

**UNIVERSIDADE ESTADUAL PAULISTA “JÚLIO DE MESQUITA
FILHO” FACULDADE DE CIÊNCIAS AGRÁRIAS E
VETERINÁRIAS CAMPUS JABOTICABAL**

**Efeitos do tratamento térmico durante o desenvolvimento
embrionário sobre a morfologia, a fisiologia respiratória e as
respostas ao estresse por calor de frangos de corte na fase inicial
de vida**

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**UNIVERSIDADE ESTADUAL PAULISTA - UNESP CÂMPUS DE
JABOTICABAL**

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DADOS CURRICULARES DO AUTOR

Isabela Maraschin Vieira, nascida em 12 de Abril de 2002, na cidade de Santa Maria, Rio Grande do Sul, é graduada em Zootecnia pela Universidade Federal de Santa Maria (UFSM). Durante a graduação, bolsista de ensino, pesquisa e extensão, atuando como integrante do Programa de Educação Tutorial (PET Zootecnia), monitora de Histologia do Departamento de Morfologia, tutora da Mostra de Ciências Morfológicas, membro do Diretório Acadêmico da Zootecnia Octávio Domingues e fundadora e presidente da Liga Acadêmica de Histologia (LAHISTO). Desde 2020 integra o Laboratório de Morfofisiologia Experimental Aplicado à Produção Animal (LABITEX/UFSM), onde desenvolve pesquisas nas áreas de morfologia, histologia, embriologia, fisiologia e produção animal. Ao longo de sua trajetória, participou de projetos envolvendo análises morfométricas, morfofisiológicas, hematológicas, seminais e embriológicas em diferentes espécies animais de produção e conservação, além de atuar em treinamento de equipes, gestão laboratorial, análise estatística e elaboração de projetos e publicações científicas. Realizou estágios extracurriculares em diferentes setores do Departamento de Zootecnia da UFSM, com destaque para as áreas de avicultura, incubação, fisiologia de peixes e nutrição de animais de companhia. Atuou profissionalmente na Associação Brasileira de Proteína Animal (ABPA), na área de marketing, e como auditora da Associação Gaúcha de Avicultura (ASGAV). Atualmente é mestranda, aprovada no doutorado, do Programa de Pós-Graduação em Ciência Animal da Faculdade de Ciências Agrárias e Veterinárias da Universidade Estadual Paulista “Júlio de Mesquita Filho” (FCAV/UNESP), Campus de Jaboticabal, sob orientação da Profª Drª Luciane Helena Gargaglioni Batalhão e coorientada do Prof. Dr. Marcos Macari. É bolsista da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). Desenvolve pesquisas na área de Biologia do Desenvolvimento e Fisiologia Animal, com ênfase nos efeitos da manipulação térmica durante a incubação sobre o desenvolvimento embrionário, a morfofisiologia pulmonar e cardiorrespiratória, o metabolismo energético e as respostas ao estresse por calor em frangos de corte. Paralelamente às atividades de pesquisa, atua como professora voluntária do Departamento de Morfologia da Universidade Federal de Santa Maria, ministrando aulas de Histologia e Embriologia para cursos das áreas das Ciências Agrárias e da Saúde. Em reconhecimento à sua trajetória acadêmica e científica, recebeu os prêmios de Aluna Destaque da Zootecnia do Rio Grande do Sul em 2024, Aluna Destaque Nacional de Zootecnia em 2025 e Zootecnista mais Influente do Brasil em 2025, concedidos pela Associação Brasileira de Zootecnistas.

Não há campo mais vasto que o pensamento
de quem sonha

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CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Avaliação da manipulação térmica embrionária no desenvolvimento do sistema cardiorrespiratório em nova linhagem de frangos de corte"**, protocolo nº 9487339468/2025, sob a responsabilidade da Profa. Dra. LUCIANE HELENA GARGAGLIONI BATALHÃO, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 22 de agosto de 2025.

(We certify that the research project entitled **"Evaluation of embryonic thermal manipulation on the development of the cardiorespiratory system in a new line of broiler chickens"**, protocol nº. 9487339468/2025, under the responsibility of Dr. LUCIANE HELENA GARGAGLIONI BATALHÃO, which involves the production, maintenance and/or use of animals belonging to the Chordata phylum, vertebrate subfile (except man), for the purposes of scientific research (or teaching) - is in accordance with the precepts of Law no. 11,794, of October 8, 2008, in Decree 6,899, of July 15, 2009, and with the rules issued by the National Council for Animal Experimentation Control (CONCEA), and was approved by the Ethics Commission on Animal Use (CEUA), from the SCHOOL OF AGRICULTURAL AND VETERINARY SCIENCES, UNESP - JABOTICABAL - SP Campus, August 22, 2025 an ordinary meeting.)

Vigência do Projeto	Após a aprovação da CEUA a 10/03/2027
Espécie / Linhagem	<i>Gallus gallus</i>
Nº de animais	200
Peso / Idade	Indiferente
Sexo	Machos e Fêmeas
Procedência	Incubatório Cobb – Vantres, Guapiaçu – SP.

Jaboticabal, 22 de agosto de 2025.



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EFEITOS DO TRATAMENTO TÉRMICO DURANTE O DESENVOLVIMENTO EMBRIONÁRIO SOBRE A MORFOLOGIA, A FISIOLOGIA RESPIRATÓRIA E AS RESPOSTAS AO ESTRESSE POR CALOR DE FRANGOS DE CORTE NA FASE INICIAL DE VIDA

Este estudo avaliou os efeitos da manipulação térmica embrionária sobre o desenvolvimento pulmonar e as respostas ao estresse térmico de frangos de corte Cobb® CDP4. Foram utilizados 200 ovos férteis, distribuídos em três tratamentos: controle (T1; 37,5–37,8 °C), manipulação térmica moderada (T2; 38,5 °C) e de maior intensidade (T3; 39,0 °C), aplicadas durante seis horas diárias entre os dias 16 e 19 de incubação. Avaliaram-se a massa corporal, a massa pulmonar relativa e a histomorfometria dos parabrônquios durante o desenvolvimento embrionário e aos 15 dias após a eclosão. As respostas ventilatórias, metabólicas e térmicas foram analisadas em aves com 1 e 15 dias de vida, em condições termoneutras, durante exposição aguda a 35 °C e na recuperação. Aos 15 dias, a função mitocondrial pulmonar foi avaliada por respirometria de alta resolução. A manipulação térmica modificou a arquitetura parabrônquica embrionária sem alterar a massa corporal ou pulmonar relativa. T2 apresentou maiores áreas total, parenquimatosa e luminal, enquanto T3 apresentou maior proporção de área de condução e menor área de troca gasosa. No primeiro dia de vida, os parâmetros ventilatórios e metabólicos basais foram semelhantes entre os tratamentos, embora T3 apresentasse menor temperatura corporal. Durante o calor, T1 e T3 reduziram a ventilação-minuto, o volume corrente e a frequência respiratória, enquanto esses parâmetros foram preservados em T2. Aos 15 dias, o remodelamento pulmonar persistiu, com maior proporção de área de troca gasosa nos grupos termicamente manipulados. T3 apresentou maior ventilação-minuto basal e, durante o calor, maior consumo de oxigênio que T1. A exposição térmica reduziu o volume corrente e aumentou a frequência respiratória em todos os grupos, mantendo a ventilação-minuto. Não houve diferenças na respiração mitocondrial pulmonar ou na produção de espécies reativas de oxigênio. Conclui-se que a manipulação térmica embrionária promove remodelamento pulmonar persistente e modifica as respostas ventilatórias, metabólicas e térmicas de maneira dependente da idade e da temperatura de incubação, sem comprometer a função mitocondrial pulmonar.

Keywords: manipulação térmica embrionária; desenvolvimento pulmonar; fisiologia respiratória; estresse térmico; bioenergética mitocondrial; frangos de corte.

EFFECTS OF THERMAL TREATMENT DURING EMBRYONIC DEVELOPMENT ON MORPHOLOGY, RESPIRATORY PHYSIOLOGY, AND HEAT STRESS RESPONSES IN BROILER CHICKENS DURING EARLY LIFE

This study evaluated the effects of embryonic thermal manipulation on pulmonary development and responses to heat stress in Cobb® CDP4 broiler chickens. Two hundred fertile eggs were assigned to three incubation treatments: control (T1; 37.5–37.8°C), moderate thermal manipulation (T2; 38.5°C), and higher-intensity thermal manipulation (T3; 39.0°C), applied for six hours daily from embryonic days 16 to 19. Body mass, relative lung weight, and parabronchial histomorphometry were evaluated during embryonic development and at 15 days post-hatch. Ventilatory, metabolic, and thermal responses were assessed in 1- and 15-day-old chicks under thermoneutral conditions, during acute exposure to 35°C, and throughout recovery. At 15 days, pulmonary mitochondrial function was evaluated using high-resolution respirometry. Thermal manipulation modified embryonic parabronchial architecture without affecting body mass or relative lung weight. T2 exhibited larger total, parenchymal, and luminal areas, whereas T3 showed a greater proportion of air-conduction area and a smaller gas-exchange area. In 1-day-old chicks, baseline ventilatory and metabolic parameters were similar among treatments, although T3 exhibited a lower body temperature. During heat exposure, T1 and T3 showed reductions in minute ventilation, tidal volume, and respiratory frequency, whereas these parameters were maintained in T2. At 15 days, pulmonary remodeling persisted, with a greater proportion of gas-exchange area in the thermally manipulated groups. T3 exhibited higher baseline minute ventilation and, during heat exposure, higher oxygen consumption than T1. Heat exposure reduced tidal volume and increased respiratory frequency in all groups, thereby maintaining minute ventilation. No differences were observed in pulmonary mitochondrial respiration or reactive oxygen species production. In conclusion, embryonic thermal manipulation induces persistent pulmonary remodeling and modifies ventilatory, metabolic, and thermal responses in an age- and incubation temperature-dependent manner without impairing pulmonary mitochondrial function.

Keywords: embryonic thermal manipulation; avian lung; pulmonary ventilation; heat stress; mitochondrial bioenergetics; broiler chickens.

CAPÍTULO 1- CONSIDERAÇÕES GERAIS E REVISÃO BIBLIOGRÁFICA

1. INTRODUÇÃO

Vivemos um cenário crítico em escala global, no qual o crescimento populacional impõe uma demanda crescente por segurança alimentar, ao mesmo tempo em que o planeta enfrenta os efeitos cada vez mais intensos do aquecimento global. Garantir segurança alimentar em meio a esse desequilíbrio climático tornou-se um desafio urgente, especialmente quando se trata da proteína animal mais consumida no mundo: a carne de frango (FAO 2023). O crescimento expressivo da produção e do consumo global de carne de frango nas últimas décadas está diretamente associado à intensificação de programas de melhoramento genético, estratégias nutricionais e ao avanço em biotecnologias aplicadas ao manejo reprodutivo (ALVES FILHO, 1999). Frangos de corte modernos, altamente selecionados para rápido crescimento e elevado rendimento de carcaça, alcançam o peso de abate em menos de seis semanas, o que exige um desenvolvimento morfofisiológico acelerado de órgãos essenciais à homeostase, como pulmões e coração (ALBERS, 1998; RONDÓN; MURAKAMI, 1998).

No entanto, este progresso produtivo vem acompanhado de desafios significativos. O rápido crescimento das aves nem sempre é acompanhado por uma adequada maturação celular do sistema cardiorrespiratório, tornando os frangos mais susceptíveis a distúrbios como estresse térmico, disfunções respiratórias, imunossupressão e até mesmo ascite (GONZALES; MACARI, 2000; JULIAN, 1987b; YAHAV, 2000a). Esses problemas se agravam frente às mudanças climáticas e ao aumento das ondas de calor, comprometendo a sustentabilidade da avicultura intensiva, setor crucial para a segurança alimentar mundial (DE VRIES; DE BOER, 2010). Com a chegada de uma nova linhagem Cobb®, uma das principais casas genéticas de frangos de corte do mundo, está em fase de desenvolvimento e lançamento de uma nova linhagem que ainda não possui nome comercial definido. De acordo com a empresa, trata-se de um produto inovador que se destaca por apresentar um equilíbrio mais evidente entre as características produtivas do frango de corte e o desempenho reprodutivo da matriz, especialmente em comparação com linhagens já consolidadas no

mercado, como a Cobb500®. Embora a ênfase da seleção genética da empresa continue voltada para os parâmetros zootécnicos do frango de corte, como conversão alimentar, ganho de peso e rendimento de carcaça. Essa nova linhagem representa um avanço importante no aprimoramento da prolificidade e viabilidade da fêmea reprodutora, trazendo um maior equilíbrio genético que é benéfico tanto para produtividade quanto prolificidade.

Segundo a Cobb, entre os diferenciais técnicos observados na matriz, destaca-se um aumento estimado de aproximadamente 10 ovos totais a mais por fêmea ao longo do ciclo produtivo, bem como uma melhora significativa nos índices de fertilidade e eclosão. Além disso, observa-se uma redução no percentual de ovos de cama, o que resulta em maior qualidade dos ovos incubáveis, e menor mortalidade durante o período de produção. Esses atributos indicam que esta linhagem foi estrategicamente desenvolvida com foco na estabilidade e eficiência reprodutiva, contribuindo para um melhor desempenho geral dos lotes.

No que se refere ao frango de corte oriundo dessa linhagem, todos os parâmetros produtivos esperados foram mantidos, incluindo alta eficiência alimentar, ganho de peso adequado e baixo índice de mortalidade. Além disso, destaca-se um ganho adicional de aproximadamente 2% no rendimento de peito, o que reforça o potencial dessa linhagem para atender às demandas da indústria de processamento. Assim, essa nova linhagem genética representa uma proposta inovadora da empresa, voltada à otimização de resultados em toda a cadeia produtiva, desde a reprodução até o desempenho final do frango de corte. Sua introdução no mercado deverá oferecer aos produtores uma alternativa com maior equilíbrio entre prolificidade, viabilidade da matriz e eficiência zootécnica, promovendo ganhos expressivos em produtividade e rentabilidade. A partir dessa novidade, surge uma oportunidade decisiva: preparar essas aves, desde a embriogênese, para o novo ambiente desafiador que irá enfrentar. Diante desse cenário, a manipulação térmica embrionária (MT) desponta como uma abordagem inovadora e de baixo custo para preparar fisiologicamente as aves para os desafios ambientais pós-eclosão (KHALIL *et al.*, 2024b; KHALIL; OTHERS, 2024; YAHAV, 2009). Essa técnica consiste na

exposição controlada dos ovos férteis a temperaturas superiores (ou inferiores) às utilizadas convencionalmente durante períodos críticos do desenvolvimento embrionário (PIESTUN *et al.*, 2008b; TAZAWA *et al.*, 1988; WEBB, 1987a). A MT promove alterações epigenéticas, neuroendócrinas e metabólicas duradouras, com potencial de melhorar a termorregulação, otimizar o metabolismo energético, aumentar a eficiência alimentar e reforçar a imunossupressão das aves (AL-ZGHOUL; EL-BAHR, 2019; COLLIN; PICARD; YAHAV, 2005a, 2005b; TZSCHENTKE; HALLE, 2009b).

Dentre os efeitos celulares mediados pela manipulação térmica durante a embriogênese, destaca-se a modulação da respiração mitocondrial, processo central na produção de ATP via fosforilação oxidativa. Essa modulação é especialmente relevante em situações de estresse térmico, onde há acúmulo de espécies reativas de oxigênio (ERO) e disfunção mitocondrial, como demonstrado por Huang e colaboradores (HUANG *et al.*, 2015), que observaram a supressão da atividade do complexo I e aumento de danos oxidativos em frangos de corte expostos ao calor. Nas mitocôndrias, a oxidação de substratos energéticos alimenta a cadeia transportadora de elétrons (CTE), estabelecendo um gradiente eletroquímico responsável pela síntese de ATP. Contudo, parte dos elétrons pode escapar da CTE, especialmente nos complexos I e III, levando à formação de espécies reativas de oxigênio (ERO), como o ânion superóxido e o peróxido de hidrogênio (MURPHY, 2009). Em condições homeostáticas, baixos níveis de ERO exercem papel sinalizador essencial para a diferenciação, proliferação celular e ativação de vias redox-sensíveis (JASTROCH *et al.*, 2010). No entanto, a exposição a desafios térmicos pode intensificar a produção de ERO, promovendo estresse oxidativo e comprometendo a integridade mitocondrial e celular, principalmente em tecidos altamente metabólicos como pulmões, coração e cérebro. A MT, ao impactar diretamente a bioenergética mitocondrial, pode induzir adaptações funcionais, como aumento da eficiência da CTE, a indução de proteínas desacopladoras e a modulação da eficiência respiratória. Esses mecanismos sugerem que parte da adaptação à temperatura pode envolver processos de termogênese mitocondrial e proteção contra sobrecarga redox, conforme

proposto por Power e Tardo-Dino (POWER *et al.*, 2014; TARDO-DINO *et al.*, 2019). Essas adaptações já foram observadas em diferentes modelos experimentais. Em frangos de corte, Li e colaboradores (LI *et al.*, 2024) demonstraram que a MT entre os dias 16 e 18 da incubação aumenta a expressão dos genes relacionados à cadeia transportadora de elétrons no músculo peitoral, reforçando a função mitocondrial e a termotolerância pós-natal. Esses efeitos estão associados à ativação de vias antioxidantes, como Nrf2/HO-1, ao aumento da biogênese mitocondrial via PGC-1 α e à maior tolerância ao estresse oxidativo (PIESTUN; DRUYAN; YAHAV, 2015; QIAO *et al.*, 2019). Além disso, alterações na densidade e morfologia mitocondrial durante a embriogênese podem repercutir na taxa metabólica basal e na eficiência da termorregulação nas fases pós-eclosão, conferindo às aves maior resiliência em ambientes hipermetabólicos ou hipoxêmicos. A hipótese de que essas alterações mitocondriais conferem maior resiliência térmica é sustentada por estudos em diferentes espécies, como o de Roussel e Voituron (ROUSSEL; VOITURON, 2020) que mostraram que alterações agudas de temperatura afetam negativamente a eficiência do ATP/O e aumentam a produção de H₂O₂ em mitocôndrias hepáticas de anfíbios. Assim, a capacidade de regular a produção de ERO e manter a homeostase redox mitocondrial emerge como um mecanismo-chave de adaptação fisiológica induzido pela manipulação térmica embrionária. Neste contexto, Bottje (BOTTJE, 2019) reforça que níveis moderados de ERO derivados da mitocôndria participam da sinalização intracelular, enquanto a produção excessiva, principalmente pelos complexos I e III, contribui para patologias como a síndrome ascítica em frangos. Esses efeitos não se restringem à fase pós-natal. Em bovinos, Payton e colaboradores (PAYTON *et al.*, 2018) demonstraram que o estresse térmico durante a maturação de oócitos altera processos mitocondriais que persistem nos embriões, indicando que os efeitos térmicos sobre a bioenergética mitocondrial são herdáveis e duradouros. O calor fisiológico também pode promover desacoplamento entre a oxidação e a fosforilação mitocondrial, reduzindo a eficiência do metabolismo de ácidos graxos em miofibrilas esqueléticas de ratos, um mecanismo possivelmente mediado por proteínas desacopladoras (TARDO-DINO *et al.*, 2019).

Os desafios/insultos ambientais que venham a ocorrer durante fases críticas de desenvolvimento pré- e pós-natal (alterações térmicas, gasosas, disponibilidade de água, agentes tóxicos, infecções/inflamação), podem remodelar dramaticamente o desenvolvimento ontogenético com consequências para aumento ou redução do risco de doenças na fase adulta (BURGGREN; CREWS, 2014) (Figura 1).

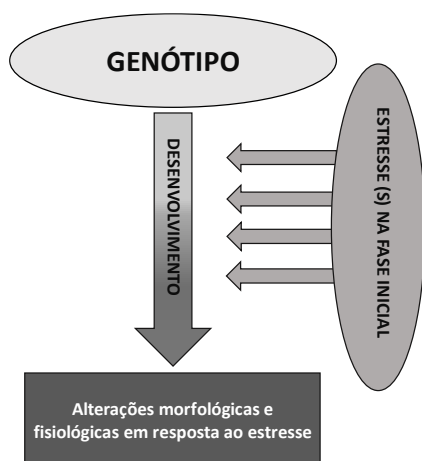


Figura 1: Esquema geral destacando que os eventos da vida, especialmente quando experimentados durante o estágio inicial de desenvolvimento, podem impactar fortemente o desenvolvimento, especialmente em indivíduos geneticamente suscetíveis. Quando não induzem morte, essas interações gene-ambiente durante o desenvolvimento podem desencadear alterações morfológicas e fisiológicas que podem persistir até a idade adulta (LOI *et al.*, 2014).

Estudos indicam que a exposição térmica entre os dias 16 e 19 de incubação, considerada fase crítica de maturação do sistema cardiorrespiratório e do eixo hipotálamo-hipófise-tireoide, pode induzir adaptações morfofuncionais que favorecem o crescimento do músculo peitoral, reduzem a temperatura corporal sob estresse térmico e aumentam a resistência ao calor em frangos adultos (MORAES *et al.*, 2003a, 2003c; PIESTUN *et al.*, 2008a; YAHAV; RATH; SHINDER, 2004a). Essas respostas parecem estar associadas a menor secreção de corticosterona, modulação dos hormônios tireoidianos e redução da expressão de proteínas de choque térmico (ALI *et al.*, 2022; SALEH; TARKHAN; AL-ZGHOUL, 2020). Além disso, Brito e colaboradores (BRITO *et al.*, 2017) evidenciaram que níveis ideais de lisina aumentam a expressão de genes da CTE, reforçando a importância da bioenergética para o desempenho zootécnico.

Um estudo anterior do nosso laboratório investigou se a manipulação térmica durante os primeiros cinco dias de incubação (organogênese) afeta o controle respiratório e o metabolismo em pintinhos de frango de 14 dias

expostos ao estresse térmico (32 °C), sob condições de ar ambiente, hipercapnia e hipóxia. Nossos dados demonstraram que alterações na temperatura durante a incubação modificam as respostas respiratórias e metabólicas à exposição ao calor em frangos de corte (machos e fêmeas) com 14 dias de idade. Sob ar ambiente, o aumento da ventilação induzido pela MT foi mais pronunciado em fêmeas do que em machos; no entanto, o consumo de O₂ foi igualmente elevado em ambos os grupos, efeito esse atribuído à massa corporal. Durante o desafio com CO₂, o estresse térmico induziu respostas opostas entre machos e fêmeas: em fêmeas, o calor intensificou a resposta hiperventilatória ao CO₂, enquanto em machos observou-se hipoventilação sob hipercapnia. Por fim, a combinação de hipóxia e calor afetou a respiração apenas nas fêmeas, sugerindo que essas são mais sensíveis à manipulação térmica durante a incubação (PATRONE *et al.*, 2023).

A aplicação estratégica da MT pode, portanto, antecipar e acelerar a maturação funcional dos pulmões, promovendo trocas gasosas mais eficientes desde o início da vida pós-eclosão, mesmo em ambientes hostis (AUTILIO *et al.*, 2018; PIESTUN *et al.*, 2009b; SANCHEZ-ESTEBAN *et al.*, 2001). Como demonstrado por Sanchez-Esteban (SANCHEZ-ESTEBAN *et al.*, 2001), estímulos físicos (incluindo temperatura e estiramento) podem modular tanto a estrutura quanto a expressão de proteínas surfactantes, indicativo de diferenciação funcional celular pulmonar. Em aves, o pulmão é rígido e não expansível, o que torna a ventilação dependente da ação dos músculos intercostais e da dinâmica dos sacos aéreos (DUNCKER, 1974; POWELL *et al.*, 1981). Dessa forma, adaptações morfológicas precoces que favorecem a integridade da estrutura pulmonar, a vascularização e o controle ventilatório são decisivas para o sucesso produtivo em condições de calor.

Apesar dos avanços genéticos, o foco da avicultura ainda se concentra majoritariamente no ganho de peso e no rendimento de carcaça (ALI *et al.*, 2022; COLLIN *et al.*, 2007; MALTBY *et al.*, 2004b, 2004c; PIESTUN *et al.*, 2009b; PIESTUN; DRUYAN; YAHAV, 2015), negligenciando aspectos fisiológicos respiratórios e deixando uma lacuna relevante no que diz respeito à valorização do sistema cardiorrespiratório, fundamental não apenas para a homeostase térmica, mas também para a manutenção do

metabolismo sob condições de estresse. É urgente, portanto, que aspectos relacionados à morfologia e à integridade funcional pulmonar recebam maior atenção, pois são esses órgãos que sustentam a resposta fisiológica essencial para o controle térmico e o equilíbrio metabólico em situações de estresse por calor (GARGAGLIONI *et al.*, 2017). O desenvolvimento de um sistema respiratório funcional e adaptado é fundamental não apenas para o desempenho produtivo, mas também para a proteção contra enfermidades respiratórias que assolam a avicultura, como a influenza aviária e a bronquite infecciosa – ameaças com impacto sanitário e econômico global (GARGAGLIONI *et al.*, 2017; OROSZ; LICHTENBERGER, 2011). Portanto, o presente estudo busca investigar os efeitos da manipulação térmica embrionária durante os dias finais da incubação sobre a morfologia pulmonar e a resposta fisiológica ao estresse térmico em frangos de uma nova linhagem Cobb®®, desenvolvida com foco em melhor desempenho e menor mortalidade. Espera-se que a aplicação da MT proporcione ganhos significativos na eficiência respiratória, na ventilação, na maturação mitocondrial pulmonar e no metabolismo energético, contribuindo com a construção de uma avicultura mais resiliente, sustentável e alinhada às demandas futuras de produção animal em um planeta em aquecimento. Investir nesse tipo de adaptação precoce é, portanto, uma medida com profundo apelo global, pois prepara a nova linhagem para suportar as pressões ambientais extremas enquanto mantém seu papel essencial na sustentação da segurança alimentar mundial.

2. OBJETIVO

3. REVISÃO BIBLIOGRÁFICA

3.1 Produção e consumo da carne de frango

Nas últimas três décadas, a produção de carne de frango ultrapassou a de carne bovina e suína. A carne de frango é destacada por vários aspectos positivos em termos de crescimento, (Windhorst, 2006). Um fator que impulsiona esse aumento no consumo são as restrições religiosas ou culturais associadas a certos tipos de carne. Por exemplo, tanto judeus quanto muçulmanos têm restrições ao consumo de carne suína, enquanto os indianos evitam a carne

bovina. Além disso, a carne de frango é mais acessível em termos de preço em comparação com outras variedades. Nos últimos anos, o preço da carne bovina e suína tem sido de 200 a 150% mais alto do que o da carne de frango no varejo, conforme relatado pelo USDA (2011). Fletcher (FLETCHER, 2002) observa que a carne de frango é reconhecida por sua qualidade e não está associada ao aumento do risco de câncer colorretal, ao contrário das carnes vermelhas, como discutido por (ENGLISH *et al.*, 2004). Adicionalmente, em comparação com outros tipos de carne, a produção de carne de frango causa menor impacto ambiental (DE VRIES; DE BOER, 2010).

No Brasil, devido à sua acessibilidade econômica, o consumo de carne de frango representa 51% do total de carne consumida no país, proporcionando à população acesso a uma fonte de proteína animal de alta qualidade. De acordo com o relatório anual de 2026 da Associação Brasileira de Proteína Animal (ABPA), prevê-se um aumento na produção de carne de frango no Brasil em 2024, alcançando até 4,5%, o que supera em 1,5 ponto percentual o valor de 2023, atingindo um volume estimado de até 15,5 milhões de toneladas. Além disso, estima-se que o consumo per capita aumente até 3% em comparação com os anos de 2022 e 2023, chegando a 47 kg por habitante em 2024. O Brasil ocupa a colocação de segundo maior produtor mundial de carne de frango, produzindo em 2023 mais de 14 milhões de toneladas (ABPA) e abatendo 16,66 milhões de frango por dia (IBGE). Além disso, desde 2004 o Brasil é líder na exportação mundial, sendo responsável atualmente por 35% das exportações totais do produto no mercado global e vendendo para mais de 150 nações. De acordo com a Agência Brasileira de Promoção de Exportações e Investimentos (ApexBrasil), a demanda pelo produto brasileiro no mercado global pode ser atribuída aos cuidados sanitários e sustentáveis empregados pelos produtores, que se dedicam para entregar produtos de qualidade. Os principais destinos de exportação da carne de frango são a China e os Emirados Árabes Unidos. No caso dos países árabes, a produção segue parâmetros de qualidade da cultura islâmica, que exige a chamada certificação Halal, obtida por auditoria do processo de abate inspecionado por câmaras árabes no Brasil.

1.2 Genética e desenvolvimento avícola

A avicultura brasileira experimentou um notável avanço a partir da década de 60, impulsionado pela introdução de materiais genéticos importados especializados na produção de carne e ovos. Além do aprimoramento genético, os avanços em áreas como sanidade, nutrição, ambiência e manejo desempenharam um papel significativo na obtenção de resultados positivos que possuem impacto direto no aumento de produção e consumo do país.

A demanda crescente por produção, decorrente do aumento populacional global, tem impulsionado o desenvolvimento contínuo da avicultura, particularmente através do melhoramento genético, visando aprimorar o desempenho dos frangos de corte. Este foco resultou em melhorias significativas no ganho de peso, rendimento de carcaça, eficiência alimentar e idade de abate das aves. Estudos como os de Alves Filho (ALVES FILHO, 1999) demonstraram um aumento substancial no crescimento dos frangos, acompanhado por uma redução considerável no consumo de ração, refletindo em ganhos econômicos para a indústria avícola. Além disso, a partir de 2004, o Brasil assumiu a posição de líder mundial nas exportações de carne de frango, superando os Estados Unidos, principal produtor global (USDA, 2012).

Ao longo das décadas, houve uma redução notável no tempo necessário para que frangos de corte atingissem o peso desejado. Um frango que levava 120 dias para atingir 1,5 Kg em 1920, precisava apenas de 44 dias em 1980 e, em 1998, de apenas 33 dias (ALBERS, 1998). Atualmente, o período de incubação de ovos de galinha é de aproximadamente 21 dias (Cobb, 2008), enquanto a idade de abate dos frangos é de cerca de 42 dias, uma marca que continua a diminuir devido ao progresso no melhoramento genético. Este encurtamento do ciclo de vida do frango destaca a importância do período de incubação na avicultura de corte. Durante a incubação, aspectos como temperatura, umidade, viragem e ventilação são cruciais para o desenvolvimento embrionário adequado, sendo essenciais para a avicultura industrial.

No entanto, esse rápido desenvolvimento também desencadeou síndromes fisiológicas, como estresse calórico (SÁNCHEZ-MARTÍNEZ; ESTÉVEZ; ARAGÓN, 2000). Isso explica-se pelo fato de que em animais selecionados para possuir maior tamanho de carcaça e rápido crescimento, há um aumento no calor metabólico embrionário durante a incubação, resultando em

uma eclosões mas precoce que pode implicar no desenvolvimento e maturação de alguns órgãos e estruturas, como os cardiorespiratórios (YAHAV, 2000a). Portanto, limitações no desenvolvimento neonatal podem implicar em um baixo desempenho do frango, por conta a imaturação anatomorfofisiologica dos órgãos cardiorespiratórios (RONDÓN; MURAKAMI, 1998), resultando em animais com alta capacidade de ganho muscular mas com carencias funcionamento basal do metabolismo do sistema respiratório.

1.3 Morfofisiologia da respiração embrionária em aves

Durante o processo de desenvolvimento embrionário em aves, a troca de gases e de água é realizada de forma restrita por difusão através dos poros presentes na casca do ovo. Este fluxo é impulsionado pela disparidade de concentração de gases entre o meio interno e externo dos ovos, com absorção destes (TAZAWA *et al.*, 1989). Respiração é definida pela quantidade de gás difundida através da casca e membrana corioalantoide, por unidade de tempo, permeabilidade do gás, área respiratória e diferença de pressão (TAZAWA; PIIPER, 1971). Inicialmente, as trocas gasosas ocorrem através do saco vitelino que em um primeiro momento se desenvolve como uma extensão do aparelho digestivo e possui uma área vascularizada dentro da camada mesodérmica que se expande rapidamente de forma centrípeta e atua como principal membrana respiratória no primeiro terço da incubação (DEEMING, 2002).

A pressão parcial de CO₂ (pCO₂) do sangue nas veias vitelínicas da membrana do saco vitelínico aumenta de 4,2 mmHG no quarto dia para 7,0 mmHg no sexto dia, e nas artérias vitelínicas de 6,9 mmHg para 10,0 mmHg (MEUER; TIETKE, 1990). Entre a segunda e a décima oitava semana, estas trocas passam a ser realizadas pelos capilares presentes na membrana corioalantoide (MORTOLA, 2009). Esta, assim como o saco vitelínico, também é uma membrana extraembrionária essencial para o desenvolvimento embrionário, surgindo nas primeiras 65h de incubação como um divertículo do intestino grosso e com sua expansão circunda o saco amniótico, a gema e o albúmen.

Ao final do quarto dia de incubação a membrana alantoide se funde ao córion para formar a membrana corioalantoide que substituirá a área vascular da

membrana vitelínica como órgão respiratório definitivo do embrião até que a respiração pulmonar comece (WANGENSTEEN; RAHN, 1970; WANGENSTEEN; WILSON; RAHN, 1970). O sangue que entra nos capilares da membrana corioalantoide através da artéria do alantoide é pobre em O_2 e rico em CO_2 . O sangue é oxigenado à medida que passa pelos capilares do alantoide, tornando-se assim rico em O_2 e pobre em CO_2 . Durante o desenvolvimento embrionário, a pCO_2 aumenta devido ao crescente metabolismo embrionário e da resistência à difusão da casca do ovo (ERASMUS; HOWELL; RAHN, 1970) na câmara de ar (EVERAERT *et al.*, 2007; TAZAWA, 1980b; WANGENSTEEN; RAHN, 1970; WANGENSTEEN; WILSON; RAHN, 1970), no sangue, nas artérias ($paCO_2$) e veias ($pvCO_2$) (MISSION; FREEMAN, 1972; TAZAWA, 1971; TAZAWA; MIKAMI; YOSHIMOTO, 1971); (BOUTILIER *et al.*, 1977). Na última semana de incubação, junto com a formação de capilares, ocorre o desenvolvimento da vascularização pulmonar, tanto por vasculogênese, quanto por angiogênese (HISLOP, 2005). A transição da respiração pela membrana corioalantoide para a respiração pulmonar é gradual, iniciando-se entre o décimo oitavo e o décimo nono dia de incubação, quando o embrião começa a bicar a câmara de ar. A mudança completa para a respiração pulmonar ocorre entre o 20º e o 21º dia, quando o embrião inicia a bicagem da casca. Durante esse período, é essencial garantir um fornecimento adequado de ar fresco para atender à crescente demanda de oxigênio do embrião, pois uma baixa oferta de oxigênio pode resultar em uma alta taxa de mortalidade (GLIGLI, 2007).

Durante o processo de incubação, a pCO_2 do sangue começa a diminuir apenas a partir do momento em que a casca é rompida (PIIPER; DREES; SCHEID, 1980), uma vez que existe elevada quantidade de CO_2 presente na câmara de ar neste momento (MISSION; FREEMAN, 1972). Por outro lado, (TAZAWA, 1980a) mostraram haver uma diminuição considerável da pCO_2 do sangue arterial e venoso com aumento no pH desde a bicagem interna até a eclosão do embrião, conhecido como alcalose respiratória, que é o resultado da ventilação pulmonar. A partir do momento em que as aves começam a respirar na câmara de ar, a pO_2 do sangue aumenta. A ventilação pulmonar provoca melhorias na oxigenação do sangue arterial embrionário e, uma vez que uma menor quantidade de sangue é transportada para a membrana corioalantoide.

O ambiente gasoso em torno do ovo antes e durante a incubação tem um impacto significativo na sobrevivência e no desenvolvimento dos embriões, bem como na eclosão dos ovos e na qualidade dos pintinhos recém-nascidos (DECUYPERE *et al.*, 1991). Durante a embriogênese, o embrião é sensível à baixa pressão parcial de oxigênio (hipóxia) e requer uma incubação artificial precisa para garantir uma sincronização adequada dos fatores envolvidos. Os quimiorreceptores periféricos, localizados nos corpos aórticos (no arco aórtico) e carotídeos (na bifurcação das artérias carótidas), são responsáveis por detectar as alterações na pressão parcial arterial de oxigênio (PaO_2) (JONES; CARTER, 2000). Além de sensíveis à PaO_2 , esses quimiorreceptores também detectam variações no pH sanguíneo e na pressão parcial arterial de dióxido de carbono (PaCO_2), contribuindo para regular a respiração e a circulação (JONES; CARTER, 2000)..

O desenvolvimento morfológico dos corpos carotídeos inicia-se durante o terço inicial da incubação, que tornam-se funcionais na última parte do período de incubação (MORTOLA, 2009).

Nas aves, ao contrário de outros vertebrados, as áreas quimiossensíveis periféricas não se encontram na bifurcação da artéria carótida. Durante o desenvolvimento embrionário, os arcos aórticos são deslocados para trás, mantendo a bifurcação da carótida próxima ao coração. As carótidas externas sofrem atrofia, enquanto as carótidas internas (também conhecidas como carótidas comuns) se dividem secundariamente na região da cabeça (MILSOM; JONES; GABBOTT, 1981). Os principais agrupamentos de tecido quimiossensível são encontrados no arco aórtico e são inervados por um ou mais ramos vagais do gânglio nodoso (ABDEL-MAGIED; KING, 1978).

Além dos quimiorreceptores periféricos, as aves possuem quimiorreceptores centrais localizados no sistema nervoso central, embora não estejam tão precisamente identificados anatomicamente. Esses receptores detectam alterações nos níveis de CO_2 e pH (BOUVEROT; DOUGUET; SEBERT, 1979; MILSOM; JONES; GABBOTT, 1981). Nos mamíferos, esses receptores estão localizados na superfície ventrolateral do bulbo, na ponte e em áreas prosencefálicas, detectando alterações no pH do líquido e na PaCO_2 , o que resulta em um aumento na ventilação quando os níveis de PaCO_2 aumentam (LI; OTHERS, 2013). As aves também possuem quimiorreceptores intrapulmonares

altamente sensíveis ao CO₂. Esses receptores são estimulados por baixos níveis de CO₂ e podem ser inibidos por níveis elevados de CO₂ pulmonar. Suas fibras aferentes vagais controlam o volume corrente e a frequência respiratória (HEMPLEMAN *et al.*, 2006).

Essas mudanças na termossensibilidade dos neurônios hipotalâmicos após exposição a diferentes temperaturas durante a incubação podem ser resultado de adaptação epigenética. A exposição à variação de temperatura durante a incubação no final do desenvolvimento embrionário pode induzir adaptações epigenéticas em aves, mantendo um condicionamento térmico durante o período de crescimento (MINNE; DECUYPERE, 1984; TZSCHENTKE, 1997). Por outro lado, estudos mostram que a exposição ao frio durante a fase tardia da embriogênese pode aumentar a capacidade de termogênese em pintos, que passam de ectotérmicos para endotérmicos. Um dos principais desafios enfrentados pelos frangos de corte após o nascimento está relacionado à sua capacidade limitada de atender às demandas de oxigênio do corpo em baixas temperaturas ambientais (MINNE; DECUYPERE, 1984).

1.4 Estrutura e mecânica respiratória no frango

O sistema respiratório dos frangos consiste principalmente em pulmões rígidos, cujo ducto aéreo se divide em brônquios primários intrapulmonares, brônquios secundários médio-dorsais, médio-ventrais, laterodorsais, latero-ventrais, além de parabrônquios, paleopulmonar e neopulmonar (DUNCKER, 1974). Adicionalmente, eles possuem nove sacos aéreos, incluindo um saco aéreo interclavicular, um par de sacos aéreos cervicais, um par torácico cranial, um par torácico caudal e um par abdominal (MAINA; KING; SETTLE, 1989). Esses sacos aéreos estão conectados aos brônquios primários ou secundários através de óstios, facilitando a ventilação superficial e rápida dos pulmões e dos capilares aéreos (DUNCKER, 1974). Embora não realizem trocas gasosas, eles aumentam a capacidade de oxigenação do sangue, permitindo aos frangos capturar um volume de ar corrente maior em relação aos mamíferos de peso equivalente (DUNCKER, 1978; FARMER, 2006a, 2006b; POWELL, 2015a, 2015b). Além dessas características, ao contrário dos mamíferos, os frangos não

possuem um diafragma muscular separando a cavidade torácica da abdominal. Em vez disso, eles têm três septos circundantes (DUNCKER, 1974), incluindo um septo que cobre a superfície ventral dos pulmões ligado às fibras do músculo costo-pulmonar, localizado entre a 3ª e 7ª costela, que se ativa durante a expiração, um septo oblíquo que separa os sacos aéreos das vísceras e um septo pós-hepático (GARGAGLIONI *et al.*, 2017). Isso resulta em uma cavidade toracoabdominal chamada celoma, cujas pressões são relativamente uniformes.

As mudanças de pressão na cavidade celomática, devido à contração ativa e ao recuo elástico dos músculos das costelas e esterno, geram os movimentos respiratórios (FEDDE, 1998). Durante a inspiração, a cavidade celomática se expande, reduzindo sua pressão interna a níveis inferiores aos atmosféricos, permitindo a entrada de ar. O ar leva cerca de dois ciclos respiratórios para percorrer todo o circuito respiratório das aves (COHN; SHANNON, 1968). Na primeira inspiração, o ar entra pelas narinas ou pelo bico, passa pela cavidade nasal, pelas conchas nasais para filtragem, aquecimento e umidificação, e em seguida chega à laringe, onde é filtrado novamente, impedindo a entrada de corpos estranhos na traqueia. No final da traqueia, o ar passa pela siringe, uma bifurcação que leva aos brônquios primários intrapulmonares, penetrando nos pulmões esquerdo e direito (POWELL *et al.*, 1981). Esses brônquios primários intrapulmonares conduzem o ar aos sacos aéreos caudais, incluindo os abdominais e torácicos caudais (POWELL *et al.*, 1981). O ar chega aos sacos aéreos abdominais diretamente dos brônquios primários, bem como através dos brônquios secundários laterodorsais e médio-dorsais, passando pelos parabônquios neopulmonares. Para os sacos aéreos torácicos caudais, o ar é conduzido pelos brônquios secundários latero-ventrais (COHN; SHANNON, 1968). Parte do ar contido nos brônquios secundários médio-dorsais é conduzido aos parabônquios paleopulmonares, onde ocorre a troca gasosa principal. Na primeira expiração, o ar sai dos sacos aéreos caudais pelos mesmos caminhos e é conduzido ao parabônquio paleopulmonar.

Na segunda inspiração, o ar sai dos parabônquios paleopulmonares, passa pelos brônquios secundários medioventrais e chega aos sacos aéreos craniais, incluindo os craniais, cervicais e claviculares (BECH; JOHANSEN, 1980). Finalmente, na segunda expiração, o ar é expelido dos sacos aéreos craniais pelos brônquios secundários medioventrais para os brônquios primários

intrapulmonares e traqueia (COHN; SHANNON, 1968). Assim, a massa de ar no sistema respiratório das aves segue um fluxo unidirecional em quase todo o trato, exceto nos parabrônquios neopulmonares, onde o fluxo é bidirecional (DUNCKER, 1974; HARVEY; BEN-TAL, 2016). O fluxo unidirecional do ar é frequentemente atribuído a válvulas aerodinâmicas e à anatomia das vias aéreas (WHANGER; BUTLER, 1988). As aves possuem um estreitamento nos brônquios primários, chamado *segmentum accelerans*, que aumenta a velocidade do ar no interior desses brônquios em direção caudal, impedindo assim a condução do ar aos brônquios secundários medioventrais (BROWN *et al.*, 1995; POWELL *et al.*, 1981). A eficiência dessa válvula aerodinâmica está relacionada à velocidade e densidade do gás (BANZETT *et al.*, 1987; FEDDE, 1998). No entanto, essa topografia não é o único fator responsável pelo fluxo unidirecional, já que os crocodilianos, com uma ancestralidade semelhante, apresentam a mesma dinâmica de fluxo, mas não possuem essa constrição (SANDERS; FARMER, 2012).

A dinâmica do ar no trato respiratório aviário garante que quase todo o ar inspirado passe pelos parabrônquios, possibilitando que as trocas gasosas sejam realizadas tanto na inspiração quanto na expiração o que as torna mais eficientes na captura de oxigênio e eliminação de CO₂ em comparação aos mamíferos (OROSZ; LICHTENBERGER, 2011; POWELL *et al.*, 1981).

1.5 Manipulação Térmica na Embriogênese de aves

Por muitos anos, a indústria avícola manteve as temperaturas de incubação relativamente estáveis ao longo de todo o período embrionário para evitar possíveis impactos negativos na formação do embrião, na taxa de eclosão e na qualidade dos pintinhos (KRAUSOVÁ; PETERKA, 2007). Entretanto, na natureza, a temperatura durante o período embrionário sofre variações (WEBB, 1987b). Nos primeiros estágios, os embriões das aves domésticas apresentam características poiquilotérmicas, adquirindo gradualmente a habilidade de regular sua temperatura corporal, alcançando a homeotermia logo após a eclosão (NICHELMANN; TZSCHENTKE, 2002; TAZAWA *et al.*, 1988). A maturidade termorregulatória varia entre as espécies, e até mesmo entre diferentes raças de aves domésticas. Assim, o protocolo ideal de manipulação

térmica (MT) deve ser desenvolvido considerando o estágio de desenvolvimento e a maturação dos sistemas orgânicos específicos da espécie.

No caso das aves, o período embrionário representa cerca de 35% a 40% da vida total, desde a postura até o abate comercial (KHALIL *et al.*, 2024a). Durante a incubação, um desenvolvimento intenso e exigente ocorre dentro do ovo, principalmente em espécies precoces. Portanto, qualquer fator que influencie positiva ou negativamente o crescimento durante a embriogênese pode ter efeitos duradouros no desempenho e performance do frango (HULET *et al.*, 2007). O momento da aplicação da MT deve ser estrategicamente selecionado de acordo com o desenvolvimento do sistema fisiológico que se deseja impactar. Por exemplo, a indução da termotolerância está relacionada ao desenvolvimento do eixo hipotálamo-hipófise-tireoide e do eixo hipotálamo-hipófise-adrenal (PIESTUN; HALEVY; YAHAV, 2009; PIESTUN; YAHAV; HALEVY, 2015; YAHAV *et al.*, 2004). Segundo Piestun e colaboradores (PIESTUN *et al.*, 2008a; PIESTUN; HALEVY; YAHAV, 2008), para frangos, o período ideal para a MT situa-se entre os dias embrionários 7 e 16, enquanto para perus essa janela é entre os dias 10 e 22 (PIESTUN; YAHAV; HALEVY, 2015). Pesquisas com embriões de frangos indicam que a manipulação térmica durante a maturação do eixo hipotálamo-hipófise-tireoide pode reduzir o metabolismo embrionário e dos pintinhos, além de favorecer o crescimento e a eficiência alimentar após a eclosão (COLLIN; PICARD; YAHAV, 2005a, 2005b; PIESTUN *et al.*, 2008a; PIESTUN; HALEVY; YAHAV, 2008). Outros estudos com frangos e perus indicam que os embriões são mais suscetíveis a temperaturas elevadas nos estágios iniciais da incubação do que nos finais (COLLIN *et al.*, 2007; MALTBY *et al.*, 2004a; NARINÇ *et al.*, 2016; YAHAV; RATH; SHINDER, 2004b). Possivelmente se deve ao aumento do calor metabólico produzido conforme o desenvolvimento avança. Durante a metade do período de incubação, o calor gerado pelo embrião ultrapassa as perdas por evaporação, tornando a tolerância ao calor nos estágios finais dependente da capacidade dos embriões de dissipar calor e lidar com a alta demanda energética (FRENCH, 1997). Além disso, a sensibilidade à MT varia entre espécies. Por exemplo, o pato-mula é extremamente sensível a aumentos moderados e contínuos (24 h/dia) ou intermitentes (16 h/dia) de +1 °C na temperatura de incubação durante a segunda metade da embriogênese, entre os dias 13 e 27 (MASSIMINO *et al.*,

2019) . Em contrapartida, embriões de codorna japonesa (ALKAN *et al.*, 2013) (Alkan *et al.*, 2013) e de frango (YAHAV *et al.*, 2004) toleram incrementos maiores, até +3,5 °C, no mesmo período embrionário. Curiosamente, outros estudos (ABUOGHABA, 2018; ELSAYED, 2016) demonstraram que a MT intermitente (+2–3 °C por 3 h) durante a embriogênese precoce (ED6–8) ou tardia (ED15–17) resultou em maior peso do embrião e menor peso do saco vitelínico, sugerindo um desenvolvimento embrionário mais acelerado, possivelmente mediado por aumento da taxa metabólica e maior troca gasosa (WALSTRA *et al.*, 2010).

Considerando a diversidade nos tempos de incubação entre as espécies domésticas, (HAMBURGER; HAMILTON, 1992) dividiram o desenvolvimento embrionário do pintinho em três fases principais: as duas primeiras englobam a organogênese, enquanto a última é caracterizada pelo crescimento e maturação. Vários estudos demonstram que uma manipulação térmica cuidadosa durante a organogênese pode modificar o metabolismo e melhorar o desempenho dos animais (ALKAN *et al.*, 2013; COLLIN; PICARD; YAHAV, 2005b; NARINC *et al.*, 2012; PIESTUN; YAHAV; HALEVY, 2011). Contudo, a exposição a temperaturas elevadas durante a incubação também pode provocar malformações e disfunções em órgãos vitais, como coração, fígado e glândulas endócrinas (BUCKIOVÁ *et al.*, 1998), além de causar eclosão prematura (LEKSRIOMPONG *et al.*, 2007) e prejudicar a qualidade dos pintinhos (COLLIN; PICARD; YAHAV, 2005b; PIESTUN *et al.*, 2009b, 2009a; PIESTUN; HALEVY; YAHAV, 2009).

A manipulação térmica (MT) durante a incubação não se limita apenas ao uso de temperaturas elevadas, mas também abrange a exposição a temperaturas reduzidas em determinadas fases da embriogênese (IPEK; SAHAN; SOZCU, 2015; IPEK; SOZCU, 2015; MAATJENS *et al.*, 2016; ROMANOFF, ; YALÇIN; SIEGEL, 2003). De modo geral, tanto o aumento quanto a redução das temperaturas padrão de incubação podem representar estratégias para aprimorar o desempenho dos pintinhos após a eclosão. Entretanto, qualquer modificação na temperatura influencia diretamente a taxa metabólica do embrião e pode acarretar perdas significativas de água, especialmente em temperaturas elevadas (COLLIN; PICARD; YAHAV, 2005b). Por isso, é essencial que a manipulação térmica seja acompanhada de ajustes adequados na

umidade relativa, a fim de evitar a desidratação excessiva dos ovos (PIESTUN; ZIMMERMAN; YAHAV, 2015).

Outro fator é a duração da exposição ao tratamento térmico (PIESTUN *et al.*, 2008a; PIESTUN; HALEVY; YAHAV, 2008; WALSTRA *et al.*, 2010), pois é necessário equilibrar os benefícios da MT com os riscos envolvidos. Alterações na temperatura durante a incubação devem ser cuidadosamente planejadas para não prejudicar a embriogênese nem a qualidade dos pintinhos, ao mesmo tempo que a duração do tratamento precisa ser suficiente para gerar os efeitos desejados nos embriões e nos frangos após eclosão. Por exemplo, a aplicação intermitente de aumento térmico (+2 °C por 12 horas diárias) durante o desenvolvimento e maturação do eixo tireoidiano tem se mostrado eficaz para melhorar o balanço energético e a termotolerância dos pintinhos (PIESTUN *et al.*, 2008a; PIESTUN; HALEVY; YAHAV, 2008). Em contrapartida, o uso contínuo da MT (24 horas diárias) no mesmo período pode reduzir a taxa de eclosão e comprometer a qualidade dos pintinhos (COLLIN; PICARD; YAHAV, 2005b; NARINÇ *et al.*, 2016; PIESTUN; YAHAV; HALEVY, 2015). Além disso, uma exposição breve, como a elevação de +2,2 °C por apenas 4 horas, mostrou-se insuficiente para promover melhorias significativas nos embriões e pintinhos (WALSTRA *et al.*, 2010), evidenciando a importância de definir protocolos que otimizem o desempenho sem comprometer o sucesso da incubação e que permitam o tempo fisiológico celular de se modular ao tratamento térmico desejado. Muitos estudos demonstram que a manipulação térmica TM durante a incubação pode promover efeitos benéficos ao desempenho produtivo e à termorregulação de frangos de corte, sem comprometer o desenvolvimento embrionário. A aplicação de TM intermitente (+1 a +1,5 °C por 18 h/dia) entre os dias embrionários ED 2 a 18 resultou em aumento significativo do peso corporal aos 35 dias de idade, sem prejuízos à eclodibilidade ou ao desenvolvimento dos embriões (AL-ZGHOUL; EL-BAHR, 2019). De forma semelhante, a TM intermitente (+2 °C por 3 h/dia) entre ED7-16 não afetou o peso corporal final dos frangos, mas promoveu aumento significativo no peso relativo do músculo peitoral em comparação aos controles (COLLIN *et al.*, 2007). Quando aplicada nos últimos dias da embriogênese ED16-18, a TM intermitente (+2 °C por 3–6 h/dia) aumentou tanto o peso corporal quanto o crescimento absoluto do músculo peitoral (PIESTUN *et al.*, 2008b; PIESTUN; HALEVY; YAHAV, 2008).

Complementarmente, Tzschentke e colaboradores (TZSCHENTKE; HALLE, 2009a) observaram que machos submetidos a aumento térmico de curto prazo (+1 °C por 2 h/dia) entre ED18-21 apresentaram ganho de peso médio diário 2,9% superior ao grupo controle.

Um dos efeitos mais consistentes e amplamente documentados da TM embrionária é o aprimoramento da termotolerância em aves, por meio da exposição dos embriões a temperaturas elevadas durante a incubação (ALI *et al.*, 2022; ALKAN *et al.*, 2013; JANKE *et al.*, 2002). Essa adaptação pode perdurar por dias ou até semanas após a eclosão, conferindo maior resiliência ao estresse térmico, seja por calor ou frio (PIESTUN *et al.*, 2008a; PIESTUN; HALEVY; YAHAV, 2008; SALEH; TARKHAN; AL-ZGHOUL, 2020). Notavelmente, a TM de +1,7 °C aplicada entre ED7-16, tanto de forma intermitente (12 h/dia) quanto contínua (24 h/dia), resultou em maior sobrevivência dos frangos expostos a um desafio térmico de 5 horas aos 35 dias (PIESTUN *et al.*, 2008a; PIESTUN; HALEVY; YAHAV, 2008), além de favorecer a manutenção de temperatura corporal mais baixa sob calor intenso (PIESTUN; YAHAV; HALEVY, 2011). Ademais, pintinhos submetidos à TM apresentaram níveis plasmáticos reduzidos de corticosterona e hormônios tireoidianos após o desafio térmico, sugerindo alterações duradouras nos eixos hipotálamo-hipófise-tireoide e adrenal (PIESTUN *et al.*, 2008a; PIESTUN; HALEVY; YAHAV, 2008). Os mecanismos fisiológicos que sustentam a termotolerância adquirida por meio da manipulação térmica (TM) durante a incubação tem sido amplamente estudados, evidenciando alterações hormonais e metabólicas que favorecem a adaptação térmica pós-eclosão. Moraes e colaboradores (MORAES *et al.*, 2003b, 2004) demonstraram que a TM de curto prazo (+1,4 °C por 2 h/dia), aplicada no final do desenvolvimento embrionário (ED13-17), não alterou significativamente as concentrações plasmáticas de tiroxina (T4), corticosterona, glicose, ácido úrico e creatina quinase. No entanto, após desafios térmicos sucessivos (30 °C por 4 h) aos 33, 35, 37, 39, 41 e 43 dias de idade, frangos TM apresentaram níveis plasmáticos reduzidos de triiodotironina (T3) e triglicerídeos, sugerindo uma adaptação metabólica que favorece a eficiência energética sob estresse térmico. Resultados semelhantes foram observados por diversos autores (YAHAV *et al.*, 2004; YAHAV; RATH; SHINDER, 2004b) que aplicaram TM de menor intensidade (+0,7 °C por 3 h/dia) durante o período

embrionário tardio (ED16-18), observando redução significativa na temperatura corporal dos pintinhos e nos hormônios tireoidianos, sem alteração nos níveis plasmáticos de corticosterona. A intensificação da TM para +1,7 °C (3 h/dia), tanto no início (ED8-10) quanto no final da embriogênese (ED16-18), promoveu redução significativa na temperatura corporal ao nascimento (YAHAV; RATH; SHINDER, 2004b). Esses autores também relataram que pintinhos submetidos à TM no período tardio da embriogênese apresentaram a menor razão T3/T4 e níveis de corticosterona significativamente menores quando expostos a um estressor térmico severo (41 °C por 6 h) no terceiro dia de vida. Tais achados reforçam a hipótese de que a TM embrionária induz ajustes duradouros nos eixos neuroendócrinos, especialmente por meio da modulação dos hormônios tireoidianos, promovendo maior termotolerância após a eclosão. Adicionalmente, Al-Zghoul (AL-ZGHOUL, 2018) demonstrou que a exposição térmica intermitente dos ovos (+1 a +2,5 °C por 18 h/dia, entre ED12-18) resultou em melhor resposta termorregulatória aos 28 dias de vida, quando os frangos foram submetidos a estresse térmico. Essa melhora foi atribuída à redução nos níveis basais de proteínas de choque térmico (HSPs) durante os primeiros dias pós-eclosão, indicando uma possível antecipação adaptativa.

1.6 Estresse por calor em frangos de corte

Os efeitos adversos do calor excessivo na produção de frangos de corte são amplamente conhecidos. A suscetibilidade elevada do frango moderno ao estresse térmico é parcialmente atribuída ao subdesenvolvimento dos sistemas cardiovascular e respiratório em comparação com seu alto potencial de crescimento (YAHAV, 2000b). Além disso, o aumento na ingestão de ração contribui para a geração de calor metabólico endógeno. A temperatura ideal para o conforto térmico varia de 18,3 a 23,9°C nas primeiras semanas após o nascimento (KESHAVARZ; FULLER, 1980). Geralmente, a zona de conforto térmico dos frangos diminui de 32,0°C, logo após o nascimento, para cerca de 24,0°C nas terceira e quarta semanas de vida, e para 21,1°C após esse período (OJANO-DIRAIN; WALDROUP, 2002; TEETER, 1996). Sob estresse térmico, as aves podem demonstrar uma variedade de comportamentos, sendo a inibição do apetite e a redução no consumo de ração indicadores comuns da intensidade

do estresse. Potter (POTTER, 1985) observou que cada aumento de 1°C na temperatura ambiente acima da zona de conforto resulta em uma redução de 1% no consumo de ração. A redução no consumo pode chegar a 30% em temperaturas de 32°C (POLIN, 1983). Estudos como os de Cerniglia e Farrell (CERNIGLIA; HEBERT; WATTS, 1983; FARRELL; SWAIN, 1977) mostraram que o aumento da temperatura na criação de frangos leva a uma diminuição no consumo de ração, o que, por sua vez, está associado a menor ganho de peso. Os frangos de corte vêm sendo selecionados em programas de melhoramento para elevado peso vivo, o que vem resultando em um substancial aumento na velocidade de crescimento. As limitações anatômicas e fisiológicas da circulação sanguínea nos pulmões levam a ave a desenvolver a síndrome de hipertensão pulmonar (PHS), que pode levar a um grande acúmulo de fluido na cavidade abdominal, quadro este denominado de ascite (HUCHZERMEYER; DE RUYCK, 1986; JULIAN, 1993). O processo que mais frequentemente leva os frangos à ascite já foi muito bem descrito (GONZALES M. MACARI, 2000; JULIAN, 1987a). Devido a uma oxigenação insuficiente, a frequência cardíaca aumenta na tentativa de suprir mais oxigênio para o metabolismo oxidativo dos tecidos em rápido crescimento, causando uma hipertensão pulmonar. O quadro é agravado ainda mais pelo aumento da resistência ao fluxo sanguíneo no pulmão, desequilíbrio entre a necessidade e o fornecimento de oxigênio e insuficiência cardíaca direita. A predisposição à ascite é maior nos frangos porque o pulmão é rígido e fixo na cavidade torácica e o peso do órgão em relação ao peso corporal diminui em função da idade (GONZALES M. MACARI, 2000).

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INTEGRATIVE EFFECTS OF EMBRYONIC THERMAL MANIPULATION ON PULMONARY MORPHOLOGY, RESPIRATORY FUNCTION, METABOLISM, AND MITOCHONDRIAL RESPIRATION IN CHICKENS

Abstract

Heat stress is a major challenge to broiler production, prompting the development of strategies to enhance thermotolerance. This study evaluated the effects of embryonic thermal manipulation during late incubation on pulmonary development, ventilatory and metabolic responses to heat, and lung mitochondrial bioenergetics in broiler chickens. Fertile eggs were assigned to three incubation treatments: control (T1; 37.5–37.8°C), moderate thermal manipulation (T2; 38.5°C), and higher-intensity thermal manipulation (T3; 39.0°C), applied for 6 h daily from embryonic days 16 to 19. Pulmonary histomorphometry was assessed during late embryonic development and at 15 days post-hatch. Ventilatory and metabolic responses were evaluated under thermoneutral conditions, during an acute thermal challenge, and during recovery using whole-body plethysmography and oxygen consumption measurements. Pulmonary mitochondrial function was assessed by high-resolution respirometry. Thermal manipulation altered parabronchial architecture during embryogenesis without affecting body mass or relative lung weight. T2 embryos exhibited larger total parabronchial, parenchymal, and luminal areas, whereas T3 embryos showed a greater proportion of the parabronchial area devoted to air conduction and a relative reduction in gas-exchange area. At 15 days post-hatch, pulmonary remodeling persisted, with thermally manipulated groups exhibiting a greater relative gas-exchange area and a proportional reduction in the air-conduction region. Under baseline conditions, oxygen consumption, ventilatory equivalent, and body temperature were similar among treatments, although T3 showed greater minute ventilation than T1. During heat exposure, tidal volume decreased and respiratory frequency increased in all groups, maintaining minute ventilation. T3 retained the greatest absolute tidal volume and exhibited higher oxygen consumption than T1, while the ventilatory equivalent remained similar among treatments. Despite persistent structural and functional alterations, pulmonary mitochondrial respiratory parameters

did not differ among groups, indicating preserved oxidative capacity. In conclusion, embryonic thermal manipulation during late incubation induced persistent, temperature-dependent remodeling of parabronchial architecture and modified ventilatory and metabolic responses to acute heat exposure without impairing pulmonary mitochondrial function. These findings indicate that the embryonic thermal environment coordinately programs pulmonary development and respiratory physiology during early post-hatch life.

Keywords: embryonic thermal manipulation; avian lung; pulmonary ventilation; heat stress; broiler chickens.

1. Introduction

Broiler farming is one of the most efficient sectors of global animal production, providing an important source of high-biological-value protein for a constantly growing population (BRUGALETTA et al., 2022). However, increased productivity achieved through intensive genetic selection for weight gain and feed efficiency has also made modern broilers more susceptible to environmental challenges, especially heat stress (LARA; ROSTAGNO, 2013; NARDONE et al., 2010). Among the various factors affecting productive performance, heat stands out for compromising growth, metabolic efficiency, physiological homeostasis, and bird survival, generating significant economic impacts for the poultry industry (EMAMI et al., 2021; LIU et al., 2022; ST-PIERRE, COBANOV, SCHNITKEY, 2003).

In birds, maintaining homeothermia depends strongly on the efficiency of the respiratory system (KLEIN; PEREIRA RIBEIRO; BUENO DE SOUZA, 2025; SCANES, 2015). Unlike mammals, the avian lung has a rigid, highly specialized architecture composed of parabronchi and air capillaries, organized to maximize gas-exchange efficiency through a unidirectional airflow mechanism (MAINA, 2006a, 2017; POWELL, 2015a, 2015b). In this system, the cranial and caudal air sacs act as fans that continuously propel air caudocranially along the parabronchi, giving the avian lung gas-exchange efficiency greater than that of the mammalian bronchioalveolar lung (MAINA, 2023, 2025). In addition to ensuring high aerobic capacity, this anatomical organization plays a fundamental role in heat dissipation, as respiratory evaporation is the primary route of thermal loss in birds exposed to high temperatures (PIESTUN; DRUYAN; YAHAV, 2015; YAHAV, 2009). Disturbances in lung development during embryonic life influence ventilatory capacity and heat tolerance after hatching (HARDING; MARITZ, 2012; ROCHA et al., 2021; TRACHSEL et al., 2022).

The development of the avian respiratory system occurs predominantly during incubation and involves a complex sequence of morphogenetic events culminating in the formation of the parabronchi and the compartments responsible for gas exchange (HSIA; HYDE; WEIBEL, 2013; MAINA, 1984, 1993; MAKANYA et al., 2006; MAKANYA; HLUSHCHUK; DJONOV, 2007, 2011; WEST, 2009). From embryonic day 15, the atria become visible, followed by the infundibula on day 16 and the air capillaries on day 18. At birth, on days 20 to 21

in chickens, the air and blood capillaries are profusely anastomosed, and the blood-gas barrier is remarkably thin (MAINA, 2003b). During the final days of incubation, the lung parenchyma expands intensely, the air capillaries differentiate, and the respiratory structures necessary to support the transition from chorioallantoic respiration to pulmonary respiration after hatching mature (GERNER-MAURO et al., 2025; MAKANYA et al., 2016). During this period, the embryo exhibits high phenotypic plasticity, becoming particularly sensitive to environmental stimuli capable of modifying developmental trajectories, as has already been described in avian species such as the turkey *Meleagris gallopavo* (TIMMWOOD; HYDE; PLOPPER, 1987) and the ostrich *Struthio camelus* (MAKANYA et al., 2012), as well as, in a comparative context, in mammals such as the mouse, where analogous processes of parenchymal maturation occur predominantly in the postnatal period (MUND; STAMPANONI; SCHITTNY, 2008; SCHITTNY, 2017).

Embryonic thermal manipulation has been proposed as a strategy to induce lasting physiological adaptations through controlled exposure of embryos to elevated temperatures during specific periods of incubation. Studies have shown that this procedure can modulate thermoregulation, energy metabolism, hormone secretion, and the response to thermal stress in broiler chickens after hatching (AL-ZGHOUL et al., 2015; MORAES et al., 2003; PIESTUN; YAHAV; HALEVY, 2011; YAHAV et al., 2004; YAHAV; RATH; SHINDER, 2004). The most effective time window for thermal manipulation generally coincides with the development and maturation of the hypothalamic-pituitary-thyroid or adrenal axis, which explains the greater responsiveness of embryos between embryonic days 7 and 18 (AL AMAZ et al., 2024; AL AMAZ; MISHRA, 2024; GOEL; NCHO, 2024).

Although the effects of thermal manipulation on productive performance, metabolism, and thermotolerance have been extensively investigated, studies that comprehensively assess its repercussions on the structural development of the lung and its functional consequences for pulmonary ventilation during the early life phase remain scarce. Recent evidence from our laboratory indicates that environmental challenges, such as temperature and gases applied during incubation, can modify the morphology of the parabronchi and the organization of the regions intended for gas exchange, as well as the ventilatory and metabolic responses to heat, indicating that the lungs of chickens exhibit considerable

plasticity during incubation (AMARAL-SILVA; DANTONIO; BRANCO, 2017, 2019; DO AMARAL-SILVA et al., 2019). Rocha and colleagues (ROCHA et al., 2021), when evaluating embryos subjected to different incubation temperatures, observed, for the first time, phenotypic plasticity in ventilation, respiratory variability, and metabolic rate in newborn and juvenile chicks subjected to thermal manipulation, suggesting that such structural modifications persist after hatching and are associated with changes in the respiratory and metabolic physiology of birds. Complementarily, Al Amaz and colleagues (AL AMAZ et al., 2025) demonstrated that embryonic thermal manipulation also modulates early immune development, reinforcing the notion that the effects of this intervention extend beyond energy metabolism and the heat response.

Therefore, the present study aimed to evaluate the effects of late-embryonic thermal manipulation on lung development in Cobb broiler chickens, investigating morphological changes in the parabronchi during incubation and their repercussions on lung architecture, pulmonary ventilation, oxidative metabolism, and mitochondrial bioenergetics in the early stages of life. The hypothesis tested was that controlled heat exposure during the final period of incubation alters the conventional morphology of the parabronchi, thereby altering the ventilatory response of these chicks at birth and in the first two weeks of life.

2. Materials and Methods

2.1 Animals and embryonic thermal manipulation

Two hundred fertile Cobb® broiler eggs, obtained from a breeder flock at peak egg production and weighing between 60 and 65 g, were incubated in automatic incubators (Premium Ecologica®, Belo Horizonte, MG, Brazil) at a standard temperature of 37.5°C and 60% relative humidity, with automatic turning every two hours. Experiments were conducted in accordance with CONCEA (National Council for Animal Care Control, Brazil) and approved by the local animal care committee (CEUA – Comissão de Ética no Uso de Animais – FCAV-UNESP; Protocol: 9487339468/2025).

The eggs were distributed across three heat-treatment temperatures: control (T1), 37.5°C; mild heat stress (T2), 38.5°C; and severe heat stress (T3), 39°C. Heat manipulation was applied between days 16 and 19 of incubation, a

critical period for the maturation of the avian respiratory system (Maina 2003; Buggren and Elmonoufy 2017; Maina 2023). Groups T2 and T3 were exposed daily to their respective temperatures for 6 consecutive hours (from 12 pm to 6 pm), whereas the control group remained at the conventional incubation temperature. On day 18, the eggs were transferred to individual hatchers, and the respective treatments were maintained until day 19 of incubation.

2.2 Embryo Assessments

2.2.1 Body mass and Relative Lung Weight

On the 17th day of incubation, eight embryos from each experimental group (n = 16 per group) were removed from the eggs, weighed individually on an analytical balance, and their lungs were collected. The relative lung weight was calculated as the ratio of the organ weight to the embryo's total body weight and expressed in g.

$$\text{Relative lung weight} = \text{Lung weight} / \text{Body mass}$$

2.2.2 Embryonic Parabronchial Histomorphometry

On the 17th day of incubation, after two daily thermal-manipulation exposures, eight embryos from each experimental group (n = 8 per group) were weighed, and the lungs were collected and fixed in 10% buffered formalin for 48 h. After fixation, the biopsies were dehydrated in alcohol, cleared in xylene, and embedded in paraffin for sectioning on a manual rotary microtome (Microm HM 340E, Thermo Scientific™, Waltham, MA, USA). 5-µm histological sections were stained with hematoxylin-eosin (HE). Slides were photographed using an optical microscope (Zeiss Axio Scope A1, Germany) and analyzed with ImageJ software (National Institutes of Health, USA) as shown in Figure 1. The total area of the parabronchi, the area of the parabronchial parenchyma, the luminal area, the air-conduction area, and the gas-exchange area were measured. Additionally, the relative proportions of the conduction and gas-exchange areas to the total area of the parabronchi were calculated.

2.3 Assessments on the first and 15th day of life

2.3.1 Measurements of pulmonary ventilation ($\dot{V}_E$) and oxygen consumption ($\dot{V}O_2$) of 1- and 15-day-old (1d and 15d) chickens

$\dot{V}_E$ was assessed using whole-body barometric plethysmography in a closed system, as described by ESPINHA et al. (2014) and ROCHA et al. (2020). Freely moving 1d and 15d chickens were housed in 1.5-L and 5-L chambers, respectively, and the chambers were ventilated with humidified room air for one hour. Pulmonary ventilation and oxygen consumption were evaluated in eight chickens from each experimental group ($n = 8$ per group). Ambient temperature was controlled to maintain thermal comfort ($\sim 30^\circ\text{C}$ for 1d and $\sim 27^\circ\text{C}$ for 14d) (COBB, 2008). During measurements, flow was interrupted, the chamber was sealed for short periods (~ 2 min), and breathing-induced pressure oscillations were monitored with a differential pressure transducer (TSD 160A, Biopac Systems, Santa Barbara, CA, United States). The signals were fed into a differential pressure signal conditioner (DA 100C, Biopac Systems, United States), then passed through an analog-to-digital converter and digitized on a microcomputer equipped with data acquisition software (MP100A-CE, Biopac Systems, United States). After calibration, respiratory rate (f_R ; breaths min^{-1}) and tidal volume (V_T ; mL) were determined using the equation proposed by Drorbaugh and Fenn (DRORBAUGH; FENN, 1955). V_T and V_E values were expressed under BTPS (Body Temperature and Pressure Saturated) conditions, corresponding to the animal's body temperature, ambient barometric pressure, and complete water vapor saturation.

$\dot{V}O_2$ was measured by open respirometry using a PA-10 oxygen analyzer (Sable Systems, USA) (ROCHA et al., 2020). Animals were housed individually in metabolic chambers connected to the gas analysis system. $\dot{V}O_2$ was calculated following Koteja (1996) and expressed under STPD conditions. The ventilatory equivalent was calculated as the ratio of minute ventilation to oxygen consumption ($\dot{V}_E/\dot{V}O_2$).

In the first hour, measurements were taken under thermoneutral conditions ($\sim 30^\circ\text{C}$ for 1 day and $\sim 27^\circ\text{C}$ for 15 days) to establish baseline values; the animal was then exposed to acute heat stress (35°C for 1 h) and subsequently allowed to recover under thermoneutral conditions. Ventilatory variables and $\dot{V}O_2$ were

measured throughout all experimental phases. To minimize behavioral interference, records used for analysis included only periods when the animals remained at rest and exhibited no visible body movements in the chamber.

2.3.1.1 Body temperature measurement

Body temperature ($T_{(b)}$) was measured using a temperature-sensitive biotag (BioTherm13, 134.2 kHz FDX-B; 12.5 mm length $\times$ 2.1 mm diameter; Biomark, Boise, ID, USA) implanted into the coelomic cavity before the plethysmographic experiments. For implantation, the chickens were anesthetized with isoflurane delivered in 100% O₂ through a face mask connected to an open anesthetic circuit and an agent-specific vaporizer (VetCase Brasmed, São Paulo, Brazil). Isoflurane was administered at 3% for induction and 1% for maintenance. After loss of the withdrawal reflex, the biotag was inserted into the coelomic cavity using a PIT-tag applicator (Animalltag, São Carlos, Brazil), and the incision was closed with 2-octyl cyanoacrylate surgical adhesive (Dermabond Topical Skin Adhesive; Johnson & Johnson, São Paulo, Brazil). Enrofloxacin (10 mg kg⁻¹, intramuscularly) and flunixin meglumine (2.5 mg kg⁻¹, subcutaneously) were administered for antimicrobial prophylaxis and postoperative analgesia, respectively. The procedure lasted approximately 6 min, and the animals were monitored until complete recovery from anesthesia before being returned to a clean housing box. Body temperature was measured during the plethysmographic protocol by scanning the implanted biotag with a compatible reader. Measurements were obtained during the thermoneutral baseline, acute heat exposure, and recovery phases, and the temperature readings were recorded using BioTerm software (Biomark).

2.4 Pulmonary histopathology and histomorphometry at 15 days of life after heat stress

After euthanasia, lung fragments from 8 to 16 animals per experimental group (n = 8–16 per group) were fixed in 10% buffered formalin, processed for paraffin embedding, and stained with HE. Each animal was represented by one histological slide containing three to four tissue sections, all of which were examined. Slides were evaluated in a double-blind manner for the integrity of the parabronchi and parabronchial septa, vascular congestion, inflammatory

infiltrate, cellular degeneration, and other histopathological alterations. Photomicrographs were obtained systematically across the entire tissue area using an optical microscope, resulting in approximately 30 to 40 images per slide and, consequently, per animal. A randomization grid was applied to the digitized images to randomly select the fields used for morphometric measurements, thereby minimizing selection bias. Morphometric analyses were performed using ImageJ software. For the parabronchi of two-week-old animals, the total parabronchial area, air conduction area, gas exchange area, atrial area, and luminal area were measured. The relative proportions of the conduction and gas exchange areas to the total parabronchial area were also calculated.

2.5 High-resolution mitochondrial respirometry

Pulmonary mitochondrial function was assessed in fresh lung samples from six 15-day-old chicks from each experimental group (T1, T2, and T3; n = 6 per group), which were maintained under thermoneutral conditions. Measurements were performed using a high-resolution respirometry system (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria). Lung tissue fragments were homogenized in MiR05 respiratory medium and analyzed at 41°C. Mitochondrial respiration was evaluated using a substrate–uncoupler–inhibitor titration (SUIT) protocol, allowing determination of Leak (L) respiration, Complex I-supported respiration (CI), OXPHOS-CI, combined Complex I and II-supported respiration (CI+II), maximal electron transport system (ETS) capacity, and Complex IV (CIV) activity. Reactive oxygen species (ROS) production was measured simultaneously during each respiratory state.

2.6. Statistical analyses

The data are presented as mean $\pm$ SD. The effects of thermal manipulation during incubation on HS were analyzed. For lung morphometry, body mass, lung relative weight, and mitochondrial respiration were analyzed using One-way ANOVA. The treatment effects on ventilation, metabolism, and temperature were evaluated with a two-way repeated-measures analysis of variance (ANOVA) with incubation temperature and heat stress as factors. If significant differences were present, means were compared using Tukey's test. Values of $P \leq 0.05$ were considered statistically significant. In all analyses, a significance level of 5% ($p <$

0.05) was adopted.

3. Results

3.1. Embryonic and Early Post-Hatch Phases

3.1.1. Body mass and relative lung weight of embryos at 17 days of incubation

At 17 days of incubation, embryonic thermal manipulation did not affect body mass (Table 1). Although embryos in the heated groups tended to have lower body mass than those in the control group, Tukey's post hoc test revealed no significant differences among treatments.

Similarly, relative lung weight was not affected by the experimental treatments (Table 1). The T2 group showed the highest mean relative lung weight ($0.872 \pm 0.247\%$), whereas the T1 and T3 groups had comparable values ($0.577 \pm 0.276\%$ and $0.543 \pm 0.058\%$, respectively). Although the mean relative lung weight in T2 was approximately 51% and 61% greater than in T1 and T3, respectively, these differences were not statistically significant according to Tukey's multiple-comparison test.

3.1.2. Parabronchial morphometry of 17-day incubation embryos

Representative photomicrographs of the parabronchi at embryonic day 17 revealed no evident qualitative differences in pulmonary morphology among the experimental groups following embryonic thermal manipulation (Figure 2). The morphometric analysis of the parabronchi demonstrated that embryonic thermal manipulation promoted discrete but significant structural changes, as shown in Figure 3. The total area of the parabronchi differed significantly between treatments; the T2 group showed a larger total area than the T1 and T3 groups (ANOVA: $F_{(2, 205)} = 4.603$; $p = 0.0124$ for T1 and $p = 0.0349$ for T3) (Figure 3A). Similarly, the parabronchial parenchyma area differed significantly between T1 and T2 treatments (ANOVA: $F_{(2, 206)} = 3.970$; $p = 0.052$) (Figure 3B).

For the parabronchial lumen area, T2 presented the highest area compared to T1 and T3 (ANOVA: $F_{(2, 206)} = 12.38$; $p = 0.0415$ for T1 and $p < 0.0001$ for T3), whereas for the parabronchial lumen/area relation, the T3 group presented the highest value compared to T1 and T2 (ANOVA: $F_{(2, 206)} = 16.18$; $p = 0.0004$ for T1 and $p < 0.0001$ for T2). The T3 group also exhibited the largest conduction area

(ANOVA: $F_{(2, 206)} = 16.18$; $p = 0.0004$ for T1 and $p < 0.0001$ for T2) and the smallest gas-exchange zone compared with T1 and T2 (ANOVA: $F_{(2, 206)} = 16.18$; $p = 0.0004$ for T1 and $p < 0.0001$ for T2). No difference among treatments was observed in the thickness of the parabronchial parenchyma (Figure 3C).

3.1.3. Body mass of chicks on day 1 after heat treatment

Table 2 shows that body mass on the first day post-hatch differed among the experimental treatments ($F_{2,105} = 4.44$, $p = 0.0141$). Chicks in the T1, T2, and T3 groups had mean body masses of 47.97 ± 3.31 g, 48.29 ± 3.91 g, and 45.95 ± 3.66 g, respectively. Tukey's post hoc test revealed that chicks in the T3 group had lower body mass than those in the T2 group (mean difference = 2.34 g, $p = 0.0201$). No significant differences were detected between T1 and T2 ($p = 0.9277$) or between T1 and T3 ($p = 0.0502$), although the latter comparison approached statistical significance.

3.1.4. Effect of heat treatment on the ventilatory, metabolic, and thermal responses of day-old chicks under thermoneutral conditions and acute heat stress at 35°C

Under basal conditions, no differences among groups were observed in $\dot{V}_E$, V_T , f_R , $\dot{V}O_2$, or $\dot{V}_E/\dot{V}O_2$ (Figure 4A-E). T_b was lower in the T3 group than in the T1 and T2 groups (Two-way ANOVA: $F_{(2,48)} = 4.787$, T1: $p = 0.0084$; T2: $p = 0.0236$) (Figure 4F).

Heat exposure reduced $\dot{V}_E$ in the T1 and T3 groups compared with their respective basal values, whereas no significant change was observed in the T2 group (Two-way ANOVA: $F_{(2,14)} = 4.086$; T1: $p = 0.0014$; T3: $p = 0.0106$; Figure 4A). This reduction in $\dot{V}_E$ was accompanied by decreases in both V_T and f_R in the T1 and T3 groups, while neither parameter was significantly affected in T2 (V_T : Two-way ANOVA: $F_{(2,14)} = 7.916$; T1: $p = 0.027$; T3: $p = 0.0016$; f_R : Two-way ANOVA: $F_{(2,14)} = 4.668$; T1: $p = 0.0341$; T3: $p = 0.0228$; Figure 4B-C). Heat stress reduced $\dot{V}O_2$ only in the T2 group compared to baseline conditions (Two-way ANOVA: $F_{(2,14)} = 17.36$, $p = 0.0084$; Figure 4D). Heat exposure also reduced $\dot{V}_E/\dot{V}O_2$ in the T3 group compared to basal conditions (Two-way ANOVA: $F_{(2,14)} = 2.567$, $p = 0.0437$; Figure 4E). T_b increased similarly across all groups, with no differences among treatments (Two-way ANOVA: $F_{(2,48)} = 231.6$, $p < 0.0001$;

Figure 4F).

During recovery, all parameters returned to basal values. The only difference was in Tb of the T3 group, which remained higher than baseline conditions (Two-way ANOVA: $F_{(2,48)} = 231.6$, $p = 0.0002$; Figure 4F).

3.2. 15 days phase

3.2.1. *Body mass and relative lung weight of embryos at 15 days after hatch*

Body mass and relative lung weight at 15 days of age did not differ across the experimental treatments, indicating minimal impact of embryonic thermal manipulation on lung development during this period (Table 3).

3.2.2. *Parabronchial morphometry at 15 days of age*

Histopathological evaluation of the lungs at 15 days post-hatch revealed overall preservation of pulmonary architecture in all experimental groups (Figure 5). Parabronchial parenchyma and septal integrity were largely maintained, with only mild focal alterations observed in 20% of birds in T1 and 40% in T2 and T3, with no significant differences among treatments.

Pulmonary congestion was the most common histopathological finding. Moderate to marked parenchymal congestion occurred in 60% of birds at T1 and T2 and in 40% at T3 (Figure 5). A similar pattern was observed in the parabronchial and air vesicle lumina, although the frequency of congestion did not differ among treatments. Notably, T3 showed a tendency toward less air vesicle congestion, with 60% of birds showing no detectable alterations, compared with 20% in T1 and T2.

Inflammatory cell infiltration was generally minimal. Luminal inflammatory infiltrates were absent in most T1 birds (80%), whereas mild-to-moderate infiltrates were more common in T2 and T3 birds. Likewise, parenchymal cellular infiltration was predominantly absent or mild across all groups, with no treatment effect. Degenerative changes were infrequent and limited to T3, where discrete foci of epithelial or pneumocyte degeneration were observed in 40% of samples (20% mild and 20% moderate to marked), although this difference was not statistically significant ($p=0.2829$). Similarly, inflammatory lesions were virtually absent, with only one T3 bird exhibiting a moderate inflammatory focus.

Overall, the lungs of 15-day-old chicks maintained normal parabronchial

architecture, exhibiting only mild vascular congestion and focal inflammatory infiltrates (Figure 5). Although cellular degeneration and focal inflammation were observed more frequently in T3, these lesions were sporadic and of low severity, indicating that embryonic thermal manipulation did not induce substantial histopathological damage to pulmonary tissue.

3.2.3. Parabronchial histomorphometry of chicks at 15 days of age

Morphometric analysis of the parabronchi at 15 days of age revealed that embryonic thermal manipulation significantly altered the internal organization of the lung tissue, as illustrated in Figures 6 and 7.

Specifically, analysis of the compartments involved in air conduction and gas exchange showed that the total parabronchial area did not differ among the experimental groups (Figure 7A). In contrast, the area occupied by the parabronchial parenchyma was significantly affected by embryonic thermal manipulation (ANOVA: $F_{(2,110)} = 21.94$, $p < 0.0001$; Figure 7B). Birds in the control group (T1) exhibited the largest parenchymal area, whereas both thermally manipulated groups (T2 and T3) showed significantly smaller values (T1 vs. T2, $p < 0.0001$; T1 vs. T3, $p = 0.0008$), with no difference between T2 and T3.

The parabronchial lumen area also differed among treatments (ANOVA: $F_{(2,110)} = 8.764$, $p = 0.0003$; Figure 7C). The T3 group displayed a larger lumen area than both T1 ($p = 0.0003$) and T2 ($p = 0.0028$). Accordingly, the lumen-to-total parabronchial area ratio was highest in T3 (ANOVA: $F_{(2,110)} = 10.92$, $p < 0.0001$; Figure 7D), differing significantly from both T1 ($p < 0.0001$) and T2 ($p = 0.0028$).

When the relative proportions of the conduction and gas-exchange compartments were evaluated, the control group (T1) exhibited the largest conduction area and the smallest gas-exchange area (ANOVA: $F_{(2,110)} = 52.53$, $p < 0.0001$; Figures 7E and 7F). Both T2 and T3 showed a reduced conduction area and a corresponding increase in the gas-exchange area compared with T1 ($p < 0.0004$ for both comparisons). In addition, T3 retained a larger conduction area and a smaller gas-exchange area than T2 ($p = 0.005$ for both comparisons).

3.1.4. Effect of heat treatment on the ventilatory, metabolic, and thermal responses of 15 day-old chicks under thermoneutral conditions and acute heat

stress at 35°C

Under basal conditions, V_E was higher in the T3 group than in the T1 group but did not differ from the T2 group (Two-way ANOVA: $F_{(2,13)} = 3.550$, $p < 0.05$) (Figure 8A). This increase was likely driven by higher V_T and f_R (Figure 8B, C), both of which tended to be elevated in T3. No differences among groups were observed in $\dot{V}O_2$, $\dot{V}E/\dot{V}O_2$, or T_B under basal conditions (Figures 8C-E).

Heat exposure reduced V_T in all groups compared with basal conditions, with the greatest reduction observed in T3 relative to both T1 and T2 (Two-way ANOVA: $F_{(2,13)} = 4.549$, $p < 0.05$) (Figure 8A). In contrast, f_R increased in all groups (Two-way ANOVA: $F_{(2,13)} = 26.6$, $p < 0.05$), compensating for the decrease in V_T and resulting in no significant change in V_E . During heat stress, $\dot{V}O_2$ was higher in T3 than in T1 but did not differ from T2 (Two-way ANOVA: $F_{(2,13)} = 2.736$, $p = 0.031$) (Figure 8D), whereas $\dot{V}E/\dot{V}O_2$ remained unchanged (Figure 8E). T_B increased significantly in all groups compared with basal values (Two-way ANOVA: $F_{(2,13)} = 204.4$, $p < 0.001$). However, the hyperthermic response was greater in T1 than in T2 ($p = 0.0357$), whereas T3 did not differ between the groups.

During recovery, V_T was higher in T3 than in T1 (Two-way ANOVA: $F_{(2,13)} = 4.549$, $p < 0.05$; Figure 8B) but remained similar to T2 and did not differ from basal values. Respiratory frequency returned toward baseline, with T1 and T3 exhibiting lower f_R than during basal conditions ($p = 0.0088$ for T1 and $p = 0.0064$ for T3) and no differences among incubation groups (Figure 8C). T_B also returned to basal levels across all groups, although birds in T2 exhibited T_B values lower than their basal values ($p = 0.0005$; Figure 8F).

3.1.5. Mitochondrial respiration of lung tissue from 15-day-old chicks following acute heat exposure at 35°C

The assessment of mitochondrial bioenergetics in lung tissue samples from chicks revealed no statistically significant differences between treatments in any of the respiratory states analyzed (Table 4). In the routine state (Leak, L), a trend toward lower oxygen consumption was observed in group T2 (0.0060 ± 0.0034 pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$) compared to the control group T1 (0.0129 ± 0.0034 pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$), while group T3 showed intermediate values (0.0086 ± 0.0055 pmol $O_2 \cdot s^{-1} \cdot mg^{-1}$). The other respiratory states evaluated, including CI,

OXPHOS-CI, CI+II, ETS, and CIV, showed no differences between treatments, indicating that pulmonary mitochondrial oxidative capacity was preserved. Reactive oxygen species (ROS) production was not affected by embryonic thermal manipulation across all respiratory states evaluated. In the Leak state (ROS-L), a trend toward higher ROS production was observed in the control group T1 (0.0042 ± 0.0015) compared to groups T2 (0.0013 ± 0.0006) and T3 (0.0027 ± 0.0025); however, this difference was not statistically significant (ANOVA, $p = 0.0645$). For the Complex I-linked states (ROS-CI), the values remained similar across T1 (0.0003 ± 0.0002), T2 (0.0002 ± 0.0001), and T3 (0.0003 ± 0.0001) ($p = 0.8514$). Similarly, no differences were observed for ROS-C ($p = 0.1977$) or ROS-CI+II ($p = 0.3400$).

Discussão

Manipulação térmica durante a incubação e remodelamento pulmonar embrionário na fase final da incubação

The first evidence of the effects of embryonic thermal manipulation observed in the present study emerged during the final stages of pulmonary development. Although embryo body mass and relative lung weight at embryonic day 17 did not differ among treatments, consistent with previous studies (COLLIN et al., 2007; LOYAU et al., 2013, 2014; PIESTUN et al., 2008; PIESTUN; HALEVY; YAHAV, 2008; YALÇIN et al., 2008), histomorphometric analysis revealed statistically significant alterations in the internal architecture of the parabronchi. Thus, thermal manipulation affected the structural organization of the lungs without altering their relative weight. At this developmental stage, incubation temperature appears to exert a morphogenetic effect, primarily influencing the structural plasticity of the avian respiratory system. The experimental period during which the different temperatures were applied corresponds to one of the most critical windows of avian lung development (MAINA, 2003). Between embryonic days 16 and 19, the parabronchial network expands, the atria, infundibula, and air capillaries undergo differentiation, and the pulmonary vasculature that will support gas exchange after hatching develops rapidly (MAINA, 2003b; MAKANYA; HLUSHCHUK; DJONOV, 2007). Intussusceptive angiogenesis reaches its peak during this period, making the pulmonary parenchyma particularly sensitive to changes in the incubation environment. Consequently, even relatively small variations in

temperature may modulate cellular processes involved in the proliferation, differentiation, and remodeling of pulmonary tissue (AMARAL-SILVA; DANTONIO; BRANCO, 2019; MAKANYA; HLUSHCHUK; DJONOV, 2007), potentially producing structural alterations that persist after hatching (DZIALOWSKI et al., 2002).

An important aspect of our findings is that the two intensities of thermal manipulation did not elicit equivalent morphological responses. Embryos incubated at 38.5°C (T2) exhibited larger total parabronchial, parenchymal, and luminal areas than control embryos incubated at 37.5–37.8°C, suggesting expansion of the parabronchial compartments at this stage of development. In contrast, embryos incubated at 39.0°C (T3) showed a greater proportion of the parabronchial area devoted to air conduction and a relative reduction in the gas-exchange area, indicating a remodeling pattern distinct from that observed in T2. Therefore, the pulmonary response to the thermal environment did not follow a linear relationship with stimulus intensity but appeared to depend on a specific thermal threshold, resulting in different morphostructural phenotypes among treatments (WILLEMSSEN et al., 2010; AL-ZGHOUL; EL-BAHR, 2019; LEÃO et al., 2024). Previous studies have demonstrated that environmental stimuli applied during embryonic development can modify pulmonary architecture independently of changes in organ mass (AL AMAZ; MISHRA, 2024), consistent with our findings. Similarly, Amaral-Silva et al. (2019) demonstrated that hypoxia imposed during parabronchial formation induced structural remodeling characterized by an expansion of the regions involved in gas exchange. This finding indicates that the avian lung responds to different environmental challenges including changes in the gaseous environment by reorganizing its internal architecture. Comparable results were reported by Copeland and Dzialowski (2009), who observed an increase in the proportion of pulmonary tissue occupied by parabronchi following manipulation of the gaseous environment during late incubation, without changes in total lung mass.

Post-hatch metabolic and ventilatory responses to heat

Although thermal manipulation induced significant remodeling of the parabronchial architecture during embryogenesis, its immediate functional effects on the respiratory physiology of newly hatched chicks were more subtle. Under

thermoneutral conditions, no statistically significant differences among treatments were observed in minute ventilation, tidal volume, respiratory frequency, oxygen consumption, or ventilatory equivalent. This indicates that the structural alterations established in the embryonic lung were not immediately accompanied by marked changes in respiratory function after hatching.

This dissociation between embryonic alterations and basal respiratory function in neonates has also been reported following other environmental stimuli applied during incubation. Mortola and Toro-Velasquez (2013) demonstrated that chicks incubated at 35°C, despite exhibiting an approximately 30% reduction in embryonic oxygen consumption throughout incubation, hatched with normal basal minute ventilation and oxygen consumption on the first day post-hatch. Similarly, embryonic thermal manipulation protocols often preserve neonatal basal metabolism despite inducing substantial physiological alterations during embryonic development (YAHAV et al., 2004; PIESTUN et al., 2008, 2009; PIESTUN; HALEVY; YAHAV, 2009; ROCHA et al., 2020, 2021). One possible explanation is that, after hatching, the respiratory system undergoes an intense period of adaptation characterized by the definitive transition from chorioallantoic to pulmonary respiration, progressive lung expansion, recruitment of the air capillaries, absorption of the residual yolk sac, and maturation of the neural mechanisms that control ventilation (DZIALOWSKI 2025; SZDZUY, 2025). Therefore, the morphological alterations established during embryogenesis and detected in the present study may remain functionally silent during this initial period because of the substantial compensatory capacity of the respiratory system before it reaches functional maturity (YAHAV et al., 2004; PIESTUN et al., 2008, 2009). In previous studies conducted by our research group, Rocha et al. (2020, 2021) also reported that chicks subjected to thermal manipulation during organogenesis, from embryonic days 0 to 5, maintained their oxygen consumption under normoxic conditions during the first days post-hatch.

Although ventilation did not differ among treatments under thermoneutral conditions, chicks incubated at 39°C exhibited a lower body temperature than those in the other groups. This finding may indicate an early adaptation of thermoregulatory mechanisms, even in the absence of detectable changes in the ventilatory pattern (COLLIN et al., 2007; MORTOLA; TORO-VELASQUEZ, 2013). Because the neural centers responsible for regulating body temperature

and respiration share pathways located in the hypothalamus and brainstem, it is plausible that temperature-induced embryonic programming initially affected the central circuits that integrate thermal and respiratory responses. This hypothesis is consistent with the high sensitivity of these systems to environmental conditions during development (TZSCHENTKE; BASTA, 2002; BURGGREN; MUELLER; TAZAWA, 2015; BAARENDSE et al., 2007).

During heat exposure, minute ventilation, tidal volume, and respiratory frequency decreased relative to their respective baseline values in the T1 and T3 groups, whereas these variables remained unchanged in T2. Oxygen consumption, in contrast, decreased only in the T2 group. The ventilatory equivalent ($\dot{V}E/\dot{V}O_2$) decreased in T3 relative to its baseline value, indicating that, despite the reductions in $\dot{V}E$, V_T , and f_R , chicks incubated at 39°C maintained oxygen consumption while adjusting ventilation more economically relative to metabolic demand during heat exposure. This index is conventionally used as an inverse measure of gas-exchange efficiency: the lower the volume of air moved per unit of oxygen consumed, the greater the efficiency with which ventilation supports metabolic oxygen requirements. This principle is widely applied in comparative studies of avian respiratory physiology (MORTOLA, 2009; BÍCEGO; MORTOLA, 2017).

The specific reduction in $\dot{V}E/\dot{V}O_2$ in chicks subjected to 39°C provides functional evidence that this group maintained its metabolic requirements at a proportionally lower ventilatory cost than would be expected from the concurrent reductions in $\dot{V}E$, V_T , and f_R . This finding is consistent with the principle of symmorphosis (WEIBEL, 1991; WEIBEL; TAYLOR; HOPPELER, 1991), according to which structures that are better matched to oxygen transport requirements can sustain a given metabolic demand at a lower ventilatory cost. It is also consistent with the central hypothesis of the present study that the embryonic thermal environment induces persistent programming of the relationship between pulmonary structure and respiratory function. Body temperature increased similarly in all groups during the acute thermal challenge at 35°C, and most variables returned to their baseline values during recovery. The exception was body temperature in T3, which remained elevated relative to its baseline value. At first glance, this result contrasts with previous studies of thermal manipulation in broilers, in which postnatal thermal-conditioning

protocols initiated a few days after hatching and frequently repeated generally attenuated, rather than prolonged, the increase in body temperature during and after subsequent thermal challenges by reducing metabolic and non-shivering heat production (OUCHI et al., 2020, 2021).

One plausible explanation for this apparent discrepancy is that those studies evaluated birds that had already experienced at least one previous thermal exposure. In the present study, however, the thermal challenge represented the chicks' first postnatal exposure to heat, at a time when the mechanisms responsible for heat dissipation were encountering the environment outside the egg for the first time (WHITTOW; TAZAWA, 1991; NICHELMANN; TZSCHENTKE, 2002). Thus, late embryonic thermal manipulation at 39°C may have programmed a response involving lower ventilatory and metabolic costs during the acute thermal challenge, although potentially at the expense of slower heat dissipation during immediate recovery. This physiological trade-off may diminish as thermoregulatory mechanisms mature. This interpretation is consistent with reports that thermal manipulation during the same late developmental window (E16–E18) results in a lower basal body temperature at hatching (COLLIN et al., 2007; YAHAV et al., 2004), indicating that a change in the thermal set point does not necessarily accelerate the return to that point after an acute disturbance. Furthermore, the direction of the ventilatory response observed in the present study warrants careful interpretation because it differs from the pattern commonly described in adult birds and even in broilers only a few weeks old. In these animals, heat exposure induces thermal tachypnea, characterized by a marked increase in respiratory frequency as the primary mechanism of evaporative heat dissipation through the upper respiratory tract (MACARI; FURLAN; GONZÁLES, 2002; BÍCEGO; MORTOLA, 2017). For example, in four-week-old broilers subjected to acute heat stress, the respiratory response is dominated by continuous panting, which may be sufficiently intense to induce respiratory alkalosis (TEETER et al., 1985).

In the present study, however, one-day-old chicks responded to heat with reductions in minute ventilation, tidal volume, and respiratory frequency, a pattern opposite to that expected based on studies of older birds. This discrepancy probably reflects the physiological immaturity of the neonate. Thermoregulatory competence is not fully established at hatching, and precocial species undergo a

gradual transition during late incubation and the first days post-hatch from a thermolabile state resembling that of an ectotherm to full homeothermy. In chicks, this process is not completed until the second or third week post-hatch (WHITTOW; TAZAWA, 1991; NICHELMANN; TZSCHENTKE, 2002).

During this period of immaturity, the classical effectors of evaporative heat loss, particularly the respiratory muscles recruited during panting, may not operate with the same efficiency observed in older broilers. Consequently, one-day-old chicks may rely more heavily on behavioral responses than on active ventilatory adjustments when exposed to excessive heat (NICHELMANN; TZSCHENTKE, 2002). The reduction in ventilation observed during heat exposure may therefore represent an energy-conserving strategy in the presence of an incompletely developed effector system, preventing an additional metabolic cost that could compromise growth even when chicks experience high environmental temperatures for a relatively short period (WHITTOW; TAZAWA, 1991; MISSON, 1976; OSTROWSKI et al., 2022).

Functional immaturity of thermolytic effectors has also been described in neonatal pigs. Panting is a major mechanism of evaporative heat dissipation in older animals, whereas newborn piglets, whose thermoregulatory systems remain immature, prioritize responses that protect against cold and do not possess the same repertoire of active heat-dissipation mechanisms observed in adults (GÓMEZ-PRADO et al., 2022). Similarly, hypometabolic responses to thermal and hypoxic challenges, rather than the classical activation of defensive effectors, have been reported in neonatal rodents (BLUMBERG; ALBERTS, 1991). In newly hatched chicks, an analogous phenomenon of metabolic depression without compensatory activation of respiratory effectors has also been demonstrated, although predominantly in response to hypoxia rather than heat (MORTOLA; BESTERMAN, 2007). Comparable limitations in neonatal thermoregulatory capacity have also been reported in nondomestic precocial birds (DURANT et al., 2012). In broilers specifically, there is evidence that thermal experience during early developmental windows shapes the ventilatory response to subsequent thermal challenges. Ouchi et al. (2020) demonstrated that chicks subjected to repeated thermal conditioning between the second and fourth days post-hatch exhibited an attenuated increase in respiratory frequency when exposed to an acute heat challenge at 14 days of age compared with

nonconditioned birds. Collectively, these findings suggest that suppression, rather than full activation, of the classical thermolytic effectors during acute challenges may be a shared characteristic of neonates across different vertebrate classes. This response may reflect the immaturity of autonomic control circuits and the limited energy reserves available during this stage of life. Moreover, even within the species investigated here, the ventilatory response to heat is not fixed and may be shaped by thermal experiences occurring during sensitive developmental windows. To the best of our knowledge, however, a specifically hypoventilatory, rather than merely hypometabolic, response to acute heat exposure has not previously been reported in one-day-old chicks, representing an original contribution of the present study.

The reduction in oxygen consumption observed in T2 during heat exposure occurred without any concurrent change in minute ventilation, tidal volume, or respiratory frequency. In contrast to T1 and T3, whose responses to heat involved ventilatory adjustments, chicks incubated at 38.5°C exclusively reduced their metabolic demand without any detectable change in their respiratory pattern. This represents an isolated metabolic-depression response, dissociated from a compensatory ventilatory response, similar to that previously described in chicks exposed to other acute environmental challenges, such as hypoxia (MORTOLA; BESTERMAN, 2007). This finding is consistent with the hypothesis that the structural remodeling of the parabronchi already established in T2 during late incubation—characterized by larger total parabronchial, parenchymal, and luminal areas than those of the control group—may have provided, at hatching, an oxygen-supply capacity that exceeded tissue demand. Even under basal conditions, a more structurally developed respiratory system may accommodate transient reductions in metabolism within its existing functional reserve without requiring compensatory changes in ventilatory mechanics (MAINA, 2006, 2025). Indeed, the structural capacity of the lungs for oxygen transport generally exceeds resting requirements, creating a functional reserve that is recruited only when metabolic demand increases (HSIA; HYDE; WEIBEL, 2016).

Although thermal manipulation at both 38.5°C and 39°C induced embryonic pulmonary remodeling, these treatments programmed distinct functional responses immediately after hatching. T2 exhibited a predominantly metabolic response, potentially supported by the greater structural reserve for gas

exchange already demonstrated in this group at embryonic day 17. In contrast, T3 showed a predominantly ventilatory response, characterized by a more economical relationship between ventilation and oxygen consumption. This response may have been associated with adjustments in central respiratory and thermoregulatory control programmed during embryogenesis (NICHELMANN; TZSCHENTKE, 2002; TZSCHENTKE; BASTA, 2002; LOYAU et al., 2015; MAINA, 2006; BAARENDSE et al., 2007), because morphometric analysis at embryonic day 17 did not indicate an expansion of the gas-exchange area in this group. Instead, T3 exhibited a greater proportion of the parabronchial area devoted to air conduction.

Persistence of parabronchial reorganization during the early post-hatch period in chicks incubated at different temperatures during late incubation

The results obtained at 15 days of age demonstrate that the pulmonary remodeling induced during embryogenesis was not transient. Instead, it persisted after hatching and modified the internal organization of the parabronchi even during a period of rapid body growth. This pattern is consistent with the findings of Amaral-Silva et al. (2019), who described parabronchial remodeling in chicks exposed to hypoxia between embryonic days 12 and 18, with morphological alterations remaining detectable at 10 days post hatch. The authors demonstrated that an environmental stimulus applied during the critical window of parabronchial development induces structural reorganization that persists throughout postnatal growth, rather than producing a transient effect limited to the embryonic period. These findings support our interpretation that thermal manipulation exerted a programming and long-lasting effect on pulmonary architecture. Although body mass, relative lung weight, and total parabronchial area did not differ among treatments, histomorphometric analysis revealed marked changes in the distribution of the respiratory compartments, indicating architectural reorganization of the lungs rather than pulmonary growth itself. This dissociation between organ mass or size and internal composition was also reported by Lewallen and Burggren (2015). When chicken embryos were incubated under chronic hypoxic or hyperoxic conditions, the authors found no differences in lung mass among groups but observed significant changes in the proportions of the

total pulmonary cross-sectional area occupied by parabronchial gas-exchange tissue and the parabronchial lumen. Together with our results, these findings reinforce the concept that the avian lung responds to early environmental stimuli primarily through plasticity in the relative allocation of its internal compartments, rather than through changes in the overall growth of the organ (MAINA, 2006; MAKANYA et al., 2007).

Although embryos subjected to thermal manipulation exhibited distinct morphological responses during incubation, these differences converged toward a common remodeling pattern at 15 days of age, characterized by a reduction in the area occupied by the parabronchial parenchyma and a proportional increase in luminal area, particularly in the group incubated at 39°C. Lewallen and Burggren (2015) reported a similar pattern, demonstrating that oxygen availability during incubation directly alters the relative proportions of gas-exchange tissue and parabronchial lumen. This suggests that hypoxic and thermal stimuli may converge on common regulatory pathways of pulmonary remodeling during the critical period of parabronchial formation. Similarly, experimental models of hyperoxia in rodents have demonstrated reorganization of the pulmonary parenchyma characterized by a relative reduction in gas-exchange area and expansion of the air spaces. These findings further indicate that different developmental stressors may ultimately result in persistent alterations in the structural organization of the lungs (RANDELL et al., 1990; YEE et al., 2017).

The morphological changes induced during embryogenesis did not disappear after hatching but continued to undergo remodeling throughout postnatal development, resulting in a pulmonary architecture distinct from that observed in birds incubated under conventional thermal conditions. This long-lasting effect is consistent with the concept of epigenetic adaptation to temperature described by Tzschentke and Basta (2002) and Nichelmann and Tzschentke (2002), according to which thermal stimuli applied during critical windows of embryonic development alter the set points of physiological control systems, with effects that persist throughout postnatal life. From a functional perspective, the greater relative proportion of gas-exchange area observed in the groups subjected to thermal manipulation may represent an adaptive response that enhances oxygen diffusion capacity, analogous to the increased anatomical diffusion factor reported by Amaral-Silva et al. (2019) in chicks incubated under

hypoxic conditions. Conversely, pulmonary alterations induced by elevated incubation temperatures have also been associated with a higher incidence of ascites and cardiopulmonary disorders in broilers (MOLENAAR et al., 2011). This emphasizes the need for functional assessments including ventilatory capacity, gas-exchange efficiency, and thermal tolerance to determine whether the remodeling observed in the present study represents an **adaptive advantage or a physiological cost associated with embryonic thermal stress**.

Pulmonary remodeling and adaptations in ventilatory and metabolic responses to thermal stress at 15 days of age

Although structural remodeling of the parabronchi persisted until 15 days of age, its functional effects became more evident only when the birds were exposed to a thermal challenge. Under baseline conditions, the groups exhibited similar oxygen consumption, ventilatory equivalent, and body temperature, indicating that embryonic thermal manipulation did not affect resting respiratory physiology. Baseline minute ventilation was higher in T3 than in T1, supported by a tendency toward greater tidal volume and respiratory frequency in this group, although T3 did not differ from T2. During heat exposure, however, tidal volume decreased relative to baseline in all groups, whereas respiratory frequency increased similarly across treatments. This increase compensated for the reduction in tidal volume, resulting in no significant change in minute ventilation. This pattern is consistent with the classical thermal tachypnea response described in Galliformes, in which an increase in respiratory frequency facilitates evaporative heat dissipation through the upper respiratory tract (MACARI; FURLAN; GONZÁLES, 2002; BÍCEGO; MORTOLA, 2017).

The reduction in tidal volume relative to baseline was greater in the group incubated at 39°C than in those incubated at 37.5 and 38.5°C. Importantly, this reduction occurred from a higher baseline. At all time points, including during heat exposure, T3 maintained the greatest absolute tidal volume among the three groups and, therefore, did not exhibit the shallowest ventilatory pattern. The greater relative decrease primarily reflected the fact that this group began the thermal challenge with a higher baseline tidal volume (POWELL, 2007; MORTOLA, 2009; FEDDE, 1987). Despite exhibiting the greatest relative reduction in tidal volume, T3 showed higher oxygen consumption than the control

group during heat exposure, although it did not differ from T2, whereas the ventilatory equivalent remained similar among treatments. These findings indicate that chicks incubated at 39°C were able to maintain, and even increase, oxygen uptake without a proportional increase in ventilation relative to metabolic demand. This suggests that the coupling between ventilation and gas exchange was maintained during the thermal challenge, possibly because of structural and/or functional adaptations of the respiratory system similar to those described in birds and other vertebrates (MAINA, 2006; MORTOLA; FRAPPELL, 2000; FRAPPELL et al., 1992). The structural reorganization of the parabronchi observed in this same group, characterized by a greater proportion of area devoted to gas exchange relative to the control group, may have contributed to sustaining the combination of a comparatively high tidal volume, greater $\dot{V}O_2$, and unchanged ventilatory cost during thermal stress. Notably, the most distinct functional response to the thermal challenge did not occur in the group with the greatest expansion of the gas-exchange area. Although birds incubated at 38.5°C exhibited the greatest proportion of area devoted to gas exchange at 15 days of age, the group incubated at 39°C displayed the most distinct ventilatory and metabolic pattern during heat exposure.

This dissociation is consistent with previous reports indicating that structure and function may respond independently depending on the developmental window during which the stimulus occurs (BURGGREN; REYNA, 2011). It suggests that, although structural reorganization of the parabronchi probably contributes to respiratory performance, it does not fully explain the functional adaptations observed. Instead, the response to thermal stress likely results from the integration of pulmonary architecture and the central mechanisms controlling ventilation, both of which may be programmed during embryonic development (BURGGREN; FLORES-SANTIN; ROJAS-ANTICH, 2016). Our findings suggest that different intensities of thermal manipulation induce distinct but complementary adaptive mechanisms. Incubation at 38.5°C induced more pronounced structural remodeling of the gas-exchange region, whereas incubation at 39°C was primarily associated with changes in the ventilatory pattern during heat exposure. Rather than representing opposing responses, these phenotypes appear to reflect distinct physiological strategies triggered by different intensities of thermal stimulation during a critical period of

embryogenesis.

Integration of pulmonary remodeling, ventilatory responses, and mitochondrial bioenergetics

The integrated analysis of the data demonstrates that heat exposure during late incubation triggered a coordinated process of respiratory-system programming involving persistent remodeling of the parabronchial architecture, changes in the ventilatory pattern during thermal stress, and preservation of pulmonary mitochondrial bioenergetics. Although the parabronchial remodeling observed at 15 days of age may enhance gas-exchange efficiency, the respiratory physiology results demonstrate that this structural reorganization did not result in increased oxygen consumption or ventilatory equivalent under baseline conditions. **This finding suggests that the greater proportion of area devoted to gas exchange represents an increase in the potential functional capacity of the lungs**, but not necessarily an increase in the organism's metabolic demand. According to Weibel and colleagues (WEIBEL, 1991; WEIBEL; TAYLOR; HOPPELER, 1991; WEIBEL; RICHARD TAYLOR; HOPPELER, 1992), the components of the oxygen transport cascade are adjusted in an integrated manner to the functional demands of the organism. In contrast to the predictions of this model under conditions of increased maximal aerobic capacity, however, our findings indicate that thermal manipulation did not induce an overall increase in oxidative metabolism. Instead, it promoted a reorganization of pulmonary architecture associated with changes in the ventilatory pattern.

The adaptations observed appear to reflect more efficient use of the existing respiratory capacity rather than an increase in metabolic capacity. This interpretation is supported by the mitochondrial respiration profile, which indicated that pulmonary oxidative capacity was preserved despite the structural remodeling induced during embryogenesis, with no evidence of mitochondrial dysfunction or oxidative stress in birds subjected to thermal manipulation.

Thermal stress is widely recognized as one of the primary factors capable of impairing mitochondrial bioenergetics in broilers by increasing electron leakage from the electron transport chain and promoting excessive production of reactive oxygen species (ROS) (LIN; DECUYPERE; BUYSE, 2006; MUJAHID et al.,

2006, 2007; BOTTJE, 2019; ARYAL et al., 2025). This impairment partly involves modulation of the avian uncoupling protein, the downregulation of which during heat exposure promotes ROS accumulation (ABE et al., 2006; KIKUSATO; TOYOMIZU, 2013). Conversely, early thermal-conditioning protocols appear to act through this same pathway, subsequently conferring greater thermotolerance (TAOUIIS et al., 2002; PIESTUN et al., 2008). The absence of pulmonary mitochondrial dysfunction in the present study is consistent with this framework, suggesting an adaptive and protective effect of embryonic thermal programming rather than a pathological condition. The group incubated at 39°C exhibited the most distinct ventilatory response during heat exposure, maintaining the greatest absolute tidal volume and the highest oxygen consumption among treatments, without any associated mitochondrial alterations. This indicates that the adaptations arose primarily from changes in ventilatory strategy rather than from changes in pulmonary metabolic capacity. Therefore, the respiratory programming induced by thermal manipulation appears to involve functional control mechanisms capable of optimizing ventilation without increasing the energetic cost of the respiratory system. The morphological and functional alterations, however, did not correspond directly across treatments. Birds incubated at 38.5°C exhibited the greatest expansion of the gas-exchange area, whereas those incubated at 39°C displayed the most distinct ventilatory and metabolic pattern during thermal stress. This dissociation indicates that, although parabronchial remodeling probably contributes to respiratory performance, it does not independently explain the adaptations observed. Instead, the response to heat is more likely to result from the integration of pulmonary architecture, ventilatory mechanics, and the programming of the neural centers that control respiration and thermoregulation, all of which are sensitive to environmental stimuli during embryogenesis (TAZAWA, 2015).

Conclusão

Thermal manipulation during late incubation acts as an important modulator of respiratory-system development in broilers. Heat exposure during this period induced persistent remodeling of the parabronchial architecture, the effects of which extended into post-hatch life and were accompanied by changes in the ventilatory response to thermal stress in newly hatched and young chicks.

However, these alterations occurred without impairment of pulmonary mitochondrial function, indicating that temperature-induced programming represents an adaptive process. Overall, our findings demonstrate that embryonic thermal manipulation can coordinately modulate pulmonary development and respiratory physiology in broilers.

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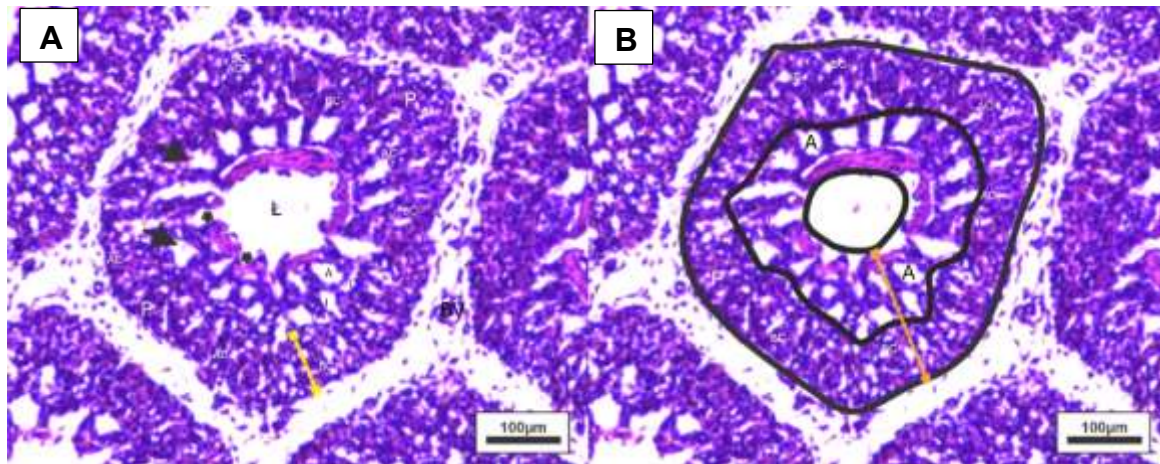


Figure 1. Photomicrographs of parabronchi from chick embryos at 17 days of incubation subjected to embryonic thermal manipulation. (A) Delimitation of air-conducting areas (black arrows and *). The yellow line indicates the gas-exchange area. (B) Example of digital segmentation performed using ImageJ software to quantify the total parabronchial area, air-conducting area, and parabronchial luminal area. Sections stained with hematoxylin-eosin (HE). 40× objective. Scale bar = 100 µm. Abbreviations: L, parabronchial lumen; A, atrium; I, infundibulum; AC, air capillary; BC, blood capillary; BV, blood vessel.

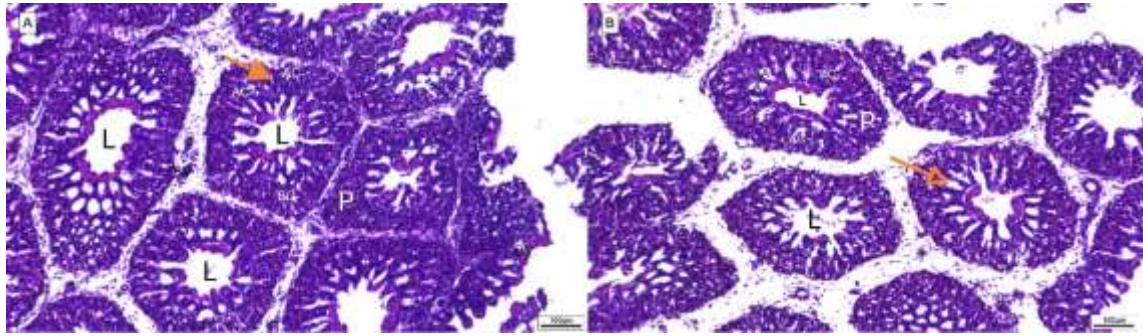


Figure 2. Photomicrograph of parabronchi from embryos subjected to thermal manipulation at 17 days of embryonic development. (A) Group T2 (38.5°C). (B) Group T3 (39.0°C). Differences in the relative proportion between the air-conducting and gas-exchange regions are observed, consistent with the obtained morphometric results. Histological sections stained with hematoxylin-eosin (HE). 20× objective. Scale bar = 100 μm. Abbreviations: L, parabronchial lumen; A, atrium; I, infundibulum; AC, air capillary; BC, blood capillary; BV, blood vessel.

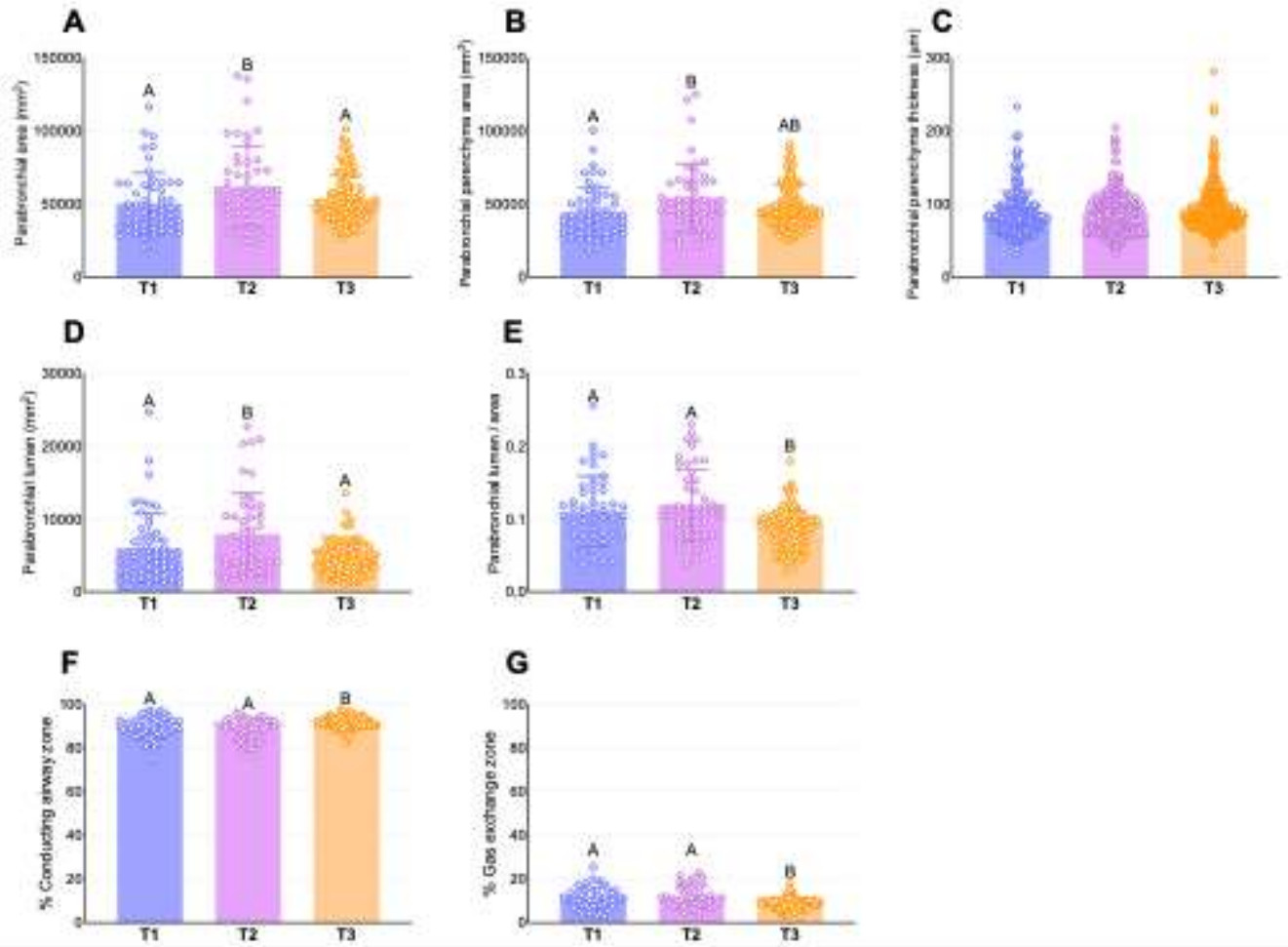


Figure 3. Morphometric parameters of parabronchi at 17 days of incubation subjected to embryonic thermal manipulation. Shown are (A) total parabronchial area (mm²), (B) parabronchial parenchyma area (mm²), (C) parabronchial parenchyma thickness (μm), (D) parabronchial luminal area (mm²), (E) lumen/total area ratio, (F) percentage of air-conducting area, and (G) percentage of gas-exchange area. T1 = control (37.5°C); T2 = 38.5°C; T3 = 39.0°C. Data are presented as mean ± SD. Different upper-case letters indicate significant differences among incubation temperature treatments ($P < 0.05$).

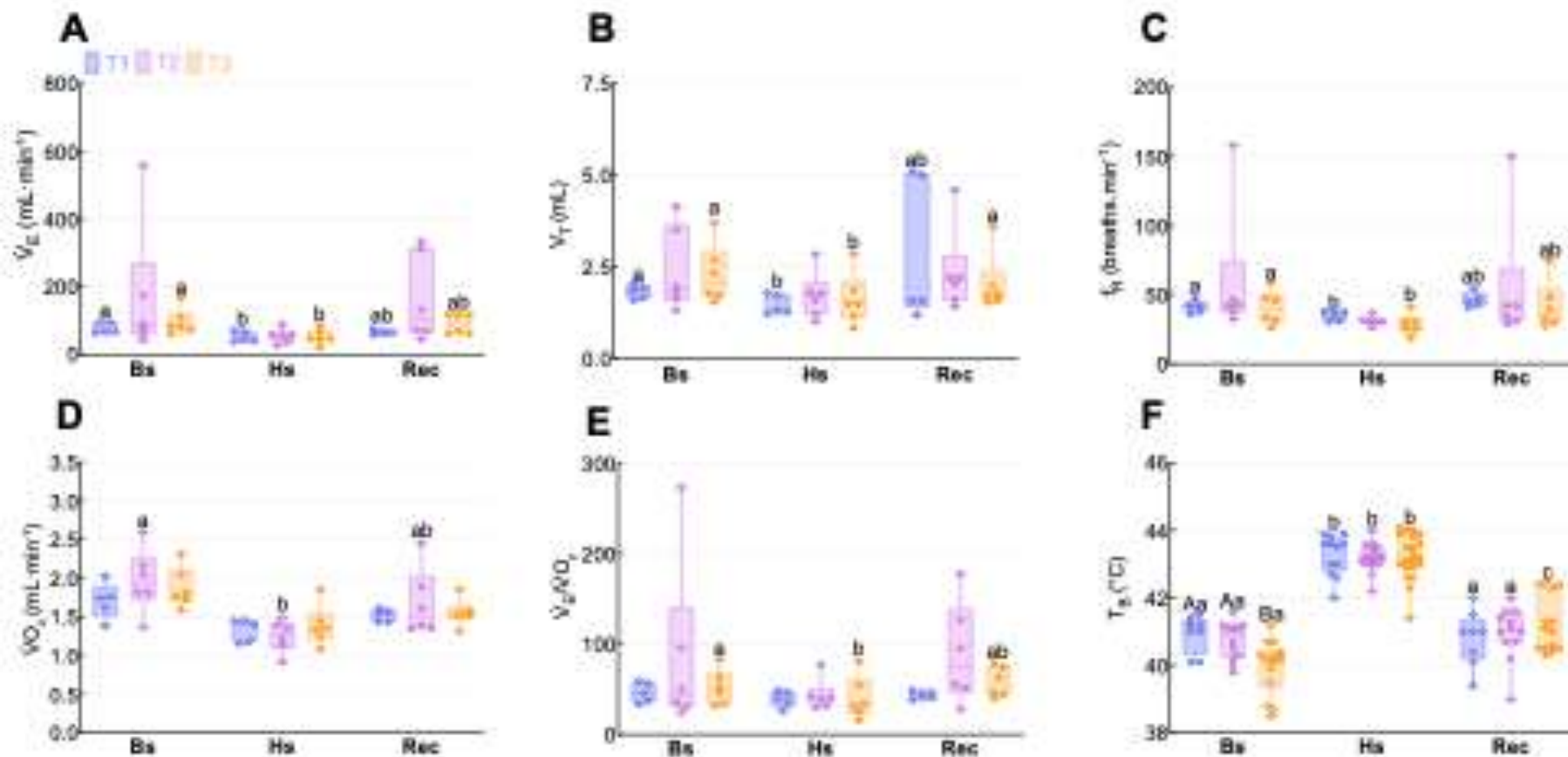


Figure 4. Ventilatory, metabolic, and thermal parameters of chicks on the first day of life subjected to embryonic thermal manipulation, evaluated under basal conditions (Bs), during thermal stress (Hs), and during the thermal stress recovery period (Rec). The following are presented: (A) minute ventilation ($\dot{V}_E$, $\text{mL} \cdot \text{min}^{-1}$), (B) tidal volume (V_T), (C) respiratory rate (f_R), oxygen consumption ($\dot{V}O_2$), (E) ventilatory equivalent ($\dot{V}_E/\dot{V}O_2$), and body temperature (T_B). T1 = control (37.5°C); T2 = 38.5°C ; T3 = 39.0°C . Boxplots represent the median, quartiles, and range of the data. Data are presented as mean $\pm$ SD. Different uppercase letters indicate significant differences among incubation temperature treatments, whereas different lowercase letters indicate significant differences among Bs, Hs, and Rec conditions within the same group ($p < 0.05$).

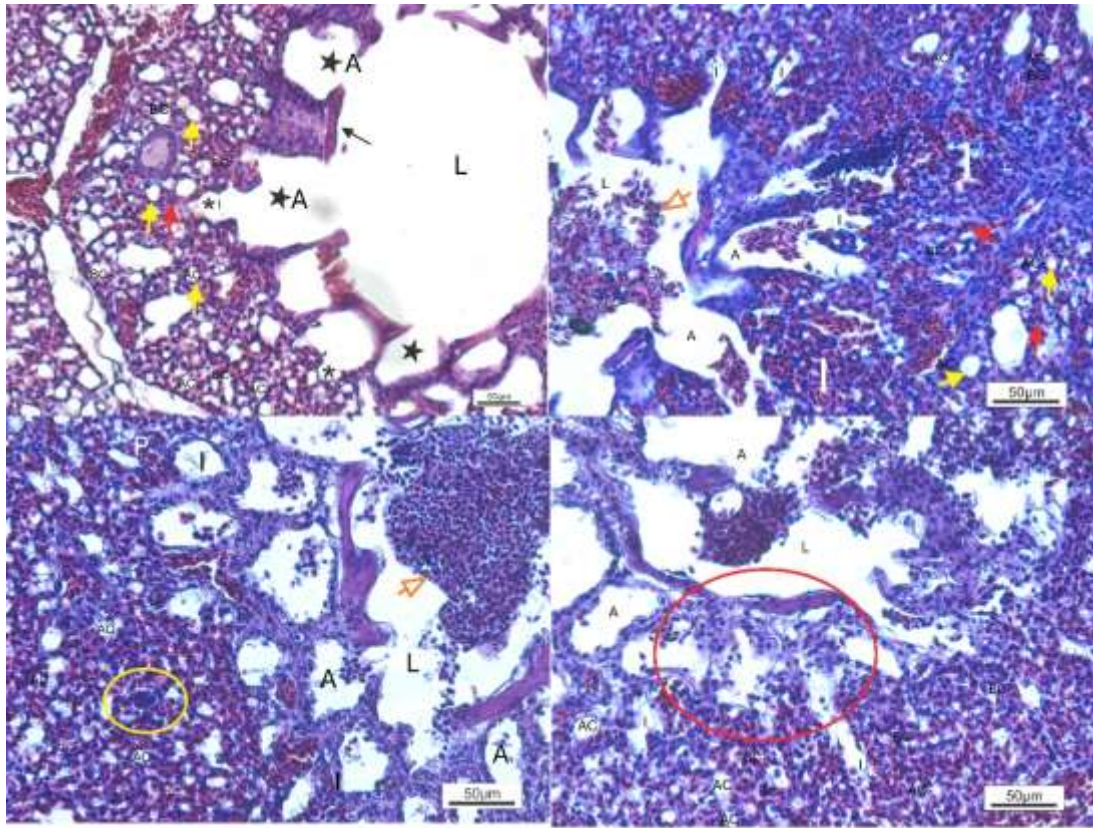


Figure 5. Representative photomicrographs of histopathological changes observed in the lungs of broiler chickens subjected to embryonic thermal manipulation, examined during the second week of life. (A) Identification of key parabronchial structures, including atria (★), infundibula (*), atrial smooth muscle (black arrow), air capillaries (yellow arrows), and blood capillaries (red arrows). (B) Blood extravasation into the luminal space (orange arrow with open arrowhead), air capillaries (yellow arrows), and blood capillaries (red arrows). (C) Accumulation of macrophages in the pulmonary parenchyma (yellow circle). (D) Area of parabronchial parenchymal degeneration, characterized by tissue disorganization and loss of the normal architecture of the conducting areas. Histological sections stained with hematoxylin and eosin (H&E). 20× and 40× objectives. Scale bar = 50 μm. Abbreviations: L, parabronchial lumen; A, atrium; I, infundibulum; AC, air capillary; BC, blood capillary; BV, blood vessel.

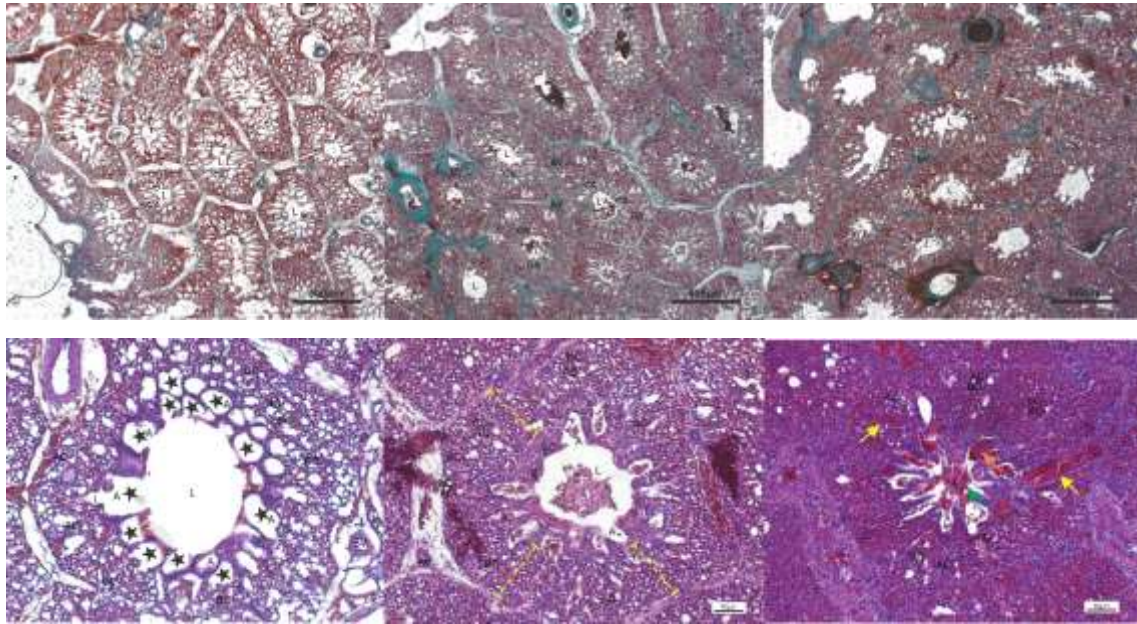


Figure 6. Photomicrographs of histological sections of the parabronchus from 15-day-old broiler chickens subjected to embryonic thermal manipulation. (A) Group T1 (control, 37.5°C), (B) Group T2 (38.5°C), and (C) Group T3 (39.0°C), showing the general organization of the parabronchial parenchyma in sections stained with Masson-Goldner trichrome. (D) Detail of a Group T1 parabronchus, highlighting conducting structures (★). (E) Detail of a Group T2 parabronchus, showing the gas-exchange region (yellow line). (F) Detail of a Group T3 parabronchus, highlighting blood extravasation in the parabronchial parenchyma (yellow arrows). Blood edema in the atria (orange arrow) and a large volume of blood cells in the parabronchial lumen (green arrow). Histological sections stained with hematoxylin-eosin (HE). Photomicrographs taken with a 5x objective; scale bars = 500 μm (A–C). Images taken with a 20x objective; scale bars = 100 μm (D–F). Abbreviations: L, parabronchial lumen; A, atrium; I, infundibulum; AC, air capillary; BC, blood capillary; BV, blood vessel.

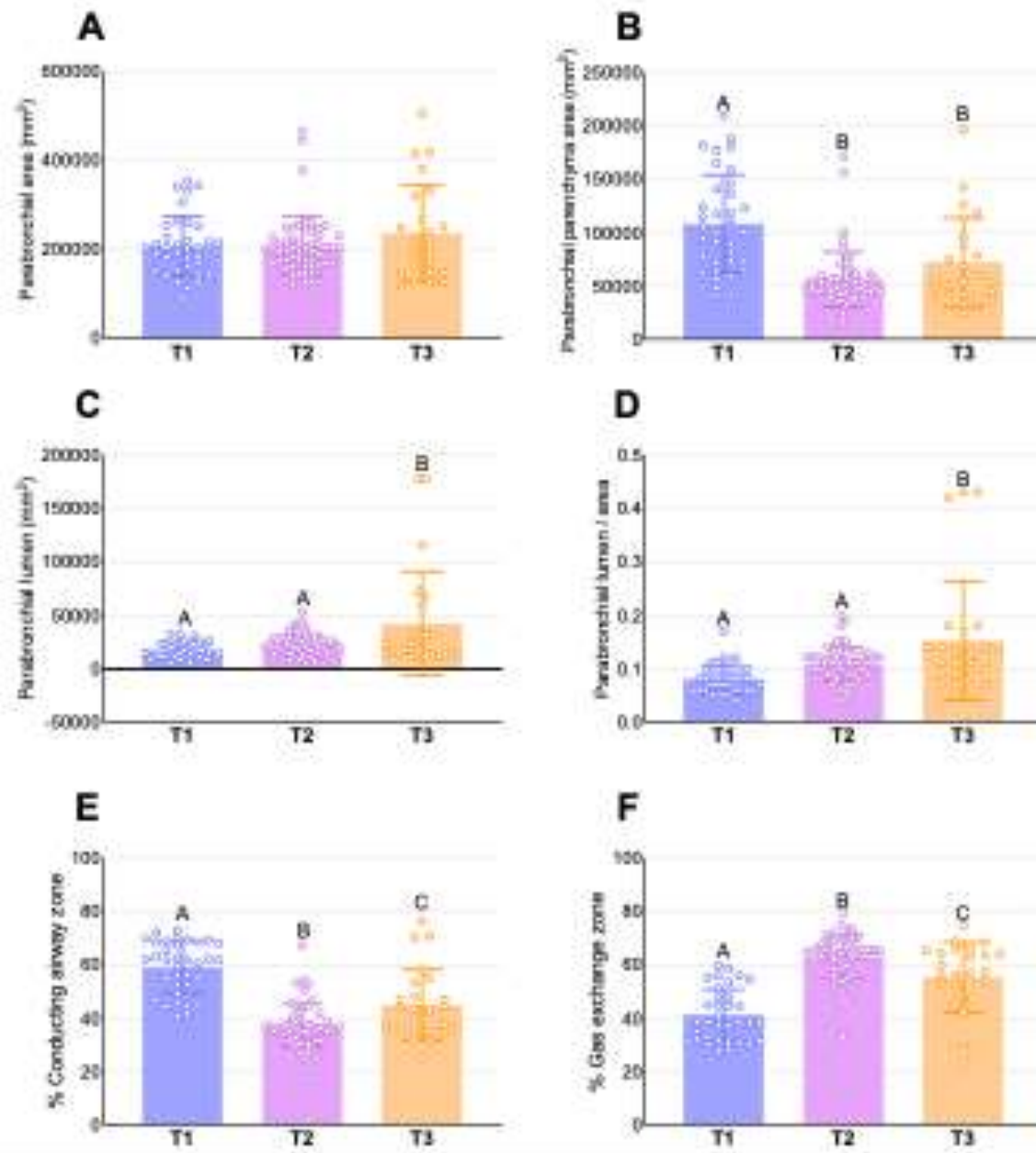


Figure 7. Morphometric parameters of parabronchi at 15 days of life subjected to embryonic thermal manipulation. Shown were (A) total parabronchial area (mm²), (B) parabronchial parenchyma area (mm²), (C) parabronchial luminal area (mm²), (D) lumen/total area ratio, (E) percentage of air-conducting area, and (F) percentage of gas-exchange area. T1 = control (37.5°C); T2 = 38.5°C; T3 = 39.0°C. Data are presented as mean $\pm$ SD. Different upper-case letters indicate significant differences among incubation temperature treatments ($P < 0.05$).

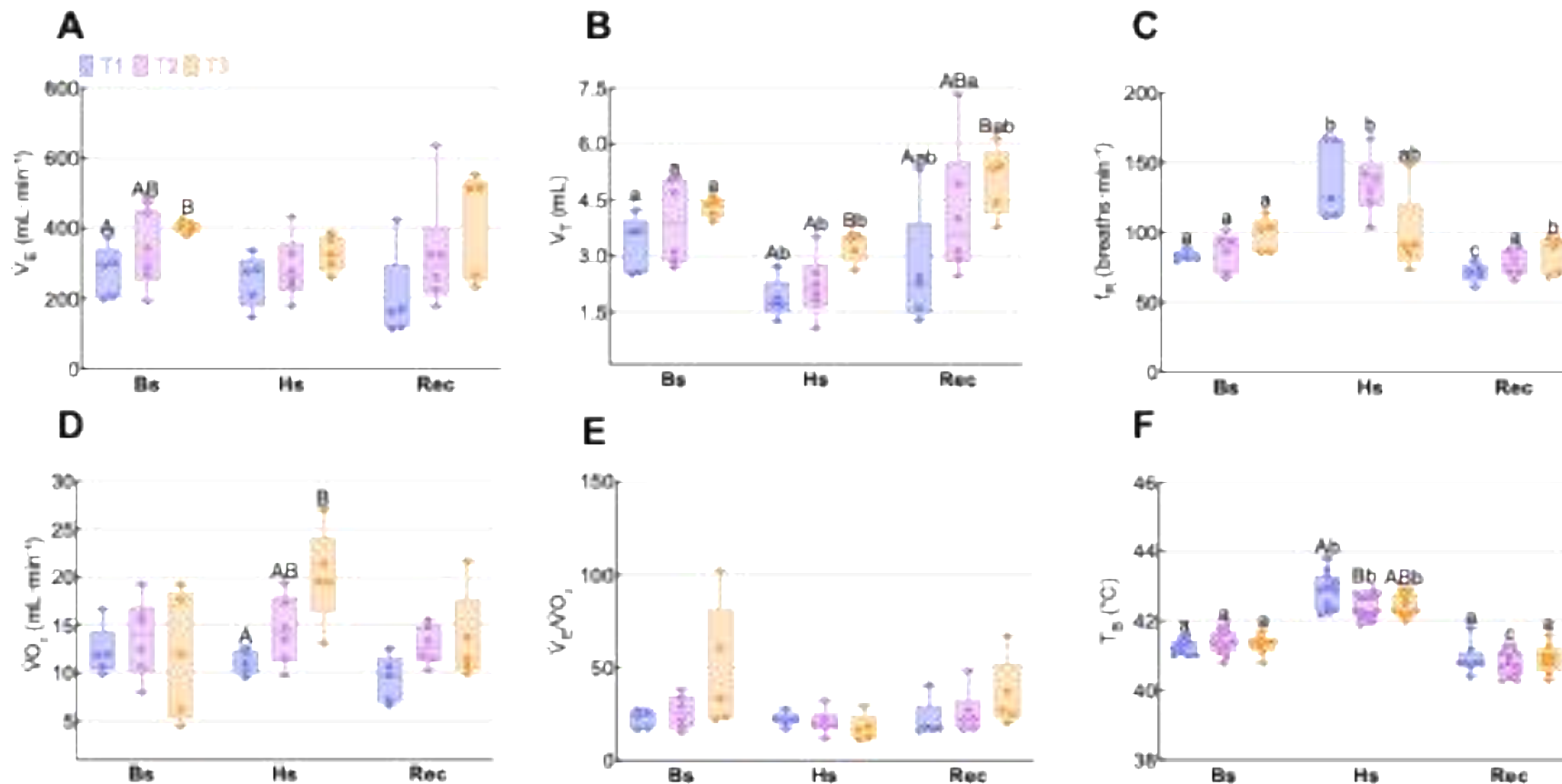


Figure 8: Ventilatory, metabolic, and thermal parameters of chicks on the 15th day of life subjected to embryonic thermal manipulation, evaluated under basal conditions (Bs), during thermal stress (Hs), and during the thermal stress recovery period (Rec). The following are presented: (A) minute ventilation ($\dot{V}_E$, mL·min⁻¹), (B) tidal volume (V_T), (C) respiratory rate (f_R), oxygen consumption ($\dot{V}O_2$), (E) ventilatory equivalent ($\dot{V}_E/\dot{V}O_2$), and body temperature (T_B). T1 = control (37.5°C); T2 = 38.5°C; T3 = 39.0°C. Boxplots represent the median, quartiles, and range of the data. Data are presented as mean $\pm$ SD. Different uppercase letters indicate significant differences among incubation temperature treatments, whereas different lowercase letters indicate significant differences among Bs, Hs, and Rec conditions within the same group ($p < 0.05$).

Table 1. Body mass and relative lung weight of embryos at day 17 of incubation following embryonic thermal manipulation

Variable	T1	T2	T3	<i>p</i>
Body mass (g)	28.13 ± 3.95	25.27 ± 3.56	24.46 ± 1.54	0.3449
Relative lung weight (%)	0.577 ± 0.276	0.87 ± 0.28	0.54 ± 0.06	0.1600

Data are presented as mean ± SD. T1 = control (37.5°C); T2 = 38.5°C; T3 = 39.0°C.

Table 2. Body mass of chicks on the first day post-hatch following embryonic thermal manipulation

Variable	T1	T2	T3	<i>p</i>
Body mass (g)	47.97 ± 3.06 ^{ab}	48.29 ± 3.91 ^a	45.95 ± 3.66 ^b	0.0141

Data are presented as mean ± SD (n = 36, 35, and 37 for T1, T2, and T3, respectively). Different superscript letters indicate significant differences among treatments according to Tukey's multiple-comparison test ($p < 0.05$). T1 = control (37.5°C); T2 = 38.5°C; T3 = 39.0°C

Table 3. Body mass and relative lung weight of 15-day-old chicks following embryonic thermal manipulation

Variable	T1	T2	T3	<i>p</i>
Body mass (g)	242.58 ± 57.53	281.78 ± 73.05	282.71 ± 69.40	0.1971
Peso pulmonar relativo (g)	0.734 ± 0.114	0.724 ± 0.173	0.677 ± 0.128	0.5409

Data are presented as mean ± SD. T1 = control (37.5°C); T2 = 38.5°C; T3 = 39.0°C.

Table 4. Mitochondrial respiration and reactive oxygen species (ROS) production in lung tissue of 15-day-old broiler chickens subjected to embryonic thermal manipulation.

Parameter	T1	T2	T3	p
Leak (L)	0.0129 ± 0.0034	0.0060 ± 0,0034	0.0086 ± 0.0055	0.0736
CI	0.0058 ± 0.0032	0.0063 ± 0,0014	0.0092 ± 0.0053	0.2939
OXPHOS-CI	0.0035 ± 0.0031	0.0083 ± 0,0058	0.0044 ± 0,0032	0.1945
CI+II	0.0033 ± 0.0033	0.0061 ± 0,0033	0.0062 ± 0,0041	0.4390
ROS-L	0.0042 ± 0.0015	0.0013 ± 0,0006	0.0027 ± 0,0025	0.0645
ROS-CI	0.0003 ± 0.0002	0.0002 ± 0,0001	0.0003 ± 0,0001	0.8514
ROS-C	0.0002 ± 0.0002	0.0004 ± 0,0003	0.0002 ± 0,0001	0.1977
ROS-CI+II	0.0001 ± 0.0001	0.0003 ± 0,0003	0.0005 ± 0,0004	0.3400

Values are expressed as mean ± standard deviation. T1 = control (37.5°C); T2 = 38.5°C; T3 = 39.0°C. Respiratory fluxes (Leak [L], CI, OXPHOS-CI, and CI+II) are expressed as pmol O₂·mg⁻¹·s⁻¹. Leak (L) = non-phosphorylating respiratory state; CI = respiratory flux supported by Complex I substrates; OXPHOS-CI = Complex I-dependent phosphorylating oxidative capacity; CI+II = maximum oxidative capacity supported by Complexes I and II. Reactive oxygen species production (ROS-L, ROS-CI, ROS-CIV, and ROS-CI+II) is expressed as pmol H₂O₂·mg⁻¹·s⁻¹. ROS-L, ROS-CI, ROS-CIV, and ROS-CI+II represent reactive oxygen species production in the respective respiratory states. No significant differences were observed among treatments (p > 0.05).

CAPÍTULO 3- EMBRYONIC THERMAL CONDITIONING MODULATES HEPATIC STRUCTURE IN BROILER CHICKENS

Embryonic thermal manipulation (ETM) has been proposed as a strategy to modulate heat tolerance in broiler chickens; however, its effects on liver development and function remain incompletely understood. This study evaluated the effects of late ETM on the morphology, histopathology, oxidative stress, and liver function of broiler chickens during embryonic development and the early post-hatch phase. Fertile eggs were incubated under three thermal conditions between days 16 and 19 of incubation, for 6 h/day: control (T1; 37.8 °C), moderate ETM (T2; 38.5 °C), and intense ETM (T3; 39.0 °C). Liver samples were collected at 17 days of incubation and at 15 days of age for morphometric, histopathological, and biochemical analyses. Exposure to 38.5°C promoted moderate hepatic remodeling during embryogenesis, characterized by morphological alterations of hepatocytes without evidence of severe tissue damage. In contrast, exposure to 39.0°C induced an acute histopathological response including hepatocellular degeneration, inflammatory infiltrate, vascular congestion, and focal areas of necrosis. At 15 days of age, birds in group T2 showed an increase in the nuclear area of hepatocytes, indicating persistent cellular adaptations, while group T3 maintained signs compatible with hepatic impairment. Oxidative stress markers showed discrete responses to the thermal treatments. Taken together, the results demonstrate that the hepatic response to embryonic thermal manipulation depends on the intensity of the applied stimulus. While exposure to 38.5°C induced morphofunctional adaptations consistent with physiological remodeling, exposure to 39.0°C led to persistent liver damage. These findings demonstrate that the liver is a target organ sensitive to embryonic thermal programming and reinforce the importance of carefully defining thermal manipulation protocols during incubation.

Keywords: embryonic thermal manipulation; liver; developmental programming; broiler chickens; heat stress.

1. Introduction

Embryonic thermal manipulation (ETM) involves the controlled exposure

of eggs to temperatures above the standard during critical periods of incubation to induce persistent physiological adaptations after hatching (PIESTUN et al., 2008; PIESTUN; HALEVY; YAHAV, 2008; YAHAV; MCMURTRY, 2001). The concept is grounded in developmental plasticity, which holds that environmental stimuli applied during sensitive developmental windows can promote lasting phenotypic adjustments that influence the structure and function of organs throughout life (BARKER, 2004; GLUCKMAN, HANSON, 2004).

Perinatal epigenetic programming is a key mechanism by which environmental changes during embryogenesis modify gene expression patterns and developmental trajectories without altering the DNA sequence (DAVID et al., 2019; TZSCHENTKE; BASTA, 2002a, 2002b). Among the mechanisms proposed to explain the effects of thermal manipulation, the following stand out: modulation of heat shock protein expression, neuroendocrine alterations, and epigenetic adaptations that increase responsiveness to subsequent thermal challenges (AL-ZGHOUL et al., 2015; AL-ZGHOUL; SUKKER; ABABNEH, 2019; TZSCHENTKE, 2007; YAHAV et al., 2004; YAHAV; RATH; SHINDER, 2004). Previous studies have demonstrated that MT can improve post-hatch thermotolerance, modulate muscle growth, alter the hormonal profile, and influence the energy metabolism of broiler chickens (COLLIN et al., 2007; LOYAU et al., 2013, 2014a, 2014b; PIESTUN; YAHAV; HALEVY, 2011). More recently, transcriptomic evidence indicates that MT at 38.5°C promotes subtle yet persistent changes in the hepatic expression profile, with a reorganization of structural, cell-signaling, and ion-transport pathways associated with thermal adaptation (AL-ZGHOUL et al., 2026).

The liver is the primary metabolic organ in birds, responsible for lipid, carbohydrate, and protein metabolism, plasma protein synthesis, and regulation of cellular redox balance (ZAEFARIAN et al., 2019). During embryonic development, this organ plays a central role in mobilizing and processing lipids from the yolk sac, which provide the energy required for fetal growth and preparation for hatching (COGBURN et al., 2018; SPEAKE; MURRAY; C. NOBLE, 1998; UNI et al., 2005). The final phase of incubation is marked by intense hepatic metabolic remodeling, characterized by activation of β -oxidation pathways, gluconeogenesis, and utilization of vitelline nutrients—processes essential for the transition from embryonic to post-hatching life (YADGARY et al.,

2010; YADGARY; UNI, 2012; YADGARY; YAIR; UNI, 2011). Therefore, alterations in the thermal environment during this critical period can permanently modify hepatic development and function.

Post-hatch heat stress is known to induce metabolic and structural changes in the liver of broiler chickens, including impaired mitochondrial function, increased production of reactive oxygen species, lipid peroxidation, hepatic steatosis, and elevated serum activities of enzymes indicative of hepatocellular injury (AKBARIAN et al., 2016; EMAMI et al., 2021; HABASHY et al., 2017; MA et al., 2021; NAWAB et al., 2018; TANG et al., 2022; TEYSSIER et al., 2022, 2023). Considering the liver's importance in energy homeostasis and physiological adaptation to heat, it is plausible that embryonic thermal manipulation directly influences the structural and functional organization of this organ, thereby impacting the health and performance of the birds after hatching. This hypothesis is reinforced by studies in aquatic birds showing that changes in incubation temperature modify hepatic lipid metabolism during embryonic development (WANG et al., 2014) and influence the capacity for hepatic lipid deposition in adulthood (MASSIMINO et al., 2019).

Despite the growing use of embryonic thermal manipulation in experimental research and its potential for commercial application, most studies focus on productive, thermoregulatory, and molecular parameters, leaving a limited understanding of its effects on liver structure and function throughout development. Studies integrating morphological, morphometric, histopathological, oxidative, and biochemical assessments of the liver from the embryonic stage through the post-hatching period, as well as those comparing different intensities of thermal stimulation during the final stage of incubation, are scarce (DAVID et al., 2019; LOYAU et al., 2013, 2014a; PIESTUN et al., 2009). Given this context, the present study aimed to evaluate the effects of embryonic thermal manipulation at 38.5°C and 39°C on morphometry, histopathology, oxidative stress, and liver enzymes in broiler embryos and chicks, to understand how different intensities of thermal stimulation modulate liver development and function during the perinatal period.

2. Material and Methods

2.1. Experimental design and embryonic thermal manipulation

Fertile Cobb® CDP4 broiler eggs were incubated in automatic incubators (Premium Ecologica®, Belo Horizonte, MG, Brazil) at a standard temperature of 37.5°C and 60% relative humidity, with automatic turning every two hours. Experiments were conducted in accordance with CONCEA (National Council for Animal Care Control, Brazil) and approved by the local animal care committee (CEUA – Comissão de Ética no Uso de Animais – FCAV-UNESP; Protocol: 9487339468/2025).

The eggs were distributed across three heat-treatment temperatures: control (T1), 37.5°C; mild heat stress (T2), 38.5°C; and severe heat stress (T3), 39°C. Heat manipulation was applied between days 16 and 19 of incubation, a critical period for the maturation of the avian respiratory system (Maina 2003; Buggren and Elmonoufy 2017; Maina 2023). Groups T2 and T3 were exposed daily to their respective temperatures for 6 consecutive hours (from 12 pm to 6 pm), whereas the control group remained at the conventional incubation temperature. On the 17th and 19th embryonic days, 10 eggs from each treatment were removed from the incubator, and the embryos were retrieved. The livers were collected, weighed to determine relative weight, and processed for histological analysis.

2.2 Acute thermal challenge protocol at 15 days

At 15 days of age, the animals were subjected to an acute thermal challenge in a climate-controlled chamber at 35°C for 1 hour. They were then euthanized to collect blood and liver samples for histopathological, morphometric, and biochemical analyses.

2.3. Hepatic histological processing and evaluation

Liver fragments were fixed in 10% buffered formalin for 48 hours. After fixation, the samples underwent conventional dehydration in increasing concentrations of ethanol, clearing in xylene, and embedding in histological paraffin. Serial sections of 5 µm thickness were obtained using a rotary microtome and subsequently stained with hematoxylin and eosin (H&E). The slides were analyzed using an optical microscope coupled to a digital image capture system.

Histopathological evaluation was performed using a double-blind method

to assess the integrity of the hepatic parenchyma, the organization of hepatocyte cords, vascular alterations, the presence of an inflammatory infiltrate, cellular degeneration, cytoplasmic vacuolization, and areas of necrosis. Observations were recorded and compared across experimental treatments.

Morphometric analyses were performed using ImageJ® software (National Institutes of Health, USA). For each animal, random images of the liver parenchyma were captured at 40x magnification. The following parameters were evaluated: liver cell density (cells/mm²); nuclear area of hepatocytes (µm²); and cytoplasmic area of hepatocytes (µm²).

2.4. Evaluation of hepatic oxidative stress and inflammatory activity

Liver fragments were collected immediately after euthanasia and stored at -80°C until analysis. For biochemical determinations, samples were homogenized in phosphate buffer (50 mM, pH 7.4) with a mechanical homogenizer, then centrifuged at 10,000 × g for 15 minutes at 4°C. Supernatants were used to assess oxidative stress and enzymatic activity. Hepatic lipid peroxidation was quantified by measuring thiobarbituric acid-reactive substances (TBARS) using the method of Ohkawa et al. (1979). The assay is based on the reaction between malondialdehyde (MDA), the main product of lipid peroxidation, and thiobarbituric acid, forming a chromogenic complex measured spectrophotometrically. Results were expressed as nmol of MDA per mg of protein. Myeloperoxidase (MPO) activity served as an indirect marker of inflammatory cell infiltration and oxidative activity in tissues. The determination was performed using a methodology adapted from Bradley et al. (1982), which involves oxidation of the o-dianisidine substrate with hydrogen peroxide. Enzymatic activity was determined spectrophotometrically and expressed as MPO units per mg of protein. The total protein concentration of the homogenates was determined by the Bradford method (1976), using bovine serum albumin as a standard, for subsequent normalization of the results.

2.5. Determination of tissue liver enzymes

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured using commercial kits from Merck® (Alanine Aminotransferase Activity Assay Kit, MAK052, and Aspartate Transaminase

Assay Kit, MAK467; Sigma-Aldrich, St. Louis, MO, USA) following the manufacturer's instructions. ALT catalyzes the transfer of an amino group from alanine to α -ketoglutarate, forming pyruvate and glutamate, while AST catalyzes a similar reaction using aspartate as the amino donor. Both enzymes served as indicators of hepatocellular integrity and liver metabolism. Readings were obtained with an automated spectrophotometer, and results were expressed in units per liter (U/L).

2.6. Serum biochemistry

After blood collection, samples were centrifuged at 3,000 rpm for 10 minutes to obtain serum, which was stored at -80°C until analysis. Serum levels of ALT, AST, gamma-glutamyltransferase (GGT), creatinine, creatine kinase (CK), uric acid, and albumin were determined by enzymatic and colorimetric methods using commercial Merck® kits validated for veterinary use. Serum activities of ALT, AST, and GGT were used as indicators of liver function and hepatocellular integrity. Creatinine was used as an indirect marker of renal function and muscle metabolism, while creatine kinase was used as an indicator of muscle damage. Uric acid was used as a marker of nitrogen metabolism and systemic antioxidant capacity in birds. Serum albumin was used as an indicator of hepatic synthetic capacity and the overall metabolic status of the animals. All analyses were performed on a semi-automated biochemical analyzer.

2.7. Statistical Analysis

Statistical analyses were conducted in R (R Core Team, version 4.6.0). The packages readxl, dplyr, tidyr, stringr, purrr, and tibble were used for data import and organization. The packages ggplot2, ggpubr, scales, flextable, and officer were used to create tables and graphs. Statistical tests and effect measures included car (Levene's test), rstatix (Welch's ANOVA, Games-Howell, Dunn's test, Kruskal-Wallis, Cramer's V, nonparametric effect sizes), effsize/effectsize (Cohen's d, η^2), nortest (Lilliefors' test), outliers (auxiliary routines for detecting extreme values), and basic R procedures (Shapiro-Wilk, Student's t-test, Mann-Whitney/Wilcoxon, chi-square, Fisher's exact test). Results were considered significant at $p < 0.05$.

3. Results

3.1. Embryonic phase

3.1.1. Relative liver weight at 17 days of incubation

At 17 days of incubation, relative liver weight did not differ among treatments (Welch's ANOVA, $F_{2,85} = 1.733$; $p = 0.2705$). Embryos in group T1 had a relative liver weight of $2.25 \pm 0.27\%$, whereas T2 and T3 groups had values of $1.97 \pm 0.22\%$ and $2.20 \pm 0.08\%$, respectively. Although no statistically significant differences were observed among groups in multiple comparisons ($p > 0.05$), a large overall effect size was observed ($\eta^2 = 0.3078$).

3.1.2. Embryonic hepatic morphometry after thermal manipulation

Figure 1 shows a photomicrograph from a histological analysis of embryonic liver tissue at E17. At 17 days of incubation, significant differences in hepatic cell density were observed between treatments (ANOVA, $F_{2,21} = 8.61$; $p = 0.0019$; $\eta^2 = 0.450$), indicating a significant effect of thermal manipulation on the cellular organization of hepatic tissue (Figure 2A-C). T2 group showed lower cell density ($8,625 \pm 1,876$ cells/mm²) compared to T1 ($11,242 \pm 1,675$ cells/mm²; $p = 0.0132$) and T3 ($11,850 \pm 1,447$ cells/mm²; $p = 0.0038$), while T1 and T3 did not differ from each other ($p = 0.8032$) (Figure 2A). The effect sizes of the T1–T2 ($d = 1.45$) and T2–T3 ($d = 1.85$) comparisons were classified as large.

At 17 days, the area of hepatocyte nuclei differed among treatments (Kruskal–Wallis, $H = 28.80$; $p < 0.0001$) (Figure 2B). The largest values were observed in the T2 group (12.18 ± 3.62 μm^2), followed by T1 (11.77 ± 3.56 μm^2), while the T3 group had the smallest hepatocyte nuclei (10.78 ± 3.18 μm^2). Post hoc comparisons revealed a significant reduction in nuclear area in T3 compared with T1 ($p = 0.0024$) and T2 ($p < 0.0001$), whereas T1 and T2 did not differ ($p = 0.1965$) (Figure 2B). The mean area of hepatocyte cytoplasm at 17 days of incubation was influenced by embryonic thermal manipulation (Kruskal–Wallis, $H = 11.60$; $p = 0.0030$; $\epsilon^2 = 0.356$), indicating a large overall effect (Figure 2C). Embryos in T2 group showed a larger cytoplasmic area (109.25 ± 29.97 μm^2) than in T1 group (81.21 ± 11.51 μm^2 ; $p = 0.0391$) and T3 (76.75 ± 10.66 μm^2 ; $p = 0.0046$) (Figure 2C). There was no difference between T1 and T3 ($p = 1.000$). The effect sizes for T1 versus T2 ($r = 0.685$) and T2 versus T3 ($r = 0.778$) were classified as large, indicating a significant increase in the cytoplasmic area of

hepatocytes in embryos subjected to thermal manipulation at 38.5°C.

After 19 days of incubation, liver cell density showed no differences between the treatments evaluated (ANOVA, $F_{1,19} = 0.0029$; $p = 0.9578$) (Figure 2D-F). T2 and T3 groups yielded practically identical values, with averages of $8,060 \pm 1,721$ and $8,003 \pm 2,763$ cells/mm², respectively, and a negligible effect size ($\eta^2 = 0.0002$) (Figure 2D). However, the nuclear area of the hepatocytes differed among treatments (Kruskal–Wallis test, $p < 0.05$). The T2 group had a larger average nuclear area (12.03 ± 3.85 µm²) than the T3 group (11.31 ± 3.63 µm²) (Figure 2E). Although the absolute difference between the groups was slight, the analysis indicated a significant change in the distribution of nuclear values after removing two outliers using the ROUT method ($Q = 1\%$).

The mean area of hepatocyte cytoplasm did not differ between the evaluated groups (Kruskal–Wallis, $H = 0.25$; $p = 0.6171$; $\epsilon^2 = -0.026$) (Figure 2F). The observed values were 111.93 ± 28.54 µm² for the T2 group and 99.59 ± 4.84 µm² for the T3 group, with no significant difference between treatments ($p = 0.6171$). The effect size was small ($r = 0.133$), indicating that the increase observed at 17 days was not maintained until the end of the heat treatment.

3.1.3. Analysis of oxidative stress and hepatic inflammatory activity in 17-day-old embryos subjected to different incubation temperatures

The hepatic concentration of thiobarbituric acid reactive substances (TBARS) in embryos was affected by thermal manipulation during incubation (Kruskal–Wallis, $H = 7.78$; $p = 0.0205$; $\epsilon^2 = 0.578$), indicating a large overall effect. T1 and T2 groups showed similar TBARS values (0.2562 ± 0.0724 and 0.2458 ± 0.0013 nmol MDA·mg⁻¹ protein respectively), with no statistically significant difference between them ($p = 1.000$) (Figure 3A). In contrast, the T3 group showed a significant reduction in TBARS levels (0.0897 ± 0.0001), which was significantly lower than that of the T1 ($p = 0.0283$) and T2 group ($p = 0.0441$) (Figure 3A). The hepatic myeloperoxidase (MPO) activity of the embryos did not differ among the experimental groups (one-way ANOVA, $F_{2,8} = 0.066$; $p = 0.9367$; $\eta^2 = 0.0162$) (Figure 3B). The observed values were 2.8540 ± 0.7323 for T1, 2.6438 ± 0.8367 U·mg⁻¹ protein for T2, and 2.9227 ± 1.6800 for T3, with no differences detected in the multiple comparisons ($p > 0.05$) (Figure 3B).

3.2. 15 days phase

3.2.1. Relative liver weight at 15 days of life

The relative liver weight showed a trend toward an effect of thermal manipulation, but no significant difference was observed overall (Kruskal–Wallis, $H = 4.89$; $p = 0.0867$; $\epsilon^2 = 0.0688$). The mean values were $3.86 \pm 0.66\%$ for the T1 group, $3.19 \pm 0.71\%$ for the T2 group, and $3.72 \pm 0.74\%$ for the T3 group. Although the post hoc comparisons did not reach statistical significance after correction for multiple comparisons ($p > 0.05$), a numerical reduction in relative liver weight was observed in group T2 compared with the control, accompanied by a moderate effect size ($r = 0.495$).

3.2.2. Histopathological Results of the Liver of Chicks in the Early Stage

Histopathological analysis of the liver revealed qualitative differences in the organization of the hepatic parenchyma among the experimental treatments (Figure 4). In the T1 control group, the hepatic tissue showed preserved architecture, with well-organized cords of hepatocytes, well-shaped nuclei, and homogeneous parenchymal distribution (Figure 4A). Slight dilation of the hepatic sinusoids was observed, without evidence of vascular congestion, inflammatory infiltrate, or areas of necrosis, indicating maintenance of the organ's structural integrity.

In the T2 group, the overall parenchymal architecture was preserved, but foci of foamy cells were observed, primarily in the periportal region adjacent to the portal triads (Figure 4B). These changes were consistent with mild focal fatty degeneration, characterized by localized lipid accumulation in hepatic tissue. No diffuse degenerative changes, hepatocellular necrosis, or marked impairment of hepatic cord organization were observed, suggesting that the changes remained localized and of low intensity. By contrast, animals in the T3 group exhibited more pronounced histological changes (Figure 4C).

A marked increase in hepatic vascularization was observed, associated with loss of lobular organization and disruption of hepatocyte cords. Foci of tissue destruction were identified in several areas of the parenchyma, accompanied by impairment of hepatic architecture and reduced structural integrity (Figure 4C). These changes were consistent with marked hepatocellular degeneration and vascular reorganization of the parenchyma, representing the most severe

histopathological pattern observed among the treatments.

3.2.3. Liver Histomorphometry of Chicks at 15 Days of Age Subjected to Different Incubation Temperatures

Liver cell density in the second week of life was not affected by embryonic thermal manipulation at 15 days of age (ANOVA, $F_{2,48} = 0.280$; $p = 0.7572$; $\eta^2 = 0.0115$) (Figure 5A). The mean values were $6,346.95 \pm 1,852.62$ cells/mm² for the T1 group, $6,021.27 \pm 1,113.89$ cells/mm² for the T2 group, and $5,965.10 \pm 1,776.57$ cells/mm² for the T3 group. Multiple comparisons did not reveal significant differences among treatments ($p > 0.05$), indicating that hepatic cellularity was maintained regardless of incubation temperature (Figure 5A). However, the hepatocyte nuclear area differed significantly among the experimental groups (Kruskal–Wallis test, $H = 6.404$, $p = 0.0407$). Hepatocytes in the T2 group had a larger nuclear area (17.79 ± 4.46 μm^2) than those in the T1 control group (16.68 ± 4.02 μm^2), while the T3 group showed an intermediate value (17.03 ± 4.81 μm^2) (Figure 5B). Post hoc analyses showed a significant increase in nuclear area in T2 compared to T1 ($p_i = 0.0342$), whereas no differences were observed between T1 and T3 ($p_i = 0.9064$) or between T2 and T3 ($p_i = 0.2251$) (Figure 5B). The overall effect size was small ($\epsilon^2 = 0.0047$), as were the effect sizes of the pairwise comparisons ($r < 0.15$). Despite this, the results indicate a slight increase in hepatocyte nuclear area in animals subjected to thermal manipulation at 38.5°C during incubation.

At 15 days, the mean area of the hepatocyte cytoplasm was not affected by the thermal manipulation during incubation (Kruskal–Wallis, $H = 1.01$; $p = 0.6048$; $\epsilon^2 = -0.010$) (Figure 5C). The mean values were 159.61 ± 51.09 μm^2 for T1, 147.36 ± 23.48 μm^2 for T2, and 167.31 ± 54.24 μm^2 for T3. Multiple comparisons showed no differences among treatments ($p > 0.05$), with only small or negligible effect sizes ($r \leq 0.152$).

3.2.4. Biochemical, oxidative, and inflammatory profile of the liver at 15 days of age

At two weeks of age, no significant differences were observed in hepatic TBARS levels among treatments (Kruskal–Wallis test, $H = 2.22$; $p = 0.3303$, $\epsilon^2 = 0.0144$) (Figure 6A). The mean values were 0.2403 ± 0.1098 for T1, $0.1330 \pm$

0.0215 for T2, and 0.2454 ± 0.1604 for T3, indicating no persistent effect of thermal manipulation on hepatic lipid peroxidation in the post-hatch phase. Similarly, at 15 days of age, hepatic MPO activity did not differ among treatments (one-way ANOVA, $F_{2,11} = 1.639$; $p = 0.2383$; $\eta^2 = 0.2296$) (Figure 6B). Groups T1, T2, and T3 had mean values of 6.2649 ± 3.0927 , 3.2326 ± 0.0001 , and 5.2795 ± 2.0560 , respectively. Despite the relatively high overall effect size, post hoc comparisons did not reveal significant differences among groups ($p > 0.05$), indicating that embryonic thermal manipulation did not alter hepatic neutrophilic inflammatory activity as assessed by MPO.

Embryonic thermal manipulation significantly affected liver enzyme activity measured at 15 days of age (Figure 6C). For hepatic ALT, a significant treatment effect was observed ($F_{2,29} = 6.79$; $p = 0.0038$), with a large effect size ($\eta^2 = 0.319$). The highest values were observed in the T3 group (88.55 ± 7.89 U/L), followed by T2 (85.00 ± 8.63 U/L) and T1 (78.75 ± 5.54 U/L). Post hoc analysis revealed a significant increase in hepatic ALT in the T3 group compared to the T1 group ($p = 0.0031$), whereas the T1 and T2 groups ($p = 0.1983$) and the T2 and T3 groups ($p = 0.6122$) did not differ (Figure 6C). The effect size for the comparison between T1 and T3 was large ($d = 1.49$). A similar pattern was observed for hepatic AST, which showed a strong treatment effect ($F_{2,30} = 22.12$; $p < 0.0001$; $\eta^2 = 0.596$) (Figure 6D). The heat-treated groups showed higher enzymatic activities than the control, with averages of 68.00 ± 5.34 U/L for T2, 67.64 ± 4.86 U/L for T3, and 54.94 ± 5.95 U/L for T1 (Figure 6D). Multiple comparisons showed significant differences between T1 and T2 ($p = 0.0002$) and between T1 and T3 ($p < 0.0001$), whereas there was no difference between the groups subjected to the two elevated temperatures ($p = 0.9918$). The effect sizes observed for T1 versus T2 ($d = 2.24$) and T1 versus T3 ($d = 2.28$) were very large.

3.2.5. Biochemical profile of chicks in the early stages

Heat treatment during incubation resulted in discrete adjustments in the serum biochemical profile of the chickens evaluated in the second week post-hatch (Table 1). Among the parameters analyzed, only the serum activity of aspartate aminotransferase (AST) was influenced by the treatments. The T1 control group had higher AST values (457.20 ± 102.08 U/L) than the groups subjected to heat manipulation, with averages of 293.67 ± 111.31 U/L in T2 and

299.20 ± 39.01 U/L in T3. Post hoc analysis revealed a significant difference between T1 and T3 ($p = 0.0365$), whereas the comparison between T1 and T2 showed a trend towards significance ($p = 0.0601$). The overall treatment effect was high ($\eta^2 = 0.521$). For the other biochemical markers, no significant differences were observed between treatments ($p > 0.05$). Alanine aminotransferase (ALT) values were 78.20 ± 57.36 U/L at T1, 128.67 ± 131.79 U/L at T2, and 44.40 ± 13.24 U/L at T3 ($p = 0.2976$). Serum gamma-glutamyl transferase (GGT) activity was 13.90 ± 1.75 U/L, 12.19 ± 1.08 U/L, and 14.14 ± 3.10 U/L for T1, T2, and T3, respectively ($p = 0.5013$). Similarly, serum creatinine levels remained similar between groups, with mean values of 0.324 ± 0.220 mg/dL at T1, 0.337 ± 0.107 mg/dL at T2, and 0.374 ± 0.114 mg/dL at T3 ($p = 0.8856$). Serum creatine kinase (CK) activity showed mean values of 2.35 ± 0.02 U/L at T1, 2.56 ± 0.46 U/L at T2, and 2.98 ± 0.74 U/L at T3, with no differences between treatments ($p = 0.364$). Serum uric acid levels also remained unchanged, corresponding to 2.62 ± 0.23 mg/dL at T1, 2.73 ± 0.45 mg/dL at T2, and 2.64 ± 0.40 mg/dL at T3 ($p = 0.9047$). For albumin, after excluding two outliers using the ROUT method ($Q = 1\%$), concentrations were 9.60 ± 1.00 g/dL at T1, 10.46 ± 1.41 g/dL at T2, and 10.02 ± 0.04 g/dL at T3, with no significant treatment effect ($p = 0.5249$).

Discussion

In our study, the heat treatment applied during the final stage of incubation altered the hepatic morphophysiology of embryos and early-stage chicks, consistent with previous studies (AL-ZGHOUL et al., 2026; ANDRIEUX et al., 2023; and LI et al., 2024) that demonstrated that the liver is a primary target organ for programming induced by the embryonic thermal environment. This response has been observed in domestic birds, wild birds, and mammals alike (LEBIGRE et al., 2025; JARRETT et al., 2025; TAO et al., 2018; HE et al., 2019; FRONTIERS IN VETERINARY SCIENCE, 2026).

Studies across species indicate that the embryonic liver is highly responsive to the thermal environment, with the magnitude of the response depending on the intensity and duration of the thermal stimulus. These responses range from alterations in mitochondrial metabolism in quail (JARRETT et al., 2025) and changes in the expression of genes involved in oxidative stress and

metabolism in chickens (AL-ZGHOUL et al., 2019, 2026; NCHO et al., 2022, 2023) to morphological and epigenetic remodeling in mammals (TAO et al., 2018). Consistent with these findings, the present study demonstrates that embryonic thermal manipulation induces cellular, biochemical, and histopathological alterations in the liver of broiler chickens without affecting overall liver size. This dissociation between structural and functional changes suggests that embryonic thermal programming primarily influences tissue organization and cellular physiology rather than gross organ growth. Similar observations have been reported in poultry, where embryonic thermal manipulation did not alter liver weight or relative liver weight in laying-type chickens (TONG et al., 2022; XU et al., 2024) or broiler chickens (LEKSRI SOMPONG et al., 2007a). Together, these findings support the view that liver morphometric parameters are relatively insensitive indicators of embryonic thermal programming, whereas histological, biochemical, and molecular markers provide a more sensitive assessment of hepatic adaptations, as also highlighted in the review by Khalil et al. (2024).

However, the persistence and direction of hepatic responses to embryonic thermal manipulation remain inconsistent across studies, suggesting that the magnitude of these effects depends on factors such as species, sex, developmental stage, and the intensity and duration of the thermal stimulus (AL AMAZ et al., 2024; KHALIL et al., 2024; NCHO et al., 2022, 2023). In the present study, after 12 h of thermal treatment (E17), embryos incubated at 38.5°C exhibited reduced hepatic cell density and increased hepatocyte cytoplasmic area. This pattern is consistent with hepatocellular hypertrophy, in which cellular expansion occurs without a proportional increase in cell number, resulting in lower nuclear density per unit tissue area (HALL et al., 2012; MIYAOKA et al., 2012). In metabolically active tissues, hepatocyte hypertrophy is commonly associated with enhanced biosynthetic capacity, increased mitochondrial content, and the accumulation of intracellular energy reserves (BEGRICHE et al., 2011). Such adaptations are particularly relevant during late embryogenesis, when the avian liver undergoes rapid functional maturation and plays a central role in processing and redistributing yolk-derived lipids to support the high energetic demands preceding hatch (MORAN, 2007). Consequently, an increase in cytoplasmic volume likely reflects an expansion of the organelle compartment,

particularly mitochondria and endoplasmic reticulum, thereby enhancing the cell's metabolic and synthetic capacity (DEGLI ESPOSTI et al., 2012; MIETTINEN & BJÖRKLUND, 2016; SHARMA et al., 2024).

The concomitant increase in hepatocyte nuclear area further supports this interpretation. Nuclear size is widely recognized as a morphometric indicator of cellular functional status, with enlarged nuclei typically exhibiting a higher proportion of decondensed euchromatin and increased transcriptional activity (Bhatt et al., 2024; Junatas et al., 2017; Schmidt & Schibler, 1995; Webster et al., 2009). Together, the simultaneous enlargement of the cytoplasmic and nuclear compartments in embryos incubated at 38.5°C suggests that moderate thermal manipulation of embryos accelerated hepatocyte differentiation and functional maturation rather than inducing pathological hypertrophy. This interpretation is consistent with previous reports demonstrating that controlled thermal manipulation enhances hepatic metabolic programming and promotes the expression of genes involved in oxidative metabolism and cellular adaptation during the final stages of embryogenesis (AL AMAZ et al., 2024; AL-ZGHOUL et al., 2026; KHALIL et al., 2024).

In contrast, exposure to 39°C resulted in a reduction in nuclear area compared with the other groups, indicating that higher temperatures can trigger distinct cellular responses, possibly associated with energy conservation mechanisms, limitations on cell growth, or chromatin reorganization in response to thermal stress (RICHTER et al., 2010; GASCH et al., 2003), or even deleterious effects (GONZÁLEZ-REIMERS *et al.*, 1987; MACKEL *et al.*, 2020).

By day 19 of incubation, the differences in cell density and cytoplasmic area were no longer present, leaving only subtle nuclear alterations; this indicates the presence of compensatory mechanisms during the final phase of incubation that can partially restore tissue organization prior to hatching, even after a cumulative 24 hours of thermal stress. Hepatic tissue plasticity and the capacity for morphological normalization in response to transient stimuli have been documented in both avian and mammalian models (MIYAOKA et al., 2012; LOPES et al., 2016). A similar phenomenon has been described in studies involving embryonic thermal manipulation, where early-induced structural alterations tend to partially normalize by the end of embryonic development (LOYAU et al., 2013, 2014a, 2014b; PIESTUN et al., 2011).

The results regarding oxidative stress reinforce this hypothesis of physiological adjustments. At 19 days of incubation, embryos in the 39°C group exhibited a significant reduction in hepatic TBARS levels, indicating lower lipid peroxidation intensity; given that embryonic metabolism increases progressively during the final incubation phase, accompanied by an exponential rise in oxygen consumption and heat production (LOURENS et al., 2007), one would expect elevated temperatures to result in increased production of reactive oxygen species (Yang et al., 2010; Mujahid et al., 2007). However, the observed reduction in TBARS levels reveals the activation of compensatory antioxidant responses induced by prior thermal exposure. Several studies demonstrate that moderate thermal challenges during incubation can stimulate adaptive responses involving heat shock proteins (HSPs), antioxidant enzymes such as SOD, CAT, and GPx, and mitochondrial protection mechanisms, thereby enhancing cellular resistance to subsequent oxidative insults (AL-ZGHOUL; EL-BAHR, 2019; NCHO et al., 2022, 2023; PIESTUN et al., 2008; SALEH; TARKHAN; AL-ZGHOUL, 2020; YAHAV et al., 2004). This antioxidant modulation induced by thermal manipulation has been documented at the hepatic level, showing reduced NOX4 expression and modulation of CAT, SOD2, and GPx genes in chickens subjected to thermal manipulation followed by a heat challenge (AL-ZGHOUL; EL-BAHR, 2019; KHALIL et al., 2024; NCHO et al., 2022, 2023). Additionally, the increased expression of HSP70 and HSP90 in embryonic tissues following thermal exposure has been associated with mitochondrial protection and the attenuation of lipid peroxidation (VINOTH et al., 2015; YAHAV et al., 2004). The absence of changes in myeloperoxidase (MPO) activity in both embryos and early-stage chicks indicates that the thermal treatments did not trigger a significant hepatic inflammatory response. MPO is an enzyme predominantly expressed in activated neutrophils, and its elevated activity in hepatic tissue is considered a marker of leukocyte infiltration and active neutrophilic inflammation (JAESCHKE; SMITH, 1997; KLEBANOFF, 2005; RENSEN et al., 2009). The lack of an increase in hepatic MPO across all groups indicates that the observed morphological and metabolic changes were not accompanied by recruitment of inflammatory cells. This is consistent with physiological remodeling responses, in which structural and functional modifications occur through cellular plasticity adjustments independent of the inflammatory cascade (JAESCHKE, 2006; RAMADORI et al.,

2008).

By the second week of life, many of the structural reorganizations of hepatocytes quantified during embryogenesis were no longer detectable. At 15 days of age, hepatocyte cell density and cytoplasmic area remained similar across treatments, indicating that embryonic structural remodeling did not produce permanent alterations to hepatic architecture. These results are consistent with those reported by Li et al. (2024) and Wang et al. (2014). In contrast, other studies report that embryonic thermal manipulation can induce long-lasting functional and transcriptional hepatic changes (AL-ZGHOUL et al., 2026; LOYAU et al., 2013, 2014a, 2014b), suggesting that the persistence or reversal of effects depends on the intensity and duration of the thermal stimulus, as well as the parameters evaluated. We quantified a slight but significant increase in hepatocyte nuclear area in animals incubated at a 1°C higher temperature; this aligns with the concept of perinatal epigenetic programming, whereby environmental stimuli applied during critical developmental windows can generate lasting restructuring of transcriptional regulation without necessarily producing abrupt morphological changes (TZSCHENTKE & BASTA, 2002b; YAHAV et al., 2004).

Despite relative morphological normalization, histopathological analyses revealed striking qualitative differences among the treatments. The livers of control animals exhibited preserved architecture, as expected under normal incubation and development conditions (ZAEFARIAN et al., 2019). Animals exposed to 38.5°C showed mild focal periportal fatty degeneration. Focal lipid accumulation in periportal hepatocytes is a low-grade alteration frequently described in birds subjected to mild metabolic imbalances; it may reflect a transient change in hepatic lipid metabolism without necessarily indicating significant hepatocellular injury (EMAMI et al., 2021; ZAEFARIAN et al., 2019). Post-hatch heat stress has previously been linked to increased *de novo* hepatic lipogenesis and triglyceride accumulation in broiler livers, processes mediated by regulation of the FAS and ACC genes (LIAO et al., 2022; MA et al., 2021). Embryonic heat programming may have predisposed hepatocytes in the T2 group to increased lipogenesis, resulting in low-grade focal steatosis (CHEN et al., 2024; MASSIMINO et al., 2019, 2021; ZHANG; FAN; PENG, 2018).

By contrast, animals incubated at 39°C exhibited more pronounced

pathological changes, even after two weeks of life, including increased hepatic vascularization, disorganization of hepatocyte cords, and tissue degeneration. These alterations are consistent with a tissue response to severe stress and resemble the histopathological pattern described in chickens subjected to chronic post-hatch heat stress, characterized by vascular congestion, inflammatory infiltration, and parenchymal disorganization (TANG et al., 2022; JIANG et al., 2022). Notably, in the T3 group, these changes occurred without MPO activation, suggesting that hepatic disorganization resulted from mechanisms independent of neutrophilic infiltration. Literature indicates that incubation temperatures exceeding 38.7–39°C can compromise the development of internal organs, including the liver, even without causing embryonic mortality (AL-ZGHOUL et al., 2026; LEKSRIOMPONG et al., 2007b; MAATJENS et al., 2016a, 2016b). In contrast, studies employing 39°C for short periods (3 h/day) during the final stages of incubation reported improved thermotolerance without compromising performance or hepatic morphology (LEÃO et al., 2024), suggesting that the duration of exposure is a critical factor in determining the hepatic outcome.

This interpretation is reinforced by the hepatic biochemical results obtained in the present study. A significant increase in hepatic ALT activity was observed exclusively in chicks from the T3 group, whereas AST activity increased in both thermally manipulated groups. ALT and AST are intracellular enzymes released into the extracellular compartment following disruption of hepatocyte membrane integrity (Giannini et al., 2005; Ozer et al., 2008). Because ALT is predominantly localized in the hepatocyte cytosol, it is generally considered a more specific indicator of hepatocellular injury than AST, which is also present in skeletal and cardiac muscle and has both cytosolic and mitochondrial isoforms (Harr, 2002; Lumeij, 2008; Stockham & Scott, 2013). The selective elevation of ALT in the T3 group suggests that incubation at 39°C resulted in greater compromise of hepatocyte cytoplasmic integrity, whereas the increase in AST observed in both T2 and T3 may reflect an earlier or less severe cellular disturbance that did not necessarily progress to extensive cytoplasmic damage at 38.5°C. This differential enzymatic response is consistent with previous descriptions in avian hepatic pathology, in which AST tends to increase during milder or earlier stages of hepatocellular injury, whereas ALT elevations are associated with more pronounced hepatocellular damage (Harr, 2002; Lumeij,

2008). Moreover, the enzymatic profile closely parallels the histopathological findings observed in the present study, with the T2 group exhibiting only mild focal fatty degeneration, while the T3 group displayed marked disruption of hepatic architecture, hepatocellular degeneration, and vascular remodeling. Similar associations between increased transaminase activity and histopathological evidence of liver injury have been reported in broilers exposed to post-hatch heat stress (Tang et al., 2022). However, unlike that study, no increase in hepatic MPO activity was detected in our experiment, indicating that the observed hepatic lesions were predominantly degenerative rather than inflammatory. The avian liver is widely recognized as a primary target of metabolic and thermal stressors (Hubbard et al., 2019; Zaefarian et al., 2019), and the present findings demonstrate that relatively small increases in incubation temperature can produce persistent functional consequences that remain detectable during the early post-hatch period. Notably, the increase in hepatic transaminase activity, particularly the selective elevation of ALT in the T3 group, was not accompanied by increases in hepatic TBARS or MPO levels at two weeks of age. This dissociation suggests that hepatocellular enzyme leakage and oxidative damage are not necessarily concomitant events. Experimental studies have shown that plasma membrane alterations may be sufficient to promote AST and ALT release without inducing detectable lipid peroxidation (Comporti et al., 2010; Domínguez et al., 2019). Alternatively, endogenous antioxidant systems may have efficiently prevented the accumulation of lipid peroxidation products at this stage. This interpretation agrees with previous studies demonstrating that embryonic thermal manipulation enhances basal hepatic antioxidant capacity through long-term modulation of genes involved in antioxidant defense, including *NRF2*, *SOD*, *CAT*, and *GPx*, thereby reducing susceptibility to oxidative injury later in life (Al-Zghoul & El-Bahr, 2019; Saleh, Tarkhan & Al-Zghoul, 2020; Ncho et al., 2022, 2023). Likewise, the absence of differences in MPO activity among treatments indicates that the morphological and biochemical alterations observed were predominantly non-inflammatory in nature (Jaeschke, 2006; Rensen et al., 2009).

Conclusion

Thermal stimuli during late incubation produced intensity-dependent hepatic responses. Incubation at 38.5°C induced predominantly adaptive structural changes, whereas incubation at 39°C promoted more pronounced histopathological alterations, accompanied by increased hepatic aminotransferase activity. These effects persisted after hatching, demonstrating long-lasting morphological and biochemical hepatic programming in the absence of detectable oxidative stress or inflammatory activation

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Figures

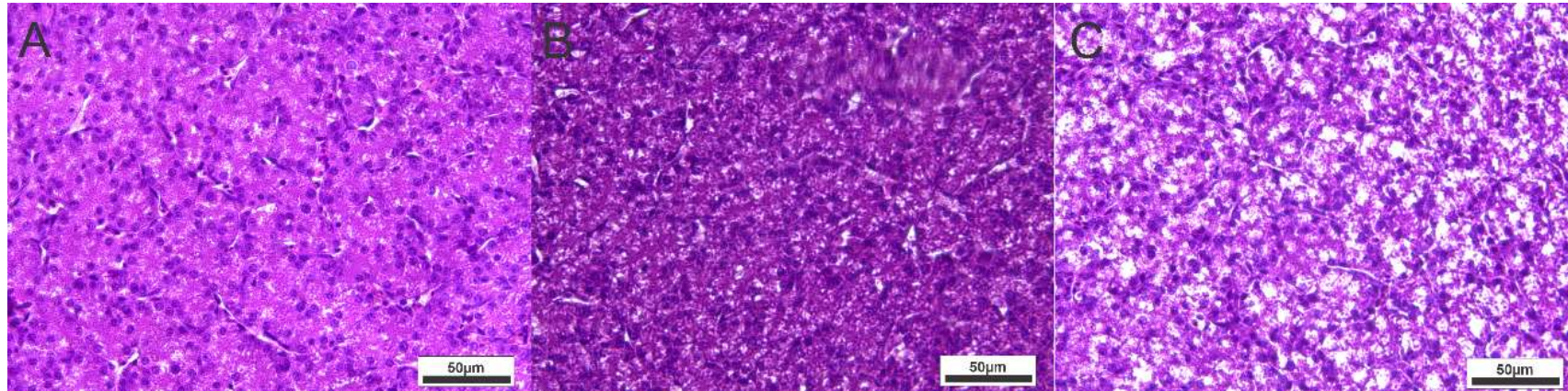


Figure 1. Representative photomicrographs of embryonic liver tissue at embryonic day 17 (E17) following incubation at different temperatures. (A) T1 (control; 37.5°C), showing preserved hepatic architecture with well-organized hepatocyte cords and regularly distributed hepatic sinusoids. (B) T2 (38.5°C), exhibiting overall preservation of liver architecture, with a slight increase in hepatocyte size. (C) T3 (39.0°C), showing preserved hepatic morphology and cell density, with no evident histopathological alterations. Sections were stained with hematoxylin and eosin (H&E). Original magnification: 40×. Scale bar = 50 µm.

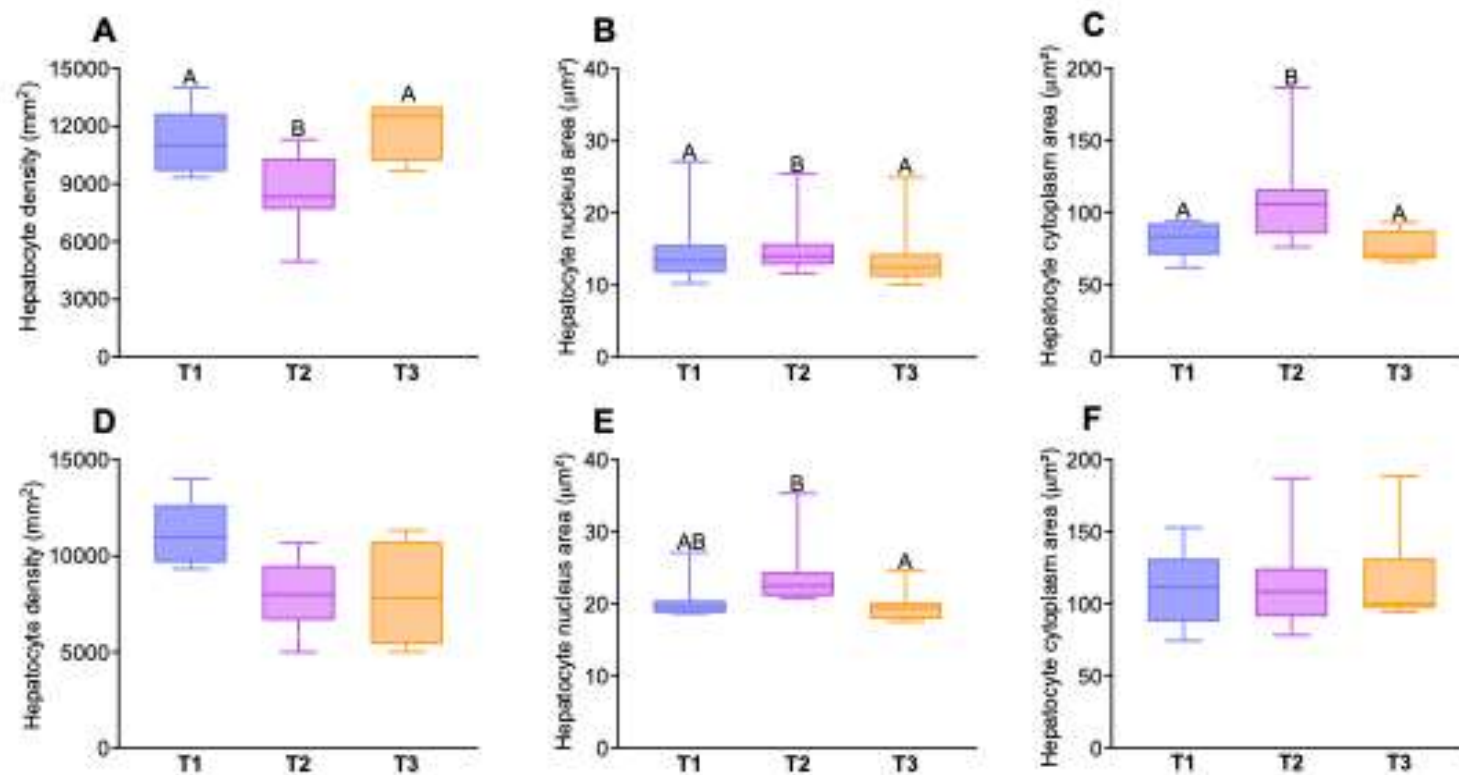


Figure 2. Morphometric analysis of the livers of chick embryos incubated at different temperatures during the final stage of incubation and evaluated between embryonic days (E) 17 and 19. (A–C) Morphometric parameters measured at E17, including cell density (A), hepatocyte nuclear area (B), and hepatocyte cytoplasmic area (C). (D–F) Morphometric parameters measured at E19, including cell density (D), hepatocyte nuclear area (E), and hepatocyte cytoplasmic area (F). T1 = control (37.5°C); T2 = 38.5°C; T3 = 39.0°C. Data are presented as mean $\pm$ SD. Different letters indicate significant differences among incubation temperature treatments ($P < 0.05$).

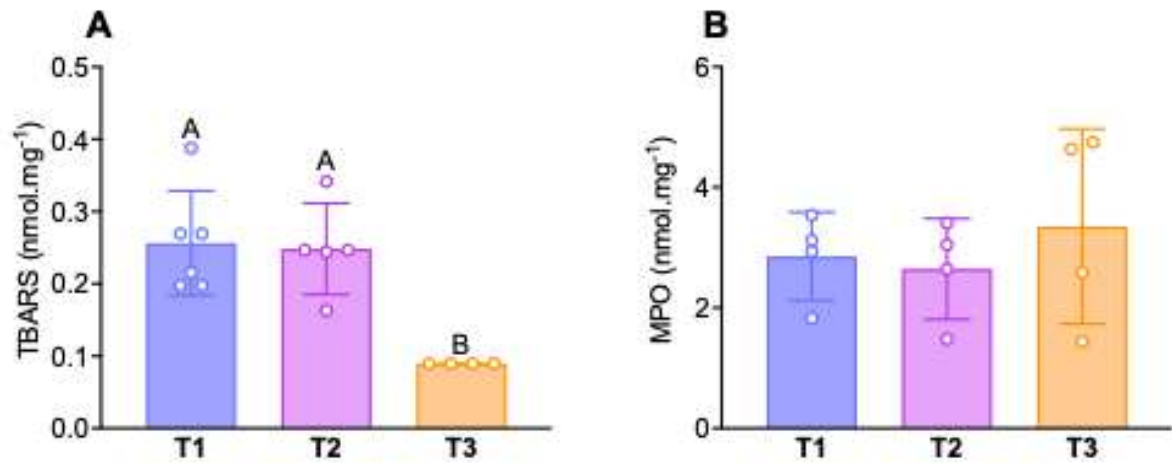


Figure 3. Effects of embryonic thermal manipulation on hepatic markers of oxidative stress and inflammation in embryos at 17 days of incubation. (A) Concentration of thiobarbituric acid-reactive substances (TBARS) is used as an indicator of lipid peroxidation. (B) Myeloperoxidase (MPO) activity is used as an indicator of tissue inflammatory activity. T1 = control (37.5°C); T2 = 38.5°C; T3 = 39.0°C. Data are presented as mean $\pm$ SD. Different letters indicate significant differences among incubation temperature treatments ($P < 0.05$).

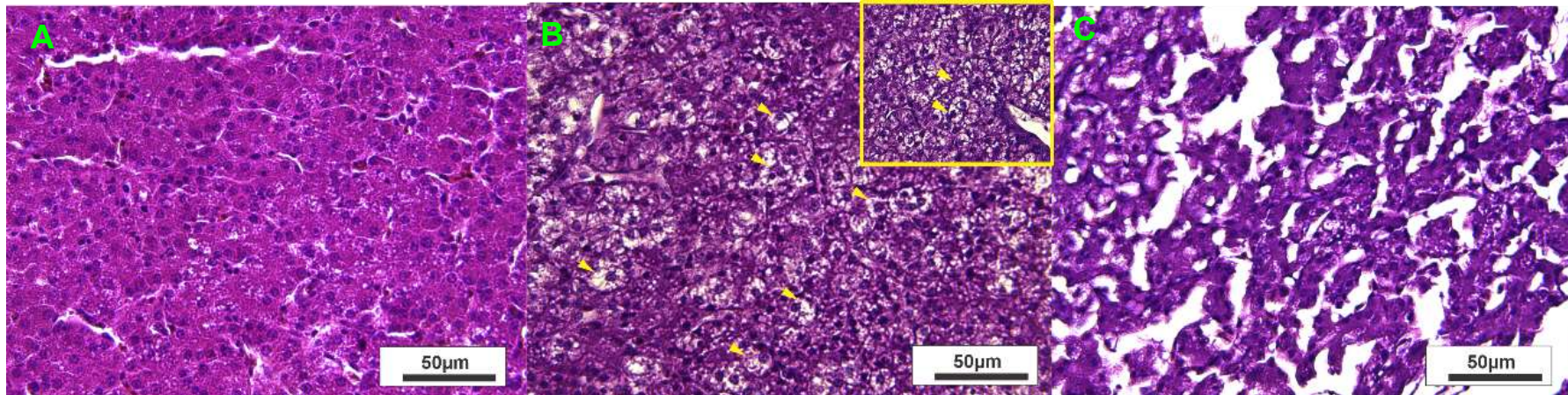


Figure 4. Representative histopathological sections of the liver from 15-day-old chicks incubated at different temperatures. (A) T1 (control; 37.5°C), showing preserved hepatic architecture, with well-organized hepatocyte cords and regularly distributed sinusoids, without evidence of tissue damage or hemorrhage. (B) T2 (38.5°C), showing focal accumulation of foamy cells (yellow arrows), predominantly in the periportal region, as highlighted in the inset, while overall hepatic architecture remained preserved. (C) T3 (39.0°C), exhibiting marked histopathological alterations, including disruption of hepatic architecture, disorganization of hepatocyte cords, and extensive hepatocellular vacuolization, indicative of hepatic degeneration. Histological sections were stained with hematoxylin and eosin (H&E). Original magnification: 40×. Scale bar = 50 µm.

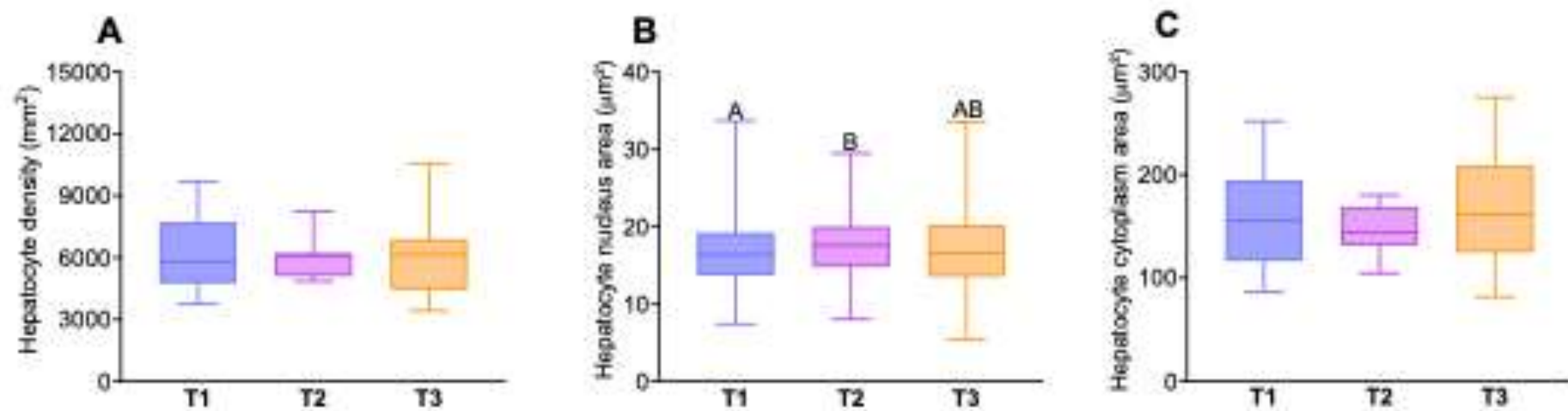


Figure 5. Effects of embryonic thermal manipulation on liver histomorphometric parameters in broiler chickens at 15 days post-hatch. (A) Hepatic cell density, (B) hepatocyte nuclear area, and (C) hepatocyte cytoplasmic area. T1 = control (37.5°C); T2 = 38.5°C; T3 = 39.0°C. Data are presented as the mean $\pm$ SD. Different letters indicate significant differences among incubation temperature groups ($P < 0.05$).

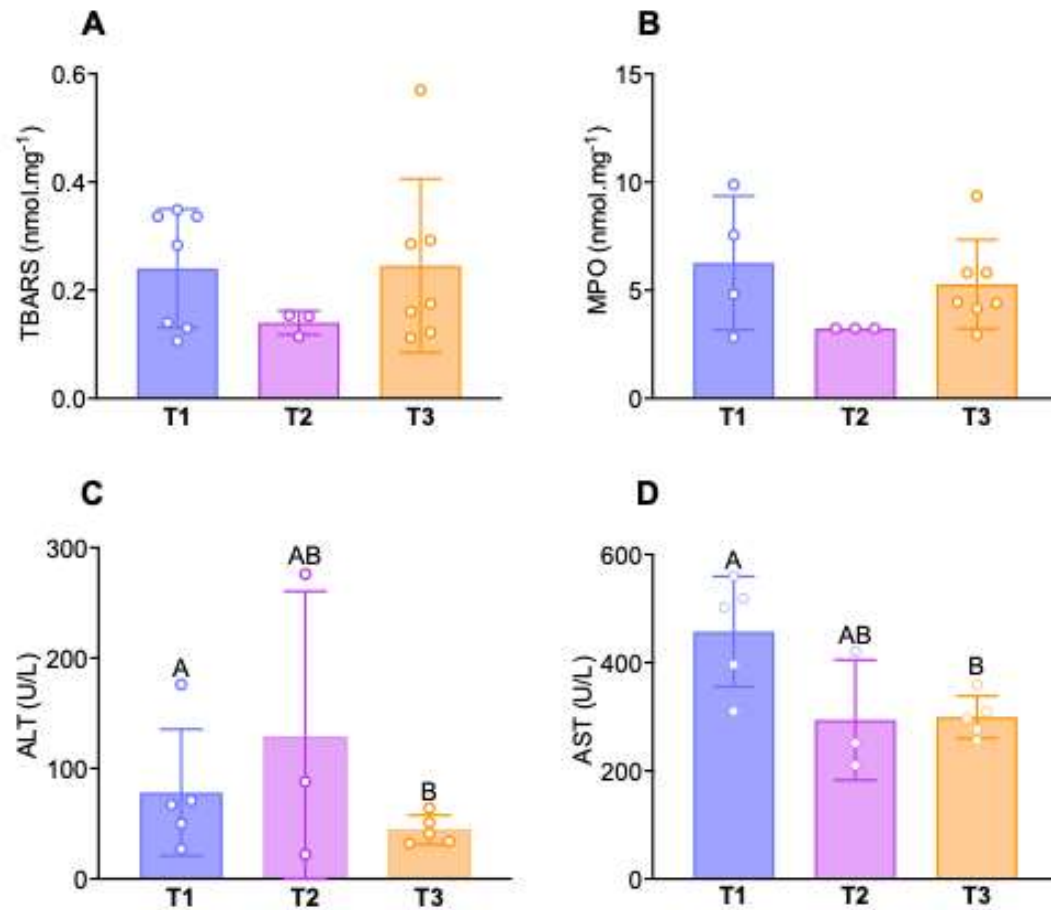


Figure 6. Effects of embryonic thermal manipulation on hepatic oxidative stress, inflammation, and tissue integrity in broiler chickens at 15 days post-hatch. (A) Thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation; (B) myeloperoxidase (MPO) activity, a marker of inflammatory cell infiltration; (C) hepatic alanine aminotransferase (ALT) activity; and (D) hepatic aspartate aminotransferase (AST) activity. T1 = control (37.5°C); T2 = 38.5°C; T3 = 39.0°C. Data are presented as mean $\pm$ SD. Different letters indicate significant differences among treatments ($P < 0.05$).

Table 1. Serum biochemical parameters of broiler chickens at 15 days post-hatch following embryonic thermal manipulation (T1 = control, 37.5°C; T2 = 38.5°C; T3 = 39.0°C; 6 h/day from embryonic days 16 to 19). Data are presented as mean $\pm$ SD.

Parâmetro	T1 (Controle)	T2 (38.5°C)	T3 (39°C)	p-value
AST (U/L)	457.20 $\pm$ 102.08 ^A	293.67 $\pm$ 111.31 ^{AB}	299.20 $\pm$ 39.01 ^B	0.0365*
ALT (U/L)	78.20 $\pm$ 57.36	128.67 $\pm$ 131.79	44.40 $\pm$ 13.24	0.2976
GGT (U/L)	13.90 $\pm$ 1.75	12.19 $\pm$ 1.08	14.14 $\pm$ 3.10	0.5013
Creatinine (mg/dL)	0.324 $\pm$ 0.220	0.337 $\pm$ 0.107	0.374 $\pm$ 0.114	0.8856
CK (U/L)	2.35 $\pm$ 0.02	2.56 $\pm$ 0.46	2.98 $\pm$ 0.74	0.3640
Uric acid (mg/dL)	2.62 $\pm$ 0.23	2.73 $\pm$ 0.45	2.64 $\pm$ 0.40	0.9047
Albumine (g/dL)	9.60 $\pm$ 1.00	10.46 $\pm$ 1.41	10.02 $\pm$ 0.04	0.5249

Different letters within a row indicate significant differences among treatments ($P < 0.05$). AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transferase; CK, creatine kinase.

Table 2. Effects of embryonic thermal manipulation on relative liver weight (%) at embryonic day 17 and 15 days post-hatch.

Age	T1 (%)	T2 (%)	T3 (%)	<i>p</i>-value
E17 embryo	2.25 ± 0.27	1.97 ± 0.22	2.20 ± 0.08	0.2705
15-day-old chicks	3.86 ± 0.66	3.19 ± 0.71	3.72 ± 0.74	0.0867

Values are expressed as mean ± SD. Relative liver weight is presented as a percentage of body weight. Welch's ANOVA was used for embryonic data (E17), whereas the Kruskal–Wallis test was used for 15-day-old chicks. No significant differences were detected among treatments ($p > 0.05$). Although pairwise comparisons were not significant, the E17 data showed a large overall effect size ($\eta^2 = 0.3078$), while at 15 days a numerical reduction in relative liver weight was observed in T2 compared with T1, with a moderate pairwise effect size ($r = 0.495$).