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**Efeitos da exposição ao frio na reprogramação metabólica de camundongos
obesos: o papel da microbiota intestinal**

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ABSTRACT

Obesity represents a systemic dysregulation of neuro-metabolic integration, characterized by the synchronized dysfunction of adipose tissue, neural circuits, and the gut microbiome. This study establishes intermittent cold exposure as an environmental perturbation capable of reprogramming this pathological state into a lean-like phenotype. Using a systems biology framework, we subjected C57BL/6 mice of both sexes to a chronic high-fat diet (HFD) combined with intermittent cold conditioning, followed by a profiling of bioenergetic, microbial, and behavioral parameters. Cold exposure effectively reversed the obese phenotype in males, restoring a composite Metabolic Score to chow-fed control levels, normalizing visceral adiposity and circulating cholesterol, and reinstating cognitive discrimination. This systemic rescue was driven by the recruitment of brown adipose tissue (BAT), evidenced by elevated surface temperature and the induction of UCP1-dependent mitochondrial respiration. Parallel 16S rRNA sequencing revealed that cold stress restructured the dysbiotic gut microbiome into a distinct "cold-adapted" configuration, enriching short-chain fatty acid producers (e.g. *Lachnospiraceae*) while suppressing typical inflammatory groups (e.g. *Clostridium sensu stricto 1*). Structural equation modeling suggested the expansion of the latter as a causal vector linking dietary lipid load to systemic hypercholesterolemia. A second cohort exposed to microbiota depletion via antibiotic administration demonstrated that the therapeutic efficacy of cold exposure is microbiota-dependent: microbial suppression prevented cold-induced fat loss and blunted *in vivo* thermogenesis. Paradoxically, this occurred despite elevated *ex vivo* mitochondrial respiration, revealing a functional dissociation where the microbiota is a necessary driver for translating cellular oxidation into systemic heat dissipation. These findings demonstrate that intermittent cold exposure reconfigures the neuro-metabolic-microbial Network, marking the gut microbiota as a regulator for BAT-mediated recovery of obesity and its neurocognitive comorbidities.

Keywords: Thermogenesis; Brown Adipose Tissue; High-Fat Diet; UCP; Dysbiosis; Systems Biology

RESUMO

A obesidade representa uma desregulação sistêmica da integração neurometabólica, caracterizada pela disfunção sincronizada do tecido adiposo, dos circuitos neurais e do microbioma intestinal. Este estudo estabelece a exposição intermitente ao frio como uma perturbação ambiental capaz de reprogramar esse estado patológico para um fenótipo semelhante ao magro. Utilizando uma estrutura de biologia de sistemas, submetemos camundongos C57BL/6 de ambos os sexos a uma dieta hiperlipídica (HFD) crônica combinada com exposição intermitente ao frio, seguido pelo perfilamento de parâmetros bioenergéticos, microbianos e comportamentais. A exposição ao frio reverteu efetivamente o fenótipo obeso nos machos, restaurando um Escore Metabólico composto aos níveis do controle alimentado com ração padrão, normalizando a adiposidade visceral e o colesterol circulante, e restabelecendo a discriminação cognitiva. Esse resgate sistêmico foi impulsionado pelo recrutamento do tecido adiposo marrom (BAT), evidenciado pela temperatura superficial elevada e pela indução da respiração mitocondrial dependente de UCP1. O sequenciamento paralelo de rRNA 16S revelou que o frio reestruturou o microbioma intestinal disbiótico em uma configuração distinta "adaptada ao frio", enriquecendo produtores de ácidos graxos de cadeia curta (e.g. Lachnospiraceae) enquanto suprime grupos tipicamente inflamatórios (e.g. *Clostridium sensu stricto* 1). A modelagem de equações estruturais sugere que a expansão deste último atue como um vetor causal ligando a carga lipídica dietética à hipercolesterolemia sistêmica. Uma segunda coorte exposta à depleção da microbiota via administração de antibióticos demonstrou que a eficácia terapêutica da exposição ao frio é dependente da microbiota: a supressão microbiana impediu a perda de gordura induzida pelo frio e atenuou a termogênese *in vivo*. Paradoxalmente, isso ocorreu apesar da elevada respiração mitocondrial *ex vivo*, revelando uma dissociação funcional onde a microbiota é um fator necessário para traduzir a oxidação celular em dissipação sistêmica de calor. Estes achados demonstram que a exposição intermitente ao frio reconfigura a rede neurometabólica-microbiana, marcando a microbiota intestinal como um regulador para a recuperação da obesidade mediada pelo BAT e suas comorbidades neurocognitivas.

Palavras-chave: Termogênese; Tecido Adiposo Marrom; Dieta Hiperlipídica; UCP1; Disbiose; Biologia de Sistemas

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Lista de abreviações

27-OHC – 27-hidroxicolesterol

ABX – Antibióticos (ou Coorte de Depleção da Microbiota)

ADP – Adenosina Difosfato

ANOVA – Análise de Variância

ARRIVE – Pesquisa com Animais: Relato de Experimentos *In Vivo*

ASV – Variante de Sequência de Amplicon

ATP – Adenosina Trifosfato

BAT – Tecido Adiposo Marrom

BBB – Barreira Hematoencefálica

BSH – Hidrolase de Sais Biliares

CCCP – Cianeto de Carbonila m-Clorofenil Hidrazona

CD – Dieta Controle

CE – Exposição ao Frio

CEUA-UnB – Comissão de Ética no Uso de Animais da Universidade de Brasília

CLR – Razão Logarítmica Centralizada

CNS – Sistema Nervoso Central

DNA – Ácido Desoxirribonucleico

ER α – Receptor de Estrogênio alfa

FGF21 – Fator de Crescimento de Fibroblastos 21

FXR – Receptor X de Farnesoide

GDF15 – Fator de Diferenciação de Crescimento 15

GDP – Guanosina Difosfato

GGM – Modelo Gráfico Gaussiano

GLM – Modelo Linear Generalizado

GPR – Receptor Acoplado à Proteína G

HDAC – Histona Desacetilase

HFD – Dieta Hiperlipídica

IQR – Intervalo Interquartil

LDA – Análise Discriminante Linear

LEfSe – Análise Discriminante Linear com Tamanho de Efeito

NIH – Institutos Nacionais de Saúde

NOR – Reconhecimento de Novo Objeto

Nrg4 – Neuregulina-4

NST – Termogênese Sem Tremor

OF – Campo Aberto

OOB – *Out of Bag*

OTU – Unidade Taxonômica Operacional

PCA – Análise de Componentes Principais

PCoA – Análise de Coordenadas Principais

PM – Piruvato + Malato

PPAR γ – Receptor Ativado por Proliferador de Peroxissomo gama

QIIME – Percepções Quantitativas sobre Ecologia Microbiana

RB – Tampão de Respiração

RI – Índice de Reconhecimento

RNA – Ácido Ribonucleico

rRNA – RNA Ribossômico

RT – Temperatura Ambiente

SCFA – Ácido Graxo de Cadeia Curta

SEM – Modelagem de Equações Estruturais

SUIT – Titulação de Substrato-Desacoplador-Inibidor

TGR5 – Receptor de Ácido Biliar Acoplado à Proteína G

TNZ – Zona de Termoneutralidade

UCP1 – Proteína Desacopladora 1

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1. INTRODUÇÃO

1.1 Obesidade como uma disfunção neurometabólica sistêmica

A obesidade representa uma desregulação sistêmica da integração neuro-metabólica, caracterizada pela disfunção sincronizada do tecido adiposo, dos circuitos neurais e do microbioma intestinal. No Brasil, a condição atinge 55% da população adulta, exibindo uma correlação inversa com o nível educacional: a prevalência de obesidade é de 25% em indivíduos com baixa escolaridade (0–7 anos), comparada a 17% naqueles com ensino superior (>12 anos). Esse gradiente reflete o impacto de determinantes sistêmicos, como a desigualdade social e o ambiente físico, que restringem o acesso a padrões alimentares saudáveis e modulam a expressão fenotípica da doença (Ferreira *et al.*, 2024). Esta complexidade exige que a obesidade seja estudada como um estado de carga alostática elevada, onde o excedente energético crônico exaure a capacidade do organismo de manter a homeostase (Mcewen & Stellar, 1993; Ferreira *et al.*, 2024).

A fisiopatologia da obesidade origina-se na falha da plasticidade do tecido adiposo. Sob condições de excesso de nutrientes, a capacidade do tecido adiposo subcutâneo de expandir de forma segura — denominada "expansibilidade do tecido adiposo" — torna-se exausta (Virtue & Vidal-Puig, 2010; Corvera *et al.*, 2025). Esta falha resulta em adipócitos hipertróficos, hipóxia e inflamação, forçando a deposição ectópica de lipídios no fígado e músculo esquelético, o que desencadeia esteatose hepática e dislipidemia sistêmica (Frayn & Karpe, 2014; Miranda *et al.*, 2025).

Esta desregulação estende-se ao Sistema Nervoso Central (SNC), onde o hipotálamo atua como o regulador central do balanço energético. No núcleo arqueado (ARC), neurônios POMC (anorexígenos) e AgRP (orexígenos) integram sinais de leptina e insulina para coordenar a ingestão alimentar e o gasto energético (Engel & Velloso, 2022). O consumo de uma dieta rica em gordura (HFD) dispara uma resposta inflamatória hipotalâmica rápida, com ativação de microglia e astrócitos detectável em 24 horas (Thaler *et al.*, 2012). A exposição prolongada a ácidos graxos saturados induz estresse de retículo endoplasmático e apoptose neuronal, resultando em resistência central à leptina e insulina (Moraes *et al.*, 2009; Engel & Velloso, 2022).

Além disso, a obesidade impacta funções cognitivas superiores. Evidências apontam uma relação etiológica entre o consumo de gordura saturada e o crescimento da prevalência da doença de Alzheimer e declínio cognitivo (Berrino, 2002; Pasinetti & Eberstein, 2008). O consumo de HFD associa-se a déficits de memória e aprendizado em crianças, adultos e idosos, demonstrando que o prejuízo cognitivo é um componente integral do fenótipo obeso (Baym *et al.*, 2014; Gonçalves *et al.*, 2023). A susceptibilidade a obesidade depende também do sexo biológico. Fêmeas de roedores frequentemente apresentam proteção relativa contra o ganho de peso e prejuízos comportamentais induzidos por HFD em comparação aos machos, devido a diferenças na plasticidade do tecido adiposo e vias centrais de detecção de energia (Britton & Fox, 2011; Edlow *et al.*, 2016).

Para investigar estes mecanismos, o modelo experimental de HFD em camundongos C57BL/6 é utilizado para mimetizar o fenótipo humano. A resposta a este modelo é temporalmente definida: uma fase aguda (1–15 dias), com pico inflamatório hipotalâmico, e uma fase crônica (>15 dias), onde a inflamação de baixo grau coexiste com alterações estruturais persistentes (*i.e.* angiopatia hipotalâmica, recrutamento de células mieloides periféricas, retração dos processos astrocitários e apoptose neuronal) tornando o fenótipo refratário à reversão (Engel & Velloso, 2022).

1.2 Termogênese induzida pelo frio e o tecido adiposo marrom como regulador metabólico sistêmico

Enquanto o excesso crônico de nutrientes representa um estresse mal-adaptativo que impede a homeostase metabólica, pressões evolutivas moldaram respostas adaptativas ao estresse térmico que podem ser recrutadas para contra-regular a disfunção metabólica. A zona de termoneutralidade (TNZ) denota a faixa de temperatura ambiente na qual um organismo mantém sua temperatura corporal central apenas através do metabolismo basal, sem a necessidade de produção ativa de calor (Kaiyala & Schwartz, 2011; Speakman & Keijer, 2012). Abaixo deste limiar, o organismo ativa respostas termorreguladoras adaptativas que engajam reservas energéticas sistêmicas para alimentar a geração de calor, alterando a bioenergética de todo o corpo (Cannon & Nedergaard, 2012; Hanssen *et al.*, 2015; Ying *et al.*, 2023).

O elemento central desta resposta adaptativa é o tecido adiposo marrom (BAT) — um tecido oxidativo especializado que é o principal efetor da termogênese sem tremor (NST) em mamíferos (Cannon e Nedergaard, 2004; Cypess *et al.*, 2009). O BAT saudável é caracterizado por uma morfologia celular distinta: adipócitos que contêm múltiplas gotículas lipídicas pequenas (multiloculares), uma densidade mitocondrial alta e uma grande rede de capilares que garante o suprimento contínuo de oxigênio e a rápida dissipação do calor gerado (Bartelt & Heeren, 2014). A capacidade termogênica do BAT é governada por um mecanismo molecular impulsionado pelo sistema nervoso simpático. Sob estimulação pelo frio, a norepinefrina liberada dos terminais simpáticos liga-se aos receptores β 3-adrenérgicos nos adipócitos marrons, ativando a adenilato ciclase e aumentando os níveis intracelulares de AMP cíclico (AMPC). Esta cascata ativa a proteína quinase A (PKA), que fosforila a lipase sensível a hormônio (HSL) e a perilipina 1, promovendo a lipólise e a mobilização de ácidos graxos livres para a mitocôndria (Bartness *et al.*, 2010).

A Proteína Desacopladora 1 (UCP1), uma proteína transmembrana localizada na membrana mitocondrial interna, é o efetor final deste processo. A UCP1 atua como um canal de prótons que permite o retorno dos mesmos à matriz mitocondrial, desviando-os da ATP sintase (Fig. 1). Este processo desacopla a fosforilação oxidativa da síntese de ATP, dissipando o gradiente eletroquímico diretamente na forma de calor (Nedergaard *et al.*, 2007; Brondani *et al.*, 2012; Fedorenko *et al.*, 2012). A atividade da UCP1 é ativada por ácidos graxos de cadeia longa e inibida por nucleotídeos de purina (como o GDP), garantindo um controle preciso da produção térmica (Stanford *et al.*, 2012).

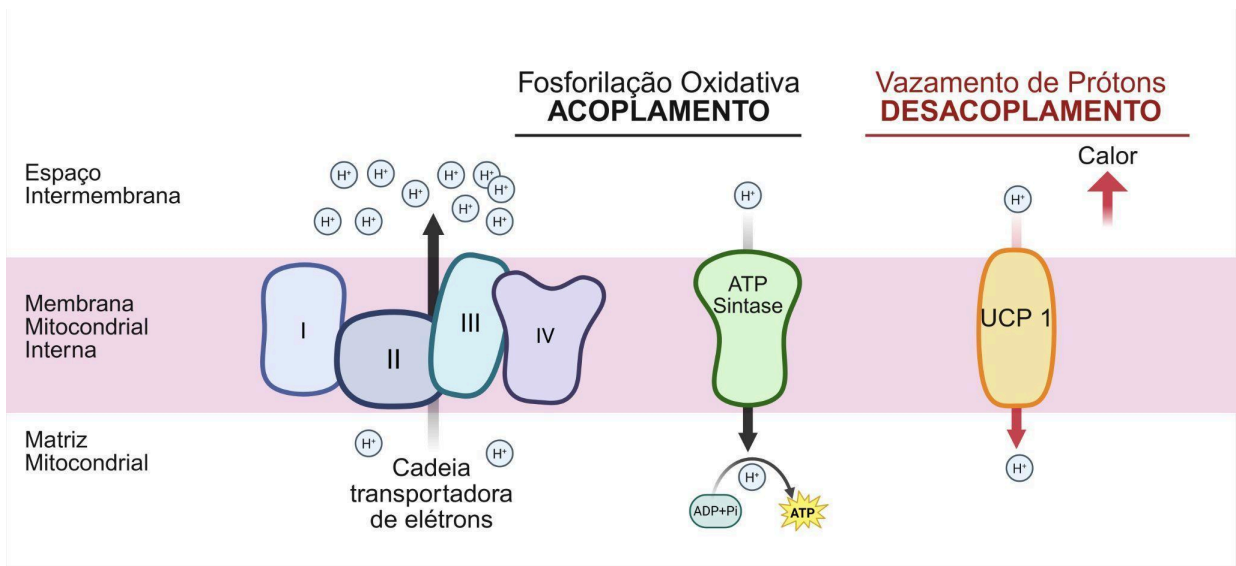


Figura 1 | Localização e função UCP1 na cadeia transportadora de elétrons. Os números I-IV correspondem aos complexos da cadeia transportadora de elétrons. A ATP-sintase é o quinto complexo da cadeia transportadora de elétrons. Durante a respiração, os prótons são bombeados através dos complexos e um gradiente de prótons é gerado. A energia do gradiente de prótons conduz a síntese de ATP pelo complexo ATP-sintase. A UCP1 catalisa uma reentrada regulada de prótons na matriz, desacoplando a cadeia transportadora de elétrons e, consequentemente, reduzindo a síntese de ATP e gerando calor. Adaptado de Brondani *et al.*, 2012.

A obesidade frequentemente compromete a integridade funcional deste sistema através de um processo de remodelagem patológica conhecido como "branqueamento" (*whitening*) do BAT. Neste estado, os adipócitos marrons adquirem características morfológicas e funcionais de tecido adiposo branco (WAT): as gotículas lipídicas tornam-se uniloculares e aumentadas, ocorre uma redução no conteúdo mitocondrial e na expressão de PGC-1 α (coativador 1-alfa do receptor ativado por proliferador de peroxissoma gama), e a capacidade oxidativa declina (Shimizu & Walsh, 2015; Alcalá *et al.*, 2017; Vilela *et al.*, 2023). Esta transição fenotípica correlaciona-se inversamente com a adiposidade sistêmica, indicando que a obesidade induzida pela dieta suprime o potencial de dissipação de energia do organismo e agrava o balanço energético (Lichtenbelt *et al.*, 2009; Chondronikola *et al.*, 2014).

Neste contexto, a exposição intermitente ao frio é uma possível contramedida terapêutica. Ao fornecer um estímulo adrenérgico, a exposição ao frio neutraliza o processo de branqueamento, promove a biogênese mitocondrial e reativa a respiração mediada por UCP1. Esta reativação resulta em melhorias metabólicas sistêmicas consequentes da termogênese, como a redução do peso corporal, o

aumento da sensibilidade à insulina e a normalização dos perfis lipídicos circulantes (Van Der Lans *et al.*, 2013; Yoneshiro *et al.*, 2013; Wang *et al.*, 2015; Carpentier *et al.*, 2018).

Finalmente, o BAT funciona como um transdutor fisiológico e um órgão endócrino complexo, que converte sinais térmicos ambientais em adaptações sistêmicas através da secreção de batoquinas. Entre elas, o GDF15 (*Growth Differentiation Factor 15*) destaca-se como um marcador de estresse mitocondrial que atua no núcleo do trato solitário, no tronco encefálico, para inibir o apetite e promover a perda de peso (Villarroya *et al.*, 2017). Outras batoquinas, como o FGF21 (Fator de Crescimento de Fibroblastos 21) e a Neuregulina-4 (Nrg4), exercem efeitos pleiotrópicos, regulando a gliconeogênese hepática, a captação de glicose no músculo esquelético e a sinalização em circuitos hipotalâmicos de apetite (Wang *et al.*, 2014; Villarroya *et al.*, 2017). Assim, a ativação do BAT representa uma estratégia integrada para restaurar o diálogo fisiológico entre a periferia e o sistema nervoso central, combatendo a obesidade e suas comorbidades associadas (Saito *et al.*, 2009).

1.3. A Microbiota intestinal como moduladora da adaptação metabólica induzida pelo frio

A microbiota intestinal constitui uma interface metabólica dinâmica e essencial, atuando como um sensor biológico que integra sinais dietéticos e pistas ambientais para moldar a homeostase energética do hospedeiro. Operando como um órgão metabólico virtual, este ecossistema complexo desempenha funções bioquímicas que transcendem a capacidade genômica do hospedeiro, incluindo a fermentação de polissacarídeos não digeríveis e a biotransformação de metabólitos que regulam a oxidação lipídica, a sensibilidade à insulina e a função mitocondrial em tecidos periféricos (Sonnenburg & Bäckhed, 2016; De Vos *et al.*, 2022). Através desta rede de sinalização química, as comunidades microbianas modulam a eficiência com que o organismo extrai, armazena e dissipa energia (Chevalier *et al.*, 2015; Fig. 2).

Um dos principais eixos desta comunicação é mediado pelos ácidos graxos de cadeia curta (SCFAs) — acetato, propionato e butirato — gerados pela fermentação bacteriana de fibras dietéticas (Koh *et al.*, 2016). Estes metabólitos atuam como

ligantes específicos para receptores acoplados à proteína G, como o GPR41 (FFAR3) e o GPR43 (FFAR2), localizados em enterócitos e adipócitos, onde promovem a secreção de incretinas como o GLP-1 e suprimem a lipogênese hepática (Tolhurst *et al.*, 2012). Crucialmente, os SCFAs exercem uma função neuroprotetora ao atravessar a barreira hematoencefálica e atuar como inibidores de histonas desacetilases (HDACs) na microglia. Esta modulação epigenética atenua a sinalização pró-inflamatória induzida pela dieta hiperlipídica, preservando a integridade dos circuitos hipotalâmicos e oferecendo uma via de resgate para a disfunção neuro-metabólica central (Rutsch *et al.*, 2020).

A temperatura ambiental atua como uma pressão seletiva que induz uma reestruturação programada da arquitetura microbiana. A exposição ao frio promove a expansão de uma "microbiota adaptada ao frio", caracterizada pelo enriquecimento de táxons como *Akkermansia muciniphila* e membros da família Lachnospiraceae (Chevalier *et al.*, 2015). Esta transição fenotípica da microbiota é funcionalmente ligada à termogênese através de múltiplos mecanismos. Primeiro, o aumento na produção de SCFAs ativa o receptor PPAR γ no tecido adiposo branco, impulsionando o programa de "*browning*" e a expressão de UCP1 (Li *et al.*, 2019). Segundo, a microbiota altera o *pool* de ácidos biliares através da atividade de hidrolase de sais biliares (BSH), aumentando a disponibilidade de ácidos biliares secundários que ativam o receptor de membrana TGR5 no BAT. Esta ativação amplifica a sinalização de AMPc e a atividade da deiodinase tipo 2 (D2), potencializando a capacidade termogênica do tecido (Thomas *et al.*, 2009).

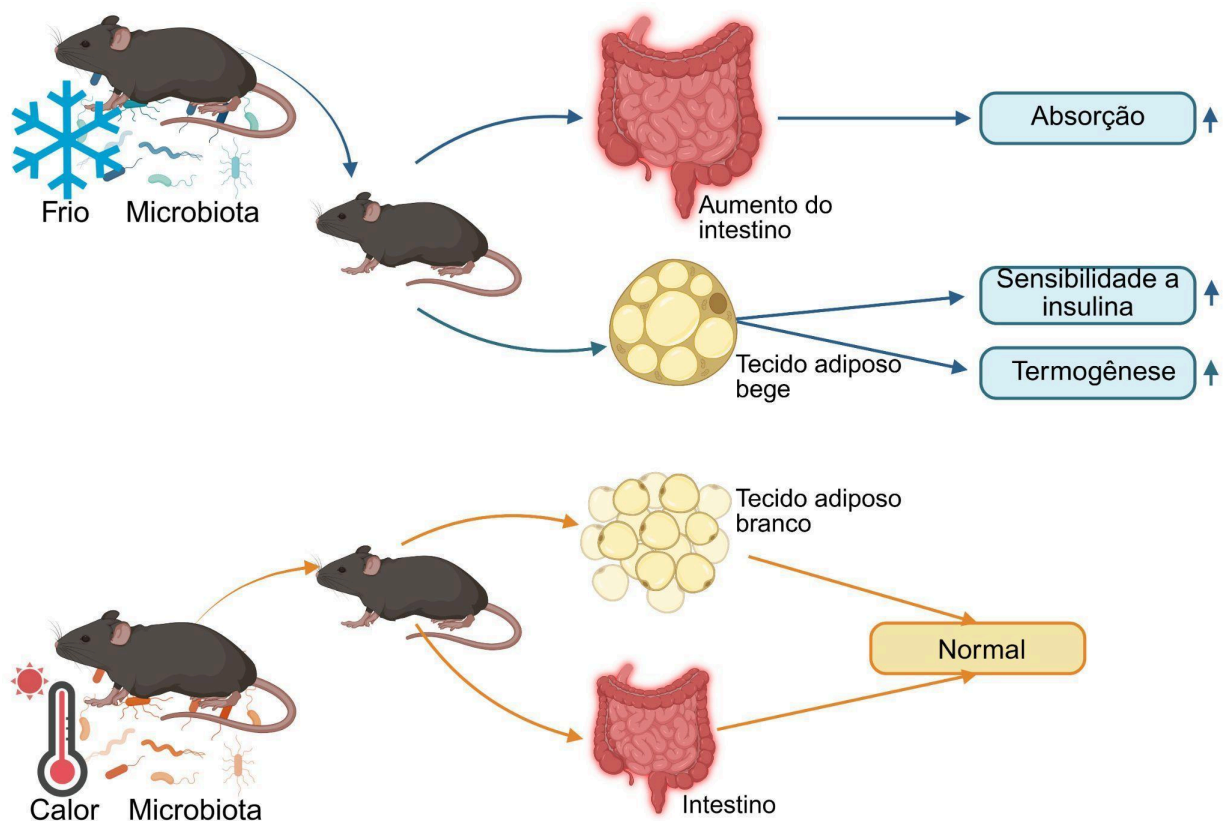


Figura 2 | O eixo microbiota-intestino-tecido adiposo na regulação da termogênese. A exposição ao frio ou a dieta induzem alterações na composição da microbiota intestinal. Metabólitos bacterianos e componentes da parede celular atuam como moléculas sinalizadoras para o sistema imune do hospedeiro. No tecido adiposo, estes sinais promovem o recrutamento de eosinófilos e a polarização de macrófagos para o fenótipo anti-inflamatório (M2). Estas células imunes secretam citocinas (como IL-4 e IL-13) e catecolaminas que estimulam os receptores adrenérgicos nos adipócitos. Esta cascata de sinalização induz a expressão de UCP1 e a biogênese mitocondrial, resultando no escurecimento (*browning*) do tecido adiposo branco e na ativação da termogênese no tecido adiposo marrom. Adaptado de Chevalier *et al*, 2015.

Estudos demonstram que a depleção da microbiota via antibióticos prejudica a capacidade termogênica do BAT e o escurecimento do tecido adiposo branco, resultando em menor gasto energético. Este fenômeno sugere que a microbiota é necessária para traduzir a oxidação celular em dissipação de calor sistêmica. Mecanicamente, este controle pode envolver a regulação da composição de cardiolipina na membrana mitocondrial interna — essencial para a estabilidade e função da UCP1 — ou a modulação do particionamento de substratos através do eixo

acetato-malonil-CoA, que regula a eficiência do desacoplamento mitocondrial (Worthmann *et al.*, 2017).

Diante da natureza sistêmica da obesidade e de suas comorbidades neurocognitivas, a investigação de estratégias que reprogramem a rede neuro-metabólica-microbiana torna-se imperativa. A integração das evidências atuais aponta para a exposição intermitente ao frio como uma proposta promissora para a modulação metabólica. Esta dissertação investiga precisamente essa intersecção, postulando que a exposição ao frio recruta a microbiota intestinal para resgatar não apenas a homeostase térmica, mas a integridade neuro-metabólica perdida na obesidade, estabelecendo a microbiota como um regulador indispensável para a recuperação mediada pelo BAT.

1.4 Hipotese

A exposição intermitente ao frio reverte a disfunção neuro-metabólica induzida por dieta hiperlipídica através do recrutamento do tecido adiposo marrom, e essa recuperação sistêmica — que abrange a normalização da adiposidade visceral, do colesterol circulante e do desempenho cognitivo — é dependente da microbiota intestinal, que atua como mediador necessário para a tradução da capacidade oxidativa mitocondrial em dissipação de calor e reprogramação metabólica *in vivo*.

2. JUSTIFICATIVA

A trajetória fisiológica de um organismo sob estresse metabólico não pode ser plenamente compreendida por meio da análise de variáveis isoladas em um contexto desvinculado do sistema como um todo. Os fenótipos metabólicos emergem da integração não linear entre domínios neurocomportamentais, microbianos e bioenergéticos. A comunicação cruzada entre a microbiota intestinal, o tecido adiposo marrom e os circuitos cognitivos centrais implica que alterações em um único ponto, como a depleção de bactérias intestinais ou a ativação da termogênese, modificam a homeostase sistêmica.

Dessa forma, análises univariadas de parâmetros isolados, como peso corporal, comportamento ou taxonomia bacteriana, são intrinsecamente limitados para capturar a complexidade dessas interdependências entre órgãos. Embora tais abordagens possam identificar diferenças estatisticamente significativas em marcadores individuais, frequentemente deixam de revelar a estrutura latente da disfunção sistêmica e os padrões combinatórios que definem estados de saúde versus doença.

Para superar essas limitações, torna-se necessária a adoção de uma estrutura computacional integrativa. A aplicação de metodologias multivariadas, incluindo técnicas de agrupamento não supervisionado e classificadores baseados em aprendizado de máquina, permite a identificação não enviesada de estados fenotípicos e o ranqueamento de características fisiológicas de acordo com sua contribuição preditiva para o perfil metabólico global. Além disso, a construção de índices compostos — como um *Score Metabólico* — fornece uma métrica quantitativa da saúde sistêmica, mitigando o ruído estocástico inerente às variáveis individuais.

Assim, o presente trabalho adota uma perspectiva de biologia de sistemas ao sintetizar dados de alta dimensionalidade provenientes das camadas comportamental, termogênica e microbiana. Esta dissertação tem como objetivo desconstruir o “fenótipo de resgate” induzido pela exposição ao frio, oferecendo uma compreensão mecanística de como sinais ambientais reprogramam a rede neurometabólica.

3. OBJETIVOS

3.1 Objetivo Geral

Investigar a eficácia sistêmica da exposição intermitente ao frio como estratégia terapêutica para reverter a disfunção neuro-metabólica induzida pela dieta hiperlipídica, avaliando os mecanismos dependentes da plasticidade do tecido adiposo marrom e da modulação da ecologia microbiana intestinal.

3.2 Objetivos Específicos

Em um modelo experimental de obesidade induzida por dieta hiperlipídica em camundongos:

Caracterizar o impacto da exposição ao frio sobre desfechos metabólicos (peso corporal, adiposidade e perfil lipídico) e comportamentais (locomoção, cognição e comportamento tipo-depressivo) e sobre a modulação da microbiota intestinal,

Avaliar a capacidade funcional do tecido adiposo marrom (BAT), por avaliação da temperatura superficial do BAT e respirometria de alta resolução, correlacionando a eficiência da respiração dependente de UCP1 com o fenótipo metabólico;

Identificar assinaturas taxonômicas específicas associadas ao “fenótipo de resgate” induzido pelo frio e determinar seu papel na mediação dos efeitos termogênicos, por meio depleção microbiana por antibióticos.

MANUSCRITO PARA SUBMISSÃO

Manuscrito Intitulado “*Effects of Cold Exposure on Metabolic Reprogramming in Obese Mice: The Role of the Gut Microbiota*” para submissão na revista “*Molecular Biology*”

Effects of Cold Exposure on Metabolic Reprogramming in Obese Mice: The Role of the Gut Microbiota

INTRODUCTION

Obesity is a chronic disorder of energy homeostasis that now affects more than one billion individuals worldwide, with rising prevalence across all income levels and demographic groups (Ferreira et al., 2024). Its pathophysiology cannot be reduced to a simple imbalance between caloric intake and expenditure; rather, it emerges from the synchronized failure of multiple physiological systems — adipose tissue, hypothalamic circuits, and the gut microbiome — whose dysfunction is mutually reinforcing (McEwen & Stellar, 1993). At the tissue level, chronic positive energy balance exhausts the expansion capacity of subcutaneous adipose depots, forcing ectopic lipid accumulation in the liver and skeletal muscle, and driving systemic dislipidemia (Virtue & Vidal-Puig, 2010; Frayn & Karpe, 2014; Miranda et al., 2025). In the central nervous system, consumption of a high-fat diet (HFD) initiates a rapid hypothalamic inflammatory response — detectable within 24 hours — involving microglial activation, endoplasmic reticulum stress, and neuronal apoptosis in the arcuate nucleus, ultimately producing central resistance to leptin and insulin (Thaler et al., 2012; Moraes et al., 2009; Engel & Velloso, 2022). These neuroendocrine defects propagate beyond homeostatic circuits: dietary fat excess and the attendant neuroinflammation impair hippocampal-dependent memory in children, adults, and aged individuals, establishing cognitive impairment as an integral feature of the obese phenotype rather than an incidental comorbidity (Baym et al., 2014; Berrino, 2002; Pasinetti & Eberstein, 2008; Gonçalves et al., 2023). Importantly, biological sex

modulates nearly every dimension of this pathology. Female rodents show relative resistance to HFD-induced weight gain and behavioral deficits compared to males, reflecting differences in adipose plasticity, hypothalamic lipid sensing, and the neuroprotective actions of estrogen (Britton & Fox, 2011; Edlow et al., 2016), and this sexual dimorphism must be explicitly addressed in any mechanistic study of obesity.

Brown adipose tissue (BAT) is the primary effector of non-shivering thermogenesis (NST) in mammals and represents a functionally relevant target for counter-acting obesity (Cannon & Nedergaard, 2004; Cypess et al., 2009). Its thermogenic capacity depends on Uncoupling Protein 1 (UCP1), a mitochondrial inner-membrane channel that dissipates the proton electrochemical gradient as heat instead of driving ATP synthesis, consuming glucose and fatty acids in the process (Nedergaard et al., 2007; Fedorenko et al., 2012). Obesity induced by HFD suppresses this system through a process of BAT whitening: brown adipocytes accumulate unilocular lipid droplets, lose mitochondrial density, and downregulate PGC-1 α and UCP1 expression, diminishing the tissue's capacity to act as a metabolic sink (Shimizu & Walsh, 2015; Alcalá et al., 2017; Vilela et al., 2023). The inverse correlation between BAT activity and adiposity documented in humans underscores that this functional deterioration is not merely a consequence but a driver of the obese energy imbalance (Lichtenbelt et al., 2009; Chondronikola et al., 2014). Cold exposure, by activating the sympathetic–adrenergic axis and the downstream cAMP–PKA–UCP1 cascade, reverses the whitening phenotype, promotes mitochondrial biogenesis, and produces measurable metabolic benefits: reduced adiposity, improved insulin sensitivity, and normalized lipid profiles (Van der Lans et al., 2013; Yoneshiro et al., 2013; Carpentier et al., 2018; Hanssen et al., 2015). Beyond its thermogenic function, activated BAT operates as a secretory organ, releasing batokines including FGF21, Neuregulin-4 (Nrg4), and GDF15 that act on the hypothalamus, liver, and skeletal muscle to coordinate systemic metabolic and neurobehavioral adaptations (Villarroya et al., 2017; Wang et al., 2014; Wang et al., 2015; Saito et al., 2009).

The gut microbiota constitutes an additional regulatory layer for energy homeostasis. Operating as a virtual metabolic organ, this microbial community ferments dietary fiber into short-chain fatty acids (SCFAs) — principally acetate, propionate, and butyrate — which signal through GPR41 and GPR43 receptors on enteroendocrine and adipose cells to promote incretin secretion, suppress hepatic lipogenesis, and regulate

mitochondrial function in peripheral tissues (Sonnenburg & Bäckhed, 2016; Koh et al., 2016; De Vos et al., 2022). SCFAs also traverse the blood-brain barrier and act as histone deacetylase inhibitors in microglia, attenuating HFD-induced neuroinflammation and preserving hypothalamic circuit integrity (Rutsch et al., 2020; Cani et al., 2008; Dinan & Cryan, 2017). The gut microbiota further modulates bile acid pools through bacterial salt hydrolase activity, generating secondary bile acids that activate the membrane receptor TGR5 in BAT to amplify cAMP signaling and thermogenic capacity (Thomas et al., 2009; Worthmann et al., 2017). Critically, environmental temperature is itself a selective pressure on microbial community composition. Chronic cold promotes expansion of a distinct cold-adapted microbiota enriched in *Akkermansia muciniphila* and members of Lachnospiraceae — SCFA-producing taxa whose abundance correlates with metabolic improvement — while suppressing pro-inflammatory lineages (Chevalier et al., 2015). Microbiota depletion by broad-spectrum antibiotics impairs BAT thermogenesis and prevents white adipose tissue browning, demonstrating that the microbiota is required for the full translation of adrenergic stimulation into systemic heat dissipation (Li et al., 2019; Suárez-Zamorano et al., 2015).

Despite these advances, several fundamental questions remain unresolved. First, the causal relationship between gut microbiota remodeling and the systemic efficacy of intermittent cold exposure in established diet-induced obesity has not been directly interrogated under a chronic conditioning paradigm. Prior studies investigating microbiota depletion and BAT activation have employed acute cold protocols or thermoneutral conditions, leaving the functional dependency of cold-induced metabolic recovery on an intact microbiome insufficiently defined (Suárez-Zamorano et al., 2015; Chevalier et al., 2015). Second, whether the metabolic rescue driven by cold extends to neurocognitive outcomes — and whether this extension is itself microbiota-dependent — has not been established. Third, the sex-specificity of the BAT–microbiota–brain axis has been largely overlooked, despite compelling evidence that males and females employ distinct adaptive strategies in response to both dietary challenge and thermal stress (Britton & Fox, 2011; Edlow et al., 2016; Corvera et al., 2025). Fourth, characterizing which specific microbial taxa and functional pathways mediate the metabolic phenotype requires integrative approaches beyond standard taxonomic profiling.

Here, we address these questions using a systems biology framework applied to C57BL/6 mice of both sexes subjected to chronic HFD and a five-week intermittent cold exposure protocol. We combined high-resolution BAT respirometry, infrared thermography, behavioral profiling, and 16S rRNA gut microbiome sequencing with multivariate analytical tools — including composite metabolic scoring, unsupervised clustering, Random Forest classification, physiological network topology, and structural equation modeling — to characterize the systemic phenotypic response to cold exposure and its microbial determinants. To causally evaluate the functional requirement of the gut microbiota, a distinct cohort underwent broad-spectrum antibiotic depletion concurrent with cold exposure. We report that intermittent cold exposure reverses the obese phenotype in male mice, restoring BAT-mediated thermogenesis, normalizing visceral adiposity and circulating cholesterol, and recovering hippocampal-dependent cognitive performance. This systemic rescue is accompanied by a restructuring of the gut microbiome into a cold-adapted configuration enriched in SCFA-producing Lachnospiraceae, while structural equation modeling identifies *Clostridium sensu stricto* 1 as a causal mediator linking dietary lipid load to hypercholesterolemia. Microbiota depletion abolishes the *in vivo* thermogenic and fat-reducing effects of cold despite preserving *ex vivo* mitochondrial respiratory capacity, revealing the gut ecosystem as a necessary gating element for the translation of cellular oxidative potential into whole-body heat dissipation and metabolic recovery.

MATERIALS AND METHODS

Animals and Ethical

This study utilized 3–6-month-old C57BL/6 mice ($n = 123$) of both sexes. Animals were housed in ventilated cages (Alesco system; 3–4 mice per cage) under controlled environmental conditions (23–25°C) with a 12-h light/dark cycle (lights on at 6:00 AM) at the Faculty of Medicine, University of Brasília. Water and diet were provided *ad libitum*. Mice were assigned to either a standard control diet (CD: 63.95% carbohydrates, 20.3% proteins, 15.75% lipids) or a high-fat diet (HFD: 27.6% carbohydrates, 15.2% proteins, 45% lipids), both supplied by Pragsoluções Biociências® (Jaú, SP, Brazil).

To investigate the causal role of the gut microbiome, a distinct subset of male C57BL/6 mice was utilized. Males were selected for this cohort based on the metabolic phenotype observed in the main experiments. Animals were divided into four groups (CD, CD+ABX, HFD, HFD+ABX; n=42) and subjected to the same dietary and thermal protocols as the main cohort. Microbiota depletion was achieved via a broad-spectrum antibiotic cocktail administered in the drinking water. Antibiotic treatment commenced at the sixth week of the dietary protocol and was maintained continuously throughout the cold exposure period until euthanasia. Dosage was calculated based on an estimated daily intake of 5 mL per animal.

All experimental procedures were conducted in strict accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and reported following the ARRIVE guidelines. The protocols were approved by the Ethics Committee on the Use of Animals of the University of Brasília (CEUA-UnB; Protocols No. 23106.107690/2023-49).

Experimental Design and Protocols

Diet-Induced Obesity And Intermittent Cold Exposure Protocol

The experimental timeline was divided into two phases: a Diet-Induced obesity period (Weeks 1–5) and a subsequent Cold Exposure intervention period (Weeks 6–10). In the first phase, male and female C57BL/6 mice were randomly assigned to receive either the control diet (CD: 63.95% carbohydrates, 20.3% proteins, 15.75% lipids) or the high-fat diet (HFD: 27.6% carbohydrates, 15.2% proteins, 45% lipids), both supplied by Pragsoluções Biociências® (Jaú, SP, Brazil). At the beginning of the sixth week, animals were stratified into subgroups to receive the thermal intervention (Room Temperature vs. Cold Exposure) while maintaining their respective dietary regimens (Fig. 3)

The cold exposure (CE) protocol was adapted from Lim *et al.* (2012). Animals in the CE groups were transferred to a climatized incubator (Eletrolab EL202/3), maintained at 4 °C for 4 hours daily, 5 days per week, for a total of 5 weeks. To prevent circadian disruption, the chamber was equipped with a ventilation system and lighting synchronized with the facility's light/dark cycle. To minimize potential confounding

effects of thermal stress on the female estrous cycle, a temporal separation was implemented: females were exposed during the morning phase (08:00–12:00), while males were exposed during the afternoon phase (13:00–17:00). Control animals (RT) remained in their home cages at standard ambient temperature (23–25 °C) throughout the study.

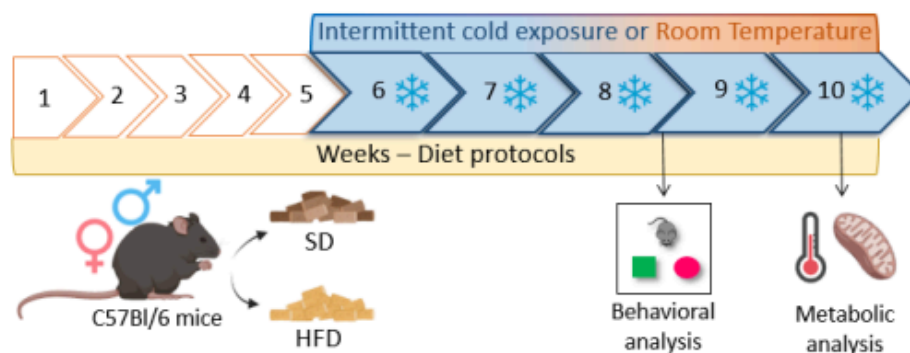


Figure 3 | Experimental design and timeline of cold exposure protocol.

Microbiota Depletion (ABX Cohort)

To assess the functional requirement of the gut microbiome in cold-induced metabolic adaptations, a distinct cohort of male C57BL/6 mice was subjected to antibiotic depletion. Using the same dietary and thermal timeline described above, animals received a broad-spectrum antibiotic cocktail in their drinking water starting from the sixth week (coinciding with the onset of cold exposure) until euthanasia (Fig. 4). The cocktail consisted of ampicillin (0.5 g/L), neomycin (1.0 g/L), vancomycin (0.5 g/L), and ciprofloxacin (1.0 g/L). Dosages were calculated based on an estimated daily water intake of 5 mL per animal. All animals in this cohort were subjected to the intermittent cold exposure protocol.

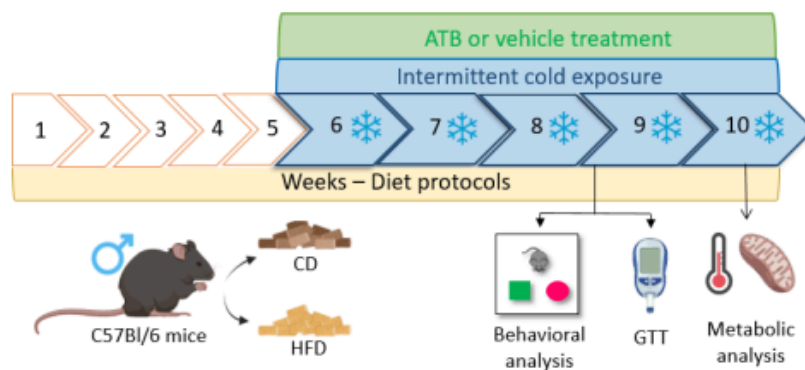


Figure 4 | Experimental design and timeline of ABX protocol.

Behavioral Assessment

General Conditions

Behavioral tests were conducted during the light phase of the cycle (08:00–12:00) in a sound-attenuated room maintained at 23 °C. To eliminate olfactory cues, all apparatuses were cleaned with a 10% (v/v) ethanol solution between trials.

Open Field Test (OF)

Locomotor activity was assessed in a cubic arena (30 × 30 × 30 cm) with a white background and spatial cues on the sidewalls. Mice were placed individually in the center of the arena and allowed to explore freely for 10 minutes. The total distance traveled and time spent in the center zone were recorded and analyzed using *ANY-maze software* (Stoelting Co., IL, USA).

Novel Object Recognition (NOR)

Short-term recognition memory was evaluated 24 hours after the OF test (which served as the habituation phase). The protocol consisted of two sessions separated by a 90-minute inter-trial interval. In the training phase, animals were exposed to two identical objects (Familiar, 'F') positioned 5 cm from the walls for 5 minutes. In the test phase, mice were reintroduced to the arena containing one familiar object (copy of 'F') and one novel object ('N') for 5 minutes. Exploration was defined as directing the nose

toward the object at a distance ≤ 1 cm. A Recognition Index (RI) was calculated as: $RI = [T_{\text{novel}} / (T_{\text{novel}} + T_{\text{familiar}})]$.

Splash Test

To assess depressive-like behavior and self-care motivation, the splash test was performed as previously described. A 10% sucrose solution was sprayed on the dorsal coat of the mouse, which was then placed in a glass funnel placed over a mirror to facilitate observation. The latency to initiate grooming and the total duration of grooming behavior were manually recorded over a 5-minute period.

***In Vivo* Thermogenesis Assessment**

To evaluate brown adipose tissue (BAT) thermogenic activity in response to the thermal intervention, interscapular surface temperature was measured immediately after the final cold exposure session (or equivalent time for RT controls). Infrared thermal images were captured using a FLIR T420 camera (FLIR Systems, UK) positioned perpendicular to the dorsal region at a fixed distance of ~50 cm. Images were processed using FLIR QuickReport 1.2 *software* to determine the maximum temperature within the interscapular area.

Sample Collection and Euthanasia

At the end of the experimental period, mice were fasted for 6 hours (08:00–14:00) during the light phase. Anesthesia was induced with isoflurane. Specifically, animals from the C57BL/6 cohorts were anesthetized using the open-drop method (gauze saturated with isoflurane). Following the confirmed loss of pedal reflexes, animals were euthanized by decapitation.

Whole blood was immediately collected and centrifuged at $1000 \times g$ for 10 minutes at 4 °C. Serum aliquots were stored at -80 °C for biochemical profiling. Interscapular BAT was rapidly dissected; a portion was immediately processed for high-resolution respirometry (see below), while the remaining tissue, along with the hippocampus,

visceral and subcutaneous white adipose depots, and intestinal contents, were weighed, flash-frozen in liquid nitrogen or dry ice, and stored at -80°C .

BAT High-Resolution Respirometry

Mitochondrial respiration in BAT was assessed using an OROBOROS Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria). Freshly dissected BAT fragments (~1 mg) were weighed and homogenized in respiration buffer (RB) using a glass-Teflon homogenizer. Samples were transferred to the oxygraph chambers containing 2 mL of air-saturated RB at 37°C under constant stirring.

A Substrate-Uncoupler-Inhibitor Titration (SUIT) protocol was applied to evaluate mitochondrial function:

Leak Respiration: Addition of pyruvate (5 mM) and malate (2.5 mM) to assess complex I-linked respiration in the absence of ADP.

UCP1-Dependent Respiration: Titration with Guanosine Diphosphate (GDP; 1–3 mM) to inhibit UCP1 activity, allowing the quantification of thermogenic capacity.

Maximal Uncoupled Respiration: Stepwise titration with the protonophore CCCP (0.5 μM steps) to determine the maximal capacity of the electron transport system.

Non-Mitochondrial Respiration: Inhibition of Complex I with rotenone (0.5 μM) and Complex III with antimycin A (2.5 μM). Oxygen flux was normalized to tissue wet weight and expressed as $\text{pmol O}_2/\text{s}/\text{mg}$.

Microbiota Profiling and Bioinformatics

Metagenomic DNA was extracted from intestinal samples using a commercial kit, following the manufacturer's protocols. The V3–V4 hypervariable regions of the bacterial 16S rRNA gene were amplified and sequenced on an Illumina MiSeq platform. Raw reads were processed using the QIIME 2 pipeline to generate Amplicon Sequence Variants (ASVs). The microbiome analysis was performed using Python (v3.12) and R ecosystems (via rpy2 interface). ASV tables were pre-processed to

remove samples with low sequencing depth (<100 reads) and transformed to relative abundance or Centered Log-Ratio (CLR).

Diversity and Composition Analysis

Alpha diversity metrics (Shannon entropy, Observed OTUs, and Faith's Phylogenetic Diversity) and beta diversity matrices (Bray-Curtis dissimilarity, Weighted and Unweighted UniFrac) were computed using the scikit-bio library. Principal Coordinate Analysis (PCoA) was employed to visualize community structure. Taxonomic composition was analyzed at the Phylum, Class, and Family levels.

Biomarker Discovery and Differential Abundance

Taxonomic biomarkers characterizing specific group differences were identified using Linear Discriminant Analysis Effect Size (LEfSe). Features were first filtered using the Kruskal-Wallis test, and significant features were subjected to Linear Discriminant Analysis (LDA) to estimate effect size (threshold score > 2).

To reconstruct the unobserved functional potential of the bacterial community, Amplicon Sequence Variants (ASVs) were processed via the PICRUSt2 pipeline (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). Sequences were aligned to a reference phylogenetic tree to predict the abundance of orthologous gene families, annotated according to the Enzyme Commission (EC) nomenclature.

Statistical Analysis and Mathematical Modeling

All data analyses were conducted using Python (v3.12).

Data Preprocessing and Univariate Analysis

Prior to hypothesis testing, outliers were identified and removed using the Interquartile Range (IQR) method ($1.5 \times \text{IQR}$ rule). The normality of residuals was assessed via the Shapiro-Wilk test.

To account for the non-normal distributions, we employed an Adaptive Generalized Linear Model (GLM) framework. Variables following a normal distribution were modeled using a Gaussian family with an identity link function, while positively skewed non-normal variables were modeled using a Gamma family with a log link function. Main effects (Diet, Temperature, Sex/Genotype) and their interactions were evaluated. Pairwise post-hoc comparisons were corrected for multiple testing using the Benjamini-Hochberg method. For the Novel Object Recognition (NOR) task, performance was compared against the theoretical chance level (50%) using a one-sample t-test.

Dimensionality Reduction and Machine Learning

To evaluate systemic phenotypic shifts, we performed Principal Component Analysis (PCA) on standardized physiological variables to reduce dimensionality while preserving variance. Unsupervised clustering was conducted in the PC space using the K-means algorithm ($k=3$). The non-random distribution of experimental groups across these topological clusters was statistically validated using a Chi-square test of independence.

To identify the specific physiological drivers of the phenotypic rescue, we utilized a Random Forest Classifier (100 estimators, Gini impurity criterion). The models were trained separately for males and females to respect sexual dimorphism. Model performance and generalizability were rigorously evaluated using Out-of-Bag (OOB) accuracy estimates to prevent overfitting. Feature importance scores were extracted to rank predictors (e.g. mitochondrial respiration, lipid profiles, thermogenesis) based on their mean decrease in impurity, quantifying their relative contribution to the classification of the rescued state.

Network Topology

To reconstruct the functional architecture of the physiological system, we employed a network analysis approach based on partial correlations. A Gaussian Graphical Model (GGM) was constructed where nodes represented physiological variables and edges represented partial correlation coefficients ($\rho > 0.6$), thereby filtering out indirect associations. The topological properties of the resulting networks were quantified using graph theory metrics, including:

Network Density: The ratio of actual connections to potential connections, representing systemic interconnectivity.

Modularity: A measure of the network's division into distinct functional modules (calculated via greedy modularity optimization).

Assortativity: The tendency of nodes to connect with nodes of similar functional domains (e.g., metabolism connecting to metabolism), quantifying systemic segregation versus integration.

Eigenvector Centrality: To identify influential nodes within the rescued phenotype.

Composite Metabolic Indices

The metabolic health was quantified using a composite Metabolic Health Score, calculated as the inverted mean of Z-scores for key pathological markers—specifically visceral and subcutaneous adiposity, circulating glucose, cholesterol, triglycerides, and weight variation. In this index, higher values indicate a healthier, leaner systemic profile, while lower negative values characterize a dysregulated metabolic state.

Correlation Matrix and Structural Equation Modeling (SEM)

To explore the functional interplay between gut microbial ecology and host metabolic physiology, we generated Spearman's rank correlation matrices. We specifically assessed the associations between the abundance of targeted microbial and metabolic features. To further investigate mechanistic hypotheses suggested by these correlations, we performed Path Analysis using a regression-based SEM approach. We constructed causal models to test specific mediation hypotheses, estimating direct (c') and indirect ($a \times b$) effects between Diet and systemic cholesterol, mediated by *Clostridium sensu stricto 1* abundance.

RESULTS

Cold Exposure Reverses HFD-Induced metabolic changes in a Sex-Dependent Manner

We evaluated the effects of diet, temperature, and gender on metabolic parameters using a three-way ANOVA. We found significant interactions between Sex and Temperature for body weight variation ($F_{1,87} = 22.24$, $p < 0.001$) and cholesterol levels ($F_{1,92} = 9.59$, $p = 0.003$). Visceral adiposity was influenced by a Diet $\times$ Temperature interaction ($F_{1,76} = 13.15$, $p < 0.001$). At room temperature, post-hoc analysis revealed that the HFD induced a pathological metabolic phenotype in male mice compared to those fed the control diet (CD). We observed a significant increase in body weight ($p = 0.0001$, $d = -2.04$; Fig. 5a), expansion of visceral adipose depots ($p = 0.002$, $d = -1.73$; Fig. 5c), and elevated cholesterol plasma levels ($p < 0.001$, $d = -2.67$; Fig. 5e). In contrast, while HFD-fed females exhibited significant weight gain ($p = 0.013$, $d = -1.03$; Fig. 5a), their overall weight gain was consistently lower when compared to males ($F_{1,87} = 63.51$, $p < 0.001$). Also, HFD did not cause significant changes in their visceral adiposity ($p = 0.13$) nor plasma cholesterol levels ($p = 0.39$).

Comparing HFD-fed animals at room temperature versus cold (HFD-RT vs. HFD-CE) revealed that cold exposure prevented weight gain ($p = 0.002$, $d = 1.17$; Fig. 5a), reduced visceral fat ($p = 0.020$, $d = 1.18$; Fig. 5c), and normalized cholesterol levels ($p < 0.001$, $d = 1.98$; Fig. 5e) in male mice. Only in CD-fed males, however, cold exposure significantly increased plasma triglycerides levels ($p = 0.011$, $d = -1.04$; Fig. 5g).

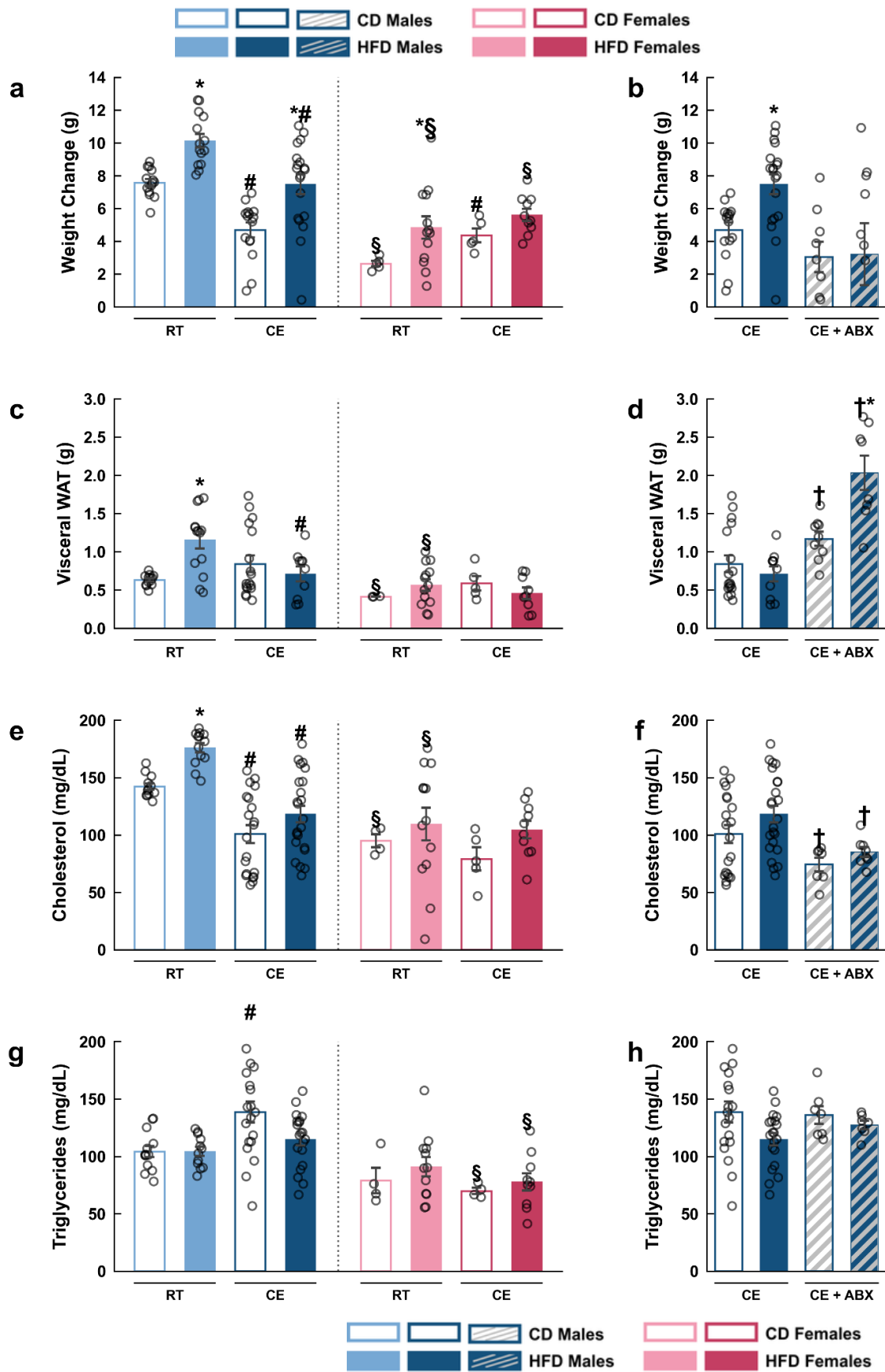


Figure 5 | Cold exposure and microbiota depletion modulate metabolic profile in diet-induced obesity. Body weight change (**a**, **b**), visceral white adipose tissue mass (**c**, **d**), total cholesterol (**e**, **f**), and plasma triglycerides levels (**g**, **h**). Left panels (**a**, **c**, **e**, **g**) present the main cohort, comparing males and female mice kept at room temperature (RT) or exposed to cold (CE), fed a control diet (CD) or

high-fat diet (HFD). Right panels (**b, d, f, h**) detail the cold-exposed male mice cohort treated with antibiotics (ABX) for microbiota depletion. Data are presented as mean $\pm$ s.e.m. Statistical symbols: * $p < 0.05$ vs corresponding CD (diet effect); # $p < 0.05$ vs corresponding RT (temperature effect); † $p < 0.05$ vs control without antibiotics (ABX effect); § $p < 0.05$ vs male counterpart (sex effect); adaptive GLM.

We investigated the role of the microbiota in the response to cold exposure using an antibiotic cocktail for microbiota depletion (ABX). Two-way ANOVA identified a significant main effect of ABX treatment on visceral fat ($F_{1,40} = 31.09$, $p < 0.001$; Fig. 5b) and cholesterol levels ($F_{1,54} = 10.36$, $p = 0.002$; Fig. 5c). Microbiota depletion blocked the beneficial effect of cold on adiposity, resulting in higher visceral adipose tissue accumulation in both CD ($p = 0.043$, $d = -0.81$, Fig 5d) and HFD ($p = 0.001$, $d = -2.75$, Fig 5d) mice compared to controls without antibiotic treatment. Contrary to this expansion, ABX treatment significantly reduced plasma cholesterol levels. Specifically, both CD ($p=0.026$; Fig. 5f) and HFD-fed ($p = 0.002$) mice treated with antibiotics exhibited lower plasma cholesterol concentrations compared to their untreated counterparts.

Cold exposure rescues BAT's thermogenesis impaired by high-fat diet in a sex-dependent manner

We further assessed brown adipose tissue (BAT) thermogenic activity by measuring interscapular surface temperature (Fig. 6c) and oxygen consumption respiration. In the main cohort, a Three-Way ANOVA revealed a main effect of Temperature ($F = 50.22$, $p < 0.001$) and Diet ($F=12.09$, $p < 0.001$) across both sexes. *Post-hoc* analysis confirmed that cold exposure elevated BAT temperature in both males – CD ($p = 0.003$, $d = -1.84$) and HFD ($p = 0.009$, $d = -1.69$) – and females – CD ($p=0.035$) and HFD ($p=0.014$) – compared to their room temperature counterparts. To validate this functional recruitment, we performed high-resolution respirometry on BAT explants (Fig. 6d). While in male mice HFD did not alter BAT bioenergetics, we observed an induction of UCP1-dependent respiration by cold ($F_{1,72} = 17.17$, $p < 0.001$), with significant increases in cold-exposed males on CD ($p = 0.033$, $d = -0.91$) and HFD ($p = 0.018$, $d = -1.15$). Basal mitochondrial respiration (PM-sustained respiration) followed a similar pattern, driven largely by temperature ($F_{1,72} = 21.19$, $p < 0.001$). In females, however, HFD feeding at room temperature blunted UCP1-dependent

respiration compared to controls ($p < 0.001$, $d = -3.19$), with a significant rescue modulated by cold exposure ($p = 0.026$, $d = 1.30$).

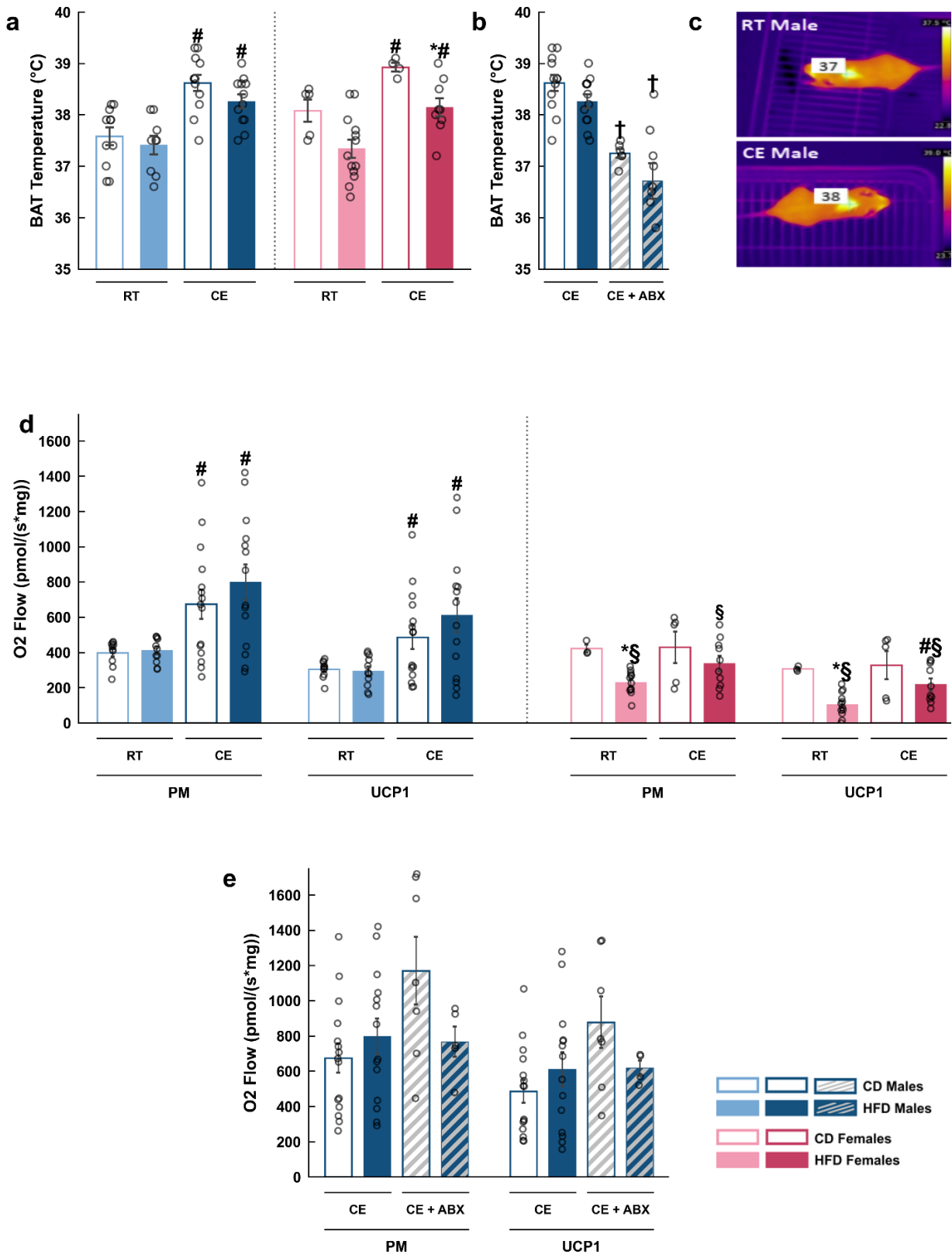


Figure 6 | Thermogenic profile and mitochondrial bioenergetics of brown adipose tissue (BAT).

Interscapular BAT surface temperature measured by infrared thermography in the main cohort (A) and the antibiotic-treated cohort (B), accompanied by a representative thermal image of a RT and CE male mice (C). Oxygen flux (high-resolution respirometry) in BAT homogenates, assessing pyruvate/malate-sustained respiration (PM), maximal uncoupled respiratory capacity by CCCP, and UCP1-dependent respiration in the main cohort (D) and the ABX cohort (E). Data are presented as mean $\pm$ s.e.m. Statistical symbols: * $p < 0.05$ vs corresponding CD (diet effect); # $p < 0.05$ vs corresponding RT (temperature effect); † $p < 0.05$ vs control without antibiotics (ABX effect); § $p < 0.05$ vs male counterpart (sex effect).

Microbiota depletion significantly altered the thermogenic profile. Two-way ANOVA revealed a main effect of antibiotic treatment on BAT temperature ($F_{1,35} = 47.81$, $p < 0.001$; Fig. 6b). Specifically, ABX treatment blunted the thermogenic response to cold, resulting in significantly lower surface temperatures in HFD-fed animals ($p = 0.003$, $d = 2.04$) compared to untreated controls. This reduction in surface temperature was not accompanied by a decrease in mitochondrial respiration parameters in the ABX groups.

Cold Exposure Restores Cognitive Performance in Obese Male Mice

We expanded phenotypic profiling to behavioral and cognitive domains by assessing locomotor activity and recognition memory. In the main cohort, open field exploration revealed a significant effect of sex in locomotion ($F_{1,87} = 8.41$, $p = 0.005$; Fig. 7c), with *post-hoc* analysis confirming that HFD-fed females displayed increased total distance traveled compared to males ($p = 0.003$). Cognitive evaluation via the Novel Object Recognition (NOR) test identified a Sex $\times$ Temperature interaction ($F_{1,51} = 7.00$, $p = 0.011$; Fig. 7a). HFD-fed males at room temperature failed to discriminate against the novel object (56.4%, $p = 0.16$ vs. 50%), indicating a cognitive deficit. Cold exposure restored statistical discrimination in the HFD CE group (55.0%, $p = 0.031$). In females, animals CD at room temperature exhibited intact memory (58.9%; $p = 0.011$), while HFD consumption blunted this performance to near-chance levels (54.3%, $p = 0.068$). However, cold exposure restored this discriminative capability in HFD-fed females (60.5%, $p = 0.024$) and further enhanced performance in controls (68.9%, $p = 0.005$). Microbiota modulation via ABX did not alter this behavioral profile. In the NOR test, we

observed a main effect of diet ($F_{1,45} = 5.04$, $p = 0.030$; Fig. 7b), but microbiota depletion did not cause cognitive deficit in both HFD (55.0%, $p = 0.031$) and CD group (56.5%, $p = 0.007$).

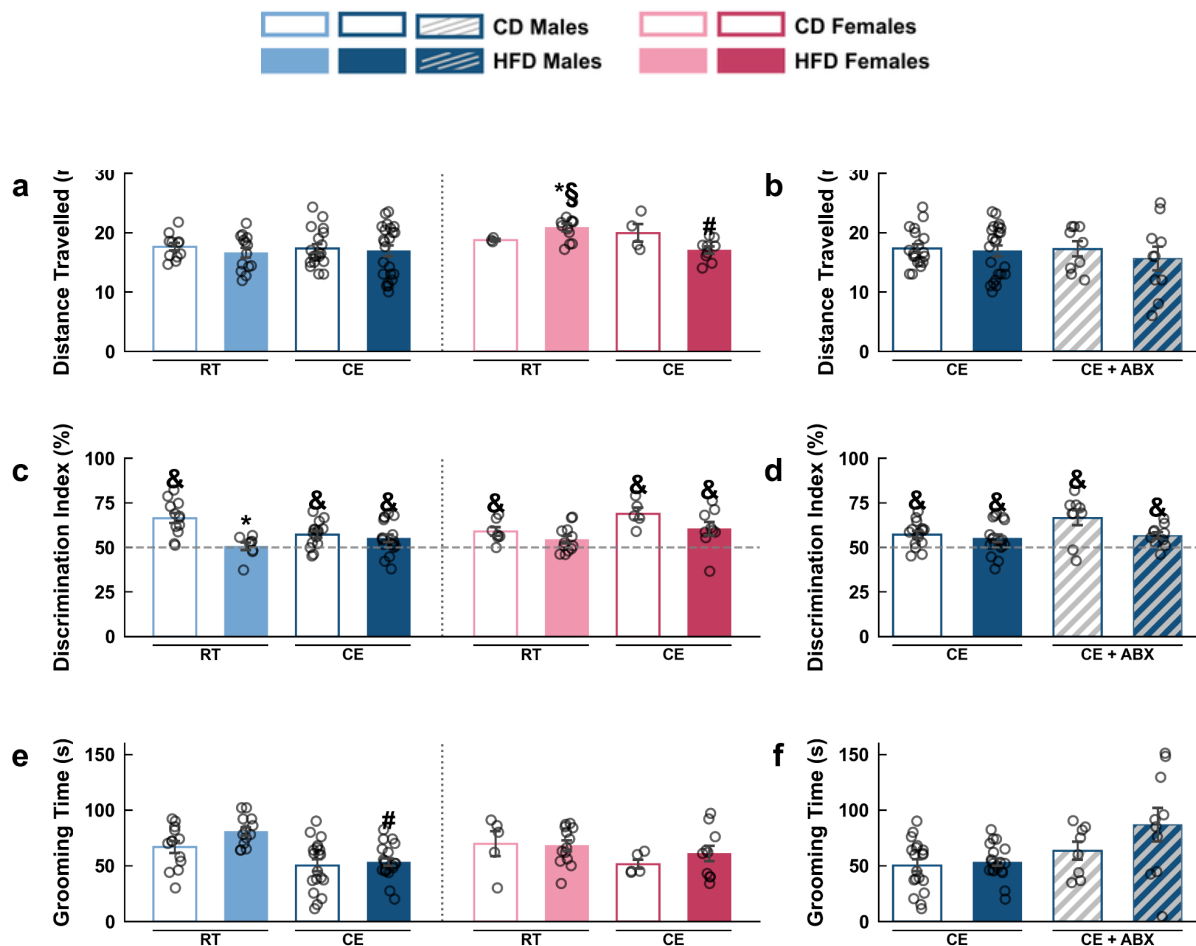


Figure 7 | Impact of cold exposure and microbiota on cognition. Total distance travelled in the Open Field test (**a**, **b**). Discrimination index in the Novel Object Recognition (NOR) test –the dashed line indicates the 50% chance level (**c**, **d**). Total grooming time in the splash test (**e**, **f**). Group organization (Main on left, ABX on right). Data are presented as mean $\pm$ s.e.m. Statistical symbols: * $p < 0.05$ vs corresponding CD (diet effect); # $p < 0.05$ vs corresponding RT (temperature effect); † $p < 0.05$ vs control without antibiotics (ABX effect); § $p < 0.05$ vs male counterpart (sex effect); & $p < 0.05$ vs 50% chance level

Cold Exposure Reprograms the Obese Phenotype Toward a Lean-Like State

To quantify the efficacy of cold exposure in reversing the obese phenotype, we constructed a composite Metabolic Score integrating all physiological variables

(variables described in material and methods). This score collapsed in the HFD male group ($M = -1.00$) compared to CD-RT controls ($M = -0.08$), characterizing a dysregulated metabolic state ($t(16.2)=5.46$, $p=0.0003$; Fig. 8a). The cold exposure restored the metabolic score of HFD-fed male mice to levels indistinguishable from healthy controls ($p = 0.54$), suggesting a phenotypic rescue ($t(21.5)=-3.51$, $p=0.0041$). In contrast, females were resistant to this metabolic collapse caused by HFD and maintained Metabolic Scores consistently higher than their male counterparts, impeding the observation of a rescue effect as seen in males.

Unsupervised K-means clustering provided topological validation of this rescue. HFD-RT males clustered almost exclusively in a distinct pathogenic niche (Cluster 0, 10/14 animals), whereas HFD-CE animals migrated entirely out of this cluster, co-localizing with CD-CE animals in the healthy Cluster 2 (8/10 animals). This topological shift was confirmed to be non-random by a Chi-Square test of independence ($\chi^2 = 94.70$, $p < 0.0001$; Fig. 8b), demonstrating that cold exposure reprograms the systemic phenotype of obese mice towards a lean-like profile.

Beyond positional shifts, the internal architecture of the metabolic network underwent a significant reconfiguration. As detailed in table 1, the transition from the obese state to the rescued phenotype was marked by a two-fold increase in Network Density (+100%) and Total Edges (+100%), indicating a substantial rise in systemic interconnectivity. In parallel, the network increased in Modularity (+50%), suggesting a more efficient functional compartmentalization. This reintegration was accompanied by a decline in Assortativity (-42.1%), signaling the dissolution of the rigid segregation observed in the obese state in favor of a more cohesive, integrated physiological network.

Network Metric	HFD-RT (Obese)	HFD-CE (Rescued)	% Change
Network Density	0.026	0.051	+100.0%
Modularity	0.500	0.750	+50.0%
Assortativity	1.000	0.579	-42.1%
Avg Degree	0.308	0.615	+100.0%
Avg Eigenvector	0.154	0.218	+41.4%
Total Edges	2.000	4.000	+100.0%

Table 1 | Global topological properties of neuro-metabolic correlation networks. Comparison of structural metrics (Density, Total Edges, Modularity, and Assortativity) between the Obese and Rescued phenotypes. The data indicate a significant reconfiguration of network architecture in the rescued state, characterized by increased systemic interconnectivity (Density and Total Edges) and functional compartmentalization (Modularity), alongside a reduction in network segregation (Assortativity).

To identify the physiological drivers of this rescue, we utilized a Random Forest machine learning classifier. In males, the model achieved high predictive accuracy (OOB = 87.5%; Fig. 8c), ranking circulating cholesterol (importance = 0.179) and mitochondrial capacity (importance = 0.167) as the top predictors of metabolic recovery, followed by weight variation (importance = 0.119). In contrast, the female metabolic landscape showed lower classification accuracy (OOB = 60.9%; Fig. 8d) and was dominated by thermogenic variables (importance = 0.175; 0.156), reinforcing the sexual dimorphism observed in the resolution of the obese phenotype.

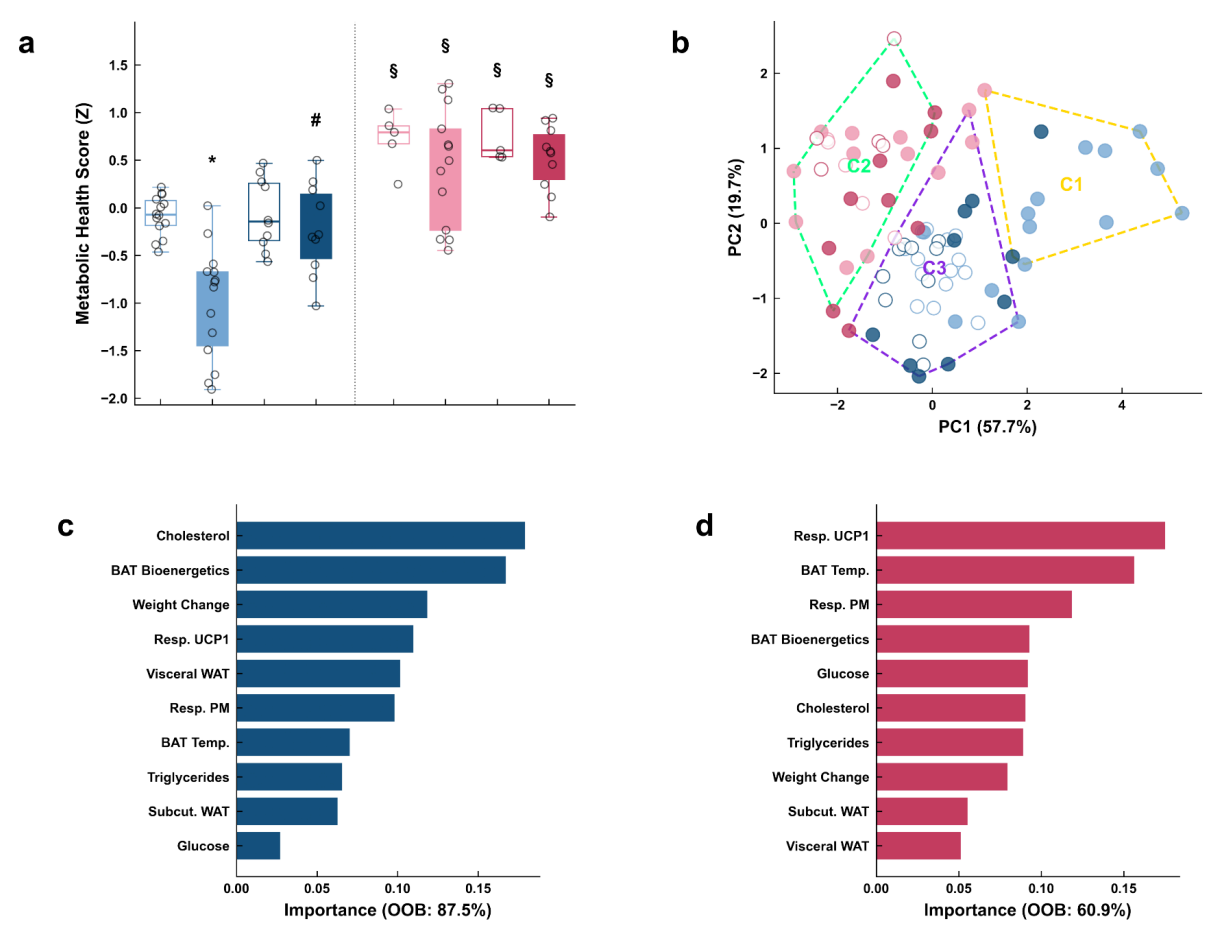


Figure 8 | Integrative analysis reveals metabolic rescue and network reconfiguration by cold exposure. Composite Metabolic Health Score calculated from the inverted Z-scores of adiposity, lipid, and glycemic profiles, indicating the degree of metabolic restoration (a). Principal Component Analysis

(PCA) of metabolic variables, where convex hulls delineate phenotypic clusters C1–C3 (**b**). Random Forest variable importance plots ranking the predictors that distinguish the rescued phenotype (HFD-CE) from the obese state (HFD-RT) in Males (**c**) and Females (**d**). Network topology metrics comparing graph density, modularity, and centrality between HFD-RT and HFD-CE conditions (**e**). Data are presented as boxplots (median, first and third quartiles) with individual values. Statistical symbols: * $p < 0.05$ vs corresponding CD (diet effect); # $p < 0.05$ vs corresponding RT (temperature effect); § $p < 0.05$ vs male counterpart (sex effect).

A Cold-Adapted Gut Microbiome Mediates Thermogenic Recovery in Obesity

To evaluate the impact of environmental temperature on HFD-induced obesity, we analyzed alpha diversity metrics using a permutation-based three-way ANOVA. HFD consumption alone did not significantly alter Shannon diversity compared to the control diet at room temperature ($p > 0.05$, Fig. 9b), suggesting that diet-induced obesity does not collapse microbial richness by itself in this model. However, cold exposure induced shifts dependent on sex.

In males, cold exposure increased microbial diversity within the HFD group (Shannon index: 3.13 ± 0.29 , Fig. 9b) compared to room temperature (2.47 ± 0.41 ; $p = 0.031$). This diversity was also significantly higher than that of cold-exposed males on a control diet (2.31 ± 0.38 ; $p = 0.0001$). In females, we observed a specific difference under cold exposure: HFD-fed mice exposed to cold displayed higher diversity (2.75 ± 0.16) compared to their cold-exposed control diet counterparts (1.99 ± 0.16 ; $p = 0.004$). Consistently, phylogenetic diversity (Faith's PD, Fig. 9c) mirrored this pattern specifically in males.

To visualize community compositional shifts, we utilized Principal Coordinate Analysis (PCoA). PERMANOVA confirmed that experimental factors explained 59% of the total variance ($p = 0.0001$), with cold exposure establishing a distinct community state in HFD males ($R^2 = 0.46$). LEfSe analysis (Fig. 9d) identified the specific drivers of these shifts: while *Clostridium sensu stricto* 1 characterized the dysbiotic HFD-RT phenotype, the cold-adapted HFD group was defined by the bloom of Lachnospiraceae genera (NK4A136 group).

To elucidate the functional consequences of taxonomic shifts, we inferred the functional metagenome using PICRUST2. A three-way ANOVA revealed that intermittent cold exposure reprogrammed the gut microbiome's metabolic potential on

males, specifically enriching pathways involved in the synthesis of bioactive metabolites. We observed a significant upregulation of genes encoding enzymes for butyrate synthesis (e.g., butyryl-CoA:acetate CoA-transferase) in HFD-Cold males compared to HFD-RT counterparts ($p = 0.0006$; Fig. 9g). Furthermore, consistent with the observed clearance of plasma cholesterol, the HFD-Cold microbiome exhibited an expanded capacity for secondary bile acid biosynthesis ($p < 0.0001$ Fig. 9h). In females, we observed an enriched butyrate synthesis in CD-CE group, when compared to their RT counterpart ($p = 0.03$).

To determine whether these taxonomic shifts were linked to host physiology, we performed a Spearman correlation analysis across the full cohort. This revealed a functional dichotomy. The genus *Clostridium sensu stricto* 1, a hallmark of HFD-induced dysbiosis, exhibited positive correlations with circulating cholesterol ($p = 0.51$, $p < 0.01$) and adiposity parameters. Conversely, *Akkermansia* and the cold-induced Lachnospiraceae NK4A136 group formed a beneficial cluster, negatively associating with visceral fat ($p = -0.30$) and positively correlating with BAT thermogenesis ($p = +0.24$).

Given the strong association between *Clostridium* and lipid dysfunction, we hypothesized that this specific taxon might act as a driver of the metabolic phenotype. We tested this hypothesis via Structural Equation Modeling (SEM, Fig. 9i). The model confirmed a statistical mediation: HFD feeding expanded *Clostridium* abundance ($\beta = 0.47$, $p = 0.003$), which in turn significantly predicted cholesterol levels ($\beta = 0.42$, $p = 0.012$). After adjusting for *Clostridium* load, the direct effect of diet on cholesterol became non-significant ($p = 0.265$), suggesting the abundance of *Clostridium sensu stricto* 1 as a key mechanistic vector linking high-fat intake to systemic hypercholesterolemia.

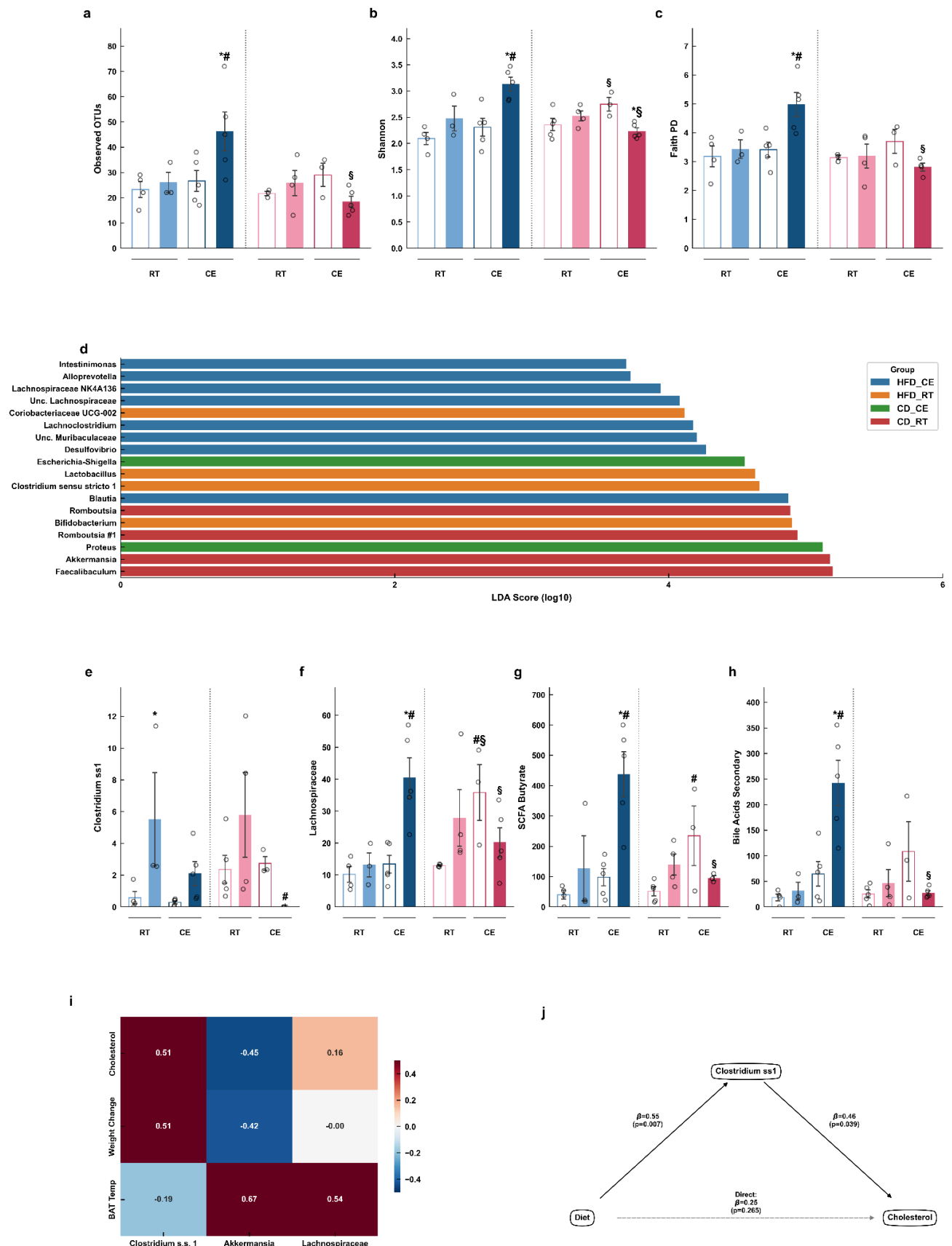


Figure 9 | Cold exposure reprograms the gut microbiome architecture and functional potential in diet-induced obesity. Alpha diversity metrics, including Observed OTUs (**a**), Shannon Entropy (**b**), and Faith's Phylogenetic Diversity (**c**), revealing a sex-specific restoration of microbial richness and complexity in HFD-fed males exposed to cold. Linear Discriminant Analysis Effect Size (LEfSe) identifying taxonomic biomarkers (LDA > 2.0) that distinguish the dysbiotic HFD-RT phenotype (**d**).

Relative abundance validation of the key discriminatory taxa, showing the suppression of *Clostridium sensu stricto 1* **(e)** and the expansion of the family Lachnospiraceae **(f)**. Predictive functional profiling (PICRUSt2) demonstrating the upregulation of metagenomic pathways associated with Butyrate synthesis **(g)** and Secondary Bile Acid biotransformation **(h)** in the cold-adapted microbiome. Structural Equation Modeling (SEM) diagram validating the abundance of *Clostridium sensu stricto 1* as a causal mediator linking dietary lipid load to systemic hypercholesterolemia (i). Data are presented as mean $\pm$ s.e.m. Statistical symbols: * $p < 0.05$ vs corresponding CD (diet effect); # $p < 0.05$ vs corresponding RT (temperature effect); § $p < 0.05$ vs male counterpart (sex effect).

DISCUSSION

Cold Exposure as a Systems-Level Intervention for Diet-Induced Neuro-Metabolic Dysfunction

The present work demonstrates that intermittent cold exposure effectively reverses the phenotype induced by chronic high-fat diet (HFD), shifting obese male mice toward a lean-like systemic state. This intervention restored the composite Metabolic Score in HFD males to values comparable to cold-exposed controls, normalizing visceral adiposity, circulating cholesterol. These findings frame thermogenesis as an actuator that re-establishes coordinated regulation across metabolic, neural, and microbial compartments, consistent with frameworks that characterize obesity as a disorder of integrated network homeostasis (Kitano, 2002; Barabási *et al.*, 2011).

Brown adipose tissue (BAT) functions as the primary effector of this systemic reprogramming. Cold exposure increased interscapular surface temperature and induced UCP1-dependent mitochondrial respiration, demonstrating successful recruitment of the sympathetic–thermogenic axis (Cannon & Nedergaard, 2004; Cypess *et al.*, 2009). These data align with established models wherein BAT dissipates chemical energy via UCP1-mediated proton leak, serving as a metabolic sink for circulating glucose and lipids (Nedergaard *et al.*, 2007; Fedorenko *et al.*, 2012; Stanford *et al.*, 2012). Reactivation of BAT in an HFD context reverses diet-induced “whitening,” reduces visceral fat, and normalizes cholesterol levels, recapitulating the metabolic profile of lean animals despite a persistent obesogenic diet (Shimizu & Walsh, 2015; Alcalá *et al.*, 2017).

The impact of cold exposure extends to neurobehavioral domains. HFD-fed males maintained at room temperature exhibited significant cognitive deficits in the Novel Object Recognition (NOR) task, a characteristic impairment in obesity models (Pistell *et al.*, 2010; Miller & Spencer, 2014). Intermittent cold exposure restored discrimination indices to control levels, implying that thermogenesis rapidly modulates cognitive and affective circuits. Mechanistically, this likely involves the normalization of circulating lipids to attenuate neuroinflammation (Gregor & Hotamisligil, 2011; Guillemot-Legris & Muccioli, 2017), the secretion of batokines such as FGF21 and neuregulin-4 (Fisher & Maratos-Flier, 2016; Jiang *et al.*, 2021), and improved microbiota-derived signaling (Cani *et al.*, 2008; Dinan & Cryan, 2017). This rapid recovery of recognition memory

stands as a distinct neuroplastic response, consistent with the view that certain hippocampal synaptic remodeling may require prolonged interventions relative to metabolic or affective normalization (Harvey, 2013; Thaler *et al.*, 2012).

Sexual dimorphism presents a distinct layer of complexity. Females exhibited baseline metabolic protection against HFD and demonstrated enhanced recognition memory following cold exposure. This observation accords with the role of estrogens in supporting thermogenic competence and neuroprotection: ER α signaling facilitates adipose mitochondrial biogenesis and sustains hippocampal plasticity (Morselli *et al.*, 2014; Mauvais-Jarvis, 2015). This shows that female mice appear to utilize cold exposure to augment an existing favorable baseline, whereas males depend on the thermal intervention to rescue a neuro-metabolic dysregulation.

The Gut Microbiota as a Functional Bottleneck in Thermogenic Metabolic Recovery

The antibiotic-depletion experiments identify the gut microbiota as a necessary functional node for the systemic efficacy of cold exposure. Broad-spectrum antibiotics prevented cold-induced visceral fat loss and suppressed the elevation of BAT surface temperature, despite increasing basal and UCP1-dependent mitochondrial respiration *ex vivo*. This dissociation between elevated mitochondrial oxygen consumption and diminished *in vivo* heat production implies that microbial signals facilitate the translation of thermogenic potential into effective energy dissipation.

While Suárez-Zamorano *et al.* (2015) previously reported that microbiota depletion promotes browning and improves tolerance to acute cold stress, it is imperative to distinguish their experimental context from our chronic conditioning paradigm. In their model, the phenotypic benefits were driven predominantly by the browning of white adipose tissue (WAT) via an immune-mediated mechanism, with negligible enhancement of UCP1 expression in the BAT itself. In contrast, our data reveal that under chronic thermal challenge, the absence of microbiota renders the BAT inefficient. We therefore propose a reinterpretation: the WAT browning observed by Suárez-Zamorano *et al.* likely represents a compensatory peripheral mechanism triggered to offset a silenced central thermogenic system. Our findings suggest that

while such compensation may suffice for acute survival, the microbiota is essential for the sustained, BAT-mediated adaptation required to reverse the obese phenotype.

Consistent with this role, Zhang *et al.* (2023) argues that the microbiota-derived short-chain fatty acids (SCFAs) function as critical molecular signal in this axis. Specifically, acetate and butyrate have been shown to bind G-protein-coupled receptors (GPR43/FFAR2) on brown adipocytes, activating the cAMP-PKA-p38 MAPK signaling cascade. This pathway is essential for upregulating UCP1 transcription and identifying the specific metabolic bottleneck induced by antibiotics. The antibiotic-induced loss of these ligands leads to a distinct mechanistic basis for the observed reduction in *in vivo* thermogenic efficiency.

Taxonomic profiling of the main cohort corroborates this mechanism, revealing a distinct cold-adapted microbial configuration defined by the specific enrichment of Lachnospiraceae (notably the NK4A136 group). As established producers of butyrate, the abundance of Lachnospiraceae tracks directly with the restoration of thermogenic competence, paralleling the "cold microbiota" effect described by Chevalier *et al.* (2015) and the SCFA-mediated BAT activation detailed by Liu *et al.* (2024). Conversely, the HFD-RT phenotype featured a predominance of *Clostridium sensu stricto* 1. Structural equation modeling validated a causal pathway wherein the diet-induced abundance of this taxon mediates hypercholesterolemia. Indeed, controlling for *Clostridium* rendered the direct effect of diet on cholesterol statistically non-significant, consistent with the capacity of specific *Clostridium* species to deconjugate bile acids and modulate hepatic cholesterol synthesis via FXR signaling.

Behavioral Phenotyping as a Mechanistic Readout of Neuro-Metabolic Integration

Behavioral metrics function as mechanistically informative readouts of systemic metabolic health. In the present study, HFD feeding induced a reduction in the discrimination index, indicative of hippocampal-dependent memory deficits. Cold exposure restored cognitive performance in HFD males, a rescue effect abolished by antibiotic treatment. This dependency supports models wherein metabolic endotoxemia, adipokine imbalance, and SCFA depletion converge to drive

neuroinflammation and impair synaptic plasticity within hippocampal circuitry (Cani *et al.*, 2008; Dinan & Cryan, 2017; Kelly *et al.*, 2016). The restoration of microbial SCFA production and BAT-mediated anti-inflammatory signals likely re-establishes a permissive physiological environment for neuronal signaling and integrity, thereby reversing HFD-induced cognitive decline (Kawada *et al.*, 2017; Arpaia *et al.*, 2013).

Locomotor profiling revealed a pervasive sexual dimorphism: females consistently maintained higher basal activity than males, independent of diet or thermal conditions. This observation accords with the established estrogenic enhancement of nigrostriatal dopamine neurotransmission and the intrinsically higher physical activity levels characteristic of female rodents (Gillies & McArthur, 2010; Mauvais-Jarvis, 2015).

Multivariate Integration

The multivariate analyses implemented elucidates the architecture of the phenotypic response to thermal stress. Principal Component Analysis (PCA) and K-means clustering define a specific topological region for HFD-RT males, defined by high adiposity, dyslipidemia, and impaired thermogenesis. Cold exposure effectively moves HFD males from this "pathological cluster" into a healthy phenotypic space shared with cold-exposed controls, demonstrating a coordinated, system-wide state shift.

This topological migration validates systems biology conceptualizations of disease as a stable state within a high-dimensional network. Within this framework, cold exposure moves the neuro-metabolic parameters from an obese phenotype into a lean-like state. This transition is mathematically corroborated by the restructuring of the physiological network: the rescued state exhibits a two-fold increase in network density and a 50% rise in modularity, coupled with a reduction in assortativity. These metrics indicate a departure from the fragmented, segregated architecture typical of the obese state toward a highly integrated, cohesive homeostatic network.

Random Forest feature-importance profiles further resolve the distinct architectures of this reorganization across sexes. In males, the classification of the "rescued" versus "pathological" phenotype is driven primarily by the normalization of circulating cholesterol and mitochondrial respiratory capacity. This priority implies a feed-forward

mechanism wherein systemic lipid homeostasis and mitochondrial efficiency reinforce one another to consolidate the lean state.

Conversely, the female metabolic landscape is dominated by thermogenic effectors, specifically UCP1 respiration and BAT temperature. However, the lower predictive accuracy of the female model (61%) suggests a fundamental biological distinction: females exhibit an intrinsic resilience to diet-induced obesity. Unlike males, they do not undergo a metabolic dysregulation on HFD, rendering the "rescued" state phenotypically less distinct from the "pathological" one.

Implications and Future Directions

These findings validate the conceptualization of obesity as a dysregulation of the integration across adipose tissue, liver, brain, and microbiota. Cold-induced thermogenesis works as a regulator within this network, synchronizing the restoration of BAT oxidative capacity with microbial remodeling to rebalance neuroimmune and behavioral states.

Translationally, the data indicate that the efficacy of BAT-targeted interventions—whether via thermal conditioning or pharmacological β 3-adrenergic stimulation—depends on the preservation of a functional microbiome. Therapeutic strategies should integrate thermogenic stimuli with targeted prebiotic or probiotic support to maintain the SCFA and secondary bile acid pools required for optimal uncoupling. For phenotypes characterized by hypercholesterolemia, thermogenic therapies may independently confer neurocognitive benefits, though they likely require adjunctive lipid-lowering agents to resolve vascular risk profiles.

Future mechanistic inquiry must define the minimal microbial consortia sufficient to rescue thermogenic competence in dysbiotic or antibiotic-treated hosts. Integrating gnotobiotic models with targeted metabolomics will isolate the specific microbial metabolites that synergize with BAT activation while genetic or pharmacological manipulation of batokines, such as FGF21 and NRG4, will establish their necessity and sufficiency in mediating the neurobehavioral recovery observed herein.

Finally, the robust sexual dimorphism observed necessitates focused investigation into the estrogenic modulation of the BAT–microbiota–brain axis. Experimental designs leveraging ER α agonists or hormone replacement will delineate how estrogen

signaling dictates the magnitude and bioenergetic quality of the thermogenic response. Such precision is essential for developing sex-specific therapeutic protocols that respect the distinct adaptive strategies employed by males and females.

CONCLUSION

This work establishes intermittent cold exposure as a systemic intervention capable of reversing diet-induced obesity through the reprogramming of integrated physiological networks. The activation of BAT thermogenesis effectively normalizes adiposity and serum cholesterol while restructuring the gut microbiome into a cold-adapted community enriched in SCFA and bile acid producers. This cascade restores metabolic stability and cognitive performance, delivering a rescue phenotype particularly in males exhibiting metabolic impairment. The therapeutic efficacy of this axis requires an intact microbiota, identifying the gut ecosystem as an essential gating mechanism for thermogenic metabolic recovery.

Behavioral and multivariate analyses validate cognitive decline, coupled to lipid status, thermogenic capacity, and microbial ecology. Topologically, cold exposure drives the obese animals from a pathological phenotype toward a lean-like state. This transition demonstrates the capacity of a singular environmental cue to reconfigure the topology of the complex neuro-metabolic–microbial network.

These findings suggest the development of strategies that integrate BAT activation, microbiota modulation, and lipid control to ameliorate obesity and its neurocognitive comorbidities.

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