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**SEXUAL SELECTION IN THE SPOTTED CUCUMBER
BEETLE, *DIABROTICA UNDECIMPUNCTATA HOWARDI*
(COLEOPTERA: CHRYSOMELIDAE):
MALE TRAITS FAVORED BY CRYPTIC FEMALE
CHOICE**

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Abstract

In the spotted cucumber beetle *Diabrotica undecimpunctata howardi* (Coleoptera: Chrysomelidae), the female determines the final outcome of mating; internal muscle control allows her the capability of blocking the transfer of sperm from the male. Males respond to this “cryptic female choice” by rhythmically stroking the female with their antennae in a distinctive pattern. The rate of this “antennation” is the best known predictor of a successful outcome for the mating, males that have a higher antennation rate are more likely to pass a spermatophore to the female. I examined a number of male traits testing for correlations with antennal stroking behavior, and found two significant results: (1) Antennation rates increased in captivity despite very high levels of artificially induced inbreeding, the opposite of expectation, probably due to heightened sexual selection for mating display rate. And (2) males with fast antennal stroking rates had a greater propensity to fly rather than run in a simulated escape test. Fieldwork indicated a high proportion of the beetles that appeared in squash fields early in the season were “flyers”, females may therefore prefer fast antennating males because they develop faster or because flight might confer an ecological advantage in foraging ability.

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Chapter 1.

INTRODUCTION

Charles Darwin is famous for discovering natural selection as a mechanism of evolutionary change (1859). The concept of sexual selection, which Darwin characterized as competition for mates either in the form of male-male combat or female choice, was presented as another evolutionary force inside the framework of natural selection, and he stated that the two could either work in combination or oppose one another (1871). Darwin used sexual selection to explain the existence of ornate secondary sexual characteristics, such as the male Peacock's tail, that are an advantage when mating but significantly decrease fitness at all other times during an organism's life (Pilastro *et al.* 2005, Langerhans *et al.* 2005). He pointed out that while natural selection tends to stabilize around an optimal condition, sexual selection is open-ended, and so can lead to extremely elaborate traits.

However Darwin found his concept of sexual selection to be even more controversial than that of natural selection. For example, Alfred Russel Wallace, who independently discovered natural selection and later joined Darwin as a collaborator, always excluded the theory of sexual selection (Brackman 1980). J.S. Huxley, otherwise

a staunch supporter of Darwin, also saw very little need for the concept as separate from natural selection (Huxley 1938).

The few early sexual selection studies that were done focused entirely on males, either on their flashy dominance displays or showy ritualized competition. Darwin included female choice in his theory, but then called sexual selection "... a constantly recurring struggle between the males for the possession of the females" (1871). The "coy" female was presumed to have only a passive role in mating, her 'choice' basically limited to accepting the contest winner (Wallace 1889, Fisher 1930, Huxley 1938, Trivers 1972, see review in Andersson 1994). It was also assumed that both intra- and intersexual selection took place only before copulation occurred. Darwin wrote: "...as far as selection is concerned, all that is required is that choice should be exerted before the parents unite." (1871).

This male oriented pre-copulatory bias, however, only holds true if the copulation in question inevitably leads to fertilization of the female's eggs; this generalization is very often not true (Eberhard 1996, Hellriegel & Ward 1998, Jennions & Petrie 2000). Males and females often have quite different and contradictory 'agendas' for sex (Partridge & Hurst 1998, Jones 2002, Bussiere *et al* 2006), and females of many species evidently retain a large measure of control over the fertilization of their gametes, both during and after copulation. Eberhard lists twenty possible post-intromission mechanisms by which females can bias paternity toward a particular male (1996). These mechanisms are often internal and hidden from view, and so are termed "cryptic female choice" (Thornhill 1983). For example, females can bias paternity cryptically by blocking the transfer of sperm from the male, discarding sperm and mating again with a

second male, failing to transport or store sperm internally as expected, failing to ovulate, or aborting zygotes. In response, males have developed courtship behaviors that they exhibit before, during and even after copulation that attempt to induce the female to use their sperm to fertilize her eggs. Actions such as these have been termed “copulatory courtship behavior” if they occur after the actual act of intromission has begun (Otronen 1992, Wcislo *et al.* 1992, Eberhard 1993, Eberhard & Kariko 1996, Eberhard 1996).

The ‘female choice’ component of sexual selection can be difficult to isolate and analyze, especially when it occurs within the body of the female. The benefits to the female that result from mating choice are categorized as either direct or phenotypic, such as nuptial gifts of defensive plant-derived chemicals or protein, or indirect so they only appear in her offspring, e.g. “good genes”. Sometimes cryptic female choice has been demonstrated to occur, and yet no benefit for the female, direct or indirect, could be identified (Fleischman & Sakaluk 2004). Shine *et al.* (2003) closely examined what appeared originally to be an example of female choice in a snake, and found it actually to be forcible insemination by the male. In other cases females have been shown to prefer male traits that do not occur naturally in that species. Experimenters have artificially added and found a predisposition for certain calls in tungara frogs (Ryan 1985), swordtails on fish (Basolo 1990) and crests on finches (Burley, in Futuyma 1998), This phenomenon was explained as a preexisting or hidden female mating preference (Ryan 1990, 1997; Basolo 1994).

A number of other hypotheses have been offered to explain the existence and evolution of female choice. The “Chase Away” sexual selection model (Rice 1996, Holland & Rice 1998) starts with a preexisting female mating preference, and then

theorizes a cyclic build up of antagonistic (rather than reinforcing) coevolution between the sexes. This would have the effect of leaving the male burdened with numerous traits, both the most recent and the older ones as well, none of which he can afford to lose (but see Eberhard 2004).

Another hypothesis used to explain female choice is ‘runaway sexual selection’ (Fisher 1930, O’Donald 1962, Lande 1981, Kirkpatrick 1982). Here the selection advantage is indirect and so is only reflected in the F1 generation. If a female mating preference occurs in the population for whatever reason, arbitrary or not, and it is heritable, it can theoretically lead to runaway selection. It is a mating advantage for a male to have this particular trait and if a female can pass down these “good attractiveness genes” to her sons, she will benefit through their mating success. There is selection pressure for both the male trait and the female preference for the trait, and so it is a runaway process, or ‘self-reinforcing choice’ (Maynard Smith 1976). Runaway selection has been difficult to evaluate empirically, possibly because selection occurs so rapidly. Support has been found, however, in the form of a genetic correlation between the male trait and the female preference in stalk-eyed flies (Wilkinson and Reillo 1994).

A fourth hypothesis accounting for female choice is the ‘good viability genes’ model (Kirkpatrick 1996, Kirkpatrick & Barton 1997, Moller & Alatalo 1999; Lailvaux *et al.* 2006). It postulates that female mating preferences arise to procure superior survival or viability genes for offspring. Males that are healthy and disease free presumably have better genes to combat parasites and fight infection. This idea is in concordance with the ‘handicap theory’ (Zahavi 1975), which states that males with huge, ornate secondary sexual characteristics are more desirable to females because they

are signaling a superior genotype by just surviving with such a handicap. As with good attractiveness genes, good viability genes would only provide benefits for choosy females indirectly through the success of her offspring. ‘Good genes’ hypotheses have found empirical support in a number of studies showing offspring had superior viability and general fitness (Knapp and Kovach 1991, Welch *et al.* 1998, Hoikkala *et al.* 1998, Sheldon *et al.* 1999; Lailaux *et al.* 2006) or were good in terms of attractiveness (Jones *et al.* 1998, Tomkins & Simmons 1999) and so provided a mating advantage for the F1 males.

If the acquisition of either good attractiveness or good viability genes results in more of a choosy female’s offspring surviving and mating, there will be a clear fitness advantage to that particular female’s mating preference. One theoretical model, however, predicts that selection involving the acquisition of indirect genetic benefits should be quite weak in relation to selection dealing with direct phenotypic benefits such as those from the acquisition of nuptial gifts, parental care, or access to rich territory (Kirkpatrick & Barton 1997). Furthermore, in lek or lek-like situations where the male does not provide the female with nutritional nuptial gifts or parental assistance but contributes only his genes, female choice was thought to be theoretically impossible because it would provide strong directional selection for those ‘good genes’ and rapidly lead to their fixation (Fisher 1930, Maynard Smith 1978, 1991). If all males have the same good genes, why would females bother to discriminate (Pomiankowski 1987, Zeh & Zeh 2003)? If this “Lek Paradox” (Borgia 1979, Kirkpatrick & Ryan 1991, Ritchie 1996) were real, there would not be any advantage to choosing one male over another because the preferred trait would be genetically similar in all males. The paradox was resolved

however, by studies in a number of diverse taxa that indicated that sexually selected traits had greater rather than less genetic variance than traits under natural selection (Pomiankowski & Moller 1995). One possible explanation for such unexpected variation in traits under directional selection is that the expression of sexually selected traits has fitness costs and so depends on condition, and since condition is determined by gene by environment interactions and the environment is always changing, large amounts of genetic variation are favored (Rowe & Houle 1996, Wilkinson & Taper 1999).

The Study Organism

Diabrotica undecimpunctata howardi Barber, (Coleoptera: Chrsomelidae), is a member of the subfamily Galerucinae and the tribe Luperini, which consists of more than 3950 species (Wilcox 1972a,b). The spotted cucumber beetle, also known as the southern corn rootworm, is well known as an agricultural pest with a broad distribution (most of the U.S. east of the Rockies as well as southern Canada and Mexico) and a wide range of host plants (Metcalf & Metcalf 1993, Olkowski *et al.* 1991). These beetles eat the foliage, pollen and roots of beans, soybeans, peanuts, cucurbit crops such as cucumbers and melons, corn and a number of other vegetables, and can even become a problem late in the season on ornamentals such as asters and dahlias (Stefferdud 1952), and have been found on more than 200 grasses, common weeds and cultivated crops (Metcalf & Metcalf 1993).

Adults over-winter in protected areas like the bases of plants, and in the spring and summer each female may lay more than 2600 eggs (Tallamy *et al.* 2000). The eggs

are laid in the ground and the larvae, upon hatching, chew into the roots and underground structures of plants; hence the second common name for this species, southern corn rootworm. There are three instars, with larval development taking about 14-24 days. New adults start to emerge during July in the mid Atlantic region. In the southern part of its range there are normally two generations a year; farther north at least a partial second generation is produced. An adult can live as long as 300 days and are fairly capable fliers; there is some evidence that they migrate northward in the early spring (Metcalf & Metcalf 1993).

D. undecimpunctata in the lab were kept at approximately 25C on a 14 L /10 D hourly lighting schedule and fed Bio Serv diet # F 9760 B (www.bio-serv.com) and slices of squash, replaced as needed. Eggs to be reared were collected on cotton oviposition pads, approximately 25 by 25 mm, cut from seed germination sheets (KimPak # K- 22, Seedburo.com). The pads were dampened daily and changed every 2-3 days; pads with eggs were placed in potting soil along with corn kernels. Flowerpots measuring 22cm in diameter and 22 cm tall containing the potting soil, a total of a few hundred eggs on several pads, and at least 50 kernels of corn, were watered lightly every 1-2 days; adult beetles emerged after approximately 28 days. Hatching rates of eggs on these pads in my previous work (Tallamy *et al* 2002A) were 60 to 75%, and Jackson *et al* (1996) measured up to 94%. However the number of adult beetles emerging indicated an overall success rate of only a few percent despite numerous modifications and technique changes.

“Antennation”

Spotted cucumber beetle males rhythmically stroke females during copulation with their antennae. Earlier work indicated that the rate of this “antennation” did vary among males, and was correlated with mating success (Tallamy *et al.* 2002a), and this has been confirmed by recent studies (Darlington Submitted). Males with more rapid antennation were more likely to pass a complete spermatophore, indicating that antennation was a trait being modified by female choice, and represents an example of copulatory courtship behavior.

The male, while riding on the back of the female, strokes her with his antennae across her pronotum, head and eyes, and sometimes hits the outer portions of her antennal scapes. This antennation behavior occurs in a very consistent and stereotyped pattern. The male lowers his head and his antennae and always shakes his head back and forth three times, representing one ‘triplet’. I quantified the antennation rate as the number of triplets that occurred during the first minute of mating; this rate was normally distributed in the population and ranged from a low of 40 to a high of about 130 (Darlington Submitted).

The antennation rate of individual males was always measured at 7-10 days after the beetle emerges as an adult. By this age the male is fully mature and has generated a spermatophore. Individual males tested again a week later had the same antennation rate; the rate did not vary with age and so was highly “repeatable” ($r = 0.97$, $F_{1,29} = 57.2$, $p = 0.0001$). Repeatability (r) is a quantitative measure of the total phenotypic variance among individuals rather than within individuals, and ranges from zero to one (Lynch & Walsh 1998). It is the ratio of the among-individual variance to the total variance (among

and within individuals) as estimated from the mean squares of a one-way ANOVA (Lessells & Boag 1987). Also a linear regression of the two rates, taken 7 days apart, showed a very high correlation ($r^2 = 0.86$; $P = 0.0001$) (Tallamy *et al.* 2002a).

In a successful mating (one in which a spermatophore is passed) the male promptly stops stroking the female with his antennae and instead holds them at a low angle over his back. This posture is distinctive and is always associated with complete aedeagus insertion. In contrast, during a rejection, the female refuses to allow the male to insert his aedeagus completely into the bursa copulatrix, thus demonstrating “cryptic female choice”, and so the male either continues to antennate the female or gives up and drops off her back. If the female does not accept the male quickly and refuses to allow complete intromission, the rate of antennation gradually slows over time. After 10 minutes duration this slowing averages 24% overall, however the endurance is significantly better in accepted males (decline of 4.2 ± 1.1 strokes/min.), than in rejected males (decline of 9.9 ± 2.5 strokes/min.) (Tallamy *et al.* 2002a,b).

I tested the hypothesis that antennation is energetically costly for males, and so represents a mating display which is a demanding test of physiological condition (Mather & Robertson 1993, Welch *et al.* 1998), in the light of this difference in the endurance of the stroking rate between fast and slow antennating males. I attempted to quantify the energetic costs of antennation for a male beetle by measuring the increase in the amount of CO₂ expelled during mating. As I was able to induce antennation in these beetles only when the male was copulating with a live female, I measured the total CO₂ for a pair of beetles, quantifying the difference in the levels before and during mating. The few data collected indicated that antennation represented approximately a three-fold increase over

the lowest resting level, equivalent to the level of a beetle walking at a moderate pace, and so not exceedingly taxing, although the muscles powering the antennae would be smaller than the leg musculature (Darlington Unpublished). Metabolic levels measured for flying *D. undecimpunctata* were not accurate, as flight was invariably intermittent and mixed with periods when the beetle was stationary inside the respirometer chamber; however flight can represent a much higher metabolic output, up to 100 times the basal level for some insects (Casey 1988, Kammer & Heinrich 1978). Although the actual energetic output of mating displays are not often quantified, roosters when crowing are using only 15% more oxygen than resting levels, over 10 times less than the maximum sustainable metabolic rate (Horn *et al.* 1996).

The existence of cryptic female choice has been well documented in the spotted cucumber beetle; during copulation the female can reject the male by refusing to accept his spermatophore (Fine 1996, Tallamy *et al.* 2002a,b). The morphological mechanism that makes cryptic female choice possible in the SCB has been identified. Beetles in copula were flash frozen in supercooled ethanol and then dissected; the female blocks access to her bursa copulatrix by tightening sphincter muscles in her vaginal duct. I have also captured on videotape one occasion when the female everted the posterior section of her reproductive tract while she was simultaneously blocking insertion of the male's aedeagus. It is possible to see the sphincter muscles in this semitransparent "ovipositor" are constricted, closing off access to the bursa copulatrix. The rejected male is therefore unable to achieve complete penetration and is prevented from transferring his spermatophore.

Cryptic female choice has now been found in a few other organisms, such as dragonflies that dump unwanted sperm while ovipositing (Cordoba 2006), and a spider that manipulates paternity by means of multiple sperm storage organs (Snow & Andrade 2005). In one case, a guppy with internal fertilization, the actual physiological mechanism that allowed female choice was not identified, the authors only wrote that the female controlled "...the number of sperm transferred or retained" (Pilastro *et al.* 2003). The term 'cryptic female choice' was used in two studies on crickets in which the female removed the spermatophore "prematurely" (Fleischman & Sakaluk 2004, Bussiere *et al.* 2006), but as the spermatophore is large and external I don't think that the word 'cryptic' applies. Recent work on the Red Flour Beetle found cryptic female choice favoring males with vigorous leg rubbing mating behavior (Edvardsson & Arnqvist 2006); however this significant result was not replicated (Fedina & Lewis 2006), and the morphology that allows cryptic female choice has also not been identified.

The spermatophore in *D. undecimpunctata* is quite large, representing approximately 7% of the male's body weight (Tallamy *et al.* 2000). It is transferred in liquid form and congeals in the female's bursa to a gelatinous consistency. I examined spermatophore size as a measure of nuptial gift quality, and found it was not correlated with the size of the male, but weakly correlated ($R^2 = 0.21$) with the size of the female. Evidently males routinely fill the female's bursa, which then determines the final size of the spermatophore. Once a complete spermatophore transfer does occur the female beetle does not mate again, possibly due to the presence of receptivity-suppressing hormones contained in the spermatophore (Tallamy *et al.* 2000). Males can mate more than once, up to at least 16 times, if given a day or two to regenerate a new

spermatophore. Copulation is typically long in the SCB; six hours or more is not unusual, and some of that time is evidently just the male physically “riding” the female to preventing other matings (Bussiere *et al.* 2006), until his hormones have reduced her receptivity. By refusing to accept one male’s spermatophore, a female gives herself the option of copulating again with a different male; female beetles have been known to refuse up to 15 mating attempts before finally accepting a “desirable” male (Tallamy *et al.* 2000).

A female that is allowed no more matings after she rejects a single male can still lay a small number of viable eggs. This means that either a small quantity of sperm can still be passed during a rejected mating, or that these beetles are capable of “occasional” parthenogenesis (see Chapman 1998). Tests of this hypothesis were inconclusive (Darlington Unpublished).

In nature this species mates in cucurbit flowers, copulation evidently occurs during the heat of the day after the bloom has closed, and so is rarely observed. These beetles aggregate in flowers and so are found in a clumped distribution pattern (Tallamy *et al.* 2000); such a group mating system is unusual for beetles, somewhat similar to a mating swarm or lek as seen in Diptera (Tallamy *et al.* Submitted), also see Alcock 1998, Hoglund & Alatalo 1995). In the lab I initially simulated the mating arena with yellow coloration, artificial flower volatiles, and had multiple males and females present. It is known that females initiate mating and signal receptivity with a sex pheromone (Guss *et al.* 1983); although a number of males were present, females did not appear to be choosing one male over another for copulation, and only one male initiated mating by leaping on the female’s back. There was no male-male hierarchical competition detected

and yet females accepted the spermatophore of some males and refused that of others. I consequently discovered mating behavior would still occur about 2/3 of the time with one female given a single male and without the artificial arena. Here I randomly paired beetles of the appropriate age (female 5-7 days, male 7-10) in test tubes and videotaped them for one hour.

Previous experiments have identified the male antennation rate as the only good predictor of mating success. SCB antennae are sexually dimorphic, males have approximately 18% longer antennae than females, and when one antenna is surgically removed a male's chances of being accepted as a mate for the second time drops from at least 50% to zero. Some other obvious parameters such as male body size and aedeagus length have been shown not to be important determinants (Fine 1997, Tallamy *et al.* 2002b). I found no correlation between the male mass and the outcome of the mating. In this Thesis I also report that the level of inbreeding is a factor in the mating outcome (Darlington Submitted).

Spotted cucumber beetles are also known to have a chemical ecology based on secondary plant defense chemicals (Metcalf & Metcalf 1994, Price 1997). Males will preferentially feed on squash containing cucurbitacins, which are extremely bitter and toxic to most other organisms (Quiring & Timmins 1990). They then modify the 'cucs' into a less toxic form, and pass them to the female during mating. Females incorporate cucs into their eggs and in their own tissues as a defense against predation (Ferguson *et al.* 1985, Nishida & Fukami 1990, Tallamy *et al.* 1997, 2000) and to confer an antibiotic effect (Tallamy *et al.* 1998). Detailed analysis has shown that males retain proportionally more cucs in their antennae than in their other body parts (Tallamy, Personal

Communication), and so it is possible that this complex antennation behavior has evolved under sexual selection from the male simply rubbing the female with his antennae so that she could ‘taste’ the cucs.

Surprisingly males were unable to differentiate bitter squash from squash that did not contain cucs in lab simulations (Tallamy *et al.* 2002b), and also males that have fed on cucs have been shown not to have a mating advantage over males that cannot pass along these defensive chemicals as a nuptial gift to the female (Tallamy *et al.* 1998).

Using the spotted cucumber beetle as a model organism, my work examines the possible benefits of female choice. In previous work, (Tallamy *et al.* 2002a) I examined the effect on female fitness of cryptic female selection for rapid male antennal stroking by mating females with males exhibiting either high or low antennation rates. I examined direct phenotypic benefits by measuring female longevity, age at first reproduction, fecundity and egg hatch. I then looked at both aspects of good genes models, ‘good viability’ and ‘good attractiveness’ genes by measuring the offspring survivorship and the mating success of the generation that followed female preference decisions. I also looked in the next generation for indirect genetic benefits for females mated to fast and slow antennators by measuring maturation times, body size, female age at first reproduction and fecundity.

I found no evidence for direct benefits for a choosy female, but did see a trend toward higher survivorship ($19.1\% \pm 3.2$ vs. $11.6\% \pm 1.9$) in the offspring of fast antennating males, a possible indirect ‘good genes’ effect (Tallamy *et al.* 2002a). However the trend of higher survivorship has been rejected by more recent work reported here (Darlington Submitted).

I did discover a highly significant ‘good attractiveness genes’ effect (Tallamy *et al.* 2002a), in that the sons of fast antennators were fast antennators as well (72.5 strokes/min \pm 0.9, N = 38 vs. 56.7 \pm 1.7 strokes/min, N = 29, $F_{1,22.3} = 74.85$, $P = 0.0001$). A half-sib analysis (Tallamy *et al.* 2002a) has verified the heritability of the antennation stroking rate in the narrow sense (Falconer and Mackay 1996), the amount of phenotypic variance due to additive genetic variance ($h^2 = 0.62 \pm 0.20$). The additive genetic coefficient of variation was also calculated ($CV_A = 13.2\%$), as Houle (1992) has argued it can be a better indicator for examining genetic evolution and variation because it is standardized by the mean. These results, and the fact that antennation rates show a normal distribution in the population, suggest that the trait of antennation is controlled by multiple genes (Falconer & Mackay 1996, Darlington Submitted). Female spotted cucumber beetles can therefore benefit (indirectly) from exercising female choice, in that they obtain ‘good attractiveness genes’ for their male offspring, who then inherit this mating advantage (Edvardsson & Arnqvist 2006).

More recent work has focused on the possible benefits of female choice by differentiating between males on traits that correlate with antennation rate. Why do female *D. undecimpunctata* bother to discriminate between males, and what actual fitness advantage might a male with fast antennal stroking convey?

Chapter 2.

Inbreeding, Male Display Rate and Female Choice in the Spotted Cucumber Beetle,

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Abstract

Female spotted cucumber beetles, *Diabrotica undecimpunctata howardi*, exert “cryptic female choice” by selectively blocking some males from passing a spermatophore during mating. Males stroke the female with their antennae in a distinctive pattern during copulation, and the rate of this antennal stroking, which has been previously shown to be heritable and highly repeatable in individuals, is the only known predictor of female receptivity. I tested the hypothesis that this trait is a marker that females use to avoid inbreeding depression. However, the rate of antennal stroking did not decrease but instead increased significantly over 6 generations of full-sibling matings. Out-bred populations had a similar increase in rate, an effect perhaps explained by heightened sexual selection under laboratory rearing conditions. An inclusive fitness theory is discussed that posits that close inbreeding can be advantageous, as mating with a relative passes down more of the individuals’ genes, up to an optimum level just before the onset of inbreeding depression.

Introduction

Darwin's theory of sexual selection included the concept of female choice (1871). Thornhill (1983) postulated that the physiological mechanism that allows a female to choose one mate over another could be located internally, hidden from view, and therefore "cryptic". Eberhard (1996) listed 20 different post-intromission mechanisms whereby a female could bias paternity toward a particular male, including controlling the transfer of sperm. He also emphasized that males and females often have quite different requirements from sex, and so their 'agendas' can clash. In the spotted cucumber beetle, *Diabrotica undecimpunctata howardi*, females often copulate with several males, but can determine the outcome of each mating by selectively tightening a sphincter muscle at the base of the bursa copulatrix that blocks transfer of the spermatophore. Once a female accepts a spermatophore she does not mate again. In contrast, males can mate successfully 16 times or more, needing about two days in between matings to rebuild spermatophores (Tallamy *et al.* 2002a, b).

Mating in the spotted cucumber beetle is initiated by the female, who releases a pheromone to signal receptivity (Guss *et al.* 1983). Males then rapidly approach, mount and initiate copulation. From the very onset of copulation the male strokes the females with his antennae. The distinctive pattern of antennal stroking, which consists of "triplets" of three strokes, is very consistent, but the time interval between triplets varies considerably between males. The rate of this "antennation" is the only predictor known for the outcome of mating, with a faster rate significantly correlated with a higher probability of a successful spermatophore transmission. In addition the duration of

antennation, i.e. time until acceptance by the female, is shorter with a faster stroking rate. Other factors that could determine mating outcome, such as the mass of the male, aedeagus dimensions, or the quantity of cucurbitacins, (a toxic plant compound used by these beetles for defense against predation and passed in the spermatophore), have been shown not to be significantly correlated with female acceptance of spermatophore transfer (Tallamy *et al.* 2002a, b).

Antennation rates show a normal distribution in the population, suggesting that the trait is polygenic (Falconer & Mackay 1996), and earlier work has shown antennation rates to be highly repeatable in individuals and also to be heritable (Tallamy *et al.* 2002a).

I have previously found a significant correlation between high antennation rates and flight ability (Darlington, unpublished data); males with high stroking rates are much more likely to fly rather than run in a simulated escape test. Besides demonstrating a very high level of physiological fitness (Kammer & Heinrich 1978; Casey 1988), flight ability might also be ecologically useful for feeding and dispersal in these beetles. Flight duration and patterns in this and several other *Diabrotica* species have been studied and related to field movements (Lawrence & Bach 1989), and there is evidence that *D. undecimpunctata* migrates, flying northward early in the spring (Metcalf & Metcalf 1993).

Inbreeding is a factor known to be important for both fitness and mating decisions; many species avoid inbreeding as it can lead to reduced fitness. Inbreeding depression, the reduction in fitness of inbred progeny relative to out-bred progeny, has been studied for over a century (Darwin 1868, 1876; Crow & Kimura 1970; Thornhill 1993; Falconer & Mackay 1996; Frankham 2005) and has been documented both in lab

studies (Charlesworth & Charlesworth 1987; Saccheri *et al.* 1996) and from fieldwork (Keller *et al.* 1994; Madsen *et al.* 1996; Saccheri *et al.* 1998; Gage *et al.* 2006). For example significant deleterious effects appeared 5 generations after an artificial population bottleneck in the housefly (Bryant *et al.* 1986), and reduced fitness has been documented in aphids that ‘selfed’ instead of mating with other individuals (Akimoto 2006).

There is experimental support for the theory that inbreeding depression has a greater impact on traits that are more closely associated with fitness. For example, in *Drosophila*, fertility is affected more than bristle number (Falconer & Mckay 1996; DeRose & Roff 1999). In male song sparrows higher levels of inbreeding are correlated with both declining immune response and also a reduction in the size of their song repertoire, a secondary sexual trait comparable to antennal stroking rates (Reid *et al.* 2005). However, inclusive fitness theory predicts that inbreeding is advantageous, in the absence of deleterious alleles, as matings between similar genotypes passes down more of the shared genes. If this is the case we might see a preference for closely related mates, up to the highest level of inbreeding possible before the onset of inbreeding depression.

METHODS

Spotted cucumber beetles, *Diabrotica undecimpunctata howardi* were reared and maintained as per Tallamy *et al.* (2000). Eggs were placed in different rearing containers so that siblings could be identified. Emerging adults were separated by sex and so were known to be virgins. Because a low rate of reproduction is typically realized in the lab, with only about 1% of the eggs surviving to adulthood, I mated all the suitable beetles that emerged. I was able to maintain three of the resulting fully inbred paternal lines for six generations.

Full siblings of the proper age for mating, 3-7 days after eclosion for females and 7-10 days for males, were paired in test tubes for one hour. The rate of mating antennal stroking, if any, was recorded on videotape under magnification and then quantified with a hand counter. This “antennation” consists of a stereotyped pattern (Tallamy *et al.* 2002a). The duration and location of the actual stroking can be seen clearly in slow motion video playback and is very consistent across males, but the time interval between the “triplets” (set of three strokes) of antennal stroking varies greatly between males; antennal stroking rates are always presented here as the number of triplets per minute.

The outcome of the mating was scored positive if, during the one-hour test period, the male was able to achieve access to the females’ bursa with his aedeagus. This is indicated by an immediate cessation of antennation; the antennae are instead held stationary and low over the males’ back. The male then proceeds to pass the spermatophore, it is pumped over in liquid form and jells inside the bursa. The male continues to ride on the back of the female for several hours, and after that time the

female is never receptive again. A negative mating outcome was recorded if: (1) The male gives up and drops off the females' back in the course of the one-hour test period, or (2) the antennation is still occurring at the end of the hour, so the eventual outcome is unknown at that time. Because males can antennate up to 8 hours, logistics required this simplification even though as a result some potential positive matings are recorded as negatives.

The out-bred groups were made up of randomly paired adults of the proper age from my lab colony. This rearing population was established in 2002 with wild beetles from the Pennsylvania State University test plots at Rock Springs, University of Delaware plots, and from several local PA organic farms, and so should represent a diverse genetic pool. Here in central PA the 2003 season was poor and so the original population suffered a complete collapse during 2004. It was re-established with wild beetles, collected mostly during the 2004 season (as 2005 was again a year with low population numbers), and also lab-reared beetles from the University of Delaware and the USDA ARS Vegetable Lab in SC.

I used JMP statistical software to perform ANOVA, ANCOVA, t tests and calculate standard errors.

RESULTS

Males with the highest antennation rates were more likely to achieve a positive outcome, i.e. pass a spermatophore to the female (Fig. 1, t test, $N = 655$, $R^2 = 0.08$, $P < 0.0001$). A multivariate analysis indicated the duration of antennation was highly correlated with the antennation rate and the outcome of the mating attempt (ANCOVA, $N = 576$, $R^2 = 0.299$, F Ratio = 81.27, Prob > F = < 0.0001), and also with the interaction term 'mating outcome by antennation rate' (F Ratio = 12.93, Prob > F = 0.0004).

An ANOVA indicated no difference between the five inbred lines, and a significant effect of generation in the male mating antennation rate (ANOVA F Ratio = 2.6856, Prob > F = 0.0149). Over time the mean rate increased despite 6 generations of full sibling inbreeding (Fig. 3); the mean antennation rate during inbreeding was significantly different from both the initial and the final out-breeding group mean (Table 1). Note that this increase in antennation rate was not due to simple natural or artificial selection, as faster antennators did not produce more offspring (Fig. 4); and also that the rates were normally distributed in the population (Fig. 5).

Figure 6 shows the time until antennation was initiated, which is dependent on the female signaling receptivity with a pheromone (Guss *et al.* 1983), was shorter when the eventual outcome of the mating was positive (t test, $N = 646$, $R^2 = 0.041$, $P < 0.001$). During the study's inbreeding phase this measure was found to be significantly different from the out-bred phase (Fig. 7, t test, OUT BRED $N = 270$, INBRED $N = 376$, $R^2 =$

0.017 $P = 0.001$), as was the duration of antennation (Fig. 8, t test, OUT BRED $N = 196$, INBRED $N = 380$, $R^2 = 0.036$, $P = < 0.0001$).

DISCUSSION

Inbreeding depression is commonly explained by one of two subtly different theories: (1) the “overdominance” hypothesis assumes that heterozygotes have higher fitness than homozygotes, and because inbreeding results in more homozygotes the population shows a reduction in fitness (East 1908; Shull 1908), or (2) the “partial dominance” theory posits that the reduction in fitness is due to the greater exposure of deleterious alleles. Such deleterious alleles would be hidden if recessive and in the heterozygotic condition, but appear in the phenotype when mating with close relatives results in more homozygotes (Davenport 1908; Bruce 1910; Keeble & Pellew 1910; Jones 1917). These theories are not mutually exclusive and both have support in the recent literature, (see Crnokrak & Barrett 2002 for a review). The amount of actual fitness loss due to inbreeding, if any, depends on the genetics involved. If dominance is present in the genes for the trait, a linear decline in fitness is seen, while epistatic interactions between loci result in a quadratic fitness decrease, and if there are only additive effects for the trait in question then inbreeding depression does not occur at all (Crnokrak & Barrett 2002).

It is also possible for sustained inbreeding to eventually result in a direct increase in individual fitness. Most organisms carry a number of deleterious genes that are not subject to selection as they are recessive and carried in the heterozygous condition. For

example, in *Drosophila* approximately 40% of the third chromosomes found in nature carry lethal genes (Simmons & Crow 1977). This “genetic load” (Haldane 1957; Lincoln *et al.* 1998) becomes visible when inbreeding increases the number of homozygotes in the population, individuals that are indeed less fit. This then is inbreeding depression, as explained by the “partial dominance theory”. Thereafter continued inbreeding can result in an actual increase in fitness, as selection has “purged” these deleterious genes from the population after they have appeared in the phenotype (Charlesworth & Charlesworth 1999; Crnokrak & Barrett 2002; Jamieson *et al.* 2006).

I was originally concerned that the spotted cucumber beetles would be able to recognize close kin and refuse to mate with brothers or sisters, as found in the ant *Iridomyrmex humilis* (Keller & Passera 1993), and field crickets *Gryllus bimaculatus* (Simmons 1989, 1991; Bretman *et al.* 2004). However, there was no problem obtaining matings between full siblings in this species; the percentage of beetle pairs that did initiate mating in the inbred lines was approximately 60-70%, similar to that observed for out crossed matings. Yet female spotted beetles are not simply mating indiscriminately. Females must first signal receptivity with a sex pheromone (Guss *et al.* 1983), and the mean elapsed time until antennation was initiated is significantly correlated with the eventual outcome of the mating (Fig. 6). So the female is evidently able to judge the quality of a male even before releasing the pheromone and allowing the male to start antennation. When I attempted to pair wild field-caught males with colony-reared females, mating was not initiated and antennation did not occur. After several weeks of keeping the males in the colony room on the lab diet some mating did finally occur, but the acceptance rate was very low (Darlington, unpublished data). Evidently the females

were rejecting the wild beetles, presumably by smell, and not releasing the sex pheromone that indicates receptivity so that the antennation phase can occur. Evidence for this idea can be seen in the relatively low coefficients of determination, (Fig. 1 total $R^2 = 0.075$, Fig. 2 total $R^2 = 0.0186$), indicating that antennation alone does not determine mating outcome. Also note the differences in the relationship as indicated by the ANOVA results given in the legend for Fig. 1, antennation rate determined 20% of the mating outcome result for the out-bred group ($R^2 = 0.20$), but less than 3% ($R^2 = 0.026$) for the inbred beetles. It appears that the relatedness of the male is another factor involved in female mating choice, and that antennation rate is not the trait used to assess relatedness or homozygosity.

Inbreeding is the norm rather than the exception in some arthropods, such as social spiders (Reichert & Roeloffs 1993), bark beetles (Jordal *et al.* 2002) and termites (Goodisman & Crozier 2002), and a lack of inbreeding avoidance has been seen in other insects: Glanville fritillary butterflies Haikola *et al.* 2004), black field crickets *Teleogryllus commodus* (Jennions *et al.* 2004). There is theoretical and experimental support for the concept that close inbreeding is actually advantageous and preferred under some conditions. Mating with a related individual, with a similar genotype, means more of an individual's genes get passed down to their offspring (Parker 1979). This inclusive fitness concept may explain why Colorado Potato beetles siblings mate more often in the lab than non-siblings (Kokko & Ots 2006), and the probability of mating in flour beetles increases when the male and female share the same genotype (Nilsson *et al.* 2002).

D. undecimpunctata appears to be quite resistant to the negative effects of inbreeding, as after 6 generations of full sib matings two beetles would be inbred as to

share almost 3/4 of their genes, $F = 0.734$ (Falconer & Mackay 1996). Yet this high level of inbreeding clearly did not decrease the fitness of the males in terms of the antennal stroking rate, a character that is significantly correlated with mating success and also flight ability. Traits such as these, that have a high fitness component, are expected to display larger negative effects under inbreeding than neutral traits (Falconer & Mackay 1996). A review study of 54 animal species, using a much lower level of inbreeding, $F = 0.25$, found that life-history traits (closely related to fitness, e.g. fecundity) declined in value 11.8% while morphological traits (less closely related to fitness, e.g. adult body mass) showed a reduction in value of only 2.2% (DeRose & Roff 1999).

The complete lack of inbreeding depression found for antennation rate indicates that this behavioural trait has additive genetic effects only, with no dominance or epistatic allele interactions; the fact that it is normally distributed in the population (Fig. 5) suggests that it is polygenic (Falconer & Mackay 1996). Note that the antennal stroking rate increase was not due to greater survivorship in the offspring of fast antennators (Fig. 4), and so does not represent natural or artificial selection. The rate increased in two outbred groups as well, one before and one after the inbreeding (Table 1), therefore the rise in rate is most likely due to adaptation to the lab rearing conditions (Willis 1999; Lima *et al.* 2004; Miyatake & Matsumura 2004). Additional evidence of heightened sexual selection for antennal stroking rates in the colony can be seen in Figs. 7 and 8 as inbred beetles had both significantly longer time until antennation was initiated and longer duration of antennation. This effect might just an effect of inbreeding on the females, or it might indicate females became more choosy in the lab, were testing prospective mates

more carefully, and appear to have been more discriminatory against mating with close kin.

So female spotted cucumber beetles appear to reject unrelated wild males, evidently preferring mates with more similar phenotypes, and they tested males that were very closely related for longer, yet still accepted them as mates. This is in line with the inclusive fitness theory that it is advantageous to mate incestuously, so that more of the shared genes are passed down to the offspring, as long as the deleterious effects of inbreeding depression are avoided. The optimum level of inbreeding can theoretically exceed 67% under some conditions (Kokko & Ots 2006), approaching the very high F figure of 0.734 eventually achieved here. Note the highest antennation rates were seen in the 4th generation of inbreeding, with a lower rate found in the most inbred 5th and 6th generations (Fig. 3). It is possible the negative effects of inbreeding depression were just starting to appear, although inbreeding depression might not be clearly seen under benign lab conditions.

In conclusion, another factor important for female choice in this species has been found; in addition to the antennal stroking rate, the relatedness of the male must be considered as well. And as closely inbred male spotted cucumber beetles do not show inbreeding depression in the lab and are preferred over unrelated mates, it is clear that, in general, one cannot say that inbreeding is always deleterious.

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TABLES

Table #1

INBREEDING AND ANTENNATION RATE

TIME	N	MEAN RATE	S.E.
OUTBREEDING 2002-2003	325	76	1.3
INBREEDING (9/03 to 5/04)	296	87	2.4
OUTBREEDING 2004-2005	158	93	2.6

FIGURE LEGENDS

Table 1.

Three breeding groups: (1) An initial out-bred group, (2) Six generations of full sibling inbreeding in five lines, and (3) A final out-bred group, all have significantly different mean antennation rates; the rate increased over time.

Fig. 1.

The mean antennal stroking rate for mating male spotted cucumber beetles was positively and significantly correlated with a positive mating outcome, one in which a spermatophore was passed to the female. A negative outcome was scored if the male stopped antennating and dismounted the female or the antennation continued beyond a one-hour total elapsed time limit so the eventual outcome was unknown. The figure represents all matings (t test, $N = 655$, $R^2 = 0.075$, $P < 0.0001$), the results were also significant for out bred matings alone (t test, $N = 274$, $R^2 = 0.200$, $P < 0.0001$) and inbred alone (t test, $N = 381$, $R^2 = 0.026$, $P < 0.015$).

Fig. 2.

Antennation rate and its duration, i.e. display time until female acceptance or male withdrawal. Females accepted males more quickly if they antennated faster. An open dot indicates a negative mating outcome, closed represents positive. The dashed

line shows no relationship between rate and duration in the negative matings, the solid line indicates a significant correlation between the duration and rate in the matings with a positive outcome (ANCOVA, N = 576, $R^2 = 0.299$, F Ratio = 81.27, Prob > F = <0.0001).

Fig. 3

The antennal stroking rates for each inbred paternal line, with the number of matings in parenthesis. Paternal line “E” starts at the F1 generation because the P Generation individual had an unknown antennation rate. The antennation rate increased significantly during the 6 generations of inbreeding, with generation treated as ordinal data (ANOVA RSquare = 0.07, F Ratio = 2.15, Prob > F = 0.021).

Fig. 4

The number of offspring sired compared to the antennal stroking rate for inbred male cucumber beetles. Beetles with higher rates did not produce more offspring that survived to adulthood than males with lower stroking rates. N = 205.

Fig. 5

The antennal stroking rate was normally distributed in the population (Mean = 85.4, St. Dev. 16.0, St. Error = 0.063, N = 655).

Fig. 6

The elapsed time until antennation was initiated, after the male and female beetles were paired together, was significantly correlated with the eventual outcome of the mating. The female first releases a pheromone to signal receptivity, then the male responds with antennal stroking.

Fig. 7

Inbreeding had a significant effect on the elapsed time until mating was initiated.

Fig. 8

Inbreeding had a significant effect on the duration of antennal stroking during mating.

FIGURES

Fig. 1

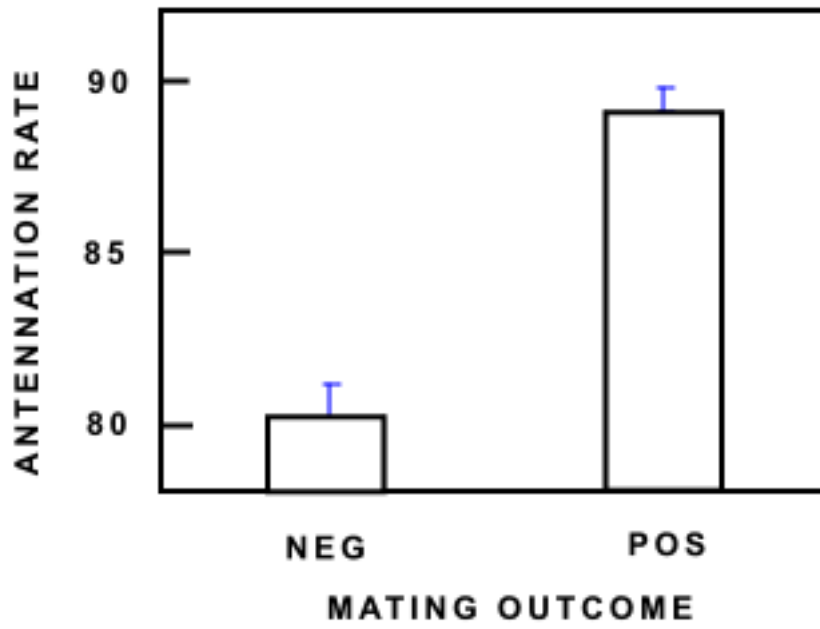


Fig. 2

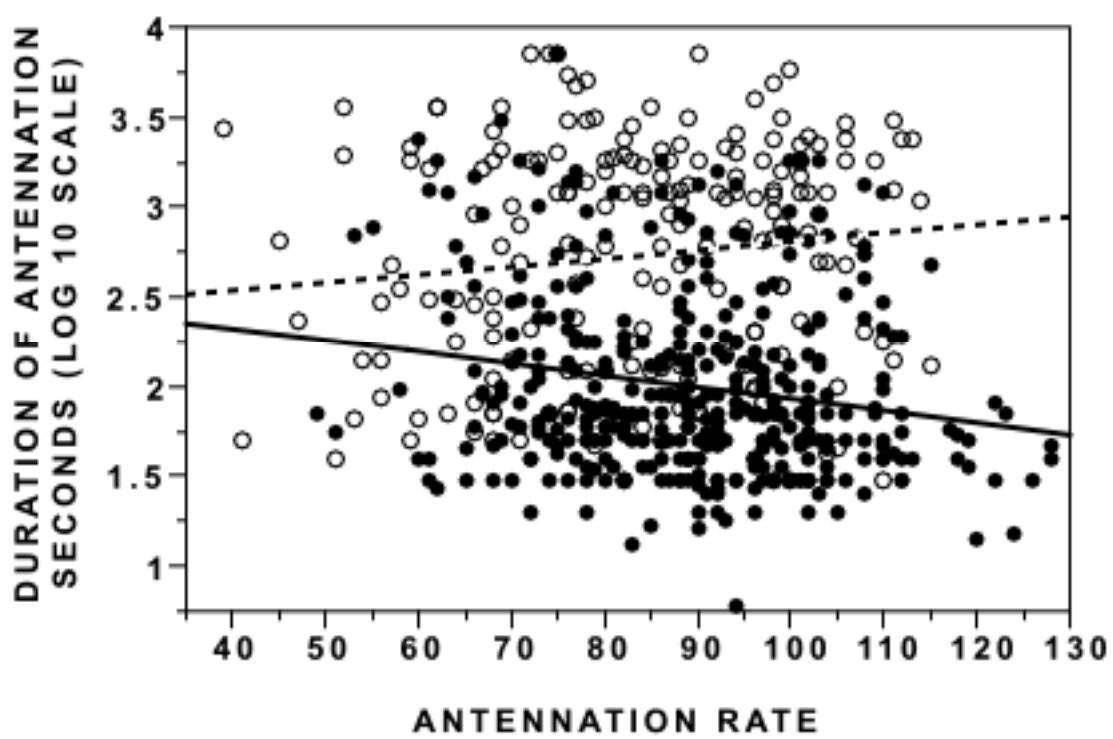


Fig. 3

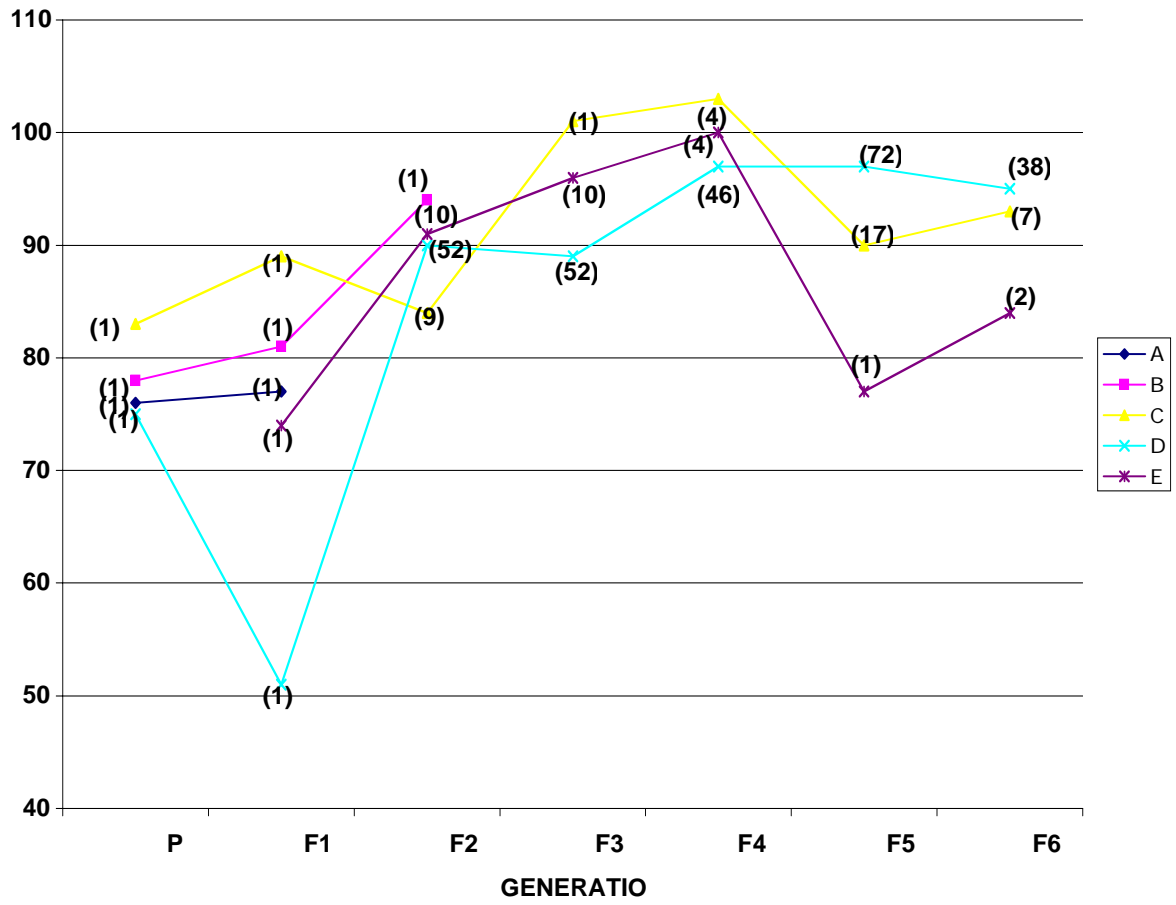


Fig. 4

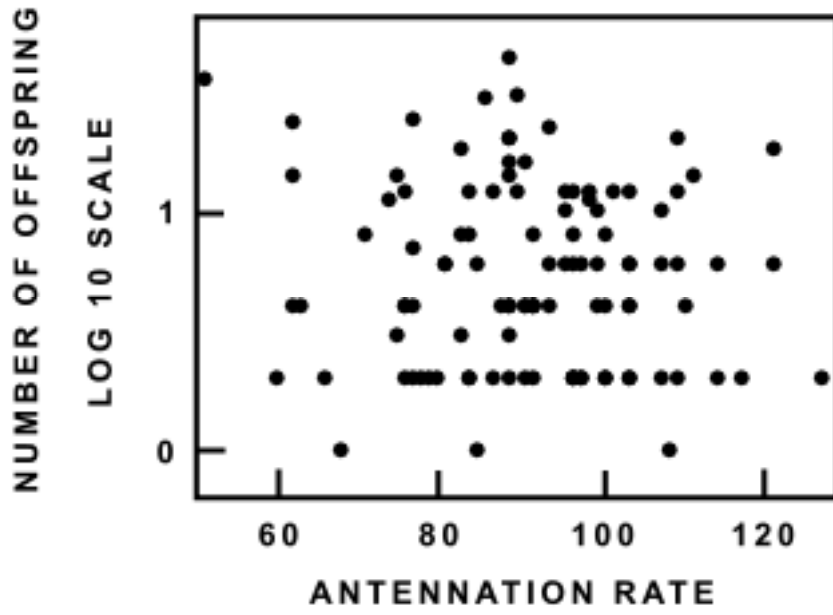


Fig. 5

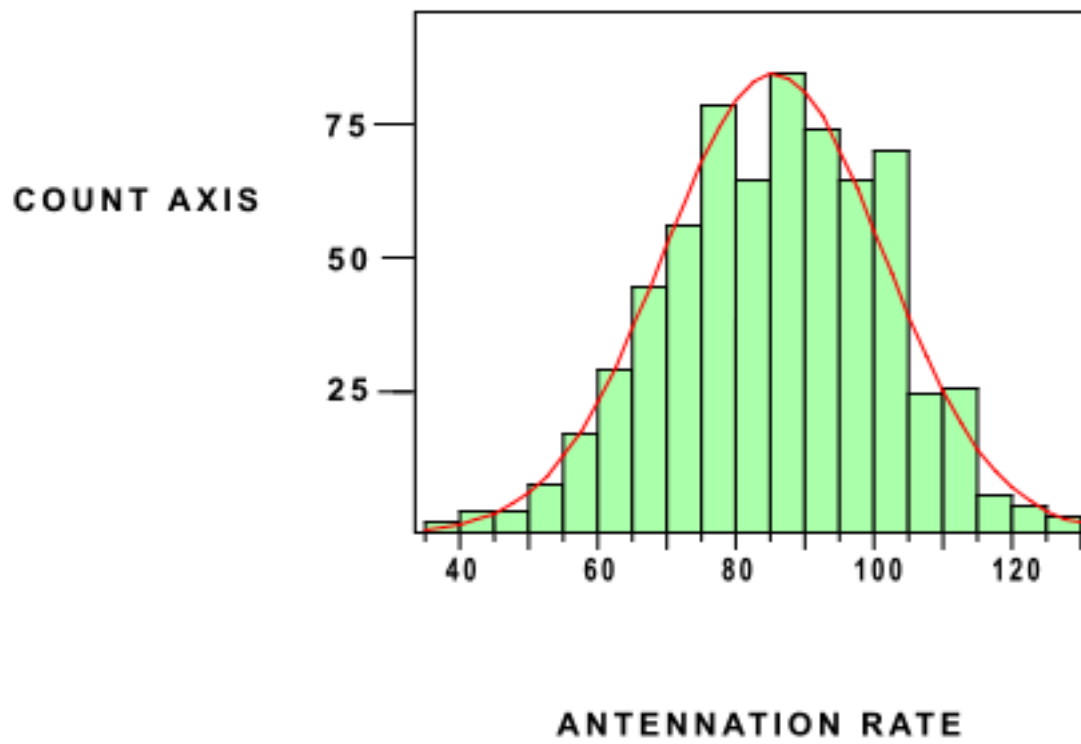


Fig. 6

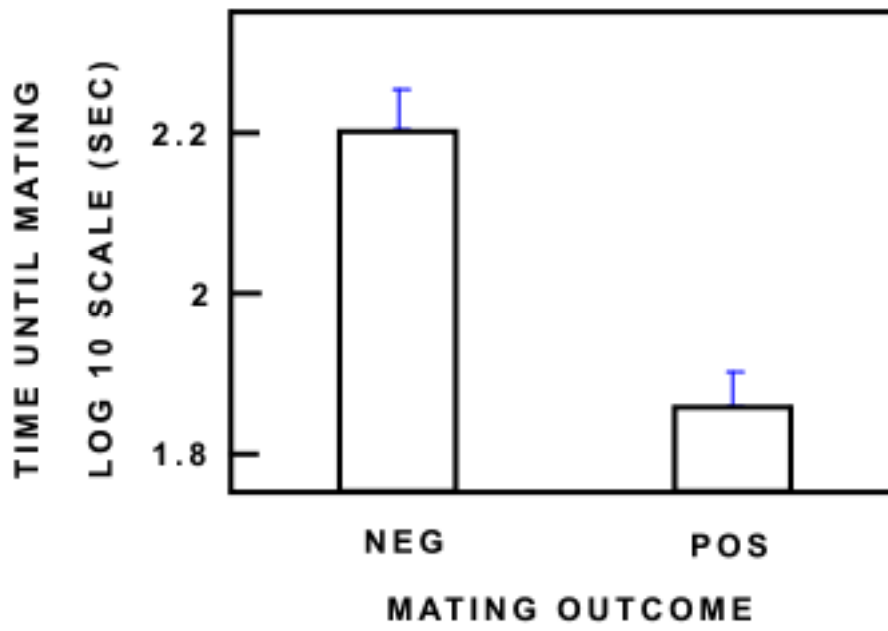


Fig. 7

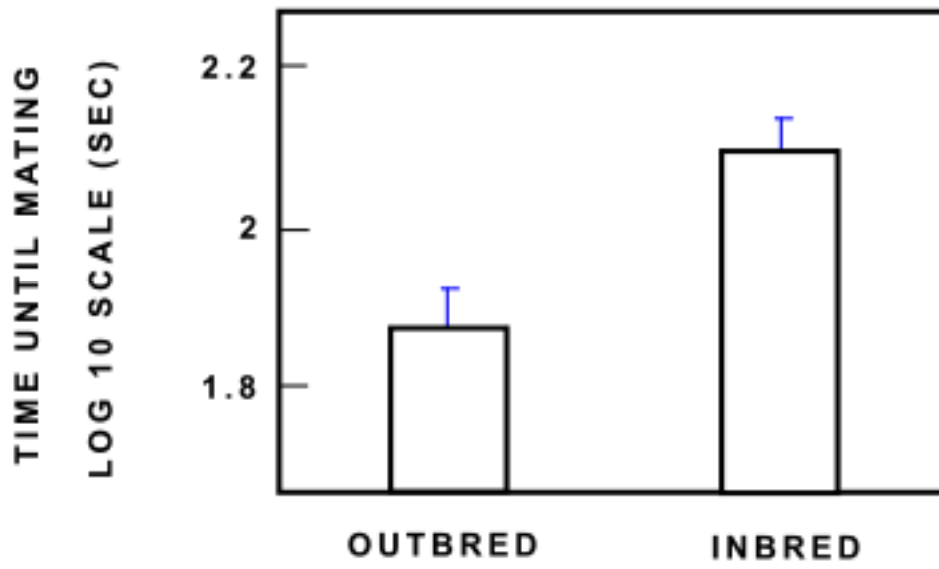
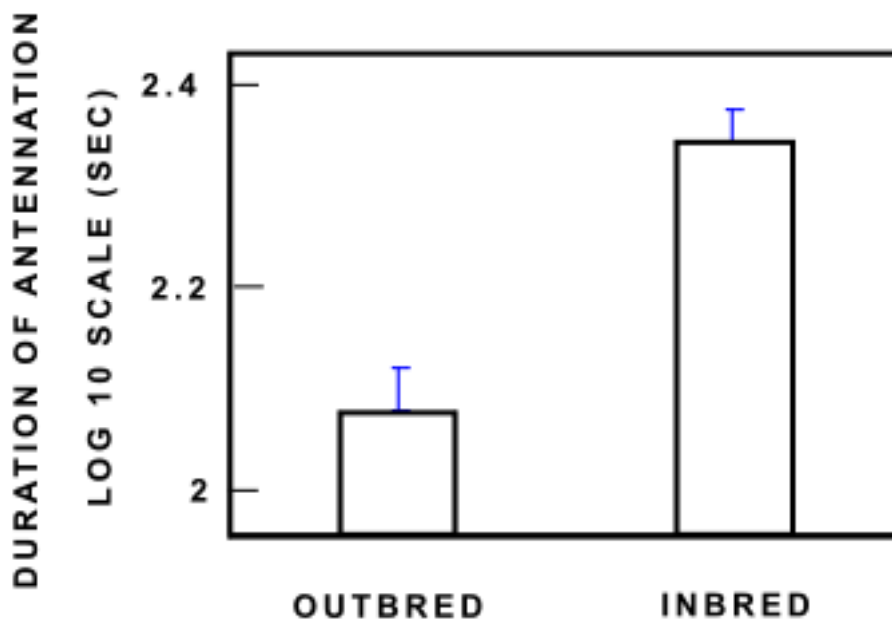


Fig. 8



Chapter 3.

Flight, Male Display Rate and Female Choice in the Spotted Cucumber Beetle,

DIABROTICA UNDECIMPUNCTATA HOWARDI.

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Abstract

Female spotted cucumber beetles, *Diabrotica undecimpunctata howardi*, demonstrate “cryptic female choice” during mating by using muscle control to selectively block male access to the bursa copulatrix. Males stroke the female with their antennae in a distinctive pattern as a mating display. The rate of this rhythmic antennal stroking, which has been previously shown to be heritable and highly repeatable in individuals, is the best predictor known of female receptivity. I tested the hypothesis that antennal stroking rate is a marker used by females to assess mate quality, and found a highly significant correlation between faster antennal stroking rates and flight (vs. running) in a simulated escape test. Flight ability requires a high level of physiological fitness, is potentially useful for foraging, dispersal and evading predation, and might be an advantage for males in obtaining cucurbitacins, i.e. a nuptial gift of defensive plant chemicals. After discovering this flying vs. running dichotomy in lab-reared beetles, I found the same behavioural response in wild beetles. Fieldwork indicated a higher proportion of beetles found in squash fields early in the season were flyers. A number of morphological and physiological parameters were examined to locate a proximal cause of the ‘flight no-flight’ difference, however no character was found that separated males and correlated with antennal stroking rate. Evidence is presented that flight is not highly advantageous in male spotted cucumber beetle life history and field ecology, and I conclude that flight propensity might not be a reliable indicator of mate quality.

Introduction

The concept of female choice during mating was part of Darwin's original theory of sexual selection (1871). The term "cryptic female choice" was coined by Thornhill to encompass internal physiological mechanisms that would allow a female to bias paternity toward a particular mate (1983). Eberhard's work has expanded the concept of female choice, and made it clear that during and even after sex the outcome can still be in question, due to competition as well as cooperation between the two mates (1996). Cryptic female choice has been documented in the spotted cucumber beetle, *Diabrotica undecimpunctata howardi*; females selectively tighten a sphincter muscle at the base of the bursa copulatrix that blocks transfer of the spermatophore (Fine 1997). Female beetles often mate with multiple males but only ever accept one spermatophore, while in contrast males can mate successfully at least 16 times, rebuilding a new spermatophore every two days (Tallamy *et al.* 2002a, b).

Female spotted cucumber beetles initiate mating by releasing a pheromone to signal receptivity (Guss *et al.* 1983). From the very onset of copulation the male rhythmically strokes the female with his antennae. The stroking is invariably in a distinctive pattern of sets of three strokes or "triplets", with the time interval between triplets varying greatly between males. The rate of this "antennation" is the best predictor known for the outcome of mating, with a faster rate significantly correlated with a higher probability of a successful spermatophore transmission (Tallamy *et al.* 2002a).

In addition the duration of antennation, i.e. time until acceptance by the female, and the time until mating is initiated (after the beetles are randomly paired together), are both shorter with a faster stroking rate (Darlington Submitted). Also *D. undecimpunctata* antennae are sexually dimorphic, males have approximately 18% longer antennae than females, and when one antenna is surgically removed a male's chances of being accepted as a mate for the second time drops from at least 50% to zero (Tallamy *et al.* 2002b, also see Fonseca & Bennet-Clark 1998). Antennation rates have been shown to be highly repeatable in individuals, heritable ($h^2 = 0.62 \pm 0.20$, Tallamy *et al.* 2002a, with Houle's (1992) Additive Coefficient of Genetic Variation or $CV_A = 16.7\%$), and be polygenic with a normal distribution in the population (Darlington Submitted).

The level of inbreeding has also been found to be important in female choice (Darlington Submitted), but other factors that might determine mating outcome, such as the mass of the male (Iyengae 1998a), aedeagus dimensions, or the quantity of cucurbitacins, (a toxic plant compound used by these beetles for defense against predation and passed in the spermatophore), have been shown not to be significantly correlated with female acceptance of spermatophore transfer (Tallamy *et al.* 2002a, b).

In an attempt to explain the female preference for high antennation rate, I tested the hypothesis that fast antennation is associated with other male trait that have benefits for females. Mating displays in other species have been found to be resource-dependent, and so represent an "honest signal" of male condition. This was verified when supplemental feeding of the male increased the display rate *in vivo*, e.g. the salivary secretion nuptial gifts of scorpionflies (Engels & Sauer 2006) and the mud pillar building by male fiddler crabs (Backwell *et al.* 1995). Other studies, in birds, showed resource

manipulations during development later affected adult song quality (Spencer *et al.* 2005, Buchanan *et al.* 2003). Earlier work in *D. undecimpunctata* found no difference in adult antennation rate when larvae were raised under food-restricted conditions (Tallamy *et al.* 2002a).

The hypothesis was that the speed of this mating display truly indicates male physiological condition, such as drumming in male wolf spiders (Mappes *et al.* 1996), courtship rates in damselfish (Kovath 1991), and bird song complexity (Spencer *et al.* 2005, Buchanan *et al.* 2003). I tested for between-male variation that correlated with antennal stroking rate, and found a highly significant behavioural difference involving flight rather than running in a simulated escape test. This behavioural response pattern was then found to be present in both lab-reared and wild beetles.

Methods

To quantify antennal stroking rates during mating, beetles were randomly paired in test tubes for one hour. Insects of specific ages were used; 7-10 days after eclosion for males and 3-7 days for females. Males need to build a spermatophore before mating, and in females the refusal rate during mating has been found to drop beyond that age (Tallamy *et al.* 2002a). Male beetles can be identified by an extra ventral plate on the posterior end of the abdomen (Krysan 1986). The rate of antennal stroking, if mating occurred, was recorded on videotape under low magnification and then quantified with a hand counter. This “antennation” consists of a stereotyped pattern of “triplets” (set of three strokes) that is very consistent across males, but the time interval between triplets

varies greatly between individuals. Antennal stroking rates are given here as the number of triplets per minute, ranging from approximately 40 to 130.

A simple 'fly vs. no-fly' test was found to separate males; when released from an aspirator tube held vertically at least 15 cm from any surface, some individuals invariably flew up and away while other males always ran down the aspirator tube (Fig. 1).

I examined male beetles for morphological differences that correlated with flight vs. running. The dry mass of the thorax, which is composed almost entirely of muscles used for locomotion, was examined as a proportion of the total (dry) mass of the insect (Fig. 2). Wing loading was measured, calculated as the weight of the insect divided by the area of the wings (Fig. 3). I quantified body fat by subtracting the lean dry body mass of the beetle from its dry body mass. The lean mass was determined by dissolving the fat with refluxing chloroform in a Soxhlet apparatus for 4 hours (Marden 1989) (Fig. 4). An open flow respirometer was used to quantify resting metabolism rate, measured as the residuals of the rate of the CO₂ volume expelled per bodyweight (Fig. 5).

I examined flight at different temperatures (Meinke & Gould 1987), testing the hypothesis that males were adapted to different optimum temperatures (Rank & Dahloff 2002, Hughes & Zalucki 1993, Watt *et al.* 1985, 2003, Zera 1987). I first tested at 25C, then included 20C and 30C, and later added 15C and 35C as per Naranjo (1991). The temperatures were T_e, or "effective environmental" temperature, (sometimes called T_o, or "operative" temperature), as they were ambient air temperatures in an environmentally controlled room, with no exposure to sunlight (Heinrich 1993). This insured constant experimental conditions without the need to directly measure body temperatures, which

could have damaged these small, soft-bodied beetles. All insects tested were reared at 25 C (and 50% relative humidity), and were kept at these conditions except for a one-hour exposure period, at the test temperature, just before the experiment began. This insured that the insect's body temperature had stabilized at the new figure but should also have limited any possible adaptation or acclimation effects (Willmer *et al.* 2000). Beetles tested were either captured wild in the Penn State University Rock Springs fields, or colony reared from the University of Delaware and the USDA lab in South Carolina. Individual beetles were repeatedly tested for flight attempts at various temperatures, with any one beetle being tested only once per day (Table 1).

I also tested wild male beetles to see if they displayed the same flight-running behavior; during the 2005 and 2006 seasons I collected all the beetles found in six central Pennsylvania squash fields, and tested for flight in the lab. Collecting was done at least weekly, starting in the early morning hours; every plant was examined in random order. I captured beetles off squash flowers and squash fruit with a homemade aspirator, not with a net, and so my sample would not be biased toward flyers or runners. To examine the ecological significance of flight in these beetles, I tested the idea that the distance to the nearest cornfield, where this season's adult beetles would emerge after developing on corn roots, was correlated with flying in males. I sampled (without replacement) all the spotted cucumber beetles found in six squash fields; the location of capture within the field was recorded, later in the lab sex ratio was determined and flight propensity was tested in males and some females. Four of the fields were Penn State University test plots, and two were local (central Pennsylvania) organic farm fields (Tait Farm, Boalsburg PA.) The two organic farm fields were yellow crookneck summer squash,

(*Cucumis pepo*) grown for consumption, while the four Penn State fields were experimental plots of *Cucurbita pepo* var. *Texana* squash that was bitter, containing cucurbitacins.

JMP statistical software was used to perform t tests and calculate standard errors.

Results

I found that some individuals were consistently taking off and flying away, while others always ran and were never attempting flight, even when escaping. This led to the discovery of a highly significant correlation between males that flew rather than ran and fast antennation rate during mating (Fig. 1, t test, N = 563 male beetles, $P = < 0.0001$). 'Flyers' made up 46% of the total, 'runners' 54% of these lab-reared beetles. I sampled another colony by means of a shipment of beetles from the USDA ARS US Vegetable Lab colony in Charleston SC on July 24, 2006, and found that fourteen males out of 35, or 40% were fliers.

The non-flyers appeared healthy and were indistinguishable from other males, yet they made no attempt to fly. They could not be induced to even spread their wings; when thrown into the air they simply fell to the ground. Dissection indicated that the wings and flight muscles of 'runners' were of normal size and appearance, and no parasites e.g. gregarines (Schilder & Marden 2006) were found to reduce fitness levels (Kolluru *et al.* 2002, Zuk 1987). I examined the dry mass of the thorax, which is composed almost entirely of the muscles used for flight, as a proportion of the total (dry) mass of the insect, and found no significant difference between flyers and non-flyers (Fig. 2). Wing loading

measurements, calculated as the weight of the insect divided by the area of the wings, showed no distinction between flyers and non-flyers (Fig. 3).

In damselflies, flight ability demonstrates energy reserves in the form of body fat deposits (Marden & Waag 1990). However, no correlation was found between body fat and flight in these beetles (Fig. 4).

An open flow respirometer was used to quantify resting metabolism, measured as the residuals of the rate of CO₂ volume expelled per bodyweight. The basal levels for flyers and runners showed no significant differences (Fig. 5).

I examined flight at different temperatures (Meinke & Gould 1987, Naranjo 1991), testing the hypothesis that males were adapted to different optimum temperatures (Rank & Dahloff 2002, Hughes & Zalucki 1993, Watt *et al.* 1985, 2003, Zera 1987). I first tested at 25C, then included 20C and 30C, and later added 15C and 35C. However an individual male that flew at 25C also flew at almost all of the other temps where flight was seen, and no runners became flyers at different temps (Table 1).

I tested wild male beetles to see if they displayed the same behavior, checking that it was not just an artifact of the artificial colony rearing. During the 2005 and 2006 seasons I collected all the beetles found in six central Pennsylvania squash fields, and tested for flight in the lab. There were very few spotted cucumber beetles until late in the 2005 season. All the beetles I collected were male, and my colony females rejected these unrelated males as mates (Darlington Submitted). During the 2006 field season I collected at least weekly, and tested all the wild beetles found in four Penn State University and two local organic farm squash fields. Wild beetles showed the same fly or

run behaviour, however there was a strong seasonal difference with more flyers early and runners later (Fig. 6).

To examine the ecological significance of flight in these beetles, I tested the idea that the distance to the nearest cornfield, where this season's adult beetles would emerge after developing on corn roots, was correlated with flying in males. I sampled (without replacement) all the spotted cucumber beetles found in six fields here in central PA and recorded location of capture, and tested for flight ability in males and some females. I found that flyers were significantly more common in the first half of the season (Fig. 7), while runners dominated later in the year (Fig. 8). There was a non-significant trend early in the field season for beetles to be found in the southern end of the fields (Fig. 9), verifying that the direction to the nearest cornfield was not a factor as in all cases the corn was not located to the south. Collecting later, after September 7th, represents insects found on squash fruit, as the bloom was mostly gone from the plants by that date; these data show no North-South or other distribution pattern (Fig. 10). The sex ratio was very skewed toward males, with only a small number of females collected in the squash fields early in the season (Fig. 11).

Discussion

Female benefits from mating choice are characterized as 'direct' (or 'phenotypic') such as nuptial gifts of protein or defensive chemicals, or 'indirect', e.g. "good genes" benefits that only appear in the offspring of a choosy female (Kirkpatrick 1996, Moller

& Alatalo 1999). Earlier work on spotted cucumber beetles failed to find any direct benefits in the form of increased fecundity, age at first oviposition, egg hatch rates or longevity for female beetles that mated with fast antennating males (Tallamy *et al.* 2002a), and a recent study on the protein content in spermatophores also had a negative result (Darlington Unpublished). Indirect benefits were examined by quantifying maturation times, body size, age at first oviposition, and fecundity in the offspring, and again no differences were found (Tallamy *et al.* 2002a). Early evidence that offspring survivorship differed based on the antennation rate of the male parent (Tallamy *et al.* 2002a), a possible indirect ‘good genes’ benefit (Knapp and Kovach 1991, Welch *et al.* 1998, Hoikkala *et al.* 1998) has since been discounted with more research (Darlington Submitted).

It is logical to assume that female choice during mating would result in some benefit that could be measured, and so would require no additional explanation. However, in a few cases females have been found to prefer traits that do not exist in males of that species, such as artificial crests on finches (Burley, in Futuyma 1998). This is thought to be due to a pre-existing genetic disposition for that character, which would then presumably lead to the eventual evolution of the trait in that species (Ryan 1990, 1997; Basolo 1994).

However, previous work did find one indirect ‘good genes’ benefit (Watt *et al.* 1986, Hoikkala *et al.* 1998, Iyengae 1998b), in that fast antennation rates were inherited by the male offspring (Tallamy *et al.* 2002a), thereby making them more attractive to females (Jones *et al.* 1998, Tomkins & Simmons 1999). This may represent an example of ‘runaway sexual selection’ (Fisher 1930, Jones *et al.* 1998) whereby the preference for

the trait is passed down to the females of the next generation, and the attractive trait itself is inherited by the male offspring. As a ‘self-reinforcing choice’ (Maynard Smith 1978), a trait with these characteristics would spread very rapidly through the population (O’Donald 1962, Kirkpatrick 1982).

I examined a number of physiological and morphological measures in an attempt to differentiate flyers and non-flyers, and so explain this behavioural difference: % thorax mass, (as a measure of flight muscle, N = 27, Fig. 2), wing loading, N = 27 (Fig. 3), % body fat N = 27 (Fig. 4), resting metabolism N = 87 (Fig. 5), and flight at different temperatures (Table 1), but found no significant differences.

The likelihood of attempting flight is not a function of the age of the individual, as has been found in the Milkweed Bug (Dingle 1965); males retested over several weeks did not change categories (Darlington Unpublished). Naranjo (1991) found also that a percentage of male *Diabrotica virgifera virgifera* did not fly at all and that flight activity in the Western Corn Rootworm males changed little with age. He did find an effect of larval rearing density on adult flight, with the highest percentage of ‘trivial’ flight in males from “moderate” rearing densities of 500-750 larvae per 6.3 by 15.3 cm diameter container. Higher densities, up to 1200 eggs per container, resulted in fewer flyers, a decrease he attributed to physiological stress from over-crowding. Naranjo suggested the high densities might be too high to represent natural conditions (1991). These densities are far higher than I used for rearing, as I used much larger 22cm by 22cm diameter pots with a maximum of approximately 500 eggs in an attempt to raise the survival rate, get more adults for testing and keep my colony viable. In one study I examined survivorship in the offspring of spotted cucumber beetles as related to antennation rates in the parental

generation, and placed only 20 eggs in each container. I included the same amount of corn roots as food, to reduce stress on the larvae (Spencer *et al.* 2005, Buchanan *et al.* 2003); this resulted in no change in flight behaviour or antennation rate (Darlington Submitted).

Other male insects have been shown to undertake flights (of a significant duration), which were thought to be associated with a failure to mate (Dingel 1965; Naranjo 1990), but this is not an issue here as I tested for flight attempt before mating, and also re-tested some individuals for flight tendency multiple times after they mated.

Flight would be necessary for the early spring northern migration that has been proposed in *D. undecimpunctata* (Metcalf & Metcalf 1993), and requires a very high level of physiological fitness in an individual (Casey 1988, Kammer & Heinrich 1978). It is known that male spotted cucumber beetles will preferentially feed on squash and other plants containing cucurbitacins and pass these to the female during mating (Tallamy *et al.* 2000). In a field study Tallamy *et al.* found that 99% of the beetles that arrived at bitter fruit were male (2002b). Cucurbitacins are secondary plant defensive chemicals that are extremely bitter and toxic to other organisms, and are incorporated into the tissues of eggs, immatures and adults of both sexes to deter predation (Nishida & Fukami 1990) and confer an antibiotic effect (Tallamy *et al.* 1998). A nuptial gift of these chemicals might be easier to obtain for a male foraging by air if the squash is widely dispersed, and so mating with a flyer would potentially be an advantage to the female. Yet a female evidently cannot detect whether a male has “cucs” or not, as it is not a factor in mating outcome (Tallamy *et al.* 2002a). Also Tallamy *et al.* found that fast stroking

males were not more likely to find and sequester cucurbitacins, based on a lab simulation with 6 slices of bitter and non-bitter squash in a 1 M³ enclosure. It is therefore possible that females are using antennation rate, which is correlated with flight propensity, as a measure of the likelihood of obtaining a nuptial gift of defense chemicals. However this hypothesis would have to be tested.

The literature indicates that the predation on adult spotted cucumber beetles is mostly by ground arthropods (Brust 1991, Barbour & Brandenburg 1996, Eben & Barbercheck 1996); feeding on squash containing toxic and bitter cucurbitacins is evidently an effective protection against vertebrate predation, e.g. birds (Gould & Massey 1984). Two large wolf spiders: *Hogna hellupo* and *Rabidosa rabida* (Aranaea: Lycosidae), and a ground beetle *Cyclotrachelus sodalis* (Coleoptera: Carabidae) were identified as the main predators on adult beetles (Synder & Wise 1999). Escape by means of flight should be a highly effective defensive strategy when threatened by a ground-dwelling predator. However field data indicated a reduction in population density greater than 50% due to predation (Synder & Wise 1999), and lab simulations recorded significant losses from *H. hellupo* (Synder & Wise 2000). Williams *et al* found a strong effect when they took into account the gender of the beetles, males were 16 times more likely to be preyed upon than females, as they did not modify their behaviour in the presence of a predator. The authors conclude that sexual selection pressure on the males to not miss mating opportunities may lead them to ignore the risk of predation (2001).

During the 2006 field season I collected all the beetles I could find (“sampled without replacement”) in six squash fields, four Penn State University plots and two at a local organic farm. The two organic farm fields were yellow crookneck summer squash,

(*Cucumis pepo*) grown for consumption, while the four Penn State fields were experimental plots of *Cucurbita pepo* var. *Texana* squash that was bitter, containing cucurbitacins. The ratio of flyers and runners was not different between the different types of fields.

Early in the field season some females were caught in the squash fields, but later on it was all males (Fig. 11). The few females tested indicate that females fly until they start to mature eggs and obviously gain weight, either after mating or having achieved a certain age regardless of reproductive status; individual female flight is comparable to that of male flyers. I found I could not separate the sexes by flight in the field. The very skewed sex ratio found (fig. 11) might represent the fact that the beetles aggregate in squash blooms to mate, and the females may leave after accepting their one spermatophore while the males remain in an attempt to get more matings. In the fields examined the males stay on the squash plants, which do not have a thick vegetative undercover suitable for overwintering, until they are plowed under for the season.

The proportion of males that flew, rather than ran away, in a simulated escape test was much higher early in the season, from August 1 until September 5, while later on the percentage of runners increased greatly (Fig. 6). I attempted to verify that the flight propensity of the wild beetles was positively correlated with antennation rate as in the lab-reared beetles (Fig. 1), but found that colony females largely rejected the wild males as mates as so I could not quantify antennal stroking rates (Darlington Submitted).

Immature *D. undecimpunctata* feed on corn roots, and so I tested whether the location of capture within the field was correlated with the orientation of the nearest cornfield, where the adult beetles might have emerged. However no association was

found between the location of capture in the field and the direction to the nearest cornfield in any of the 6 fields tested. During the first half of the 2006 field season, there was a non-significant tendency that more beetles were found in the southern end of all the fields (Fig. 7), which verifies no general effect for location of capture as in all cases the corn was not to the south. Later in the field season there was no dispersal pattern (Fig. 8). However there was a significant effect that beetles were predominately flyers early in the season, until the end of August (Fig. 9). After the squash bloom was mostly gone, by early September, the beetles were found in semi-rotten squash, which was not nearly as numerous or evenly distributed as the flowers were earlier in the year; these beetles would have been all runners (Fig. 10). So flyers appeared in the squash fields before runners, either emerging earlier or traveling faster (my weekly sampling may not have been frequent enough to differentiate). Earlier work, in the lab, did not find a difference in maturation time for males based on antennation rate (Tallamy *et al.* 2002a). The sex ratio found indicates that females may be under selection pressure to emerge early and so reach the squash bloom with the flyers (Fig. 11).

Flight duration patterns in this and several other *Diabrotica* species have been studied and related to field movements (Naranjo 1991). Data indicate that these cucumber beetles are more likely to fly during the morning hours (Lawrence & Bach 1989). This effect did not appear in my lab studies. In the wild, it has been my experience that they do fly early in the day when the sun has started to warm the fields, and then retreat into the bloom that closes in the heat of the day. These beetles, like many insects, are attracted to light, and so it is possible that after eclosion they just flew toward the sun, in a generally southern direction. *D. undecimpunctata* don't seem to fly

when the winds are strong, and as these dispersal data were collected in a valley with the prevailing winds west to east, wind direction doesn't seem to be a factor.

From what is known of the life history of the spotted cucumber beetle, they overwinter as adults (Meinke & Gould 1987), and there is evidence of migration towards the north early in the spring (Metcalf & Metcalf 1993). We see only a very few spotted cucumber beetles in the squash fields here early in the year, often for just a single day (A. Stephenson, Penn State Univ., Personal Communication). Although I have not been able to sample these beetles, this flight might represent arrival of migrating individuals from the south. Here in central PA there would be only one generation a year, while farther south at least a partial second generation is produced. I assume that the spotted cucumber beetles I have sampled in central PA are emerging as adults here, as they appear in the fields late in the summer rather than in the spring; according to Metcalf & Metcalf new adults start to emerge during July in the mid Atlantic region (1993). Certainly the beetles that show no tendency to fly at all, that are more common late in the year, do not appear to be migratory.

Female cucumber beetles prefer mates that antennate rapidly, and this is correlated with flight in males, at least lab-reared males. Flying, even for the short term, requires an individual to be excellent physical condition and so would be a good mate choice. However I quantified flight propensity, and did not formally examine flight duration. The question of whether this fly-run behavioural difference is just an escape maneuver or is indicative of long-term flight ability is unresolved; the lack of physiological and morphological differences found between the males does not point to a

major distinction between ‘flyers’ and ‘runners’. I collected some data on tethered insects that indicated that the duration of flight was short, less than 15 minutes (Darlington Unpublished). Naranjo found in *D. virgifera virgifera* that most flights were ‘trivial’, and sustained flights (>20 min) were rare (1991). There were no dispersal patterns quantified here, and no other data that indicate flying ability would be an advantage, if indeed the beetles are emerging as adults this season and are not migratory. The low level of anti-predator response in the males due to sexual selection, as found by Williams *et al.*, would mean also that flight as an escape behaviour would not be highly advantageous (2001).

There is strong selection pressure on males for high antennation rate, however if there is only weak selection for flight ability, then the question of why runners are so common in the population is easier to solve. Females that mate later in the year would likely encounter runners rather than flyers (Fig. 10), and older females (> 7 days as adults) have been found to be less choosy and more often accept males with lower antennation rates.

In conclusion, flight does not appear to be highly advantageous for male *D. undecimpuncta*, and there was no evidence found that flight propensity represents an “honest signal” of the true physiological fitness in a male spotted cucumber beetle.

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TABLE 1 INDIVIDUAL BEETLE FLIGHT AT VARIOUS TEMPERATURES

BEETLE #	15C	20 C	25C	30 C	35C
892		FLY	FLY	FLY	
893		FLY	FLY	FLY	
894		-	-	-	
895		-	-	-	
896		-	-	-	
897		-	-	-	
898		-	-	-	
899		-	-	-	
900		FLY	FLY	FLY	
901		-	-	-	
903		-	-	-	
904		FLY	FLY	FLY	
905		FLY	FLY	FLY	
906		-	-	-	
907		-	-	-	
908		FLY	FLY	FLY	
909		FLY	FLY	FLY	
910	-	-	-	-	-
911	-	FLY	FLY	FLY	-
914	-	FLY	FLY	FLY	FLY
915			-		-
916	FLY	FLY	FLY	FLY	FLY
917	-	-	FLY	FLY	-
918	FLY	FLY	FLY	FLY	FLY
919	-	-	-	-	-
920	FLY	FLY	FLY	FLY	FLY
922	-	-	-	-	-
WILD	-	FLY	FLY	FLY	
WILD	-	FLY	FLY	FLY	
WILD	-	FLY	FLY	FLY	
WILD	-	FLY	FLY	FLY	

Figure legends.

Fig.1. The antennal stroking rate during mating was significantly correlated with the behaviour of the male spotted cucumber beetles in a simulated escape test, fast “antennators” flew away while slow “antennators” ran to escape (T test, $P = < 0.0001$, $N = 563$). Flyers made up 46% of the total in these beetles from my rearing colony and non-flyers 54%.

Fig. 2. The dry mass of the thorax, primarily muscle tissue, as a percentage of the total dry body mass, was found not to differ between male beetles that fly or run away in a simulated escape test ($N = 27$).

Fig. 3. The wing loading of male spotted cucumber beetles, the insect’s wing area divided by its weight, was not significantly different when flyers were compared with runners ($N = 27$).

Fig.4. The percentage of body fat did not differentiate flyers and non-flying male beetles. The fat content was determined by subtracting the lean dry body mass from the dry body mass, after dissolving the fat in refluxing chloroform ($N = 27$).

Fig. 5. The basal, or resting, metabolism of male beetles was not correlated with flight attempt ($N = 87$).

Fig. 6. Flight behaviour in wild beetles, sampled without replacement from six central PA squash fields.

Fig. 7. The mean number of beetles collected per week (N = 7). Immature *D. undecimpunctata* or “Southern Corn Rootworm” develops on corn roots; however there was no pattern based on the compass direction to the nearest cornfields, none of which were located south of the squash fields where the beetles were found.

Fig. 8. No pattern was seen in the dispersal pattern late in the season. (N = 5).

Fig. 9. Early in the 2006 season, before September 10, beetles were significantly more likely to be flyers than runners. These beetles would likely have been captured on bloom. The mean number of beetles caught in squash fields per week ($P = 0.0052$, $RSquare = 0.44$, $S.E. = 13.7$, $N = 16$).

Fig. 10. After September 10, 2006, beetles found were runners rather than flyers. These beetles would have been mostly captured on squash fruit.

Fig. 11. Spotted cucumber beetles aggregate in squash flowers to mate. It is a lek-like situation, with a very skewed male biased sex ratio.

Figures

Fig. 1

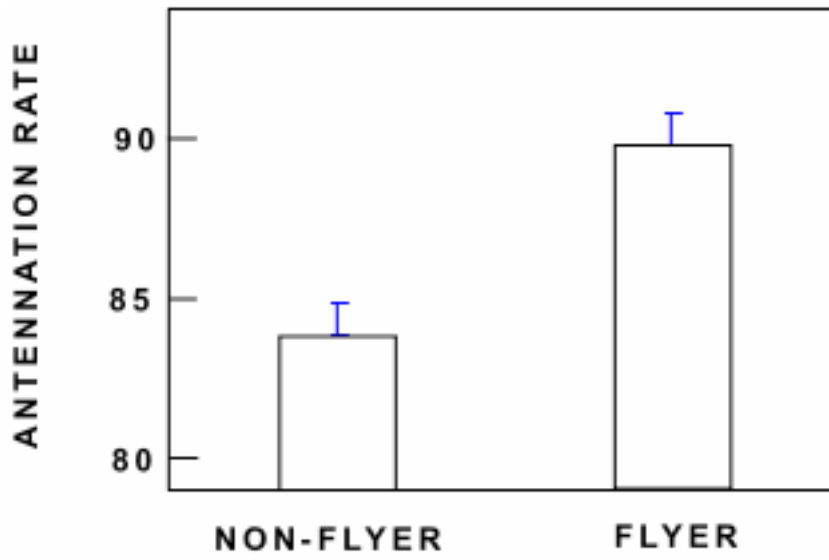


Fig. 2.

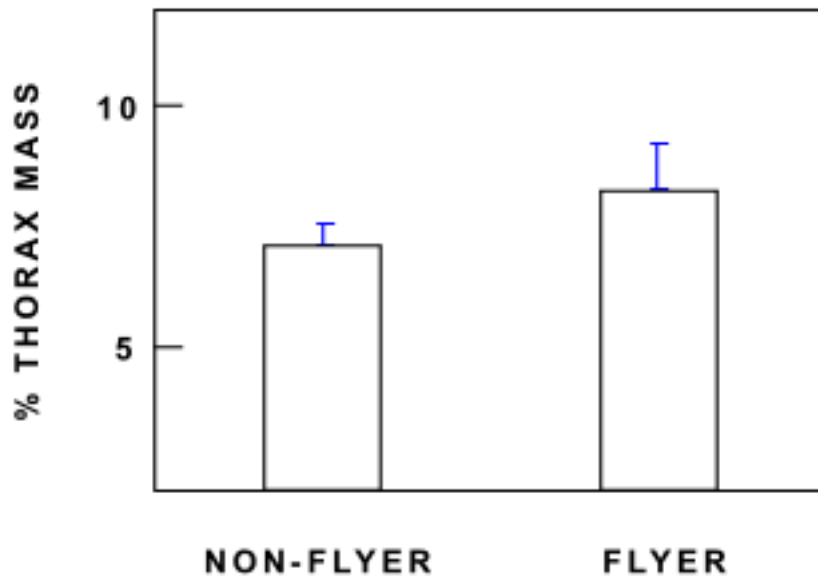


Fig. 3.

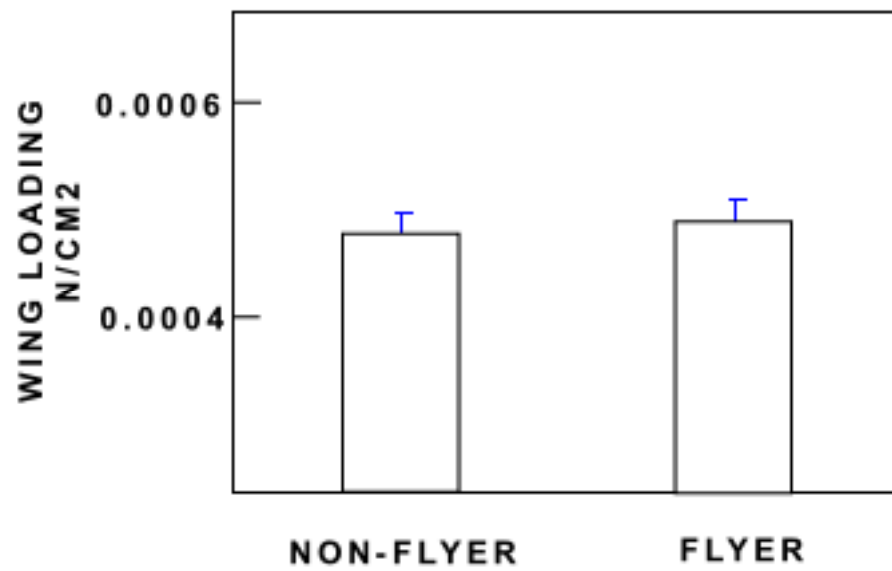


Fig. 4.

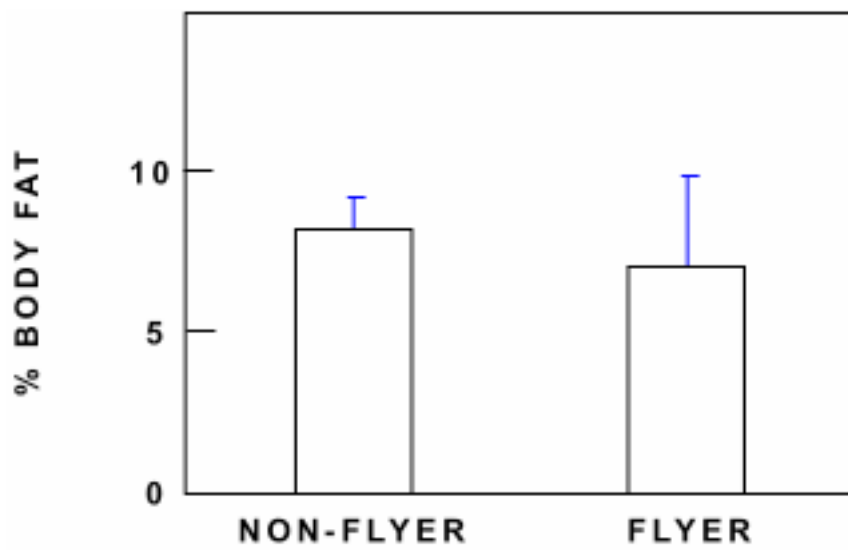


Fig. 5.

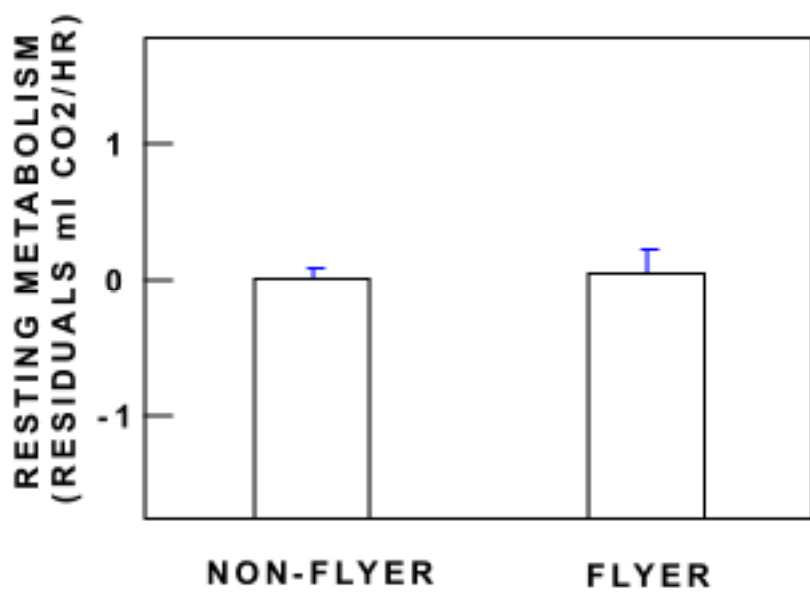


Fig. 6.

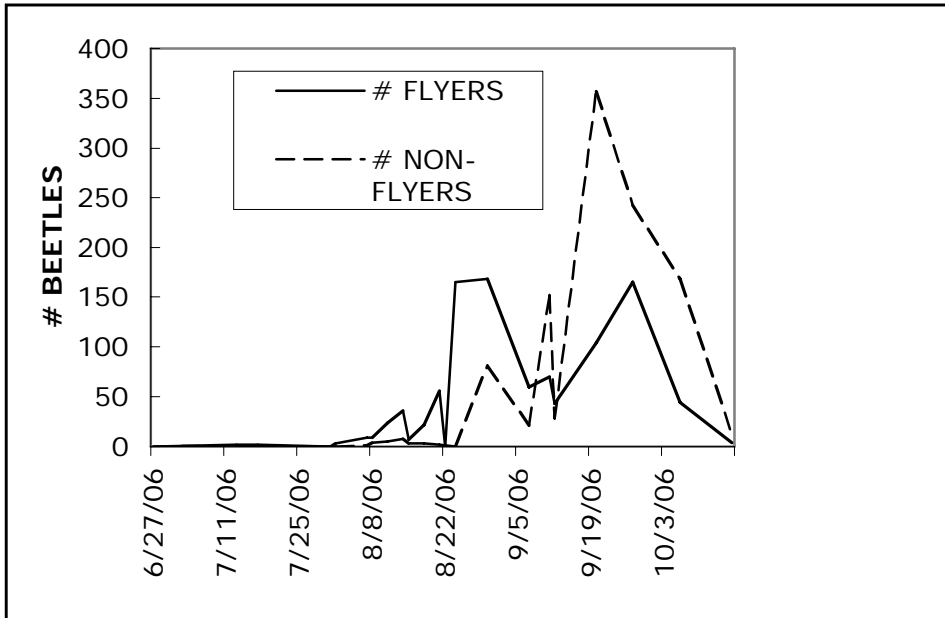


Fig. 7.

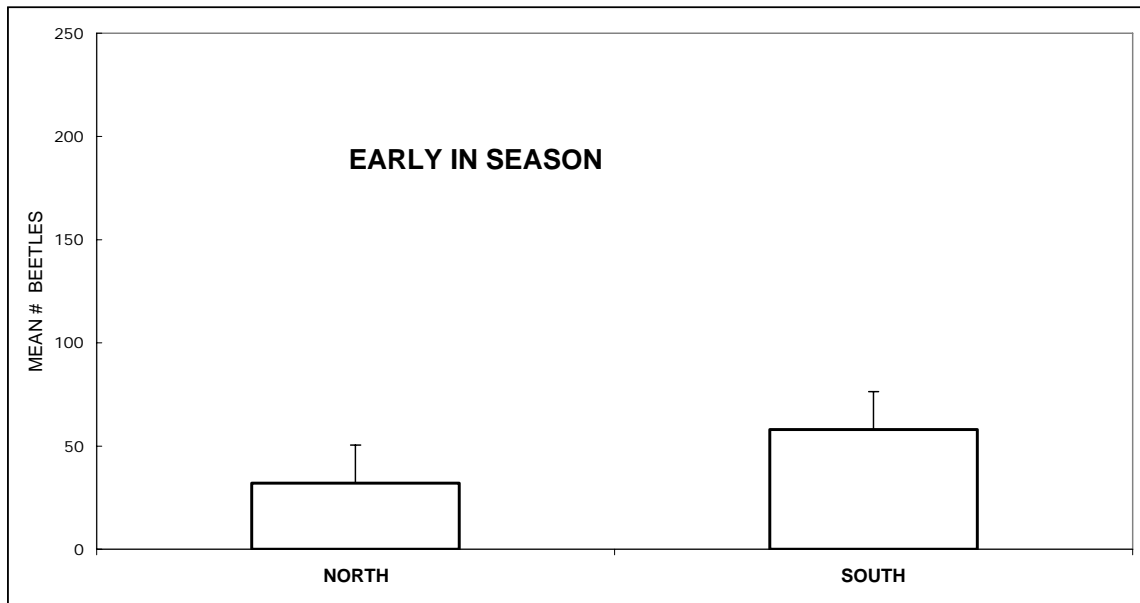


FIG. 8

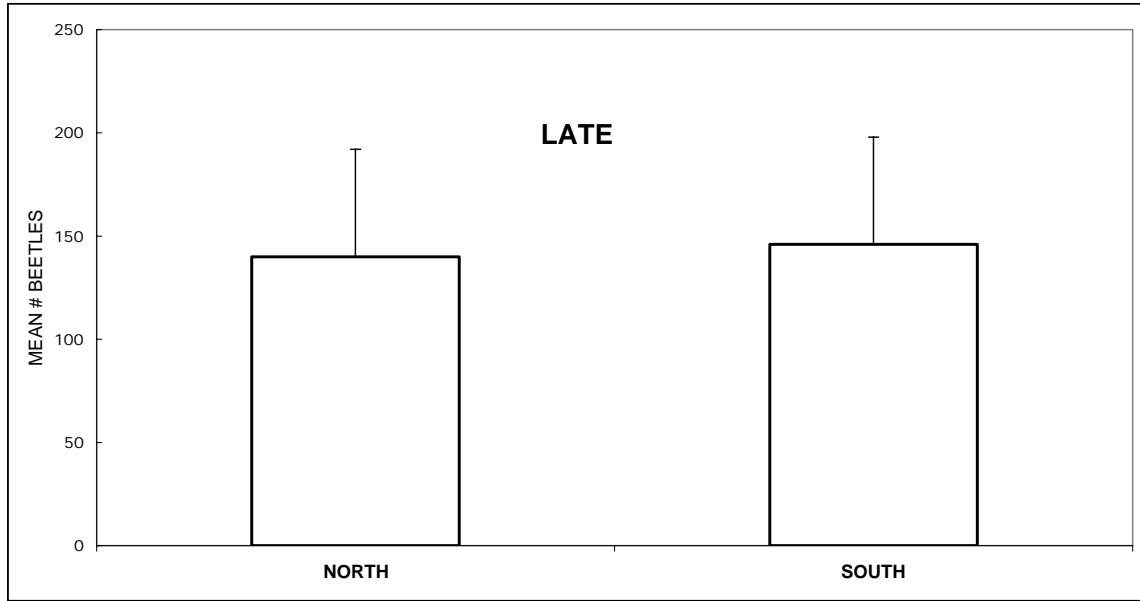


FIG. 9

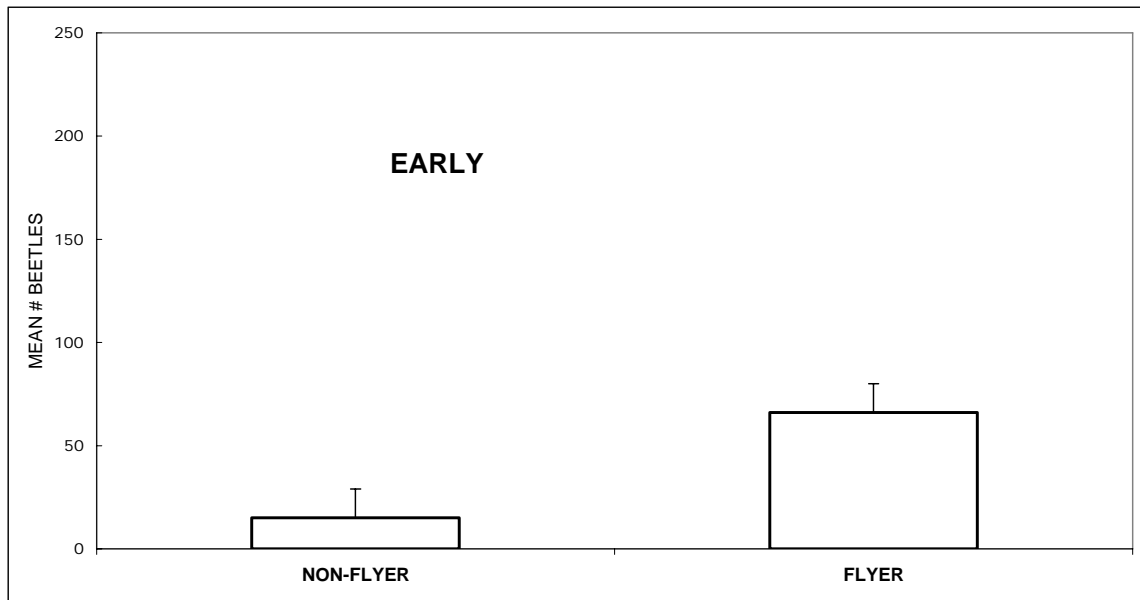


FIG. 10

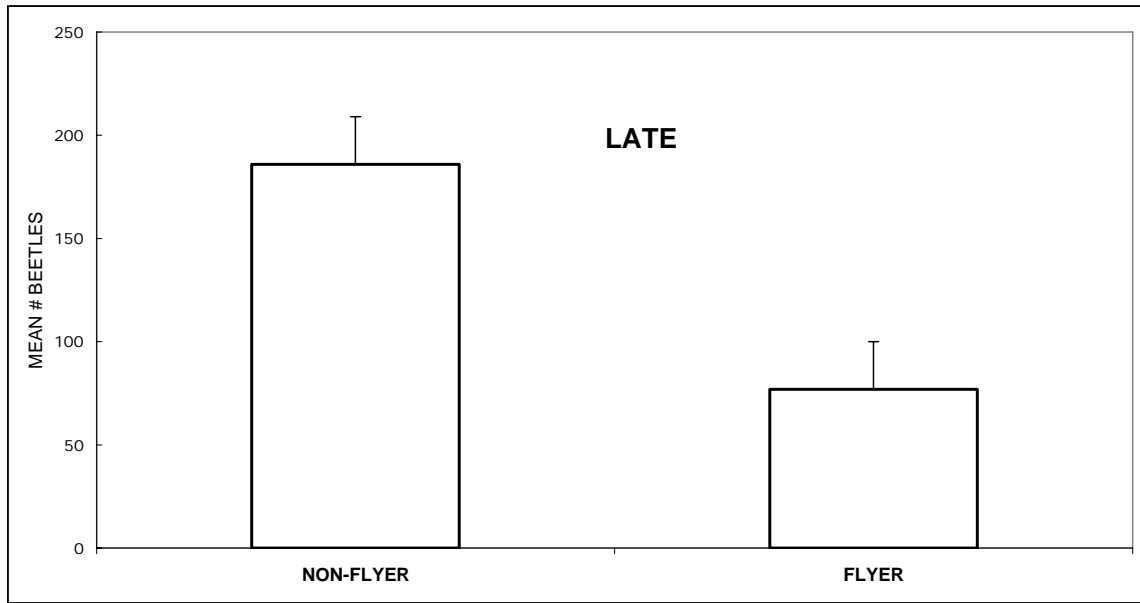
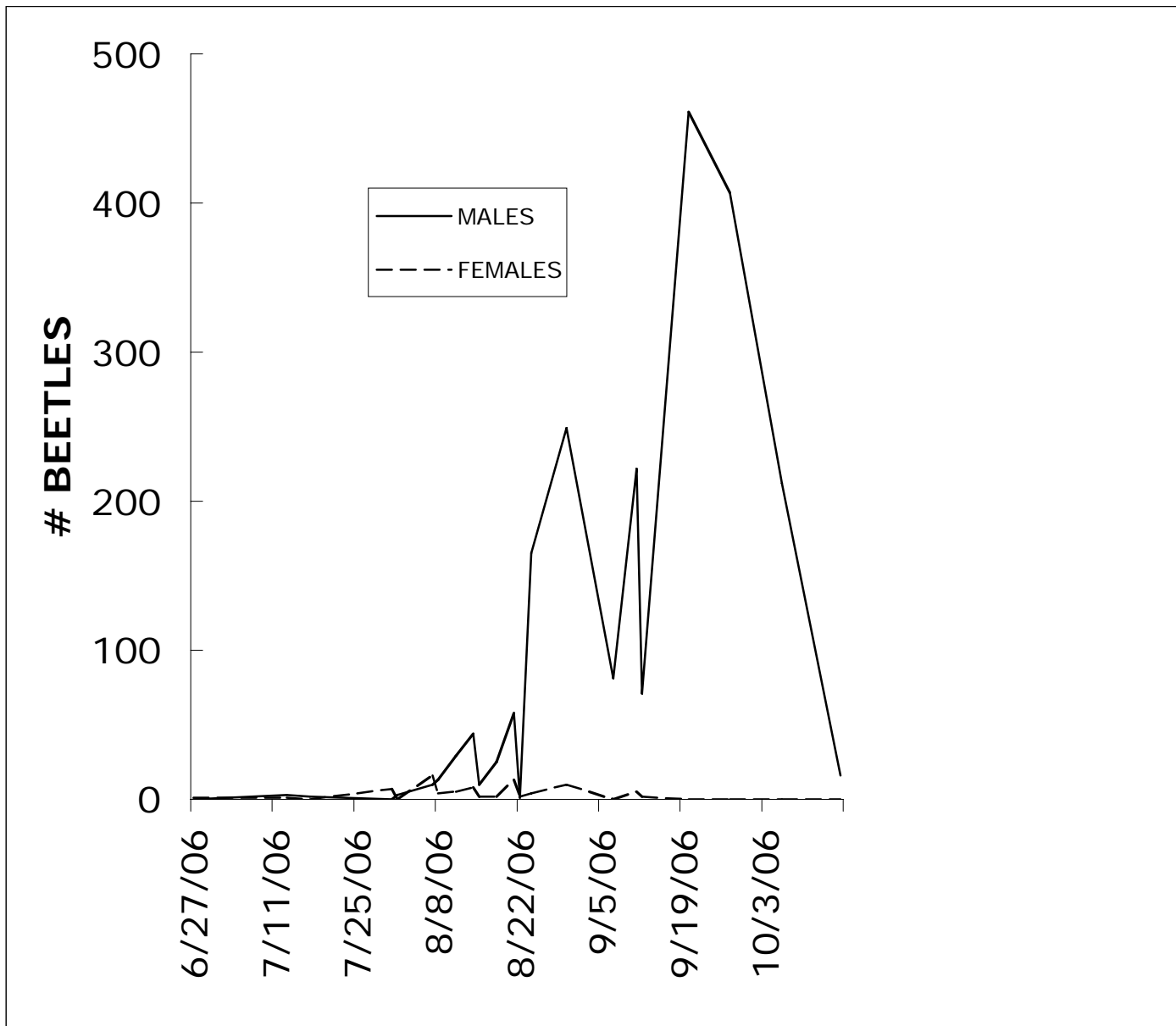


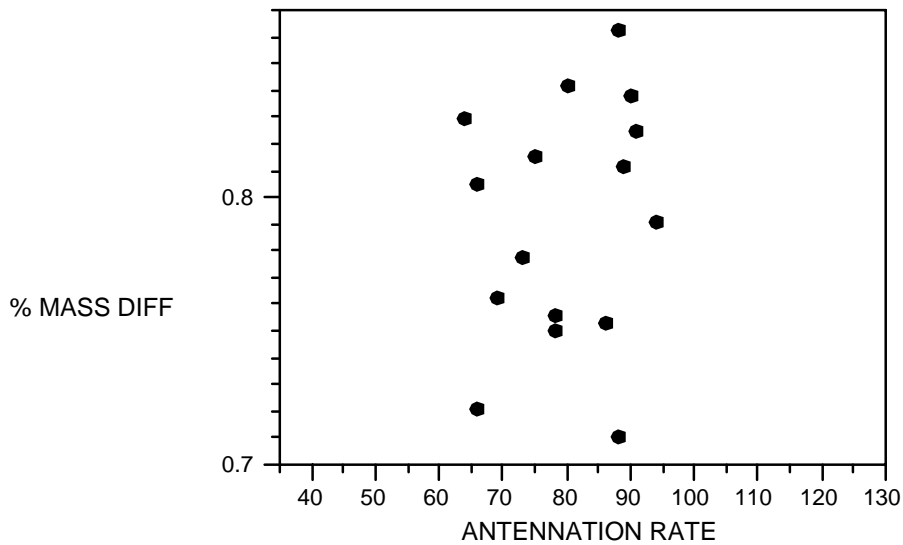
Fig. 11



Appendix A: Spermatophore Production time, Size, and Protein Content.

One measure of male fitness is the time required to produce a spermatophore (Kolluru *et al* 2002, Ernsting and Isaaks 2002, Wedell 1993, Anstensrud 1990, Zuk 1987, Giojalalas and De-Montenrgro 1986). Previous work indicated male *D. undecimpunctata* can mate again after 1-2 days (Tallamy *et al.* 2000). The hypothesis of a correlation between a high antennation rate and short spermatophore production time was tested by weighing males just before mating, just after mating and then again at several hour intervals until the original weight was regained. However when mating the male can remain on the female from just a few minutes to 8 hours or more, and cannot be dislodged without physical damage. During this time the male actively antennates or is passive, and does not eat or drink. A preliminary study showed that the mass lost by the males in copula (and taken to be the mass of the spermatophore transferred) showed great variation; any differences in production time were lost in “noise” as the males lost variable amounts of mass while “riding” the female for different lengths of time (Bussiere *et al* 2006).

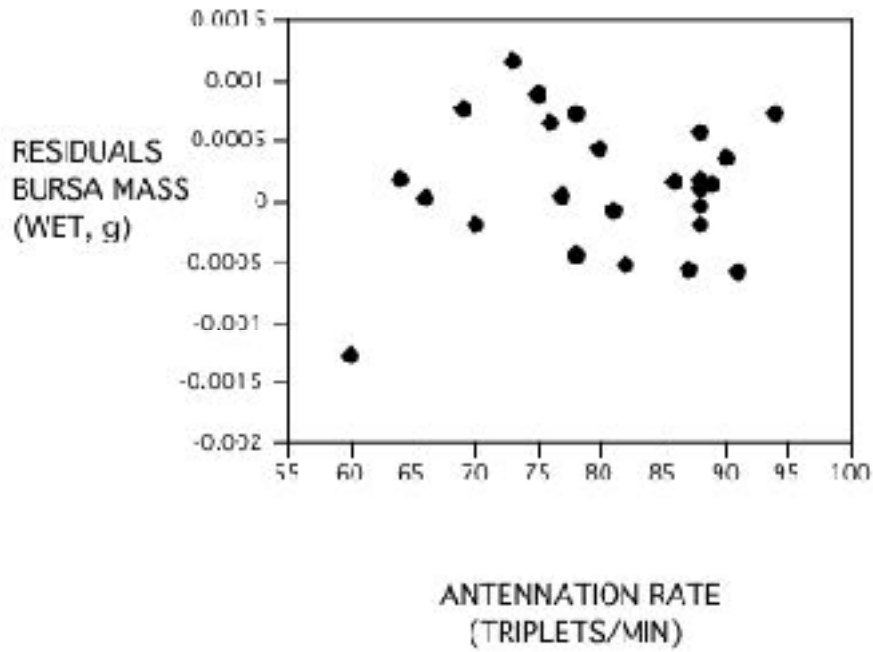
I therefore weighed males before mating and again 24 hours later, and calculated the percentage of change in mass. The hypothesis was that a fast antennator would be able to rebuild a new spermatophore quicker, and so recover more of its original mass in that time. However Figure 1 shows no relationship between antennatal stroking rate and the percentage of mass difference, and also shows most of the original mass has been recovered in 24 hours in all males tested (N = 12).



Also this test may be unrealistic as there was unlimited access to food and water available in the lab, while in nature a better forager might indeed rebuild a spermatophore more rapidly than a less efficient herbivore.

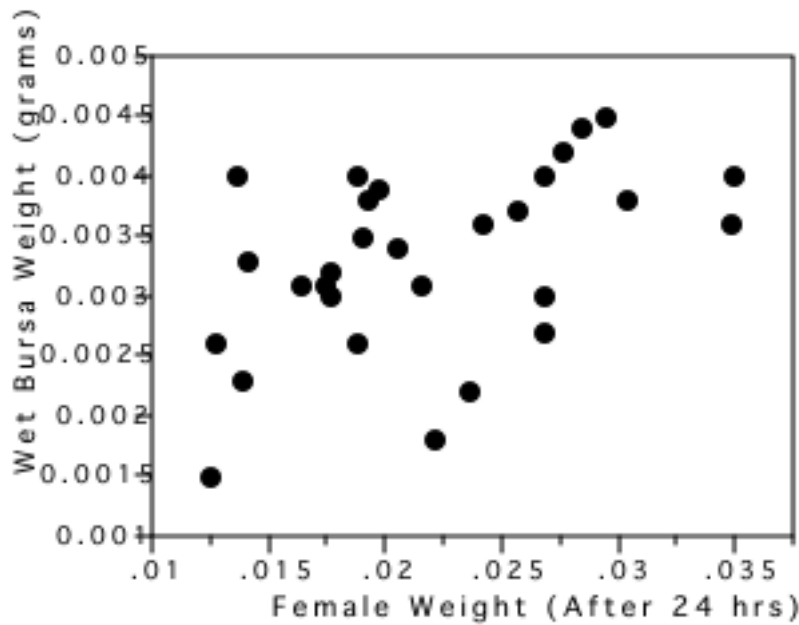
The size of the spermatophore produced is an obvious measure of a male's Darwinian fitness in many organisms (Farmer and Barnard 2000, Hiroki and Obara 1997, Simmons and Kvarnemo 1997, Quiring and Timmins 1990), and the spermatophores in this species are quite large, averaging about 7% of the male's mass (spermatophore mass was calculated as residuals of the male's mass in case it is not a simple proportion of body mass). However, the size of the spermatophore was found not to be correlated with male mass (Tallamy *et al.* 2000), or antennation rate. (The bursa mass was used as the size of the spermatophore it was not practical to separate the two by dissection).

Fig. 2.



The SCB spermatophore is pumped into the female's bursa in liquid form, and so I looked for a correlation between the mass of the spermatophore and the mass of the female. This factor alone explains 21% of the variation in size. (R square = 0.21)

Fig. 3.

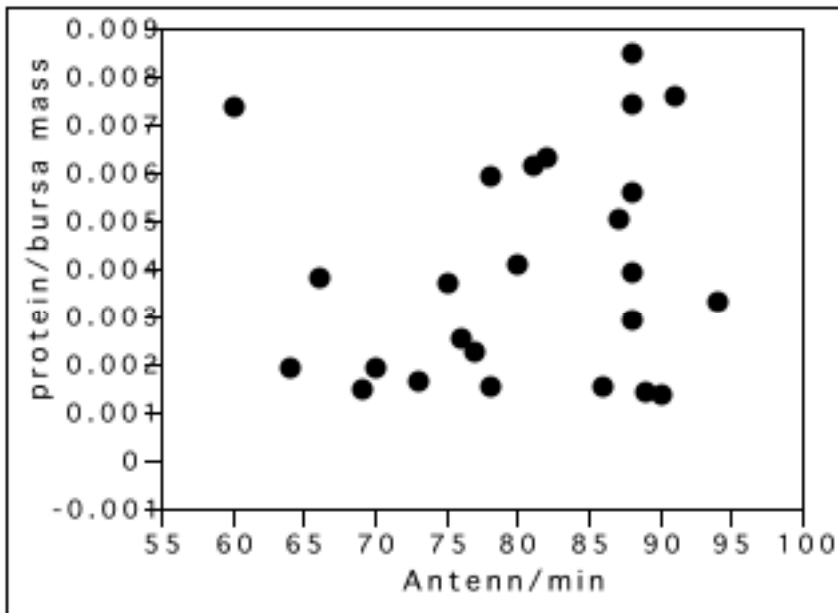


Presumably then the male has the capacity to pump more but only transfers enough liquid spermatophore to fill the female's bursa, which is why the eventual size of the spermatophore is more closely associated with the mass of the female than the male.

I also checked the total protein content of the spermatophores, thinking that a fast antennating male might transfer more protein than a slow stroking male, and so the female is discriminating in order to receive more nutritional material in a better quality 'nuptial gift'. This was a more direct check on my previous work, which failed to find an

association between antennation rate and female longevity, age at first reproduction, fecundity or egg hatch rate. To study the protein content of the spermatophore vs. antennation rate, after mating the bursa was dissected out of the female and the total protein content was quantified by BCA protein assay. I tested the bursa and spermatophore together as it was extremely difficult to separate the two. The total protein contained in the bursa/spermatophore was not found to be tied to antennation rate, so this parameter would then not be a good explanation for female choice.

Fig. 4



Appendix B: Parthenogenesis?

Female spotted cucumber beetles that have mated but rejected the male can still lay a few viable eggs. It was thought that rejected males were able to transfer small numbers of sperm (Tallamy *et al.* 2002 a), but an alternative explanation would be that these beetles are capable of “occasional” parthenogenesis (see Chapman 1998).

Female beetles raised in the lab and so known to be virgins were isolated, and after several months a small number of eggs were produced. I attempted to rear the eggs with the standard colony procedure (Tallamy *et al.* 2000). One possible offspring was found in the cage, a male. The beetle did not appear to have just emerged, and it is possible that it escaped another cage and chewed its way through the screen into this rearing cage.

The most common method of parthenogenesis is termed “thelytoky”, whereby the female clones herself (by modifying or eliminating the process of meiosis), and so produces only female offspring. There is one beetle species known to practice “arrhenotoky” in which unfertilized eggs develop into haploid males and fertilized eggs into diploid females (Chapman 1998). This is unlikely to have occurred here and would certainly need confirmation, and so the question of parthenogenesis in spotted cucumber beetles is still open.

Appendix C: Flight Propensity

I quantified free flight speed in individual male beetles, under the hypothesis that the fastest flyers would also have high antennation rates. The equipment utilized consists of two computer-coordinated cameras to record a three-dimensional flight path and so calculate the maximum flight velocity achieved. This apparatus has been used successfully on *Drosophila* with the insects flying up to an ultraviolet light (Montooth *et al* 2003), however the response in spotted cucumber beetles was only intermittent. Various attractants included: ultraviolet light, incandescent light, food (squash slices and corn sprouts), female beetles (both live and crushed to release pheromones), and squash blooms that were real and artificial (yellow paper with artificial flower volatiles as per Lampman and Metcalf, 1987; Metcalf and Lampman, 1991). As a repellent I created a crowded situation with a number of other beetles, however none of these inducements succeeded in motivating the majority of the males to fly.

Next I tried suspending the insect above the substrate, which is known to induce flapping in flies (Dethier, 1962), by means of a small glass rod super-glued to the pronotum. The beetle was then placed in front of a pair of vertical rotating cylinders with black and white bands and a fan to simulate forward motion (Dickinson and Lighton, 1995, Vogt *et al*, 2000). High-speed video was used to record wing beat frequency and amplitude; however a large percentage of the trails were failures as the beetles could still not be induced to flap their wings, and no trends were found in the data from insects that did attempt flight (Fig. 1, N = 16).

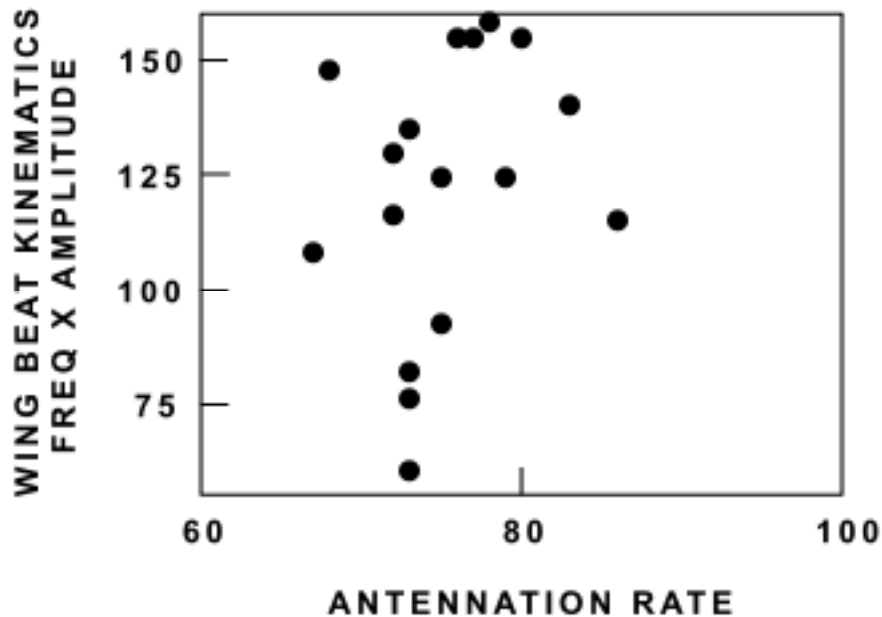


Fig. 1. Wing beat kinematics in male spotted cucumber beetles, the flapping frequency times the amplitude, did not correlate with antennal stroking rates, a mating display (N = 16).

Running speed was examined next, with electric shocks to insure maximum velocities. I had a shock plate fabricated with 1mm. wide copper bands separated by 1mm. of non-conducting material. Adjacent bands were powered by one of two wires connected to different sides of the plate (Wallace & Sperlich, 1988). I experimented with AC and different voltages up to 20, but found that 3 volts DC gave the best results, as higher shock values actually paralyzed the insect. I videotaped the beetles running over multiple trials, and calculated their best speed as grid distance, covered on a calibrated VCR screen, over time in frames per second. No correlation was found between antennal stroking rates and running speed, (Fig. 2, N = 46).

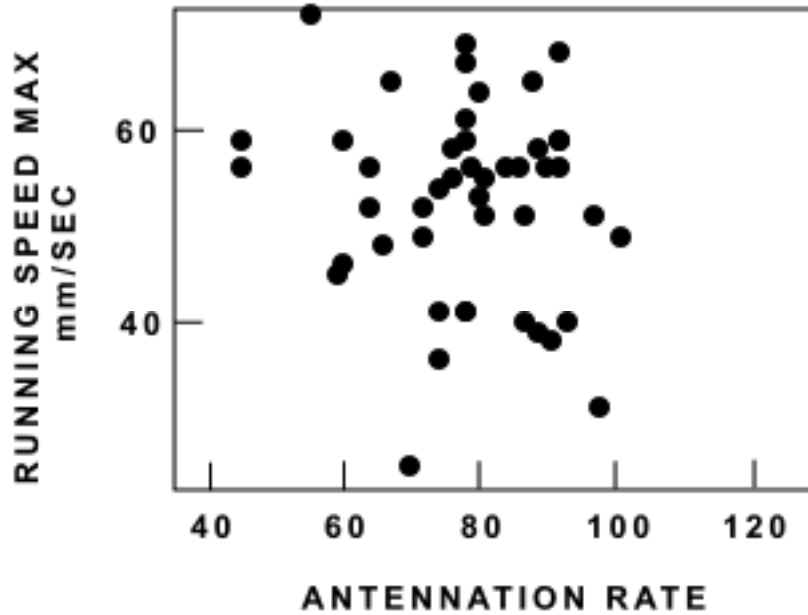


Fig. 2. The running speed of male beetles, on an electric shock plate to insure maximums, showed no connection with high antennation rates (N = 46).

I noticed a pattern in this species, individual males tend to be either “flyers” or “non-flyers”, some consistently attempt to fly while others will always run instead and never try to take flight. Even when escaping from a shock-plate, some always ran and some always flew. This led to the discovery of a highly significant correlation between high antennation stroking rate and flight attempt.

Appendix D: Field traps.

To test the hypothesis that wild male ‘flyers’ have a higher mean antennation rate than runners, I designed and built a ground trap to catch ‘runners’, and a second trap suspended on thin wires to catch only ‘flyers’; the expectation was that flyers would be found to have a higher average antennation rate. As bait I used artificial flower volatiles, as these beetles aggregate in squash flowers to mate and feed. (E)-cinnamaldehyde has been extracted from *Cucurbita* blossom volatiles and has been shown to be the major long-distance attractant for *D. undecimpunctata* (Lampman and Metcalf, 1987; Metcalf and Lampman, 1991). Here it was released slowly from cotton wicks that were first soaked in mineral oil. Male spotted cucumber beetles are known to be highly polygynous (up to 15 matings in the lab) as long as they are given 2 days to produce a new spermatophore before mating again (Tallamy *et al* 2002 b), so the experimental design was to test the flight ability of the captured wild males, hold them in the lab for 3 days and then mate them to virgin lab-reared females to quantify antennation rates. However I got no trap results from the 2003 field season because the wild population crashed and very few beetles appeared in the fields, and in 2004 I also got no data as my trap harmed the beetles. The (E)-cinnamaldehyde is evidently toxic when the fumes are concentrated, as the insects within the holding section of the trap were not in good condition. Other researchers using the artificial flower volatilities as attractants were using a sticky trap to sample beetle numbers, not trying to keep the insects alive and healthy (Andersen and Metcalf, 1986; Lampman and Metcalf, 1987; Metcalf and Lampman 1991). I modified the trap design but was not confident that the beetles were not harmed so that their behaviour was affected.

A redesigned trap with the (E)- cinnamaldehyde located well away from the beetle retaining area didn't catch any beetles. The trap was yellow in color to simulate squash flowers and used a plastic funnel mounted vertically (Hoffman, et al 1996; Jackson et al 2004); orientating the funnel down or horizontally had no effect. I wasn't sure the artificial flower volatiles would draw *D. undecimpunctata* if there were a lot of natural squash blossoms in the area that the bait would have to compete against, so then I set up the traps in different locations. I tried a grassy area between the squash field and a cornfield (after mating the females lay eggs in corn where the larvae feed underground) at Penn State and at similar spots at two different local farms. But I still had no success, and because it was also a wet season I had difficulties with flooding in the ground traps. For the 2005 and 2006 season I abandoned the traps and captured beetles by hand, testing for flight and sex ratio back in the lab.

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