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A method for evaluating hunger and thirst in monkeys by measuring blood ghrelin and osmolality levels

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Short title: Measurements of hunger and thirst states in monkeys

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Author Contributions

H.Y. designed the study; Y.S. conducted measurement experiments; J.K., A.K., and H.Y. conducted animal experiments; Y.S. and H.Y. analyzed the data; and H.Y., J.K., and Y.S. wrote the manuscript. All authors have edited and approved the final manuscript.

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32 **Data availability:** All data used in this study are presented in the manuscript.
33 **Declaration of generative AI**: During the preparation of this study, we did not use any generative
34 artificial intelligence (AI) or AI-assisted technologies.
35 **Keywords:** Monkey, Hunger, Thirst, Satiety, Reward
36

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37 **Significance statement (120)**

38 Standard methods for behavioral and neurophysiological experiments in non-human primates rely
39 on controlled access to food or fluid rewards to motivate their performance. We previously
40 assessed the thirst state of monkeys by measuring blood osmolality, the most widely used
41 hematological index of hydration status. Here, we assessed the hunger state of monkeys by
42 measuring blood ghrelin levels, a widely measured hunger-related peptide hormone in humans,
43 using an enzyme-linked immunosorbent assay. We measured these indices and found that they
44 reflected the hunger and thirst states of the monkeys before and after consuming dry meals, with
45 no relation to each other. Thus, these two physical indices can be utilized to monitor hunger and
46 thirst in primates.
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48 **Abstract**

49 Hunger and thirst drive animals' consumption behavior and regulate their decision-making
50 concerning rewards. We previously assessed the thirst states of monkeys by measuring blood
51 osmolality under controlled water access and examined how these thirst states influenced their
52 risk-taking behaviour in decisions involving fluid rewards. However, hunger assessment in
53 monkeys remains poorly performed. Moreover, the lack of precise measures for hunger states
54 leads to another issue regarding how hunger and thirst states interact with each other in each
55 individual. Thus, when controlling food access to motivate performance, it remains unclear how
56 these two physiological needs are satisfied in captive monkeys. Here, we measured blood ghrelin
57 and osmolality levels to respectively assess hunger and thirst in four captive macaques. Using an
58 enzyme-linked immunosorbent assay, we identified that the levels of blood ghrelin, a widely
59 measured hunger-related peptide hormone in humans, were high after 20 h of no food access
60 (with *ad libitum* water). This reflects a typical controlled food access condition. One hour after
61 consuming a regular dry meal, the blood ghrelin levels in three out of four monkeys decreased to
62 within their baseline range. Additionally, blood osmolality measured from the same blood sample,
63 the standard hematological index of hydration status, increased after consuming the regular dry
64 meal with no water access. Thus, ghrelin and osmolality may reflect the physiological states of
65 individual monkeys regarding hunger and thirst, suggesting that these indices can be used as
66 tools for monitoring hunger and thirst levels that mediate an animal's decision to consume rewards.

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68 **Introduction**

69 Hunger and thirst are fundamental constituents of the physiological needs that drive an animal's
70 consumption behavior to maintain its physical state (Mattes, 2010). Controlled access to food or
71 drink is commonly utilized in standard experimental procedures to motivate performance in non-
72 human primates. Hunger drives the consumption of food that maintains the metabolic state, while
73 thirst drives the intake of fluid that maintains the hydration state (Berne and Levy, 1993). Although
74 these physiological needs are not independent of each other (e.g., food items contain both energy
75 and fluid) (Ramachandran and Pearce, 1987; Watts, 1999; Betley et al., 2015). For instance,
76 thirsty animals are unlikely to eat dry food, even if they are hungry (Eiselt et al., 2021), which
77 implies that the reward values of food and beverages may be linked to the reward circuitry (Haber
78 and Knutson, 2010). Hunger and thirst levels are subjectively evaluated and controlled by
79 experimenters, yet these states vary individually due to differences in homeostatic conditions. As
80 such, establishing a method to reliably evaluate the hunger and thirst states of animals is useful,
81 as it drives their consumption behavior to appropriately maintain their physical state.

82 In the experimental testing of behavior in monkeys and rodents, typical procedures generally
83 involve the control of either of these physiological needs to motivate the animal's performance,
84 especially in experiments involving electrophysiology in monkeys (Evarts, 1968; Wurtz, 1968;
85 Watanabe, 1996). To assess thirst, the blood osmolality level can be used as the standard
86 hematological index to detect hydration status in mammals (Rolls and Rolls, 1982; Stedman,
87 2006). We previously used the osmolality level to relate the behavioral characteristics of economic
88 decisions such as risk preferences (Yamada et al., 2013), instrumental performance of actions to
89 earn fluid rewards (Minamimoto et al., 2012), and simple eye movements for fluid reward intake
90 (Yamada et al., 2010) in monkeys, which are close relatives of humans. To assess hunger,
91 various physical measures can be employed (Mattes, 2010), such as blood sugar levels and

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insulin as standard measures and leptin and ghrelin as recently developed measures. These physical indices of hunger and thirst are useful for assessing and controlling the motivational state of primates, in addition to other possible measures, such as accumulated earned rewards (Minamimoto et al., 2012; Pastor-Bernier et al., 2021). However, the lack of a simultaneous assessment of hydration and metabolic status leads to difficulty in evaluating neural correlates for hunger and thirst in behaving monkeys, as control of either hunger or thirst can affect the status of the other (Ramachandran and Pearce, 1987; Watts, 1999; Betley et al., 2015).

This aforementioned limitation introduces several potential problems in neuroeconomic studies (Camerer et al., 2005; Glimcher et al., 2008), that employ experimental testing of reward valuation systems for economic choices. When measuring neural activity in the reward circuitry, the subjective values of any reward depend on the physical state of the subject (Nakano et al., 1984; Critchley and Rolls, 1996; de Araujo et al., 2006; Pritchard et al., 2008), even for money (Symmonds et al., 2010). Hunger and thirst may affect neural activity in specific brain regions (Yaxley et al., 1985; Rolls et al., 1988), or dramatically change the activity in many parts of the brain (de Araujo et al., 2006). Therefore, the control and monitoring of these states are insufficient for precisely assessing the neural valuation system embedded in the reward circuitry. The subjective values of the items used in the monkey experiments depend on these physiological statuses. For instance, the intake of juice rewards must elicit changes in both statuses since it contains both sugar and liquid. Thus, assessing hunger and thirst with controlled access to either food or beverages is worthwhile.

In the present study, we measured blood ghrelin levels to assess hunger (Kojima et al., 1999) and osmolality to assess thirst (Rolls and Rolls, 1982) in captive monkeys. We evaluated the physical status of the monkeys using a typical experimental procedure to control food and fluid

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115 access with assessments of food and water intake behavior. Our results indicate that these

116 indices can be used to monitor hunger and thirst in individual monkeys.

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117 **Materials and Methods**

118 **Experimental subjects**

119 Five macaque monkeys were used in this study (*Macaque fuscata*; monkey MON, male, 7.8 kg,
120 4 years; monkey Y1, male, 6.4 kg, 2 years; monkey MIY, female, 5.8 kg, 4 years; monkey Y23,
121 male, 6.0 kg, 4 years; *Macaque mulatta*; monkey SUN, male, 7.5 kg, 13 years). Blood samples
122 were collected from all four monkeys, excluding monkey Y23. The Animal Care and Use
123 Committee of the University of Tsukuba approved all the experimental procedures (protocol no.
124 H30.336), which were also performed in compliance with the United States Public Health
125 Service's Guide for the Care and Use of Laboratory Animals.

126

127 **Blood Collection**

128 The monkeys were anesthetized using medetomidine (0.03 mg/kg, intramuscularly [i.m.]) and
129 midazolam (0.3 mg/kg, i.m.), except monkey SUN who was seated in a standard primate chair
130 and desensitized to leg restraint through positive reinforcement with food rewards before starting
131 the blood collection procedure. Blood samples (2.0 mL) were drawn from the saphenous vein
132 using a butterfly needle (22 gauge) in a single collection. Atipamezole (0.024 mg/kg, i.m.) was
133 administered, and 30 min after recovery, the monkeys were fed a dry meal at approximately 10:00
134 until 11:30 (1.5 h), after which any remaining food was extracted. After 1 h (total 3 h from the first
135 blood collection), a second blood sample was collected using the same procedure as the first
136 sample collection. Monkey SUN was sampled while awake, exhibiting no distress during the
137 sampling procedure after desensitization. Approximately 1.0 mL plasma was extracted in each
138 collection from the 2.0 mL blood sample in ethylene diamine tetra-acetic acid 2K-containing tubes

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139 by centrifugation at 2000 × *g* for 10 min at 4 °C. The total amount of blood extracted within any 2-
140 week experimental period did not exceed 5% of the total blood volume (total blood volume was
141 estimated at 65 mL/kg weight).

142 Sixteen blood collections were performed over 8 days for each monkey (two blood collections
143 per day, before and after food intake). A total of 64 blood samples were therefore collected from
144 four monkeys.

145

146 **Regular feeding**

147 In the approved controlled food access protocol, the monkeys received a fixed daily allocation of
148 dry meals (certified diet, PS-A, Oriental, Japan), containing water (8.0 ± 0.7 g), protein (21.5 ± 0.3
149 g), fat (6.8 ± 0.3 g), ash (7.7 ± 0.2), fabric (3.1 ± 0.3), soluble nitrogen-free (52.9 ± 0.7), calorie
150 (359.1 ± 3.5 kcal) per 100 g. The diet also contains sodium (0.66 g per 100 g), vitamins, and other
151 minerals. This diet was provided at least once a day at approximately noon, except during the
152 periods before and after surgery. From 10:00 to 11:00, food and water were restricted, although
153 before 10:00 some monkeys had finished eating food and drinking beverages on the day after the
154 last feeding. The fixed daily allocation of food was determined by veterinary stuffs as follows:
155 monkey MON, 140–160 g; monkey Y1, 140 g; monkey MIY, 130–160 g; and monkey SUN, 140
156 g; monkey Y23, 130 g. The average amount of dry meal consumed in a day was as follows:
157 monkey MON, 140 g; monkey Y1, 136 g; monkey MIY, 153 g; monkey SUN, 134 g; and monkey
158 Y23, 129 g.

159

160 **Controlled access to food and water**

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161 Under the experimental conditions employed (Fig. 1A, blood test), all leftover food was removed
162 at 14:00 on the day before blood collection, resulting in approximately 20 h of no food access.
163 Food was delivered after the first blood collection in each individual, and the amount was allocated
164 daily to each monkey at approximately 10:00 am. The average amount of food consumed by each
165 monkey during the test period was as follows: monkey MON, 159 g or 20 g/kg/day; monkey Y1,
166 140 g or 22 g/kg/day; monkey MIY, 127 g or 22 g/kg/day; and monkey SUN, 134 g or 18 g/kg/day.
167 If the monkeys did not start eating the dry meal quickly, they were fed small pieces of sweet
168 potatoes (< 10 g). The monkeys were free to access a 500-mL bottle of water in a day, and no
169 water access control was performed throughout the entire period of the test that the monkeys
170 engaged in, except for the period between the first and second blood collections. This procedure
171 mimicked a typical experimental procedure for controlling food access, which motivated subjects
172 to obtain food rewards, but not for liquid rewards in the experimental room.

173

174 **Test schedules for the control of hunger and thirst levels**

175 We prepared the blood test schedule as described above with anesthesia (Fig. 1A, blood test),
176 and further conducted a food/water intake test without anesthesia at the same feeding interval,
177 but without performing blood collection (Fig. 1A, intake test). In these tests, controlled access to
178 food and water was evaluated by using control conditions (Fig. 1A, control 1 and 2). 20 h of no
179 food and free access to water was followed by food access without water for blood collection (Fig.
180 1A 2nd row). The same feeding interval was used in the food and water intake tests without
181 anesthesia (Fig. 1A, 3rd row). Under control condition 1 (Fig. 1A, fourth row, control 1), monkeys
182 were not provided food to measure water intake, which confirmed that the monkeys became
183 thirstier after consuming a dry meal. In control condition 2 (Fig. 1A, bottom row, control 2), a short

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184 feeding delay (17 h) was used to pre-check the amount of food intake while drinking water when
185 eating dry meals, which confirmed whether blood and food intake test conditions yield hunger in
186 monkeys.

187

188 **Body weight measurements**

189 The body weight of each monkey was measured before the first blood collection.

190

191 **Measuring the blood ghrelin levels using enzyme-linked immunosorbent assay (ELISA)**

192 Unacylated ghrelin levels were measured using ELISA (Unacylated Ghrelin (human) Express
193 Enzyme Immunoassay kit, Bertin Pharma, #A05119.96 wells, France). All immunoassays were
194 performed according to the manufacturer's instructions. The plates coated with the primary
195 antibody in each well were rinsed five times with the wash buffer (300 μL/well) included in the kit.
196 Standards, controls, and samples were added to each well. Additionally, 100 μL of a tracer was
197 dispensed to the wells, and the plates were incubated at room temperature (RT) for 3 h
198 (approximately 20 ~ 25 °C). After rewashing, 200 μL of the detection reagent was dispensed into
199 the wells. The plates were subsequently incubated in the dark at RT for 1 h, and absorbance was
200 measured at 414 nm using a microplate reader (Varioskan LUX multimode microplate reader,
201 Thermo Fischer Scientific, USA). The ghrelin concentration in each plate was estimated from a
202 standard curve. We used a linear fit because almost no curvilinear fit exists in standard measures.

203

204 **Measuring blood osmolality**

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205 Blood osmolality was measured for each sample; 250 μL plasma was extracted from each sample
206 and the osmolality was measured using a freezing point method (Advance 3250, Advanced
207 Instruments Inc., USA), as described in our previous studies (Yamada et al., 2010; Minamimoto
208 et al., 2012; Yamada et al., 2013). The measurement error, evaluated as the value range for the
209 same blood sample, was almost 2 mOsm/kgH_2O. This error was determined by measuring three
210 samples from a single collected sample, with 2 mOsm/kgH2O being the maximum difference
211 observed in our previous study (Yamada et al., 2010).

212

213 **Statistical analysis**

214 Changes in food and water intake were analyzed using a two-way analysis of variance (ANOVA)
215 at $P < 0.05$, and the amount of dry meal consumed (Y) was analyzed using the following equation:

216 $$Y = b_1 \text{ Duration} + b_2 \text{ Monkey} + b_3 \text{ Interaction} + \text{error} \quad (1)$$

217 where errors are the residuals, "Duration" is a categorical variable used to define the duration
218 without foods, "Monkey" is a categorical variable identifying the four monkeys, and "Interaction"
219 is the interaction between "Duration" and "Monkey." If b_1 or b_3 was not 0 at $P < 0.05$, we concluded
220 that food duration did not significantly affect monkey food intake.

221 The amount of consumed water (Y) was analyzed using the following equation:

222 $$Y = b_1 \text{ Condition} + b_2 \text{ Monkey} + b_3 \text{ Interaction} + \text{error} \quad (2)$$

223 where errors are the residuals, "Condition" is a categorical variable to define the test condition of
224 the intake test and its control (control 1), "Monkey" is a categorical variable identifying the four
225 monkeys, and "Interaction" is the interaction between "Condition" and "Monkey." If b_1 or b_3 was

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226 not 0 at $P < 0.05$, we concluded that the test conditions (i.e., food intake before the water intake
227 test) significantly affected the water intake of the monkey.

228 Changes in plasma ghrelin levels before and after eating the dry meal were analyzed using
229 two-way ANOVA at $P < 0.05$, and plasma ghrelin (Y) was fitted using the following equation:

230 $$Y = b_1 \text{ Hunger} + b_2 \text{ Monkey} + b_3 \text{ Interaction} + \text{error (3)}$$

231 where errors are the residuals, “Hunger” is a categorical variable to define the hunger state,
232 differentiated by the conditions before or after food intake on a test day, “Monkey” is a categorical
233 variable identifying the four monkeys, and “Interaction” is the interaction between “Hunger” and
234 “Monkey.” If b_1 or b_3 was not 0 at $P < 0.05$, we concluded that the hunger state significantly
235 affected plasma ghrelin levels.

236 Changes in plasma osmolality levels were analyzed using two-way ANOVA at $P < 0.05$. The
237 plasma osmolality (Y) was fitted using the following equation:

238 $$Y = b_1 \text{ Hunger} + b_2 \text{ Monkey} + b_3 \text{ Interaction} + \text{error (4)}$$

239 where errors are the residuals, “Hunger” is a categorical variable to define the hunger state,
240 differentiated by the conditions before or after food intake on a test day, “Monkey” is a categorical
241 variable identifying the four monkeys, and “Interaction” is the interaction between “Hunger” and
242 “Monkey.” If b_1 or b_3 was not zero at $P < 0.05$, we concluded that the hunger state significantly
243 affected plasma osmolality.

244

245 **Influence of plasma concentration on the optical density measurements**

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246 The nonspecific effect of plasma on the measured optical density was evaluated by adding 5%
247 and 10% plasma to the standard concentration. All these plasma samples were obtained from a
248 monkey on a single day. We partially evaluated the degree of noise from the plasma sample by
249 comparing the fitted curves to these data (5% and 10%) with a standard curve without plasma
250 (0%). In this evaluation, we used a sample from monkey SUN, which contained relatively low
251 ghrelin levels among the four monkeys.

252 The influence of the plasma concentration on the optical density was evaluated using a
253 general linear model. The optical densities (Y) were fitted using the following equation:

254 $$Y = b_0 + b_1 \text{ COS} + b_2 \text{ PC} + b_3 \text{ Interaction} + \text{Error}, \quad (5)$$

255 where b_0 and the error are the intercept and residual, respectively. "COS" is a concentration of
256 the standard, "PC" is the plasma concentration (0%, 5%, or 10%), and "Interaction" is the
257 interaction between "COS" and "PC." If b_2 was not zero at $P < 0.05$, we concluded that the plasma
258 concentration significantly affected the optical density. b_2 should be significantly different from
259 zero if the plasma sample contains endogenous ghrelin. If b_3 was not zero at $P < 0.05$, it indicated
260 that a nonspecific effect of the plasma was present on the measured optical density in the sample.

261 We suspected a nonspecific effect of the plasma on the measured optical density in the
262 following way. Assuming no endogenous ghrelin was present in the sample and that all the
263 measured optical densities came from the plasma, the increase in the intercept values from 0%
264 to 10% plasma indicated the amount of noise from the plasma sample. Because it is not possible
265 to extract endogenous ghrelin from the sample, the maximum possible noise level was estimated
266 in this way.

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268 **Results**

269 **Effect of controlled food and water access on consumption behavior in monkeys**

270 To confirm that our experimental protocol obeying controlled access schedules adequately
271 evoked hunger and thirst in monkeys, we prepared five different conditions in our experiment (Fig.
272 1A). First, in the blood test condition (Fig. 1A, second row), no food access was provided to the
273 monkeys for 20 h. This controlled food access was started at 14:00 the day before the test in all
274 test condition, except control 2 (Fig. 1A, bottom row). This feeding interval of 20 h no-food yielded
275 almost the same, but the slightly different amount of dry meal's intake as that in regular feeding
276 condition in some monkeys (Fig. 1B, see 20 h and Reg, two-way ANOVA, n = 104, d.f. = 92,
277 duration, F = 13.6, $P < 0.001$; monkey, F = 3.10, $P = 0.032$), while the intake duration was 1.5 h
278 for food consumption (Fig 1A, 2nd row, yellow bar). Indeed, 17 h of no food access we made as
279 a pre-check (Fig 1A, bottom row, control 2) seemed to be sufficient to elicit this level of
280 consumption behavior (Fig. 1B, see 17 h), although the effect of the no food duration differed
281 between monkeys (interaction, F = 6.60, $P < 0.001$). Thus, our controlled food access protocol
282 used for the blood test (20h of no food access) adequately elicited a hunger state in the monkeys.

283 In the blood test, the monkeys consumed dry meals without any liquid reward (Fig. 1A,
284 second row, gray bar for water). Under this feeding condition, monkeys became thirstier as
285 indicated by the increased water intake (Fig. 1C, see test, see also Fig 1A, middle row) than those
286 when they did not consume the dry meal (Fig. 1C, see con1, see also Fig 1A, fourth row, two-way
287 ANOVA, n = 24, d.f. = 16, condition, F = 58.3, $P < 0.001$, monkey, F = 78.9) maybe because the
288 dry meal contained almost no water but some sodium (See Materials and Methods). Conformation
289 of this consumption behavior without anesthesia in the intake test indicated that under the blood
290 test condition, which employs the same schedule as the intake test condition, monkeys were
291 hungry and thirsty at the first and second blood collections, respectively.

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292

293 **Effect of dry meal intake on blood level measurements of ghrelin and osmolality**

294 We measured the blood levels of ghrelin and osmolality before and after consuming the dry meal
295 under the no water access conditions (Fig. 1A, second row) during an approximately 3-h period
296 until the second blood collection, consisting of periods of 0.5 h before start eating, 1.5 h during
297 eating, and 1.0 h after eating the meal. This controlled food access procedure mimicked a typical
298 experimental condition for measuring animal behavior in an experimental room that motivates
299 subject performance to obtain food rewards, whereas the hydration status was not controlled to
300 motivate subject performance. Two consecutive blood collections were performed on each test
301 day with controlled food access, which was started a day before the test day and continued until
302 the second blood collection (Fig. 1A, second row, blood test). Throughout the test period for each
303 monkey, water access was not controlled, except for the period between the first and second
304 blood collections. Plasma was extracted from whole blood samples using a standard procedure
305 (See Materials and Methods).

306 We identified that the unacylated ghrelin levels tested using ELISA (See Materials and
307 Methods) decreased on average after consuming the dry meal compared to those before intake
308 (Fig. 2A, two-way ANOVA, $n = 64$, $df = 56$; Hunger, $F = 5.44$, $P = 0.023$; Monkey, $F = 29.8$, $P <$
309 0.001) in three out of the four monkeys. Additionally, blood ghrelin levels were vastly different
310 among the monkeys (pg/mL: monkey SUN, 54–350; monkey MIY, 79–380; monkey Y1, 174–
311 1,122; monkey MO, 206–1,123). Monkey Y1 exhibited a decrease in value in all eight tests, while
312 monkeys MO and MIY exhibited a decrease in six of the eight measurements. In contrast, monkey
313 SUN, who exhibited the lowest blood ghrelin levels, demonstrated the opposite effect, exhibiting
314 an increase in blood ghrelin levels (Interaction, $F = 3.61$, $P = 0.019$) in seven of the eight

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315 measures. Thus, although individual differences existed, food intake consistently decreased
316 blood ghrelin levels, except in one monkey.
317 To examine the effect of dry-meal intake on thirst levels, we measured blood osmolality using
318 the same blood samples described above. Our simple prediction for this result was that the
319 monkeys would become thirstier after eating a dry meal without fluid intake, which is a typical
320 condition when using food rewards to motivate monkeys in an experimental room. After the dry
321 meal intake without water intake, all four monkeys exhibited consistent increases in blood
322 osmolality (Fig. 2B, two-way ANOVA, n = 64, df = 56; Hunger, F = 45.7, $P < 0.001$). The effect of
323 dry meal intake on blood osmolality differed from that on blood ghrelin levels, with no significant
324 individual differences in osmolality levels, and no significant interaction between food intake and
325 the monkeys (monkey, F = 2.61, $P = 0.060$; Interaction, F = 0.41, $P = 0.746$). Indeed, none of the
326 samples collected over the 32 testing days (8 testing days × four monkeys) demonstrated a
327 decrease in osmolality. Thus, the blood osmolality level was capable of consistently reflecting the
328 internal state of thirst in all four animals (Figs. 1C and 2B), which is consistent with the results of
329 our previous studies (Yamada et al., 2010; Minamimoto et al., 2012; Yamada et al., 2013).
330 We further examined the relationship between changes in blood osmolality and ghrelin levels
331 to determine whether these values were related, identifying no relation between these changes
332 (Fig. 3A; linear regression, n = 32, df = 30; intercept, t = 9.54, $P < 0.001$; ghrelin difference, t =
333 1.30, $P = 0.20$). Furthermore, we visualized the relationship between the changes in osmolality
334 and ghrelin levels in each period before and after consuming the dry meal (Fig. 3B), and examined
335 whether a relationship existed between osmolality and ghrelin levels using regression analysis
336 (see the regression slope in Fig. 3B). Overall, we noted no consistent effects among the monkeys.
337 Monkeys Y1, MO, and MIY did not demonstrate consistent regression slopes, although these
338 three individuals consistently exhibited decreased blood ghrelin levels and increased blood

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339 osmolality (Figs. 2A and B). Monkey SUN, which exhibited the opposite effect on blood ghrelin
340 levels, demonstrated regression coefficients similar to those of monkey MO. We further examined
341 whether a behavioral relationship was present between food intake and an increase in water
342 intake under non-anesthetized conditions (Figs. 4A and B), but did not detect any such
343 relationship (Fig. 4C). Thus, blood ghrelin and osmolality levels did not co-vary with each other
344 either before or after consuming dry meals, indicating that these two physical measures can be
345 used to evaluate hunger and thirst in animals.

346 In summary, after dry meal intake without water, blood ghrelin levels decreased, except in
347 monkey SUN, whereas osmolality increased in all four monkeys. However, we did not observe a
348 clear relationship between blood osmolality and ghrelin levels. These data indicated that the
349 accuracy of the measurements and physical changes differed for each individual.

350

351 **Assessing the influence of plasma concentration on optical density measurement**

352 We further evaluated the degree of measurement error (or noise) included in our ghrelin
353 measurements from the plasma, enabling us to compare the two different physical measures of
354 the indices for hunger and thirst, accounting for both measurement errors. For the osmolality
355 measures we employed, we previously evaluated the measurement error of osmolality, as the
356 value range from a single sample was within almost 2 mOsm/kgH2O in our procedure. Here, we
357 assessed the degree of error in the optical measurement of unacylated ghrelin by ELISA. As
358 plasma must induce a nonspecific effect on optical absorption, such as the nonspecific binding of
359 proteins (Neutens et al., 2021), we evaluated the noise as follows: plasma samples were
360 compared with different concentrations obtained from one monkey (SUN) among a single
361 collected samples after the standard was added to them at different dilutions (0% or no
362 endogenous plasma, 5%, and 10%). We compared these three optical measures, which included

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363 the original standard and endogenous ghrelin in the 5% and 10% plasma samples. Ideally, the
364 fitted lines should change consistently as the percentage increases. We note that removing
365 endogenous ghrelin from plasma is impossible, and optical absorption is derived from
366 endogenous ghrelin, plasma, and standard ghrelin.

367 All three curves were well described with a linear fit because the R-squared values were >
368 0.99, indicating that 5% and 10% plasma and endogenous ghrelin elicited a consistent increase
369 in optical absorption (Fig. 5). The background optical absorption without ghrelin or plasma was
370 approximately 0.141, as estimated from the fitted curves (intercept of the black regression slope).
371 These values increased to 0.192 and 0.269 in 5% and 10% ghrelin-containing buffers,
372 respectively, representing increments of 0.051 and 0.077 from 0% to 5% and 5% to 10%,
373 respectively. This nonlinear effect was a 1.5-fold increase (0.077/0.051) and may be elicited by a
374 5% point increase in the plasma concentration from 5% to 10%. Moreover, the relative influence
375 of the additive plasma on the optical measurements decreased as the amount of standard ghrelin
376 increased (almost no differences were observed at 125 pg/mL): the optical densities were 0.949,
377 0.952, and 0.966 in 0%, 5%, and 10% plasma at 125 pg/mL, respectively. The slopes of the
378 regression coefficients in these three linear fits became slightly shallow as the plasma
379 concentration increased (general linear model: $n = 24$, $df = 20$, the concentration of standard:
380 regression coefficient, 0.0128, $t = 55.8$, $P < 0.001$; plasma concentration, regression coefficient,
381 0.0066, $t = 13.7$, $P < 0.001$; interaction, regression coefficient, −0.00008, $t = -4.6$, $P < 0.001$).
382 Thus, the blood ghrelin levels in our samples were reliably measured by ELISA using 10% plasma.
383 In addition, some small nonspecific optical signals were derived from the plasma.

384 Finally, the maximum possible noise levels in the optical measurements were estimated. If
385 we assume that no endogenous ghrelin is present in the sample, the increase in optical
386 measurements could serve as noise from the plasma (i.e., the maximum possible noise). This

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387 increase was estimated from the intercept differences between 0% (i.e., standard) and 10%
388 plasma concentration and can serve as the maximum level of noise derived from the plasma
389 under this assumption. This increase was 0.128 (0.141 to 0.269) for 10% plasma, and thus, the
390 value in normal plasma was 12.8 pg/mL. As plasma contains endogenous ghrelin, which
391 increases optical absorption, we could only conclude that the maximal noise originating from the
392 plasma must be smaller than this value. Thus, the nonspecific optical absorption from 10% plasma
393 was relatively small for measuring the blood ghrelin level within the range observed in these
394 monkeys. For instance, 12.8 pg/mL noise may come from 10% plasma within the entire range of
395 0–1,200 pg/mL in our measurements.

396

397 **Discussion**

398 In this study, the consumption of food after 20 h of no-food access elicited a decrease in blood
399 unacylated ghrelin levels in three out of four monkeys. Blood ghrelin levels were further observed
400 within various value ranges across individual monkeys, with most demonstrating a decrease in
401 the value, whereas one monkey exhibited an increase in the value. This increase is surprisingly
402 consistent in this monkey, indicating that the increase of blood ghrelin predicts food consumption
403 in this particular monkey. In contrast, the blood osmolality level perfectly reflected the change in
404 fluid balance in all monkeys after dry meal intake; the blood osmolality level consistently increased
405 after dry meal intake for approximately 3 h without fluid intake. Although blood osmolality and
406 unacylated ghrelin levels can be used as indices for thirst and hunger, respectively, we need to
407 consider the different characteristics of physical pressure and peptide hormone levels as
408 hematological indices when evaluating the two satiety states.

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410 **Measuring ghrelin as a physical index of hunger levels**

411 In this study, we obtained evidence that ghrelin levels was high before food intake, and then,
412 decreased after the food intake, reflecting hunger (Figs. 2A and 1B). This result is consistent with
413 that of human studies demonstrating the role of ghrelin in food intake and its changes throughout
414 the day (Spiegel et al., 2011). For instance, Spiegel et al. reported that an increase in unacylated
415 ghrelin levels between meals in humans occasionally covaries with acylated ghrelin levels. Most
416 human studies have reported average changes across participants (Spiegel et al., 2011; Marchio
417 et al., 2019). In contrast, our repeated measurements from each monkey revealed individual
418 changes in ghrelin levels and demonstrated the reliability of blood ghrelin levels as a physical
419 index of hunger. However, it might be possible that the high ghrelin level before the first blood
420 collection reflected an anticipatory response to food (Schussler et al., 2012).

421 Ghrelin plays a pivotal role in regulating food intake, body weight, and glucose metabolism in
422 the brain (Tschop et al., 2000; Nakazato et al., 2001). Additionally, ghrelin is released from the
423 stomach, where it transmits satiety signals to the central nervous system (Sato et al., 2014).
424 Acylated and unacylated ghrelin in humans, monkeys, and rodents comprises 28 amino acid
425 residues. In humans, acylated ghrelin is the active form that modifies the third serine with the fatty
426 acid n-octane. The amino acid sequence of mammalian ghrelin is highly conserved in humans,
427 monkeys, rats, mice, cows, pigs, and sheep. Furthermore, 10 amino acids from the N-terminus
428 constitute the active region (Kaiya et al., 2011), which is used in ELISA to detect endogenous
429 ghrelin in humans and monkeys. Unacylated ghrelin is detected in the stomach and blood at
430 certain concentrations, and does not bind to the growth hormone secretagogue receptor;
431 however, some studies have reported that unacylated ghrelin enhances eating behavior (Toshinai
432 et al., 2006).

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433 Acylated and unacylated ghrelin (or total ghrelin) changed similarly in relation to consumption
434 behavior in humans, whereas unacylated ghrelin was identified at a 10-fold higher concentration
435 than acylated ghrelin (Spiegel et al., 2011). In addition, acylated ghrelin was discovered to be
436 highly unstable in the whole blood and plasma. To acquire accurate ghrelin level data, blood
437 samples were collected with ethylenediaminetetraacetic acid-aprotinin and centrifuged under
438 cooled conditions within 30 min of collection (Hosoda and Kangawa, 2004). Following this
439 protocol, we opted to measure unacylated ghrelin, which may serve as a physical index of hunger.
440 We noted that acylated ghrelin, which is present at a concentration of approximately 100 pg/mL
441 in humans, was not measured in this study. We also noted that whether acylated ghrelin can be
442 used as an index of hunger levels remains unclear.

443

444 **Blood osmolality measurement as a physical index of thirst levels**

445 We discovered that the fluid balance of monkeys after eating a dry meal was reflected in their
446 blood osmolality. In the water intake test we confirmed that the monkeys consumed more water
447 under the same conditions than that under the control condition, in which the monkeys ate no dry
448 meal before water intake (Fig. 1C). The dry meal intake evoked the increase in the extracellular
449 dehydration state, as measured by the osmolality level, indicated how thirsty the monkeys were
450 after eating the dry meal (Fig. 2B). This increase in osmolality (7 mOsmo/kgH2O) was almost
451 equal to the osmolality increase observed under regular controlled water access in our previous
452 study (8 mOsmo/kgH2O, Fig. 3, Yamada et al., 2010). In our previous study, we established that
453 animals worked harder to earn more water on days when they exhibited lower hydration states
454 (i.e., higher osmolality) than when they exhibited higher hydration states (i.e., lower osmolality).
455 This relationship between osmolality level and water consumption behavior reflects the

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456 physiological condition of fluid balance, and it was observed as the increased osmolality and more
457 water intake in the present study (Figs. 2B and 4B).

458 It is well known that osmoreceptors are located in the anterior-ventral third ventricle of the
459 hypothalamus, where they detect changes in blood osmolality (Buggy et al., 1979). Neurons in
460 the organum vasculosum of the lamina terminalis change their firing rates according to blood
461 osmolality (Thrasher and Keil, 1987) to stimulate or suppress water and salt ingestion. In the
462 present study, fluid balance changed after consuming a dry meal because the monkeys were not
463 allowed access to water. We fed the monkeys certified diets containing water (8%), protein (21%),
464 fat (7%), coarse ash (8%), crude fiber (3%), soluble nitrogen (53%), salt (0.7%), and vitamins.
465 Monkeys consumed more than 130–160 g of this meal, which contained > 1 g of salt. Salt intake
466 without concurrent fluid intake must stimulate and change the body's fluid balance. Another
467 important mechanism involves a reduction in blood volume, stimulates fluid ingestion, and
468 suppresses diuresis via the renin-angiotensin system. (Berne and Levy, 1993). Thus, osmolality
469 is a critical component of the hydration state, and direct blood osmolality measurements provide
470 a reliable index for quantifying the hydration state. We note that an increase in ghrelin levels may
471 affect the drinking behavior of monkeys (Hashimoto et al., 2007).

472

473 **Age, sex, species, and experimental procedures**

474 In our experiment, we used four macaque monkeys for blood tests, one of which exhibited distinct
475 changes in blood ghrelin levels (Fig. 2A, Monkey SUN). We further analyzed the relationship
476 between blood ghrelin and osmolality changes before and after dry meal intake (Fig. 3A),
477 identifying no relationship, as these changes depended on the individual monkeys (Fig. 3B).
478 Although ghrelin plays essential roles in adiposity and metabolism in vertebrates (Kaiya et al.,

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479 2013), the ghrelin levels were heterogeneous and differed between individuals. Multiple factors
480 may be related to this discrepancy, and we discuss the potential factors that affect blood ghrelin
481 levels. First, monkey SUN, which exhibited increased blood ghrelin levels, differed from other
482 monkeys in terms of age, species, and blood collection procedures. The monkey SUN was a
483 rhesus macaque, whereas the others were Japanese macaques, although they are closely related
484 species. This species difference is one possible explanation for this distinction. Sex differences
485 cannot explain our results, as the monkey MIY was the only female among the four monkeys. As
486 such, age is the most reasonable explanation for the difference: monkey SUN was middle-aged
487 (13 years old) compared to the other young monkeys (2–4 years old, see Materials and Methods).
488 Indeed, ghrelin is a growth hormone, and total ghrelin and unacylated ghrelin levels decrease in
489 an age-dependent manner (Wilasco et al., 2012). In aged frail adults, reduced levels of total
490 ghrelin and impaired response to a meal test have been observed, suggesting that ghrelin
491 contributes to the anorexia mechanisms associated with aging (Serra-Prat et al., 2009). Blood
492 collection from monkey SUN was performed while awake; however, age-related changes in
493 ghrelin levels are one possible explanation of our results observed in monkey SUN. In addition, a
494 large number of individual observations across different ages are required to elucidate this issue.
495 This inconsistency observed in one monkey (SUN) may limit the utility of ghrelin measures in
496 evaluating hunger states.

497

498 **Neuroeconomic perspectives and satiety states**

499 Hunger and thirst are distinct psychological (Powloski, 1953; Zimbardo and Montgomery, 1957;
500 Ramachandran and Pearce, 1987) and physiological phenomena (Berne and Levy, 1993).
501 However, they are interdependent (McKiernan et al., 2009), as observed in mammals and flies

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502 (Jourjine, 2017). From an economic perspective, this interdependence may explain why foods
503 and beverages are complementary goods (Mas-Colell et al., 1995). From a food science
504 perspective, foods and beverages contain similar components such as energy, water, salt, and
505 other essential nutrients. The pathological association is often linked to obesity (Martens and
506 Westerterp-Plantenga, 2012). From a neuroscientific perspective, interdependency is explained
507 by the fact that the neural circuits underlying hunger and thirst interact with each other (Rolls,
508 1975; Jourjine, 2017; Eiselt et al., 2021; Tan et al., 2022). The interdependence was not observed
509 in this study (Fig. 3), maybe because the interdependence might be observed when animals are
510 both hungry and thirsty.

511 In neuroeconomic studies, hunger, thirst, and satiation have been demonstrated to have a
512 significant impact on animal behavior (Minamimoto et al., 2012; Yamada et al., 2013; Yamada,
513 2017), although thirst and hunger may affect neural representations categorized as taste in the
514 brain (Augustine et al., 2020; Pastor-Bernier et al., 2021). Physiologically, the hypothalamus plays
515 an integral role in energy and water homeostasis by sensing and reacting to systemic calorie and
516 osmotic fluctuations (Lee, 2016). Maintaining the energy and fluid balance is fundamental to
517 survival; thus, a rebalance of these physiological states occur, such as dehydration after
518 consumption of a dry meal in the present study.

519 For the reliable estimation of animal states, both indices should be measured with similar
520 levels of precision. Herein, we aimed to compare the measurement precision and demonstrate
521 the index reliability within their value ranges (Fig. 5) under the assumption. We still need to
522 improve this evaluation, as well as the ELISA procedure. Additionally, easy access to blood
523 samples is required to understand the relationship between neurophysiological measures and
524 indices such as trial-by-trial correlations. However, blood ghrelin and osmolality measurements
525 allow for an improved understanding of the neural basis of decision-making as a physical index

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526 of satiety. These measurements may be an easy and efficient way to evaluate controlled access
527 to food and fluids in home cages.

528

529

530 **References**

531 Augustine V, Lee S, Oka Y (2020) Neural Control and Modulation of Thirst, Sodium Appetite, and Hunger.
532 Cell 180:25-32.

533 Berne RM, Levy MN (1993) In: Physiology, 3rd Edition, pp 754-783. St. Louis: Mosby-Year Book, Inc.

534 Betley JN, Xu S, Cao ZFH, Gong R, Magnus CJ, Yu Y, Sternson SM (2015) Neurons for hunger and thirst
535 transmit a negative-valence teaching signal. Nature 521:180-185.

536 Camerer C, Loewenstein G, Prelec G (2005) Neuroeconomics: How Neuroscience Can Inform Economics
537 Journal of Economic Literature 43: 9-64.

538 Critchley HD, Rolls ET (1996) Hunger and satiety modify the responses of olfactory and visual neurons in
539 the primate orbitofrontal cortex. J Neurophysiol 75:1673-1686.

540 de Araujo IE, Gutierrez R, Oliveira-Maia AJ, Pereira A, Jr., Nicolelis MA, Simon SA (2006) Neural ensemble
541 coding of satiety states. Neuron 51:483-494.

542 Eiselt AK, Chen S, Chen J, Arnold J, Kim T, Pachitariu M, Sternson SM (2021) Hunger or thirst state
543 uncertainty is resolved by outcome evaluation in medial prefrontal cortex to guide decision-making.
544 Nat Neurosci 24:907-912.

545 Evarts EV (1968) A technique for recording activity of subcortical neurons in moving animals.
546 Electroencephalogr Clin Neurophysiol 24:83-86.

547 Glimcher PW, Camerer CF, Fehr E, Poldrack RA (2008) Neuroeconomics: Decision Making and the Brain.
548 New York: Elsevier.

549 Haber SN, Knutson B (2010) The reward circuit: linking primate anatomy and human imaging.
550 Neuropsychopharmacology 35:4-26.

551 Hashimoto H, Fujihara H, Kawasaki M, Saito T, Shibata M, Otsubo H, Takei Y, Ueta Y (2007) Centrally and
552 peripherally administered ghrelin potently inhibits water intake in rats. Endocrinology 148:1638-

eNeuro Accepted Manuscript

553 1647.

554 Hosoda H, Kangawa K (2004) GHRELIN MEASUREMENT: PRESENT AND PERSPECTIVES. In:
555 GHRELIN (Ghigo E, Benso A, Broglio F, eds): Springer.

556 Jourjine N (2017) Hunger and thirst interact to regulate ingestive behavior in flies and mammals. Bioessays
557 39.

558 Kaiya H, Miyazato M, Kangawa K (2011) Recent advances in the phylogenetic study of ghrelin. Peptides
559 32:2155-2174.

560 Kaiya H, Kangawa K, Miyazato M (2013) What is the general action of ghrelin for vertebrates? -
561 comparisons of ghrelin's effects across vertebrates. Gen Comp Endocrinol 181:187-191.

562 Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K (1999) Ghrelin is a growth-hormone-
563 releasing acylated peptide from stomach. Nature 402:656-660.

564 Lee P (2016) Quenching the thirst for hunger. Am J Physiol Endocrinol Metab 311:E950-E951.

565 Marchio M, Roli L, Giordano C, Trenti T, Guerra A, Biagini G (2019) Decreased ghrelin and des-acyl ghrelin
566 plasma levels in patients affected by pharmacoresistant epilepsy and maintained on the ketogenic
567 diet. Clin Nutr 38:954-957.

568 Martens MJ, Westerterp-Plantenga MS (2012) Mode of consumption plays a role in alleviating hunger and
569 thirst. Obesity (Silver Spring) 20:517-524.

570 Mas-Colell A, Whinston M, Green J (1995) Microeconomic Theory. New York: Oxford University Press, Inc.

571 Mattes RD (2010) Hunger and thirst: issues in measurement and prediction of eating and drinking. Physiol
572 Behav 100:22-32.

573 McKiernan F, Hollis JH, McCabe GP, Mattes RD (2009) Thirst-drinking, hunger-eating; tight coupling? J Am
574 Diet Assoc 109:486-490.

575 Minamimoto T, Yamada H, Hori Y, Suhara T (2012) Hydration level is an internal variable for computing
576 motivation to obtain water rewards in monkeys. Exp Brain Res 218:609-618.

577 Nakano Y, Oomura Y, Nishino H, Aou S, Yamamoto T, Nemoto S (1984) Neuronal activity in the medial
578 orbitofrontal cortex of the behaving monkey: modulation by glucose and satiety. Brain Res Bull
579 12:381-385.

580 Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, Matsukura S (2001) A role for ghrelin
581 in the central regulation of feeding. Nature 409:194-198.

eNeuro Accepted Manuscript

582 Neutens P, Jansen R, Woronoff G, Rutowska M, Hosseini N, Buja F, Humbert A, Colle F, Stakenborg T, Van
583 Roy W (2021) Waveguide-based absorption measurement system for visible wavelength
584 applications. Biomed Opt Express 12:2041-2053.

585 Pastor-Bernier A, Stasiak A, Schultz W (2021) Reward-specific satiety affects subjective value signals in
586 orbitofrontal cortex during multicomponent economic choice. Proc Natl Acad Sci U S A 118.

587 Powloski RF (1953) The effects of combining hunger and thirst motives in a discrimination habit. J Comp
588 Physiol Psychol 46:434-437.

589 Pritchard TC, Nedderman EN, Edwards EM, Petticoffer AC, Schwartz GJ, Scott TR (2008) Satiety-
590 responsive neurons in the medial orbitofrontal cortex of the macaque. Behav Neurosci 122:174-
591 182.

592 Ramachandran R, Pearce JM (1987) Pavlovian analysis of interactions between hunger and thirst. J Exp
593 Psychol Anim Behav Process 13:182-192.

594 Rolls BJ (1975) Interaction of hunger and thirst in rats with lesions of the preoptic area. Physiol Behav
595 14:537-543.

596 Rolls BJ, Rolls ET (1982) Thirst. Cambridge: Cambridge University Press.

597 Rolls ET, Scott TR, Sienkiewicz ZJ, Yaxley S (1988) The responsiveness of neurones in the frontal opercular
598 gustatory cortex of the macaque monkey is independent of hunger. J Physiol 397:1-12.

599 Sato T, Ida T, Nakamura Y, Shiimura Y, Kangawa K, Kojima M (2014) Physiological roles of ghrelin on
600 obesity. Obes Res Clin Pract 8:e405-413.

601 Schussler P, Kluge M, Yassouridis A, Dresler M, Uhr M, Steiger A (2012) Ghrelin levels increase after
602 pictures showing food. Obesity (Silver Spring) 20:1212-1217.

603 Serra-Prat M, Palomera E, Clave P, Puig-Domingo M (2009) Effect of age and frailty on ghrelin and
604 cholecystokinin responses to a meal test. Am J Clin Nutr 89:1410-1417.

605 Spiegel K, Tasali E, Leproult R, Scherberg N, Van Cauter E (2011) Twenty-four-hour profiles of acylated
606 and total ghrelin: relationship with glucose levels and impact of time of day and sleep. J Clin
607 Endocrinol Metab 96:486-493.

608 Stedman TL (2006) Stedman's Medical Dictionary. In, 28th Edition. Philadelphia: Lippincott Williams &
609 Wilkins.

610 Symmonds M, Emmanuel JJ, Drew ME, Batterham RL, Dolan RJ (2010) Metabolic state alters economic

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611 decision making under risk in humans. PLoS One 5:e11090.

612 Tan B, Nobauer T, Browne CJ, Nestler EJ, Vaziri A, Friedman JM (2022) Dynamic processing of hunger
613 and thirst by common mesolimbic neural ensembles. Proc Natl Acad Sci U S A 119:e2211688119.

614 Toshinai K, Yamaguchi H, Sun Y, Smith RG, Yamanaka A, Sakurai T, Date Y, Mondal MS, Shimbara T,
615 Kawagoe T, Murakami N, Miyazato M, Kangawa K, Nakazato M (2006) Des-acyl ghrelin induces
616 food intake by a mechanism independent of the growth hormone secretagogue receptor.
617 Endocrinology 147:2306-2314.

618 Tschop M, Smiley DL, Heiman ML (2000) Ghrelin induces adiposity in rodents. Nature 407:908-913.

619 Watanabe M (1996) Reward expectancy in primate prefrontal neurons. Nature 382:629-632.

620 Watts AG (1999) Dehydration-associated anorexia: development and rapid reversal. Physiol Behav 65:871-
621 878.

622 Wilasco MI, Goldani HA, Dornelles CT, Maurer RL, Kieling CO, Porowski M, Silveira TR (2012) Ghrelin,
623 leptin and insulin in healthy children: Relationship with anthropometry, gender, and age distribution.
624 Regul Pept 173:21-26.

625 Wurtz RH (1968) Visual cortex neurons: response to stimuli during rapid eye movements. Science
626 162:1148-1150.

627 Yamada H (2017) Hunger enhances consistent economic choices in non-human primates. Sci Rep 7:2394.

628 Yamada H, Louie K, Glimcher PW (2010) Controlled water intake: a method for objectively evaluating thirst
629 and hydration state in monkeys by the measurement of blood osmolality. J Neurosci Methods
630 191:83-89.

631 Yamada H, Tymula A, Louie K, Glimcher PW (2013) Thirst-dependent risk preferences in monkeys identify
632 a primitive form of wealth. Proc Natl Acad Sci U S A 110:15788-15793.

633 Yaxley S, Rolls ET, Sienkiewicz ZJ, Scott TR (1985) Satiety does not affect gustatory activity in the nucleus
634 of the solitary tract of the alert monkey. Brain Res 347:85-93.

635 Zimbardo PG, Montgomery KC (1957) The relative strengths of consummatory responses in hunger, thirst,
636 and exploratory drive. J Comp Physiol Psychol 50:504-508.

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638 **Figure Legends**

639

640

641 **Fig. 1. Controlled food and water intake and behavioral measures.**

642 (**A**) Five different experimental schedules for the control of food and water intakes: Top row,
643 regular feeding condition for daily allocation of the regular dry meal and water (Reg in **B**); 2nd
644 row, blood test condition for collecting blood for testing the influence of hunger and thirst (20 h in
645 **B**); middle row, intake test condition to measure the food and water intake without anesthesia
646 (test in **C**); Fourth row, control condition for evaluating thirst to measure the effect of dry meal
647 intake on water intake (con1 in **C**); bottom row, control condition to pre-check whether 17 h of no-
648 food access was enough to induce the hunger, compared to the 20 h (17 h in **B**). The controlled
649 food and water access is initiated on the day before the test day, at 14:00, except control 2
650 condition. (**B**) The amount of food intake in four monkeys during the blood test condition (20 h),
651 control 2 (17 h), and regular feeding with a constant dry meal applied at 11:00 (Reg). (**C**) Amount
652 of water intake in four monkeys during the intake test (test) and control condition 1 (con 1). In **B**,
653 the result from monkey Y23 was not demonstrated because his blood data was not collected.

654

655

656 **Fig. 2. Blood ghrelin and osmolality levels before and after dry meal intake.**

657 (**A**) Box plot of blood ghrelin levels in the four monkeys before (pre) and after (post) consuming
658 the regular dry meal. (**B**) Box plot of blood osmolality levels before (pre) and after (post) the
659 consumption of regular dry meals in four monkeys. In A and B, the mean is indicated by a cross.
660 Consecutive measurements are obtained daily using a line.

661

662

663 **Fig. 3. No consistent relationship between the changes in blood ghrelin and osmolality**
664 **levels.**

665 (**A**) No significant relationship observed between changes in blood ghrelin and osmolality levels
666 before (pre) and after (post) the food intake. The dashed line represents the regression slope. (**B**)
667 Two consecutive measurements per day are represented by dotted black lines. The thick gray
668 and orange lines indicate the regression lines for the data in the pre-and post-periods, respectively.

669

670

671 **Fig. 4. Amounts of food and water intake without anesthesia.**

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672 (**A**) The amount of the dry meal intake during the 1.5 h of the food intake test. (**B**) The increase
673 in water intake amount during the 1.5 h of the food intake test compared to that during control
674 condition 1, in which no food was provided. (**C**) No significant relationship observed between the
675 increase in water intake compared to control condition 1 (vertical axis) and the amount of the food
676 intake during the intake test (horizontal axis). The dashed line represents the regression slope.

677

678

679 **Fig. 5. Assessing the influence of plasma concentration on measuring optical density.**

680 Plots of the measured optical density (vertical axis) against standard concentrations. Each point
681 is obtained from the average of two wells: no plasma (black), 5% plasma (blue), and 10% plasma
682 (green). The colored dotted lines indicate a linear fit to the data. R-squares are indicated.

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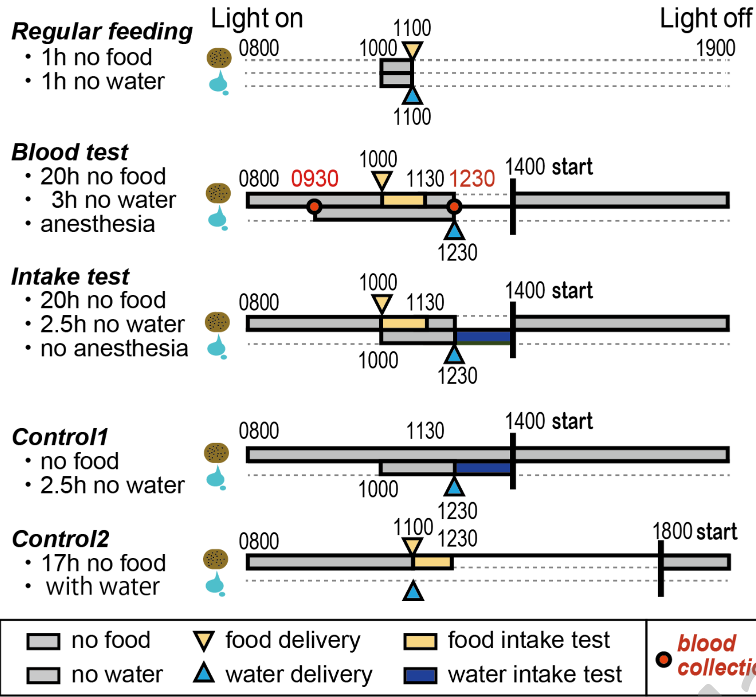

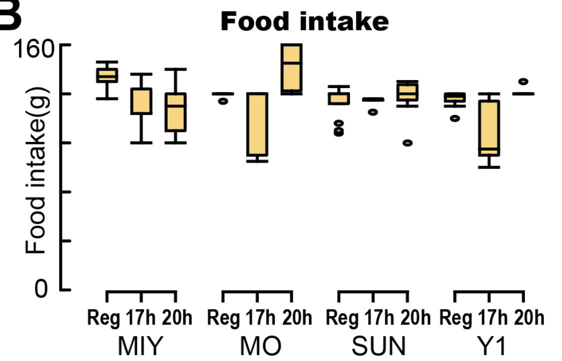

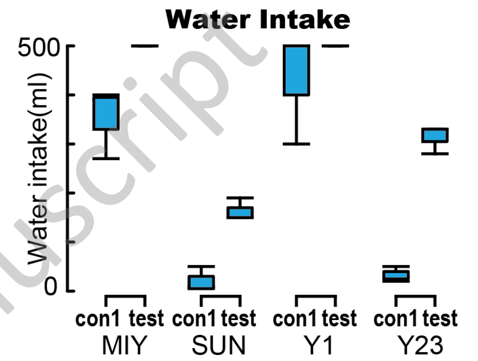

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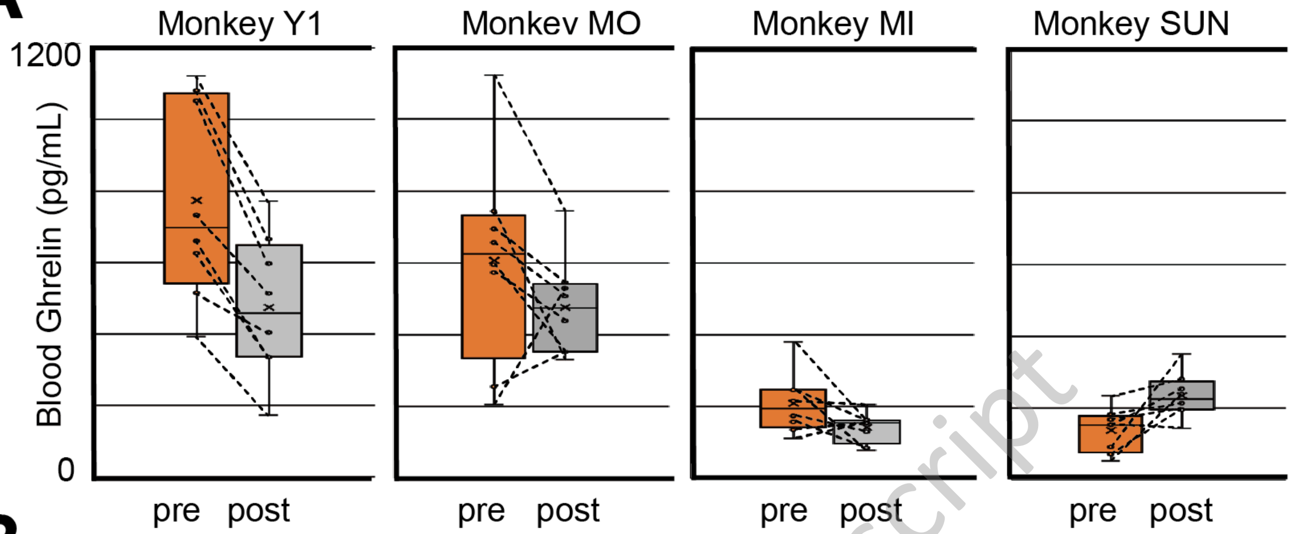

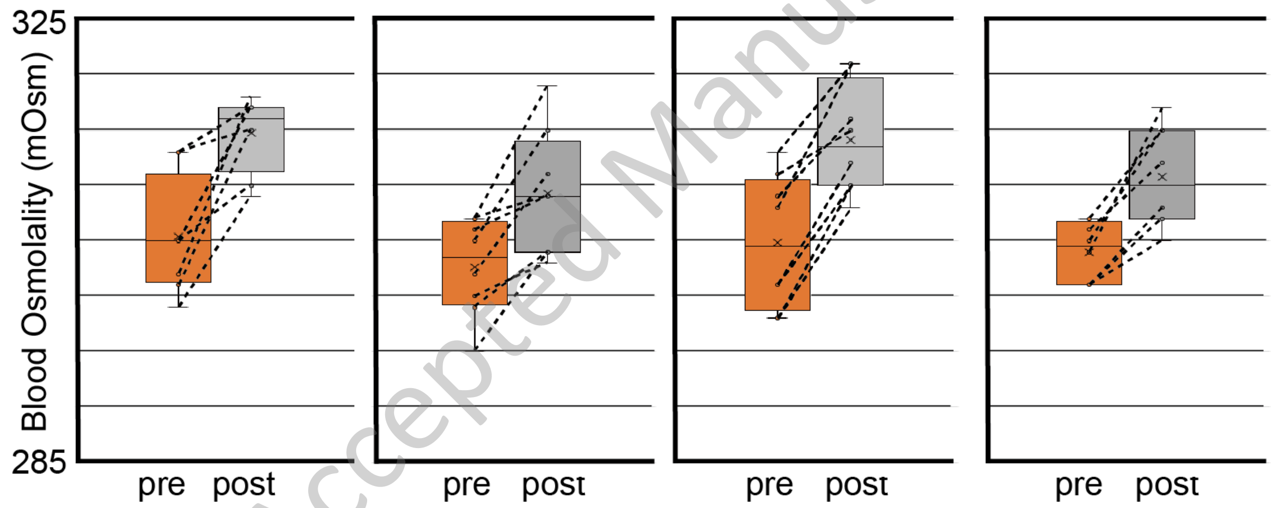

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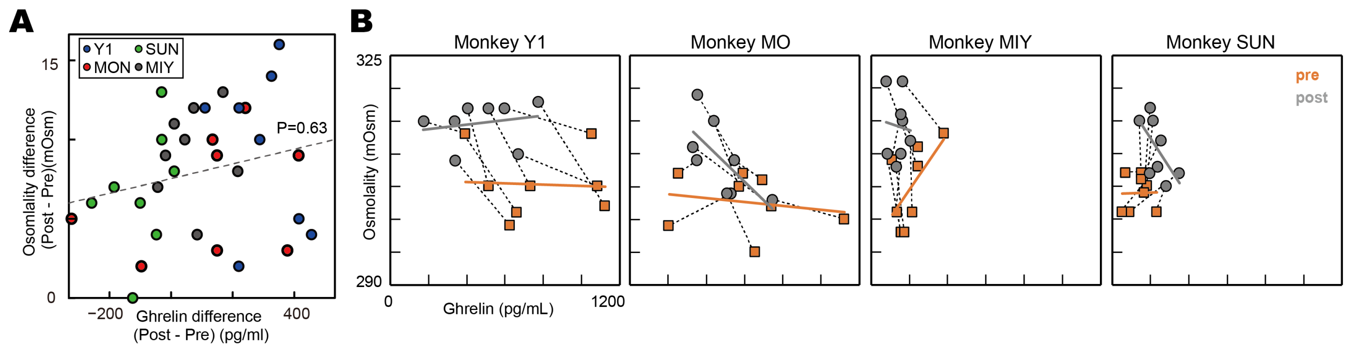

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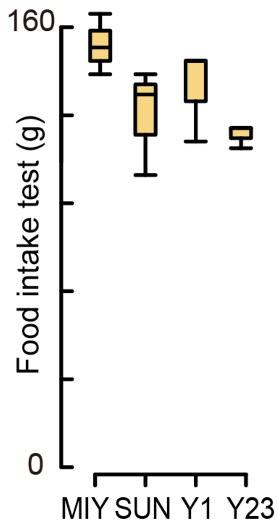

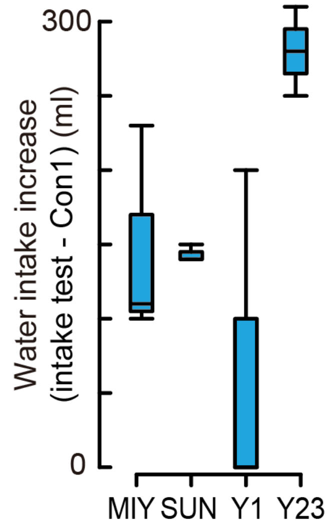

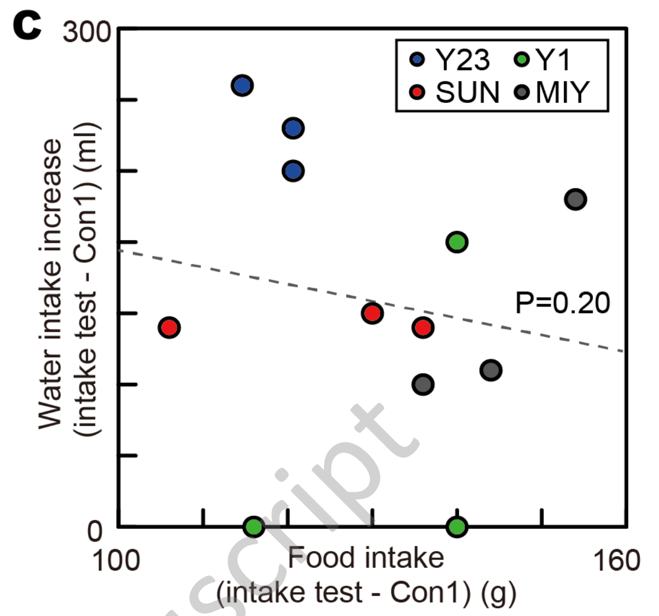

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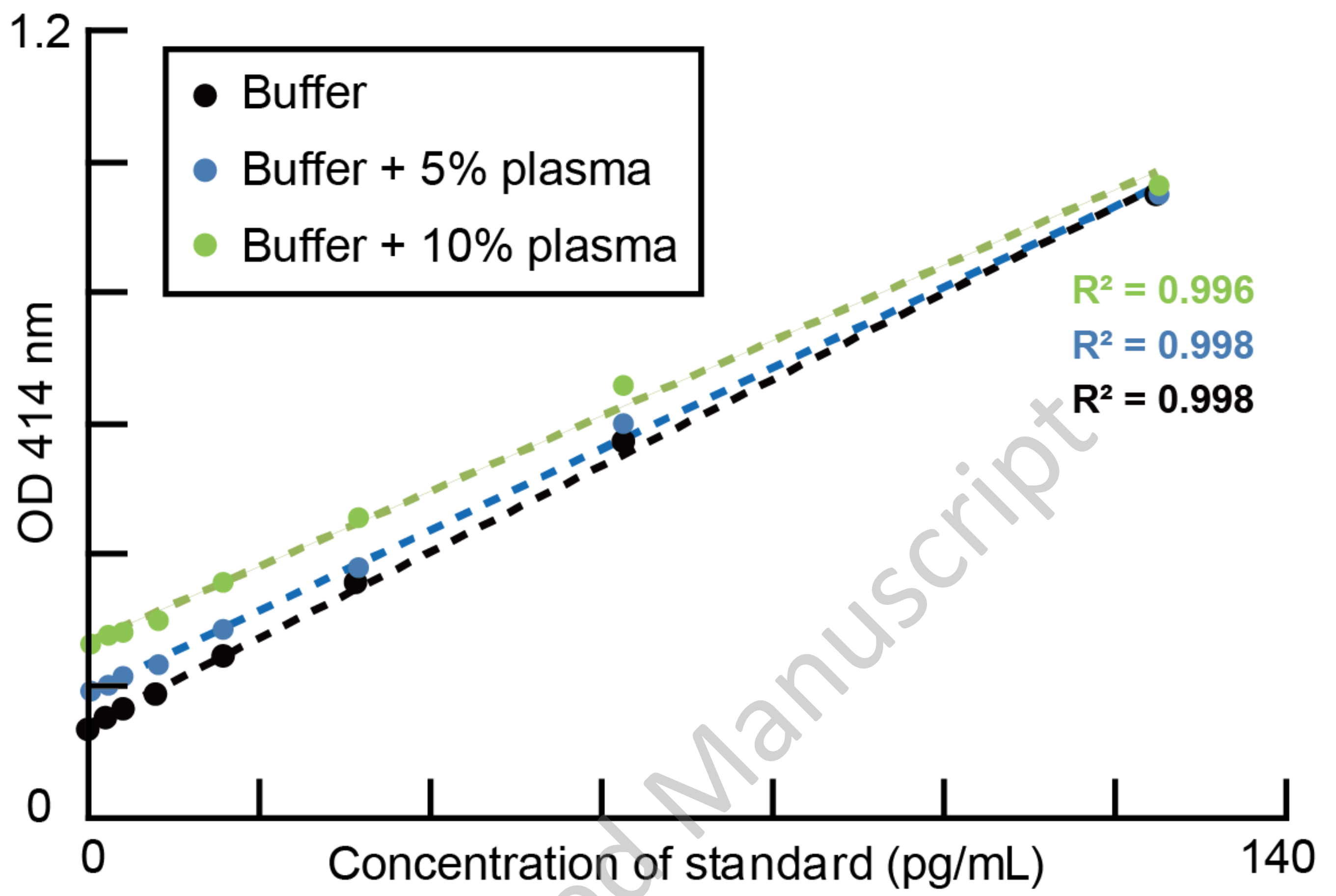

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