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Function of the mating plug in *Drosophila hibisci* Bock

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Abstract The mating plug in *Drosophila hibisci* Bock is a firm, gelatinous structure that forms within the female's uterus during copulation. Two non-mutually exclusive hypotheses for the function of the plug were evaluated. The plug may serve as a nutritional gift that females digest, using the constituents for somatic maintenance or to provision eggs as they mature within the ovaries. Alternatively, the plug may act as a chastity enforcement device by preventing subsequent copulations, and thereby reducing sperm competition. Plug size did not decrease within females over a period of 2 days, and dietary treatment in females did not affect plug size. The extent of ovarian provisioning was also not related to plug size. These results weaken the nutritional gift hypothesis. In contrast, the probability of a second copulation increased sharply with an experimental decrease in plug size. Moreover, females with plugs experimentally reduced in size were courted significantly more and mated significantly faster than females with larger plugs. These results support the chastity enforcement hypothesis. The plug retains the ejaculate and concentrates sperm at the anterior end of the uterus near the apertures of the sperm storage organs. The presence of the plug thus probably facilitates the movement of sperm into storage by retaining sperm at the anterior end of the uterus near the apertures of the sperm storage organs, which may be especially important for *D. hibisci*, in which sperm length is nearly twofold greater than ventral receptacle length. Matings with newly eclosed virgin females were significantly shorter than with older virgins, and copulations with the younger virgins ended more often without any sperm having yet entered into storage. The effectiveness of the plug in safeguarding a male's ejaculate may have favoured the evolution of shortened copula durations with young virgins. One fitness advantage of shortened copula duration could be time liberated for the pursuit of further mating opportunities.

Keywords Nuptial gift · Mating plug · *Drosophila hibisci* · Mating effort · Parental investment · Sperm competition

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Introduction

Insects have evolved an impressive diversity of nuptial gifts that males transfer to females before, during or immediately after copulation. Nuptial gifts may be in the form of prey items (Downes 1970), parts or most of the male's body (Elgar 1992), salivary secretions (Thornhill 1976), accessory gland secretions (Leopold 1976), spermatophores (Sakaluk 1986), spermatozoa (Hinton 1964) and mating plugs (Parker 1970). Deciphering the function of nuptial gifts is crucial for understanding the selective forces responsible for their maintenance, and for reconstructing their evolutionary origin (Thornhill and Alcock 1983; Wickler 1985; Simmons and Parker 1989). Two main functions of nuptial gifts have been hypothesized: parental investment and mating effort.

A nuptial gift is parental investment if its constituents are used by the female to elevate progeny number or quality (Trivers 1972; Alexander and Borgia 1979; Simmons and Parker 1989). In the katydid, *Requena verticalis*, for example, females feeding on the spermatophore increase the number and size of eggs produced (Gwynne 1984). In certain species of *Drosophila*, nutrients in the male's ejaculate are absorbed from the reproductive tract and incorporated into both the ovaries and somatic tissues (Markow and Ankney 1984; Pitnick et al.

1997). But whether ejaculate-derived material in *Drosophila* indeed constitutes mating effort is not resolved. For example, Markow et al. (1990) found that in *Drosophila mojavensis*, these materials enhance female productivity over the first 10 days of life, but lifetime reproductive success was unaffected by levels of incorporation.

A gift is mating effort when it increases the gift-giving male's probability of fertilizing the eggs of the female, or his number of mating opportunities (Simmons and Parker 1989). Such gifts are especially likely to be positively selected in species whose females mate multiply because of the relative fitness gains accrued by a male through outcompeting sperm of rival males. In *D. melanogaster*, for example, components of the male's seminal fluid increase a male's ability to displace rival sperm already in storage (Clark et al. 1995), and increase female egg-laying rates (Kalb et al. 1993), but also possibly harm the female through toxic effects on longevity (Chapman et al. 1995; Holland and Rice 1999).

Mating plugs are typically interpreted as mating effort (Parker 1970; Boorman and Parker 1976; Thornhill and Alcock 1983; Simmons and Parker 1989; Simmons and Siva-Jothy 1998). Indeed, much evidence supports the view that mating plugs block the female reproductive tract, thereby erecting a physical barrier to the sperm of rival males, and reducing sperm competition. Mating plugs, which are induced by components of the male's ejaculate within or at the opening of the female genital tract, occur in a wide range of animal taxa, including nematodes, insects, reptiles and mammals (Blum et al. 1962; Giglioli and Mason 1966; Devine 1975; Fenton 1984; Dickinson and Rutowski 1989; Orr and Rutowski 1991; Barker 1994; Duvoisin et al. 1999).

Whether mating plugs can also play a nutritive role, and hence potentially influence progeny number or survival, is less clear, although some theory (Thornhill 1976), and a few empirical observations argue that this might be so. For example, in fox squirrels (*Sciurus nigra*) and eastern gray squirrels (*S. carolinensis*), females frequently use their incisors to remove and consume the mating plug from their genital tract (Koprowski 1992). In some insects, including *Aedes* mosquitoes and *Drosophila* (Wheeler 1947; Parker 1970), an opaque viscous material, that may represent a mating plug, is relatively transient within the female reproductive tract, and disappears within a few hours of copulation. Although disappearance probably results from ejaculate outflow from the reproductive tract (e.g. Wheeler 1947), the mating plug is conceivably digested and absorbed by the female (Thornhill 1976), although this has not been tested.

The mating plug in *D. hibisci* is a firm, gelatinous structure that completely fills and seals the uterus at copulation (Polak et al. 1998). The sperm mass is held at the anterior end of the uterus, adjacent to the openings of the sperm storage organs (ventral receptacle and paired spermathecae). Thus, by holding and concentrating sperm at this position, and possibly also by dilating the apertures of the storage organs (and see Bairati and Perotti 1970), the plug may facilitate sperm movement into storage. Characteristics of the plug in *D. hibisci* are consistent with the defining criteria for a mating plug within the genus *Drosophila* as set forth by Alonso-Pimentel et al. (1994).

The present paper evaluates two non-mutually exclusive hypotheses for the function of the plug in *D. hibisci*: (1) the plug serves as a nutritive gift to the female and (2) the plug serves to prevent subsequent matings, thereby acting as a chastity enforcement device. The nutritive gift hypothesis has two subsidiary hypotheses, namely that females assimilate plug constituents either for their own somatic maintenance, or for provisioning eggs within the ovaries. We tested the following predictions to evaluate these possibilities. If plug constituents are used for female nourishment, plug size within newly eclosed and once-mated females will decrease with time after copulation (i.e. as plug constituents are broken down within the female's reproductive tract and assimilated) and, more critically, this decrease will be more pronounced in females maintained on a restricted diet, relative to that in well-fed individuals. Alternatively, given that ovarian development is arrested in nutritionally stressed females (see Methods), we predicted that if plug constituents provision ovaries, there will be (1) a more pronounced decrease in plug size within well-fed females with active egg production relative to nutritionally stressed females and (2) a negative relationship between plug size and extent of ovarian maturation. Lack of decrease in plug size with time after copulation, and lack of an effect of dietary restriction on plug size would be results failing to support the nutritive gift hypothesis.

The chastity enforcement hypothesis predicts that if plug size is experimentally decreased, the probability of a second copulation will increase either linearly or in threshold manner. We manipulated copula duration, thereby creating the needed experimental variation in plug size to evaluate this prediction. Nearly all experimental females that carried a plug also carried some ejaculate, which was a crucial component of the experimental design because it eliminated the potentially confounding effect of absence of ejaculate on remating by females. However, we were unable to control for the abundance or concentration of ejaculate constituents outside the plug, the ramifications of which are discussed. To help alleviate this problem, we tested whether previously mated females would remate soon after oviposition. Although sperm numbers may have decreased somewhat due to oviposition, sperm number in storage should nevertheless have been similar to that in females with intact plugs and that had not yet oviposited.

Information is also provided on characteristics of male and female reproductive physiology (sperm and ventral receptacle length) and sperm storage dynamics, which are then related to the function of the mating plug.

Methods

Fly rearing and husbandry

Flies were reared from native flowers of *Hibiscus heterophyllus* collected at Bungwahl Creek (32°3.353′ S, 152°26.528′ E) and Bellingen (30°25.155′ S, 152°49.425′ E), NSW, Australia. On the day of collection, flowers were inserted into 200-ml glass bottles containing moistened sand and sealed with foam stoppers. Bottles were held in an incubator at 25°C and a 12:12 h light:dark cycle. Virgin flies were harvested with an aspirator from these bottles no more than 2 h post-emergence. Females were used on the morning they emerged, whereas males were held for at least 3 days until sexual maturity in 200-ml bottles or in a population cage (38×38×20 cm) supplied daily with open *Hibiscus* flowers from plants growing in a greenhouse. Matings were conducted in vials containing sucrose (5%)–agar (1.5%) substrate. Unless otherwise mentioned, experiments were conducted at 20–22°C in a laboratory at the University of New England during October and November 1997.

Reproductive physiology

To estimate the number of sperm ejaculated by males, teneral virgin females were allowed to copulate with virgin, sexually mature males, and immediately after copulation, the female was killed with ether, and her reproductive tract dissected into a drop of physiological saline. The uterus was ruptured and the sperm mass pulled apart with fine dissecting probes. Sperm were released from the uterus within 3 min after copulation. The preparation was oven-dried, fixed in a 3:1 methanol:acetic acid solution, stained using a 5×10^{-7} M Hoechst solution (Sakaluk and O'Day 1984), washed, and oven dried. Sperm were visualized and counted using epifluorescence under a Zeiss Axioskop.

To measure sperm length, the sperm mass was released from the uterus of a recently mated female and gently teased apart. The slide was fixed as above and oven dried. Individual sperm cells were visualized using Nomarski optics of a Zeiss Axioskop and length measured using NIH Object Image 1.60 software. To determine the length of the ventral receptacle, measurements were made on freshly dissected female reproductive tracts immersed in a drop of physiological saline.

Sperm storage dynamics and female age

We examined the proportion of teneral and older females that had sperm in storage immediately upon termination of copulation; these females were used because previous work (Polak et al. 1998) has shown that males copulate significantly longer with older than with teneral virgins. We determined whether in these short copulations with young females, sperm had sufficient time to enter into storage, a question with important implications for understanding the function of the plug. Teneral virgins were allowed to mate within 2 h of emergence with virgin males. Older female virgins were held in a population cage with flowers for 3–9 days prior to mating. For each mating, we timed copula duration, and immediately after copulation, the intact female reproductive tract was dissected into a drop of saline under a Wild M3Z stereomicroscope, and the spermathecae separated from the rest of the reproductive tract. The presence/absence of sperm within the ventral receptacle and spermathecae was determined by gently rupturing and teasing apart these organs; the sperm are relatively long and coiled, and hence readily distinguished.

Nutritional gift hypothesis

We tested the hypothesis that the plug serves as a nutritional gift to the female. Plug materials may be assimilated by the female for either of two reasons, somatic maintenance and/or ovary provisioning (i.e. oogenesis). Two experiments were performed to evaluate these possibilities. Each experiment consisted of first allowing virgin, newly eclosed (teneral) females to mate without interruption at room temperature in 8-fluid-dram sucrose-agar vials. Males used in any replicate of these experiments were the same age, and male age across replicates was 3–8 days. Following mating, females were held in groups of five on sugar-agar vials for 24 h at 25°C, and assigned at random to either of two dietary treatments. Females on a high-quality diet were placed into a population cage containing moistened, sterilized sand, seven open sugar-agar vials, and one fresh *Hibiscus* flower supplied daily. Females on a low-quality diet were held in a cage with similar materials but without flowers. Flies feed on floral exudate in nature, and on the micro-organisms that inhabit the flowers. If females are given access to fresh *Hibiscus* flowers, they rapidly undergo ovarian maturation and begin ovipositing within 3–5 days post-eclosion in the laboratory at 25°C. Ovarian development is arrested when teneral, mated females are denied flowers.

In the first experiment, which consisted of two replicates 7 days apart, plug size was determined in females 3 and 5 days post-mating. At day 3, a randomly selected subset of females from each high- and low-stress cage was dissected, and at 5 days, all remaining females were dissected. The plug was gently teased from the uterus into a drop of physiological saline on a microscope slide, and the length (l), width (w) and depth (d) of the plug were measured with an ocular micrometer on a Wild M3Z strereomicroscope. Plug volume was calculated as lwd. In a previous study (Polak et al. 1998), we measured only plug length and width, but in the present context, it was possible to gently manoeuver the plug within the saline onto its side using a fine probe, so that its third dimension could be measured. To estimate female body size, thorax length (mm) was measured using a digital filar eyepiece on a stereomicroscope. In this first experiment, there was a significant relationship between plug volume and female thorax length (cubed) (regression slope $\hat{b}$=0.00721, SE=0.00264, t=2.73, P=0.0088, n=49). Factorial ANOVA was therefore performed on residuals in plug volume from this regression, with replicate (Rep: 1 and 2), treatment (Trt: high- and low-quality diet) and time since mating (Time: 3 and 5 days) as factors. Previous work has shown that male body size and plug size are not related (r=–0.0064, n=9) (M. Polak, W.T. Starmer, J.S.F. Barker, unpublished data).

In the second experiment, which consisted of three replicates (3 and 6 days apart), plug volume was determined in 5-day post-mating females only. We eliminated the 3-day post-mating category in this experiment because the Time factor was not significant in the first experiment (see Results), and because there was greater ovarian development, and a greater range of egg stages among 5-day post-mating females, which afforded a stronger test of the relationship between plug size and oogenesis. The regression between plug volume and female thorax length (cubed) was not significant $\hat{b}$=–0.0017, SE=0.00212, t=–0.786, P=0.44, n=55), so factorial ANOVA was performed on uncorrected values of plug volume, with replicate (Rep: 1, 2 and 3) and treatment (Trt: high- and low-quality diet) as factors. Females used in the second experiment were significantly larger than those in the first experiment [mean±SE=0.856±0.011 (n=49) and 0.960±0.010 (n=55), respectively; t=7.78, df=102, P<0.0001], which translated to a greater plug size in the second than in the first experiment [mean± SE=0.0105±0.00049 (n=49) and 0.0118±0.00042 (n=55), respectively; t=2.02, df=102, P=0.046].

To increase the power of our statistical test to detect differences between Trt categories, volume data across the above experiments were pooled. Each experiment was treated as a Block, and although Rep and Trt were retained, the Time effect was dropped (Time did not significantly affect plug volume; see Results). Female thorax length was entered as a covariate. Given the reported sample size and variance within Trt categories, power analysis (Cohen 1988) revealed that we should be able to detect a 12% or greater decrease in plug volume with 80% power, which we view as reasonable.

The developmental stage of all eggs within each ovariole was scored using the criteria of Mahowald and Kambysellis (1980).

Only eggs greater than stage 5 were scored. Total egg volume was determined by summing egg volume over all ovarioles in both ovaries, and used as an approximation of total egg volume, or the total amount of oogenenis undergone by that female since eclosion since no eggs had yet been laid (because the plug was still present). If all eggs within a female were less than or equal to stage 5, total egg volume was set to zero for that female. Length and width of each egg at stage >5 were measured using a digital filar eyepiece, and egg volume was estimated using the equation for the volume of a prolate spheroid (e.g. Montague et al. 1981). The relationship between plug volume and total egg volume was determined among females from the high-quality diet only; all eggs in all females subjected to the low-quality diet were at stage <5 (i.e. ovarian development was arrested in these nutritionally stressed females).

Chastity enforcement hypothesis

Flies were reared from flowers collected in the field, and males were held with flowers but without females for 7–12 days prior to use in any phase of this experiment. Within 2 h of emergence, females were placed individually into sugar-agar vials, and each female was allowed to acclimate for 10 min at room temperature. Two males were then aspirated into the vial (two males were used to increase the frequency of male-female interactions). Latency to copulation (time from introduction of males to copulation) and courtship duration (duration of time the female was courted by the males) were determined for each copulation, all of which occurred within 30 min of introducing the males. The proportion of time that males courted was calculated as courtship duration divided by latency to copulation.

Thirty-nine (78%) of 50 total first copulations were interrupted by gently separating the pair using a fine paintbrush. Eleven (22%) first copulations were allowed to progress to completion without interruption. Copulation duration was the time from initiation of copula until the pair was experimentally separated or, in the case of uninterrupted copulations, until the male dismounted. Fig. 1 shows that manipulated copula duration and size of the resultant plug were positively correlated (n=23 interrupted copulations that resulted in at least some plug production); plug size ranged from very small quantities of plug material to plugs that were similar in size to those formed during full-length copulations with the sperm localized at the anterior end of the uterus (see Fig. 1 in Polak et al. 1998). The relationship between female thorax length and plug size resulting from interrupted copulations was not significant (r=–0.009, n=22, one female was not measured), nor was there a relationship between female thorax length and copula duration in the case of uninterrupted copulations (r=0.32, n=11, P>0.2).

Following the first copulation, both males were removed from the vial, and within an average of 3.3±0.092 min (n=50) after the end of copulation, a new pair of males was introduced into the vial. Latency to copulation, courtship duration, and proportion of time spent courting were determined as above. If a second copulation did not occur within 0.5 h of introducing the second set of males, males were discarded and the female dissected as described below. When no second copulation occurred, the proportion of time spent courting was calculated as courtship duration/0.5 h.

When a second copulation did occur, the pair was immediately separated by vigorously shaking the vial; all second copulations were thus interrupted in 5 s or less. Because we know that minimum copula duration required for the transfer of any sperm, or production of a plug of any size, is 23 s (see Results), any plug material or sperm found within the female could be attributed safely to the first copulation. Each female was then dissected in a drop of physiological saline to determine the presence/absence of a plug and, if present, plug dimensions (i.e. length, width and depth) were measured using an ocular micrometer of a Wild M3Z stereomicroscope. Presence or absence of sperm within the uterus was also determined. This experiment was conducted on five mornings, with 8–12 females tested per morning.

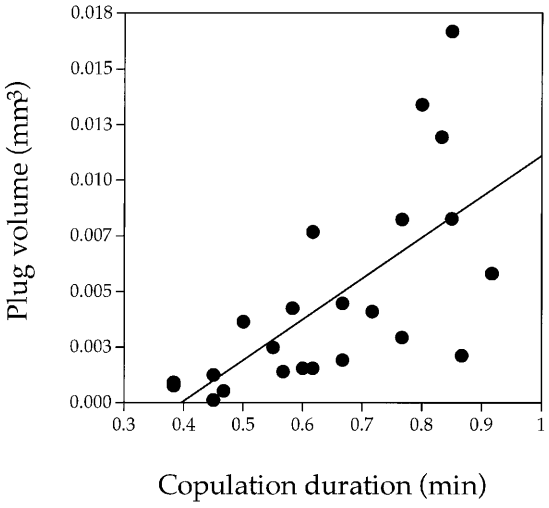

Fig. 1 Effect of experimentally determined copula duration on plug volume. Equation: plug volume=–0.0073 (SE=0.0029)+0.018 (SE=0.0044) (copulation duration); $F_{1,22}$=17.4, P=0.0004

We conducted an experiment (two replicates) during October and November 1996 to test whether females would remate soon after oviposition. Newly eclosed virgin females reared in the laboratory from population cages were each mated to males that had been held without females for at least 24 h. After copulation, females were held for 3 days in 200-ml glass bottles supplied each day with an open *Hibiscus* flower and without males. As noted above, females reach sexual maturity and begin to oviposit at 3–5 days of age if maintained on fresh flowers (Polak et al. 1998). Thus, among 3-day-old females, only a subset will be ready to lay eggs. On the morning of day 3 (11.30 a.m.), females (n=11 and 16 females on replicate morning one and two, respectively) were removed from their bottle containing an open flower, placed with two males in a sugar-agar vial, and observed continuously for 1.5 h, or until a copulation occurred. By using females at this age, we helped to ensure that any females that had oviposited and hence expelled the plug had done so on the morning of the experiment, shortly before being exposed to males. For each copulation, latency to copulation was determined, and all females that did not copulate were dissected to determine presence/absence of a plug or egg within the uterus.

Results

Reproductive physiology and sperm storage dynamics

After copulation the mean (±SE) number of sperm within the uterus of teneral previously virgin females was 3.69×10^3 (0.14×10^3, n=2). Sperm length was 0.431± 0.011 mm (n=5 sperm cells across two males), whereas ventral receptacle length was 0.265±0.0050 mm (n=2 females). Of the 12 teneral females dissected within 2 min after the end of copulation, 5 (42%) had sperm within their ventral receptacle, whereas no female had sperm within the spermathecae. Of the 20 older females (3–9 days old) dissected within 2 min after copulation, 18 (90%) had sperm within the ventral receptacle, and 5 of these had sperm within the spermathecae (of the 2 females without sperm in the ventral receptacle, neither had sperm within the spermathecae); the proportion of

older females with sperm in the ventral receptacle was significantly greater than that in teneral females (χ^2=10.97, *df*=1, *P*<0.001). This difference may be explained by the nearly fourfold longer copulation durations with older females compared to teneral females [mean±SE for teneral females=3.30±0.59 min (*n*=12); for older females=11.83±1.17 min (*n*=20); *t*=5.4, *df*=30, *P*<0.0001] (discussed in Polak et al. 1998).

Thus, in *D. hibisci*, sperm are stored within both the ventral receptacle and spermathecae, although sperm move more rapidly into the ventral receptacle. Matings with teneral females are more likely to end with no sperm yet in storage, whereas nearly all matings with older females end with at least some sperm in the ventral receptacle.

Nutrient donation hypothesis

If females utilize the plug as a source of food, we predicted that the plug would decrease in size with time after mating. However, plug digestion may depend on the nutritional state of the female, so we predicted that if females assimilate plug constituents for their own somatic maintenance and survival, the plug would be significantly smaller in females following a period of dietary restriction relative to that in well-fed individuals. In contrast, if females utilize plug constituents to provision eggs (i.e. oogenesis), we predicted that the plug would be significantly smaller in females that had undergone ovary maturation relative to females with quiescent ovaries, and that there would be a negative relationship between plug size and total egg volume.

In the first experiment, factorial ANOVA on residuals in plug volume indicated that the Rep, Trt, Time, and Trt×Time interaction effects were not statistically significant (all *P*>0.25). Likewise, in the second experiment, factorial ANOVA showed that the Trt effect was not significant (*P*=0.24). A significant effect of Rep in this experiment resulted from plug volume being largest in the third replicate [for replicates 1, 2 and 3, mean±SE= 0.0107±0.000596 mm^3 (*n*=20), 0.0100±0.000527 mm^3 (*n*=16), 0.0145±0.000576 mm^3 (*n*=19)]. Data from these experiments were also pooled, and plug volume analysed with factorial ANOVA in which experiments was treated as blocks. Although the effect of Rep on plug volume was significant (as above), neither the effects of Block (*P*=0.12) or Trt (*P*=0.914) were significant. In fact, plug volume (corrected for thorax length) was marginally *larger* in stressed females [mean±SE=0.01186±0.000456 mm^3 (*n*=48)] than in well-fed individuals [0.01179± 0.000407 (*n*=56)].

The critical result of the first experiment is that plug size did not decrease with time since mating, which alone indicates that the plug is probably not digested by the female. However, we also found that plug size was insensitive to female nutritional state, at least to the extent experienced in the present study. Moreover, the relationship between plug size (corrected for thorax length) and $\log_{10}$-transformed total egg volume was not significant ($\hat{b}$±SE=–0.000478±0.00053, *P*=0.37, *n*=49), which argues against the possibility that females provision their ovaries with plug-derived material. These results do not support the nutritional gift hypothesis.

Chastity enforcement hypothesis

Uninterrupted copulations lasted on average 2.29± 0.33 min (*n*=11, range=1.28–4.82 min), all of which resulted in sperm transfer and plug production. Experimentally interrupted copulations lasted 0.58±0.031 min (*n*=39, range=0.22–0.97 min). Sixteen (41%) of these interrupted copulations resulted in no plug production or sperm transfer, whereas of the 23 (59%) interrupted copulations that resulted in plug production, all except 2 resulted also in sperm transfer. Duration of interrupted copulations that did not result in plug production (0.472±0.047 min, *n*=16, range=0.22–0.97 min) was significantly less than that for copulations that did result in the formation of at least some plug material within the uterus (0.646±0.034, *n*=23, range=0.38–0.92 min; *t*=3.07, *df*=37, *P*=0.004). Thus, a male must copulate for a minimum duration of about 0.38 min (23 s) before a plug begins to form within his female.

We tested the hypothesis that the plug deters subsequent copulations by evaluating the prediction that the probability of a second copulation will increase with an experimental decrease in plug size. All 11 females that carried a plug from an uninterrupted copulation failed to copulate with a second male within 30 min (log-likelihood ratio test, *G*=15.3, *df*=1, *P*<0.001). In contrast, of the 16 interrupted copulations that did not result in plug formation, 15 (94%) females copulated with a second male (*G*=14.7, *df*=1, *P*<0.001). These data are consistent with the chastity enforcement hypothesis, and indicate that the mere act of copulating (albeit briefly) is not sufficient to deter subsequent matings.

When a second male succeeded in copulating with a female that did contain a plug derived from an interrupted first copulation, the plug was significantly smaller than when a second male failed to copulate with a female containing a plug (Fig. 2, t=3.96, *df*=32, *P*=0.0004). Logistic regression (Hosmer and Lemeshow 1989) was performed using a full data set including females with and without a plug; the continuous independent variable was plug volume and the dichotomous dependent variable was whether or not a second copulation occurred. Plug volume from a first copulation had a significant effect on the probability of copulating with a second male (χ^2=7.66, *df*=1, *P*=0.006), with the estimated logit ($\hat{g}$) given by the equation $\hat{g}$=–1.70 (SE=0.55)+737.7 (SE=266.6) (plug volume) (Fig. 3). The goodness-of-fit statistic was not significant (χ^2=1.39, *df*=6, *P*>0.97), indicating that the data fit the model well. These results indicate that the likelihood of a second copulation increased significantly with decreasing plug volume. The plug volume which gives a 95% and 99% probability of copulation inhibition, calculated using the linear logistic

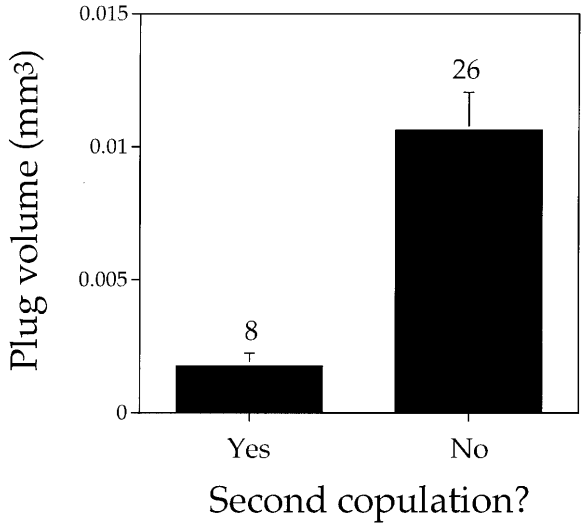

Fig. 2 Mean plug volume in females that did and did not copulate a second time. Females in which no plug material was found are excluded. *Numerals* represent sample sizes and *errors bars* are +1 SE

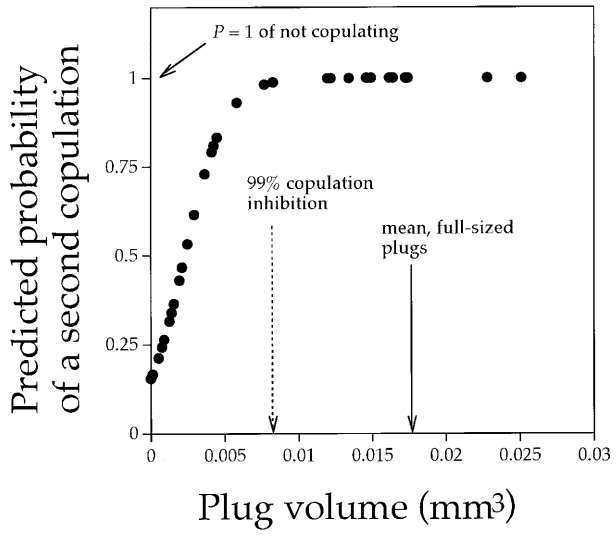

Fig. 3 Effect of experimentally manipulated plug volume on the predicted probability of not engaging in a second copulation, obtained from logistic regression

model, is ≈0.0063 mm^3 and 0.0085 mm^3, respectively. Thus, given that the mean volume of full-sized plugs was found to be 0.0173±0.0011 mm^3 (n=11), these threshold plugs are 36% and 49% that of an average full-sized plug. The maximum size of a plug is probably set by the distensibility of the uterus. During normal copulation, males probably need to fill the uterus completely with plug material, above the amount required for immediate chastity enforcement. This excess filling probably ensures that the plug remains firmly in place, and is not pushed out prematuraly, for example, by female muscular contraction. The above results support the chastity enforcement hypothesis for plug function.

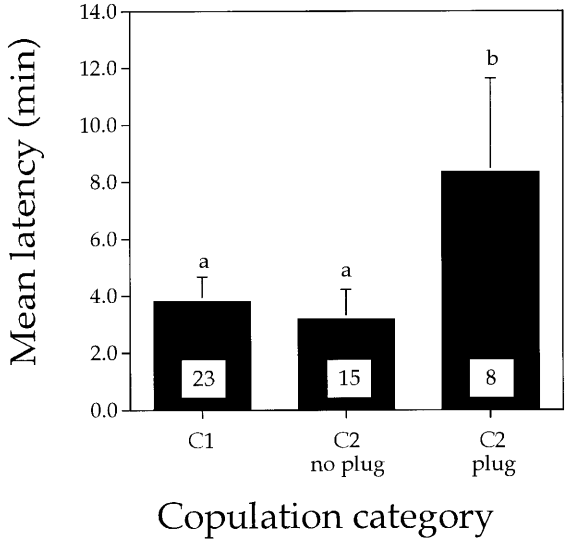

Fig. 4 Mean copulation latency in a female's first copulation (*C1*), second copulation when she did not contain a plug (*C2 no plug*) and second copulation when she did contain a plug (*C2 plug*). Means not sharing a letter are significantly different at α=0.05 using Fisher's least-significant method for multiple contrasts. *Numerals* are sample sizes, and *error bars* represent +1 SE

We also evaluated the relationship between plug size and latency to copulation. Mean latency was contrasted between a female's (1) first copulation, (2) second copulation in which she did not carry a plug and (3) second copulation in which she did carry a plug. Single-factor ANOVA showed non-significant differences between these categories ($F_{2,43}$=2.72, P=0.077), but multiple-comparison testing using Fisher's least-significant-difference method indicated that copulation latency was significantly greater for females that carried a plug (Fig. 4). Latency to a second copulation was linearly related to plug volume for data that included females without a plug ($F_{1,22}$=20.92, P=0.0002, R^2=0.50; Fig. 5a); data without these females were best described by an exponential function ($F_{1,6}$=16.63, P=0.007, R^2=0.74; Fig. 5b). The slope of a linear regression relating copulation latency to female thorax length was not significant for either first ($\hat{b}$±SE=–13.99±9.52, $F_{1,48}$=2.16, P=0.15) or second ($\hat{b}$±SE=–14.51±20.90, $F_{1,22}$=0.48, P=0.50) copulations.

There was no difference in the proportion of time males courted virgin females compared to females that had mated previously, but that carried neither sperm nor plug (t=0.283, df=64, P>0.7). Thus, the mere act of copulating did not appear to alter the attractiveness of females to males. We contrasted courtship of second males toward females that either (1) carried no plug, (2) carried a small partial plug from an interrupted first copulation (and that did eventually copulate a second time), (3) carried a larger plug from an interrupted first copulation (and that failed to copulate a second time) and (4) a plug from a full-length copulation. Partial plugs were distinguished by their irregular reticulated structure (see Fig. 1 in Polak et al. 1998). Significant differences in courtship

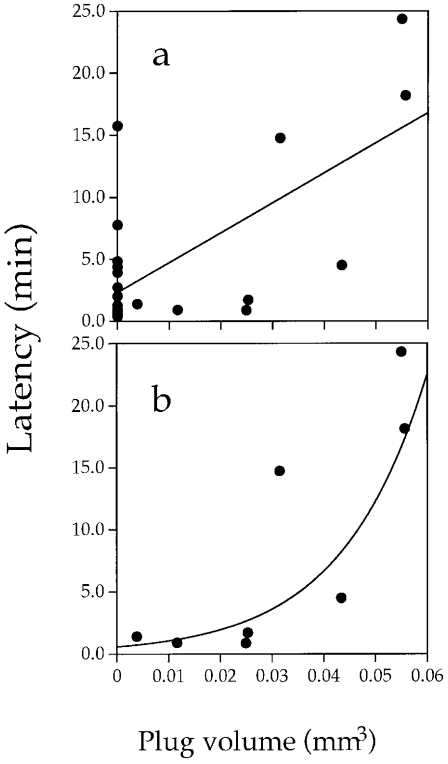

Fig. 5a,b Effect of plug volume on latency to second copulation. **a** Data include copulations with females without a plug (i.e. volume=0). Latency=2.44 (SE=1.15)+4,080.86 (SE=892.24) (plug volume). **b** Data do not include copulations with females without a plug. Latency=0.83 (SE=0.98)×$10^{25.15}$ (SE=9.83) (plug volume)

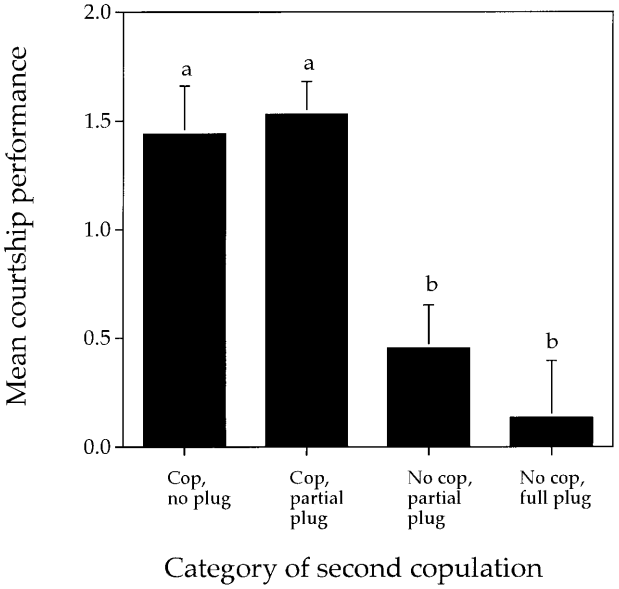

Fig. 6 Mean courtship performance by second males (log_{10}-transformed courtship rate) when the female copulated a second time and did not carry a plug from her first copulation (*Cop, no plug*), copulated a second time and carried a small partial plug (*Cop, partial plug*), failed to copulate a second time and carried a larger partial plug (*No cop, partial plug*) and failed to copulate a second time and carried a large plug derived from an uninterrupted first copulation (*No cop, full plug*). Means not sharing a letter are significantly different at α=0.05, and *error bars* represent +1 SE

performance between these categories were detected (one-way ANOVA, $F_{3,45}$=8.66, P=0.0001; Fig. 6). Females that carried either a full-sized plug or a large partial plug were courted significantly less than females that either carried a small partial plug or no plug at all (Fig. 6).

We also determined whether previously mated females would remate soon after being given an opportunity to oviposit and, hence, to expel the plug. Of the 27 females tested, 6 (22%) remated with a copulation latency of 49.57 min (SE=4.2). Of the females that did not mate with a second male, 17 (63%) carried a plug within the uterus, 3 (11%) carried an egg, and 1 (4%) carried neither a plug nor egg within the uterus.

Discussion

Two hypotheses were tested regarding the functional significance of the mating plug in *D. hibisci*. Whereas the nutritional donation hypothesis was not supported, the data were consistent with the chastity enforcement hypothesis, namely, that the plug functions as a physical barrier against matings with subsequent males, and thus represents a form of male mating effort. However, females may leach nutrients from the plug without noticeably reducing its volume. To test this idea, for example, detailed chemical analysis of the plug shortly after copulation, and at subsequent post-mating intervals, would be needed. Nevertheless, since these hypotheses are non-mutually exclusive, positive results from such an experiment would not compromise support for the chastity enforcement hypothesis.

The plug also holds and concentrates the ejaculate at the anterior end of the uterus, thereby preventing backflow of sperm, and probably facilitating entry of sperm into storage (Polak et al. 1998). This function may be especially important in *D. hibisci*, in which sperm length is nearly twice ventral receptacle length. This disparity is a gross exception to the pattern observed within the genus *Drosophila*; in 45 of 46 species examined in a comparative study of female sperm storage organs (Pitnick et al. 1999), ventral receptacle length exceeded sperm length, with the mean ratio (±SE) being 1.7±0.08. The only exception was *D. wassermani*, in which sperm length (4.52±0.03 mm, n=5) was also nearly twice that of the ventral receptacle (2.63±0.09 mm, n=10). However, in contrast to *D. hibisci*, *D. wassermani* rarely uses the receptacle to store sperm (Pitnick et al. 1999). This length mismatch may be an impediment to sperm movement into and/or packaging within the ventral receptacle, and the mating plug in *D. hibisci* possibly serves to alleviate this problem by mechanical means, for example by concentrating sperm and anchoring sperm tails, thereby allowing them to "push" and fold their way into storage. It is therefore noteworthy that *D. wassermani* does not pos-

sess a plug (S. Pitnick, personal communication), which may in part explain the lack of use of its ventral receptacle to store sperm.

The plug's effectiveness at protecting a male's ejaculate may explain the significantly shorter copula durations with young virgins than with older virgin females. In young females, the plug will remain within the uterus for several days after copulation if a female is mated soon after eclosion. The plug will be expelled only by an oocyte descending into the uterus from the ovaries, when the female reaches reproductive maturity. Thus, since there will also be no chance of sperm displacement by rivals in these females due to the plug, a male needs only copulate long enough for his ejaculate and plug to be placed within the uterus, since there will be ample time for sperm to fully move into storage over the several days that the female is maturing oocytes. Indeed, the data presented show that copulations with young females typically end before sperm have reached any storage organ, as opposed to copulations with older females, most of which (90%) terminated with sperm already in the ventral receptacle. These relatively prolonged copulations with older, reproductively mature, females probably help ensure that at least some sperm are in storage before a male accepts the risk of having his ejaculate and plug expelled from the female, which can occur immediately after copulation should an oocyte descend into the uterus from the ovaries. Moreover, since the risk of sperm competition in young females will essentially be nil (due to the presence of the plug), sperm competition theory predicts that males will pass fewer sperm to these females; but this remains an untested prediction.

The experiment to test the chastity enforcement hypothesis was predicated on variation in plug size generated by manipulating copula duration. With this tool in hand, we evaluated the prediction that as plug volume decreased, there would be a concomitant increase in the probability of a second copulation. The design controlled for presence of ejaculate across levels of plug size, because sperm (and presumably other components of the ejaculate) were almost always present, regardless of plug size; only when no plug was formed were no sperm found within the uterus. The key prediction was borne out by the data: once plug volume fell below a threshold, the probability of a second copulation rose sharply. The threshold plug volume, at which there was a 99% chance of copulation inhibition, was approximately 8.5×10^{-3} mm^3. This evidence, together with the finding that a decrease in plug size was also significantly associated with reduced copula latency, supports the chastity enforcement hypothesis for plug function.

The proportion of time that previously mated females not carrying a plug were courted was similar to that for females mating for the first time. Thus, mere physical contact during copulation is not sufficient to reduce the attractiveness of females to subsequent males, which might occur, for example, if males applied an antiaphrodisiac to the female's terminalia. In *D. melanogaster*, copulating, and even courting males transfer the antiaphrodisiac compound 7-tricosene to the females' cuticle, which renders them less attractive to subsequent males (elicits less courtship in these males; Scott 1986). Male *D. hibisci* probably detect and respond to the presence of the plug itself, with the relevant gustatory or olfactory cues received during courtship. Courting males pursue females closely and with brisk upwards strokes "lick" the female's terminalia with their proboscis, while often pushing it between the female's terminal sternites and into the opening of the vagina. To the extent that the pursuit of females, courtship, and copulation attempts will be costly to males in terms of energy and time expenditure, selection will favour judicious allocation of mating effort, reflected in *D. hibisci* males in their decision to forgo courtship and mounting attempts upon detecting the presence of the plug. A similar argument was made by Orr and Rutowski (1991) in relation to the sphagris in the butterfly *Cressida cressida*. The sphagris is hard and highly visible, and experiments have shown that males detect it using visual cues (Orr and Rutowski 1991). Males make no attempt to mate with females carrying the sphagris, presumably because it signals the presence of an effective barrier to intromission.

Another pertinent observation consistent with a threshold effect of plug size on female remating is that females with very small plugs were courted as frequently as females with no plug or ejaculate. This lack of a difference occurred despite the presence of at least some ejaculate within females carrying these small plugs. Thus, the presence of ejaculate within the female is not sufficient to deter male courtship (or copulation, see above); small plugs probably go undetected. An alternative, albeit less likely explanation, is that males can assess the size of the plug, and hence the probability of a successful mating, and attempt copulations only when the plug is perceived to be sufficiently small.

In the chastity enforcement experiment, we were unable to control for quantitative variation in components of the ejaculate, such as sperm numbers, or concentration of accessory gland or ejaculatory bulb products (e.g. Wolfner 1997). Thus, males possibly do not respond to the plug per se, but to the concentration or abundance of components of the seminal fluid. Indeed, the abundance of these materials probably covaried with manipulated plug size, since the amount of these materials is also expected to increase with copula duration. However, males are perhaps unlikely to have been selected to respond to such stimuli under normal social conditions, because within females carrying normal, full-sized plugs, the sperm mass, and probably most of the ejaculate, are firmly sandwiched at the anterior end of the uterus, where they may normally be inaccessible to the probing proboscis of a courting male.

Admittedly, our interpretation of the results thus far has excluded female control mechanisms. For example, a possible explanation for the increased probability of copulating as plug size was experimentally decreased is simply that the very short copulations used to generate small plugs did not render females unreceptive. Females there-

fore allowed a subsequent copulation because of insufficient receipt of stimuli (e.g. uterine stretching) from either the plug, sperm, or other components of the ejaculate. Sugawara (1979), for example, showed that stretching of the bursa copulatrix was responsible for loss of female receptivity in the butterfly *Pieris rapae*. Likewise, in *D. hibisci*, aberrant packaging of sperm, or sperm backflow away from the anterior end of the uterus where sperm are normally packed, leading to insufficient filling of the storage organs, could have induced females with the smallest plugs to seek a further copulation by giving males the appropriate behavioural or chemical signal of their receptivity. By similar logic, females that carried a larger or full-size plug did not permit remating because they did receive enough sperm. But the remating experiment showed that older, mature females, allowed a bout of oviposition (the number of eggs layed was unknown), would remate with males. This observation strengthens the chastity enforcement hypothesis because sperm numbers stored in these females are expected to be relatively similar to those in females still carrying their plug and who do not remate. Moreover, these females that recently oviposited and remated should have had *more* sperm in storage than females that received larger partial plugs, yet females with these partial plugs still did not mate.

Further support for the chastity enforcement hypothesis comes from the observation that males voluntarily abandoned courtship and copulatory attempts with females carrying full-size plugs, despite the absence of any observable rejection behaviour by females. In this experiment, we used very young females to help eliminate any effect of a female propensity to control copulation. Previous observations document that although newly emerged, virginal females sometimes attempt to resist male mating advances, males are nevertheless readily able to grasp and to mate with these females (Polak et al. 1998; and see Manning 1967; Thornhill and Sauer 1991). Thus, we favour the interpretation that the observed lack of remating in females with plugs above threshold size is probably not due to female control of the mating process, but largely to an effect of the plug itself on the behaviour of subsequent males.

A question of importance to the evolution and maintenance of the mating plug in *D. hibisci*, and other animal species, is the fitness consequences of the plug to females. If the plug imposes non-trivial costs to the female, we might expect the female to have the capacity to expel or chemically dissolve the plug (at least partially, which might represent evidence for some form of coevolutionary "arms race" between the sexes). By this logic, the presence of a stable plug implies that females derive some adaptive benefit from it. One possible advantage to females may be that the plug discourages courtship by subsequent males; the presence of courting males decreases female lifespan in *D. melanogaster*, perhaps because of the energetic costs that accrue from being pursued and hence prevented from resting and feeding (Partridge and Fowler 1990). Thus, one possible evolutionary scenario is that the plug was selected due to fitness gains acquired through chastity enforcement and/or mechanical assistance with sperm storage, with its presence then favouring the evolution of female mating as near as possible to eclosion, which is rare in most other *Drosophila* species. Early mating would alleviate energetic costs to females associated with harassment throughout their entire juvenile life, i.e. until the onset of oviposition, because at this point, the plug is pushed out by the first mature oocyte that descends into the uterus for fertilization (Polak et al. 1998).

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