Best Practices for Diet-Induced NASH and DIO Studies: How Housing and Husbandry Can Impact Outcomes

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Agenda

Diet-Conditioned Rodent Model Fundamentals

NASH and DIO Models at Taconic

Husbandry and Housing

Experimental Design Considerations

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Diet-Conditioned Obese and NASH Rodent Models

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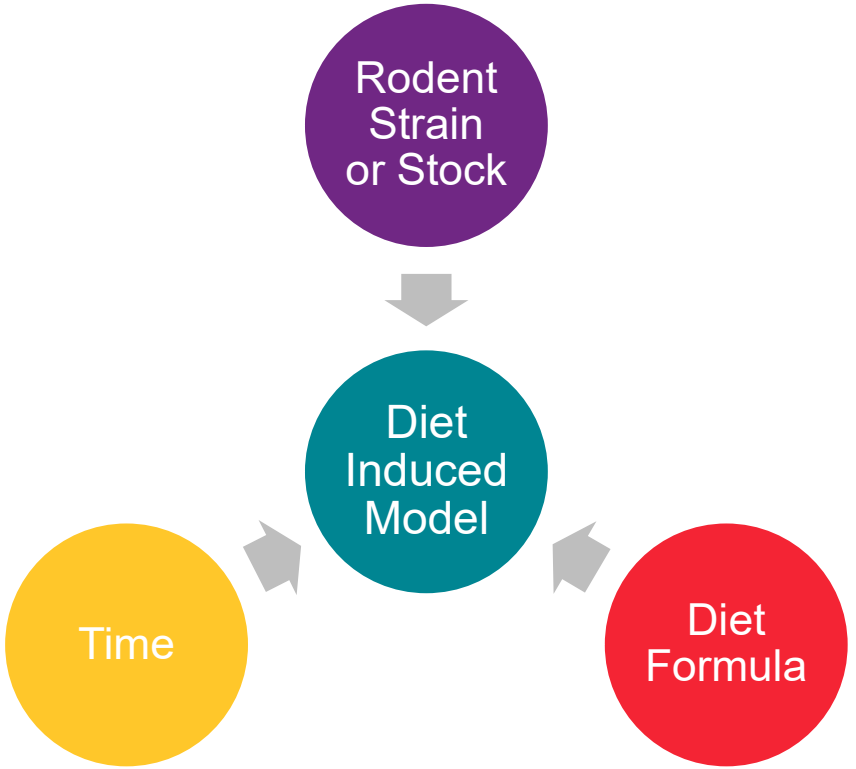
What Are Diet-Induced Metabolic Disease Models?

- Animal models used to study diseases such as obesity or non-alcoholic fatty liver disease where phenotype onset is caused by long term administrations of special rodent diets
  - High fat and/or high sugar diets
  - Nutrient-restricted or modified diets
- Diets are on a purified ingredient background
  - Original diets were made by mixing additional ingredients (lard, for example) into an existing chow diet
  - Purified diets allow for simple or complex modifications to one or more nutrients
- Certain rodent models are more susceptible to diet-induced phenotypes
  - Strain and sex are both factors to consider
- Timeframe of induction and disease severity depends on rodent model and diet formulation



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Formulations for Diet-Induced Obesity

- Diet is a primary environmental factor that contributes to obesity onset in humans;
  - Rodent models of diet-induced obesity (DIO) serve as an excellent tool to study mechanisms and develop mitigation techniques
- Elevated fat content:
  - 30-60 kcal%
  - Typically saturated sources like lard, beef tallow, or coconut oil
- Higher fat content (60 kcal% fat) reduces induction timeframe, but is less physiologically relevant
  - May also be more difficult to prevent or reverse phenotype compared with a moderately-high fat diet (45 kcal% fat)

(DIO) Formulas

Product #	D12451		D12492	
	gm%	kcal%	gm%	kcal%
Protein	24	20	26	20
Carbohydrate	41	35	26	20
Fat	24	45	35	60
Total		100		100
kcal/gm	4.73		5.24	
Ingredient	**gm**	**kcal**	**gm**	**kcal**
Casein, 80 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	72.8	291	0	0
Maltodextrin 10	100	400	125	500
Sucrose	172.8	691	68.8	275
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	177.5	1598	245	2205
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Red Dye #40	0.05	0		
FD&C Blue Dye #1			0.05	0
Total	**858.15**	**4057**	**773.85**	**4057**

Wang, et al. PLoS One. 2009; 4:e6884; Moschonas, et al. Exp Biol Med. 2014; 239:937-947; Ikemoto, et al. Metabolism. 1996; 45: 1539-1546.

Formulations for Dietary and Non-Alcoholic Fatty Liver Disease (NAFLD)

Formulations have differe
translatability

Nutrient-Deficient Diets

- Methionine and ch
- Choline deficient

Obesogenic Diets

- High fat
- High fructose
- High fat, high fructo

Chemical Induction

- CCl_4

Combinations

- Methionine restrict
- High fat diet + fruct

Diet	Rodent Model	Body Weight	Fasting Plasma Glucose/ Insulin	Steatosis	Steato Hepatitis	Fibrosis	Time Frame (Fibrosis) #
Methionine Choline Deficient Diet (MCD)	Rats and Mice	↓	↓	+	+	+	4-8 weeks
0.1% Methionine Choline Deficient High-Fat Diet (CDAHFD)	Mainly Mice	↓*	No Change	+	+	+	6-12 weeks
Choline Deficient Amino Acid Diet (CDAA)	Rats and Mice	No Change	↑ Mainly Mice	+	+	+	4-8 weeks (rats) 12weeks (mice)
Choline Deficient High-Fat Diet (CD)	Mainly Mice	↑	↑	+	+	+	12 months
High-Fat Diet (HFD)	Rats and Mice	↑	↑	+	+	+ (Mild)	24 weeks (rats) 16 weeks (mice)
High Fructose Diet (HFR)	Mainly Rats	No Change	↑	+	+	+	12 weeks
High-Fat, High Fructose, High-Cholesterol Diet	Rats and Mice	↑	↑	+	+	+ Mainly Mice	20-30 weeks (mice)

Fructose, High NAFLD Diet

	D09100310	
	gm%	*kcal%*
	22.5	*20*
	44.9	*40*
	19.9	*40*
		100
	4.49	
	gm	***kcal***
	200	*800*
	3	*12*
	100	*400*
	200	*800*
	96	*384*
	50	*0*
	25	*225*
	20	*180*
	135	*1215*
	10	*0*
	13	*0*
	5.5	*0*
	16.5	*0*
	10	*40*
	2	*0*
	18	*0*
	0	*0*
	0.025	*0*
	0.025	*0*
Total	**904.05**	***4056***

Reproduced with permission from Research Diets, Inc

Radhakrishnan et al. Curr Dev Nutr. 2020; 4(6):nzaa078

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Rodent Models for DIO and NASH

DIO:

C57Bl/6 mouse

Sprague-Dawley rat

Wistar rat

NASH:

A/J mouse

C57Bl/6 mouse

Wistar rat

F1B hamsters

Golden Syrian hamsters

Guinea pigs

C57BL/6 MALES ARE THE MOST COMMONLY USED STRAIN FOR DIO AND NAFLD STUDIES

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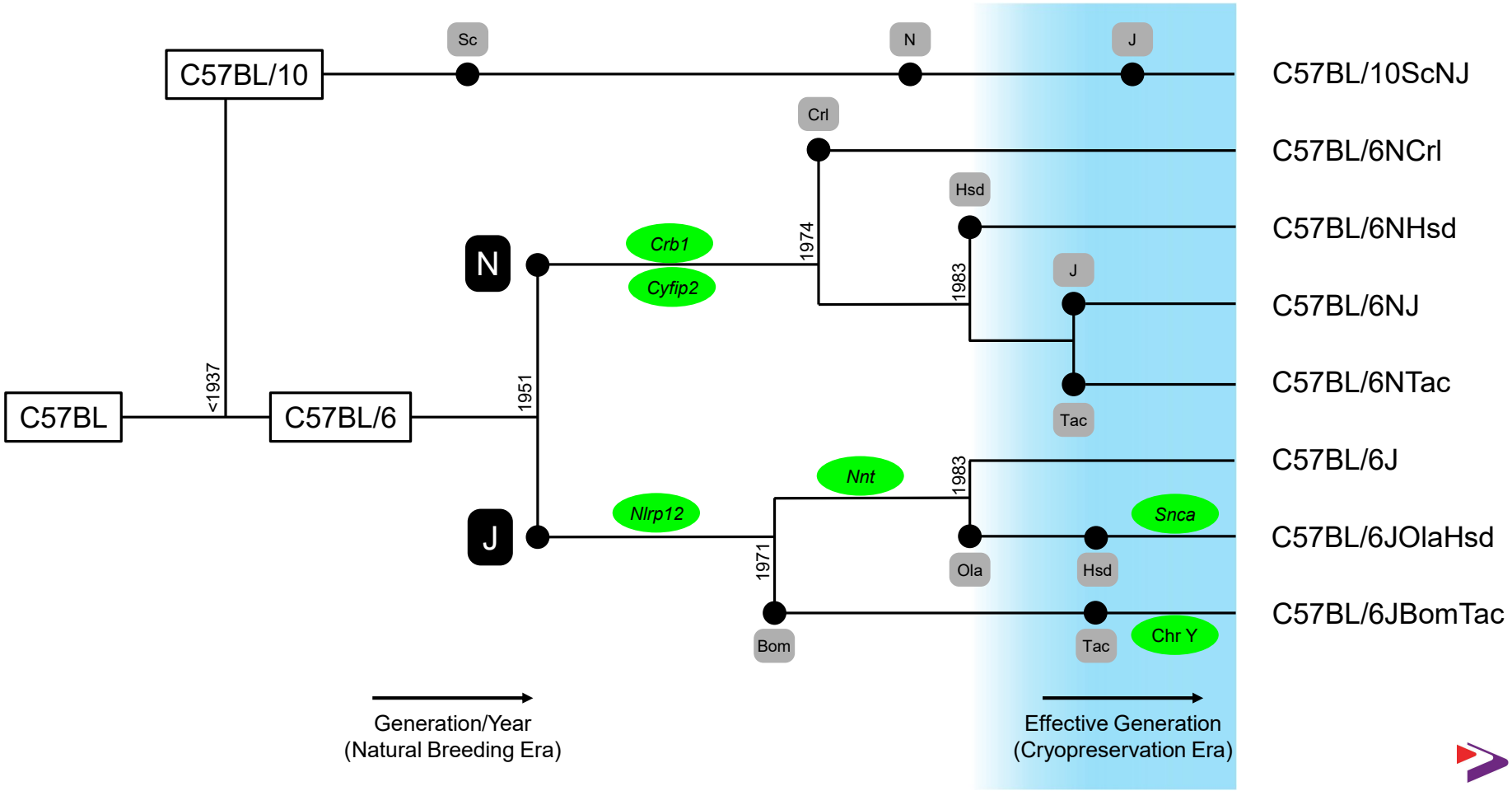
Most Common Obesogenic Diet Models Leverage C57BL/6

BEWARE: Divergence in B6 colonies produced two major substrains N (NIH) and J (JAX)



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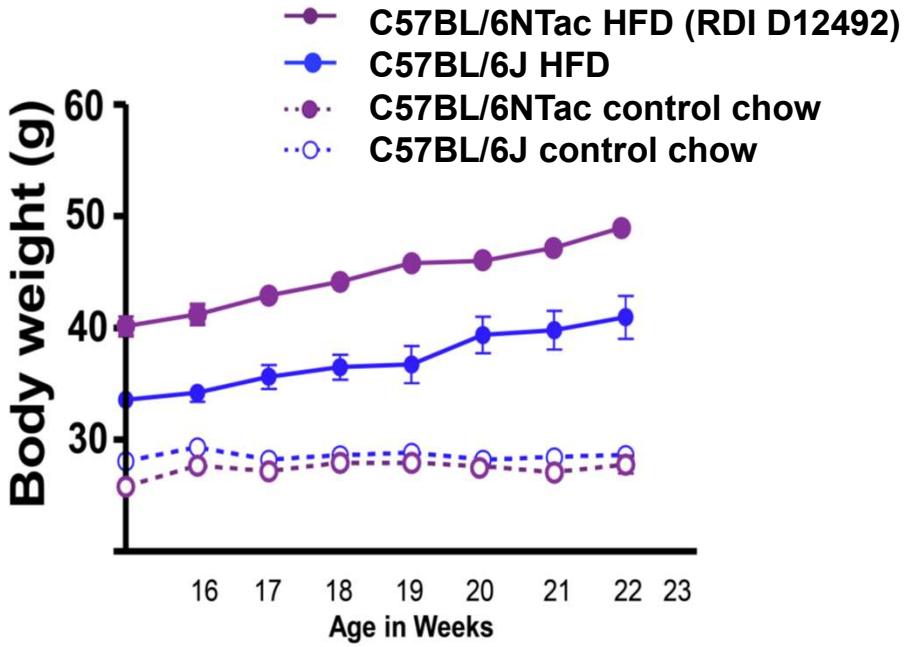
Most Common Obesogenic Diet Models Leverage C57BL/6

Genetic divergence and microbiome differences influence DIO phenotype in B6 Mice

Nicotinamide nucleotide transhydrogenase (NNT):

- *Nnt*$^{C57BL/6J}$ – delete exons 7 to 11 in J substrains
- Mitochondrial inner membrane protein
- Regulates redox state between NADP/NADPH and NAD/NADH pools
- Linked to carbohydrate metabolism, insulin secretion, and defense against oxidative stress



B6NTac MICE GAIN MORE WEIGHT RELATIVE TO B6J ON HIGH FAT DIET

Data from internal study

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Health Standard Should Be Carefully Selected

GERM FREE (GF)
- Axenic
- Free of all microorganisms

DEFINED FLORA (DF)
- Only permitted microorganisms are Altered Schaedler Flora, dimorphic yeast, mold, aerobic spore-forming bacteria, and anaerobic spore-forming bacteria
- No excluded pathogens are allowed
- All organisms in isolator are known

OPPORTUNIST FREE™ (OF™)
- Free of rodent pathogens plus the opportunists *Klebsiella oxytoca* and *K. pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, Beta hemolytic *Streptococcus* (non-Group D), and Proteus species

RESTRICTED FLORA™ (RF™)
- Free of rodent pathogens plus the opportunists *Klebsiella oxytoca* and *K. pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, Beta hemolytic *Streptococcus* (non-Group D)

MURINE PATHOGEN FREE™ (MPF™)
- Free of rodent pathogens
- Appropriate for many types of experiments and may be preferred for certain immunological studies

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Diet-Induced Models

More than simply adding special diet to the feed hoppers!

- Fundamental Considerations
  - Choice of strain/stock + diet
  - Phenotype onset timeframe
- Other Considerations
  - Freezer space
  - Vivarium capacity; husbandry
  - Thermoneutrality
- Challenges
  - Lengthy conditioning times
  - Difficulty in adapting study designs
  - Inconsistent diet response

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Off-the-Shelf NASH and DIO B6 Models

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Diet-Induced Obese (DIO) B6

Description: Male B6 mice fed DIO diet starting at 6 wks of age

Control: Age-matched male B6 mice fed NIH-31M diet

Background: C57BL/6NTac

Health Standard: Murine Pathogen Free™

- more stringent options by custom order

Diet: D12492 from Research Diets, Inc.

Availability: diet conditioned for 3-20 weeks

- longer by custom order

Production Locations: USA (NY) and Denmark

D12492 Formula

Product #	D12492	
	gm%	kcal%
Protein	26.2	20
Carbohydrate	26.3	20
Fat	34.9	60
Total		100
kcal/gm	5.24	
Ingredient	**gm**	**kcal**
Casein, 80 Mesh	200	800
L-Cystine	3	12
Corn Starch	0	0
Maltodextrin 10	125	500
Sucrose	68.8	275.2
Cellulose, BW200	50	0
Soybean Oil	25	225
Lard*	245	2205
Mineral Mix, S10026	10	0
DiCalcium Phosphate	13	0
Calcium Carbonate	5.5	0
Potassium Citrate, 1 H2O	16.5	0
Vitamin Mix, V10001	10	40
Choline Bitartrate	2	0
FD&C Blue Dye #1	0.05	0
Total	**773.85**	**4057**

Diet-Induced NASH B6

Description: Male B6 mice fed NASH diet starting at 6 wks of age

Control: Age-matched male B6 mice fed NIH-31M diet

Background: C57BL/6NTac

Health Standard: Murine Pathogen Free™

- more stringent options by custom order

Diet: D09100310 from Research Diets, Inc.

Availability: diet conditioned for 3-26 weeks

- longer by custom order

Production Locations: USA (NY and CA) and Denmark

D09100310 Formula

Product#	D09100310	
	gm%	*kcal%*
Protein	22.5	*20*
Carbohydrate	44.9	*40*
Fat	19.9	*40*
Total		*100*
kcal/gm	4.49	
Ingredient	**gm**	***kcal***
Casein	200	*800*
L-Cystine	3	*12*
Maltodextrin 10	100	*400*
Fructose	200	*800*
Sucrose	96	*384*
Cellulose	50	*0*
Soybean Oil	25	*225*
Lard	20	*180*
Palm Oil	135	*1215*
Mineral Mix S10026	10	*0*
DiCalcium Phosphate	13	*0*
Calcium Carbonate	5.5	*0*
Potassium Citrate, 1 H2O	16.5	*0*
Vitamin Mix V10001	10	*40*
Choline Bitartrate	2	*0*
Cholesterol	18	*0*
FD&C Yellow Dye #5	0	*0*
FD&C Red Dye #40	0.025	*0*
FD&C Blue Dye #1	0.025	*0*
Total	**904.05**	***4056***

Advantages of Off-the-Shelf Diet-Conditioned Models

1. Pre-induced cohorts available when you need them
2. Animals can start on study after moderate acclimation period
3. Save space, time and husbandry efforts in your vivarium
4. Flexibility in study design

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Husbandry and Housing

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Diet Considerations

- Once animals are on diet, they must stay on diet!
- D12492 and D09100310 (Research Diets, Inc.) are popular diets available with minimal lead time
  - **Have on hand prior to study start / arrival of animals** and reorder before you run out!
  - Taconic uses irradiated version → check if necessary in your facility
  - 5 gm/mouse/d + 30% overage
  - Customizations possible but require longer lead time
- Preferred storage
  - NASH: cool dry place
  - DIO: frozen
  - Shelf life of 6 months
- Administration
  - Consider refreshing diet twice weekly
  - Discard old diet and replace with new → do not “top off”

Control Diets

Taconic uses grain-based control diet by default; purified control diets are available

Nutrient Type	NIH-31M	Purified Diet
Carbohydrate	Ground whole wheat, ground whole yellow corn, ground whole oats, wheat middlings	Corn starch, maltodextrin, sucrose, cellulose, fructose
Protein	Fish meal, soybean meal, alfalfa meal, corn gluten meal	Casein
Fat	Soybean oil, fish meal	Soybean oil, lard, palm oil
Vitamins and Minerals	Brewer's dried yeast, ground limestone, salt, vitamin and mineral mixes	Vitamin and mineral mixes

PURIFIED CONTROL DIETS ARE INGREDIENT AND CALORIE MATCHED TO HIGH FAT COUNTERPARTS

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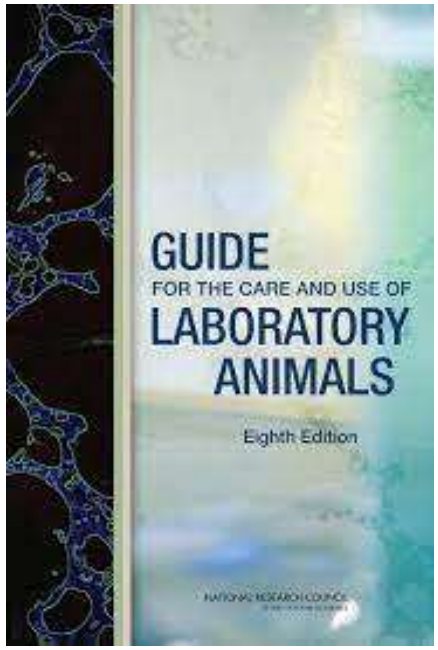
Thermoneutral Housing

- The Guide recommends dry bulb temperature of 20-26°C (68-79°F) for rodents
- The TNZ (thermoneutral zone) for rodents is 26-34°C (79-93°F)
  - Taconic does not apply thermoneutral housing for its DIO / NASH products
  - Working in the TNZ for rodents would not be comfortable for animal care staff
- At lower temperatures mice are able to thermoregulate their environments
  - Includes building nests, utilizing shelters, congregating in groups
- During activity, mice prefer temperatures below the TNZ
- The macroenvironment and microenvironment play a role in temperature
  - ACH, density, humidity, type of caging, etc.



National Research Council. 2011. Guide for the Care and Use of Laboratory Animals: Eighth Edition. Washington, DC: The National Academies Press. https://doi.org/10.17226/12910.
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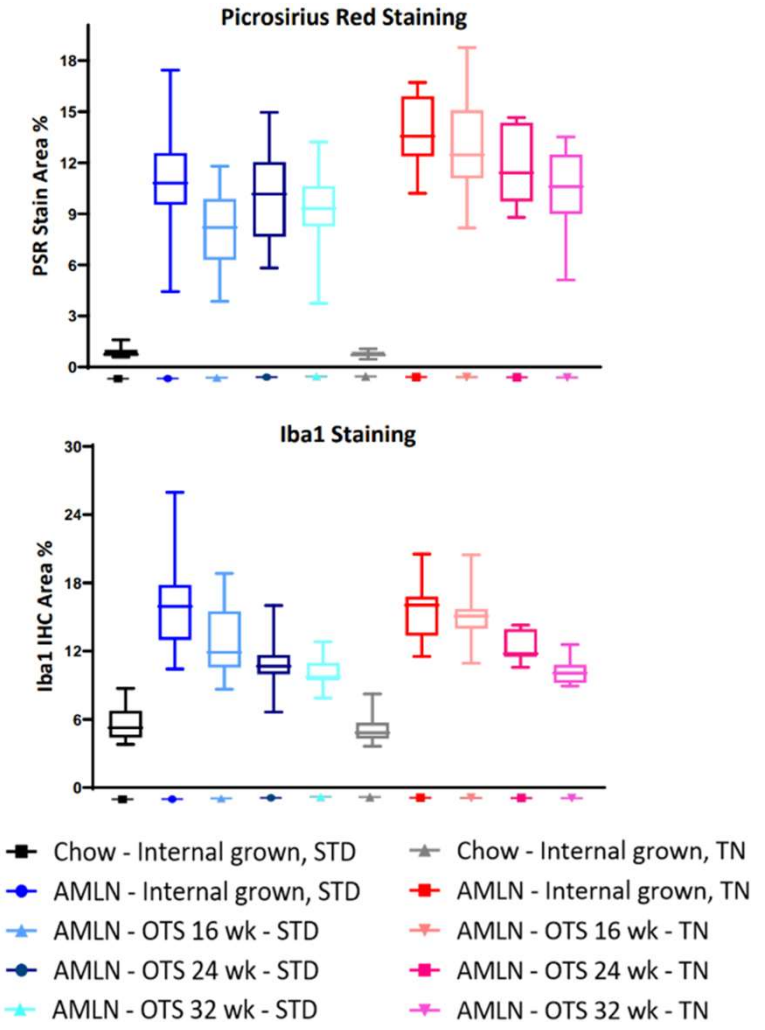
Thermoneutral Housing

Murine setpoint differs from vivarium setpoint

- Vivarium temperatures are 21°C but 30°C is closer to murine thermoneutral zone
- Cold and stress activate β-andrenergic receptors
  - Epinephrine: anorexia, lipolysis, glyconeogenesis
  - Cold: non-shivering thermogenesis
- Reducing caloric imbalance is clinically best way to resolve obesity, steatosis, NASH
- Raising ambient temperature accentuates obesity, steatosis, NAS

Giles, et. al. Nat Med. 2017; 23:829



Bernardo, et al. Poster presented at AASLD 2020

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Housing density

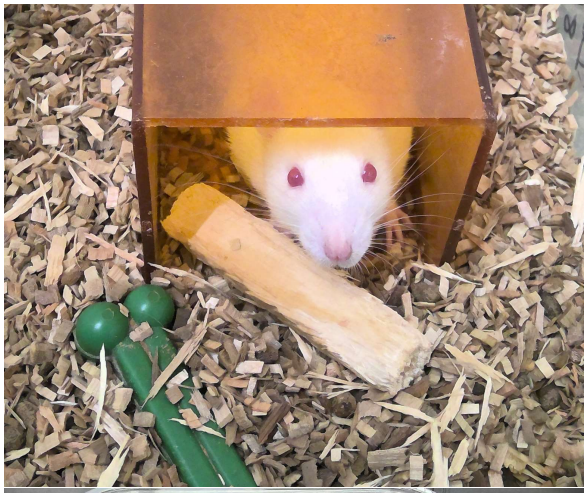
Impacts weight gain and aggression

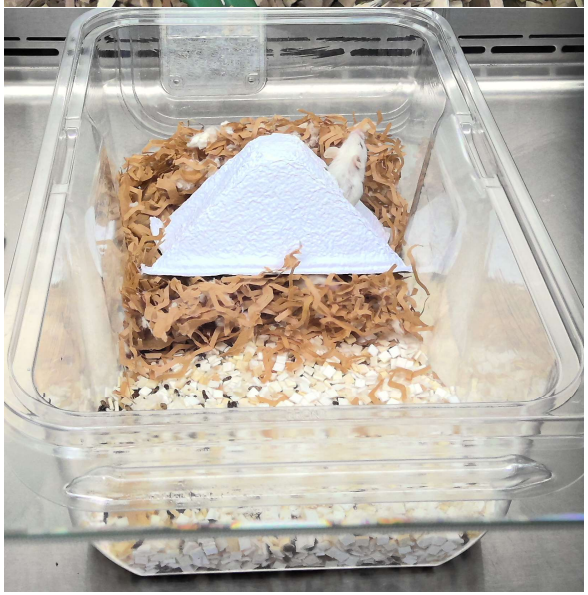
- Guidelines (per cage):
  - 1: only with justification; thermal challenge
  - 2: low aggression risk, high thermal risk
  - 3: good
  - **4: moderate aggression risk, low thermal risk**
  - 5: high aggression risk
- Best practice to define cage density for the duration of a specific project
- Open top cages vs IVC
  - Potential for increased cold stress in IVC
  - Provide additional bedding/nesting material to offset

David, et al. J Am Assoc Lab Anim Sci. 2013; 52(6): 738-744

Practical Notes on Husbandry

- Enrichment can mitigate aggression
  - Nesting material + chewstick works well
  - Shelters may increase aggression due to resource guarding
- Bedding
  - Consider cellulose bedding if clinical concerns develop
- Water treatment
  - Taconic recommends hyperchlorinating or autoclaving
    - Acidified water can have a significant impact on mouse microbiome
  - Taconic animals are provided water bottles
    - If animals are switched to an automatic water system, ensure they transition





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Challenges and Clinical Concerns

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Unpacking DIO and NASH mice from Taconic

- Taconic packs as NASH, DIO and control mice as cagemates
  - One cage per Taconic Transit Cage™ (TTC™)
- While unpacking observe body condition, fight wounds, other clinical abnormalities
- **To reduce potential aggression, do not ever recombine mice from different housing groups!**
- Unpack and house the mice in their final study configurations. Options:
  - House exactly as received, with contents of one TTC placed in one cage
  - Break down into smaller groups, unpacking a single TTC into several cages
    - **Never** recombine mice from different TTCs
  - Singly house one mouse per cage
    - With prior approval by IACUC or oversight body
    - Provide extra enrichment

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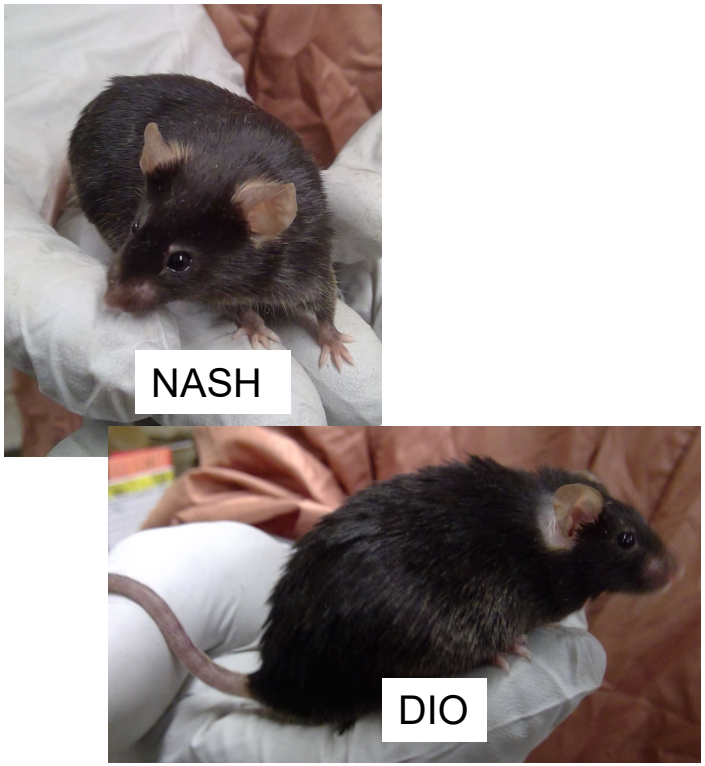
Normal for diet-induced, odd for other models

- Overweight
- Greasy/poor hair coat
- Less active
- Less friendly/social
- Increased aggression even compared to a normal C57BL/6 background
- Increased skin issues in the summer
  - Fluctuations in temperature and humidity can affect



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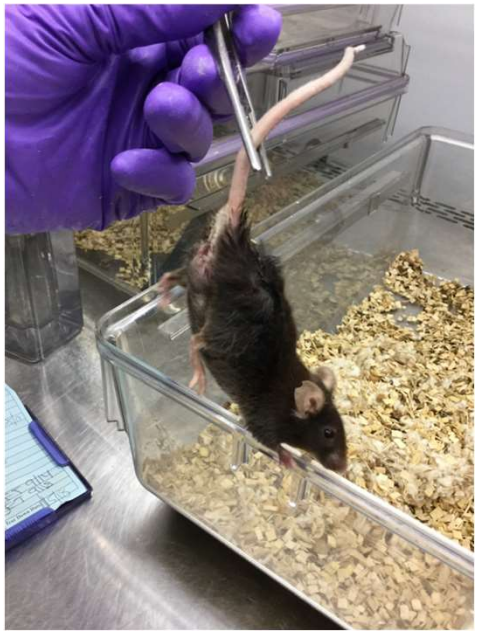
Aggression Can Occur at any Time

Monitor for signs of distress and aggression throughout study

- Aggression is common in B6 males
- Avoid and minimize:
  - Disturbances during acclimation
    - Recommend extending period to 2-4 weeks
  - Shelters (provide at least 2 if you do)
- Support low stress handling methods
- Observe animals for:
  - Active aggression when housing after arrival and at every cage change
  - Superficial fight wounds that can progress to -> Deep wounds
  - Injuries to preputial region
- Can remove aggressor from study or singly house



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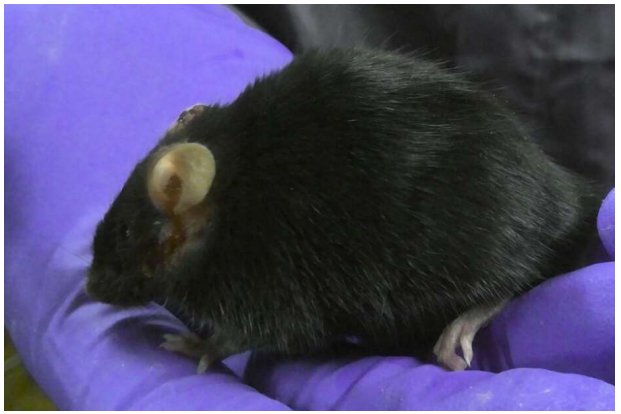
Other Clinical Concerns

- Alopecia
  - Can occur in NASH mice
  - Treatment not generally required as long as skin appears normal
- Urine scalding
  - Topical treatment to affected area
  - Increase cage change frequency
  - Decrease cage density
  - Change cage substrate
- Ulcerative dermatitis
  - Ranging in severity
  - Treatment typically consists of nail trims



Ulcerative Dermatitis

Experimental Design Considerations

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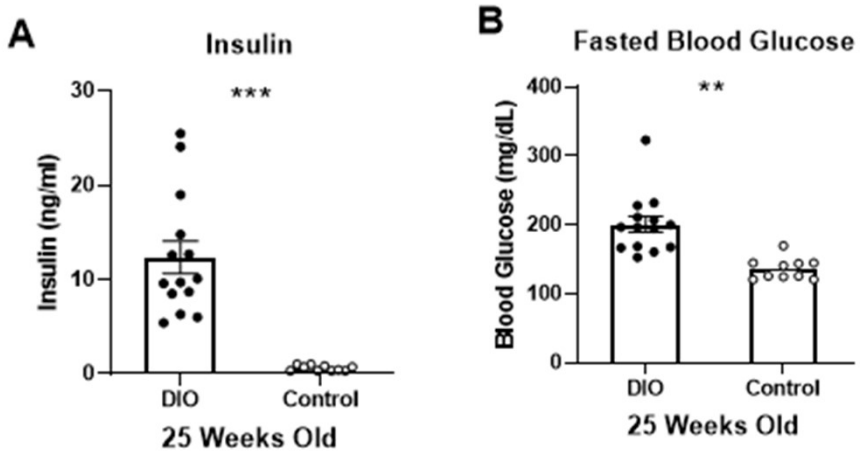
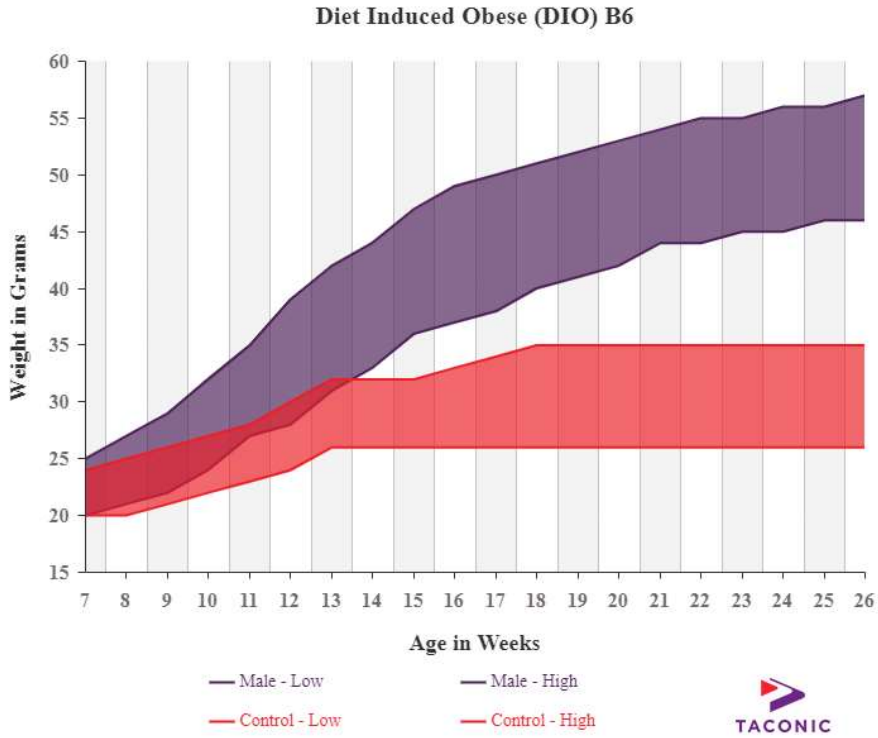
What Age Should I Request?

DIO phenotype onset in 10-12 weeks





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PHENOTYPE DEVELOPMENT OF THE DIET-INDUCED OBESE C57BL/6NTac

6 weeks	14 weeks	16–18 weeks	20 weeks	22 weeks
Start diet	Marked weight difference vs. controls	Metabolic syndrome Neuropathy Insulin resistance Elevated glucose	Elevated leptin and corticosterone	Mild liver complications

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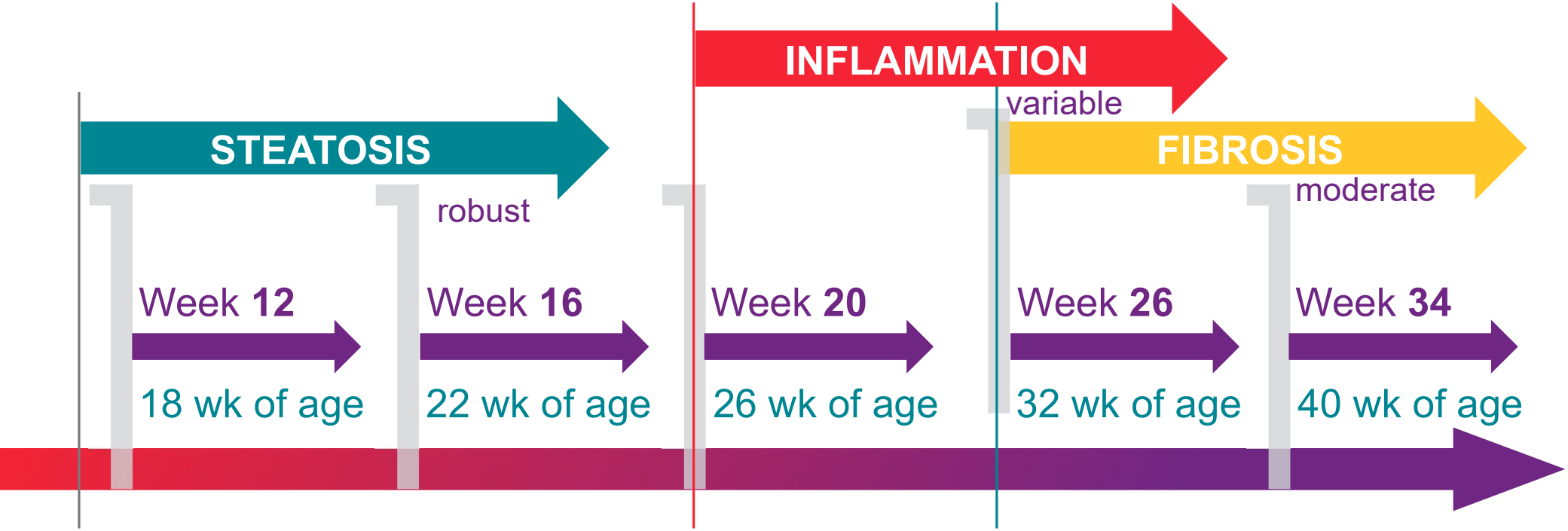
What Age Should I Request?

NASH phenotype is a spectrum and is affected by shipment



SOLD BY WEEK OF AGE

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Markers of NASH Progression

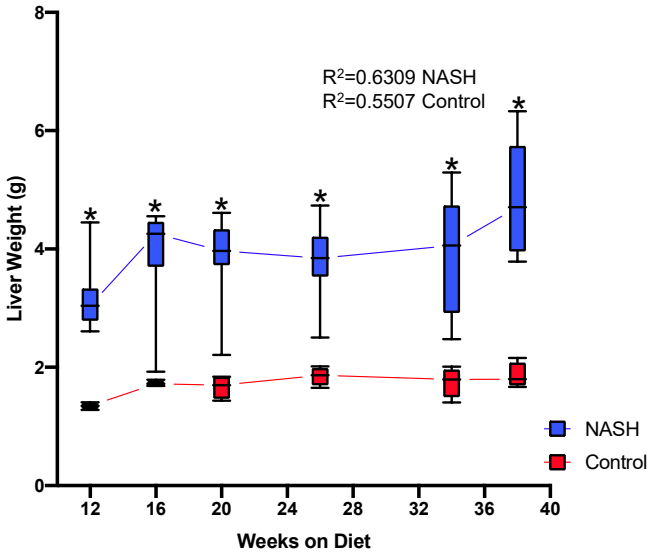
Two-way ANOVA: Diet x Time

Geisser-Greenhouse correction

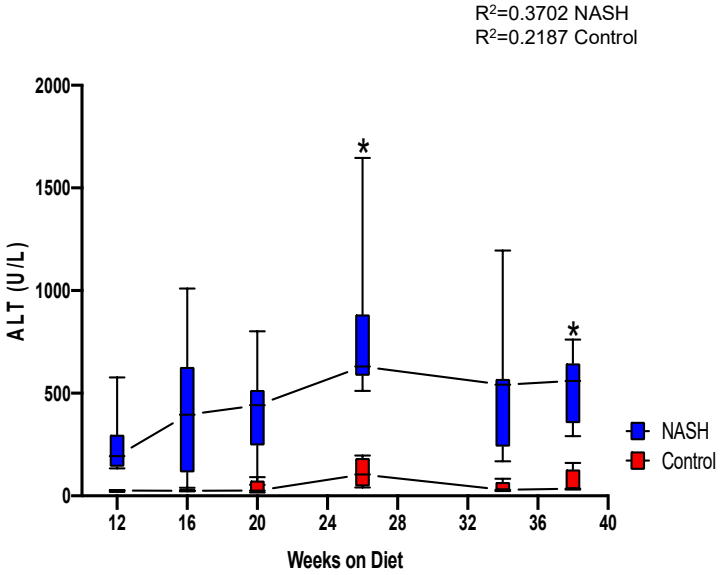
Sidak's multiple comparisons test

P-value < .01

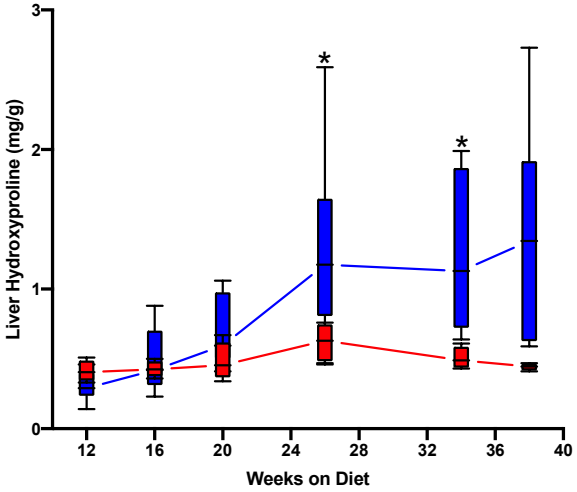
LIVER WEIGHT



ALT



LIVER HYDROXYPROLINE



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Acclimation Period

Consider BW before commencing study

- Weight loss is a stress response in diet-induced obese mice
- Transit times > 48hrs by ground or air can cause bodyweight reductions of 10-20% (NASH) or 20-25% (DIO)
  - Best practice to account for in study design
  - A consideration when selecting cohort age when ordering
- Weigh animals upon arrival and monitor weight gain in over time
- Resume NASH or DIO diet immediately
- May choose to acclimate until body weight is restored
  - 2 weeks minimum
  - 4 weeks common
  - 6 weeks in certain cases

MINIMIZE HANDLING DURING ACCLIMATION PERIOD

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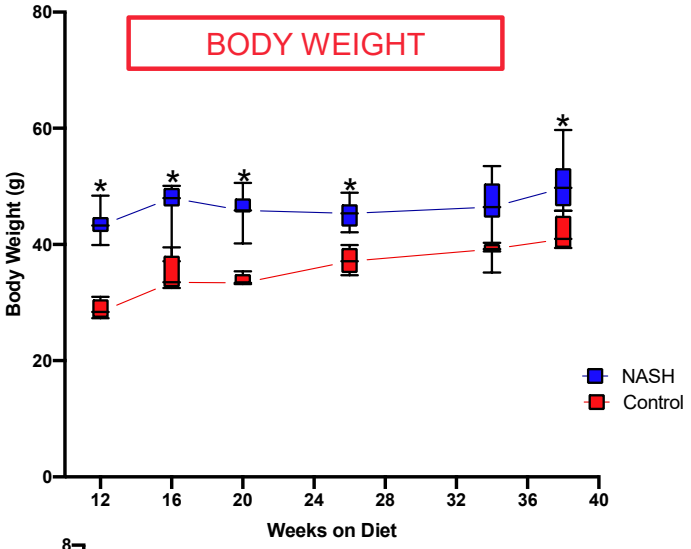
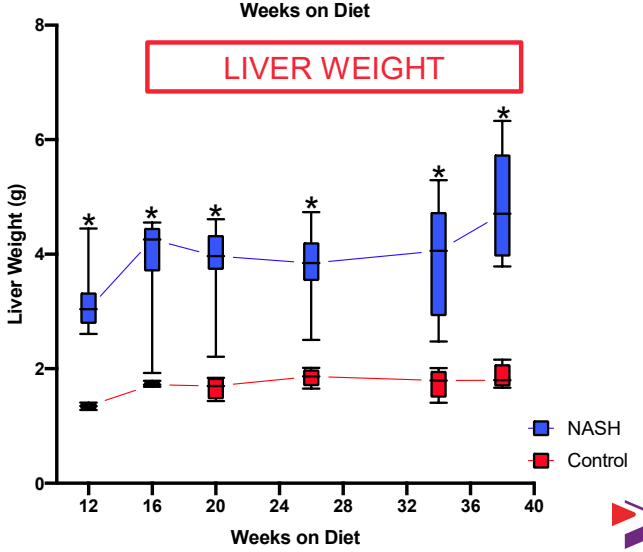
How Many Animals To Order?

Account for biological variability in models

- Plan for BW losses due to transportation and potential failure to regain weight in DIO mice
  - How far away is your facility from the nearest production location?
- Incomplete penetrance of NASH phenotype
  - BW alone is not a good indicator of phenotype
- Best practice for determining *N:*
  - 10-20% overage per treatment arm; *n* = 10-12
  - Consider conducting a pilot study and adjust accordingly





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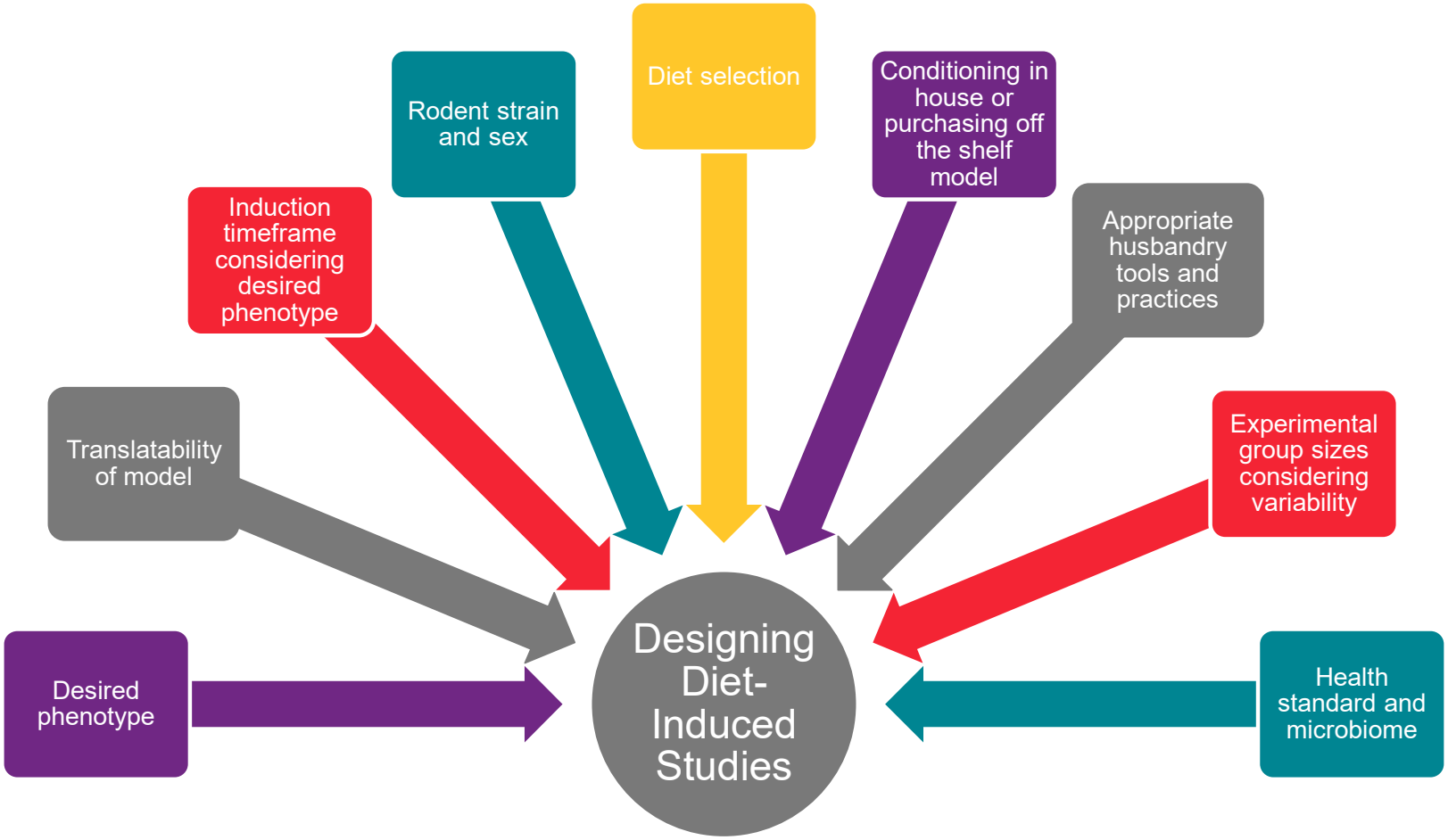
Consistency is Key

Taconic Field Application Scientists are available to help!



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Questions?

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