

# Tortoise beetle genitalia and demonstrations of a sexually selected advantage for flagellum length in *Chelymorpha alternans* (Chrysomelidae, Cassidini, Stolaini)

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## Abstract

Rapid divergent evolution in the morphology of male genitalia is one of the most ubiquitous trends in animal evolution. Several studies suggest that male intromittent organs are under sexual selection, yet direct evidence supporting this view is meager. The authors show that the length of male and female genital tubes varies by nearly two orders of magnitude within a suite of 56 Neotropical tortoise beetle species, are poorly related to adult size, but are highly correlated between the sexes. Within a single species of tortoise beetle, *Chelymorpha alternans*, they additionally show that males with a longer genitalic flagellum father more offspring in multiple mating tests with virgin females. In addition, virgin females mated with males with a longer flagellum less often emitted sperm droplets during mating, and sperm emission was negatively correlated with numbers of sperm stored in the spermatheca. Further, virgin females mated with males whose flagellum was artificially shortened emitted greater quantities of sperm during mating, and stored smaller quantities of sperm than virgin females mated with unoperated males. These results suggest that the length of the flagellum in tortoise beetles can be an important factor determining male mating success, and may be a far more important paternity-determining factor than order of mating, duration of mating, various indices of body size, or length of the aedeagus.

## Introduction

The male genitalia of a sweeping range of animals, including planarians, nematodes, spiders, insects, snakes, and mammals, are used by taxonomists to distinguish closely-related species (Mayr, 1963; Shapiro & Porter, 1989), and several types of data suggest that male intromittent organs are often under sexual selection by female choice (Arnqvist & Danielsson, 1999; Danielsson & Askenmo, 1999; Dixson, 1987; Eberhard, 1985, 1993; Parker, 1970;

Simmons, 2001). The tendency for male genitalic structures in different species to diverge, especially compared with other body parts, is probably one of the most widespread and general trends in all animal evolution (Eberhard, 1985). There are several theories that attempt to explain why this should be. One idea is that male genitalia diverge rapidly, due to sexual selection by cryptic female choice (Eberhard, 1985; Thornhill, 1983). This hypothesis proposes that male genitalia often function as internal courtship devices that induce a female in order to increase the chances that their sperm, rather than that of other males with which she mates, will be used to fertilize her eggs. However, to date, most of the evidence favoring this idea has been indirect (Eberhard, 1996). There have been few direct tests in which the effects of male genitalic traits on particular female reproductive processes have been tested. The few direct studies in this field (Rodríguez, 1993; Arnqvist & Danielsson, 1999; Danielsson & Askenmo, 1999; Tadler, 1999) have supported the sexual selection hypothesis.

Variation among species in female remating and sperm precedence in several families was reviewed by Dickinson (1997). Both published and unpublished results of sperm transfer experiments in the cassidine chrysomelid beetle, *Chelymorpha alternans* Boheman (Stolaini) were reviewed. Earlier, the complicated spermathecal morphologies in 33 species of European Cassidinae (tribe Cassidini) were described by Bordy & Doguet (1987). Many, but not all, of these species had extremely long and highly coiled spermathecal ducts. However, the nature of the corresponding sclerites in males was not examined.

Below, we compare the lengths of male and fe-

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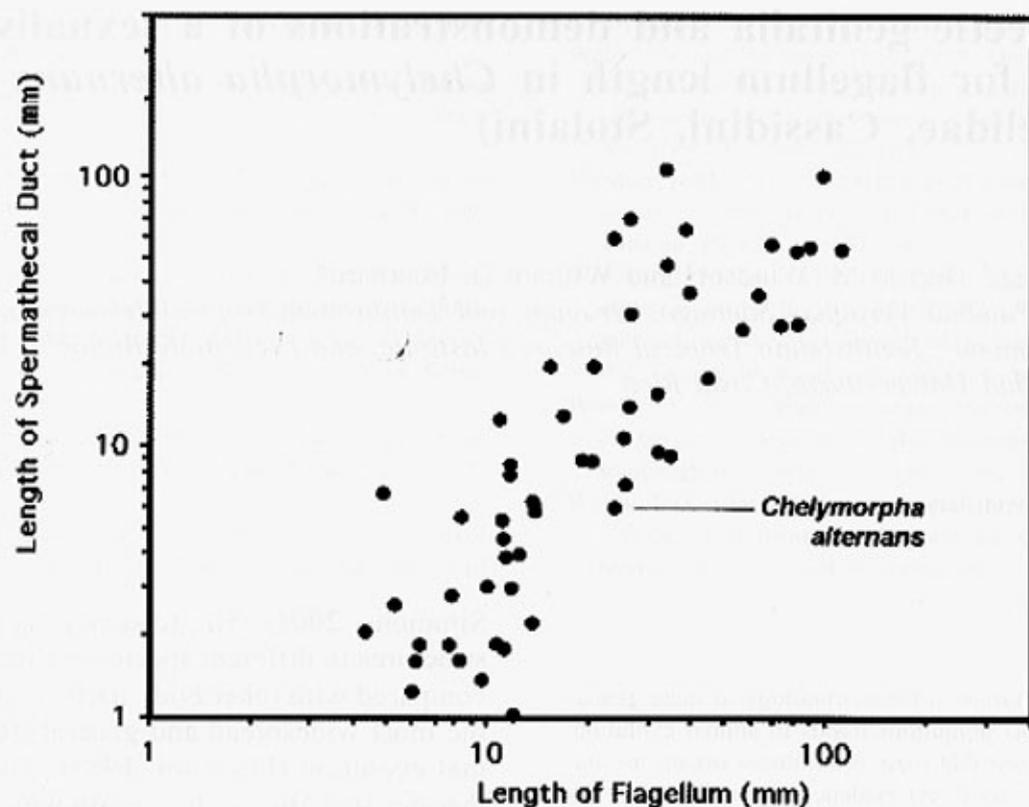


Fig. 1. Log-log plot of interspecific variation in average flagellum and spermathecal duct length in 57 species (eight tribes) of Panamanian Cassidinae s.l. (i.e., Hispinae + Cassidinae).

male genitalic structures that come into close contact during mating in a broad selection of Neotropical tortoise beetles. We then review experimental evidence from one of these species, *C. alternans*, a species whose flagellum measurements relative to spermathecal duct measurements are among the highest in the group of taxa examined. Multiple lines of experimental evidence indicate that greater insemination and fertilization success accrues to *C. alternans* males with a relatively longer genitalic 'flagellum'.

## Methods

All specimens examined in the survey were collected in Panama under annual collecting permits provided to the investigators by the Panamanian National Authority on the Environment (ANAM). Voucher specimens are stored at the Smithsonian Tropical Research Institute in Panama City.

Genitalic structures were removed by dissection from fresh specimens or from dried specimens allowed to masserate for 48 hours in water in a closed chamber. After cleaning in fresh water, dissected parts were mounted on microscope slides in Hoyer's medium. Tracings of the spermathecal duct and flagellum were made using a camera lucida, and lengths determined using a map measuring device or digitizing tablet (Jandel Corporation) and digitizing software (Sigmascan). The 'flagellum', the tubular, open-ended, sclerotized prolongation of the ejaculatory duct (Lindroth, 1957; below), was measured from the point where the ejaculatory duct emerges from the heavily thickened muscular tissues of the

seminal vesicle ('0' in Fig. 2) to its tip ('1'). The tightly coiled and often heavily sclerotized spermathecal ducts were measured by breaking them into pieces so that they could be flattened and measured with greater precision.

The positions of male and female reproductive structures during copulation were determined by immersing mating beetles in liquid nitrogen. Sperm were counted by breaking apart the distal spermathecal duct, the ampulla, and the proximal duct with the spermatheca. Each section was opened in 100 ml of saline solution with the dispersant 'Tween' added (20 drops/100 ml). After homogenizing the solution by agitation with the tip of an insect pin, a drop was placed in a hemacytometer (Neubauer, 0.1 mm in depth), and sperm were counted at 400x in four areas of 1 mm<sup>2</sup>.

Paternity tests were conducted according to the following protocol. Each of 32 one-to-two-week-old double recessive females (metallic color form) was mated in rapid succession (three to five minutes between matings) with two ( $n = 10$ ; experiment 1) or three ( $n = 22$ ; experiment 2) one-to-two-week-old virgin males homozygous for the metallic color, black striped, and red elytral color alleles. Offspring were raised to maturity to determine paternity ( $x = 179 \pm 74$  offspring/female). Previous test crosses showed Mendelian ratios of offspring, which indicated a genetic model involving three alleles at a single locus: metallic: rr; red: Rr or RR; black-striped a: Tr or TT; and black-striped b: TR. No intermediate color forms occurred in >13,000 beetles.

The spermathecal muscles of living females were severed in the following way. A one-to-two-week-old virgin female was pinned on her back unanes-

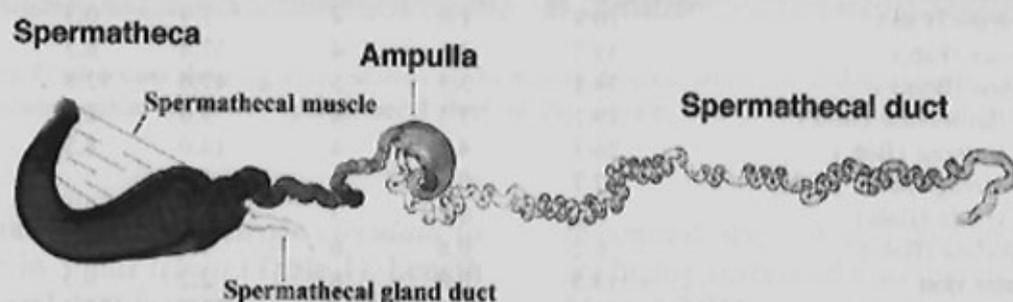
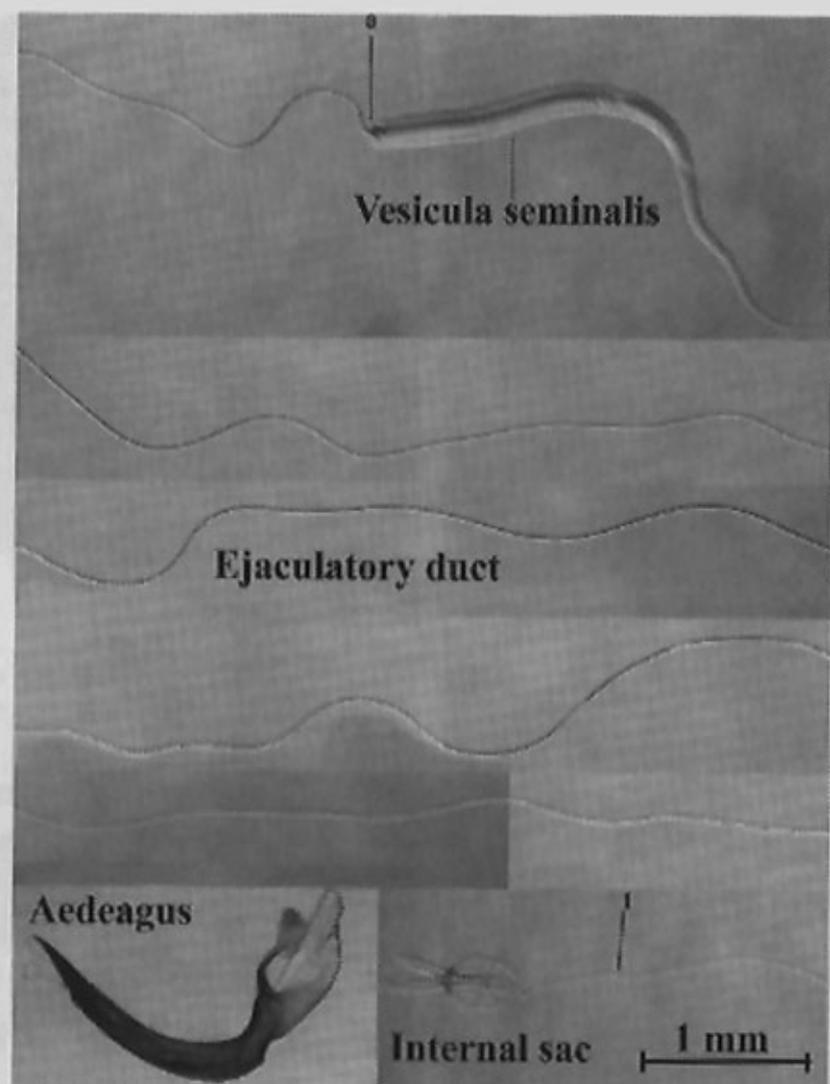


Fig. 2 Above: Ejaculatory apparatus of a male (minus testes and vas deferens). Below: spermatheca, ampulla and spermathecal duct of a female *C. alternans*. 0: marks the basal end of the flagellum; 1: the apical end. Note that the tip of the flagellum ends at 1, within a membranous sheath that is continuous with the eversible sac, through which the flagellum is extended during copulation.

thetized, a small flap of her third abdominal sternite was raised, and the spermatheca was teased into an exposed position. The muscle was then either severed and removed, or, in sham operations, manipulated but not cut. Finally, the flap was lowered and sealed with cyanoacrylate glue. The female was allowed to recover for seven days before being mated.

The flagellum was artificially shortened by interrupting copulation, causing the male to climb off the female. His flagellum was withdrawn only slowly, and during this process was severed with fine scissors. The free end was then pulled from the female and measured. Following the experiments, the males were sacrificed and the remaining basal portion measured.

Tests of normality, heteroscedascity and statistical comparisons were performed using the program Systat version 9.0 for Windows (SPSS, 1998). We

used nonparametric tests when normal distributions and unequal variance could not be eliminated by arc sin square root and log transformations.

## Results

### Variation in genitalic measurements

#### Among species

Female tortoise beetles, like most Chrysomelidae, have a rigid, sclerotized, 'c'-shaped spermatheca, joined to the oviduct-bursa by a long, sometimes highly-coiled and rigid spermathecal duct. The average length of spermathecal ducts in 56 species of Panamanian tortoise beetles varied over two orders of magnitude: from less than  $1.03 \pm 0.06$  mm (mean

Table 1. Variation among species in the length of the male flagellum and female spermathecal duct (mm)

Species	Male Flagellum Length (mm)			Female Spermathecal Duct Length (mm)		
	Avg	std dev	N	Avg	std dev	N
1 <i>Acromis sparsa</i> Boh.	11.9	1.2	11	3.0	0.7	5
2 <i>Agroiconota judaica</i> (Fab.)	11.2	1.2	6	1.8	0.6	7
3 <i>Akantaka godmani</i> (Baly)	90.1	31.9	7	54.4		1
4 <i>Aslamidium semicircularum</i> (Oliv.)	11.7	1.0	5	8.5	1.6	4
5 <i>Calyptocephala antenuata</i> Spaeth	8.3	0.1	2	5.4	0.3	3
6 <i>Calyptocephala brevicornis</i> (Boh.)	20.1	1.0	3	8.8	1.8	3
7 <i>Agroiconota propinqua</i> (Boh.)	33.6	12.6	9	108.1	38.2	5
8 <i>Charidotella sinuata</i> (Champ.)	15.4	7.8	4	19.6	2.9	7
9 <i>Charidotella sexpunctata</i> (Fab.)	20.5	4.1	5	19.5	3.4	7
10 <i>Charidotella sp.1</i>	5.3	0.3	14	2.6		1
11 <i>Charidotella ventricosa</i> (Boh.)	16.9	5.3	4	12.9	4.0	11
12 <i>Charidotella zona</i> (Fab.)	11.7	0.8	9	7.8	2.2	6
13 <i>Charidotis abrupta</i> Boh.	25.5		1	7.3	0.1	3
14 <i>Charidotis incincta</i> (Boh.)	11.4	1.1	3	3.9	0.4	4
15 <i>Charidotis aurofasciata</i> Erich.	11.0		1	5.4		1
16 <i>Charidotis vitriata</i> (Perty)	26.6		1	30.5		1
17 <i>Chelymorpha alternans</i> Boh.	24.1	4.1	38	5.9	1.2	27
18 <i>Chelymorpha gressoria</i> Boh.	8.3	1.5	5	1.6	0.2	2
19 <i>Chelymorpha sp. 1</i>	11.3	1.4	9	4.5	1.3	4
20 <i>Chelymorpha cribraria</i> (Fab.)	13.8	1.5	4	5.8		1
21 <i>Cistudinella foveolata</i> Champ.	32.0	5.3	5	15.8	1.9	2
22 <i>Coptocycla leprosa</i> (Boh.)	81.6	12.0	3	52.7	0.9	2
23 <i>Coptocycla rufonotata</i> Champ.	69.4		1	56.4	5.3	4
24 <i>Cyelocassis circulata</i> (Boh.)	12.0	0.7	2	1.0	0.1	2
25 <i>Discomorpha salvini</i> (Baly)	25.3	6.0	3	10.8	2.5	3
26 <i>Echoma anaglyptoides</i> Boroweic	56.6	2.2	2	26.7		1
27 <i>Eugenysa coscoroni</i> Viana	74.1	32.4	2	27.9	1.2	2
28 <i>Hybosa mellicula</i> Boh.	9.7	1.1	4	1.4	0.3	2
29 <i>Imatidium thoracicum</i> (Fab.)	10.9	1.8	4	1.9	0.3	4
30 <i>Ischnocodia annulus</i> (Fab.)	39.7	3.8	4	37.4	6.3	8
31 <i>Metrionella erratica</i> (Boh.)	34.1	3.5	5	47.6	12.8	4
32 <i>Microctenochira flavonotata</i> (Boh.)	19.1	3.3	4	9.0	2.9	3
33 <i>Microctenochira fraterna</i> (Boh.)	26.3	4.7	4	14.0	4.3	5
34 <i>Microctenochira hieroglyphica</i> (Boh.)	7.7	0.8	4	1.8		1
35 <i>Microctenochira vivida</i> (Boh.)	6.1	0.3	3	1.3	0.1	2
36 <i>Ogdoecosta catenulata</i> (Boh.)	6.2	0.6	6	1.6	0.4	10
37 <i>Omaspides bistrata</i> Boh.	13.5	0.9	8	2.2	0.5	8
38 <i>Parachirida subirrorata</i> (Boh.)	38.8	6.2	5	63.7	25.4	5
39 <i>Paraselenis tersa</i> (Boh.)	4.4	0.2	2	2.1	0.0	2
40 <i>Polychalma multicava</i> (Latr.)	112.2	19.8	4	53.7	5.9	5
41 <i>Prosopodonta dorsata</i> Baly	26.4		1	69.7		1
42 <i>Prosopodonta limbata</i> Baly	62.5		1	36.0		1
43 <i>Spaethiella flexuosa</i> (Champ.)	14.0	3.8	6	6.0	0.2	2
44 <i>Spaethiella sp. 1</i>	82.0		1	28.5		1
45 <i>Spaethiella sp. 2</i>	23.6		1	58.5	7.3	4
46 <i>Spaethiella sp. 3</i>	34.8	4.6	2	9.1		1
47 <i>Stolas cucullata</i> (Boh.)	44.8	9.1	7	17.9	1.9	4
48 <i>Stolas epiphium</i> (Lichtenstein)	13.5	0.9	5	6.2	1.6	7
49 <i>Stolas extricata</i> (Boh.)	12.4	0.4	6	4.0		1
50 <i>Stolas lebasii</i> (Boh.)	98.8	10.0	6	101.8	4.0	5
51 <i>Botanochara ordinata</i> (Boh.)	32.3	5.2	2	9.6		1
52 <i>Stolas pictilis</i> (Boh.)	6.4	0.7	5	1.8	0.2	9
53 <i>Stolas xanthospila</i> Champ.	10.0	0.7	7	3.0	0.3	4
54 <i>Terpsis quadrivittata</i> (Champ.)	7.9	0.6	2	2.8	0.7	3
55 <i>Xenocassis ambita</i> (Champ.)	10.9	0.8	2	12.5	0.2	2
56 <i>Xenocassis puella</i> (Boh.)	4.9	0.5	5	6.8	1.2	7

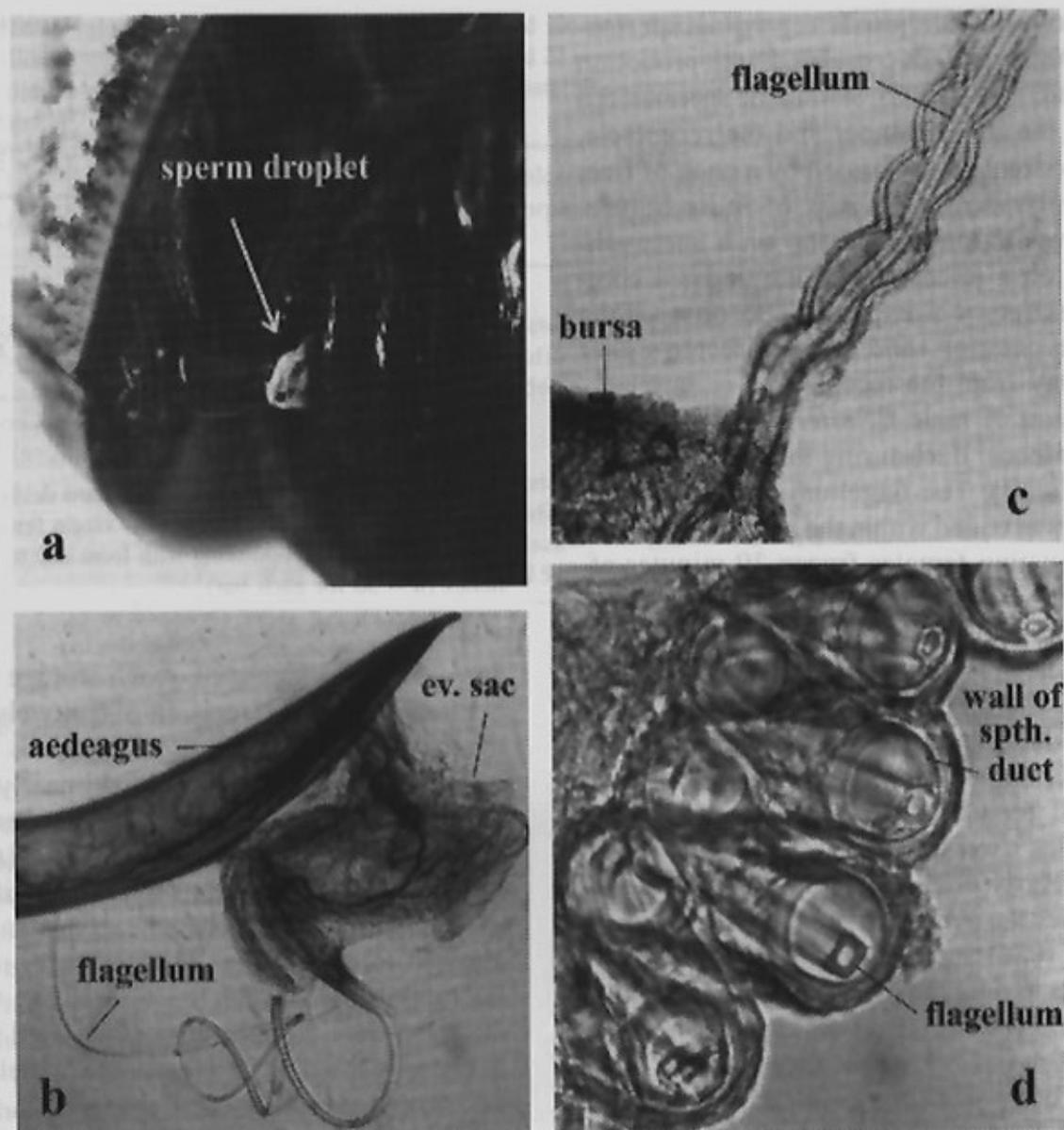


Fig. 3. *a*. Copulating *C. alternans* showing accumulation of the mating droplet (arrow); *b*. aedeagus with everted sac and extended flagellum; *c*. flagellum within the lower spermathecal duct of the female, and *d* cross-sections of the flagellum and mid-spermathecal duct.

$\pm$  standard deviation) in *Cyclocassis circulata* to  $101.8 \pm 4.0$  mm in *Stolas lebasii* (Table 1). Length of the spermathecal duct is uncorrelated with body length (Spearman rank correlation index,  $r_s = 0.06$ ,  $n = 56$ ,  $p = \text{NS}$ ). The morphology of spermathecal ducts varied greatly among species. Some were essentially linear, others loosely looped, and the majority were tightly coiled over a portion or their entire length. A spermathecal muscle joined the tip to the base of the spermatheca in all species observed by us. In *C. alternans*, its contraction and relaxation appear to influence, by an unknown mechanism, the uptake of sperm during fertilization and the delivery of sperm during oviposition (see below, and Villavaso, 1975).

Male tortoise beetles have a long, open-ended, tubular, lower ejaculatory duct or 'flagellum' which is threaded up the spermathecal duct of the female during copulation in most if not all Cassidinae. Flagellum length varies greatly among species, with the shortest in our sample of taxa occurring in *Paraselenis tersa* ( $4.37 \pm 0.19$  mm), the longest in *Polychalma multicava* ( $112.2 \pm 19.8$  mm). Flagellum length is weakly correlated interspecifically with average adult body size ( $r_s = 0.30$ ,  $n = 56$ ,  $p = 0.05$ ).

In contrast, spermathecal duct and flagellum lengths are highly correlated interspecifically ( $r_s = 0.85$ ,  $n = 56$ ,  $p < 0.01$ ).

#### Within species

The spermathecal duct in *C. alternans* includes a highly coiled 'lower spermathecal duct' with a smooth inner wall, a short expanded section or 'ampulla' located approximately two-thirds of the distance to the spermatheca, and a thicker walled, tightly coiled 'upper spermathecal duct' which joins the ampulla to the base of the spermatheca (Fig. 2). Of the 56 species examined, only two species, *C. alternans* and *Chelymorpha* sp.1 had ampullae in their spermathecal ducts. The spermathecal ducts in *C. alternans* contained on average  $54.5 \pm 6.9$  coils ( $n = 14$ ). The direction of coiling of the lower duct changed (e.g., from clockwise to counter-clockwise) an average of  $12.4 \pm 3.3$  times ( $n = 8$ ) in its  $39.7 \pm 5.7$  coils ( $n = 12$ ). Due to lack of transparency, we were unable to determine the number of reversals accurately for the upper duct, but they occurred there as well. The entire length of the *C. alternans* spermathecal duct averaged  $5.9 \pm 1.2$  mm ( $n = 27$ ).

Variation between individuals regarding spermathecal duct length was not correlated with pronotum width (Eberhard *et al.*, 1998). Within the spermatheca and between the first chamber and the receptacle, there is an apparent valve formed by a cone of fine, flexible cuticular strips (Fig. 3, p. 65 in Rodríguez, 1993). By pressing on a coverslip on a spermatheca mounted on a microscope slide under a compound microscope, it was possible to observe the strips change position, and to be directed either toward or away from the receptacle.

The flagellum of male *C. alternans* is threaded up the spermathecal duct during the early stages of copulation (Fig. 3). The flagellum tip and most of the flagellum was coiled within the ampulla in twelve of 21 (57%) mating females frozen 30 minutes after the initiation of copulation (average total duration about 90 minutes). Significantly, in one of these twelve pairs, the apical part of the male's flagellum passed out of the ampulla and through the upper spermathecal duct, and the tip was in the first chamber of the spermatheca.

The overall length of the flagellum in the *C. alternans* males used in the paternity experiments below averaged  $21.46 \pm 2.67$  mm ( $n = 86$ ), more than three times the average male body length ( $7.48 \pm 0.45$  mm,  $n = 86$ ) and nearly four times the average spermathecal duct length of females. Flagellum length was weakly correlated with elytron length ( $r_s = 0.227$ ,  $p = 0.04$ ;  $n = 86$ ), but not with two other estimators of body size (body length,  $r_s = 0.132$ ,  $n = 86$ , NS; pronotum width,  $r_s = -0.137$ ,  $n = 86$ , NS). Flagellum length varied significantly among the three color morphs (Kruskal Wallis = 8.62,  $df = 2$ ,  $p < 0.02$ ) with an average of  $22.77 \pm 3.38$  ( $n = 30$ ) for metallic males,  $20.52 \pm 1.38$  ( $n = 31$ ) for red males, and  $20.98 \pm 2.27$  ( $n = 25$ ) for black-striped males.

### Experimental manipulations

#### Sperm storage and delivery

Cassidine males store sperm in the seminal vesicle, a dilated portion of the upper ejaculatory duct which is thickly sheathed in muscle. The seminal vesicle ends abruptly at a sclerotized junction with the flagellum ('0' in Fig. 2). Seminal vesicles dissected from virgin *C. alternans* males contained  $129,600 \pm 66,480$  sperm ( $n = 30$ ). Seminal vesicles removed from thirty males of the same age allowed to mate once before being frozen ten to twenty seconds after copulation contained  $71,210 \pm 29,610$  sperm. Thus, in a single ejaculation, *C. alternans* males deliver about 58,000 sperm to females, about 45% of their available sperm. Judging by the lack of overt female resistance during copulation, males appear to determine copulation duration.

The numbers of sperm in the spermathecae of once-mated females frozen 0, 12, 24 and 48 hours after copulation were, respectively,  $5161 \pm 4886$ ;  $21,130 \pm 9313$ ;  $21,520 \pm 10,830$  and  $23,660 \pm 7719$  ( $n = 30$  in each case). Thus, approximately 22,000 sperm,

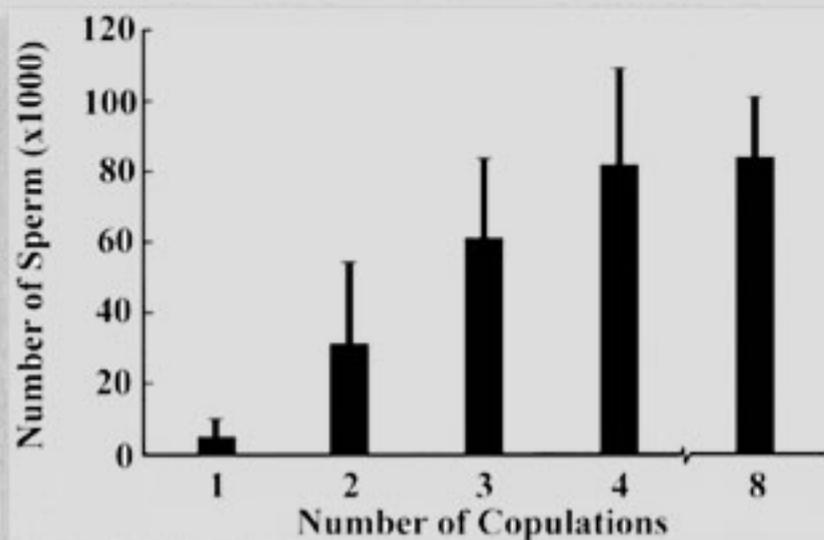


Fig. 4. Number of sperm (mean, standard deviation) observed in the spermathecae of *C. alternans* virgin females two hours after they finished copulating with from one to four and eight males ( $n = 30$  for each bar).

38% of those ejaculated, reach storage in the spermatheca, and are largely in place twelve hours after the end of copulation.

Additional copulations added linearly to the content until, after four matings, the number of spermathecal sperm averaged  $81,683 \pm 17,285$  ( $n = 30$ ) (Fig. 4). Additional copulations did not significantly add to this quantity: the spermathecal content of females allowed to copulate eight times with no intervening oviposition was  $83,783 \pm 17,285$  ( $n = 30$ ). Thus, the maximum number of sperm stored in the spermatheca is about 80,000, approximately the number stored after four copulations.

The process of sperm transfer is complex and poorly understood. A gelatinous spermatophore is present in the bursa near the entry of the spermathecal duct in 90% of females ( $n = 29$ ) dissected shortly after copulation is initiated. Within one to five minutes after spermatophore deposition, sperm exit and begin ascending the spermathecal duct. Apparently, during this same period, immediately following spermatophore deposition, the bursa and exit sclerites of the eversible sac are aligned with the entrance to the spermathecal duct, and the flagellum is threaded up the spermathecal duct of the female. Although sperm were observed in the lumen of the inserted flagellum, and emerging from the tip of the single flagellum observed in the spermatheca, the relative contributions of sperm from the spermatophore and from the flagellum to sperm stored in the spermatheca are not clear. In most cases, the flagellum was looped in the ampulla, and it is not certain whether large numbers of sperm are deposited into the spermatheca and the spermathecal duct directly from the tip of the flagellum.

#### Effect of flagellum length on paternity in two- and three-male mating experiments

Flagellum length was not correlated with duration of mating either ( $r_s = 0.27$ ,  $-0.07$ ), but it was related to the probability of fathering offspring (Fig. 5). The males with a longer flagellum sired a median of 75%

Table 2. The median value of mating duration, mating order, and six male morphological attributes for low, intermediate, and high ranking males allowed to mate sequentially with each of the ten and 22 virgin females in experiments 1 and 2, respectively. The Kruskal-Wallis test was used to compute the significance of differences in paternity accruing to males according to their rank with regard to each of the eight attributes

	Mating duration (min)	Order of mating	Flagellum length (mm)	Color morph	Body length (mm)	Pronotum width (mm)	Elytral length (mm)	Aedeagus length (mm)
Experiment 1: (n=10)								
High	162.5	1	23.9	Metal	7.6	4.9	5.9	1.8
Low	93.0	2	19.8	Rufi	7.2	4.7	5.6	1.6
P	0.019	0.59	0.003	0.56	0.99	0.36	0.65	0.24
Experiment 2: (n=22)								
High	151.5	1	24.1	Metal	7.7	4.9	6.0	1.9
Medium	104.0	2	20.4	Rufi	7.4	4.7	5.7	1.8
Low	58.5	3	19.4	Black	6.9	4.4	5.3	1.6
P	0.27	0.41	0.03	0.15	0.98	0.39	0.13	0.34

of offspring in the two-male experiment (Mann-Whitney  $U = 89$ ,  $df = 1$ ,  $p = 0.003$ ). In the three-male experiment, the male with the longest flagellum sired a median of 44% of offspring, the second longest male 26%, and the male with the shortest flagellum, 30% (Kruskal-Wallis statistic = 7.01,  $df = 2$ ,  $p = 0.03$ ). Of the three possible contrasts in the three-male experiment, the male with the longest flagellum sired significantly more offspring than the male with the intermediate length flagellum (Mann-Whitney  $U = 347$ ,  $df = 1$ , Bonferonni adjusted  $p = 0.038$ ). Although the male with the longest flagellum also sired more offspring than the male with the shortest flagellum, the difference was not significant (Mann-Whitney  $U = 319$ ,  $df = 1$ , NS). Similarly, there was no significant difference in the percentages of offspring sired by males with the intermediate and shortest flagellum (Mann-Whitney  $U = 200$ ,  $df = 1$ , NS). Thus, in both multiple mating experiments, males with longer flagella sired a greater percentage of offspring.

Neither differences in length of male body, aedeagus, elytra, width of pronotum, order of mating, nor male body color were correlated with paternity (respective  $p$  values for two- and three-male experiments, Kruskal-Wallis Test, were 1.0, 0.97; 0.25, 0.34; 0.65, 0.13; 0.36, 0.39; 0.60, 0.41; and 0.56, 0.15). However, males that copulated longer in the two-male experiment also sired a significantly greater percentage of offspring (median = 76%; Mann-Whitney  $U = 81$ ;  $p = 0.019$ ). A weak and insignificant trend in the same direction was present in the three-male experiment (Fig. 5) (Kruskal-Wallis = 2.64,  $p = 0.27$ ).

#### Flagellum length, sperm dumping and length of copulation

A second indication of the importance of flagellum length comes from the droplets of sperm which sometimes emerge from the female's external genital opening during copulation (Fig. 3a). Large numbers

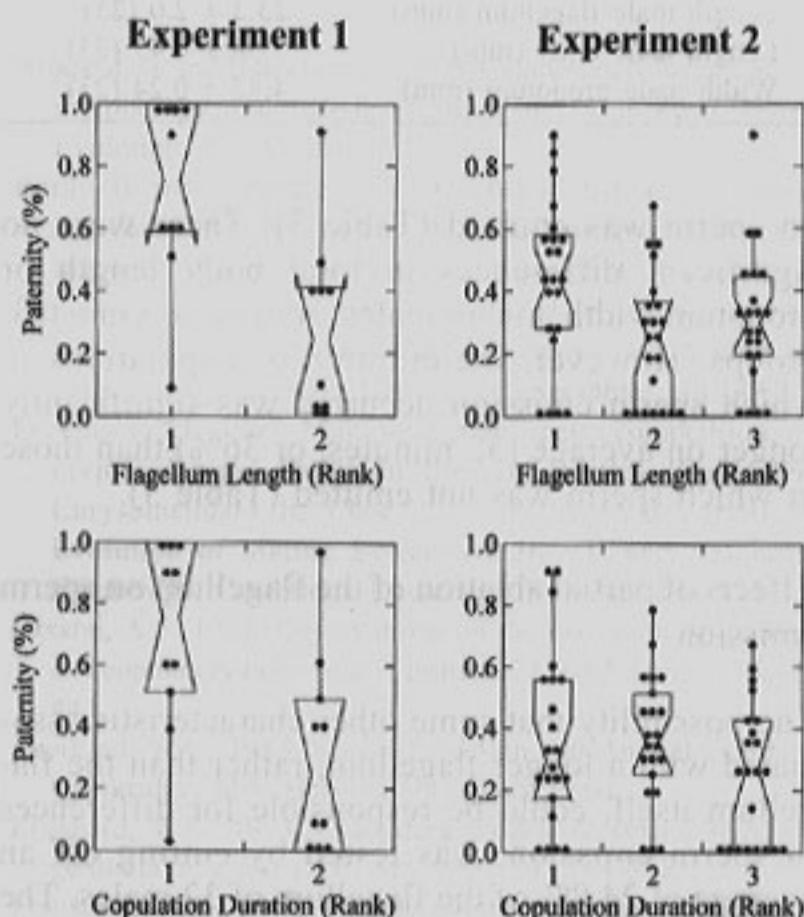


Fig. 5. The effects of male flagellum length and copulation duration on paternity, the percentage of offspring sired by either of the two males or three males mating with each virgin female (experiment 1,  $n = 10$ ; experiment 2,  $n = 22$ ). The males that mated with each female were ranked among themselves with regard to the character of interest (independent variable). Box plots display the median, first and third quartiles, and range, as well as the data points.

of living sperm were present in 137 of 141 droplets (97%) examined under a microscope. Sperm was present in all pairs in Table 3; however, because they were densely clumped, their numbers could not be determined by the dilution. Females that emitted a sperm droplet during copulation contained 9640 fewer sperm on average in their spermatheca than females who did not emit a droplet, a 35% reduction. When one-to-two-week-old virgin males and females were paired, the flagellum lengths of the males from 23 pairs in which sperm was emitted were shorter than those of males from 31 pairs in which

**Table 3.** Comparison of mating pairs of female virgins in which sperm droplets were, and were not, formed during copulation. Significant differences occurred between the two groups regarding length of copulation ( $p < 0.001$ ), numbers of spermathecal sperm 24 hours after copulation ( $p < 0.001$ ), and length of male flagellum ( $p < 0.05$ ) (Mann-Whitney U test), but not in measurements of male body size or pronotum width

	<i>mn ± sd (n)</i>
<i>Pairs in which a drop of sperm was not emitted</i>	
Duration of copulations (min)	89 ± 44 (73)
Number of spermathecal sperm 24 hours later	27900 ± 10450 (31)
Length male flagellum (mm)	24.8 ± 2.6 (31)
Length male body (mm)	7.50 ± 0.41 (31)
Width male pronotum (mm)	4.81 ± 0.38 (31)
<i>Pairs in which a drop of sperm was emitted</i>	
Duration of copulations (min)	121 ± 30 (11)
Number of spermathecal sperm 24 hours later	18260 ± 6825 (23)
Length male flagellum (mm)	23.3 ± 2.0 (23)
Length male body (mm)	7.56 ± 0.47 (23)
Width male pronotum (mm)	4.82 ± 0.24 (23)

no sperm was emitted (Table 3). There were no significant differences in total body length or pronotum width among males from these same two groups. However, the duration of copulations in which sperm emission occurred was significantly longer on average (32 minutes, or 36%) than those in which sperm was not emitted (Table 3).

#### Effects of partial ablation of the flagellum on sperm emission

The possibility that some other characteristic associated with a longer flagellum, rather than the flagellum itself, could be responsible for differences in sperm emission, was tested by cutting off an average of 24.8% of the flagellum of 32 males. The length of the portion of the flagellum left after the operation averaged  $17.1 \pm 2.8$  mm; the length removed,  $5.7 \pm 2.2$  mm. When the male was allowed to mate with a virgin female one day later, the proportion of females emitting sperm increased from 13.1% ( $n = 84$ ) in controlled pairings to 87.5% ( $n = 32$ ) (chi-square test,  $df = 1$ ,  $p < 0.001$ ).

The number of sperm in the spermatheca 24 hours after copulation with a male with a shortened flagellum was typical of that for an intact male when sperm emission occurred. In females paired with experimental males, there was no correlation between the length of the shortened flagellum and the number of sperm in the spermatheca 24 hours later ( $r_s = 0.157$ ,  $p = 0.381$ ,  $n = 32$ ).

#### Effects of the spermathecal muscle

The influence of the female's spermathecal muscle on sperm movement out of the spermatheca was studied by severing the muscle in 22 virgin females (Rodríguez, 1994). All these females emitted sperm

when subsequently mated with an intact male; while only three (13.6%) of 22 similarly-mated, sham-operated females emitted a sperm mass (chi-square test,  $df = 1$ ,  $p < 0.001$ ). Copulation duration for sham-operated females that emitted sperm was  $114 \pm 37$  minutes, significantly longer than the  $86 \pm 34$  minutes spent in copulation by sham-operated females that did not emit sperm ( $p = 0.04$  with the Mann-Whitney U test).

#### Changes in paternity with time and number of ovipositions

Sperm mixing within the spermatheca was apparently incomplete. The proportion of offspring fathered by the male with the most offspring in a female's first egg mass decreased significantly over her next ten masses in nine females in the experiment with two males ( $r_s = -0.759$ ,  $p = 0.007$ ), and also decreased (but not significantly) in eighteen females in the experiment with three males ( $r_s = -0.240$ ,  $p = 0.48$ ). Moderate sperm clumping was also suggested by the fact that, in the first eleven egg masses of the nine females in the two-male experiment and the eighteen females in the three-male experiment that laid at least eleven egg masses, the mix of paternity was significantly different from the total mix for the female (chi-square test,  $p < 0.05$ ) in 43 of 277 masses. Such differences would be expected by chance in only 5% of these masses (13.85 masses). Significant differences were thus more common than expected by chance (chi-square test,  $df = 1$ ,  $p < 0.001$ ).

#### Discussion

The flagellum (lower ejaculatory duct) of the male *Chelymormpha alternans* may exceed three times its overall body length, by any measure an exaggerated morphological characteristic. In flash-frozen mating pairs, the flagellum of the males was often deeply inserted into the spermathecal duct of the females. The distal portion of the flagellum was coiled within the ampulla in over half the females examined. Thus, it appears that the flagellum may stay for a considerable period of time in the ampulla, located approximately two-thirds along the 6-mm distance to the spermatheca. One flagellum extended beyond the ampulla and passed through the entrance to the spermatheca, indicating that the flagellum is sufficiently long (despite its many loops within the ampulla), and at least occasionally (or alternatively, often, but for brief periods), it reaches the spermatheca. The ampulla may act as a trap causing the flagellum to coil up on itself (the flagellum coils tightly up on itself when unrestrained), and thereby seldom find the entrance to the final section of spermathecal duct leading to the spermatheca. If the ampulla does function in this manner, then selection may have favored females that can control male

access to the spermatheca. On the other hand, males with longer flagella could be favored because every turn within the ampulla would provide an additional opportunity to find the entrance to the distal section of spermathecal duct leading to the spermatheca. While it seems fairly certain that the ampulla must impede access to the spermatheca, the advantage to females of doing this is less clear.

Our observations do not allow us to say whether the flagellum functions primarily to introduce sperm or to remove sperm deposited in the spermatheca by previous matings (Waage, 1979). However, the sperm that is discharged from the female during copulation is probably not the result of sperm removal, because droplets containing sperm occurred in 13% of matings with virgin females. Once-mated females, from which sperm-containing droplets emerged during copulation, had significantly fewer sperm in their spermathecae afterwards than others.

Sperm usage patterns in *C. alternans* are apparently largely determined by relative numbers of sperm in the spermatheca, with only moderate clumping. Thus, the positive effect of shortening a male's flagellum on the emission of sperm by the female (thus reducing the number of his sperm stored in her spermatheca) probably influences his chances of paternity.

Details of the mechanism by which males with a longer flagellum achieved greater paternity are not known. Females probably play an active role, and sperm emission, or perhaps sperm uptake involving contractions of the spermathecal muscle, as well as the length of the spermathecal duct, may be involved. The significance of differences in copulation duration is unclear, but copulation duration was not related to flagellum length in intact males ( $r_s = 0.027$ ,  $n = 86$ ,  $p > 0.1$ ). Cues used by females to discriminate between males could be purely mechanical (perhaps the long spermathecal duct and ampullar expansion make penetration to the spermatheca more difficult for a shorter flagellum), or possibly sensory (e.g., the female might sense the pressure exerted by the folded flagellum in the ampulla). Removal of the tip of the flagellum may have made it more difficult for the male to thread his flagellum up the spermathecal duct.

The strong correlation between spermathecal duct length and male flagellum length in different species of Cassidinae (Fig. 1) suggests that our detailed observations of *C. alternans* are of general significance for this subfamily. Presumably, the especially long spermathecal ducts, together with their flagellum-trapping ampullae and their reversals of coiling direction, evolved to enable females to bias or otherwise reduce the paternity of some males. In response, the long genitalic flagella of males evolved in order to enable them to improve their chances of paternity when mating with females that possessed such ducts.

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