

1 Avid lysosomal acidification in fibroblasts of the Mediterranean mouse *Mus*  
2 *spretus*

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11

## 12 Abstract

13

14 Failures of the lysosome-autophagy system are a hallmark of aging and many disease states.  
15 As a consequence, interventions that enhance lysosome function are of keen interest in the  
16 context of drug development. Throughout the biomedical literature, evolutionary biologists have  
17 discovered that challenges faced by humans in clinical settings have been resolved by non-  
18 model organisms adapting to wild environments. Here, we used a primary cell culture approach  
19 to survey lysosomal characteristics in selected species of the genus *Mus*. We found that cells  
20 from *M. musculus*, mice adapted to human environments, had weak lysosomal acidification and  
21 high expression and activity of the lysosomal enzyme  $\beta$ -galactosidase, a classic marker of  
22 cellular senescence. Cells of wild relatives, especially the Mediterranean mouse *M. spretus*, had  
23 more robustly performing lysosomes and dampened  $\beta$ -galactosidase levels. We propose that  
24 classic laboratory models of lysosome function and senescence may reflect characters that  
25 diverge from the phenotypes of wild mice. The *M. spretus* phenotype may ultimately provide a  
26 blueprint for interventions that ameliorate lysosome breakdown in stress and disease.

27

## 28 Introduction

29

30 Many aspects of metazoan health hinge on the ability of the lysosome-autophagy pathway to  
31 recycle damaged macromolecules and to direct cell growth decisions (Shin & Zoncu 2020).  
32 Indeed, interventions that act broadly to promote health and longevity often require the  
33 lysosome-autophagy system (Aman *et al.* 2021; Hansen *et al.* 2018; Bareja *et al.* 2019). More  
34 specific mechanisms to boost autophagy are also of potential clinical interest, particularly for  
35 treatment of proteinopathies and aging etiologies (Bonam *et al.* 2019; Hansen *et al.* 2018). In  
36 practice, whether and how to stimulate proteostasis machinery to advance organismal health  
37 remains an open question, and the literature describing such manipulations is in its infancy  
38 (Simonsen *et al.* 2008; Pyo *et al.* 2013; Pickford *et al.* 2008; Leiva-Rodríguez *et al.* 2018; Shin  
39 *et al.* 2013; Liu *et al.* 2023; Bhuiyan *et al.* 2013).

40

41 Cellular senescence is a tumor-suppressive program characterized by cell cycle arrest,  
42 resistance to apoptosis, and activation of immune signaling in response to stress (Campisi &  
43 d'Adda di Fagagna 2007; Campisi 2005; Hornsby 2002). Though senescence mitigates cellular  
44 stress and safeguard health, the accumulation of senescent cells in aged tissues leads to  
45 dysfunctional tissue remodeling and chronic inflammation (Krtolica *et al.* 2001; Parrinello *et al.*  
46 2005; Davalos *et al.* 2010; Olivieri *et al.* 2018; Wan *et al.* 2021). In senescent cells, lysosomes  
47 increase in size, and lysosomal  $\beta$ -galactosidase becomes active at sub-optimal pH (Robbins *et al.*  
48 1970, Magalhães & Passos 2018, Dodig *et al.* 2019, Curnock *et al.* 2023). Though the exact  
49 role of lysosomes in senescence has not been fully elucidated, enhancements to their function  
50 have been associated with reduced cellular degeneration and diminished inflammation (Green  
51 *et al.* 2011, Carmona-Gutierrez *et al.* 2016, Rovira *et al.* 2022).

52

53 Against a backdrop of decades of work in laboratory systems, ecologists have cataloged stress-  
54 and disease-resistance traits in wild genotypes from unusual niches, perhaps most famously in

55 long-lived animal species (Oka *et al.* 2023; Chusyd *et al.* 2021; Finch 2009). Cell-based surveys  
56 represent a powerful approach to find and dissect these natural resilience programs. One  
57 particularly fruitful discipline has profiled the variation in chemical stress resistance across  
58 primary cells from panels of non-model animal species, including, in landmark cases, discovery  
59 of the underlying mechanisms (Tian *et al.* 2019; Harper *et al.* 2007; Attaallah *et al.* 2020; Sulak  
60 *et al.* 2016). Cellular senescence has also been shown to vary across animal species in *in vitro*  
61 models (Attaallah *et al.* 2020; Kang *et al.* 2023; Zhao *et al.* 2018; Gomez *et al.* 2012).

62  
63 In this study, we aimed to leverage species-specific variation in lysosomal markers of cellular  
64 senescence within the *Mus* genus to investigate how evolutionary processes have shaped  
65 lysosomal function. Mouse species in this genus shared a common ancestor 7-8 million years  
66 ago, and as they radiated across Eurasia, *M. musculus* subspecies came to occupy human-  
67 associated niches, whereas other taxa are still found exclusively in the wild (Pagès *et al.* 2015,  
68 Smissen & Rowe 2018). Our previous case study (Kang *et al.* 2023) found differences in  
69 senescence behaviors, including lysosome markers, across fibroblasts from *M. musculus*  
70 subspecies and *M. spretus*, a wild relative that diverged 1-3 million years ago (Morgan *et al.*  
71 2022; Dejager *et al.* 2009). Here, we aimed to build on these observations to gain a deeper  
72 understanding of lysosomal programs in non-model mice, focusing on the contrasts between the  
73 genotypes of human commensal and laboratory mice and those of their wild relatives.

## 74 75 Results

### 76 77 ***M. spretus* fibroblasts exhibit reduced lysosomal $\beta$ -galactosidase activity**

78  
79 To extend our initial observation of divergent cellular senescence behaviors across *Mus* (Kang  
80 *et al.* 2023), we established a panel of primary tail skin fibroblasts from four wild-derived strains  
81 of *M. m. musculus* (PWK/PhJ, BLG2/Ms, CHD/Ms, MSM/Ms), two wild-derived strains of *M. m.*  
82 *domesticus* (ManB/NachJ, TUCA/NachJ), an admixed classic laboratory strain of mostly *M. m.*  
83 *domesticus* origin (C57BL/6J), a wild-derived strain of the steppe mouse *M. spicilegus* (ZRU),  
84 and two wild-derived strains of the Mediterranean mouse *M. spretus* (STF/Pas, SFM/Pas). We  
85 subjected each culture to 15 Gy of infrared irradiation and incubated for 10 days, which is  
86 sufficient to induce DNA damage response, arrested cell growth, and senescence expression  
87 programs in fibroblasts from across the genus (Kang *et al.* 2023). We first assayed each culture  
88 for lysosomal  $\beta$ -galactosidase, as a standard marker of senescence that reports exhaustion of  
89 protein quality control in the lysosome (Campisi & d'Adda di Fagagna 2007; Kang *et al.* 2023;  
90 Casella *et al.* 2019; Guerrero-Navarro *et al.* 2022; Carmona-Gutierrez *et al.* 2016). Fibroblasts  
91 of each genotype displayed the anticipated increase in  $\beta$ -galactosidase activity following  
92 irradiation, compared to their respective control counterparts (Figure 1B). Whereas cells from  
93 strains of each species behaved similarly, we observed significant differences between species:  
94 irradiated *M. spretus* fibroblasts exhibited  $\beta$ -galactosidase signal ~2-fold lower than did cells  
95 from *M. m. musculus* and *M. m. domesticus*, and staining of *M. spicilegus* cells was between the  
96 two extremes (Figure 1B). To follow up on this difference, we used an alternative approach for  
97 senescence induction with the radiomimetic drug neocarzinostatin, which induces senescence  
98 in mouse cells at 3.6  $\mu$ M (Ito *et al.* 2018, Correia & Melo *et al.* 2016). Fibroblasts treated with

99 neocarcinostatin exhibited a pattern mimicking that we had seen under radiation, with the  
100 treatment inducing elevated  $\beta$ -galactosidase activity in all cultures, and cells from *M. spretus*  
101 staining lower than those of sister taxa (Figure 1C). Furthermore, the same trend was  
102 detectable in cells cultured in the absence of DNA damage: fibroblasts from *M. musculus*  
103 subspecies exhibited higher  $\beta$ -galactosidase staining than those of *M. spicilegus*, which stained  
104 higher than *M. spretus*. The latter species differences from untreated cells were of smaller  
105 magnitude than those manifesting after stress, and this dependence on treatment was  
106 statistically significant (Figures 1 and S1). Thus, *M. spretus* fibroblasts exhibited dampened  $\beta$ -  
107 galactosidase activity relative to other genotypes, with amplification of the effect under stress.  
108 Controls ruled out culture passage number as a driver of the variation (Figure S2).

109  
110 In principle, differences in the senescence-apoptosis fate choice after DNA damage (Childs *et al.*  
111 *et al.* 2014; Zhao *et al.* 2018; Attaallah *et al.* 2020) could contribute to the divergence between  
112 mouse genotypes that we had seen in fibroblast  $\beta$ -galactosidase staining. To explore this, we  
113 measured Caspase 3/5 activity in fibroblasts in response to irradiation (Figure S3A) and  
114 neocarcinostatin treatment (Figure S3B). The results showed no consistent patterns of  
115 apoptosis either within or between species' genotypes, arguing against a role for apoptosis  
116 activation in species differences in fibroblast  $\beta$ -galactosidase activity.

117  
118 To establish further the robustness of *Mus* species differences in fibroblast  $\beta$ -galactosidase  
119 activity, we considered the dose-response relationship with stress. We compared primary  
120 fibroblasts from *M. m. musculus* PWK and *M. spretus* STF as representatives of their respective  
121 species, in each case assaying  $\beta$ -galactosidase upon irradiation at increasing doses. The  
122 results revealed a consistent ~2-fold difference between the genotypes at each dose (Figure  
123 S4), a magnitude slightly exceeding the species divergence in the untreated control, consistent  
124 with our survey across genotypes at fixed stress doses (Figure 1). These data ruled out a switch  
125 by *M. spretus* fibroblasts into a high-amplitude, *M. musculus*-like program above a certain  
126 threshold of stress exposure. We conclude instead that *M. spretus* cells are hard-wired for lower  
127  $\beta$ -galactosidase activity in all tested conditions—including the resting state.

128  
129 **No consistent divergence in senescence signaling across *Mus* species fibroblasts**

130  
131 We reasoned that the differences in  $\beta$ -galactosidase activity in fibroblasts among the *Mus*  
132 species we tested were likely mediated by mechanisms unrelated to senescence itself. As a first  
133 investigation of this idea, we explored senescence signaling, using as a readout P21, a  
134 regulator of immune recruitment and cell cycle arrest in senescence (Gu *et al.* 2013; Yew *et al.*  
135 2011). We quantified levels of P21 protein in response to either irradiation or neocarcinostatin,  
136 with fibroblasts from *M. m. musculus* PWK and *M. spretus* STF as a testbed. Results showed  
137 the expected induction of P21 in fibroblasts from both species in response to irradiation or  
138 neocarcinostatin (Figure S5). In investigating quantitative patterns among these P21 induction  
139 behaviors, we noted some species-specific differences, though they were not of consistent  
140 direction: *M. spretus* cells induced P21 protein abundance more than did *M. musculus* under  
141 neocarcinostatin treatment but less following irradiation and in untreated controls (Figure S5).  
142 These changes could reflect the differential activity of alternate senescence signaling pathways

143 (Saul *et al.* 2023) between species in some treatments. However, given our focus on lysosomal  
144  $\beta$ -galactosidase differences between *M. musculus* and *M. spretus* across all tested conditions  
145 (Figure 1), we concluded that P21 regulation was not a consistent correlate of this phenotype  
146 and not a compelling candidate for its underlying mechanism. Indeed, in analysis of mRNA  
147 expression profiles (Kang *et al.* 2023), we detected no differences in induction of the P21 gene  
148 (*CDKN1A*) between *M. musculus* and *M. spretus* fibroblasts in response to stress (Figure S6).

149

### 150 ***M. spretus* fibroblasts exhibit strongly acidified lysosomes**

151

152 To advance our search to understand *Mus* species differences in  $\beta$ -galactosidase activity in the  
153 fibroblast model, we next focused on regulation of the enzyme itself. Transcriptional profiling  
154 data from fibroblasts revealed 1.5 to 2-fold higher expression of the lysosomal  $\beta$ -galactosidase  
155 *GLB1* in *M. musculus* fibroblasts than in those of *M. spretus*, regardless of treatment (Figure  
156 S7A). Allele-specific expression measurements in stressed and control fibroblasts from an  
157 interspecies F1 hybrid made clear that this change was regulated in *cis*: that is, the *M. musculus*  
158 allele of *GLB1* drove higher expression of its own encoding locus than the *M. spretus* allele,  
159 when both were in the same nucleus (Figure S7B). Thus, the species changes in  $\beta$ -  
160 galactosidase activity we had noted in terms of cell biology in fibroblasts (Figure 1) were  
161 mirrored by regulation of gene expression, including robust differences between genotypes in  
162 unstressed control conditions.

163

164 We hypothesized that the higher  $\beta$ -galactosidase expression and activity we had seen in *M.*  
165 *musculus* fibroblasts could be a consequence of heightened need owing to failures elsewhere in  
166 the proteostasis system, relative to cells of *M. spretus*. To explore this, we assayed lysosomal  
167 acidity with the LysoTracker stain, again making use of *M. m. musculus* PWK and *M. spretus*  
168 STF as a test system. Conforming to our prediction, *M. spretus* cells exhibited 3-fold stronger  
169 LysoTracker signal relative to cells of *M. musculus*, in untreated cultures (Figure 2, left). The  
170 species difference persisted in cultures induced to senesce with irradiation (Figure 2, middle)  
171 and neocarzinostatin (Figure 2, right). Taken together, our results establish a trait syndrome of  
172 elevated lysosomal acidification and low  $\beta$ -galactosidase expression and activity in *M. spretus*  
173 fibroblasts relative to those of other mice, whether the cells are senescent or not.

174

## 175 Discussion

176

177 For decades, biomedical researchers have relied on strains from the *M. musculus* clade as the  
178 standard for studying lysosomal and senescence biology. In this work, we have demonstrated  
179 that lysosomal phenotypes vary quantitatively across *Mus* in fibroblast culture models, and that  
180 in contrast to *M. musculus*, cells from the non-commensal mouse *M. spretus* exhibit a lysosomal  
181 acidity and  $\beta$ -galactosidase program of the kind that, in the experimental literature, has been  
182 associated with healthy aging and disease resistance.

183

184 When experimentally induced in laboratory cell models, a backup in the lysosomal-autophagy  
185 system triggers compensatory increases in lysosomal mass and number, for which high  $\beta$ -  
186 galactosidase activity is a robust marker (Curnock *et al.* 2023; Delfarah *et al.* 2021; Lee *et al.*

187 2006, Dimri *et al.* 1995, Rovira *et al.* 2022). We propose a similar causal relationship between  
188 the two phenotypes we have observed as they vary across *Mus* fibroblasts. Under this model,  
189 the elevated  $\beta$ -galactosidase expression and activity in *M. musculus* cells would represent  
190 compensation for their weak lysosomal acidification in comparison to *M. spretus* genotypes,  
191 even in the absence of experimental stress. The *M. musculus* *cis*-regulatory allele upregulating  
192  $\beta$ -galactosidase that we have noted in fibroblasts could well represent a genetically encoded  
193 component of such a program, a constitutive boost in protein degradation capacity by a  
194 regulatory mechanism, in the face of lysosomal acidification defects.

195  
196 Our work leaves open the question of whether weak lysosomal acidity and high  $\beta$ -galactosidase  
197 in fibroblasts represent a state ancestral to *Mus* that was resolved in the *M. spicilegus*-*M.*  
198 *spretus* lineage, or an evolutionary novelty that arose with commensalism in *M. musculus*. In  
199 either case, the evidence that we have seen for conservation across *M. musculus* strains and  
200 subspecies strongly suggests a history of constraint in this lineage. If the lysosomal behaviors  
201 we have studied here prove to have evolved under selection, they could conceivably relate to  
202 body size, response to immune challenges, or association with human ecology as they differ  
203 between *M. musculus* and *M. spretus* (Dejager *et al.* 2009, Mahieu *et al.* 2006, Kawakami &  
204 Yamamura 2008, Staelens *et al.* 2002, Blanchet *et al.* 2010, Pérez del Villar *et al.* 2013, Harr *et*  
205 *al.* 2016).

206  
207 Ultimately, as its mechanism is revealed, the high-acidification phenotype of *M. spretus*  
208 fibroblasts may prove to be well suited for the development of mimetics that would boost  
209 lysosomal function in a human clinical setting. More broadly, our results provide a way forward  
210 for the use of wild mouse species as models for lysosomal function and senescence, without the  
211 peculiarities of commensal lineages.

212

## 213 Methods

214

215 Primary tail fibroblasts were extracted as described by Khan and Gasser (2016). Subsequent  
216 culture and experiments used complete medium (DMEM, 10% FBS, 1% pen-strep) in T25 flasks  
217 at 37°C, 3% O<sub>2</sub>, and 10% CO<sub>2</sub>. Cells were passaged based on confluence using trypsin.  
218 Experimental treatments included 15 GY of ionizing irradiation or 3.6  $\mu$ M neocarzinostatin.  
219 Analysis kits used were the Abcam Ltd. Senescence Detection Kit (Cat. #ab65351), ApoTox-  
220 Glo™ Triplex Assay (Promega Cat. #G6321), and LysoTracker™ Green DND-26 (Thermo  
221 Fisher Cat. #L7526). Primary and secondary antibodies used in western blots were the rabbit  
222 monoclonal Anti-p21 antibody (ab188224, Abcam, 1:1000), mouse monoclonal anti- $\beta$ -tubulin  
223 (T9026, Sigma-Aldrich, 1:1000), Goat Anti-Mouse IgG(H+L) Human ads-HRP (Cat#1031-05,  
224 Southern Biotech, 1:5000), and Goat Anti-Rabbit IgG(H+L) Human ads-HRP (Cat#4050-05,  
225 Southern Biotech, 1:5000). Additional details of methods available in Supplementary Materials.

226

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228



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266 Figure captions

267

268 **Figure 1. Low  $\beta$ -galactosidase activity in *M. spretus* fibroblasts regardless of treatment.**

269 In a given panel, each bar length displays the mean percentage of primary fibroblasts of the  
270 indicated strain and species that stained positive after administration of the colorimetric  $\beta$ -  
271 galactosidase substrate X-Gal. From top to bottom, complete names of the strains are as  
272 follows: *M. m. musculus* (PWK/PhJ, BLG2/Ms, CHD/Ms, MSM/Ms), laboratory strain of mostly  
273 *M. m. domesticus* origin (C57BL/6J), *M. m. domesticus* (TUCA/NachJ, ManB/NachJ), *M.*  
274 *spicilegus*, and *M. spretus* (SFM, STF/Pas). Panels report results from (A) untreated cells, (B)  
275 cells treated with infrared irradiation followed by a 10-day incubation, or (C) cells after 1 hour of  
276 neocarzinostatin treatment followed by 24 hours of incubation. Points report biological and  
277 technical replicates collected over at least two separate days. Error bars report one standard  
278 error above and below the mean. \*\*\*, two-tailed Wilcoxon  $p < 10^{-7}$  comparing *M. spretus* with all  
279 other genotypes; *M. spretus* and *M. spicilegus* also differed in each panel (Wilcoxon  $p < 0.05$ ).  
280 For (B) and (C), in a comparison between the respective treatment and the untreated control in  
281 (A), a two-factor ANOVA with treatment and genotype as factors yielded  $p < 2e^{-16}$  for the  
282 interaction between the two factors.

283

284 **Figure 2. High lysosomal acidity in *M. spretus* cells across all treatments.**

285 Shown are results from assays of the lysosomal acidity reporter Lysotracker on primary  
286 fibroblasts of the indicated genotype (*M. spretus*, strain STF; *M. m. musculus*, strain PWK). The  
287 y-axis reports mean fluorescence of the indicated culture normalized to the control *M. musculus*  
288 sample for each experiment. Pairs of bars report results from untreated cells (left), or cells  
289 treated with irradiation followed by a 10-day incubation (middle) or after 1 hour of  
290 neocarzinostatin treatment followed by 24 hours of incubation (right). Data points correspond to  
291 biological and technical replicates collected over at least two different days, and the bar height  
292 reports their mean. Error bars report one standard error above and below the mean. \*, Wilcoxon  
293  $p < 0.05$ , \*\*, Wilcoxon  $p < 0.01$ .

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## 307 Supplementary figure captions

308

### 309 **Figure S1. Low normalized $\beta$ -galactosidase activity in *M. spretus* fibroblasts.**

310 Data are as in Figure 1 of the main text, except that measurements from the culture of a given  
311 genotype and DNA damage treatment were normalized to the average of all measurements  
312 from untreated controls of that genotype. \*\*\*, two-tailed Wilcoxon  $p < .001$  comparing *M. spretus*  
313 with all other genotypes for both treatments.

314

### 315 **Figure S2. Fibroblast passage number has no detectable effect on X-Gal staining.**

316 In a given panel, each row reports results from assays of the  $\beta$ -galactosidase substrate X-Gal  
317 on fibroblasts of the indicated genotype shown in Figure 1 of the main text, and each point  
318 reports one replicate culture. The x-axis reports the passage number of the respective culture  
319 and the y-axis reports X-Gal staining. (A) Cells induced to senesce with irradiation (right) and  
320 their paired controls (left). (B) Cells induced to senesce with neocarzinostatin (right) and their  
321 paired controls (left).

322

### 323 **Figure S3. No consistent variation between *Mus* species fibroblasts in apoptosis activity.**

324 Data are as in Figure 1 of the main text, except that for a given genotype and treatment, the x-  
325 axis reports caspase activity of the culture measured with the Apotex-Glo assay, normalized to  
326 the average from untreated cultures of the respective genotype for each experiment. (A)  
327 Outliers trimmed for visualization. (B) Full data set view.

328

### 329 **Figure S4. Dose dependence of $\beta$ -galactosidase staining in *Mus* fibroblasts.**

330 Each trace shows results from assays of the  $\beta$ -galactosidase substrate X-Gal on primary  
331 fibroblasts of the indicated genotype (*M. spretus*, strain STF; *M. m. musculus*, strain PWK). For  
332 a given trace, the x-axis reports irradiation dosage (in centigray, cGy) at 0 (Control), 1000, 1500,  
333 2000, and 2500 cGy, and the y-axis reports the percentage of X-Gal positive cells in untreated  
334 controls or in irradiated cells after 10 days of incubation. Smaller points represent biological and  
335 technical replicates, and larger points indicate their mean values. \*\*\*, two-factor ANOVA with  
336 dosage and species as factors yielded  $p < 2e^{-16}$  for each factor.

337

### 338 **Figure S5. No consistent variation between *Mus* fibroblasts in P21 abundance.**

339 Shown are results of Western blot assays of abundance of the senescence regulator P21 in  
340 primary fibroblasts of the indicated genotype (*M. spretus*, strain STF; *M. m. musculus*, strain  
341 PWK). (A) Top, representative blot showing tubulin (55 kDa) and P21 (21 kDa) levels in 25  $\mu$ g of  
342 whole cell lysate (WCL) from untreated control cells or those irradiated (Xray) and incubated for  
343 10 days to establish senescence. Bottom, blot as at top except that cells were treated with 1  
344 hour of neocarzinostatin (NCS) and incubated for 24 hours to establish senescence. (B) Each  
345 column reports quantification of P21 protein abundance normalized to tubulin for the indicated  
346 genotype and treatment. Data points correspond to biological and technical replicates collected  
347 over at least two different days, and the bar height reports their mean. Error bars report one  
348 standard error above and below the mean. No comparisons were significant in pairwise  
349 Wilcoxon tests. \*, two-factor ANOVA with condition and species as factors yielded  $p < 0.05$  for  
350 species.

351 **Figure S6. No significant variation between *Mus* fibroblasts in *Cdkn1a* mRNA expression.**

352 (A) Shown are measurements of mRNA expression of the  $\beta$ -galactosidase gene *Cdkn1a*, from  
353 profiling of fibroblasts of the indicated genotype (*M. spretus*, strain STF; *M. m. musculus*, strain  
354 PWK), untreated or treated with irradiation followed by a 10-day incubation (Kang *et al.* 2023).  
355 Data points within each column represent biological and technical replicates and the bar height  
356 reports their mean. Error bars report one standard error above and below the mean. Only  
357 condition had a significant impact on expression in a two-factor ANOVA with treatment and  
358 genotype as factors ( $p < 0.005$ , \*\*).

359

360 **Figure S7. Variation between *Mus* fibroblasts in *Glb1* mRNA expression.**

361 (A) Shown are measurements of mRNA expression of the  $\beta$ -galactosidase gene from profiling of  
362 fibroblasts of the indicated genotype (*M. spretus*, strain STF; *M. m. musculus*, strain PWK),  
363 untreated or treated with irradiation followed by a 10-day incubation (Kang *et al.* 2023). Data  
364 points within each column represent biological and technical replicates and the bar height  
365 reports their mean. Only the effect of genotype was significant in a two-factor ANOVA with  
366 condition and species as factors (\*,  $p < 0.05$ ). (B) Data are as in (A), except that allele-specific  
367 mRNA expression of the  $\beta$ -galactosidase gene was measured from profiling of fibroblasts of an  
368 F1 hybrid between STF and PWK. (*M. spretus*, strain STF; *M. m. musculus*, strain PWK)(Kang  
369 *et al.* 2023). In a two-factor ANOVA with condition and genotype as factors, both had significant  
370 effects (species  $p < 0.0005$ , \*\*\*; condition  $p < 0.05$ , \*).

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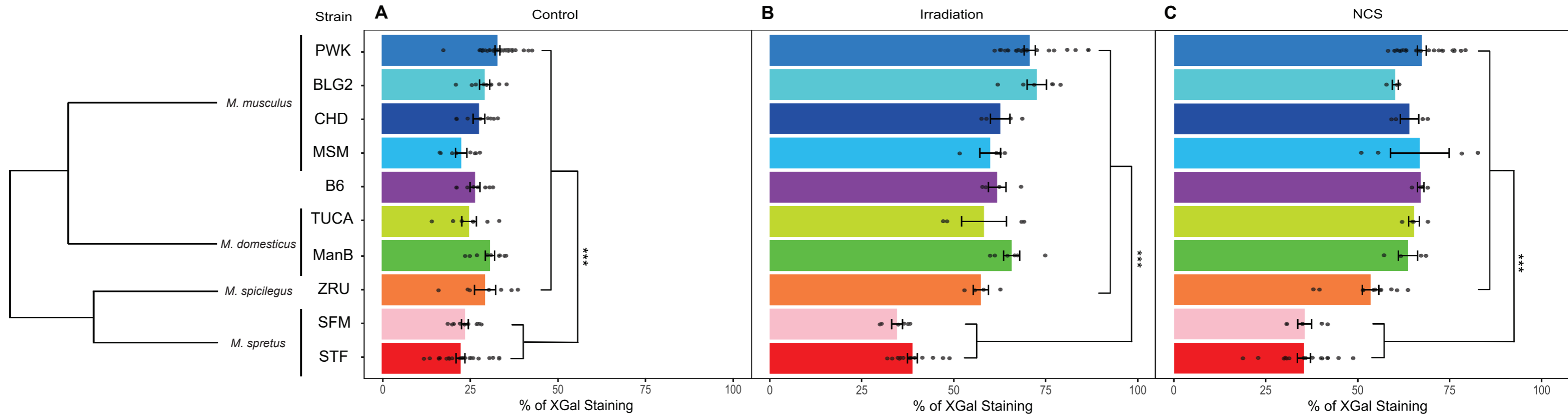
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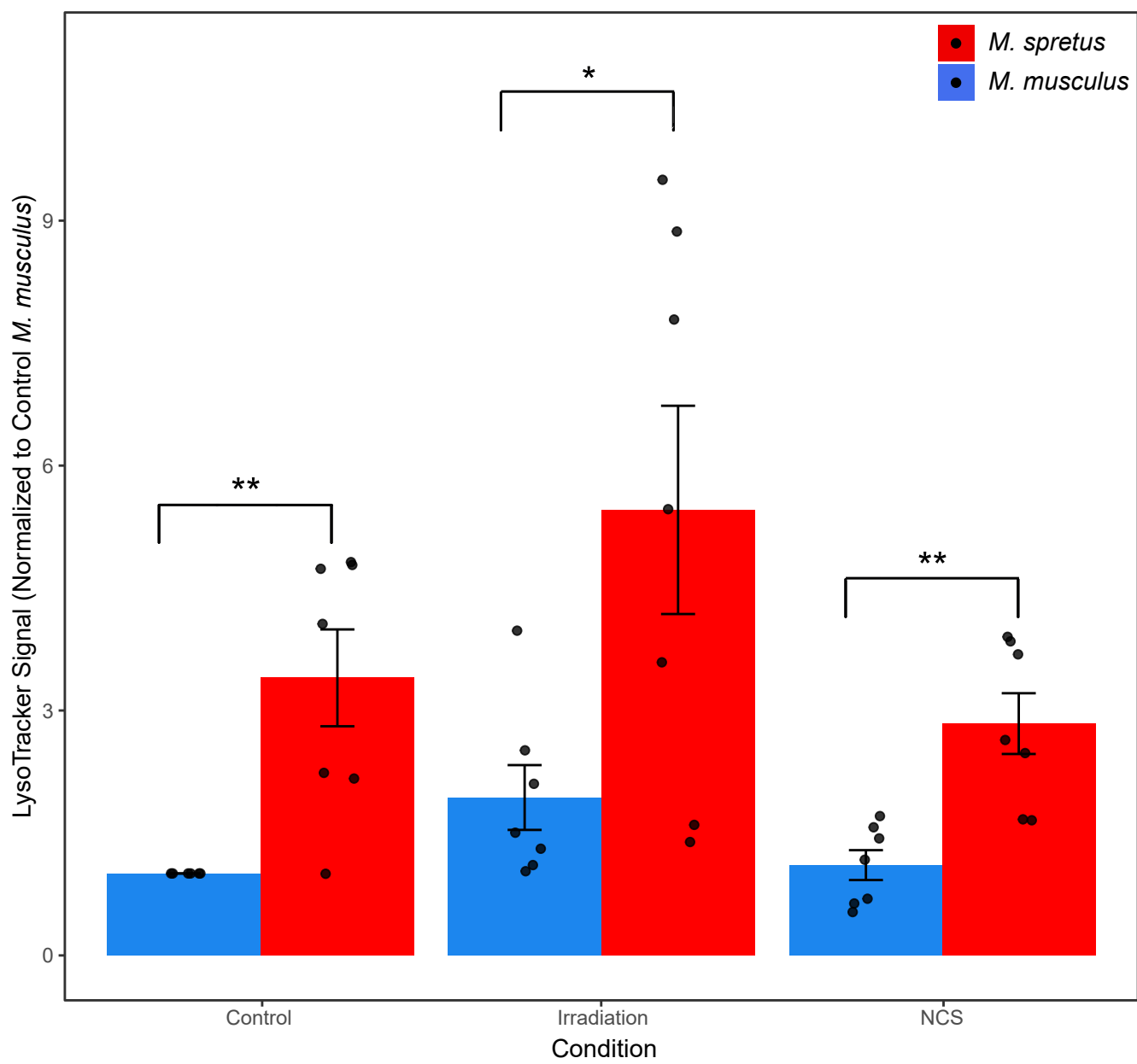
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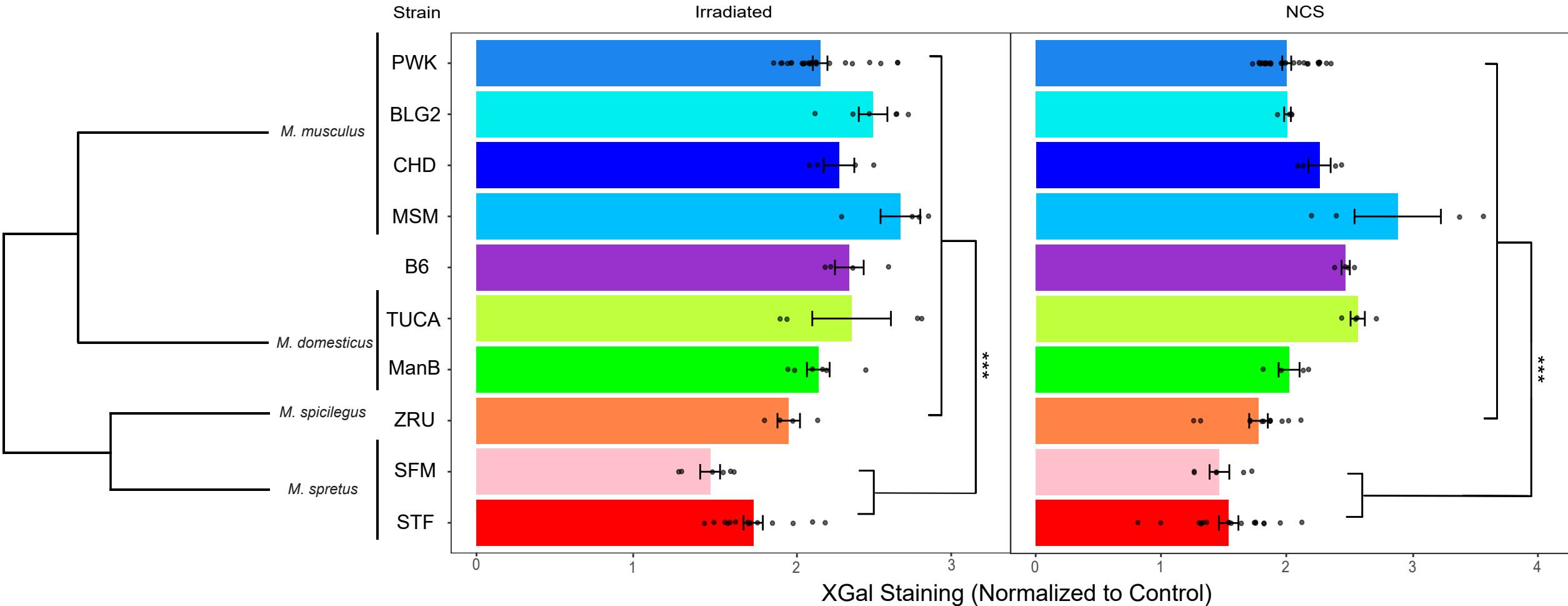


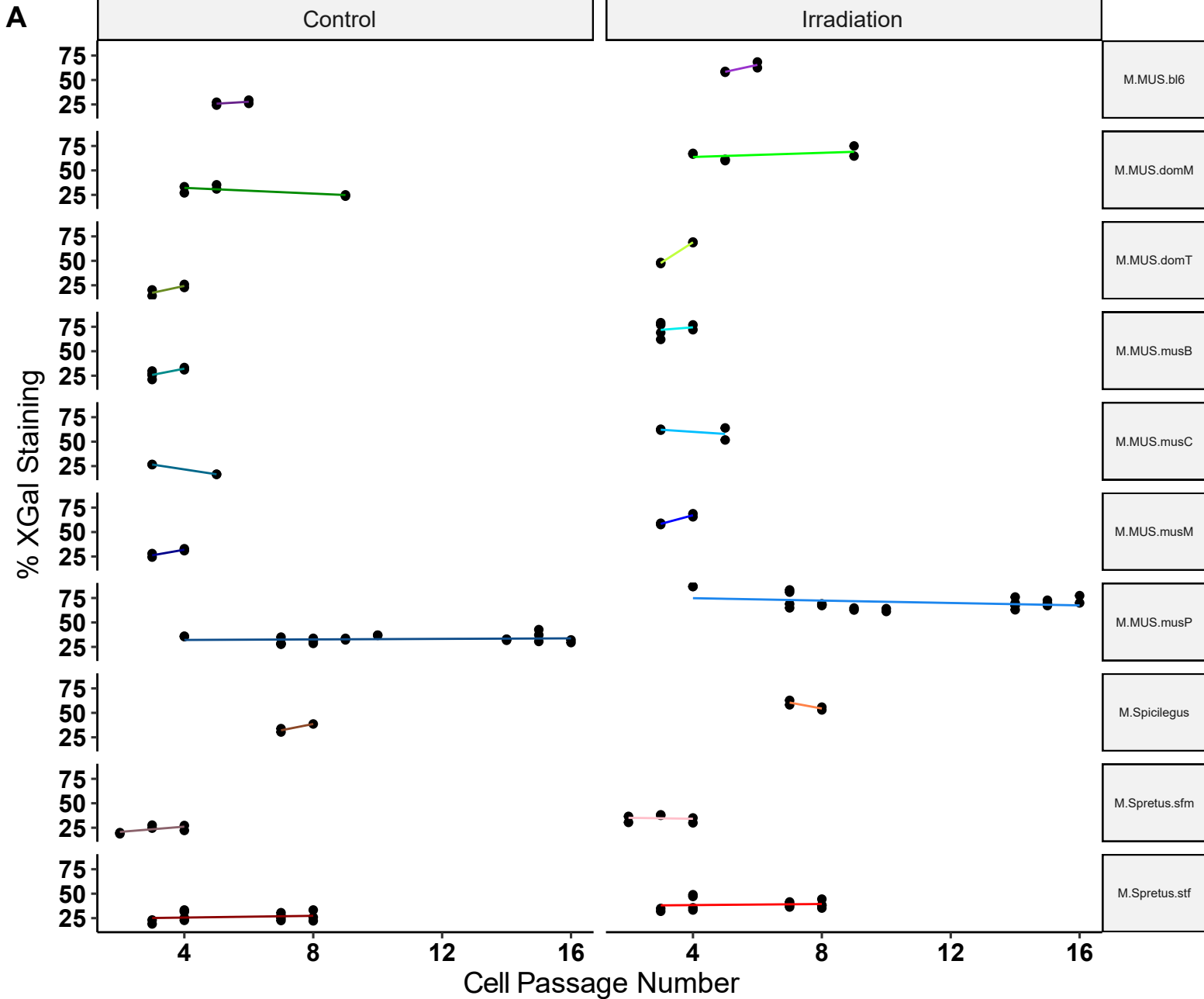
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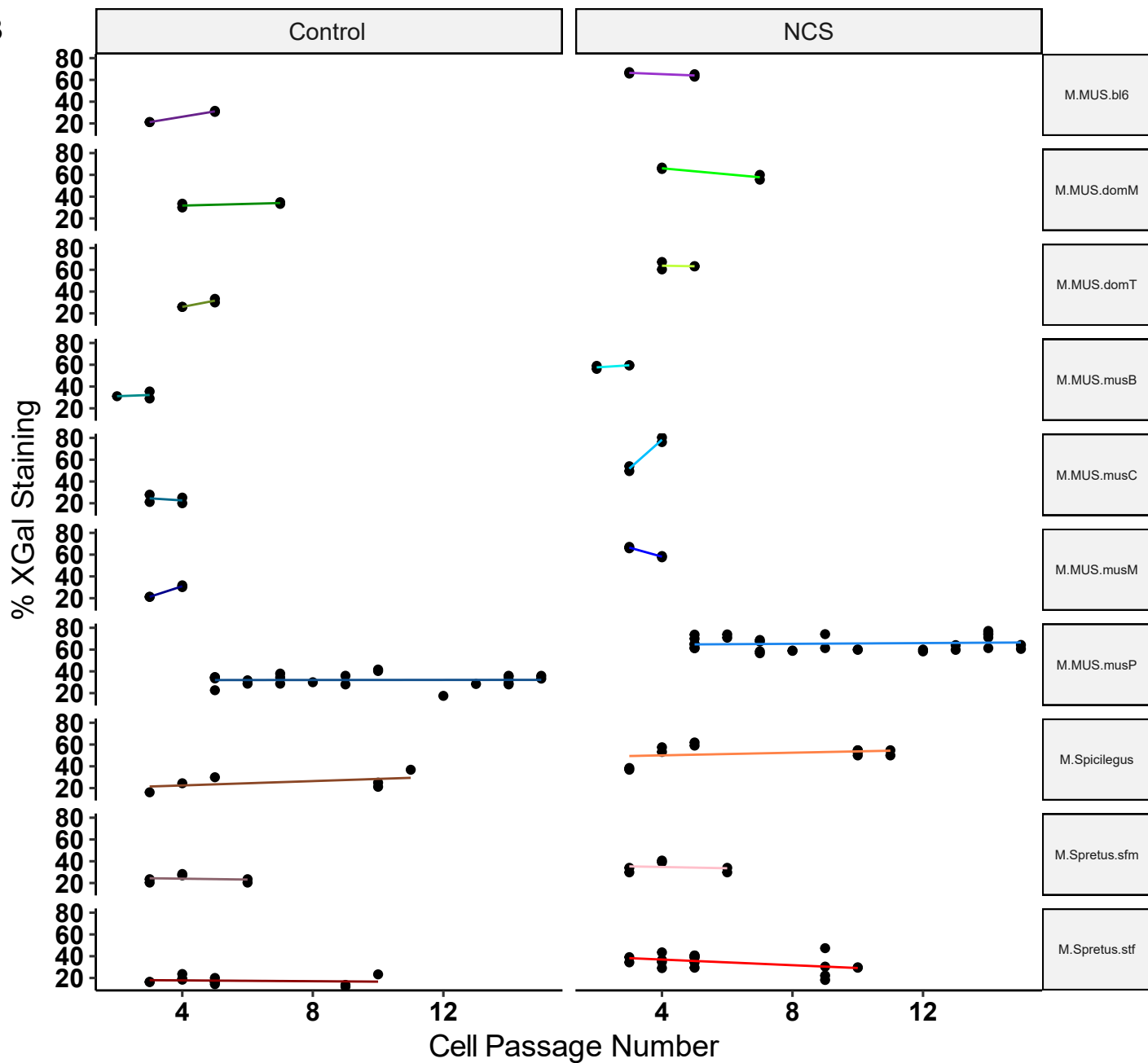


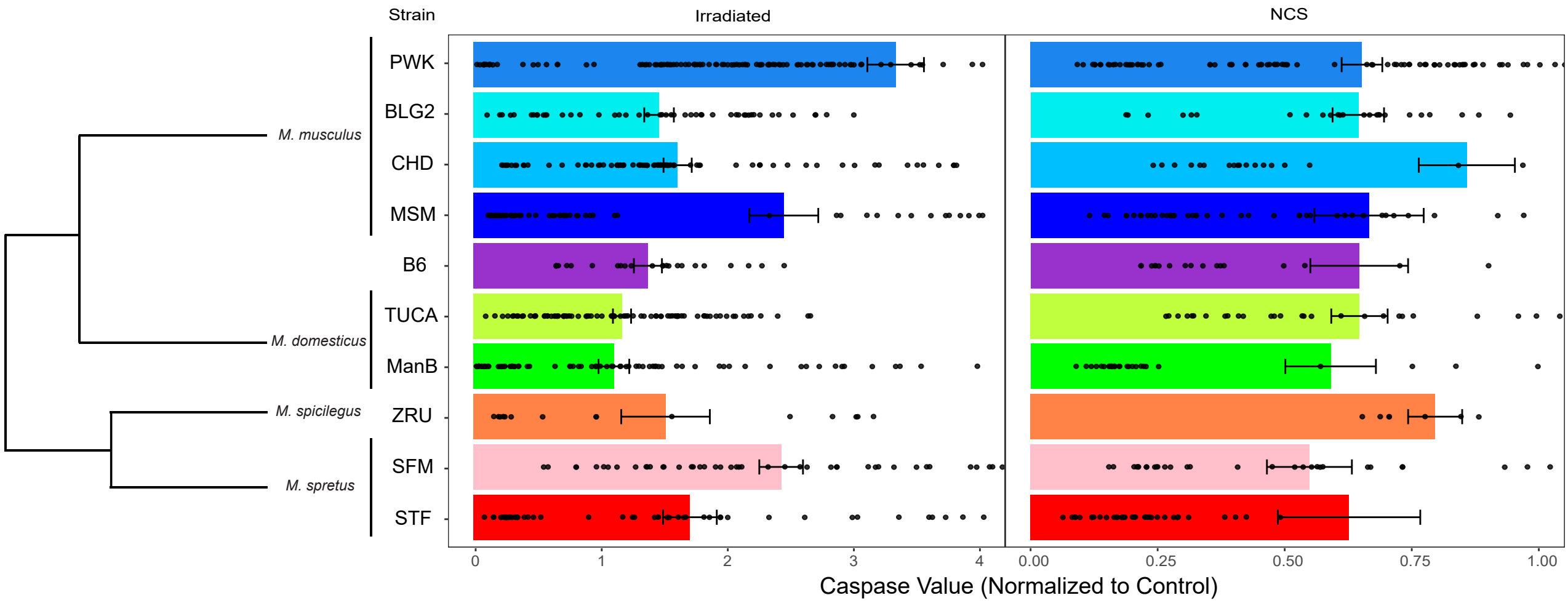


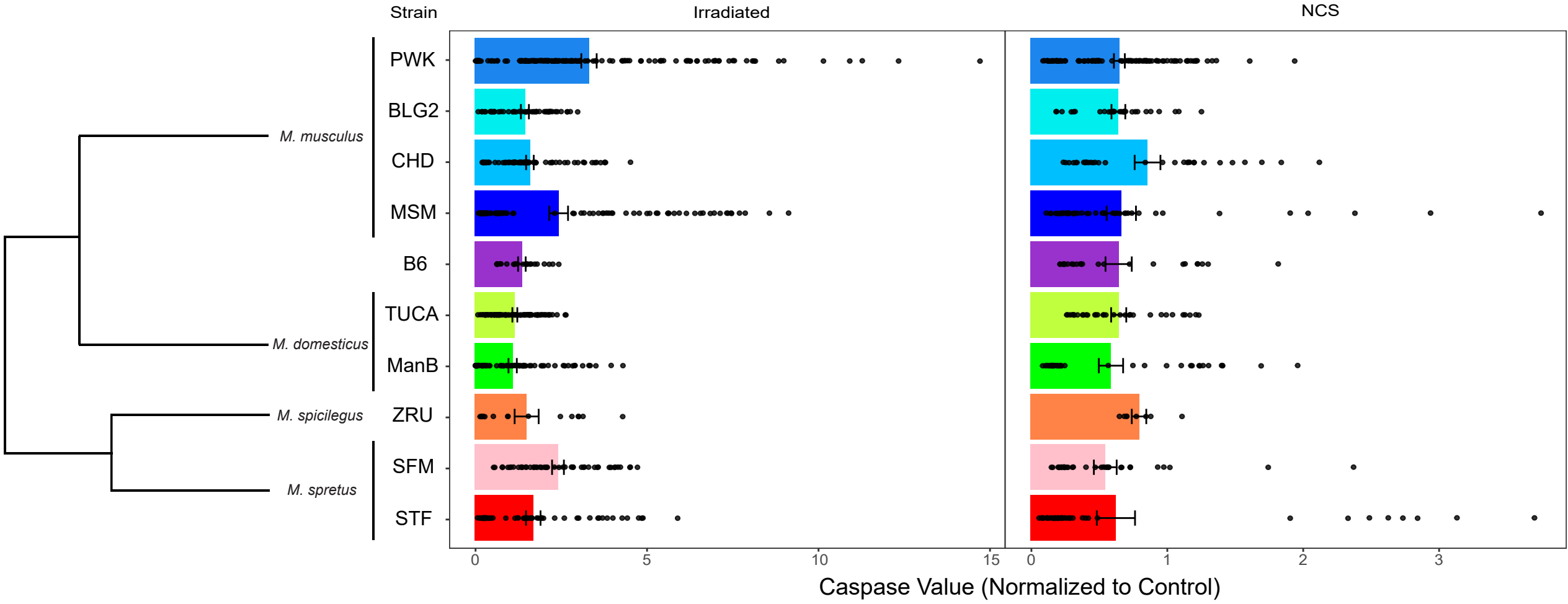


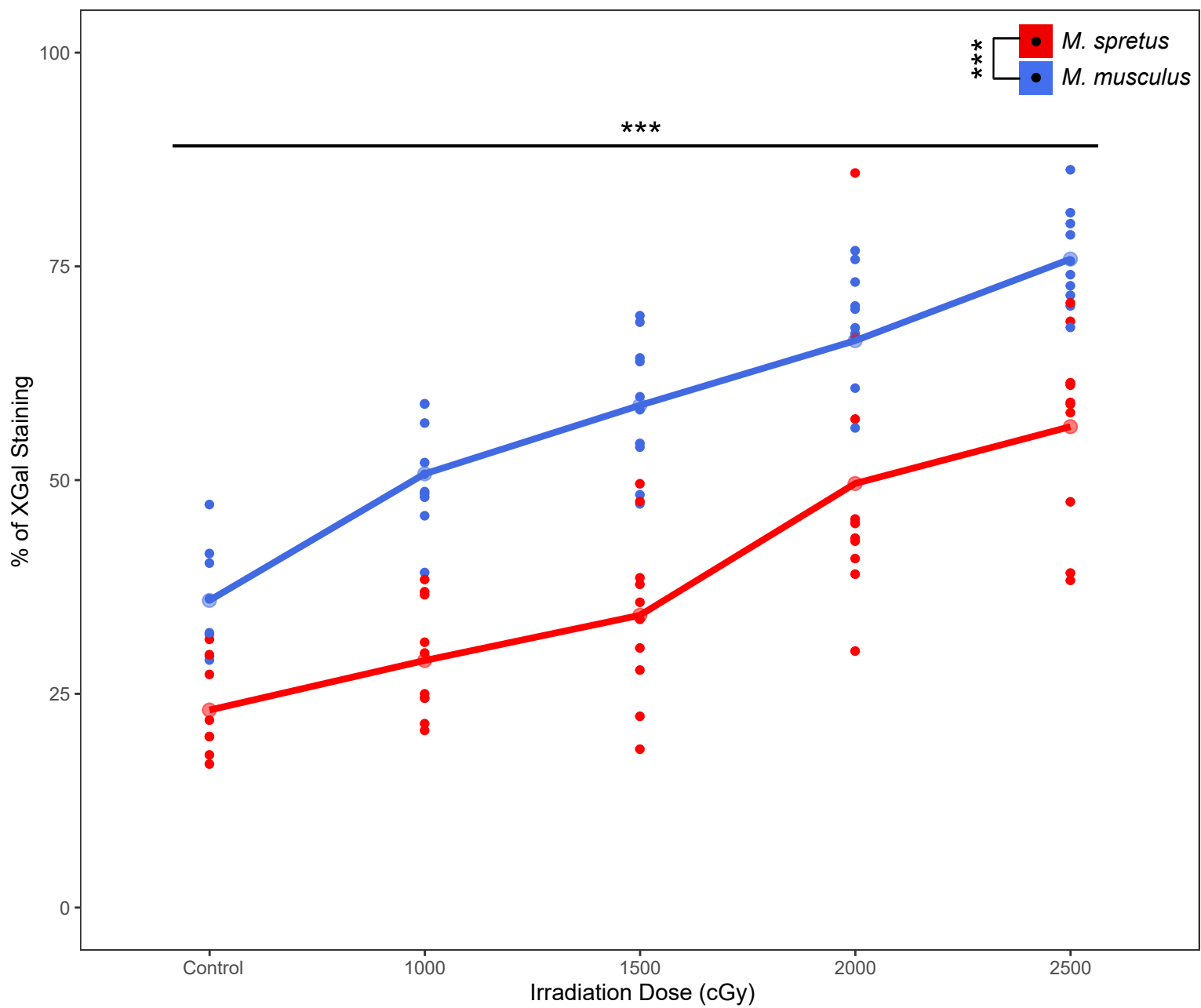




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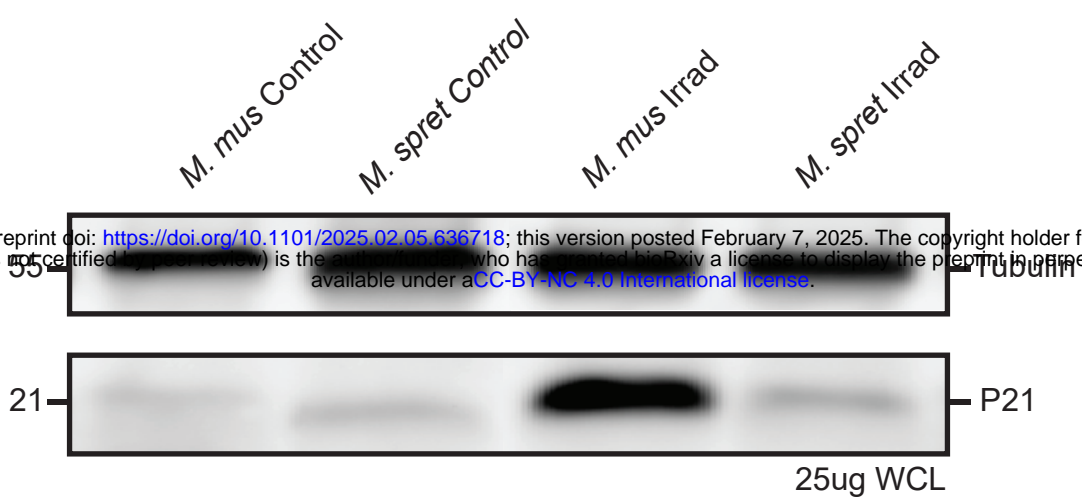
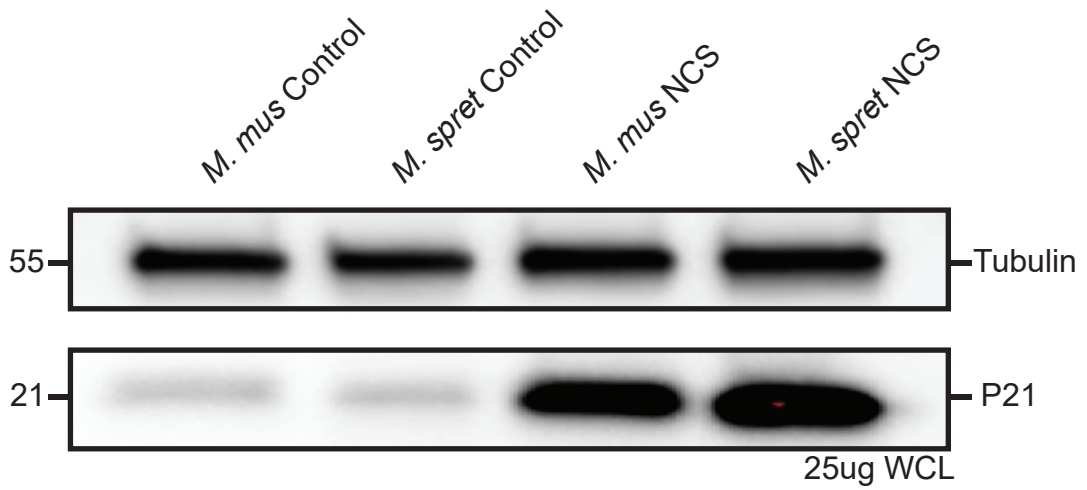
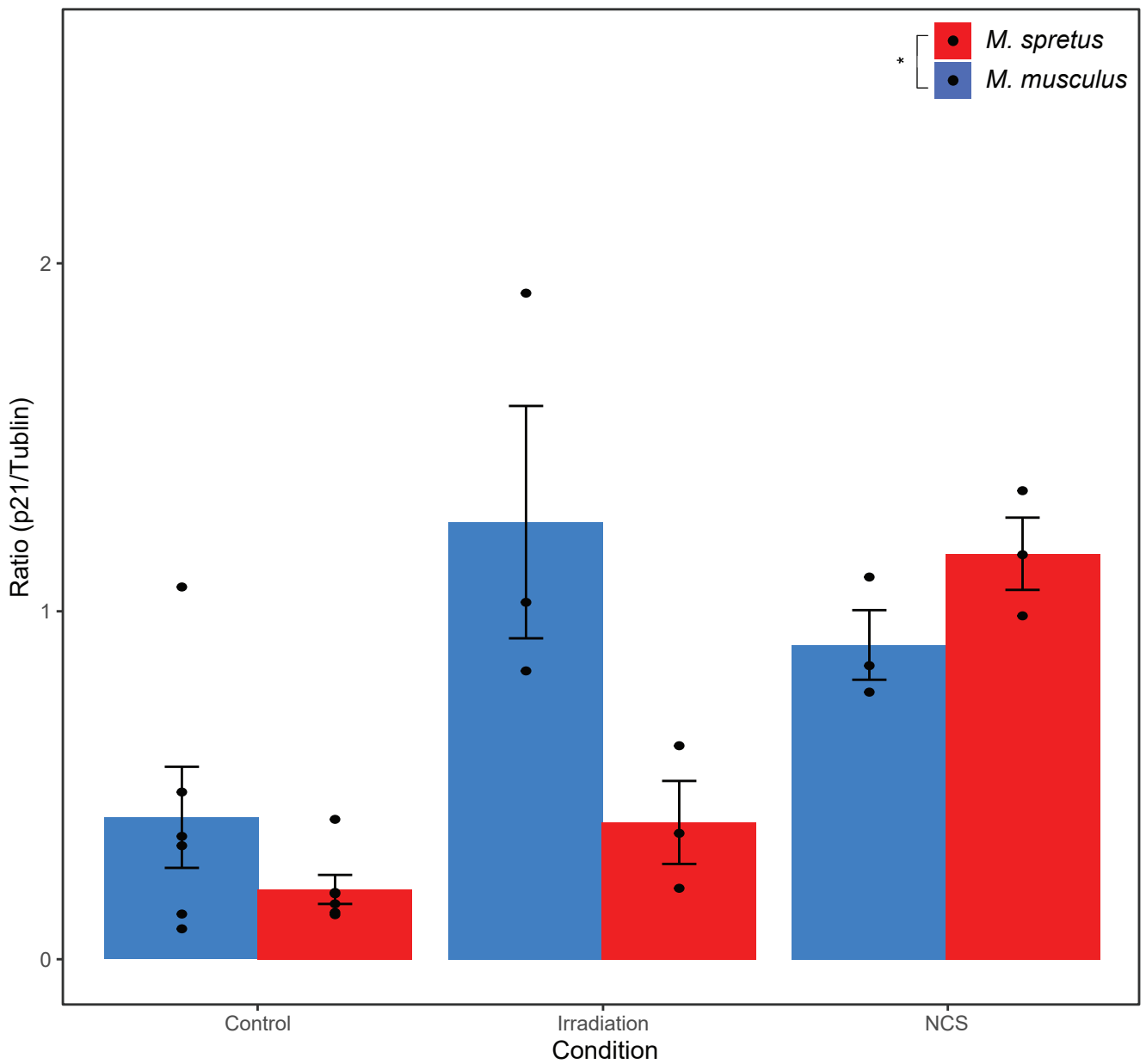
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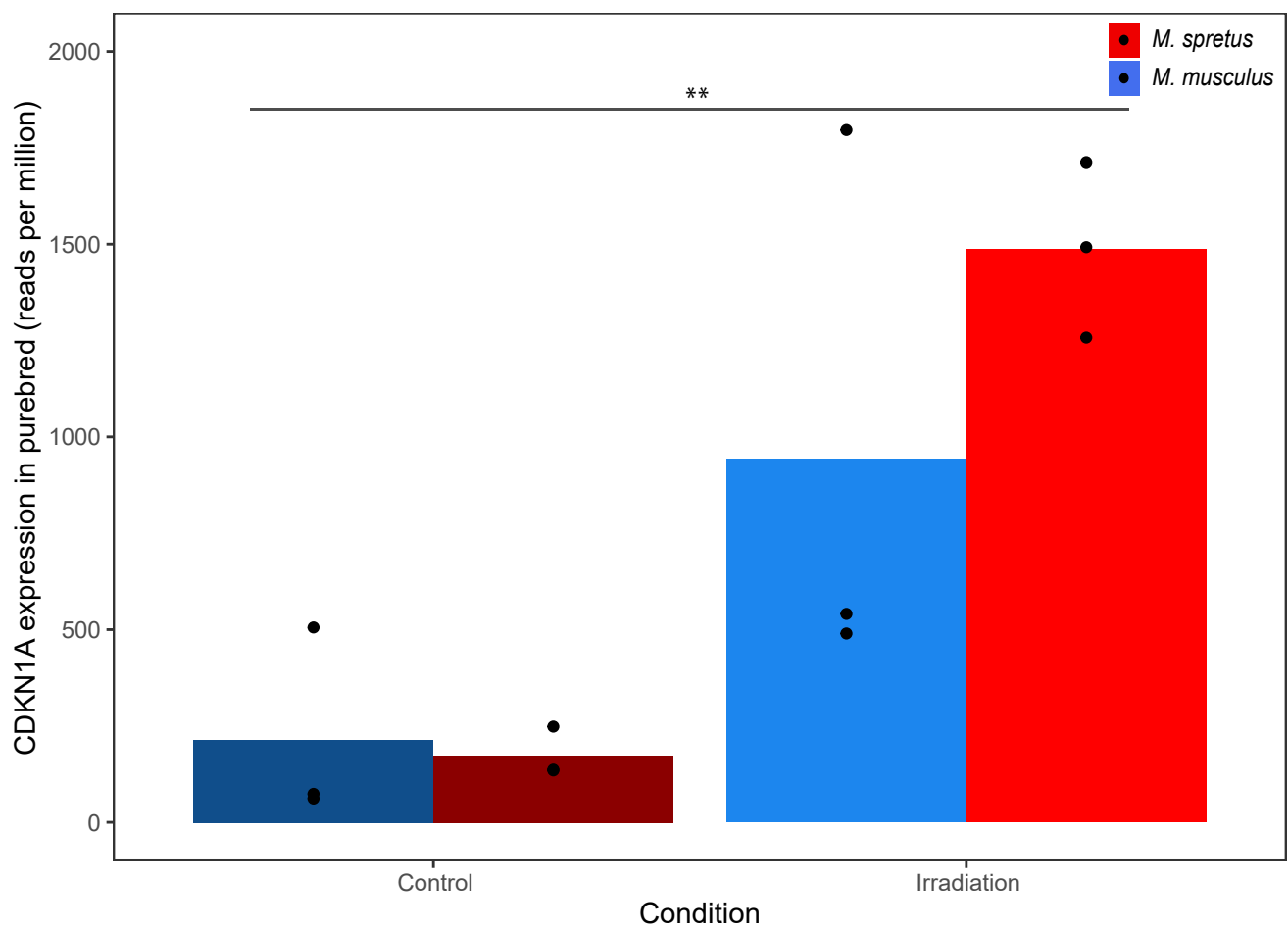
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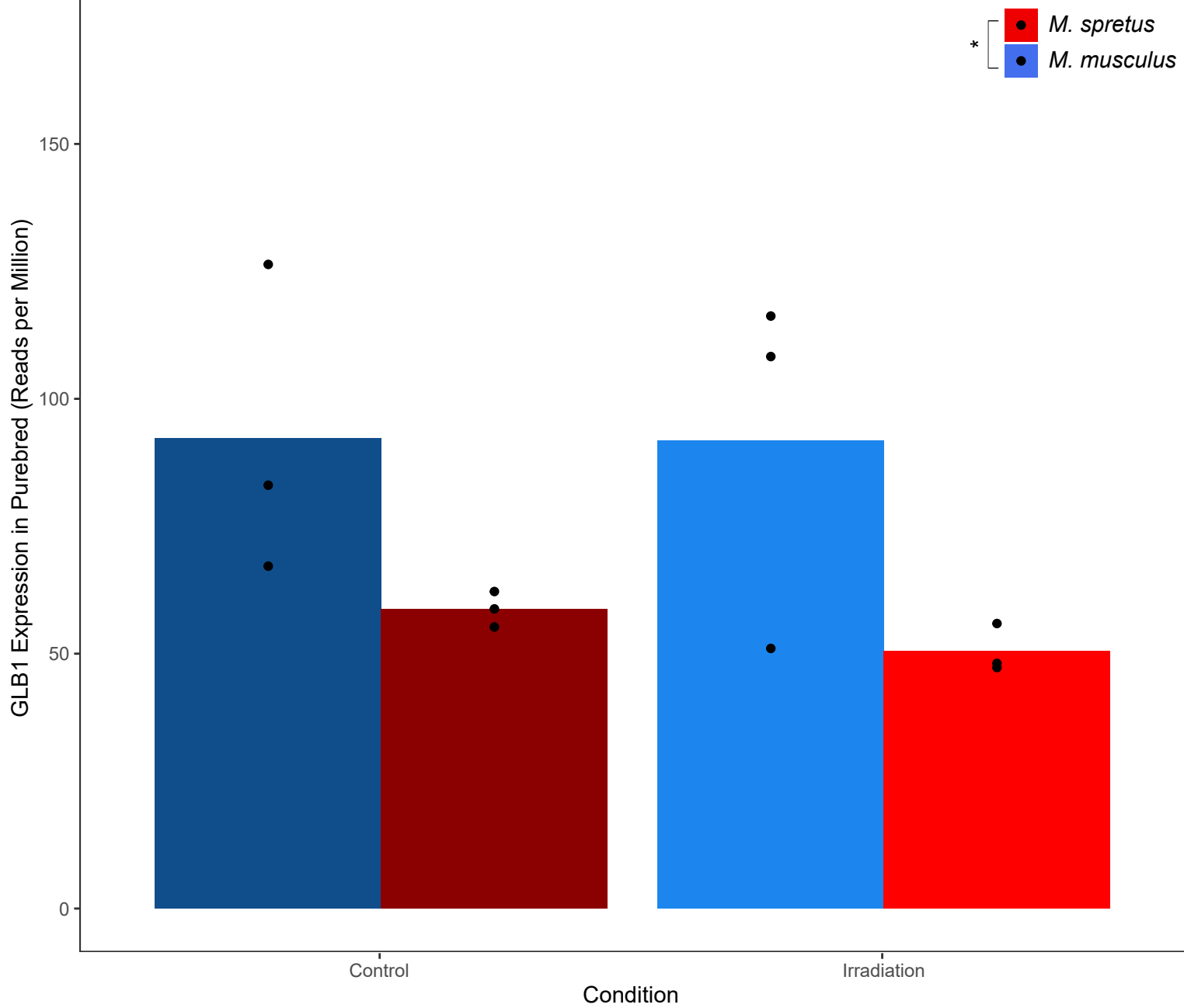
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**B****C**





**A**

**B**