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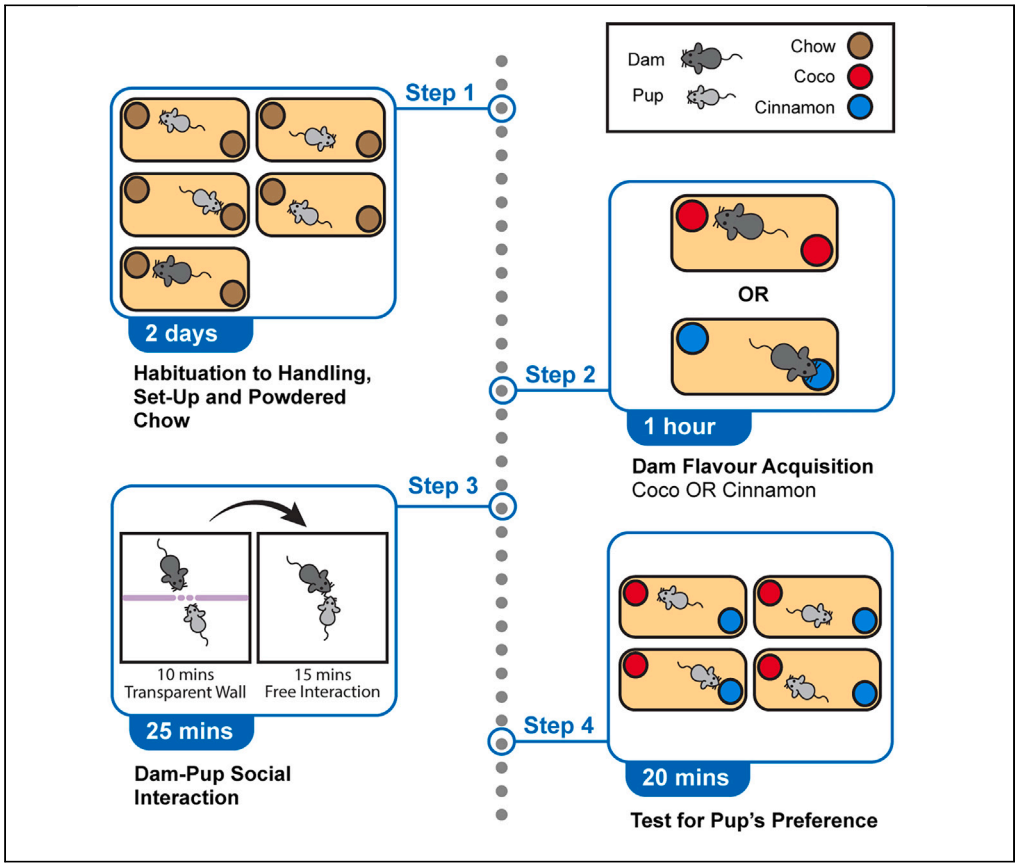
Protocol to study dam-pup social transmission using a modified paradigm for transmission of food preference



The social transmission of food preference, a rudimentary form of social learning, has primarily been studied in pairs of adult rodents. Here, we present a protocol to explore the parent-offspring context in social learning using an adaptation of this classic paradigm for rodent dam-pup dyads. We describe steps for studying weanling mice from the same mother and present a worked example using weight-based (food consumption) and time-based (exploration) indices of social learning.

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

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Highlights

Social transmission of food preference protocol for dam-pup dyads

Steps for habituating and assessing dam-pup interaction and transmission

Food consumption and exploration time to evaluate social learning

Compatible with modern tools like optogenetics and AI-based modeling

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Protocol to study dam-pup social transmission using a modified paradigm for transmission of food preference

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SUMMARY

The social transmission of food preference, a rudimentary form of social learning, has primarily been studied in pairs of adult rodents. Here, we present a protocol to explore the parent-offspring context in social learning using an adaptation of this classic paradigm for rodent dam-pup dyads. We describe steps for studying weanling mice from the same mother and present a worked example using weight-based (food consumption) and time-based (exploration) indices of social learning.

BEFORE YOU BEGIN

Healthy development relies on early life experiences that are socially nourishing. Mutually-coordinated parent-child interactions facilitate the transmission of information through social learning[1,2] and represent crucial scaffolds for social learning capacities later in life.[3–5] In rodents, a robust form of social learning is the social transmission of food preference (STFP), where a naive "observer" rodent learns to prefer consuming novel foods that their "demonstrator" peer has eaten following a brief bout of social interaction. This paradigm has been routinely used to study the neural underpinnings of learning,[6,7] memory,[8] and several neurological diseases[9,10] in adult rodents.

The seminal procedure by Galef and Wigmore[11] which described STFP in adult rats has been adapted for use in adult mice,[12] however, no such procedure exists for the unique dam-pup context. We present an adaptation of this classic STFP protocol[11] to allow the study of food preference transmission from dam-to-pup, bringing forth the unique context of early development. We designed the current protocol to be compatible with modern tools including neural activity recording, optogenetics, and AI-based modeling of both social behaviors and learning outcomes. While STFP has been classically indexed by the amount of the demonstrator's diet eaten relative to the total amount eaten, our protocol introduces an exploration (time)-based metric that may be a more robust measure of social learning outcomes in the context of pup social learning.

Institutional permissions

Prior to performing animal-related procedures listed in this protocol, approval must be obtained with all animal care and ethics committees as well as any regulatory agencies. All animal studies described here were approved by the Nanyang Technological University Institutional Animal Care and Use Committee (NTU-IACUC).

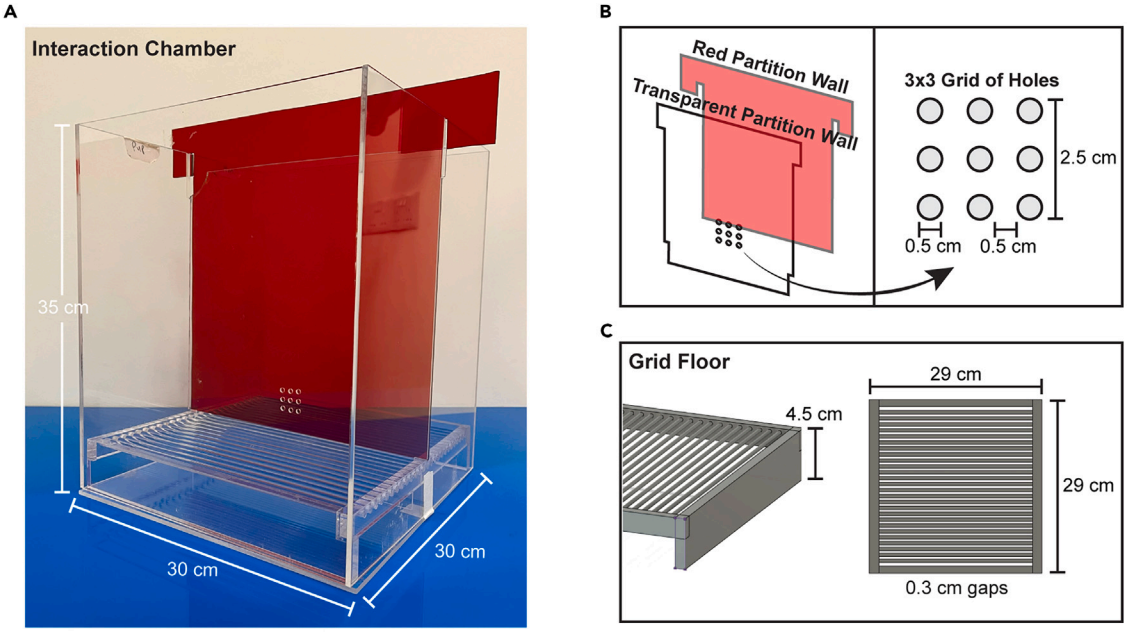

Figure 1. Equipment set-up for dam-pup social interactions and STFP testing

(A) Image of the interaction chamber which can be built in-house using materials described in the key resources table.

(B) Schematic representation of the custom-designed partitions, and the measurements of the 3 × 3 grid configuration of holes within the transparent partition.

(C) Side and aerial views of the grid floor with measurements.

Experimental setup

This procedure requires an enclosed room, at least 2 surfaces (for placing mouse home cages and weighing of food), 5 opaque enclosures (feeding chambers) for individual food sampling, 1 transparent enclosure (interaction chamber) for mouse dyad interactions, and 5 pairs of food bowls (one mouse dyad per feeding chamber). To reduce animal anxiety, the room should be dimly lit (ideally <~30 lux) during procedures – achieved through adjustable room lighting or via lamps directed towards wall corners (preferable). Red lighting can also be used to supplement white light, enabling better video capture of rodent behavior. The room should be thoroughly cleaned prior to testing. Good air filtration (>15 air change per h) is also highly recommended.

The enclosures can be modified from commercially available boxes of the correct dimensions (i.e., Ikea or Muji) or custom-built from acrylic manufacturers (Refer to key resources table). Ensure that the height of enclosures is at least 28 cm to prevent escape. The feeding chambers should be at least 30 cm × 20 cm to allow sufficient distance between the placement of food bowls. Ensure that the feeding chambers are not placed directly under overhead lighting, as light reflection may interfere with automated animal tracking.

The interaction chamber should be 30 cm × 30 cm to allow adequate roaming space while increasing the probability of interaction between dyads during free interaction (Figure 1A). Partitions can be custom-made to place constraints on interactions. A red translucent partition, opaque to mice (mice have reduced perception for red light), effectively blocks physical and visual interactions while allowing researchers to observe their movements. Additionally, a transparent partition featuring a 3 by 3 grid configuration of holes (each 0.5 cm in diameter and spaced 0.5 cm apart) at mouse height, limits the range of social behaviors to face-to-face interactions and accommodates procedures that involve tethered recordings of brain activity (Figure 1B). Based on our pilot experiments, pup sniffing of dam feces and urine may affect the success of STFP in pups. Therefore, the floor of the interaction chamber should be replaced with a grid to allow droppings and urine to fall

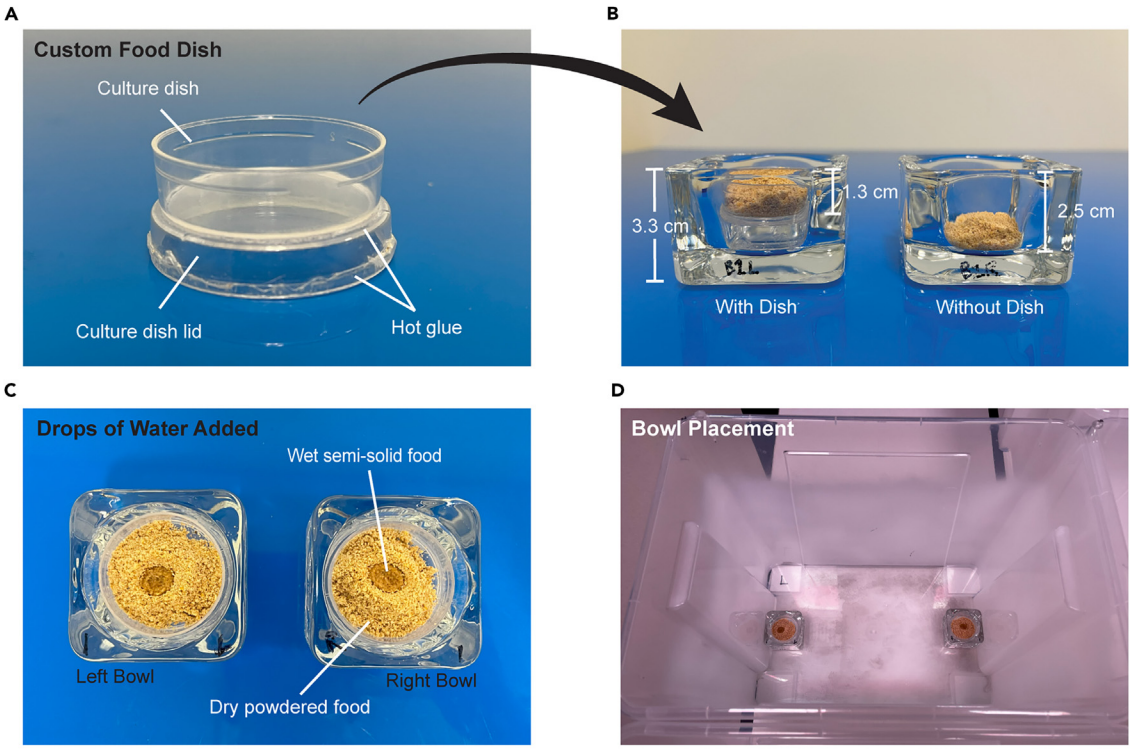

Figure 2. Custom-designed food bowls

(A) An elevated dish crafted by gluing a small culture dish and its lid together.

(B) Placing an elevated dish into a candle bowl allows pups easy access to the food pile.

(C) Adding drops of water to the powdered food pile creates a semi-solid texture that resembles food pellets. This helps to ease mice into consuming powdered food.

(D) The left and right food bowls are placed on their respective sides in the feeding chamber.

through the gaps (Figure 1C). The distance between grids should be 0.3 cm edge to edge, to prevent tripping yet allow waste to fall through.

Food bowls were handcrafted by hot gluing the flat surfaces of a 35 mm cell culture dish and its lid together, and then placing this elevated dish within a candle bowl (Figure 2). A layer of hot glue is also applied under the lid base to prevent wobbling. We opted for higher glass bowls so that mice will have to expend energy to reach the powdered food, ensuring that bowl visits are motivated behaviors. The plastic dishes that hold the powdered food are appropriately elevated to allow easy access and minimize spillage from food digging. For full supplier details, see the key resources table.

Animal breeding and food restrictions

Animals should be bred in-house as dams have to be kept with their offspring from birth. Breeding can be performed with 1 male and 2 females per cage, but females have to be individually housed nearing parturition to prevent dams from getting pregnant whilst STFP is ongoing (males can inseminate females immediately after parturition), and to ensure clear distinction of biological relationships (rodents typically co-parent and synchronize their breeding cycles, hence dams would likely give birth at the same time, making it difficult to identify and re-house pups with their birth mothers). It typically takes ~6 weeks before a litter is born following the first introduction of mating partners. A maximum of 4 pups (dependent on lab housing limits, in our case 5; 4 pups + 1 dam) should be kept with the dam post-weaning (P21), while the remaining can be separated into cages of 5 and used as controls. These housing arrangements ensure that dam-pup relationships remain clear throughout

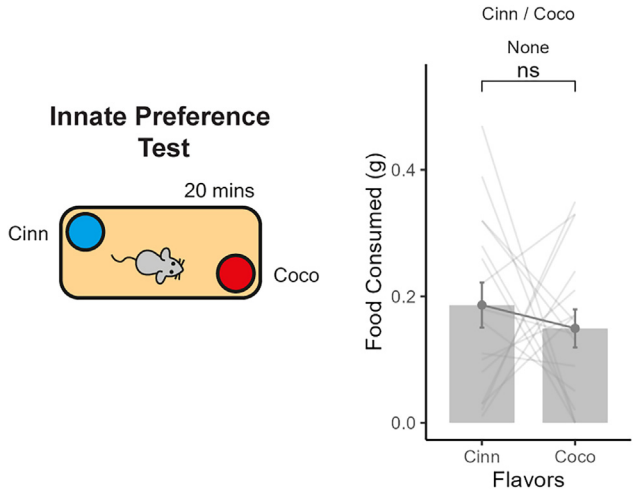

Figure 3. Innate preference test

Flavored food consumed by pups in each bowl (n = 16). Data are represented as mean ± SEM. Linear mixed model fit by restriction maximum likelihood (REML). T-tests were performed using Satterthwaite's method. ns, $p > .05$.

early development and that animals have sufficient cage space for their well-being post-weaning. Pups of both sexes were equally used.

Food and water should be supplied *ad libitum* in home cages up until the beginning of the protocol (~P19). A few pellets can be placed on the floor from ~P14 onwards to facilitate the acclimatization of pups to solid food. A food restriction schedule is implemented upon the start of the procedure. Water access is only restricted for the mother during the interaction phase (to prevent washing out of novel food odor). For our experiments, mice were housed on a reverse cycle (12-h dark/light; 9 am lights off/9 pm lights on) and were supplied with corncob bedding. All testing occurs during the dark cycle between 9 am and 6 pm. 4 litters, 16 pups (8 males and 8 females) per experimental group will provide enough statistical power based on our preliminary results (see Figure 2).

Powdered food preparation

This procedure uses powdered chow to enable ease of infusing different store-bought flavors. Batches of powdered chow can be made ahead of time by crushing, blending, and sieving standard pellets used by the animal facility. The use of standard facility pellets as a base ingredient for flavored chow reduces the need to habituate animals to a novel diet prior to the STFP procedure. Different flavors of chow can be made by infusing commercially available spices with powdered chow through simple mixing – typically in the range of 1%–2% w/w. Flavor pairings should be tested on a batch of 10–15 naïve pups to ensure even preference for both flavors (Figure 3).

Habituation to handling

Timing: 1 h 15 mins

Note: As this procedure involves mice completing successive stages in different setups, mice (and experimenters) should be habituated to handling for ease of transfer and to minimize stress from handling.

1. Transport all mice to a behavioral testing room and allow them to settle down and acclimatize to the room in their home cage for at least 30 min.
2. In the first encounter, approach the mice with an inflated glove for 5 min to accustom them to an approaching hand and the foreign smell of gloves.

3. Before handling mice, begin by rubbing hands with some bedding to imbue the home cage scent onto palms.
4. Slowly and gently nudge the dam to a corner with both approaching hands.
5. Create a flat platform with both hands so that the curious mouse steps onto palms.

⚠ CRITICAL: We generally recommend that mice be handled by encouraging them to step onto palms, instead of employing potentially stress-inducing techniques such as tail grabbing which may alter behavioral and cognitive performance.[13]

a. To handle the dam, create a cave with one hand without fully enclosing her to allow free exploration.

Note: The dam should begin to self-groom after about 5 mins, in which case, habituation to handling is considered successful.

6. For handling of pups (>P21), follow step 4. If pups (<P21), pick pups up by pinching their nape (simulating how dams would carry their pups) and placing the pup on the palm.
7. Once in the palm, hands should be fully enclosed as pups may jump off.
   a. Once pups have settled down on palm, gently stroke them periodically at the cheek to mimic grooming.

Note: Stroking can be performed until pups no longer jerk in response to touch.

8. Return the mice to their housing room.

Food deprivation & habituation to powdered food and interaction chamber

Timing: 30 min for Steps 9–10 (setup food deprivation)

Timing: 18 h gap between Steps 10 and 11 (passive; food deprivation duration)

Timing: 2 h 30 min for Steps 11–23

Timing: 18 h gap between Step 23 and social transmission of food preference (passive; food deprivation duration)

Note: Refer to Figure 4A for graphical timeline.

⚠ CRITICAL: Begin the food deprivation schedule and habituation to powdered food and the interaction chamber at least 2 days before the social transmission of food preference protocol to ensure adequate time for the mice to acclimate.

9. Before initiating food deprivation, transfer the mice to a new cage as there could be food pieces hidden in the bedding.

Note: Whenever mice are transferred to a new cage, spread some bedding and nesting material (not food or mouse wastes) from the original cage into the new home cage to reduce stress from relocation to a foreign environment.

10. Initiate food deprivation by limiting food provision to 4.6 *g* of food pellets over the period of 18 h. Troubleshooting 3.
    a. Mice should be weighed at the start and end of this food deprivation to ensure that weight is lost (but not >20% of their baseline weight).

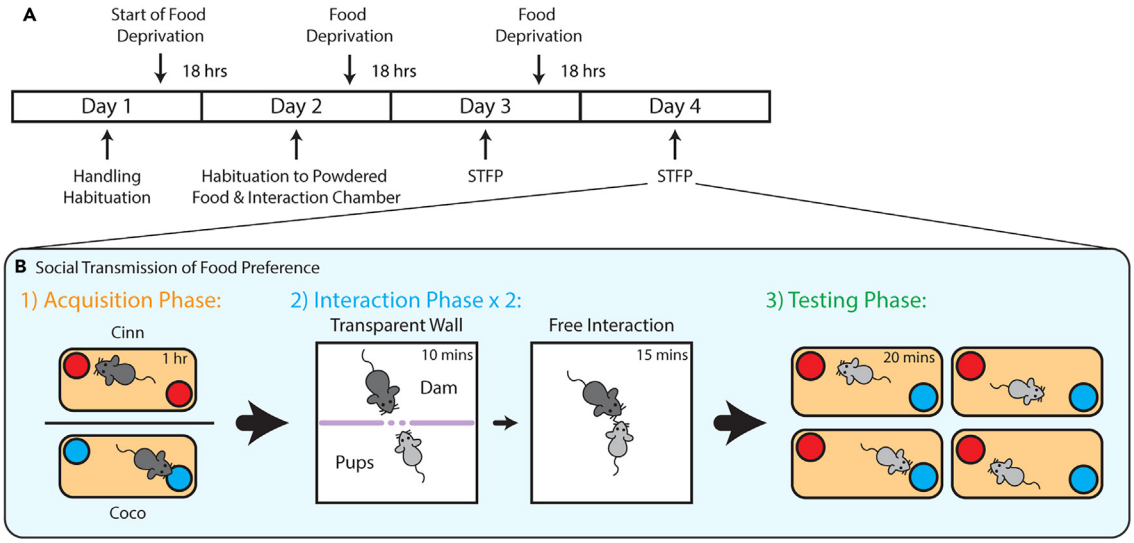

Figure 4. Detailed protocol timeline

(A) Timeline depicting protocol sequence across days for a typical set of experiments.

(B) Graphical representation of STFP sequence and duration of each phase: Acquisition, Interaction and Testing.

b. Provide free access to water.

Note: Food deprivation entails restricting the food allocation for a mouse to 15% of its baseline weight. This percentage assumes that a mouse typically consumes 20% of its body weight per day. Thus, over an 18-h period, a mouse would consume 15% of its body weight (calculated as 0.2 × body weight × 0.75 day). When mice are housed together as a family in the same cage, the total amount of food provided is 15% of their combined baseline body weight. The food allocation for the family is standardized at 4.6 grams per family to simplify the experimental process, though this step is optional. New standards are necessary when testing different mouse lines or developmental stages.

Note: Leaving behind approximately 4.6 *g* of food pellets in the home cage was found to induce sufficient hunger the following day to facilitate the consumption of powdered food chow, while limiting weight loss to not more than 20% of their body weight to meet ethical standards (may vary across institutions). Mice that lost more than 20% of their body weight should be excluded from the study and allowed to feed immediately.

Note: From a practical perspective, it is important to develop an experimental schedule that accommodates this food deprivation period. It is recommended to initiate the 18-h food deprivation in the late afternoon so that the subsequent day of experimentation can begin in the morning or at noon.

⚠ CRITICAL: Subsequent experiments should be performed at a consistent time of day and maintained throughout the experiment.[14]

11. After 18 h of food deprivation, retrieve mice from the housing room and allow them to acclimate to the testing room for at least 30 min.
12. Prepare plain powdered food:
    a. Scoop ~2 *g* (3 tiny spatulas) of plain powdered food into the dam's and pup's bowl.
    b. Create a well in each food pile using the same spatula.
    c. Add 3 drops of water into the well within the dam's bowls and 2 drops of water into the well within each pup's bowls using a plastic dropper.

Note: Adding water droplets into the food wells creates a small patch of semi-solid food that is of similar texture to food pellets. This minor step eases the transition to the consumption of powdered food (Figure 2C).

13. Weigh the glass bowls with food on a balance sensitive to 0.01 g and record individual values as pre-habituation food weight according to box and bowl assignment (E.g., Box1-L, Box1-R, …).
14. Place bowls in the feeding chambers where the habituation will take place and ensure that each chamber has two bowls positioned at opposite ends, placed in the middle of the opposing walls (Figure 2D).

Note: Bowl positions can be adjusted based on experimental conditions, but do not place bowls at the corners of the enclosure, as rodents tend to use corners for defecation and relief.

Note: The feeding chambers are not enclosed to allow video recording from an overhead camera which can be used for tracking mouse movement trajectories (see Figure 3).

15. Prior to beginning the habituation to powdered food, weigh the mice to ensure that it has lost weight.

Note: Weight loss is used as a proxy for mice hunger which ensures that the mice are sufficiently motivated to consume food of a different texture. The weight measurement marks the end of the 18-hr food deprivation.

⚠ CRITICAL: Weight loss may not be as apparent for pups due to natural weight gain as a result of developmental growth. If there are minute increases in weight (<20% increase from baseline) the experiment should continue, though the habituation phase may have to be extended to allow extra eating time.

16. Place each mouse into their individual feeding chambers and initiate the 1 h habituation period.
17. At the end of the 1 h habituation to powdered chow, return the mice to their home cage.
18. Calculate the amount of powdered chow eaten by each mouse. Troubleshooting 1.
    a. Scoop any spillage back into the glass bowls with a tiny spatula.
    b. Remove any defecation material inside the bowl with a tweezer.
    c. Weigh the bowls to compute the amount of powdered chow eaten.
19. Transfer the mice into the interaction chamber and allow them to interact as a family for 20 min to become accustomed to the chamber and grid.
20. Transfer the mice back to their home cage.
    a. Supply them with food pellets for at least 1 h.

Note: This free-feeding phase allows mice to recover from their weight loss, maintaining healthy body weights even after 18 hr food deprivations.

21. Clean the feeding and interaction chambers.
    a. First wipe urine and collect feces with dry paper towels.
    b. Spray water onto paper towels and wipe the chambers thoroughly, ensuring that corners are cleaned too.
    c. Repeat the process with 60% ethanol.
    Clean food bowls, plastic dishes, and spatulas with soap and water.
22. Prepare 4 new cages.
    a. Socially isolate the dam and pups #1 and #2, which will undergo STFP the following day, by single housing each of these mice.
    b. Pups #3 and #4 should be housed together and given free access to food pellets and water.

Note: Social isolation ensures that dyads are sufficiently motivated to elicit social behaviors during the interaction phase. In addition, isolation helps control deprivation levels as nursing may still occur.

23. Initiate another 18-h food deprivation for the dam and pups #1 and #2 by providing food pellets equivalent to about 15% of the baseline weight (from Day 1) of the mouse. Troubleshooting 3.
24. Return the 4 cages to the animal housing room.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
Mice: male or female C57Bl6/J mice, aged 21–30 days, strain 000664	The Jackson Laboratory	RRID: IMSR_JAX:000664 https://www.jax.org/strain/000664
Software and algorithms		
Bonsai	Bonsai	https://bonsai-rx.org/
"lme4" package in R	Bates et al.[15]	https://doi.org/10.18637/jss.v067.i01
"lmerTest" package in R	Kuznetsova et al.[16]	https://doi.org/10.18637/jss.v082.i13
Other		
Mouse food pellets	Altromin	Cat #1314 – irradiated https://altromin.com/products/standarddiets/mice/1310#produkt_1314
Ground cinnamon	Tesco	https://www.tesco.com/groceries/en-GB/products/254920403
Unsweetened coco powder	Hershey's	https://www.hersheyland.com/products/hersheys-cocoa-100-cacao-natural-unsweetened-8-oz-can.html
Feeding chamber – opaque enclosures (39 × 28 × 28 cm/22 L) with sides spray painted white	IKEA	Cat #101.809.44 https://www.ikea.com/sg/en/p/samla-box-transparent-10180944/
Food bowls (5 × 5 × 3.3 cm)	IKEA	Cat #202.901.26 https://www.ikea.com/sg/en/p/glasig-tealight-holder-clear-glass-20290126/
Elevated food dish (diameter of 3.5 cm)	Corning	Cat #430165 https://ecatalog.corning.com/life-sciences/b2b/US/en/Surfaces/Advanced-Cell-Culture-Surfaces/Corning%C2%AE-Treated-Culture-Dishes/p/430165
Custom-built acrylic transparent enclosure (30 × 30 × 35 cm)	Custom-built (Dama Trading, Singapore)	Refer to Figure 1 & Experimental Setup
Custom-built acrylic transparent partition (29 × 0.3 × 34 cm)	Custom-built (Dama Trading, Singapore)	Refer to Figure 1 & Experimental Setup
Custom-built acrylic red partition (29 × 0.3 × 34 cm)	Custom-built (Dama Trading, Singapore)	Refer to Figure 1 & Experimental Setup
Custom-built acrylic grid (29 × 29 × 4.5 cm)	Custom-built (Dama Trading, Singapore)	Refer to Figure 1 & Experimental Setup

MATERIALS AND EQUIPMENT

Stock Flavored Powdered Chow (80 g per Flavor)		
Ingredients	Final concentration	Amount
Standard food pellets, grounded	99%	79.2 g
Ground cinnamon/coco	1%	0.8 g
Deionized water	-	2–3 drops added upon serving
Total	N/A	80 g

An 80 *g* batch can be filled into two 50 mL Falcon tubes and stored in a Ziplock bag at 22–25°C. All ingredients should also be stored in their respective Ziplock bags at 22–25°C. To make unflavored powdered chow, grind 80 *g* of food pellets without adding cinnamon or coco.

⚠ CRITICAL: Flavored powdered chow should not be made in bulk as the scent of the added flavors may dissipate over time. We recommend using a freshly made mixture that is not

over a month old. An 80 *g* batch per flavor should be sufficient for a month of experiments. About 2 *g* of food is used per serving during the food habituation, dam flavor acquisition and pup testing phases.

Alternatives: We do not anticipate that the brand of the added flavor will be a critical factor; however, it is recommended to use the same brand throughout the study.

STEP-BY-STEP METHOD DETAILS

Social transmission of food preference

Timing: 4 h / day for full protocol (2 pups / day, but can be extended to test a max of 3 pups / day)

Timing: 1 h 30 mins for Phase 1 - dam flavor acquisition phase

Timing: 1 h 30 mins for Phase 2 - dam-pup interaction phase (~45 mins per dyad)

Timing: 30 min for Phase 3 - pup testing phase

Note: Refer to Figure 4 for graphical timeline.

This section elucidates the protocol for the social transmission of food preference for dam-pup dyads. The protocol begins with the learning phase in which the dam or "demonstrator" will be fed either cinnamon (Cinn) or chocolate (Coco) flavored powdered food. The dam will then interact with two of her pups or "observers" sequentially in the interaction phase, after which the pups will be presented with both Cinn and Coco flavor options in the testing phase.

Pause point: Due to cage size restrictions, our protocol pairs a maximum of four pups to each dam. The number of pups per dam can be increased based on housing conditions, however, as dams interact with their pups sequentially, dam fatigue and flavor dissipation could affect the experimental results. Our protocol was therefore designed to possibly span two days, with two to three pups being tested per day.

Note: Prior to this day of testing, the dam demonstrator is randomly assigned to either Cinn or Coco conditions. The dam is assigned to the same flavor on both days to preserve the integrity of the food preference being transmitted.

1. Retrieve the dam and pups #1 and #2 from the housing room and allow the mice to rest for 30 min in the experiment room after the move.
2. Dam Flavor Acquisition Phase.
   a. Prepare powdered flavored food for the dam demonstrator.
      i. Fill two plastic dishes within glass bowls with ~2 *g* of Cinn/Coco-infused food.
      ii. Create a well in each food pile and add 3 drops of water using the plastic dropper.
      iii. Weigh each bowl.
      iv. Place bowls into the dam's feeding chamber, centralized and flushed to each wall on opposite ends.

⚠ CRITICAL: Before and after food preparation, store each flavored food in designated Ziplock bags to preserve smell and prevent smell from escaping into the room, which could interfere with STFP. Different flavored powdered foods should be handled separately to prevent cross-contamination. Designate separate areas on a table to prepare Cinn and Coco flavored foods. These areas should also be at a distance from the mice.

b. Record the weight of the dam. This marks the end of the 18 h food deprivation period for the dam.
c. Begin the acquisition phase of the dam demonstrator.
   i. Place the dam into the feeding chamber.
   ii. Allow her to consume the flavored food for 1 h with no perturbations.
d. Return the dam back to her home cage but first ensure that any source of water has been removed to prevent potential dilution of the recently acquired food odors.
e. Calculate the amount of flavored food eaten by the dam. Troubleshooting 1 and 2.
   i. Scoop any spillage from digging or movement back into the respective food bowls.
   ii. Remove any defecation material inside the bowl with a tweezer.
   iii. Measure the weight of the food bowls to compute the amount of flavored food eaten by the dam.

3. Dam-Pup Interaction Phase.
   a. Prepare the interaction chamber. Place both the transparent and red partitions to split the interaction chamber into two halves.
   b. Initiate the 25 min interaction phase for the first dyad. Troubleshooting 5.
      i. Transfer the dam into one side of the interaction chamber, followed by pup #1 in the other.
      ii. After 5 min, remove the red partition while leaving the transparent partition intact.

Note: This first 5 mins allows both animals to acclimatize to the chamber and can be used to quantify general locomotor activity or record baseline neural activity in implanted animals.

      iii. Remove the transparent partition after 10 min.

Note: The transparent partition has a 3 by 3 grid of holes that only permits face-to-face social interactions. This partition narrows down the range of social behaviors and can remain throughout the interaction phase if tethered animals are used in the experiment.

      iv. Remove the transparent partition to enable 15 min of free interaction between the dam and pup.

Note: The free interaction phase allows quantification of naturalistic behaviors and social engagement between dam and pup.

   c. Transfer the dam and pup #1 back to their respective home cages. The dam must not have access to water, and both the dam and pup #1 should be without food.

⚠ CRITICAL: Removing dam water access during this period prevents washing out of the demonstrated flavor.

   d. Using the same cleaning procedure as before, wipe the interaction chamber and partitions thoroughly using paper towels and water and subsequently paper towels and 20% ethanol.

Note: 60% ethanol should only be used for end-of-day cleaning as stronger ethanol concentrations will introduce a lingering scent in the interaction chamber. Thus, 20% ethanol is suitable for cleaning between social interactions.

   e. Begin the interaction phase for pup #2 following the same steps 3a-d.
   f. Transfer the dam and pup #2 back to their respective home cages. Allow the dam free feeding of food pellets and water for at least an h. This allows weight recovery as the next interaction phase only takes place the next day. Pup #2 should not be supplied any food.

Note: As dams may hide food pellets during the free feeding period, it is recommended to supply the dam with a fixed number of pellets (approx. 5 pellets) to enable ease of retrieval when initiating deprivation for the next day. Alternatively, one may transfer the dam to a new cage when initiating food deprivation later in the day.

Pause point: The duration between the interaction phase and testing phase may be extended to probe remote long-term memory.[17] However, if the testing phase is delayed, the duration of food deprivation for pups should be adjusted accordingly.

4. Pup Testing Phase.
   a. Prepare Cinn and Coco flavored foods for pups #1 and #2.
      i. Fill the respective bowls with flavored foods.
      ii. Weigh each bowl before placing them into the feeding chambers.

   Note: To account for the potential effects of food locations, the location of the demonstrator's diet should be counterbalanced across pups. For instance, the demonstrator diet could be placed in the left food bowl for pup #1 and in the right food bowl for pup #2.

   b. Record the weights of pups #1 and #2. This marks the end of the food deprivation period for the pups, which should have lasted for about 20 h.
   c. Begin the 20-min testing phase for pups #1 and #2. Troubleshooting 4.
      i. Begin video recording from an overhead-mounted camera.
      ii. Transfer the pups to their respective feeding chambers. Ensure that they are placed in the middle of the chamber to reduce any directional biases.
      iii. Allow the pups to feed for 20 min.

   Note: Based on our experience 20 mins is the optimal duration to access food preference with pups, as pups tend to eat from both bowls equally if left for longer durations (i.e., 1 hr).

   d. Transfer both pups back to one cage.
      i. Provide free access to food pellets and water as these pups have completed the STFP task.
   e. Calculate the amount of flavored food eaten by the pup. Troubleshooting 1 and 2.
      i. Scoop any spillage from digging or movement back into the respective food bowls.
      ii. Remove any defecation material inside the bowl with a tweezer.
      iii. Measure the weight of the food bowls to compute the amount of flavored food eaten by the pup.
   f. Clean the feeding chambers, interaction chambers, food bowls, and tools thoroughly in preparation for the next day of experimentation.
5. Return all mice to the mouse housing room.
6. Split pups #3 and #4 and single house them in separate cages.
   a. The dam should remain isolated as well.
7. Initiate the 18-h food deprivation period for the dam and pups #3 and #4. Troubleshooting 3.
8. Repeat social transmission of food preference protocol the following day with pups #3 and #4.

EXPECTED OUTCOMES

Food consumption (weight)-based index of STFP

The weight of each food option eaten by the observer mouse is measured following the Testing Phase. After the 18 h of food deprivation, the weanling pups should be sufficiently motivated to explore their food options. The STFP effect is observed when the pup consumes more of the food fed to the demonstrator compared to the other option. As shown in Figure 5, pups that interacted with dams that were fed the Cinn-flavored food consumed significantly more of the Demo- than the NonDemo-flavored food (Figure 5A – right plot).

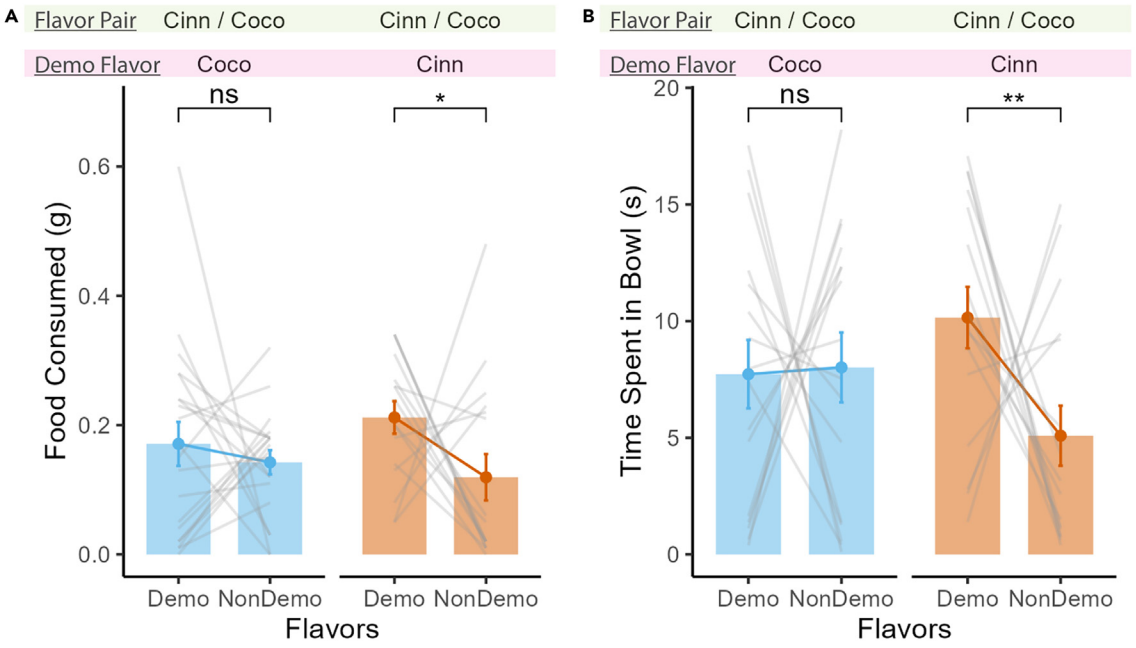

Figure 5. Measures of social learning - Food eaten and time spent

(A) Food consumed and (B) Time spent in each food bowl (Demo and NonDemo) under different demonstrated flavors: Coco (blue, $n = 20$), Cinn (orange, $n = 16$). Demonstrated flavors (pink) and flavor pairings (green) are indicated above graphs. Data are represented as mean ± SEM. Linear mixed model fit by REML for each demonstrated flavor. T-tests were performed using Satterthwaite's method. ns $p > .05$, *$p < .05$, **$p < .005$.

> ***Note:*** Some demonstrated flavors may not work for STFP in the context of dam-pup dyads (i.e., Figure 5A – left plot). This may be due to an intrinsic lack of volatility in specific flavors or pup insensitivity to particular smells that hinder effect size. Pilot trials should be performed when testing new flavor combinations.

Exploration (time)-based index of STFP

Using the videos captured during the testing phase, we can also quantify the amount of time spent by pups exploring each bowl (Figure 6) as an alternative measure of social knowledge transmission (i.e., pups spent more time interacting with and exploring a location marked with the learned flavor). When pups interacted with dams that were given cinnamon, pups spent significantly more time in the bowl containing the dam demonstrated flavor compared to non-demonstrated flavor (Figure 5B – right plot). Exploration time may be a more robust measure of social learning as this is not confounded with hunger levels in the pup.

> ***Note:*** Exploration (time spent) is a better measure of social learning for pups, partially due to the minute amount of food eaten by pups, making food measurements prone to measurement errors from food spillage, digging, etc.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantification of food eaten and exploration time

The amount of both food option eaten by pups can be easily quantified using a weighing scale sensitive to 0.01 *g*. On the other hand, the time spent by the pup exploring each food bowl can be recorded over the 20-min testing phase using a camera mounted directly above the feeding chambers. These recordings may be analyzed using various video quantification software such as Bonsai, Anymaze, or Ethovision. The following outlines our analysis pipeline using bonsai (Figure 6). The locations of the feeding chambers and food bowls are first delineated in bonsai (Figures 6A and 6C, Box ROIs and Bowl ROIs). Using pixel value thresholding, mice are visually separated from the

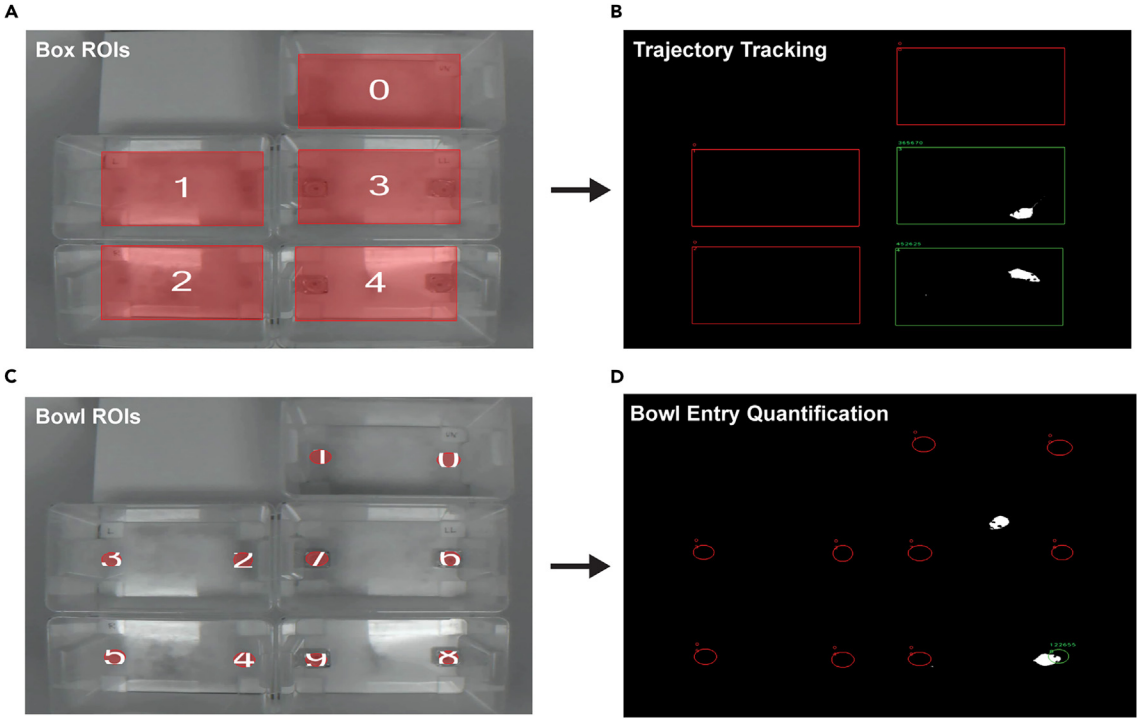

Figure 6. Video quantification of rodent trajectory and time spent visiting food bowls using Bonsai

(A) The perimeter of each opaque enclosure is defined in Bonsai as Region of Interests (ROIs).

(B) Bonsai detects the presence of mice within regions 3 and 4 (green box). Further data extraction is constrained within these regions.

(C) The circumference of each food bowl is defined in Bonsai.

(D) Bonsai records the timestamps corresponding to pup entries and exits for individual food bowls. The green circumference indicates pup entry into bowl 8.

background to allow the automated tracking of pup movements (Figures 6B and 6D, Trajectory Tracking and Bowl Entry Quantification). When a pup enters or exits the opening of each food bowl, a precise timestamp is generated to mark these events. Timestamps are subsequently processed in R to calculate the total duration pups spent exploring each food bowl.

Hierarchical structure and linear mixed effect modeling

Observations from pups that are born and raised by the same dam introduces a hierarchical structure to the data. Therefore, systematic variations in STFP across mothers may exist, otherwise known as the random effects of family. When comparing the amount of Cinn and Coco-flavored food eaten by pups, a linear mixed effect model should be fitted for each maternal condition (Innate Preference Test, Cinn, or Coco) to account for this random effect of family (e.g., by fitting a random intercept). Linear mixed effects models are robust against violations in assumptions for parametric testing.[18] Restricted maximum likelihood (REML) was used in the models to obtain less biased estimates of variance components while Satterthwaite's method was used to obtain more accurate approximations of degrees of freedom, especially given our small sample size.[15,16]

LIMITATIONS

Although differences across families can be accounted for through statistical means, this effect may very well represent meaningful variations in maternal care in the postnatal period. Indeed, research has shown that the licking and grooming behaviors of the dam can scaffold the development of social cognitive capacities, including those required for STFP.[4,5] This protocol, while offering a promising start, warrants further adaptation to include the quantification of early maternal care to truly uncover the complex relationship between the dam-pup context and the pup's social learning.

TROUBLESHOOTING

Problem 1

Step 18 of the food deprivation & habituation to powdered food and interaction chamber section, and Steps 2e and 4e of the social transmission of food preference Section: Mouse movement leading to food spillage and defecation into food bowls.

Potential solution

Ensure that any spillage is thoroughly scooped back into corresponding food bowls. Mice may also defecate into the food bowls, in which case, wastes should be carefully removed using a tweezer, without spilling any food.

Problem 2

Step 18 of the food deprivation & habituation to powdered food and interaction chamber section and Step 2e of the social transmission of food preference Section: Mouse has not eaten at least 0.2 *g* (~10%) of the given food, suggesting that habituation to powdered food or acquisition of the novel food flavor is unsuccessful.

Potential solution

Place the mouse back into its feeding chamber with the same food bowls until it has eaten at least 0.2 *g*. If the mouse is unable to achieve the minimum of 0.2 *g*, remove the animal from the study. Ensure mice are properly habituated to handling, as rodent anxiety may prevent consumption of food. A few drops of water can encourage eating of powdered chow in the initial Powdered Food Habituation stage (Figure 2C).

Problem 3

Steps 10 and 23 of the food deprivation & habituation to powdered food and interaction chamber section and Step 7 of the social transmission of food preference Section: Due to variability in experimenter and their availabilities, the duration of food deprivation can fall short of or exceed 18 h.

Potential solution

The start and end time of food deprivation should be properly documented. Further, it may also be helpful to measure pup weight at the start and end of this deprivation as an indicator of hunger. Variations in the duration of food deprivation and hunger levels can be accounted for statistically by including them as covariates.

Problem 4

Step 4c of the social transmission of food preference Section: The quantification of mouse trajectories using video recordings of the Testing Phase is unsuccessful or incomplete due to occlusions caused by experimenters blocking the camera's view.

Potential solution

When placing each mouse into their respective feeding chamber, experimenters should position themselves directly in front of the respective chamber. It is important to extend the arm directly into the chamber being accessed and avoid extending arms across other chambers. During the testing phase, it is crucial for experimenters to remain seated and refrain from walking around the feeding chambers to ensure uninterrupted observation and recording.

Problem 5

Step 3b of the social transmission of food preference Section: Dyads may interact at the edge of the partition instead of at the designed grid of holes.

Potential solution

Ensure that the partition fits nicely with the enclosure. Small gaps may entice dyads to interact at the edges instead. In this situation, quantification of social interactions should include instances of edge interaction.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gao-Xiang Ham (gaoxiang90@gmail.com).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Gao-Xiang Ham (gaoxiang90@gmail.com).

Materials availability

3D drawings of the custom interaction chamber are available at https://github.com/GxHam/STFP_dam-pup (https://doi.org/10.5281/zenodo.10983377). All other required materials are provided in the key resources table.

Data and code availability

Bonsai quantification pipelines are available at https://github.com/GxHam/STFP_dam-pup (https://doi.org/10.5281/zenodo.10983377). The data is available in NTU research data repository DR-NTU (Data) at https://doi.org/10.21979/N9/JLGVSL. R scripts for analysis are available from the technical contact upon request.

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AUTHOR CONTRIBUTIONS

The protocol was conceptualized and designed by G.H., V.L., and G.J.A. The manuscript was written by G.H., J.Z.O., V.L., and G.J.A. Representative data were generated by G.H. and J.Z.O.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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