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Ingestion of male haemolymph and mating propensity of female sagebrush crickets: no evidence of a male-derived antiaphrodisiac

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Male sagebrush crickets, *Cyphoderris strepitans*, offer an unusual nuptial food gift to females during copulation: females feed on the hindwings of males and ingest haemolymph seeping from the wounds they inflict. Previous work has shown that females prevented from wing feeding during initial copulations are more receptive to subsequent matings than females permitted to wing feed. In the present study, we tested the hypothesis that hormonal substances contained in the haemolymph of males, and ingested by females during copulation, function to decrease female receptivity to further matings. We tested this hypothesis by providing females, experimentally prevented from wing feeding, with male-derived haemolymph or appropriate control substances (female-derived haemolymph or cricket Ringer's solution), and recording their propensity to mate (in 1999) or remate (in 2000). Female mating propensity was not affected by ingestion of male haemolymph in either experiment. Although these results are inconsistent with the male manipulation hypothesis, it is possible that putative receptivity-inhibiting substances are sequestered in the integument of males' hindwings, rather than contained in male haemolymph per se. Alternatively, both the results of the present study and those of previous studies are consistent with the hypothesis that wing feeding leads simply to satiation of females, and thereby diminishes their motivation to seek out additional matings.

In various insect species, females frequently exert greater control over the reproductive process than males because females often retain direct control over the transfer of sperm and may also be able to selectively recruit sperm of desired sires stored from previous matings (Thornhill & Alcock 1983; Sakaluk 1984; Sakaluk & Eggert 1996; Eberhard 1996). Hence, female mate choice can occur not only in the context of precopulatory decisions, but also during or after copulation via processes that bias the paternity of offspring in favour of particular males. Such preferences have been termed 'cryptic' female choice because they normally remain concealed to casual observation even after the more obvious decision to copulate has been made (Thornhill 1983; Eberhard 1996). As is the case with more overt forms of female choice, cryptic female choice invariably leads to a sexual conflict of interest with males because, while females often benefit by being selective of the potential sires of their offspring, males' reproductive success is contingent upon maximizing the returns from their gametic investment by ensuring that their sperm are used in fertilizations.

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Sexual conflict over the use of sperm for fertilizations has led to the evolution of various male counter-adaptations that function to neutralize female mating preferences, cryptic or otherwise. Such adaptations include structures designed to impose forced copulations on females (Thornhill 1980; Arnqvist 1989; Sakaluk et al. 1995), behaviours such as mate guarding that function to prevent remating by females (Alcock 1994; Johnsen et al. 1998; Jormalainen 1998), and substances in males' ejaculates that reduce female receptivity to subsequent courtship attempts (Eberhard 1996). The use of anti-aphrodisiacs, such as hormones transferred from the male to the female during copulation, has been reported for several insect species (reviewed in Eberhard 1996; Wolfner 1997; Miyatake et al. 1999). Although the emphasis has been on receptivity-inhibiting substances contained in males' ejaculates, this is not the only conduit through which males might attempt to alter the subsequent receptivity of their mates. In various insect species, males provide females with nuptial food gifts at mating, which can take the form of insect prey captured by the male, glandular secretions, or even portions of the male's body (Thornhill 1976; reviewed in Vahed 1998). One of the pervasive features of such gifts is that they afford males direct access to the female's physiology. In a

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recent meta-analysis of female multiple mating in gift-giving insects, Arnqvist & Nilsson (2000) reported that although female fitness increases markedly with increased mating rate, females of most species mate at a lower than optimal rate; this finding led them to suggest that food 'gifts' are in fact 'Medea' gifts, male refractory-inducing substances disguised as nutritional offerings.

The sagebrush cricket, *Cyphoderris strepitans*, is a member of the Haglidae, a relatively obscure family within the ensiferan Orthoptera, now nearly extinct. The species occurs exclusively in mountainous regions of the western U.S.A., where it primarily inhabits high-altitude sagebrush meadows (Morris & Gwynne 1978). In Grand Teton National Park where the present study was conducted, sagebrush crickets have a relatively short breeding season, commencing in early spring and lasting for approximately 4–6 weeks. Male *C. strepitans* offer an unusual nuptial food gift to females during copulation: females feed on the fleshy hindwings of males and ingest haemolymph seeping from the wounds they inflict. Because the wounds are not fatal and only a portion of the hindwing material is consumed in a given copulation, males are not precluded from mating again (Dodson et al. 1983).

In a recent study, Johnson et al. (1999) allowed female *C. strepitans* to mate initially with virgin males that had been subjected to either of two experimental treatments: (1) males' hindwings were left intact, such that copulatory wing feeding was possible; or (2) males' hindwings were surgically removed to prevent copulatory wing feeding. Following this initial copulation, females were given the opportunity to remate with a virgin male with intact hindwings, and latency to remating was recorded as a measure of female sexual receptivity. Females that were experimentally prevented from wing feeding during initial copulations remated significantly sooner than females that were allowed a normal wing meal during their initial mating.

Johnson et al. (1999) interpreted these results as evidence of a passive form of cryptic female choice, in which females that remain unsatiated following copulation seek out additional matings (or more precisely, the additional wing meals that such matings provide) until their intrinsic motivation to feed has been abated. Alternatively, the decreased receptivity of females permitted to feed normally on males during initial matings could be interpreted as evidence of male manipulation, if substances passed in a male's haemolymph contain receptivity-inhibiting substances (Johnson et al. 1999). The objective of this study was to test the hypothesis that chemical substances contained in the haemolymph of males and ingested by females during copulation, function to decrease female receptivity to subsequent copulations and thereby protect the paternity of investing males.

We tested this hypothesis by providing females, experimentally prevented from wing feeding, with male-derived haemolymph or appropriate control substances (female-derived haemolymph or cricket Ringer's solution), and recording their propensity to mate (year 1 of study) or remate following an initial mating (year 2 of study). The 'male manipulation' hypothesis predicts that ingestion of adult male haemolymph should increase female latency to subsequent copulation relative to that of females that ingest either the haemolymph of adult females or cricket Ringer's solution.

METHODS

The study was conducted over two breeding seasons at the University of Wyoming–National Park Service Research Center, where previous studies of this species have been conducted (Snedden & Sakaluk 1992; Eggert & Sakaluk 1994; Sakaluk et al. 1995). Early in the breeding season (mid-May 1999 and 2000, respectively), adult males and adult females were collected from several populations within Grand Teton National Park and transported to the field station. Experimental subjects were maintained at the field station according to standard procedures (Snedden & Sakaluk 1992; Eggert & Sakaluk 1994). All males used in the experiment were virgins, as indicated by intact hindwings. The mating status of experimental females was unknown, but as most were captured early in the breeding season orienting to calling males, the majority were presumed virgin. Females found with a spermatophore attached, indicative of recent mating, were excluded from experimental trials.

Summer 1999

Crickets were maintained in the laboratory on a diet of fresh, moistened sagebrush clippings offered daily. On the evening following their capture, we randomly assigned females to one of three experimental treatments in which they were permitted to consume 20 µl of one of the following substances: (1) haemolymph derived from an adult male (N=16), (2) haemolymph derived from an adult female (N=16), or (3) cricket Ringer's solution (N=17). Aliquots of the respective treatment substances were presented on small portions of iceberg lettuce, and the time at which females consumed the experimental substances was recorded using time-lapse video photography.

We extracted haemolymph from males by severing one of the hindwings and drawing haemolymph from the wound using a microhaematocrit capillary tube. Extraction of haemolymph from adult females involved making a small incision in the cuticle of the pronotum and then, using the same technique, suctioning haemolymph from the wound once it began to bleed. Wounds caused by these procedures normally healed very rapidly and the insects fully recovered from their operations. None of the males used to obtain haemolymph were used in mating trials.

Twenty-four hours after the treatments had been established, mating trials were staged in which females were each randomly paired with a virgin male. Experimental pairs were placed into specially constructed Plexiglas viewing chambers divided into two equal compartments ($10 \times 6.8 \times 4.4$ cm). Each compartment was equipped with a calling perch in the form of a short stick. Pairs were established early in the evening when the crickets normally become sexually active, and mating behaviours

were monitored over the next 12 h using time-lapse video recording. No food or water was provided during mating trials.

Upon review of the video recordings, we recorded the following measures of female receptivity relative to the start of the trial: (1) total number of occurrences in which males were mounted by females and (2) time at which successful copulation occurred as indicated by the transfer of the spermatophore.

Summer 2000

Animals were maintained in the laboratory on a diet of fresh, moistened sagebrush, supplemented with leaf galls formed by a parasitic wasp in sagebrush foliage and upon which crickets have frequently been observed to feed in nature (S. K. Sakaluk, personal observation). Twenty-four hours after their initial capture, we randomly paired experimental females with virgin males whose hindwings had been surgically removed to prevent wing feeding by females. Hindwings of males were removed by clipping the wings at their point of insertion, resulting in minimal bleeding. Although wounds from this procedure heal very rapidly, surgically altered males were allowed 24 h to recover prior to use in mating trials (Eggert & Sakaluk 1994; Johnson et al. 1999).

Initial mating trials were staged in small, glass viewing jars equipped with a short stick as a calling perch for males. We established pairs early in the evening when the crickets normally become sexually active and monitored mating behaviours over the next 6 h via direct observation. Females that did not mate successfully during the first night were allowed the opportunity to mate the next night, and every night thereafter, until successful initial copulation had occurred. No food or water was provided during initial mating trials.

Immediately following successful copulation with the wingless male, we established experimental treatments in which females were allowed to consume 20 µl of one of the following substances: (1) haemolymph obtained from an adult male (N=8), (2) haemolymph obtained from an adult female (N=8), or (3) cricket Ringer's solution (N=8). All treatment substances were pipetted onto a small piece of puffed rice, which readily absorbed the liquid, and presented to females on the tip of a dissecting probe. Females readily consumed food items presented to them in this manner. Haemolymph extractions were performed following the procedures established in the summer of 1999.

Immediately following the ingestion of their prescribed substances, we assigned females to subsequent mating trials with virgin males whose hindwings had been left intact. Experimental pairs were placed into Plexiglas viewing chambers equipped with a short stick as a calling perch and monitored via time-lapse video recording until dawn, at which time the crickets cease to be active. Females that had not mated successfully by dawn were paired with another virgin male the following evening for 12 h, and every evening thereafter, until successful copulation occurred or the study had ended. As before, no food or water was provided during mating trials.

Upon review of the video recordings of subsequent mating trials, we recorded the following measures of female remating propensity relative to the start of the trial: (1) total number of occurrences in which males were mounted by females and (2) time at which successful copulation occurred as indicated by transfer of the spermatophore.

Data Analysis

Despite attempts at transformation, the data obtained for the number of female mounts in the summer of 1999 failed to meet the assumptions of parametric analysis of variance (ANOVA). We therefore used a Kruskal–Wallis test to compare the total number of mountings across treatments. The data for the same measure obtained in the summer of 2000 did meet parametric assumptions and ANOVA was used to compare number of female mounts across treatments. Nonparametric failure-time analysis was used in comparisons of latency to mating for both breeding seasons. Failure-time analysis provides a method for accommodating censored data, observations in which an event may not have occurred before the end of the study (Fox 1993). Data such as these are often incorrectly omitted from subsequent analyses, which can lead to highly biased statistical comparisons (Fox 1993).

In the summer of 1999, we used a regression analysis to test for a relationship between the time at which females consumed treatment substances and their latency to subsequent mating. If the efficacy of any putative antiaphrodisiac substances in the haemolymph of males decreases over time, latency to mating should covary positively with the time of treatment ingestion. Alternatively, if such substances begin to degrade upon exposure to air, there should be a negative relationship between time of ingestion and latency to mating. Any relationship between treatment ingestion and subsequent copulation was controlled for in the summer of 2000 by feeding females the prescribed treatment immediately after initial copulation and immediately prior to subsequent mating trials.

RESULTS

Summer 1999

There were no significant differences in female sexual receptivity across treatments. Median latency to successful copulation was not significantly different across treatments (failure-time analysis: χ^2_2=0.27, P=0.87; Fig. 1). Only one female in the experiment (female haemolymph treatment) failed to mate before the end of the study. Total number of female mounts also did not differ significantly across treatments (Kruskal–Wallis test: H_2=2.73, P=0.26; Fig. 2). For all treatments, latency to mating was not significantly related to the time at which treatment substances were consumed (linear regression: female haemolymph: R^2=0.01, $F_{1,14}$=0.14, NS; male haemolymph:

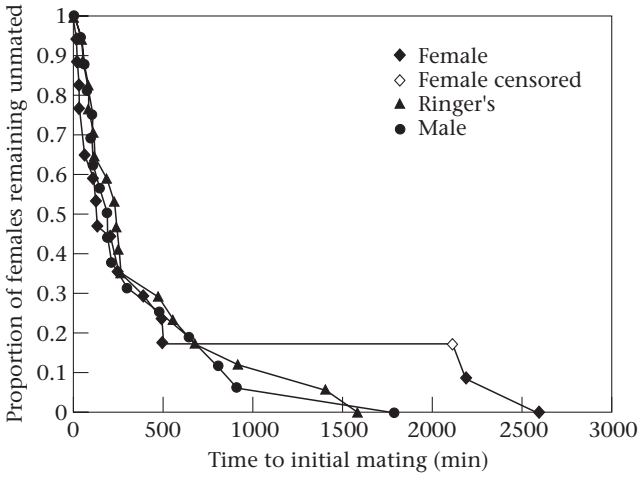

Figure 1. Survival distribution of latency to initial copulation for female *C. strepitans* permitted to consume various treatment substances prior to mating. There was no significant difference in latency to mating across treatments.

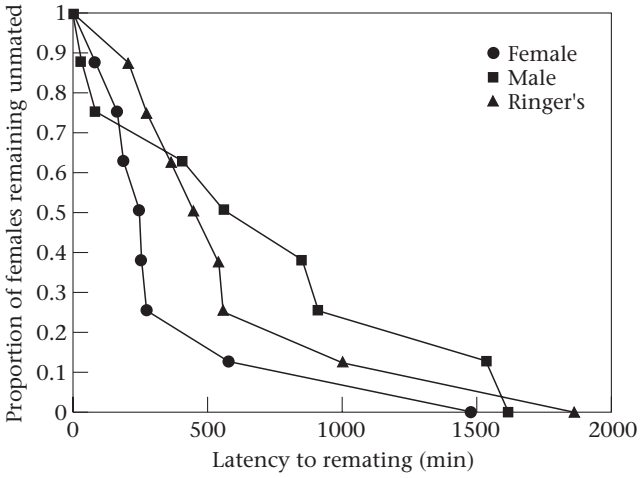

Figure 3. Survival distribution of latency to remating for female *C. strepitans* following initial copulations with de-winged males. There was no significant difference in latency to remating across treatments.

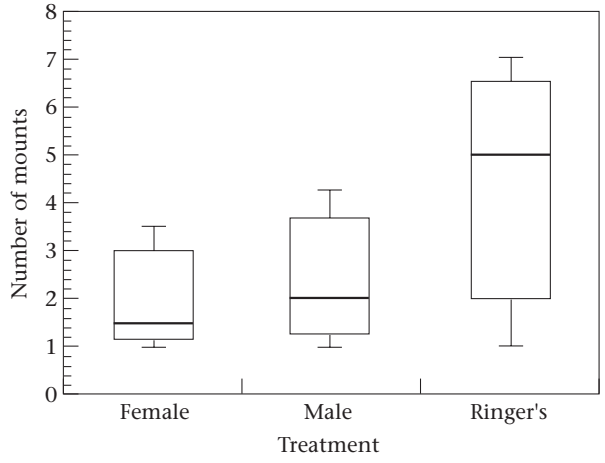

Figure 2. Number of times females mounted males in each treatment. The lower and upper boundaries of the boxes represent first and second interquartile ranges, respectively. The median is represented by the horizontal bisecting a box. The lower and upper bars represent the 10th and 90th percentiles, respectively. There was no significant difference in the number of female mounts across treatments.

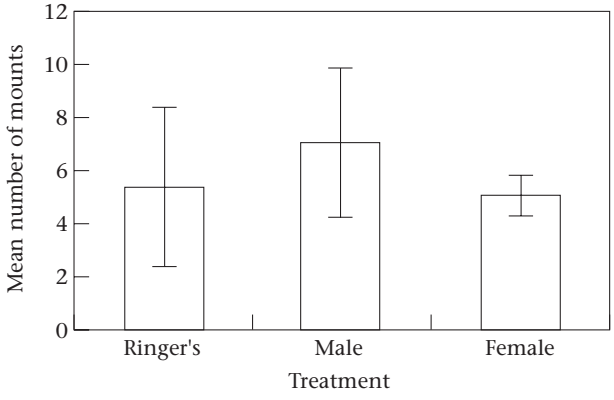

Figure 4. Mean number of female mounts (±SE) following initial copulations with de-winged males. There was no significant difference in mean number of female mounts across treatments.

R^2=0.16, $F_{1,14}$=2.69, NS; Ringer's solution: R^2=0.11, $F_{1,15}$=1.8, NS).

Summer 2000

There were no significant differences in female remating propensity across treatments. Females that ingested male haemolymph had a slightly higher median latency to remating than females that received Ringer's solution and those that received female haemolymph (Fig. 3), but these differences were not statistically significant (failure-time analysis: χ^2_2=2.68, P=0.262). All females successfully copulated in remating trials prior to the end of the study. Mean number of female mounts for each treatment did not differ significantly across treatments (ANOVA: $F_{2,21}$=0.19, P=0.825; Fig. 4).

DISCUSSION

Female propensity to mate was not influenced by ingestion of male haemolymph prior to mating, a result that is inconsistent with the male manipulation hypothesis. While we do not know precisely how much haemolymph females consume at mating, it is probably much less than the prescribed treatment volume, so that the lack of any treatment effect cannot be attributed to insufficient haemolymph ingestion prior to copulation. Also, although we cannot be certain that all of the females used for haemolymph extractions were virgins, the Ringer's solution treatment controls for the possibility that some of the female haemolymph may have contained some unknown quantity of male-derived antiaphrodisiac.

It is also possible that putative hormonal substances contained in male haemolymph degraded over the time

between presentation of treatment substances and their subsequent ingestion. However, there was no significant relationship between the time at which male haemolymph was consumed and female latency to mating. It is still conceivable, however, that male-derived hormonal substances influence female receptivity only in the short term, in which case their efficacy may have dissipated in the 24 h between female consumption of male haemolymph and experimental pairings.

Alternatively, it may be that any effect of male haemolymph on the subsequent receptivity of females must first be primed by copulation, or by the receipt of sperm or other substances in males' ejaculates. In the study by Johnson et al. (1999), females were first mated to a de-winged male before they were given the opportunity to mate with an unmanipulated male, whereas in our 1999 experiment, most of the experimental females were presumably virgin. It was to assess this very possibility that treatment regimes were established simultaneously with initial copulations in our 2000 experiment. However, just as in the 1999 experiment, female receptivity to remating was not affected by ingestion of male haemolymph immediately after copulation with a de-winged male. These results are also inconsistent with the predictions of the male manipulation hypothesis.

One explanation for the discrepancy between the results of the present study and those of Johnson et al. (1999) is that receptivity-inhibiting hormonal substances may be sequestered in the integument of males' hindwings, rather than contained in male haemolymph. In the Johnson et al. (1999) study, females that were permitted to feed on males undoubtedly ingested hindwing material along with the haemolymph and would, therefore, have consumed any antiaphrodisiacs contained in the wings. There is some indirect support for this possibility. In the present study, females of all three treatments remated within a time period (pooled median=7.13 h; Fig. 3) comparable to those of females in the Johnson et al. (1999) study that were prevented from wing feeding during their initial copulation (median=6 h). In contrast, females in the Johnson et al. (1999) study that were permitted to feed on males' wings at their initial mating took much longer to remate (median=17 h). While the results of the two studies cannot validly be compared statistically, having been conducted in different breeding seasons and with females maintained on slightly different diets, the difference is, none the less, suggestive of an effect of consumption of hindwing material on female remating propensity.

Both the results of the present study and those of Johnston et al. (1999) remain consistent with the female satiation hypothesis, namely that wing feeding leads to satiation of females, and thereby diminishes their motivation to seek out additional matings. While females in the Johnston et al. (1999) study either received a wing meal or no wing meal at their initial mating, we were hesitant to add a treatment in which females received nothing at all (i.e. a negative control) due to constraints of sample size and statistical power. However, Johnson et al.'s (1999) study suggests that had we established a negative control, these females would have remated sooner than females of the other three treatments due to the absence of any feeding treatment and the corresponding lack of satiation. Indeed, Johnston et al. (1999) demonstrated that female *C. strepitans* maintained on a low-nutrient diet mounted males in mating trials significantly sooner than females maintained on a high-nutrient diet. This seems to indicate that underfed, or unsatiated females are more motivated to seek out matings to gain the wing meal offered by males during copulation.

Any effects of copulatory wing feeding on female reproductive success or offspring survival have yet to be demonstrated in *C. strepitans* because of the inherent difficulty in tracking females and their offspring in the field; all attempts at rearing *C. strepitans* in the laboratory environment to date have failed. However, the evidence obtained thus far indicates that in this species variation in the capability of males to feed females may have favoured females that seek out additional matings when receiving inadequate nourishment in previous matings. Such a passive mechanism of mate choice would be amplified under conditions of nutrient limitation. Variation in male investment might be expected to arise as males continue to secure copulations over the course of the breeding season and are gradually depleted of wing material and energy reserves (Sakaluk et al. 1987; Sakaluk & Snedden 1990; Snedden 1996). Indeed, the results of the present study and those of Johnson et al. (1999) suggest that it is the nutritional contribution that males make at mating, rather than male manipulation via substances in the haemolymph, that influences the remating propensity of females.

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