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Sucrose Preference and Novelty-Induced Hypophagia Tests in Rats Using an Automated Food Intake Monitoring System

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Vineeta Bajaj, M.S., Ph.D.
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January 9, 2020

Dear Dr. Bajaj,

please find enclosed the revised article entitled 'Performing Anhedonia Tests, Sucrose Preference and Novelty Induced Hypophagia, Using an Automated Food Intake Monitoring System in Rats' co-authored by Martha A. Schalla, Stepahnie G. Kühne, Tiemo Friedrich, Peter Kobelt, Miriam Goebel-Stengel, Matthias Rose and Andreas Stengel that we would like to resubmit for publication in Journal of Visualized Experiments.

We thank the editor for the thoughtful comments. We made changed in the manuscript accordingly. We hope that we sufficiently addressed all points and that the manuscript will be considered favorably for further processing in its present form.

We are looking forward to hearing from you.

Sincerely,

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TITLE:

Sucrose Preference and Novelty-Induced Hypophagia Tests in Rats Using an Automated Food Intake Monitoring System

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anhedonia tests, animal model, behavior, depression, automated food and liquid intake system, rats

SUMMARY:

Presented here is a protocol to study depression-like and anhedonic behavior in rats. It combines two well-established behavioral methods, the sucrose preference and novelty-induced hypophagia tests, with an automated food and liquid intake monitoring system, to indirectly investigate rodent behavior using surrogate parameters.

ABSTRACT:

The prevalence and incidence of depressive disorders are rising worldwide, affecting about 322 million individuals, underlining the need for behavioral studies in animal models. In this protocol, to study depression-like and anhedonic behavior in rats, the established sucrose

preference and novelty-induced hypophagia tests are combined with an automated food and liquid intake monitoring system. Prior to testing, in the sucrose preference paradigm, male rats are trained for at least 2 days to consume a sucrose solution in addition to tap water. During the test, rats are again exposed to water and sucrose solution. Consumption is registered every second by the automated system. The ratio of sucrose to total water intake (sucrose preference ratio) is a surrogate parameter for anhedonia. In the novelty-induced hypophagia test, male rats undergo a training period in which they are exposed to a palatable snack. During training, rodents show a stable baseline snack intake. On test day, the animals are transferred from home cages into a fresh, empty cage representing a novel unknown environment with access to the known palatable snack. The automated system records the total intake and its underlying microstructure (e.g., latency to approaching the snack), providing insight into anhedonic and anxious behaviors. The combination of these paradigms with an automated measuring system provides more detailed information, along with higher accuracy by reducing measuring errors. However, the tests use surrogate parameters and only depict depression and anhedonia in an indirect manner.

INTRODUCTION:

On average, 4.4% of the world's population is affected by depression. These account for 322 million people worldwide, an 18% increase compared to ten years ago[1]. According to estimates by the World Health Organization, depression will be second in the ranking of Disability Adjusted Life Years in 2020[2]. To address the rising prevalence of affective disorders and establish new interventional strategies, it is necessary to further study this behavior. Prior and in addition to examination in humans, animal studies are necessary.

Several models have been established to study components of depressive behavior (i.e., forced swim test, tail suspension test, sucrose preference test, and novelty-induced hypophagia)[3,4]. The sucrose preference test (SPT) and novelty-induced hypophagia (NIH) can detect depression-like behavior in animals. These tests themselves do not induce a state of depression in rodents but depict acute changes in behavior. Both the SPT and the NIH assess a characteristic trait of depression known as anhedonia, which is the loss of interest in the following: rewarding activities, activities that were once enjoyed by the individual[5], and one aspect of the complex phenomenon of processing and responding to reward[6]. Both tests study the response to a rewarding stimulus in the form of palatable food. The extent of consumption serves as a surrogate parameter for anhedonia[7-9].

The value of tests investigating anhedonia is strongly dependent on the accurate determination of consumption resulting from precise measurement of the substance's weight. Conventionally, this measurement is conducted manually once before and once after the test. However, this is prone to erroneous measurements for several reasons. First, rodents tend to hoard food, meaning that they remove food without consuming it immediately then hide it in a safe place. Thus, this loss of food may be included in the calculation of total consumption. Second, rats spill food and water, resulting in weight loss without respective consumption. Third, unintentional

loss of liquid occurs due to the handling of the bottles by inserting and removing them from cages.

In an approach to reduce these sources of error, we combined the two common tests assessing anhedonia (SPT[3,4] and NIH[9]) with measurement of food and water intake using an automated food and liquid intake monitoring system. This procedure allows accurate investigation of the consumption of palatable substances as well as provides information about the experience of pleasure in rats as a feature of depression-like behavior. The abovementioned errors associated with manual measurement are reduced by using different approaches, which are illustrated later in more detail.

To provide information about microstructure, the automated intake monitoring system used in this protocol[10] weighs the food (±0.01 g) every second. Thus, a stable weight is documented as "not eating", and an unstable weight as "eating". A "bout" is defined as change in stable weight before and after an event. A meal consists of one or more bouts and its minimum size in rats was defined as 0.01 g. A meal is separated from another meal in rats by 15 min (standardized value). Thus, food intake is considered to be one meal when the bouts occurred within 15 min and the weight change is as equal to or greater than 0.01 g. Meal parameters assessed in this protocol include meal duration, time spent in meals, bout size, bout duration, time spent in bouts, latency to first bout, and number of bouts.

PROTOCOL:

Animal care and experimental procedures followed the specific institutional ethics guidelines and was approved by the state authority for animal research.

1. Operation of the automated monitoring system

NOTE: When operating the automated monitoring system, it is crucial to document every action in the comment box included in the software immediately prior to the action. The description should be typed into the comment box, and by pressing **Save**, it is saved with a specific timepoint. The timepoints are significant when analyzing the data, since the system records continuously, and the period of interest must be indicated for analysis.

1.1. Installing, using and maintaining the automated monitoring system

NOTE: This protocol uses adult male Sprague Dawley rats weighing 250–300 g (~10 weeks old). It is recommended to house rats in groups during the acclimatization period. The environmental conditions should be controlled with the following parameters: 12 h/12 h dark/light cycle with lights on at 6:00 A.M., humidity of 45%–65%, and temperature of 21–23 °C, and *ad libitum* access to water and standard rodent diet. Daily handling enables the animals to become accustomed to the investigators.

1.1.1. Separate the rats so that every animal has an individual cage. Ensure that every rat stays separated during the protocol.

1.1.2. Fill the housing cages with regular bedding with a 1–2 cm thick layer. This (reduced) amount decreases the possibility of contamination of microbalances and hoppers with spillage, thereby reducing measuring errors. Add plastic tubes (e.g., a 20 cm long piece of a plastic drainpipe with diameter of 8 cm) and gnawing wood as enrichment, while omitting paper tissues to reduce measuring errors.

1.1.3. Prepare the cages for the automated solid and liquid food intake measurement by attaching two closed cage mounts with microbalances to custom-made holes in the front side of the cages. Place two empty hoppers on the cage mounts, one for the chow and one for the bottle.

NOTE: The microbalances are connected via cables to a recording system attached to a computer and the respective software is installed on the computer.

1.1.4. To start recording, open "**Monitor**" and press "**Start**", then choose a place to save the data.

1.1.5. Using the calibration (press "**Calibrate**") function of the automated intake monitoring system, calibrate every balance by removing the hoppers and placing two different gauged weights on the cage mounts with balances. Do this at regular time intervals (weekly is recommended)**.**

1.1.6. Fill one hopper completely with chow (~100 g) and remove chow pieces and crumbles that are too small in size. Fill water into the bottle (~100 mL) and place it into the other hopper.

1.1.7. Document the position of food and water (e.g., balance 1: food animal 1, balance 2: water animal 1).

1.1.8. Place the rat in the cage and open all gates of the cage mounts so that it can eat and drink *ad libitum*.

NOTE: For an accurate measurement, it is necessary to maintain the balances and hoppers daily by cleaning gently with a brush from spillage and removing small food crumbs from the food container. This will greatly reduce erroneous measurement. Close all gates during the daily maintenance.

1.2 Accessing data after the experiments

1.2.1. Search in the comment box for the beginning and end timepoints of a period (e.g., training, test) that needs to be analyzed.

1.2.2. Click on "**View data**" on the software to open the Data Viewer.

1.2.3. Insert the timepoints in the boxes below "**Begin time**" and "**End time**". Press the square in the left upper corner indicating the balance that recorded the information.

1.2.4. Click on "**PSC Totals**" to access the microstructure data. Press the button "**Export PSC Table**" to export the data.

NOTE: To compare the microstructure of individual animals (e.g., unstressed vs. stressed) using automated monitoring, individual animals can be selected in the "**Data viewer"** by pressing the appropriate square in the left upper corner. The **PSC Totals** shows only the microstructure for the selected animal. Statistical analysis cannot be performed with the system. The data needs to be extracted into a spreadsheet program/analyzing software.

2. Implementation of the sucrose preference test

2.1. Conducting the training period

NOTE: Prior to the test, animals must be accustomed to the availability of two bottles for liquids on hoppers through the gates, while food should be provided from the tops of cages (set-up is shown in **Figure 1**). This training period should last for at least 2 days. It is performed in the home cages in the room where animals are held.

2.1.1. Close all the gates. Remove the water bottle and food container from the microbalances.

2.1.2. Place pre-weighed food (~50 g) on the top of the cage and document its weight daily using a regular balance to assess daily food consumption. Refill, if necessary.

2.1.3. Clean a bottle with clear water and refill with around 100 mL of water. Place it back on the hopper.

2.1.4. Fill a second clean bottle with 100 mL of freshly made 1% sucrose solution. Place it on the hopper.

NOTE: Mark the bottles carefully and document their locations (e.g., balance 1: water animal 1, balance 2: sucrose solution animal 1).

2.1.5. Open all gates. Document the start of training in the monitoring system. Leave the gates open for 24 h, resulting in *ad libitum* access to both bottles. After 24 h, close the gates and document the end of the training. Data from the 24 h interval can be assessed using the automated monitoring system by inserting the "**begin time**" and "**end time**". The procedures are the same when a 1 h test interval is assessed.

2.1.6. Clean and refill the bottles every 24 h. Prepare fresh 1% sucrose solution daily. Switch the position of the water and sucrose solution bottle daily to avoid habituation effects.

NOTE: Conduct the training in all animals at least 48 h until the preference ratios reache ~1. The sucrose preference ratio is assessed directly after training using the "**Data viewer**". It is calculated as the ratio of sucrose intake to overall intake (water plus sucrose intake).

2.2.1. 24 h before the test, remove the bottle with the sucrose solution so the rat has access to standard chow and water only.

2.2.2. Prepare one fresh bottle filled with tap water and one filled with a 1% sucrose solution, both with ~100 mL.

2.2.3. Prior to testing, close all gates.

2.2.4. Remove the bottle filled with tap water from the hopper and place the two fresh bottles, one filled with tap water and one filled with a 1% sucrose solution, on the hopper.

2.2.5. Open all gates, document the start of the test in the monitoring system. Leave the gates open for 60 min. Close the gates after 60 min and document the end of the test.

2.2.6. Assess the data (e.g., the sucrose/total fluid intake ratio).

NOTE: The test can be repeated several times with intervals of training (at least 2 days) in between.

3. Implementation of the novelty-induced hypophagia test

3.1. Conducting the training period

NOTE: Prior to testing, a daily 30 min training period of 5 days is recommended (set-up is shown in **Figure 2**). The aim is to achieve a stable baseline of palatable snack intake before the experiment. It is performed in the home cages in the room where animals are held.

3.1.1. Close all gates and remove the hopper with standard chow.

3.1.2. Fill a fresh hopper with the palatable snack (~50 g). Insert the crackers carefully into the hopper to prevent crumbling. Place the hopper on the cage mount on top of the microbalance.

3.1.3. Open the gates for 30 min so that the rat has *ad libitum* access to the snack and water. Document the beginning of training in the monitoring system.

NOTE: The rat should have no access to standard chow during the training period.

3.1.4. Close the gates after 30 min and document the end of training in the monitoring system. Replace the snack with standard chow.

3.1.5. Repeat this daily until a 1) stable baseline palatable snack intake is achieved (e.g., 1.5–2.0 g/30 min) and 2) intake does not statistically differ between training days.

3.2. Performing the novelty-induced hypophagia test

3.2.1. Prepare an empty, freshly cleaned cage without bedding or enrichment attached to the automated food intake monitoring system. Place a hopper with a bottle of tap water and hopper with a palatable snack on the cage mounts.

NOTE: The novel cage should placed in the same room where the rats are held and training conducted. Keep the gates closed.

3.2.2. Remove the rat from the home cage and place in the novel cage.

3.2.3. Open all gates for 30 min. Document the beginning of testing in the monitoring system.

NOTE: During the 30 min of access to the snack, the size of snack intake and underlying microstructure parameters (e.g., latency to first meal) are recorded using the automated food intake monitoring system.

3.2.4. Close the gates after 30 min and document the end of testing. Place the rat back into the home cage.

NOTE: The test can be repeated several times with intervals of training (at least 5 days) in between.

REPRESENTATIVE RESULTS:

To test data distribution, the Kolmogorov-Smirnov test was used. T-tests were used when data were normally distributed and Mann-Whitney-U test was used, if not. One-way ANOVA followed by Tukey post-hoc test was used for normally distributed multiple group comparison. One-way ANOVA followed by Dunn's multiple comparison test was used in cases of non-normal distribution. Differences between groups were considered significant when $p < 0.05$.

The SPT was performed on naïve rats in this study. The consumption of sucrose solution increased and intake of water decreased over the training period (**Figure 3**). On the first day of training, rats drank 24.40 mL ± 3.48 mL of sucrose solution (**Figure 3A**) and 4.83 mL ± 0.89 mL of regular water (**Figure 3B**), yielding a sucrose preference ratio of 0.80 ± 0.06 (**Figure 3C**). On the second day of training, rats increased the sucrose solution consumption up to 33.77mL ± 4.49 mL (not significant, $p = 0.17$ *vs*. day 1) and decreased water intake to 0.42 mL ± 0.13 mL ($p < 0.001$ *vs*. day 1), resulting in a ratio of 0.99 ± 0.004 ($p < 0.05$ *vs*. day 1; **Figure 3C**). Eight rats

were studied here; thus, each datapoint is derived from eight animals. Fluid intake including its microstructure was recorded automatically. The data was extracted from **PSC Totals** using the **Data viewer**.

During the 60 min testing, the animals consumed between 0–6.18 mL of sucrose solution with a mean value of 2.12 mL ± 0.07 mL without consuming water, resulting in a sucrose preference ratio of 0.99 ± 0.00. The animals that did not consume any liquid during the test were excluded from the analysis. Eight rats were studied. Fluid intake was recorded automatically.

The automated intake monitoring system provided data about sucrose intake microstructure assessed automatically during testing, which were extracted from **PSC Totals** using the **Data viewer**. These parameters were meal size (**Figure 4A**), meal duration (**Figure 4B**), time spent in meals in seconds (**Figure 4C**) and percentages (**Figure 4D**), meal frequency (**Figure 4E**), latency to first meal (**Figure 4F**), inter-meal interval (**Figure 4G**), drinking rate (**Figure 4H**), bout duration (**Figure 4I**), bout size (**Figure 4J**), and time spent in bouts in seconds (**Figure 4K**) and percentages (**Figure 4L**).

To further study advantages of the automated intake monitoring system, the data illustrated above were compared to data gained using conventional manual assessments (weighing bottles manually before and after training/test period, **Table 1**). On the first day of training, sucrose ($p < 0.01$) and water intake ($p < 0.05$) were significantly higher when assessed manually compared to automatically. On the second day of training, water intake ($p < 0.001$) and sucrose preference ratio ($p < 0.01$) differed between the two groups and during testing. All parameters, namely sucrose intake ($p < 0.001$), water intake ($p < 0.001$), and sucrose preference ratio ($p < 0.001$) were different between groups, possibly due to erroneously high measurement or spillage when assessed manually.

Overall intake of the palatable snack during the training steadily increased: 0.48 g ± 0.14 g (day 1), 1.05 g ± 0.32 g (day 2), 1.48 g ± 0.56 g (day 3), 1.1 g ± 0.39 g (day 4), and 1.91 g ± 0.68 g (day 5), indicating an adaptation during the first 2–3 days. Similarly, meal size trended towards an increase between training days (**Figure 5A**, $p = 0.12$), whereas meal duration (**Figure 5B**) did not ($p > 0.05$). Likewise, other microstructural parameters such as time spent in meals (**Figure 5C**), latency to first bout (**Figure 5D**), bout size (**Figure 5E**), bout duration (**Figure 5F**), time spent in bouts (**Figure 5G**), and number of bouts (**Figure 5H**) were not significantly different between these days ($p > 0.05$). Eight rats were studied; thus, each datapoint is derived from eight animals. Snack intake including the microstructure was recorded automatically. The data was extracted from **PSC Totals** using the **Data viewer**.

On the test day, naïve rats exposed to the snack in a novel environment showed an intake of the palatable snack of 0.98 g ± 0.34 g (**Figure 6A**). Parameters of food intake microstructure on the test day, including meal duration (**Figure 6B**), time spent in meals (**Figure 6C**), latency to first bout (**Figure 6D**), bout size (**Figure 6E**), bout duration (**Figure 6F**), time spent in bouts (**Figure 6G**), and number of bouts (**Figure 6H**), were assessed automatically and extracted from **PSC Totals** using the **Data viewer**.

To study specificity of the novelty-induced hypophagia test, the data described above from naïve and unstressed rats were compared to those that received an intracerebroventricular injection of corticotropin-releasing factor, which stimulates the hypothalamus-pituitary-adrenal stress axis and induces stress and anxiety[11].The individual data for every animal was obtained using the "**Data viewer**" and "**PSC Totals**", as described in the protocol section. The individual data was then assembled according to groups in a spreadsheet program and analyzed for statistical differences. A significant difference in meal size ($p < 0.01$) and bout size ($p < 0.01$) was detected between both groups (**Table 2**). The difference in bout size would not have been detectable using manual assessment.

FIGURE AND TABLE LEGENDS:

Figure 1: Set-up of sucrose preference test.

Figure 2: **Set-up of novelty-induced hypophagia test**.

Figure 3: **Training period for the sucrose preference test.** The consumption of sucrose solution (**A**) and water (**B**) was assessed over 24 h for 2 days. The sucrose preference ratio (**C**) was calculated accordingly. Data are presented as mean ± SEM from eight rats (*$p < 0.05$, ***$p < 0.001$).

Figure 4: **Sucrose preference test.** On test day, the liquid intake microstructure (here, shown for sucrose intake) was analyzed over 1 h for encompassed meal size (**A**), meal duration (**B**), time spent in meals in s (**C**), time spent in meals in % (**D**), number of meals (**E**), latency to first meal (**F**), inter-meal interval (**G**), drinking rate (**H**), bout duration (**I**), bout size (**J**), time spent in bouts in s (**K**), and time spent in bouts in % (**L**). Data are presented as mean ± SEM, n = 8 rats.

Figure 5: **Training period for the novelty-induced hypophagia test**. Meal size (**A**), meal duration (**B**), time spent in meals in s (**C**), latency to first bout (**D**), bout size (**E**), bout duration (**F**), time spent in bouts (**G**), and number of bouts (H) were assessed over a period of 5 days. Data are presented as mean ± SEM, n = 8 rats.

Figure 6: **Novelty-induced hypophagia test**. On test day, the food intake microstructure was analyzed over 1 h for encompassed meal size (A), meal duration (B), time spent in meals (C), latency to first bout (D), bout size (E), bout duration (F), time spent in bouts (G) and number of bouts (H). Data are presented as mean ± SEM, n = 8 rats.

Table 1: Sucrose preference test in naïve rats using manual assessment vs. automated intake monitoring system. Distribution of the data was determined using the Kolmogorov-Smirnov test. Data are expressed as mean ± SEM and differences were analyzed using t-tests or the Mann-Whitney U test depending on distribution of the data (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ *vs*. manual assessment).

Table 2: Novelty-induced hypophagia test in naïve unstressed and stressed (CRF-injected) rats. In the stress group, icv-cannulated rats were injected with 0.6 µg/5 µL CRF and subjected to novelty-induced hypophagia afterwards. Distribution of the data was determined using the Kolmogorov-Smirnov test. Differences were analyzed using t-tests or the Mann-Whitney U test depending on distribution of the data. For better comparability, all data are expressed as mean ± SEM (**$p < 0.01$ *vs*. unstressed).

DISCUSSION:

The sucrose preference and novelty-induced hypophagia tests are two established techniques for evaluating anhedonia in rats. Their combination with the automated food intake monitoring system allows for more detailed analysis in undisturbed rats and reduces erroneous measurement.

The incidence of errors is reduced by different approaches. First, to address the error occurring due to spillage, the gap between the food hopper and gate allows crumbs generated during gnawing to fall onto the integrated tray. By collecting this spillage on cage mounts, they are included in the measurement (since spillage is still on the balance, it does not affect the measurement). Second, to prevent hoarding, the rat cage mount opening is large enough to allow the animal to eat from the food hopper with its head inside the opening, but small enough to limit the animal's ability to use its hands while eating. This limits their ability to remove food and bring it into the cage.

Third, the system reduces unintentional loss of liquid because the liquid bottle, after priming, does not leak, and evaporation occurs only slowly (approximately <10 mg/h) at the precision stainless steel ball/sipper tube interface. Additionally, because the system weighs the bottles automatically, handling of the bottles during recording is not necessary, which is a common cause of errors. The differences seen in the comparison between manual and automated measurements (**Table 1)** are suspected to be due to unintentional loss during manual handling of the bottles.

The use of the automated food intake monitoring system provides several advantages, such as detailed analysis of solid and liquid food intake and assessment of the underlying food intake microstructure[10]. The term "microstructure" describes the pattern of food or fluid intake in more detail. In studies without an automated intake monitoring system, intake is measured by weighing food/fluid at the beginning and end of a timepoint of interest. The only information gained by this approach is the total consumption over a certain period of time.

In contrast, the automated monitoring system provides more information about consumption during this period, because it records changes in weight of the microbalances every second. The recordings can detail when the rodent starts to eat, how often it eats, how long it eats for, how much it eats, how long the breaks between eating are, etc. To obtain data similar to the automated system using manual measurement, users would have to measure the content

frequently during testing/training and thereby significantly disturb the animals. With the automated system, rodents remain undisturbed during the training sessions and testing.

Considering the large number of automated intake systems available for rodents, it is important to note that no specified model is required for the protocol described here. However, this system is very sensitive. To avoid errors due to environmental noise (low level vibration or shaking of the mass on the scale), the system's algorithm evaluates the values collected on a second-by-second basis, and it only accepts those that are below a set point for “noise” in order to average 10 values. If the system exceeds this noise threshold, the values are not used to calculate stable weights, which are used to calculate bouts of feeding.

With regards to execution of the tests, several critical points should be kept in mind. All behavioral tests should be performed at the same time of day, as circadian alterations may affect the behavior of the animals[12,13]. Most studies perform behavioral testing during the light phase, while here, all tests were performed during the dark phase. Rodents are nocturnal animals and therefore active in the dark phase, while they are asleep or less active[12] with lower exploratory activity[13] during the light phase. Thus, behavioral testing is more physiologically appriopriate during the dark photo period.

It is important to note that for the sucrose preference test, different concentrations of the sucrose solution have been used, ranging from 0.5%–10%[4,7,14]. They are primarily chosen depending on species, strain, sex, and age, but especially based on observed drinking behavior during training (all animals should drink approximately the same amount before treatment/intervention). However, high concentrations (e.g., 10 %) may override anhedonia, since even animals with depression-like behavior still drink very sweet liquids[4].

Additionally, high caloric content due to high concentrations may more prominently affect the preference for sucrose solution. Therefore, a 1% sucrose solution was chosen for this protocol. Some studies recommend the use of saccharin instead of sucrose[15] to avoid any caloric influence. However, the mean caloric content of the consumed amount of % sucrose solution (2 g of 1% sucrose solution contains 0.08 kcal) is considerably lower than that of the same amount of standard chow (2 g contains 7.8 kcal). Thus, this point seems secondary.

It is also important to note that the baseline sucrose preference ratio assessed here using the automated food intake monitoring system is higher (0.99) compared to previous studies employing a manual assessment (with 0.7 in mice[8], 0.8 in young adult rats, 0.6 in aged male Sprague Dawley rats[16]). This may be due to handling of the bottles, since conventional weighing likely causes loss of fluid during inserting into and removing from the cages. This is further substantiated by the results shown in **Table 1.** Therefore, use of the automated monitoring system may be more suited to detecting anhedonia, while further stimulation of hedonic aspects of food intake may be missed due to ceiling effects.

With regards to the novelty-induced hypophagia test, it is crucial to allow rats to develop a stable baseline for intake of the palatable snack before performing the test. Only when a stable

baseline is reached within and between the rats should the actual test be performed. Otherwise, effects of the intervention (i.e., drug, stress, etc.) may be missed, or fluctuations of the baseline may be misinterpreted. It is also important to make sure that the novel cage induces a novelty stress resulting in hypophagia. Although several protocols suggest that the use of a new cage is sufficient by itself, we observed that cages not used before but containing bedding and enrichment may fail to induce stress, since rats are often used to weekly cage cleaning/changing. Therefore, an empty, new cage should be used. Since pre-test food intake may also affect the results, food intake should be monitored before the test. This can be easily done in an automated manner.

In the literature, various alternative tests are used to assess different aspects of depression-like behavior (often despair instead of anhedonia); however, the methods illustrated within this manuscript have several advantages. An alternative commonly used method to assess behavioral despair as part of depression-like behavior is the forced swim test[4]. Hereby, no food consumption is evaluated; thus, there is no risk of measuring inaccuracy.

This protocol bears several other disadvantages. A recent review concluded that the forced swim test is a test that actually measures stress coping strategies and not depression-like behavior[17]. In addition, if a crossover design is preferred to reduce the number of animals according to the "three R's" of animal welfare, the forced swim test cannot be applied, since it may exert a long-lasting (traumatizing) effect on the behavior of tested animals[18].

In contrast, the SPT and NIH both have no traumatizing aspects and can be repeated. Also, of note, the training phase before the SPT and NIH establish a habituation to the consumption; therefore, repeating the protocol is possible. After the test, the palatable food (sucrose solution or snack) is removed, and training is reintroduced approximately 24 h after testing; thus, the rodents have a break without access to the palatable stimulus. It is assumed that after the break, a new training period with adaptation should occur to ensure a preference ratio of around 1 or that a stable baseline snack intake is achieved before repeating the tests.

A test similar to the forced swim test is the tail suspension test, a short-term and inescapable stress period in which animals are suspended by their tail and develop an immobile posture interpreted as a sign of depression-like behavior[19]. This test can only be used in mice, since rats should not be suspended by their tails due to a higher average weight [20], while the SPT and NIH can be used in both mice and rats.

Further advantages of the tests presented in this manuscript are that the novelty-induced hypophagia test displays good construct validity; therefore, it measures up to its claims well[21,22]. Consequently, the amount of the palatable substance consumed correlates with the intensity of anhedonia, corroborated by conformity of SPT and NIH results with other behavioral tests[12]. Additionally, both the sucrose preference test and novelty-induced hypophagia test have good face validity. They are subjectively viewed as measuring what is intended (here, anhedonia assessed as reduced intake of palatable substances)[21,22].

Major limitations of the automated intake monitoring system are the requirements for proper training and daily maintaining/cleaning of the system, which makes it labor-intensive than manual protocols. In previous experiments[10], it has been observed that although young rats adapt to the automated system, older rats sometimes do not. Those animals should obviously be excluded from the experiment.

Regarding limitations of the behavioral test, it should be mentioned that training is also time-consuming, especially the NIH. Additionally, the protocols are both short-term, and long-term application may lead to malnourishment. Thus far, the literature does not report the usage of these tests in states of starvation (e.g., a model for anorexia nervosa, an eating disorder commonly associated with symptoms of depression), so there is no recommendation their use for states of starvation.

With this protocol, it is only possible to detect whether there is anhedonia (reduced intake of fluid/snack). However, it is not able to specifically quantify the degree of anhedonia. In the future, the introduction of several bottles with different sucrose concentrations could be a possible addition to further quantify anhedonia. Overall, use of the automated intake monitoring can be useful in any experiment where an accurate detection of intake is necessary, such as by monitoring the oral intake of medication dissolved in the drinking water for pharmaceutical studies.

In summary, the sucrose preference test and novelty-induced hypophagia test are well-established protocols to assess anhedonia as a part of depression-like behavior in rodents. When combined with the automated food intake monitoring system, even subtle differences can be detected in a reliable and repeatable fashion.

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DISCLOSURES:
A.S. is consultant for a & r Berlin, Boehringer-Ingelheim, Takeda and Schwabe. No conflicts of interest exist.

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Fig. 1

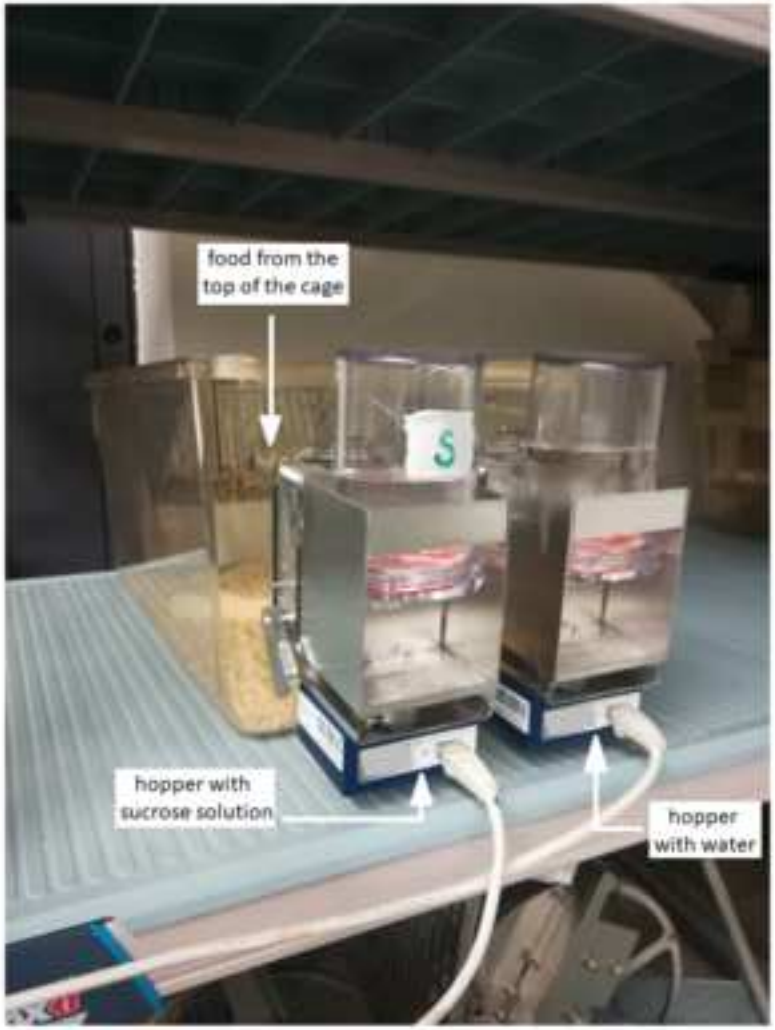
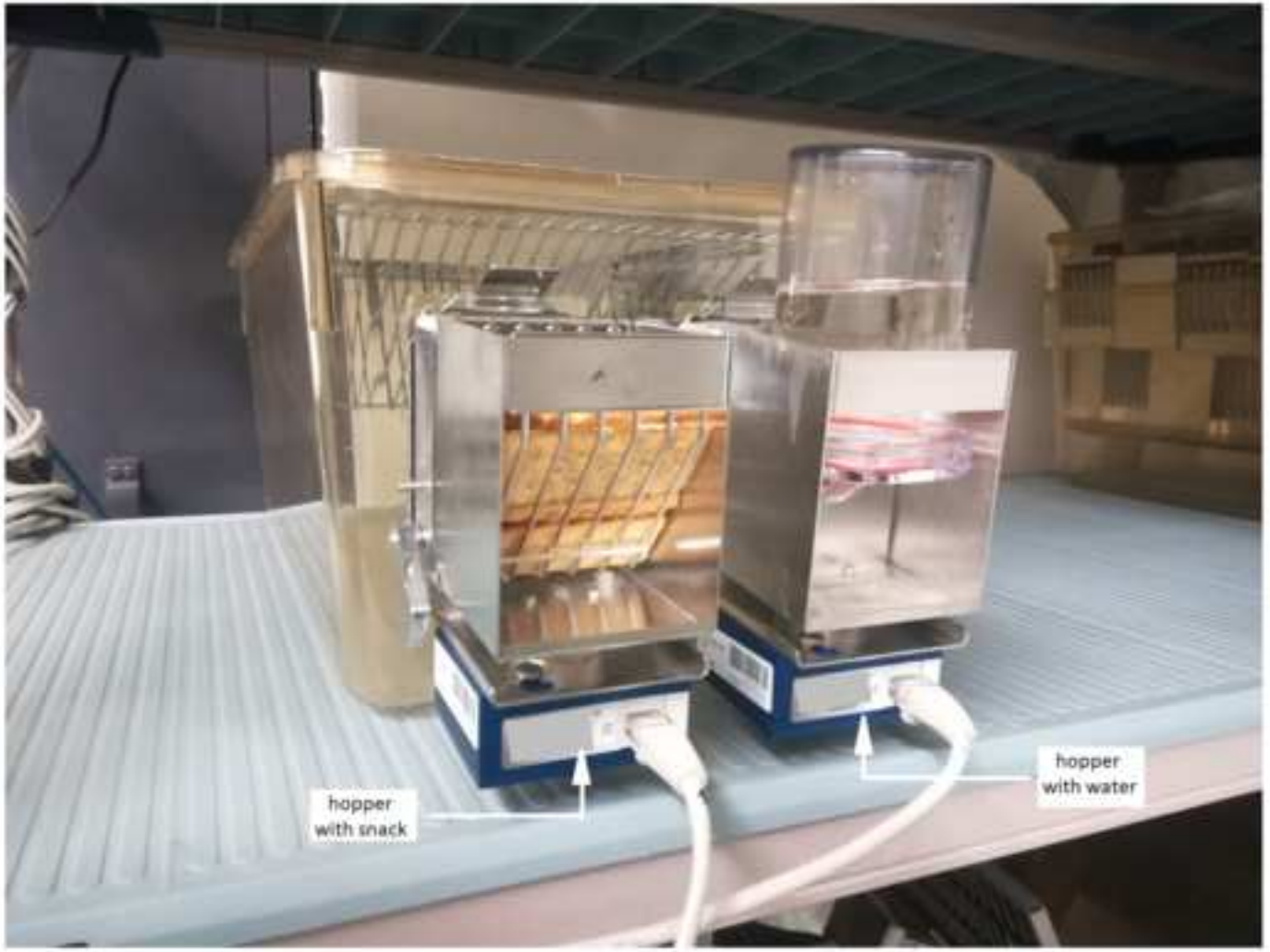


Fig. 2



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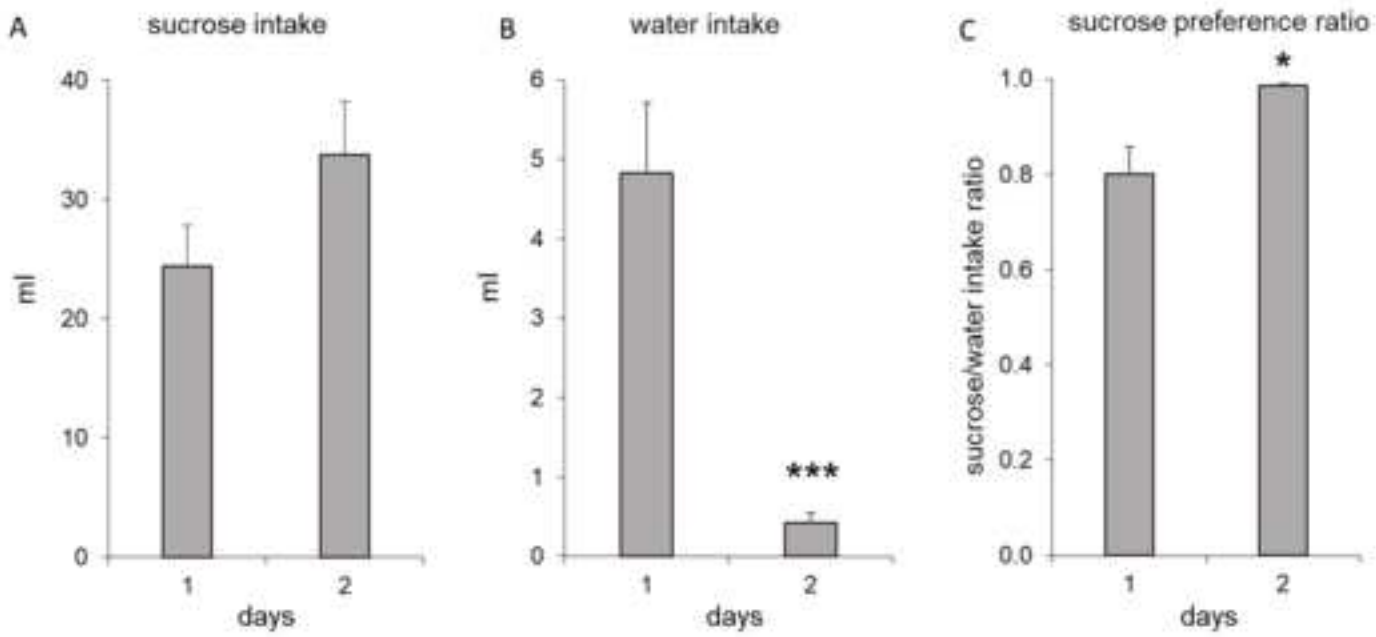

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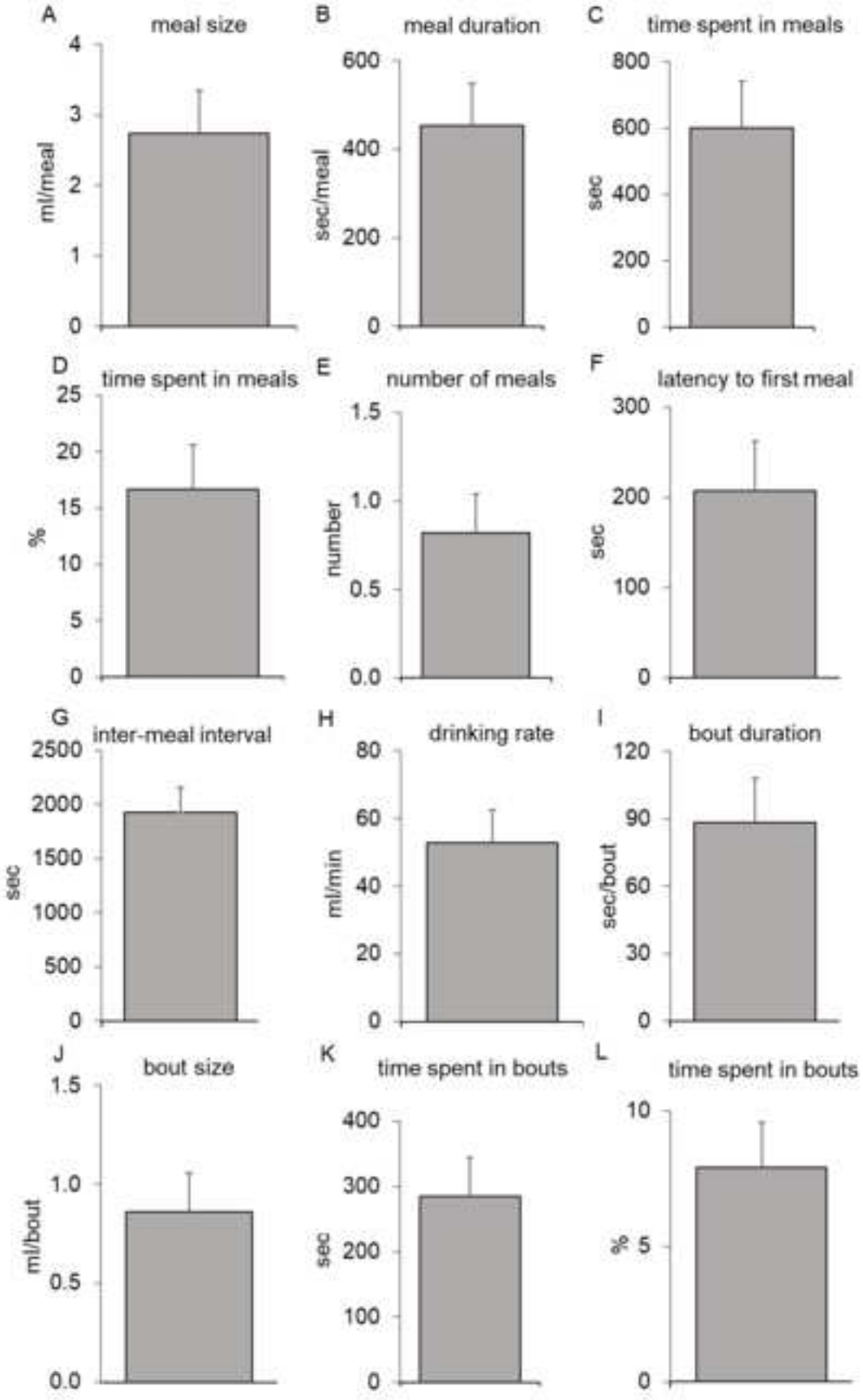

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Fig. 5

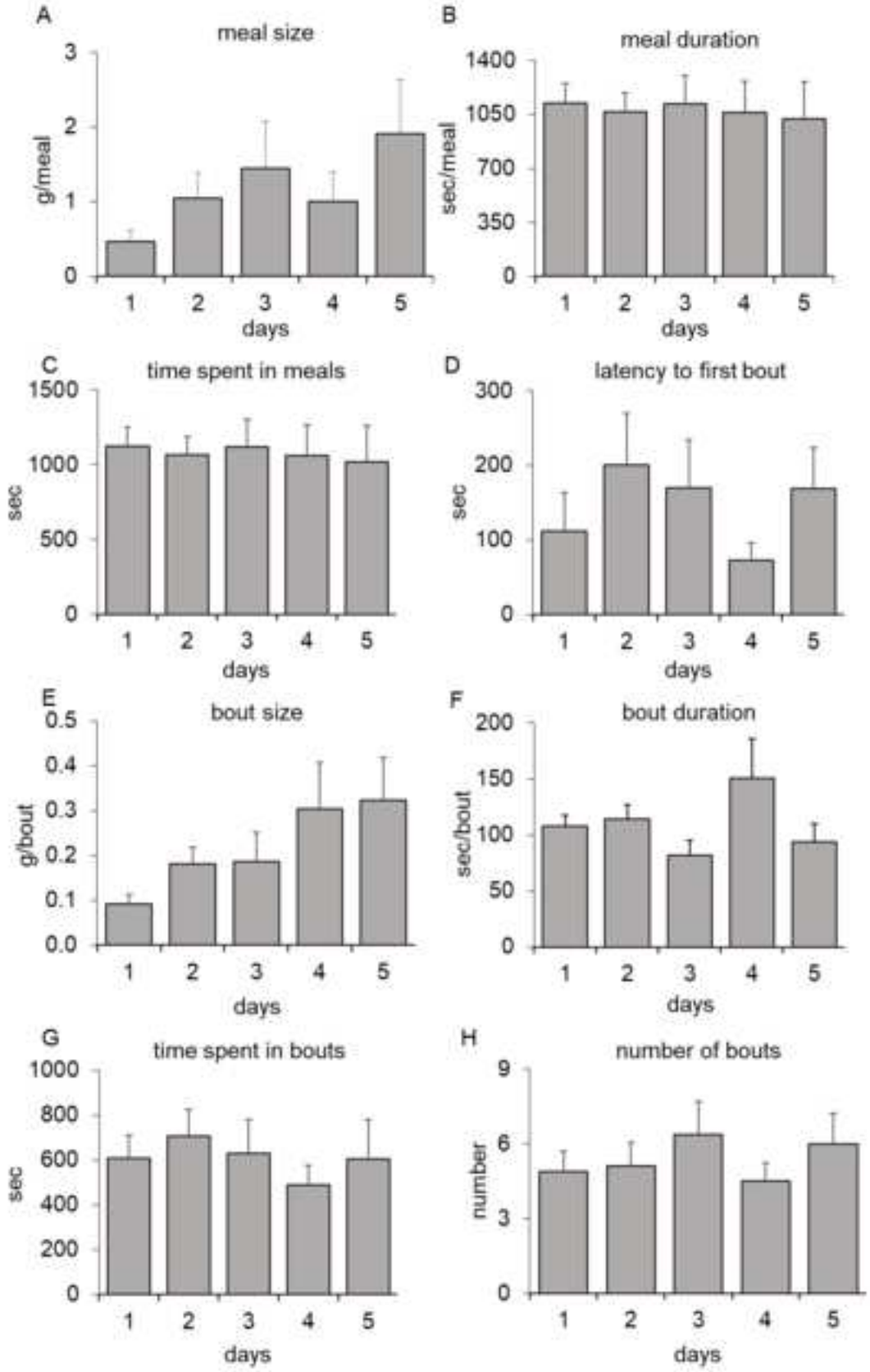

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Fig. 6

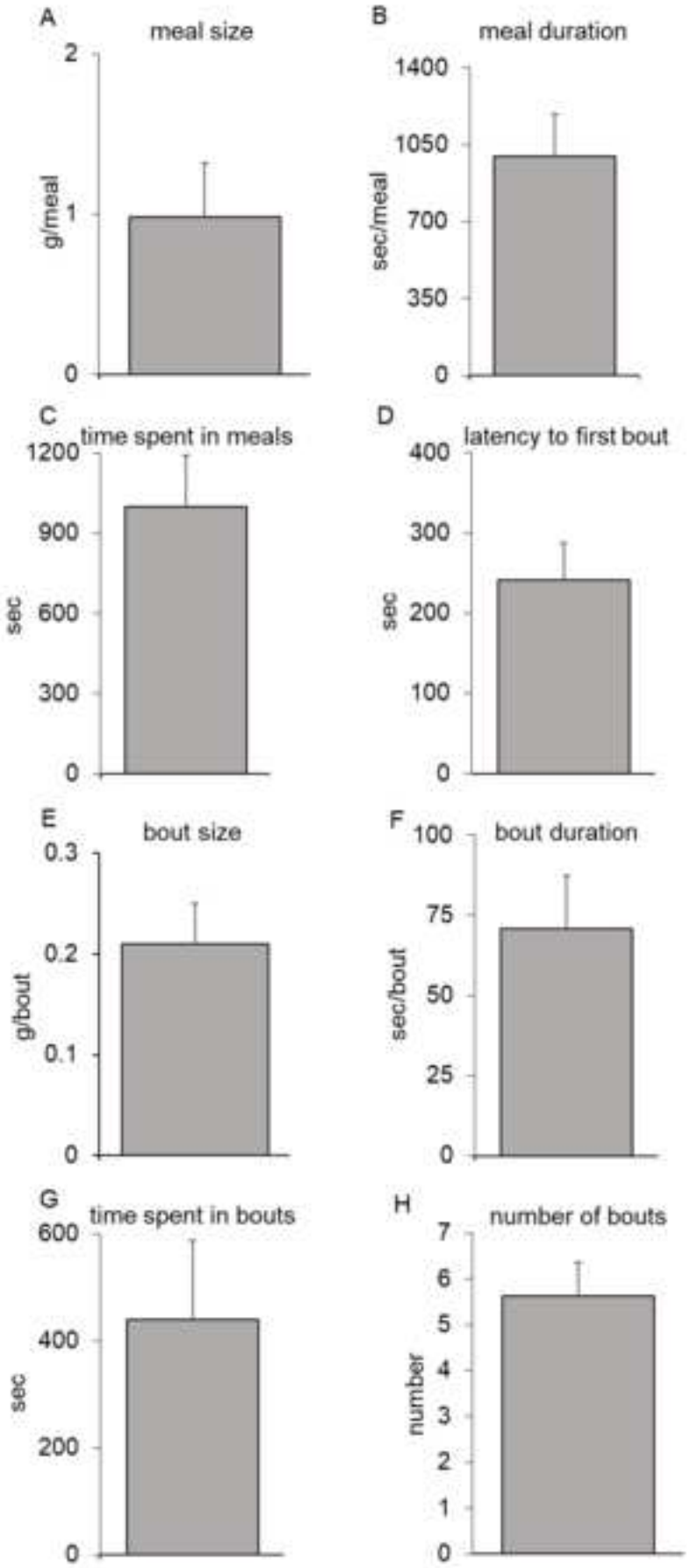

Table 1. Sucrose preference test in naïve rats using manual assessment vs. an au

Parameter	Manual assessment (n=6)	Automated monitoring (n=8)
Training period (day 1)		
Sucrose intake (mL)	63.46 ± 10.2	24.4 ± 3.48**
Water intake (mL)	11.08 ± 2.33	4.83 ± 0.89*
Sucrose preference ratio	0.84 ± 0.04	0.8 ± 0.06
Training period (day 2)		
Sucrose intake (mL)	44.99 ± 5.56	33.77 ± 4.49
Water intake (mL)	5.92 ± 1.03	0.42 ± 0.13***
Sucrose preference ratio	0.87 ± 0.03	0.99 ± 0.00**
Sucrose preference test		
Sucrose intake (mL)	10.65 ± 0.84	2.12 ± 0.68****
Water intake (mL)	6.65 ± 0.68	0.00 ± 0.00***
Sucrose preference ratio	0.61 ± 0.04	0.99 ± 0.00***

ıtomated intake monitoring system

Table 2. Novelty-induced hypophagia test in naïve unstressed and in stressed (C

Parameter	unstressed (n=8)	stressed (n=11)
Meal size (g/300g bw)	0.98 ± 0.29	0.35 ± 0.07**
Meal duration (sec)	998.29 ± 163.87	1209.11 ± 114.67
Time spent in meals (sec)	998.29 ± 163.87	989.27 ± 174.73
Time spent in meals (%)	55.46± 9.10	54.96 ± 9.71
Latency to first bout (sec)	241.25 ± 45.96	185.50 ± 57.52
Bout size (g)	0.21 ± 0.03	0.08 ± 0.01**
Bout duration (sec)	70.70 ± 14.12	45.59 ± 4.20
Time spent in bouts (sec)	439.75 ± 125.94	208.73 ± 45.01
Time spent in bouts (%)	24.43 ± 7.00	11.6 ± 2.50
Bouts (number)	5.63 ± 0.67	4.64 ± 0.80

RF injected) rats.

Table of Materials

Name of Material/Equipment	Company
Assembly LH Cage Mount - RAT-FOOD - includes Stainless cage mount, hopper, blocker, coupling	Research Diets, Inc.,Jules Lane, New Brunswick, NJ, USA
Assembly LH Cage Mount unplugged - RAT - FOOD includes stainless steel cage mount, hopper, blocker, unplugged adapter, coupling	Research Diets, Inc.,Jules Lane, New Brunswick, NJ, USA
cage w/ 2 openings - RAT - costum modified cage - includes cage top and standard water bottle	Research Diets, Inc.,Jules Lane, New Brunswick, NJ, USA
Data collection Laptop Windows - Configured w/ BioDAQ Software	Research Diets, Inc.,Jules Lane, New Brunswick, NJ, USA
enrichment (plastic tubes, gnawing wood)	
HoneyMaid Graham Cracker Crumbs	Nabisco, East Hanover, NJ, USA
low vibration polymer rack	Research Diets, Inc.,Jules Lane, New Brunswick, NJ, USA
male Sprague Dawley rats	Envigo
Mode#2210 32x Port BioDAQ Central Controller - includes cables, and calibration kit	Research Diets, Inc.,Jules Lane, New Brunswick, NJ, USA
Peripheral sensor Controller - includes cable	Research Diets, Inc.,Jules Lane, New Brunswick, NJ, USA
SigmaStat 3.1	Systat Software, San Jose, CA, USA
Stainless steel blocker	Research Diets, Inc.,Jules Lane, New Brunswick, NJ, USA
standard rodent diet with 10 kcal% fat	Research Diets, Inc.,Jules Lane, New Brunswick, NJ, USA
sucrose powder	Roth

Catalog Number	Comments/Description
BCMPRF01	
BCMUPRF01	
BCR02	single housing
BLT003	
	distributed by the animal facility
ASIN: B01COWTA98	palatable snack for NIH test
BRACKR	
Order Code: 002	
BCC32_03	
BPSC01	
	statistical analysis
BBLKR	
D12450B	
4621.1	for SPT

We thank the editor for their thoughtful comments. We provided a point-by-point reply and made changes in the manuscript accordingly (in track).

1. *Title reworded to bring out clarity. Please check.*
   The title was checked.

2. *This part is not clear in the protocol and the results. Please include how the system was used to compare the depressed and non depressed rats. Please inlcude results for depressed rats for sucrose preference test.*
   We added a description to the protocol and results section that explains how data can be compared using the automated monitoring system
   We have no unpublished results for depressed rats for SPT to include.

3. *Both food and water are provided from the top? Please mark these in the figure to show how this is done. Also please mark the gates as well to bring out clarity, in both Figure 1 and 2.*
   For better understanding this sentence was rephrased. We added descriptions to Figures 1 and 2.

4. *How the readings are taken after 24 h in this case?*
   We added a description to the protocol that explains how data of a 24h interval is obtained.

5. *How do you check this?*
   Do you let the preference level for stressed rats reach to 1 as well?
   We added a description to the protocol that explains how the sucrose preference level is obtained and calculated.

6. *How many rats were studied in this case? Are all data recorded automatically? How do you obtian data for each parameters presented in the result?*
   Include the numbers here and in all the other results. If possible please comment why different numbers were used in each case.
   Were these provided automatically? How did you obatin these results?
   How did you study these?
   How did you get these results?
   More detailed information about how the results were obtained, the n, the usage of the automatic system was added to the results section.

7. *Please include another column for stressed rats as well to show that indeed this system is able to assess anhedonia.*
   We have no unpublished results for depressed rats for SPT to include.

8. *Citation for this to show that this method induces stress.*
   An appropriate citation was added.

9. *Please do not embed the table in the text. Please upload the table as .xlsx file individually to your editorial manger account and leave the legend here.*
   The tables were uploaded as separate .xlsx files.

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