

The evolutionary ecology of cheating: does superficial oviposition facilitate the evolution of a cheater yucca moth?

KARI A. SEGRAVES¹, DAVID M. ALTHOFF¹ and OLLE PELLMYR²

¹Department of Biology, Syracuse University, Syracuse, New York, U.S.A. and ²Department of Biological Sciences, University of Idaho, Life Sciences South, Moscow, Idaho, U.S.A.

Abstract. 1. A major question in the study of mutualism is to understand how mutualists may revert to antagonists that exploit the mutualism (i.e. switch to *cheating*). In the classic pollination mutualism between yuccas and yucca moths, the cheater moth *Tegeticula intermedia* is sister to the pollinator moth *T. cassandra*. These moth species have similar ovipositor morphology, but *T. intermedia* emerges later, oviposits into fruit rather than flowers, and does not pollinate.

2. We tested if the pollinator, *T. cassandra*, was pre-adapted to evolve a cheater lineage by comparing its emergence and oviposition behaviour on yucca fruit to a distantly related pollinator, *T. yuccasella*, that differs in ovipositor morphology and oviposition behaviour. We predicted that if *T. cassandra* was pre-adapted to cheat, then these pollinators would emerge later and be able to oviposit into fruit in contrast to *T. yuccasella*.

3. Contrary to expectations, a common garden-rearing experiment demonstrated that emergence of *T. cassandra* was not significantly delayed relative to *T. yuccasella*. Moth emergence patterns overlapped broadly.

4. No choice oviposition experiments with female moths demonstrated that both pollinator species attempted to oviposit into fruit, but only *T. cassandra* was successful. Four out of 84 *T. cassandra* successfully oviposited into older fruit, whereas zero out of 79 *T. yuccasella* oviposited into older fruit. The rarity of the cheating behaviour in pollinators, however, meant that no significant difference in oviposition ability was detected.

5. The results suggest that a shift in emergence phenology is likely not a pre-adaptation to the evolution of cheating, but that the ability to successfully lay eggs into fruit may be. The results also demonstrate that cheating attempts are rare in these pollinator species and, hence, the evolutionary transition rate from pollinator to cheater is likely to be low.

Key words. Behaviour, emergence, exploitation, obligate pollination mutualism, oviposition.

Introduction

Mutualisms are interspecific interactions where the participants gain a net benefit (Boucher *et al.*, 1982; Thompson, 1982; Herre *et al.*, 1999). As mutualisms often generate resources, they can also be exploited. Exploiters or cheaters are individuals that reap the benefits of the mutualism at a cost to the mutualistic

partners (Bronstein, 2001). In many instances, cheaters are opportunists from outside of the focal mutualism – for example, nectar robbing bees that can not legitimately pollinate or collect nectar but, instead, steal nectar by biting through floral tissue (e.g. Irwin & Maloof, 2002). Cheaters may also originate from the focal interaction. In this case, cheaters are mutualists that do not cooperate. This second type of cheating may result in the evolution of cheater species that were once mutualistic and now depend on mutualists for a resource.

Although cheaters are conspicuously present in all classes of mutualism (Bronstein, 2001) and should readily evolve unless

Correspondence: Kari A. Segraves, Department of Biology, Syracuse University, 130 College Place, Syracuse, NY 13244, U.S.A. E-mail: ksegrave@syr.edu

there are sanctions regulating their origins (Addicott, 1998; Bronstein, 2001; West *et al.*, 2002), we know very little about the mechanisms that drive the evolution of cheating. For example, is one mutualistic partner more likely to evolve into a cheater species? Are there particular traits that facilitate the evolution of cheating from mutualistic species? Answers to questions such as these will help us to discern the ecological circumstances that may promote the evolution of cheaters from within mutualistic lineages. These studies will be paramount to our understanding of the dynamics of mutualistic interactions.

Yuccas and their pollinator moths have been an established model for studies of mutualism for well over a century (Riley, 1871; Baker, 1986; Pellmyr, 2003). In this interaction, yucca moths are the sole pollinators of yuccas and yucca seeds serve as the food source for yucca moth larvae. A female moth oviposits into a yucca flower and then actively pollinates using specialised mouthparts unique to yucca moths. The larvae eat only a small portion of the developing ovules, thus making this interaction one of mutualism (reviewed by Pellmyr, 2003). In addition to the pollinator moths, there are also two cheater moth species (Pellmyr *et al.*, 1996). Cheaters lack the mouthparts used for pollination and lay their eggs directly into fruit. In this manner, cheaters exploit yucca seeds without providing any benefit to the plant (Addicott, 1996; Pellmyr *et al.*, 1996).

Extensive phylogenetic analyses of the yucca moths have shown that cheaters evolved from pollinators (Pellmyr *et al.*, 1996, 2008; Pellmyr & Leebens-Mack, 1999; Althoff *et al.*, 2006), and that there are at least two independent origins of cheating (Pellmyr & Leebens-Mack, 1999; Althoff *et al.*, 2006). Thus, cheater moths once possessed the ability to pollinate and lay eggs into flowers and, sometime during their evolution, cheaters lost the ability to pollinate and started exclusively using fruit as oviposition sites. For one of the cheater lineages, a phylogenetic analysis indicated that the cheater moth *Tegeticula intermedia* Riley is sister to the pollinator moth *T. cassandra* Pellmyr (Pellmyr & Leebens-Mack, 2000; Seagraves, 2003). These species diverged very recently and differ little in morphology with the exception of the specialised mouthparts used for pollination (Pellmyr & Leebens-Mack, 2000; Seagraves & Pellmyr, 2004). Some *T. intermedia* only possess rudiments of these structures, and many individuals lack them entirely. These sister species represent a situation where a mutualistic species (ancestral *T. cassandra*) very recently split into a descendant mutualistic species (*T. cassandra*) and an antagonistic cheater species (*T. intermedia*). Both species lay their eggs superficially in the plant tissue (superficial-oviposition), and only differ in oviposition site preference (flowers versus fruit) and in whether or not they pollinate. As these species are young and strikingly similar in morphology, they offer an opportunity to examine the ecological attributes of the shift to antagonism.

The evolution of cheating required three crucial steps: a delayed emergence time corresponding with the appearance of fruit in the population, the ability to recognise fruit as an oviposition substrate, and the ability to oviposit into fruit tissue (Pellmyr & Leebens-Mack, 2000). Here we test whether *T. cassandra* possesses a suite of traits that may have predisposed it to evolve a cheating lineage by comparing *T. cassandra* with another pollinator species, *T. yuccasella*, that is not evolutionarily

associated with cheaters. *Tegeticula yuccasella* also differs from *T. cassandra* in ovipositor shape and oviposition behaviour, as *T. yuccasella* use a thin, narrow ovipositor to lay their eggs deep inside the flower locule next to the ovules (locule-oviposition). We examine the emergence phenology and oviposition behaviour of these two pollinator species to address the following questions:

- 1 Does *T. cassandra* emerge later than *T. yuccasella*, as expected if *T. cassandra* is pre-adapted to evolve a cheating lineage?
- 2 Do both moth species recognise that yucca fruit are suitable oviposition sites? We predict that *T. cassandra* must recognise fruit as a potential substrate to have been predisposed to cheating.
- 3 Can both pollinators successfully oviposit into fruit? We predicted that only *T. cassandra* would be successful because *T. cassandra* has a short, thick ovipositor similar to the cheaters. Furthermore, using fruit would require no behavioural changes for *T. cassandra*, as females would perform the same puncture into young fruit as they do in flowers, whereas *T. yuccasella* may need to change oviposition behaviour to penetrate a thicker and harder fruit wall with their relatively thin ovipositors.

Methods

Moth rearings

We conducted rearing trials in 2001–2003 to determine whether *T. cassandra* and *T. yuccasella* differ in emergence timing. Here we define emergence time as the date at which adult moths emerge from the soil. This measure is intended as a comparison of the moth species and is independent of the flowering stage of the local yucca population. Moth larvae were collected from mature fruit from two populations each year of the study. Larvae feed within the fruit for approximately 30 days and, once mature, they emerge from the fruit, burrow into the soil, and diapause underground. *Yucca filamentosa* fruit were collected at Lake Placid, FL (27°11'18"N, 81°20'16"W) to obtain *T. cassandra* larvae and at the Eglin Air Force Base near Crestview, FL (30°44'00"N, 86°22'00"W) to obtain *T. yuccasella* larvae. Although *T. yuccasella* occurs in Lake Placid, this species is extremely rare on *Y. filamentosa*. Thus, fruit were collected from *Y. filamentosa* at Eglin Air Force Base, as this is the nearest site where *Y. filamentosa* is pollinated by *T. yuccasella*. Single populations of each species were used, because these sites were the only populations large enough to remove large numbers of larvae without detrimental effect. In addition to having pollinator larvae, fruit also invariably contained cheater yucca moth larvae. As the species are not distinguishable by larval morphology, we waited until they emerged as adults to identify them to the species level.

Fully developed larvae that failed to exit fruit naturally were manually extracted from fruit via dissection. Larvae that had not exited the fruit were fully developed as they had finished feeding and spun cocoons within their feeding paths. As larvae emerged or were extracted, they were placed into rearing chambers made from lined 3.8-litre metal containers (19.4 cm height,

16.5 cm diameter). The chambers had lids and bottoms fitted with metal screening to allow water drainage, but prevent access by predators. The rearing chambers were filled with approximately 3 litres of sterilised soil taken from the same sites as the larvae, except in the first year when all of the soil was purchased. The soil was sterilised to avoid including any pre-existing larvae or other organisms in the rearings. Fifty or fewer larvae were placed into each chamber, and all larvae within a single chamber were from the same location and were collected in the same year. The larvae immediately dug into the soil and spun cocoons. The chambers were then sealed and buried at a site on the property of the Archbold Biological Station in Lake Placid, FL. The top of each chamber was placed ~15 cm from the soil surface and containers were spaced ~5 cm apart. Chambers were haphazardly arranged in the common garden burial site. Chambers from previous years were returned to the common garden along with the new chambers at the end of the emergence season, because yucca moth larvae may diapause for more than 1 year (Riley, 1892). As a consequence, the moths that emerged, for example, in 2003 resulted from larvae that had first been placed in the common garden during 2000, 2001, and 2002.

The moths were allowed to over-winter in the common garden until the following spring when the rearing chambers were excavated and kept indoors at the field station. The lids were removed from the chambers, and replaced with cages made from metal screen hoops placed on the top of each chamber and topped with a clear plastic petri dish (15-cm diameter). Moths were allowed to emerge naturally, and the number emerging per species was determined daily. Moths were removed from the rearing chambers upon emergence and used in subsequent behaviour trials (see below). We determined whether *T. cassandra* emerges later than *T. yuccasella* by comparing the mean date of moth emergence for males, females, and both sexes combined using Wilcoxon's rank tests.

Behaviour trials

As the moth rearings generated a large number of naïve moths, we used these in a subsequent behaviour experiment. We hand-pollinated flowers and covered them with inflorescence bags made from white mesh fabric (~0.2-mm mesh) to prevent naturally occurring pollinator and cheater moths from accessing the flowers and developing fruit. Fruit were collected after 2, 4, or 6 days of maturation and used in the behaviour trials. On the night of emergence, *T. cassandra* and *T. yuccasella* females were paired with conspecific males in small screen cages made from a 6-cm wide screen hoop fitted between the halves of a plastic petri dish (15-cm diameter). The moths were placed in the dark for approximately 10 h and allowed to mate. The success of mating, which takes several hours, was determined via direct observation. On the second night, females were presented with either a fruit or a 'virgin' flower in a no-choice oviposition trial. The flowers and fruit were collected from the hand-pollinated plants (see above) immediately before the start of the trials. Females were placed into shell vials (23 × 85 mm) with a fresh flower or fruit suspended by its pedicel in the top of the

vial. Vials were capped with foam stoppers. Females were allowed access to the flowers and fruit for 10 h and single females were presented with plant material of the same age (flower, 2-, 4-, or 6-day-old fruit) each night of the trial. At the end of the evening, the plant material was removed and females were left in the shell vials. Flowers and fruit were examined to determine the number of oviposition attempts (visible as scars) per female per night, and dissections were performed to determine the number of eggs deposited per female per night. Females that failed to survive for at least two nights were excluded from the analysis. We used Pearson's χ^2 -tests to determine whether the number of females leaving scars and eggs were dependent on moth species. Separate tests were conducted for each level of fruit age.

Results

The 2001 rearings were unproductive, with only 40 moths emerging. For the 2002 and 2003 rearings, we used local soil from the populations where the moths were collected and rearing success improved greatly. In total, we used 43 rearing chambers over 3 years, and 859 moths emerged during this time span (279 *T. cassandra*, 237 *T. yuccasella*, 343 *T. intermedia*). In 2002, 25 female *T. cassandra*, 45 male *T. cassandra*, 14 female *T. yuccasella*, and 11 male *T. yuccasella* emerged. In 2003, 58 female and 148 male *T. cassandra* emerged and there were 83 female and 102 male *T. yuccasella*. Some moths emerged after 2 or 3 years. Because so few moths emerged in 2001, we excluded those data from further analyses.

Within the rearings, the pollinator species tended to emerge simultaneously and there was no difference in the timing of emergence between *T. cassandra* and *T. yuccasella*, with the exception of females in 2003. In this case, *T. yuccasella* females emerged significantly later than *T. cassandra* females (~3.8 day later, $Z = -2.88$, $P = 0.004$). When the pollinators were compared with the cheaters emerging in the rearings, the cheaters emerged significantly later than the pollinators (8–9 day later, $\chi^2 = 198.14$, $P < 0.0001$). The distribution of emergence timing overlapped for all species (Fig. 1).

Both pollinator species recognised yucca fruit, attempted to deposit eggs into fruit, and both were successful at ovipositing into flowers and 2-day-old fruit (Fig. 2). The number of females leaving scars and eggs on flowers and fruit, however, was not statistically dependent on moth species (Fig. 2). Although not statistically significant, *T. cassandra* alone was successful in ovipositing into 4- and 6-day old fruit. Four out of 84 *T. cassandra* successfully oviposited into older fruit (4- and 6-day-old fruit) whereas zero out of 79 *T. yuccasella* oviposited into older fruit. Given the data at hand, we cannot rule out the possibility that *T. yuccasella* can also use 4- and 6-day-old fruit. A power analysis indicated that more than 600 trials would be needed for significance of this comparison. This is not surprising given the infrequency of female attempts and successful egg depositions into fruit. A post-hoc analysis excluding females that did not attempt to oviposit on 6-day-old fruit was marginally significant and suggests that *T. cassandra* may have an increased ability to use these fruit ($\chi^2 = 2.955$, $P = 0.085$, $n = 30$).

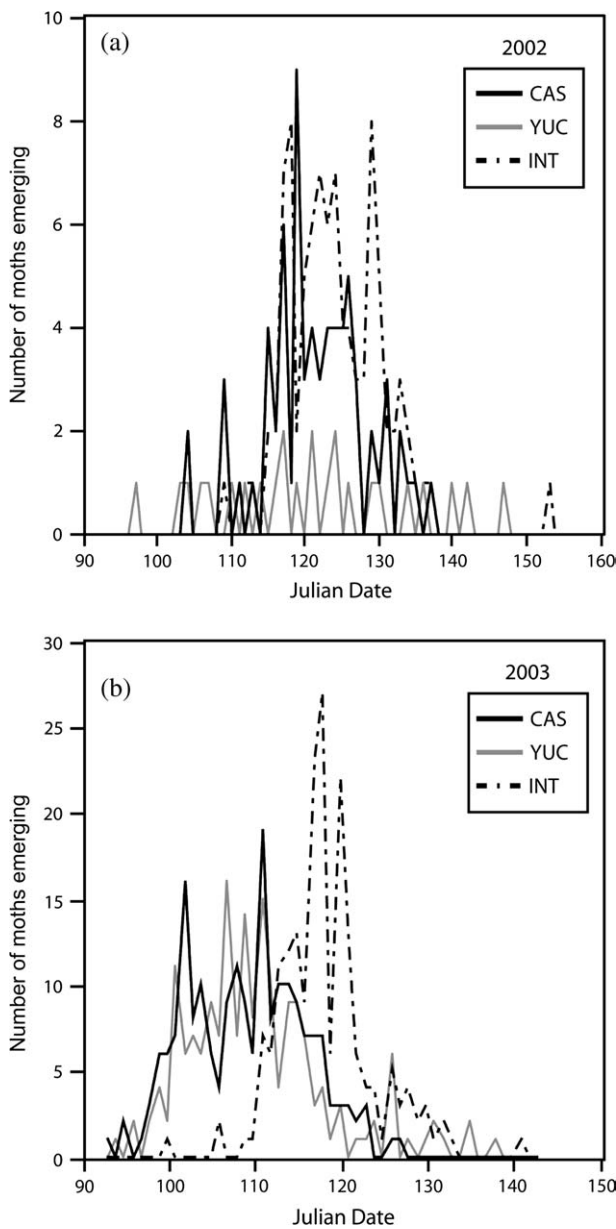


Fig. 1. Comparison of reared moth emergence for pollinator moths *Tegeticula cassandra* (CAS), *T. yuccasella* (YUC) and the cheater moth *T. intermedia* (INT). (a) Moth emergence in 2002. (b) Moth emergence in 2003.

Discussion

Cheaters are a prominent aspect of mutualism and may either be opportunists from outside of the mutualistic interaction, or may be uncooperative mutualist partners. In this second instance of cheating, cheater species may evolve from mutualistic lineages. Although the evolution of cheaters from mutualist species has happened frequently (e.g. Janzen, 1975; Compton *et al.*, 1991; Pellmyr *et al.*, 1996), we know little about the underlying mechanisms that cause this shift from mutualism to antagonism. Here

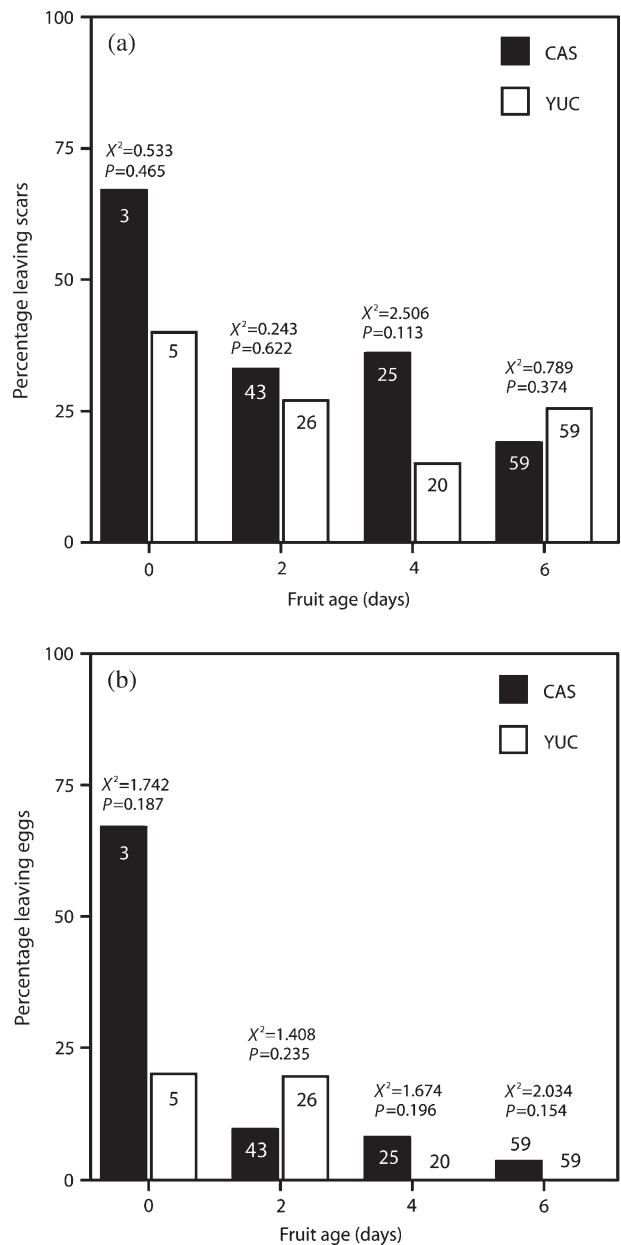


Fig. 2. Percentage of female moths leaving scars and eggs on flowers and fruit. Black bars represent *Tegeticula cassandra* (CAS), white bars *T. yuccasella* (YUC). Numbers on the bars indicates the number of females used in each trial. Fruit age of zero indicates that flowers were used in behaviour trials. Fruit age of two, four, and six indicates the number of days the fruit was allowed to grow after pollination. (a) Percentage of females leaving scars on flowers or fruit. (b) Percentage of females leaving eggs within flowers or fruit.

we test whether some mutualist yucca moth lineages may be predisposed to evolve cheater species.

Variation among yucca moths in oviposition habit may allow particular moth lineages to evolve into cheater species. Phylogenetic evidence demonstrates that the two lineages of cheater yucca moths have independently evolved from a clade

of superficially-ovipositing pollinators (Althoff *et al.*, 2006; Pellmyr *et al.*, 2008), suggesting that this oviposition behaviour may pre-adapt superficially-ovipositing moths to cheat. There are three factors that may have lead to this pattern. First, superficially-ovipositing species may emerge later in the flowering season, thus increasing the probability of contact with fruit. Second, superficially-ovipositing species may have a greater tendency to recognise fruit as an oviposition site. Finally, superficially-ovipositing moths may have the physical capacity to lay eggs into fruit, whereas species that lay their eggs deep inside the flower locule may not.

Comparisons of emergence timing between *T. cassandra* and *T. yuccasella* indicate that these pollinators emerge nearly simultaneously when placed into a common environment. In 2002, there was no difference in emergence phenology between the species, and in the following year, there was a significant difference, but in the opposite direction as predicted: *T. yuccasella* was delayed in comparison to *T. cassandra*. As the distribution of the two moth species required that we collect *T. cassandra* and *T. yuccasella* from geographically separate populations, there is the possibility that emergence timing is a result of local adaptation in different populations. Although this caveat should be kept in mind, observations of the emergence timing of the cheaters argues against local adaptation, as cheater moths from the two populations emerged simultaneously in our common garden experiments (data not shown).

Although the data clearly rejects the hypothesis that delayed emergence was an existing trait in the pollinator *T. cassandra* that could facilitate the shift to cheating, the data have important bearing on the future potential for evolution in these moths. The distribution of emergence timing for both pollinators was broad, and largely overlapped that of the cheater species (Fig. 1). The overlapping phenology of these moth species show that pollinators encounter fruit during the same period as cheaters and, thus, pollinators have the opportunity to cheat using fruit. Indeed, behavioural cheating by the pollinator yucca moth *T. maculata* has been frequently observed (Aker & Udovic, 1981). Furthermore, the substantial variation in emergence time within each species suggests the potential for selection to act in these pollinator species. This study shows that these species have the phenotypic variation required for selection to shift the timing of emergence although, certainly, other factors may constrain the evolution of emergence phenology.

Changes in moth emergence patterns are not the only factors involved in the shift to cheating in yucca moths. Cheaters must also recognise that fruit are suitable places to lay eggs and they must possess the ability to oviposit into fruit. We tested these ideas via no-choice behaviour trials in which the two pollinators *T. cassandra* and *T. yuccasella* were presented with flowers or fruit of varying age. Both species attempted to lay eggs and left scars on flowers, and 2-, 4-, and 6-day-old fruit. In no choice situations, both *T. cassandra* and *T. yuccasella* recognise fruit as a potential oviposition site. Thus, if pollinator moths of either species were to emerge late in the season and only fruit were present, there is the possibility that they would attempt to cheat.

Although both pollinator species attempted to oviposit into fruit, only *T. cassandra* successfully deposited eggs into fruit older than 2 days. The difference in successful oviposition be-

tween *T. cassandra* and *T. yuccasella* was not statistically different. However, given the rarity of oviposition success (two *T. cassandra* on 6-day-old fruit and two on 4-day-old fruit), this was not surprising. The present data show that the superficially-ovipositing species *T. cassandra* is capable of using 4- and 6-day-old fruit as oviposition sites, but at this time, we cannot rule out whether the locule-ovipositing species *T. yuccasella* is similarly capable of cheating. Although these data demonstrate that these moths recognise fruit as oviposition sites and may be able to also lay eggs into fruit, the question remains whether they use fruit under natural circumstances. Hence, if given a choice to oviposit into fruit or flowers, do moths ever choose to cheat? Field observations of these species suggest that this is not the case, but if they did, this could provide a direct link between cheating behaviour and the evolution of cheater yucca moths.

Previous work on the evolution of cheating has indicated that ecological interactions among mutualist partners may lead to the evolution of cheaters (Pellmyr *et al.*, 1996; Stanton *et al.*, 1999; Pellmyr & Leebens-Mack, 2000; Sachs *et al.*, 2004). Here we demonstrate that although mutualists are capable of cheating when forced, cheating behaviours are relatively rare among mutualistic yucca moths. This suggests that the evolutionary transition rate between mutualism and antagonism is likely to be low.

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