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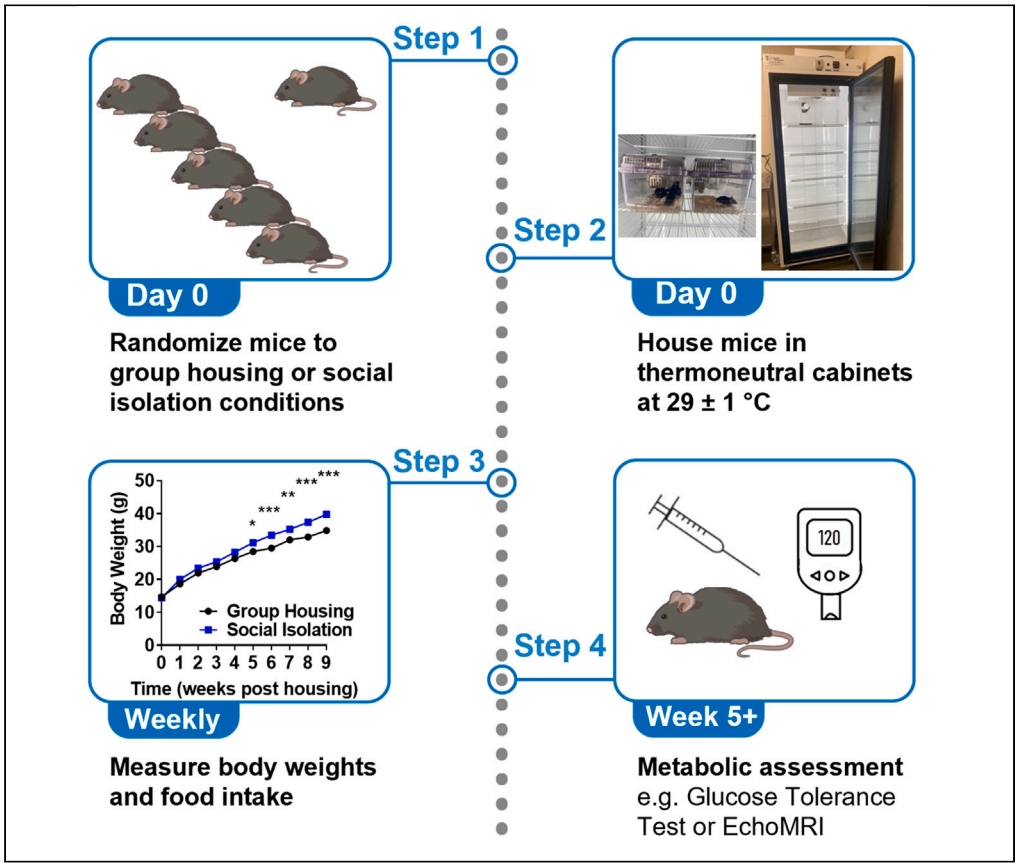
Protocol to minimize the confounding effect of cold stress on socially isolated mice using thermoneutral housing



Social isolation, a risk factor for mortality and various disease states, in mice remains poorly understood, due in part to under-consideration of housing temperature and the murine thermoneutral zone. Here, we present a housing protocol to minimize the confounding effect of chronic cold stress on socially isolated mice that are unable to socially thermoregulate. We describe steps for allocating mice to group housing or social isolation conditions, housing mice in thermoneutral cabinets, feeding mice with high-fat diet, and measuring body weight, food intake, and metabolic indicators.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Under-consideration of temperature interferes with murine social isolation experiments

Steps for setting up a thermoneutral cabinet to control temperature and humidity levels

Maintenance care to minimize confounding variables

Measurement of metabolic function to assess effect of social isolation

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Protocol to minimize the confounding effect of cold stress on socially isolated mice using thermoneutral housing

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SUMMARY

Social isolation, a risk factor for mortality and various disease states, in mice remains poorly understood, due in part to under-consideration of housing temperature and the murine thermoneutral zone. Here, we present a housing protocol to minimize the confounding effect of chronic cold stress on socially isolated mice that are unable to socially thermoregulate. We describe steps for allocating mice to group housing or social isolation conditions, housing mice in thermoneutral cabinets, feeding mice with high-fat diet, and measuring body weight, food intake, and metabolic indicators.

For complete details on the use and execution of this protocol, please refer to Queen et al..[1]

BEFORE YOU BEGIN

In humans, social isolation and loneliness serve as risk factors for mortality[2] and various disease states—including depression, anxiety, obesity, type 2 diabetes, cardiovascular disease, and certain types of cancer.[3] Researchers have attempted to study social isolation's influence on disease states, though the current literature remains limited and conflicted. Dissonance within the literature may stem from the under-consideration of murine housing temperatures in social isolation experiments.

Mice are typically housed at ambient temperature of 20°C–24°C, which provides comfort for human experimenters. Although commonplace, room temperature housing remains below the murine thermoneutral zone, which is defined as the ambient temperature for which energy is expended only to maintain the basal metabolic rate (29°C–33°C for mice).[4–7] At 20°C–24°C, mice burn additional energy to maintain the core body temperature. Thus, housing at 20°C–24°C induces a chronic cold stress that can influence metabolic outcomes in experiments. Group-housed mice can counter this cold stress through social thermoregulation (e.g., huddling for warmth). In contrast, socially isolated mice lack this option and must therefore burn additional energy to maintain body temperature. The resulting extra cold stress may interfere with investigations on how social isolation affects physiology and pathophysiology in murine models.

This protocol describes the best practices for experiments probing social isolation outcomes in mice. Notably, housing should be maintained under thermoneutral conditions to eliminate the chronic cold stress confound and the metabolic differences derived from inability to perform social thermoregulation.

Social isolation experiment outcomes may be influenced by animal strain, age, sex, vendor, etc. Our lab has validated the social isolation protocol in males of two strains, C57BL/6 and BALB/c mice,

when initiated at 4 weeks of age. Social stress during the developmental periods can cause functional and behavioral alterations that persist into adulthood.[8–12] For our characterization of the model, we initiated social isolation in juveniles during the so-called "critical period" of rodent development. The protocol below describes a social isolation experiment for mice on a high-fat diet. However, we have also used normal chow diet and observed metabolic disturbance in isolated mice. Variations to the below protocol should be validated by each individual lab as necessary.

In general, animals should be minimally disturbed until they are humanely euthanized. For this reason, all animals should be weighed once each week and food should be added once per week as well. It is important to ensure the thermoneutral cabinets are placed in a room that has minimal noise disturbance and to minimize opening and closing of the cabinet doors. Doing so has the potential to cause swings in temperature and humidity. The thermoneutral cabinets used herein have ventilation systems, reducing the need for human interference.

As a general note, group housing is defined as 5 mice within a cage. Socially isolated housing refers to 1 mouse housed with the same type of cage, nestlet, and bedding as the group housed mice. Multiple group-housed cages should be used to minimize cage effects. It may be necessary to use in excess of 15 socially isolated mice to achieve proper statistical power.

Institutional permissions

All animal experiments followed The Ohio State University's Institutional Animal Care and Use Committee's (IACUC) regulations. Readers should pursue similar approvals from their institution and regulatory bodies.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
D-(+)-Glucose	Sigma Life Science	Cat#1002148718
Pharmaceutical-grade saline	Baxter	Cat#2B1322Q
Experimental models: Organisms/strains		
Mice	Charles River Laboratory	C57BL/6 or BALB/c
Other		
Thermoneutrality cabinet	Powers Scientific, Inc	Cat#IS33SD
Bed-o'cobs $^{1}/_{4}$ corncob bedding	The Andersons	Cat#4B
High-fat diet (60% kcal from fat; caloric density 5.2 kcal/g)	Research Diets, Inc.	Cat#D12492
Nesting square (approx. 1″ x 1″)		
400 mL water bottles or similar	Allentown	
Thermometer/humidity meter	ThermoPro	Cat#TP49
3 in 1 analyzer	EchoMRI, LLC	3-in-1 Analyzer
Bayer Contour Next EZ Blood Glucose Monitoring System	Bayer	Cat#193725201
Contour Next Blood Glucose Test Strips	Ascensia Diabetes Care	Cat#0382465
0.5 mL insulin syringes	Beckton, Dickinson, and Company	Cat#329461
Mouse cages (194 mm × 181 mm × 398 mm)	Allentown	Cat#223581-4
Kwik-Stop Powder	ARC Laboratories	Cat#463522

MATERIALS AND EQUIPMENT

- 10% w/v glucose solution: add 5 g glucose in 50 mL pharmacological-grade saline.

  Note: Make the solution on the day of the GTT. If the test is to expand across multiple days, make the solution fresh each morning.

Figure 1. Housing conditions
(A) Empty thermoneutral cabinet.
(B) Group housed cage and socially isolated cage in cabinet.
(C) Close-up of group and isolated cages.

STEP-BY-STEP METHOD DETAILS

Initiating housing conditions

Timing: 2 days (for steps 1 to 3)

This step outlines how to randomize and initiate social isolation experiments when using the thermoneutral cabinets. Refer to Figure 1.

Note: The below protocol describes how our lab has been able to establish a murine model that recapitulates social isolation-induced propensity for obesity. Variations in the housing temperature, age/sex/line/vendor of mice, diet, and housing conditions may limit reproducibility. It remains likely that variations in other aspects of the protocol (e.g. brand and model of the thermoneutral chamber) are more amenable to substitutions. Please consider all the above when designing experiments.

Note: This protocol has been validated to induce obesity in C57BL/6 and BALB/c male mice from Charles River Laboratories when initiated at 4 weeks of age, with the birth of a mouse defined as postnatal day 0.

1. Allow the mice to acclimate to the animal facility for at least 2 days after arriving to your institution before moving the mice to the thermoneutral housing cabinets.

Note: All our experiments in social isolation have used animals purchased at 4 weeks of age. No special cautions were taken to minimize shipping stress from the vendor. Though we have no data to advise, shipping mice at an earlier post-wean date would provide more time to habituate and could be considered.

Note: Other researchers have used male C57BL/6 mice born to the facilities in social isolation initiated post-weaning and have noted behavioral deficiencies in isolated mice, though metabolic data was not presented.[13]

2. While the mice are habituating, set up the thermoneutral cabinet (Powers Scientific, Inc) to equilibrate temperature (29 ± 1°C) and humidity (30%–70%) levels.
   a. Cabinets are static housing conditions, which means all mice can smell each other. We do not use physical or visual barriers between cages. Up to 24 cages (Allentown, 194 mm × 181 mm × 398 mm) can fit inside one cabinet.
   b. Set up the cabinet's light timer such that the lights are on a 12 h light/12 h dark cycle such that the lights are on between beginning at 06:00. and 18:00.
   c. Place a digital thermometer inside the cabinet to monitor the internal temperature and humidity. This thermometer should be capable of (1) continuous reporting of the cabinet temperature/humidity or (2) storing the maximum and minimum temperature/humidity levels of the cabinet.
3. After 2 days of habituation and the equilibration of cabinet conditions, the mice can be randomized into groups, assigned experimental IDs, and placed in the proper housing conditions.
   a. Perform a body composition scan using an EchoMRI 3-in-1 analyzer (EchoMRI LLC, Houston, TX) to obtain fat and lean mass data for each mouse. Randomize the mice into a socially isolated housing condition and the group housing condition such that there are no significant differences in fat and lean mass between the two housing groups.
   b. Assign mouse IDs using ear tags, ear punches, or similar methods.
   c. Place mice into housing according to their randomized experimental groups.
      i. Group-housed mice should be placed into a cage with 5 total mice, corncob bedding, and a single nestlet.
      ii. Socially isolated mice should be place into a cage with 1 total mouse, corncob bedding, and a single nestlet.
   d. Add 60% high-fat diet (Research Diets, Inc.) to the overhead food hopper of each cage. Aim to have around 300 g of food at the start of each week.
   e. Fill water bottles with 400 mL of reverse osmosis water. Ensure that the bottles do not leak and place them inside each cage.

Weekly maintenance

Timing: 1 h

Note: Minimize the amount of time the cabinet door is open and the amount of time each cage is outside of the cabinet. To do this, record body weight one cage at a time and close the cabinet door between each measurement. Cage locations should remain constant throughout the entirety of the experiment to avoid introducing confounding variables.

4. Record body weights for each mouse.
5. Determine cage-by-cage food consumption once per week.
   a. Record the concluding week's final food weight.
   b. Add additional food to the food hopper, placing new food on top of older food.
   c. Measure the coming week's initial food weight.

Note: 60% high-fat diet crumbles easily, especially at temperatures exceeding 22°C. Be gentle when refilling cages to minimize the amount of diet that falls on the bedding and to improve the accuracy of food intake measurements.

6. Change water bottles weekly.
7. On a daily basis, monitor the temperature and humidity within the cabinet with a digital thermometer. This can be done by animal husbandry staff if permitted by the local institution. Record the maximum and minimum temperature and humidity. Reset the thermometer to wipe data and initiate recording for the coming day.
   a. If target temperature and humidity ranges are not met, review the thermoneutral cabinet settings and inform the attending veterinarian as warranted.

Model validation

Timing: >5 weeks

Timing: 16 h (for steps 8 to 10)

Timing: 3 h (for steps 11 to 21)

Body weights will diverge around 5–8 weeks post housing (strain-dependent; we have observed divergence by 5 weeks in C57BL/6 mice and 8 weeks in BALB/c mice). At this point, perform a measure of metabolic function to assess whether social isolation has promoted metabolic dysfunction. An EchoMRI can be performed to assess body composition, or a Glucose Tolerance Test (GTT) can be performed to assess glucose processing.

Note: Mice tend to lose 1–2 g of body weight due to the GTT fasting period (described below). Recovery of this body weight can take up to one week. For this reason, it is recommended that the weekly body weight measurements needing to occur the same week as the GTT occur immediately before the fasting period.

8. Fast mice for 16 h overnight.
9. Upon initiation of the fast, remove mice from their home cage and place them in a new cage that contains new bedding and lacks food.

Note: Mice are coprophagic, hence the need to move them to a new cage during the fast. Coprophagic behavior can influence blood glucose levels.

Note: We typically fast overnight from 17:30 to 09:30.

10. Ensure water bottles are filled and remain accessible.
11. On the morning of the test, prepare a 10% w/v solution of glucose in pharmaceutical-grade saline.

Note: The dose of glucose used depends on the diet or metabolic state of the mouse. For obese mice, a dose of 1g/kg body weight is used. For mice with normal weight, a higher dose of 2g/kg body weight is used. If wanting to make comparisons between two different food types in one experiment, a median dose should be used.

12. Remove all mice from the thermoneutral cabinet. All mice will remain outside the thermoneutral cabinet for the entirety of the GTT.
13. Weigh each mouse. Glucose doses will be normalized to this weight.
14. Tail mark each mouse with a marker to allow for easy identification within each cage.

Note: If you keep group housed mice in their home cages and do not tail mark each mouse, it will be nearly impossible to keep on-pace during the upcoming sequence. If permitted by IACUC, we recommend keeping the mice in their home cages and using a sharpie to temporarily tail mark the mice.

15. Obtain baseline fasting blood glucose levels.
    a. Remove <1 mm of the mouse tail tip to elicit blood flow. Alternatively, use a small needle to pierce the tail vein to elicit blood flow.
    b. Insert a measurement strip (Contour Next Blood Glucose Test Strips, Ascensia Diabetic Care) into a portable glucometer (Contour Next EZ Blood Glucose Monitoring System, Bayer).
    c. Elicit blood flow and measure the blood glucose level by placing a drop of blood on the measurement strip.
    d. Record the blood glucose level as t = 0 min.
    e. Discard the used measurement strip.

Note: At this point, blood flow can be elicited as needed throughout the test. If blood flow remains limited, the tail can be massaged to elicit enough blood for measurement of blood glucose levels. In rare cases, the above steps can be repeated to continue blood flow.

Note: Next, the experimenters will initiate the GTT. Keep in mind that only one mouse can be injected with a glucose bolus and measured at any given time. Accordingly, it will be necessary to stagger injections and blood glucose measurements in intervals to allow for assessments of multiple mice. The timing of each injection must be staggered such that every mouse is able to be injected within 15 min and each blood glucose measurement is able to be recorded at consistent intervals. 30 s is typically the smallest possible interval between injections. Large groups may necessitate multiple test dates. If this is done, ensure that animal representatives from each experimental group are present on each test day.

16. For each mouse, prepare a 0.5 mL syringe with a weight-normalized bolus of glucose solution at a total dose of 1.0 g glucose per kg body weight. Place each syringe in an easy-to-handle location.

Note: Arrange the environment to your success. Place each syringe in order from left to right in the front of each corresponding cage. Physically space each cage across a countertop to allow for quick movement between cages and animals. Leave enough room to allow for cage lid removal and replacement.

17. Intraperitoneally inject the weight-normalized glucose bolus. Start a timer with a count-up function. Stagger mouse injections by a previously-defined interval (see above notes). Continue intraperitoneal injections with each mouse's corresponding pre-measured glucose bolus at the defined interval. All injections should occur within 15 min.
18. At t = 15 min, begin sequential measurements of the blood glucose levels. Measure the first mouse by performing the below steps. Wait the appropriate staggering interval and then continue with the next mouse in line until a t = 15 min measurement has been obtained from each mouse.
    a. Circulate blood to the bottom of the tail by massaging the tail from the proximal end to the distal end. Wipe the elicited blood onto a paper towel. Repeat this three times.
    b. Insert a measurement strip (Contour Next Blood Glucose Test Strips, Ascensia Diabetic Care) into a portable glucometer (Contour Next EZ Blood Glucose Monitoring System, Bayer).
    c. Return the mouse to its cage.
    d. Record the blood glucose level.

Note: Since the glucose injections are staggered, the blood glucose measurements can be staggered and later synced during data analysis.

19. Repeat the above steps to obtain blood glucose measurements at t = 0 min, 15 min, 30 min, 60 min, 90 min, and 120 min post glucose injection.
20. After obtaining each measurement for every mouse, you must stop blood flow. Dip the snipped tails in Kwik-Stop powder (ARC Laboratories) to help clot the blood.
21. Return mice to their home cages with food and water in the thermoneutral cabinet.

Note: The insulin tolerance test (ITT) and pyruvate tolerance test (PTT) are two variations of the above test that can be used to assess metabolic function. Refer to the literature to see how these tests differ in their fasting and administration protocols.

Note: The GTT induces stress in mice, and isn't recommended to be performed frequently. An EchoMRI body composition analysis can be used repeatedly as a non-invasive method to assess metabolic changes due to housing condition as a supplement to a GTT.

EXPECTED OUTCOMES

We expect body weights will diverge around 5–8 weeks post housing (strain-dependent; we have observed divergence by 5 weeks in C57BL/6 mice and 8 weeks in BALB/c mice). The socially isolated mice are expected to have increased body weight as compared to group-housed mice.

We expect socially isolated mice to exhibited worsened metabolic function following a similar timeline. Regarding body composition, we expect socially isolated mice will show increased fat mass and reduced lean mass as compared to group-housed counterparts. We additionally anticipate that socially isolated mice will have exhibit worsened glycemic processing as compared to group-housed control counterparts.

Within socially isolated mice, we have observed greater intragroup variation—perhaps due in part to individual differences in stress adaptive responses. It is advisable to use larger group sizes (n >= 15) and perform the proper power analyses to ensure the samples are statistically relevant.

Note: Our lab has not used social isolation housing models on litters of mice. However, other groups have described their social isolation experimental set up using litters of C57BL/6 mice and should be referred to for suggestions. Pietropaolo and group suggests using at least two litters per cage for group housed mice and to aim for an even distribution between housing condition per litter.[13]

QUANTIFICATION AND STATISTICAL ANALYSIS

Food intake should be reported on a per-cage basis. Food will be weighed at the beginning and end of each week and the two values will be subtracted to calculate the weekly food intake. Divide the per-cage weekly food intake by the number of mice in each cage. It is advisable to report data on a per-cage basis to provide a more conservative statistical estimate of the mean weekly food intake. We prefer to take this approach, as it is impossible to accurately measure the food intake on a per-mouse basis for group-housed mice without a period of individual housing.

For the glucose tolerance test, determine the area under the curve to report glycemic processing ability.

All other measures can be analyzed with a Student's t-test, ANOVAs, or mixed models as appropriate. Statistical significance is reached when $P < 0.05$.

LIMITATIONS

Our lab has exclusively studied social isolation in male mice. There are known differences in huddling behavior due to cold stress at ambient temperature of 20°C–24°C between sexes. Female mice tend

to huddle more than males at 20°C–24°C,[14] which may alter the response to social isolation at thermoneutral conditions in females. Previous work has shown that singly-housed female mice increase energy expenditure to maintain the same body temperature as group-housed mice; this was not observed in males.[15] It remains to be seen whether such sexual dimorphisms affect metabolic outcomes in the thermoneutral-social isolation paradigm; it is feasible that the two sexes may interpret and manifest psychosocial stress in different manners.

Social stress during the developmental periods can cause functional and behavioral alterations that persist into adulthood.[8–12] Accordingly, this protocol was implemented in younger mice at 4-weeks old during a so-called murine development "critical period". Psychosocial stress being implemented at a younger age may produce stronger, more long-term effects on biological activity than stress being implemented at older age. It remains to been seen whether the age of social isolation initiation is essential to replicate our findings.

The method of measuring food intake described in this protocol is what we have done for our recent publications, but it poses limitations regarding accuracy. In group-housed mice, the weekly food intake per mouse can only be estimated. Food can be lost to the cage, may vary among animals, and could be measured more frequently. More accurate measurements can be obtained by singly-housing mice in metabolic chambers to obtain daily food intake per mouse. However, since this introduces short bouts of social isolation for grouped mice, we suggest measuring food intake on a per-cage basis for a conservative estimate of weekly food intake that does not overinflate statistical power.

TROUBLESHOOTING

Problem 1

No differences in body weight are observed after the suggested housing period.

Potential solution

- Determine whether your experiment differs from the described protocol. Remember, this protocol may not be reliable with deviations of the strain, sex, age, and vendor of the mice.
- House the mice for additional time and continue weekly body weight and food intake measurements. Generally, an additional 4 weeks is advisable. Housing beyond that poses no known risks but has not been done by our lab in the past.

Problem 2

Alterations in glycemic processing are not observed as expected.

Potential solution

- Allow the mice to be housed for an additional 2 weeks or until body weights diverge, and then repeat the test. Allow the mice at least one week to recover between tests.
- A clear spike of blood glucose levels and subsequent clearance is necessary to assess glycemic processing capability. It may be necessary to alter the dose of the glucose bolus to achieve this goal. For mice on a high-fat diet, the standard glucose tolerance dose is 1.0 g/kg body weight. The dose may be lowered if the peak blood glucose levels (1) exceed the measurable range of the glucometer or (2) do not return to near-fasting levels by the conclusion of the test. Alternatively, the dose can be increased if the glucose bolus does not initiate a sufficient peak in blood glucose levels.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Lei Cao (lei.cao@osumc.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This experiment did not generate datasets or code.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

B.A. and N.J.Q. wrote and revised the manuscript. L.C. revised the manuscript and provided the funding.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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