UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"

FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS

CÂMPUS DE JABOTICABAL

PAPEL DOS RECEPTORES DE GRELINA GHS-R1a NA RESPOSTA TÉRMICA AO DESAFIO INDUZIDO POR LPS

Thais Fortunato Oliveira

Médica Veterinária

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Orientadora: Profa. Dra. Kênia Cardoso Bícego

Dissertação apresentada à Faculdade de Ciências Agrárias e Veterinárias – UNESP, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Mestre em Ciência Animal.

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Jaboticabal, 01 de março de 2024

DADOS CURRICULARES DO AUTOR

Thais Fortunato Oliveira, nascida em 21 de fevereiro de 1996 na cidade de Ouroeste-SP, filha de Carmo Donizete Oliveira e Neide Fortunato Oliveira, ingressou no curso de medicina veterinária na universidade Brasil, campus Fernandópolis-SP em 2014. Durante a graduação a aluna fez estágios em clínica médica de pequenos animais, inseminação artificial de tempo fixo em bovinos de corte, e o estágio obrigatório foi realizado no frigorífico Ouroeste - LTDA de abate de bovinos, em dezembro de 2018 defendeu o trabalho de conclusão de curso e em janeiro de 2019 colou grau. Em agosto de 2019 foi beneficiada a uma bolsa de auxílio técnico no laboratório de ciências Avícolas da Unesp- Jaboticabal e em novembro de 2020 deu início a Bolsista nível III da FAPESP no laboratório de Fisiologia Animal da Unesp- Jaboticabal onde ingressou no curso de mestrado em 03 de janeiro de 2022 pelo programa de pós-graduação em Ciência animal sendo beneficiada pela bolsa de estudos CAPES. Durante o curso apresentou poster na XXXVII Reunião Anual da FeSBE intitulado “Effect of ghrelin antagonist on lps-induced regulated hypothermia in chicken chicks”, e em dezembro de 2023 apresentou poster no 6° international course in comparative Physiology of respiration, intitulado “Effect of [D-Lys3] GHRP-6 on respiratory parameters and feed intake in 5-day-old chicks challenge with LPS”, onde também fez parte da comissão organizadora do curso. A aluna submeteu-se ao exame geral de qualificação dia 13 de dezembro de 2022.

DEDICATÓRIA

À minha mãe Neide Fortunato que mesmo não entendendo muito sobre pós-graduação sempre acreditou e nunca mediu esforços para que eu chegasse academicamente até aqui; ao meu pai Carmo Donizete que mesmo não estando presente fisicamente sempre estará vivo em meu coração; a minha irmã Flavia que mesmo a quilômetros de distância sempre se faz presente.

A vocês dedico!

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Agradeço a minha irmã Flavia que mesmo distante sempre se faz presente me ajudando em absolutamente TUDO!

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Aos meus primos Luiza e Murilo que mesmo tão pequeninos me inspiram e me motivam a dar cada passo, eles são as “coisas” mais preciosas da minha vida;

À Carol Silva que me ensinou tanto e teve muita paciência no meu início de laboratório, como bolsista TT3 e toda dedicação para que esse trabalho de mestrado fosse concluído;

Ao Lango e à Alana por ter me atendido e ajudado todas as inúmeras vezes que precisei, tanto no laboratório quando fora de lá;

Às minhas amigas que se fizeram minha família Jaboticabalense Ane, Bia, Livia e Maria por todo companheirismo dentro e fora do laboratório, com certeza vocês foram essenciais para todo desenvolvimento nesse trabalho, e meu crescimento como pessoa, agradeço a vocês por todas as vezes que me ouviram e aconselharam, por todas as vezes que ajudaram com meus animais, e principalmente agradeço por cada rolê;

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À Jéssica e Mari por toda parceria e conversas;

Aos demais membros do laboratório;

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001. (88887.662086/2022-00).

À Faculdade de ciências agrárias e Veterinárias de Jaboticabal pela estrutura;

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E por último e mais importante aos meus animais, sem eles nada disso teria sido realizado.

Sumário

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Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Efeito do antagonista de grelina sobre a resposta térmica bifásica ao LPS em pintainhos de corte"**, protocolo nº 1861/2023, sob a responsabilidade da Profa. Dra. Kênia Cardoso Bicego, 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 15 de março de 2023.

Vigência do Projeto	18/03/2023 a 20/03/2024
Espécie / Linhagem	*Gallus gallus domesticus*
Nº de animais	180
Peso / Idade	70-100g / 5 dias
Sexo	Machos e fêmeas
Origem	Fornecedor comercial

Jaboticabal, 15 de março de 2023.

Profa. Dra. Paola Castro Moraes
Vice coordenadora – CEUA/FCAV

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PAPEL DOS RECEPTORES DE GRELINA GHS-R1a NA RESPOSTA TÉRMICA AO DESAFIO INDUZIDO POR LPS

RESUMO

Um desafio importante para a avicultura, interferindo tanto na produção quanto no bem-estar animal é a possibilidade de infecção por agentes agressores tais como bactérias e vírus. Esse cenário é especialmente impactante no início da vida, quando os sistemas de termorregulação e defesa imunológica estão em desenvolvimento. Diante de um desafio imunológico severo, o animal pode desenvolver uma resposta oposta à febre, chamada hipotermia regulada. Além disso, sabe-se que a grelina é um peptídeo encontrado em aves e mamíferos, principalmente no estômago e no encéfalo. Dentre os efeitos da grelina, temos a estimulação da ingestão de alimentos e inibição de febre induzida por endotoxina em mamíferos. Em contraste, em aves este peptídeo diminui a ingestão de alimento, o que poderia influenciar a anorexia que acompanha a resposta de fase aguda induzida por endotoxina. Alguns resultados anteriormente realizados no laboratório indicam que a grelina encefálica pode reduzir a temperatura corporal (Tc) em pintainhos, mas não parece afetar a resposta térmica ao LPS. Em mamíferos há comprovações que o antagonista de grelina atenua o comportamento de apatia provocado pela administração do LPS, mas o consumo de alimento não é alterado. Com base nisso, hipotetizamos que a grelina é um mediador químico envolvido na hipotermia regulada induzida por LPS em pintainhos de 5 dias de idade. Para isso coletamos dados de temperatura corporal, consumo de oxigênio (índice de termogênese), ventilação pulmonar, consumo de ração e perda de calor cutânea diante do antagonista de receptor GHS-R1a de grelina, [D-Lys³] GHRP-6 (intracerebroventricular, icv). O pré-tratamento com [D-Lys³] GHRP-6, intensificou a queda de Tc corporal pelo LPS (intramuscular, IM) e retardou o retorno da Tc ao valor inicial. O consumo de oxigênio ($\dot{V}O_2$), no tratamento [D-Lys³] GHRP-6 + LPS mostrou uma queda (de 60-120 min) quando comparado ao tempo inicial. Em relação ao consumo de ração, o [D-Lys³] GHRP-6 (0,5nmol) não interferiu com o efeito inibidor da ingestão de alimento pelo LPS. Por fim, o índice de perda de calor cutâneo aumentou, 300 min após a

administração de LPS, indicando indução de vasodilatação cutânea. Os resultados do presente estudo indicam que em conclusão a grelina parece ter influência nos termoefetores periféricos.

Palavras chaves: Antagonista de grelina- LPS- hipotermia regulada- termogênese.

Capítulo 1: Revisão de literatura

1. Considerações gerais

A produção de aves e ovos no Brasil demonstra uma atividade importante na agricultura sempre ficando entre os 3 maiores produtores, em 2023 segundo a ABPA o país ficou em 2° lugar produzindo 14,524 milhões de toneladas de carne de frango sendo 33% dessa quantidade, destinadas à exportação, movimentando cerca de US$ 9,7 bilhões. Esses dados comprovam o quão a cadeia avícola é importante para o Brasil, logo podemos afirmar que entender e descrever as bases fisiológicas das respostas desses animais a desafios ambientais é de grande valia para a evolução dessa cultura.

2- Revisão de literatura

2.1- Mecanismos termorreguladores nas aves

A temperatura é um fator importante para todos os seres vivos, e sabemos que as mudanças na temperatura ambiente (Ta) levam a alterações fisiológicas em animais ectotérmicos e endotérmicos, afetando-os direta e indiretamente (Bícego & Gargaglioni, 2020). Os animais endotérmicos frente a mudanças na Ta ativam mecanismos termorreguladores com o objetivo de manter a homeostase térmica dentro de certos limites, visto essa relevância, e levando em consideração também as mudanças climáticas no aumento importante da temperatura global que estão ocorrendo (IPCC, 2022), os estudos envolvendo a termorregulação vem se expandindo nas últimas décadas, com o intuito de aprofundar os conhecimentos na área.

As aves possuem a maior Tc e a maior taxa metabólica dentre os vertebrados (Legendre e Davesne, 2020) isso indica uma alta demanda energética, e consequentemente um alto consumo de alimento. Além disso as aves também possuem eficientes isolantes térmicos, a camada de gordura subcutânea e as penas que auxiliam na conservação de calor corporal (Bícego., et al 2017).

A endotermia, no entanto, vai sendo adquirida ao longo de seu desenvolvimento ontogenético (Sbong et al., 2007; Price e Dzialowski, 2018; Seebacher, 2009), antes da eclosão, aves precoces apresentam um sistema termorregulador funcional, porém com eficiência reduzida e sem um valor

adaptativo imediato, até os 10 dias de idade após a eclosão, há um aumento na eficiência do sistema regulatório, marcado por uma colaboração entre os sistemas autônomos e comportamentais (Nichelmann e Tzschentke 2002). A fase inicial é caracterizada por essa cooperação intensa entre diferentes sistemas efetores, resultando em uma homeotermia plenamente eficiente após os 10 dias de idade nas aves precoces, mas por se tratar de uma espécie de desenvolvimento precoce, é possível observar capacidade de termorregulação e locomoção nos primeiros dias pós-eclosão (Mortola et al., 2009).

A regulação da temperatura depende de sensores periféricos que captam as variações da Ta e enviam informações para o sistema nervoso central, onde respostas autonômicas e comportamentais são ativadas a fim de produzir e conservar calor ou facilitar a perda dele (Bícego et al., 2007; IUPS Thermal Comission, 2001).

A área pré optica hipotalâmica (POA) possui papel importante na termorregulação não só em aves, mas também em mamíferos por conter neurônios sensíveis a elevação da temperatura, aumentando assim sua taxa de disparo e ativando mecanismos de combate ao calor, e neurônios insensíveis a alteração de temperatura, que se conectam por meio de sinapses excitatórias e inibitórias com os neurônios efetores de produção e de perda de calor (Morrison & Nakamura, 2011; Matsuda et al., 1992; Boulant, 1998).

Em casos de queda da Ta os sensores periféricos sensíveis a temperatura, captam e ativam respostas relacionadas a produção e conservação do calor, autonômicas (elevação da taxa metabólica, tremores musculares e vasoconstrição periférica) e comportamentais (se agrupam, aumentam o consumo de alimento, buscam lugares mais quentes) (Carlton & Marks, 1958; Tan & Knight, et al 2018).

Já quando há um aumento da Ta o sistema nervoso central ativa mecanismos de perda de calor podendo ser sensível comportamental, onde o animal procura ambientes com temperaturas mais amenas, quando em grupo esses animais se afastam uns dos outros, ocorre uma vasodilatação periférica a fim de facilitar a troca de calor com o ambiente e há uma maior ingestão de água, ou latente que consiste na perda de calor evaporativa pela respiração (taquipneia térmica ou ofêgo), aumentando a ventilação pulmonar sem alterar o

volume corrente, podendo assim se por um longo período se tornar patológico (alcalose respiratória) pela alta eliminação de CO_2 (Menuan e Richards, 1975; Mortola e Maskey, 2011; Romanovsky, 2018). O ofego tem capacidade de diminuir em até 2°C a Tc de frangos, demonstrando um importante papel para o resfriamento encefálico, pois mantém o sistema nervoso central com a temperatura mais amena que o restante do corpo, esse evento é chamado de resfriamento encefálico seletivo (Arad et al., 1984).

2.2- Termorregulação associada ao desafio imunológico

Ao longo da vida dos animais, a possibilidade de se infectar com algum agente agressor é significativa, e quando ocorre, a febre é uma das principais respostas térmicas de defesa contra microrganismos infectantes (Blatteis., 2011). Autores discutem há vários anos sobre duas alternativas para justificar a eficácia da febre no papel de defesa do hospedeiro, a primeira consiste em que o aumento regulado da temperatura corporal é fundamental para eliminar patógenos, especialmente aquelas sensíveis a temperatura. Já a segunda discute que a febre estimula o sistema imunológico do animal, permitindo que ele combata até mesmo patógenos resistentes a temperaturas mais altas. Essa compreensão é respaldada por estudos de Blatteis (2003), Evans et al. (2015) e Kluger et al. (1998).

A febre nos animais endotérmicos se dá pela ativação de mecanismos produtores e/ou conservadores de calor (Romanosvky et al., 2005; Bícego et al, 2007). Os benefícios da febre para a proteção do hospedeiro são tão significativos que ela é observada em diversos grupos de invertebrados e em todos os grupos de vertebrados, sejam ectotérmicos ou endotérmicos, como indicado em estudos de Bicego et al. (2007), Evans et al. (2015), Kluger et al. (1996) e Rakus et al. (2017) Dantonio et al. (2016) e Amaral-Silva (2020 e 2021). Portanto, junto com as demais respostas mobilizadas pelo sistema imunológico inato, a febre se torna um processo energeticamente custoso. O custo associado à febre é, assim, identificado como um de seus principais efeitos colaterais, podendo levar à morbidade e até mesmo à mortalidade do hospedeiro quando ultrapassa os benefícios esperados, conforme destacado em estudos como os de Blatteis (2003), Garami et al. (2018), Gray et al. (2013) e Kluger et al. (1996).

A ativação da febre pode acontecer em resposta a vários agentes externos, como bactérias Gram positivas, Gram negativas, vírus ou fungos, conforme documentado em estudos como os de Bicego et al. (2007) e da Comissão de Fisiologia Térmica da IUPS (2001).

Em modelos experimentais, o método mais utilizado para expor os animais a essa condição é a injeção de lipopolissacarídeo (LPS), que se trata de um componente da membrana celular de bactérias Gram negativas (Gray et al, 2013). De fato, vários exemplos existem na literatura, mostrando alterações térmicas em passarídeos (Lee et al, 2005), patos (Marais et al., 2011), galinhas (Qu et al., 2020), frangos de corte adultos (De Boever et al, 2008), e pintainhos (Dantonio et al, 2016; Amaral-Silva et al, 2020; 2021) frente ao LPS. No caso de infecções mais severas, ou sepse, no entanto, a resposta térmica pode ser oposta à febre, ou seja, uma queda de Tc, chamada de hipotermia regulada ou anapirexia.

Pelo menos em mamíferos, o LPS induz a liberação de pirogênios endógenos por células do sistema imune, as citocinas (interleucinas IL – 1 e 6, TNF-alfa). Essas citocinas sinalizam o SNC, induzindo produção de prostaglandinas (PG), principalmente PGE_2, que atuam na área pré optica (POA) inibindo atividade de neurônio sensível ao calor e, consequentemente ativando mecanismos de conservação e/ou produção de calor (Kluger, 1991; Bícego et al., 2007; Morrison & Nakamura., 2011, 2019). Alguns estudos em roedores indicam que há conexão entre a POA e a rafe bulbar, e que essa última teria os neurônios pré-motores para ativação das vias simpáticas para o tecido adiposo marrom e a vasoconstrição periférica (Morrison & Nakamura, 2011).

Em aves desafiadas com LPS, também ocorre liberação de citocinas na corrente sanguínea, principalmente IL-1 e IL-6, que transmitem a informação para o SNC da presença de um agente pirogênio exógeno no organismo (Macari et al.,1993; Marrais et al., 2011; Gray et al., 2013). A partir do SNC, mecanismos (autonômicos e/ou comportamentais) de produção e/ou conservação de calor são ativados, evoluindo assim para febre (Bicego et al., 2007; Gray et al, 2013). De fato, existem evidências da elevação da concentração PGE_2 durante a resposta febril ao LPS em pintainhos de corte

(Dantonio et al, 2016) e a injeção intracerebroventricular de prostaglandina em frangos causa aumento de Tc (Macari et al.,1993). Esses dados em aves indicam mecanismos comuns de indução de febre aos de mamíferos.

Para pintainhos nos primeiros dias de vida, a febre é uma resposta de defesa importante, mas com alto custo energético, o que se torna desafiador nessa fase em que esses animais apresentam alta taxa metabólica para crescimento e desenvolvimento dos sistemas fisiológicos (Evans et al., 2017; Dantonio et al 2016; Amaral-Silva et al, 2021; 2022). De fato, a simulação de uma condição mais severa de infecção com alta dose de LPS em diferentes condições de demanda e oferta energéticas (frio, jejum e associação entre os dois) mostra priorização da resposta hipotérmica regulada em pintainhos (Amaral-Silva et al., 2022).

2.2- Anapirexia

A anapirexia ou hipotermia regulada é uma resposta oposta a febre, quando o animal se encontra em algum desafio ambiental, sendo ele hipóxia, hipercapnia, desidratação, intoxicação, fome e hiploglicemia, trauma e sepse induzida por LPS por exemplo (Bícego, et al, 2020).

Evidências da literatura apontam que a hipotermia regulada ocorre tanto em mamíferos (Almeida et al., 2006; Steiner e Romanovsky, 2019; Morrison&Nkamura.,2011) quanto em aves (Amaral-Silva et al., 2020; 2021; 2022), e que parece ser relevante para proteção contra lesões e hipóxia teciduais nesses casos graves (Liu et al., 2012).

Os mamíferos quando expostos a estímulos graves apresentam comportamentos termorreguladores que facilitam a perda de calor para o ambiente, estudos comprovam que os ratos de laboratório (*ratus novergicus*) (neonatos e adultos) expostos a hipóxia 7% O_2 ativam respostas que facilita a perda de calor, como por exemplo a vasodilatação de cauda e o afastamento quando em grupo em Ta baixa para a idade (20°C) fazendo com que os animais apresentem uma Tc baixa, porém regulada (Pereira et al, 2006; Scarpelini et al, 2010). Autores discutem que especialmente para mamíferos a resposta de anapirexia induzida por hipóxia ocorre somente em animais de pequeno porte onde há uma alta relação $\dot{V}O_2$/Kg, esses animais começam a

reduzir a Tc por mecanismos facilitadores de perda de calor, antes de haver uma redução na taxa metabólica, e que após esse início da redução de Tc ela continua pela queda da taxa metabólica (Bícego et al, 2020).

Em aves a redução da Tc também ocorre pela mudança de concentrações de gases quando esses animais são expostos, em 2008 Scott et al, demonstrou que três espécies de patos (pato de cabeça listrada, pato bravo e pato pequim) aumentaram a temperatura do bico, que é uma janela térmica e com isso facilita a perda de calor para o ambiente quando os níveis de O_2 foi reduzido de ~21% a 5% de O_2. Além de patos, respostas termogênicas induzidas pela queda de O_2 são apresentadas por pombos (*Columbia Livia*) que demonstraram a queda de termogênese por tremor quando expostos a 10-7% de O_2 (Barnas & rautemberg,1990; Gleeson et al,1986). Há uma redução de Tc e taxa metabólica em codornas (*Coturnix Coturnix*) quando essas foram expostas de 1h a 11 a 9% de O_2 (Atchey et al, 2008). Mortola e Labbè, em 2005 também mostraram a diminuição da taxa metabólica frente a desafios gasosos em embriões de galinhas (*Gallus Domesticus*) de 11 dias expostos a 16% de O_2 durante 30-60min.

Em relação a inflamações sistêmicas sabe-se que a resposta de fase aguda quando o hospedeiro é submetido a uma inflamação branda trata-se da febre, mas quando há um desgaste energético devido a respostas da fase aguda, o indivíduo já apresenta um enfraquecimento por uma patologia preexistente ou quando há um número expressivo de microrganismos circulantes e a inflamação sistêmica se torna severa como por exemplo também alta doses de LPS, a resposta apresentada é a anapirexia (Bícego et al, 2020).

Para essas repostas de defesa (febre e/ ou anapirexia) sejam apresentadas são necessárias alguns mediadores químicos, a interleucina 1 beta (IL-1β), interleucina 6 (IL-6) prostaglandinas E_2 (PGE_2) são mediadores químicos tipicamente relacionados a respostas febris, já a prostaglandina D2 (PGD_2) e o fator de necrose tumoral α (TNFα) podem ter ações criogênicas, sendo que o aumento das concentrações de TNFα devido a severidade da inflamação sistêmica pode ser determinante para a mudança de febre para anapirexia (Bícego et al, 2020).

2.4- Grelina

A grelina foi primeiramente descoberta no estômago de ratos (KOJIMA et al., 1999) e, posteriormente, por meio de análises de RT-PCR, foi confirmado uma baixa expressão desse peptídeo também no intestino, pulmão e encéfalo (KAIYA et al., 2002). Esse peptídeo está relacionado com atividade orexigênica (nos mamíferos), liberação do hormônio do crescimento (GH), e possui relação em outras funções que garantem a homeostase, como por exemplo, durante uma inflamação (KOJIMA et al., 1999; WREN et al., 2000; GUYON et al., 2008; WANG et al., 2009).

Inúmeros estudos têm mostrado uma considerável complexidade dentro do eixo grelina/receptor de grelina, indicando que um aspecto importante da biologia da grelina pode ser a identificação de subtipos de receptores que participa das diversas funções dessa família de peptídeos (SIBILIA et al, 2012).

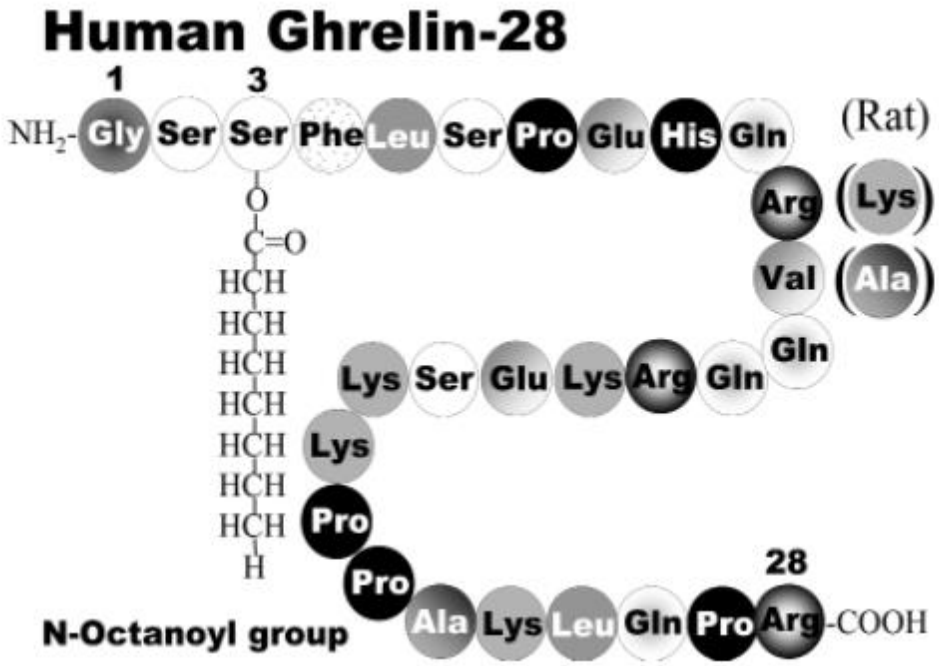

Figura 1: Representação da grelina de humanos, note a diferença octanoil na posição do resíduo Ser_3 e as duas alterações nos aminoácidos nas posições 11 e 12 para a grelina de ratos. (ST-Pierre et al, 2003).

O ligante endógeno dos receptores GHS-R1a, que participa do estímulo de secreção do hormônio do crescimento (GH) liberado pela hipófise foi descoberto em 1999, por meio de purificação do estomago de ratos, recebendo o nome de GHrelin (KOJIMA et al, 1999).

A grelina deriva de um pré-pró-hormônio de 117 aminoácidos, que é clivado em um peptídeo pró-grelina de 94 aminoácidos. Esse peptídeo pró-grelina é posteriormente clivado e dá origem ao peptídeo da grelina, composto por 28 aminoácidos. Foram identificadas duas principais formas de grelina de 28 aminoácidos (Hosoda et al. 2000). A grelina, geralmente referida na literatura, é acilada no serina-3 pela grelina-O-aciltransferase (GOAT) (Gutierrez et al. 2008), enquanto existe uma forma não acilada (DAG) que circula em níveis dez vezes maiores do que a grelina (Holmes et al. 2009; Patterson et al. 2005).

Além de sua atividade de liberação do hormônio do crescimento, a grelina influencia uma ampla variedade de processos biológicos, como ingestão de alimentos e gasto energético, proliferação celular e funções gastrointestinais, cardiovasculares, pancreáticas, pulmonares e imunológicas (Van der Lely et al. 2004; Cummings 2006; Leite-Moreira & Soares 2007).

Estudos recentes têm se concentrado no papel da grelina no controle da percepção da dor. A expressão da grelina foi encontrada em várias áreas do cérebro envolvidas no controle da nocicepção, como o hipotálamo, a área sensório-motora do córtex, o mesencéfalo e a medula espinhal. O mRNA do GHS-R também é amplamente expresso em várias regiões do encéfalo associadas à transmissão da dor. (Hou et al. 2006; Vergnano et al. 2008; Zigman et al. 2006).

Além disso, a interação da grelina com neurônios contendo opioides endógenos no núcleo arqueado hipotalâmico e o sistema endocanabinoide, conhecidos por exercerem um papel modulador na regulação central e periférica da percepção da dor, reforçam a ideia de que a grelina pode atuar como moduladora da dor (Nakazato et al. 2001; Zigman et al. 2006).

Em ratos em jejum ocorre o aumento das concentrações de grelina na corrente sanguínea estimulando a fome nesses animais; após alimentados, os

valores reduzem e os animais tornam-se saciados (TOSHINAI et al., 2001). Além disso há trabalhos que demonstram que tanto injetada intracerebroventricularmente (icv) como perifericamente a grelina promove um efeito orexígeno nos ratos (TSCHÖP et al, 2000; WREN et al., 2000), o que não ocorre nas aves (ver a seguir).

Autores mostraram evidências indicando a associação da grelina em casos de depressões graves em mulheres na fase de pós menopausa, mostrando que nesses casos os níveis de grelina total eram elevados, os autores discutem que essa associação acontece por distúrbios metabólicos e obesidade (Naufel et al, 2021).

Em 2022 de Paula Jr. e seus colaboradores mostraram que ratos tratados com LPS e [D-Lys^3] GHRP-6, (20nmol) atenuaram o comportamento de apatia causado pelo LPS, mostrando ação referente a hábitos comportamentais desses animais quando desafiados.

Desde a descoberta da grelina em aves, aumentou-se o interesse pela sua ação, por se tratar de um hormônio que expressa algumas funções diferente das encontradas em mamíferos. A grelina nas aves é composta por 26 aminoácidos, e exibe funções de liberação de GH, aumento na motilidade gastrointestinal, que são semelhantes aos mamíferos, porém tanto administrada icv como perifericamente demonstra efeito anorexígeno (Furuse et al., 2001).

Saito., et al (2005) enfocou a participação do eixo Hipotálamo-Hipófise-Adrenal, devido a evidências no aumento das concentrações de corticosterona quando grelina é administrada perifericamente. Além disso também se notou que ao administrar grelina ocorre o aumento da vocalização, que sugere um comportamento de ansiedade nesses animais (Kaya et al., 2002; Saito et al., 2002).

A microinjeção icv de grelina em pintainhos suprime o consumo de ração, e que provavelmente é mediada pelos sistemas dopaminérgicos e canabinoidérgicos (especificamente receptores D1 e CB1 respectivamente), que estão distribuídos em diversas áreas encefálicas (Zendehdel et al, 2016).

Estudos realizados anteriormente em nosso laboratório mostrou que a grelina icv em pintainhos causa uma queda de Tc dose dependente na primeira hora pós microinjeção, o que coincide com o tempo da queda de Tc causada pela administração IM de alta dose de LPS (100µg/kg). Ao associar uma dose de grelina (0,005µg/µL) que não afeta a Tc dos animais com o LPS (100µg/kg), nenhuma alteração na resposta térmica ocasionada pelo LPS foi observada (dados não publicados). Entretanto, não há dados sobre o efeito da inibição do receptor de grelina, que possa indicar participação na queda de Tc induzida por LPS. Diante disso, no presente trabalho objetificou-se investigar o efeito do antagonista de grelina [D-Lys^3] GHRP-6 sobre a resposta térmica induzida por LPS em pintainhos de 5 dias de idade.

3- Referências

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CAPÍTULO 2: **Ghrelin GHS-R1a receptors as modulators of the thermal response to systemic inflammation induced by LPS.**

ABSTRACT

An important challenge for poultry farming, affecting both production and animal welfare, is the possibility of infection by aggressive agents such as bacteria and viruses. This scenario is particularly impactful in early life when thermoregulation and immune defense systems are still developing. Faced with a severe immune challenge, the animal may develop a response opposite to fever, called regulated hypothermia. Additionally, ghrelin is known to be a peptide found in both birds and mammals, primarily in the stomach and brain. Among the effects of ghrelin, there is the stimulation of food intake and inhibition of endotoxin-induced fever in mammals. In contrast, in birds, this peptide reduces food intake, which could influence the anorexia accompanying the acute-phase response induced by endotoxin. Some previous results from the laboratory indicate that brain ghrelin may reduce body temperature (Tc) in chicks but does not seem to affect the thermal response to LPS. In mammals, there is evidence that ghrelin antagonists attenuate the apathetic behavior caused by LPS administration, but food consumption is not altered. Based on this, we hypothesize that ghrelin is a chemical mediator involved in LPS-induced regulated hypothermia in 5-day-old chicks. For this, we collected data on body temperature, oxygen consumption (thermogenesis index), lung ventilation, food consumption, and cutaneous heat loss in the presence of the ghrelin receptor antagonist, [D-Lys³] GHRP-6 (intracerebroventricular, icv). Pre-treatment with [D-Lys³] GHRP-6 intensified the drop in body temperature caused by LPS (intramuscular, IM) and delayed the return of Tc to the initial value. Oxygen consumption ($\dot{V}O_2$) in the [D-Lys³] GHRP-6 + LPS treatment showed a decrease (60-120 min) compared to the initial time. Regarding food consumption, [D-Lys³] GHRP-6 (0.5nm) did not interfere with the inhibitory effect of LPS on food intake. Finally, the cutaneous heat loss index increased 300 minutes after LPS administration, indicating induction of cutaneous vasodilation. The results of this study suggest that, ghrelin appears to influence peripheral thermoeffectors.

Keywords: Ghrelin antagonist - LPS - regulated hypothermia - thermogenesis.

1- Introduction

Throughout animal's life, the probability of infection by some aggressive agent is high. In this case, one of the organism's primary defense responses is fever, which occurs through the activation of thermogenesis and/or inhibition of thermolysis mediated by autonomic and behavioral mechanisms, via the central nervous system (CNS) (Bícego et al., 2007). Besides the undoubted importance of fever as a defense response, it imposes a high energy cost to the organism. This becomes more challenging in early life, a phase when animals have a high metabolic rate for growth and development of physiological systems, leading to a greater need for feed intake (Evans et al., 2017; Dantonio et al., 2017; Amaral-Silva et al., 2021; 2022). Thus, in cases of more severe infection or sepsis, the thermal response may be opposite to fever, resulting in a drop in body temperature, known as regulated hypothermia or anapyrexia. Literature evidence indicates that regulated hypothermia occurs in both groups of endotherms, i.e., mammals (Almeida et al., 2006; Steiner and Romanovsky, 2019; Morrison & Nakamura, 2011) and birds (Amaral-Silva et al., 2020; 2021; 2022), as a response to save energy. Indeed, simulation of a more severe infection condition with high dose of endotoxin under different energy demand and supply conditions (cold, fasting, and a combination of both) shows prioritization of the regulated hypothermic response in chicks (Amaral-Silva et al., 2022). This hypothermia is also shown to play a relevant role in protecting against tissue injuries and hypoxia in severe systemic inflammation, as demonstrated in rats (Liu et al., 2012). In any case, fever or regulated hypothermia, when exposed to a pathological agent, animals also generally exhibit sickness behavior, with includes loss of appetite and reduced locomotor activity (Soriano et al., 2011).

The peptide ghrelin, which is well known for its orexigenic effect in mammals (KOJIMA et al., 1999, WREN et al., 2000; TSCHÖP et al, 2000), has been also documented to play roles in thermoregulation under low-energy states, such as fasting (Sato et al., 2021), and in modulating inflammation (Gonzalez-Rey et al., 2006; Guyon et al., 2008, Mafra et al., 2011, Wang et al., 2009). Increased plasma concentration of ghrelin is observed during endotoxemy and injection of exogenous ghrelin decreases the plasma

concentration of cytokines (TNF-α, IL-1β and IL-6) induced by the administration of lipopolysaccharide (LPS, endotoxin of Gram-negative bacteria) (Wang et al., 2009). Moreover, ghrelin attenuates LPS-induced fever in rats, a response that is associated with the further increase of plasma corticosterone (an antipyretic pathway) and the reduction of prostaglandin E_2 (PGE_2) concentration in the preoptic region (a pyrogenic pathway) (Soriano et al., 2011).

After the discovery of two ghrelin receptors, GHS-R1a, which is fully functional, and GHS-R1b, which is a truncated splice variant with unclear functionality (Muccioli et al., 2001), authors suggest that the functions of this peptide are entirely related to the ghrelin-receptor interaction. Antagonism of the receptors GHS-R1a is demonstrated to be involved in the modulation of sickness behavior induced by LPS in rats (de Paula Jr et al., 2021). Moreover, both ghrelin and des-acyl ghrelin (DAG), which does not bind to GHS-R1a receptors, have the ability to inhibit the apoptosis of cardiomyocytes and endothelial cells (Baldanzi et al., 2002), even in cells that do not express GHS-R1a. This suggests the existence of an alternative and functionally active binding site, yet unidentified, that could mediate some of the effects of both receptors (Seim et al., 2011).

Birds intriguingly contrast with mammals regarding the effect of ghrelin in food intake, as this activity is inhibited by brain administration of ghrelin in different species, such as chicken, Japanese quail and duck hatchlings (Kaiya et al., 2002; 2007; 2008; 2009; Saito et al., 2002; 2005; Furuse et al., 2001; Shousha et al., 2005). Despite this fact, mammals and birds share many of the ghrelin functions. For example, ghrelin increases corticosterone levels in chicks (Kaiya et al., 2002) and in LPS-treated rats (Soriano et al., 2011), and corticosterone is suggested to play an antipyretic role in both mammals (Coelho et al., 1992) and birds (Gray et al., 2013). Moreover, ghrelin injected in the brain lateral ventricle of 5-day-old chicks decreases Tb one hour after injection (unpublished data), the same time when the Tb reduction occurs after LPS treatment (Dantonio et al., 2017; Amaral-Silva, 2021; 2022). Regarding the receptors for ghrelin in birds, the GHS-1a seems to be involved in the

hypophagic effect of ghrelin in chicks (Farrolkhi et al., 2021), but no information exists about a role in thermoregulation and systemic inflammation.

Thus, based on the considerations above, we aimed to investigate the hypotheses that ghrelin acting on the receptors GHS-R1a modulates the LPS-induced regulated hypothermia in early life in birds. For this purpose, we investigated the effect of intracerebroventricular (icv) injection of the ghrelin receptor antagonist, [D-Lys^3] GHRP-6, on body temperature, oxygen consumption (thermogenesis index), lung ventilation, food consumption, and cutaneous heat loss index in control and LPS-treated 5-day-old chicks.

2-Material and Methods

2.1- Animals

Five-day-old chicks of the Cobb®500 lineage, non-anesthetized, were utilized and provided by a local company (Pluma Agroavícola, Descalvado SP). For experiments involving the measurement of O_2 consumption, pulmonary ventilation, body temperature, cumulative feed intake, and heat loss index, fertile eggs were incubated at 37°C, 65% relative humidity, with turning every two hours in electric incubators (Premium Ecológica- Belo Horizonte, Brazil) until the nineteenth day of incubation. After this period, the eggs were transferred to a hatcher (Premium Ecológica- Belo Horizonte, MG) under the same controlled conditions, but without turning. Upon hatching, chicks were housed in climatic chambers (Premium Ecológica, Belo Horizonte, MG) maintaining temperature controlled at 30-32.5°C (decreasing according to age) (Oliveira et al., 2006; Cristina-Silva et al., 2021) under a light-dark cycle of 14-10h, where lights turned on at 6:00h. Animals had ad libitum access to water and standard chick feed (Rostagno et al. 2011). Procedures involving experimentation with the chicks were conducted after approval by the local Animal Care and Use Committee (CEUA) of FCAV-UNESP No. 1861/2023.

2.2- Surgical Procedure for Mini Temperature Sensor Implantation

On the second day after hatch, chicks were anesthetized via inhalation using isoflurane (Cristália LTDA, Itapira-SP) (3% for induction and 1% for maintenance) to undergo a surgical procedure where a mini temperature sensor (12.5 in length by 2.1 in diameter; BioTherm13, 134.2 kHz FDX-B; Biomark,

Boise ID, USA) was implanted near the liver into the celomic cavity of the animals through an incision ~1cm in the skin, muscle, and peritoneum. After implantation, the openings of peritoneum, muscle, and skin were closed with surgical glue (Dermabond Topical Skin Adhesive; Johnson & Johnson, São Paulo, Brazil). During the procedure, animals received anti-inflammatory (flunixin meglumine 2.5 mg/kg) and antibiotic (enrofloxacin 10 mg/kg) agents to prevent any post-surgical discomfort.

2.3- Oxygen Consumption ($\dot{V}O_2$)

The $\dot{V}O_2$ was measured using an open-flow respirometry system. Chicks were individually placed in a chamber (3L), where air was continuously sucked from the experimental chamber or ambient air (baseline) and directed through a column for CO_2 absorption (Atrasorb, CO_2 absorbers, São Roque SP), by a flowmeter (SS4; Sable Systems, USA) at a rate of 1000 mL/min. Then, an air subsample (180 mL/min) continued to flow to a water vapor pressure analyzer (RH-300; Sable Systems, USA), and dried by a moisture absorber (drierite; Sigma, Germany), before reaching the O_2 analyzer (PA-10, Sable Systems, USA). The analyzer was properly calibrated on the day of the experiment with nitrogen (zero) ambient air (20.95% O_2). The system was connected to an analog-to-digital converter and to a computer for data recording and storage in an appropriate software (Labchart; ADInstruments, Australia). Thus, the $\dot{V}O_2$ was determined based on the air flow rate and the difference between gas concentrations at the chamber's inlet and outlet, [FRe*(FiO2 – FeO2)/(1-FiO2)], where FRe= Air flow passing through the chamber; FiO2= Fraction of oxygen input (baseline); FeO2= Fraction of oxygen output (from the respirometer) (Lighton, 2018). The values are presented in STPD (standard temperature, pressure, and dry air conditions)

2.4- Pulmonary Ventilation ($\dot{V}E$)

Concomitant with the measurements of $\dot{V}O_2$, pulmonary ventilation $\dot{V}\mathrm{E}$ measurements were taken using the barometric method (revised by Mortola and Frappell, 1998). This method is based on the principle that the gas volume within a closed chamber containing the animal expands during inspiration

because the air is warmed and humidified as it passes from the chamber into the airways. The opposite occurs during expiration, and it can be detected by a pressure transducer connected to the experimental chamber, which captures the oscillatory ventilatory waves and thus determines the tidal volume and respiratory frequency. Thus, the following were determined: respiratory frequency (fR; counting peaks of pressure) and tidal volume (Vt), which the product (fR x Vt) is $\dot{V}E$. The Vt is determined using the formula Vt= P(dV/dP) [Tb(Pbaro - Pch_{H2O})] / [Tb(Pbaro - Pch_{H2O})] - [Tch(Pbaro - Pb_{H2O})], where P is the calibration pressure (resulting from the injection of a known volume of air into the chamber), dV/dP is the air compliance of the chamber, Pbaro is barometric pressure, PchH2O is the water vapor pressure inside the chamber, Tch is the temperature inside the chamber, and Pb_{H2O} is the animal's water vapor pressure. The $\dot{V}E$ was recorded periodically for a maximum of 2 minutes each time the respirometer was sealed for analysis of the incoming air (baseline). The temperature within the respirometer (31°C) was monitored by thermissor Pod (MLT415/A adinstruments Australia) connected to analog-to-digital converter.

2.5- Cutaneous Heat Loss Index (HLI)

For the measurement of skin temperature (Ts), a thermal camera (Flir E40 - Switzerland) was used to capture thermal images of the chicks' feet, which represent important thermal windows in these animals (Amaral-Silva et al., 2021; 2022, and Cristina-Silva et al., 2021). These thermal windows demonstrate vasodilation (facilitating heat loss to the environment) and vasoconstriction (reducing heat loss to the environment). The HLI ranges from 0 to 1, where zero indicates maximum vasoconstriction and one indicates total vasodilation. The HLI was calculated using the formula proposed by Romanosysky et al. (2002): HLI= (Ts-Ta)/(Tb-Ta), where: Ts= Animal's skin temperature; Ta= Ambient temperature; Tb= Animal's body temperature.

2.6- Intracerebroventricular (icv) Injection

For injections into the lateral brain ventricle, each chick was restrained in an acrylic apparatus during 20 seconds, with an additional 20 seconds interval

to prevent reflux. Drugs were diluted in 1% Evans Blue solution in saline 0.9% (vehicle) and icv injected (1 μL) in non-anesthetized chicks, as previously described (Coleone et al., 2009; Dantonio et al., 2016). The drugs used were ghrelin (0.5 μg/μl, for chicken, Phoenix Pharmaceuticals, Inc.) and the ghrelin receptor GHSR-1a antagonist [D-Lys^3] GHRP-6 (5 nmol/μl; Sigma-Aldrich, St Louis, MO, USA). The concentration of the antagonist was chosen based on previous studies (Farrokhi et al. 2021; Paula Jr et al., 2022) and in pilot experiments, for not showing changes in feed consumption and Tb per se. After the completion of experimental protocols, animals were euthanized with an overdose of Thiopental (Crístalia LTDA Itapira SP 40mg/kg) intraperitoneally to confirm the injection site. Only animals with microinjections into the lateral ventricle were included in the analysis.

2.7- Lipopolysaccharide treatment

Lipopolysaccharide (LPS) from Escherichia coli, O127: B8 (Sigma-Aldrich), or vehicle (0.9% saline) was intramuscularly (IM) injected in five-day-old chicks at a dose of 100 μg/kg to induce regulated hypothermia (Dantonio et al., 2016; Amaral-Silva et al., 2021). LPS was dissolved in sterile 0.9% saline and kept in a -20°C freezer until the day of the experimental protocol.

2.8- Cumulative feed intake

To measure cumulative feed intake, a feeder with standard feed for the chicks' age was used, placed in the apparatus during the HLI protocol (item 2.3), and the weight of the food was measured in grams using the BELL® Engineering Mark 3100 Class II scale.

3- Experimental Protocols

The experiments were conducted on 5-day-old chicks between 7:00 AM and 6:00 PM (light phase) to avoid interference of the light-dark cycle. The ambient temperature (Ta) used was 31°C, inside the thermoneutral zone for this age (Amaral-Silva et al., 2021)

3.1- Protocol 1: Effect of the ghrelin antagonist [D-Lys3] GHRP-6 preceding ghrelin treatment on Tb.

Ghrelin injected in the brain lateral ventricle (icv) causes a drop in Tb one hour after microinjection (0.1 µg/µL: -0.9 ± 0.2°C; 0.5 µg/µL: -1.4 ± 0.1°C; 1.0µg/µL: -1.5 ±0.3°C; $p < 0.05$; n= 6-12) (unpublished data; see Figure 1 of the appendix). Based on this dose-response curve, 0.5 ug/uL of ghrelin was chosen to test the effectiveness of the ghrelin antagonist [D-Lys3] GHRP-6 in chicks. Experiments were conducted to test the effect of the pretreatment with the antagonist (5 nmol/µl; icv), 40 minutes before the ghrelin (0.5 µg/µL, icv) microinjection on Tb. Measurements of Tb were done every 30 minutes.

3.2- Protocol 2: Effect of the ghrelin antagonist [D-Lys3] GHRP-6 on Tb, $\dot{V}O_2$ and $\dot{V}E$ during a systemic inflammation induced by LPS.

Five days after hatching, chicks equipped with intracoelomic temperature sensors were individually placed in a respirometry chamber (3L) and maintained at 31°C, with access to water and food ad libitum. After 30 minutes for habituation, measurements of $\dot{V}O_2$ and $\dot{V}E$ were taken every 30 minutes, as described in sections 1.3 and 1.4, respectively. After the initial measurements, the animals received an icv injection of the ghrelin antagonist (5 nmol), followed 30 minutes later by an intramuscular injection of LPS (100 µg/kg) or saline. The animals were then returned to the respirometry chamber, where measurements of oxygen consumption, ventilation, and body temperature were continued for about 3 hours, corresponding to the duration of the LPS-induced regulated hypothermia (Amaral-Silva et al., 2021).

3.3- Protocol 3: Effect of the ghrelin antagonist [D-Lys3] GHRP-6 on cutaneous HLI and cumulative feed intake during a system-wide inflammation induced by LPS

Five-day-old chicks equipped with intracoelomic temperature sensors were paired (to mitigate stress) and placed in an apparatus with a mesh floor for

thermal imaging acquisition and Feed consumption measurements (item 1.8). After an initial 30 min habituation time, Tb was measured and the animals received an icv microinjection of [D-Lys3] GHRP-6 (5 nmol), and the feeders were weighed. The animals returned to the apparatus, and 30 minutes later, LPS was administered (100 µg/kg IM). Then, tb was measured each 30min and feeders were weighed just after, 60 and 300 min after LPS. At the end of the protocol, the animals were killed with a high dose of thiopental (Cristália LTDA 40mg/kg) to confirm the injection in the ventricle. Only animals with microinjections into the lateral ventricle of the brain were included in the analysis.

4- Statistical Analysis

The results are presented as mean ± SEM. Repeated measures analysis of variance (Two-Way ANOVA) was used to analyze the effect of treatment and time on Tb, oxygen consumption, pulmonary ventilation, cutaneous heat loss index, and cumulative feed intake. As body mass influences $\dot{V}E$ and $\dot{V}O_2$, a covariance analysis (ANCOVA) was performed, with body mass as a covariate. A Tukey´s test was used for multiple comparisons (R software 4.2.1). Differences among means were considered significant at $p<0.05$.

5- Results

The figure 1 shows a representat mage of the blue label spread in the ventricular system after injection of the elin antagonist in a 1% Evans blue solution was done correctly in the lateral ventricle. It can be observed in figure 1A the spread of dye into the lateral ventricle, and in figure 1 B, the representation of a coronal section showing the spread of the solution through the brain ventricles.

The absolute initial values for each treatment before any intervention were not different among groups (Table 1). Only body mass was lower in the group antagonist + saline compared to the other ones (protocol 2; p=0.025), but it did not affect $\dot{V}E$ and $\dot{V}O_2$, based on the analysis of covariance.

5.1- Effect of the pre-treatment with ghrelin antagonist on the temperature drop caused by ghrelin.

Intracerebroventricular (icv) administration of ghrelin results in a significant decrease in body temperature (Tb) between 30-150 minutes after injection ($p<0.001$). The pre-treatment with the ghrelin antagonist [D-Lys^3] GHRP-6 (5 nmol/100nL), administered 40 minutes earlier, inhibits this ghrelin-induced reduction in Tb (Fig. 2).

5.2- Effect of ghrelin antagonist + LPS treatment on core body temperature (Tb) and respiratory parameters, $\dot{V}E$, $\dot{V}O_2$, *f* and Vt.

The combination of intracerebroventricular (icv) microinjection of the ghrelin antagonist and LPS (A + LPS) resulted in a decrease in Tb from 60 to 270 minutes post intramuscular LPS injection compared to the initial Tb ($p< 0.001$) and hindered the return of individuals' Tb ($p<0.001$), showing a significant difference in Tb drop compared to the V + LPS treatment from 90 to 240 minutes ($p<0.001$ to 0.004) post LPS injection. Treatments with vehicle + saline (V + S) and ghrelin antagonist + saline (A + S) showed no significant difference between them ($p= 0.540$) (Fig. 3).

The metabolic rate (MR) presents a significant reduction ($P=0.018$) in the first hour post-injection compared to the initial MR in the vehicle + LPS treatment (V + LPS). This reduction is also observed in the A + LPS treatment; however, RM remains lower than the initial value between 60-120 minutes after LPS injection ($p=0.002$ to 0.030). After 120 minutes, the results indicate a return to the initial TM. No statistical differences were observed between the V + S and A + S treatments ($p=0.205$) (Figure 4A and B). $\dot{V}E$ was higher in the A + LPS treatment 120 minutes after LPS injection compared to the V + LPS treatment ($p=0.003$) (Fig. 4C). No difference was demonstrated between the control treatments, but there is a reduction over time (240-300 min) in the control treatment (V+S) ($p=0.007$ to 0.025). Despite body mass being significant among treatments, in the covariance model described in the statistical analysis, it was not significant in relation to MR ($p=0.386$) (Fig. 4D).

In the context of respiratory frequency (f), a significant reduction over time was observed in the V + S treatment compared to the initial value between 240 and 300 minutes (p=0.004-0.015). This decrease was also evidenced in the A + S treatment, presenting a statistically significant difference at 300 minutes compared to the initial value (p=0.038). In contrast, no statistical differences were identified in the groups treated with LPS (p=0.646) (Fig. 4E and F).

Regarding the respiratory quotient ($\dot{V}E\ /\dot{V}O_2$), the results showed that over time (60 to 180 minutes) in the A + LPS group, there is an increase compared to the initial (p<0.001 to 0.035), with the mean being significantly higher than the V + LPS group (p<0.001 to 0.026). No statistical differences were identified in the groups that received saline treatment (p=0.331) (Fig. 4G and H). No significant difference in relation to Vt was observed (p=0.748) Fig. 4I and J).

5.3- Effect of ghrelin antagonist + LPS treatment on HLI, Tb, and cumulative feed intake

The results from experimental protocol 3 showed a reduction in Tb in the LPS-treated groups 90 and 120 minutes after im LPS injection (p≤ 0.001), with the A + LPS group maintaining lower Tb until 210 minutes (p<0.001 to 0.007). The VEH + LPS group shows a significant increase compared to the A + LPS group, indicating fever 300 minutes after LPS treatment (p=0.007). No significant difference was identified between the saline-treated groups (p=0.850) (Fig. 5A).

Cumulative feed intake showed no difference between the groups and over time for saline-treated animals (p=0.437 to 0.826), nor between the groups and over time for LPS-treated animals (p=0.696 to 0.874). However, there is a difference between the control groups (VEH + S and A + S), showing higher feed intake when compared to the LPS-treated groups (VEH + LPS and A + LPS) (p<0.001) (Fig. 5B).

The cutaneous HLI reveals vasoconstriction in the V + LPS group 270 and 300 minutes after LPS administration (p< 0.001 and 0.016). Additionally, a significant difference is observed 300 minutes after LPS injection compared to the initial time in the VEH + LPS group (p=0.006). In contrast, animals in the saline-

treated groups showed no difference between them, nor over time (p=0.850 to 1.000) (Fig. 5C and 5D).

6- Discussion

The present study demonstrates that the ghrelin receptor GHS-R1a seems to be involved in the return to normothermia after LPS-induced regulated hypothermia, by mainly interfering with thermogenesis and the air convection requirement ($\dot{V}E$ /$\dot{V}O_2$). This was evidenced by the effect of the pretreatment with the ghrelin antagonist delaying the increase in Tb after hypothermia in LPS-treated animals and reducing the metabolic rate in relation to pulmonary ventilation. The GHS-R1a antagonist also inhibited cutaneous vasoconstriction associated to the later febrile response, about 4-5 hours after the LPS injection.

In previous experiments, ghrelin centrally injected per se decreased the Tb of chicks, depending on their concentration, approximately 1-2 hours after microinjection (unpublished data; see Figure 6 of the appendix). The oxygen consumption was also reduced after icv administration of 0.5 µg/µL ghrelin (see Figure 7 of the appendix), but a little earlier, around 30 minutes after microinjection. Therefore, it is possible to observe that the decrease in Tb was preceded by a decrease in O2 consumption, indicating that the thermogenic effector was inhibited by the central ghrelin, causing the decrease of Tb. In mammals, although there is data showing hyperthermia induced by ghrelin (Jaszberényi et al., 2006), the evidence is stronger for a hypothermic effect of this peptide (Lawrence et al., 2002; Yassuda et al., 2003; Mano Otagiri et al., 2009). Lawrence et al. (2002) demonstrated in rats that 0.1 and 1 µg of ghrelin icv cause transient Tb decrease, a similar effect to that in chicks. This effect in rats may be related to the reduction of sympathetic activity (Yasuda et al., 2003) and the secretion of noradrenaline (NA) to brown adipose tissue (BAT) (Mano OtagirI et al., 2009), a tissue known to be involved in heat production in rodents. In this case, ghrelin appears to act on suprachiasmatic and arcuate hypothalamic nuclei to decrease NA secretion to the BAT (Mano Otagiri et al., 2009), which may be due to the activation of neurons producing neuropeptide Y (NPY) (Morton and Schwartz, 2001). These nuclei could have a connection with the medial dorsal hypothalamus and medullary raphe, known to be involved in

thermogenic responses and activation of the sympathetic-BAT pathway during cold exposure or LPS (Nakamura and Morrison, 2011). In contrast to placental mammals, birds lack BAT and rely mainly on thermoregulatory mechanisms associated with skeletal muscle (Bícego et al., 2007; Rowland et al., 2015). The regulation of Tb by ghrelin in birds is not yet fully understood, but studies suggest that ghrelin may participate in adrenergic pathways, influencing heat production by skeletal muscle (Joubert et al., 2010). This mechanism may involve a neural pathway that includes the medullary raphe, as this region was recently demonstrated to contribute to thermogenic responses in chicks (Cristina-Silva et al., 2021).

Other mechanisms for ghrelin action may involve modulation of cytokines. In rats, it is known that exogenous ghrelin (dose-dependent) plays a role in modulating inflammation caused by LPS, inhibiting some LPS-induced cytokines released by macrophages (IL-6β and TNFα) (Waseem, et al. 2008). In mice models for Parkinson's disease, ghrelin plays a protective role against cell death by blocking microglial activation, which is a component of the brain's immune system, showing to be a favorable mechanism against the deleterious effects of the disease (Moon, et al. 2009). Regarding birds, adult chickens with a viral infection induced by Gumboro disease in the Bursa of Fabricius shows an stimulation for the Bursa to release pro-inflammatory cytokines. In this case, considered low (0.5 nmol/100g) and high (10 nmol/100g) doses of ghrelin injected intraperitoneally reduced the expression of the cytokines induced by infection (Yu et al., 2020), indicating similar results to mammals.

In the present study, the icv injection of the antagonist of the ghrelin receptor GHS-R1a, [D-Lys^3] GHRP-6, alone did not affect Tb of chicks, but inhibited the temperature drop caused by ghrelin. This finding confirmed that the GHS-R1a receptor is involved in the reduction of Tb induced by ghrelin in chicken. This fact also allowed us to use the antagonist combined with LPS to test the involvement of the ghrelin receptor GHS-R1a in hypothermic response to an immune challenge. Surprisingly, contrary to our expectation, the group treated with the antagonist + LPS showed a delay in the return of Tb to normothermia after initial hypothermia. In this case, the relationship between ventilation and oxygen consumption also showed a transient increase at the

same time when Tb was still lower in the antagonist + LPS compared to the vehicle + LPS group. This indicates that a delay in post-hypothermia increase in Tb is caused by a delay in $\dot{V}O_2$ increase, as it is the main mechanism involved in the return to normothermia after LPS-induced regulated hypothermia in chicks (Dantonio et al., 2016; Amaral-Silva et al., 2020; 2022). These results suggest a more complex scenario involving the ghrelin-receptor interactions in the modulation of thermal responses to immune challenges in birds.

At least for sickness behavior in rats, the receptor GHS-R1a seems to have similar effects in its neuromodulation, as a study using Wistar rats with behavioral tests and sucrose preference, which showed that the antagonist [D-Lys^3] GHRP-6 attenuates sickness behavior induced by LPS (de Paula Jr. et al., 2022). In birds, behavioral experimental protocols are not defined, and the variable used to assess the attenuation or not of sickness behavior of LPS was cumulative feed intake. In this case, the ghrelin antagonist, in a concentration that does not affect food ingestion alone (Fig.5B.; Farroki et al., 2020) did not interfere with the LPS anorexic effect.

In the animals for the protocol 3, for HLI, it was possible to observe the presence of fever induced by LPS 4-5 hours after injection, a response that was suppressed by the ghrelin antagonist. The inhibition of peripheral vasoconstriction involved in LPS-induced febrile response (Amaral-Silva et al., 2021) (Fig. 5D) by the antagonist of GHS-R1a provides an explanation for maintaining body temperature below the levels observed in the vehicle + LPS 4-5 hours after injection. This suggests that central ghrelin acting on GHS-R1a receptors plays a significant role in peripheral thermoeffector mechanisms.

7- Conclusion

In summary, the results suggest a role for the ghrelin receptor GHS-R1a in modulating thermal responses to inflammation, indicating an excitatory influence on metabolism for returning to normothermia after LPS-induced regulated hypothermia. Furthermore, the blockade of GHS-R1a receptors appears to inhibit LPS-induced vasoconstriction.

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Appendices

Table

Table 1: Pre-injection values of physiological variables for all treatment groups of 5-day-old chicks

Protocol 1					
Variable	Vehicle + Vehicle	Vehicle + Ghrelin	D-Lys³ [GHRP-6] + Ghrelin		P value
T*b* (°C)	40.8 ± 0.2	40.7 ± 0.2	40.9 ± 0.2		0.854
Variable	Vehicle + Saline	Vehicle + LPS	D-Lys³ [GHRP-6] + Saline	D-Lys³ [GHRP + LPS	P value
Protocol 2					
T*b* (°C)	41.2 ± 0.2	41.0 ± 0.2	41.2 ± 0.2	40.8 ± 0.2	0.692
$\dot{V}O_2$	3.01 ± 0.33	3.08 ± 0.12	3.68 ± 0.25	3.4 ± 0.22	0.278
$\dot{V}E$	133.19 ± 13.07	116.08 ± 14.38	108.35 ± 10.90	112.77 ± 8.22	0.474
V_t (ml)	1.91 ± 0.12	2.04 ± 0.11	1.77 ± 0.12	1.94 ± 0.14	0.527
f (breaths min $^{-1}$)	69.93 ± 6.5	56.31 ± 5.0	62.23 ± 7.7	58.15 ± 2.3	0.367
$\dot{V}E/\dot{V}O_2$	36.1 ± 2.4	31.3 ± 2.7	35.6 ± 4.2	33.0 ± 2.2	0.735
Body mass (g)	90.0 ± 4.0 a	91.0 ± 3.0 a	79.0 ± 3.0 b	93.0 ± 2.0 a	0.025
Protocol 3					
HLI	0.6 ± 0.0	0.7± 0.0	0.7 ± 0.1	0.7 ± 0.1	0.994
Tb (°C)	41.0 ± 0.2	41.0 ± 0.1	40.6 ± 0.2	41.3 ± 0.2	0.723
Body mass (g)	83.9 ± 2.4	87.0 ± 3.0	84.7 ± 2.5	77.2 ± 1.7	0.074

Abbreviations in the table: Tb, body temperature; $\dot{V}O_2$, Oxygen consumption; $\dot{V}E$, pulmonary ventilation; Vt, tidal volume; f, frequency respiratory; HLI, Heat index loss.

Figures legend

Figure 1. **A.** Image of a representative brain showing the blue label spread in the ventricular system after injection in the lateral ventricle. **1 B** is the representation of a coronal section showing the spread of the solution through the brain ventricles.

Figure 2. Effect of the intracerebroventricular (icv) pre-treatment with the ghrelin antagonist [D-Lys3] GHRP-6 (0.5 nmol/µL) on the decrease in body temperature (Tb) caused by ghrelin (0.5 µg/µL; icv). The dashed arrow indicates the moment of [D-Lys3] GHRP-6 injection, and the solid arrow indicates ghrelin injection. Volume of icv injection 1 µL. The numbers (n) of individuals are in parentheses. Open symbols represent statistical difference compared to the initial time (-60 min). *Represents statistical difference with vehicle + vehicle treatment at the same time.

Figure 3. Effect of the intracerebroventricular (icv) pre-treatment with the ghrelin antagonist [D-Lys3] GHRP-6 (0.5nmol/µL) on LPS (100ug/kg, im)-induced change in Tb (experimental protocol 1). The dashed arrow indicates the moment of [D-Lys3] GHRP-6 injection, and the solid arrow indicates the LPS injection. Volume of icv injection 1 µL. The numbers (n) of individuals are in parentheses. Open symbols represent statistical difference compared to the initial time (-30 min). *Represents statistical difference of the 'GHRP-6+LPS' group compared to all the other 3 groups at the same time.

Figure 4. Effect of the intracerebroventricular (icv) pre-treatment with the ghrelin antagonist [D-Lys3] GHRP-6 (0.5 nmol/µL) on LPS (100 mg/kg, im)-induced changes in oxygen consumption (A and B), pulmonary ventilation (C and D), respiratory frequency (E and F), tidal volume (G and H) and respiratory coeficient (I and J) (experimental protocol 2). The dashed arrow indicates the moment of [D-Lys3] GHRP-6 injection, and the solid arrow indicates the LPS injection. Volume of icv injection 1 µL. The numbers (n) of individuals are in parentheses. Open symbols represent statistical difference compared to the initial time (-30 min). *Represents statistical difference between treatments at the same time.

Figure 5. Effect of the intracerebroventricular (icv) pre-treatment with the ghrelin antagonist [D-Lys3] GHRP-6 (0.5 nmol/µL) on LPS (100 mg/kg, im) -induced changes in body temperature (A), feed intake (B), and HLI (experimental protocol 3). The dashed arrow indicates the moment of [D-Lys3] GHRP-6 injection, and the solid arrow indicates the LPS injection. Volume of icv injection 1 µL. The numbers (n) of individuals are in parentheses. Open symbols represent statistical difference in the same group compared to the initial time (-

30 min). *Represents statistical difference with vehicle groups (A and B) and between treatments (D)

Figure 6. Body temperature of Ghrelin- or Vehicle-treated chicks. Time course for the effect of Intracerebroventricular microinjection of Ghrelin (0.005, 0.05, 0.1, 0.5, 1µg/µL) or Vehicle (Evans Blue) 1 µL on body temperature in 5-day-old chicks. Numbers of animals are indicated within parentheses. Arrow indicates Ghrelin or Vehicle microinjections.

Figures

Figure 1 A

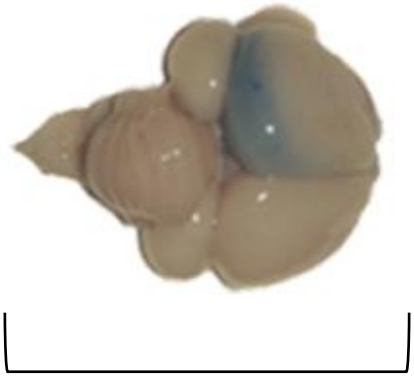


Figure 1 B

Figure 2

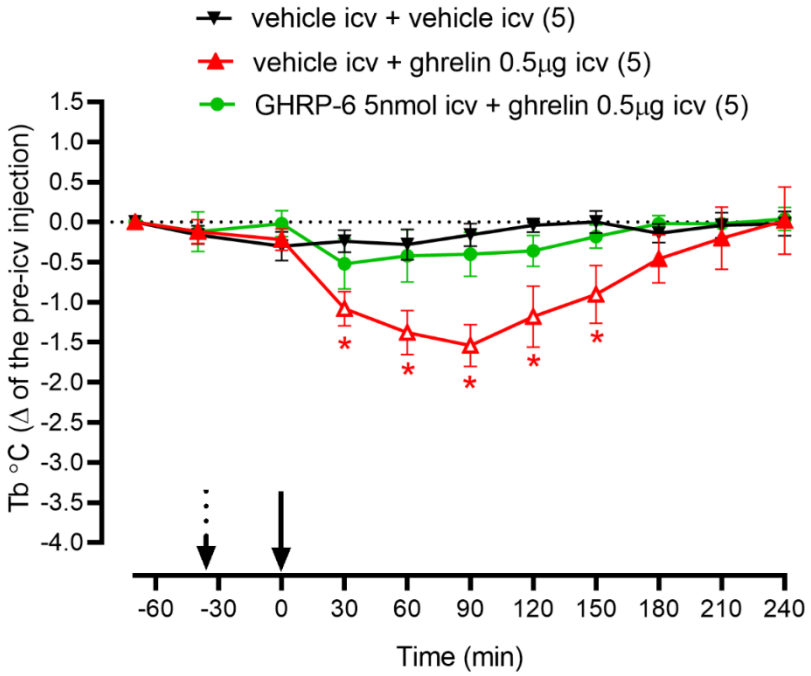


Figure 3

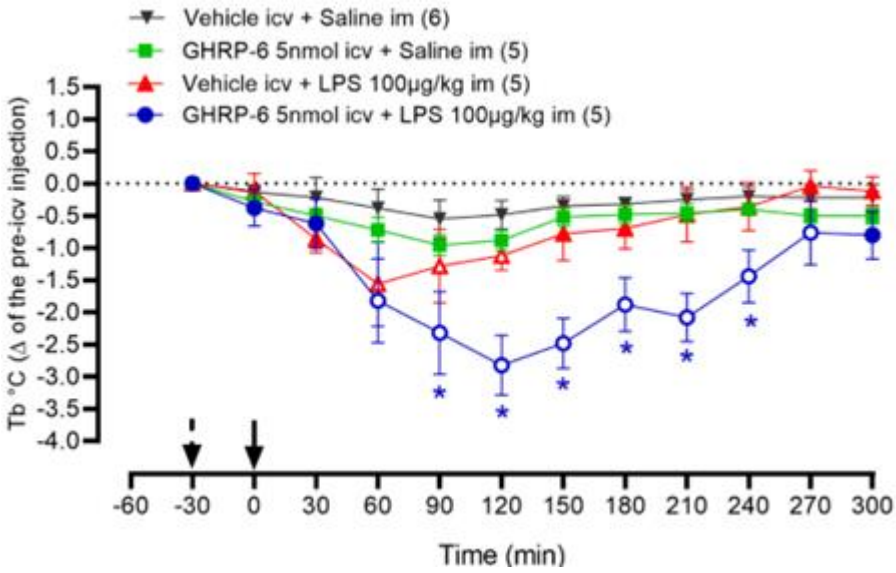

Vehicle icv + Saline im (6)
GHRP-6 5nmol icv + Saline im (5)
Vehicle icv + LPS 100µg/kg im (5)
GHRP-6 5nmol icv + LPS 100µg/kg im (5)
Tb °C (Δ of the pre-icv injection)
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
-3.5
-4.0
-60
-30
0
30
60
90
120
150
180
210
240
270
300
Time (min)

Figure 4

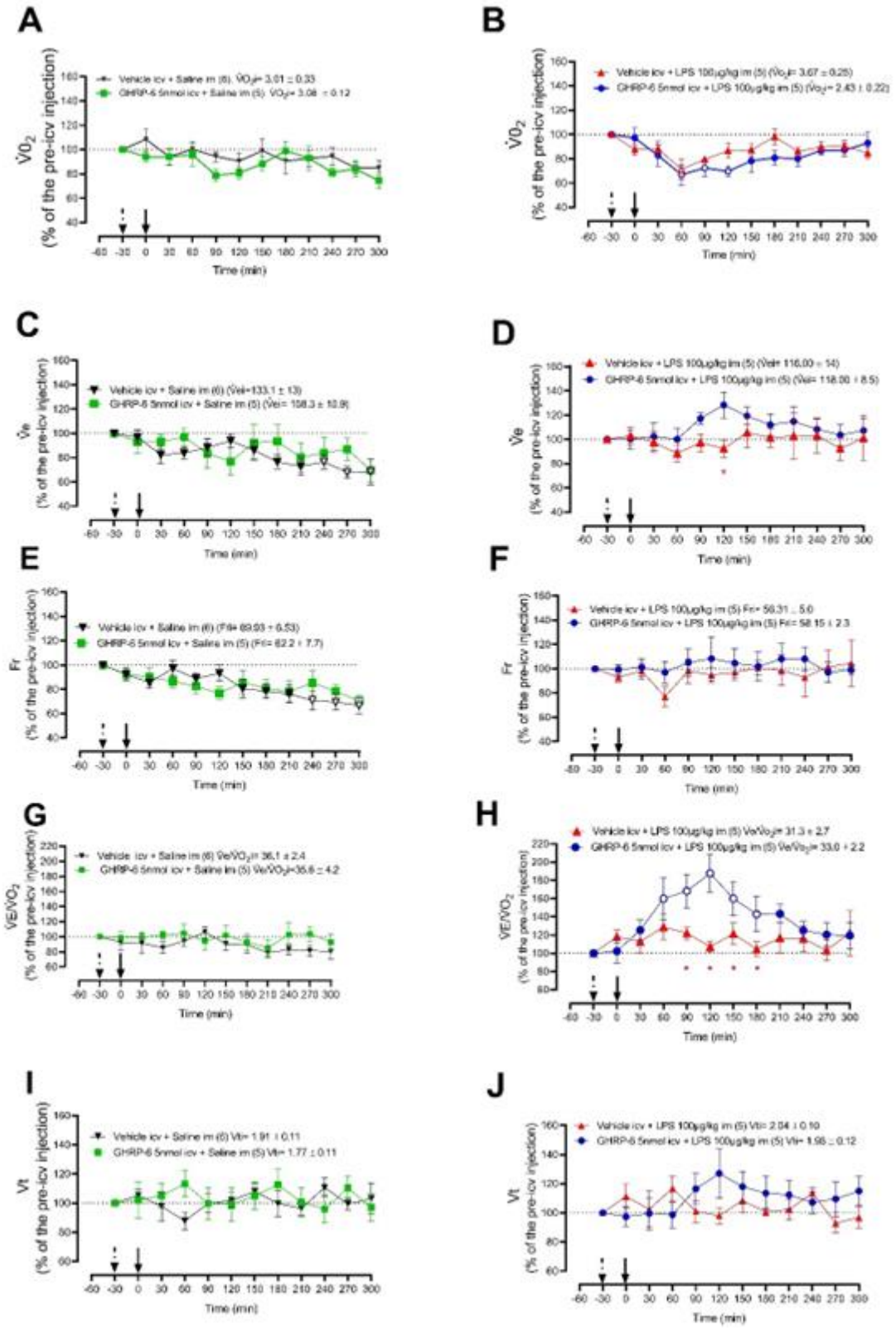
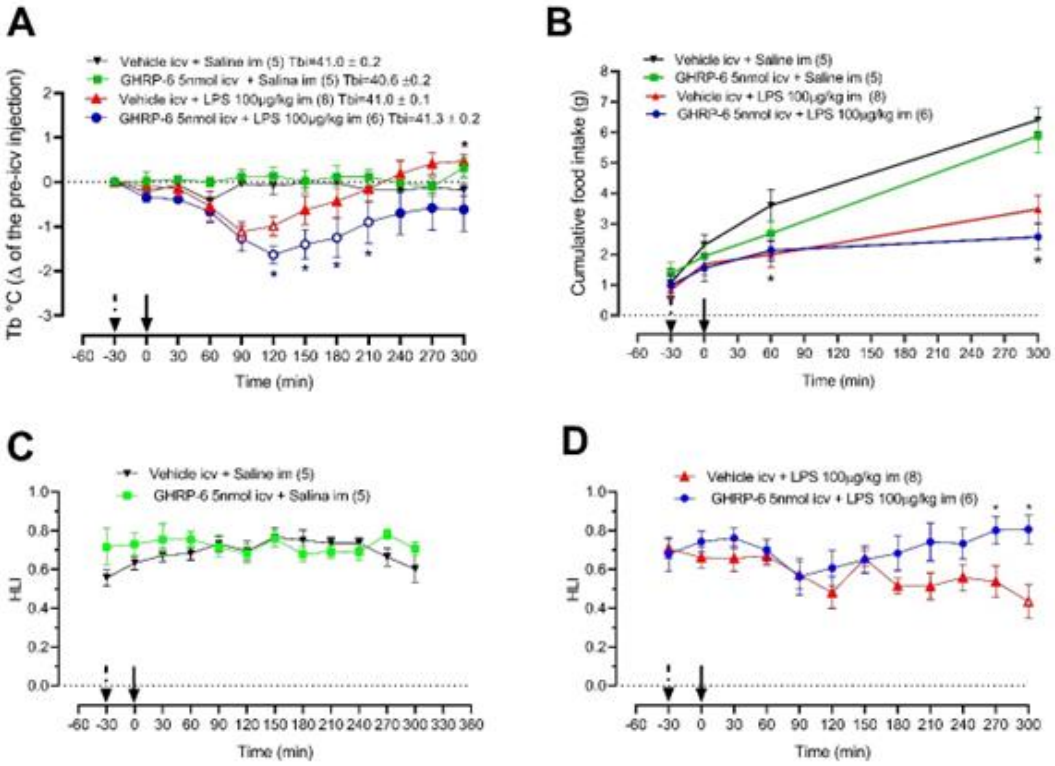


Figure 5


A
Vehicle icv + Saline im (5) Tbi=41.0 ± 0.2
GHRP-6 5nmol icv + Salina im (5) Tbi=40.6 ±0.2
Vehicle icv + LPS 100µg/kg im (8) Tbi=41.0 ± 0.1
GHRP-6 5nmol icv + LPS 100µg/kg im (6) Tbi=41.3 ± 0.2
Tb °C (Δ of the pre-icv injection)
Time (min)
B
Vehicle icv + Saline im (5)
GHRP-6 5nmol icv + Saline im (5)
Vehicle icv + LPS 100µg/kg im (8)
GHRP-6 5nmol icv + LPS 100µg/kg im (6)
Cumulative food intake (g)
Time (min)
C
Vehicle icv + Saline im (5)
GHRP-6 5nmol icv + Salina im (5)
HLI
Time (min)
D
Vehicle icv + LPS 100µg/kg im (8)
GHRP-6 5nmol icv + LPS 100µg/kg im (6)
HLI
Time (min)

Supplementary

Figure 6.

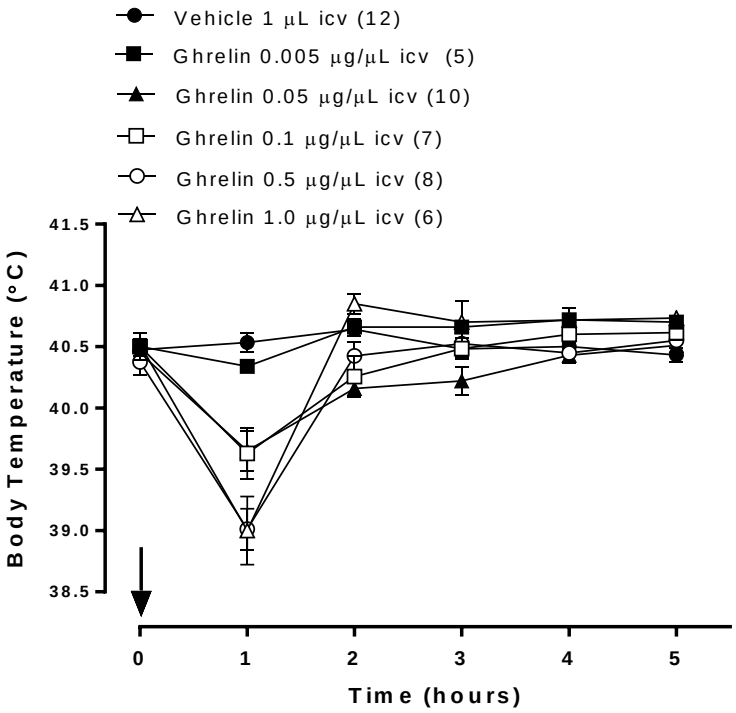


Figure 7: