STUDY SUMMARY REPORT

STUDY NUMBER: 138_L

GENERATION AND BASELINE NATURAL HISTORY OF FOUR NEW MOUSE MODELS

FOR

CALPAIN 3 DEFICIENCY

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COALITION TO CURE CALPAIN 3, INC

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I. Introduction

Limb girdle muscular dystrophy 2A (LGMD2A), caused by mutations in Calpain 3, has been difficult to model in mouse, limiting the use of current models for preclinical drug development. Current knock out models of Calpain3 deficiency on a C57BL/6J genetic background are relatively mild in their phenotype with only minor histological and locomotor deficits. Moreover, the mice are not readily accessible to the scientific community. We created and characterized Calpain3 knockouts in three different, widely used inbred strains: 129/Sv (reference strain), DBA2/J, and FVB/NJ. In addition, we also created a knock out in a strain from the Collaborative Cross, CC041/UncJ. The goal was to develop new LGMD2A models with a more clinically-relevant phenotype, while observing if differences in genetic background affect the phenotype, and to make this new resource available to the community from a public mouse Repository. The different strains were compared for in vivo phenotype severity, serum creatine kinase, and post-mortem muscle histology.

II. Crispr/Cas9-mediated Calpain 3 mutations and protein studies

We used CRISPR/Cas9 microinjected in embryos to create a 1759 nucleotide deletion for strains 129-Capn3, and FVB-Capn3, a 1748 nucleotide deletion in CC041-Capn3, and a 1711 nucleotide deletion in DBA-Capn3. All deletions removed exons 2 and 3 and resulted, in silico, in premature stop codons. We crossed heterozygous mice of each strain to generate homozygous, heterozygous mutants and wild-type littermates in each strain, and validated Calpain 3 deficiency on their tissues.

Protein studies were performed with the WES Simple system, a capillary-based, fluorescence detection system that is more quantitative than traditional western blots. To carry out Calpain 3 protein quantification, we first validated for specificity and sensitivity a commercial polyclonal antibody (ProteinTech, 10415-1-AP). Mouse Capn3 was successfully detected as two proteins, the active (at ~60 kDa) and inactive (full length at ~93 kDa) forms, in a linear fashion over a range of tissue extract dilutions, thus validating the quality of the antibody (slide 3).

The validated antibody was used to probe tissue extracts from the homozygous, heterozygous mutants and wild-type littermates in each strain. For all 4 strains, we observed a 50-70% reduction in the amount of Calpain 3 in the heterozygous mice and a complete absence in the homozygous mice (slide 4), hence demonstrating that the mutations in all strains result in a complete loss of Calpain 3.

III. In vivo phenotyping.

Cohorts of homozygous mutants and wild type littermates in all three strains were then raised for longitudinal phenotyping and, terminally, histopathology. Over 160 mice were used in the study. Body weight was recorded as mice aged, and the only difference noted was CC041 homozygous females weighed more than wildtype females; no difference between homozygous and wild type mice of either sex, was observed in the other strains up to 14 months of age (slides 6-7). Mice were tested

longitudinally on a variety of locomotor tests for neuromuscular phenotypes. 129-Capn3, FVB-Capn3 and DBA-Capn3 were tested on the open field, rotarod, wheel running and treadmill exhaustion tests at 4-6 months of age. Few clinically-relevant phenotypes were observed at this age with these assays, and mice, including the CC041-Capn3 cohorts, were aged further to 12-16 months of age, to allow for a phenotype to manifest. At this age, we replaced treadmill running, a low throughput assay, with grip strength testing, more amenable to battery testing in drug studies, in 129-Capn3, FVB-Capn3 and CC041-Capn3. CC041-Capn3, which had not been tested on treadmill at 6 month, was tested at this age. Locomotor phenotypes were still mild in these strains at this age, thus, mice were aged a few more months, and tested with a more sensitive technique, the in vivo force recording.

Breeding and husbandry of strain DBA-Capn3 proved challenging and the cohorts were generated later than for the three other strains. At the time of the first report, cohorts from this strain had reached the 7-month testing point. To provide an element of comparison with the other strains, the DBA-Capn3 were tested on the complementary, in vivo force tests above at 6 months, then again at 20 months

A. Locomotor phenotyping in all strains at 4-6 months of age

1. Description of the tests.

For rotarod testing, mice were acclimated to the testing room for at least 30 min before the test. Mice were then placed on a rod starting at 4 rpm, which accelerates to 40 rpm over 300 sec. Latency to fall was recorded. Three trials were administered with a 45 sec rest between trials. The rotarod tests a broad range of locomotor features including coordination (neuronal phenotype), and axial and limb muscle strength (muscle phenotype).

In the open field test, mice were acclimated to the test room for at least 30 min before being placed individually in 40cmx40cm open arenas. Movements were tracked for a period of 60 min, and automated analysis of the video-recordings returned the total distance travelled, the time spent walking, and the time spent resting, in the horizontal dimension; light beams positioned above the area provide vertical activity measures through beam-breaks, and the number of rearings was also quantified. These measures were taken in 12 intervals of 5 min each. The open field tests the spontaneous activity, which can be affected by either muscle weakness, or neurobehavioral phenotypes such as anxiety, which reduce the overall locomotion and time spent in the center of the arena (also recorded), respectively.

The running wheels were placed in the home cage of mice individually housed, for at least three days. Each wheel is wirelessly connected to a computer to which it feeds the number of wheel rotations and the time the mouse spends on the wheel, by 15 min intervals. The first day of monitoring was not analyzed, as it represents the habituation period. For the two last nights and last days, activity on the wheel was recorded and analyzed. The running wheels can detect muscle weakness, as well as other neurobehavioral deficit in mice such as hyperactivity or depression.

For the treadmill test, mice were placed in individual lanes, and were acclimated on the stopped treadmill for at least 15 min. The treadmill was then set in motion at 5 m/min for five minutes, and the

speed was increased by steps of 5 m/min every five minute to a maximum speed of 25 m/min. The treadmill was gently inclined to a 5 degrees angle; at the base of the treadmill, an electrical prodding pad was set to deliver unpleasant pulses. Mice were deemed to have reached exhaustion when they stayed on the prodding pad for more than 15 consecutive seconds, at which time they were removed and returned to their cage. The treadmill tests locomotor coordination, muscle fatigue, and resistance of muscles to a mild exercise.

2. Results of the Rotarod, open field, running wheels and treadmill testing at 4-7 months

On the rotarod, at 4-7 months, both male and female mutants of Strain 129-Capn3 had a slightly lower latency to fall than their wild type controls (slides 9-10). In the FVB-Capn3 (slides 11-12), male mutants had a slightly lower latency than their controls, while females were normal. None of these differences reached statistical significance, but suggested that at an older age, the phenotype would become stronger. In DBA-Capn3 (slides 13) males and females were normal.,

On the open field test, 129-Capn3 females presented a slight hyperactivity in average, compared to their controls. In-depth analysis revealed that this effect was caused by a single, outlier female with high hyperactivity. 129-Capn3 males had a lower locomotor activity than their controls. These differences were significant, both for the horizontal and vertical activities (slide 14). FVB-Capn3 mice showed signs of hyperactivity as well, with a higher horizontal activity in males, and more time in the center, for females (slide 15). One hyperactive, outlier female caused the difference in the group average. Males, with a higher horizontal activity, showed a decreased vertical activity, which could be interpreted as a weakening of the axial muscles. In DBA-Capn3 mice, the males only, but not the females, had a slightly reduced activity (slide 16).

Home cage wheel running revealed a slight hyperactivity of the 129-Capn3 (slide 17) and FVB-Capn3 (slide 18) males and females compared to their wild type controls, at 4 months, who ran twice to three times more during the active nocturnal period for 129, and about 50% more for FVB. There was however an important inter-individual variability between mutant mice of each group, and the difference were not significant. Last, a strong, significant hyperactivity was observed in the females of DBA-Capn3 (slide 19), who ran twice as much as their wild type controls, but not in the males.

On the treadmill test, two protocols were used. For most of the experiments, treadmill speed was increased by 5m/min increments every 5 minutes to a maximum speed of 25 m/min reached at 35 min of testing (JAX protocol). A separate cohort of 129-Capn3 mice was run on a protocol adapted from the Spencer lab with speed increased by 1m/min increments every minute (Spencer protocol, slide 21). No difference in the time and distance to exhaustion was observed at 4-7 months in either of the strains with the JAX protocol (slides 20-22). The only, minor weakness observed was in FVB-Capn3 (slide 21), whose females, specifically, reached exhaustion slightly faster, but not in a significant manner. The slightly more aggressive test (Spencer protocol) unmasked a mild weakness in the 129-Capn3 males when tested at 9 months of age (slide 20)

3. Conclusion of the in vivo phenotyping at 4-7 months

Overall, none of the Calpain 3 deficient groups in the 129, FVB and DBA background displayed a strong locomotor phenotype at 4-7 months of age. The only significant deficits found were for 129-Capn3 males in the open field, where they displayed a slightly reduced locomotion; and, at 9 months with a special treadmill protocol (Spencer protocol), a reduced exercise tolerance on the treadmill.. An unexpected finding consisted in the higher spontaneous locomotion for all strains observed on the running wheels, which may suggest that Calpain 3 deficient mice present behavioral phenotypes.

B. Locomotor phenotyping in all strains at 12 months of age

1. Description of the additional test: grip strength.

Subjects are weighed and acclimated to the testing room for at least 60 min. The equipment is a Bioseb grip strength meter equipped with a bar for grasping that is suited for mice. Mice are lowered towards the bar by their tails to allow for visual placing and for the mouse to grip the bar with their forepaws. Subjects are firmly pulled horizontally away from the bar (parallel to the bench) for 3 consecutive trials with a brief (approximately 30 sec) rest period on the bench between trials. The average strength over the 3 trials is used for graphs and statistics.

2. Results of the Rotarod, open field, running wheels and grip strength testing at 12-16 months

At 12-16 months, male, but not female mutants of Strains 129-Capn3 and FVB-Capn3 had a slightly lower latency on the rotarod than their wild type controls (slide 24-27), with a non-significant difference. There was no phenotype in the DBA-Capn3 males (slide 28) but DBA-Capn3 females had a shorter latency to fall (slide 29). In the CC041-Capn3 strain, male mutants had a lower latency than their controls, on average, but a closer analysis of the results showed that this was caused by a difference during the 2nd trial, which disappeared in the third trial, indicating a procedural learning delay instead of true muscle weakness (slide 30).

On the open field test, 129-Capn3 females presented a slight hyperactivity in average, compared to their controls. In depth analysis revealed that this effect was caused by a single, outlier female with high hyperactivity. 129-Capn3 males had a lower vertical activity than their controls but their horizontal activity was normal (slide 31). FVB-Capn3 males presented an overall depressed activity, both vertically and horizontally, which could suggest a worsening of their open field phenotype first observed at 4-6 months – however, in absence of phenotype in the other tests (see below), this result must be taken with caution (slide 32). CC041-Capn3 males (which were not tested at 4-6 months) presented with a mild hyper-activity, vertically and horizontally (slide 33).

On the running wheels, the activity of 129-Capn3 males was depressed, in sharp contrast from their hyperactivity at 4 months, while 129-Capn3 females remained slightly more active than their controls

like at 4 months (slide 34). There were no differences between mutants and wild type mice in either FVB-Capn3 or CC041-Capn3 (slide 35-36).

Grip strength showed no phenotype in either of the strains (slides 37-39).

On the treadmill, with the JAX protocol, the strains CC041-Capn3 and 129-Capn3 showed no deficit at 12 months (other strains were not tested, slides 40-41)

3. Conclusion of the in vivo phenotyping at 12 months

The 129-Capn3 males, specifically (but not the females) presented at 12 months a set of phenotypes consistent with developing neuromuscular weakness: on the rotarod (non-significant), open field (visible on the vertical activity only) and the running wheels. The use of a specific, more challenging treadmill protocol elicited signs of exercise intolerance in the on the 129-Capn3 at 9 months. Because this specific protocol (from the Spencer lab) was not used on the other strains, the observation is isolated but noteworthy. Overall, these results are consistent with the findings at earlier ages, where 129-Capn3 were the only mutants to present a meaningful locomotor weakness phenotype.

IV. In vivo specific muscle force and resistance to exercise-induced damage in late mice.

In absence of strong locomotor phenotype on the previous tests, we next tested muscle specific force and resistance to exercise in the Capn3 mutants. This measure of muscle properties is known to unmask weakness and exercise intolerance in other neuromuscular mutants with otherwise weak phenotypes on traditional tests, like the B10.mdx mutant.

1. Description of the muscle specific force system.

The measure, in vivo, of the force output of a single muscle group formed by the tibialis anterior (TA) and extensor digitorum longus (EDL), is achieved as follows. Mice are lightly sedated and lay on their back, with the left leg secured on a stand at the level of the knee. The stabilized hind limb is positioned horizontally, perpendicular to a footplate holding the foot, to measure the torque of the dorsiflexion of the ankle when the tibialis anterior (TA) muscle contracts. The foot plate opposes the dorsiflexion while measuring the torque, so that the isometric force is measured. The footplate subsequently imposes a plantar flexion of the foot while the TA is stimulated tetanically to measure force during a series of 20 eccentric contractions, mimicking exercise. Muscle contraction is elicited by transcutaneous electrical stimulation of the peroneal nerve. Torque values (Nm) are normalized by the body weight (Nm/g). Normalized torque values are plotted against stimulation frequencies between 0-150 Hz to yield a torque-frequency curve for each mouse, for the isometric measurement. Curves are averaged within genotype and sex groups and mutant groups compared to curves in the control group. For the fatigue protocol, 20 eccentric contractions are induced by imposing a plantarflexion of the foot, from 90 to 135˚, at 1200˚/sec, while the TA is tetanically stimulated at 150 Hz; a rest period of 10 sec separates

successive eccentric contractions; tetanic torques are recorded before the first contraction, and at the 20th contraction. The ratio of the last to the first contraction quantifies the percentage of loss in force due to the repeated injury: this ratio is close from 1 in normal mice, and its decrease indicates exercise-intolerance, i.e. an increased sensitivity to exercise-induced muscle damage.

2. Results of the in vivo muscle force measurements.

These measurements were administered on all available cohorts shortly before the euthanasia and tissue collection for histology. Cohort ages were: FVB-Capn3 and 129-Capn3, 20 months; CC041-Capn3, 14 months; DBA-Capn3, 7 and 20 months.

The force-frequency curves measuring isometric torques for a short contraction of the muscle showed no difference between Capn3 mutants and controls in either sex or strain (slides 43-46), with the notable exception of the DBA-Capn3 females at 20- months, that were weaker than their wild type controls

For the post/pre-exercise ratios, as expected, most wild-type mice in the FVB, 129and CC041 strains returned values close from 1, indicating the ability of their muscles to sustain, repeatedly, eccentric contractions without drop in force (slide 48-51). In the CC041-Capn3 mutants, both males and females, showed a significant drop in ratio, a sign that their muscles accumulated injuries over the repeated eccentric contractions (slide 50). In DBA, both mutant and control groups showed a similarly strong decrease of the ratio, consistent with other findings at JAX that the DBA/2J strain is naturally sensitive to exercise-induced injury; the Capn3 mutation did not sensitize the DBA muscles further (slide 51).

3. Conclusion of the in vivo force measurement.

None of the Capn3 mutant group presented a decrease in acute strength, a sign that skeletal muscles are only mildly, if at all, altered in their excitation-contraction properties. The CC041-Capn3 mutants were the only ones presenting a decrease in strength over repeated eccentric contractions, indicating that the Capn3 mutation in this genetic background either specifically alters the excitation-contraction coupling, or increases the fragility of the sarcolemma, making it more prone to contraction-induced tears. Further analyses are needed to tease out both possibilities.

V. Serum Creatine Kinase

Serum creatine kinase is a blood biomarker of the breakdown of the muscle membrane, or sarcolemma. It is naturally very low in mice with healthy muscles. There was a very significant increase in serum CK in FVB- and CC041-Capn3 mutants at 13-19 months of age (slide 53). DBA-Capn3 mutants had the most severe increase seen at 6 mo. When longitudinal CK was measured in 129-Capn3 mice from 3 to 19

months, we found a transient increase in serum CK at 3 months in males (but not in females) that normalized at later ages (slide 54). From the study of other mouse models of muscle breakdown like the mdx mouse, it is well established that younger mice with muscular dystrophy have higher CK, and serum CK decreases with age. Consistently, at the time of the measures, CC041-Capn3 and DBA-Capn3 cohorts were slightly younger than the FVB cohorts. The time course of serum CK in the 129-Capn3 strain also align with observations in other models. In addition, we observe a correlation between the sensitivity to repeated eccentric contractions in CC041, and their high CK, both findings being consistent with this strain presenting the most severe consequences of the Capn3 deficiency on sensitivity to exercise.

VI. Histopathology.

1. Description of the histology stains.

Histopathology sections were prepared for both male and female mice. Four muscles were harvested and stained: the psoas, the diaphragm, the soleus and the tibialis anterior. At the time of this report, only male histology on the psoas has been assessed; histology on the other male muscles and on females is ongoing. However, the psoas is expected, from the published Capn3 models, to be one of the most affected muscles, and male mice are more affected by neuromuscular phenotypes than females in a broad variety of models; hence the findings below, despite their interim nature, are likely the most relevant ones.
The cohorts were euthanized, and their muscles analyzed, at slightly different ages: 129S4/SvJaeJ-Capn3 (#31211) at 6, 12 and 21 months; FVB/NJ-Capn3 (#31227) and DBA-Capn3 (#31557), at 21 months; CC041-Capn3 (#31228), at 13 months.
A reticulin stain was used to measure muscle fiber sizes and quantify central nuclei; a Sirius Red stain was used to quantify interstitial fibrosis (in red) and observe general histology.
The psoas, diaphragm, soleus and tibialis anterior muscles were analyzed.

2. Results of the histology on the male psoas.

The pathology was most severe in 129-Capn3 (slides 57-64), and mild in FVB-Capn3 (slides 65-68), CC041-Capn3 (slides 69-72), and DBA-Capn3 (slides 73-76). In all strains, the leg muscles and diaphragm were less affected than the psoas.
The pathology was characteristic of LGMD2A (Rosales, 2013, PMID 23553538) and consisted in central nucleation (a sign a muscle injury and regeneration), interstitial fibrosis (deposit of collagen between the muscle fibers, a sign of inflammation), and replacement of necrosed muscle fibers by fat (or fat infiltration).
In 129-Capn3, there was a significant increase in fibrosis in the psoas, but not the diaphragm or soleus, at 12 months, which was not detected at 6 months of age (slide 57). The same observation was made in the psoas at 21 months (slides 58). Fiber hypertrophy of the psoas (but not of the other muscles, including the tibialis anterior) also developed in both sexes between 6 and 12 months (slide 59-61), along with central nucleation (slide 62-63). There was a transient increase in central nuclei in the

diaphragm and soleus at 6 months, that resolved at 12 months (slide 62). Observation also revealed fat infiltration in the psoas at 21 months (slide 64).
There was no detectable fibrotic or morphometric changes in FVB-Capn3 muscles at 21 months (slides 65-67) and the pathology was similarly mild in CC041-Capn3 (slides 69-71) although this strain was observed at a younger age of 13 months. No detectable fibrotic or morphometric changes were found in DBA-Capn3 muscles at 21 months (slides 73-75). All strains presented some fatty infiltration (slides 64, 68, 72 and 76).
While relevant to the clinical presentation, fatty infiltration and fibrosis (without experimental muscle injury) in Capn3 mouse models have not been observed before in the published knock-outs (Richard, 2000, PMC2150676). It is worth noting that Capn3 mutants have been shown before to be prone to fibrosis: fibrosis after experimental injury has been reported previously (Yalvac, 2017, PMID:29241457).

VII. General conclusion.

In this study, we generated four new mouse models for Capn3 deficiency, in four different mouse strains: FVB, 129, CC041 and DBA. The screen for in vivo locomotor phenotype at different ages, showed than the 129-Capn3 mutants were the only ones to present a robust locomotor deficit at both 4-6 and 12-16 months, specifically on the open field test. Consistent with this weakness, the psoas muscle of the 129-Capn3 mice presented the most severe histopathology. Serum CK in 129-Capn3 was normal at 19 months of age, an indication that muscle degradation had ended at this age however there was a transient increase in serum CK at 3 months of age in this strain. The same transient increase in signs of muscle damage was observed with the percentage of fibers in leg muscles, with central nuclei: it peaked at 6 months before normalizing at 12 months. Muscle force recording on the leg muscle in this strain at 20 months showed no deficit, which could be explained because the bulk of muscle degeneration happens in axial muscles and not the appendages or because because a bout of degeneration had taken place earlier in age but had been repaired by regeneration. The latter hypothesis is consistent with the observation of central nuclei in young, but not aged mice.. The recommendation from this report is therefore to use the 129-Capn3 mutants for drug testing at the earliest age possible, likely 4 months, when spontaneous locomotion is the desired outcome measure.
The other tests used, including rotarod, grip strength, force running on the treadmill and spontaneous running on the wheels did not detect meaningful differences between Capn3 mutants and strain- and age-matched controls in any of the other Capn3-deficient strains. In conclusion, the diversity in genetic background did not lead to the identification of a strain with a phenotype spectacularly stronger than the published one.
A surprising observation was the hyperactivity in young (4-6 months) Capn3-deficient mice in various genetic backgrounds, calling for further investigation of the role in Calpain 3 in the central nervous system. This observation, however, may not be relevant to the clinical presentation of LGMD2A.

A noteworthy finding of this project is the identification of strain CC041 as an enhancer of the muscle sensitivity to exercise-induced damage or fatigue associated with Capn3 deficiency. CC041-Capn3 mutants were the only ones to present a decrease in the post/pre-eccentric contraction tetanic forces. There could be at least two explanations for this observation: either an increased sensitivity of the CC041-Capn3 sarcolemma to elongation-induced tears: this could be quantified with an Evans-Blue

uptake test; or, independently of muscle break-down, a higher fatigability of the CC041-Capn3 muscles, i.e. a loss in the excitation-contraction efficacy upon repeated muscle stimulation, without structural damage to the fibers: this could be tested with a different in vivo force recording protocol whereby repeated isometric (and not eccentric) contractions are administered; or re-testing of the force after a rest period of a few minutes after the first eccentric contraction series, sufficient for a recovery from fatigue, but not from structural damage.

In conclusion, we have delivered a license-free, easily available mouse model with a documented Capn3-deficiency pathology in the 129-Capn3 mutant, which can be used for drug testing immediately. In addition, the CC041-Capn3 mutant presents with an exercise intolerance phenotype which is novel among Capn3 mouse models, but clinically-relevant, and it could support further research in the pathomechanisms of LGMD2.

Calpain3 KO Report

129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J; **JR 031211**

FVB/NJ-*Capn3*^{em9Lutzy}/J; **JR 031227**

CC041-*Capn3*^{em10Lutzy}/J; **JR 031228**

DBA/2J-*Capn3*^{em12Lutzy}/J; **JR 031557**

Update 02.02.22

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Protein Studies

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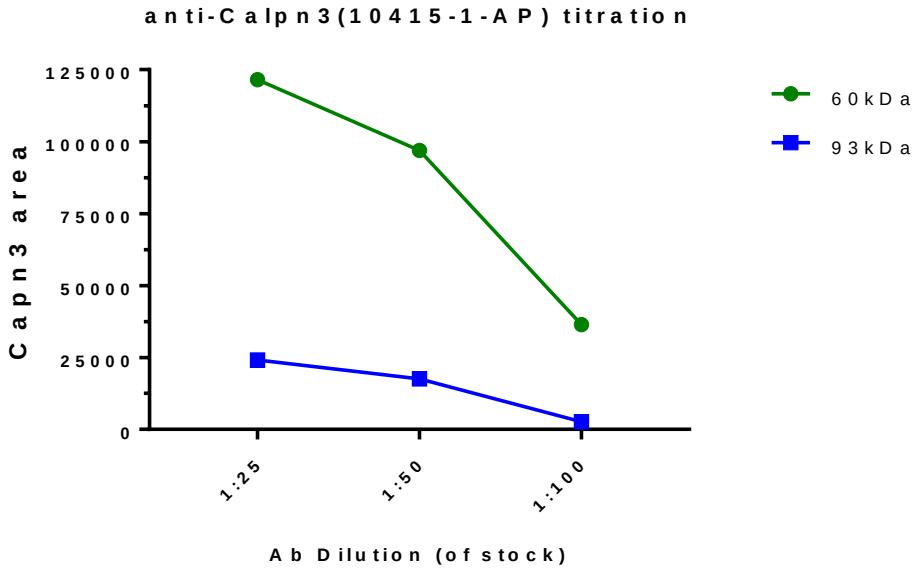
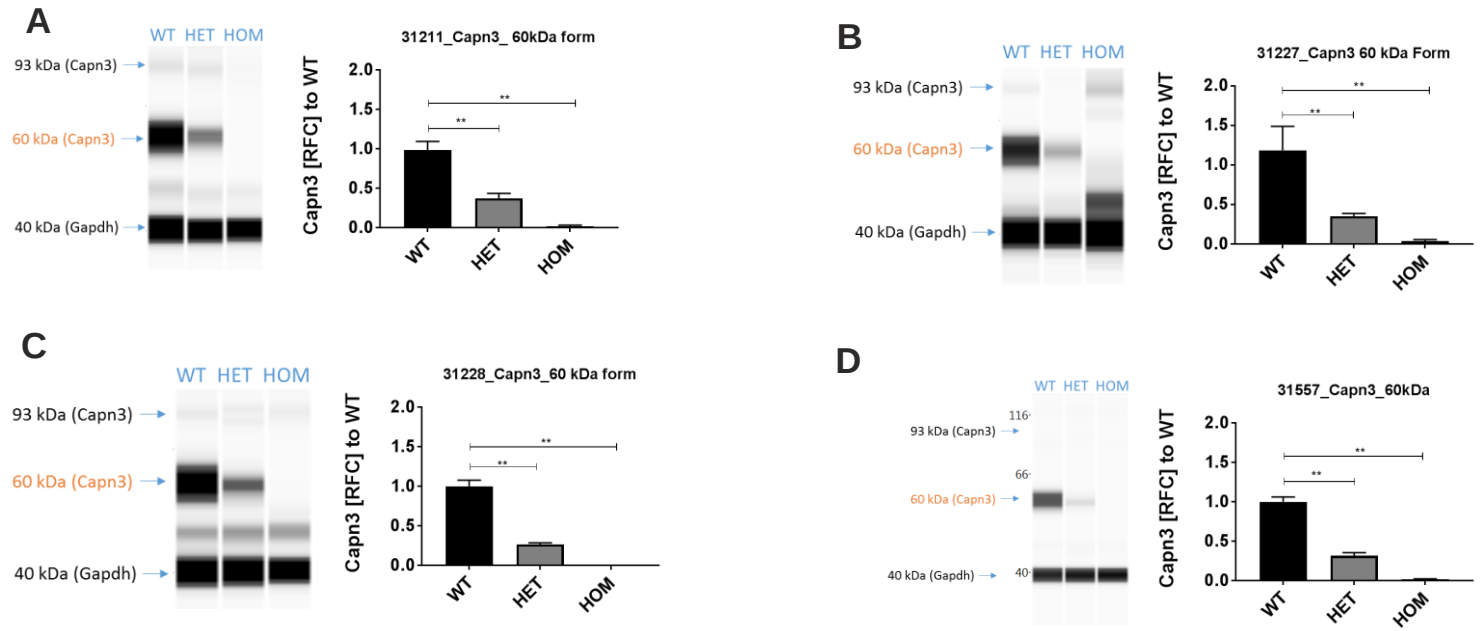
Anti-Capn3 (10415-1AP) titration:



Anti-Capn3 (10415-1AP) Titration. Mouse *Capn3* is detected as active (~60 kDa) and inactive form (full length ~93 kDa). The linear range for detection of both *Capn3* forms with anti-Capn3 (10415-AP) in the automated western blot system Simple Wes.



Western blot. Analysis shows absence of active *Capn3* (60 kDa form) in homozygous mice and a reduction of *Capn3* in heterozygous mice. n = 6(3F/3M); ***p<0.01*, Mann-Whitney t-test

Body weight

(CC041-*Capn3*, DBA-*Capn3*)

and Growth Curves

(129-*Capn3*, FVB-*Capn3*)

comparisons

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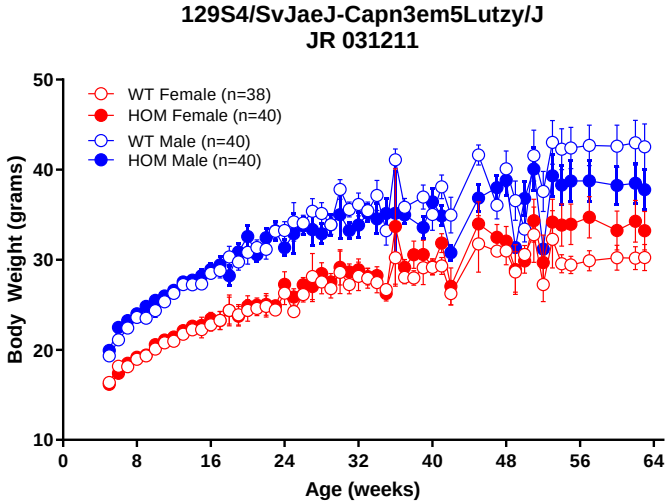
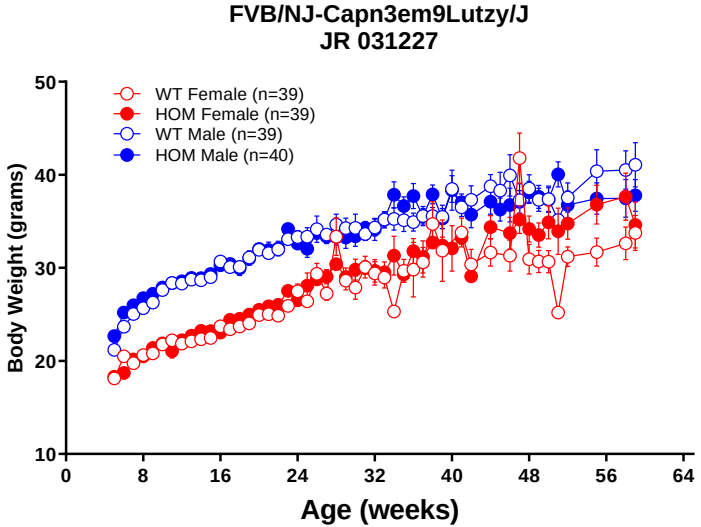
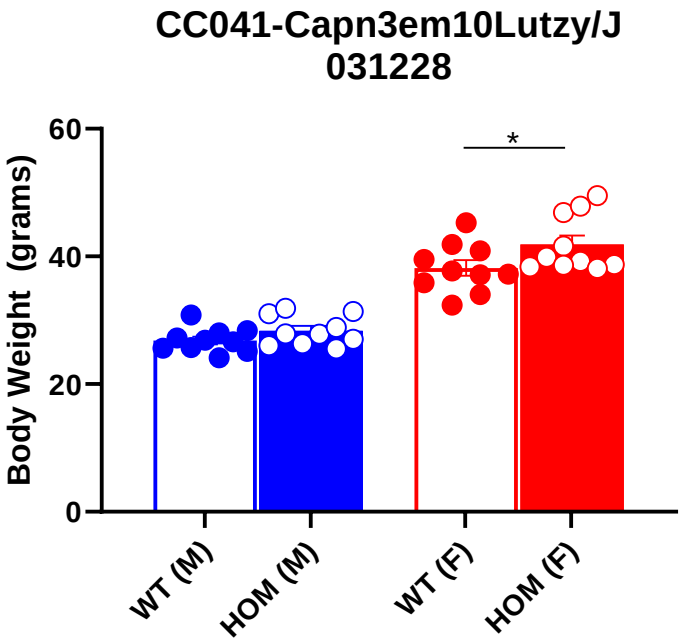
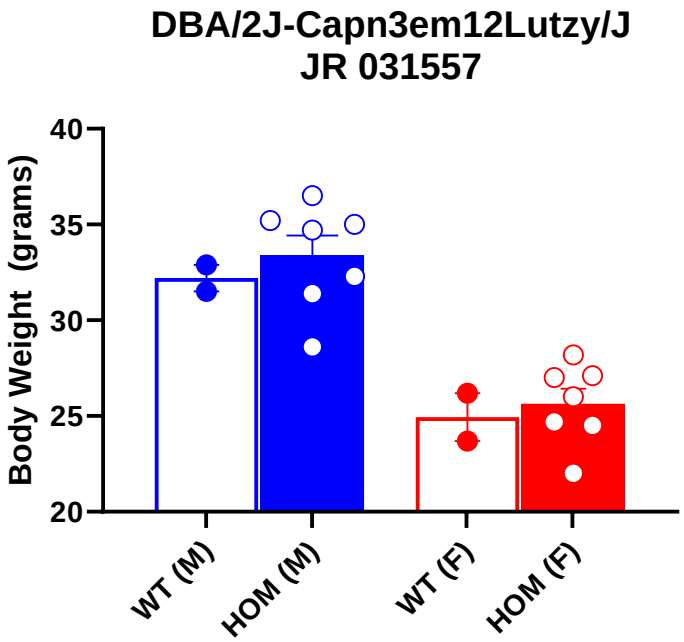




Body Weight. Body weights were collected weekly starting at 6 weeks of age. There are no significant differences in body weight for between genotype for either sex in strains 129-*Capn3*, FVB-*Capn3.* n = 38-40; *p>0.05*, Sidak's multiple comparisons test.





6 Month Body Weight. Body weights at 6 months of age. CC041-*Capn3* HOM females weighed significantly more than WT females *(p*<0.05*);* there are no other significant differences in body weight between genotype for either sex in strains 129-*Capn3*, FVB-*Capn3.* n = 2-10; *p>0.05*, Sidak's multiple comparisons test.

4-12 month locomotor assays

(Strains FVB-Capn3, 129-Capn3 and DBA-Capn3)

Rotarod, Open field,
Running Wheels
and Treadmill exhaustion testing

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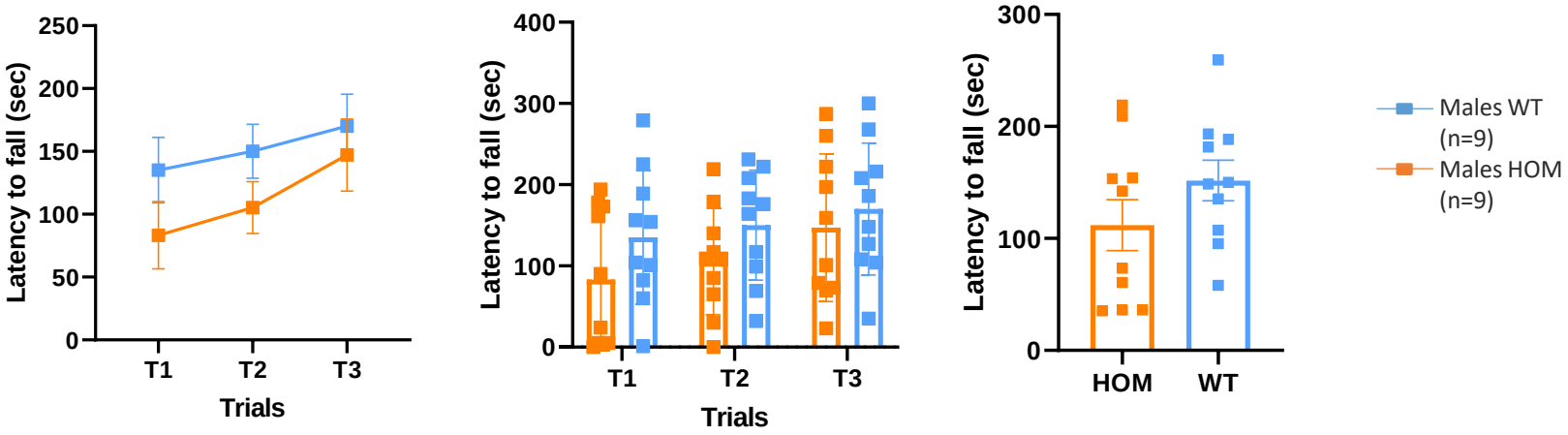
Rotarod, Males

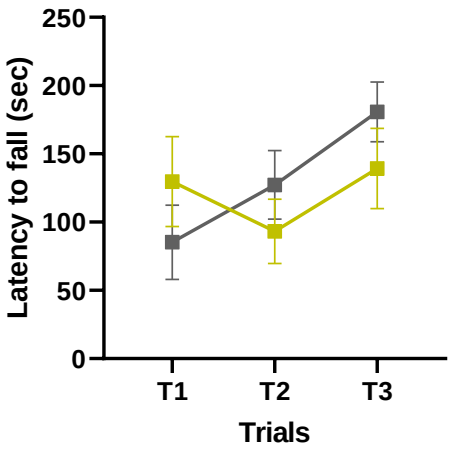
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211

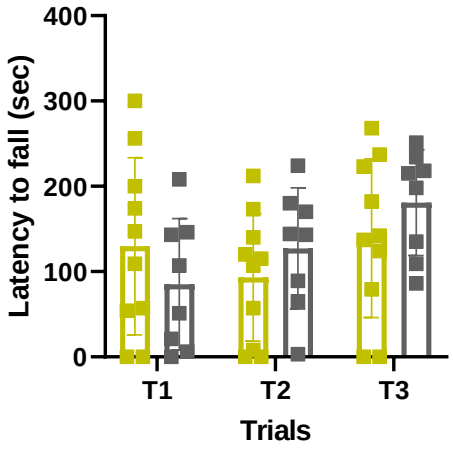


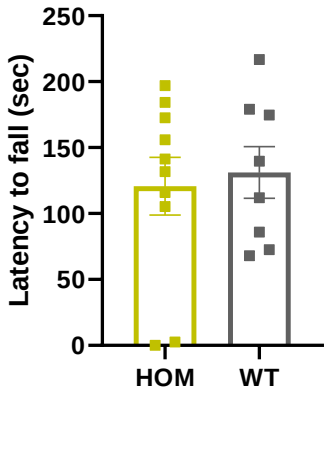
Rotarod test for JR 031211, 4 month Males. No significant differences between genotypes for latency to fall during the 3 trials and average of the 3 trials. n = 9; *p>0.05*, 2-way ANOVA and unpaired t-test.

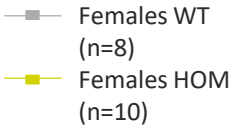
Rotarod, Females

129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211





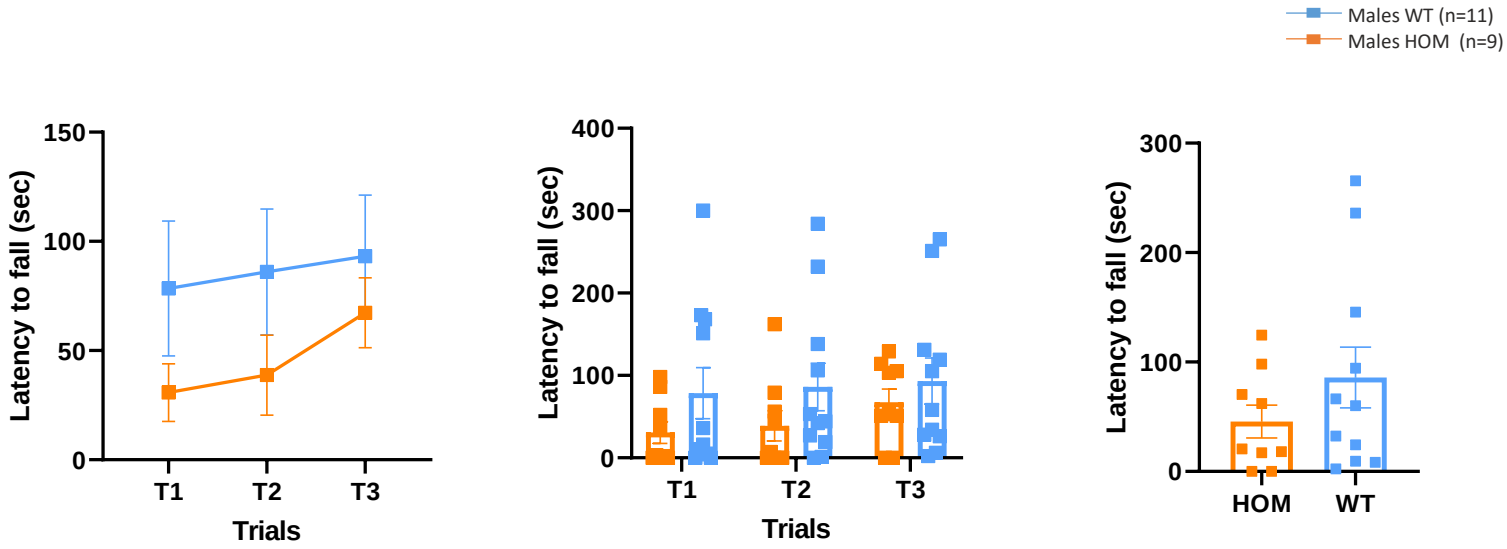




Rotarod test for JR 031211, 4 month Females. No significant differences between genotypes for latency to fall during the 3 trials and average of the 3 trials. n = 8-10; *p>0.05*, 2-way ANOVA and unpaired t-test.

Rotarod, Males

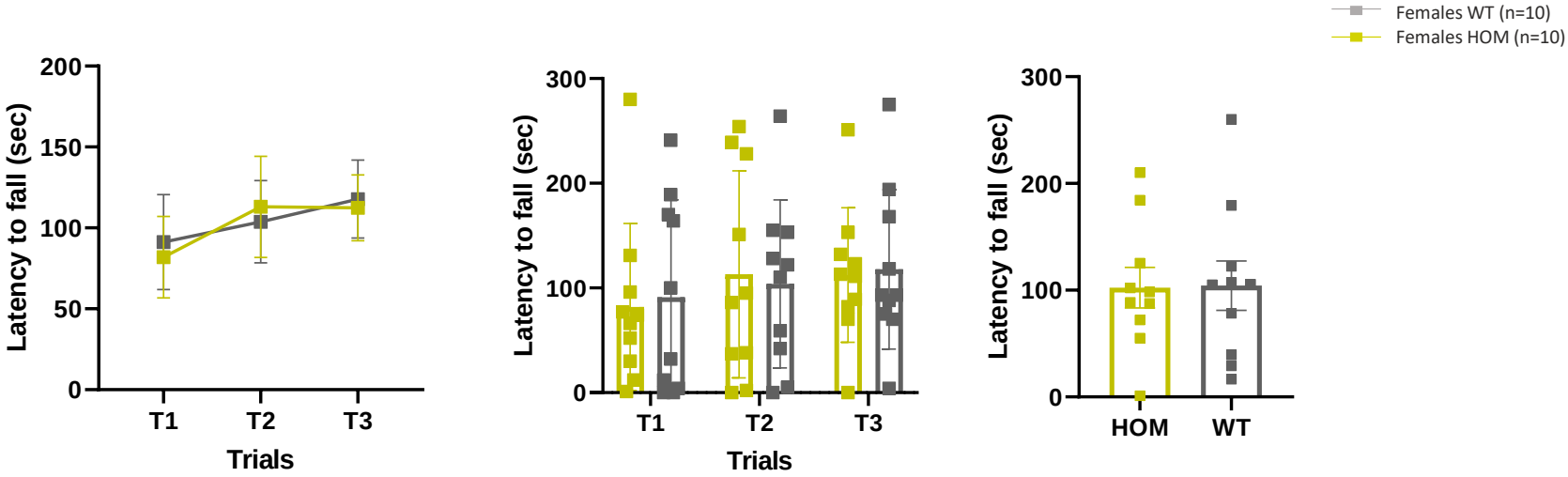
FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227



Rotarod test for JR 031227, 4 month Males. No significant differences between genotypes for latency to fall during the 3 trials and average of the 3 trials. n = 9-11; *p>0.05*, 2-way ANOVA and unpaired t-test.

Rotarod, Females

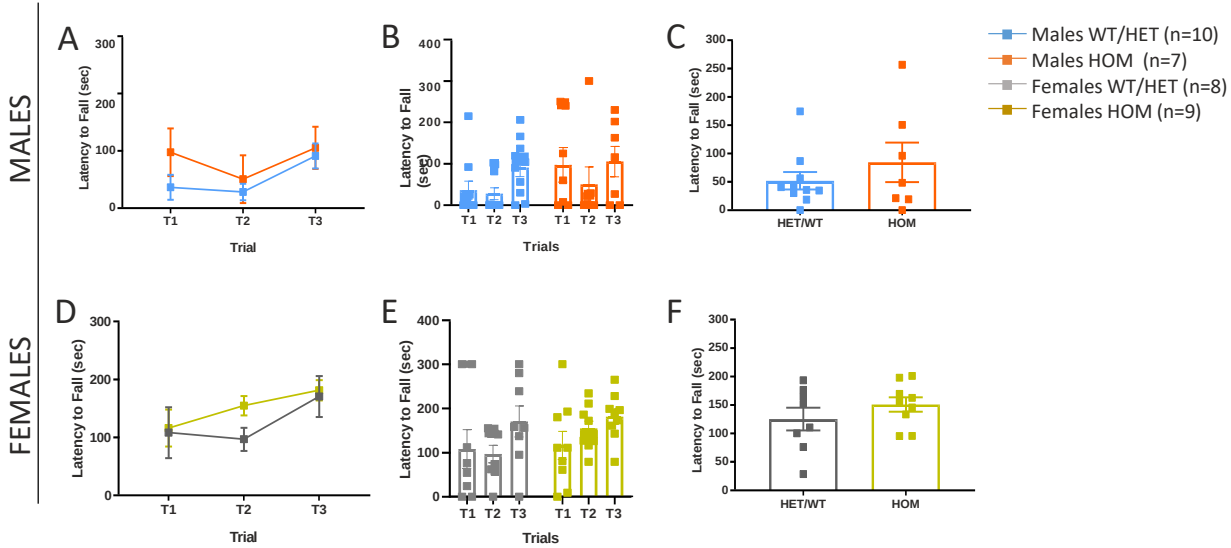
FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227



Rotarod test for JR 031227, 4 month Females. No significant differences between genotypes for latency to fall during the 3 trials and average of the 3 trials. n = 10; *p>0.05*, 2-way ANOVA and unpaired t-test.

Rotarod

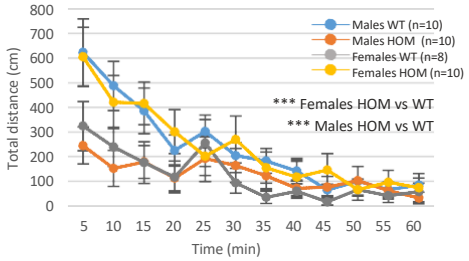
DBA/2J-*Capn3*^{em12Lutzy}/J JR 031557

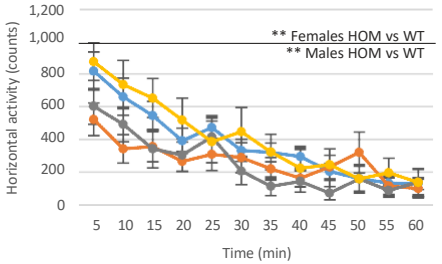


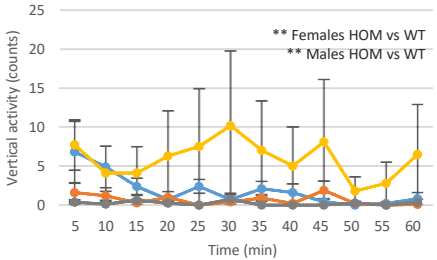
Rotarod test for JR 031557, 4 month Males & Females. No significant differences between genotypes for latency to fall during the 3 trials and average of the 3 trials. n = 7-10; *p>0.05*, 2-way ANOVA and unpaired t-test.

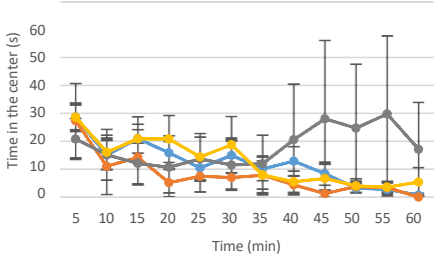
Open Field, Females & Males

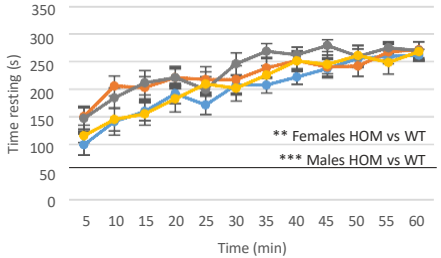
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211









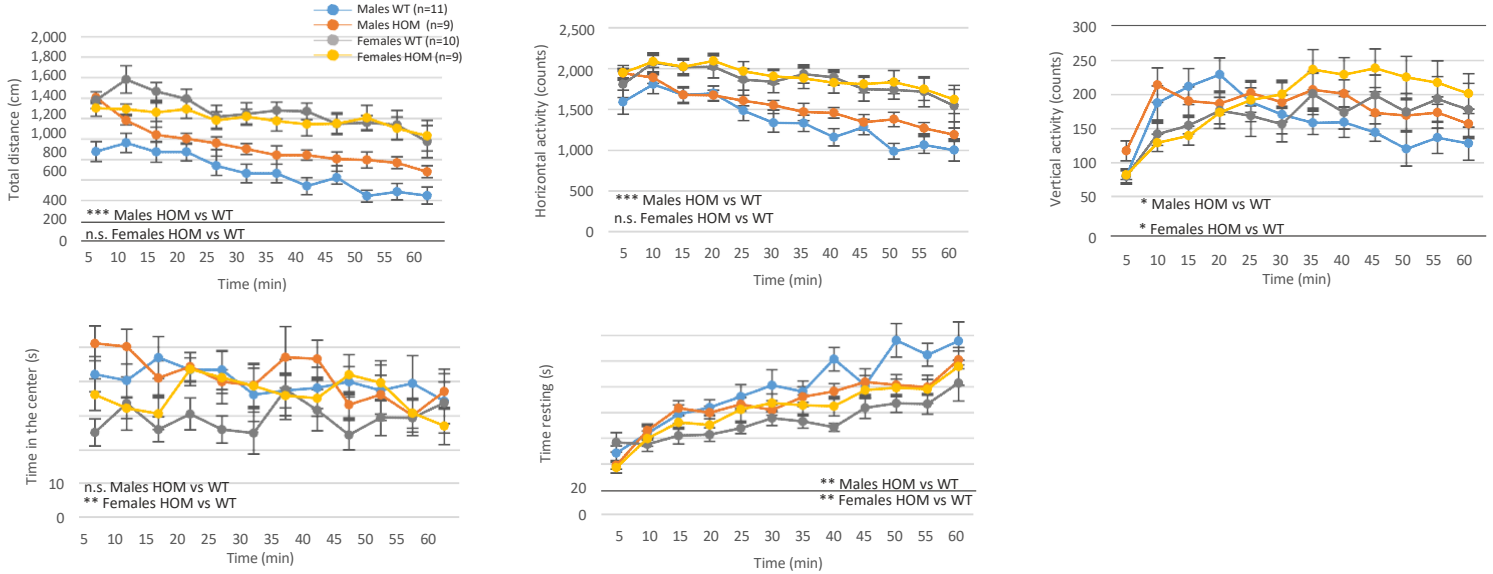


Open field test for JR 031211, Females & Males, 4 months. Total distance, horizontal and vertical activities, distance run and time spent in the center and immobility time in the arena for the entire time of the test in bins of 5 min.

Female HOMS travel more distance, have increased horizontal & vertical activity, and spend less time resting compared to WT. Male HOMS show the opposite trend; HOMS travel less distance, have decreased horizontal & vertical activity and spend more time resting compared to WT.

Open Field, Females & Males

FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227

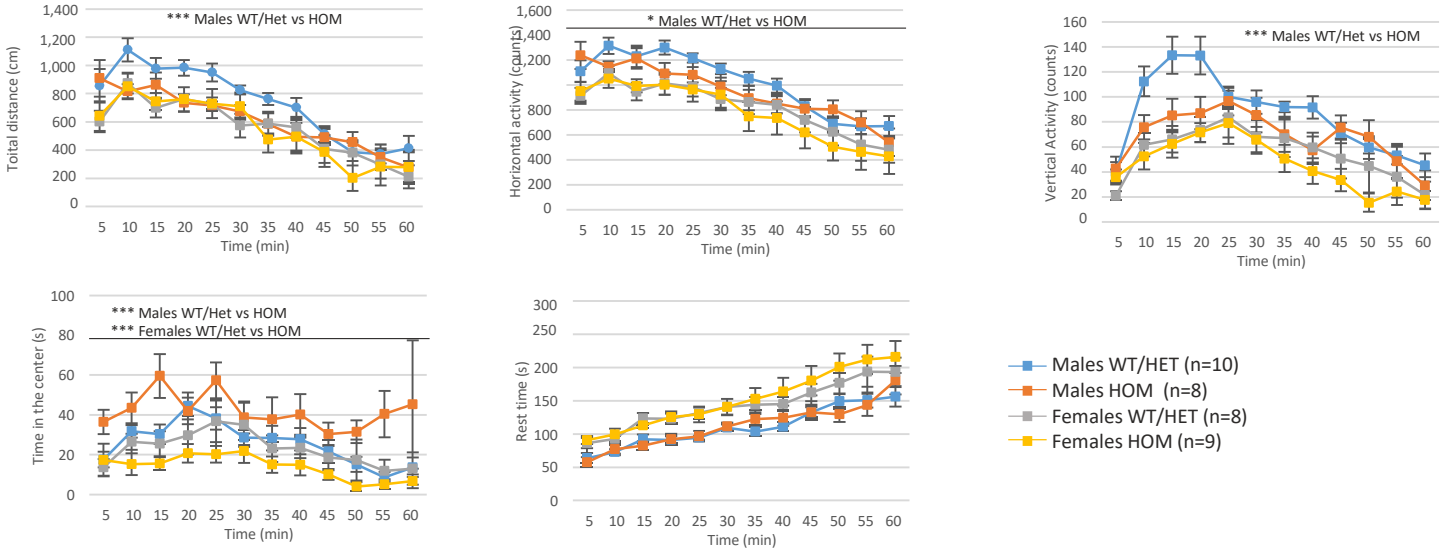


Open field test for JR 031227, Females & Males, 4 months. Total distance, horizontal and vertical activities, distance run and time spent in the center and immobility time in the arena for the entire time of the test in bins of 5 min.

Female HOMS have increased vertical activity, spent more time in the center and spent more time resting compared to WT. Male HOMS travel more distance, have increased horizontal activity, have increased vertical activity and spend less time resting compared to WT.

Open Field, Females & Males

DBA/2J-*Capn3*^{em12Lutzy}/J JR 031557

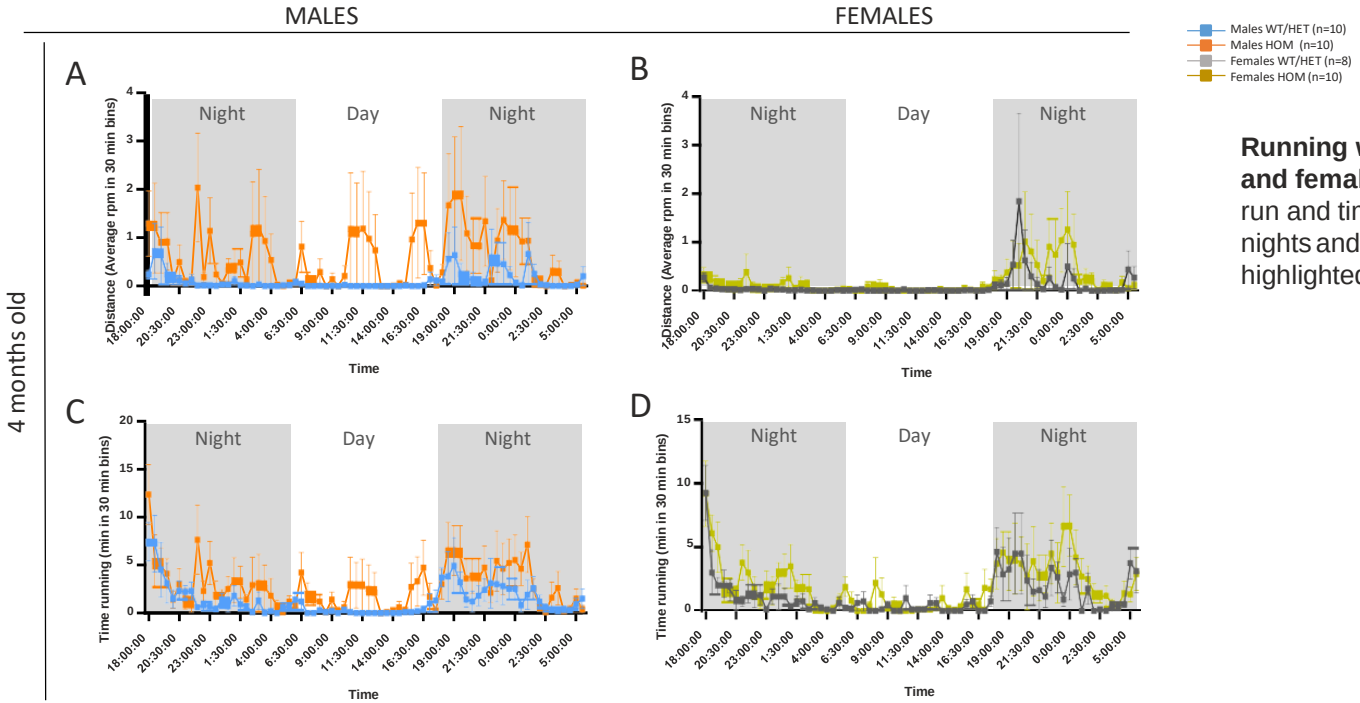


Open field test for JR 031557, Females & Males, 4 months. Total distance, horizontal and vertical activities, distance run and time spent in the center and immobility time in the arena for the entire time of the test in bins of 5 min.

Female HOMS spend less time in the center compared to WT, all other analyses are not significant. Male HOMS travel less distance, have decreased horizontal activity, decreased vertical activity and spend more time in the center compared to WT.

Wheel Running, Females & Males

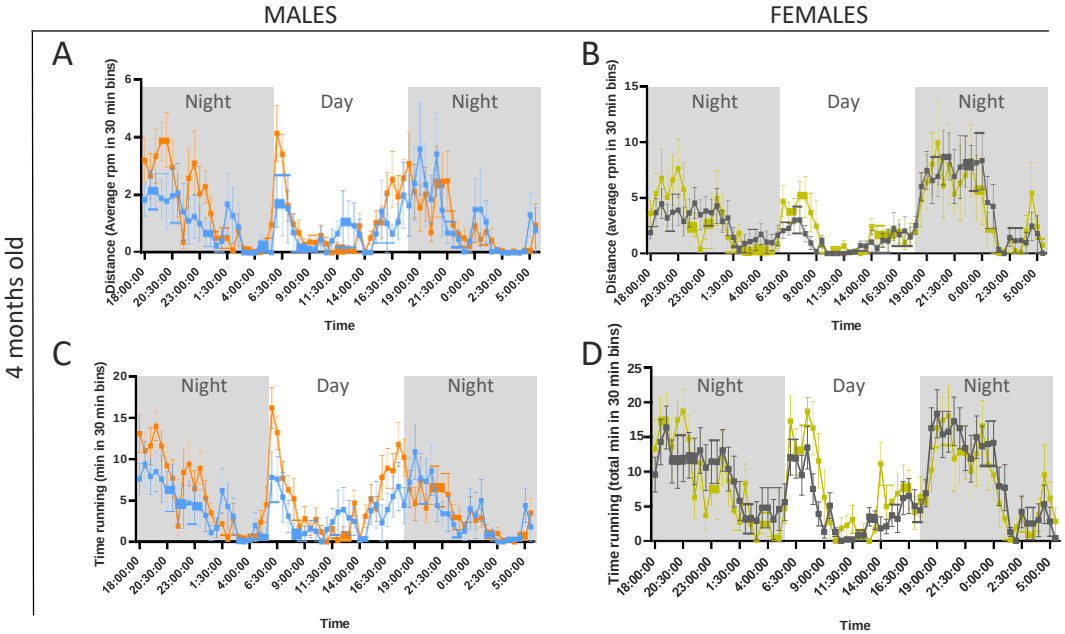
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211

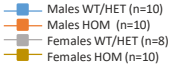


Running wheel test for JR 031211 males and females at 4 months old. Distance run and time spent running with time over 2 nights and 1 day (A-D). Nights are highlighted in the grey zones.

Wheel Running, Females & Males

FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227

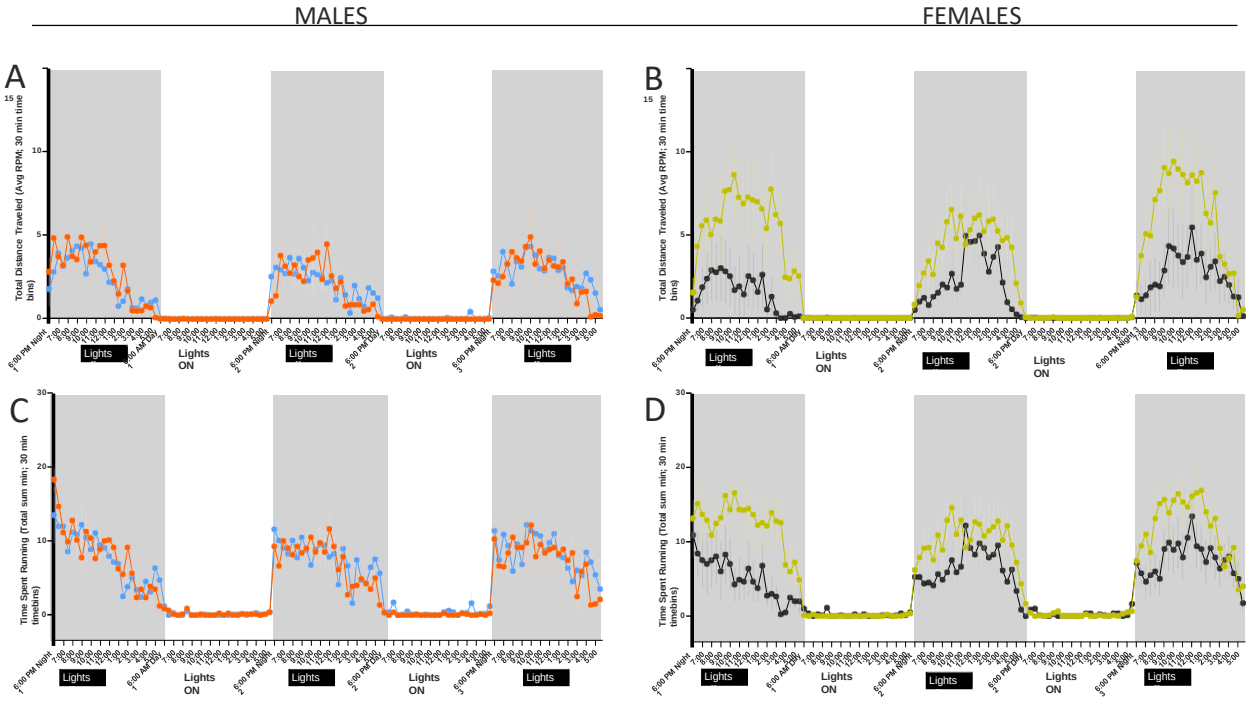




Running wheel test for JR 031227 males and females at 4 months old. Distance run and time spent running with time over 2 nights and 1 day (A-D). Nights are highlighted in the grey zones.

Wheel Running, Females & Males

DBA/2J-*Capn3*^{em12Lutzy}/J JR 031557



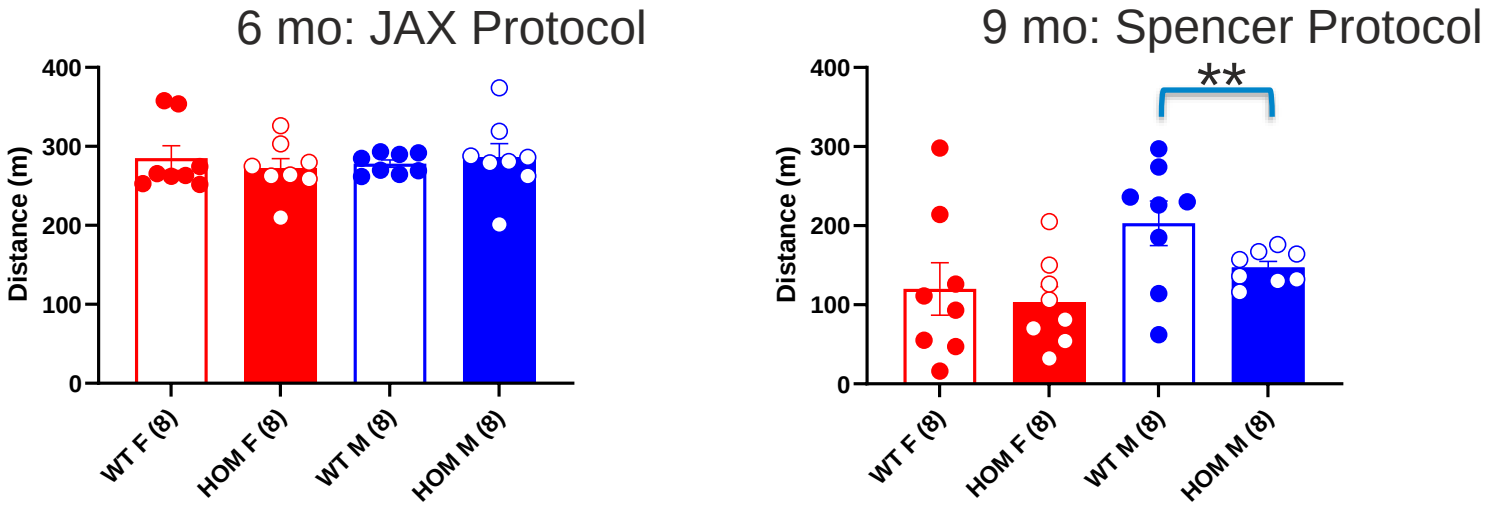
Running wheel test for JR 031557 males and females at 4 months old. Distance run and time spent running with time over 2 nights and 1 day (A-D). Nights are highlighted in the grey zones.

There is a significant difference between HOM females compared to WT

- Males WT/HET (n=10)
- Males HOM (n=8)
- Females WT/HET (n=8)
- Females HOM (n=9)

Treadmill, 6 and 9 months

129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211



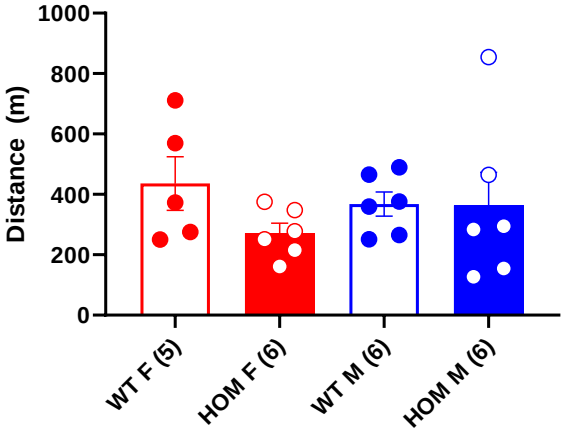
Treadmill analysis for JR 031211 males and females, 6 and 9 months.

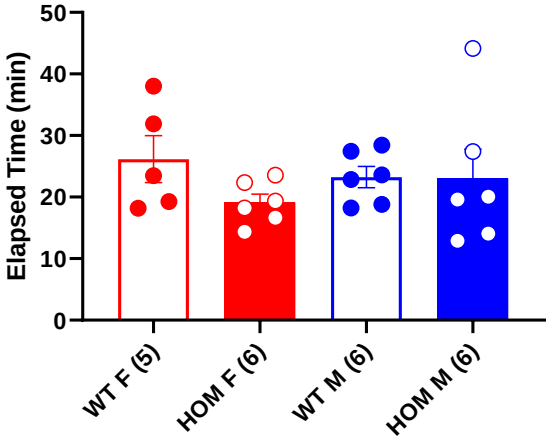
Total distance on the inclined treadmill (5%) before exhaustion. Speed was increased by 5m/min increments every 5 minutes to a maximum speed of 25 m/min reached at 35 min of testing (JAX Protocol) or increased by 1m/min increments every minute (Melissa Spenser's Protocol).

No significant differences between genotypes for either sex at 6 mo, but shorter distance in males at 9 mo with the Spencer protocol

Treadmill, 6 months

FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227





Treadmill analysis for JR 031227 males and females, 6 months.

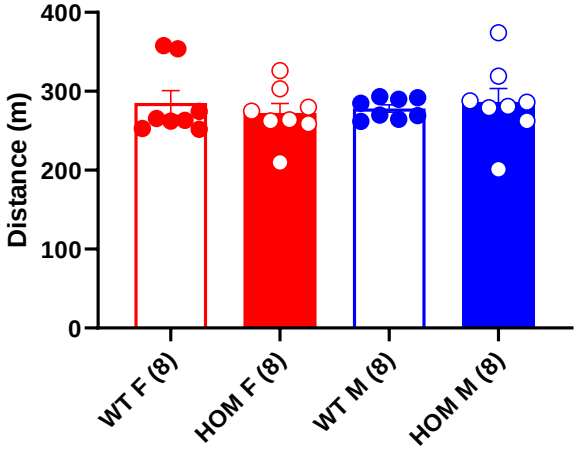
Total distance and time run on the inclined treadmill (5%) before exhaustion. Speed was increased by 5m/min increments every 5 minutes to a maximum speed of 25 m/min reached at 35 min of testing.

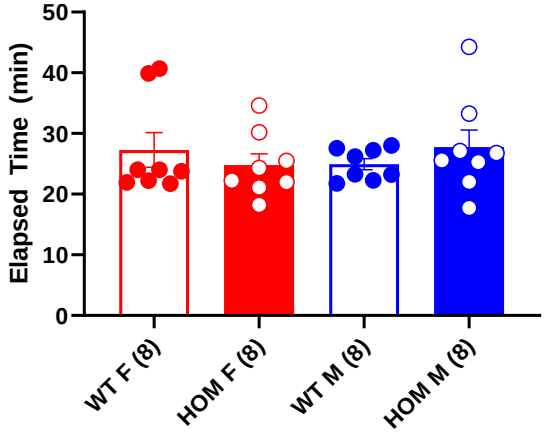
No significant differences between genotypes for either sex

*tests performed & data analyzed by CBA

Treadmill, 7 months

DBA/2J-*Capn3*^{em12Lutzy}/J JR 031557





Treadmill analysis for JR 031557 males and females, 7 months.

Total distance and time run on the inclined treadmill (5%) before exhaustion. Speed was increased by 5m/min increments every 5 minutes to a maximum speed of 25 m/min reached at 35 min of testing.

No significant differences between genotypes for either sex

12-16 month locomotor assays

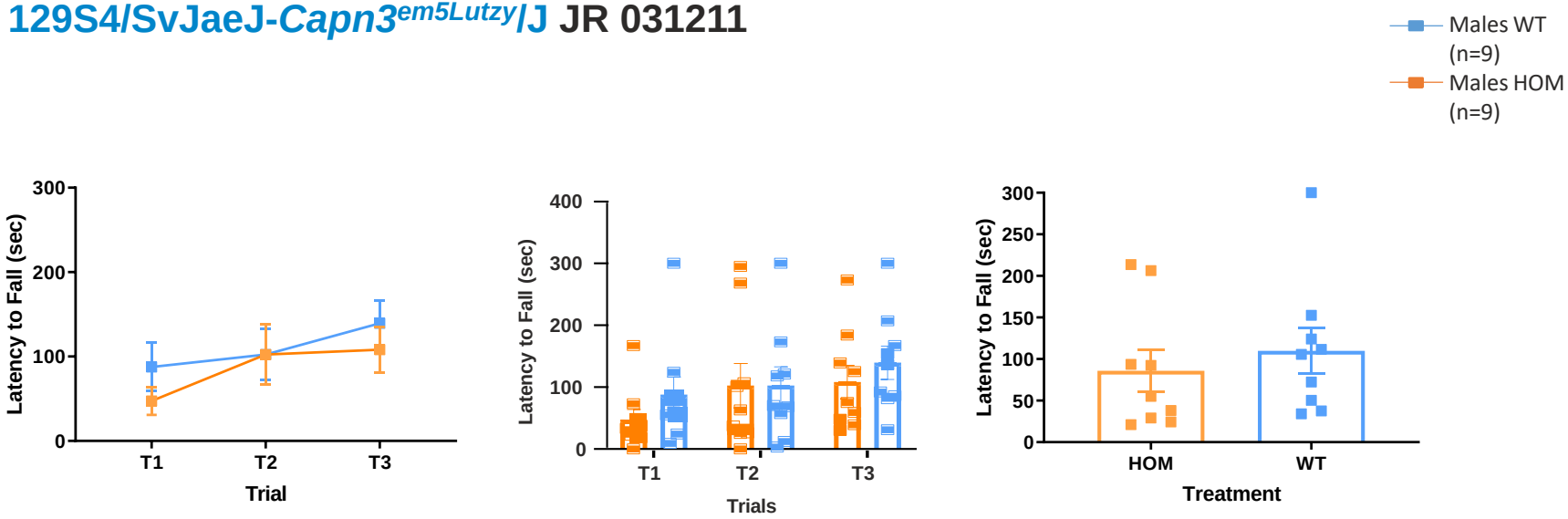
Rotarod, Open field, Running Wheels and Grip Strength testing

The Jackson Laboratory

Leading the search for tomorrow's cures

Rotarod, Males

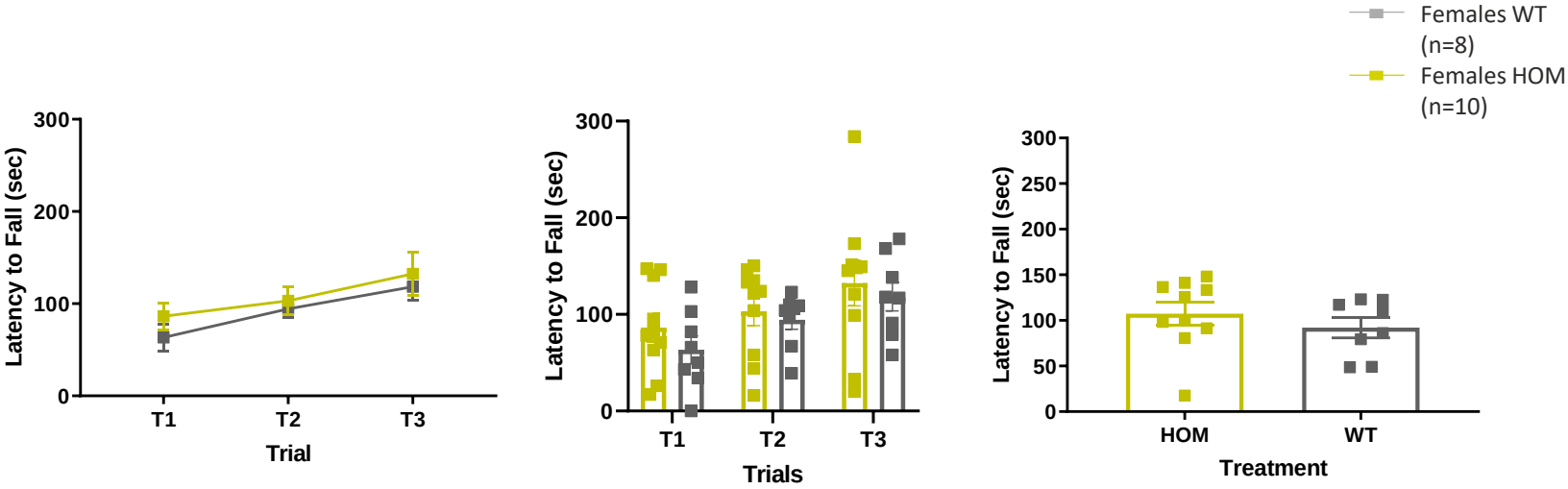
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211



Rotarod test for JR 031211, 16 month Males. No significant differences between genotypes for latency to fall during the 3 trials and average of the 3 trials. n = 9; *p>0.05*, 2-way ANOVA and unpaired t-test.

Rotarod, Females

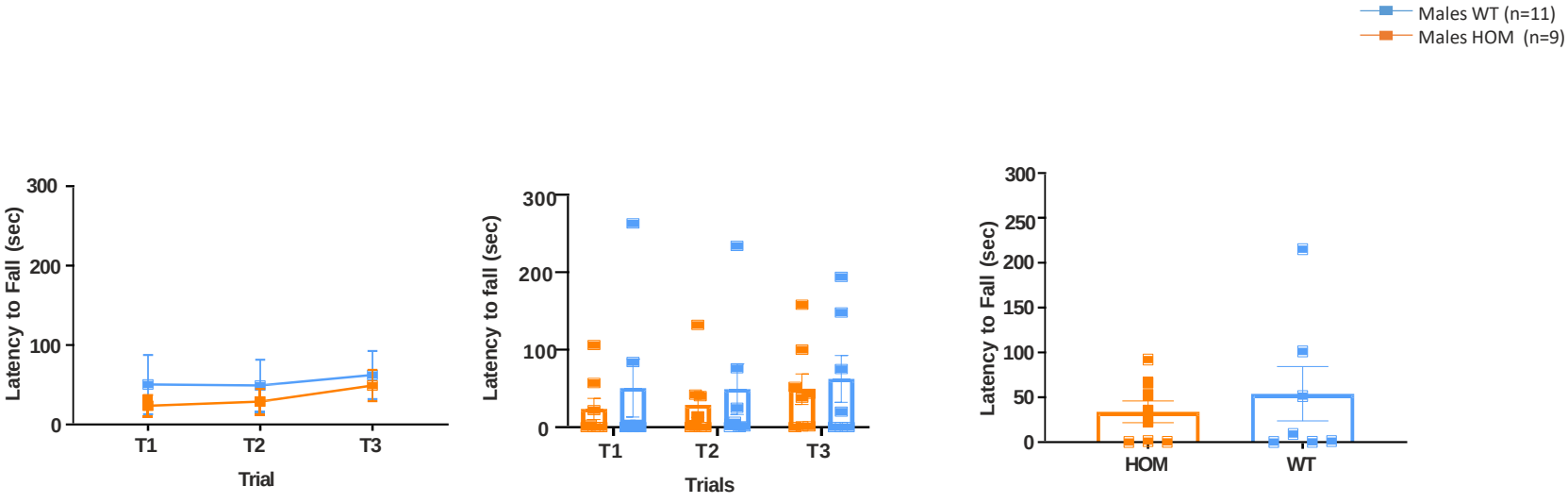
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211



Rotarod test for JR 031211, 16 month Females. No significant differences between genotypes for latency to fall during the 3 trials and average of the 3 trials. n = 8-10; *p>0.05*, 2-way ANOVA and unpaired t-test.

Rotarod, Males

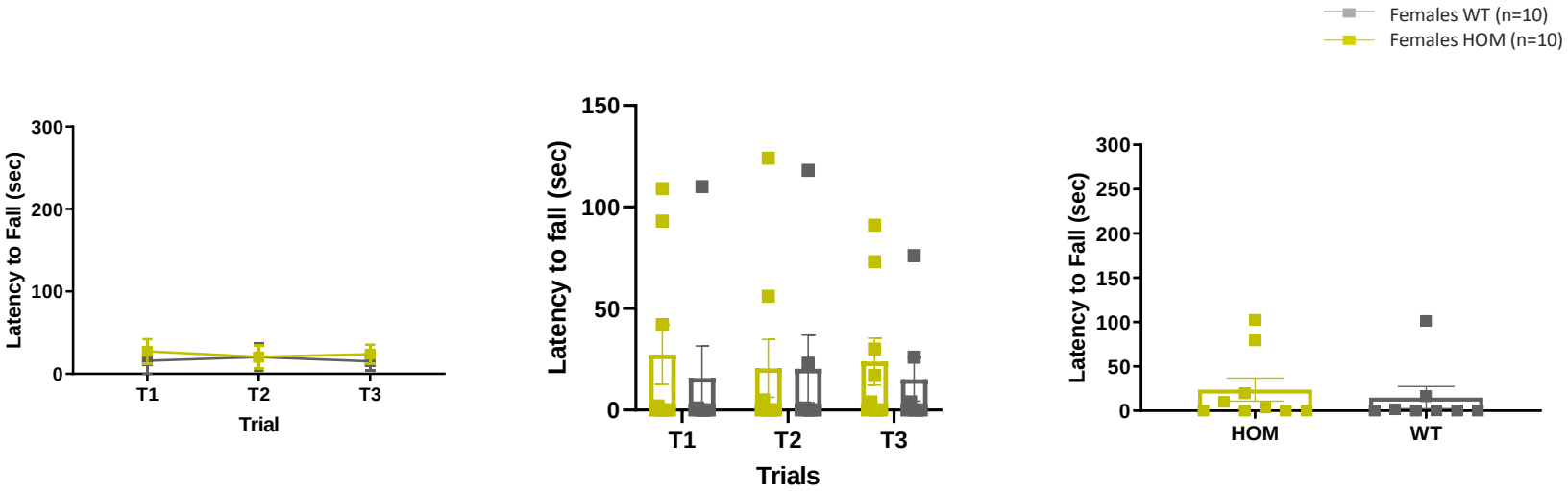
FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227



Rotarod test for JR 031211, 16 month Females. No significant differences between genotypes for latency to fall during the 3 trials and average of the 3 trials. n = 9-11; *p>0.05*, 2-way ANOVA and unpaired t-test.

Rotarod, Females

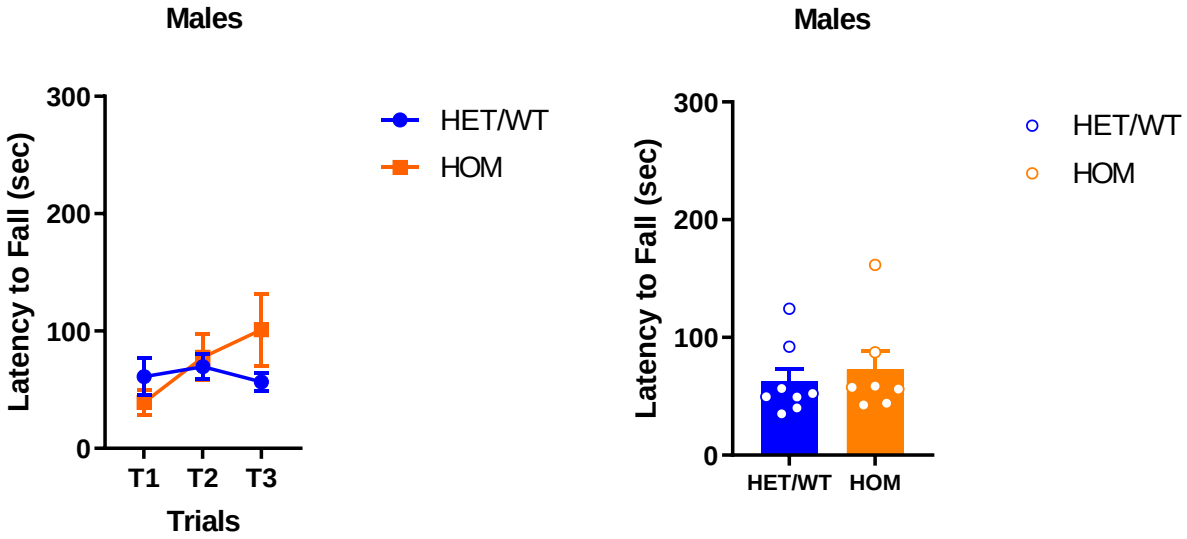
FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227



Rotarod test for JR 031227, 16 month Females. No significant differences between genotypes for latency to fall during the 3 trials and average of the 3 trials. n = 10; *p>0.05*, 2-way ANOVA and unpaired t-test.

Rotarod, Males

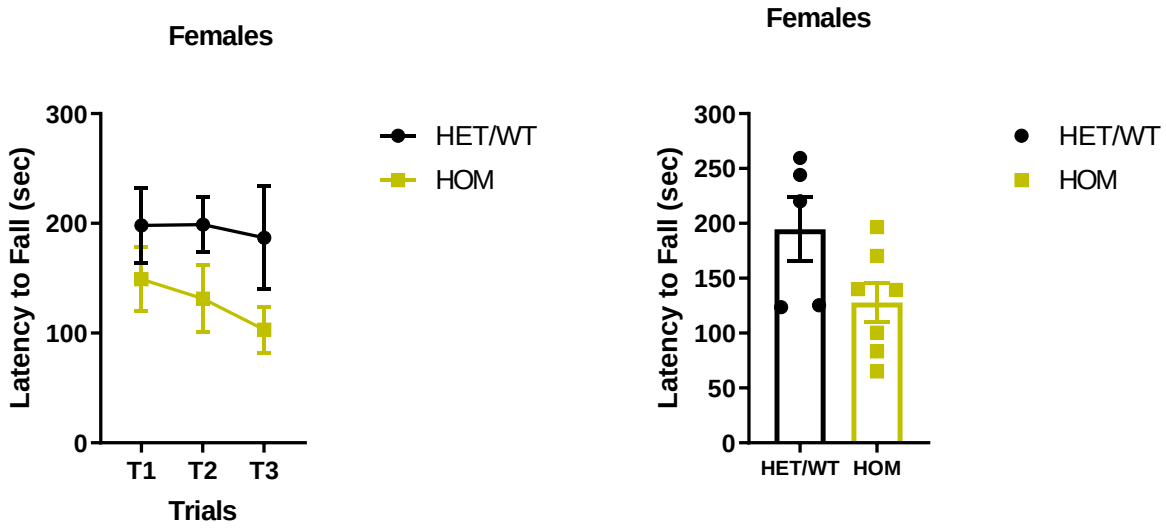
DBA/2J-*Capn3*^{em12Lutzy}/J JR 031557



Rotarod test for JR 031557, 12 month Males. No significant differences between genotypes for latency to fall during the 3 trials and average of the 3 trials. n = 8; *p>0.05*, 2-way ANOVA and unpaired t-test.

Rotarod, Females

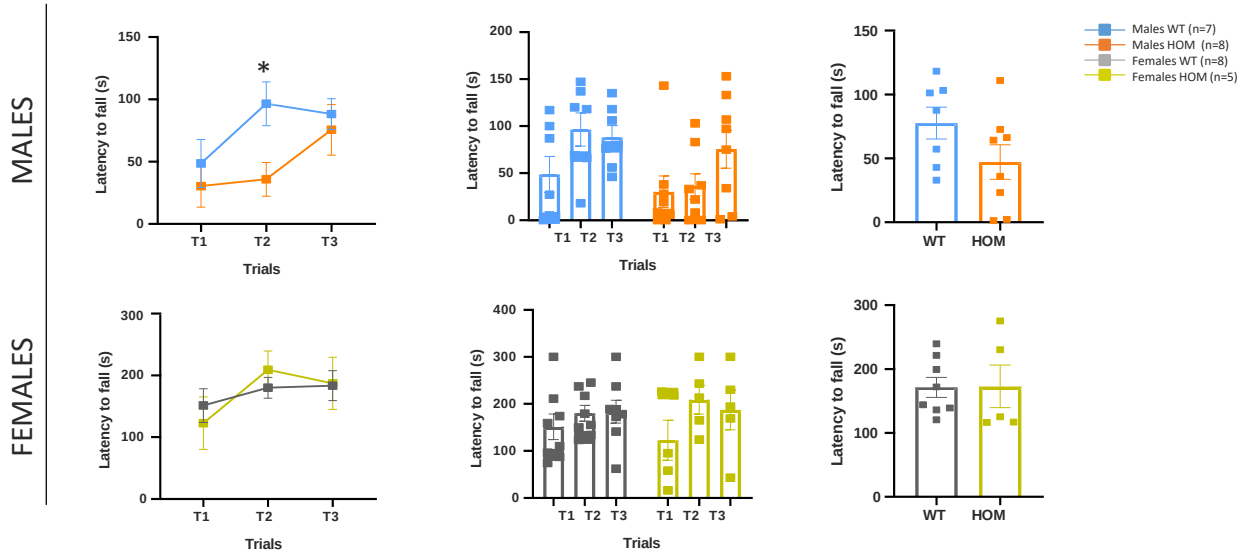
DBA/2J-*Capn3*^{em12Lutzy}/J JR 031557



Rotarod test for JR 031557, 12 month Females. Significant differences between genotypes for latency to fall during the 3 trials (p=0.014, left) but not for the average of the 3 trials (p=0.06, right). n = 8, 2-way ANOVA and unpaired t-test.

Rotarod

CC041-*Capn3*^{em10Lutzy}/J JR 031228



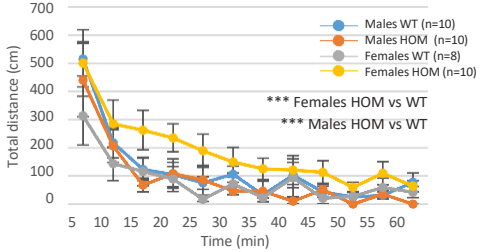
Rotarod test for JR 031228, 12 month. Latency to fall during the 3 trials and average of the 3 trials for males and females separately.

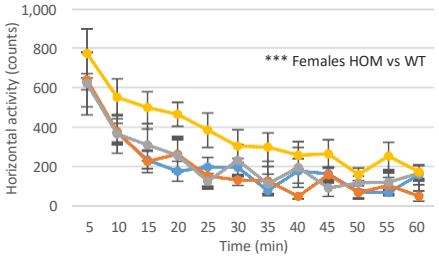
No significant difference between genotypes observed in overall motor coordination.

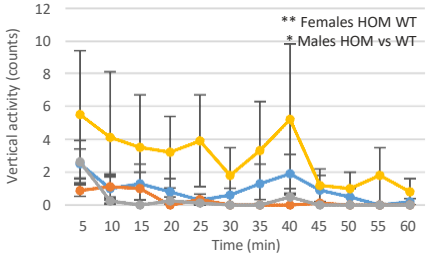
Male HOM and WT mice differed significantly for the latency to fall on the second trial of learning the test; HOM mice showed a quicker latency to fall ($p<0.05$). This could indicate a procedural learning delay.

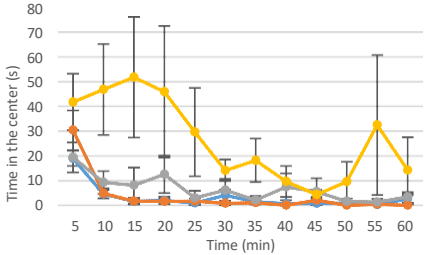
Open Field, Females & Males

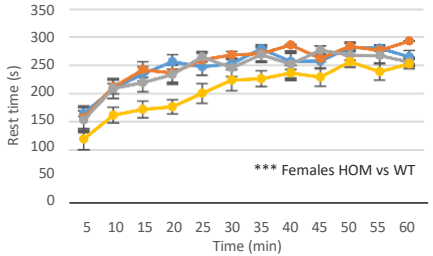
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211









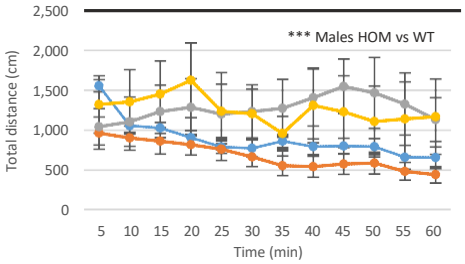


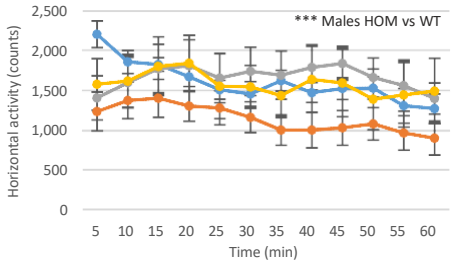
Open field test for JR 031211, Females & Males, 16 months. Total distance, horizontal and vertical activities, distance run and time spent in the center and immobility time in the arena for the entire time of the test in bins of 5 min.

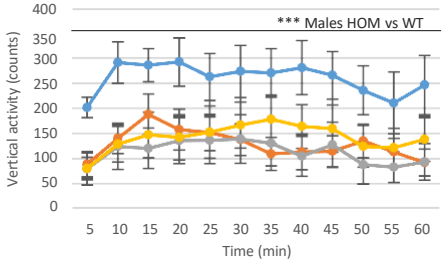
Female HOMS travel more distance, have increased horizontal & vertical activity, and spend less time resting compared to WT. Male HOMS show the opposite trend; HOMS travel less distance and have decreased vertical activity compared to WT.

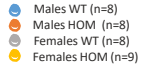
Open Field, Females & Males

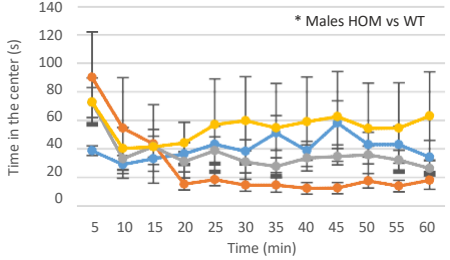
FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227

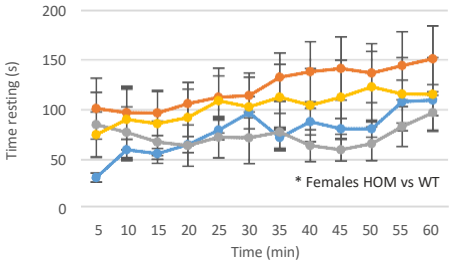










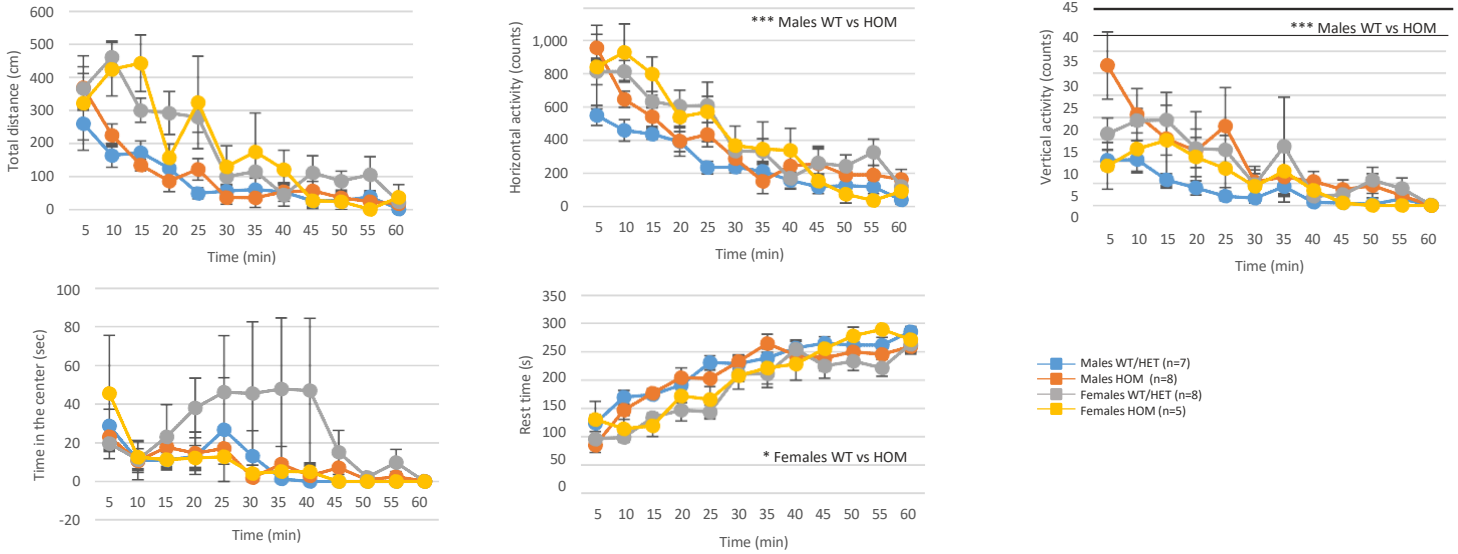


Open field test for JR 031227, Females & Males, 16 months. Total distance, horizontal and vertical activities, distance run and time spent in the center and immobility time in the arena for the entire time of the test in bins of 5 min.

Female HOMs spent more time resting when compared to WT; no other tests significant. Male HOMS show the opposite trend when compared to the 4 month test; HOMS travel less distance and have decreased horizontal activity compared to WT. Additionally, HOM males also have decreased vertical activity and spend less time in the center compared to WT.

Open Field, Females & Males

CC041-*Capn3*^{em10Lutzy}/J JR 031228

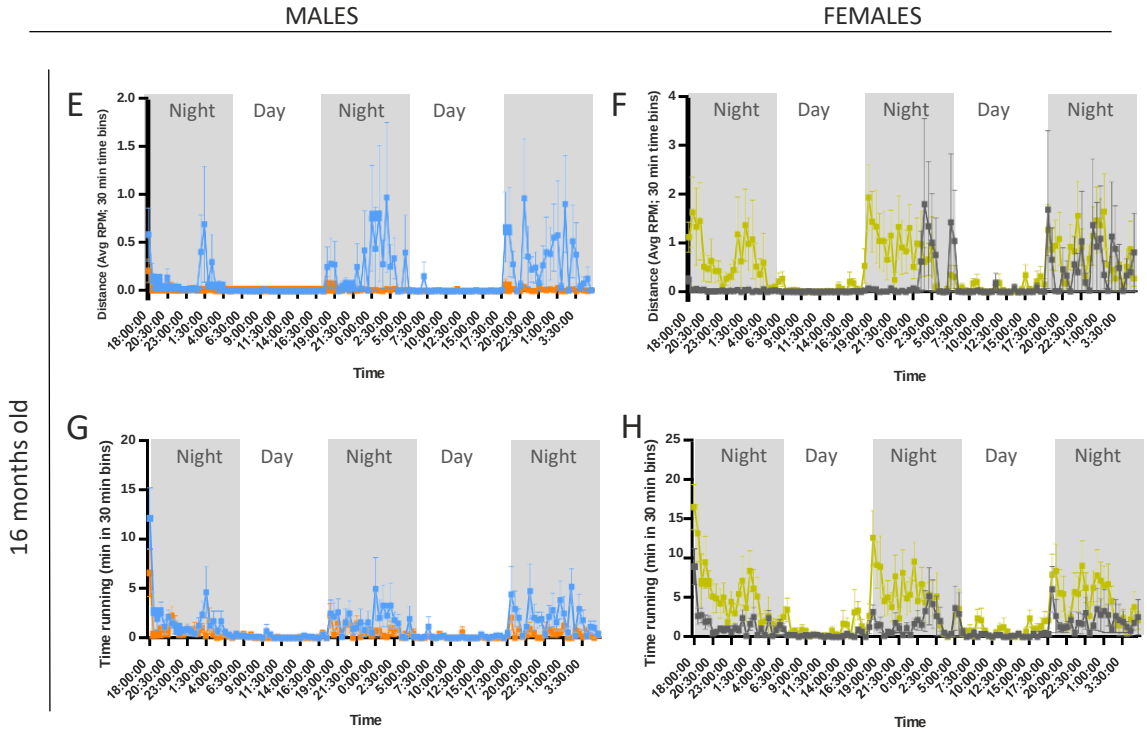


Open field test for JR 031228, Females & Males, 12 months. Total distance, horizontal and vertical activities, distance run and time spent in the center and immobility time in the arena for the entire time of the test in bins of 5 min.

Female HOMS have increased time resting compared to WT, all other analyses are not significant. Male HOMS have increased horizontal activity and increased vertical activity compared to WT.

Wheel Running, Females & Males

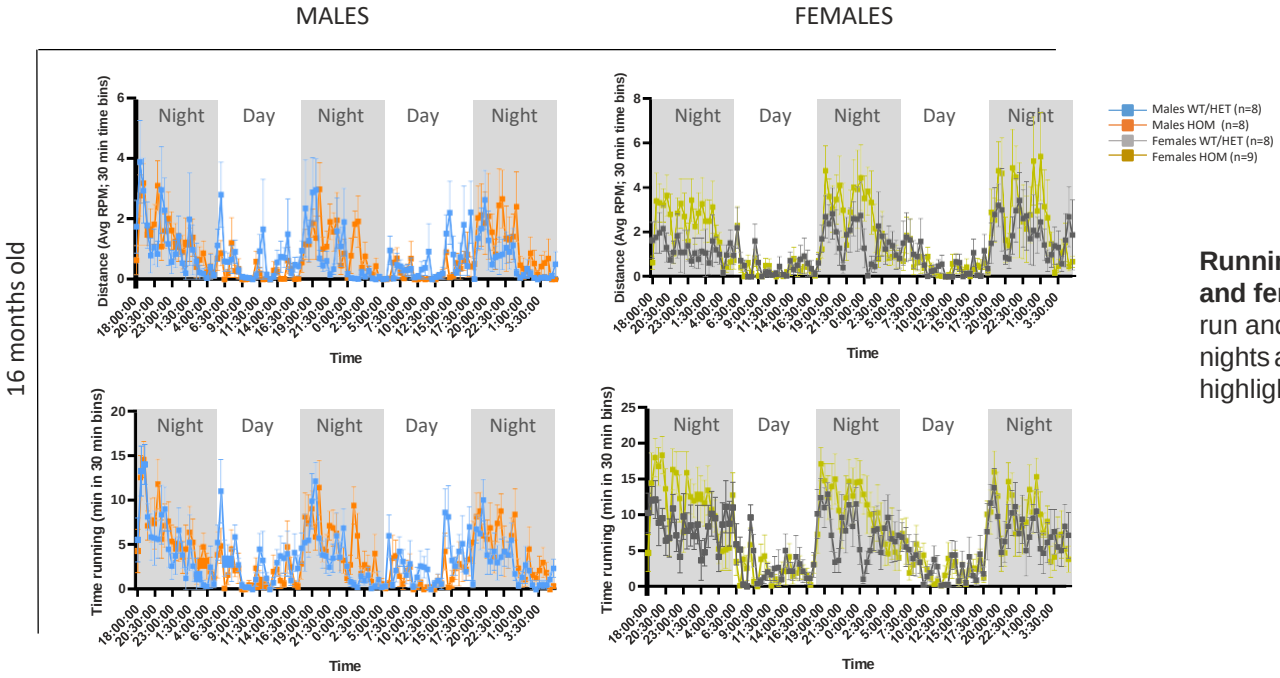
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211



Running wheel test for JR 031211 males and females at 16 months old. Distance run and time spent running with time over 2 nights and 1 day (E-H). Nights are highlighted in the grey zones.

Wheel Running, Females & Males

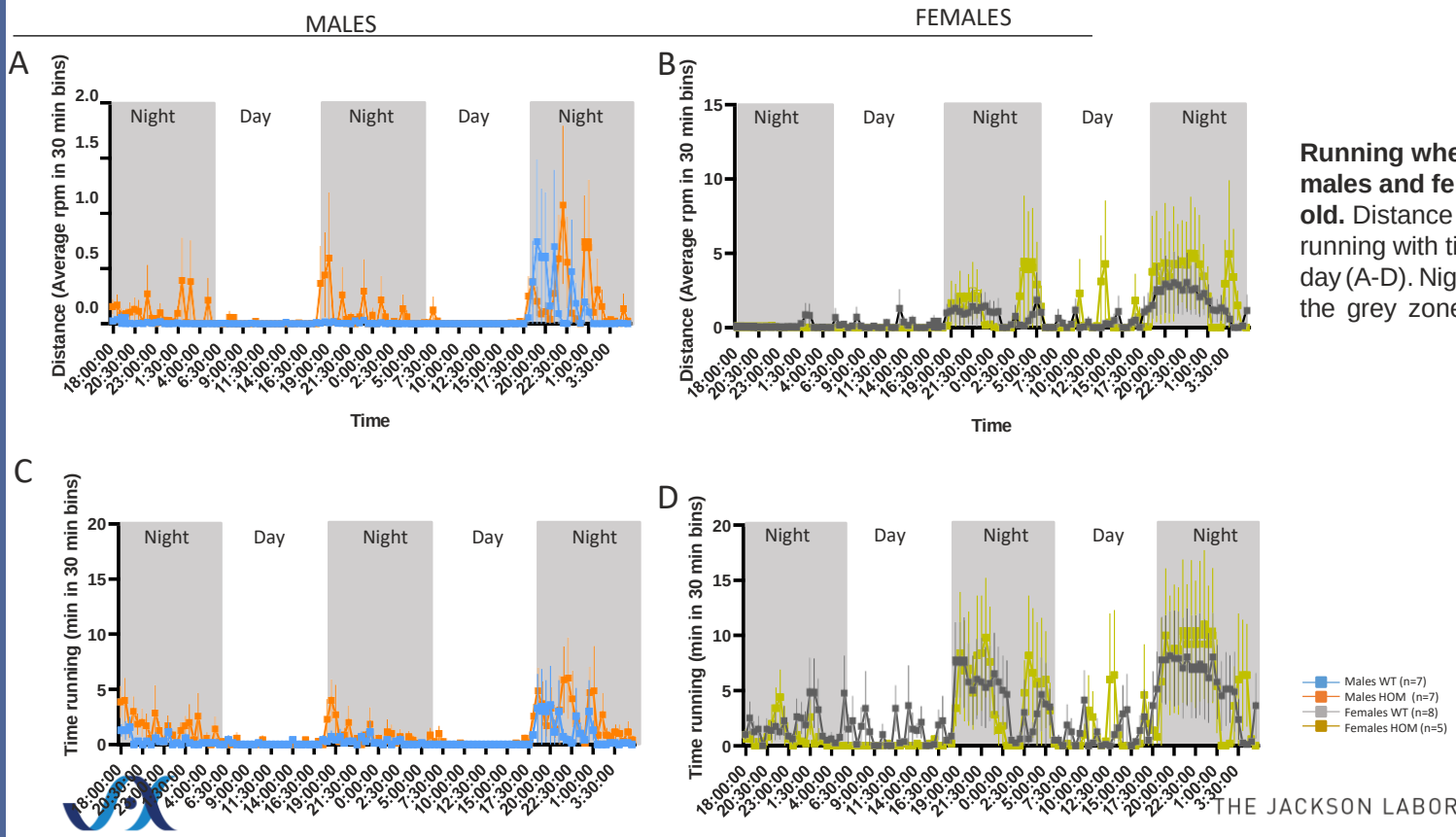
FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227



Running wheel test for JR 031227 males and females at 16 months old. Distance run and time spent running with time over 2 nights and 1 day (E-H). Nights are highlighted in the grey zones.

Wheel Running, Females & Males

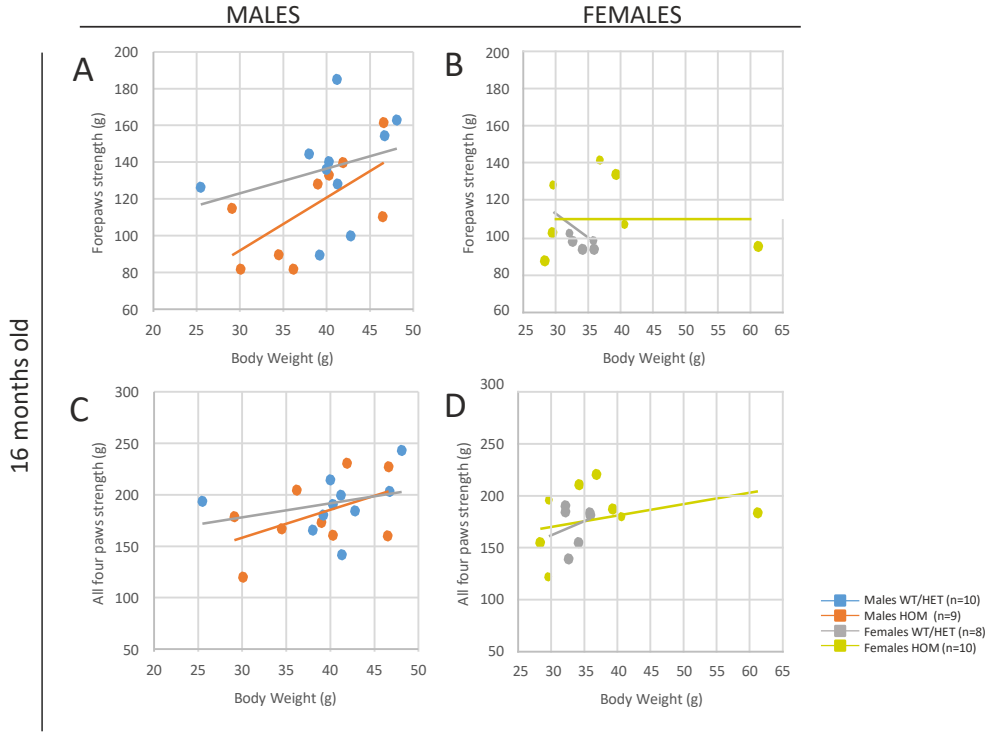
CC041-*Capn3*^{em10Lutzy}/J JR 031228



Running wheel test for JR 031228 males and females at 12 months old. Distance run and time spent running with time over 2 nights and 1 day (A-D). Nights are highlighted in the grey zones.

Grip Strength

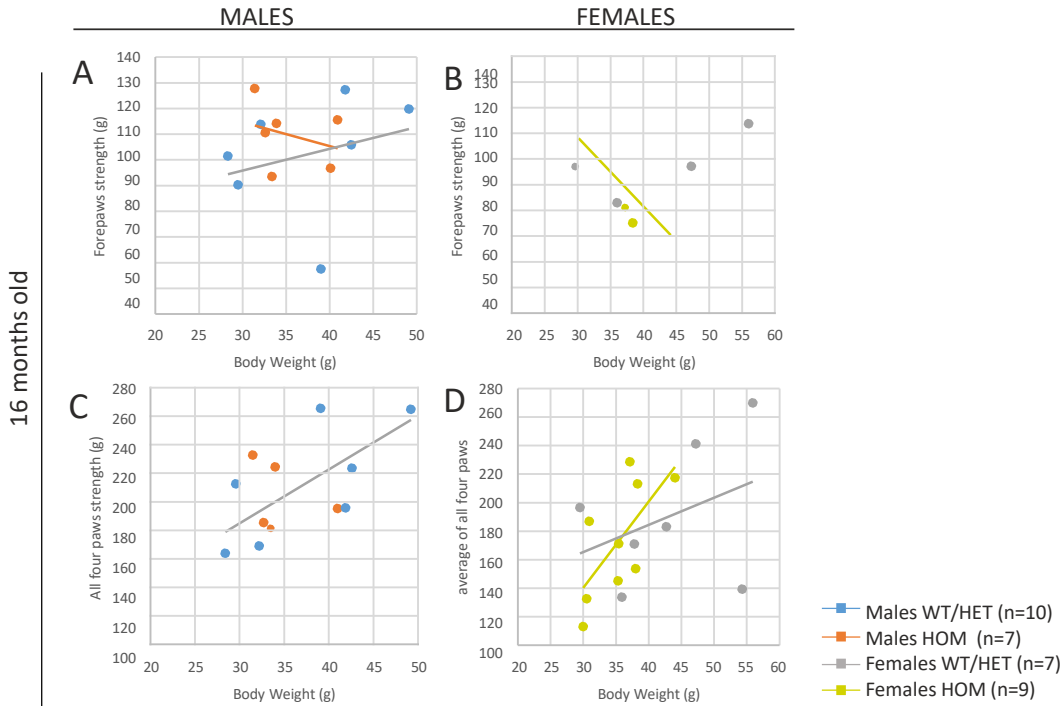
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211



Grip strength test for JR 031211, 16 months. Bodyweight, grip strength of the forelimb and of all four paws for males and females considered separately. No significant statistical differences detected between genotypes for either sex for the variables observed including when running a 2-way ANOVA with body weight as covariate.

Grip Strength

FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227

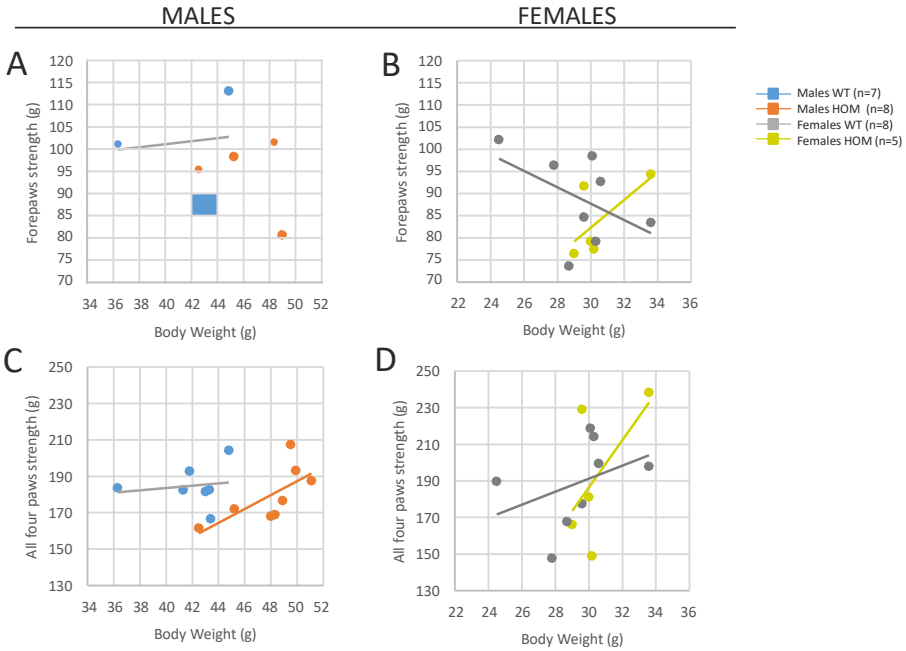


Grip strength test for JR 031227, 16 months. Bodyweight, grip strength of the forelimb and of all four paws for males. No significant statistical differences were detected between genotypes for either sex for the variables observed including when running a 2-way ANOVA with body weight as covariate.

*tests performed & data analyzed by CBA

Grip Strength

CC041-*Capn3*^{em10Lutzy}/J JR 031228

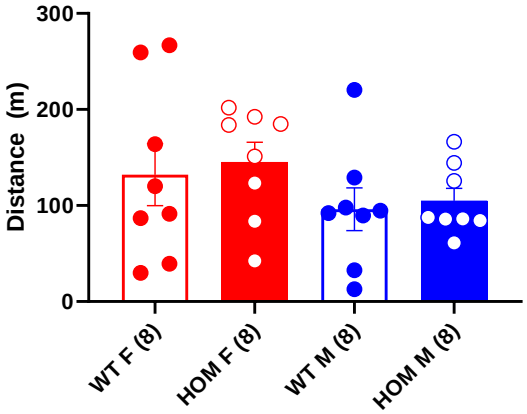


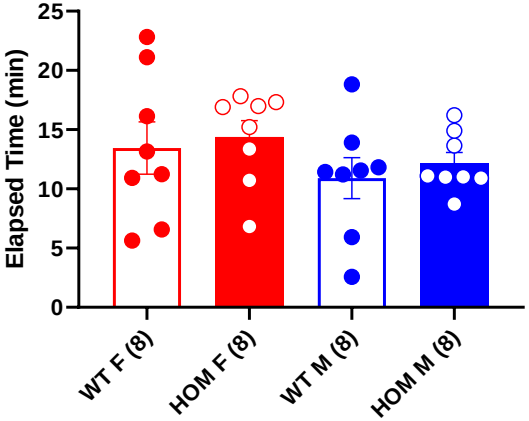
Grip strength test for JR 031228, 12 months. Bodyweight, grip strength of the forelimb and of all four paws for males. No significant statistical differences were detected between genotypes for either sex for the variables observed including when running a 2way ANOVA with body weight as covariate.

*tests performed & data analyzed by CBA

Treadmill, 14 months

CC041-*Capn3*^{em10Lutzy}/J JR 031228





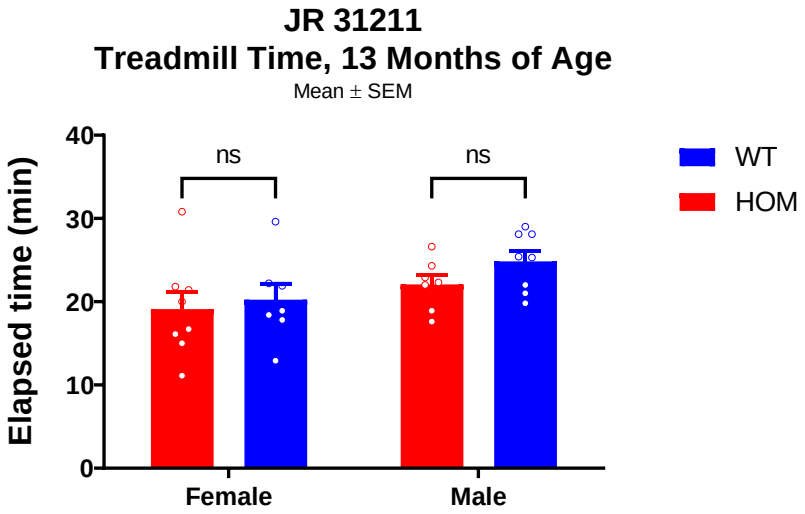
Treadmill analysis for JR 031228 males and females, 14 months.

Total distance and time run on the inclined treadmill (5%) before exhaustion. Speed was increased by 5m/min increments every 5 minutes to a maximum speed of 25 m/min reached at 35 min of testing.

No significant differences between genotypes for either sex

Treadmill, 13 months

129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211



Source of Variation	% of total variation	P value	P value summary
Interaction	0.7046	0.6337	ns
Sex	15.35	0.0331	*
Genotype	4.039	0.2587	ns

Treadmill analysis for JR 031211 males and females, 12.5 months.

Time run on the inclined treadmill (5%) before exhaustion.

Speed was increased by 5m/min increments every 5 minutes to a maximum speed of 25 m/min reached at 35 min of testing (JAX Protocol).

No significant differences between genotypes for either sex at any time point.

Late age in vivo muscle force

FVB-Capn3 and 129-Capn3 at 20 months
CC041-Capn3 at 13 months,
DBA-Capn3 at 7 months

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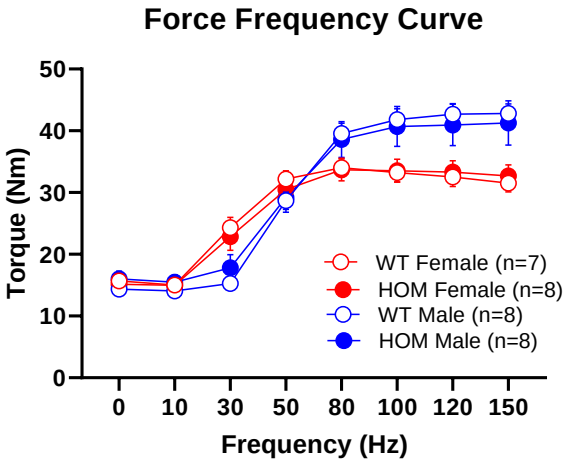
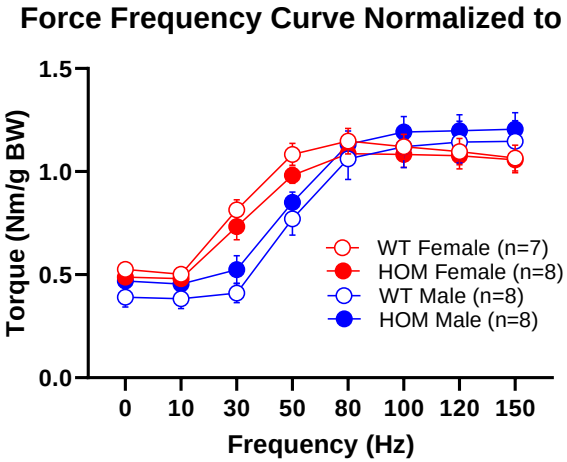
Muscle Contraction Analysis, 20 months

129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211





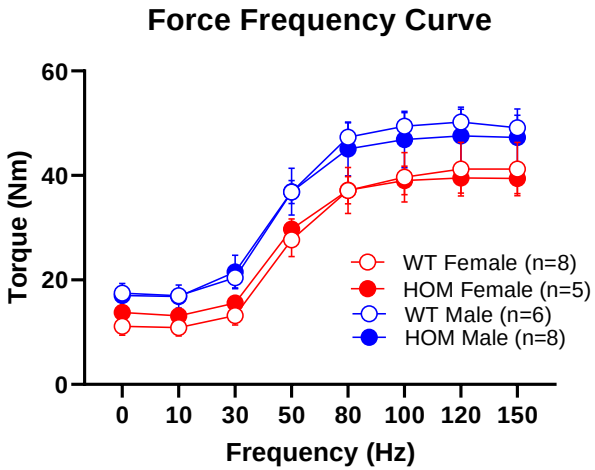
Muscle contraction analysis for JR 031211 males and females, 20 months.

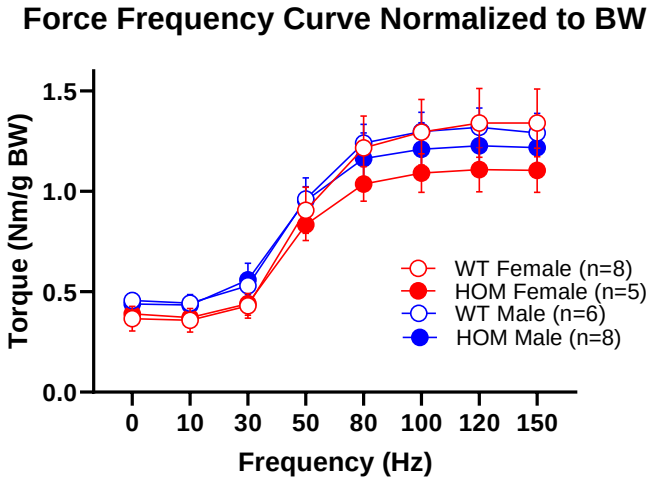
Measure of the isometric force developed by the tibialis anterior (TA) muscle upon stimulation at increasing frequencies, as an absolute value or normalized to the body weight. Force of the TA is measured as the torque of the ankle dorsiflexion. At the highest stimulation frequencies (120-150 Hz), tetanic torque represents the maximum force that the muscle can develop.

No significant differences between genotypes for either sex.

Muscle Contraction Analysis, 20 months

FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227





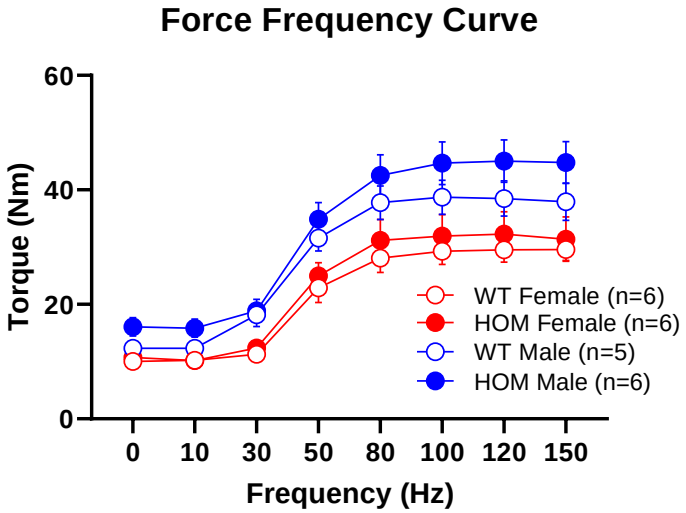
Muscle contraction analysis for JR 031227 males and females, 20 months.

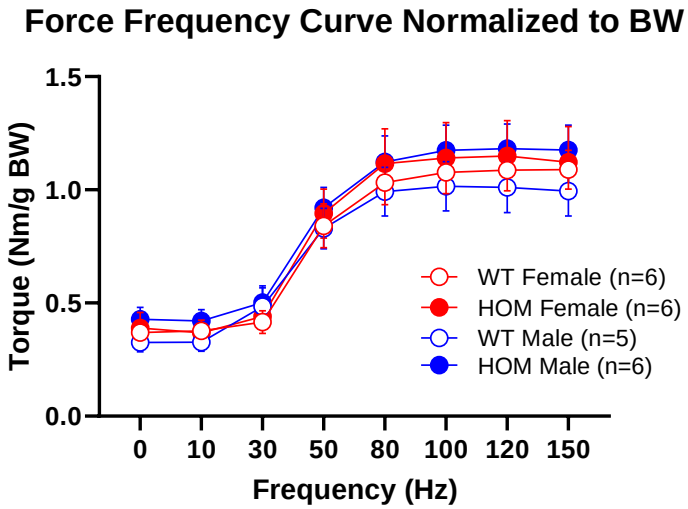
Measure of the isometric force developed by the tibialis anterior (TA) muscle upon stimulation at increasing frequencies, as an absolute value or normalized to the body weight. Force of the TA is measured as the torque of the ankle dorsiflexion. At the highest stimulation frequencies (120-150 Hz), tetanic torque represents the maximum force that the muscle can develop.

No significant differences between genotypes for either sex.

Muscle Contraction Analysis, 14 months

CC041-*Capn3*^{em10Lutzy}/J JR 031228





Muscle contraction analysis for JR 031228 males and females, 14 months.

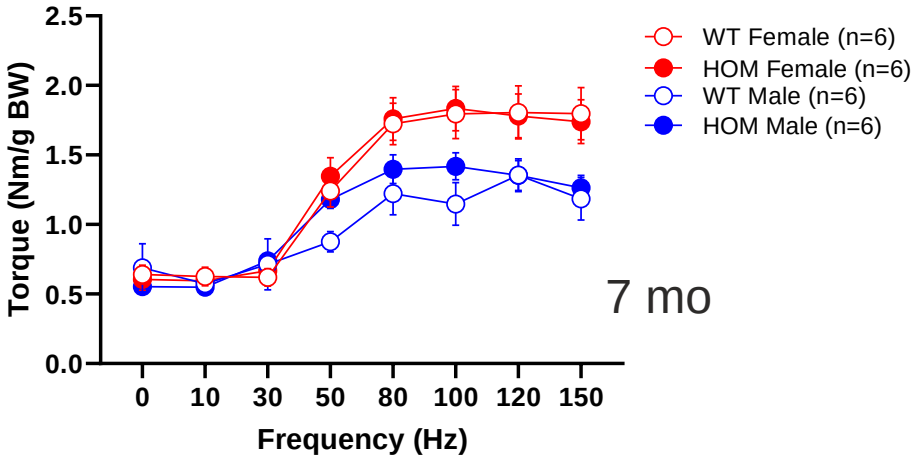
Measure of the isometric force developed by the tibialis anterior (TA) muscle upon stimulation at increasing frequencies, as an absolute value or normalized to the body weight. Force of the TA is measured as the torque of the ankle dorsiflexion. At the highest stimulation frequencies (120-150 Hz), tetanic torque represents the maximum force that the muscle can develop.

No significant differences between genotypes for either sex.

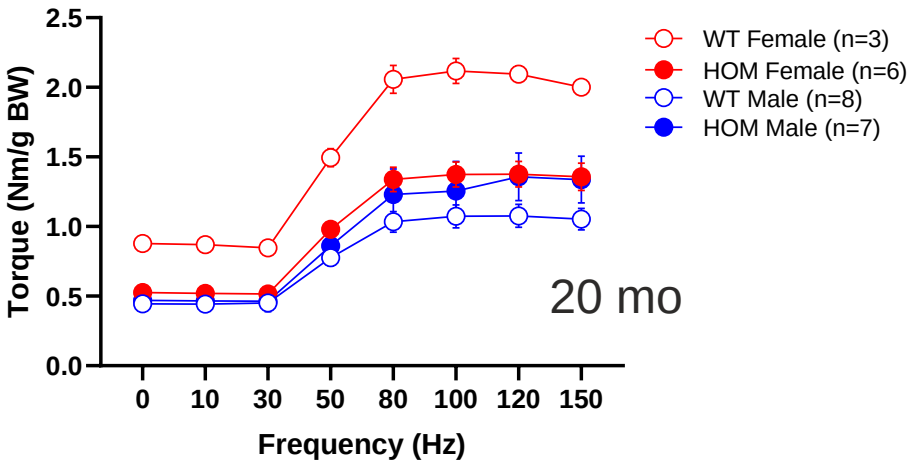
Muscle Contraction Analysis, 7 and 20 months

DBA/2J-*Capn3*^{em12Lutzy}/J JR 031557

Force Frequency Curve Normalized to BW



Force Frequency Curve Normalized to BW



Muscle contraction analysis for JR 031557 males and females, 7 and 20 months.

Measure of the isometric force developed by the tibialis anterior (TA) muscle upon stimulation at increasing frequencies, as an absolute value or normalized to the body weight. Force of the TA is measured as the torque of the ankle dorsiflexion. At the highest stimulation frequencies (120-150 Hz), tetanic torque represents the maximum force that the muscle can develop.
No significant differences between genotypes for either sex at 7mo. Apparent weakness in the Capn3 females at 20 mo, but because of the small sample size, and absence of weakness in males, this might be an artifact.

Late age in vivo muscle force

FVB-Capn3 and 129-Capn3 at 20 months
CC041-Capn3 at 13 months,
DBA-Capn3 at 7 months

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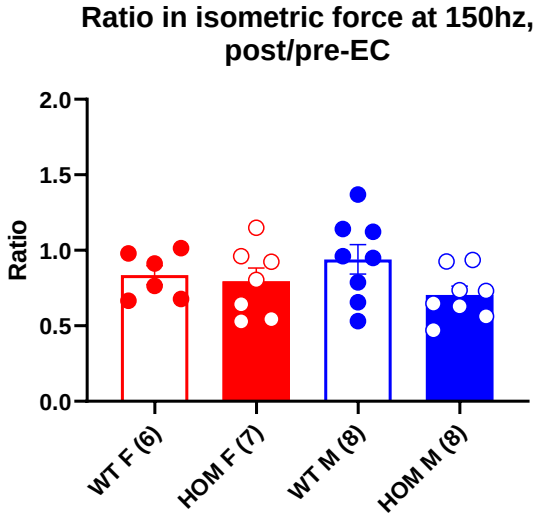
Muscle Contraction Analysis, 20 months

129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211

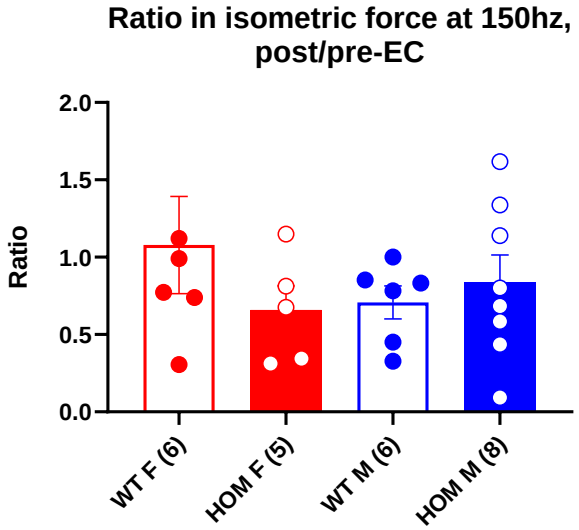


Muscle contraction analysis for JR 031211 males and females, 20 months.

Ratio of the isometric torques of the TA, measure as on the previous slide, and developed after vs. before a series of 20 eccentric contractions, modeling exercise-induced muscle injury. A ratio of 1 represents a perfect resistance to exercise; decreasing ratios are a sign of exercise intolerance caused by an abnormal sensitivity of the muscle to exercise-induced muscle damage.
No significant differences between genotypes for either sex, although 129-Capn3 males present an almost significant (p=0.06, t-test) decrease in ratio, suggesting increased fragility of their muscles.

Muscle Contraction Analysis, 20 months

FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227

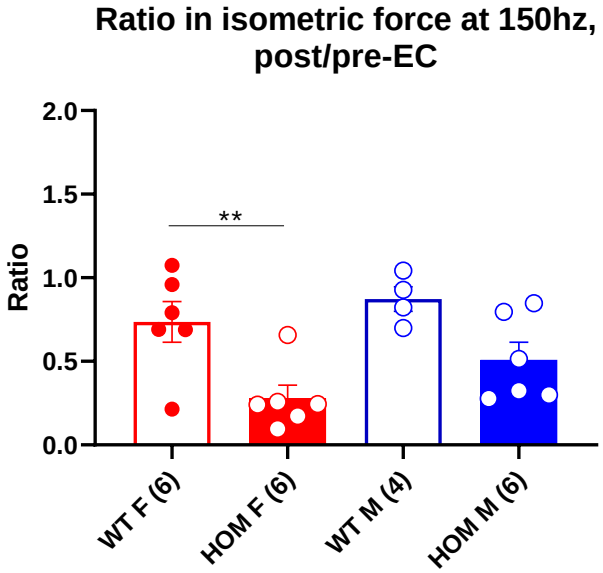


Muscle contraction analysis for JR 031227 males and females, 20 months.

Ratio of the isometric torques of the TA, measure as on the previous slide, and developed after vs. before a series of 20 eccentric contractions, modeling exercise-induced muscle injury. A ratio of 1 represents a perfect resistance to exercise; decreasing ratios are a sign of exercise intolerance caused by an abnormal sensitivity of the muscle to exercise-induced muscle damage.
No significant differences between genotypes for either sex.

Muscle Contraction Analysis, 14 months

CC041-*Capn3*^{em10Lutzy}/J JR 031228

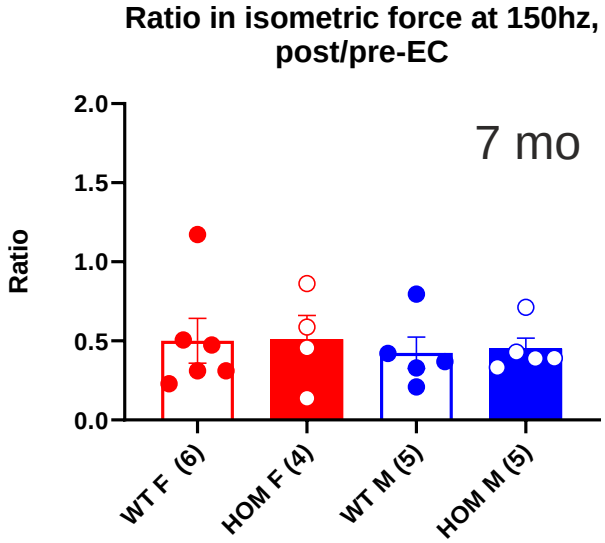


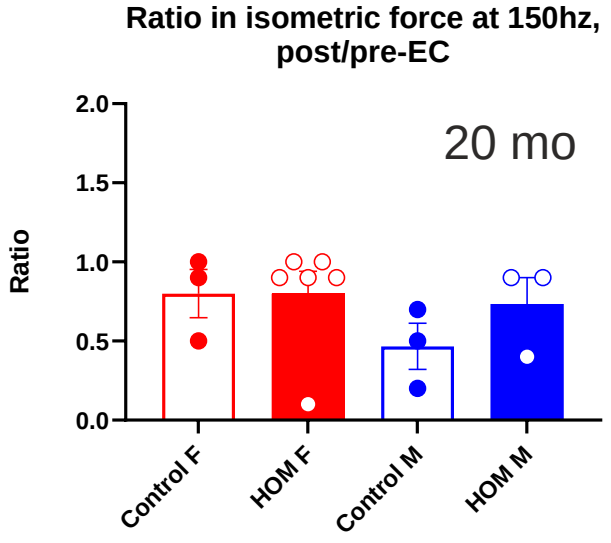
Muscle contraction analysis for JR 031228 males and females, 14 months.

Ratio of the isometric torques of the TA, measure as on the previous slide, and developed after vs. before a series of 20 eccentric contractions, modeling exercise-induced muscle injury. A ratio of 1 represents a perfect resistance to exercise; decreasing ratios are a sign of exercise intolerance caused by an abnormal sensitivity of the muscle to exercise-induced muscle damage.
CC041-Capn3 mice had overall a lower ratio (p<0.01, 2-way ANOVA for sex, genotype); with a more pronounced weakness in the females (p=0.01, Sidak's multicomparison) than in the males (p=0.17).

Muscle Contraction Analysis, 7 & 20 months

DBA/2J-*Capn3*^{em12Lutzy}/J JR 031557





Muscle contraction analysis for JR 031557 males and females, 7 and 20 months.

Ratio of the isometric torques of the TA, measure as on the previous slide, and developed after vs. before a series of 20 eccentric contractions, modeling exercise-induced muscle injury. A ratio of 1 represents a perfect resistance to exercise; decreasing ratios are a sign of exercise intolerance caused by an abnormal sensitivity of the muscle to exercise-induced muscle damage.
No significant differences between genotypes for either sex.

Serum Creatine Kinase

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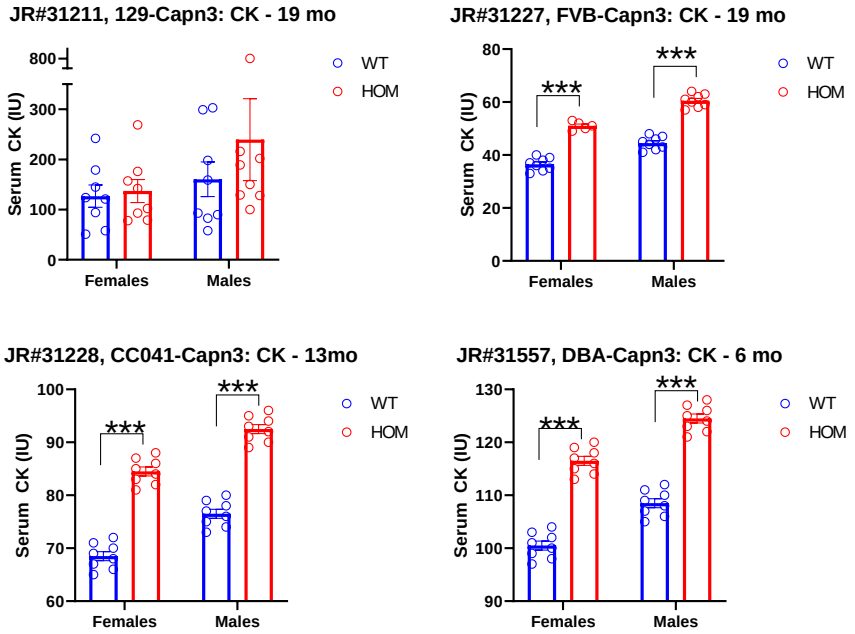
Serum Creatine Kinase

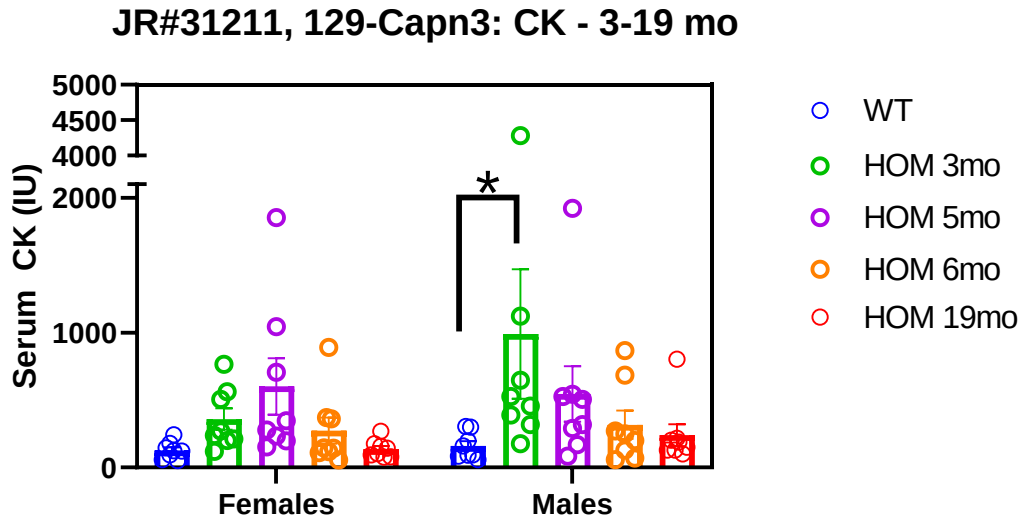


Serum Creatine Kinase for the four Capn3 strains

Serum CK measures sarcolemma breakdown (disruption of the membrane of muscle fibers). There was a very significant increase in serum CK in all strains but 129-Capn3. CC041-Capn3 and DBA-Capn3 had the most severe increases – which coud be explained by the younger age of the mice.

Serum Creatine Kinase

129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211



Longitudinal Serum Creatine Kinase for the 129.Capn3 strain

Serum CK measures sarcolemma breakdown (disruption of the membrane of muscle fibers). There was a very significant increase in serum CK at 3 months in the males. Serum CK then decreased with age and there was no difference in aged mice. The same trend was observed in females but did not reach statistical significance.

Calpain3 KO Histology

129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J; **JR 031211**

FVB/NJ-*Capn3*^{em9Lutzy}/J; **JR 031227**

CC041-*Capn3*^{em10Lutzy}/J; **JR 031228**

DBA/2J-*Capn3*^{em12Lutzy}/J; **JR 031557**

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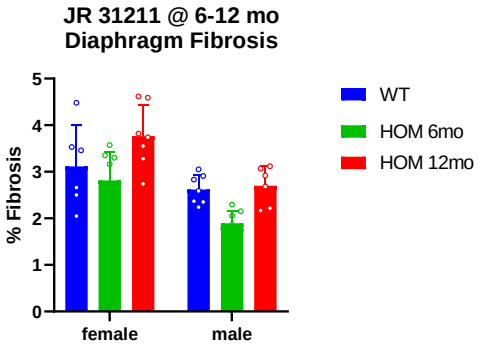
Histology, Males

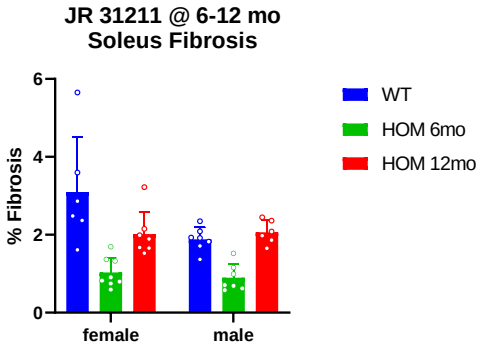
- Histopathology was assessed on male and female (not shown)
  - 129S4/SvJaeJ-Capn3^{em5Lutzy}/J (#31211), from 6 to 21 months
  - FVB/NJ-Capn3^{em9Lutzy}/J (#31227) and DBA-Capn3 (#31557), at 21 months
  - CC041-Capn3^{em10Lutzy}/J (#31228), at 13 months
- A reticulin stain was used to measure muscle fiber sizes and quantify central nuclei; a Sirius Red stain was used to quantify interstitial fibrosis (in red) and observe general histology.
- Representative pictures of the psoas muscle in males only, are shown. In each strain, WT mice represent the normal reference.
- The pathology was **most severe in 129-Capn3**, intermediate in CC041-Capn3, and mild in FVB-Capn3. In all strains, **the leg muscles and diaphragm were less affected than the psoas**.
- The pathology was characteristic of LGMD2A (Rosales, 2013, PMID 23553538) and consisted in **central nucleation** (a sign a muscle injury and regeneration), **interstitial fibrosis** (deposit of collagen between the muscle fibers, a sign of inflammation), and replacement of muscle fibers by fat (or **fat infiltration**).
- While relevant to the clinical presentation, fatty infiltration and fibrosis (without experimental muscle injury) in Capn3 mouse models have **not been observed before** in the published knock-outs (Richard, 2000, PMC2150676). Fibrosis after experimental injury was reported previously (Yalvac, 2017, PMID:29241457).

Histology: fibrosis

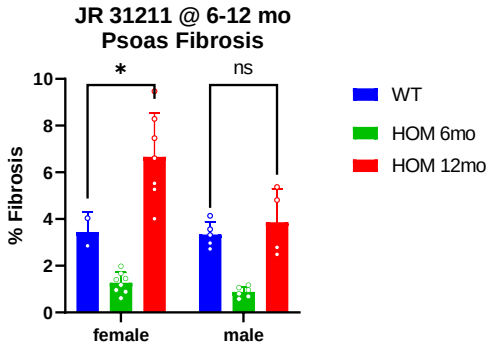
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211



Two-way ANOVA	Ordinary	
Alpha	0.05	
Source of Variation	% of total variation	P value
Interaction	3.866	0.2426
Sex	28.65	0.0035
Genotype	6.213	0.1422



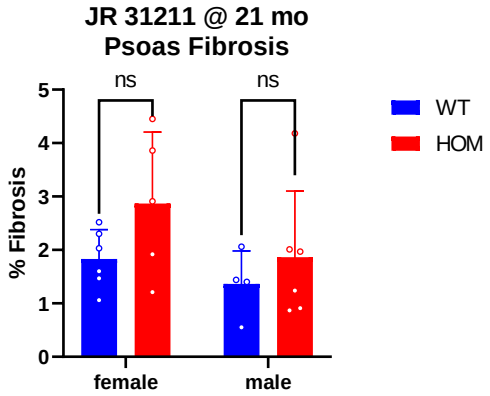
Two-way ANOVA	Ordinary	
Alpha	0.05	
Source of Variation	% of total variation	P value
Interaction	13.71	0.0483
Sex	11.67	0.0667
Genotype	6.945	0.1508



Two-way ANOVA	Ordinary	
Alpha	0.05	
Source of Variation	% of total variation	P value
Interaction	9.257	0.0981
Sex	10.76	0.0768
Genotype	17.89	0.0273

Histology: fibrosis

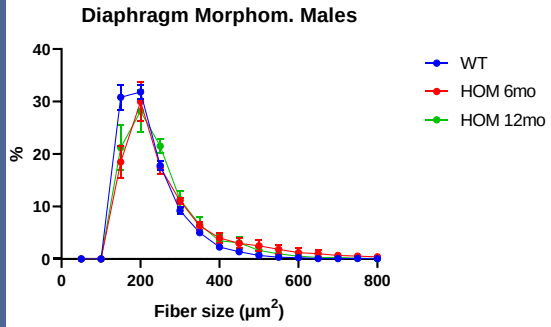
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211



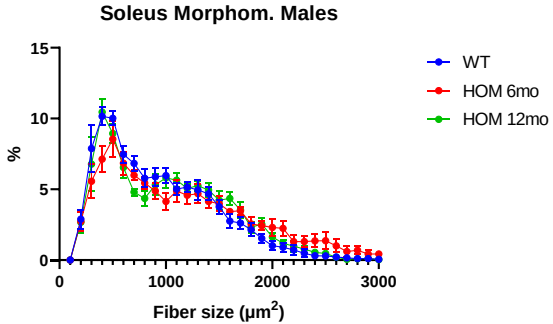
Two-way ANOVA	Ordinary	
Alpha	0.05	
Source of Variation	% of total variation	P value
Interaction	1.601	0.5560
Sex	11.97	0.1189
Genotype	13.07	0.1042

Histology: morphometry, males

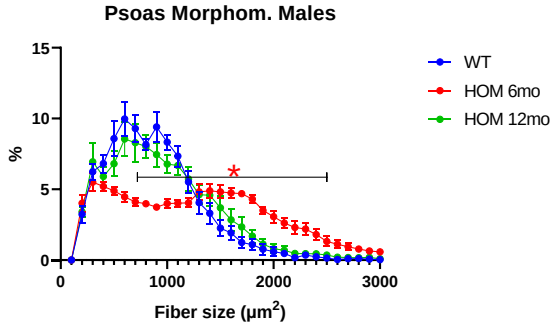
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211



Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	2.059	<0.0001
Size	84.74	<0.0001
Genotype	4.711e-007	0.2469



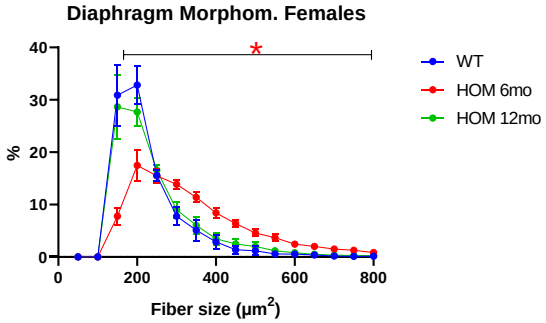
Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	2.091	0.0194
Size	84.40	<0.0001
Genotype	5.707e-015	0.2880



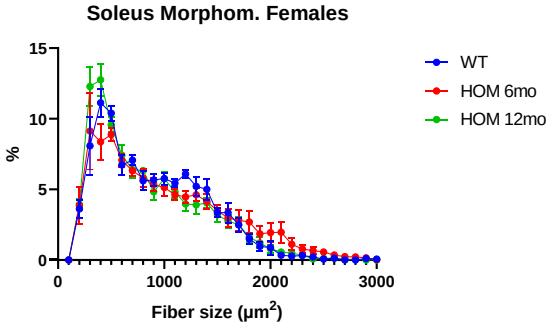
Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	10.23	<0.0001
Size	78.94	<0.0001
Genotype	5.270e-015	0.3576

Histology: morphometry, females

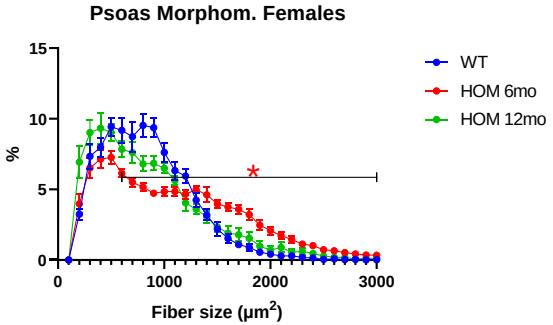
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211



Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	10.71	<0.0001
Size	79.20	<0.0001
Genotype	9.710e-016	0.7620



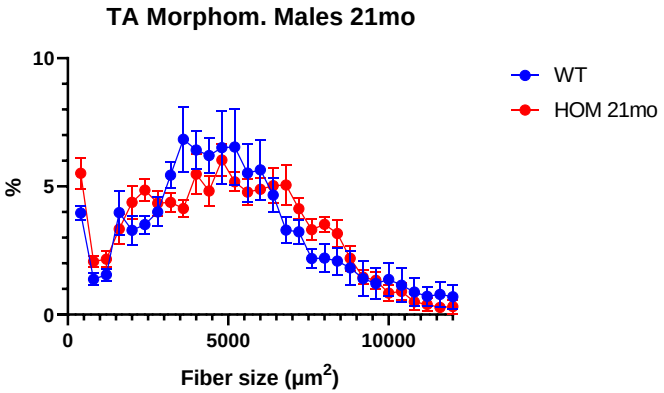
Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	2.053	0.0693
Size	81.67	<0.0001
Genotype	1.770e-015	0.6827



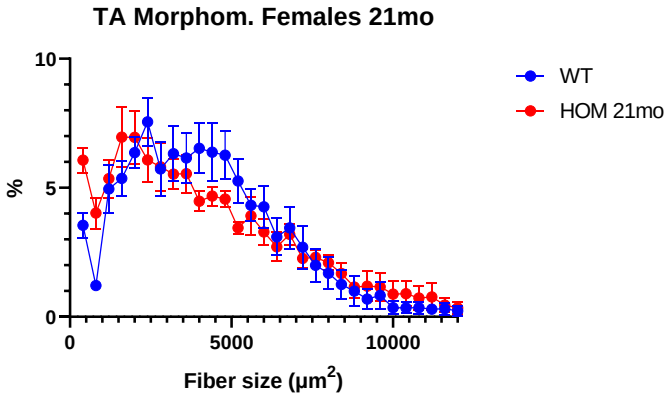
Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	5.631	<0.0001
Size	87.64	<0.0001
Genotype	5.590e-015	0.4379

Histology: morphometry, TA muscle of aged mice

129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211



Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	2.678	0.0374
Size	80.06	<0.0001
Genotype	3.520e-007	0.3893

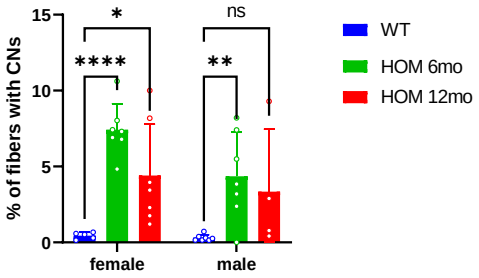


Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	2.701	0.0560
Size	78.21	<0.0001
Genotype	1.673e-006	0.1060

Histology: Central nuclei

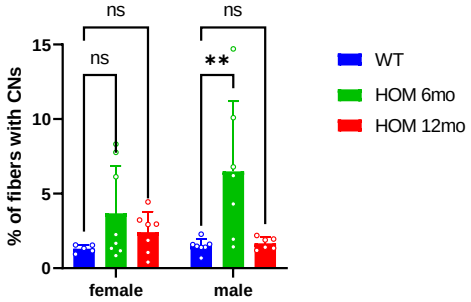
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211

JR 31211 @ 6-12 mo
Diaphragm Central Nuclei



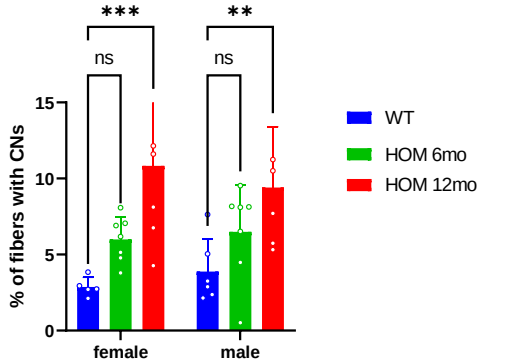
Two-way ANOVA	Ordinary		
Alpha	0.05		
Source of Variation	% of total variation	P value	P value summary
Interaction	3.385	0.3186	ns
Sex	4.179	0.0969	ns
Genotype	46.08	<0.0001	****

JR 31211 @ 6-12 mo
Soleus Central Nuclei



Two-way ANOVA	Ordinary		
Alpha	0.05		
Source of Variation	% of total variation	P value	P value summary
Interaction	6.865	0.1674	ns
Sex	1.564	0.3605	ns
Genotype	30.75	0.0011	**

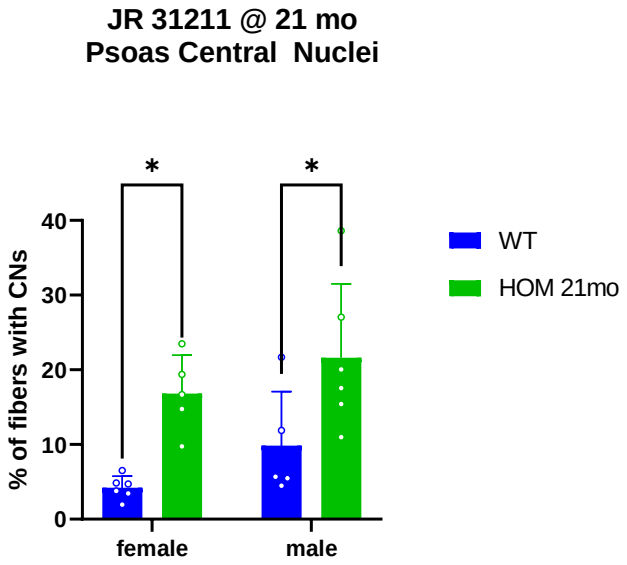
JR 31211 @ 6-12 mo
Psoas Central Nuclei



Two-way ANOVA	Ordinary		
Alpha	0.05		
Source of Variation	% of total variation	P value	P value summary
Interaction	1.725	0.5908	ns
Sex	0.001721	0.9741	ns
Genotype	46.13	<0.0001	****

Histology: Central nuclei, aged mice

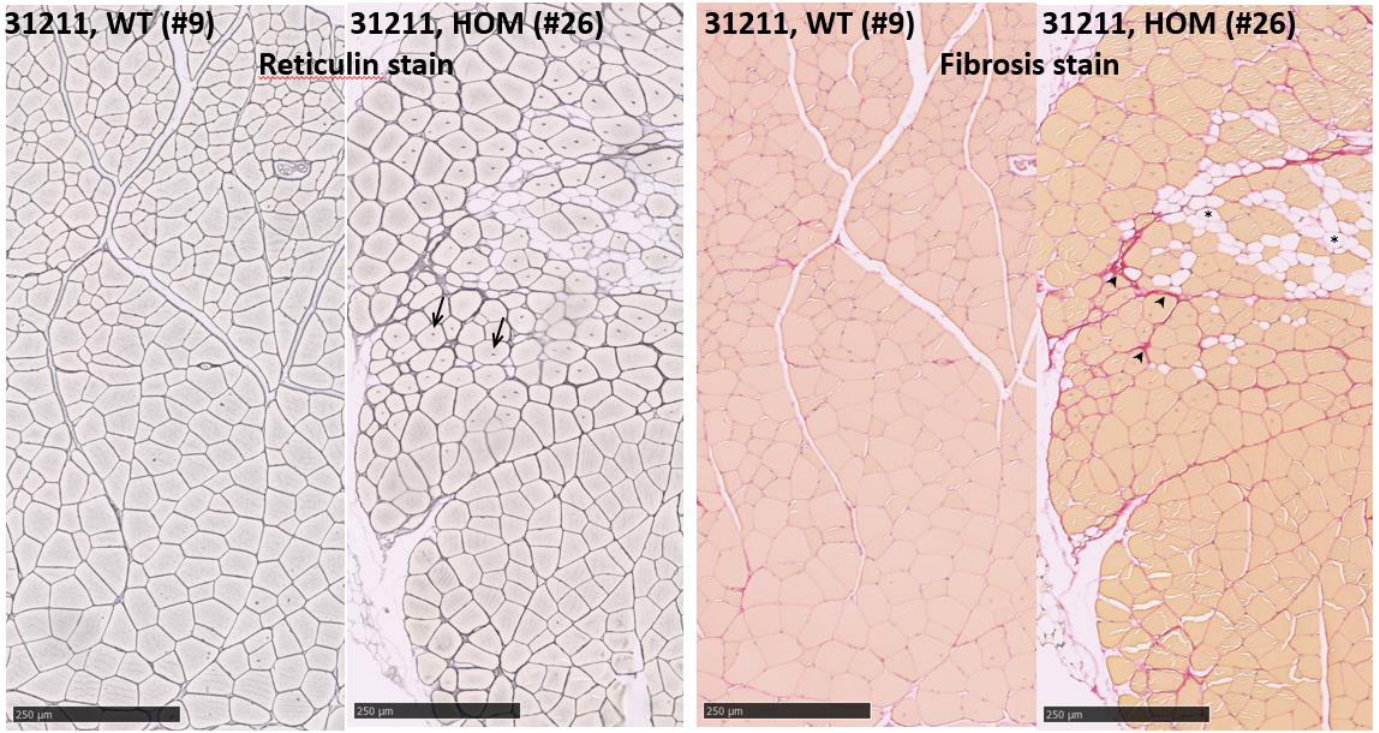
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211



Two-way ANOVA	Ordinary		
Alpha	0.05		
Source of Variation	% of total variation	P value	P value summary
Interaction	0.05037	0.8877	ns
Sex	8.024	0.0874	ns
Genotype	43.84	0.0005	***

Histology, Males

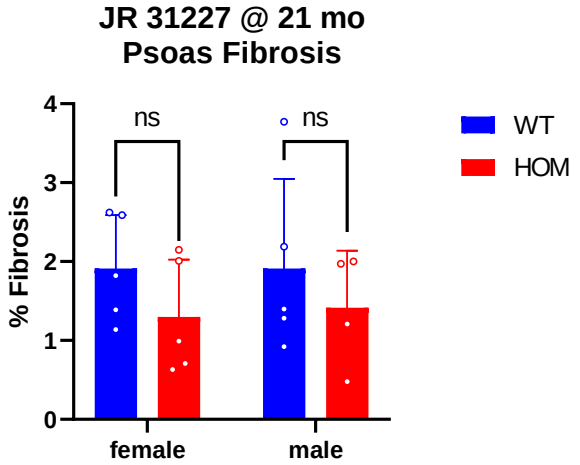
129S4/SvJaeJ-*Capn3*^{em5Lutzy}/J JR 031211



Central nucleation (arrows), interstitial fibrosis (arrowheads), fatty infiltration (stars) characterize the histopathology of the 129-Capn3 mutants at 24 months of age

Histology: fibrosis

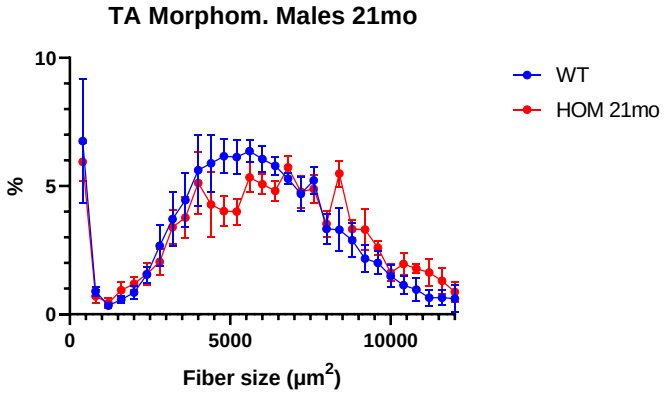
FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227

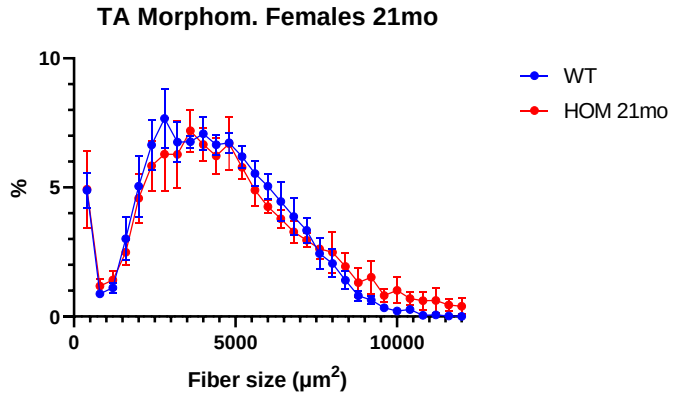


Two-way ANOVA	Ordinary	
Alpha	0.05	
Source of Variation	% of total variation	P value
Interaction	0.1317	0.8826
Sex	0.1317	0.8826
Genotype	11.88	0.1741

Histology: morphometry

FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227



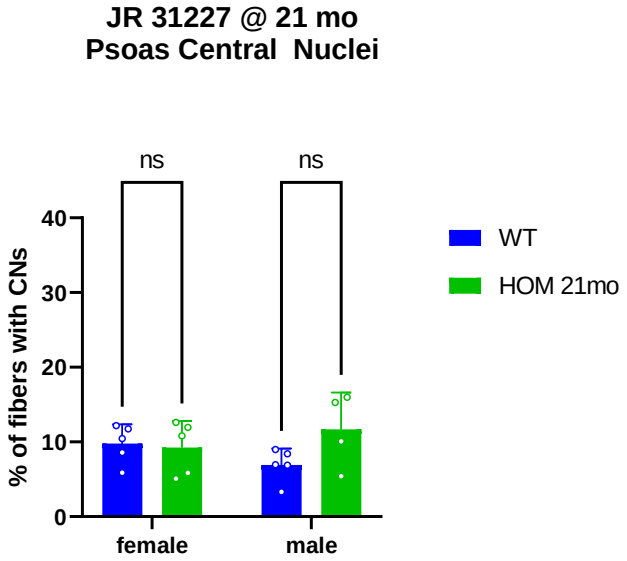


Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	2.377	0.6301
Size	77.15	<0.0001
Genotype	6.016e-016	0.7019

Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	0.6694	0.9999
Size	86.26	<0.0001
Genotype	5.346e-017	0.9512

Histology: Central nuclei, aged mice

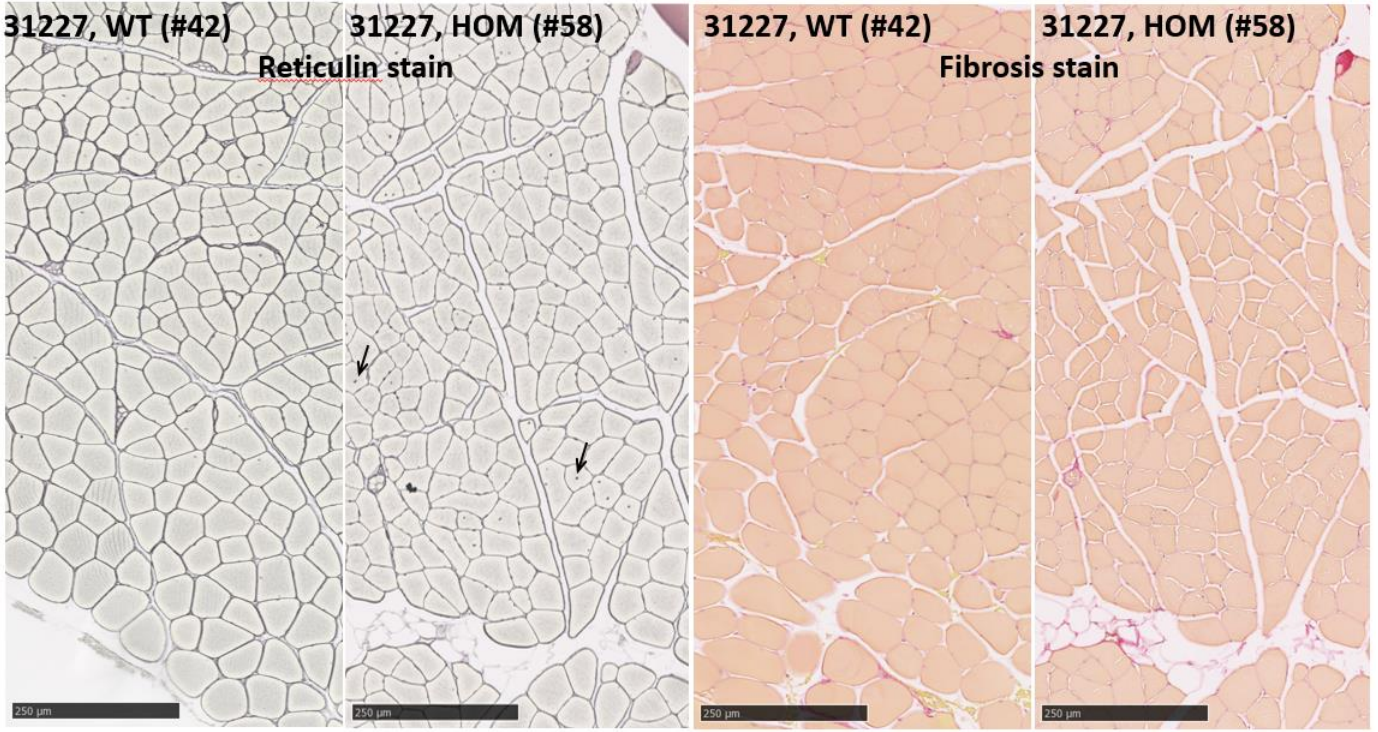
FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227



Two-way ANOVA	Ordinary		
Alpha	0.05		
Source of Variation	% of total variation	P value	P value summary
Interaction	14.79	0.1090	ns
Sex	0.09709	0.8920	ns
Genotype	9.660	0.1886	ns

Histology, Males

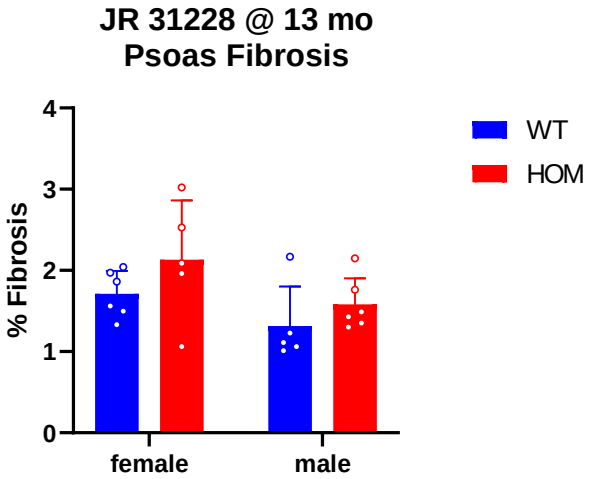
FVB/NJ-*Capn3*^{em9Lutzy}/J JR 031227



Mild central nucleation (arrows), but no interstitial fibrosis or fatty infiltration characterize the histopathology of the FVB-Capn3 mutants

Histology: fibrosis

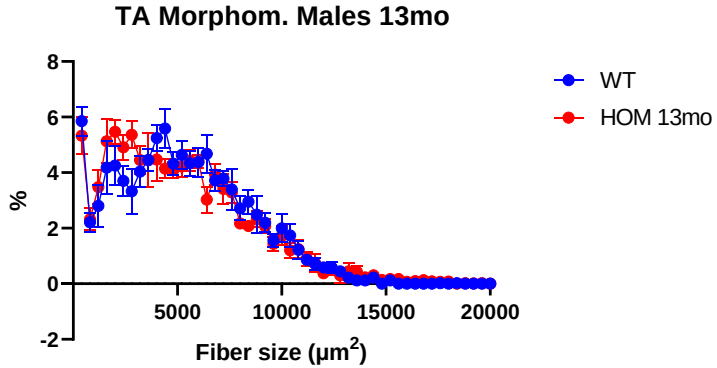
CC041-*Capn3*^{em10Lutzy}/J JR 031228

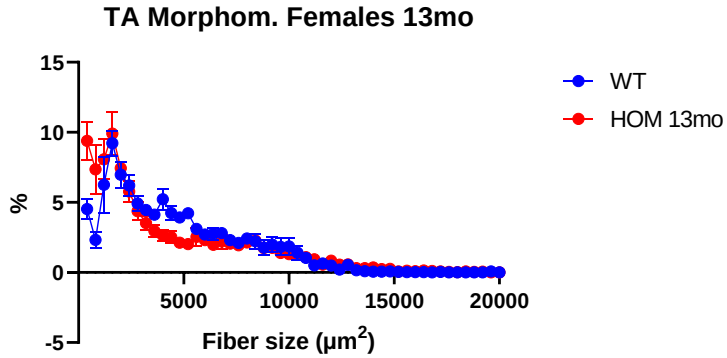


Two-way ANOVA	Ordinary	
Alpha	0.05	
Source of Variation	% of total variation	P value
Interaction	0.5926	0.6999
Sex	21.24	0.0307
Genotype	11.17	0.1063

Histology: morphometry

CC041-*Capn3*^{em10Lutzy}/J JR 031228



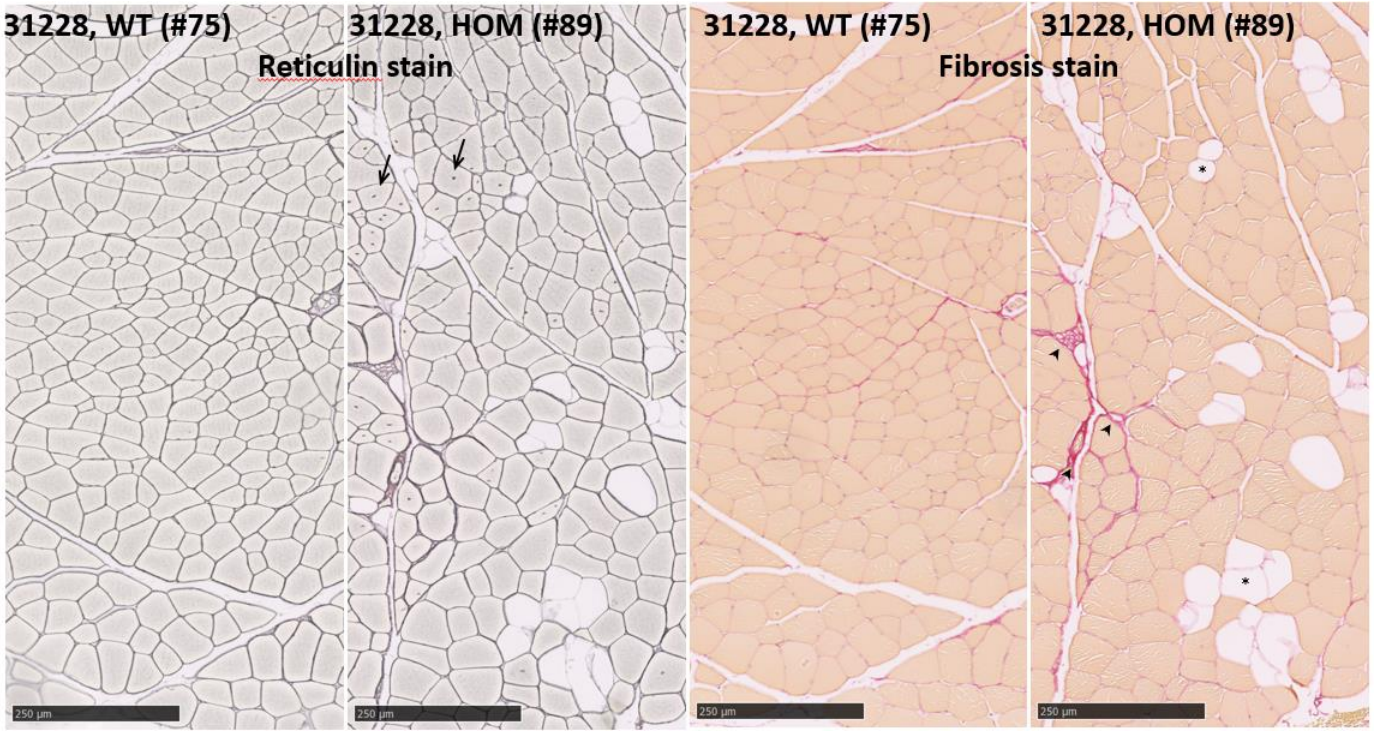


Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	1.900	0.2917
Size	82.00	<0.0001
Genotype	2.923e-005	0.3020

Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	5.711	<0.0001
Size	77.67	<0.0001
Genotype	1.411e-006	0.5647

Histology, Males

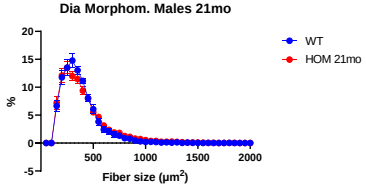
CC041-*Capn3*^{em10Lutzy}/J JR 031228



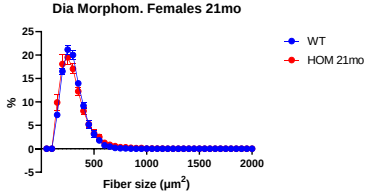
Central nucleation (arrows), mild interstitial fibrosis (arrowheads) and mild fatty infiltration (stars) characterize the histopathology of the CC041-Capn3 mutants

Histology, Morphometry

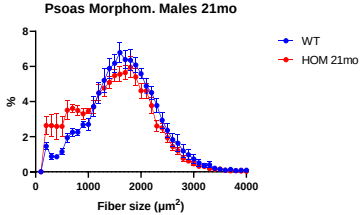
DBA/2J-*Capn3*^{em12Lutzy}/J JR 031557



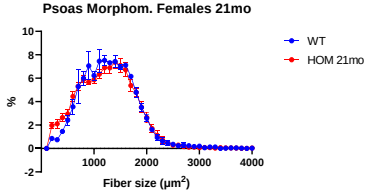
Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	0.5078	0.6102
Size	91.48	<0.0001
Genotype	6.723e-009	0.8926



Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	0.4634	0.8193
Size	86.44	<0.0001
Genotype	1.189e-009	0.9590



Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	2.312	<0.0001
Size	84.65	<0.0001
Genotype	6.844e-016	0.5452

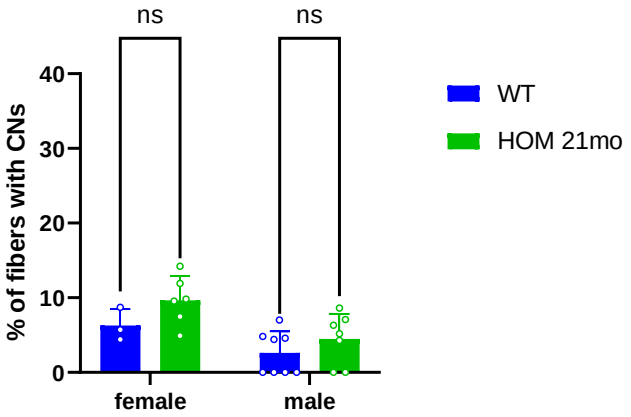


Two-way RM ANOVA	Matching: Stacked	
Assume sphericity?	No	
Alpha	0.05	
Source of Variation	% of total variation	P value
Size x Genotype	0.6205	0.6947
Size	85.67	<0.0001
Genotype	1.179e-014	0.2893

Histology: Central nuclei, aged mice

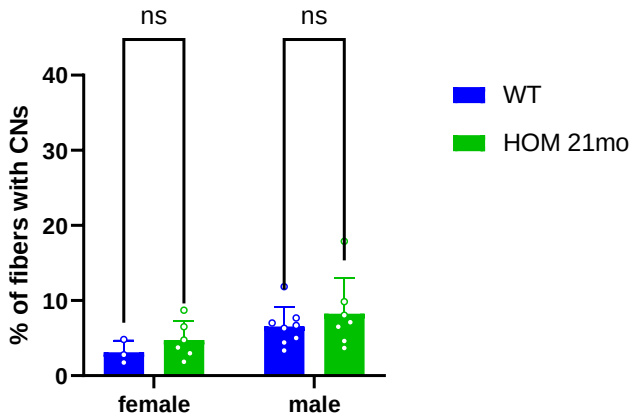
DBA/2J-*Capn3*^{em12Lutzy}/J JR 031557

JR 31557 @ 21 mo
Diaphragm Central Nuclei



Two-way ANOVA	Ordinary		
Alpha	0.05		
Source of Variation	% of total variation	P value	P value summary
Interaction	0.7624	0.5919	ns
Sex	27.68	0.0037	**
Genotype	9.735	0.0657	ns

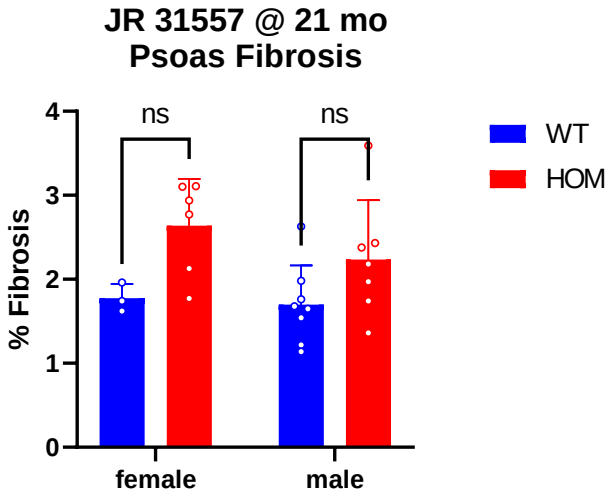
JR 31557 @ 21 mo
Psoas Central Nuclei



Two-way ANOVA	Ordinary		
Alpha	0.05		
Source of Variation	% of total variation	P value	P value summary
Interaction	0.001725	0.9831	ns
Sex	21.58	0.0266	*
Genotype	5.077	0.2592	ns

Histology, Fibrosis

DBA/2J-*Capn3*^{em12Lutzy}/J JR 031557



Two-way ANOVA	Ordinary	
Alpha	0.05	
Source of Variation	% of total variation	P value
Interaction	1.460	0.5067
Sex	3.061	0.3393
Genotype	26.63	0.0091

Histology, Males

DBA/2J-Capn3em12Lutzy/J JR 031557