Sexual Dimorphic Metabolic and Cognitive Responses of C57BL/6 Mice to Fisetin or Dasatinib and Quercetin Cocktail Oral Treatment

Yimin Fang[1], David Medina[2], Robert Stockwell[2], Sam McFadden[1], Kathleen Quinn[1], Mackenzie R. Peck[1], Andrzej Bartke[2,3], Kevin N. Hascup[1,3,4], Erin R. Hascup[1,4,*]

[1] Department of Neurology, Dale and Deborah Smith Center for Alzheimer's Research and Treatment, Neuroscience Institute, Southern Illinois University School of Medicine, Springfield, IL 62702, USA.

[2] Department of Internal Medicine, Southern Illinois University School of Medicine, Springfield, IL 62702, USA.

[3] Department of Medical Microbiology, Immunology and Cell Biology, Southern Illinois University School of Medicine, Springfield, IL 62702, USA.

[4] Department of Pharmacology, Southern Illinois University School of Medicine, Springfield, IL 62702, USA.

*Corresponding Author: Erin R. Hascup, Department of Neurology, Dale and Deborah Smith Center for Alzheimer's Research and Treatment, Southern Illinois University School of Medicine, P.O. Box 19628, Springfield, IL 62794-6928, USA Tel: 217-545-6988, Email: ehascup@siumed.edu

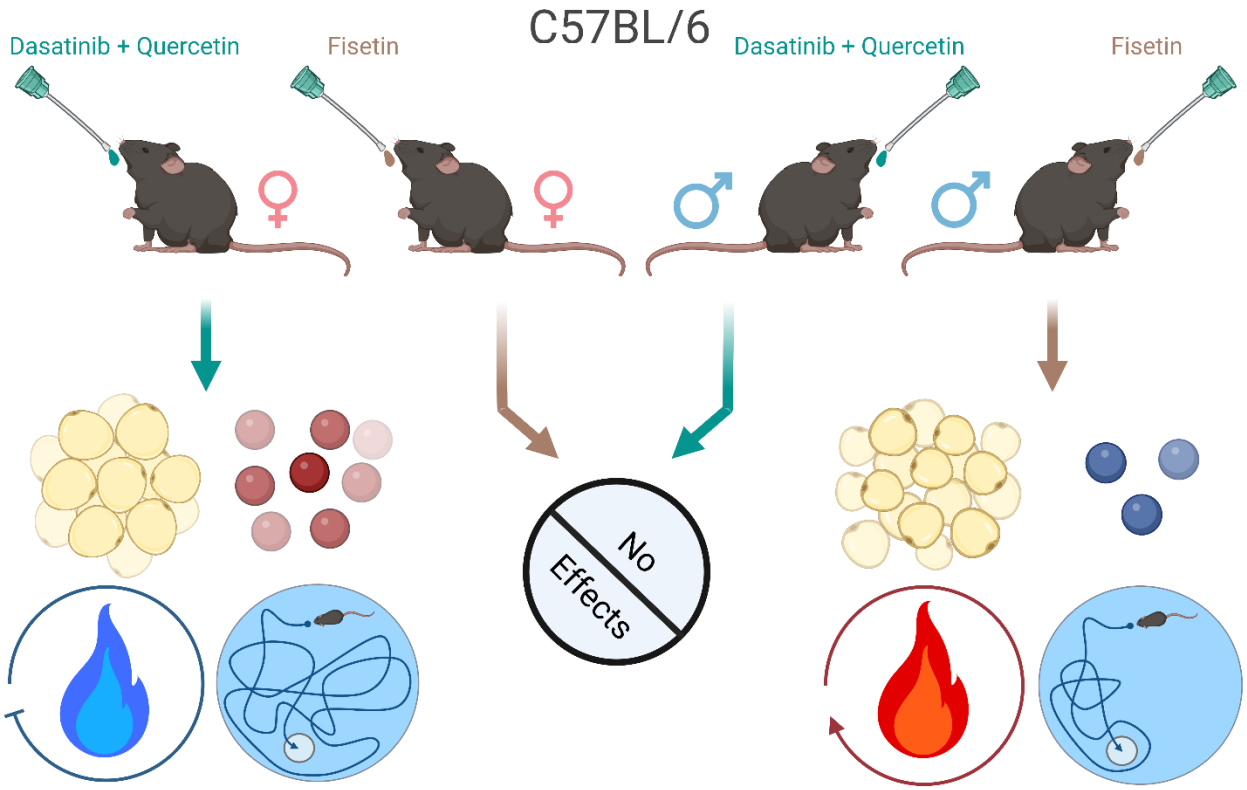
Key words: senolytic drugs, cognition, adiponectin, glucose metabolism

Abstract

Senolytic treatment in aged mice clears senescent cell burden leading to functional improvements. However, less is known regarding the effects of these compounds when administered prior to significant senescent cell accumulation. From 4-13 months of age, C57BL/6 male and female mice received monthly oral dosing of either 100 mg/kg Fisetin or a 5 mg/kg Dasatinib (D) plus 50 mg/kg Quercetin (Q) cocktail. During treatment, several aspects of healthy aging were assayed including glucose metabolism using an insulin and glucose tolerance test, cognitive performance using Morris water maze and novel object recognition, and energy metabolism using indirect calorimetry. Afterwards, mice were euthanized for plasma and tissue specific markers of senescence-associated secretory phenotype (SASP) and white adipose tissue accumulation (WAT). Sexually dimorphic treatment effects were observed. Fisetin treated male mice had reduced SASP, enhanced glucose and energy metabolism, improved cognitive performance, and increased mRNA expression of adiponectin receptor 1 and glucose transporter 4. D+Q treatment had minimal effects in male C57BL/6 mice, but was detrimental to females causing increased SASP expression along with accumulation of WAT depots. Reduced energy metabolism and cognitive performance were also noted. Fisetin treatment had no effect in female C57BL/6 mice potentially due to a slower rate of biological aging. In summary, the senolytic treatment in young adulthood, has beneficial, negligible, or detrimental effects in C57BL/6 mice dependent upon sex and treatment. These observations should serve as a note of caution in this rapidly evolving and expanding field of investigation.

42 **Graphical Abstract**

43 Male and female C57BL/6 mice were treated with once monthly oral doses of either
44 Dasatinib (D) + Quercetin (Q) or Fisetin from 4-13 months of age. Females treated with
45 D+Q had increased adiposity and SASP markers (red spheres) along with decreased
46 metabolism (blue flame) and cognitive performance. Males treated with Fisetin had
47 reduced SASP markers (blue spheres) as well as improved metabolism (red flame) and
48 cognition. No effects were observed in females treated with Fisetin or males treated with
49 D+Q.



50

Introduction

Fisetin and quercetin (Q) are plant-derived flavonoids that offer cytoprotection against cellular stress and act as anti-inflammatory, chemopreventive, chemotherapeutic, and senotherapeutic agents (1, 2). Additionally, Q incites immune response against allergic diseases (2, 3). Dasatinib (D) is a tyrosine kinase inhibitor used to treat leukemia (4) and is routinely used in combination with Q to improve the senotherapeutic potency. Although both prooxidan flavonoids are structurally analogous, Fisetin has higher senolytic activity than Q whose potency is also reliant on endogenous trace elements such as iron and copper (5).

As the first senolytic drugs, they were discovered to selectively clear senescent cells generated during cellular senescence (6). Cell senescence is a phenomenon defined as a stable cell cycle arrest, and is associated with fundamental aging processes and age-related diseases (7). Although this process is essential for preventing replication of damaged DNA thereby suppressing tumor formation for cancer, accumulation of senescent cells with aging produces senescence-associated secretory phenotype (SASP) (7, 8). SASP includes proinflammatory cytokines, chemokines, growth factors, and proteases (9, 10) caused by chronic inflammation (10-12), DNA damage (13, 14), mitochondrial dysfunction (15), immune cell dysfunction (16), ROS generation (13, 17), and brain protein aggregation (18, 19). All of which may be factors that predispose individuals to a multitude of age-related disorders (7).

Fisetin and D+Q selectively clear senescent cells (6), thereby delaying aging-associated disorders and improving healthspan and lifespan. This has been observed after reducing senescent cell burden in progeroid or aged (twenty-four-month-old)

74 C57BL/6 mice (20-22). Moreover, deletion of senescent cells from the brain genetically
75 or pharmacologically with senolytic drugs led to functional improvements in mouse
76 models of neurodegenerative diseases such as Parkinson's and Alzheimer's disease
77 (18, 23-25). These studies have shown senotherapeutics can reduce senescent cell
78 burden and have positive impacts on animals with accelerated aging, advanced age, or
79 neurodegenerative disorders. Accordingly, senotherapeutics are currently marketed as
80 anti-aging therapies where young, healthy adults can take these products as dietary
81 supplements.

82 However, less is known about the effects of these compounds when
83 administered prior to significant senescent cell accumulation. Thus, the experiments
84 were designed to examine the long-term effects of monthly oral Fisetin or D+Q
85 treatment when initiated in young (four-month-old) C57BL/6 mice. We examined
86 morphological, metabolic, physical, and cognitive components that are known to be
87 affected by senescent cell accumulation. The results presented here indicate that
88 monthly administration of Fisetin or D+Q had sexually dimorphic effects which also
89 depended on treatment type in C57BL/6 mice. We highlight a potential new mechanism
90 of action involving the beneficial roles of glucose and adiponectin signaling both
91 peripherally and centrally by early and long-term administration of Fisetin on male
92 C57BL/6 mice.

93 **Results**

94 **Administration of Fisetin or D+Q altered the SASP profile of C57BL/6 mice in a**
95 **sex dependent manner.**

96 It has been reported that senolytic drug treatment decreased senescent burden
97 in twenty-four-month-old C57BL/6 mice leading to functional improvements (20).
98 However, it was unknown whether the same treatment administered monthly initiated
99 during young adulthood (four months of age) would improve the overall health of
100 C57BL/6 mice as they aged. To examine this, Fisetin or a cocktail of D+Q were orally
101 administered in C57BL/6 mice starting at four months of age and continued once each
102 month for nine months. All mice underwent glucose tolerance test (GTT) and insulin
103 tolerance test (ITT) to evaluate glucose homeostasis, indirect calorimetry to determine
104 energy metabolism, grip force to monitor muscle strength, and Morris water maze
105 (MWM) and novel object recognition (NOR) to ascertain cognitive abilities. At
106 approximately thirteen months of age, the mice were sacrificed to determine body
107 composition and collect plasma and tissues for analyses of SASP markers, lipid
108 metabolism, glucose and adiponectin signaling, and synaptic plasticity. The
109 experimental paradigm is shown in Fig 1a.

110 To investigate whether the compounds used in this study decreased SASP
111 profiles and other senescent markers, the gene expressions were measured in gonadal
112 white adipose tissue (WAT) by RT-PCR. To evaluate aging effects, additional groups of
113 treatment naïve young (four months of age) and old (twenty-one months of age) mice
114 were studied. We assayed for SASP markers including $p16^{Ink4\alpha}$, $p21^{Cip1}$, TNFα, MCP 1,
115 IL-6, and IL-10. Although IL-10 is a potent anti-inflammatory cytokine, studies have

116 shown IL-10 activates cellular senescence mechanisms (26) and is secreted from
117 senescent cells (27). The results showed that in both the male and female mice, the
118 transcriptional levels of these SASP markers were lower in the treatment naïve young
119 compared to old C57BL/6 mice (Fig. 1b-g). The vehicle treated littermate control
120 C57BL/6 mice sacrificed at thirteen months of age had similar mRNA levels of the
121 above mentioned SASP markers as the untreated twenty-one-month-old mice (Fig. 1b-
122 g). This is consistent with recent tissue and age-specific RNA sequencing in both sexes
123 of C57BL/6 mice where a significant increase in differentially expressed genes was first
124 evident in gonadal WAT that occurred by mid-age and was maintained at ages older
125 than twenty months (28). Others have shown $p16^{Ink4\alpha}$ and $p21^{Cip1}$ have similar mRNA
126 expression levels at 12 and 24 months of age in the hypothalamus, heart, liver, and
127 kidney of female CB6F1 mice (29).

128 We also observed that C57BL/6 males had higher levels of mRNA expression of
129 TNFα and IL-6 (Fig. 1d, f) compared to littermate females. Interestingly, the effects of
130 the treatments were sex-dependent. The treatment with Fisetin or D+Q reduced mRNA
131 levels of $p16^{Ink4\alpha}$ in the male mice, but had no effects in the female mice (Fig. 1b). The
132 treatment with Fisetin reduced the mRNA levels of $p21^{Cip1}$, TNFα, and IL-6 in the male
133 mice, but had no effect in the female mice (Figs. 1c, 1d, & 1f), while the treatment with
134 D+Q raised the mRNA levels of $p21^{Cip1}$, MCP1, IL-6, and IL-10 (Figs. 1c & 1e-g) in the
135 female mice.

136 The same SASP mRNA levels were examined in the hippocampus (SI Fig. 1a-f).
137 mRNA levels were higher in both sexes of treatment naïve twenty-one-month-old
138 C57BL/6 mice compared to four-month-old littermates. $p21^{Cip1}$ was the only SASP

139 marker increased in the hippocampus of female C57BL/6 mice compared to male
140 littermates. In general, mRNA hippocampal expression patterns after senotherapeutic
141 treatment was similar to observations in gonadal WAT. In male C57BL/6 mice, Fisetin
142 treatment reduced mRNA levels of $p16^{Ink4\alpha}$ and $p21^{Cip1}$ (SI Fig 1a-b), but did not affect
143 the other assayed cytokines (Fig 1c-f). Reduced hippocampal mRNA levels of $p21^{Cip1}$
144 (SI Fig. 1b) were also observed in female C57BL/6 mice. D+Q treatment increased
145 mRNA levels of TNFα (SI Fig. 1c) and IL-10 (SI Fig. 1e) in both sexes of C57BL/6 mice.

146 Plasma concentrations of TNFα, MCP1, IL-6, and IL-10 (SI Fig. 2a-d) were
147 elevated in both sexes of treatment naïve twenty-one-month-old C57BL/6 mice
148 compared to four-month-old littermates. The plasma concentration of TNFα was
149 increased in males, while IL-10 was increased in females compared to sex-matched
150 C57BL/6 littermates. Fisetin treatment decreased the concentration of MCP1 (SI Fig.
151 2b) in males while increasing IL-10 (SI Fig. 2d) concentration in female C57BL/6 mice.
152 D+Q treatment had no effect in males, but increased the concentration of MCP1, IL-6,
153 and IL-10 (SI Fig. b-d) in female C57BL/6 mice.

154 Altogether, the data showed that Fisetin treatment reduced the levels of SASP
155 markers in the plasma as well as the peripheral and central tissue of male C57BL/6
156 mice, while little to no changes were observed in female littermates. The D+Q treatment
157 increased, rather than decreased, SASP markers in female C57BL/6 mice. In male
158 C57BL/6 mice, D+Q treatment had minimal to no effects in plasma and WAT, but
159 increased hippocampal TNFα mRNA expression.

160

161 **D+Q treatment led to increased body weight and adiposity in female C57BL/6**
162 **mice while Fisetin treatment improved glucose metabolism and increased plasma**
163 **adiponectin in male C57BL/6 mice.**

164 Given the observation that C57BL/6 mice responded to the treatment in a
165 sexually dimorphic manner, it was important to know how the physiological parameters
166 including body composition, glucose utilization, and energy metabolism were affected
167 by senotherapeutic treatment. The results showed that body weight in the males was
168 not changed by either treatment, while it was increased in the female C57BL/6 mice
169 receiving monthly D+Q administration (Fig. 2a-b). Body composition analysis indicated
170 that the increased body weight of D+Q treated female C57BL/6 mice was attributed to
171 an elevation of adiposity including subcutaneous (SC) and gonadal WAT depots (Figs.
172 2c-e). No changes in body weight or composition were observed after nine months of
173 Fisetin treatment in female C57BL/6 mice, and no changes in these parameters were
174 detected in male C57BL/6 mice receiving either of the treatments (Figs 2a-g). Body
175 composition also showed sex differences whereby female C57BL/6 mice had a higher
176 percentage of SC and gonadal WAT while males had a larger perirenal depot (Fig. 2d-
177 f).

178 The accumulation of WAT in D+Q treated female mice could result from
179 upregulated lipid deposition or downregulated thermogenic activity in brown adipose
180 tissue (BAT). Thus, the expressions of genes related to lipid metabolism in gonadal
181 WAT, and to uncoupling of the mitochondrial electron transport chain in BAT were
182 examined. The results showed that Acetyl-CoA carboxylase (ACC), the rate-limiting
183 enzyme for lipid synthesis, was up-regulated in gonadal WAT of D+Q treated female

184 C57BL/6 mice (Fig. 2h). In contrast to WAT, uncoupling protein 1 (UCP1), a key
185 regulator of non-shivering thermogenesis, and its upstream activator PPARγ (30, 31)
186 were downregulated in BAT of D+Q treated female mice (Fig. 2i-j). Sex effects were
187 observed with higher ACC in female C57BL/6 gonadal WAT and UCP1 in male
188 interscapular BAT. The data from WAT and BAT support that D+Q treated female mice
189 had higher lipid synthesis activity, mediated by increased ACC, and lower thermogenic
190 activity, via reduced UCP1 and PPARγ. These changes could cause WAT
191 accumulation subsequently leading to the observed SASP increases after D+Q
192 treatment in female C57BL/6 mice.

193 Considering adipocytes are involved in glucose metabolism, we wanted to
194 determine treatment effects on peripheral glucose utilization. The results showed that
195 Fisetin treatment improved glucose clearance as measured by GTT (Figs. 2k, m) in
196 male mice. Although D+Q treated female mice accumulated more adipose tissues they
197 did not have impaired glucose metabolism (Figs. 2l-m). No differences in insulin
198 sensitivity between treated and littermate control mice were observed as determined
199 with ITT (Figs. 2n-p). Additionally, plasma insulin concentrations (Fig. 2q) were similar
200 across treatments in both sexes of C57BL/6 mice. Plasma adiponectin (an adipokine
201 that is involved in regulating glucose levels) concentration was significantly increased in
202 the D+Q and Fisetin treated males, but not after either treatment in female C57BL/6
203 mice (Fig. 2r). The concentration of plasma insulin was elevated while adiponectin was
204 decreased in male C57BL/6 mice compared to female littermates. This is consistent
205 with the literature where adiponectin levels in male mice (32) and humans (33) are
206 lower compared to females. The data indicated that monthly Fisetin treatment improved

207 peripheral glucose metabolism in the male mice with increased adiponectin levels and
208 enhanced glucose clearance. In female C57BL/6 mice, D+Q treatment increased WAT
209 accumulation without affecting glucose metabolism. This may be reflective of the GTT
210 assay being conducted in the middle rather than at the end of the experimental design.

211

212 **Fisetin treatment enhanced energy metabolism in male C57BL/6 mice, while D+Q**
213 **treatment reduced energy metabolism in female C57BL/6 mice.**

214 Energy metabolism is strongly associated with aging and is a contributing factor
215 to adipose accumulation (34, 35). In the present study, the treatment effects on energy
216 metabolism were assessed by indirect calorimetry. Oxygen consumption (VO_2) (Figs.
217 3a, c) and energy expenditure (EE) (Fig. 3d, f) were increased after Fisetin treatment in
218 male C57BL/6 mice, while both parameters were decreased by D+Q treatment in
219 female littermates (Figs. 3b-c, e-f). Thus, Fisetin enhanced energy metabolism in male
220 C57BL/6 mice, while D+Q reduced energy metabolism in the female mice, potentially
221 contributing to their WAT accumulation. Respiratory quotient (RQ) is the ratio of carbon
222 dioxide produced and oxygen consumed by the body, reflecting substrate utilization for
223 energy generation (36). In the current study, RQ for Fisetin treated male C57BL/6 mice
224 was reduced indicating a shift in metabolic substrate utilization from mixed lipids and
225 carbohydrates (RQ ~ 0.8) to predominantly lipids (RQ ~ 0.7) (36) (Fig. 3g, i). The RQ of
226 female C57BL/6 mice treated with D+Q did not change (Fig. 3f). Female C57BL/6 mice
227 had higher levels of all three measures compared male littermates. In summary,
228 monthly Fisetin treatment in male C57BL/6 mice increased energy metabolism as
229 indicated by enhanced VO_2 and EE and shifted substrate utilization to predominantly

230 lipids. In contrast, D+Q treatment decreased energy metabolism in female C57BL/6
231 mice as indicated by reduced VO_2 and EE without a concomitant change in substrate
232 utilization.

233

234 **Fisetin treatment improved spatial cognitive performance in male C57BL/6 mice,**
235 **while D+Q treatment impaired object recognition in female C57BL/6 mice.**

236 Although deletion of senescent cells by the senolytic compounds led to functional
237 improvements in models of neurodegenerative diseases (23, 24), it remains to be
238 elucidated whether the monthly senolytic treatment could affect cognitive performance
239 in normal aging mice. To investigate the effects of the treatment on cognitive functions,
240 the MWM spatial learning and memory paradigm and a NOR task were performed. The
241 results from MWM indicated that over 5 training sessions, the path efficiency was
242 increased (Fig. 4a, c), and the corrected integrated path length (CIPL) (Fig. 4d, f) was
243 reduced significantly in the Fisetin-treated male C57BL/6 mice indicative of improved
244 learning. Neither Fisetin nor D+Q treatment affected MWM learning in the female
245 C57BL/6 mice (Figs. 4b-c, e-f). Platform entries during the delayed probe challenge was
246 not affected by either treatment in both sexes (Fig. 4g) suggesting spatial memory recall
247 was unaffected. The results of NOR showed that the exploration preference for the
248 novel object measured by retention index was decreased in the D+Q treated female
249 mice (Fig. 4h), indicating reduced memory recall in this group. Since senotherapeutic
250 treatment had differential effects on energy expenditure between the sexes, we wanted
251 to verify changes to cognitive performance were not due to motor function. Mean
252 swimming speed was similar for all treatment groups and across sexes for the training

253 sessions and probe challenge (SI Figs. 3a-d). Additionally, no treatment differences in
254 grip strength were observed (SI Fig. 3e). Female C57BL/6 mice had better grip strength
255 compared to littermate males, which was consistent with other reports in C57BL/6 mice
256 (37).

257 Considering the effects of the treatments on cognitive performance, several
258 genes related to memory formation and synaptic plasticity (38) were examined by RT-
259 PCR in the hippocampus. Genes associated with synaptic vesicle release and
260 glutamatergic signaling were not affected by either treatment in male or female C57BL/6
261 mice (SI Fig. 4). Next, we examined glucose transporter 4 (Glut4) hippocampal mRNA
262 expression because central glucose metabolism is directly related to cognitive
263 performance. Glut4 was increased in the hippocampus of Fisetin treated male C57BL/6
264 mice while D+Q had no effect in either sex (Fig. 4i). Peripherally, D+Q and Fisetin
265 treatment increased Glut4 liver expression in male C57BL/6 mice only, but neither
266 treatment altered expression in gonadal WAT (Figs. 4j-k). Since plasma adiponectin
267 concentration was increased after senotherapeutic treatment in male C57BL/6 mice, we
268 also examined adiponectin receptor 1 (AdipoR1) mRNA expression in both the
269 hippocampus and peripheral tissues. Adiponectin signaling modulates glucose
270 metabolism while providing anti-inflammatory effects both of which improve cognitive
271 performance. Fisetin treated male C57BL/6 mice had increased hippocampal AdipoR1
272 expression, but D+Q treatment did not alter expression levels of either sex (Fig. 4l).
273 Peripherally, neither senotherapeutic treatment affected AdipoR1 expression in the liver
274 nor gonadal WAT of male C57BL/6 mice (Figs 4m-n). D+Q treatment reduced AdipoR1
275 expression in the liver while increasing expression in gonadal WAT of female C57BL/6

276 mice (Figs. 4m-n). This dichotomy is a result of tissue specific AdipoR1 regulation on
277 lipid storage and utilization (39). Decreased AdipoR1 signaling in the liver increases
278 hepatic lipogenesis causing triglyceride release and accumulation. Increased AdipoR1
279 expression in WAT reduces lipolysis. Together, this helps explain the increased body
280 and WAT weight seen in C57BL/6 females mice receiving D+Q treatment. Overall, the
281 effects of serotherapeutic treatment on Glut4 and AdipoR1 expression is consistent with
282 changes to peripheral glucose metabolism (Figs. 2) and energy expenditure (Figs. 3).

283 **Comparing the rate of biological aging between sexes of C57BL/6 mice.**

284 Finally, to better understand why senotherapeutic treatment, particularly Fisetin,
285 was only effective in males, we compared physical (SI Fig. 5a), metabolic (SI Fig. 5b-f),
286 and cognitive (SI Fig. 5g-i) in four and thirteen-month-old C57BL/6 mice to determine
287 potential differences in the rates of biological aging. Compared to female littermates,
288 four-month-old mice male C57BL/6 mice have worse metabolic parameters as observed
289 by GTT, oxygen consumption, and energy expenditure. Physical and cognitive
290 parameters are similar between sexes at this age. By 13 months of age, an aging effect
291 is observed whereby physical, metabolic, and cognitive parameters were reduced in
292 both sexes of C57BL/6 mice. Since swimming speed declines with age this can
293 influence the duration to the platform and the number of platform crosses on the MWM
294 (SI Figs. 5g, i, j). Although the CIPL calculation avoids variability in results due to
295 swimming speed differences, both sexes of 13-month-old C57BL/6 mice had worse
296 spatial navigation learning and memory recall compared to their younger sex-matched
297 littermates (SI Fig. 5h, k). These baseline differences between sexes of C57BL/6 mice
298 may partially explain why Fisetin was effective only in males.

299 **Discussions and conclusions**

300 Administration of senolytic compounds is a novel approach to removing
301 senescent cell accumulation and thereby reducing inflammation. These compounds are
302 being investigated as potential lifespan and healthspan extending therapies and may
303 also have applications in the treatment of numerous metabolic and neurodegenerative
304 disorders (18, 23-25). Since Fisetin and Q are naturally occurring plant flavonoids, they
305 are readily available as dietary supplements for use without a prescription. There is little
306 information on the efficacy of these compounds when taken at young ages and for
307 prolonged periods of time. The data presented here indicates that these compounds
308 have sexually dimorphic effects, each with health benefits and risks. The monthly
309 dosing strategy used in this study was based on previous publications showing that
310 markers of cell senescence are still reduced after a four week off-treatment period (7).
311 Prior research suggests that senolytic compounds work either by clearing senescent
312 cells or reversing cellular senescence rather than preventing cells from entering a
313 senescent state. Since senescent cell accumulation occurs over weeks, maintaining an
314 effective concentration dosage is not required. Rather, intermittent dosing may be
315 preferable thereby avoiding adverse effects.

316 Monthly Fisetin treatment was efficacious in male C57BL/6 mice when started at
317 four months of age. This dosing strategy had little to no effect in female littermates
318 when compared to vehicle treatment. Nine months of Fisetin treatment efficiently
319 reduced SASP in WAT (Fig. 1), hippocampus (SI Fig. 1), and plasma (SI Fig. 2), of
320 thirteen-month-old male C57BL/6 mice as lower mRNA expression of $p16^{Ink4\alpha}$, $p21^{Cip1}$,
321 TNFα, and IL-6 were observed. Male C57BL/6 mice receiving Fisetin treatment also had

322 improved glucose metabolism as supported by better glucose clearance (Figs. 2k, m)
323 after five treatments and elevated plasma adiponectin (Fig. 2r) collected upon
324 euthanization at thirteen months of age. Improved energy metabolism was also
325 supported by increased VO_2 and EE (Figs. 3a, c-d, f) along with reduced RQ (Fig. 3g, i)
326 when measured at 12 months of age. Monthly Fisetin treatment also reduced SASP
327 markers to similar levels as four-month-old treatment naïve male C57BL/6 mice. The
328 metabolic improvements also mirrored levels observed in younger littermates. But
329 physical parameters (swimming speed and grip strength) as well as learning and
330 memory recall was still worse after Fisetin treatment when compared with younger male
331 C57BL/6 mice. This suggests administering Fisetin treatment at younger ages has a
332 stronger anti-inflammatory (potentially mediated through increased plasma adiponectin
333 signaling) rather than an overall anti-aging effect. This cannot be definitively concluded
334 without frailty or lifespan measurements which were outside the scope of the study.
335 Also, inflammation and aging are not mutually exclusive whereby chronic inflammation
336 contributes to worse health and the onset numerous age-related ailments and
337 neurodegenerative disorders. Approaches to reduce chronic inflammation have the
338 potential to extend quality of life during aging.

339 In the present study, D+Q treatment showed minimal efficacy in male C57BL/6
340 mice and was detrimental to female mice. In females, SASP markers (Fig. 1) and body
341 weight were increased attributed to larger WAT depots (Fig. 2a-e). Several factors
342 contributed to this increased abdominal adiposity. These include increased ACC (the
343 rate-limiting enzyme in lipid synthesis) and AdipoR1 in WAT (Fig. 2h and Fig. 4n),
344 reduced UCP1 and PPARγ activity in BAT (Figs. 2i-j), reduced energy metabolism and

345 liver AdipoR1 expression (Fig. 3 and Fig. 4m). Because obesity can lead to adipose
346 tissue entering a senescent-like state at a young age (40), the accumulation of
347 abdominal WAT in D+Q treated female mice could result in increased SASP. This
348 possibility is supported by expression of SASP-related genes in D+Q treated females
349 resembling or even exceeding the values measured in much older (twenty-one-month-
350 old) untreated females (Fig. 1, SI Fig. 1, and SI Fig. 2). The reasons for the detrimental
351 outcome of D+Q treatment in the female mice remain to be fully elucidated, but are not
352 unique to this study. A recent publication using a similar D+Q dosing regimen reported
353 similar observations in a C57BL/6 mouse model of hepatocellular carcinoma (41).
354 Others have proposed that continuous senescent cell removal beginning at young ages
355 does not always activate cell-replacement mechanisms, but rather induces regenerative
356 responses causing fibrosis (42). A better understanding of cell-type specific effects of
357 senescent cell removal is warranted.

358 Sexually dimorphic biological aging may partially explain the senotherapeutic
359 differences observed in C57BL/6 mice. Four-month-old male and female C57BL/6
360 treatment naïve mice have similar SASP mRNA and plasma concentrations (Fig. 1 and
361 SI Fig. 2), but males have worse metabolism (SI Figs. 5b, d-e). SASP became more
362 prominent in male C57BL/6 mice compared with age-matched thirteen-month females,
363 particularly proinflammatory cytokines TNFα and IL-6 (Fig. 1d, f and SI Fig. 2a, c).
364 Since Fisetin shows therapeutic efficacy by reducing inflammation leading to metabolic
365 improvement, these SASP factors were not at harmful levels in female C57BL/6 mice.
366 This suggests male C57BL/6 mice age biologically faster and may explain why Fisetin
367 treatment was ineffective at the ages tested in female littermates. Unfortunately, data is

368 only beginning to emerge regarding sexually dimorphic aging characteristics due in
369 large part from male centric sampling in both preclinical and clinical research. Sparse
370 evidence suggests females maintain better cellular health throughout the majority of
371 their life, but become frailer when approaching death compared with males (43).

372 In C57BL/6 mice, cell senescence usually becomes prominent by approximately
373 14 months of age (7), and treatment with senolytic drugs at this or a later ages cleared
374 senescent cells and led to various beneficial effects (20, 21). However, it was unclear
375 whether chronic treatment with senolytic drugs started at an earlier age (prior to
376 reported senescent cell accumulation) in C57BL/6 mice would have similar effects as
377 the reported observations during later age (20, 21). This gap in knowledge is particularly
378 relevant given that Fisetin and Q are available without a prescription and may be taken
379 by younger adults. Our data revealed that such treatment indeed had impacts on the
380 mice as they reached median lifespan. However, the senotherapeutic treatment effects
381 were sexually dimorphic. C57BL/6 male, not female, mice receiving Fisetin treatment
382 had beneficial responses mediated through reduced SASP and improved glucose and
383 energy metabolism. Female, not male, mice receiving a D+Q cocktail treatment had
384 detrimental responses with reduced energy metabolism, increased WAT accumulation,
385 and elevated SASP.

386 D+Q was the first well-characterized senotherapeutic agent proven to reduce
387 senescent cell burden and extend lifespan. Despite Q and Fisetin being structural
388 analogs, Q had minimal to no effects when initiated at a young age in male C57BL/6
389 mice. Although both are potent redox-active flavonoids, Fisetin has higher prooxidant
390 activity. Furthermore, Q's senolytic potency is augmented by trace elements such as

391 copper and iron that are known to accumulate during aging and in senescent cells (5).
392 Since senolytic drugs were administered at a young age prior to reported senescent cell
393 accumulation, iron and copper accumulation was not a threshold to enhance Q's
394 potency. Fisetin's prooxidant activities are not amplified in the presence of copper or
395 iron, which may explain why health benefits were only observed with this
396 senotherapeutic in male C57BL/6 mice.

397 Moreover, Fisetin-treated male C57BL/6 mice not only had improved markers of
398 peripheral health, but also their spatial learning during the MWM training sessions (Figs.
399 4a, c, d, f) compared to vehicle treated C57BL/6 mice. These cognitive improvements,
400 however, did not reach levels observed in 4-month-old treatment naïve mice (Fig. 4g
401 and SI Fig. 5i). Although we did not observe changes in markers of synaptic plasticity
402 associated with hippocampal memory formation (SI Fig. 4), we did observe elevated
403 expression of Glut4 (Fig. 4i-j), hippocampal AdipoR1 (Fig. 4l), and plasma adiponectin
404 concentrations (Fig. 2r) suggesting enhanced glucose transport and adiponectin
405 signaling in the brain. This novel finding that Fisetin treatment concurrently improved
406 peripheral as well as central glucose and adiponectin signaling has important
407 implications for metabolic and cognitive function. In the periphery, adiponectin increases
408 insulin sensitivity, stimulates fatty acid oxidation and glucose uptake, suppresses
409 hepatic glucose production (44), and inhibits inflammation (45). In the brain, adiponectin
410 signaling via AdipoR1 has anti-inflammatory effects and is involved with cognitive and
411 neuroprotective mechanisms (46). Monthly Fisetin treatment in male mice led to
412 increased adiponectin levels in circulation, which likely contributed to improvements of
413 peripheral glucose and lipid metabolism. Circulating adiponectin passes through the

414 blood brain barrier where it can enhance cognitive function by upregulating glucose
415 transport and AdipoR1 signaling (46). However, neither hippocampal Glut4 (Fig. 4i),
416 AdipoR1 (Fig. 4l), nor the examined synaptic plasticity markers (SI Fig. 4) were affected
417 by D+Q treatment in the female mice even though object recognition memory retention
418 was reduced (Fig. 4h). The reduced cognitive performance of these animals might be
419 attributed to the increased adiposity after D+Q treatment, but future studies are needed
420 to identify the mechanisms involved.

421 Cell senescence is strongly correlated with aging, but currently no diagnostic
422 testing is available to quantify senescent cells accumulation. It seems possible that the
423 earlier in life senotherapeutics are taken, the better likelihood of delaying age-related
424 decline and disease risk might be expected. Senolytics such as Fisetin and D+Q are
425 known to reduce senescent cell burden (7), but the optimal age to begin treatment is
426 currently unknown. Our study indicates that both biological aging and sex may also
427 determine the therapeutic outcomes of senolytic treatment. When the treatment was
428 started at 4 months of age, before the reported senescent cell accumulation, Fisetin had
429 beneficial effects in male C57BL/6 mice while a D+Q cocktail had adverse
430 consequences in female C57BL/6 mice.

431 These observations provide novel information with translational relevance. First,
432 senolytic drugs taken at an age before significant senescent cell burden to reduce or
433 prevent their prevalence later in life may be detrimental to overall health. Second, males
434 and females can have differential responses to the same senolytic treatment when
435 initiated at younger ages. Third, a particular senolytic treatment may have beneficial,
436 negligible or detrimental effects depending on the age, sex, or disease state. Fourth,

437 oral senolytic treatment not only impacted peripheral physiological responses, but also
438 affected cognitive performance, possibly via increased central adiponectin signaling and
439 glucose transport. Overall, these observations should serve as a note of caution in this
440 rapidly evolving and expanding field of investigation.

441 Designing senotherapeutics with the role of sex and age of onset could
442 conceivably provide a useful preventative treatment strategy for dealing with
443 development of neurodegenerative disorders. Supporting this possibility, development
444 of metabolic syndrome in midlife was reported to increases the risk for Alzheimer's
445 disease (AD) later on (47). Besides clearance of senescent cells, senotherapeutics may
446 ameliorate or delay cognitive decline through mechanisms associated with improved
447 peripheral and central glucose metabolism. Considering metabolic dysregulation has
448 been observed in the APP/PS1 model of AD (48), Additional studies are warranted to
449 determine if prodromal senolytic treatment could provide cognitive benefits in
450 neurodegenerative diseases.

451 **Material and methods**

452 **Chemicals**

453 Unless otherwise noted, all chemicals were obtained from Sigma-Aldrich (St. Louis,
454 MO) including Quercetin (Cat# RHR1488). Fisetin was purchased from Selleckchem
455 (Houston, TX; Cat #S2298), and Dasatinib from LC laboratories (Woburn, MA; Cat# D-
456 3307).

457 **Animals**

458 Male and female C57BL/6 mice were maintained in our established breeding colonies.
459 Protocols for animal use were approved by the Institutional Animal Care and Use
460 Committee at Southern Illinois University School of Medicine. Mice were group housed
461 on a 12:12 h light-dark cycle, and food (Chow 5001 with 23.4% protein, 5% fat, and
462 5.8% crude fiber; LabDiet PMI Feeds) and water were available ad libitum. Sentinel
463 mice located in the same rooms as our breeder and experimental mice were tested
464 triannually for pyrogens and pathogens and none were detected. All assays, including
465 tissue collection, were conducted one week after monthly senotherapeutic dosing. As
466 shown in Fig 1a, glucose metabolism was assessed via ITT and GTT after six
467 treatments at the animal age of nine months. Grip Strength was measured after seven
468 treatments while NOR and MWM was performed after eight treatments. Energy
469 metabolism was measured by indirect calorimetry at the animal age of twelve. A week
470 following the final senotherapeutic treatment at 13 months, body weight was recorded
471 then euthanized with an overdose of isoflurane. A cardiac puncture was used to collect
472 blood for assessment of circulating SASP markers and proteins. Mice were rapidly

473 decapitated and the peripheral tissues were weighed to calculate their contribution to
474 total body weight. Tissue was immediately flash frozen and stored at -80˚C until
475 processing as previously described (49).

476 **Senolytic drug treatment**

477 Senotherapeutic concentrations and dosing strategy were based on previous
478 publications (20, 50-52). C57BL/6 mice were dosed with 100 mg/kg of Fisetin while a
479 cocktail of 5 mg/kg of D + 50 mg/kg of Q, or vehicle (2% DMSO in canola oil) by oral
480 administration (53). The treatments were given once per month from 4-13 months of
481 age.

482

483 **Glucose tolerance test (GTT) and insulin tolerance test (ITT)**

484 GTT or ITT was carried out as described previously (49). Sixteen-hour-fasted mice
485 underwent GTT by intraperitoneal (i.p.) injection with 1 g glucose per kg of body weight
486 (BW). Blood glucose levels were measured at 0, 15, 30, 45, 60, and 120 min with a
487 PRESTO glucometer (AgaMatrix). For ITT, nonfasted mice were injected i.p. with 1 IU
488 porcine insulin from sigma (St. Louis, MO) (Cat# I5523) per kg of BW. Blood glucose
489 levels were measured at 0, 15, 30, 45, 60, and 120 min. The data for GTT are presented
490 as absolute value, and for ITT are presented as a percentage of baseline glucose.

491

492 **Indirect calorimetry**

493 Indirect calorimetry was performed as previously described (49) using AccuScan
494 Metabolic System (AccuScan Instruments). In this system, mice are housed individually
495 in metabolic chambers with ad libitum access to food and water. After a twenty-four-hour
496 acclimation period, VO_2, VCO_2, EE and RQ measurements were collected every ten min
497 per animal and averaged for each hour.

498

499 **Morris water maze (MWM) training and probe challenge**

500 The MWM was used to assess spatial learning and memory recall, and performed as
501 previously described (54). Mice were trained to utilize visual cues placed around the room
502 to repeatedly swim to a static, hidden escape platform (submerged one cm below the
503 opaque water surface), regardless of starting quadrant. The MWM paradigm consisted of
504 5 consecutive training days with three, 90 s trials/day and a minimum inter-trial-interval of
505 20 min. Starting quadrant was randomized for each trial. After two days without testing,
506 the escape platform was removed and all mice entered the pool of water from the same
507 starting position for a single 60 s probe challenge to test long-term memory recall. The
508 ANY-maze video tracking system (Stoelting Co., Wood Dale, IL) was used to record
509 mouse navigation during the training and probe challenge. The three trials for each
510 training day were averaged for each mouse.

511

512 **Novel Object Recognition (NOR)**

513 The NOR was used to evaluate memory retention based on the premise that mice
514 spend more time exploring a novel object rather than a familiar object if memory

515 capabilities remain intact. Mice were habituated in the open field chamber for 30 min on
516 the first day. Twenty-four hours after, the mouse was returned to the chamber and
517 presented with two similar objects for 5 min. A 24-hour inter-session-interval was used
518 between introduction and retention phases to assess long-term memory retrieval.
519 During the retention phase one of the familiar objects is replaced with a novel object
520 and the mouse is given 5 minutes of exploration. The ANY-maze video tracking system
521 (Stoelting Co., Wood Dale, IL) was used to record mouse navigation during the
522 familiarization and retention phases and time spent at each object. The time spent
523 exploring the novel object was divided by the total time spent exploring both objects to
524 calculate the retention index.

525

526 **Grip Strength**

527 Using their forepaws, mice grasp a wire grid connected to an isometric force transducer
528 (Harvard Apparatus; Holliston, MA) and are gently pulled horizontally away by the tail.
529 Force is measured in grams and normalized according to body weight. Three trials with
530 a minimum inter-trial-interval of twenty minutes are averaged per mouse.

531

532 **Assessment of blood chemistry**

533 Plasma was collected from animals anesthetized with isoflurane by cardiac puncture at
534 sacrifice. The blood was mixed with EDTA, followed by centrifugation at 10,000 g for 15
535 min at 4°C for plasma collection. Per the manufacturer's protocol, insulin or adiponectin
536 was measured with respective ELISA kits (Crystal Chem, Elk Grove Village, IL; Cat#

537 90080 and 80569). Plasma cytokine levels were assayed using a multiplex immunoassay
538 on a MESO QuickPlex SQ 120 with accompanying software (Meso Scale Diagnostics,
539 LLC).

540

541 **RT–PCR**

542 mRNA expression was analyzed by quantitative RT–PCR as previously described (49)
543 using cDNA synthesis kits and SYBR green mix from Bio-Rad (Cat# 1708897 and
544 1725121) performed with the StepOne Real-Time PCR System (Thermo Fisher
545 Scientific). RNA was extracted using an RNeasy mini kit or RNeasy Lipid Tissue Mini Kit
546 (Qiagen) following the manufacturer's instructions. Relative expression was calculated as
547 previously described and primers were purchased from Integrated DNA Technologies
548 (Supplemental Information Table 1).

549

550 **Statistical analysis**

551 Statistical analyses were conducted using a two-way ANOVA to test for significance of
552 sex (males × females), treatment (Vehicle × Fisetin × D+Q), and an interaction between
553 sex and treatment. Difference between two groups were assessed with unpaired two-
554 tailed Student's t tests with significance defined as $p \leq 0.05$. The area under the curve
555 was calculated using the two-way ANOVA. Data are presented as means ± SEM. All
556 statistical analyses and graphs were completed using Prism 9 (GraphPad Inc, La Jolla,
557 CA, USA).

Acknowledgements

This work was supported by the National Institutes of Health NIA R01-AG057767 and NIA R01-AG061937, Dale and Deborah Smith Center for Alzheimer's Research and Treatment, Kenneth Stark Endowment (YF, SF, KQ, MRP, KNH, ERH), NIA R21-AG062985, and American Diabetes Association 1-19-IBS-126 (DM, RS, AB). We would like to thank Melissa Roberts for performing and analyzing the plasma cytokine multiplex assay and Lisa Hensley for editorial support.

Author Contributions:

YF, DM, RS, SM, KQ, and MRP conducted the experiments and analyzed the data. YF, AB, KNH, and ERH conceived the study, designed the experiments, interpreted the data, and wrote the manuscript. All authors approved the final version of the manuscript.

Data Availability

All data is available upon reasonable request.

Data Citation

All data was generated by the authors. No external sources of data were used to create any figures presented in this manuscript.

Conflict of Interest

The authors declare no conflicts of interest.

581 ## Reference

582 1. Grynkiewicz G & Demchuk OM (2019) New Perspectives for Fisetin. *Front Chem* 7:697.
583 2. Jafarinia M*, et al.* (2020) Quercetin with the potential effect on allergic diseases. *Allergy Asthma*
584 *Clin Immunol* 16:36.
585 3. Mlcek J, Jurikova T, Skrovankova S, & Sochor J (2016) Quercetin and Its Anti-Allergic Immune
586 Response. *Molecules* 21(5).
587 4. Aguilera DG & Tsimberidou AM (2009) Dasatinib in chronic myeloid leukemia: a review. *Ther Clin*
588 *Risk Manag* 5(2):281-289.
589 5. Wang Y, He Y, Rayman MP, & Zhang J (2021) Prospective Selective Mechanism of Emerging
590 Senolytic Agents Derived from Flavonoids. *J Agric Food Chem* 69(42):12418-12423.
591 6. Zhu Y*, et al.* (2015) The Achilles' heel of senescent cells: from transcriptome to senolytic drugs.
592 *Aging Cell* 14(4):644-658.
593 7. Kirkland JL & Tchkonia T (2020) Senolytic drugs: from discovery to translation. *J Intern Med*
594 288(5):518-536.
595 8. Campisi J (2005) Senescent cells, tumor suppression, and organismal aging: good citizens, bad
596 neighbors. *Cell* 120(4):513-522.
597 9. Kuilman T & Peeper DS (2009) Senescence-messaging secretome: SMS-ing cellular stress. *Nat Rev*
598 *Cancer* 9(2):81-94.
599 10. Coppe JP, Desprez PY, Krtolica A, & Campisi J (2010) The senescence-associated secretory
600 phenotype: the dark side of tumor suppression. *Annu Rev Pathol* 5:99-118.
601 11. Lasry A & Ben-Neriah Y (2015) Senescence-associated inflammatory responses: aging and cancer
602 perspectives. *Trends Immunol* 36(4):217-228.
603 12. Freund A, Orjalo AV, Desprez PY, & Campisi J (2010) Inflammatory networks during cellular
604 senescence: causes and consequences. *Trends Mol Med* 16(5):238-246.
605 13. McHugh D & Gil J (2018) Senescence and aging: Causes, consequences, and therapeutic avenues.
606 *J Cell Biol* 217(1):65-77.
607 14. Chen JH, Hales CN, & Ozanne SE (2007) DNA damage, cellular senescence and organismal ageing:
608 causal or correlative? *Nucleic Acids Res* 35(22):7417-7428.
609 15. Chapman J, Fielder E, & Passos JF (2019) Mitochondrial dysfunction and cell senescence:
610 deciphering a complex relationship. *FEBS Lett* 593(13):1566-1579.
611 16. Prata L, Ovsyannikova IG, Tchkonia T, & Kirkland JL (2018) Senescent cell clearance by the immune
612 system: Emerging therapeutic opportunities. *Semin Immunol* 40:101275.
613 17. Passos JF, Miwa S, & von Zglinicki T (2013) Measuring reactive oxygen species in senescent cells.
614 *Methods Mol Biol* 965:253-263.
615 18. Musi N*, et al.* (2018) Tau protein aggregation is associated with cellular senescence in the brain.
616 *Aging Cell* 17(6):e12840.
617 19. Ogrodnik M*, et al.* (2019) Obesity-Induced Cellular Senescence Drives Anxiety and Impairs
618 Neurogenesis. *Cell Metab* 29(5):1233.
619 20. Yousefzadeh MJ*, et al.* (2018) Fisetin is a senotherapeutic that extends health and lifespan.
620 *EBioMedicine* 36:18-28.
621 21. Xu M*, et al.* (2018) Senolytics improve physical function and increase lifespan in old age. *Nat Med*
622 24(8):1246-1256.
623 22. Baker DJ*, et al.* (2011) Clearance of p16Ink4a-positive senescent cells delays ageing-associated
624 disorders. *Nature* 479(7372):232-236.
625 23. Bussian TJ*, et al.* (2018) Clearance of senescent glial cells prevents tau-dependent pathology and
626 cognitive decline. *Nature* 562(7728):578-582.

627 24. Zhang P, *et al.* (2019) Senolytic therapy alleviates Abeta-associated oligodendrocyte progenitor
628 cell senescence and cognitive deficits in an Alzheimer's disease model. *Nat Neurosci* 22(5):719-
629 728.
630 25. Khatoon S, Agarwal NB, Samim M, & Alam O (2021) Neuroprotective Effect of Fisetin Through
631 Suppression of IL-1R/TLR Axis and Apoptosis in Pentylenetetrazole-Induced Kindling in Mice. *Front*
632 *Neurol* 12:689069.
633 26. Huang YH, *et al.* (2020) Interleukin-10 induces senescence of activated hepatic stellate cells via
634 STAT3-p53 pathway to attenuate liver fibrosis. *Cell Signal* 66:109445.
635 27. Maciel-Baron LA, *et al.* (2016) Senescence associated secretory phenotype profile from primary
636 lung mice fibroblasts depends on the senescence induction stimuli. *Age (Dordr)* 38(1):26.
637 28. Ou MY, Zhang H, Tan PC, Zhou SB, & Li QF (2022) Adipose tissue aging: mechanisms and
638 therapeutic implications. *Cell Death Dis* 13(4):300.
639 29. Hudgins AD, *et al.* (2018) Age- and Tissue-Specific Expression of Senescence Biomarkers in Mice.
640 *Front Genet* 9:59.
641 30. Cannon B & Nedergaard J (2004) Brown adipose tissue: function and physiological significance.
642 *Physiol Rev* 84(1):277-359.
643 31. Kalinovich AV, de Jong JM, Cannon B, & Nedergaard J (2017) UCP1 in adipose tissues: two steps
644 to full browning. *Biochimie* 134:127-137.
645 32. Gui Y, Silha JV, & Murphy LJ (2004) Sexual dimorphism and regulation of resistin, adiponectin, and
646 leptin expression in the mouse. *Obes Res* 12(9):1481-1491.
647 33. Isobe T, *et al.* (2005) Influence of gender, age and renal function on plasma adiponectin level: the
648 Tanno and Sobetsu study. *Eur J Endocrinol* 153(1):91-98.
649 34. Azzu V & Valencak TG (2017) Energy Metabolism and Ageing in the Mouse: A Mini-Review.
650 *Gerontology* 63(4):327-336.
651 35. Westbrook R, Bonkowski MS, Strader AD, & Bartke A (2009) Alterations in oxygen consumption,
652 respiratory quotient, and heat production in long-lived GHRKO and Ames dwarf mice, and short-
653 lived bGH transgenic mice. *J Gerontol A Biol Sci Med Sci* 64(4):443-451.
654 36. Prentice RL, *et al.* (2013) An exploratory study of respiratory quotient calibration and association
655 with postmenopausal breast cancer. *Cancer Epidemiol Biomarkers Prev* 22(12):2374-2383.
656 37. Baumann CW, Kwak D, & Thompson LV (2019) Sex-specific components of frailty in C57BL/6 mice.
657 *Aging (Albany NY)* 11(14):5206-5214.
658 38. Reshetnikov VV, *et al.* (2020) Genes associated with cognitive performance in the Morris water
659 maze: an RNA-seq study. *Sci Rep* 10(1):22078.
660 39. Lee B & Shao J (2014) Adiponectin and energy homeostasis. *Rev Endocr Metab Disord* 15(2):149-
661 156.
662 40. Tchkonia T, *et al.* (2010) Fat tissue, aging, and cellular senescence. *Aging Cell* 9(5):667-684.
663 41. Raffaele M, *et al.* (2021) Mild exacerbation of obesity- and age-dependent liver disease
664 progression by senolytic cocktail dasatinib + quercetin. *Cell Commun Signal* 19(1):44.
665 42. Grosse L, *et al.* (2020) Defined p16(High) Senescent Cell Types Are Indispensable for Mouse
666 Healthspan. *Cell Metab* 32(1):87-99 e86.
667 43. Hagg S & Jylhava J (2021) Sex differences in biological aging with a focus on human studies. *Elife*
668 10.
669 44. Roy B & Palaniyandi SS (2021) Tissue-specific role and associated downstream signaling pathways
670 of adiponectin. *Cell Biosci* 11(1):77.
671 45. Bloemer J, *et al.* (2018) Role of Adiponectin in Central Nervous System Disorders. *Neural Plast*
672 2018:4593530.
673 46. Rastegar S, *et al.* (2019) Expression of adiponectin receptors in the brain of adult zebrafish and
674 mouse: Links with neurogenic niches and brain repair. *J Comp Neurol* 527(14):2317-2333.

675 47. Baumgart M*, et al.* (2015) Summary of the evidence on modifiable risk factors for cognitive decline
676 and dementia: A population-based perspective. *Alzheimers Dement* 11(6):718-726.
677 48. Hascup ER*, et al.* (2019) Diet-induced insulin resistance elevates hippocampal glutamate as well
678 as VGLUT1 and GFAP expression in AbetaPP/PS1 mice. *J Neurochem* 148(2):219-237.
679 49. Fang Y*, et al.* (2020) Lifespan of long-lived growth hormone receptor knockout mice was not
680 normalized by housing at 30 degrees C since weaning. *Aging Cell* 19(5):e13123.
681 50. Palmer AK*, et al.* (2015) Cellular Senescence in Type 2 Diabetes: A Therapeutic Opportunity.
682 *Diabetes* 64(7):2289-2298.
683 51. Saccon TD*, et al.* (2021) Senolytic Combination of Dasatinib and Quercetin Alleviates Intestinal
684 Senescence and Inflammation and Modulates the Gut Microbiome in Aged Mice. *J Gerontol A Biol*
685 *Sci Med Sci* 76(11):1895-1905.
686 52. Roos CM*, et al.* (2016) Chronic senolytic treatment alleviates established vasomotor dysfunction
687 in aged or atherosclerotic mice. *Aging Cell* 15(5):973-977.
688 53. Dalterio S & Bartke A (1979) Perinatal exposure to cannabinoids alters male reproductive function
689 in mice. *Science* 205(4413):1420-1422.
690 54. Hascup KN, Findley CA, Sime LN, & Hascup ER (2020) Hippocampal alterations in glutamatergic
691 signaling during amyloid progression in AbetaPP/PS1 mice. *Sci Rep* 10(1):14503.

692

693 **Figure legends**

694 **Figure 1: Senotherapeutic treatment altered the SASP profile in C57BL/6 mice.** (a)
695 Experimental paradigm. (b-g) Gene expressions in gonadal fat depot. Data are
696 represented as means ± *SEM* (*n* = 8–12). A two-way ANOVA was used to determine P-
697 values for the categorial variables (S = Sex and T = Treatment) and their interaction (S
698 x T), which are shown for each graph. **p* ≤ 0.05, ***p* ≤ 0.01, ***p ≤ 0.001 based on a
699 two-tailed Student's *t* test.

700 **Figure 2: D+Q treatment increased abdominal WAT in female mice while Fisetin**
701 **treatment improved glucose metabolism and increased plasma adiponectin in**
702 **male mice.** Mouse body weight (a-b, BW) along with percentage of total (c) and
703 different adipose tissue depots present in relation to BW (d-g). Gene expression of ACC
704 in gonadal WAT (h) as well as UCP1 and PPARγ in interscapular BAT (i-j) depots.
705 Glucose tolerance test (GTT), insulin tolerance test (ITT), and their respective area
706 under the curve (AUC) after five treatments (k-p). Plasma insulin and adiponectin
707 concentrations (q-r) taken at time of euthanization after 10 treatments. Data are means
708 ± SEM (n = 8–20). A two-way ANOVA was used to determine P-values for the
709 categorial variables (S = Sex and T = Treatment) and their interaction (S x T), which are
710 shown for each bar graph. *p ≤ 0.05, **p ≤ 0.01 based on a two-tailed Student's t test.

711 **Figure 3: Fisetin treatment enhanced energy metabolism in male mice, while D+Q**
712 **treatment decreased energy metabolism in female mice.** Twenty-four-hour male and
713 female oxygen consumption (a-c; VO_2), energy expenditure (d-f; EE), and respiratory
714 quotient (g-i; RQ) along with their corresponding AUC after 9 treatments. Data are
715 presented as means ± SEM (n = 16-20). A two-way ANOVA was used to determine P-

716 values for the categorial variables (S = Sex and T = Treatment) and their interaction (S
717 x T), which are shown for each bar graph. **p ≤ 0.01 based on a two-tailed Student's t
718 test.

719 **Figure 4: Fisetin treatment improved spatial learning in male mice, while D+Q**
720 **treatment impaired object recognition memory recall in female mice.** Assessment
721 of cognition and memory recall was determined by MWM and NOR. MWM path
722 efficiency (a-c) and corrected integrated path length (d-f; CIPL) in male and female
723 C57BL/6 mice during 5 training sessions. Number of platform crosses during the
724 delayed probe challenge (g). Retention index of the familiar object after a delayed
725 novelty introduction (h). Hippocampal, liver, and gonadal WAT mRNA expression levels
726 of Glut4 (i-k) and AdipoR1 (l-n) were measured at time of euthanization after 10
727 treatments. Data are presented as means ± SEM (n = 16-20). A two-way ANOVA was
728 used to determine P-values for the categorial variables (S = Sex and T = Treatment)
729 and their interaction (S x T), which are shown for each bar graph. *p ≤ 0.05, **p ≤ 0.01
730 based on a two-tailed Student's t test.

731 **Supplemental Information: Figure 1: Effects of senotherapeutic treatment on the**
732 **hippocampal SASP profile in C57BL/6 mice.** mRNA expression levels of
733 hippocampal SASP markers (a-f) were measured at time of euthanization after 10
734 treatments. Data are presented as means ± SEM (n = 8-12). A two-way ANOVA was
735 used to determine P-values for the categorial variables (S = Sex and T = Treatment)
736 and their interaction (S x T), which are shown for each bar graph. *p ≤ 0.05, **p ≤
737 0.01, ***p<0.001 based on a two-tailed Student's t test.

738 **Supplemental Information Figure 2: Senotherapeutic treatment altered plasma**
739 **cytokine concentrations in C57BL/6 mice.** Plasma concentrations of TNFα, MCP1,
740 IL-6, and IL-10 (a-d) from time of euthanization after 10 senotherapeutic treatments.
741 Data are represented as means ± SEM (n = 8). A two-way ANOVA was used to
742 determine P-values for the categorial variables (S = Sex and T = Treatment) and their
743 interaction (S x T), which are shown for each bar graph. *p ≤ 0.05, **p ≤ 0.01,
744 ***p<0.001 based on a two-tailed Student's t test.

745 **Supplemental Information Figure 3: Effects of senotherapeutic treatment on**
746 **physical performance in C57BL/6 mice.** MWM swimming speed during the training
747 days and probe challenge (a-d) and forepaw grip strength (e). Data are presented as
748 means ± SEM (n = 16-20). A two-way ANOVA was used to determine P-values for the
749 categorial variables (S = Sex and T = Treatment) and their interaction (S x T), which are
750 shown for each bar graph.

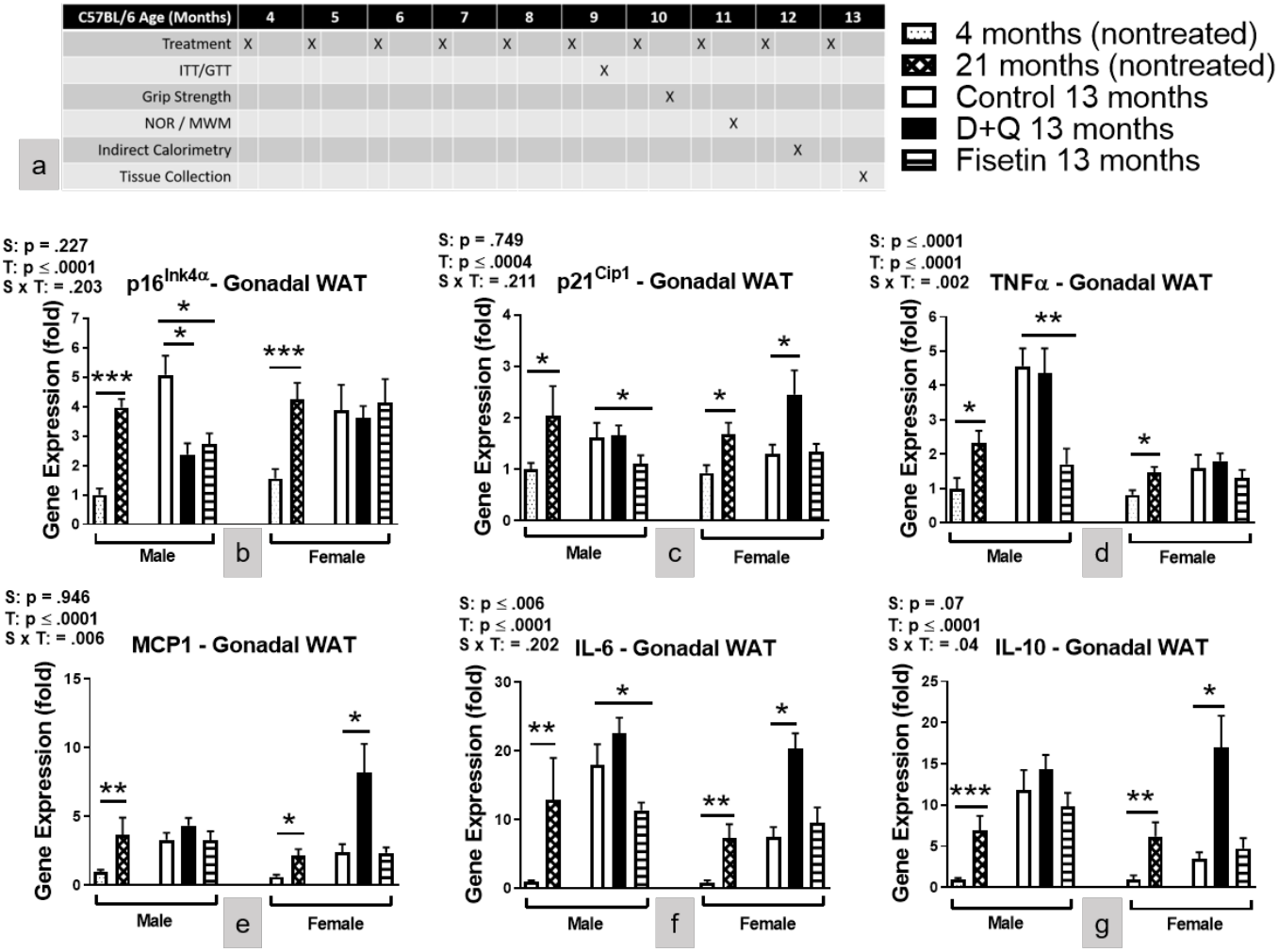
751 **Supplemental Information Figure 4: Hippocampal mRNA expression of genes**
752 **involved with synaptic plasticity were unaltered after senotherapeutic treatment.**
753 Hippocampal mRNA expression from time of euthanization after 10 senotherapeutic
754 treatments in C57BL/6 mice. Data are represented as means ± SEM (n = 16-20). A
755 two-way ANOVA was used to determine P-values for the categorial variables (S = Sex
756 and T = Treatment) and their interaction (S x T), which are shown for each bar graph.
757 **p ≤ 0.01 based on a two-tailed Student's t test.

758 **Supplemental Information Figure 5: Effects of aging on physical, metabolic, and**
759 **cognitive parameters.** Nontreated four-month-old C57BL/6 mice were compared to
760 sex-matched control treated mice. Data are represented as means ± SEM (n = 16-20).

761 A two-way ANOVA was used to determine P-values for the categorial variables (S =
762 Sex and A = Age) and their interaction (S x A), which are shown for each bar graph.
763 *$p \leq 0.05$, **$p \leq 0.01$, ***$p < 0.001$ based on a two-tailed Student's t test.

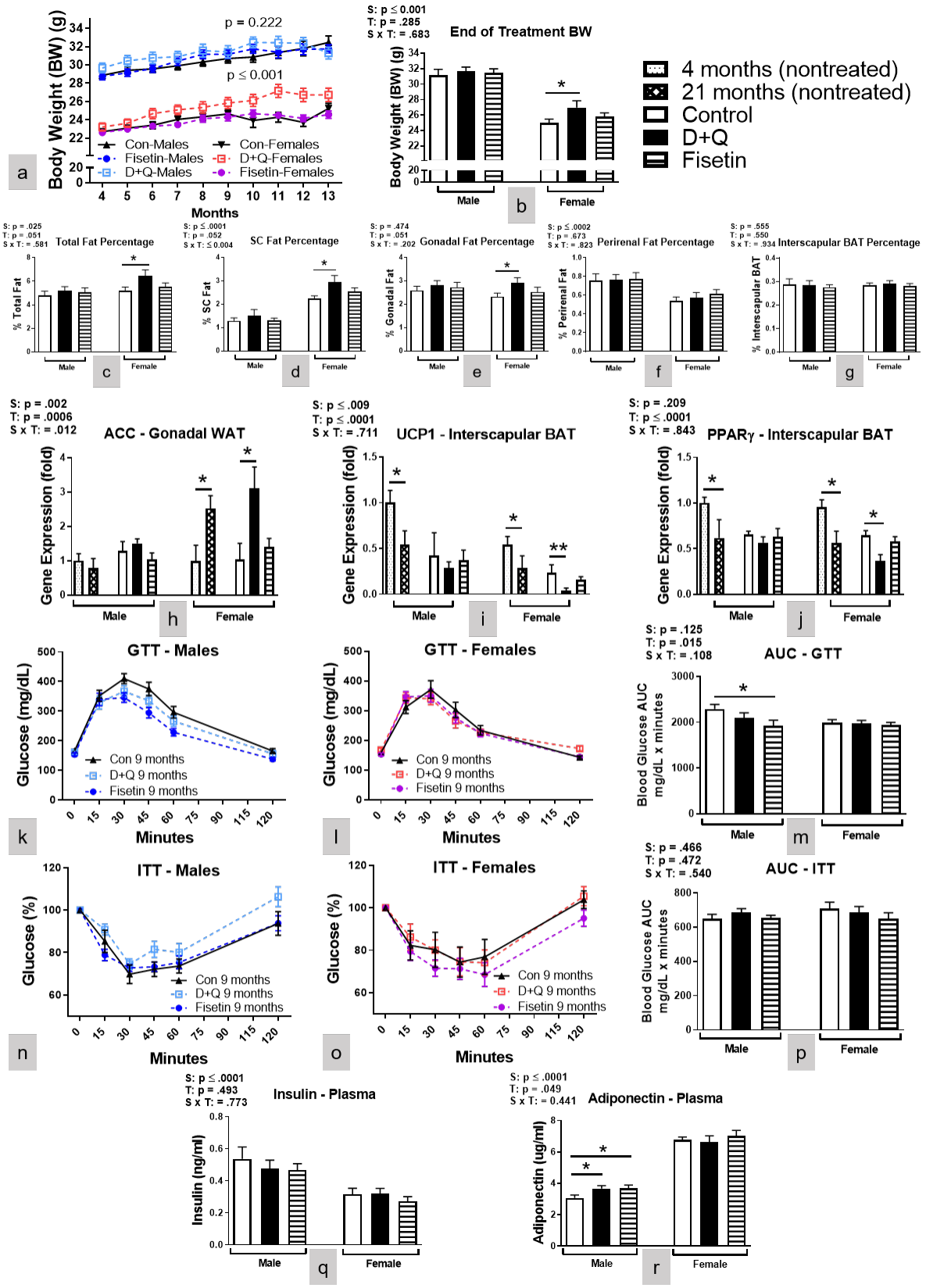
764 **Supplemental Information: Table 1:** A list of forward and reverse primers used in this
765 study.

766 **Figure 1**

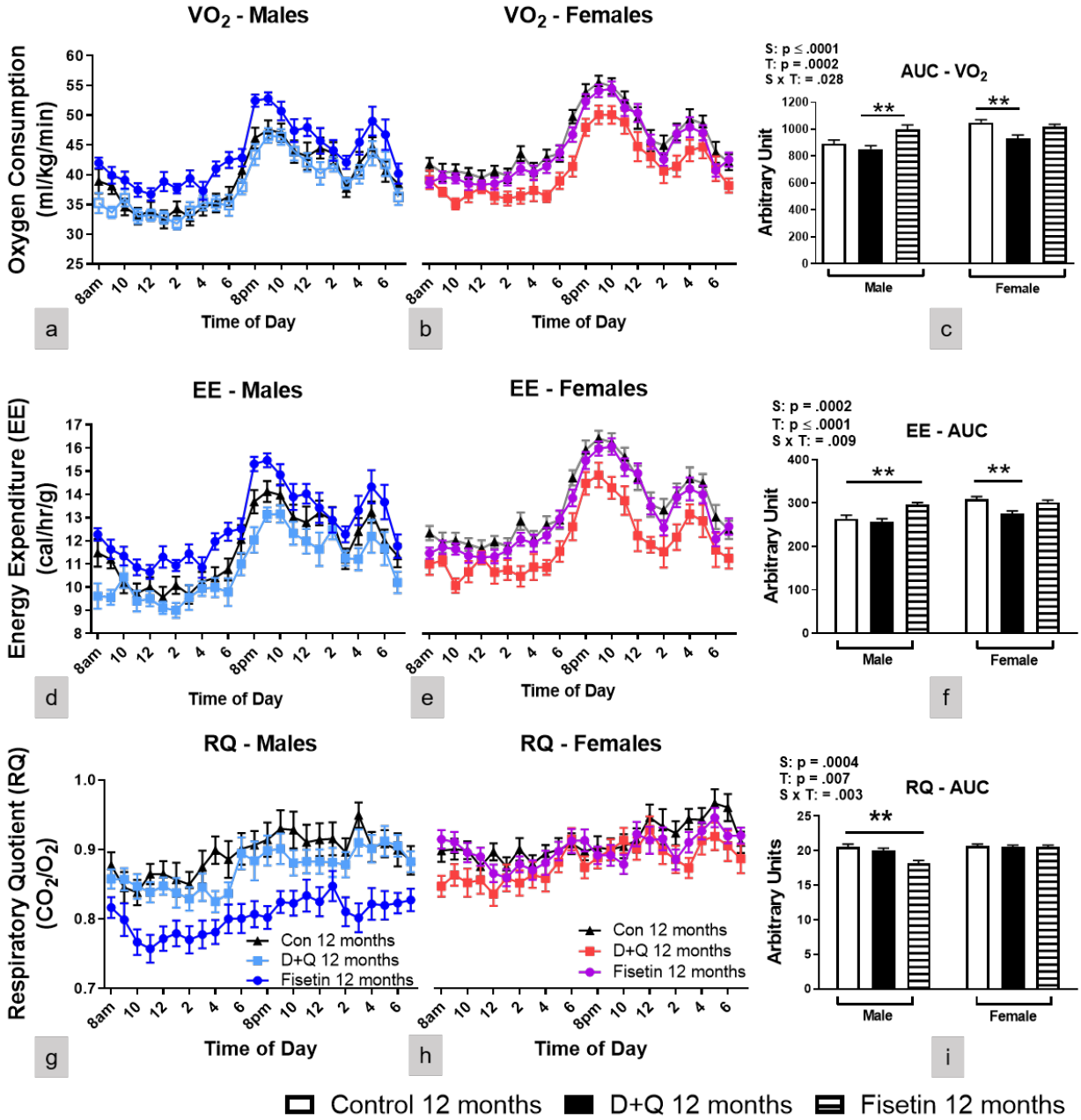


767

Figure 2

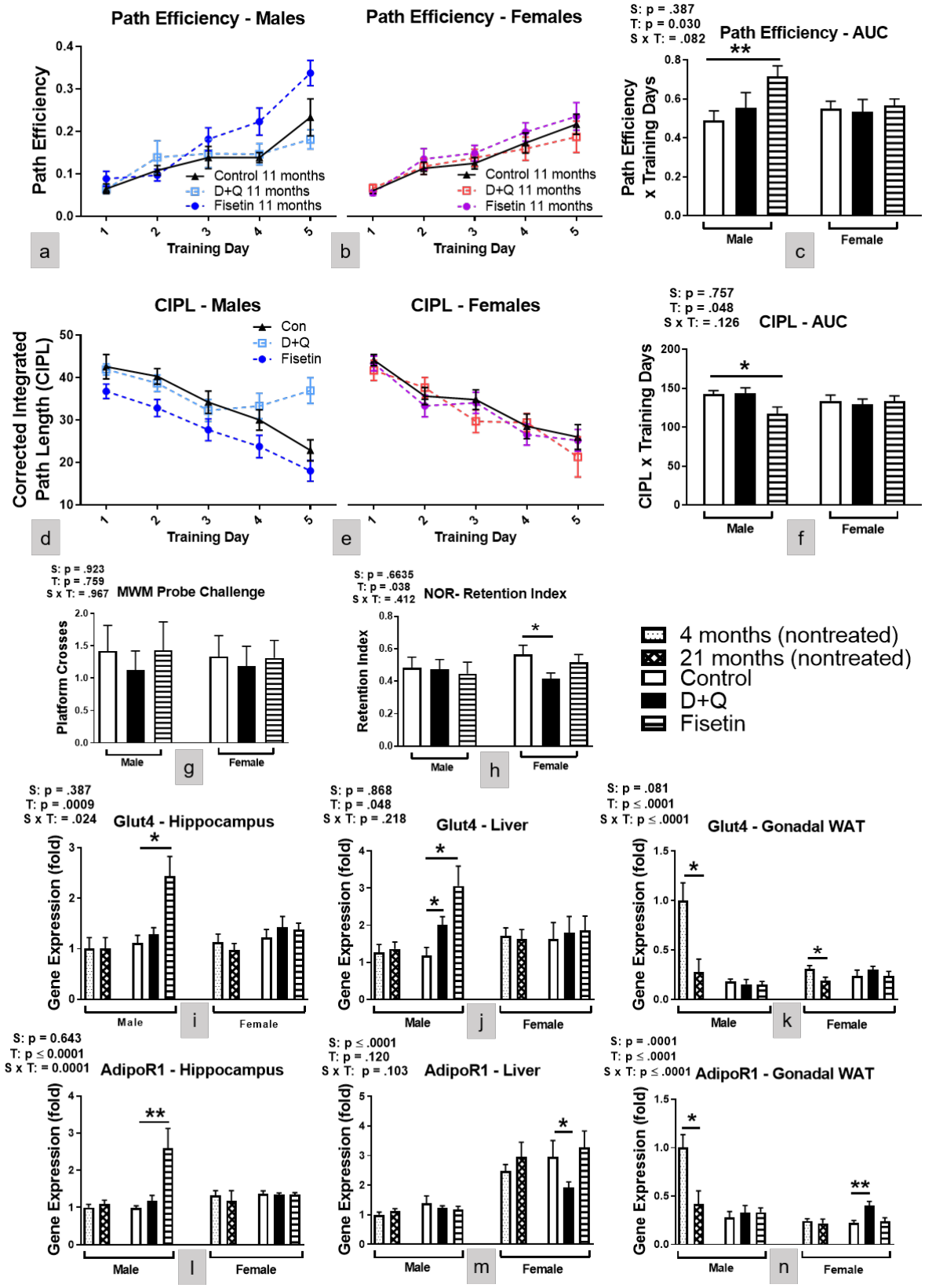

a
Body Weight (BW) (g)
p = 0.222
p ≤ 0.001
Con-Males
Fisetin-Males
D+Q-Males
Con-Females
D+Q-Females
Fisetin-Females
Months
S: p ≤ 0.001
T: p = .285
S x T: = .683
End of Treatment BW
b
Male
Female
4 months (nontreated)
21 months (nontreated)
Control
D+Q
Fisetin
S: p = .025
T: p = .051
S x T: = .581
Total Fat Percentage
% Total Fat
c
S: p ≤ .0001
T: p = .052
S x T: ≤ 0.004
SC Fat Percentage
% SC Fat
d
S: p = .474
T: p = .051
S x T: = .202
Gonadal Fat Percentage
% Gonadal Fat
e
S: p ≤ .0002
T: p = .673
S x T: = .823
Perirenal Fat Percentage
% Perirenal Fat
f
S: p = .555
T: p = .550
S x T: = .934
Interscapular BAT Percentage
% Interscapular BAT
g
S: p = .002
T: p = .0006
S x T: = .012
ACC - Gonadal WAT
Gene Expression (fold)
h
S: p ≤ .009
T: p ≤ .0001
S x T: = .711
UCP1 - Interscapular BAT
i
S: p = .209
T: p ≤ .0001
S x T: = .843
PPARγ - Interscapular BAT
j
GTT - Males
Glucose (mg/dL)
Con 9 months
D+Q 9 months
Fisetin 9 months
Minutes
k
GTT - Females
l
S: p = .125
T: p = .015
S x T: = .108
AUC - GTT
Blood Glucose AUC mg/dL x minutes
m
ITT - Males
Glucose (%)
n
ITT - Females
o
S: p = .466
T: p = .472
S x T: = .540
AUC - ITT
p
S: p ≤ .0001
T: p = .493
S x T: = .773
Insulin - Plasma
Insulin (ng/ml)
q
S: p ≤ .0001
T: p = .049
S x T: = 0.441
Adiponectin - Plasma
Adiponectin (ug/ml)
r

770 **Figure 3**

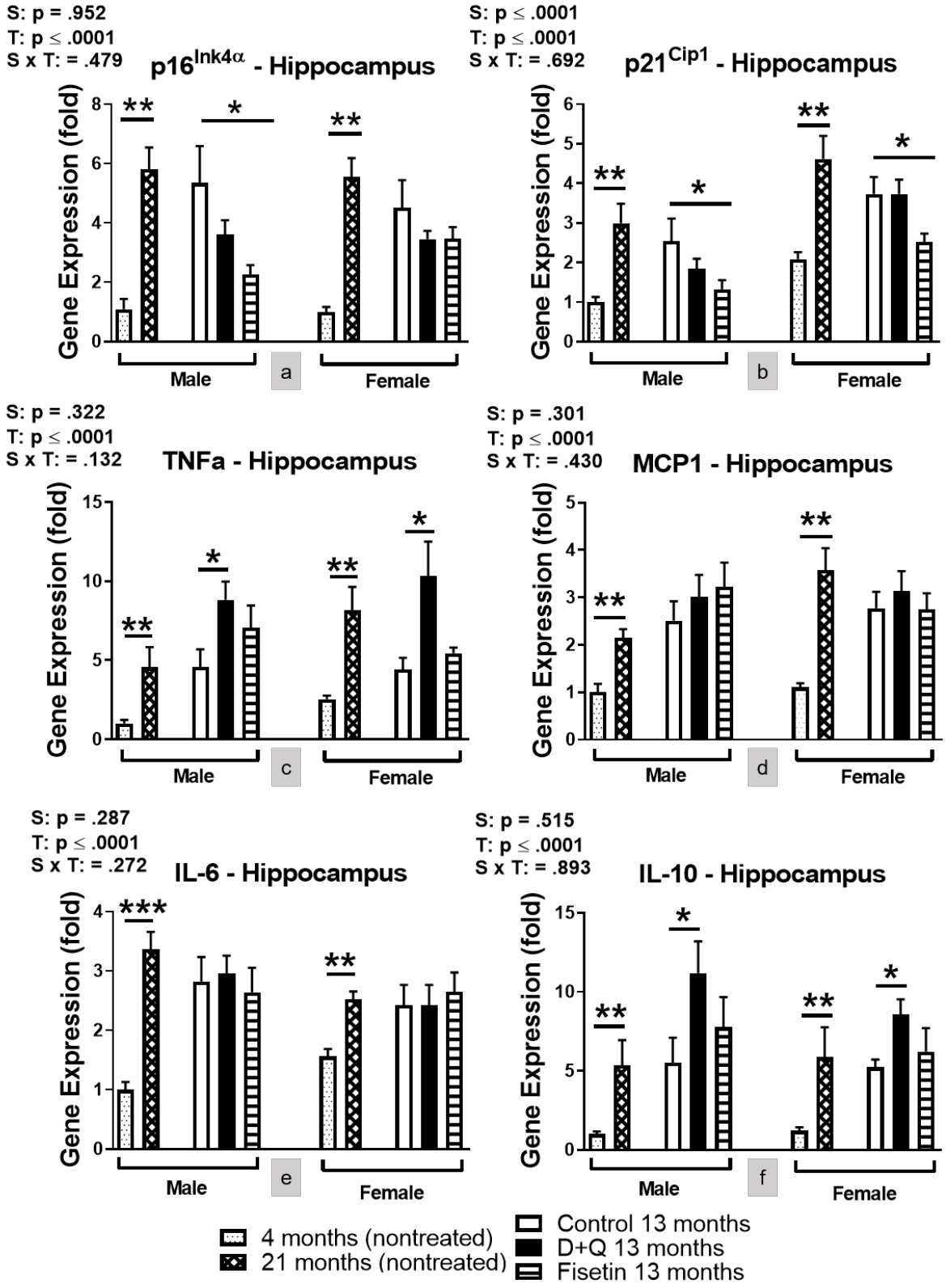


771

Figure 4

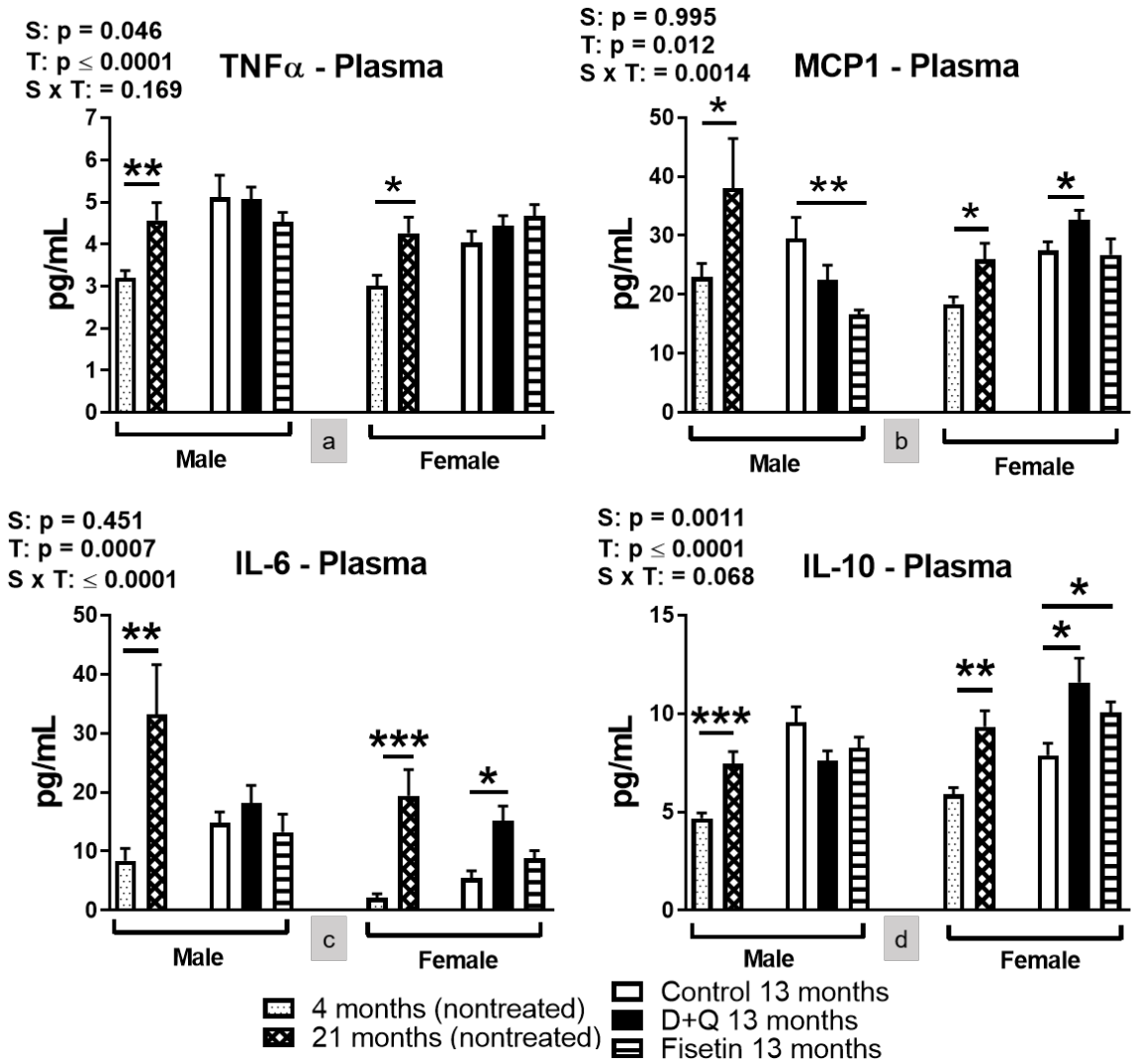

Path Efficiency - Males
Path Efficiency
Training Day
Control 11 months
D+Q 11 months
Fisetin 11 months
a
Path Efficiency - Females
b
S: p = .387
T: p = 0.030
S x T: = .082
Path Efficiency - AUC
Path Efficiency x Training Days
Male
Female
c
CIPL - Males
Corrected Integrated Path Length (CIPL)
Con
D+Q
Fisetin
d
CIPL - Females
e
S: p = .757
T: p = .048
S x T: = .126
CIPL - AUC
CIPL x Training Days
f
S: p = .923
T: p = .759
S x T: = .967
MWM Probe Challenge
Platform Crosses
g
S: p = .6635
T: p = .038
S x T: = .412
NOR- Retention Index
Retention Index
h
4 months (nontreated)
21 months (nontreated)
Control
D+Q
Fisetin
S: p = .387
T: p = .0009
S x T: = .024
Glut4 - Hippocampus
Gene Expression (fold)
i
S: p = .868
T: p = .048
S x T: p = .218
Glut4 - Liver
j
S: p = .081
T: p ≤ .0001
S x T: p ≤ .0001
Glut4 - Gonadal WAT
k
S: p = 0.643
T: p ≤ 0.0001
S x T: = 0.0001
AdipoR1 - Hippocampus
l
S: p ≤ .0001
T: p = .120
S x T: p = .103
AdipoR1 - Liver
m
S: p = .0001
T: p ≤ .0001
S x T: p ≤ .0001
AdipoR1 - Gonadal WAT
n

774 **Supplemental Information: Figure 1**



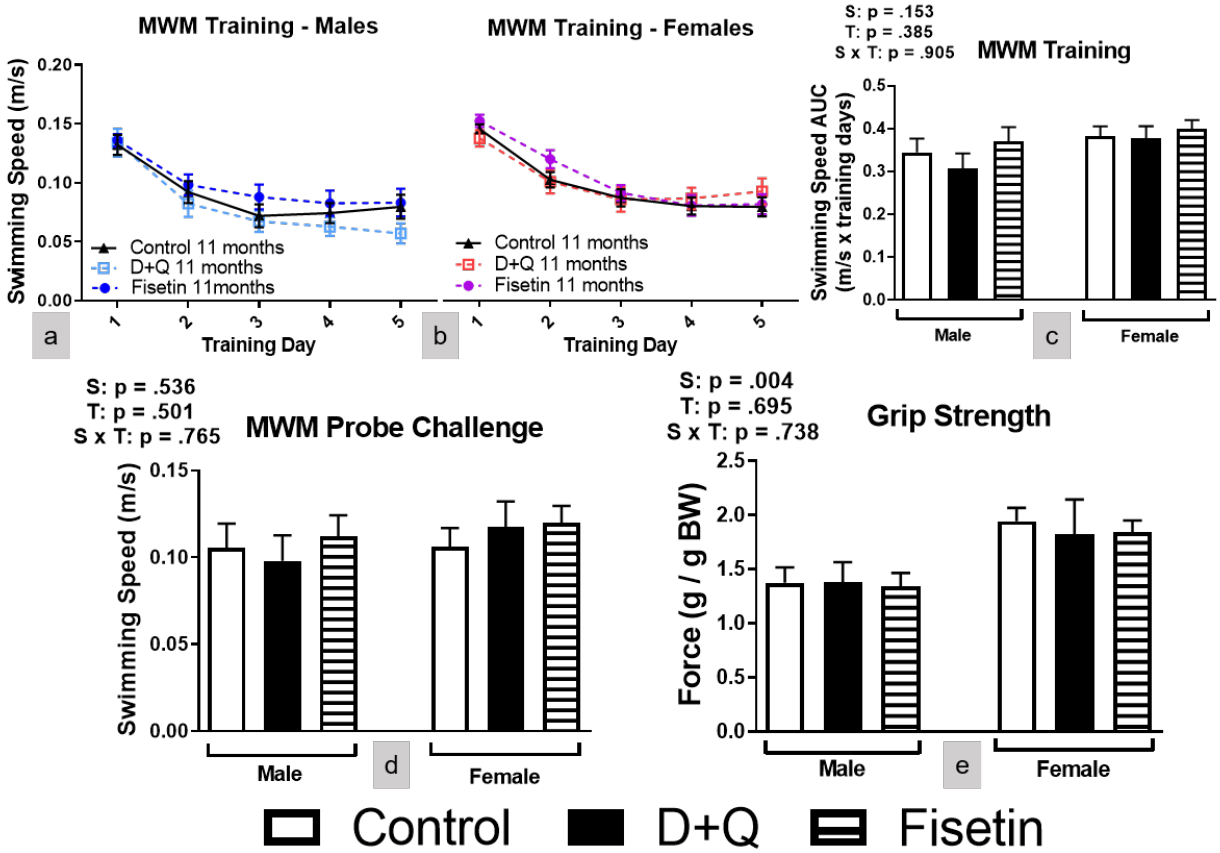
775

776 **Supplemental Information: Figure 2**

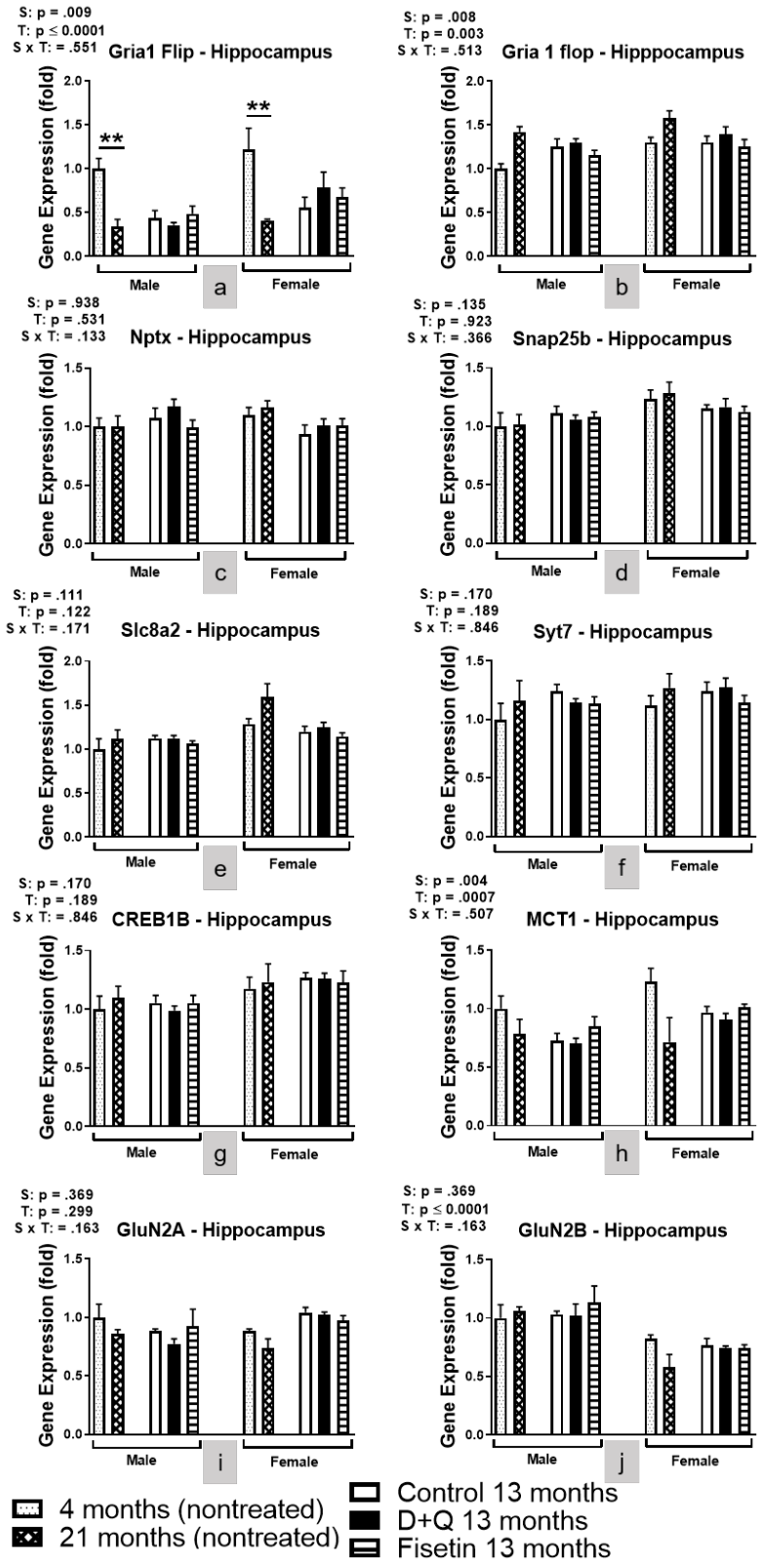


777

Supplemental Information: Figure 3



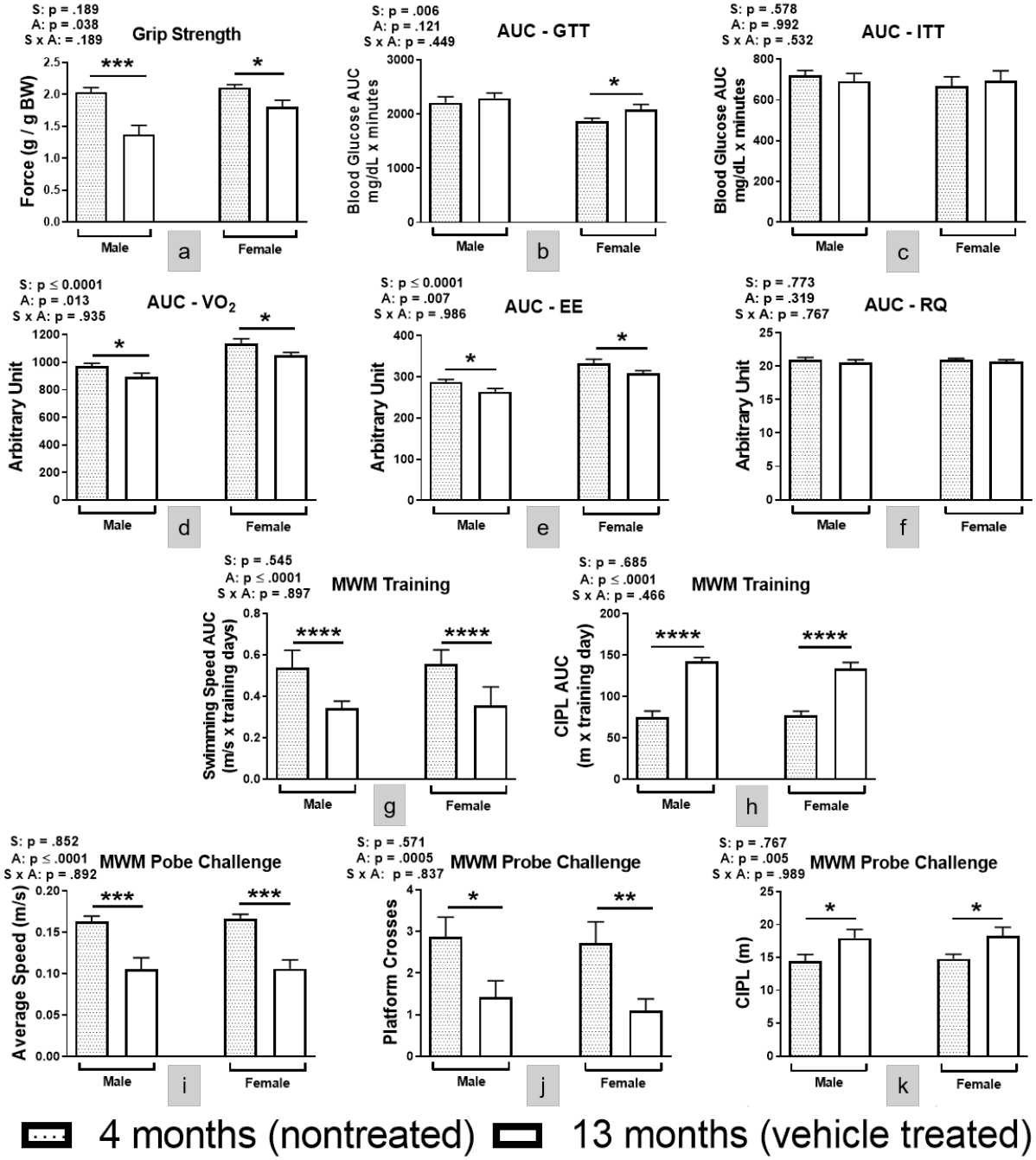
780 **Supplemental Information: Figure 4**



781

782

Supplemental Information: Figure 5



785 **Supplemental Information: Table 1**

Gene	Protein	Forward	Reverse
CDKN2A	$p16^{Ink4\alpha}$	TACCCCGATTCAGGTGAT	TTGAGCAGAAGAGCTGCTACGT
CDKN1A	$p21^{Cip1}$	CCTGGTGATGTCCGACCTG	CCATGAGCGCATCGCAATC
TNFα	TNFα	GCCTCTTCTCATTCCTGCTTG	CTGATGAGAGGGAGGCCATT
CCL2	MCP1	CCACTCACCTGCTGCTACTCAT	TGGTGATCCTCTTGTAGCTCTCC
IL6	IL-6	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
IL10	IL-10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
ACACA	ACC	ATGGGCGGAATGGTCTCTTTC	TGGGGACCTTGTCTTCATCAT
UCP1	UCP1	AGGCTTCCAGTACCATTAGGT	CTGAGTGAGGCAAAGCTGATTT
PPARG	PPARγ	ACCCCCTGCTCCAGGAGAT	TGCAATCAATAGAAGGAACACGT
NPTX	Nptx	ACACCATGAAAGTGGGAGGTAACT	ACTGGTCTTGTCCTTACTTCCGGA
SNAP25	Snap25b	CTCATCGAGTGGGGCAACA	GTGATGCCATTTGCCATCGTT
Syt7	Syt7	ACTCCATCATCGTGAACATCATC	TATGTCGAAGGCGAAAGAC
Slc8a2	Na^{+} / Ca^{++} Exchanger, Member 2	AACAGCACCTTCTACGTGG	GTCCTTCTGCGTCTCCC
GRIN2A	GluN2A	ACGTGACAGAACGCGAACTT	TCAGTGCGGTTCATCAATAACG
GRIN2B	GluN2B	CAGCAAAGCTCGTTCCCAAAA	GTCAGTCTCGTTCATGGCTAC
CREB1	Creb1b	AGCAGCTCATGCAACATCATC	AGTCCTTACAGGAAGACTGAACT
SLC16A1	MCT1	TGTTAGTCGGAGCCTTCATTTC	CACTGGTCGTTGCACTGAATA
GRIA1	Gria1 Flip	ACACCATGAAAGTGGGAGGTAACT	ACTGGTCTTGTCCTTACTTCCGGA
GRIA1	Gria1 Flop	GTCCGCCCTGAGAAATCCA	GCACTCGCCCTTGTCGTA
SLC2A4	Glut4	ACACTGGTCCTAGCTGTATTCT	CCAGCCACGTTGCATTGTA
ADIPOR1	AdipoR1	ACGTTGGAGAGTCATCCCGTAT	CTCTGTGTGGATGCGGAAGAT
B2M	B2M	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTCGGCTTCCCATTC

786