

## Chemical Ecology of Yucca Moth (*Tegeticula yuccasella*) Mating Systems

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### Abstract

The yucca moth, *Tegeticula yuccasella*, is an obligate pollinator of the yucca plant (*Yucca glauca*). Female moths collect and pollinate yucca flowers before laying their eggs within the ovule. Larvae do not consume all the developing fruit, completing the mutualistic interaction. Yucca moths mate inside the flowers, but little else is known about their reproductive behavior. The goal of this study was to determine if yucca moths, an archaic branch of Lepidoptera, use the sex pheromones common in modern moth species. Modern female moths release species-specific blends of chemicals from a gland that archaic moth species lack. Male moths only fly towards the blend produced by conspecific females. Prior research identified (Z)-11-hexadecenol (Z11-16:OH), a typical moth pheromone structure, from female *T. yuccasella* abdomens. In 2018, traps baited with Z11-16:OH and four similar odors were compared to Hexane control lures for attraction to *T. yuccasella*. Across all lure types, more males were caught compared to females. Statistical analysis by ANOVA indicated no impact of lure type on female trap catches. Lure type was a significant factor in trap catches of male moths, based on an ANOVA F-test. Results support that yucca moths, members of the archaic Lepidoptera species, do use sex pheromones similar to those known from modern species.

**Keywords:** Chemical Ecology, Yucca Moth, Mating Systems

### 1. Introduction

The yucca moth, *Tegeticula yuccasella* (Lepidoptera: Prodoxinae), is the obligate pollinator of the yucca, or soapweed, plant (*Yucca glauca*). The moths and plants are mutualists in a specialized brood pollination system<sup>1</sup> and a common example of plant-pollinator coevolution<sup>10</sup>. The yucca moth is found in an archaic branch of the Lepidopteran phylogeny; its basal position within the order was recently confirmed with genetic data<sup>5</sup>. Unlike modern Lepidopterans, female yucca moths have tentacle-like structures originating near their mouthparts that they use to gather and hold yucca pollen<sup>15</sup>. Female moths move to different plants from which they collected pollen from, and determine if the flowers have been previously oviposited in. If too many eggs are laid in a flower or if the flower's ovules have been damaged and the flower has not been pollinated, then the plant will preferentially abort the flowers, and the female moth's offspring will have nothing to feed on when they hatch<sup>9</sup>. If the flower is acceptable, the female moths lay their eggs in the ovules of the flowers<sup>7</sup>. The females then deposit some of their collected pollen on the stigma of the flower, to ensure the flowers will produce fruit. Larvae hatch and feed on the developing yucca fruit, but they don't consume all of it, thus the interaction is mutualistic<sup>1</sup>. After several weeks, larvae complete development and drop to the ground where they build cocoons and pupate<sup>7</sup>. Adult moths emerge in spring-summer, in sync with the flowering of the yucca plant.

Adult moths mate inside yucca flowers, and there is some evidence that both sexes rely on flower scent to identify their hosts<sup>14</sup>. Little else is known about yucca moth reproductive behavior. In modern Lepidopteran species, the use of sex pheromones for chemical communication in mating systems is common<sup>6</sup>. Female moths release species-specific volatile fatty acid type pheromone blends, which conspecific males detect during flight with highly sensitive antennae<sup>13</sup>. The purpose of this study was to determine if *Tegeticula yuccasella* moths use sex pheromones like modern

Lepidopterans. The yucca moth/yucca plant mutualism is unique to North America, and recent studies have investigated the specific number of species involved in such relationships. Recent revision of the previously called *Tegeticula yuccasella* species has indicated that at least 13 species of yucca moth exist in the complex<sup>8,11</sup> including the *Tegeticula yuccasella* species analyzed in this study. Recent research has also investigated patterns of speciation of the yucca moth in North America<sup>2</sup>. If results of this study indicate species preferential sex pheromone use in mating systems, this research hints at and can be used to justify further investigation on the potential patterns of sympatric speciation of yucca moths.

Unpublished findings from preliminary experiments conducted by the Wanner lab (described as follows) also guided this study. Potential sex pheromones were identified by performing solvent washes on abdomens of female moths of the later identified species *T. yuccasella* and *T. corruptrix*. (Z)-11-hexadecanol, Z11-16:OH and (Z)-11-hexadecenyl acetate, Z11-16:OAc respectively were identified. Rubber septa formulated with different ratios of those pheromones were used as lures in Delta Traps (triangular pheromone traps with sticky bases that trap insects) at a Toston Dam, MT, site in the summer of 2012, but few moths were caught and all flower spikes in area had been eaten by deer. To determine if potential sex pheromones are attractive only if associated with floral odor, in the summer of 2014 traps were placed at two sites in Townsend, MT: Exit 350 East and Tosten Dam. Six scent treatments were applied at each site, with some traps containing yucca flowers as well (Table 1).

Table 1. Scent lure combinations tested in 2014, in Townsend, MT.

Lure #	Exit 350 East	Tosten Dam
1	100% Alcohol (OH)	Z11-16:OH + Flower
2	100% Aldehyde (Ald)	Z11-16:OAc + Flower
3	25% OH, 75% Ald	25% OH, 75% Ald + Flower
4	25% Ald, 75% OH	25% Ald, 75% OH + Flower
5	Blank Solvent	Blank Solvent
6	Blank Solvent + Flower	Blank Solvent + Flower

Traps with flowers successfully caught yucca moths, which were not identified by species. It was determined that yucca flowers were needed in future traps, as potential pheromones were only attractive when associated with floral odor. This discovery was consistent with recent literature<sup>14</sup>.

## 2. Methods

### 2.1. Collecting Samples

Moths analyzed in this study were captured in the summer of 2018. Great Falls, MT, is warmer than Townsend, MT, so yucca flowering is about one week ahead. A brief experiment to provide observations prior to primary data collection in Townsend was therefore conducted in Great Falls: Delta Traps with combinations of yucca flowers/none and 12 potential pheromones were placed on one to two meter tall stakes at a location with yucca in Great Falls at noon on June 13th, and moths in traps were collected at seven am the morning of the 14th. Small, though notably different, counts of moths were lured by potential pheromones.

On the nights of June 25th and 26th, and on July 5th, four repetitions of six Delta Traps (Figure 1) with lures (Table 2) were set in Townsend.

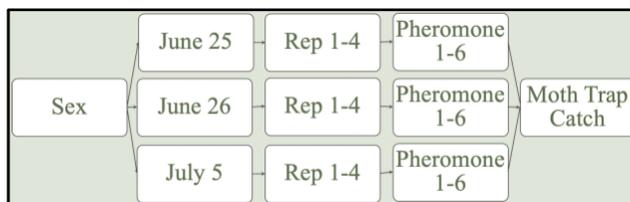


Figure 1. Generalized experimental design. Moths, by sex, were counted from each trap.

Table 2. Scent lure combinations used in 2018 Townsend, MT traps.

Lure #	Scent Combination
1	Z11-14:OH + Flower
2	Z11-16:OH + Flower
3	Z11-16:Ald + Flower
4	Z11-16:Oac + Flower
5	Lures 1-4 Mix + Flower
6	Hexane + Flower + Flower

1 uL of each potential pheromone in 50 uL hexane was applied to rubber septa for these traps. At the end of each repetition collecting period, sticky cards with caught moths were labeled appropriately and stored in 20 mL Falcon tubes or small plastic cups, then transferred back to the lab and stored at -20 degrees Celsius to preserve samples' genetic material.

## 2.2. Sample Processing and Identification

For the purpose of species and sex identification, abdomens from each moth were dissected off, then boiled for 15 minutes in 10% KOH solution in 1-dram (4 mL) glass bottles with loose screw tops. The abdomens were transferred to deionized water in ceramic twelve cavity spot plates, and hairs, innards, and residual glue from the sticky traps were gently removed with fine camelhair brushes and dissecting tools. The abdomens were then transferred to 0.6 mL microcentrifuge tubes filled with red Eosin Y Stain and dyed for at least four hours. Genitalia were then dissected out from the abdomens in 20% ethanol in spot plate cavities using a Motic dissecting scope and fine dissecting tools. Abdomens were stored in glycerol in 0.6 mL microcentrifuge tubes, for potential future slide mounting.

Species identifications were supported using dichotomous keys from two papers<sup>8,11</sup> on phylogeny of the *T. yuccasella* complex. Genitalia (Figure 2) were identified by sex and species visually at at least 12x magnification.

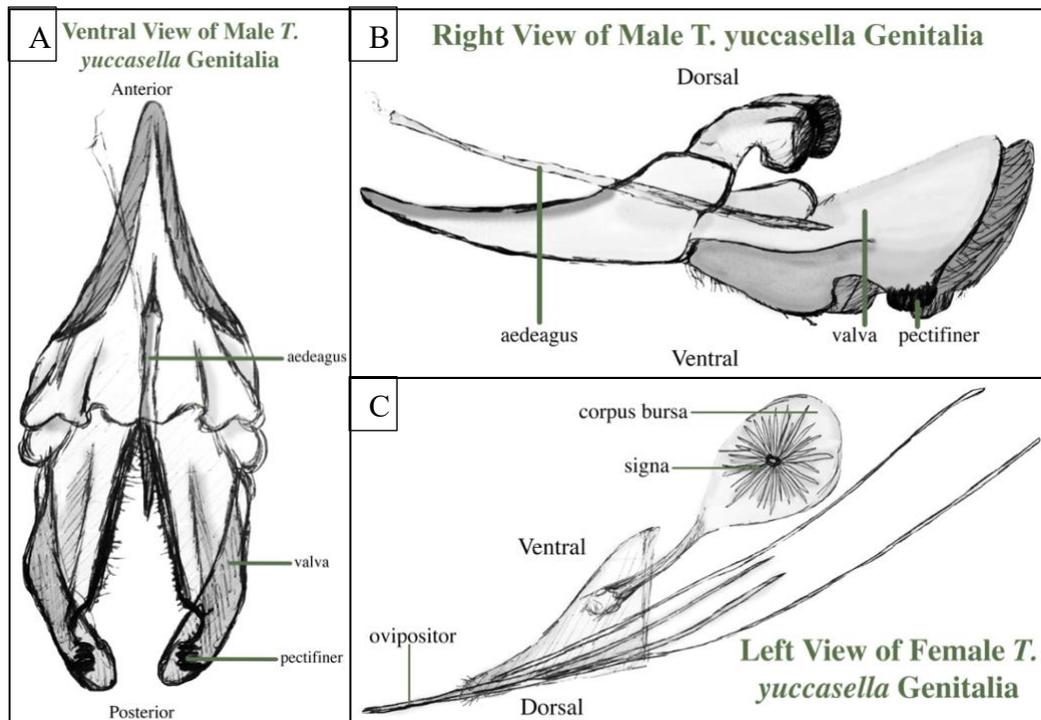


Figure 2. General structures of male (A, B) and female (C) *T. yuccasella* genitalia.

Visual identifications distinguishing *T. yuccasella* from other species collected were confirmed with DNA analysis. Genetic data of moth leg samples was obtained using PCR, and results were run through the BOLD Systems database<sup>4</sup>.

### 2.3. Statistical Procedures Used

The male moth count for this study was equal to 228 moths, while the female moth count was equal to 102 moths. Control traps, which included Hexane (not a potential pheromone candidate) and flowers caught an average of 2.58 males and 1.5 females. Because of the male bias for moths present when traps were set, Male and Female *Tegeticula yuccasella* counts were analyzed separately. All analysis, model fitting, and data visualizations were completed using R software<sup>12</sup>. Raw data are visualized in Figure 3.

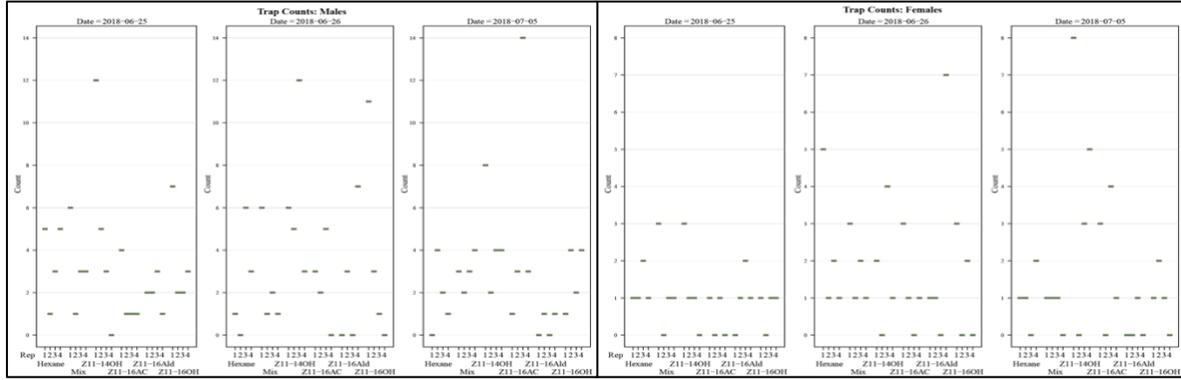


Figure 3. Male and female moths caught, per trap, by date, pheromone, and repetition.

Two mixed effects models were fit, which account for the random effects of Rep nested in Date, to analyze the relationship between potential pheromone and moth count for male and female moths separately, see equation (1).

$$\text{Moth Count}_{ijk} = \beta_0 + \beta_1 I_{\text{Mix}} + \beta_2 I_{14\text{OH}} + \beta_3 I_{16\text{Ac}} + \beta_4 I_{16\text{Alc}} + \beta_5 I_{16\text{OH}} + \text{Date}_i + \text{Rep}_{ij} + \varepsilon_{ijk}; \quad (1)$$

where:

$$i = 1, 2, 3 \text{ Dates}; j = j^{\text{th}} \text{ Rep. in } i^{\text{th}} \text{ Date}; \text{Date}_i \sim N(0, \sigma_{\text{Date}}^2); \text{Rep}_{ij} \sim N(0, \sigma_{\text{Rep}}^2); \varepsilon_{ijk} \sim N(0, \sigma^2_{\varepsilon});$$

and:

$$I_{\text{pheromone}} = 1 \text{ when pheromone was used as lure, } 0 \text{ if else.}$$

Effects plots for these models indicate more potential for significant differences in counts for male moths (Figures 4 and 5).

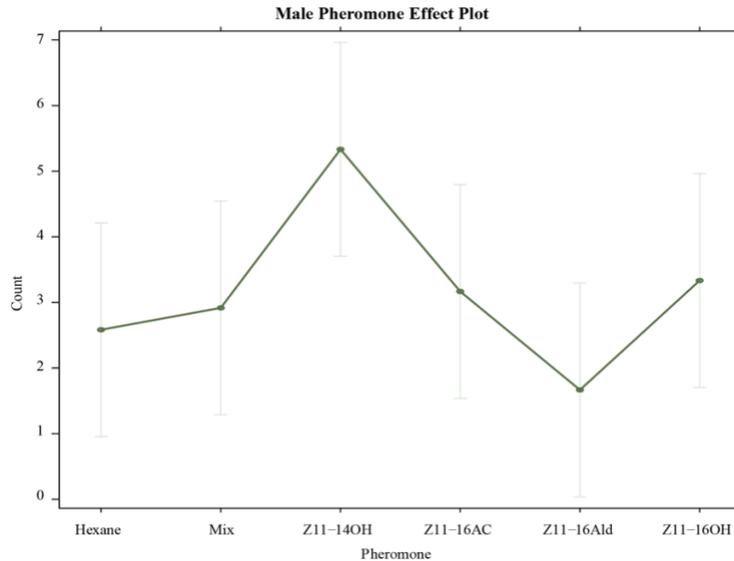


Figure 4. Effects of potential pheromones on average male moth count per trap.

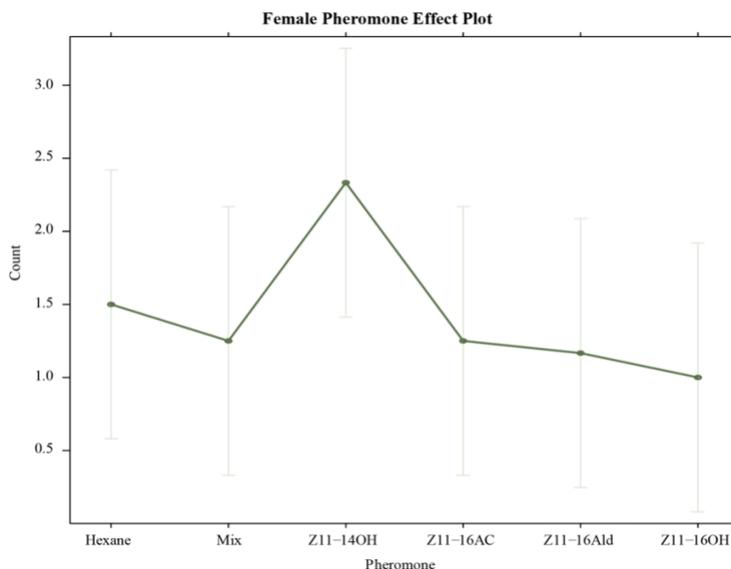


Figure 5. Effects of potential pheromones on average female moth count per trap.

ANOVA tests were conducted to determine if the Male and Female models were better fits for the data than mean only models, and provided evidence that the Male Count model was a better fit for the data than a model not considering the impacts of pheromone to trap count (p-value=0.0013) but that the Female Count model was not a better fit for the data than a model not considering the impacts of pheromone to trap count (p-value=0.125). Analysis proceeded with one-way ANOVA testing on both Male and Female models, with null hypotheses of no difference in the true trap count across pheromones, equation (2), and alternative hypotheses of some difference in the true mean count across pheromones, equation (3).

$$H_0: \mu_{\text{Hexane}} = \mu_{\text{Mix}} = \mu_{\text{Z11-14OH}} = \mu_{\text{Z11-16AC}} = \mu_{\text{Z11-16Ald}} = \mu_{\text{Z11-16OH}} \quad (2)$$

$$H_a: \text{at least one } \mu_{\text{Hexane}}, \mu_{\text{Mix}}, \mu_{\text{Z11-14OH}}, \mu_{\text{Z11-16AC}}, \mu_{\text{Z11-16Ald}}, \mu_{\text{Z11-16OH}} \text{ is different} \quad (3)$$

Diagnostic plots for both the Male and Female models indicate some evidence against the assumption of normality of residuals, little to no evidence against the assumption of normality of the random effects, and little to no evidence against the assumption of constant variance for the models used. Tukey's Post-hoc Comparison of Means for male counts were conducted to determine which potential pheromone trap counts were significantly different from others.

### 3. Results

The mean count of male moths per trap was 3.16 and the mean count of female moths per trap was 1.42. Traps with the potential pheromone Z11-14OH on average caught the most males, 5.33, compared to Hexane control traps which caught an average of 2.58 males. Z11-14OH traps also caught on average more females, 2.33, while Hexane control traps caught an average of only 1.5 females. One-way ANOVA F-testing on the Male model yielded an F-statistic of  $F(5,55)=2.31144$ , and a p-value of 0.056, while testing on the Female model yielded  $F(5,55)=1.15938$  and a p-value of 0.341. Tukey's Post-hoc Comparison of Means yielded a p-value of 0.0148 for the comparison of counts from Z11-16Ald and Z11-14OH potential pheromone baited traps, accounting for the random nested effects of Date and Rep and adjusting for multiple testing. The next smallest p-value was from the comparison of Z11-14OH and our control

lure, Hexane (p-value=0.1445). Tukey's comparisons of all other pheromone candidates also yielded p-values of >0.05.

#### 4. Discussion

We have no evidence to reject the null hypothesis for Female Count, that there is no difference in true trap count across potential pheromones (p-value=0.341). We have some evidence to reject the null hypothesis for Male Count (p-value=0.056), thus we conclude that there is a true difference in male trap count between at least two potential pheromones. This indicates that *Tegeticula yuccasella* yucca moths do use sex pheromones in their mating system. In this case, females release pheromones (but are not attracted by them) and males detect them and thus find conspecific mates. Further analysis of sensilla on yucca moth antennae could clarify the use of pheromones in mating systems, as male moths should have more structures for detecting the specific chemicals<sup>13</sup>.

Tukey's comparisons indicate that there is strong evidence for a difference in male counts for traps baited with Z11-16Ald and Z11-14OH (p-value=0.0148), and a little evidence for a difference in counts between Z11-14OH and our control Hexane (p-value=0.1445). This indicates that *Tegeticula yuccasella* yucca moths use a pheromone similar in structure to Z11-14OH, a fourteen-carbon chain alcohol. The small number of male *T. yuccasella* moths caught using potential pheromone Z11-16Ald, a sixteen-carbon chain aldehyde, may indicate that pheromones similar in structure are in fact repellent to *T. yuccasella* males, particularly if they are the primary pheromones used by another yucca moth species<sup>13</sup>. Further research into this finding is needed. While the models used in this study treat Date as a random effect, dates sampled for this study were not randomly selected therefore we cannot generalize our conclusions to all yucca moths of the summer of 2018 and other years. Further analysis of the species present throughout the summer, dates of peak occurrence, and times of peak mating activity would allow broader generalizations of our conclusions and might hint at the yucca moth's use of temporal niche differentiation in mating systems. Such research would provide further avenues of explanation for the potential sympatric speciation of yucca moths in North America.

#### 5. Conclusion

This study investigated if the yucca moth *Tegeticula yuccasella*, an archaic branch of Lepidoptera, uses sex pheromones in their mating systems as modern Lepidopterans do, and if so which potential pheromones they use. Traps baited with five potential pheromones and a control to catch yucca moths were set in the summer of 2018. The hypothesis stated that the true mean count of yucca moth males (which would be the sex attracted to conspecific pheromones) would be different between at least two potential pheromone baited traps. This study did not find evidence that yucca moth females are attracted to potential pheromones, but there was evidence to support that male moths are. Based on analysis, male *Tegeticula yuccasella* moths detect a pheromone similar in structure to Z11-14OH, a fourteen-carbon chain alcohol, to detect conspecific mates. Further analysis both using methods in this study and other avenues of investigation should proceed to confirm this finding and better explain the development of use of sex pheromones in yucca moth mating systems. Significance of these findings supports further investigation of the potential of species-specific sex pheromone use as a mechanism for the sympatric speciation of yucca moths in North America.

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