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TribUTE-F assay: Fluorescence-based rapid quantification of dietary intake in the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae), facilitates evaluation of antifeedant inhibitory effects

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ABSTRACT

Tribolium castaneum (Herbst.) is an important pest of stored grains, nuts, and cereals worldwide. Different methods have been used to estimate dietary intake and feeding preferences of this beetle. A previously described TribUTE (*Tribolium* Urges To Eat) assay estimates its dietary intake by measuring the amount of gypsum excreta in beetles fed gypsum, a non-digestible and non-toxic compound, using an analytical microbalance. The method revealed the sweet preferences of *T. castaneum* adults. However, the measurements based on microbalance require individual quantitation of each sample, and the weight of the samples is sometimes below the detection limit of the microbalance. Here, an improved TribUTE assay, designated TribUTE-F assay, that uses gypsum labeled with the fluorescence dye ROX as an endogenous tracer is described. The fluorescence intensity remains constant during the consumption and excretion of the labeled gypsum and it is strongly correlated with the amount of gypsum. This approach was then used to evaluate the inhibitory effect of an antifeedant, mulberry latex, on dietary intake of *T. castaneum* adults. Compared with microbalance measurements, the new method enables more accurate measurement of near the minimum detectable quantities and is a suitable tool for the discovery of antifeedants.

1. Introduction

Red flour beetle *Tribolium castaneum* (Herbst.) is a serious, cosmopolitan pest that recognizes and feeds on a wide range of foods, including wheat flour, chocolate, dry fruits, nuts, pasta, germs of cereals, and processed foods (LeCato, 1975; Hagstrum, 2013). Experiments using wheat flour-based disks have shown that its feeding behavior is activated by carbohydrates (Krishna and Saxena, 1963; Xie et al., 1996). However, evaluation of the feeding preferences of *T. castaneum* has been limited because the organic compounds in wheat flour cannot be separated completely. Furthermore, the antifeedant and toxic activity of volatile compounds from plants and insecticides, such as fumigants, have been assessed using filter paper and a flour disk bioassay to estimate the mortality, growth rate, and progeny emergence in beetles (Chiam et al., 1999; Xie et al., 1996), but these assays are time-consuming. Previously, a method termed TribUTE (*Tribolium* Urges To Eat) assay has been developed for dietary intake estimation of *T. castaneum* adults using gypsum composed of calcium sulfate dihydrate without added organic compounds (Takada et al., 2017).

Dietary intake of *T. castaneum* adult beetles was facilitated with added sweeteners. The gypsum eaten by *T. castaneum* is then directly excreted without digestion, and thus, the amount of gypsum excreted as waste corresponds to an insect's feeding behavior. The dietary intake assay was conducted by measuring excreted gypsum waste using an analytical microbalance (Takada et al., 2017). However, the use of microbalance poses several problems. First, the amounts of excreted gypsum in the absence of sweeteners are sometimes below the minimum detectable weight of the balance. Second, for accurate quantification, errors due to humidity and electrostatic charge while using analytical microbalances should be minimized (Reichmuth et al., 2004). Finally, these measurements are time-consuming because the gypsum excreted by *T. castaneum* adults is weighted for each individual separately. An improved TribUTE assay will overcome these difficulties and can be applied to conduct high-throughput chemical screening. This study

Abbreviations: ROX, 5(6)-Carboxy-X-rhodamine.

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presents an improved quantification method for gypsum excreta using fluorescence dye as a tracer.

This new method was tested to evaluate the inhibitory effect of antifeedants, such as plant latex, on dietary intake in *T. castaneum* because it is sensitive enough to quantify a few granules of gypsum excreta. Plants exude latex from an injured spot upon mechanical damage or after attacks by herbivorous insects. The exuded latex contains sugar-mimicking alkaloids and defense proteins, such as cysteine proteases, that show toxic and defensive activities against polyphagous insects (Konno, 2011). A study on enzymatic activities and gene expression of *T. castaneum* adult beetles fed diets containing mulberry latex extracts revealed that mulberry latex inhibits glycosidase activities in this species (Tatun et al., 2014). However, it remains unknown whether mulberry latex acts as an antifeedant against *T. castaneum* adults. In the present study, the TribUTE-F assay was performed to examine whether mulberry latex acts as an antifeedant and effective inhibitor of *T. castaneum* dietary intake. This approach will facilitate evaluation of the inhibitory effects of antifeedants on dietary intake of *T. castaneum* adults.

2. Materials and methods

2.1. Insect

Red flour beetles (*Tribolium castaneum* Herbst.) were cultured in a whole-wheat flour (Pioneer-kikaku, Kanagawa, Japan) containing 5% yeast (Saf-instant®, Lesaffre, Marcq-en-Baroeul, France) at 29 ± 1 °C and 70% relative humidity under a 16:8 h light:dark cycle. Newly-emerged adults were kept for a week and used for the TribUTE assay. Male and female insects were not separated, as described in a previous study (Takada et al., 2017).

2.2. TribUTE assay

The dietary intake method was performed as described in the previous study (Takada et al., 2017). Briefly, gypsum diets were comprised of dry gypsum powder and water mixed in a ratio of 1.3:1 (w/w). The gypsum diets were not supplemented with any organic compounds. *T. castaneum* adult beetles were starved for 1 week as no food was provided, and they were kept in cages at 30 °C to facilitate their feeding behavior. The gypsum excreted as waste by *T. castaneum* adults was collected from each individual to estimate their dietary intake. At least five beetles were used to evaluate their dietary intake for each assay. All TribUTE assays were repeated at least three times as biological replicates, and the representative data are shown here. The complete set of data obtained for the biological replicates are given in supplemental data. All data were deposited in Mendeley Data.

2.3. Quantification of the amount of excreta based on fluorescence intensity

A fluorescence dye, ROX, 5-Carboxy-X-rhodamine (Thermo Fisher Scientific, Carlsbad, CA, USA) at 2.5 μM was added to the gypsum diet, and the mixture was vortexed to ensure complete dispersion. The gypsum was kept at 25 °C for 4 days in the dark until it solidified, and then stored under the same conditions until use to prevent fluorescence extinction. To quantify both the amount of gypsum and fluorescence intensity, gypsum was dissolved in 100 μL of distilled water using an ultrasonic bath (ASU-10; AS-ONE, Osaka, Japan) at middle oscillations for 10 min. The gypsum pellet was collected by centrifugation at 5000 × *g* for 10 min. The supernatant was used to measure fluorescence intensity using a plate reader Infinite® 200 (TECAN Trading AG, Männedorf, Switzerland) installed with filters (excitation 560/20 nm, emission 610/20 nm) at 20 °C. The amount of gypsum pellet was measured using an analytical microbalance (AT201, Mettler-Toledo, Columbus, OH, USA). Gypsum without ROX was used to measure endogenous fluorescence intensity, which, although almost negligible, was subtracted as non-labeled food from experimental readings. Statistical analyses were conducted in Prism 6 (GraphPad, San Diego, CA, USA).

2.4. Inhibitory effect of mulberry latex on dietary intake of T. castaneum

Mulberry latex was collected by cutting the petioles of the well-developed mulberry tree growing at Tokyo University of Agriculture and Technology (Koganei, Tokyo, Japan). The latex was independently collected several times from different trees and stored at −30 °C until use. The latex was diluted with distilled water containing ROX and mixed with gypsum powder in different ratios to prepare 100-, 10-, and 3-fold dilutions. Gypsum mixture with no latex added (organic-free gypsum) was used as a control. These gypsum dilutions were fed to beetles for 48 h. Dietary intakes of *T. castaneum* were examined based on fluorescence measurements as described above.

3. Results and discussion

The fluorescence in gypsum diet was preserved after a suite of processes, from the time adult beetles bit into gypsum and swallowed it, to the time it was excreted. The gypsum excreta emitted fluorescence that could be observed under a microscope (Fig. 1A), which was then quantified by measuring the fluorescence intensity. The coefficient of determination between the amount of gypsum and fluorescence intensity caused by the ROX dye as a tracer was defined using a linear correlation of the variables (Fig. 1B). The change in relative fluorescence intensity emitted by ROX correlated strongly with the amount of gypsum. In *Drosophila*, quantification of dietary intake was based on colorimetry (Deshpande et al., 2014; Masek et al., 2014). For color spectrometry, individual *Drosophila* flies fed dye-labeled food were homogenized to measure the absorbance by subtracting the absorbance of non-dye-fed flies used as controls from experimental readings (Masek et al., 2014). The present study was inspired by this approach; however, the colorimetry is restricted by the wavelength derived from each dye, which must have a considerably higher signal-to-noise ratio, a property that cannot be achieved in gypsum.

The required characteristics of a tracer dye applicable in the TribUTE assay are as follows: (i) high stability even during water loss; (ii) non-toxic to and not absorbed by the body; and (iii) low adsorptivity by tissues and proteins. These requirements were met by the ROX dye. The relative fluorescence intensity of the ROX dye

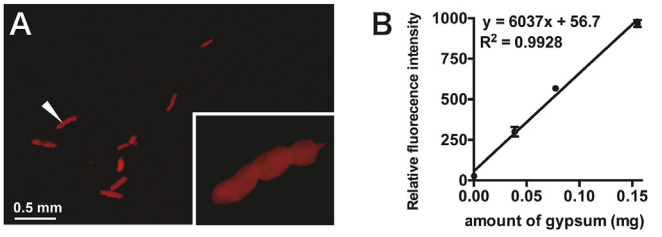

Fig. 1. Gypsum staining with ROX fluorescence dye. A. Gypsum excreted by *Tribolium castaneum* adult beetle. Excreta were observed using fluorescence microscopy with RFP filter. Scale bar shows 0.5 mm. Arrowhead indicates the inset. B. Quantification based on the fluorescence intensity. The fluorescence intensity emitted by gypsum represents relative fluorescence intensity. The means are for gypsum in the range from 0 to 0.15 mg. Standard error bars are SEM ($n = 3$).