favoured a breathing pattern with large tidal volume and low f (Mortola and Limoges, 2006; Mortola and Seguin, 2009). The current result in the

ducklings, if representative of other aquatic hatchlings, would indicate that the low f has evolved to be part of the genetic makeup of aquatic species, expressed at birth prior to any diving experience.

4.3. Ventilatory chemosensitivity

The $\dot{V}E$ responses to hypoxia and hypercapnia were clear in both ducklings and chicks. This is not surprising, given that at birth hatchlings have the full complement of chemoreceptors (Hempleman and Pilarski, 2011). The $\dot{V}E$ response to CO_2 had been measured before in chicken hatchling at ~19 h of age (Mortola, 2009): the increase to 220% of the air value was almost identical to the increase (226%) here observed. Larger increases in $\dot{V}E$ (to more than three times the air values) have been measured in the only other hatchling studied to date, the Wedge-tailed shearwater (Pettit and Whittow, 1982). These results contrast the modest $\dot{V}E$ response of the ducklings, caused by the small increase in tidal volume (Figs. 1 and 2). Butler and Taylor (1974) compared the $\dot{V}E$ response to hypercapnia between adult chickens and mallard ducks and concluded that it was similar. Other investigators (Tenney and Boggs, 1986), from a review of the literature, concluded that the $\dot{V}E$ response to hypercapnia was somewhat depressed in diving mammals and birds, while differences in the $\dot{V}E$ response to hypoxia were less clear. Human elite free-divers have a blunted response to inspired CO_2 but a normal response to hypoxia (Grassi et al., 1994). Hence, it is possible that the lower $\dot{V}E$ response to CO_2 in the ducklings, by comparison to chicken and shearwater hatchlings, was not simply the accidental manifestation of species differences but represented the trend of some diving animals.

5. Conclusions

The ventilatory equivalent of the duckling was significantly lower than that of the chicken hatchling, confirming in the newborns the difference present in the adults of the two species. Nevertheless, in either species the ventilatory equivalents were not as low as in the adults, perhaps because mechanical factors may have lowered the efficiency of the hatchlings' respiratory apparatus. The breathing pattern with low f and the blunted $\dot{V}E$ response to hypercapnia of the newborn duck could be related to the aquatic habitat of the species. If this was the case, it would mean that these breathing characteristics are genetic traits, the phenotypic expression of which does not require the diving experience.

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APÊNDICE 3- The thermal preference of the chicken hatchling: below thermoneutrality

Paula Andrea Toro-Velasquez[1,2], Kênia C. Bícego[2] and Jacopo P. Mortola[1]*

[1]Department of Physiology, McGill University, Montreal, QC, Canada,

[2]Department of Animal Morphology and Physiology, College of Agricultural and Veterinarian Sciences, Sao Paulo State University, Jaboticabal, SP, Brazil

Running title: Behavioural and autonomic thermogenesis in the chicken hatchling

* Corresponding author. Address: Dept. of Physiology, McGill Univ., 3655 Promenade Sir William Osler, room 1121, Montreal, Quebec, H3G 1Y6 Canada - Fax: +1 514 3987452 - *E-mail address*: jacopo.mortola@mcgill.ca

Abstract

We asked whether or not the preferred ambient temperature (Ta*pref*) of the 1-day old chicken hatchling, a precocial neonate with excellent locomotory capacity, clearly identifiable thermogenesis and independence from maternal care, coincides with the lower critical temperature (LCT) of thermoneutrality and minimal oxygen consumption ($\dot{V}_{O_2}$). Ta*pref* of single chicks measured in a thermocline (N=16) averaged 33.5±0.3°C (mode, 33.3±0.4°C). The same value was obtained in hatchlings studied in pairs. LCT was computed from the ambient temperature (Ta)- $\dot{V}_{O_2}$ relationship, constructed by slowly decreasing the Ta of a respirometer from 38 to 29°C over 2.5 hrs, while continuously measuring $\dot{V}_{O_2}$ by an open-flow methodology; it averaged 36.4°C ±0.3 or 36.8°C ±0.4, depending on the method of computation. In all hatchlings Ta*pref* was lower than LCT ($P<0.001$), by a magnitude that depended on the method of computation of the two variables, 2.8°C ±0.3 ($P<0.001$) or 3.9°C ±0.5. The Ta*pref*-LCT difference implied that, at Ta*pref* , $\dot{V}_{O_2}$ was substantially higher than at thermoneutrality. We conclude that in the chicken hatchling thermal preference does not coincide with thermoneutrality, probably because during development what seems optimal from a thermoregulatory viewpoint may not necessarily be so for other regulatory functions.

Key words: Bird.Newborn. Thermoneutrality. Thermoregulation

1. Introduction

The broad question of whether or not thermoneutrality[1] corresponds to the individual's thermal preference can be difficult to answer, and particularly so in case of neonates. In fact, preference can be ascertained through direct questioning only in adult humans. In infants and in animals preference is inferred from behavioral responses; of these, probably the most quantifiable is the thermal preference within a range of Ta, or thermocline. In adult laboratory rodents, comparisons of the preferred Ta (Ta*pref*), assessed in the thermocline, and the thermoneutral lower critical temperature[2] (LCT), usually measured from the changes in oxygen consumption ($\dot{V}_{O_2}$) at various Ta, have indicated that Ta*pref* can fall close to or below LCT (Gordon, 1990, for review). In young rabbits the preferred Ta (Ta*pref*) appeared to be similar to LCT (Hull et al., 1986); however, the authors recognized that LCT could not be resolved clearly because $\dot{V}_{O_2}$ was measured at few, and too far apart, Ta intervals. In neonatal guinea pigs Ta*pref* averaged 30°C (Clark and Fewell, 1996) or 32°C (Fewell et al., 1997), respectively, slightly below or coincident with LCT. In newborn piglets Ta*pref* varied between 32 and 30°C, depending on whether they were placed singly or in groups within a thermocline; these values would be 2-4°C degrees below the piglets' LCT (Mount, 1979).

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1 'Thermoneutrality' is the range of ambient temperature at which temperature regulation is achieved only by control of sensible heat loss, i.e., without regulatory changes in metabolic heat production or evaporative heat loss (IUPS Thermal Commission, 2001)

2 'Lower critical temperature' is the ambient temperature below which the rate of metabolic heat production of a resting thermoregulating tachymetabolic animal must be increased by shivering and/or nonshivering thermogenesis in order to maintain thermal balance (IUPS Thermal Commission, 2001).

Several factors complicate the assessment of the relationship between Ta*pref* and thermoneutrality in neonatal birds and mammals. Newborns of the commonest laboratory species have a limited locomotory ability, which defeats the use of the thermocline in the assessment of Ta*pref*. Some neonates lack thermogenesis or present a very narrow range of thermoneutrality (Mortola, 2001); in these cases a clear definition of LCT requires metabolic measurements at small Ta intervals. Piglets and neonates of other precocial species have manifest thermogenesis and good locomotion; however, their needs for maternal care and suckling and their explorative behavior are competing drives that can override thermoregulatory needs and blurry the determination of Ta*pref* (Ingram and Legge, 1970; Mount, 1979). Occasionally, numerous animals have been tested simultaneously (e.g., Mount, 1979; Nichelmann, 2004); in these cases, the tendency to huddle or to establish a social hierarchy could condition their choice for Ta*pref*. In an attempt to lower the impact of these modifiers, in this study we have measured Ta*pref* and LCT in 1-day old chicken hatchlings.

Chickens are precocial birds with excellent locomotory capacity at birth and clearly identifiable thermogenesis (Mortola, 2009, for review). Because of the incorporation of the residual yolk inside the abdomen during hatching (Williams, 1994), unlike neonatal mammals the 1-day old hatchlings have neither the urge nor the needs to be fed. Their independence from maternal care diminishes the possibility that external drives may provide competing cues, which simplifies the physiological interpretation of Ta*pref*.

2. Methods

Experiments were conducted on chicken hatchlings (*Gallus gallus,* White Leghorn variety), at about 17 hrs post-hatching, n=16. Freshly laid fertilized eggs were obtained from a

local supplier. After noting the weight, the eggs were placed in incubators set at the temperature (T) of 37.5°C and 60% relative humidity, with a 45^{o} egg rotation four times a day. Incubation T and relative humidity were monitored every 10 min by a data logger placed inside the incubator. At end incubation, when embryos approached the internal pipping phase, eggs were transferred to a hatchery-incubator with no rotation. All measurements were conducted within the first 24 hrs from hatching.

2.1. Thermocline

The thermocline consisted of a 102 x 10 x 20 cm (length, width, height), 14 mm thick, Styrofoam column, covered by an acrylic panel. Numerical markings on the floor and sides indicated the horizontal distance (D) from one end. The thermocline was warmed by external heat sources empirically spaced so as to generate an end-to-end temperature difference of about 15°C (from 43.9 to 28.4°C), with an average gradient of 0.15°C/cm. Before each experiment, the relationship between D and Ta was checked with a thermocouple (previously calibrated against a mercury-thermometer) located on the internal lateral side, at about 4 cm from the floor of the thermocline (corresponding to the approximate center of mass of hatchling), in 5 cm steps. The D-Ta (X-Y) data points were fit by a polynomial of the type $Y=a\cdot X^2 + b\cdot X +c$; the correlation coefficient of the fitting averaged 0.98 and was never below 0.96. The T at the floor of the thermocline averaged 1°C less than the T on the lateral side of the thermocline, which was the one considered most representative of the T felt by the hatchling. The thermocline was located in an isolated temperature controlled room, with no people in it. At the time of the experiment, the hatchling was placed at the center of the thermocline and left undisturbed for at least one hr before data collection. Then, a digital camera on top of the thermocline automatically took a picture every minute for 60 min.

Analysis by two independent investigators of more than 100 frames picked at random produced an average discrepancy of only 0.1°C, indicating that the subjective component of the analysis had no impact on the results of the study.

2.2. Measurements of $\dot{V}_{O_2}$

Oxygen consumption ($\dot{V}_{O_2}$) was measured by an open-flow methodology (Szdzuy et al., 2008). The hatchling was placed in a sealed 200-ml plastic container; this was inside a second container almost entirely submerged into water and maintained at the desired temperature (37.5 ^{o} C) by a water bath. A steady gas flow of 150 ml/min was continuously delivered through the respirometer, passed through a drying column, and the outflow O_2 and CO_2 concentrations were recorded continuously by calibrated gas analyzers (Sable Systems International Fox, Henderson, NV). The inflow concentrations were monitored intermittently. After mathematical correction of the gas concentrations for a respiratory quotient different from unity (Depocas and Hart, 1957;Mortola and Besterman, 2007), $\dot{V}_{O_2}$ was computed from the flow rate and the inflow-outflow gas concentration difference, averaged over each 10 min time interval. The ambient temperature (Ta) of the respirometer was collected by telemetry via a transmitter placed at the height of the hatchling powered by an energizer-receiver unit (4000E, Minimitter, Sunriver, OR). The concentrations of O_2 and CO_2, flow and Ta were continuously collected and displayed on a computer monitor. The wash-out time of the respirometer and connecting tubings in the conditions of the experiment was measured by rapidly injecting a bolus of CO_2 and measuring the time of its detection by the analyzer; it was 22 sec.

At the time of the measurements, the hatchling was left in the respirometer at 37.5^{o} C for

at least half hour before data collection. Then, data were collected for 3 hrs, of which the first 30 min were in warm conditions (37.5°C) and the remaining 2.5 hrs during cooling. The decrease in Ta was achieved by adjusting the T of the water bath so as to reach ~29°C in the respirometer with an approximately linear T decline of 0.06°C/min (see RESULTS). Data of Ta and $\dot{V}_{O_2}$ were computed every 10 min by averaging the data sampled during the preceding 5 min. Cloaca temperature was measured at the beginning and the end of the experiment with fine tungsten-constantan thermocouple connected to a digital thermometer (Omega 871A, Omega Engineering Inc., Stanford, CT), and taken as representative of body temperature (Tb).

2.3. Protocols

Most of the hatchlings (11 of 16) had the thermocline test performed in the late morning followed by the Ta-$\dot{V}_{O_2}$ relationship, because we were concerned that the alternate approach may have had some carry-over effect of the cooling phase. However, in 5 animals the protocol was reversed, with ~2 hrs between tests; the results were identical to those of the majority of the group. In the end, we made sure that the postnatal hours of the two tests coincided (Table 1), to avoid the possibility that the changes in thermoregulatory capacity during the first postnatal day (Szdzuy et al., 2008) may complicate the interpretation of the results.

In all cases of the respirometry measurements and in 12 of the 16 measurements in the thermocline the hatchlings were studied individually. In the remaining four cases of the thermocline test the hatchlings were studied in the presence of a sibling; this was done to consider whether or not the hatchling's preferred ambient temperature (Ta*pref*) differed when studied individually or in pairs.

In an additional group of hatchlings we measured the $\dot{V}_{O_2}$ response to a sudden drop in

Ta. This was done to examine the possibility that a rapid drop in Ta would entail a different thermogenic response than the slow cooling adopted in the main protocol. Six hatchlings (17.3 ±3 hrs old, 41.8 ±0.8 g), first, were exposed for 30 min to the same warm condition as the main group (Ta=37.5°C); then, they were immediately exposed to Ta=35°C for an additional 30 min.

2.4. Analysis

For each frame, first, the distance D of the hatchling from one end of the thermocline was recorded based on the numerical scale. From the min-by-min ΔD it was possible to calculate the average and maximal velocities of the hatchling's horizontal travel. The min-by-min thermocline Ta at the location of the hatchling was computed from the polynomial equation of the Ta-D curve established prior to each experiment.

The Ta-$\dot{V}_{O_2}$ relationship around LCT often had a curvilinear shape, owing to the fact that the rise in $\dot{V}_{O_2}$ below thermoneutrality is a gradual process. Therefore, to calculate LCT, first, we identified the $\dot{V}_{O_2}$ data points during the cooling process that were statistically higher than the data points during warm conditions (by paired two-tailed analysis). Then, a regression line was constructed through the first four of these Ta-$\dot{V}_{O_2}$ data points (X and Y, respectively); from the regression function, LCT was the X-value where Y equaled the average $\dot{V}_{O_2}$ in warm condition. With a slightly different approach, the regression line also included the Ta-$\dot{V}_{O_2}$ pair with Ta=Tb and $\dot{V}_{O_2}$=0, based on the consideration that, for Tb to remain as at thermoneutrality with $\dot{V}_{O_2}$=0, Ta must correspond to Tb (Gordon, 1990). The two approaches produced very similar results (see below).

Group data are reported as means ±1SEM. Statistical comparisons between two sets of data were evaluated by two-tailed *t* test, paired or unpaired as appropriate, with statistical significance at $P<0.05$.

3. Results

The characteristics of the hatchlings (N=16) are in Table 1. The age of the chicks at the time of the two tests (thermocline and respirometer) was comparable, 16.9 hrs and 17.3 hrs, respectively ($P>0.05$).

3.1. Preferred temperature

The hatchlings moved substantially within the thermocline; on average the total distance traveled in the hour of the test was 270 cm (±46), which corresponded to an average speed of 4.5 cm/min (±0.8), with a peak speed of 28 cm/min (±4). The most frequent Ta during the hour of the test when all data were pooled together (60 data points for each of the 16 hatchlings) was 32.5°C (Fig. 1, top panel). The Ta average and mode were, respectively, 33.5°C±0.3 and 33.3°C±0.4. The figure panel also presents the frequency distribution of the first half hour and of the second half hour of the test; they were virtually identical.

To consider the possibility that isolation or distraction may have altered the results, eight hatchlings (four of the main group and four additional ones) were tested in four groups of two. The results of these pairs were not significantly different from those of the main group, whether analysed by histogram distribution (Fig. 1, bottom panel) or by Ta average or mode.

3.2. $\dot{V}_{O_2}$ during warm and cooling phases

Fig. 2 gives the time profile of ambient temperature (top) and $\dot{V}_{O_2}$ (bottom) during the

warm and cooling phases. The value of $\dot{V}_{O_2}$ in warm condition (0.79 ml·min^{-1}) was very close to the values collected previously on chicken hatchlings of similar age (Mortola, 2009, for review). As we started the cooling protocol, Ta significantly decreased, while a significant rise in $\dot{V}_{O_2}$ occurred only 30 min into the cooling phase. At the peak of the response $\dot{V}_{O_2}$ averaged 1.68 ml·min^{-1}, or 212% of the warm value.

From the Ta-$\dot{V}_{O_2}$ curve (Fig. 3), LCT, computed as described in METHODS, averaged 36.4°C ±0.3 when the regression line included the Ta-$\dot{V}_{O_2}$ pair for $\dot{V}_{O_2}$=0 (where Ta=body temperature). Without inclusion of that data point LCT was a little higher (36.8°C ±0.4), although not statistically different from 36.4°C (P>0.05, paired *t*-test).

In all hatchlings Ta*pref* was lower than LCT, by a magnitude that varied slightly depending on the method of computation of these two variables. With Ta*pref* measured as the average of the 1-hr observation and LCT computed including the Ta-$\dot{V}_{O_2}$ pair for $\dot{V}_{O_2}$=0, the group LCT-Ta*pref* difference was 2.8°C ±0.3 (P<0.001; Fig. 4). Had the LCT computation not included the Ta-$\dot{V}_{O_2}$ pair for $\dot{V}_{O_2}$=0 and with Ta*pref* equal to the mode of the histrogram distribution, the LCT-Ta*pref* would have been 3.9°C ±0.5 (P<0.0001).

3.3. Sudden drop in ambient temperature

The average thermogenic response to an acute drop in Ta (from 37.5 to 35°C) is represented in Fig. 5. Sudden cold exposure raised $\dot{V}_{O_2}$ to about 180% of the warm value. At the same Ta, the gradual cooling adopted in the main protocol (Fig. 3) had raised $\dot{V}_{O_2}$ by only 135%. Hence, the acute drop in Ta almost doubled the thermogenic response by comparison to gradual cooling.

4. Discussion

The results of this study indicated that in the 1-day old chicken hatchling Ta*pref* and LCT did not coincide, the former averaging ~3°C lower than the latter. Before discussing the potential mechanisms and implications, we will consider aspects of the methodology that may be prone to experimental errors.

4.1. Methodological concerns

The recording of Ta was instantaneous, while that of $\dot{V}_{O_2}$ had a delay related to the washout of the apparatus, which for our setup was 22 sec. Because Ta changed very slowly (9°C over 150 min, or 0.06°C/min), the 22 sec asynchrony between the two signals meant that the Ta temporal misalignment was <0.03°C, too small to cause any significant modification of the Ta-$\dot{V}_{O_2}$ relationship.

Previously, based on steady state measurements of $\dot{V}_{O_2}$ at four-to-six ambient temperatures chosen to be around thermoneutrality, the LCT in 1-day old White Leghorn strain was taken as 35°C (Meltzer et al., 1982), or about 1.5°C less than here computed. However, the accurate measurement of LCT stands on frequent $\dot{V}_{O_2}$ samplings at small Ta intervals, which in our case was not a concern given the very slow change in Ta. The computation of LCT as outlined in METHODS (*2.4.*) was a conservative measure to avoid overestimation of LCT.

The almost identical Ta*pref* between the first and the second half of the 1-hr data collection (Fig. 1, top panel) was reassuring on several grounds. First, it meant that the hour

preceding the experiment was sufficient time to habituate the hatchling to the thermocline environment. In addition, it indicated that the 1-hr data collection was sufficient to gather consistent results and external factors potentially disturbing Ta*pref* either remained constant throughout the hour or, more likely, played an insignificant role. This latter conclusion is consistent with the observation that Ta*pref* did not differ between hatchlings studied individually and those studied in pairs (Fig. 1, lower panel).

4.2. $\dot{V}_{O_2}$ *at* Ta*pref*

Because Ta*pref* was 33.5°C, or 2.8°C lower than LCT, $\dot{V}_{O_2}$ at Ta*pref* exceeded its minimal value. The magnitude of the $\dot{V}_{O_2}$ increase at Ta*pref* would be ~70% if computed from the Ta-$\dot{V}_{O_2}$ curve (Fig. 3). However, the Ta-history is likely to have a profound influence on the $\dot{V}_{O_2}$ response to cold, as indicated by the much higher $\dot{V}_{O_2}$ reached during a sudden, rather than gradual, drop in Ta (Fig. 5). Because in the thermocline the hatchlings could move at leisure through a range of ambient temperatures, it is possible that at Ta*pref* the rise in $\dot{V}_{O_2}$ was less than apparent from the Ta-$\dot{V}_{O_2}$ curve obtained by gradual cooling. In any case, the question arises on what may be the benefit of $\dot{V}_{O_2}$ higher than minimal when Ta*pref* is lower than LCT.

The hatchling's preference for a Ta lower than LCT may be appropriate to maintain a thermogenic stimulus at a time when thermoregulation is in its developmental phase. This would be on line with the general principle that stimuli are important in the development of a function (Kandel et al., 2000), like visual inputs for the development of the visual cortex (Wiesel, 1982) or chemical information for the development of ventilatory chemosensitivity

(Carroll, 2003). A $\dot{V}_{O_2}$ above its minimal level forces the cardio-respiratory O_2 convection to perform above the absolute minimum, which could be analogous to setting the idle higher to keep the motor from stalling. For example, in the case of neonatal breathing, the metabolic drive above its minimum lowers the chances of breathing irregularities and apnea (Cameron et al., 2000). In addition, a $\dot{V}_{O_2}$ idling at higher than its minimal level leaves open the possibility of its decrease in case of heat exposure as a safety margin against hyperthermia, which could be particularly relevant in newborns because of their limited heat dissipation mechanisms. In an organism with minimal heat loss capacity, was metabolism at its minimum level (that is, if Ta*pref* was the same as LCT) heat exposure would cause an immediate rise in body temperature. Finally, it is conceivable that optimality takes into account many regulatory systems, not only thermoregulation. In this respect, it is of interest that various companies of the North-American egg-laying poultry industry, based on large scale empirical criteria for health, disease resistance and growth, suggest to maintain the hatchlings at Ta of 32-35°C (Hy-Line, 2010; Bovans Brown, 2012), which is lower than LCT and close to Ta*pref*. For newborn broilers, with LCT of 35°C (Meltzer, 1983), the recommended Ta is 30-34°C (Cobb, 2012). Similarly, housing recommendations for mice suggest a temperature below thermoneutrality (Gordon, 2012).

Locomotion in the thermocline requires some energy expenditure, and the associated heat production may have induced the hatchling to opt for a Ta*pref* lower than LCT; this would be reminiscent of the activity-thermogenesis substitution of some endotherms (Humphries and Careau, 2011). However, velocity determines the cost of terrestrial locomotion (Taylor et al., 1982) and the hatchlings' velocity was quite small. From the energetic data provided by Fedak et al. (1974) for 42 g adult painted quails it is possible to calculate that the hatchling's

locomotion should have raised $\dot{V}_{O_2}$ by no more than 2%. Even if it is probable that bipedal locomotion in hatchlings is clumsier, and therefore more expensive, than in adults it remains unlikely that locomotion in the thermocline caused an important rise in $\dot{V}_{O_2}$. However, the state of wakefulness and explorative alertness may have contributed to a general condition of stress with increased $\dot{V}_{O_2}$ and heat production, requiring a drop in Ta*pref*. In summary, it is conceivable that two sets of mechanisms may have resulted in Ta*pref* lower than LCT, the possible advantages of having a $\dot{V}_{O_2}$ higher than minimal and the behavioral control of body temperature, which requires a drop Ta*pref* whenever $\dot{V}_{O_2}$ is higher than minimal.

As hypothetical as the above conjectures may be, the fact that in the hatchlings Ta*pref* was lower than LCT prompts the general consideration that optimality can (Gordon, 1990; Almeida et al., 2006), but does not have to, coincide with minimal cost and that thermoneutrality does not necessarily equate with thermal-comfort zone (Mount, 1979). Taylor (1980) argued that "to a certain extent the concept of a thermoneutral zone is a physiologist's artifact. Being enamored with this concept (probably because it is so measurable), physiologists tend to forget that it really has very little to do with animals in nature".

4.3. Conclusions

In 1-day old chicken hatchling Ta*pref* in a thermocline was some 3°C lower than LCT. This was the first experiment where the two variables were measured in a newborn bird; therefore, it is impossible to say whether they represent an exception or a general pattern. Nevertheless, the results give fuel to the concept that optimality is not necessarily coincident with minimal cost, probably because what seems optimal from a thermoregulatory viewpoint

may not necessarily be so for other regulatory functions.

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Table 1. Characteristics of the main group of hatchlings

	Hatchlings (N=16)
Fresh egg weight, g	58.2 ± 0.8
Post-hatching age, hours	16.9 ± 1.5 ; 17.3 ± 1.4[1]
Body weight, g	40.6 ± 1.0
Body temperature in warm, °C	39.5 ± 0.2
Body temperature at end of cooling protocol, °C	35.4 ± 0.5
Oxygen consumption, in warm condition, $ml \cdot min^{-1}$	0.79 ± 0.04
$ml \cdot kg^{-1} \cdot min^{-1}$	19.59 ± 1.18
Oxygen consumption at peak cold response, $ml \cdot min^{-1}$	1.68 ± 0.11
$ml \cdot kg^{-1} \cdot min^{-1}$	41.48 ± 2.84

Values are means ±1 SEM. [1]The two values refer to the postnatal hours of the chicks when studied in the thermocline and in the respirometer, respectively.

Figure legends

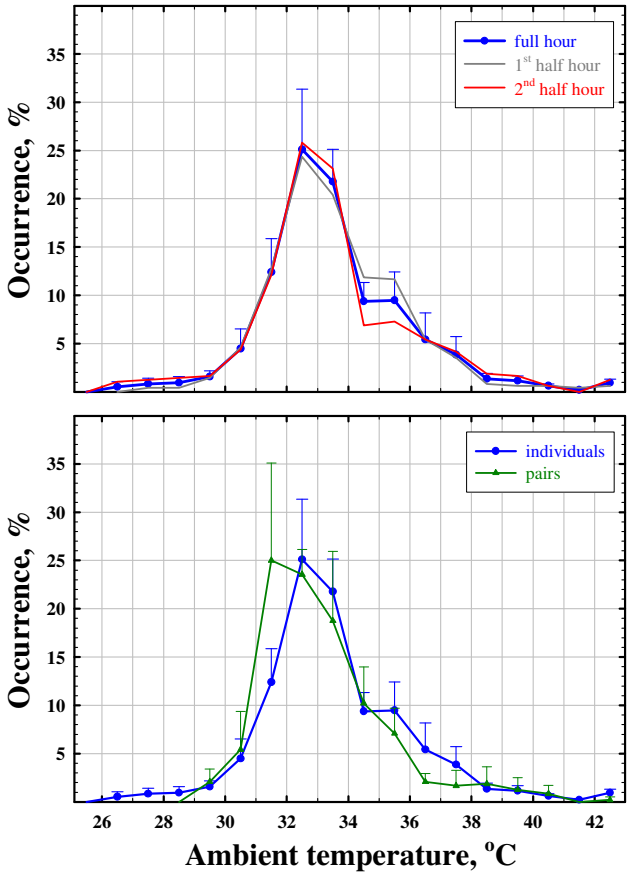

Fig. 1. Frequency distribution of the ambient temperatures at the hatchlings' locations on a min-by-min basis during the thermocline test. **Top**: results of the first and second half hour and of the whole hour. **Bottom**: results of the hatchlings placed singly (N=12) or in groups of two (N=8). Symbols are mean values, bars 1 SEM.

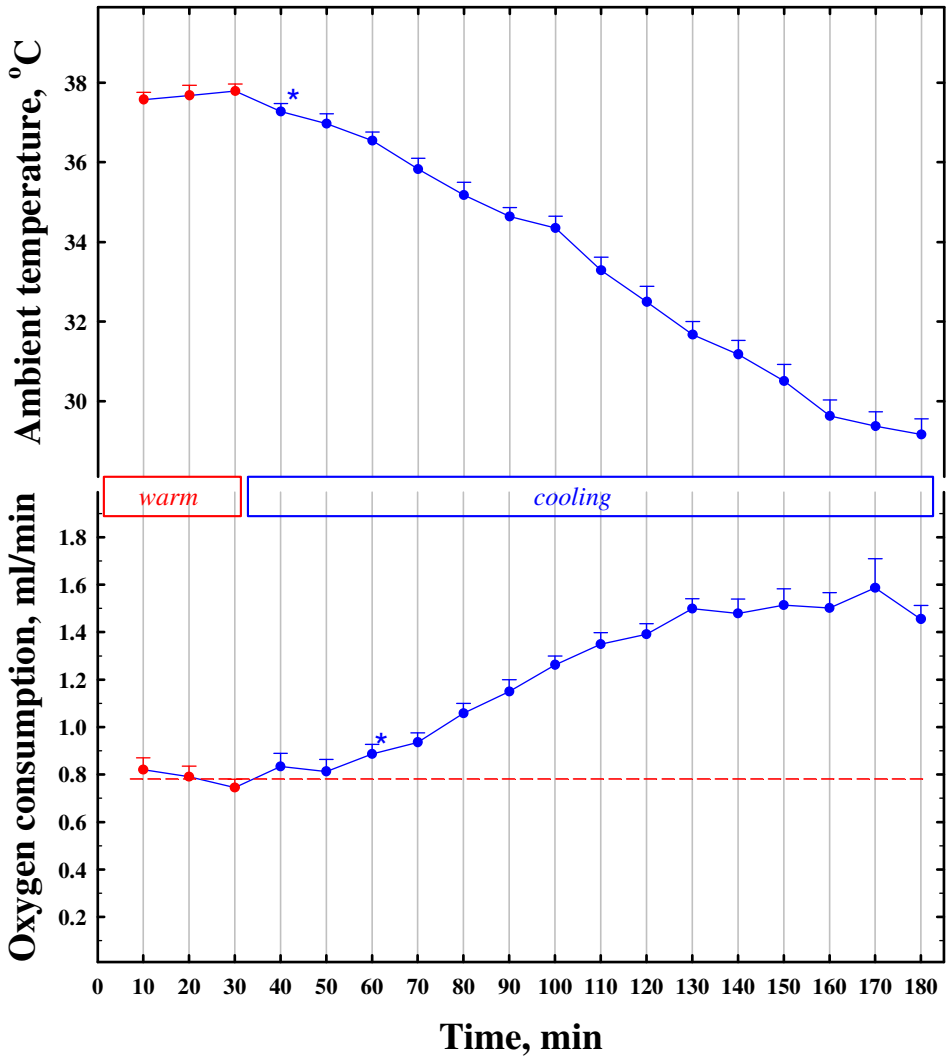

Fig. 2. Average time profile of the ambient temperature (**top**) and oxygen consumption ($\dot{V}_{O_2}$, **bottom**) during the warm phase (first 30 min, red symbols) and the cooling phase (remaining 2.5 hrs, blue symbols) of the experiment. Dashed horizontal line represents the mean $\dot{V}_{O_2}$ in warm conditions. Symbols are group averages (N=16), bars are 1SEM. Asterisks indicate the first values during cooling significantly different from the average warm.

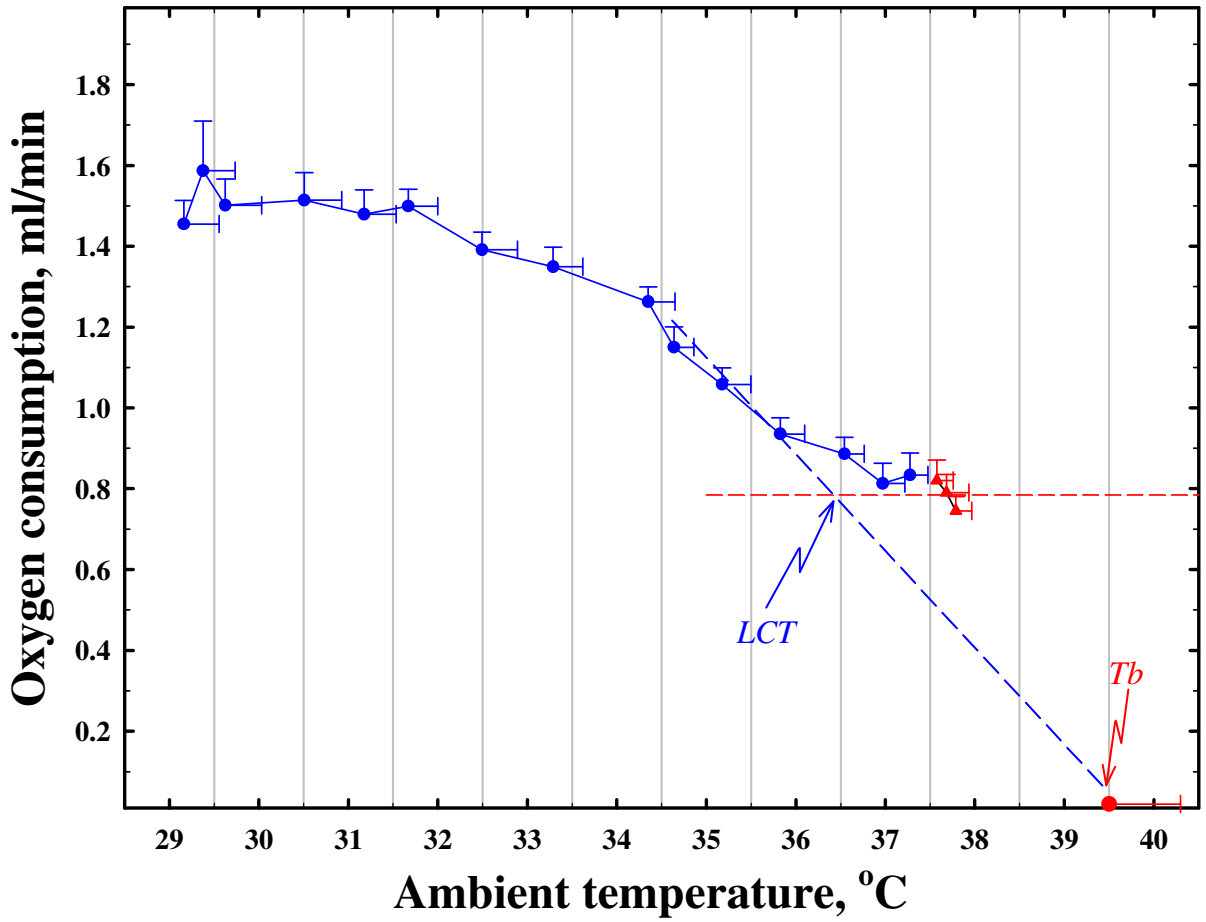

Fig. 3. Ambient temperature (Ta) -Oxygen consumption ($\dot{V}_{O_2}$) relationship in warm (red triangles) and during progressive cooling (solid circles, blue line). Tb, body temperature in warm conditions. LCT, Lower Critical Temperature, which is the Ta where the linear regression through the thermogenic points meets the value of $\dot{V}_{O_2}$ in warm conditions. Symbols are group averages (N=16), bars are 1 SEM.

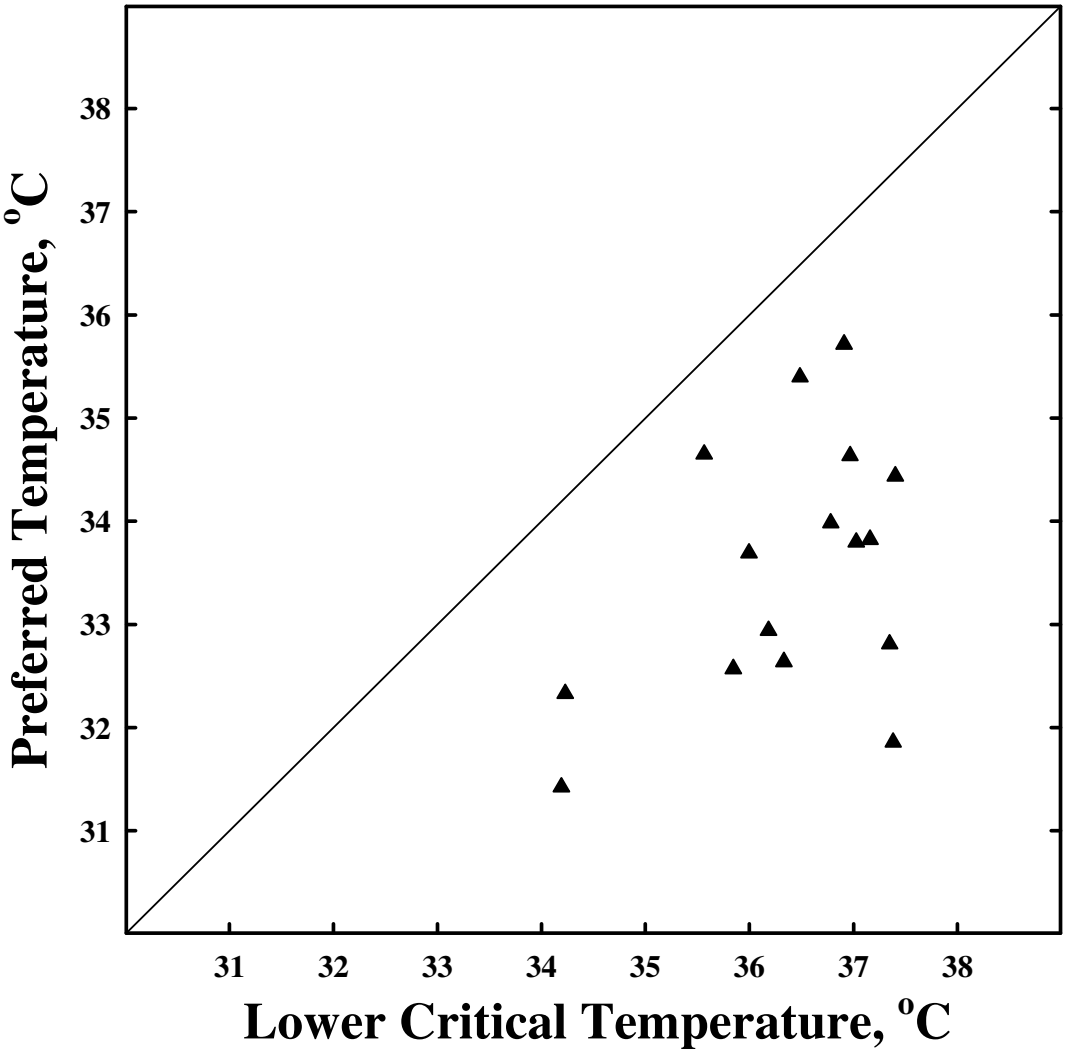

Fig. 4. Identity plot of the Lower Critical Temperature (LCT) versus the preferred ambient temperature (Ta*pref*) for each of the hatchlings studied (N=16). Ta*pref* was measured as the average of the 1-hr observation and LCT was computed with the inclusion of the Ta-$\dot{V}_{O_2}$ pair for $\dot{V}_{O_2}$=0 (see Methods). The oblique line represents the identity line. In all cases Ta*pref* was lower than LCT.

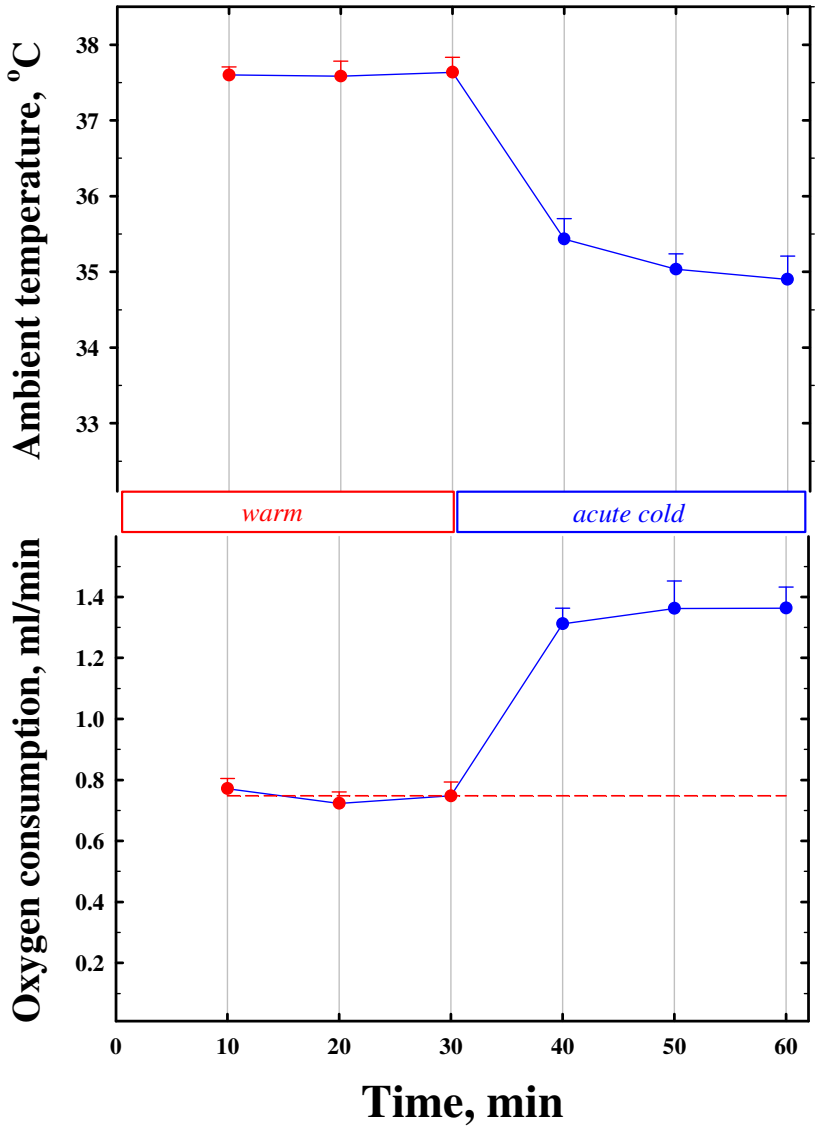

Fig. 5. Acute cold exposure. **Top**: time course of the change in ambient temperature within the respirometer during the warm phase (red) and during the cold exposure (blue). **Bottom**: oxygen consumption during warm and cold exposure. Dashed horizontal line represents the mean $\dot{V}_{O_2}$ in warm conditions. Symbols are group averages (N=6), bars are 1 SEM.

APÊNDICE 4- Thermogenesis, vocalization and temperature preference of 1-day old chicken hatchlings after cold-exposure in late embryogenesis

Paula Andrea Toro-Velasquez[1,2] and Jacopo P. Mortola[1]*

[1]Department of Physiology, McGill University, Montreal, QC, Canada,

[2]Department of Animal Morphology and Physiology, College of Agricultural and Veterinarian Sciences, Sao Paulo State University, Jaboticabal, SP, Brazil

Running title: Behavioural and autonomic temperature control in the chicken hatchling

* Corresponding author. Address: Dept. of Physiology, McGill Univ., 3655 Promenade Sir William Osler, room 1121, Montreal, Quebec, H3G 1Y6 Canada - Fax: +1 514 3987452 - *E-mail address*: jacopo.mortola@mcgill.ca

Abstract

In a thermal gradient the preferred ambient temperature (Ta*pref*) of chicken hatchlings is a few degrees lower than thermoneutrality. To investigate whether or not a correlation may exist between Ta*pref* and the autonomic thermogenic capacity we studied a group of hatchlings (N=15) exposed to cold at end-incubation, a procedure known to increase their postnatal thermogenesis. Chicken embryos were exposed to cold (34.5 instead of 38°C) at days 18-20 of incubation. They hatched a few hrs after Controls (N=15), with similar body weight, body temperature (Tb), vocalization (measured as number of sounds produced per unit time) and oxygen consumption ($\dot{V}_{O_2}$, measured in a respirometer by an open-flow methodology). Upon exposure to cold, these hatchlings had a brisker thermogenic response (higher Lower Critical Temperature [LCT] of thermoneutrality and higher $\dot{V}_{O_2}$) and slightly higher vocalization than Controls. In a thermal gradient, Ta*pref* averaged 34.3±0.3°C, or 1°C higher than in Controls (33.4°C±0.3; $P<0.05$), in proportion with their higher LCT (38±0.1°C instead of 36.7°C ±0.3; $P<0.001$), so that the Ta*pref*-LCT difference (-3.6±0.3°C) was similar to Controls (-3.3±0.3°C). From various considerations it was estimated that, at Ta*pref*, $\dot{V}_{O_2}$ was ~20% higher than at thermoneutrality in both sets of hatchlings. Such metabolic increase could carry some physiological advantage and it is possible that the hatchling's choice of Ta*pref* lower than LCT reflects the needs to maintain $\dot{V}_{O_2}$ slightly elevated.

Key words: Bird.Newborn. Behaviour. Thermoneutrality. Thermoregulation

1. Introduction

Endotherms (birds and mammals) control body temperature (Tb) through the concerted action of autonomic and behavioral mechanisms. In the cold, autonomic regulation consists in a reduction of heat loss and an increase in heat production (thermogenesis). Behavioral means aim to decrease the temperature gradient between the body and the environment, for example by huddling or through heat-conserving postures, or by searching warmer locations. Behavioral reduction in heat loss is extensively adopted by birds and mammals exposed to cold (Bicego et al., 2007), probably because it can be less expensive than autonomic heat production and lowers the needs for autonomic activation. At the thermal comfort-zone, which is the ambient temperature preferred by an animal free to move within a thermal gradient (Ta*pref*) (Gordon, 2012), one may expect thermogenesis to be absent and oxygen consumption ($\dot{V}_{O_2}$) to be minimal; in other words, Ta*pref* may correspond to thermoneutrality[3]. However, when tested experimentally, the coincidence between Ta*pref* and thermoneutrality not always has been confirmed, Ta*pref* being lower than the lower critical temperature (LCT[4]) of thermoneutrality (Mount, 1979; Gordon, 1985; Gordon, 1987). Whenever Ta*pref* is lower than LCT energy production exceeds its minimum, meaning that the behavioral control of Tb has not eliminated the needs for some thermogenesis.

The question of whether or not Ta*pref* coincides with thermoneutrality is interesting biologically and clinically important especially in the neonatal period. In the case of

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[3] '*Thermoneutrality*' is defined as range of ambient temperature at which temperature regulation is achieved only by control of sensible heat loss, i.e., without regulatory changes in metabolic heat production or evaporative heat loss (IUPS Thermal Commission, 2001).

[4] 'Lower critical temperature' is the ambient temperature below which the rate of metabolic heat production of a resting thermoregulating tachymetabolic animal must be increased by shivering and/or nonshivering thermogenesis in order to maintain thermal balance (IUPS Thermal Commission, 2001).

premature human infants the temperature of the incubator is set to coincide with the neonate's thermoneutrality, on the assumption that the temperature of minimal $\dot{V}_{O_2}$ corresponds to the infant's comfort temperature (Friedman and Baumgart, 2005). Yet, proof that this is so is lacking and can be difficult to approach experimentally. In newborns of the most common laboratory species Ta*pref* cannot be evaluated because of their minimal locomotory capacity and frequent needs for suckling or feeding. Equally, LCT is difficult to measure in animals with limited heat loss mechanisms and a narrow range of thermoneutrality.

Differently from the human infant and most neonatal animal models, the hatchlings of a precocial avian species like the chicken at birth have excellent motility, clear thermogenesis and no needs of maternal care; hence, they provide a rare opportunity to study the relationship between Ta*pref* and thermoneutrality in a neonate of an endothermic species. Recently, in a group of 1-day old chicken hatchlings we noted that Ta*pref* was about 3°C below LCT (Toro-Velasquez et al., 2013). The possibility of Ta*pref* being lower than LCT may depend on the thermogenic capacity, and hatchlings with brisk response to cold could defend body temperature (Tb) with a Ta*pref* substantially lower than LCT (Fig. 1; case 1). However, if the energetic cost was a constraint in the choice of Ta*pref*, hatchlings with high thermogenic responses may opt for higher Ta*pref* in order to limit the increase in $\dot{V}_{O_2}$ (Fig. 1; case 2). In the present work we studied a group of chicken hatchlings exposed to cold during the late phase of embryogenesis. In fact, cold exposure during the last days of incubation, which is the time when thermogenic competence begins to develop, results in some increase in the thermogenic capacity of the newborn (Minne and Decuypere, 1984; Tzschentke and Nichelmann, 1999; Tzschentke et al. 2001; Nichelmann, 2004; Mortola, 2006). The first goal was to measure the Ta*pref* of hatchlings born with a thermogenesis higher than normal and to

explore the possibility of a correlation, if any, between Ta*pref* and thermogenesis.

Vocalization could be used by neonatal animals to attract parental attention whenever the synergism between behavioral and autonomic responses fails to protect Tb. In such a case, hatchlings with high Ta*pref* or increased thermogenesis in the cold may make less use of vocalization. However, the behavioral origin of sound production calls has been questioned (Blumberg and Alberts, 1990). In addition, no correlation was found between vocalization and the degree of autonomic thermogenesis when this latter was depressed by acute hypoxia (Al Awam et al., 2011). Hence, a second goal of the current study was to examine the possibility of a correlation between the hatchling's vocalization, their Ta*pref* and thermogenesis in hatchlings born with thermogenesis higher than normal.

2. Methods

Experiments were conducted on chicken hatchlings (*Gallus gallus,* White Leghorn variety), about 15-18 hrs post-hatching. Freshly laid fertilized eggs were obtained from a local supplier. After noting the weight, the eggs were placed in incubators set at the temperature of 37.5°C and 60% relative humidity, with a 45^{o} egg rotation at least four times a day. The start of incubation was denoted embryonic day 0 (E0). Incubation temperature and relative humidity were monitored every 10 min by a data logger placed inside the incubator. At embryonic day 18 half of the eggs were placed in an incubator set at 34.5°C ("Embryonic Cold", from here on labeled EC, N=15), while the remaining ones ("Controls", N=15) continued in control conditions. At end incubation (E20, internal pipping phase) all eggs were transferred to a hatchery-incubator with no rotation; hatching day and time were noted.

The main measurements consisted of determining the preferred ambient temperature

(Ta*pref*) within a thermocline and in measuring oxygen consumption ($\dot{V}_{O_2}$) and vocalization during a slow cooling protocol.

2.1. Thermocline

The thermocline was the same as previously described (Toro-Velasquez and Mortola, 2013). It consisted of a 102 x 10 x 20 cm (length, width, height), 14 mm thick, Styrofoam column, covered by an acrylic panel. Numerical markings on the floor and sides indicated the horizontal distance (D) from one end. The thermocline was warmed by external heat sources spaced so as to generate an end-to-end ambient temperature (Ta) difference of about 16°C (from 44.8 to 28.8°C), with an average gradient of 0.16°C/cm. Consistency of the thermal gradient was checked before each experiment. A digital camera on top of the thermocline was programmed to take a picture every minute. The tests were performed in an isolated T-controlled room under dim light, with no people in it. First, the hatchling was placed at the center of the thermocline and left undisturbed for at least one hr; then, data collection started and continued for 60 min. From each frame, the min-by-min thermocline Ta at the location of the hatchling was computed from D and the D-Ta function (Toro-Velasquez and Mortola, 2013).

2.2. Measurements of $\dot{V}_{O_2}$

Oxygen consumption ($\dot{V}_{O_2}$) was measured by an open-flow methodology (Szdzuy et al., 2008). The hatchling was placed in a 200-ml plastic container maintained at the desired temperature (37.5 ^{o} C) by a water bath. A steady gas flow of 150 ml/min was continuously delivered through the respirometer, passed through a drying column, and the outflow O_2 and CO_2 concentrations were recorded continuously by calibrated gas analyzers (Sable Systems International Fox, Henderson, NV). The inflow concentrations were monitored intermittently.

After mathematical correction of the gas concentrations for a respiratory quotient different from unity (Depocas and Hart, 1957;Mortola and Besterman, 2007), $\dot{V}_{O_2}$ was computed from the flow rate and the inflow-outflow gas concentration difference, averaged over each 10 min time interval. The ambient temperature (Ta) of the respirometer was collected by telemetry via a transmitter placed at the height of the hatchling.

2.3. Vocalization

A microphone was fit through the upper lid of the respirometer, just above the hatchling, to monitor the hatchling's vocalization. Mono-channel sound acquisition was obtained by use of a commercially available software (WavePad Sound Editor®, NCH Software Inc, Greenwood Village, CO), at 44100 Hz.

Play back of the audio file was performed at low speed with synchronous visual display of the sound waves to facilitate the separation from occasional noise. Each individual sound was counted and the results grouped for each 10-min bin, by analogy with the analysis of $\dot{V}_{O_2}$. It is worth noting that the number of sounds counted in this manner can greatly exceed what could be recognized by the human ear during play back at the natural speed.

2.4. Protocols and Data Analysis

The cooling protocol was identical to that recently performed. After a period of habituation, during which the hatchling was left undisturbed in the respirometer for at least half an hour at 37.5^{o} C, recording started for the duration of 3 hrs; the first 30 min were in warm conditions (37.5°C) and the remaining 2.5 hrs during gradual cooling (Toro-Velasquez and Mortola, 2013). The decrease in Ta was approximately linear at 0.06°C/min (see RESULTS). Data of Ta and $\dot{V}_{O_2}$ were computed every 10 min. Cloacal temperature, taken as representative of body temperature (Tb), was measured at the beginning and the end of the

experiment with a fine tungsten-constantan thermocouple connected to a digital thermometer (Omega 871A, Omega Engineering Inc., Stanford, CT).

Most of the hatchlings (20 of 30) had the thermocline test performed in the late morning followed by the Ta-$\dot{V}_{O_2}$ relationship, because we were concerned that the alternate approach may have had some carry-over effect of the cooling phase. However, in 10 animals (5 Controls and 5 EC) the protocol was reversed, with ~2 hrs between tests; the results were undistinguishable from those of the majority of the group. To calculate LCT, we computed the 2nd-order polynomial function of all the Ta-$\dot{V}_{O_2}$ data points (X and Y, respectively) during cooling with $\dot{V}_{O_2}$ values that exceeded the warm values by at least 5%; from the function, LCT corresponded to the X-value where Y equaled the average $\dot{V}_{O_2}$ in warm condition. The function included the Ta-$\dot{V}_{O_2}$ pair with Ta=Tb and $\dot{V}_{O_2}$=0, based on the consideration that, for Tb to remain at the thermoneutral value when $\dot{V}_{O_2}$=0, Ta must equal Tb (Gordon, 1990).

Group data are reported as means ±1SEM. Comparisons of the time trajectory of $\dot{V}_{O_2}$, Ta-$\dot{V}_{O_2}$ and Ta-sound curves between EC and Controls were done by paired analysis of the Y-values at any given X. The statistical significance of the comparisons between two sets of data was evaluated by two-tailed *t* test, paired or unpaired as appropriate, rejecting the null hypothesis at a probability value $P<0.05$.

3. Results

The characteristics of the hatchlings and resting values during warm conditions are given in Table 1; none differed significantly between the two groups except for the hatching time,

which on average in EC happened ~10 hrs after Controls.

3.1. Thermogenic response to cooling

Ambient temperature (Ta) was controlled by the water bath and programmed to drop slowly, by about 10°C in 2.5 hours, in order to facilitate the detection of LCT. However, during the first 30 min of the cooling phase, the time-course of Ta within the respirometer was not identical between the two groups (Fig.2, top panel). This was due to the fact that the thermogenic response of the EC group was brisker than that of Controls, which temporarily offset the cooling process in the respirometer. In fact, with the onset of cooling, the first rise in $\dot{V}_{O_2}$ occurred earlier ($P<0.001$; top panel) in EC than in Controls (Fig. 2, bottom). The overall thermogenic response (Ta- $\dot{V}_{O_2}$ curve) during the 2.5 hrs was significantly higher in EC, although this was due to the early portion of the response; later, the $\dot{V}_{O_2}$ values of EC fell within the 95% CI of the Control curve (Fig. 3, top). LCT, computed as indicated in METHODS (arrow), was significantly higher in EC (38.0°C ±0.1) than in Controls (36.7°C ±0.3; $P<0.001$), confirming the brisker response to cold in the EC hatchlings.

By the end of the cold-exposure, Tb had decreased similarly in both groups, averaging 35.5 (±0.5°C) and 35.1 (±0.4°C) in Controls and EC, respectively ($P>0.05$).

3.2. Preferred ambient temperature

During the 60 min of the measurements in the thermocline, which occurred one hour after the habituation period, both groups of hatchlings moved greatly and by similar amounts; the total distance traveled was 285 cm (±47) and 280 cm (±47) in Controls and EC, respectively, which in either group corresponded to an average speed of 4.7 cm/min (±0.8).

The most frequent Ta, assessed from the peak of the histogram distribution of all data pooled together (60 data points for each of the 15 hatchlings), was 32.5°C in Controls and

34.5°C in EC (Fig. 3, middle); the corresponding data averages were 33.4°C±0.3 (Controls) and 34.3°C±0.3 (EC; P<0.05). The one degree difference in Ta*pref* between the two groups approximately corresponded to the difference in LCT reported above (*3.1.*); hence, Ta*pref* was lower than LCT by similar amounts in both groups, -3.3°C±0.3 in Controls and -3.6°C±0.3 in EC (P>0.05) (Fig. 4).

In 6 Control hatchlings Tb was measured before and at termination of the thermocline test; Tb dropped slightly in five of them and remained unchanged in one; the group averages before and after the test were, respectively, 40.8°C±0.2 to 40.4°C±0.1 (paired *t* test: P<0.01).

3.3. Vocalization in response to cooling

In warm conditions, the number of sounds did not differ between the two groups (Table 1), and was similar to what measured previously in same-age hatchlings using the identical anlytical protocol (Al Awam et al., 2001). With cooling, sounds number increased sharply in both sets of animals and reached the peak before $\dot{V}_{O_2}$ did, as noted previoulsy (Al Awam et al., 2011). At all values of Ta the number of sounds in EC was higher than in Controls (Fig. 3, bottom), so that the overall response during the 2.5 hrs was significantly (<0.001) more pronounced.

4. Discussion

The hatchlings born after a brief period of cold-exposure toward end incubation had a brisker response to the cooling protcol and higher Ta*pref* than Controls; like in Controls, their Ta*pref* was some 3°C lower than LCT.

4.1. The experimental (EC) group

Sustained cold exposure during embryogenesis typically blunts body growth, lowers embryonic metabolic rate, delays hatching and results in newborns with minimal thermogenic competence (Mortola, 2009, for references). However, when the cold exposure occurs toward the end of incubation, that is, at a time when the thermogenic competence begins to develop, the effects can be quite different. Indeed, the results of numerous experiments (Minne and Decuypere, 1984; Tzschentke and Nichelmann, 1999; Tzschentke et al. 2001; Nichelmann, 2004; Mortola, 2006) indicated that the hatchlings can present a thermogenic response higher than normal, similarly to what occurs in adult endotherms, in which sustained cold exposure enhances the thermogenic response to a new cold episode. The current results in EC confirmed this pattern; the cold exposure at end-incubation was sufficiently mild and brief to have no impact on hatching time and the newborn's body weight and $\dot{V}_{O_2}$, while it increased the hatchling's thermogenic competence, as shown by the higher LCT and the upward displacement of the Ta-$\dot{V}_{O_2}$ curve.

4.2. The preferred ambient temperature

At the onset of the study we asked what may be the consequences of higher thermogenesis on the hatchling's thermal preferences, thinking that a greater thermogenic capacity may permit a lower Ta*pref* (Fig. 1, case 1). This turned out not to be the case, because Ta*pref* of EC was about 1°C higher than in Controls; this increase was similar to that of LCT, so that the Ta*pref*-LCT interval was the same in the two groups (Fig. 4). Hence, the previous observation that the 1-day old chicken hatchlings prefer a Ta lower than theremoneutrality (Toro-Velasquez et al., 2013) has been confirmed in a group with higher thermogenic capacity. The farming industry of egg-laying and broilers, based on empirical large-scale observations of growth success, recommends to maintain the brood between 30 and 35°C

(Hy-Line, 2010; Bovans Brown, 2012; Cobb, 2012); these Ta values correspond to the range of Ta*pref* here measured and are lower than LCT.

In endotherms, at Ta lower than LCT metabolic rate exceeds the thermoneutral minimum. It is possible that this increase in energy expenditure may pose a limit on how low Ta*pref* can be relative to LCT. Hence, one interpretation of the fact that EC opted for a Ta*pref* higher than Controls (option 2 in Fig. 1) could be their attempt to limit the rise in $\dot{V}_{O_2}$. In such a case it would be of interest to know what the real increase in $\dot{V}_{O_2}$ at Ta*pref*<LCT is and what the benefits of the higher energetic cost could be.

4.3. What is the energetic cost of Ta*pref<LCT*?

The most correct answer would require simultaneous measurements of $\dot{V}_{O_2}$ and Ta*pref*, which is unpractical and has never been done in any species. From the Ta- $\dot{V}_{O_2}$ curve constructed in the respirometer (Fig. 3, top), at Ta=Ta*pref* of ~34°C $\dot{V}_{O_2}$ is ~75% higher than the thermoneutral value. However, the Ta- $\dot{V}_{O_2}$ curve was constructed with a continuous fall in Ta, which is different from the oscillations in Ta experienced by the hatchling in the free-ranging conditions of the thermocline. In this respect it is interesting to note that during the cooling protocol from 38°C to 29°C, the hatchlings' Tb dropped about 4.5°C. If we assume for simplicity that Tb decreased linearly with Ta, at the Ta*pref* of 34°C Tb should have dropped by ~2°C. This strikingly contrasts the Tb drop of only 0.2°C after two hours in the thermocline. To get some insights into the possible mechanism behind responsible for this discrepancy we measured Tb under two experimental protocols (APPENDIX), with continuous cooling (as done for the construction of the Ta- $\dot{V}_{O_2}$ curve) and when the same level of cooling was attained by oscillations above and below the mean (as it was the case in the thermocline,

where hatchlings moved below and above Ta*pref*). The results (Fig. A1) showed that alternating cooling and warming around a mean Ta had lesser consequences on Tb than maintaining a steady Ta. The most likely reason for it is the difference in the effective time constant between the hatchling's heat dissipation and heat production, where 'effective' refers to the combination of physical and regulatory mechanisms. Newborns have some thermogenic capacity, which allows some protection against cold, but very limited control of heat loss, which precludes Tb protection against heat (Mortola, 2001). The implication is that the effective time for Tb to drop in cold is longer than the effective time for Tb to rise in the heat. The oscillation between cooler and warmer regions around Ta*pref*, therefore, permits to maintain Tb values higher than with a constant exposure to Ta=Ta*pref*. In conclusion, it seems likely that in the respirometer, during the construction of the Ta- $\dot{V}_{O_2}$ relationship, the cold stimulus was greater than that sustained over time in the thermocline for similar *average* Ta, with the effect of a greater thermogenic response. The measured 0.2°C drop in Tb after two hours in the thermocline corresponds to a minimal (~0.5°C) drop in Ta, and this (Fig. 3, top) entails an increase in $\dot{V}_{O_2}$ of less than 20%.

At present, the physiological benefits in maintaining $\dot{V}_{O_2}$ slightly above its minimum are only a matter of speculation. Because of the importance of appropriate stimuli in the structural and functional development of a sensory system, it was proposed (Toro-Velasquez et al., 2013) that the Ta*pref*-related increase in $\dot{V}_{O_2}$ may be the stimulus necessary for the appropriate development of thermoregulation. Additionally, a metabolic rate slightly elevated could be advantageous to the operation of the oxygen convection systems. For example, it is known that newborns subjected to a modest cold stimulus have fewer breathing irregularities

than when their metabolic rate is kept at its minimum in the warm (Cameron et al., 2000). Finally, if Ta*pref* coincided with thermoneutrality, $\dot{V}_{O_2}$ would be at its minimum which, given the newborns' poor control of heat loss, would immediately cause hyperthermia for any elevation of Ta. Differently, when Ta*pref*<LCT, a slightly elevated $\dot{V}_{O_2}$ leaves open the possibility of lowering heat production against heat exposure. In conclusion, it is conceivable that thermoneutrality, even if it represents the minimum energetic cost, may not necessarily be optimal from the viewpoint of the integration of various regulatory systems.

4.4. Vocalization

In all hatchlings the number of sounds increased rapidly with the onset of the cold stimulus and anticipated the metabolic response. The response of EC was brisker and greater than in Controls, and this was confirmed statistically, although the inter-animal variability was very large and the EC curve was close to the Control upper 95% CI. It has been argued (Blumberg & Alberts, 1990), based on experiments with recordings of the ultrasound calls of neonatal rats, that vocalization is not a true behavioral response designed to attract the parents' attention; rather, the ultrasound calls would be simply acoustic phenomena associated with laryngeal expiratory braking through narrowing of the vocal folds. According to this view, the higher the metabolic demands and pulmonary ventilation, the higher the vocalization would be (Blumberg & Alberts, 1990, 1991). In the hatchlings, in which expiratory laryngeal braking has never been described, the vocalization response anticipated the metabolic response to cold, which should exclude the possibility of the latter being the causative event. The average energetic cost of each sound is very low (2.2 μl O_2, Al Awam et al., 2011); hence, even at the peak of the EC vocalization response (556 sounds/10 min, Fig. 3, bottom) the cost involved in sound production could not have raised $\dot{V}_{O_2}$ by more than

16%, which is much less than the increase actually measured (62%). Previously, among hatchling with different thermogenic capacities and in hatchlings with reduced thermogenesis because of hypoxia, the vocalization response to cold was found to be unrelated to thermogenesis (Al Awam et al., 2011). Finally, we could not detect any correlation between the sounds number and $\dot{V}_{O_2}$, whether at rest or during cooling, nor between sounds number and Ta*pref* or Tb. In conclusion, the hatchling's vocalization appears to be a stereotyped phenomenon not quantitatively coordinated with the behavioral and autonomic thermoregulatory control of Tb.

4.5. Conclusions

Following a 2-day period of cold-exposure at end incubation hatchlings were born a few hrs after Controls, with similar body weight, Tb and $\dot{V}_{O_2}$, and presented a brisker thermogenic response to cold, with higher LCT and $\dot{V}_{O_2}$. In a thermal gradient, Ta*pref* was higher than in Controls and in proportion with LCT, so that the Ta*pref*-LCT difference remained the same, about -3°C. Some considerations indicated that $\dot{V}_{O_2}$ at Ta*pref* may be ~20% higher than at thermoneutrality. This increase could carry some physiological advantage and it is possible that the hatchling's Ta*pref* reflects the needs of maintaining $\dot{V}_{O_2}$ slightly elevated.

5. Appendix

These additional measurements were conducted for the purpose of assessing the effect of ambient temperature (Ta) on body temperature (Tb) during two protocols of moderate cooling (see Discussion, *4.3.*). By the first protocol, Ta was lowered to a target value and maintained constant. By the second protocol, Ta oscillated above and below the target Ta. The hatchling

was placed in a respirometer that had ample windows for rapid equilibration of its internal temperature. The respirometer was transferred from a warm container to a cold container every 2 min for a total of 26 min, so as to generate cyclic oscillations in Ta of the order of ~15^{o}C (between 38° and 23°C), around the average 30.6°C. After a period of rest in the hatchery, the second test consisted of maintaining the hatchling at a Ta approximately equal to the average Ta of the previous test. These measurements were performed on four 1-day old hatchlings. In all cases, when Ta oscillated, Tb hardly changed, while with Ta maintained constant around the average value Tb invariably decreased (Fig. A1).

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Table 1. Resting values during warm conditions

	Controls (N=15)	Cold (N=15)	P
Fresh egg wt, g	57.8±0.7	59.3±0.7	ns
Incubation duration, days	20.3±0.1	20.7±0.1	<0.05
Postnatal age, hrs	18±1	15±2	ns
Body weight, g	40.2±0.9	41.7±0.7	ns
Body temperature, °C	39.5±0.2	39.9±0.1	ns
Oxygen consumption, ml/min	0.786±0.039	0.746±0.022	ns
ml/kg/min	19.8±1.2	18±0.7	ns
Sounds, N/min	17±4	15±5	ns

Values are group mean values ± 1 SEM. P, level of statistical difference between the two groups. ns, not significant (P>0.05).

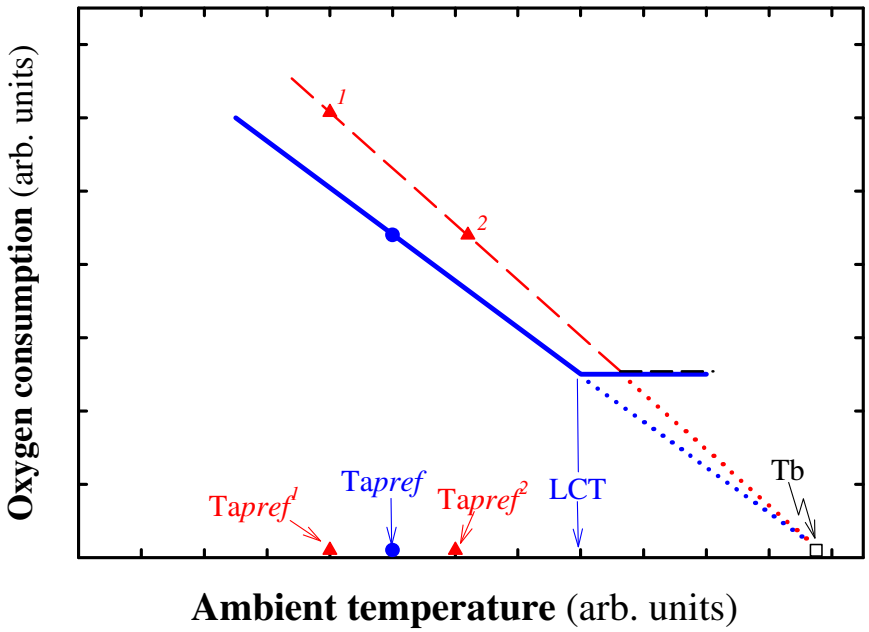

Figure 1. Schematic representation of the ambient temperature (Ta)-oxygen consumption ($\dot{V}_{O_2}$) relationship for ambient temperatures slightly above or below the lower critical temperature (LCT) of thermoneutrality (thick blue line). At Ta below thermoneutrality (Ta*pref*, in blue) $\dot{V}_{O_2}$ exceeds the minimal value (blue circle) in an effort to maintain body temperature (Tb) constant. With greater thermogenesis (dashed red line), the higher $\dot{V}_{O_2}$ could permit a lower Ta*pref* (case 1); another possibility is that, with greater thermogenesis, Ta*pref* increases to limit the rise in $\dot{V}_{O_2}$ (case 2).

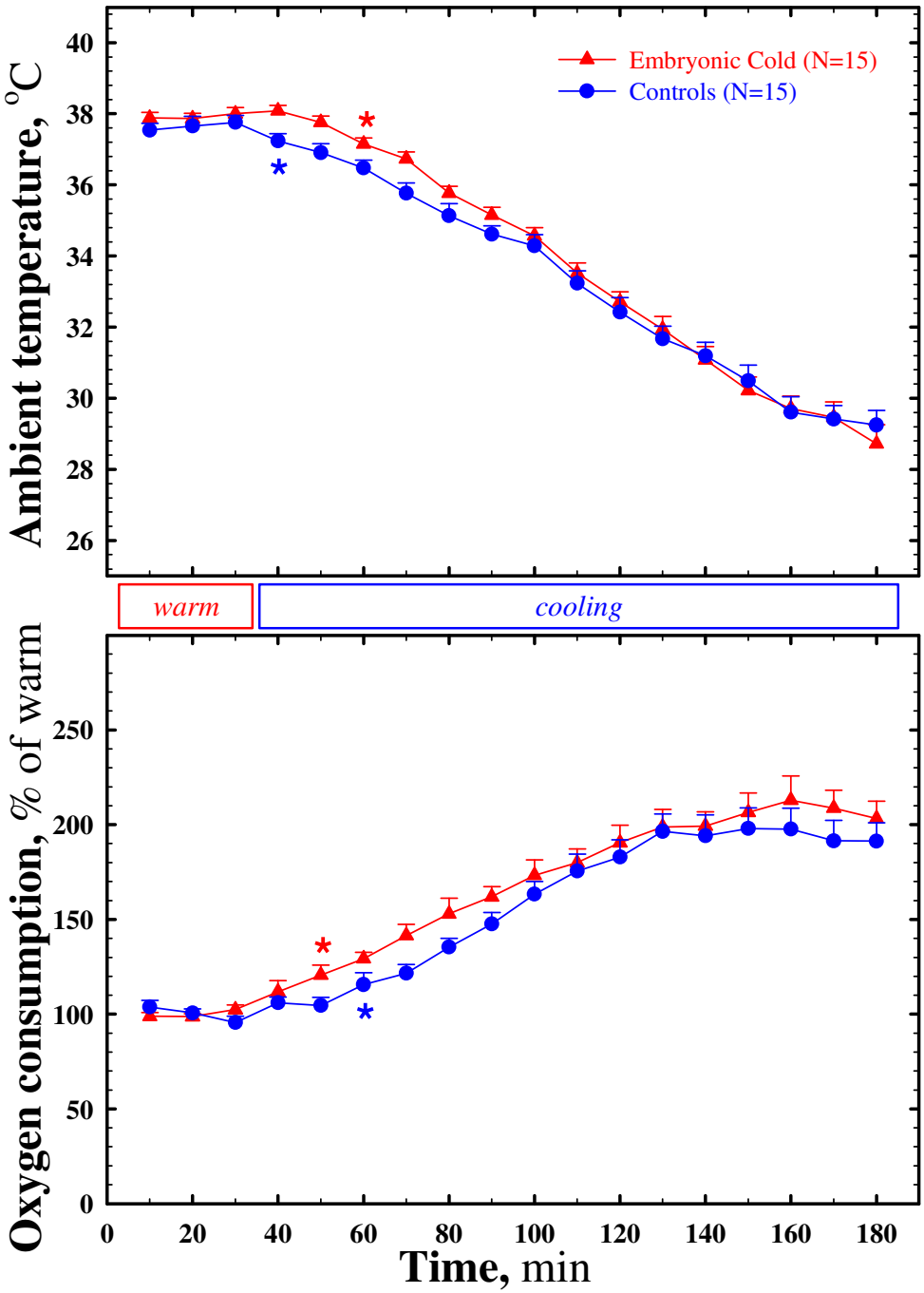

Figure 2. Time course of ambient temperature (top) and oxygen consumption (bottom) in 1-day old hatchlings exposed to cold during the late portion of embryogenesis (red triangles) and controls (blue circles). Symbols are group means ±1 SEM. Asterisks indicate the first data points during cooling significantly different from the warm.

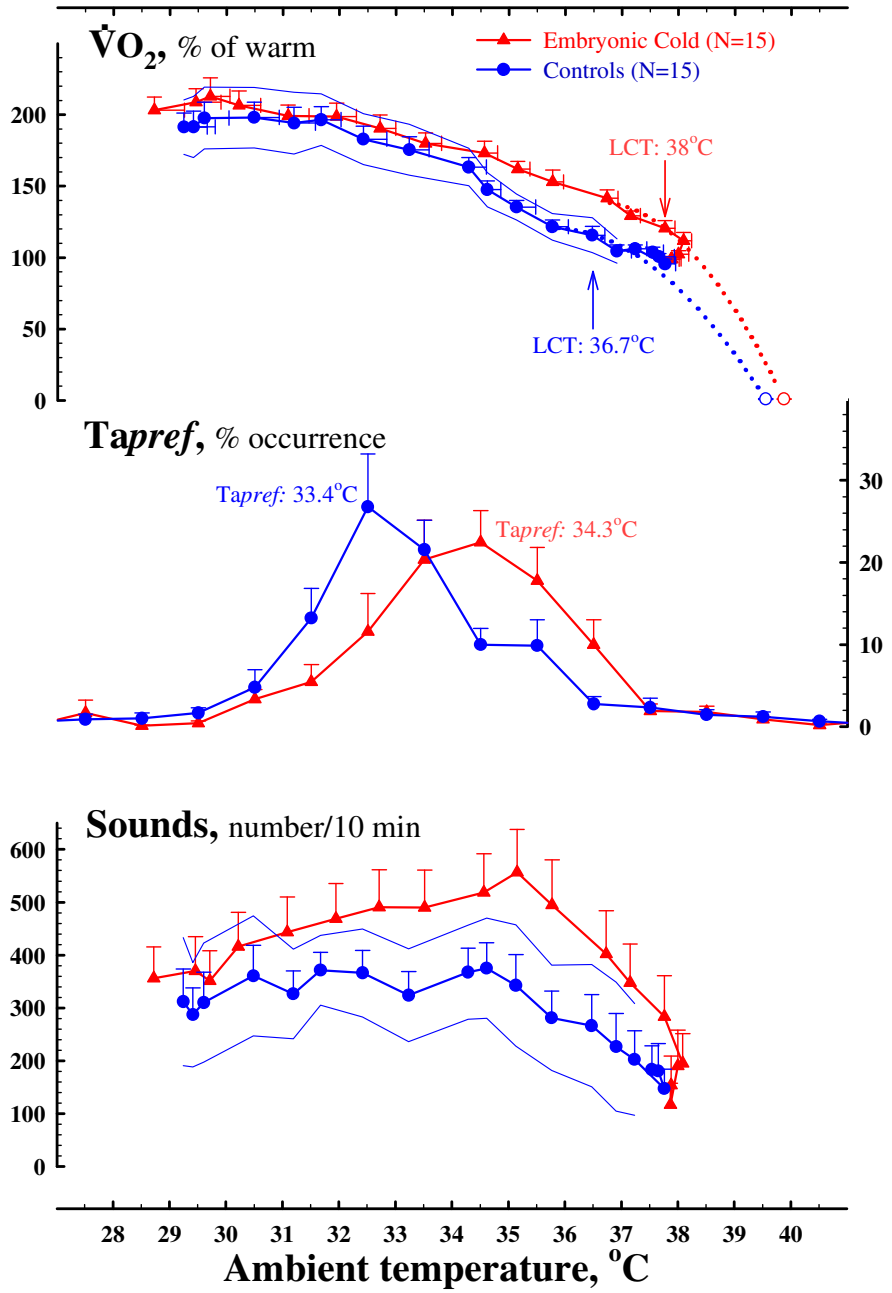

Figure 3. Plots of oxygen consumption ($\dot{V}_{O_2}$, top), preferred ambient temperature (Ta*pref*, middle) and vocalization (sounds number, bottom) as function of ambient temperature in 1-day old hatchlings exposed to cold during the late portion of embryogenesis ("Embryonic Cold", red triangles) and Controls (blue circles). Symbols are group means ±1 SEM. The thin blue lines indicate the 95% Confidence Intervals of the Controls. Arrows indicate the Lower Critical Temperatures (LCT) of the two groups. In the middle panel, values refer to the average Ta*pref* of the two groups.

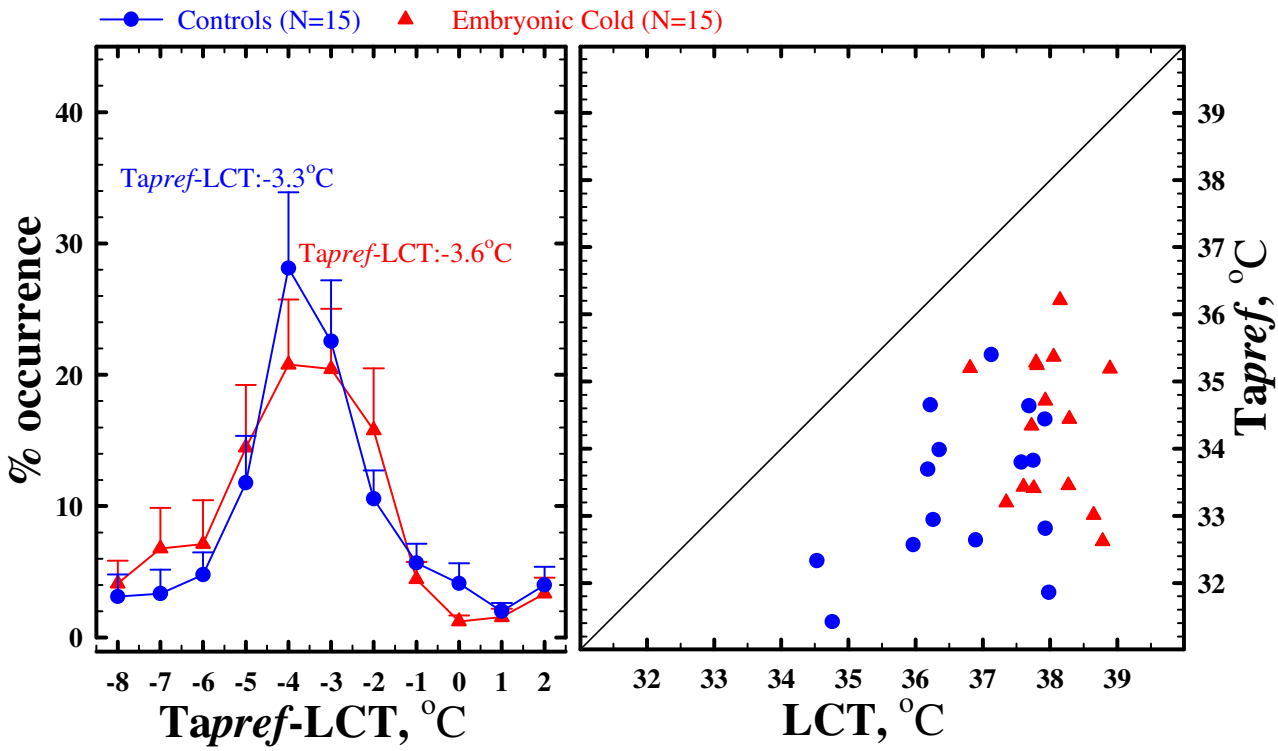

Figure 4. **Left**: Percent distribution of the differences between preferred temperature (Ta*pref*) and Lower Critical Temperature (LCT) for all hatchlings of the Control group (blue circles) and for the hatchlings exposed to cold during the last days of incubation ("Embryonic Cold", red triangles). The two temperature values in the panel indicate the average Ta*pref*-LCT of the groups. There were no statistical differences. **Right**: identity plot of the average values of LCT and Ta*pref* for each of the hatchlings of the two groups. In all of them Ta*pref* was lower than LCT.

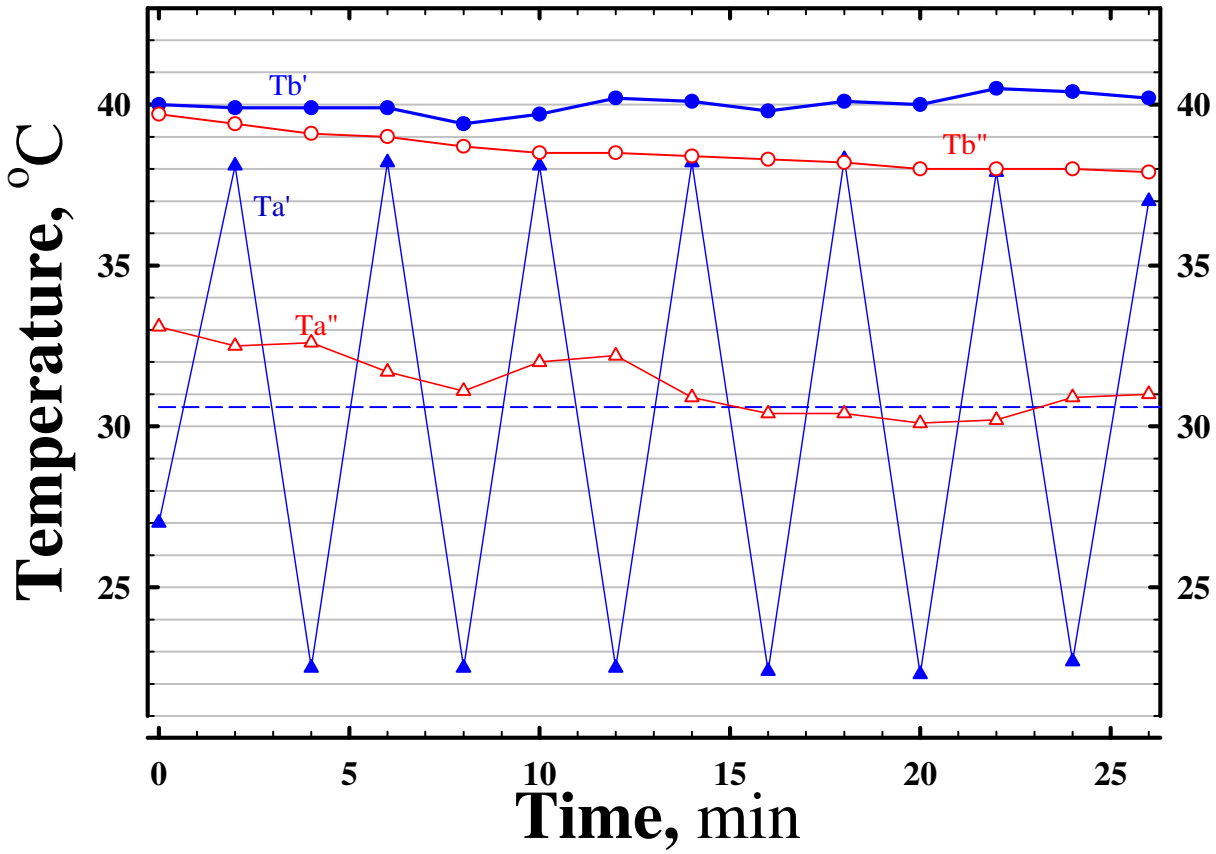

Figure A1 (Appendix). Experimental recordings of ambient temperature (Ta) and body temperature (Tb) in a 1-day old hatchling during two cooling protocols. In the first protocol, a was oscillating between 38 and 23°C (Ta', filled blue triangles) around a mean of 30.6°C (dashed line). In the second protocol, Ta was approximately steady around 30.6°C (Ta", open red triangles). With the first protocol Tb remained constant at about 40°C (Tb', filled blue circles), while with the second protocol it dropped by 2°C (Tb", open red circles).