PASS Protocol Proposal for MoTrPAC Steering Committee

Joslin Diabetes Center (Joslin), University of Iowa (Iowa) and University of Florida (UF)

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Abstract

Exercise is a powerful and pleiotropic physiological stimulus that helps prevent many chronic diseases and is used as a therapeutic for disease. While the beneficial effects of exercise are widely acknowledged, there is still very little understood about the molecular transducers of the systems-wide effects of exercise on health. The goal of MoTrPAC is to define the molecular map, including both signals and responses, that transmit the systemic health effects of physical activity, and indicate how they are altered by age, sex, body composition, and fitness level.

The Preclinical Animal Study Sites (PASS) will play a critical role in the Consortium in several aspects. First the PASS sites will be collecting tissues that cannot be obtained from humans. Second, the PASS sites will perform collections that provide more temporal resolution following an acute bout of exercise. Third, the PASS sites will collect tissues from rats at different stages of training. The data from the chemical analysis from the tissues and timepoints generated by PASS studies will tremendously expand the breadth and impact of the exercise transducer map. The outcomes of these studies will provide the basis for future mechanistic investigations leading to a vast number of novel discoveries that will ultimately transform human health.

Definition of Phase 1 and Phase 2 Studies

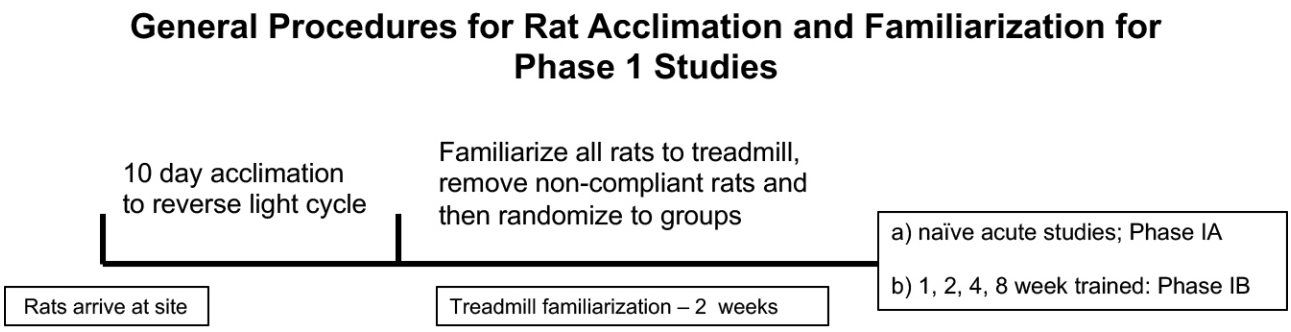
In contrast to the human studies, the PASS component of MoTrPAC is divided into two Phases. For Phase 1 studies, young adult (6 months) and late middle-aged (18 months) rats will undergo acute exercise, chronic endurance exercise training, or serve as sedentary controls. The major goal of the Phase 1 studies is to use these animals to collect as many tissues as feasible for in-depth analysis by the Chemical Analysis Sites (CAS). As described in more detail below, the PASS Phase 1 studies are divided into two parts; Phase 1A will collect tissues from acutely exercised rats and Phase 1B will collect tissue from exercise trained rats. The proposed dates for completion of the tissue collection are: Phase 1A - acute studies in September 2018 (6 months) and February 2019 (18 months), and the Phase 1B - training studies in February 2019 (6 months) and September 2019 (18 months). We note that the PASS sites are only performing treadmill training in Phase 1. There was agreement among members of the animal committee that there was no appropriate rodent model of resistance training for the goals of Phase 1. However, the Iowa PASS site will include a model of resistance exercise in their Phase 2 rodent studies.

Phase 2 studies will occur in years 3-6 of MoTrPAC. The aims of the Phase 2 studies are unique for each PASS site. For the UF and Joslin PASS, mechanistic studies will be performed using CAS data generated from Phase 1 studies. The focus of these studies will be to screen for exercise induced molecules that transduce stress resistance at UF and to test myokines/adipokines/circulating factors for health benefits at Joslin. For the Iowa PASS, the Phase 2 studies will focus on the effect of aging on adaptation to chronic endurance and resistance exercise.

General Procedures for Animal Handling and Exercise

Phase I studies will use male and female 6 month (adult) and 18 month (late-middle age) Fischer 344 (F344) rats. This is an inbred rat strain that will be provided by the NIA rodent colony. The 6 month age was selected because the rats have reached a stable lean body mass. The 18 month age was selected because the rats will be relatively healthy and will not have acquired many of the pathologies known to occur in this strain. Acclimation and familiarization will be as shown in Figure 1 and will be identical for Phase 1A and 1B.

Figure 1



Rat Housing and Chow: Rats are nocturnal; therefore animal room housing for MoTrPAC rats will be on a reverse dark-light cycle with lights off at 9 am and lights on at 9 pm. Upon arrival to each facility, rats will be acclimated to the reverse dark-light cycle for a minimum of 10 days. The reverse light cycle will allow for MoTrPAC staff to carry out all procedures and exercise sessions during the awake phase of the rat and during normal working hours for staff. Only red lights will be used during the rats' dark cycle. Red lights will be used in order to provide enough light for staff to handle rats and perform all procedures including exercise. The animal facility staff for each PASS has agreed to the reverse dark-light cycle and the use of red lights.

Housing conditions will be standardized across all three PASS sites. Animal housing room temperatures will be kept between 68° – 77° F, with humidity between 25-55%. This variability is due to locations in the Boston, Iowa City and Gainesville and is under the control of the animal facilities. Each site will record the temperature and humidity of each MoTrPAC rat room regularly on the forms developed by the Consortium Coordinating Committee (CCC). Rats will be housed in ventilated racks in cages with a minimum size of 142 sq inches. Rats will be housed 2-3 animals per cage; weight permitting. Environmental enrichment will be provided for animals that are individually housed. All sites will standardize on the same cage bedding type as used in the NIA colony (Teklad 7093 Shredded Aspen) to control for any environmental variables. Each cage will be filled to approximately 0.25-0.75 inch depth with bedding. The specific pelleted diet to be used is Lab Diet 5L79 from Lab Supply (Fort Worth, Texas), which is the same diet as is used in the NIA colony. Each site will purchase feed in reasonable quantities (e.g, 3-6 month supply) in order to minimize lot number changes during the course of the projects.

Treadmill Familiarization: *Exercise:* Treadmill exercise will be used for all studies. All sites have the Panlab 5 stall (lane) rat treadmill. As noted above, all exercise will be performed in the active, dark phase of the rats.

The rats will arrive at each of the PASS sites at least 3 weeks prior to initiation of the acute or training studies. The familiarization program has two components: one is designed to allow the rats to adjust to human handling and the second involves treadmill exposure without inducing any exercise training adaptations.

Upon arrival to the PASS sites, rats will be acclimated to the reverse light cycle and this takes 10 days. During this time the rats will undergo familiarization, which involves daily handling (Monday – Friday and not required on weekends) by the research staff. Following the intitial lighting and handling acclimation period, the rats will go through a treadmill familiarization period to identify potential non-compliant rats.

<u>The UF/Joslin Phase 1A familiarization plan will be:</u>

Days 1-2	10 minutes/day	Rats explore stationary treadmill

Days 3-5	10 minutes/day	Place rats on moving treadmill, speed 5-7 m/min, 0° incline
Days 6-10	10 minutes/day	Place rats on moving treadmill, speed 8-10 m/min, 0° incline

On day 10, rats will be assigned treadmill activity scores. Activity scores will range from 1-4, with 4 being the highest score.

4	Rats are active on the treadmill the entire activity session without assistance
3	Rats require minimal assistance, defined as, assistance for less than 25% of the time of the activity session
2	Rats require much assistance, defined as, assistance for greater than 25% of the time of the activity session
1	Rats are non-compliant and fail to complete an activity session

Iowa Phase 1B: Treadmill Familiarization Plan:

The initial familiarization plan has been adjusted to get the rats to run at speeds approximating 70% VO2 max:

1) Days 1 - 2: Put rats on treadmill at 0 m/min for 10 mins to familiarize them to the treadmill.
   a) Block the shock area, so that they won't be able to sit on the shock grid
2) Days 3 - 5: Block the shock area and run the rats at 6 m/min for 10 mins
   a) Use a pen (with a dull point) to gently prod the rats or turn their head to make them walk forward and motivate them
3) Days 6 - 12: Block the shock area and run the rats at 10 m/min for 10 mins
   a) If the rat will not run at this speed, then reduce the speed to 6 m/min. Once the rat starts walking and walks forward properly, then increase the speed to 8 m/min and let it walk for 5 mins (if they get better, increase to 10 m/min and let it walk for 3 mins)
   b) On the next day, start the rat running at 10 m/min along with the other rats and see how it reacts.
   c) If it will not run at 10 m/min, repeat the routine outlined in 3a.
4) Days 10 - 12: Only for those rats that will not run continuously, use a light shock to make them run
   a) Once a shock is used, do not let them sit on the shock grid.
   b) If they are backward walking, use a pen to turn their head and prod them to run forward.
5) On Day 11: After each familiarization session, increase grade and speed to 10 degree and 12 m/min for 2 mins.
6) Evaluation (on Day 12)
   a) Run the rats at 0 Degree and 10 m/min for 5 mins to initially evaluate them (continue encouraging the rats with a pen)
   b) Increase grade and speed to 10 degree and 12 m/min for 5 mins for final evaluation.

For the poor runners (not trying to run), separate them and run them at 10 degree and 12 m/min for 1 min using shock. For the remaining 4 minutes, turn off the shock and evaluate their running.

On day 12, rats will be assigned treadmill activity scores. Activity scores will range from 1-4, with 4 being the highest score.

Following completion of the Iowa familiarization, rats are scored using the following criteria:

4	Rats are active on the treadmill the entire activity session without assistance
3	Rats require minimal assistance, defined as, assistance for less than 25% of the time of the activity session

2	Rats require much assistance, defined as, assistance for greater than 25% of the time of the activity session
1	Rats are non-compliant and fail to complete an activity session

The results of this assessment will be entered into the rat database via the MoTrPAC website. Rats that are assigned a score of 1 will be removed from the study. Rats that score 2-4 will be used by the DMAQC group of CCC and they will be randomized to either an exercise or control group. The assignment of rats to groups will be completed within a 3-workday window and communicated to each PASS site through the online website.

MoTrPAC PASS Staff: All MoTrPAC staff have been trained, or will be trained, in standard lab safety and proper handling of rats, as required by each PASS's individual institutional animal facility (IACUC). Staff will be thoroughly trained in exercising of rats and all tissue collection procedures. Staff will be listed at the CCC and linked to their specific role within the training and tissue collection plans.

The three PASS have had one joint training session to date, and will continue to coordinate training to maximize consistent animal handling.

Phase 1A: Studies of an Acute Bout of Endurance Exercise

Phase 1A studies will be conducted at UF and Joslin. The goal of Phase 1A is to perform detailed acute exercise time course protocols in 6-month and 18-month F344 rats, and from these rats collect as many tissues as feasible in order to provide high quality samples for analysis by CAS.

Sample Size: For each acute exercise time point (immediately, 0.5, 1, 4, 7, 24 and 48 hours post exercise, a total of 12 male and 12 female rats will be used. Based on sample size analysis and consultation with CAS, it has been determined that this sample size will be sufficient to allow CAS sites to maximize the signal to noise across time series, while providing sufficient number of samples to each site (n=6 rats per site and time point) to be able to discern any potential PASS site specific effects in the CAS outcomes.

Acute Exercise Protocol and Time Course: Rats will be sedentary or perform a single 30 minute bout of treadmill exercise and sacrificed at timepoints as shown in **Figure 2**. On the day of study, food will be removed from the cages at 1 hour prior to lights off for all control and exercised rats. The exercise bout will be staggered by group and will commence within 1-3 hours after lights off (10am-1pm). We used a staggered schedule to allow for each site to collect 1 rat for each control and exercise timepoint (n=9) in a single day. In addition, we did this with one cohort of 9 males and a second cohort of 9 females so that both males and females were exposed to the exercise on the same days. To see how the PASS Phase !a teams organized the exercise interventions with tissue collections please see this outlined in **Table 1**.

The exercise bout for the 6 month rats will consist of treadmill running for 30 min at 5 degrees (8.7%) grade at 28cm/s for males and 30cm/s for females. The intensity for the 6 month male rats is > 70% VO_2 max and is close to 75-80% for 6 month males and 70-75% $V0_2$ max for the 6 month females. We used a slightly higher intensity for the males due to issues of treadmill compliance (e.g. backward running) at lower treadmill speeds.

The exercise bout for the 18 month old rats will consist of treadmill running for 30 minutes at 5 degrees grade at 20cm/s for males and 23cm/s for females. We selected these speeds to try and match the intensity of the 18month runners to the 6 month runners.

The running speeds were based on VO_2 max measurements made on male and female 6- and 18-

Table 1: Schedule for running and collections

Order of Sacrifice	Group	Time of Intervention	Last Feeding	Sacrifice Time	Time Fasted (hh:mm)
1	SED 0	9:20-9:50	8:00	9:50	1:50
2	SED 0	9:30-10:10	8:00	10:10	2:10
3	24 hours	10:00-10:30 (day prior)	8:00	10:30	2:30
4	24 hours	10:20-10:50 (day prior)	8:00	10:50	2:50
5	48 hours	10:40-11:10 (2 days prior)	8:00	11:10	3:10
6	48 hours	11:00-11:30 (2 days prior)	8:00	11:30	3:30
7	0.5 hr	10:50-11:20	8:00	11:50	3:50
8	0.5 hr	11:10-11:40	8:00	12:10	4:10
9	0 (IPE)	12:40-13:10	8:00	13:10	5:10
10	0 (IPE)	13:00-13:30	8:00	13:30	5:30
11	1 hr	12:20-12:50	8:00	13:50	5:50
12	1 hr	12:40-13:10	8:00	14:10	6:10
13	4 hr	10:00-10:30	8:00	14:30	6:30
14	4 hr	10:20-10:50	8:00	14:50	6:50
15	SED 7	9:20-9:50	11:50	16:50	5:00
16	SED 7	9:40-10:10	12:10	17:10	5:00
17	7 hr	10:00-10:30	12:30	17:30	5:00
18	7 hr	10:20-10:50	12:50	17:50	5:00

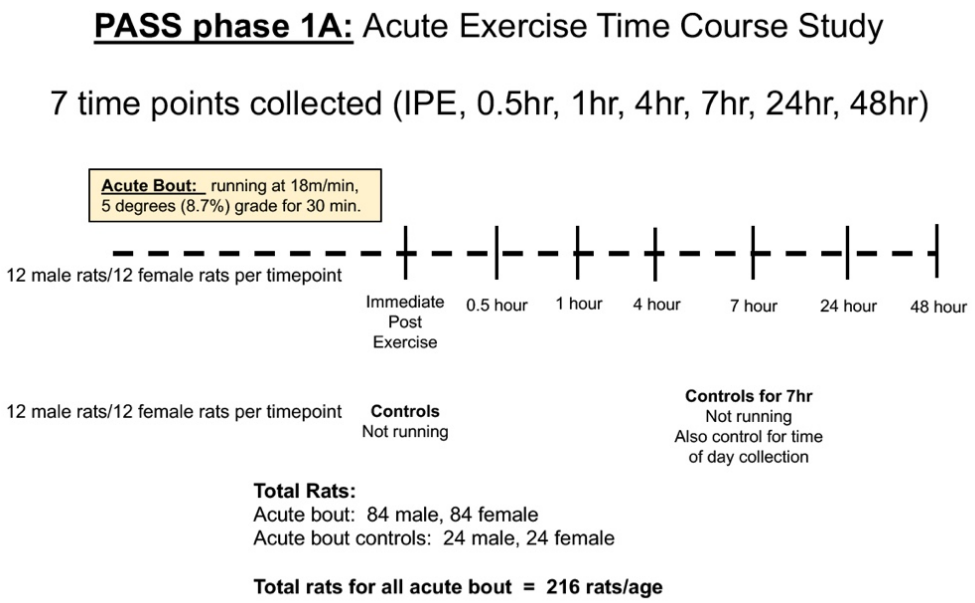
month old rats (n=5/ group) at the University of Iowa (June 2018). $\underline{VO_2 \text{ max}}$ will be determined using the following protocol. The treadmill incline will be set at 10° following a 15 minute warm up period at 0° incline and 9 m/min. Treadmill speed will be increased by 1.8 m/min every 2 minutes based on the protocol outlined in Wisloff et al. *AJP Heart* 280:H1301, 2001. Criteria for reaching VO_2max will be a leveling off of oxygen uptake despite increased workload, a respiratory exchange ratio above 1.05, and an unhemolyzed blood lactate concentration ≥6 mmol/l.

All sedentary control rats for these studies will be handled with the same frequency as the exercised rats. This will include removing them from their cages to a non-moving treadmill on the day of collections.

Tissues will be collected immediately post-exercise (IPE) and 0.5, 1, 4, 7, 24 and 48 hours after exercise.

Food availability: For the IPE and 0.5, 1, and 4 hour post-exercise groups, the rats will not have access to food between the end of exercise and before sacrifice. The exercise intervention for the 4hr group will be earlier after lights off so that these rats will not be without food for > 7hr total. For the 7 hour post-exercise group, rats will have a controlled amount of food for the 2 hours immediately following the exercise. The amount of food to be provided will be 1 pellet and the amount of the pellet eaten during this time will be recorded for each rat. The goal will be to provide enough food to the rats to prevent hypoglycemia. For the 24 and 48 hour post-exercise groups, the rats will be returned to their home cage following the exercise bout and allowed to feed ad libitum. On the day of collection, food will be removed at 8 am, which is 1 hour prior to lights off and consistent with all other exercise groups. As with the other acute exercise groups, the UF and Joslin sites will schedule the exercise bout for the 24 and 48 hour post-exercise groups in a way that limits the amount of time without food to < 7hrs.

Figure 2:



Euthanasia and Tissue Collection for Phase 1A: For the decision making process followed for the tissue collection plans see the MOP Chapter 3.

The euthanasia and tissue collection team will be made up of a minimum of 7-8 team members and each team member will have a clear outline of their duties that will be practiced with attention to time of collection/freezing at all sites prior to the start of the Phase IA studies. The procedure room for tissue/blood collections will be in a separate location from the treadmill training and rat housing.

At the specific timepoint post-exercise, the rats will be anaesthetized with isoflurane until the foot pinch/pedal reflex is lost, which takes about 1.5 to 2 minutes. For Phase 1A, rats will be anesthetized, followed by removal of 3 ml of blood via cardiac puncture. The rat will then be euthanized by decapitation

or removal of the heart and lungs. The right leg will be rapidly removed from the torso, and the head, torso and leg sections distributed to 3 different dissection stations. For tissues in which there are more than 1, the PASS sites have agreed to collect one lobe of the liver (Joslin took the right and UF the left) and the tissues from the right side of the rat (e.g. gastrocnemius, kidney, lungs, testes). For very small organs, such as the adrenal glands, both the right and left sides will be collected.

Acute exercise studies (Florida and Joslin) tissue collection protocols:

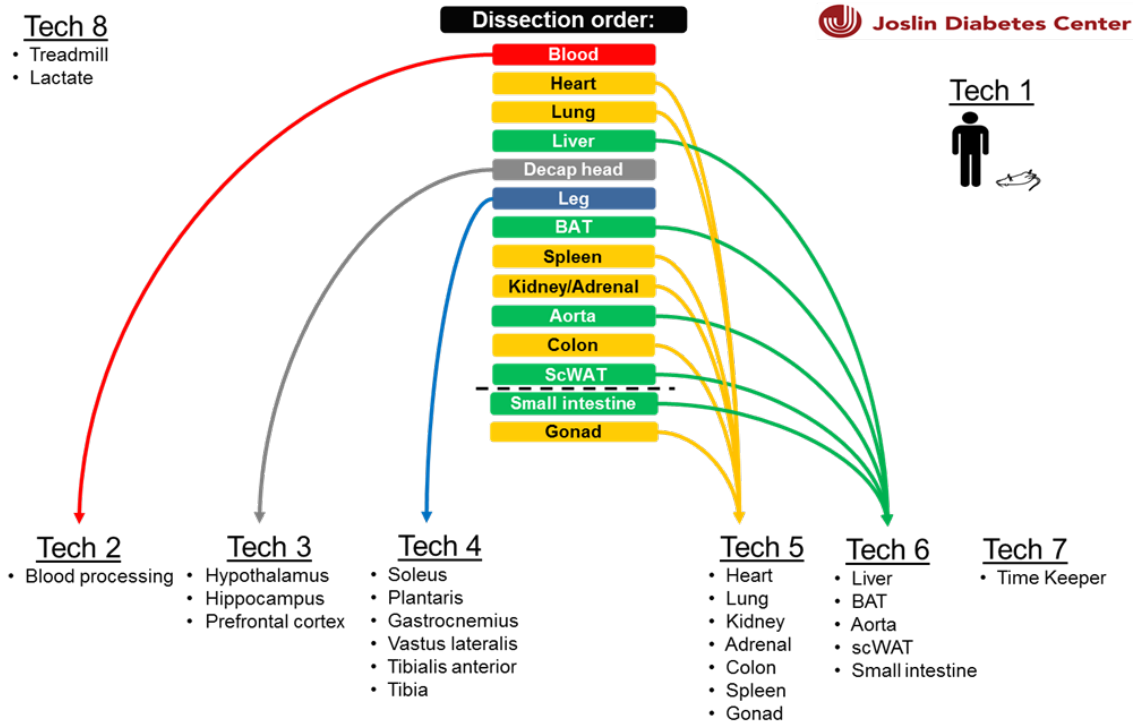
Joslin:

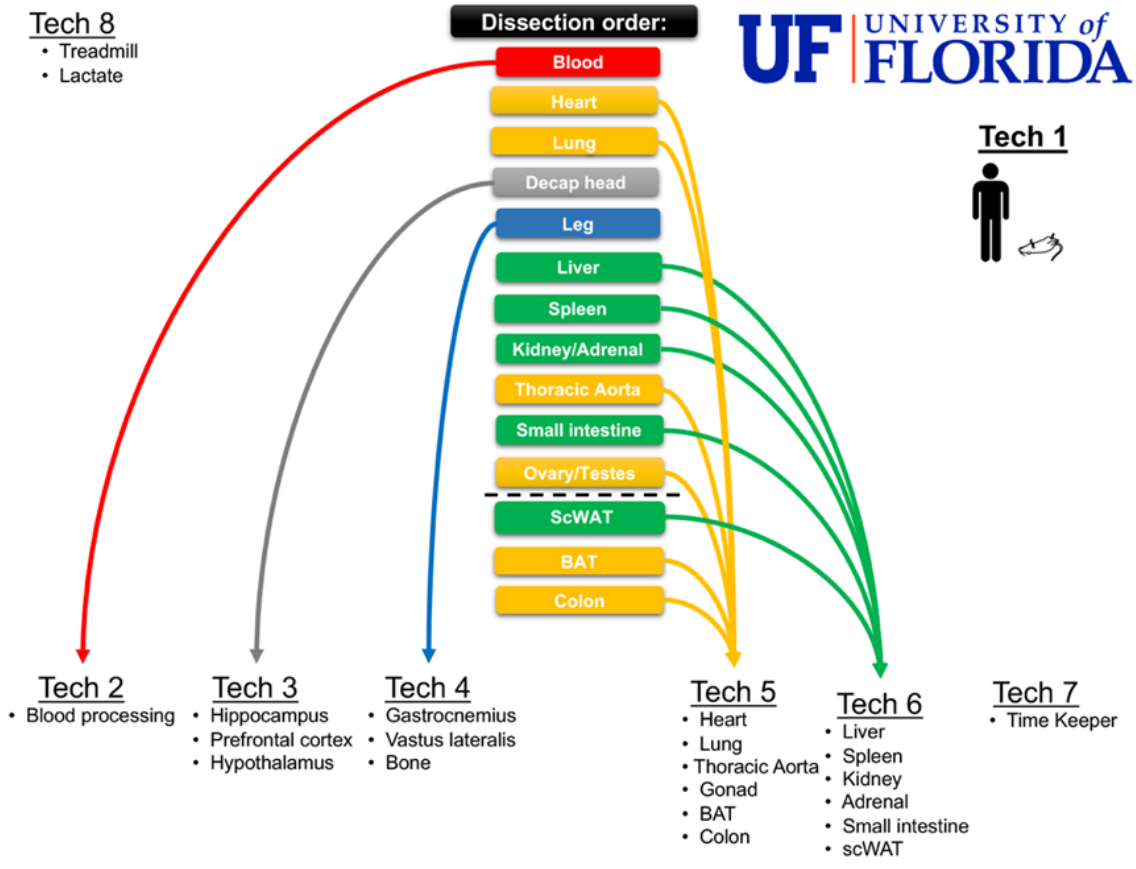
1. Under isoflurane anesthesia (1-3%), approximately 3 ml blood will be drawn via cardiac puncture by Tech 1. Blood will be handed to Tech 2 for processing (whole blood, plasma, and packed cells).
2. Immediately following removal of the blood, Tech 1 will remove the heart and lungs as one entity and give them to Tech 5 for dissection and freezing.
3. Tech 1 will dissect out the liver and give it to Tech 6 for cleaning and freezing.
4. A guillotine will be used to decapitate the rat. The head will be given to Tech 3, who will remove the brain from the skull and dissect out specific brain regions in the following order: hypothalamus, left and right hippocampus, left and right cerebral cortex.
5. Tech 1 will use a guillotine to cut off the right hind leg near the acetabulum. The leg will be given to Tech 4 for dissection and snap freezing of tissues in the following order: soleus, plantaris, gastrocnemius, vastus lateralis, and tibialis anterior. Tibia will collected and clean only for control and 48hrs post-exercise.
6. Tech 1 will dissect out the, brown adipose,spleen, kidney, adrenal glands, aorta, and colon in that order. The brown adipose, and aorta will be given to Tech 6 for cleaning and freezing, while the spleen, kidney, adrenal glands, and descending colon will be handed to Tech 5 for cleaning and freezing.
7. The rat carcass will be handed to Tech 6, who will rapidly dissect out and freeze the subcutaneous white adipose tissue.
8. Tech 1 will then remove the small intestine (duodenum), and gonad from the rat, in that order. The small intestine will be given to Tech 6 for cleaning and freezing, while the gonad will be given to Tech 5 for cleaning, freezing and storage.

Florida:

1. Under isoflurane anesthesia (1-2%), approximately 3 mL blood will be drawn via cardiac puncture by Dissector 1. Blood will be handed to the Blood Processing Technician for processing (whole blood, plasma, and packed cells).
2. Immediately following removal of the blood, Dissector 1 will remove the heart and lung as one entity and give them to Dissector 5 for dissection and freezing.
3. A guillotine will be used to decapitate the rat. The head will be given to Dissector 2, who will remove the brain from the skull and dissect out specific brain regions in the following order: hypothalamus, left and right hippocampus, left and right cerebral cortex.
4. Dissector 1 will use a guillotine to cut off the left hind leg at the acetabulum. The leg will be given to Dissector 3 for dissection and snap freezing of the gastrocnemius.
5. Dissector 1 will remove the liver (left lobe) and give it to Dissector 4 for freezing.
6. Dissector 1 will remove the heart, lungs and thoracic aorta as one entity and give them to Dissector 5 for dissection and freezing.
7. Dissector 1 will dissect additional tissues as follows: small intestine, spleen, left adrenal gland and kidney, right adrenal gland and kidney, left testes or left ovary and right ovary, and descending colon. Dissector 4 will clean and freeze the small intestine, spleen and descending colon. Dissectors 5 and 6 will clean and freeze the kidneys, adrenal glands and ovaries/testes.
8. Dissector 1 will remove and freeze the subcutaneous white adipose followed by the brown adipose. Dissector 5 will dissect and clean the brown adipose.
9. The tibias from selected rats (control and 48hrs post exercise) will be cleaned and frozen by Dissector 7.

Since time for tissue collection is a high priority, the UF and Joslin sites will prioritize tissue collections in a complementary way to get the broadest number of tissues out the fastest. The proposed plan is illustrated in the following two cartoons for each of the two sites:





The general order of removal can also be viewed in the table below with the following note. Phase 1A sites employ 3 simultaneous dissection stations, so many of these tissues will be dissected and frozen at about the same time. The goal is for the high priority tissues (listed above the dotted lines on the cartoons) to be frozen within 5 minutes of death. Data on time from death to freezing will be recorded for every sample taken from each rat, and this will be saved within the rat registry with the CCC.

Joslin	**UF**
Blood	Blood
Heart	Heart
Lung	Lung
Liver	Gastrocnemius
Gastrocnemius	Liver
Brown adipose	Spleen
Kidney	Kidney
Hippocampus	Hippocampus
Adrenal	Adrenal
Aorta	Aorta
Cortex	Cortex
Hypothalamus	Hypothalamus
Colon	Small Intestine
White adipose	Vastus Lateralis: mid-belly
Vastus Lateralis: mid-belly	White adipose

Second tier: 5-10 minutes post death.

Spleen	Brown adipose
Small Intestine	Colon
Gonad: Testes/Ovary	Gonad: Testes/Ovary
Tibia (selected timepoints only)	Tibia (selected timepoints only)

PASS PIs are committed to collecting the largest number of unique tissues as is feasible. We realize that we are collecting more than CAS can analyze and we will work with the CCC/UVM to support archiving when needed.

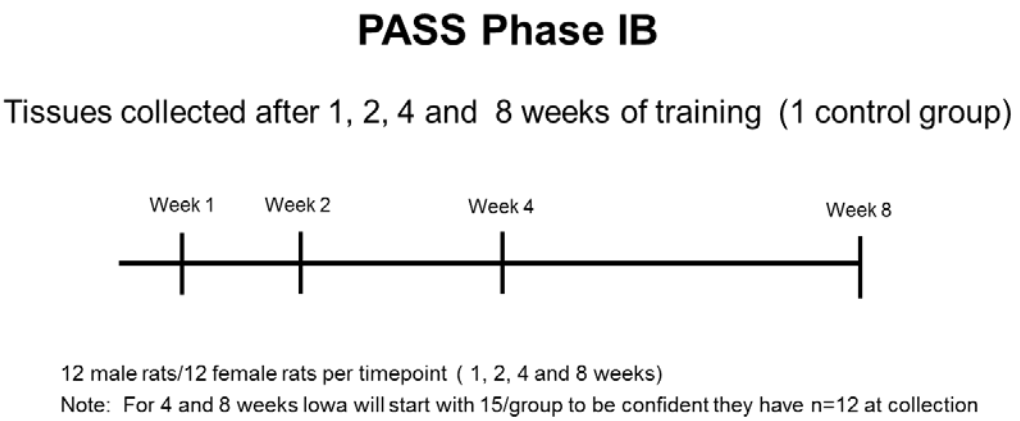
Female rats start to transition to reproductive senescence around the age of 18 months. To get a marker for reproductive status with these female rats, the PASS PIs agreed to dissect out and weigh the uterus from the females and record the weight. We expect that the range of uterine weights will be from about 200mg (senescent) to 600mg (non-senescent). The tissues are not for analysis and are not sent to UVM/CCC but the weights will provide phenotyping data for the 18 month old females that will be important for downstream analysis and interpretation.

Phase 1B: Studies of Exercise Training in Rats.

Phase 1B studies will be done at Iowa. The goal of Phase 1B is to perform exercise training studies in adult (6 month) and old (18 month) F344 rats, and from these rats collect as many tissues as feasible in order to provide high quality samples for detailed analysis by CAS.

Sample Size: Based on power calculations using outcomes including $V0_2$ max, changes in muscle hexokinase II and citrate synthase activity, and changes in cardiac biochemistry, n=10-12 males and 10-12 females are necessary for each group. For the sedentary controls and the 1 and 2 week training groups, studies will be initiated with 12 male and 12 female rats. While we do not expect significant drop out with training, the design can accommodate loss of 1-2 rats over those two weeks of training. To account for an increased rate of drop outs with the 4 and 8 week training groups, the Iowa site will initiate the training with 15 males and 15 females. Rat numbers are depicted in the diagram below.

Figure 3



Controls: Tissues will be collected from 10-12 rats sedentary control rats concurrent with the collection of the 8-week training groups. The 8-week training group and controls will be from the same cohort and will be the same age at euthanasia (either 8 or 20 months old).

For the older age group, an additional set of controls (n=5-6) will be collected with the 1-2 week training group and will ~ 18.5 months old.

Exercise Training Protocol: Rats will be sedentary or undergo an exercise training program. Rats will be exercised on the rodent treadmill 5 days per week using a progressive training protocol designed to exercise the rats at approximately 70% of VO_2max as outlined in the Table on the next page. Training will be performed no earlier than 10:00 am and no later than 5:00 pm over 5 consecutive days per week. Training will be initiated with the treadmill set at 70% of VO_2 max (see tables) and 5 degrees grade for 20 minutes. As outlined in the Table below, the duration of exercise will increase by one minute each day until day 31 of training (start of week 7), when a duration of 50 min will be reached. Speed and grade of each training session will increase in larger increments due to treadmill parameters. The highest intensity and duration of training will begin on day 31. This intensity will be maintained for the final 10 days of the protocol to ensure steady state has been achieved. If any rats are unable to perform at least 4 days of training per week they will be removed from the study and euthanized.

It is important to note that the starting treadmill speed will vary depending on the sex and age of the rat. The initial and maximum speeds will be based on VO_2max measurements obtained during the pre-training testing of the compliant rats.

For reference: 5 degrees = 8.715 % grade: 10 degrees = 17.36 % grade

Sedentary controls: Rats assigned to the control group will follow a schedule similar to the training group. They will be placed in one lane on the treadmill for 15 minutes/day, 5 days per week. The treadmill will be set at 0 m/min at an incline that corresponds to the incline being used by the training group.

PASS Progressive Training Protocol for male and female 6 month old rats

Week	Day	Speed (meters/min) Male/Female	Grade (degrees)	Duration (mins)
1	1	13/16	5	20
	2	13/16	5	21
	3	13/16	5	22
	4	13/16	5	23
	5	13/16	5	24
2	6	15/18	5	25
	7	15/18	5	26
	8	15/18	5	27
	9	15/18	5	28
	10	15/18	5	29
3	11	15/18	10	30
	12	15/18	10	31
	13	15/18	10	32
	14	15/18	10	33
	15	15/18	10	34
4	16	18/21	10	35
	17	18/21	10	36
	18	18/21	10	37
	19	18/21	10	38
	20	18/21	10	39
5	21	20/23	10	40
	22	20/23	10	41
	23	20/23	10	42
	24	20/23	10	43
	25	20/23	10	44
6	26	23/26	10	45
	27	23/26	10	46
	28	23/26	10	47
	29	23/26	10	48
	30	23/26	10	49
7	31	25/28	10	50
	32	25/28	10	50
	33	25/28	10	50
	34	25/28	10	50
	35	25/28	10	50
8	36	25/28	10	50
	37	25/28	10	50
	38	25/28	10	50
	39	25/28	10	50
	40	25/28	10	50

PASS Progressive Training Protocol for male and female 18 month old rats

Week	Day	Speed (meters/min) Male/Female	Grade (degrees)	Duration (mins)
1	1	10/13	5	20
	2	10/13	5	21
	3	10/13	5	22
	4	10/13	5	23
	5	10/13	5	24
2	6	12/15	5	25
	7	12/15	5	26
	8	12/15	5	27
	9	12/15	5	28
	10	12/15	5	29
3	11	12/15	10	30
	12	12/15	10	31
	13	12/15	10	32
	14	12/15	10	33
	15	12/15	10	34
4	16	14/18	10	35
	17	14/18	10	36
	18	14/18	10	37
	19	14/18	10	38
	20	14/18	10	39
5	21	16/18	10	40
	22	16/20	10	41
	23	16/20	10	42
	24	16/20	10	43
	25	16/20	10	44
6	26	16/20	10	45
	27	16/22	10	46
	28	16/22	10	47
	29	16/22	10	48
	30	16/22	10	49
7	31	16/24	10	50
	32	16/24	10	50
	33	16/24	10	50
	34	16/24	10	50
	35	16/24	10	50
8	36	16/24	10	50
	37	16/24	10	50
	38	16/24	10	50
	39	16/24	10	50
	40	16/24	10	50

Phenotyping: Body composition will be determined for all rats prior to training and post training in the 4 and 8-week groups only. VO_2max measurements will be determined prior to the onset of training and during the last week of training for the 4 and 8 week exercise groups. Muscle fiber type composition and fiber size will be determined for the medial and lateral gastrocnemius of the 8-week trained group.

Tissue Collection Overview: For Phase 1B, rats will be euthanized 48 hours following the last training session for all training groups (1, 2, 4, and 8-week training). On the day of sacrifice, food will be removed at 8:30 am, three hours prior to the start of dissections.

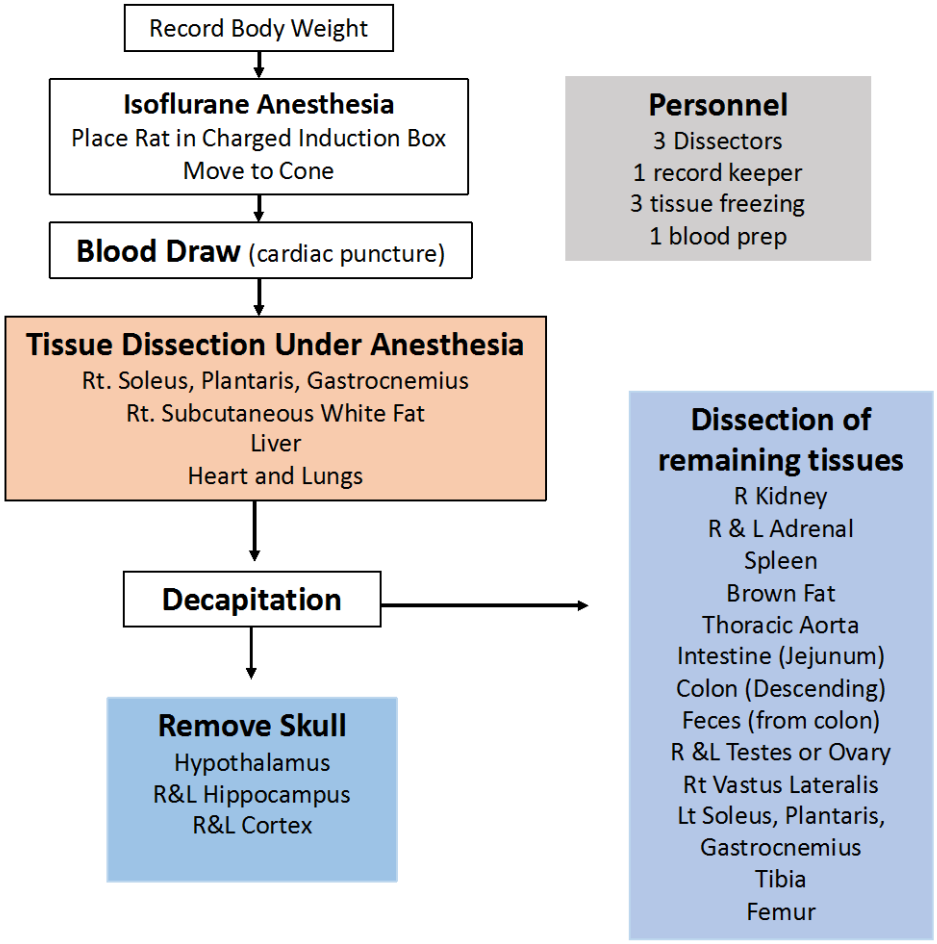
For tissues in which there are more than 1, the PASS sites have agreed to collect one lobe of the liver (right) and the tissues from the right side of the rat (e.g. gastrocnemius, kidney, white adipose). For small organs both the right and left sides will be collected (eg. adrenals, lungs).

Euthanasia and Tissue Collection Process for Phase 1B: The tissues from these rats will be collected at 48hrs after the last bout of exercise. The Iowa PASS site will follow a modified procedure for tissue collections.

1. Under isoflurane anesthesia (1-2%), blood will be drawn via cardiac puncture
2. Under continued isoflurane anesthesia, Dissector #1 will remove the following tissues in the specified order: right triceps surae muscles (soleus, gastrocnemius, and plantaris), subcutaneous white fat on right side, right lobe of the liver, heart and lungs .
3. Removal of the heart will be result in death.
4. Immediately following removal of the heart, a guillotine will be used for decapitation.
5. The brain will be given to Dissector #2 who will remove the brain from the skull and dissect out specific brain regions. The skull will be opened with rongeurs and the brain removed. Following isolation of the brain the following regions will be removed in the specified order: hypothalamus, right and left hippocampus, right and left cerebral cortex. With the ventral side of the brain facing upward the hypothalamus will be removed with a scoop. The brain will be turned so that the dorsal side is now facing upward. The right and left cerebral cortex will be separated at the midline with forceps exposing the right and left hippocampus which will be removed. Following removal of the hippocampus, the right and left cortex will be removed. Following removal of the cortices, the cerebellum will be removed.
6. Following decapitation, the body will be given to Dissector #3 who will remove organs in the following order: right kidney, right & left adrenal, spleen, brown fat, thoracic aorta, small intestine (jejunum), colon (transverse and descending) and feces, right testes or ovaries, right vastus lateralis, left triceps surae muscles (soleus, medial and lateral gastrocnemius, plantaris), tibia, femur.

All tissues will be flash frozen immediately upon removal in liquid N_2 and stored at -80^{o}C prior to shipment to the CCC.

- The left soleus, plantaris, medial and lateral gastrocnemius muscle will be weighed and frozen at a fixed length in chilled isopentane and stored at Iowa for histological analysis.
- The right femur will be cleaned and stored in 70% ethanol and stored at Iowa.
- Feces removed from the colon will be frozen and stored at Iowa.



Consistent with Phase 1A, Phase 1B will also collect female rat uterus weight from the 18month group. This provides a marker of reproductive status with these female rats and the PASS PIs agreed to dissect out and weigh the uterus from the females and record the weight. We expect that the range of uterine weights will be from about 200mg (senescent) to 600mg (non-senescent). The tissues are not for analysis and are not sent to UVM/CCC but the weights will provide phenotyping data for the 18 month old females that will be important for downstream analysis and interpretation.

PASS Blood Processing: For all 3 PASS sites.

1. Draw ~3 mL blood from the rat with a syringe via cardiac puncture, noting the start and end time of the draw.
2. Add the blood to a 3 mL EDTA vacutainer tube, taking care to avoid excess turbulence by angling the syringe toward the side of the draw tube. Note the time that the blood was transferred to the draw tube.
3. Gently invert the filled tube 8-10 times to mix the additive with the blood sample.
4. Place the tube upright in ice.
5. Remove a total of 250 uL EDTA whole blood from the draw tube to make 2 Paxgene RNA aliquots. For each aliquot transfer 125 uL rat whole blood from the EDTA tube into a 1.1 ML Micronic tube containing 345 uL PAXgene lysis buffer (2.76x ratio whole blood : lysis buffer).

6. Invert the tubes containing the whole blood and PAXgene buffer 10 times to mix, and leave the tube on the benchtop (room temp) for a minimum of 2 hours (max overnight) to allow for complete lysis

7. After leaving on ice for 20 minutes, centrifuge the EDTA draw tube containing the remainder of the rat whole blood for 30,000 g-min at 4ºC (ex. 3,000 g x 10 min or 2,000 g x 15 min).

8. Carefully remove the tube from the centrifuge to avoid disturbing the cell layer.

9. Carefully remove the draw tube closure and use a P1000 pipette (or similar) fitted with an appropriate tip to remove 3 x 0.5 mL of plasma and place each in a 0.5 mL labeled cryovial. Take care to avoid disturbing the buffy coat/cell layer and continue with the remaining aliquots.

    a. The pipette tip should be placed at an angle against the side of the tube.

    b. If the cellular layer is disturbed, the EDTA tube should be centrifuged again.

    c. To avoid disturbing the buffy coat/cell layer, it is expected that a small amount of plasma will be left behind in the tube.

10. After the plasma layer is removed, mix the buffy coat/cell layer that remains in the bottom of the draw tube and transfer 1 x 0.5 mL to a labeled 1.1 Micronic tube, and the remainder into a 2 mL labeled cryovial using a transfer pipette.

11. Cap all the aliquots and place them in a -80˚C freezer as soon as possible. If the aliquots cannot be placed in the freezer immediately after aliquotting, place them on dry ice until the transfer to the freezer can occur.The support staff will work with the dissecting teams to handle the blood processing for plasma, transfer and freeze samples, track time of death to freezing of each tissue and store tissues as they come from the dissection stations. Samples will be snap frozen first and then placed in pre-cooled bar-coded vials or cyro-freezer bags and stored at -80 until shipment to the CCC.

Timeline for rat delivery from NIA and experimental completion

PASS Phase 1A

YEAR	Month Delivered	AGE	Florida - Phase 1A	Harvard - Phase 1A	Experiment Month	
2018						
	March					
	April					
	May	4 month	15M/15F	15M/15F		
	June	5 month	15M/15F	15M/15F		
	July	4 month	15M/15F	15M/15F	July : 6 month	
	Aug	5 month	15M/15F	15M/15F		
	Sept				September: 6 month	**All 6 month rats complete**
	Oct	16 month	15M/15F	15M/15F		
	Nov	17 month	15M/15F	15M/15F		
	Dec	16 month	15M/15F	15M/15F	December: 18 month	
2019	Jan	17 month	15M/15F	15M/15F		
					February: 18 month	**All 18 month rats complete**

PASS Phase 1B

YEAR	MONTH	Age(s)	Iowa-Phase 1B	PURPOSE	Experimental Months	
2018						
	March					
	April					
	May	5 and 17 month	5M, 5F (5mon); 5M, 5F (16M)	6 and 18 month VO2	test VO2 peak in males & females	
	June	5 month	30M	8 week training, controls	July, August	
	July	5 month	30F	8 week training, controls	August, September	
	Aug	4 month	20M	4 week	October	
	Sept	4 month	20F	4 week	November	
	Oct	3 month	30M	1, 2 week	January	
	Nov	3 month	30F	1, 2 week	February	**All 6 month rats complete**
	Dec					
2019	Jan	17 month	30M	8 week training, controls	February, March	
	Feb	17 month	30F	8 week training, controls	March, April	
	March	16 month	20M	4 week	May	
	April	16 month	20F	4 week	June	
	May	15 month	30M	1, 2 week	August	
	June	15 month	30F	1, 2 week	September	**All 18 month rats complete**
	July					
	Aug					

Assessment of Safety

Rats will be monitored by the staff at each PASS site for signs of stress which include loss of body mass or discoloration of the fur at the nap of the neck or around the eyes. If any rats show significant signs of stress or discomfort during the 4 week or 8 week training, they will be removed from the study. Other features to look for include bleeding feet/toenails and abrasion of testicles with the male rats.