CITRIC ACID WATER AS AN ALTERNATIVE TO WATER SCHEDULING IN BEHAVING MICE

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ABSTRACT

Powerful neural measurement and perturbation tools have positioned mice as an ideal model species for probing the neural circuit mechanisms of cognition. Crucial to this success is the ability to motivate animals to perform specific behaviors. One successful strategy is to schedule their water intake, rewarding them with water during a behavioral task. However, water scheduling requires rigorous monitoring of animal's health and hydration status, and can be challenging for some animals.

Here, we present an alternative that allows mice more control over their water intake: free home-cage access to water to which a small amount of Citric Acid (CA) has been added. CA makes water taste slightly sour, but still palatable. Mice reduce their intake of CA water while still maintaining a healthy weight. Home-cage access to CA water has only subtle impacts on their willingness to perform a decision-making task, in which they are rewarded with sweetened water. CA water is thus a promising alternative that allows animals more control over their water intake without interference to behavioral performance.

INTRODUCTION

The mouse has been a powerful model system in biological research for decades. The rich set of available tools to record and manipulate brain structure and function, combined with the knowledge of teaching mice specific behavioral tasks, has made it an indispensable model species for current-day systems neuroscience. While long thought to be beyond the capacities of the mouse (Abbott, 2010), animals are now routinely trained to perform abstract sensory, navigational and decision-making tasks (Carandini and Churchland, 2013; The International Brain Laboratory et al., 2020). Crucial to this success is the ability to motivate animals to perform specific behaviors. One successful strategy is to schedule animals' intake of water, and reward them with water when they perform behavioral tasks (Guo et al., 2014). However, water scheduling requires rigorous monitoring of animal's health and hydration status, usually with daily weighing and precisely measured water intake.

We here consider a complementary, alternative approach: free access to water, in which a small amount of Citric Acid (CA) has been dissolved. CA is a food preservative which makes water taste slightly sour. Previous work (Reinagel, 2018) has shown that in rats, free access to CA water (2% or 5%) allowed animals to maintain a healthy weight, with only subtle impacts on their willingness to perform behavioral tasks in which they are rewarded with plain water. This strategy has significant advantages for both animal welfare and scientific throughput. However, the strategy presented by (Reinagel, 2018) has only been shown to work in rats. Moreover, continuous availability of CA water led to a reduction in trial yield of around 30%, preventing its use in high-throughput behavioral paradigms. We here set out to test the safety and efficacy of this approach in mice, and explore further strategies that combine the benefits of freely accessible CA water with high trial yields.

Mice readily consumed CA water without adverse health effects. Free access to 2% CA water resulted in stable weights, and water intake similar to commonly used water scheduling regimes. Mice were motivated to perform many trials (500-1000) evenly over the week, doing a decision-making task in which they earned sugar water. Specifically, giving mice free access to CA water on days when they are not

trained did not affect the number of trials they did, nor their task performance. Free access to 2% CA water thus allows animals more control over their water intake, without negative effects on behavioral performance.

RESULTS

MICE MAINTAIN HEALTHY WEIGHTS AND WATER INTAKE ON CA WATER

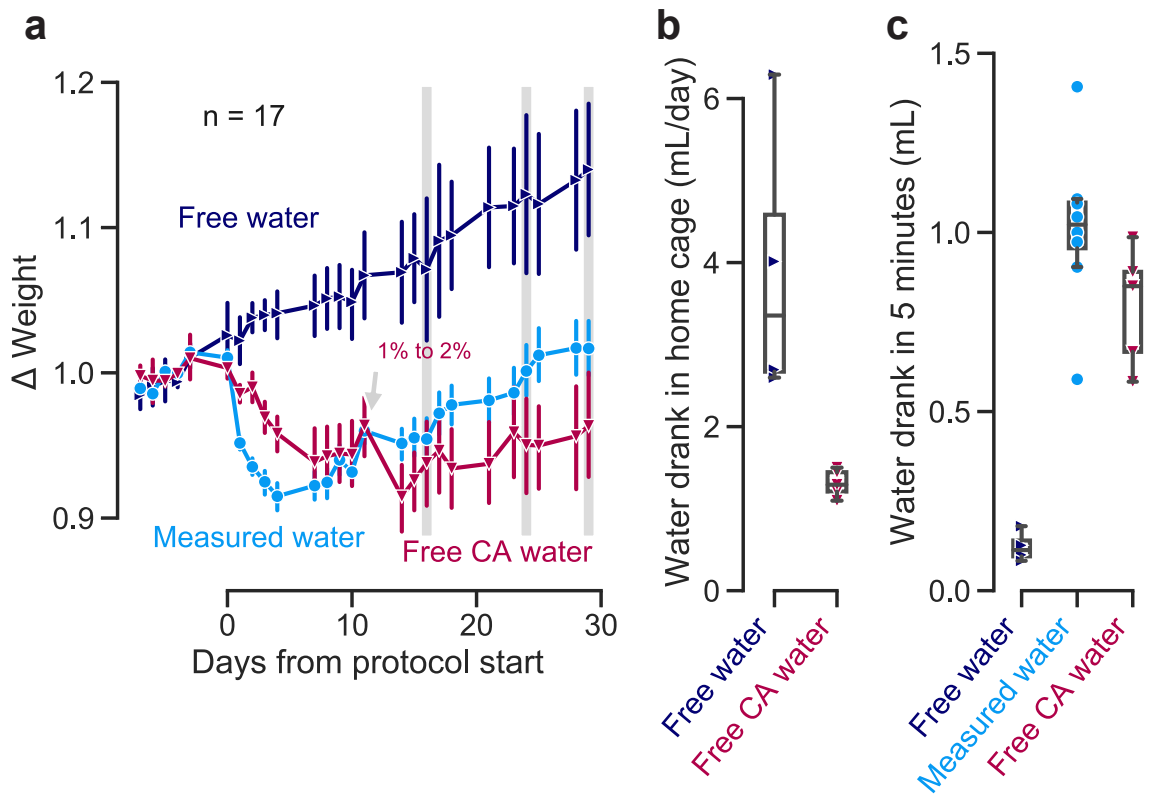

Figure 1. *(**a**) Average weight change (in grams, as a fraction of each animal's baseline weight) for each cohort of animals. On day 11 (the Friday of the second week after the intervention), the CA group was switched from 1% to 2% CA in their home-cage water bottle. Error bars show mean +- 68 confidence intervals. (**b**) Volume of water drank in home cage, estimated by measuring water bottles daily (days 11-29) for the cohorts of mice on adlib regular (blue) or 2% CA (red) water. Each data point is the average for one animal; boxplots show median and quartiles. (**c**) Plain water consumed in 5 minutes of free access, measured on days 16, 24 and 29, as indicated with grey shaded bars in (**a**). Each data point is the average for one animal; boxplots show median and quartiles.*

We first confirmed that mice remained healthy, and maintained stable weights when given adlib CA water. Three cohorts of animals (total n = 17, 2-3 months) were given either measured amounts of daily water, free plain water, or free CA water in their home cage. All animals were healthy (as judged by frequent experimenter handling and inspection) and showed no signs of dehydration. Over time, mice with access to free plain water steadily gained weight (Figure 1a, dark blue), as expected for young animals (The Jackson Laboratory, 2020). Mice given measured amounts of water rapidly lost weight over the first week, after which their weight stabilized (Figure 1a, light blue line). This pattern of initial weight loss and subsequent healthy recovery matches previous reports (Guo et al., 2014; Miles Wells, personal

communication), taking into account animal's age-appropriate growth curves. In the last cohort of mice, we started by testing the overall impact of CA water at a low concentration (1% m/v) on animals' health. Mice did not show adverse health effects nor signs of dehydration, and lost only modest amounts of weight (Figure 1a, red line). Mice also tolerated CA at a higher concentration of 2% m/v, in line with findings in rats (Reinagel, 2018). With free access to CA water, animals' weights stabilized around 95% of their pre-intervention body weight (Figure 1a: day 11-30).

As expected from CA-induced weight loss, we confirmed that animals with access to CA water reduced their fluid intake. Mice consumed 3.9 mL of plain water per day (range 2.6-6.3 mL; Figure 1b, blue), but only about half as much 2% CA water (1.3 mL, range 1.3-1.5 mL; Figure 1b, red). Importantly, mice with access to free 2% CA water were still motivated to drink plain water. In five minutes of free access, they drank 0.58-0.99 mL plain tap water, similar to animals receiving daily measured water (Figure 1c). Not only did animals tolerate 2% CA water well, these data also suggest that free access to CA water does not impede animals' motivation to perform behavioral tasks to earn more palatable liquids.

MICE PERFORM MANY TRIALS, EVEN WITH FREE ACCESS TO CA WATER

In a new cohort of animals (n = 6, 8-18 months) that had been trained on a visual decision-making task (The International Brain Laboratory et al., 2020), we tested how different liquid regimes affected animals' willingness to work for sweetened water rewards in the context of a behavioral task (Figure 2a). Animals performed the task for 45-90 minutes each weekday (with the length of each session dictated by the automated detection of the animal's engagement, see (The International Brain Laboratory et al., 2020) Supp Table 3), and earned a drop of 10% sucrose water for each correct response. Expert animals normally earn their daily water requirements in the task, and do not require supplemental fluids.

When mice have access to measured plain water on weekends (light blue circle), they perform sessions of 730-1230 trials stably over the week (Figure 2b, center). This practice is commonly used in laboratories that perform high-throughput mouse training, but is also the most labor intensive. On the contrary, free home-cage water during the weekend (blue, rightward-pointing triangle) results in lower trial counts and worse performance on Mondays and Tuesdays (Figure 2b, center and bottom; 97-420 trials on Mondays vs. 668-1140 trials on Fridays) (Busse et al., 2011). Under this regime, weights often fluctuate dramatically over the course of the week (Figure 2b, top: weight loss of around 10% of body weight (approximately 3g) between Mondays and Fridays), which can have adverse effects on animal health.

Free access to 2% CA water over the weekend (pink, downward pointing triangle) combines the best of both approaches. Without requiring experimenter intervention on days without training, animals maintained stable weights (Figure 2b, top), and consistently performed many trials (Figure 2a, center). We could not detect a significant difference in the number of trials performed after a weekend with free 2% CA water (682 trials/day, range 535-826) vs. 1mL/day (733 trials/day, range 634-817) ($t(5) = 0.880$, $p = 0.4193$, $Bf_{10} = 0.505$, Figure 2c). Similarly high trial yields were achieved by providing CA dissolved in HydroGel cups, a practical alternative in cases where bottled water is difficult to provide (Supplement 3).

When free 2% CA was available in the animals' home cage throughout the week (red, upward pointing triangle), the number of trials slightly decreased from Monday to Friday (435-1019 on Mondays vs 312-513 on Fridays). As a result, the average number of trials performed was slightly lower than when the 2% CA water bottle was removed on Monday morning (average 573 vs. 682 trials, $t(5) = -2.505$, $p = 0.0542$, $Bf_{10} = 1.971$, Figure 2c). While both approaches thus allow for stable health and fairly high motivation, a regime with continuous 2% CA water availability may not consistently yield the quantity of behavioral data required for some experimental purposes.

a

Liquid regime	*Weekend*	*Week*	
		Task	*Cage*
◀ Free water	Water bottle	10% sucrose water	Water bottle
▶ Free wknd water	Water bottle		X
● 1 mL/day	1 mL		X
▼ Free wknd CA water	2% CA water bottle		X
▲ Free CA water	2% CA water bottle		2% CA water bottle

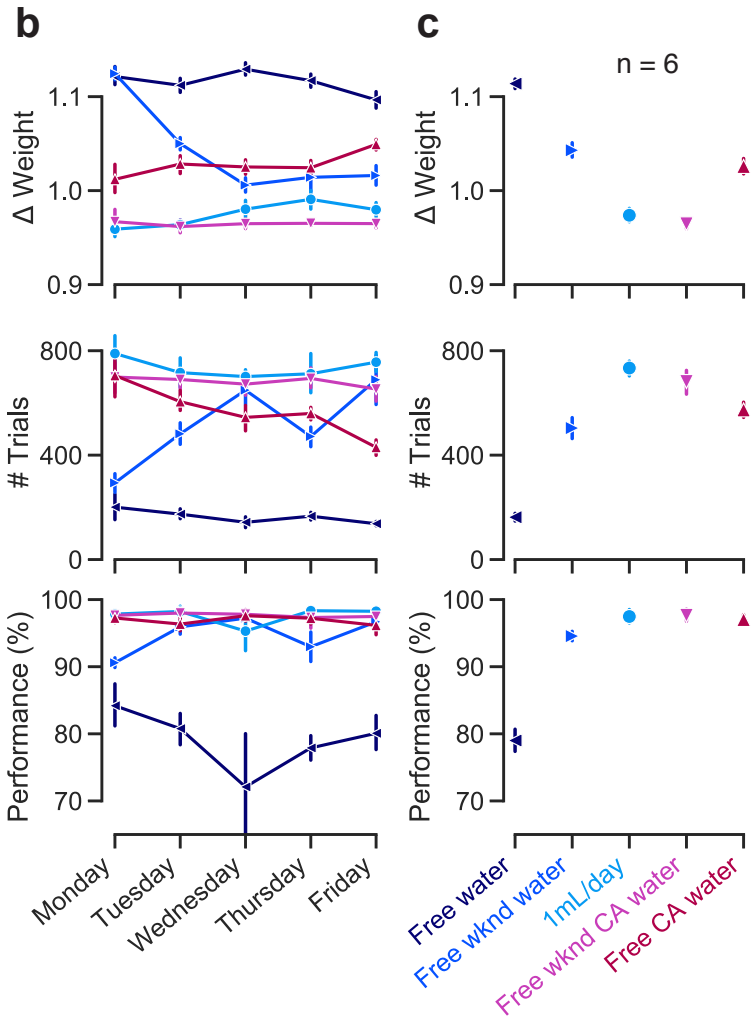

Figure 2. *(**a**) Schematic of all liquid regimes. (**b**) Average weight, trial counts and task performance over the course of the week for each liquid regime. (**c**) As (**b**), summarized over weekdays. Error bars show mean +- 68% CI across animals. See also Supplement 2.*

DISCUSSION

High-throughput, reliable behavioral training is a key requirement for the use of mice in behavioral and systems neuroscience. Here, we have shown that free access to CA water is well tolerated, and motivates mice to perform many trials of a decision-making task in which they earn sugar water. We thus consider CA water a promising alternative to water scheduling for some experimental regimes.

In contrast to commonly used water scheduling regimes, free access to CA water allows animals full control over the amount and timing of their water intake. Simultaneous home-cage access to food and CA water enables animals to eat while consuming water, which may benefit their nutrition status and metabolism overall. While we have achieved a satisfactory balance between water intake and trial counts using a concentration of 2% CA (m/v), this concentration could be further fine-tuned based on the details of each experimental setup. While we here achieved high-quality behavioral data by placing animals on free CA water over the weekend, this strategy may not be viable for all experimental setups. For example, studies investigating differences in reward and taste processing, or those requiring precise tracking of individual animal's fluid intake, may not be amenable to this approach.

Beyond benefits to animal welfare, eliminating water scheduling also benefits the quality and throughput of behavioral experiments. It removes the need to give animals supplemental fluids on days when they do not train (e.g. weekends) or days when they do not earn their water requirements in the task (as often happens early in training). Such supplements are often beyond the capacities of routine animal care procedures, and require scientific personnel's daily attention and record-keeping. Besides being highly labor-intensive, water supplements carry a risk of human error in timing and the precise delivery of required water amounts, as well as the distribution of water between co-housed mice. Unexpected absence of experimental personnel usually requires animals to be placed on free plain water, which can impede their progress in behavioral training for many days or weeks afterwards.

Free access to CA water provides a relatively non-labor-intensive, low-error option for keeping animals healthy without hindering behavioral training progress. Indeed, several laboratories currently use this method as an alternative to measured water administration on weekends, without effects on behavioral performance (The International Brain Laboratory et al., 2020). The use of CA water can make it easier to leverage the mouse as a model species for high-throughput behavioral studies.

METHODS

Experiments were approved by ORBEA Animal Welfare Body at the Champalimaud Center for the Unknown (CCU; number 2016/005; Figure 1) and the Cold Spring Harbor Laboratory (CSHL) Institutional Animal Care and Use Committee (protocol 16-13-10-7, amendment approved 2018-07-28; Figure 2 and Supplement 3).

EXPERIMENTAL DESIGN

STUDY 1

Seventeen mice (male wild-type Thy1-GCaMP6, C57BL/6 background, 2-3 months) were single-housed at CCU. Each animal's baseline weight was recorded on five consecutive days, while they had free access to plain tap water in their home cage. They were then divided into three groups, each with its own water regime. The first group (n = 8) received measured amounts of water each weekday. This was either 600 µL (the institutional minimum at CCU), or 40 µL of water per gram of body weight as measured right before water administration (range 600 - 1150 µL). On Saturday they received double this amount, while no water was administered on Sundays. The second group (n = 5) had access to free water in their home cage, in

which CA (1% or 2%) was dissolved. The last group (n = 4) remained on free water in their home-cage, and served as a control.

The home-cage bottles with regular or 2% CA water were weighed daily, and the weight change used as a proxy for the volume of water consumed. To measure thirst, each animal was placed in a cage with 2 mL of plain water for 5 minutes on days 16, 24 and 29 after the experimental intervention. For the animals on measured water, these 5 minutes replaced their daily measured water administration.

STUDY 2

Six mice (male C57BL/6, Charles River, 8-18 months) were single-housed at CSHL. Animals were trained on a visual decision-making task where they discriminated the location of an image and reported their percept (The International Brain Laboratory et al., 2020). For each correct response, they received 1.5 µL of 10% sucrose water. Early in training, they were supplemented towards the institutional minimum of 1 mL/day after the experimental session. After animals became proficient at the behavioral task, they usually performed enough trials to fully earn their water requirement in the rig on weekdays. The duration of each session was dependent on the engagement of the animal, which was determined through automated criteria ((The International Brain Laboratory et al., 2020), their Figure 1e and Supp Table 3).

We then varied the liquid regimes, with different types of water administration both during the weekend and weekdays (Supp Fig 2). Home-cage bottles were put into the cage on Friday evening and removed on Monday morning, except for the conditions where adlib water (regular or 2% CA) was also available on weekdays. When no home-cage bottle was available and mice did not earn their minimum required amount of 1 mL/day, they were supplemented with measured water or HydroGel at the end of the day.

CA WATER PREPARATION

Citric Acid (Sigma Aldric) was dissolved into tap water at 1% or 2% m/v. That is, at 2% m/v, 2g of CA powder was mixed into 100 mL water and shaken until dissolved. Bottles were replaced with fresh CA water weekly, or when empty.

DATA ANALYSIS

Data about each animal (sex, date of birth, lineage), their water intake and weights were logged in the Alyx colony management system (The International Brain Laboratory et al., 2019) and analyzed using DataJoint (Yatsenko et al., 2018). We visualized all data in python, using pandas and seaborn (Waskom et al., 2020).

CODE AND DATA AVAILABILITY

All code and instructions for reproducing figures are available at https://github.com/int-brain-lab/citricAcid.

ACKNOWLEDGEMENTS

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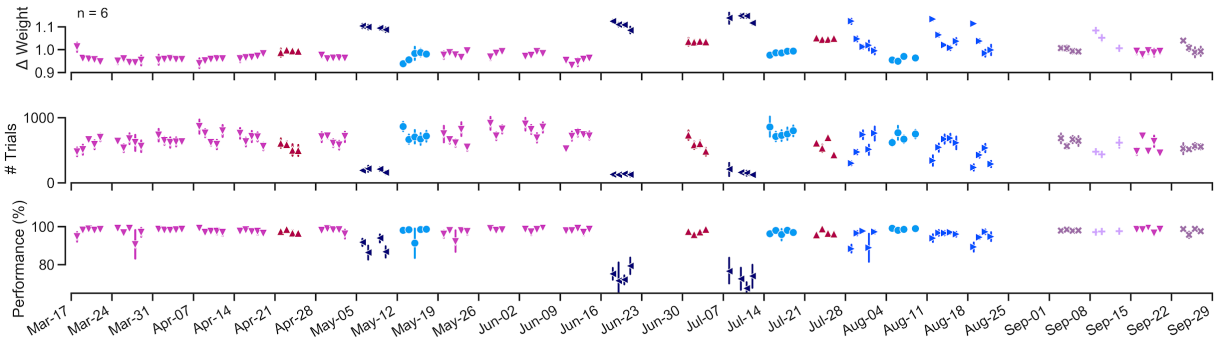
SUPPLEMENTARY MATERIALS

SUPPLEMENT 1. CONTRIBUTION TABLE

Levels of contribution: **lead**, **equal** and **support**.

	AEU	VAR	ICL	FC	IBL	ZM	AKC
Conceptualization							
Investigation							
Collected data for figure 1							
Collected data for figure 2							
Collected data for supp figure 3							
Data curation							
Formal Analysis							
Visualization							
Resources							
Writing – original draft							
Writing – review & editing							

SUPPLEMENT 2. WEIGHT, TRIAL COUNTS AND BEHAVIORAL PERFORMANCE OVER TIME

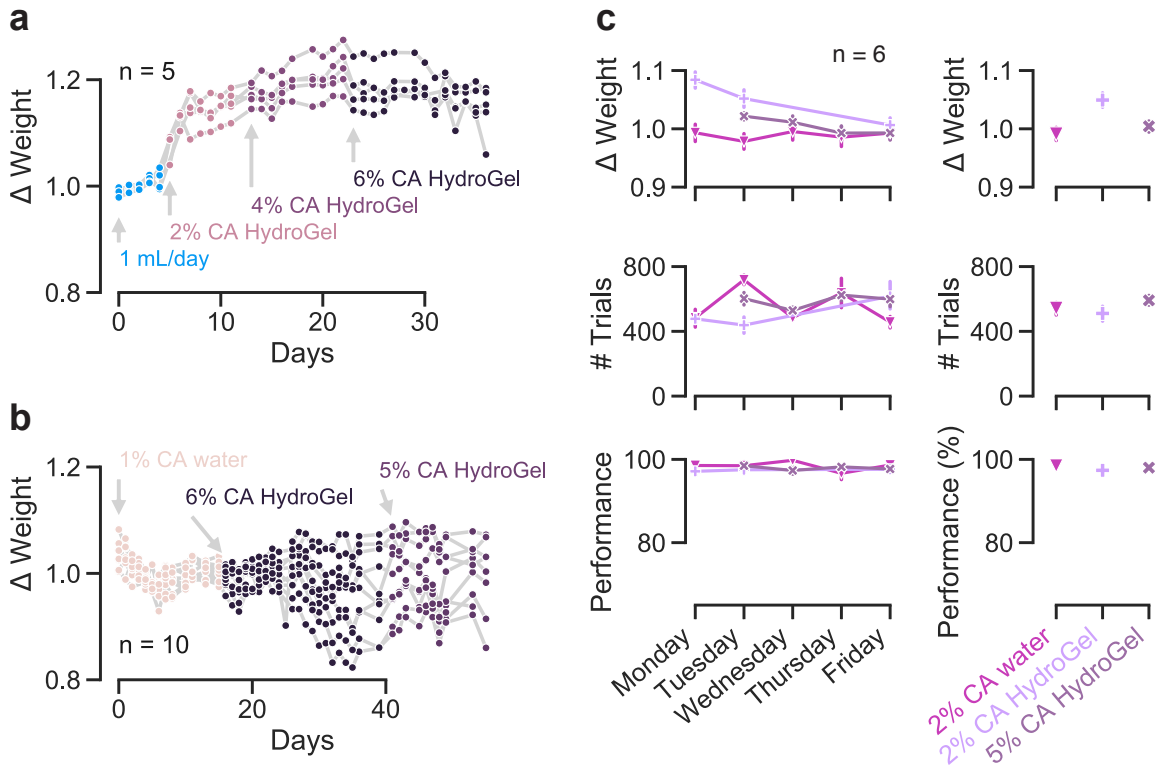


Supplementary Figure 2. *Data as in Figure 2b-c, shown over the full period of data collection. A leak in our rig tubing resulted in inaccurate reward volumes during two weeks of training (in late June and early September); these data were excluded from all analyses.*

SUPPLEMENT 3. CITRIC ACID CAN BE DISSOLVED IN HYDROGEL™ INSTEAD OF WATER

If bottled CA water cannot easily be provided (during travel, due to cage size restrictions, or when head implants preclude the use of bottle-top cages), CA can be mixed into HydroGel™ cups (https://www.clearh2o.com/product/hydrogel/) as an alternative to liquid water. HydroGel™ was melted by placing unopened 56g cups in a 60°C oven until the gel had liquified (3-5 hours). CA powder was then mixed into the liquefied gel and stirred thoroughly, before resealing the Hydrogel cups and letting them solidify at 4°C. As the flavor and perceived aversiveness of CA may differ when dissolved in water or HydroGel, we again titrated CA concentrations to achieve stable animal weights.

In a first cohort of animals (n = 5, 15 months), this required increasing concentrations from 1% to 6% m/v (Supp Figure 3a). With a second cohort of animals (n = 10, 15-16 months), switching from plain Hydrogel to 6% CA Hydrogel resulted in weights close to the institutional minimum of 80% (Supp Figure 3b). At 5% CA Hydrogel, all animals showed stable weights. In our cohort of trained animals (Figure 2), we confirmed that weekend regimes of 2% CA in water versus 5% CA in Hydrogel yielded similar trial counts and task performance (Supp Figure 3c, Suppl Figure 2). The observation that higher concentrations of CA are required in Hydrogel to achieve the same behavioral effects agreed with informal human sourness perception of both substances.



***Supplementary Figure 3**. (**a**) Weight change (from baseline on measured water), as animals were given free Hydrogel with different concentrations of CA. (**b**) As in (**a**), but with a larger cohort. (**c**) As in Figure 2, but comparing 2% CA water with 2% CA HydroGel and 5% CA HydroGel on weekends (same animals as shown in Figure 2; see Supplementary Figure 2).*