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The Role of Salivary Enzymes in the Detection of Polysaccharides in the Termite *Reticulitermes flavipes* Kollar (Isoptera: Rhinotermitidae)

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Abstract

This study tested the ability of the termite *Reticulitermes flavipes* to detect the presence of large polysaccharides when salivary enzymes catabolize them. Previous work has found that the saliva of *Reticulitermes* contains cellulase and amylase but not xylanase. In several experiments, lab colonies were given choices between glucose and starch in the presence and absence of an amylase inhibitor or a choice between xylan and xylose. The results found that there was no preference between artificial food that contained equal amounts of starch and glucose but termites did prefer food containing xylose over food containing xylan. In addition, the presence of an amylase inhibitor in an artificial food source reduced the termite's preference for food containing starch. The results confirm that the enzymes are necessary for termites to detect the presence of polysaccharides. Termites were previously found to prefer denser wood and higher concentrations of cellulose. The mechanism found here provides an explanation of how wood density is determined by termites.

Introduction

Termites are selective in which types of wood they feed on (Smythe & Carter, 1970; Waller, 1988; Waller et al., 1990; Morales-Ramos & Rojas, 2001, 2003; Ngee et al., 2004; Aihetasham & Iqbal, 2012). The mechanisms termites use to select certain wood products over others have been of interest for ecological and economic reasons. Wood species vary in chemical make-up and structure providing a number of possible cues termites could use in food selection. Natural compounds such as sugars (Abushama & Kamal, 1977; Waller & Curtis, 2003; Saran & Rust, 2005; Swoboda & Miller, 2005; Haifig et al., 2008; Haifig et al., 2010; Wallace & Judd, 2010; Castillo et al., 2013), amino acids (Swoboda & Miller, 2005; Castillo et al., 2013), urea (Swoboda & Miller, 2005; Haifig et al., 2008; Castillo et al., 2013), and phosphates (Botch et al., 2010) were previously found to increase termite feeding. It has also been suggested that wood fiber density also plays an important role in food selection. Waller et al. (1990) found that an equal or greater biomass was consumed by *Coptotermes formosanus* when they were exposed to a wood that was compressed beyond its normal density. Denser woods have a higher amount of cellulose per unit volume. Judd and Corbin (2009) found that *Reticulitermes flavipes* prefers food with higher concentrations of cellulose. However, it is unlikely that termites have a receptor for large polysaccharides such as cellulose or starch. If termites could catabolize these molecules in their saliva, then denser woods would produce higher concentrations of glucose (Judd & Corbin, 2009) and thus digestion and absorption through salivary fluid breakdown of polysaccharides may be a potential avenue for investigating how termites assess wood density.

The breakdown of polysaccharides occurs in multiple points in the termite digestive tract. Most termites have cellulose and amylase in their saliva (Hewitt et al., 1974; La Fage & Nutting, 1978; Inoue et al., 1997; Tokuda et al., 2002; Tokuda et al., 2005) but their saliva lacks hemicellulases such as xylanase (La Fage & Nutting, 1978; Inoue et al., 1997; Brune, 2014). In the hindgut, microorganisms produce multiple enzymes to digest polysaccharides including cellulase, amylase and hemicellulases such as xylanase (Brune, 2014). Thus, the saliva of termites is

more limited in its digestive ability than the hindgut. This has implications in the ability of termites to respond to various polysaccharides. The feeding responses of the genus *Reticulitermes* in response to sugars has been well studied. Both glucose and xylose were found to increase the feeding response in species of this genus (Waller & Curtis, 2003; Saran & Rust, 2005; Swoboda & Miller, 2005; Wallace & Judd, 2010). Thus, termites are able to detect both sugars. Inoue et al. (1997) found that *R. serratus* has both cellulase in their saliva whereas there is virtually no xylanase activity. Park et al. (2014) found that *R. serratus* produces α-amylase which is commonly found in insect salivary glands (Cohen, 2004) and has been found in the saliva of other termite genera (Hewitt et al., 1974; La Fage & Nutting, 1978). Thus, if *Reticulitermes* foragers are detecting polysaccharides salivary fluid then starch should be detectable to the termites whereas xylan would not be detectable.

In this study, we determined if the termite *Reticulitermes flavipes* could directly detect large polysaccharides or if they needed to break the polysaccharides down prior to detection. We used artificial food sources to compare the termites' consumption of artificial foods containing potato starch or glucose in the presence or absence of an amylase inhibitor. We also tested if xylan has a similar palatability as xylose to termites. If the termites are determining density of polysaccharides in the food by breaking them down, then 1) they should prefer xylose over xylan but should not prefer glucose to starch, and 2) the amylase inhibitor should reduce the termites' feeding rate on starch enriched foods.

Methods and Materials

Collections: Colonies were sampled from Cape Girardeau County using termite traps as described by Judd and Fasnacht (2007). Traps were placed in known location in order to collect ten colonies. In the case of Experiments 1 and 4, only eight of the ten traps had enough termites to conduct the experiment.

Experiment 1: In this experiment we tested whether *R. flavipes* showed a preference for food enriched with glucose or potato starch. *R. flavipes* has amylase in their salvia that breaks down starch (La Fage & Nutting, 1978) and thus the prediction would be that there should be no feeding preference between food enriched with starch and food enriched with glucose.

Food: Two different artificial food sources were created in a similar manner as Judd and Corbin (2009). Both foods contained 1.5 g agar heated in 100 ml of distilled water. In the starch-enriched food, 1.0 g of potato starch was added and in the glucose-enriched food 1.0 g of glucose was added (Table 1). After the potato starch or glucose was added, 7.5 g of α-cellulose was added to both foods and the mixture was stirred and poured into petri dishes. Because cellulose doesn't dissolve in water, each dish was placed on a shaker to keep the cellulose suspended until the solution solidified.

Table 1. Summary of the molecules added to the two food items (see methods for full description of food) and the number of colonies tested in each experiment.

Experiment	Food 1	Food 2	Number of Colonies
1	Potato starch	Glucose	8
2	Xylan	Xylose	10
3	Potato starch + Amylase inhibitor	Potato starch	10
4	Glucose + Amylase inhibitor	Glucose	8*

* One colony died

Trials: From each of 8 colonies, 100 worker termites were extracted and housed in 17.8 x 17.8 x 5 cm sealed plastic containers filled with approximately 200 g of topsoil. Only workers were used because soldiers do not directly feed on food sources (La Fage & Nutting, 1978). Once each container was prepared, a 3.0 g piece of each food item (starch-enriched food and glucose-enriched food) was weighed and placed on a 3.5 cm x 3.5 cm note card. The food was then placed within each container in opposite corners and each corner was labeled to indicate what food item was present. Three additional containers were created without termites as controls to measure water loss in the food (Judd & Corbin 2009).

The containers were housed in a dark area at room temperature. Because the bins were sealed the moisture levels were kept constant. The control bins were housed with the other bins. Each trial period lasted 14 days and each food square was weighed every two days to determine how much food was eaten by the termites. During the weighing process, termites and soil were brushed away from the food before the mass was determined. Throughout the experiment the food sources remained intact and thus there was no issue of over-estimation of weight-loss due to crumbs lost in the soil.

Experiment 2: *Reticulitermes* lacks xylanase in their saliva (Inoue et al., 1997) therefore *R. flavipes* should not be able to break down xylan with their saliva. *Reticulitermes* was previously shown to prefer food enriched with xylose over unenriched food (Saran & Rust, 2005; Wallace & Judd, 2010). The prediction of this experiment was that termites should preferentially feed on xylose-enriched food because they lack the salivary enzymes to free the xylose from xylan. The methods for this experiment were identical to Experiment 1 except foods were enriched with xylan or xylose, respectively, rather than potato starch or glucose. 1.0 g of xylan was added to the xylan-enriched food and 1.0 g xylose was added to the xylose-enriched food (Table 1). A total of 10 colonies were used in this experiment.

Experiment 3: If the amylase is necessary for termites to detect the presence of starch in food items, inhibiting the

function of amylase should limit their ability to detect starch in foods. This experiment was identical to Experiment 1 except the two food items contained potato starch (instead of potato starch and glucose) and an amylase inhibitor was added to one of the food items (Table 1). If amylase increases the termites' response to starch, then the termites should preferentially feed on the starch-enriched food that lacks the inhibitor. During the food preparation, 1.0 g of potato starch was added to each food item. The food was allowed to cool to room temperature before 0.5 mg of amylase inhibitor was added to one of the food solutions. This was done to prevent the amylase inhibitor from denaturing. A total of 10 colonies were used in this experiment.

Experiment 4: This experiment insured that the presence of the inhibitor itself did not negatively affect the feeding behavior of the termites. To control for this effect, two identical glucose enriched foods were used except one contained the amylase inhibiter (Table 1). The amylase inhibiter should not affect the termites' ability to detect the glucose; therefore, both food sources were predicted to be equally palatable. This experiment had the same methodology as Experiment 1 except 1.0 g of glucose was added to both food types. The amylase inhibitor (0.5 mg) was added to one of the foods in the same manner as in Experiment 3. A total of 8 colonies were used in this experiment; however, one colony died part way through the experiment and was eliminated from the analysis.

Data analysis

The data were analyzed in the same manner as Judd and Corbin (2009). In each experiment, the final mass of the food was subtracted from the average final mass of the same type of food from three control bins. This controlled for change in mass loss due to water loss. The difference between the change in mass of the food and the average change in mass for the controls was the amount eaten by the termites. Negative numbers were converted to zeros, signifying that no significant amount of food was consumed by the termites. This only happened in three cases in Experiment 1 and three case in Experiment 2, and in all cases the result was within 0.025 g of 0.0 g. For each experiment, the amount of each food type eaten by the termites (adjusted weight loss) was compared using the Wilcoxon Signed-Rank Test.

Results

Experiment 1: There was no significant difference between the adjusted weight loss of glucose or starch enriched foods (T=13, p=0.2734; Fig. 1). Thus, the termites showed no preference for glucose or starch enriched food sources.

Experiment 2: The adjusted weight loss for the xylose enriched food was significantly higher than the adjusted weight loss of the xylan enriched food (T=6, p=0.027; Fig 1). Thus, the termites preferred the xylose-enriched food source.

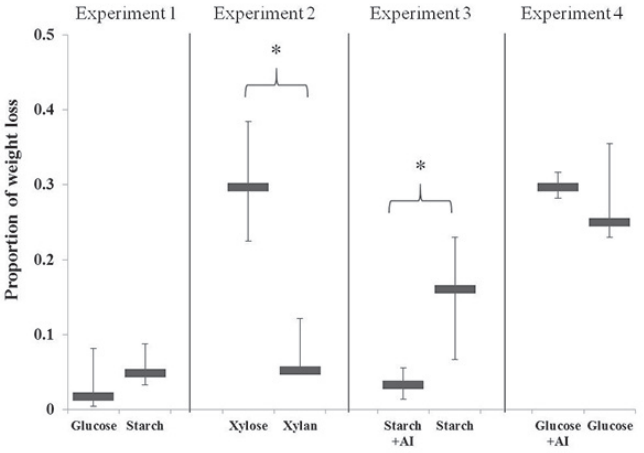

Fig 1. Median and quartiles of the percent weight loss of artificial food over a 14 day period due to termite feeding (adjusted for natural water loss from each food square) for all four experiments in this study. The four experiments were analyzed independently. An asterisk indicates a significant difference between percent weight loss ($p < 0.03$) of the two food types in the experiment.

Experiment 3: There was significantly higher adjusted weight loss in the food that lacked the amylase inhibitor compared to the adjusted weight loss in the food with the inhibitor (T=6 p=0.014; Fig. 1). These results suggest that the starch-enriched food item was preferred over the food enriched with starch and the amylase inhibitor.

Experiment 4: There was no significant difference in the adjusted weight loss between the food enriched with glucose and food enriched with glucose that included the amylase inhibitor (T=11.5, p=0.34; Fig 1). This result confirms that the amylase inhibitor did not affect the feeding behavior of the termites. Thus, the amylase inhibitor did affect the termites' ability to detect starch but not glucose.

Discussion

In this study we demonstrated that termites can detect larger polysaccharides only if the enzyme that hydrolyzes the specific polysaccharide is found in their saliva. In the experiments of this study, we provided termites food in which we controlled for the levels of a polysaccharide and its corresponding monosaccharide. Based on the fact that *Reticulitermes* has amylase in its saliva but not xylanase, we predicted that the termites should be able to detect the presence of starch but not xylan. As expected, there was no significant preference for food enriched with glucose compared to food enriched with starch but there was a significant preference for food enriched with xylose over food enriched with xylan (Experiments 1 and 2). We also predicted that the addition of an amylase inhibitor would lower the phagostimulatory response of *R. flavipes* to starch but not glucose. The results of Experiments 3 and 4 support this prediction. Thus, the results of this study suggest that termites are able to detect starch because the amylase in their saliva hydrolyzes the starch into its monomer components.

Enzymes that digest polysaccharides are produced in two major areas of the termite gut, the salivary glands and the hindgut (Brune, 2014). The latter has a larger suit of enzymes that attach large polysaccharides due to the presence of symbiotic bacteria (König et al., 2013; Li et al., 2013; Ni & Tokuda, 2013; Raychoudhury et al., 2013; Brune, 2014). The enzymes produced in the salivary glands are generated by the termites themselves (Brune, 2014). The role of the enzymes of the salivary glands is twofold: 1) they initiate the digestion of cellulose and starch from the food the termites consume (Saadeddin, 2014); free up smaller, detectable molecules (demonstrated herein). *Reticulitermes* saliva contains both amylases and cellulases (Inoue et al., 1997) and thus based on our results, termites should be able to detect the presence of cellulose. Indeed *R. flavipes* does prefer foods with higher concentrations of cellulose (Judd & Corbin, 2009). Interestingly, the saliva of the little soldier of *Macrotermes subhyalinus* has an endo-beta-D-glycosidase that is able to act on cellulose and xylan (Bedel et al., 2012). It would be interesting to see how this termite responds to xylan.

Assessing food quality is one of the many critical roles of taste. For social insect colonies, the ability to assess the quality of food allows for the colony as a whole to optimize the distribution of its foraging force. This phenomenon was previously well documented in social Hymenoptera such as honey bees and ants (Sudd & Sudd, 1985; Stein et al., 1990; Seeley, 1995; Judd, 2006; Cook et al., 2010, 2011). Subterranean termites burrow to and into food sources and thus assessing the quality of a potential food source is critical to colony success. This is especially true if one considers the energetic investment of burrowing in soil and wood (Lee et al., 2007; Bardunias & Su, 2009). Indeed, many studies have shown that termites are selective in the types of wood they feed (Morales-Ramos & Rojas, 2001; Ngee et al., 2004; Aihetasham & Iqbal, 2012). The role of small molecules in food palatability has been well studied and much like social Hymenoptera, termites are sensitive to different concentrations of sugars and regulate their feeding accordingly (Waller & Curtis, 2003; Saran & Rust, 2005; Swoboda & Miller, 2005).

It was proposed by Judd and Corbin (2009) that the preference of denser wood and higher concentrations of cellulose was a result of termite enzymes freeing up detectable sugars from polysaccharides. Higher concentrations of cellulose would produce higher levels of glucose per unit area when broken down by cellulase. Thus, the termites are detecting higher concentrations of glucose in denser wood (Judd & Corbin, 2009). Although cellulose was not directly tested in this study, the mechanism of polysaccharide detection found in this study confirms this mechanism is the likely explanation of how the concentration of cellulose is determined by the termites. The role of saliva in the regulation and enhancement of taste has been well studied in vertebrates (Pedersen et al., 2002). This study demonstrates a case for a similar role for insect saliva.

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