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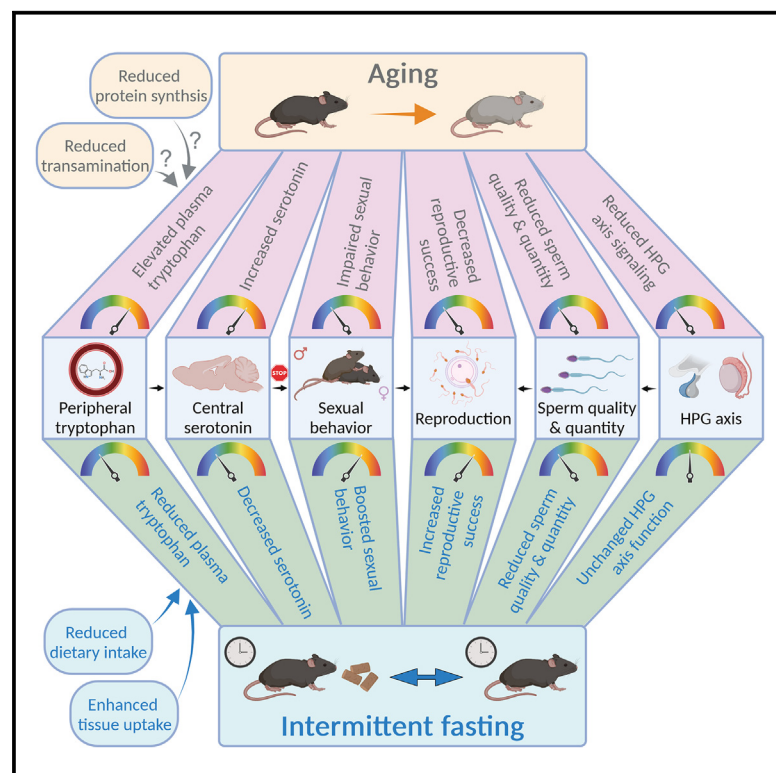
Intermittent fasting boosts sexual behavior by limiting the central availability of tryptophan and serotonin

Graphical abstract

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In brief

Xie et al. show that intermittent fasting (IF) enhances sexual behavior in male mice by reducing serotonergic inhibition through lower brain tryptophan. While IF does not improve endocrine function or sperm quality, it preserves reproductive success in aged mice, suggesting a dietary approach to counter age-related libido decline.

Highlights

- Intermittent fasting (IF) prevented aging-related fertility decline in male mice

- IF did not rescue aging-related endocrine changes or male reproductive alterations

- IF boosted sexual behavior by lowering tryptophan (Trp) and central serotonin

- IF increased Trp uptake in muscles, possibly reducing its availability in the brain

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Intermittent fasting boosts sexual behavior by limiting the central availability of tryptophan and serotonin

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SUMMARY

Aging affects reproductive capabilities in males through physiological and behavioral alterations, including endocrine changes and decreased libido. In this study, we investigated the influence of intermittent fasting (IF) on these aging-related declines, using male C57BL/6J mice. Our findings revealed that IF significantly preserved reproductive success in aged mice, not by improving traditional reproductive metrics such as sperm quality or endocrine functions but by enhancing mating behavior. This behavioral improvement was attributed to IF's ability to counter age-dependent increases in serotonergic inhibition, primarily through the decreased supply of the serotonin precursor tryptophan from the periphery to the brain. Our research underscores the potential of dietary interventions like IF in mitigating age-associated declines in male reproductive health and suggests a novel approach to managing conditions related to reduced sexual desire, highlighting the complex interplay between diet, metabolism, and reproductive behavior.

INTRODUCTION

The decline in reproductive capability with aging is a phenomenon extensively documented in females.[1] Nonetheless, this decrement is not exclusive to the female population. It is also evident in males, albeit without a definitive cessation analogous to menopause. In males, aging is associated with a spectrum of physiological alterations including changes in the endocrine system,[2,3] testicular alterations,[4] and quantitative as well as qualitative changes in sperm.[5] However, aging-associated alterations are not limited to the endocrine and testicular mechanisms sup-porting male reproduction but also affect behavioral control over sexual functions. Decreased libido is common in old males. For instance, 26% of men 70 years or older have been reported to lack sexual drive.[6] In fact, aging is the most important risk factor for male hyposexual desire disorder (HSDD),[7] a syndrome characterized by low sexual desire associated with marked distress and interpersonal difficulties.[8]

Aging-associated declines in reproductive functions are not restricted to humans but are widespread across mammalian species. For instance, male fertility is known to decrease with advancing age in laboratory mice.[9,10] Several factors have

been proposed to play a role in driving age-related subfertility in male mice. First, old male mice feature alterations in the endocrine control of spermatogenesis (e.g., reduced testosterone levels)[9,11] that are associated with testis atrophy,[9] decreased sperm counts,[9,12] an increased rate of abnormal sperm,[13] and a decreased ability of sperm to fertilize oocytes *in vivo* and *in vitro*.[12] In addition, older males exhibit changes in mating behavior, characterized by a diminished participation in sexual activities and a decreased success rate in completing mating sequences.[14] Both changes on the level of germ cells and impaired mating behavior could contribute to reduced reproductive success in older male mice.

Dietary restriction (DR) induces lifespan extension and can ameliorate age-related morbidities in a number of species, including invertebrates and mammalian species.[15] Lifespan extension can be induced with different DR regimens, including caloric restriction (CR)[16–19] and intermittent fasting (IF).[20–22] In CR experiments, restricted animals receive a specified portion (e.g., 60%) of the amount of food that control mice consume when given unlimited access to food (*ad libitum*; "AL"). In the context of IF experiments, restricted mice are subjected to periods of *ad libitum* access to food that alternate with periods of full starvation. One version of IF is every-other-day (EOD) feeding, in the context of which 24-h periods of fasting and *ad libitum* access to food alternate.[21,22] An important feature of IF paradigms is that animals adjust their feeding behavior to the availability of food. As a consequence, starvation during periods of food restriction is largely compensated for by gorging in periods when food is available.[22,23] Of note, IF extends lifespan despite only slightly decrementing average daily food intake.[21–23]

The effects of IF on the male reproductive system and reproductive behavior (if any) are still elusive. Here, we set out to assess the influences of long-term IF on age-related changes in reproductive physiology in male C57BL/6J mice. Surprisingly, we found that IF largely prevented decrements in successful reproduction of aged male mice. The IF-associated preservation of reproductive capabilities into old age was not due to effects on endocrine measures, spermatogenesis, or sperm quality metrics. Rather, IF reduced serotonergic inhibition of sexual behaviors by decreasing tryptophan (Trp) supply from the periphery to the brain, thereby strongly promoting mating behaviors in young and old IF mice relative to AL controls. Our data suggest that IF may hold potential as a therapeutic approach for conditions associated with decreased libido, such as HSDD.

RESULTS

IF prevented the aging-associated fertility decline in male mice

Consistent with prior reports, we observed that only a fraction (43%) of aged (>23 months of age) male C57BL/6J mice were able to reproduce successfully when kept with young adult females (3-month-old) in a 1:1 mating context over a period of 3 weeks (Figure 1A). To explore whether IF could modulate the age-related decline in reproductive success, we generated groups of male C57BL/6J mice subjected to either IF or AL treatment. Dietary regimes were started at 8 weeks of age and were continued throughout the course of the study. We initiated

fertility testing in groups of male mice either at 8 or 24 months of age by mating them, over a 3-week period, with young adult females (Figure 1B; for details, see STAR Methods). All males in the 8-month group were able to reproduce within the given time window (Figure 1C). Consistent with the observation described above, we found that only 38% of the 24-month-old AL controls sired offspring. By contrast, 24-month-old IF males sired offspring in 83% of cases, indicating that IF largely prevented aging-associated reproductive impairments (Figure 1C). Neither IF nor age had an influence on the number of offspring sired and the sex distribution among the F1 offspring (Figures 1D and 1E).

IF did not influence aging-associated endocrine changes and alterations in the male reproductive system

Aging is associated with decreased testis weight, reduced levels of spermatogenesis, decreased sperm counts, and altered sperm functionality, including decreased motility.[9,12,13] Therefore, we next wanted to address whether IF promotes reproductive success by improving aging outcomes in the male reproductive tract. IF did not attenuate the aging-associated decline in testis weight but instead led to further testis weight reductions (Figure 2A).

Next, we investigated whether IF modified aging-associated transcriptome changes in testis. To this end, we carried out total RNA sequencing (RNA-seq) analyses of testes derived from young (3-month-old) and old (24-month-old) males kept on either AL or IF (Figures 2B–2I; Table S1). Gene-level differential expression analysis revealed a number of age effects (Figure 2B; 67 differentially expressed genes; false discovery rate [FDR] < 0.05), including a downregulation of *Klk1b21*, *Klk1b22*, *Klk1b24*, *Klk1b27*, *Pdgfrl*, and *Osbpl1a*, as well as an upregulation of *Fetub*, *Cxcl13*, *Igf2*, *Ly6e*, and *Lyz2*, among other changes (Figure 2D; Table S1). Gene Ontology (GO) analyses revealed the expected[24] enrichment, among age-sensitive genes, of GO terms related to immune response and inflammation (Table S1). We found only one gene significantly (FDR < 0.05) affected by diet and one gene with a significant (FDR < 0.05) age x diet interaction (Figures 2B, 2E, and 2F; Table S1), indicating that IF had overall only limited effects on aging-associated transcriptome changes in testis.

In addition to a gene-level analysis of our total RNA-seq testis dataset, we also performed a differential expression analysis of repetitive elements across young (3-month-old) and old (24-month-old) mice subjected to either AL or IF (Figures 2G–2I; Table S1). These analyses showed age effects (nine differentially expressed repetitive elements; FDR < 0.05), mainly due to an age-related upregulation of repeat elements, such as internal sequence of the murine retrovirus on the Y chromosome (MURVY-int), intracisternal A particle long terminal repeat 4 (IAPLTR4), IAPLTR4_I, and internal sequence of the murine retrovirus-related sequence 4 (MuRRS4-int). Of note, there was no significant difference between IF and AL testes, supporting limited IF effects on testis repeat element expression.

Our studies also included a detailed assessment of sperm quantity and quality in 8- vs. 24-month-old C57BL/6J mice subjected to either AL or IF. These analyses showed the expected age effects on sperm counts, swim speed, swim range, and
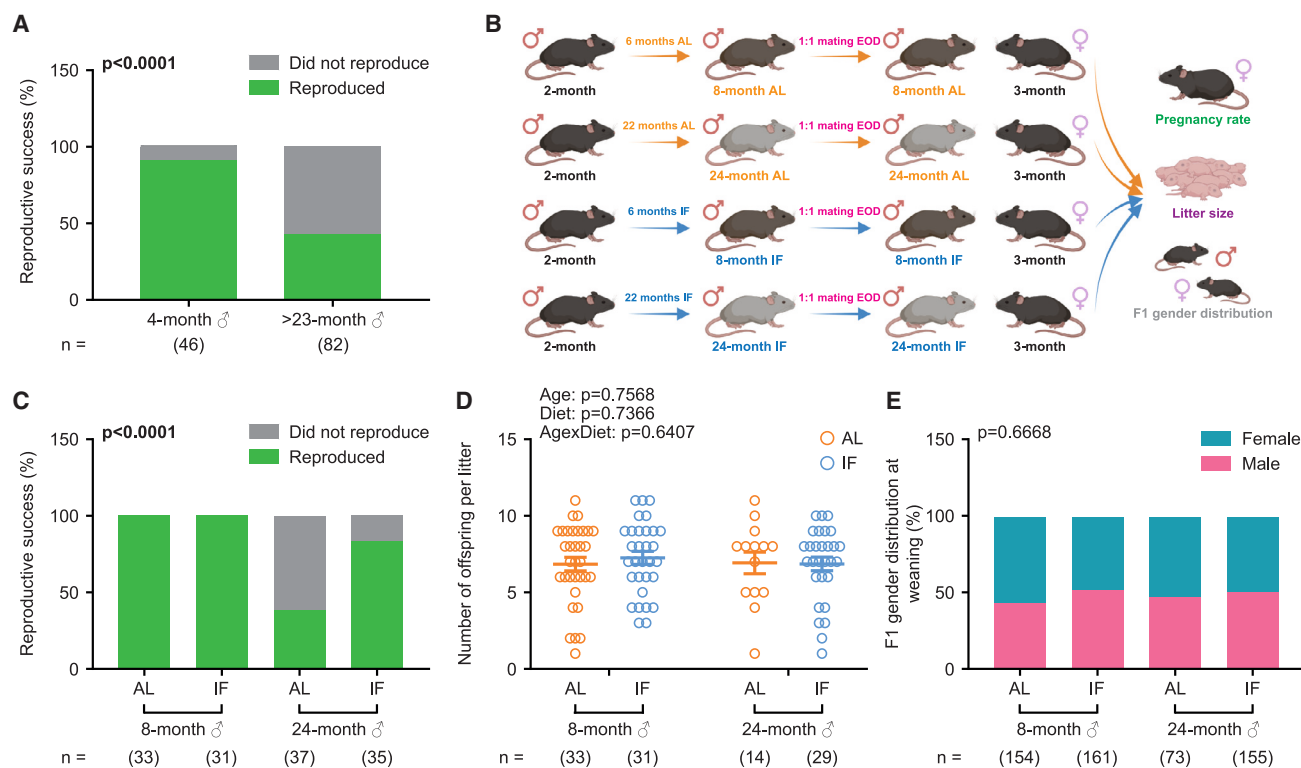
Figure 1. Intermittent fasting prevented aging-associated reductions in male reproductive success

(A) Proportion of young (4-month-old) vs. old (>23-month-old) C57BL/6J male mice that successfully sired offspring when mated with young (3-month-old) female C57BL/6J mice; sample size corresponds to the number of mating pairs examined.

(B) Schematic illustrating experimental design for data shown in (C)–(E). "1-1 mating EOD" indicates that 1:1 matings were carried out every other day (on a feeding day) to ensure that females were not subjected to food deprivation.

(C) Proportion of young (8 months)/old (24 months) IF/*ad-libitum*-fed (AL) C57BL/6J male mice that successfully sired offspring when mated with young (3 months) female C57BL/6J mice exposed to AL. Sample size corresponds to the number of mating pairs examined.

(D and E) (D) Litter size and (E) sex distribution in F1 offspring sired by young (8 months)/old (24 months) IF/AL male C57BL/6J mice mated with young (3 months) AL female C57BL/6J mice. Sample size corresponds to number of F1 litters (D) or total number of F1 offspring animals (E) examined. (D) shows means ± SEM with individual data points superimposed.

percentage of immotile sperm (Figures 3A–3E). However, IF either failed to have significant effects on these measures or aggravated the age effect (Figures 3B–3E). The acrosome reaction is a crucial process by which the spermatid penetrates the zona pellucida to fertilize the oocyte. Enzymes released from the acrosome, such as acid phosphatase, may serve as proxies for the fertilization capabilities of sperm.[25] Our acid phosphatase activity assay, carried out on sperm derived from young/old AL/IF mice, did not reveal any age or IF effects when normalized to sperm count (Figure 3F).

To be able to detect a possible modulation of age effects by IF specifically in male germ cells, we also carried out total RNA-seq analyses of sperm isolated from young (3-month-old) and old (24-month-old) mice subjected to AL or IF (Figures 3G–3K; Table S2). Consistent with the findings in whole testis (described above), our analyses of sperm revealed a number of age effects (Figure 3G; 111 differentially expressed genes, FDR < 0.05), including altered expression of *Cmklr2*, *Ugt1a1*, *Afap1l1*, *Il7*, *Gzmk*, and others (Figure 3H; Table S2). GO analyses of age-sensitive genes showed a significant enrichment of defense response, response to stress, as well as immune-related terms, among other GO terms (Table S2). Only one

gene showed a significant main effect of diet (Figure 3I; Table S2), and only two genes showed a significant age × diet interaction (Figure 3J; Table S2). In conclusion, our analyses of total RNA-seq data derived from sperm showed a considerable number of age effects but only minor influences of IF.

Finally, we also asked if aging-associated DNA methylation changes in sperm are prevented by long-term IF. To address this question, we used the SureSelectXT target enrichment system followed by bisulfite sequencing to broadly examine DNA methylation across the genome of young (3-month-old) and old (24-month-old) males subjected to either IF or AL treatment (Figures 3L–3N; Table S2). These analyses revealed 124 regions differentially methylated by age and 167 differentially methylated by diet (FDR < 0.05). Although age- and diet-sensitive regions were partially overlapping (Figure 3M; 11 regions; corresponding to ~8.9% of age-sensitive regions), age effects were not countered by fasting effects within these regions (Figure 3N). No genomic intervals with a significant age × diet interaction were identified (Figure 3M).

Taken together, these analyses indicated that IF had only minor influences on aging-associated changes in the male germ
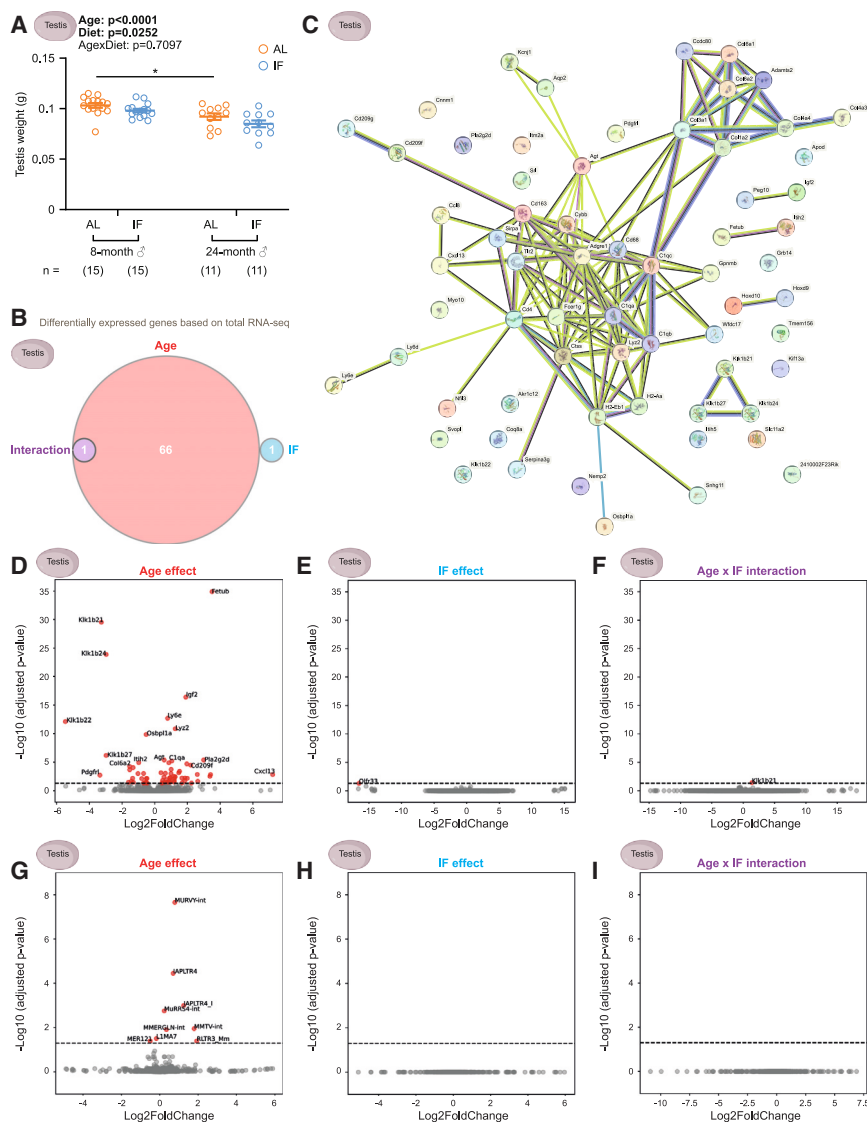
Figure 2. IF did not prevent aging-associated changes in testis

(A) Testis weight in young (8 months) and old (24 months) male C57BL/6J mice subjected to AL or IF. Sample sizes correspond to the number of male mice examined. Graph shows individual data points as well as means ± SEM. * $p < 0.05$.

(B–I) Total RNA-seq analyses of testes (gene-level analysis [B–F]; repeat-element-based expression analysis [G–I]) of young (3-month-old) and old (24-month-old) male C57BL/6J mice subjected to AL or IF. (B) Venn diagram shows the set of age-sensitive genes, diet-sensitive genes, genes subject to an age × diet interaction, as well as their overlaps. (C) Network plot details age-sensitive genes (nodes) as well as known links between them (edges). Volcano plots show log2FC vs. $-\log_{10}$(FDR) for analyses focused on the age effect (D and G), diet effect (E and H), and age × diet interaction (F and I). Red denotes genes/repeat elements with a significant effect (FDR < 0.05). Only a subset of differentially expressed genes is labeled to avoid cluttering. Note that only genes with a gene symbol annotation are included in the volcano plots to provide gene name labels instead of Ensembl IDs.

line, suggesting that the IF-mediated rescue of aging-associated reproductive decrements is unlikely to be caused by an attenuation of germline aging.

In addition to the analyses of the male reproductive tract (outlined above), we also performed assessments targeting the endocrine status of 3- vs. 24-month-old male C57BL/6J mice subjected to either AL or IF. Analyses of plasma testosterone concentrations confirmed the expected[11,26] aging-associated decline, but they revealed no influence of IF (Figure S1).

IF promoted sexual behavior in male mice by attenuating serotonergic behavioral inhibition

Next, we considered the possibility that reduced reproduction of old males may have been caused by dampened mating behavior. If this was the case, IF could potentially rescue aging-associated reproductive deficits by promoting mating behavior. To test this hypothesis, we set up mating assays in the context of which single-housed 8- and 24-month-old male C57BL/6J mice, subjected to either AL or IF, were each pre-

sented, in their home cage, with a single young adult C57BL/6J female for a 5-min mating trial (Figure 4A; for further details, see STAR Methods). Animals were sexually naive before use. Mating assays were repeated, with a fixed assignment of mating pairs, after 6 days (at day 7) and 14 days (at day 15), respectively. Analyses of total interaction times revealed significantly reduced values in old males when encountering naive females (Figure 4B; Table S3). Additional metrics (latency to first mount; mounting frequency; latency to first intromission) also were in line with diminished mating activity in old males (Figures 4C and 4D; Table S3), indicating that reduced mating behavior could be an important determinant of aging-associated decrements in reproductive success. IF effects on mating behavior were evident both in aged mice on 22-month IF and younger animals subjected to 6-month IF (Figures 4B–4D; Table S3). Compared with AL male mice, animals assigned to IF displayed increased interaction time, reduced latency to first mount, higher mounting frequency, higher frequency of mounts with intromissions, decreased latency to first intromission, and reduced latency to ejaculation (Figures 4B–4D; Table S3). In sum, these analyses showed that male mating behavior is attenuated by advanced age and is strongly promoted by IF. Hence, the data indicate that the IF-mediated rescue of aging-associated reproductive impairments (Figure 1C) is likely caused by bolstered mating behavior in fasted males.

To explore whether a shorter-term IF treatment of 6 weeks is sufficient to induce these behavioral changes, we analyzed a cohort of male C57BL/6J mice in which IF was started either in
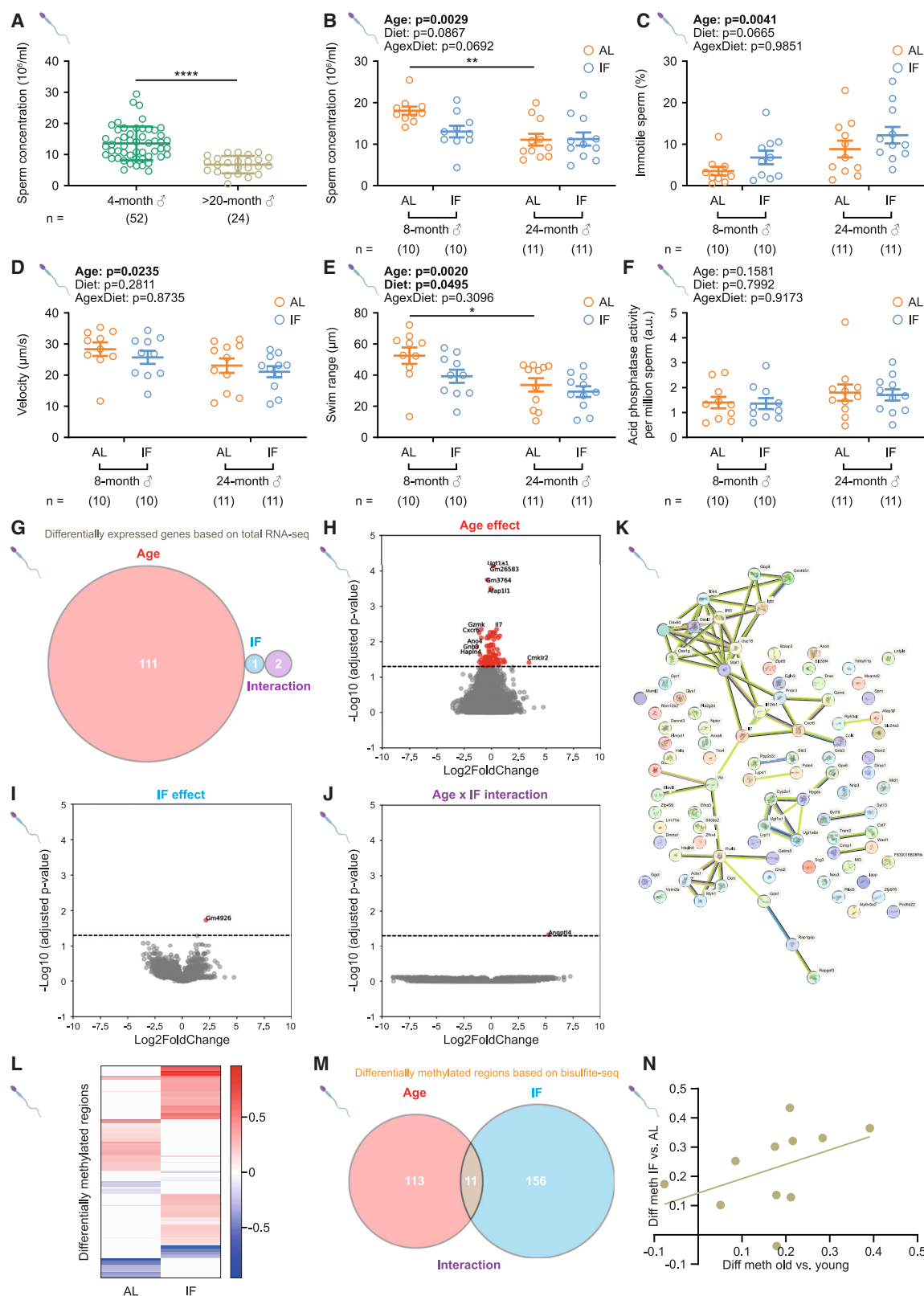
Figure 3. IF did not prevent aging-associated changes in sperm

(A) Sperm concentration measured in epididymal swim out of young (4-month-old) vs. old (>20-month-old) C57BL/6J mice. Sample sizes correspond to the number of male mice examined. Graphs show individual data points as well as means ± SEM. **** $p < 0.0001$.

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young adulthood (3 months of age) or later in life (17 months of age) (Figure S2). A 6-week IF treatment was not sufficient to boost male sexual behavior, as evidenced by unaltered interaction time (Figures S2A and S2B) and unchanged mounting frequency (Figures S2C and S2D). Additional metrics (latency to first mount, proportion of animals displaying mounting behavior) even suggested adverse effects of a 6-week IF treatment initiated in aged animals (Figures S2E and S2F). Together, our data indicate that longer-term IF is required to induce enhancements in mating behavior.

Male mating behavior is governed by a range of excitatory and inhibitory circuits utilizing different neurotransmitter systems, among which dopamine and serotonin are thought to play major roles[27] (Figure 5A). In fact, consistent with decreased sexual activity in aged males, our analyses showed decreased brain dopamine and increased serotonin concentrations with advancing age in male C57BL/6J mice (Figures 5B and 5C). We hypothesized that IF stimulates mating behavior by altering the balance between transmitter systems promoting vs. inhibiting sexual behaviors.[27] To address this possibility, we harvested whole brains from 3-, 8-, and 24-month-old male mice, subjected to either AL or IF, for the analysis of diet effects on neurotransmitter systems modulating sexual behaviors in male rodents[27] (Figures 5D–5J). These analyses confirmed the age-dependent reduction in the concentration of the stimulatory neurotransmitter dopamine (Figure 5D) as well as the aging-associated increase in serotonin concentrations (Figure 5G), which is in line with inhibitory effects on sexual behaviors in aged mice. Our analyses showed no measurable IF effects on a number of transmitter systems that either promote (dopamine, oxytocin, and pro-opiomelanocortin) or inhibit (prolactin, enkephalins, and dynorphins) male mating behavior (Figures 5D–5F and 5H–5J). IF, however, specifically dampened the aging-associated elevation of brain serotonin levels (Figure 5G), indicating that IF may exert its behavioral effects via attenuation of serotonergic inhibition of male sexual behavior. Additional assessments using either the medial prefrontal cortex or the hypothalamus as input material confirmed lower serotonin concentrations in these brain regions in long-term-treated IF mice (Figures 5K and 5L). For pharmacological validation, we carried out mating assays in AL/IF mice injected with the serotonin precursor 5-hydroxytryptophan (5-HTP) or vehicle control 15 min before behavioral analysis (Figures 6A–6E). Consistent with our prior analyses, these experiments revealed significant diet effects on mating behavior with increased mounting frequency in IF males relative to AL controls

(Figures 6B and 6D; Table S4). 5-HTP significantly attenuated mounting frequencies in a dose-dependent fashion, indicating that increasing serotonergic transmission caused decrements in male mating behavior (Figures 6D and 6E). Together, these data are consistent with a model wherein IF disinhibits male sexual behavior by reducing serotonergic neurotransmission.
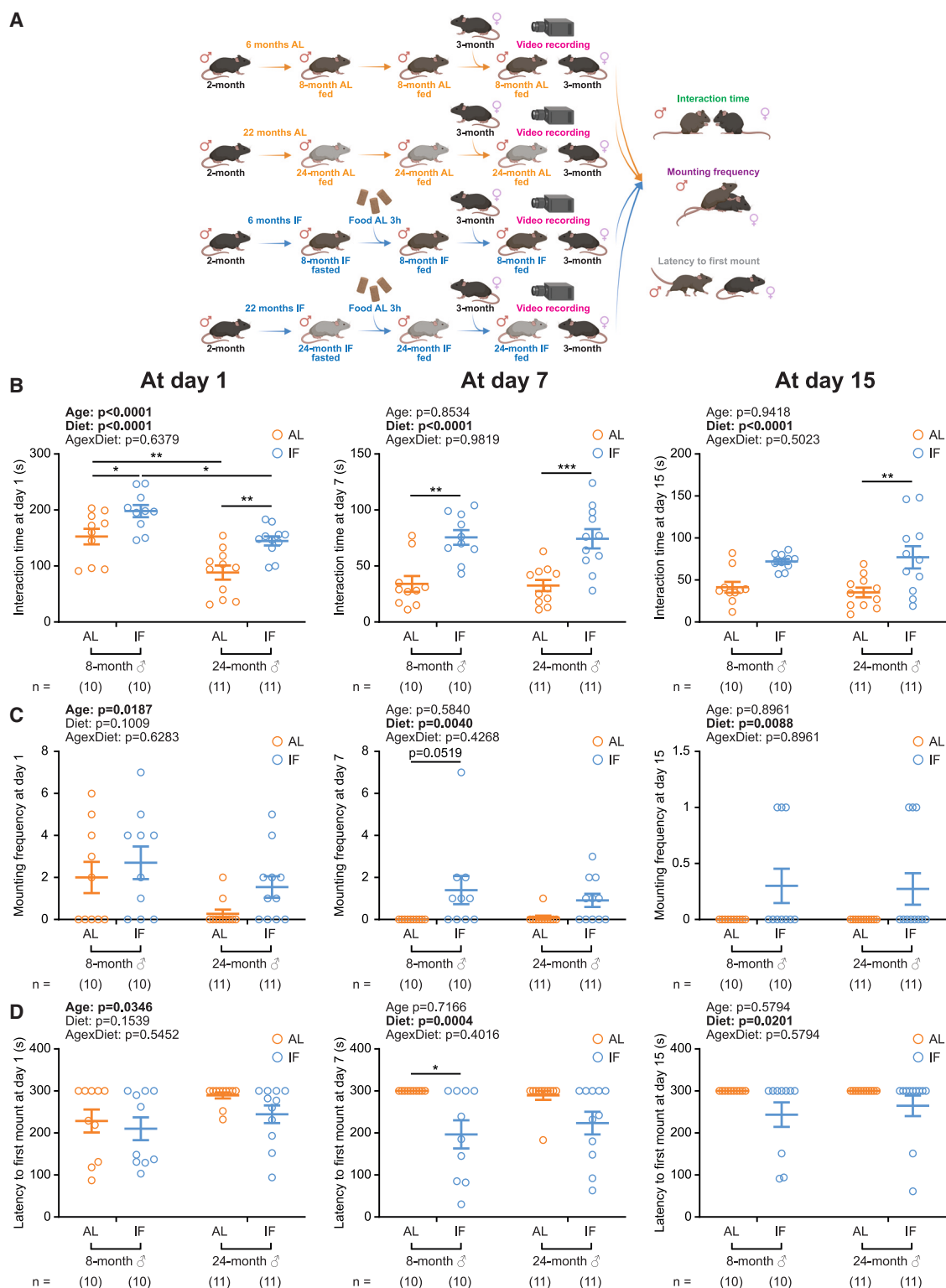
IF decreased central serotonin concentrations by limiting Trp availability

Next, we investigated the connection between IF and alterations in brain serotonin levels (Figures 7A–7H). Serotonin is synthesized in the brain from its precursor Trp via the intermediate 5-HTP in a two-step process catalyzed by the enzymes tryptophan hydroxylase and aromatic amino acid decarboxylase[28] (Figure 7A). The degradation of serotonin is mediated through different mechanisms, including its catabolism to 5-hydroxyindoleacetic acid (5-HIAA) (via monoamine oxidase and aldehyde dehydrogenase) as well as its degradation via the kynurenine pathway[28] (Figure 7A). We used ELISAs to quantify Trp, 5-HTP, and 5-HIAA concentrations in whole-brain homogenates derived from 3-, 8-, and 24-month-old male mice kept on AL or IF dietary regimes. For all studies, brains and other tissue samples were extracted at the end of an IF feeding cycle (i.e., at a time point when IF males have had free access to food for a 24-h time period; for details, see STAR Methods). Our analyses showed that IF reduced not only the brain concentration of serotonin but also that of its precursors Trp and 5-HTP as well as of its degradation product 5-HIAA (Figures 7B–7D). Western blot- and qPCR-based assessment of brain expression levels of enzymes mediating the synthesis or degradation of serotonin revealed no difference between IF and AL mice (Figures S3A–S3L). Consistent with these observations, RNA-seq-based transcriptomic analyses of brains derived from 3- and 24-month-old C57BL/6J mice subjected to either AL or IF also showed no evidence for an IF effect on the expression of genes linked to serotonin biosynthesis or degradation (Table S5). Further, qPCR analyses revealed no clear IF effect on the expression levels of any of the serotonin receptors examined (Figures S4A–S4N). Together, these data suggest that IF does not diminish brain serotonin concentrations by altering its local metabolism. Instead, IF-associated reductions in brain serotonin concentrations may be due to diminished import of its precursor Trp from the blood into the brain.

We considered two scenarios: first, there could be reduced Trp flux into the brain due to IF effects on the mechanism

(B−F) Sperm concentration (B) and sperm functional parameters (C−F) in young (8-month-old) vs. old (24-month-old) C57BL/6J mice exposed to AL or IF. The following functional analyses were carried out on spermatozoa: assessment of the percentage of immotile spermatozoa (C), velocity (D), swim range (E), and acid phosphatase activity (F). Sample sizes correspond to the number of male mice examined. Graphs show individual data points as well as means ± SEM. *$p < 0.05$, **$p < 0.01$.

(G−K) Total RNA-seq analyses of sperm of young (3-month-old) and old (24-month-old) male C57BL/6J mice subjected to AL or IF. (G) The Venn diagram shows the sets of age-sensitive genes, diet-sensitive genes, genes subject to an age × diet interaction, as well as their overlaps. (H−J) Volcano plots show log2FC vs. −log$_{10}$(FDR) for analyses focused on the age effect (H), diet effect (I), and age × diet interaction (J). Red denotes genes with a significant effect (FDR < 0.05). Only a subset of differentially expressed genes may be labeled to avoid cluttering. Note that only genes with a gene symbol annotation are included in the volcano plots to provide gene name labels instead of Ensembl IDs. (K) The network plot details age-sensitive genes (nodes) as well as known links between them (edges).

(L−N) Genome-wide differential methylation analyses of sperm derived from young (3-month-old) and old (24-month-old) male C57BL/6J mice subjected to AL or IF. (L) The clustered heatmap shows differential methylation values (old-young; IF-AL) for all differentially methylated regions (FDR < 0.05) in sperm of young/aged AL/IF mice. (M) The Venn diagram shows the number of genomic regions differentially methylated due to age and/or diet and/or subject to an age × diet interaction (none showed an interaction). (N) The scatterplot shows differential methylation due to diet (IF-AL), plotted against differential methylation due to age (old-young) for the 11 differentially methylated regions that were both age- and diet-sensitive ($r2 = 0.1865$, $p = 0.1847$).

A

B

At day 1

Age: **p<0.0001**
Diet: **p<0.0001**
AgexDiet: p=0.6379

At day 7

Age: p=0.8534
Diet: **p<0.0001**
AgexDiet: p=0.9819

At day 15

Age: p=0.9418
Diet: **p<0.0001**
AgexDiet: p=0.5023

C

Age: **p=0.0187**
Diet: p=0.1009
AgexDiet: p=0.6283

Age: p=0.5840
Diet: **p=0.0040**
AgexDiet: p=0.4268

Age: p=0.8961
Diet: **p=0.0088**
AgexDiet: p=0.8961

D

Age: **p=0.0346**
Diet: p=0.1539
AgexDiet: p=0.5452

Age p=0.7166
Diet: **p=0.0004**
AgexDiet: p=0.4016

Age: p=0.5794
Diet: **p=0.0201**
AgexDiet: p=0.5794

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involved in Trp transport across the blood-brain barrier. We used western blot and qPCR as well as targeted analyses of RNA-seq data to address whether expression levels of the key Trp transporter—large neutral amino acid transporter (Lat1, also known as Cd98)—across the blood-brain barrier were altered in brains of IF mice. Lat1 is a heterodimer composed of its subunits Slc3a2 and Slc7a5. These studies revealed no obvious and consistent differences between AL and IF animals (Figures S5A–S5E; Table S5), suggesting that IF did not influence these transport mechanisms, either at the transcriptional or protein level. Alternatively, IF could reduce brain Trp import by reducing Trp blood concentrations, thereby resulting in lower substrate availability for Trp transport across the blood-brain barrier (Figure 7A). To explore this possibility, we measured plasma Trp concentrations, using ELISAs, in 3-, 8-, and 24-month-old male mice subjected to AL or IF (again, plasma was harvested at the end of an IF feeding cycle; for details, see STAR Methods). These experiments revealed strongly attenuated plasma Trp concentrations in IF mice (Figure 7E), indicating that IF reduces brain serotonin content by limiting Trp supply from the periphery.

Trp is an essential amino acid and, as such, cannot be synthesized in mouse tissues.[28] Possible sources feeding into plasma Trp pools include recruitment from external sources, via the gastrointestinal tract (dietary sources), and mobilization from endogenous stocks (Trp-containing proteins in peripheral tissues).[28] Plasma samples for Trp measurements were harvested at the end of a feeding cycle. Food intake analyses showed that during a feeding cycle, IF mice consumed on average 1.76 times more chow than AL controls (Figure S6B). These considerations indicate that decreased dietary Trp intake during the 24-h time window prior to plasma collection does not account for reduced plasma Trp concentrations in IF animals.

IF enhanced Trp uptake into skeletal muscle

We hypothesized that to support energy production during periods of starvation in IF animals, Trp (alongside other amino acids) could get mobilized from protein pools in peripheral tissues, such as skeletal muscle. During refeeding, in turn, food-derived Trp may be taken up into these tissues to restore depleted protein pools, thereby promoting reduced plasma Trp concentrations in IF animals. If this was the case, IF animals should feature enhanced incorporation of diet-derived Trp into peripheral tissues. To test this hypothesis, we fed animals on the IF or AL regimes with chow containing ^{15}N-labeled Trp at concentrations equivalent to standard rodent chow (Figure 7G; for details, see STAR Methods). We then harvested anterior thigh skeletal muscle tissue for mass spectrometry analysis to assess the fraction of Trp-containing peptides (for details, see STAR Methods) featuring ^{15}N-Trp label. These analyses revealed an increased fraction of ^{15}N-Trp-labeled peptides in mice subjected to IF (Figure 7H). Our findings suggest that increased Trp incorporation into skeletal muscle under our fasting/refeeding scheme could contribute to reduced plasma Trp concentrations in IF mice.

Age and IF effects on central serotonin are explained by their effects on peripheral Trp

To address whether limited Trp supply could fully explain age effects and/or diet effects on whole-brain serotonin concentrations, we performed partial correlation analyses predicting brain serotonin concentrations based on age and IF while accounting for plasma and brain Trp concentrations measured in the same animals (3-, 8-, and 24-month-old male mice subjected to AL or IF; Figure 7F). These analyses revealed that there was no significant effect of age and diet when accounting for plasma and brain Trp concentrations (both of which were significantly associated with brain serotonin concentrations) (Figure 7F), implying that both age and IF effects on brain serotonin can be fully explained by their influence on the abundance of serotonin's precursor Trp in plasma and brain.

Aging-associated changes in peripheral Trp emerge in the context of broader alterations in peripheral amino acid metabolism

These findings indicate that aging-associated changes in brain serotonin concentrations are linked to elevated Trp supply in old age. We wanted to further understand the metabolic context in which elevated plasma Trp concentrations take place during aging in mice. To this end, we subjected plasma samples of 1-, 3-, 5-, 8-, 14-, and 20-month-old male C57BL/6J mice (on AL access to food) to metabolomic analyses (Figures S7A–S7I). These analyses confirmed age-related increases in plasma Trp abundance (Figure S7B). Linear regression analyses revealed 172 metabolites with differential abundance (FDR < 0.05) as a function of age (Table S6). Chemical structure-based metabolite set enrichment analyses (MSEAs) identified a significant overrepresentation of "amino acids, peptides and analogs" among age-sensitive metabolites (ASMs) (Figure S7D). KEGG-pathway-based MSEA also highlighted amino acid-related changes during aging, including arginine biosynthesis, arginine and proline metabolism, histidine metabolism, etc. (Figure S7E). In line with these results, 64 out of the 153 ASMs for which corresponding "super-pathway" annotation was available (see Table S6) were members of the "amino acid" super-pathway (Figure S7C). Together, these results are consistent with the notion that aging is associated with broader alterations in amino acid metabolism.

Next, we wanted to address whether plasma Trp abundance correlates with specific other metabolites. To this end, we performed linear regression analyses to identify metabolites significantly associated with Trp abundance while accounting for age (Table S6). Oxindolylalanine, a Trp oxidation product,[29] was significantly (FDR < 0.05) associated with Trp abundance (Figure S7F; Table S6). Exploratory analyses, focused on 23

Figure 4. IF promoted male sexual behavior
(A) Schematic illustrating experimental design.
(B−D) Total interaction time (B), mounting frequency (C), and latency to first mount (D) for young (8-month-old)/old (24-month-old) IF/AL male C57BL/6J mice subjected to a 5-min mating assay with young (3-month-old) naive female C57BL/6J mice. For each mating pair, we recorded three mating assays (performed at days 1, 7, and 15, respectively). Sample size corresponds to the number of mating pairs examined. Graphs show individual data points as well as means ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.
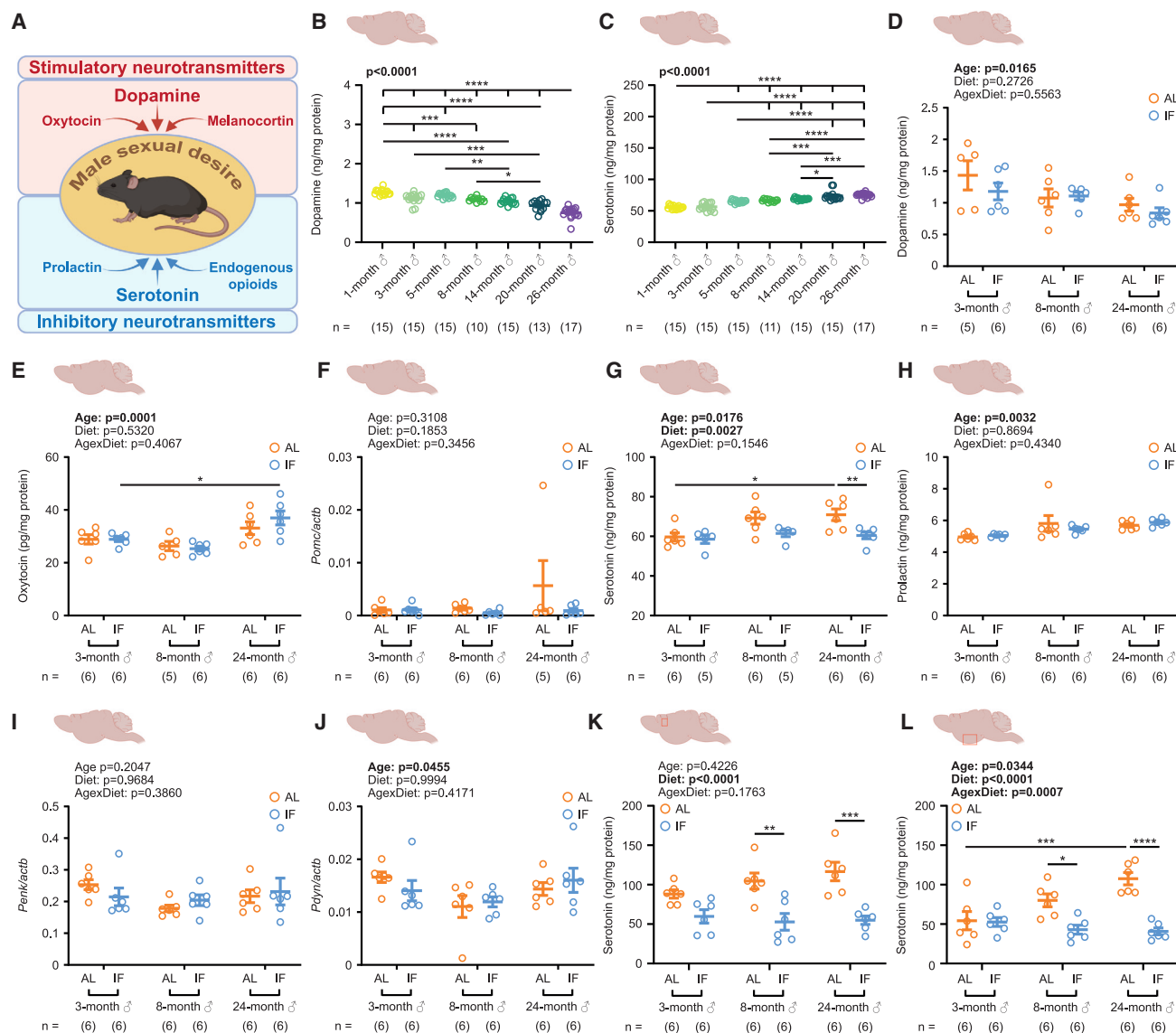
Figure 5. IF removed serotonergic inhibition of male mating behavior

(A) Schematic illustrates neurotransmitter systems involved in the regulation of male sexual behavior.

(B and C) Whole-brain dopamine (B) and serotonin (C) concentrations in 1-, 3-, 5-, 8-, 14-, 20-, and 26-month-old male C57BL/6J mice.

(D–J) Whole-brain-based assessments of neurotransmitter concentrations or related gene expression in 3-, 8-, and 24-month-old male C57BL/6J mice subjected to AL or IF: (D) dopamine, (E) oxytocin, (F) pro-opiomelanocortin, (G) serotonin, (H) prolactin, (I) proenkephalin, and (J) prodynorphin.

(K) Serotonin concentrations in the medial prefrontal cortex of 3-, 8-, and 24-month-old male AL and IF mice.

(L) Serotonin concentrations in the hypothalamus of 3-, 8-, and 24-month-old male AL and IF mice.

Sample size corresponds to the number of male mice examined. Graphs show individual data points as well as means ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

metabolites with a suggested positive association with Trp ($p < 0.01$; coefficient > 0), revealed a significant overrepresentation of "amino acids, peptides and analogs," "phenylalanine, tyrosine and tryptophan biosynthesis," and other amino acid-related terms (Figures S7H and S7I). In line with these findings, 17 metabolites out of this set had an "amino acid" super-pathway annotation, such as glycine, ornithine, phenylalanine, glutamate, tyrosine, and kynurenine (Figure S7G; Table S6). In conclusion, these analyses indicate that elevated plasma Trp abundance in aging male C57BL/6J mice was associated with

a parallel increase in additional amino acids as well as other molecules related to amino acid metabolism.

Taken together, our data are consistent with a model wherein IF lowers plasma Trp concentrations by altering Trp tissue distribution and/or by diminishing dietary Trp supply, thereby reducing brain serotonin abundance and promoting sexual behaviors in male C57BL/6J mice. Due to these behavioral effects and despite not improving sperm quality, endocrine changes, and other endpoints, IF is sufficient to substantially enhance fertility in aged male mice.
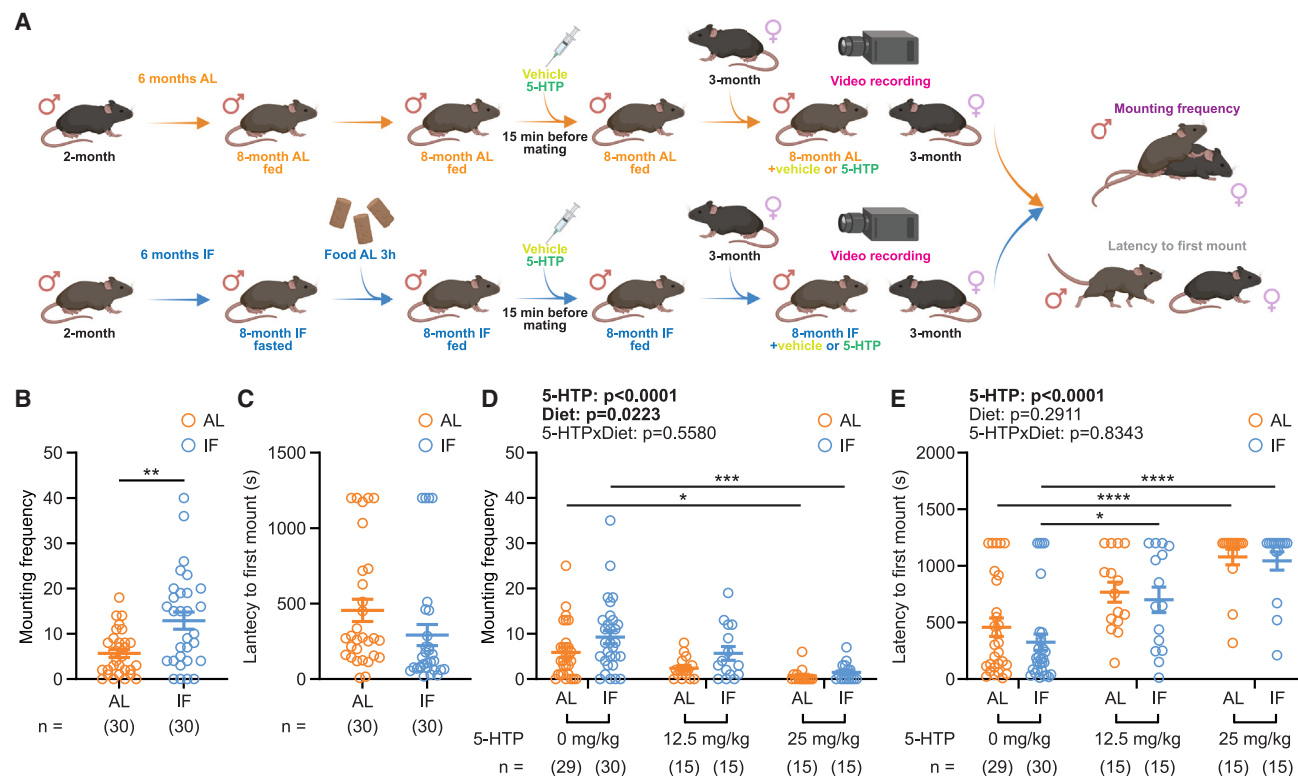
Figure 6. Pharmacologically increasing central serotonin inhibited male sexual behavior

(A) This schematic illustrates the experimental design corresponding to the results shown in (D and E).

(B and C) Mounting frequency (B) and latency to first mount (C) during the mating assessment of untreated 8-month-old IF/AL male C57BL/6J mice.

(D and E) Mounting frequency (D) and latency to first mount (E) during the mating assessment of 8-month-old IF/AL male C57BL/6J mice following treatment with either of two 5-HTP doses or vehicle injection.

Graphs show individual data points as well as means ± SEM. Sample size corresponds to the number of male mice examined. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

DISCUSSION

Here, we provide first evidence that IF strongly promotes sexual behavior and reproduction in male mice. We show that IF-mediated behavioral effects were linked to a removal of serotonergic inhibition of sexual behaviors under the fasting regime. Reduced CNS serotonin concentrations in turn were caused by diminished Trp supply from the periphery, which may be linked to elevated Trp uptake into peripheral tissues in the context of starvation/refeeding cycles.

IF decreased brain serotonin concentrations similarly in young and old mice by limiting Trp supply from the periphery to the brain. Trp is converted into serotonin, locally in the brain, in a two-step enzymatic process involving tryptophan hydroxylase and aromatic amino acid decarboxylase.[28,30] Trp hydroxylase catalyzes the rate-limiting step in serotonin synthesis but is, under normal conditions, not saturated with substrate.[31] As a consequence, a unique feature of the serotonergic system is that the abundance of the neurotransmitter (i.e., serotonin) is directly influenced by the availability of its precursor (i.e., Trp) to the biosynthetic machinery.[28,31] In other words, increased Trp availability (e.g., by increased dietary ingestion) will result in a rise in serotonin biosynthesis, whereas shortened supply will decrease brain serotonin content. Our study in IF animals

showed unaltered expression levels of components of the serotonin biosynthetic machinery, degradative pathways, and transport mechanisms across the blood brain barrier. However, IF led to decreased plasma Trp, thereby limiting Trp availability for conversion into serotonin in the brain.

The current IF regime entailed alternating 24-h cycles of full starvation (no access to food) and *ad libitum* access to food initiated at 8 weeks of age and continued throughout. Consistent with prior observations,[22] our analysis of food intake revealed that IF mice substantially increased food intake on the feeding day relative to controls, thereby largely compensating for the lack of food on the subsequent starvation day. As a result, average food intake under IF was reduced by only 13.3%. Sample collection for all molecular analyses was carried out at the end of the 24-h feeding period during which IF animals had unlimited access to food. Accordingly, any of the molecular IF effects described, including the reduced Trp/serotonin concentrations in plasma and brain, are not shaped by acute food deprivation. Rather, as outlined above, IF mice consumed more food in the 24-h time period preceding sample collection.

Whether the stimulatory effect on sexual behavior reported here is specific to IF or is also seen with other food restriction regimes (such as CR) remains to be determined in future work. It is possible that chronic low-level food deprivation per se
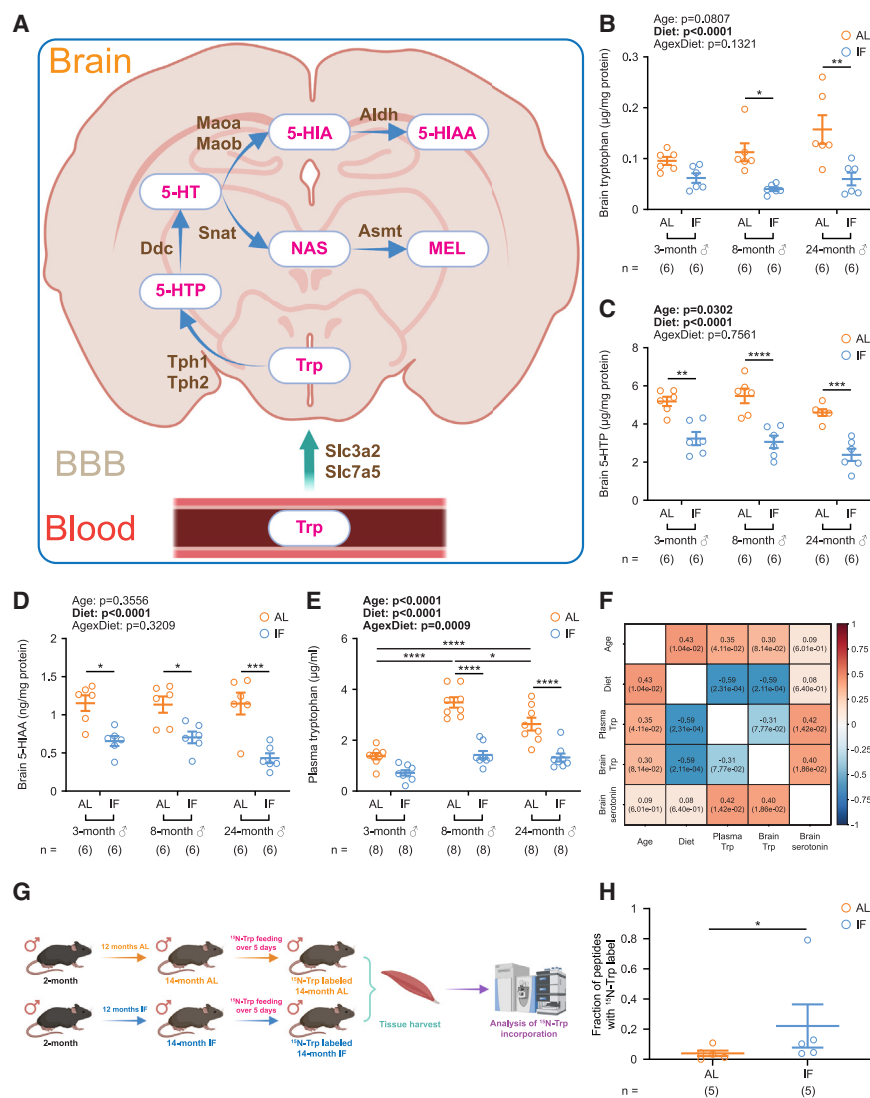
Figure 7. IF and aging influenced brain serotonin abundance by altering Trp supply from the periphery

(A) This schematic illustrates major steps in serotonin biosynthesis and metabolism following the import of its precursor Trp into the brain.

(B–E) These panels show concentrations of brain Trp (B), brain 5-HTP (C), brain 5-HIAA (D), and plasma Trp (E) measured in samples derived from 3-, 8-, and 24-month-old male C57BL6/J mice. Graphs show individual data points as well as means ± SEM.

(F) The heatmap shows results derived from partial correlation analyses where any one variable shown is predicted based on the other variables available. The column "Brain serotonin," for example, refers to results of partial correlation analyses in the context of which brain serotonin concentrations were predicted based on plasma Trp concentrations, brain Trp concentrations, age, and diet. Numbers in parentheses refer to p values and numbers not in parentheses to correlation coefficients. Brain serotonin concentrations were not significantly influenced by age and diet when accounting for plasma and brain Trp concentrations, suggesting that plasma and brain Trp concentrations can fully explain age and diet effects on brain serotonin concentrations.

(G and H) To address whether IF results in an increased uptake of Trp into peripheral tissues, we fed 14-month-old male IF/AL C57BL6/J mice a diet containing ^{15}N-labeled Trp and determined the fraction of ^{15}N-labeled peptides in skeletal muscle using mass spectrometry. The graph (H) shows individual data points as well as means ± SEM. Sample size corresponds to the number of male mice examined. $*p < 0.05$.

free amino acids for energy production.[34,35] Accordingly, in IF mice, we expect refeeding after a 24-h fasting period to lead to the replenishment of tissue protein reserves, thereby decreasing amino acid concentrations in the peripheral blood. This could be especially pronounced for Trp with its low abundance in dietary sources that are thought to be just enough to replace the amounts lost to catabolism.[36,37] To address whether reduced plasma Trp concentration in IF mice are associated with enhanced Trp accumulation in peripheral tissues, we fed animals on IF or AL regimes a diet containing ^{15}N-labeled Trp. These ^{15}N-Trp-labeling experiments showed higher ^{15}N-Trp incorporation rates in skeletal muscle of IF mice, supporting the notion that Trp redistribution from plasma to peripheral tissue pools in IF mice limits Trp availability for serotonin synthesis in the brain of IF mice.

Our work showed that aging-associated increases in brain serotonin concentrations are paralleled by age-related increases in plasma Trp concentrations. Given that central serotonin abundance is directly influenced by Trp availability,[28,31] it is possible that the aging-associated changes in brain serotonin concentrations are directly caused by altered systemic Trp availability. Indeed, our analyses indicate that aging effects on brain serotonin concentrations can be fully explained by aging effects on

(corresponding to an average calorie reduction of 13.3% under the current fasting regime) is sufficient to reduce plasma Trp concentrations and to induce the associated behavioral effects. Our observations indicate that plasma Trp and brain serotonin reductions emerged with protracted periods of fasting (with 6 months of IF producing larger effects than 1 month of IF; Figures 5G and 7E), suggesting that clear effects on sexual behavior may only be detectable with longer-term fasting. In line with this notion, our analyses showed that a 6-week IF treatment is not sufficient to promote sexual behavior in young and aged mice (Figures S2A–S2F). Similarly, available studies using short-term CR in rats did not yield consistent effects on male sexual behaviors.[32,33] Future work should explore systematically the extent to which different fasting regimes and durations impact sexually motivated behaviors.

It is also possible that the IF-associated starvation/refeeding cycles contribute to diminished plasma Trp concentrations independent of an overall reduction in food intake. Fasting periods are associated with enhanced levels of protein mobilization from peripheral tissues, such as skeletal muscle, which provide

plasma and brain Trp concentrations (Figure 7F). Our metabolomic data show that age-related plasma Trp changes take place in the context of broader aging-associated alterations in amino acid metabolism (Figures S7B–S7I). The increase in plasma amino acids during aging may be due to an age-related reduction in energy demand[38] that is paralleled by reduced transamination and diminished consumption of amino acid metabolites for cellular energy production.[39] In addition, an age-related reduction in anabolic processes associated with reduced protein synthesis[40,41] could potentially lower tissue Trp demand, thereby increasing plasma Trp concentrations and facilitating elevated levels in brain serotonin.

Dopaminergic neurotransmission is thought to be a major driver of sexual motivation through actions within mesolimbic and hypothalamic circuitry.[27,42] Excitatory influences on sexual arousal circuitry are balanced by inhibitory pathways. The major known inhibition system of sexual arousal is serotonergic.[27,42,43] Serotonin exerts these effects by regulating a number of relevant circuits, including glutamatergic projections from prefrontal cortex to the brainstem, thereby dampening dopamine release.[27,43] Our observations indicate that aging-associated reductions in male sexual behaviors may be linked to alterations in both excitatory and inhibitory neural regulation. Specifically, and in line with prior research,[44–47] we found an overall decrease in the abundance of central dopamine in aged brains, in addition to an aging-associated increase in serotonin concentrations. IF had no effect on aging-associated changes in brain dopamine, but it decreased serotonin concentrations, thereby removing inhibitory control over male sexual behaviors. Increasing serotonin, by administering 5-HTP, in turn countered IF effects on mating behavior.

Our data indicate that IF shapes systemic Trp availability and central serotonin biosynthesis, thereby coupling food intake to sexual behavior. Of note, dietary Trp intake is also known to influence appetite regulation via effects on central serotonergic systems,[48,49] suggesting a joint dietary influence, via Trp availability and serotonin metabolism, on food seeking and sexual behaviors. Future research should address to what extent fasting may couple different serotonin-regulated behaviors and emotional and cognitive states, via influencing Trp availability.

In humans, aging is well-known to confer risk for male HSDD, a relatively common syndrome characterized by low sexual desire associated with psychological strain and not better explained by any other medical conditions.[7,8,50–53] Here, we observed in old male mice, the neurochemical signature of reduced sexual motivation (low dopamine, high serotonin) that has been proposed to underlie human HSDD.[42] These observations suggest that aged male C57BL/6J mice may represent a useful model for male HSDD in humans. Moreover, our data indicate that IF and/or other dietary manipulations targeting the serotonergic system may hold promise as therapeutic interventions for male HSDD and potentially other conditions associated with reduced libido.

Besides the changes in neurotransmitter systems outlined above, we examined a range of additional aging effects potentially relevant for efficient male reproduction and aging-associated reproductive decrements. Our findings confirm age-related reductions in plasma testosterone concentrations, spermatogenesis, testis weight, sperm concentration, and sperm motility. We also demonstrate a number of alterations in testis and sperm

transcriptomes as well as the sperm methylome. IF, however, had negligible effects on most of these changes (or even aggravated them), indicating that the IF-mediated rescue of aging-associated reproductive impairments was not linked to these alterations. Given that IF-treated aged animals did efficiently reproduce despite the persistence of these alterations, our observations also indicate that the aging-associated endocrine, sperm, and testis changes outlined above are not the major determinants that limit male fertility in aging C57BL/6J mice.

Our analyses of transcriptomic changes in testis and sperm revealed a number of age effects, most of which were not prevented by long-term IF. However, we did observe a small number of IF effects and IF × age interactions that may deserve further follow-up in future studies. In testis, IF influenced the age-dependent change in *Klk1b21*, one of several age-sensitive genes encoding kallikrein 1-related peptidases. Kallikrein 1-related peptidases are thought to play a role in various physiological processes, including, among others, seminal liquefaction[54] and metabolic regulation.[55] Our total RNA-seq analyses of sperm identified *Angptl4* to feature a significant IF × age interaction. *Angptl4* has been reported to be induced by fasting, represses lipoprotein lipase, and thereby inhibits triglyceride uptake into adipocytes during fasting.[56,57] It remains to be addressed whether *Angptl4* plays a similar role in the male reproductive tract, limiting lipid uptake into germ cells during fasting to spare energy for other physiological processes.

Fasting is associated with lifespan extension under a number of experimental conditions.[16–22,58–61] Given that fasting was also found to reduce fertility (mostly supported by experiments conducted in females),[62–64] it has been proposed that fasting may promote lifespan by shifting resources away from reproduction toward the maintenance of the soma to favor survival and increase the chances for reproduction in future, more energy-favorable environments.[65] Our findings, however, indicate that when subjecting male C57BL/6J mice to IF, lifespan extension (reported in Xie et al.[22]) is associated with *increased* reproductive functions in aged mice (Figure 1C). These observations indicate that in our experimental context, reduced reproduction is not necessary for fasting-induced lifespan extension. Our findings nicely align with work performed in the neriid fly *Telostylinus angusticollis*, which demonstrated that robust lifespan extension by DR in males is associated with only subtle and context-dependent effects on fertility,[66] again indicating that lifespan extension under fasting can occur in the absence of adverse effects on reproduction. These conclusions are also consistent with the notion that lifespan extension under IF in mice is caused by cancer suppression,[22,67] which represents the main cause of death during natural aging across many mouse strains.[68–72]

It is also noteworthy that most previous studies have used measures like reduced weights of testes and epididymi,[73,74] decreased spermatogenesis,[74] or an increase in the percentage of abnormal sperm[73,75] as proxies for reduced reproductive functioning under CR in males. However, these measures may poorly predict effects on actual reproductive activity. For instance, our findings indicate that while IF decreased testis weight (Figure 2A), appeared to reduce sperm concentrations (Figure 3B), and had adverse effects on sperm motility (Figures 3C and 3E), it strongly promoted overall reproductive rates (Figure 1C) due to stimulatory effects on mating behavior.

Cell Metabolism
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These observations indicate that the proxies mentioned above are not sufficient to comprehensively evaluate fasting effects on reproduction. Moreover, they suggest that energy investment into reproduction may actually be *increased* under fasting (at least under IF examined here)——given increased mating activity. Evaluating the relative energy investment into reproduction vs. other physiological processes requires taking into account various aspects of reproduction (i.e., beyond the mere generation of germ cells) and should include behavioral aspects as well.

Whether long-term IF boosts sexual behavior in humans has not been explored yet. While acute and complete dietary Trp deprivation may induce aggression and depressive-like behaviors in humans,[76–81] commonly used DR protocols, such as chronic calorie restriction or IF, do not result in complete Trp deprivation but instead lead to a proportional reduction in daily Trp intake. To date, clinical trials employing various DR protocols over periods ranging from several weeks to 2 years have not reported adverse effects on mental or physical health.[82–87] On the contrary, several studies have associated DR with improved mood and increased vigor.[82–85] Compared with dietary supplementation with branched-chain amino acids (BCAAs), an alternative intervention known to reduce Trp levels and serotonergic neurotransmission in the brain,[88,89] IF does not lead to adverse health consequences induced by BCAA supplementation, such as metabolic dysfunction and reduced lifespan.[89] Therefore, we propose that IF may be a valuable strategy for enhancing libido in humans without causing adverse effects like depression or metabolic impairment.

In summary, our study offers compelling evidence that IF boosts sexual behavior and reproductive success in aged male mice, independent of traditional reproductive metrics such as sperm quality or endocrine functions. The observed enhancement in mating behavior is attributed to IF's ability to modulate central serotonin levels through the regulation of Trp availability, highlighting the intricate connection between dietary practices, neurotransmitter dynamics, and reproductive biology. These findings not only expand our understanding of the multifaceted roles of diet in aging and reproductive health but also open new avenues for exploring dietary interventions in addressing sexual desire disorders and possibly other conditions associated with reduced libido.

Limitations of the study

Our study has several limitations. As the first study of its kind to investigate the modulatory effects of long-term DR on male sexual behavior in mice, it remains unknown whether other common fasting protocols, such as chronic calorie restriction or time-restricted fasting, would produce similar outcomes through the same mechanisms. Additionally, our findings are based on a single inbred mouse strain (C57BL/6J), which limits the generalizability of whether long-term IF stimulates male sexual behavior across genetically diverse mouse populations. Moreover, we did not precisely identify the treatment duration at which the observed effects begin to manifest, nor did we determine if the required treatment period depends on the age at which IF is initiated. Finally, we cannot rule out the possibility that IF-induced enhancements in sociability may help counteract age-related declines in male sexual behavior in IF-treated mice.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to the lead contact, Dan Ehninger (dan.ehninger@dzne.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

RNA sequencing and genome-wide DNA methylation data are available through Sequence Read Archive (SRA) at accession number PRJNA1108267. Analysis code is available at https://github.com/ehningerd/Xie_et_al-intermittent_fasting. Scans of full-length, unmodified western blots and an Excel file containing the data used to generate the graphs shown in the paper are included in Data S1.

AUTHOR CONTRIBUTIONS

D.E. conceived the study and developed the concepts presented in this paper; K.X. and D.E. planned and prepared the study; K.X., C.W., E.S., B.P., K.H., C.W., X.M., X.T., J.J., and M.W. performed the experiments; K.X., E.S., B.P., D.R., C.W., X.M., X.T., and D.E. analyzed the data; C.M., S.B., D.B., Y.Z., and D.E. provided oversight and resources; D.E., K.X., and E.S. wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

A text-generating tool was used to confirm the correct English syntax of a few sentences.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Animals
- METHOD DETAILS
  - Intermittent fasting
  - Fertility assays
  - Mating assays and 5-HTP administration
  - Isolation and processing of blood plasma, brain tissue and sperm
  - Quantitative and qualitative analyses of sperm
  - Protein extraction
  - ELISA

- ○ Western blot
- ○ Brain RNA isolation and qPCR
- ○ RNA sequencing
- ○ Bisulfite sequencing-based analyses
- ○ Metabolomic analyses
- ○ Mass-spectrometric analyses of isotope-labeled tryptophan
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cmet.2025.03.001.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
DDC (D6N8N) Rabbit mAb	Cell Signaling Technology	Cat#13561; RRID: AB_2798255
Anti-Actin, mouse monoclonal antibody (Clone C4)	MP Biomedicals	Cat#SKU0869100-CF
Rabbit CD98 Recombinant Rabbit Monoclonal Antibody	Thermo Fisher Scientific	Cat#MA5-29814
Rabbit SLC7A5 Polyclonal Antibody	Thermo Fisher Scientific	Cat#PA5-115916
Rabbit Anti-TPH2 antibody	Abcam	Cat#ab184505; RRID: AB_2892828
Anti-Rabbit IgG (H+L), HRP Conjugate	Promega	Cat#W4011; RRID: AB_430833
Anti-Mouse Immunoglobulins/HRP	Agilent Technologies	Cat#P044701-2
Chemicals, peptides, and recombinant proteins		
4-Methylumbelliferyl phosphate	Merck	Cat#M8883
5-Hydroxy-L-Tryptophan	Merck	Cat#H9772
Calcium ionophore A23187	Merck	Cat#7522
Trypan blue solution	Merck	Cat#T8154
Complete, EDTA-free Protease Inhibitors	Merck	Cat#4693132001
PhosSTOP	Merck	Cat#4906837001
Skim milk powder	Carl Roth	Cat#T145.3
Trizol	Thermo Fisher Scientific	Cat#T9424
WesternBright Chemiluminescence HRP Substrate	Advansta	Cat#K-12045-C20
Critical commercial assays		
Bio-Rad Protein Assay Dye Reagent Concentrate	Bio-Rad	Cat#5000006
5-HIAA(5-Hydroxyindoleacetic Acid) ELISA Kit	MyBiosource	Cat#MBS2516157
General Serotonin ELISA Kit	SAB Signalway Antibody	Cat#EK2268
Mouse 5-Hydroxytryptophan(5-HTP) ELISA Kit	MyBiosource	Cat#775570
Mouse DA(Dopamine) ELISA Kit ELISA Kit	FineTest	Cat#EM1712
OT (Oxytocin) ELISA Kit	FineTest	Cat#EU2549
Mouse PRL(Prolactin) ELISA Kit	FineTest	Cat#EM0158
Testosterone Parameter Assay Kit	R&D Systems	Cat#KGE010
General Tryptophan ELISA Kit (Trp)	ABclonal	Cat#RK00727
High-Capacity cDNA Reverse Transcription Kit	Thermo Fisher Scientific	Cat#4368814
PowerUp SYBR Green Master Mix	Thermo Fisher Scientific	Cat#A25778
PureLink RNA Mini Kit	Thermo Fisher Scientific	Cat#12183018A
RNA 6000 Nano Kit	Agilent Technologies	Cat#50671511
Nextera XT DNA Library Preparation Kit	Illumina	Cat#FC-131-1096
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Cat#Q32851
Agilent SureSelect XT target enrichment kit	Agilent Technologies	Cat#G9611
Deposited data		
Mouse Reference Genome, GRcm38	Genome Reference Consortium	https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000001635.20/
RNA sequencing and genome-wide DNA methylation data	This paper	SRA: PRJNA1108267
Analysis code	This paper	https://github.com/ehningerd/Xie_et_al-intermittent_fasting
Experimental models: Organisms/strains		
Mouse: C57BL/6J	Charles River Laboratories	Strain Code: 632
Oligonucleotides		
Primer sequences utilized for qPCR-based analyses	This paper	N/A

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Image J	https://imagej.net/	https://imagej.net/software/imagej/
TrackMate	Tinerez et al.[76]	https://imagej.net/plugins/trackmate/
STAR	Dobin et al.[78]	https://github.com/alexdobin/STAR
FeatureCounts	Liao et al.[79]	https://subread.sourceforge.net/featureCounts.html
DESeq2	Love et al.[80]	https://bioconductor.org/packages/release/bioc/html/DESeq2.html
TEcounts	Jin et al.[81]	https://github.com/bodegalab/tecount
STRING	Szklarczyk et al.[82]	https://string-db.org/
Bison	Ryan and Ehninger[83]	https://github.com/dpryan79/bison
DMRSeq	Korthauer et al.[84]	https://www.bioconductor.org/packages/release/bioc/html/dmrseq.html
MetaboAnalyst	Xia and Wishart[85]	https://www.metaboanalyst.ca/
GraphPad Prism	GraphPad Software	https://www.graphpad.com/
Pinguoin	Vallat[89]	https://pypi.org/project/pingouin/
Other		
Standard rodent chow	Altromin	Cat#1314
^{15}N-labeled Trp mouse diet	Silantes	N/A
Tryptic peptides with tryptophan amino acid residues used in targeted mass spectrometry analysis, see Table S7	This paper	N/A

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

All experiments were carried out in C57BL/6J mice purchased from Charles River Laboratories (Wilmington, MA, US). Female mice, used in mating and fertility assays, were 3-month-old and sexually naïve, without prior contact to male mice, before their first use in these assays. The age of male mice used in various assays is specified in the main text.

All mice were housed in individually ventilated cages (IVC) under specific pathogen-free conditions. The animals received Altromin 1314 standard rodent chow (composition: 5.1% fat (equivalent to 14% of total metabolizable energy), 22.5% protein (equivalent to 27% of total metabolizable energy) and 40.4% carbohydrates (equivalent to 59% of total metabolizable energy)). Husbandry conditions included a constant temperature of 22 °C, a 12h:12h light/dark cycle as well as ad libitum access to food (except for IF animals as described below) and water. Animals were housed in groups of 2–5 animals per cage, unless stated otherwise. The present study was approved by the "Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen" (Recklinghausen, Germany) (in accordance with the German Animal Welfare Act) as well as the Chancellor's Animal Research Committee at Qingdao University (in accordance with National Institutes of Health guidelines).

METHOD DETAILS

Intermittent fasting

For all IF experiments performed in this study, male C57BL/6J mice were randomly assigned to either the IF or control group (ad libitum feeding). As described in the context of our prior work,[22,67] long-term IF-treated animals were subjected to alternating 24-hour cycles of free access to food (Altromin 1314 standard rodent chow, as specified above) and complete food deprivation, starting at the age of 8 weeks and continued throughout the course of the experiments (specified in detail below). The Altromin 1314 chow diet was provided in the form of 10 mm solid pellets. Our earlier work had shown that mice do not crumble these pellets. Accordingly, removing the pellets on the restriction days was sufficient to fully deprive the animals of food (no cage change required). IF mice were compared to controls with ad libitum (AL) access to food throughout the course of the study.

To determine whether short-term IF, initiated either in young adulthood or later in life, affects male sexual behavior, 3-month-old and 17-month-old male C57BL/6J mice were randomly assigned to AL feeding or IF for 6 weeks prior to behavioral testing. The remaining experimental details were consistent with those described for the long-term IF treatment.

Fertility assays

To assess IF effects on age-related changes in male fertility, we subjected an aging cohort as well as a younger cohort of male mice to either IF or AL, which were initiated at 8 weeks of age and were continued throughout aging and the course of the experiments. Males in the aging cohort were 24 months old when we started with fertility assays. Males in the younger groups of mice were 8 months old when we initiated mating experiments. Prior to the initiation of fertility assays, all males were single housed for seven consecutive days. They were maintained on their respective feeding regime throughout the course of the study. Females used in the fertility assays were young adult (3 months old), had not previously encountered male mice and were initially group-housed with other females. Before initiating fertility assays, they were pseudo-randomly assigned to one of the IF/AL males. To ensure that all females had *ad libitum* access to food at all times, each of them was placed into the assigned male's cage only during those 24-hour windows that corresponded to feeding periods in the IF groups. During IF fasting periods (which also lasted 24 hours; see above), females were then placed back to their individual home cages (where they had free access to food). This procedure was repeated either until females were pregnant or until 3 weeks elapsed, whichever came first. We report the proportion of male mice within each experimental group (young/old IF/AL) that successfully sired offspring in the context of these assays. This outcome measure was compared across groups (age, diet) via chi-square test.

Mating assays and 5-HTP administration

Mating assays were performed by presenting to single-housed IF or AL male mice young adult and sexually naïve females that were introduced to the males' home cage on a regular feeding day of the IF cohort. In order to avoid competition between food seeking behavior and sexual behavior, we granted IF male mice *ad libitum* access to food for three hours prior to performing the mating assay. For the mating experiments represented in Figure 4 and Table S3, we recorded spontaneous behavior over a period of 5 min for later offline analysis that covered various measures, including interaction time, percentage interaction time, mounting frequency, mounting frequency per minute, number of mounts with intromission, number of mounts with intromission per minute, percentage of mounts with intromission (restricted to animals showing mounting behavior), latency to first mount, latency to first intromission, latency to ejaculation, proportion of mice showing mounting behavior, proportion of mice showing intromission and proportion of mice showing ejaculation. Analysis criteria applied were published elsewhere.[90,91] For every male-female pair, mating assays were carried out 3 times with an interval of ca. 1 week between assays (day 1, day 7, day 15). Male-female pairs were kept constant across these assays. Outcome measures were compared across groups using 2-way ANOVAs with the between-subject factors age and diet, followed by Tukey's post hoc analyses where appropriate.

The effect of peripheral 5-HTP (Merck, Darmstadt, Germany) administration on male sexual behavior was investigated in groups of mice that were kept under a reversed dark/light cycle, using an extended observation period of 20 min per mating assay. Mating assays were performed by introducing young adult, sexually naïve females into the home cage of single-housed males on either AL or IF, as described above. Initially, we assessed sexual behavior in untreated male IF and AL mice (n=30 mice per group) under these conditions and compared behavioral measures using unpaired t-tests (Table S4). To examine the effects of 5-HTP, we pseudo-randomly assigned male C57BL6/J mice, subjected to AL or IF, to either one of the pharmacological treatment groups (receiving an i.p. injection of 12.5 mg/kg or 25 mg/kg 5-HTP dissolved in physiological saline; n=15 mice per group) or the corresponding control group (receiving an i.p. vehicle injection; n=29–30 mice). Injections of 5-HTP or saline were administered 15 min prior to the mating assay. During these mating assays, we recorded spontaneous behavior of vehicle/5-HTP-treated IF and AL male mice over a period of 20 min after introducing the female partner. We scored latencies to first mount and mounting frequency and compared them across groups using 2-way ANOVAs with the between-subjects factors pharmacological treatment and diet, followed by Tukey's post hoc analyses if appropriate.

Mating assays involving short-term AL- or IF-treated young (3 months of age at IF initiation) and aged male mice (17 months of age at IF initiation) were performed after 6 weeks of dietary pre-treatment. These experiments were conducted under a reversed dark/light cycle with a recording time of 30 min per mating assay. All mice were sexually naïve prior to behavioral testing. Interaction time, mounting frequency and latency to first mount were quantified.

Isolation and processing of blood plasma, brain tissue and sperm

After sacrificing the animals using an overdose of isoflurane, blood was drained from the vena cava using syringes coated with 500 mM EDTA (pH 8.0). The samples were centrifuged at 1000 x g for 8 min to pellet blood cells. The cell-free supernatant was then transferred to another vessel and kept at -80 °C for later use.

Mouse brains were rapidly separated from the skull, snap frozen in liquid nitrogen and stored at -80 °C. Afterwards, the brain tissue was immersed in liquid nitrogen and ground on dry ice using a mortar and pestle (MTC Haldenwanger, Waldkraiburg, Germany). Aliquots of the resulting tissue powder were stored at -80 °C until use.

For sperm isolation, the epididymides were detached from the testes and individually placed into Eppendorf tubes filled with 1 ml of PBS (pH 7.4), then sliced four times with scissors. The spermatids were allowed to swim out for 60 minutes at 37 °C without shaking. After the swim-out period, the supernatant was transferred to a separate tube and centrifuged at 1000 x g for 8 minutes to pellet the spermatids. Following the removal of the supernatant, the sperm pellets were snap-frozen in liquid nitrogen and stored at -80 °C until further use.

Quantitative and qualitative analyses of sperm

To determine sperm concentration, a 5-μL aliquot of spermatids was collected before pelleting and diluted 1:10 in 0.4% trypan blue (Merck, Darmstadt, Germany) and counted in a Neubauer chamber.

For sperm tracking, a 20-μl aliquot of swim-out spermatids was diluted 1:10 in HTF medium (Thermo Fisher Scientific, Waltham, MA, US) and equally divided to four wells on a sterile 96 well plate. Videos of five seconds duration were recorded at 5x magnification, 15 frames per second and a resolution of 1280 x 960 pixels (corresponding to 1800 x 1350 μm). Analysis of individual sperm tracks were performed using ImageJ's TrackMate plugin.[92] Tracks shorter than 15 frames (1 second) as well as tracks containing gaps larger than 50 pixels between two frames were excluded from analysis. Parameters measured included proportion of immotile spermatids, average swim speed and swim range. Spermatids moving <6 pixels away from their starting location were classified as immotile. Swim range is defined as the maximal linear distance away from its starting location.

Acid phosphatase activity was determined using a previously described protocol.[93] The mouse epididymis was mechanically disrupted by three cuts and swim out of the spermatids was performed at 37 °C for 1 h. Afterwards, the supernatant containing the spermatids was transferred to a new reaction tube and centrifuged at 500 xg and 4 °C for 8 min. The resulting sperm pellet was resuspended in 1 ml HM-buffer (8.33 mM Hepes, 36.33 mM NaCl, 1.59 mM KCl, 0.4 mM $MgSO_4$, 1 mg/ml glucose, 740 μl/l sodium lactate (60% syrup), 0.4 mM KH_2PO_4, 330 mg/l sodium pyruvate, 2.5 g/l $CaCl_2$) followed by an additional centrifugation step. The washing procedure was repeated twice, before the spermatids were capacitated in 1 ml HMB-buffer (HM-buffer + 2 g/l $NaHCO_3$ + 3 mg/ml bovine serum albumin fraction V) at 37 °C for 1 h. Next, 10 μM calcium ionophore A23187 (Merck, Darmstadt, Germany) was added and the suspension was incubated at 37 °C for 1 h. The capacitated spermatids were centrifuged once more and the resulting supernatant was transferred to a new reaction tube. 4-methylumbelliferyl phosphate (0.03 mg/ml; Merck, Darmstadt, Germany) was added to the supernatant and incubated at 37 °C for 1 h in dark. The enzymatic reaction was terminated by adding an equivalent volume of 400 mM glycin buffer (pH 10.4). Fluorescence intensity was measured using a Tecan Infinite 200 Pro microplate reader (Tecan, Männedorf, Switzerland) (excitation=365 nm, emission=449 nm, excitation bandwidth=9 nm, emission bandwidth=20 nm, gain=50, number of flashes=10, integration time=20 μs).

Statistical comparisons across groups were made using 2-way ANOVAs with the between-subjects factors age and diet, followed by Tukey's post hoc analyses where appropriate.

Protein extraction

500 μl tris-buffered saline (TBS, pH 7.6) supplemented with 1% triton x-100 (Merck, Darmstadt, Germany), 1x protease inhibitor cocktail (Merck, Darmstadt, Germany) and 1x phosphatase inhibitor cocktail (Merck, Darmstadt, Germany) were added to a brain powder aliquot. Homogenization was performed using 15 passages through a 24-gauge needle. The homogenates were kept on ice for 30 min before centrifugation for 10 min at 10000 x g and 4 °C. The resulting supernatant was transferred to a new vessel and kept at -80 °C until use. Protein concentrations were measured via Bradford assays (Bio-Rad Laboratories, Hercules, CA, US).

ELISA

Commercial ELISA kits were used to determine the abundance of 5-HIAA (#MBS2516157; MyBiosource, San Diego, CA, US), 5-HT (#EK2268; Signalway Antibody, Greenbelt, MD, US), 5-HTP (#MBS775570; MyBiosource, San Diego, CA, US), dopamine (#EM1712; FineTest, Wuhan, China), oxytocin (#EU2549; FineTest, Wuhan, China), prolactin (#EM0158; FineTest, Wuhan, China), testosterone (#KGE010; R&D Systems, Minneapolis, MN, US) and tryptophan (#RK00727; ABclonal, Woburn, MA, US) in blood plasma and/or brain homogenates following the manufacturers' instructions.

Western blot

30 μg of protein homogenate per sample was separated using self-cast tris-glycine gels and subsequently transferred onto a nitrocellulose membrane with a 0.1 μm pore size (GE Healthcare, Chicago, IL, US). To minimize background noise, the membrane was incubated with phosphate-buffered saline (PBS) containing 10% skim milk (Carl Roth, Karlsruhe, Germany) for one hour at room temperature. The primary antibodies, diluted in PBS with a 1% milk solution, were applied and left to incubate overnight at 4 °C. Secondary antibody solutions, which included goat anti-rabbit horseradish peroxidase conjugates (Promega, Madison, WI, US) at a 1:3000 dilution and goat anti-mouse horseradish peroxidase conjugates (Agilent Technologies, Santa Clara, CA, US) at a 1:10000 dilution in PBS with 0.5% milk, were incubated for 90 minutes at room temperature. The WesternBright ECL HRP substrate (Advansta, Menlo Park, CA, US) was then applied to detect immunoreactive targets through enhanced chemiluminescence. For densitometric analysis, ImageJ software (version 1.53q) was utilized, with the density of the target band normalized to the actin band from the same lane. In this study, the primary antibodies used targeted aromatic L-amino acid decarboxylase (Aadc) (#13561, clone D6N8N, 1:2000 dilution; Cell Signaling Technology, Danvers, MA, US), actin (#SKU0869100-CF, clone c4, 1:10000 dilution; MP Biomedicals, Santa Ana, CA, US), solute carrier family 3 member 2 (Slc3a2) (#MA5-29814, clone 021, 1:2000 dilution; Thermo Scientific, Waltham, MA, US), solute carrier family 7 member 5 (Slc7a5) (#PA5-115916, polyclonal, 1:2000 dilution; Thermo Scientific, Waltham, MA, US) or tryptophan hydroxylase 2 (Tph2) (#ab184505, clone EPR19191, 1:2000 dilution; Abcam, Cambridge, UK). Western blot data were analyzed using 2-way ANOVAs with the between-subjects factors age and diet, followed by Tukey's post hoc analyses where appropriate. Full length Western blots are shown in Data S1.

Brain RNA isolation and qPCR

Total brain RNA was extracted using Trizol (Thermo Scientific, Waltham, MA, US) following the manufacturer's protocol. The RNA concentration was measured with a NanoDrop 2000c device (Thermo Scientific, Waltham, MA, US). Subsequently, 2000 ng of total RNA was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific, Waltham, MA, US). Real-time quantitative PCR experiments, utilizing the SYBR Green method, were conducted with the PowerUp SYBR Green Master Mix (Thermo Scientific, Waltham, MA, US) on a QuantStudio 6 Flex Real-Time PCR system (Thermo Scientific, Waltham, MA, US), using 8 ng of cDNA per reaction. The cycle threshold (Ct) values of target genes were normalized to the Ct value of *Actb* detected in the respective sample. The expression level of a target gene was calculated using the formula $2^{(\Delta Ct)}$, where ΔCt is the difference between Ct(actb) and Ct(target gene). Samples displaying irregular amplifications were excluded from data analysis. The following primer sequences were used in the qPCR-based analyses: *aanat* (forward: TCCCCTCTACTTGGATGAGATC; reverse: CTGTGTAG TGTCAGCGACTC), *actb* (forward: CCCTGAAGTACCCCATTGAAC; reverse: CCATGTCGTCCCAGTTGGTAA), *asmt* (forward: GAAGTGGGACAGGAAGTGAG; reverse: CGGGAACAGGAAGTGGC), *ddc* (forward: AGTCATTCTTGGGTTGGTCTG; reverse: CAGCAAAGCGTAGCACAAAC), *fev* (forward: TGATCAACATGTACCTGCCAG; reverse: AGGAGAAACTGCCACAACTG), *htr1a* (forward: CTCCAACTCCCTGCTCAAC; reverse: CCACCACTATTACCACCACTAC), *htr1b* (forward: TCATGCATCTCTGTGTCATCG; reverse: AGGGTGGCAACGAAATAGAG), *htr1d* (forward: GGTGGATGCTGGTGATAACA; reverse: CAAAACATTCCCGTGACC AAG), *htr1f* (forward: GGAGGTGAAGTGAGAATGAAGAC; reverse: GTCAAGTTTTGGTCTGATGCG), *htr2a* (forward: CCTGAAAA TCATTGCGGTGTG; reverse: TGCCACAAAAGAGCCTATGAG), *htr2b* (forward: AACTGAACAGACACAGGACG; reverse: GATTC AGGCTCTCGAAGATGG), *htr2c* (forward: CCATTGCTGATATGCTGGTG; reverse: GGACGCAGTTGAAAATAGCAC), *htr3a* (forward: CAACTTCCCCTTTGATGTGC; reverse: CCACTCGCCCTGATTTATGAAG), *htr3b* (forward: CGAGAGGTTTGGAACGATGAG; reverse: ATAGGGCAGGTCAGGAGAT), *htr4* (forward: GTCCTACTTACCACAGCATCG; reverse: GCAGCCTCCCAACATTAATG), *htr5a* (forward: CCATCGGTCGCAAACATCTAG; reverse: TGCGGTTAGGTTCCAAAGAG), *htr5b* (forward: ACCCCTTGATTTAC ACTGCC; reverse: GAAGGTCTCGGGTGAAATGAG), *htr6* (forward: GCTGAGCATGTTCTTTGTCAC; reverse: AGGGTTCATGGT GCTATTACAG), *htr7* (forward: TCTGCAACGTCTTCATCGC; reverse: TTTCCCATTCTGCCTCACG), *maoa* (forward: TGGTTCTTGT GGTATGTGAGG; reverse: AGCTTCACTTTATCCCCAAGG), *maob* (forward: GCCCGTCCATTATGAAGAGAAG; reverse: CTGTT TCAGTGCCTGCAAAG), *pdyn* (forward: TGCAGTGAGGATTCAGGATG; reverse: AGGGTGAGAAAAGATGAGAAGC), *penk* (forward: CCAGGCGACATCAATTTCC; reverse: TGCAGGTCTCCCAGATTTTG), *pomc* (forward: GCTTCAGACCTCCATAGATGTG; reverse: TTGCCAGGAAACACGGG), *slc3a2* (forward: ACCTCACTCCCAACTACCAG; reverse: ATCAGCTTTCCCACATCCC), *slc6a4* (forward: GCTGAGATGAGGAACGAAGAC; reverse: CAAACCCAGCGTGATTAACATG), *slc7a5* (forward: TCACTACCCTCTCTACCA ACC; reverse: TGAACAGAGACCCATTGACAG), *tph1* (forward: CTTGGAGAATGAAGTCGGAGG; reverse: AACTGTTCTCGGCTG ATGTC), *tph2* (forward: GGAAGTATTTTGTGGATGTGGC; reverse: AGTCGGGTAGAGTTTGGAGAG). Data were analyzed using 2-way ANOVAs with the between-subjects factors age and diet, followed by Tukey's post hoc analyses where appropriate.

RNA sequencing

Total RNA from testis (without removal of sperm) and sperm was extracted using the PureLink RNA Mini Kit (Thermo Scientific, Waltham, MA, US). The quality of the RNA was assessed on a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, US) using the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, US). Libraries were prepared using the Nextera DNA Library Preparation Kit (Illumina, San Diego, CA, US) and sequenced on an Illumina HiSeq 2000 instrument (Illumina, San Diego, CA, US). Reads from Illumina were quality and adapter trimmed using Trim Galore! and aligned using STAR[94] against the GRCm38 genome with the Ensembl release 79 annotation. Gene expression was then quantified using featureCounts[95] and differential expression determined using DESeq2.[96] For repeat elements, the same procedure was followed, with the exception that repeat elements were quantified with TEcounts.[97] The Benjamini-Hochberg procedure was employed to adjust p-values for multiple comparisons. P-values below a false discovery rate threshold of 0.05 were considered statistically significant. Differentially expressed genes were further analyzed using STRING (version 12.0)[98] to illustrate known interactions among differentially expressed genes and to identify enriched gene ontology terms within these gene sets.

Bisulfite sequencing-based analyses

The size of the purified genomic DNA (gDNA) was analyzed on an Agilent 2100 Bioanalyzer (Agilent Technologies), using a High Sensitivity chip, and the concentration was determined with the Qubit dsDNA HS Assay Kit (Life Technologies). The fragmentation of 200 ng gDNA was performed using a Covaris (duty cycle 10%, intensity 4, 200 cycles per burst for 60 s) to obtain fragments with an average length of ∼250 base pairs (bp). The DNA sequencing library was generated using the Agilent SureSelectXT target enrichment kit and 100 ng of fragmented gDNA according to the manufacturer's instructions. The DNA sequencing library was subsequently purified, quality controlled using an Agilent Bioanalyzer 2100. The sample concentration was measured with a Qubit dsDNA HS Assay Kit prior to sequencing on an Illumina HiSeq2000 using a TruSeq SBS Kit v3 according to the manufacturer's instructions. Reads were quality and adapter trimmed using Trim Galore! and aligned against the GRCm38 genome with Bison[99] with methylation quantified using MethylDackel. Differentially methylated regions were then determined using DMRseq.[100]

Metabolomic analyses

Mouse plasma samples were submitted to Metabolon (Morrisville, NC, US) for metabolomic analyses.

Sample preparation involved the automated MicroLab STAR® system (Hamilton), with proteins precipitated by methanol for metabolite recovery. The extracts were fractionated for analysis via reverse phase (RP)/UPLC-MS/MS in both ion modes and HILIC/UPLC-MS/MS for negative ion mode. QC involved a pooled matrix, process blanks, and QC standards for instrument performance and chromatographic consistency.

UPLC-MS/MS utilized Waters ACQUITY UPLC and Thermo Scientific Q-Exactive mass spectrometers with a heated electrospray ionization (HESI-II) source. The dried extracts were reconstituted in solvents tailored for each method, containing fixed-concentration standards for consistency. Analyses were performed under conditions optimized for compound hydrophilicity or hydrophobicity, with the choice of acidic or basic conditions based on the ionization mode. The scan range covered 70-1000 m/z, and raw data files were archived for extraction.

Compound identification relied on comparing extracted peaks against a library containing over 5400 standards, using retention time, mass to charge ratio, and fragmentation patterns. Unnamed biochemicals were identified through their recurrent nature, with the library continuously updated to enhance metabolite identification.

QC procedures ensured the accuracy and consistency of chemical entity identification, removing artifacts and errors. Proprietary software was used for peak identification verification across samples.

Metabolite quantification used area-under-the-curve measurements, with data normalization steps correcting for inter-day instrument variations. Normalization ensured consistent data representation, with potential adjustments for sample-specific factors like protein content.

Batch-normalized metabolite values for plasma Trp were compared across age groups using one-way ANOVA with the between-subjects factor age.

To identify age-sensitive metabolites, we carried out linear regression analyses, in the context of which we predicted batch-normalized metabolite values based on age. Metabolites with an adjusted p-value<0.05 were considered to be age-sensitive.

To identify metabolites associated with plasma Trp, we performed linear regression analyses, predicting Trp values based on the values of any other given metabolite (one at a time), while accounting for age. We selected metabolites featuring a p-value<0.01 for further exploratory downstream analyses.

MetaboAnalyst (version 6.0)[101] was used for metabolite set enrichment analyses.

Mass-spectrometric analyses of isotope-labeled tryptophan
Mice and diet

A mouse diet based on artificial ingredients (modified from the Altromin C1069 diet), in which natural Trp was replaced by ^{15}N-labeled Trp (3 g/kg), was purchased from Silantes (Munich, Germany). After being maintained on the respective dietary regime for 12 months, single-housed 14-month-old AL and IF mice (n=5) were fed with 10 mm food pellets labeled with ^{15}N-Trp over 5 consecutive days (equivalent to 3 feeding and 2 fasting cycles for IF mice). Feeding procedures were equivalent to those described above. Animals were sacrificed at the end of a feeding day. A separate cohort of AL and IF animals being fed with the regular Altromin 1314 chow was run in parallel, serving as negative controls for quality assessment of ^{15}N-Trp incorporation into *de novo* synthesized proteins.

Sample preparation and protein extraction

Pulverized skeletal muscle tissue harvested from 5 mice from each group of either ad libitum feeding or IF feeding on tryptophan labelled mouse diet or normal diet, were utilized for total protein extraction with 200 μl lysis buffer (50 mM HEPES (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1.5% SDS, 1 mM DTT, supplemented with 1x protease and phosphatase inhibitor cocktail). Lysis was aided by 6 cycles of sonication in a water bath for 1 min at 35 kHz, followed by incubation on ice for 2 minutes. A modified protocol for Filter-aided-Sample-preparation (FASP),[102] with approximately 20 μg of total protein extracts was employed to generate tryptic peptides for targeted MS analysis. Harvested peptides were cleaned and desalted on C18 stage tips and re-suspended in 1% FA for LC-MS/MS analysis.

Selection of tryptic peptides with tryptophan amino acid residues for targeted mass spectrometry

We selected unique tryptic peptides from 10 abundantly expressed protein candidates with tryptophan amino acids in their sequence for targeted MS analysis by a modified SureQuant-based method. Amongst the selected candidates, 9 unique peptides from 6 highly abundant proteins were utilized in the targeted MS experiment (Table S7). We prioritized unique peptides of candidate proteins that were previously shown to be abundantly expressed in our label free aging mouse proteomics experiments (unpublished data). Secondly, the peptides had to be easily ionized and observed in other MS-based analyses (information from Peptide atlas). Thirdly, selected peptides were 8–25 amino acids long and without internal C residues. Based on the above criteria, we designed a method to extract endogenous tryptic peptides with both unlabelled tryptophan residues (light) and isotope labelled tryptophan (heavy), for subsequent targeted MS experiments.

Characterization of tryptic peptides with isotope labelled tryptophan

First, we performed an initial survey run with the monoisotropic mass of the precursor ion for each target "light" and "heavy" peptide and a mass range of 2–3, to capture all reference fragments at an intensity threshold of 1×10^5. In a second survey run, the mass/charge (m/z) ratios of precursor ions from the previous step were used for optimization and verification of correct fragment ions. The modified SureQuant-based analysis involved acquisition of high resolution MS1 scans to monitor the predefined optimal precursor ions of the "light" and "heavy" peptides, based on their associated m/z values and intensity thresholds. In the next step, targeted m/z from the inclusion list that met the minimum intensity threshold and short fill time requirements, were scanned at low resolution in MS2 of the "light" and "heavy" peptides in the subsequent MS cycle. Finally, scans with at least 5 of 6 specified product ions were

subjected to a high resolution MS2 scan of the "light" and "heavy" peptides at the defined mass offset, with longer fill times to improve measurement sensitivity.

Liquid chromatography and Survey MS analyses: Endogenous tryptic peptides were injected at starting conditions of 95% eluent A (0.1% FA in water) and 5% eluent B (0.1% FA in 80% ACN) for analysis on a Dionex Ultimate 3000 RSLC nanosystem coupled to an Orbitrap Exploris 480 MS. They were loaded onto a trap column cartridge (Acclaim PepMap C18 100Å, 5 mm x 300 μm i.d., #160454, Thermo Scientific) and separated by reversed-phase chromatography on an Acclaim PepMap 100 C18 75 μm X 25 cm (both columns from Thermo Scientific) using a 35 min linear increasing gradient from 5% to 25% of eluent B followed by a 5 min linear increase to 50% eluent B. The mass spectrometer was operated in data dependent and positive ion mode with a spray voltage of 1.9 kV, no sheath or auxiliary gas flow, heated capillary temperature of 300°C, MS1 spectra recorded at a resolution of 120K, mass scan range of 300–1500, automatic gain control (AGC) target value of 300% (3×10^6) ions, maximum injection time (maxIT) of 50 ms and a default charge state of 2. For every scan, a cycle time of 7 s, an isolation window of 1.0 m/z, normalized collision energy (NCE) of 28% by higher energy collisional dissociation (HCD), scan range of 100–1700 m/z, maximum IT of 10 ms, AGC target value of 1000% and mass resolution of 7500, were used.

SureQuant-based quantitation of selected tryptic peptides

SureQuant analysis was performed as previously described,[103] but with some modifications. Only peak areas with a dotp $\geq$ 0.80 were considered in the final analysis. Peak area ratios of endogenous "light" and "heavy" peptides for the 6 selected product ions were exported from Skyline software v21.1.0.278[104] and peptides filtered according to the following criteria: First, only peptides with an AUC > 0 for n $\geq$ 5 product ions were considered. Second, peak area values of the 3 highest intensity product ions from both the light/heavy peptides were summed. Quantitation was based on 3 selected product ions to balance specificity with the ability to retain lowly abundant targets. In order to estimate the overall fraction of ^{15}N-Trp-labelled peptides in our samples, we derived the following ratio (based on the sum of the top 3 peak area values as outlined above): sum of top 3 peak area values of heavy peptides summed across peptides / (sum of top 3 peak area values of heavy peptides summed across peptides + sum of top 3 peak area values of light peptides summed across peptides). Statistical comparison across groups was performed via Mann-Whitney test.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism (version 10.2.0; GraphPad Software, La Jolla, CA, US) if not stated otherwise. Statistical tests used are specified in the respective methods section above. Linear regression analyses were performed using statsmodels (version 0.14.0). Partial correlation analyses were carried out using Pingouin (version 0.3.11).[105]