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Effects on Mouse Food Consumption After Exposure to Bedding from Sick Mice or Healthy Mice

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Control mice housed in the same room as mice with pancreatic ductal adenocarcinoma (PDAC) demonstrate decreased food intake coincident with the cachexia experienced by the mice with PDAC. Mice are considered an empathetic species, and we hypothesized that the reduced food intake in normal mice was an "empathy state" that was mediated by olfactory cues. Naïve male and female C57BL/6 mice were exposed to soiled bedding from mice experiencing PDAC induced cachexia or from control mice in the PDAC study. Body weight, food intake, and food spillage were measured across 48 h. Statistically significant differences in food consumption were found at various time points in both positive and negative directions for the 2 bedding conditions, and the direction of effect was opposite for males and females. Although analysis of data from previous PDAC studies showed differences in food spillage between PDAC mice and their controls, in this study we found no correlation between food consumption and food spillage based on bedding type. Disruption of food intake due to the "empathy state" requires testing larger numbers of animals to attain appropriate statistical power, which is contrary to the goal of using fewer animals. Empathy effects require careful consideration of sample size and cautious interpretation of results. This study also highlights the importance of sex as a biologic variable and why quantifying food spillage is important in studies of food intake.

Abbreviation: PDAC, pancreatic ductal adenocarcinoma

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Cachexia is a multisystemic syndrome involving lean mass catabolism, metabolic disturbances, and behavioral changes such as fatigue and anorexia.[7,29] This wasting disease is associated with multiple underlying disease processes, including cancer, chronic kidney disease, congestive heart failure, and AIDS.[4] Cachexia may also worsen the course and prognosis of these diseases.

Among all forms of malignancy, pancreatic ductal adenocarcinoma (PDAC) is among the most highly associated with cachexia, with an estimated 83% of patients suffering from the condition.[1,6,16] A murine model of PDAC cachexia has been developed that closely models human PDAC.[15] In murine PDAC studies, one would logically assume that control mice that received heat-killed cells would continue eating the same baseline amount of food throughout the experiment; however, their food intake modestly decreases coincident with the cachexia experienced by the mice with PDAC (Figure 1). As illustrated in Figure 1, when the PDAC mice are removed from the room at the end of the study, food intake of control mice returns to baseline within 24 h. Several theories for this effect have been proposed, including an empathy-like effect transmitted by olfactory, auditory, or visual routes.

At its simplest level, empathy can be defined as the capacity to be affected by and to share in the emotional state of another individual.[21] Emotional contagion is the sharing of emotional states between individuals,[12] and this event appears to occur without conscious awareness.[25] Mice are believed to be an empathetic species, at least at the level of emotional contagion.[25] Much research has been performed on the topic of rodent empathy,[5,9,12,19,20,25] and perception of a conspecific's emotional state has been described in many species,[5,12,19,25] including mice.

Numerous studies analyzed sources of social stressors to rodents, such as olfactory cues[26] and ultrasonic vocalizations.[10,24] Pheromones seem to play a role in stress responses in mice. For example, a true pheromone was shown to be responsible for aversion to the odor of stressed conspecific C57BL/6J mice.[24] Others studied hyperalgesia communicated to bystander mice via olfactory cues from bedding exposure by placing soiled bedding in empty cages adjacent to cages of naïve mice.[26] They found that olfactory cues from soiled bedding of mice experiencing hyperalgesia from alcohol withdrawal were sufficient to rapidly provoke similar hypersensitivity in naïve mice.[26] A group of mice exposed to bedding from control mice showed no behavioral changes, indicating that the hypersensitivity could not be attributed merely to cues associated with exposure to novel mouse bedding.

Although our mice in active cancer studies experienced illness due to PDAC, rather than alcohol withdrawal, the previous findings[26] are compelling with regard to social and/or olfactory transfer of illness behaviors, especially as the study showed long-range olfactory communication of hyperalgesia (throughout a room, rather than direct bedding exposure within a cage).

In addition to the stressors described above, food intake can also decrease for many other reasons, such as infection,[17] environmental temperature,[2] and disruptions in photoperiod,[2]

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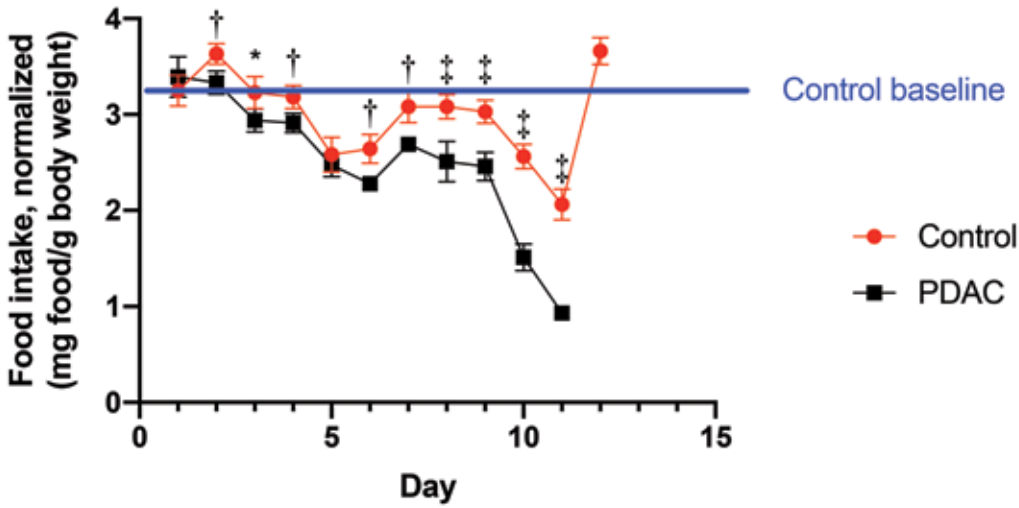

Figure 1. Food intake for mice with KxPxCx PDAC and control mice. Solid blue line indicates baseline food consumption of control mice. *, $P < 0.05$; †, $P < 0.005$; ‡, $P < 0.001$.

nutrient stores, and gastrointestinal signals.[28] However, these reasons seem unlikely to explain why our control mice had consistently decreased food intake only when PDAC mice were housed in the same room. Therefore, we considered social transfer of illness behaviors (hereafter referred to as the "empathy state") as the most likely cause of decreased food intake and the cause we chose to investigate.

In addition to changes in food consumption, food grinding or food wastage can occur for a variety of reasons, such as old age,[27] hardness or fat content of food,[3] a stereotypic or compulsive behavior due to lack of environmental enrichment,[3] or an attempt to select the more energetically profitable parts of food.[3] Based on our repeated observations, we suggest that transferred illness behavior plays a role in food grinding as well.

Another significant problem is that statistical power may fall when the empathy state occurs, such that more animals are needed to reach a statistically significant result. In research, we strive to reduce animal numbers whenever possible, whereas the empathy state requires the opposite. US Government Principle III directs researchers to work with the minimal number of animals necessary to obtain valid results,[18] however, underpowered studies are a major cause of irreproducibility.[22] In addition, the need to use more animals has an economic cost.

To date, no studies have assessed empathy-derived cachexia in control mice or examined its potential underlying mechanisms. The goal of this study is to investigate the drivers of this reduced food intake by the control mice to allow investigators to more accurately predict experimental power and take steps to reduce animal distress and animal use in these studies. The study aims to determine whether exposure to soiled bedding from cachexic mice will result in decreased food intake in control mice. We hypothesize that normal mice exposed to soiled bedding from cages of cachexic mice will consume less food than mice exposed to soiled bedding from cages of control mice. Despite observing reduced food intake in control mice, we have not observed increased food spillage in control mice. We also hypothesize that food spillage will be equivalent in mice exposed to bedding from cachectic or control mice.

Materials and Methods

Animals. Subjects were male ($n = 20$ total) and female ($n = 20$ total) C57BL/6J mice (age, 5 wk at arrival; stock no. 000664, The Jackson Laboratory, Bar Harbor, ME). Upon arrival, mice were individually housed and allowed to acclimate to the experimental room and housing conditions for 1 wk. All experimental procedures were approved by the Oregon Health and Science University IACUC and performed in an AAALAC-accredited facility. All research adhered to the guidelines of the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.[8]

To limit variables induced by housing nonexperimental animals in the experimental room, and due to the short period of time that mice were housed in the experimental rooms, no sentinel animals were in place in these rooms. Soiled bedding (described below) was collected from a conventional room of the same pathogen status as the mice in this experiment. Colony health status was monitored quarterly, and sentinel animals in the bedding collection room tested free of the following agents: Sendai virus, pneumonia virus of mice, mouse hepatitis virus, minute virus of mice, mouse parvovirus 1 and 2, parvovirus NS-1, Theiler murine encephalomyelitis virus, reovirus (types 1, 2, 3, 4), mouse rotavirus, *Mycoplasma pulmonis*, *Pneumocystis murina*, *Spironucleus muris*, *Myobia musculi*, *Myocoptes musculinus*, *Radfordia affinis*, *Aspiculuris tetraptera*, and *Syphacia obvelata*.

Husbandry. Mice were housed in standard polycarbonate rodent cages (no. 1 Maxi-Miser cages, Thoren Caging Systems, Hazleton, PA) with wire cage tops and static microisolation lids. Mice were housed on 1/4" pelleted cellulose bedding (BioFresh Performance Bedding, BioFresh, Ferndale, WA) with one cotton nesting square (Cotton square, Ancare Corp., Bellmore, NY) and one half (approximately 3.5 g) of a package of brown crinkled paper (EnviroPak containing EnviroDri, Shepherd Speciality Papers, Watertown, TN) provided per cage. All caging components were autoclaved. The animal housing rooms were maintained at 23.5 to 25.5 °C with a relative humidity of 30% to 70% and 12:12 light:dark photoperiod. Mice had unrestricted access to food (PicoLab 5L0D, LabDiet, St Louis, MO) and reverse osmosis purified, chlorinated, autoclaved water in bottles.

Mice were placed in clean cages with fresh bedding and fresh food at least 5 d before each experiment started, and cages were not changed during an experiment. At each cage change, the old nest was removed and fresh enrichment material was provided. Each housing room contained a single group of mice ($n = 5$) during an experiment, and mice were housed one per cage (5 cages per room). Up to 2 experiments were carried out concurrently, therefore 1 or 2 experimental rooms being used at

a given time. Mice remained in the same housing room through washout and the subsequent cross over experiment.

Soiled bedding. Soiled bedding (including pelleted bedding, feces, and nesting material) was collected from the cages of male and female mice experiencing PDAC ("sick bedding") or from cages of control mice in the same cohort ("control bedding"), all of which were housed in a conventional room of the same pathogen status as the mice in this experiment. Bedding was collected within 2 to 3 d of the end of the PDAC study, at the peak of signs of illness, such as weight loss and decreased food intake. PDAC was induced in mice by inoculation with KxPxCx cells, as described by others.[15] Briefly, The KxPxCx cells are derived from a tumor explant of a C57BL/6 mouse expressing pancreas specific conditional alleles $KRAS^{G12D}$ and $TP53^{R172H}$ via the Pdx1-Cre driver. C57BL/6 mice were inoculated intraperitoneally (IP) with a 0.5 mL suspension of 3 million KxPxCx tumor cells. Sham mice were inoculated with an equal volume of heat-killed KxPxCx tumor cells. Soiled bedding was stored in sealed bags at -80 °C for 9 to 68 d prior to use (mean = 40 d). The observer in this study was blind as to the source of the bedding (sick or control).

Experimental design. Experimental groups consisted of naïve mice ($n = 5$ per group) exposed to either sick bedding or control bedding in a 2 × 2 crossover design. Experiments began just before lights out (0 h). At 0 h and 24 h, approximately 4 g of soiled bedding and 1 g of soiled nesting material (sex-matched) were placed in each mouse cage. Measurements of food intake (as described below) took place over 48 h. At the end of the experiment, mice were moved to clean cages with fresh bedding, nesting material, and food and water. While nests are typically transferred with the mice during husbandry procedures, for this study the old nest was discarded and fresh nesting material was provided to prevent the possible transfer of experimentally introduced soiled bedding in the cross-over design. The mice were left undisturbed for a washout period of 5 d ($n = 30$) to 10 d ($n = 10$), before being exposed to the opposite type of soiled bedding (sick or control). Eight replicates of 5 mice each were completed (total mice males $n = 20$, females $n = 20$). Sample size was chosen based on prior studies of bedding exposure for olfactory cues.[26]

Food intake measurement. Food intake was assessed serially at 0, 2, 4, 12, 24, 36, and 48 h, and included the measurement of feed in the cage top and food granules within the cage (commonly known as orts). Cage top food was weighed in a plastic beaker, then replaced in the cage top. To obtain orts at each of the time points above, the mouse was placed in the weighing beaker and the bedding was sifted for approximately 10 to 15 s using a mesh sieve that allowed small particles to fall through onto a collection surface while retaining bedding within the sifter. The sifter was constructed from a discarded microisolation lid (19.7 × 30.6 × 10.1 cm) with mesh window screen material replacing the filter medium (Figure 2). Separate sifters were used for each bedding type ("sick" and "control") to prevent cross-contamination of olfactory cues. The sifted debris was scraped together on the collection surface with an index card, which allowed orts to fall to the bottom of the pile and fibers from bedding debris to rise to the top. Bedding debris was removed from this pile, and the remaining orts were then weighed and discarded. The food intake was defined as the difference in cage top food weight, minus the orts within the cage, over the time interval of interest. After sifting, the bedding was replaced in the cage with the nest on top of the bedding, and the mouse was returned to the cage.

Food spillage. In addition to the ort data collected in this study, ort data was analyzed from previous PDAC studies within our lab.[13,14,30] C57BL/6J mice ($n = 5$ to 8 male) per cohort were evaluated. Groups included mice inoculated with KxPxCx-derived tumors by IP injection, and control mice who received heat-killed cells IP. Food was weighed once daily for up to 16 d. Orts were collected as described above in "food intake measurement".

Analysis for minimum required mice in PDAC studies. In order to assess how the "empathy state" affects the number of mice required for a PDAC study, data from previous PDAC studies[30] were reviewed to retrospectively determine the necessary sample size and statistical power for PDAC studies. Food intake data were grouped as pre-cachexia or cachexia based on anorexia. Cachexia was defined as the interval beginning with 2 consecutive days with a greater than 10% decrease in food intake; pre-cachexia was the interval between implantation of cells and the onset of cachexia. To estimate the baseline food intake of control mice injected with heat-killed KxPxCx cells if an "empathy state" did not exist, pre-cachexia daily food intake of control mice was averaged, and this value was used as the "no-empathy" baseline daily food intake. For "empathy state" food intake, actual daily food intake across the entire experimental period was averaged for control and for PDAC mice. These food intake data were used to determine the sample size necessary at 90% power with an α value of 0.05, in order to detect a difference in mean food intake between PDAC mice and control mice. Sample sizes were calculated for "no-empathy" (theoretical food intake) and "empathy state" (actual food intake).

Animal weighing. Mice were lifted from their cage by the tail, briefly held in the palm of a gloved hand, and transferred to a plastic beaker for weighing at 0, 12, 24, 36, and 48 h. Mice remained in the beaker briefly while their bedding was sifted, as described above. Tail lift, rather than scooping, was chosen to reduce the chance of transferring bedding to the beaker.

Animal Disposition. At the conclusion of the current study, mice were transferred to a different unrelated experiment.

Statistical analysis. Food intake was normalized to mg of food consumed per g of body weight. For food intake measurement of mice exposed to soiled bedding, normalized food intake was modeled using a linear mixed model with bedding type, time, and their interaction as fixed factors. Because each mouse contributed multiple data points, a random intercept for each mouse was included in the model. A second model, including the fixed effect of gender and its interaction with the other fixed factors, was also analyzed. Statistical analysis was done using statistical packages R version 3.5.1 (R Foundation for Statistical Computing, Vienna, Austria. 2018. URL http://www.R-project.org/) and NLME (Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team (2019). *nlme: Linear and Nonlinear Mixed Effects Models*. R package version 3.1-137, https://CRAN.R-project.org/package=nlme). Food spillage analysis was performed using GraphPad Prism version 8.2.0 for MacOS (GraphPad Software, San Diego, CA USA, www.graphpad.com). A P value of less than 0.05 was considered to be statistically significant.

Results

Food intake measurement. Food intake data are shown in Table 1. Statistically significant differences in food intake were detected based on bedding type. Situations in which mice in "sick" bedding conditions ate less than mice in "control" bedding conditions were female mice at 24 h ($P = 0.0003$) (Figures 3 A and B) and male mice at 48 h ($P = 0.001$) (Figures 3 C and D). At 48 h, female mice in the "sick" bedding condi-

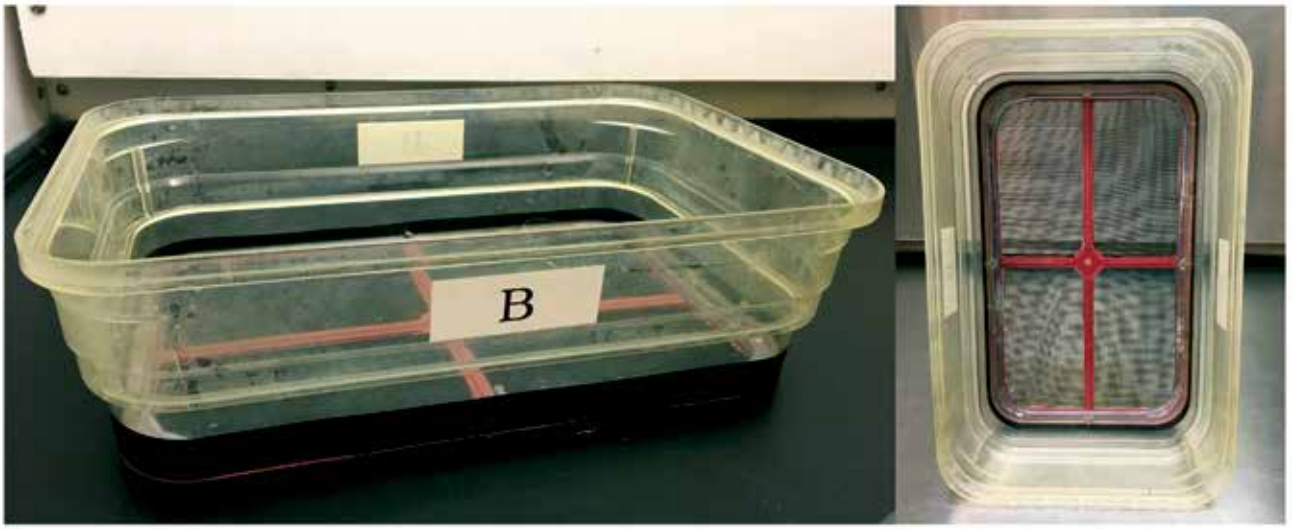

Figure 2. Two views of a bedding sifter constructed from a discarded microisolation lid with mesh window screen material replacing the filter medium. "B" indicates the code for the bedding type, as separate sifters were used for sick and control bedding.

Table 1. Food intake (mg food/g body weight) of mice exposed to sick bedding compared with mice exposed to control bedding. Only significant results shown ($P < 0.05$).

	Females		Males	
Time Point	Food Intake (mg/g)	*P* value	Food Intake (mg/g)	*P* value
24 h	−12.1	0.0003	—	—
48 h	14.2	0.0004	−22.2	0.001

tion ate more than mice in the "control" bedding condition ($P = 0.0004$). The order of exposure to each of the 2 bedding types did not affect food intake. One male mouse that had received the sick bedding was eliminated from data analysis at all time points due to the incorrect weighing of the food.

Food spillage (orts). Food spillage data are shown in Table 2. Food consumption and food spillage were strongly correlated for all groups (Table 3). Bedding type had no discernable effect on the correlation between food consumption and food spillage (Table 3). With regard to food spillage alone, statistically significant differences by bedding type were found in female mice at 12 h (Figure 4) and in male mice at 4 h (Figure 5).

Food spillage data from previous PDAC studies done in our lab[14] are shown in Figure 6. Significantly greater food spillage ($P < 0.05$) occurred on days 13, 14, 15, and 16 in mice with PDAC as compared with controls.

We also examined baseline food spillage data from 5 different previous PDAC experiments performed in our lab[13,14,30] and found great variability in the baseline ort production of individual mice (Table 4). The overall mean and standard deviation of ort production was 0.34 ± 0.15 g, and the range of baseline ort production was 0.07 to 0.84 g. Orts represented 4 to 45% of food removed from the hopper (mean 14% ± 1%), with the remaining percentage being food consumed.

Analysis for minimal necessary numbers of mice in PDAC studies. Retrospective comparisons between PDAC and control mice in the absence (equivalent food intakes) or presence (actual food intake) of an empathy state [30] revealed a minimal necessary sample size of 16 mice if the "empathy state" did not exist and 28 mice if an "empathy state" was present. Thus, 75% more mice would be necessary to detect a difference in food intake between PDAC and control mice with an "empathy state".

Discussion

Possible causes of the empathy state, demonstrated by previous studies,[5,9,12,19,20,25] include stressor-related olfactory, auditory, and visual signals. This study was focused on olfactory cues. We hypothesized that mice exposed to soiled bedding from cages of mice with illness would consume less food than mice exposed to soiled bedding from cages of control mice. The current data support our hypothesis, but only at a few of the tested time points.

This study illustrates mouse sex differences in responses to exposure to bedding from mice with PDAC. At the 24 h time point, female mice exposed to sick bedding ate less than female mice exposed to control bedding, whereas at 48 h female mice exposed to sick bedding ate more than female mice exposed to control bedding. The opposite was observed in male mice. Male mice exposed to sick bedding ate less than male mice exposed to control bedding at 48 h. Studies in rats have shown that female rats can be unresponsive to fear contagion, especially in the estrus phase of the cycle.[12] Further, estrogen has a central role in anxiety in rats, as ovariectomized rats are more anxious, and this anxiety is relieved by administration of estrogen.[12] While social factors are also likely to be a component in this innate behavior in female rodents, this study shows clear differences in food intake between the sexes. The impact of the phase of estrous cycle was not examined in the bedding exposure mice. This possible variable could be evaluated in future studies to better assess sex effects on food intake.

Stress can be defined as a state in which homeostasis is disrupted or perceived to be threatened.[11] Stress affects food

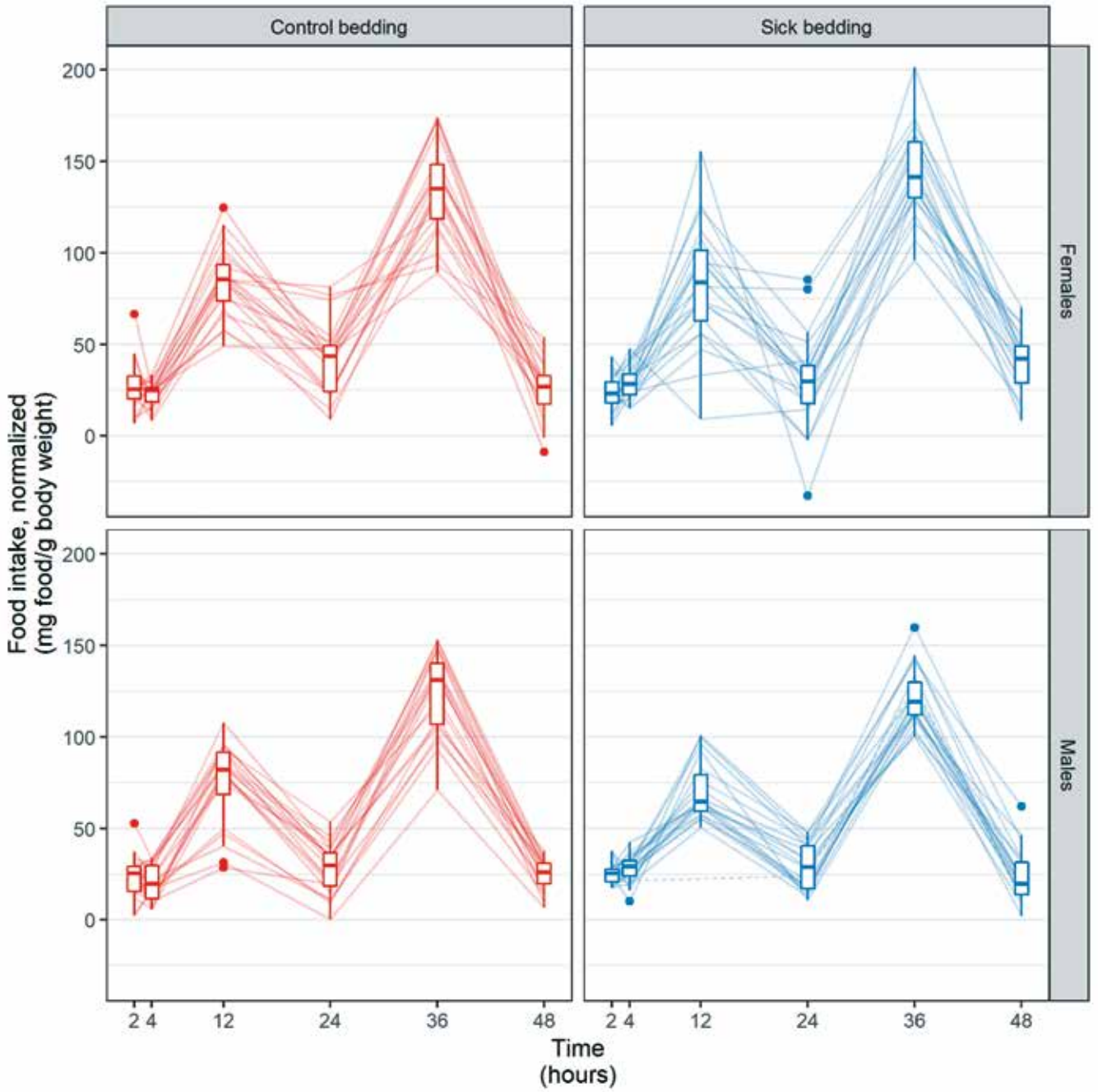

Figure 3. Food intake for female and male mice exposed to control bedding or sick bedding across time points. *, $P < 0.05$.

Table 2. Food spillage (g food) of mice exposed to sick bedding compared with mice exposed to control bedding. Only significant results shown ($P < 0.05$).

	Females		Males	
Time Point	Orts (g)	*P* value	Orts (g)	*P* value
4 h	—	—	0.05	0.0016
12 h	–0.11	0.0003	—	—

intake in a bidirectional manner, leading to either increases or decreases in food intake, as is well evidenced in both human and animal studies.[11,23] The influence of stress on feeding responses is multifactorial, reflecting a variety of intrinsic and extrinsic elements, such as type and severity of stress, availability of palatable food, and individual differences.[11,23] From an evolutionary perspective, chronic stressors may promote obesogenic mechanisms.[23] However, this simple idea is difficult to demonstrate experimentally in rodents, probably because classic rodent stressors do not mimic human situations, and direct comparisons between acute and chronic stress are difficult to find.[23]

When the empathetic control mice consume less food coincident with the decreased food intake of PDAC mice, the smaller

Table 3. Correlation between food intake and ort production.

	Females		Males	
	Food Intake (g) to Orts (g) (control bedding)	Food Intake (g) to Orts (g) (sick bedding)	Food Intake (g) to Orts (g) (control bedding)	Food Intake (g) to Orts (g) (sick bedding)
Correlations: *r* (*P*)	0.987 (0.0003)	0.944 (0.005)	0.982 (0.0005)	0.970 (0.001)
Comparison of correlations: *z* (*P*)	0.908 (0.182)		0.317 (0.376)	

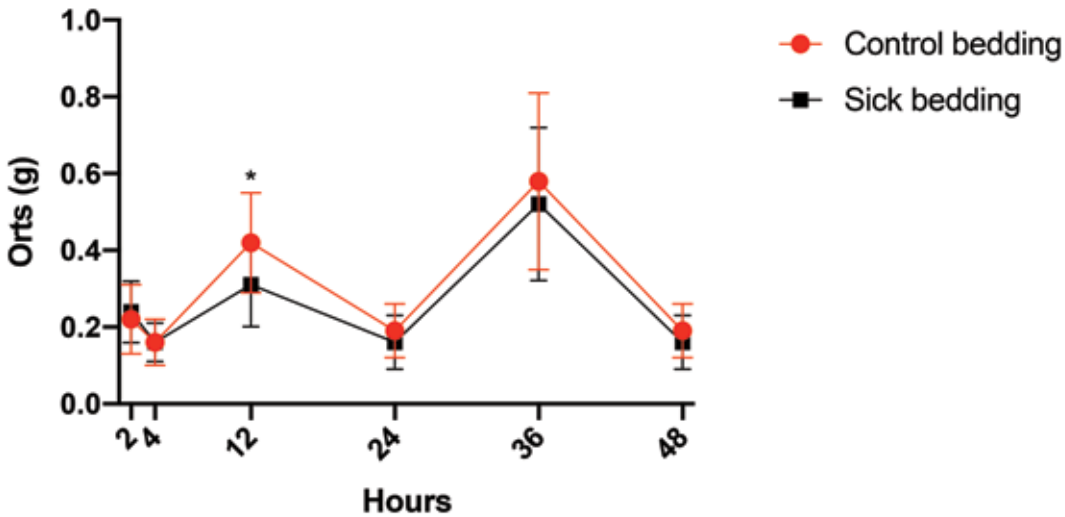

Figure 4. Food spillage of female mice. *, $P < 0.05$.

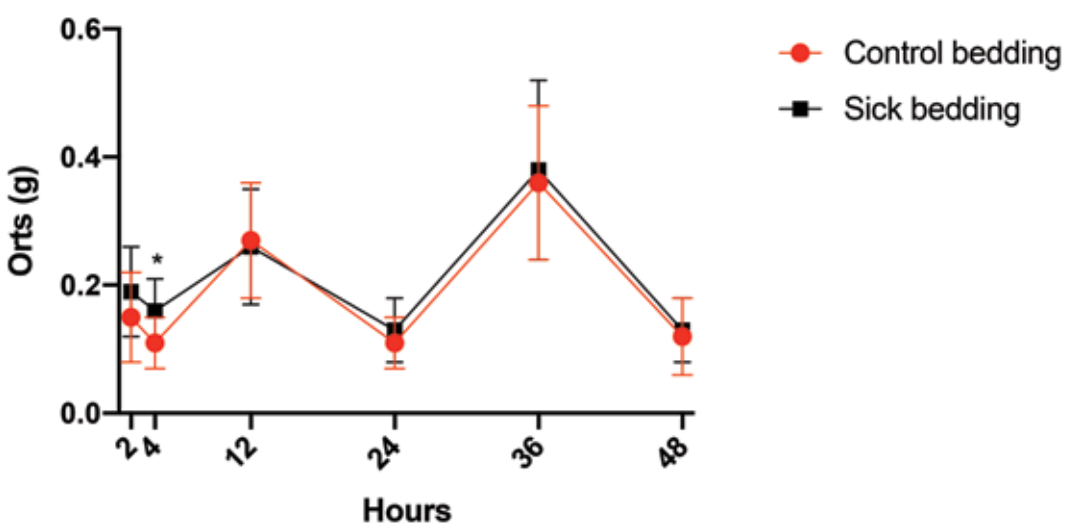

Figure 5. Food spillage of male mice. *, $P < 0.05$.

difference between the food intake of PDAC and control animals decreases the statistical power. This means that more animals are needed to reach a statistically significant result; we calculated that 75% more mice would be required. A real difference in food intake between PDAC and control mice would be masked by the empathy state. The difference in the food intake between PDAC and control mice would be less than in a state in which control mice continued to have baseline food consumption. The empathy state reduces experimental power, thereby requiring the use of more animals. A difference in food intake that is greater than what is actually measured could have implications for future studies.

As reported here, previous PDAC studies performed by our group found a clear significant difference in food spillage among mice with PDAC compared with controls as the disease state of PDAC mice progressed. In the current study, food spillage was significantly different between bedding exposure types at only one time point for each sex, and varied in direction of effect between males and females. While these significant differences in ort production occurred at different time points than did the significant differences in food consumption, this finding still highlights the variability between sexes, and validates the need to study both sexes.

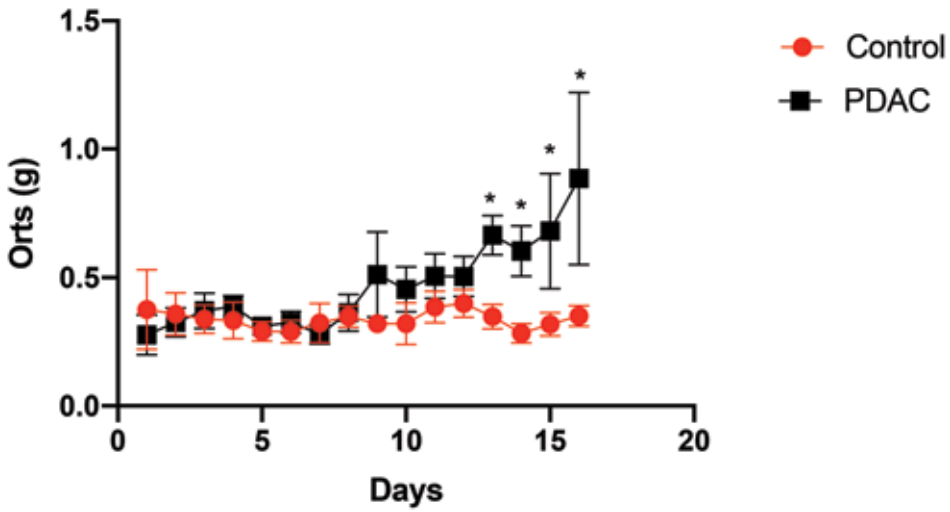

Figure 6. Food spillage of PDAC mice compared with control mice. *, $P < 0.05$.

Table 4. Baseline food spillage (g) among mice in 5 different PDAC experiments.

Mouse no.	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Overall
1	0.27	0.52	0.24	0.57	0.36	
2	0.20	0.27	0.32	0.37	0.41	
3	0.19	0.33	0.44	0.40	0.32	
4	0.84	0.31	0.31	0.22	0.31	
5	0.20	0.42	0.31	0.13	0.28	
6	0.57	0.34	0.33	0.43	0.22	
7	0.13	0.56	0.46	0.45	0.47	
8	0.18	0.57	0.50	0.14	0.16	
9	0.07	0.21	0.19	0.18	0.21	
10	0.30	0.21	0.30		0.25	
11	0.10	0.44			0.37	
12	0.66				0.52	
13					0.28	
14					0.48	
15					0.31	
Mean ± SD.	0.31 ± 0.25	0.38 ± 0.13	0.34 ± 0.10	0.32 ± 0.16	0.33 ± 0.11	0.34 ± 0.15
Range	0.07 – 0.84	0.21 – 0.57	0.19 – 0.50	0.13 – 0.57	0.16 – 0.52	0.07 – 0.84

In studies measuring food intake, bedding should be screened for ort weight, as this represents a major source of potential error. As shown in Figure 6, our group has observed that PDAC mice increasingly grind and waste their food as their disease progresses. If we weighed food in the food hopper alone, the PDAC mice would appear to be eating more over time. This effect is magnified as the disease state becomes more advanced. Failure to account for orts makes the differences between groups appear smaller, therefore requiring more animals to achieve appropriate statistical power. In addition, individual animals show baseline differences in ort production (Table 4). These reasons validate our decision to measure orts, which is essential to reducing the variability in food intake data.

Our study had several limitations. The PDAC and control mice that inspired the original question were housed in the same room, such that control mice had constant, chronic exposure from the PDAC mice. The current study provided intermittent, acute exposure of a stimulus. This difference in exposure timing likely affected our results, and our study was only 48 h in duration. This time interval was chosen based on the illness behaviors that PDAC mice normally display for the final 2 d of experiments. A longer period of experimental bedding exposure may reveal additional patterns of altered food consumption. Another confounding factor could be due to single housing, complete cage change, and replacing the nest that had familiar scent cues. Single housing is unfortunately necessary for accurate measurement of food consumption with our caging system. Cage change and nest replacement were performed 5 d prior to each experiment to allow the mice time to acclimate to the clean environment. We recognize that these husbandry procedures themselves may be stressful, even if it was unlikely to affect the experimental design after 5 d of acclimation.

Future research on this topic could be improved in a number of ways. A group of mice exposed to unsoiled bedding or no bedding could control for neophobia that may be induced by introduction of foreign bedding into the cage. Alternatively, the soiled bedding could be placed in an empty cage directly adjacent to the test subject, as was done in a study of alcohol withdrawal hyperalgesia.[26] Likewise, instead of studying olfac-

tory cues as a cause of the "empathy state", one could study auditory cues with congenitally deaf mice.

Even though our results were unanticipated, we can conclude that exposure to the bedding of sick mice, presumably via the olfactory cues it contains, affects food consumption of bystander mice both in positive and negative directions. Detecting this disruption in feeding may require the use of higher animal numbers to attain adequate statistical power, which is contrary to our goal of using fewer animals. The effect of the "empathy state" on a studied variable may vary based on numerous factors, such as study design, the specific parameter being measured, and the species or strain chosen for study. Researchers are encouraged to carefully consider sample size and to cautiously interpret results when no prior research is available to guide sample size calculations.

Acknowledgments

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