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1 **Full title: A mannitol/sorbitol receptor stimulates dietary intake in *Tribolium***

2 ***castaneum***

3 **Short title: Sweet taste preference in home-stored pest**

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14 **Abbreviation**

15 ORF, open reading frame; RT, reverse transcription; RNAi, RNA interference; dsRNA,
16 double strand RNA; cRNA, capped RNA; MBS, modified Barth's saline; CBB, Coomassie
17 brilliant blue; Gr, gustatory receptor;

18

19 **Keywords**

20 Gustatory receptor; sugar alcohol; *Xenopus* oocyte expression; gene silencing;

21

22 # Abstract

23 Perception of chemical stimuli by insects aids in accepting or rejecting food. Gustatory
24 receptors (Grs) regulating external signals in chemosensory organs have been found in
25 many insects. *Tribolium castaneum*, a major pest of stored products, possesses over 200 *Gr*
26 genes. An expanded repertoire of *Gr* genes appears to be required for diet recognition in
27 generalist feeders; however, it remains unclear whether *T. castaneum* recognizes a suite of
28 chemicals common to many products or whether it is attracted to specific chemicals, and
29 whether its Grs are involved in its feeding behavior. It is difficult to determine the food

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30 preference of *T. castaneum* based on its dietary intake due to a lack of appropriate
31 methodology. This study established a novel dietary intake estimation method using
32 gypsum, designated the TribUTE (*Tribolium* Urges To Eat) assay. *T. castaneum* adults were
33 fed gypsum block without added organic compounds. Sugar preference was determined
34 by adding sweeteners and measuring the amount of gypsum in the excreta. Mannitol was
35 the strongest attractant of *T. castaneum* dietary intake; in addition, TcGr20 was
36 responsible for mannitol and sorbitol responses in *Xenopus* oocyte expression, but did not
37 respond to any other non-volatile compounds tested. The EC_{50} values of TcGr20 for
38 mannitol and sorbitol were 72.6 mM and 90.6 mM, respectively, suggesting that TcGr20 is
39 a feasible receptor for the recognition of mannitol in lower concentrations. *TcGr20* was
40 expressed in the antennae, where the perception of mannitol would occur. We examined
41 whether *TcGr20* was involved in mannitol recognition using RNAi and the TribUTE assay.
42 The amounts of excreta in *TcGr20* dsRNA-injected adults decreased significantly despite
43 the presence of mannitol, compared to that of the control adults. Taken together, our
44 results suggest that *T. castaneum* adults recognized mannitol/sorbitol using TcGr20
45 receptors, thereby facilitating their dietary intake.

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46 # Introduction

47 Feeding behavior in insects is comprised of several processes for recognizing
48 chemical compounds, tasting, continuous feeding, and digestion [1]. Food-acceptance or
49 food-rejection actions in insects are determined by non-volatile compounds such as
50 carbohydrates and caffeine contained in host plants [2, 3]. Chemical compounds stimulate
51 the gustatory receptors (Grs) located in external sensory organs. Stimulated Grs transmit
52 electrochemical signals through sensory neurons to the sub-esophageal ganglion and
53 brain, thereby regulating the feeding behaviors of insects [4, 5]. Feeding behaviors differ
54 across diverse insect species [6]. For example, larval growth of the specialist-feeding
55 silkworm *Bombyx mori* depends solely on mulberry leaves [7]. *B. mori* perceives some
56 gustatory stimulants such as sucrose, inositol, morin and β-sitosterol contained in the
57 mulberry leaves using maxillary palps [8]. *Myo*-inositol, an indispensable nutrient and a
58 feeding behavior prolonging factor in *B. mori* larvae, was recognized via BmGr10
59 expression in the sensory organs [9]. Therefore, Grs in specialist feeders play a key role in
60 detecting specific compounds. Meanwhile, a generalist feeder, *Helicoverpa armigera*,
61 utilizes a wider range of host plants. Given that generalists feed on various plants and

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62 plant products, many types of non-volatile chemical compounds contained in foods may be
63 sensed by various Grs-expressing sensory organs [10, 11].

64 *Tribolium castaneum*, a major pest of grains, cereals, pasta, chocolates, and nuts
65 [12, 13], possesses 207 *Grs* genes available as gene models based on genome analysis [14].
66 Tissue-specific expression analyses have shown that 34 *Grs* genes in the antennae
67 associated with the gustatory perception, with many more types of Grs expressed in
68 antennae than in other insect species [15]. This expanded Gr family likely plays a
69 functional role for host selection. One pressing question pertains to whether *T. castaneum*
70 recognizes a suite of chemicals common to many products, or whether it is attracted to
71 specific chemicals. To answer this question, a dietary intake evaluation of *T. castaneum* is
72 required using an artificial diet composed of as few compounds as possible. Dietary intake
73 of *T. castaneum* has been previously examined using dried flour, but this is insufficient for
74 evaluating preferences since the organic compounds in flour cannot be completely
75 separated [16, 17]. Therefore, the establishment of a dietary intake assay in an
76 organic-free state is required. One commonly-used method involves the measurement of
77 swallowed liquid food composed of sugar and water, such as in the CAFE (Capillary

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78 Feeder) assay [18]. However, this method is only applicable for sucking or licking insects.
79 Because *T. castaneum* prefers dry products with water content less than 12% [17, 19],
80 CAFE cannot be applied for investigations into its dietary preference. Little is known about
81 the dietary intake of *T. castaneum* due to the lack of technical methods using dry
82 compounds. This study developed a novel dietary intake estimation method using gypsum
83 block without added organics. Since the gypsum eaten by *T. castaneum* adults is eventually
84 excreted without digestion as waste, the measurement of excreta allows for the
85 quantification of dietary intake in *T. castaneum*.

86 Additionally, sugar preference can be determined by adding sweeteners to act as a
87 stimulant for feeding behavior. Sweeteners would act as ligands to stimulate Grs; however,
88 to date, only few Grs have been identified as ligand-stimulated in the ectopic system in
89 generalist feeders, including *T. castaneum*. HarmGr4, found in *H. armigera*, responded to
90 fructose in the *Xenopus* oocyte expression system [11]. Furthermore, the Gr43-like clade in
91 Gr family including HarmGr4, have been found in various insects such as the Diptera and
92 Lepidoptera, where they act as receptors of fructose and *myo*-inositol [9, 20]. Since the
93 Gr43-like genes are also represented in *T. castaneum*, the candidate genes may be

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94 stimulated by any sugars/sugar alcohols. Here, we predicted that sweeteners deduced in
95 the dietary intake assay were potentially ligand stimulators of Gr43-like genes in *T.*
96 *castaneum*. This study demonstrates ligands of TcGr20 belonging in the Gr43-like gene
97 family using an exogenous expression system with a two-voltage clamp assay. We also
98 demonstrate the *in vivo* effect of the combination of RNAi and the dietary intake assay.
99

100 # Materials and methods

101 ## Insects

102 Red flour beetles (*Tribolium castaneum* Herbst.) were obtained from Sumica
103 Technoservice Co. (Hyogo, Japan) and reared on whole-wheat flour (Pioneer-kikaku,
104 Kanagawa, Japan) and yeast (Saf-instant®, Lesaffre, Marcq-en-Baroeul, France). They were
105 maintained at 29 ± 1°C under a 16 L:8 D cycle.
106

107 ## Total RNA preparation and cDNA synthesis

108 Total RNA was isolated from the antennae and proboscis of 20 adults using
109 ISOGEN II (NIPPON GENE, Tokyo, Japan) following the manufacturer's instructions. cDNA

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110 was synthesized from total RNA by ReverTra Ace® (TOYOBO, Osaka, Japan) using Oligo-dT
111 primers. Primer sequences are shown in S1 Table. The cDNA transcription reaction was
112 performed at 42°C for 90 min to produce cDNA and 99°C for 5 min to denature the
113 enzymes. Open reading frames (ORF) of *TcGr20, TcGr21, TcGr27 and TcGr28* were
114 amplified from cDNA by PCR using a high-fidelity DNA polymerase, PrimeSTAR® HS
115 (TaKaRa Bio, Shiga, Japan) with specific primers containing restriction enzyme sites at 5
116 and 3 ends of ORF (S1 Table). Kozak sequences were inserted between restriction sites
117 and primer binding sites to enhance translational efficiency. PCR reactions were
118 performed as follows: 35 cycles of denaturation at 98°C for 30 s, annealing at 55°C for 15 s,
119 and extension at 72°C for 70 s. *TcGr20*, *TcGr21*, *TcGr27* and *TcGr28* were directly
120 subcloned into expression vector pT7XbG2 (DDBJ accession number, AB255037) and the
121 sequence analyses were performed by Eurofins Genomics (Tokyo, Japan) to confirm the
122 correctness of the construct. Sequence data were analyzed using FinchTV sequence
123 scanner software.
124

125 ## Quantitative RT-PCR

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126 Total RNA was isolated from male and female adults (n = 20) with ISOGEN II
127 (NIPPON GENE, Tokyo, Japan) following the manufacturer's instructions. Gene expression
128 levels were examined in various tissues: antennae, heads (without antennae but including
129 mouthparts), thorax, abdomen and legs. First-strand cDNA was synthesized using a
130 PrimeScript™ RT reagent Kit (TaKaRa Bio) following the manufacturer's instructions. The
131 quality and concentration of synthesized cDNA were measured using a NanoPhotometer®
132 NP80 (Implen, München, Germany). Quantitative RT-PCR was performed using SYBR®
133 Premix *Ex Taq*™ II (Tli RNaseH Plus, TaKaRa Bio) with a StepOnePlus™ (Thermo Fisher
134 Scientific, Carlsbad, CA) under the following conditions: a holding cycle at 95°C for 10 min,
135 followed by 40°C cycling stage of 95°C for 15 s, 60°C for 1 min, and melting curve stages
136 were carried out at 95°C for 15 s and 60°C for 1 min to confirm the presence of nonspecific
137 PCR reactions. Relative expression levels were calculated using $\Delta\Delta$Ct. The *TcGr20*
138 expression levels in tissues were normalized using the expression levels of *T. castaneum*
139 ribosomal protein S3 (*RpS3*, NCBI accession: NM_001172392.1). *RpS3* was used as a
140 housekeeping gene due to the expression stability through the stage from larvae to adult
141 [21, 22]. Primer sequences are shown in S2 Table.

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Capped RNA synthesis and two-electrode voltage clamp electrophysiology

Procedures followed those described in a previous study [9]. Briefly, capped-RNA (cRNA) were synthesized using mMESSAGE mMACHINE® T7 kit (Thermo Fisher Scientific) according to the manufacturer's instructions, and were kept at −80°C until use. The cRNA injected oocytes were incubated in modified Barth's saline (MBS) buffer supplemented with 10 mg/mL penicillin and streptomycin for 3 days at 20°C [23]. Water-injected oocytes, which had endogenous receptor activities alone, were used as negative controls. Whole-cell current was recorded with two-electrode voltage clamp in a perfusion system using Ringer's solution [9]. Current was amplified with an OC-725C amplifier (Warner Instruments, Hamden, CT, USA) at a holding potential of −70 mV, low-pass filtered at 50 Hz, and digitized at 1 kHz. Data was acquired by software pCLAMP™ 10 (Molecular Devices, Sunnyvale, CA). Dose-dependent response curves were fitted to a hill slope curve using Prism 6 software (GraphPad, San Diego, CA).

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158 **Evaluation of dietary intake**

159 We developed a novel feeding application for *T. castaneum* adults using gypsum
160 containing a near-zero amount of organic compounds instead of artificial diets and wheat,
161 termed a TribUTE (*Tribolium* Urges To Eat) assay. The artificial gypsum diets were
162 comprised of dry powder and water mixed in a ratio of 1.3:1 (w/w) containing 200 mM
163 sugars or sugar alcohol solutions. The mixed gypsum was allowed to solidify completely at
164 65°C for 48 h. *T. castaneum* adults were starved for one week in the absence of foods and
165 held in cages at 25°C. Each gypsum block of approximately 5 mm was provided to
166 individual adult beetles in a 24-well microplate. They were kept for 48 h at 25°C. The
167 eaten gypsum by *T. castaneum* adult was eventually excreted without digestion as a waste,
168 permitting the measurement of the amount of gypsum. The excrements of the artificial
169 gypsum diet were collected in 200 μL microtubes by using a thinning silicon wire under a
170 stereoscopic microscope. The excreta were dissolved with 50 μL deionized water to
171 remove sugars or sugar alcohols, and then the precipitate of gypsum was dried thoroughly
172 at 65°C for 24 h. Excreta were weighed by microbalance (AT201, Mettler-Toledo, OH).
173

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174 **RNA interference**

175 To silence the gene expression of *TcGr20* in adults, we synthesized
176 double-strand (dsRNA) *in vitro* using MEGAscript® T7 RNAi kit (Thermo Fisher Scientific)
177 according to the manufacturer's instructions. Primers sequences are shown in S3 Table.
178 The dsRNA at 1 μg/μL was kept at –80°C until use. A 150–200 nL volume of dsRNA was
179 injected between the internode of head and thorax in adult beetles using a capillary needle,
180 Nanoject II (Drummond Scientific Company, Broomall, PA) on a cooling block at –10°C.
181 Emerald luciferase (*Eluc*, TOYOBO, Osaka, Japan) was used as a negative control. The
182 similar sequences of *Eluc* were searched in NCBI *Tribolium* genome (ID:216). Since the
183 over 22-bp identical sequences of *Eluc* and *TcGr20* could potentially function as false
184 targets, these sequences were trimmed from the dsRNA regions. The dsRNA-injected
185 adults were kept at 25°C. Adult beetles were used for the TribUTE assays and quantitative
186 RT-PCR at 48 h after injections.
187

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188 # Results

189 ## Identification of feeding promoting stimulants in *T.*
190 ## *castaneum*

191 We found that *T. castaneum* adults consumed gypsum block in the absence of organic
192 compounds (Fig 1A). To discriminate gypsum excreta from conventional excreta derived
193 from wheat flour, gypsum block stained with Coomassie Brilliant Blue (CBB) R-250 (Wako,
194 Osaka, Japan) was given to adult beetles (Fig 1A). We visualized the digestive tract from
195 the foregut to the anus under a microscope, and found that the tract was partially stained
196 by CBB (Fig 1B), while the tract was not stained using gypsum without CBB (Fig 1B'). The
197 excreta of the stained gypsum were also observed (Fig 1C). Together, these findings
198 showed that *T. castaneum* adults recognized and fed on gypsum, and excreted it as a waste.
199 We then attempted to identify feeding-promoting stimulants for *T. castaneum*. We
200 produced gypsum blocks containing mono-, di-, and tri-saccharides or sugar alcohols as
201 candidates. As the amount of excreta with these additives was higher than the amount
202 produced from sugar-free gypsum, we assumed that *T. castaneum* recognized sugars and
203 sugar alcohols as attractants. *T. castaneum* actively fed on gypsum with added

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204 sugars/sugar alcohols, and excreted the gypsum as a waste. The amount of excreted
205 sweetened gypsum was significantly greater than gypsum without sweetener (Fig 1D).
206 Additionally, gypsum with 200 mM galactose and sucrose yielded a negligible and
207 non-significant difference compared to gypsum without sweeteners (Fig 1D). The amount
208 of excreta was markedly greater in the presence of 200 mM mannitol, demonstrating that
209 feeding behavior was stimulated based on the sweetener itself. These findings indicate
210 that mannitol acts as a strong attractant for *T. castaneum*.
211
212 **Fig 1. Establishment of feeding assay in *T. castaneum* adults using gypsum block.**
213 The starved *T. castaneum* adults show in a series of the behavior from recognition, eating
214 to excretion the gypsum diet. Scale bars: 1 mm. **A**. The adults bit gypsum. The gypsum was
215 stained with Coomassie Brilliant Blue (CBB). **B, B'**. Digestive tract of adult beetle with the
216 CBB staining gypsum (**B**) and without the CBB staining (**B'**). **C**. Excreta of the CBB staining
217 gypsum by *T. castaneum* (arrowheads). **D**. The effect of gypsum intake with sugar or sugar
218 alcohol mixture. Each sugar/sugar alcohol at 200 mM was contained in the gypsum block.
219 *T. castaneum* adults fed on the artificial diet for 48 h. The amount of excretion was

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220 measured using microbalance. Each plot represents the amount of excreta of adult beetles
221 in individuals (n = 4–10). Standard error bars show S.E.M. Statistical analyses were
222 performed one-way ANOVA and the post hoc Tukey's multiple comparison test ("**"
223 P<0.01, "***" P<0.001, "****" P<0.0001, "ns" no significant). Fructose, Fru; glucose, Glu;
224 galactose, Gal; sucrose, Suc; maltose, Mal; trehalose, Tre; mannitol, Man; sorbitol, Sor.
225
226

227 Phylogenetic analysis of insect Grs

228 Mannitol, a sugar alcohol, acts as a sweetener for vertebrate chemosensation [24],
229 and other sugar alcohols are readily recognized by insect species. In *B. mori*, sugar
230 alcohols such as *myo*/*epi*-inositol were recognized by the gustatory receptor BmGr10
231 belonging to the Gr43-like clade [9]. We aimed to identify homologous genes in *T.*
232 *castaneum* belonging to the same clade as BmGr10 based on the phylogenetic analyses
233 using amino acid residues. The phylogenetic tree of the insect Grs was constructed using
234 neighbor-joining (Fig 2). TcGr20, 21, and 25–28 belonged to the clade including the
235 DmGr43a fructose receptor derived from *Drosophila melanogaster*, the BmGr9 fructose

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236 receptor and BmGr10 *myo*/*epi*-inositol receptors derived from *B. mori*, and the HarmGR4
237 fructose receptor derived from *Helicoverpa armigera* [9, 11, 20]. On the basis of these
238 results, we hypothesized that mannitol receptors belonged to the TcGr20, 21, and 25–28
239 groups.
240
241 **Fig 2. Phylogenetic analysis of deduced amino acid sequences of Gr43-like genes.**
242 Amino acid sequence alignment was generated using ClustalW, and a rooted tree of insect
243 Grs was conducted by neighbor-joining method in MEGA ver. 7 [43]. The percentage of
244 replicate trees are shown in the associated taxa clustered together in the bootstrap test of
245 500 replicates. The scale bar represents 0.2 substitutions per amino acid site. Amino acid
246 sequences of *B. mori* and *T. castaneum* Grs were obtained from [14, 44], respectively. The
247 other amino acid sequences, AmGr3 derived from *Apis mellifera*, HarmGr4 from
248 *Helicoverpa armigera*, DmGr43 and DmGr66a derived from *Drosophila melanogaster*, were
249 obtained from the NCBI public database.
250
251

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252 ## TcGr20 is a mannitol/sorbitol receptor

253 We attempted to amplify the ORF in the *TcGr20*, *21*, and *25–28* genes for
254 subcloning into the *Xenopus* oocyte expression vector. However, we failed to obtain PCR
255 products of *TcGr25* and *26* from any cDNA produced from any sampled tissues and stages.
256 Hence, we analyzed the biochemical functions of TcGr20, 21, 27 and 28 using
257 electrophysiological analysis. Two-electrode voltage-clamp recording was performed in
258 accordance with a previous study [9]. We reconfirmed a BmGr10 response to *myo*-inositol
259 using a two-electrode voltage clamp as a positive control (S1 Fig). Each *TcGr20, 21, 27* and
260 *28*-expressing oocyte was clamped with electrode capillaries filled with 3 M KCl. When
261 mannitol was added to the perfusion chamber, an inward current was observed in
262 *TcGr20*-expressing oocytes (Fig 3A), but not in *TcGr21*-, *TcGr27*- or *TcGr28*-expressing or
263 water-injected oocytes (Fig 3B, S2 Fig). TcGr20 also responded to sorbitol but not the
264 other sugars (Fig 3A). We did not observe a current response in *TcGr21*, *27* or
265 *28*-expressing oocytes for any sugars/sugar alcohols tested. The
266 mannitol/sorbitol-induced currents observed in TcGr20 expressing oocyte were
267 concentration-dependent to 20-200 mM mannitol and 40-230 mM sorbitol (Fig 4A, 4B).

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268 Based on the dose-response curves, the EC_{50} value of mannitol and sorbitol were 72.6 ±
269 9.1 mM and 90.6 ± 10.4 mM, respectively (Fig 4C, 4D). This result suggests that TcGr20
270 functions as a mannitol/sorbitol receptor.
271
272 **Fig 3. Current recording of *Xenopus* oocytes expressing TcGr20.**
273 **A**. Inward current response of *Xenopus* oocytes expressing TcGr20 to candidate tastants
274 (arrowheads). Tastants were tested at 200 mM. **B**. The current of water-injected oocytes
275 to same tastants were also recorded. The current data are representative of recordings
276 independently performed in several times.
277
278 **Fig 4. Ligand dose-dependent response of TcGr20.**
279 Two-electrode voltage clamp recordings of TcGr20-expressing *Xenopus* oocytes. **A-B**.
280 Inward current response of TcGr20-expressing oocytes with a range of 20 to 400 mM
281 mannitol (**A**), and 40 to 230 mM sorbitol (**B**). Each arrowhead represents various
282 concentrations. **C-D**. Curves were fitted with a standard slope, and EC values were

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283 calculated for mannitol (**C**) and sorbitol (**D**), respectively. Data are shown as mean ± S.E.M.
284 (n = 3).
285
286
287 ## Tissue expression of *TcGr20*

288 Previous studies have shown that *TcGr20* is predominantly expressed in the
289 antennae with RNA sequencing and *in situ* RT-PCR [15, 25]. Our findings here confirm that
290 *TcGr20* is mainly expressed in the antennae (Fig 5), as well as in head structures including
291 the mouthparts, and in the thorax, abdomen, and legs in both males and females. *TcGr20*
292 was highly expressed in antennae in both male and female adults (Fig 5A, 5B). TcGr20 on
293 the antennae likely acts as sensors for mannitol and sorbitol.
294
295 **Fig 5. Tissue expression of *TcGr20*.**
296 The relative expression level of the *TcGr20* in different tissues was determined by
297 quantitative RT-PCR. *TcGr20* expression in male (**A**) and female (**B**). An, antennae; H, head;
298 T, thorax; Ab, abdomen and L, legs. Relative expression was caluclated using $\Delta\Delta$Ct method.

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299 Ribosomal protein S3 (*RpS3*) in *T. castaneum* was used as the control to normalize the
300 amount of templates. Data are shown as mean ± S.E.M. (n = 3).
301
302

303 Effect of mannitol/sorbitol concentrations in the dietary 304 intake assay

305 We measured the dietary intake of *T. castaneum* adult beetles using gypsum containing
306 mannitol or sorbitol at various concentrations (Fig 6). The dietary intake increased in the
307 presence of 100 mM mannitol (Fig 6A) and 200 mM sorbitol (Fig 6B), respectively. The
308 amount of gypsum excreta in individuals at 48 h was 0.6 ± 0.07 mg for 100 mM mannitol,
309 and 0.26 ± 0.03 mg for 200 mM sorbitol (Fig 6). These results indicate that mannitol
310 stimulates a feeding response even at lower concentrations, promoting dietary intake by
311 the beetles.
312

313 **Fig 6. Concentration response of mannitol and sorbitol in TribUTE assay.**

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314 The dose effect of gypsum intake with sugar alcohol mixture. **A**, mannitol; **B**. sorbitol.

315 Sugar alcohols at various concentrations were contained in the gypsum block. *T.*

316 *castaneum* adults fed on the gypsum for 48 h. The amount of excreta was measured using

317 microbalance. Scatter plot represents the amount of excretion of adult beetles in

318 individuals (n = 5–7). Standard error bars show S.E.M. Statistical analyses were performed

319 one-way ANOVA and the post hoc Tukey's multiple comparison tests ("**" P<0.01, "***"

320 P<0.001, "ns" no significant).

321

322

323 Evaluation of dietary intake in *TcGr20*-silencing *T.* 324 *castaneum*

325 Our results showed that *T. castaneum* fed on gypsum in the presence of mannitol (Fig 6A),

326 and that TcGr20 was a mannitol receptor (Fig 3A). We therefore next investigated

327 whether TcGr20 was involved in mannitol recognition for feeding. One promising

328 approach for validating gene function, RNA interference (RNAi), was effective in *T.*

329 *castaneum* [26]. We injected *TcGr20* double-strand RNA (dsRNA) into *T. castaneum* adults;

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330 as a result, *TcGr20* expression was significantly suppressed compared with that of *emerald*
331 *luciferase (Eluc)*-dsRNA injected adults (Fig 7A). Using the *TcGr20* dsRNA-injected adult
332 beetles, we evaluated the dietary intake of gypsum in the presence of 100 mM mannitol.
333 The amounts of excreta from *TcGr20* dsRNA-injected adults significantly decreased in the
334 presence of 100 mM mannitol compared in comparison to that of *Eluc* dsRNA-injected
335 adults (Fig 7B). These results indicate that *TcGr20* RNAi is an effective tool, and that
336 TcGr20 is responsible for mannitol recognition *in vivo*.
337

338 **Fig 7. TribUTE assay in *TcGr20*-silencing *T. castaneum.***

339 **A**. Knockdown of *TcGr20* by using the injection of the dsRNA into the starved adult beetles.
340 The *Eluc*-dsRNA was injected as a control. *TcGr20* expression levels of whole body were
341 examined at 48 h after the dsRNA injection. Standard error bars show S.E.M. Statistical
342 significance was determined by *t*-test (P = 0.0006). **B**. Effect of gypsum intake in the
343 presence of 100 mM mannitol. The gypsum in the presence of 100 mM mannitol was given
344 to the *TcGr20*- and *Eluc*-dsRNA-injected adult beetle individuals at 48 h after dsRNA
345 injection, respectively for 48 h. Amount of gypsum was measured as excretes. Each plot

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346 represents the amount of excreta of adult beetles in individuals (n = 24). Standard error
347 bars show S.E.M. Statistical significance was determined by *t*-test ("****" $P < 0.0001$).
348
349

350 Discussion

351 Our results demonstrate that mannitol acts as a significant feeding attractant in *T.*
352 *castaneum* adult beetles, and that TcGr20 is responsible for mannitol/sorbitol recognition
353 and the promotion of dietary intake.

354 Gustatory receptor (Gr)-expressing chemosensory organs are involved in external
355 non-volatile compound recognitions. Antennae and legs recognize sucrose in *Tribolium*
356 *brevicornis* in electrophysiological analyses [27]. In *H. armigera,* the fructose receptor
357 HarmGr4 in the antenna recognized fructose [11]. Hence, *TcGr* genes expressed in
358 antennae or legs were hypothesized as being involved in the perception of mannitol as an
359 external signal. In the present study, we used qRT-PCR analysis to show that *TcGr20* was
360 expressed in the antennae. Previous analyses such as tissue-specific RNA-seq and *in situ*
361 PCR have also shown that *TcGr20* is expressed in the antennae [15, 25], and our results are

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362 consistent with these findings. We observed a difference in *Gr* expression levels between
363 males and females [28], implying the occurrence of sex-specific feeding behavior. However,
364 we observed no difference in *TcGr20* expressions between the sexes (Fig 5).
365 Electrophysiological analyses using *Xenopus* oocytes showed that TcGr20
366 contributes to responses to mannitol and sorbitol (Fig 3). The EC_{50} values of TcGr20 for
367 these sugar alcohols showed that the mannitol response was 0.8 times more sensitive than
368 the sorbitol response. Additionally, the dietary intake of *T. castaneum* adult beetles in the
369 TribUTE assay was also more sensitive to mannitol than sorbitol (Fig 6). Mannitol
370 response was significant at 100 mM, while the significance of sorbitol response was
371 observed at 200 mM (Fig 6), indicating that the concentration response of mannitol
372 appears to be correlated with that of TcGr20 response levels in the electrophysiological
373 analysis. It is likely that TcGr20 mainly regulates mannitol recognition in the gustatory
374 organs. We also examined whether TcGr20 is involved in mannitol recognition using RNAi
375 and the TribUTE assay. To explore effective RNAi in *T. castaneum*, *TcGr20*-dsRNA was
376 injected into pupae; however, the *TcGr20* expression levels in *TcGr20*-dsRNA-injected
377 adult beetles after eclosion showed a negligible difference compared to those injected with

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378 *Eluc*-dsRNA injection (S3 Fig). The 6–9-day period of pupal development prior to
379 adulthood [29] may have decreased the gene silencing effect. To test this possibility, we
380 attempted dsRNA injection into starved adult beetles. The test showed a significant RNAi
381 silencing effect (Fig 7A). Using the *TcGr20* RNAi beetles (Fig 7A), we showed that TcGr20
382 is responsible for mannitol recognition-dependent dietary intake behavior (Fig 7B). The
383 attraction of *TcGr20*-dsRNA-injected beetles to the gypsum block appeared to decrease in
384 the presence of 100 mM mannitol, resulting in a decrease in gypsum intake. It is important
385 to note that the silencing effects of RNAi were obtained temporarily. TcGr20 responds to
386 both mannitol and sorbitol, but these dietary intake assays should be further confirmed
387 using other methods such as the clustered regularly interspaced short palindromic repeats
388 (CRISPR)/Cas9 system.

389 In *T. castaneum,* six *TcGr* genes in the *Tribolium* genome (NCBI public data, ID:
390 216) were found as the Gr43-like clade (Fig 2). This study demonstrated that TcGr20 is a
391 mannitol/sorbitol receptor, but the functions of other *TcGr21-28* genes remain unclear.
392 These include *TcGr25* and *26*, for which we failed to amplify PCR products from any cDNAs.
393 The six *TcGr* genes would allow *T. castaneum*, as a generalist feeder, to be capable of

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394 recognizing a large number of natural attractants such fructose and sugar alcohols. Since
395 these *TcGr* genes were highly expressed in the antennae, contributing to the beetles'
396 ground-dwelling life style and scanning behavior [15], they may respond to non-volatile
397 stimuli produced by host plants. As alternative candidate targets, we expected that
398 compounds produced by fungi would be attractive, since beetles were strongly attracted
399 to chemical stimuli from fungi grown on flour and cotton seeds [30–33]. When the beetle
400 larvae fed on *Aspergillus niger,* suitable development occurred and the reproductive
401 potential of the females eventually increased (30-32).

402 Mannitol, the most widely distributed of the polyols, is found in more than 50
403 species of plants, algae, fungi, and lichens [34, 35]. In particular, dried seaweeds contain
404 1–1.7 M mannitol as a major carbohydrate component [36]. Sorbitol is also present in
405 prunes at 2.4 g/100 g dry weight and pears at 4.6 g/100 g dry weight [37]. *T. castaneum*
406 would recognize these stored foods because sugar alcohols are present at much higher
407 concentrations than are responded to by TcGr20. *A. niger* produces 45-210 mM mannitol
408 [38], implying that *T. castaneum* can recognize the fungi themselves using TcGr20. Fungal
409 growth is favored in high-moisture conditions in stored products and bulk grains. It is

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410 possible *T. castaneum* may be drawn to such stored products using such fungal attractants
411 as a cue. *T. castaneum* is also a harmful wheat flour pest [12, 39], attacking cake flour and
412 whole-wheat flour containing around 0.032 mg/g and 0.01 mg/g dry weight
413 mannitol/flour, respectively (S4 Table), which are levels far lower than biochemically
414 required for TcGr20 recognition. Therefore, *T. castaneum* adults are unlikely to recognize
415 wheat flour using TcGr20. Rather, they more likely recognize sugars such as glucose and
416 fructose contained in the wheat flour. Our TribUTE assay demonstrated that adult beetles
417 could, to some degree, recognize some sugars such as fructose, glucose, maltose, and
418 trehalose (Fig 1D). Based on these findings, we suggest that TcGrs in the sugar clade [15]
419 are potential candidates for recognition of these carbohydrates contained in wheat flour.
420 A novel artificial dietary method for *T. castaneum* adult beetles facilitates
421 examination of their dietary intake based on the eventual measurement of amount of
422 excreta. Gypsum, or calcium sulfate dihydrate, is an inorganic compound, formed into a
423 solid in combination with water (PubChem database, CID: 24928). The observation of
424 gypsum intake by *T. castaneum* demonstrated a series of behaviors from recognition and
425 swallowing to excretion. The TribUTE assay can quantify beetle preferences for

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426 non-volatile compounds by measuring gypsum excreta without consideration of odorant
427 stimulation. Sugars/sugar alcohols act as feeding behavior-facilitating factors in some
428 insects [40–42], and *T. castaneum* significantly preferred gypsum containing these
429 additives (Fig 1D). The TribUTE assay enabled the identification of non-volatile
430 compounds associated with the food preference of *T. castaneum.* Notably, adult beetles
431 can feed on gypsum without sweeteners (Fig 1). Thus, the TribUTE assay would be also
432 applicable for the exploring non-volatile compounds that induce a decrease in dietary
433 intake by *T. castaneum*. Such compounds would be useful in controlling *T. castaneum* in
434 the future.
435
436
437 **Author contributions**
438 TT, SK conceived and designed the experiments; TT, SK performed the experiments; TT,
439 RS, SK analyzed data; TT SK contributed reagents/materials/analysis tools; TT, SK wrote
440 the paper.
441

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442 **Conflicts of interest**

443 The authors declare no competing financial interests.

444

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449 and RNA.

450

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Supporting information

S1 Table. Primers for *Xenopus* oocyte expression

S2 Table. Primers for quantitative RT-PCR

S3 Table. Primers for double strand RNA synthesis

S4 Table. Mannitol in flours

S1 Fig. The BmGr10 response to *myo*-inositol as a positive control in a two-electrode voltage clamp

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616 **S2 Fig. Current recordings of *Xenopus* oocytes expressing TcGr21, TcGr27 and**

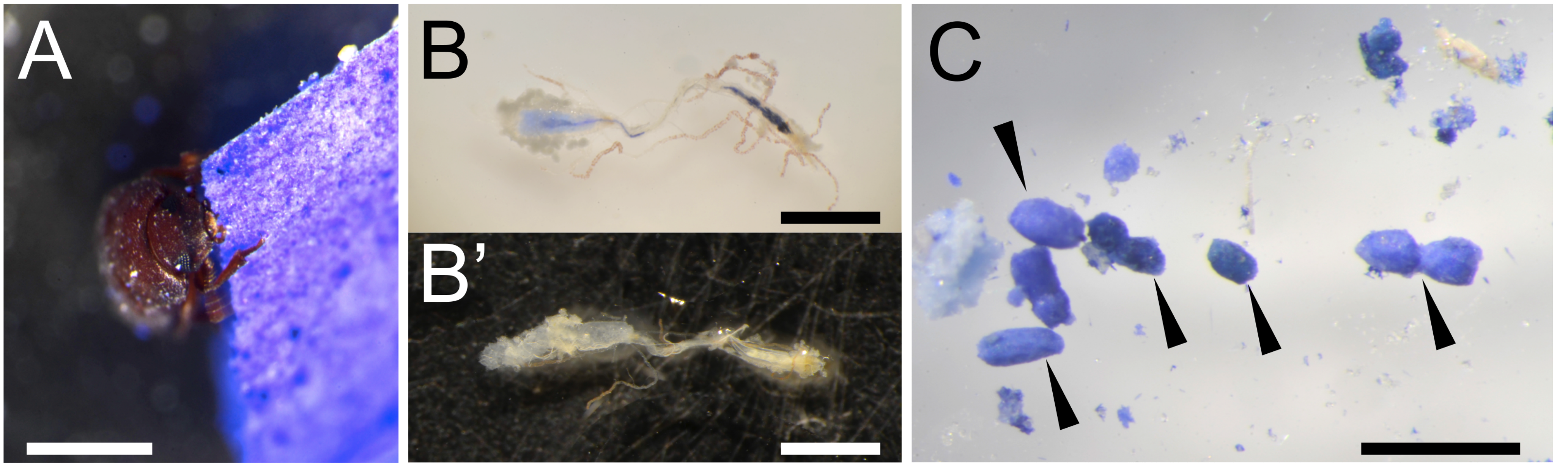
617 **TcGr28 against sugars and sugar alcohols**

618 **S3 Fig. Gene silencing effect**

619 **S1 file. Quantification of mannitol content in wheat flours**

620

1 Fig



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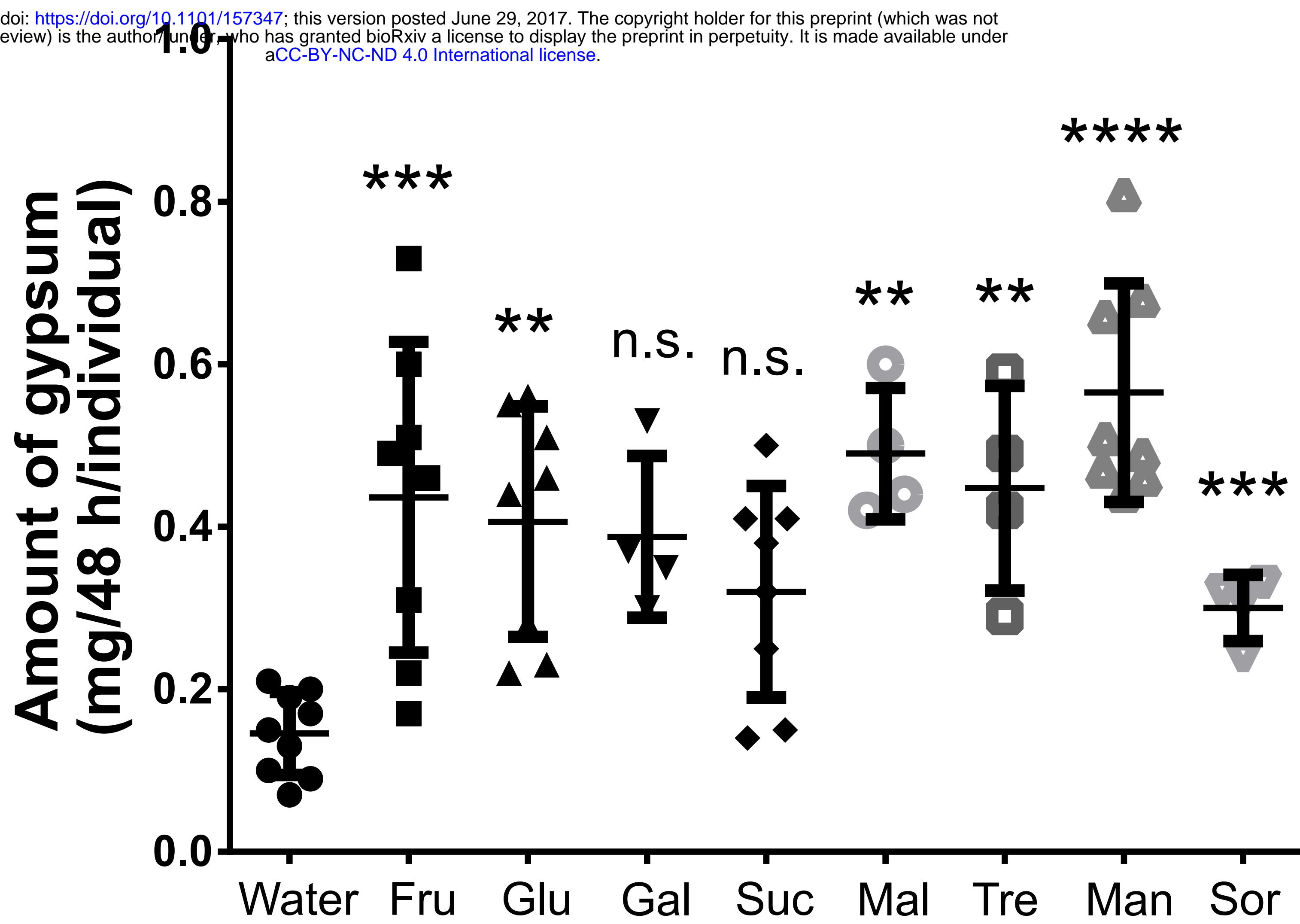
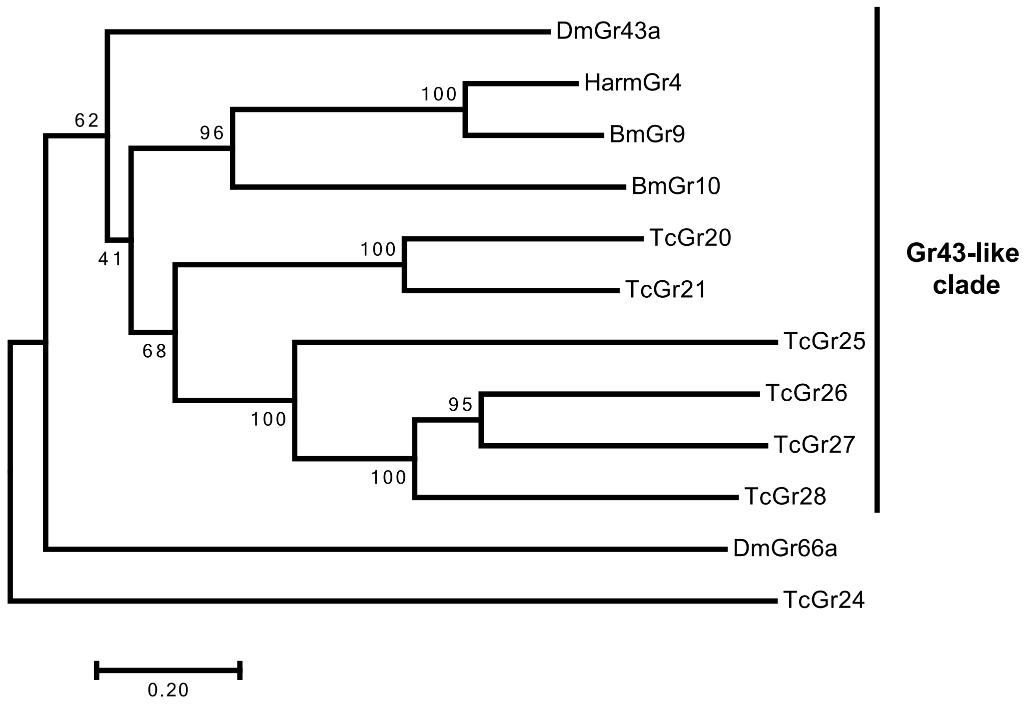


2 Fig



3 Fig

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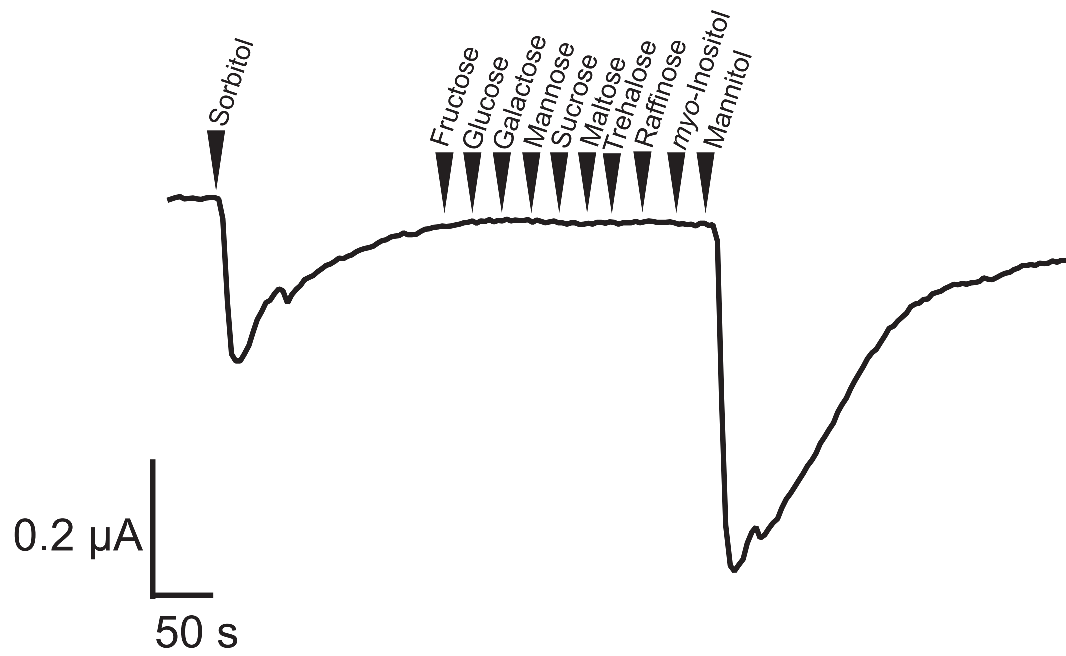

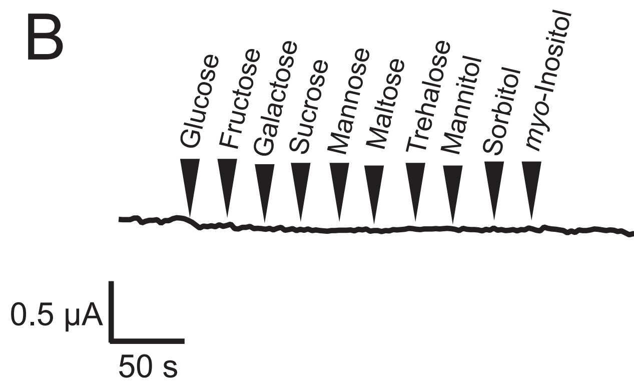

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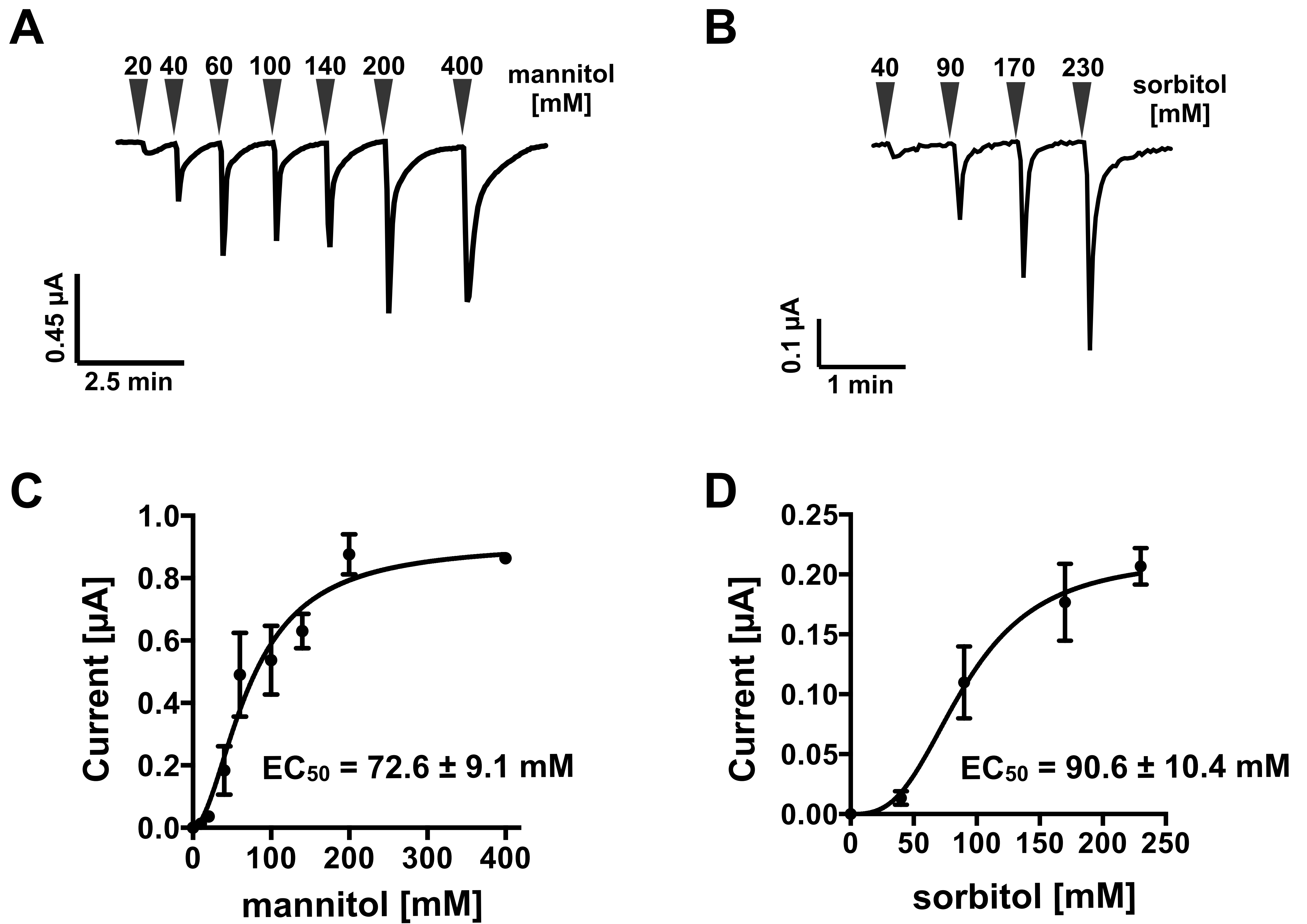
4 Fig



5 Fig

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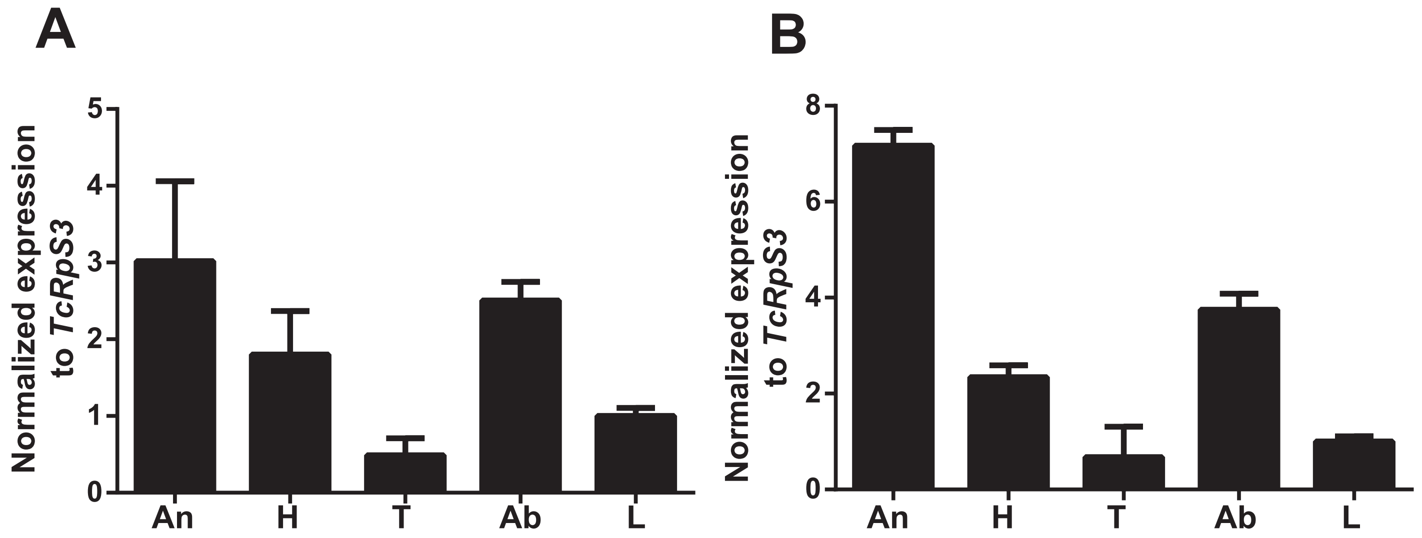

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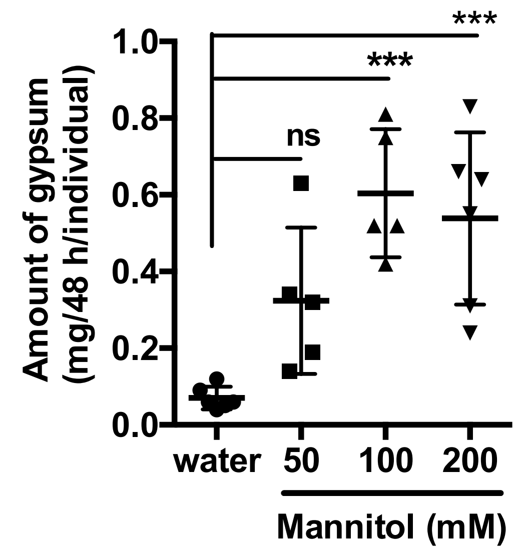
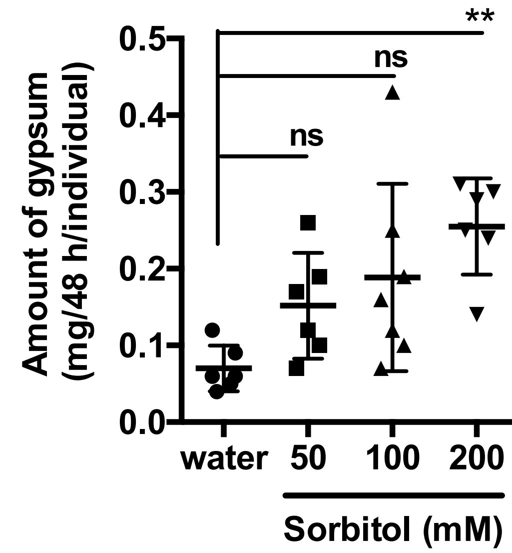
6 Fig





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7 Fig