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Should he stay or should he go: male influence on offspring sex ratio via postcopulatory attendance

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Abstract In species without nuptial gifts or parental care, postcopulatory attendance of females by males has generally been interpreted as males guarding against sperm competition. Guarding benefits may be concurrent with attendance (the guarding now hypothesis) or male behavior during attendance may make the female unreceptive (the guarding in absentia hypothesis). However, in addition to guarding functions, attendance may provide the male with an opportunity to influence the female's use of sperm. In haplodiploids such as hymenopterans, doing so may be beneficial because only daughters and not sons are produced sexually and so influence male reproductive success (the sex ratio hypothesis). In the parasitoid wasp *Urolepis rufipes*, postcopulatory attendance involved the male remaining mounted after copulation and resuming courtship. Support for the guarding now hypothesis was limited. A male's presence on a female did not reduce the probability, or quickness, of another male mounting, and second-mounted males frequently copulated. The guarding in absentia hypothesis was not supported. Females became unreceptive soon after mating even when copulation and postcopulatory attendance were experimentally prevented. The sex ratio hypothesis was supported. Postcopulatory attendance caused females to produce more daughters. They also produced more total offspring. Thus, a male should stay and should not go even in the absence of other males, at least when opportunities for other matings are absent as in the present study. Although most studies of offspring sex ratios have focused on maternal control, this study provides an example of apparently adaptive male influence on sex ratio.

Key words Haplodiploidy; Mate guarding; Parasitoid wasp; Postcopulatory courtship; Receptivity; Sex ratio; Sexual conflict

Introduction

In some animals, males continue to stay near, or even mounted on, their mate even after copulation is complete, and not just to provide paternal care (Eberhard 1996; Arnqvist and Rowe 2005). This is often called mate guarding because it has generally been interpreted as males guarding against sperm competition and protecting females from harassment, e.g., from unbeneficial suitors (Alcock 1994; Rodriguez-Munoz 2011). However, males staying on or near their mate after copulation may or may not

be effective for these functions and may serve other functions as well, so here we instead use a term that does not imply function, “postcopulatory attendance”. Guarding benefits from postcopulatory attendance may be concurrent with attendance, e.g., the male’s presence physically interferes with concurrent mating attempts by other males (here referred to as the guarding-now hypothesis). Alternatively, under the guarding in absentia hypothesis, the male’s behavior during attendance may cause the female to become less attractive or receptive to males that she encounters after the first male is gone (King and Fischer 2005; Ablard et al. 2011).

Even if guarding benefits are absent or incomplete, postcopulatory attendance may still be beneficial against male-male competition (Alcock 1994). During attendance, males may provide stimuli that facilitate the female's immediate use of sperm and her production of more or bigger current offspring. This may provide a fitness advantage to the male by causing the female to allocate resources to his offspring before she remates and some of her offspring are no longer his.

In haplodiploid organisms, postcopulatory attendance may also be beneficial if it provides an opportunity for the male to influence the female’s sex ratio (Kajita 1986), which we refer to here as the “sex ratio hypothesis for postcopulatory attendance”. In haplodiploids, haploid offspring are sons and have only mothers. Daughters are diploid, having developed from fertilized eggs. Thus male reproductive success is strictly through daughters. In contrast, a female’s reproductive success includes both sons and daughters. However, her fitness is affected not only by the total number of offspring, but also by the particular sex ratio that she produces and her ability to adjust that sex ratio with environmental conditions (Charnov 1982; Steiner and Ruther 2009; West 2009). Thus haplodiploidy provides an opportunity for sexual conflict (Hawkes 1992; Shuker et al. 2009). There have been few tests of the sex ratio hypothesis for postcopulatory attendance specifically (see Discussion). More generally, male influence on offspring sex ratio (Shuker et al. 2009) and the possibility of sexual conflicts between mates over sex ratio have “gained negligible empirical attention” (West 2009). Even for vertebrates, “both empirical and theoretical work has traditionally concentrated on the female and dismissed any potential paternal contribution as minor and irrelevant” (Saragusty et al. 2012).

The guarding hypotheses and the sex ratio hypothesis for postcopulatory attendance were examined here, using the parasitoid wasp *Urolepis rufipes* (Pteromalidae). This 2-3 mm long wasp develops within the pupa of certain flies that live along shore lines or in and on decaying organic matter (Rueda and Axtell 1985). Generally, only one wasp develops per host, but hosts can be highly clumped. Sex ratios are female-biased, and adult males emerge from hosts first and are smaller than females (Powell et al. 2003).

Male and female *U. rufipes* noticeably respond to each other only once they are within a couple of wasp lengths of each other (Cooper 2010). Upon reaching the female, the male mounts her dorsally (Fig. 1). Her body becomes still, and she bats his face with her antennae, and he extends his antennae and mouthparts, presumably releasing a pheromone as in confamilials (van den Assem et al. 1980; van den Assem 1986). If she opens her genital orifice, i.e., is receptive, he backs up and they copulate. After copulation is postcopulatory attendance, during which the male stays mounted but not in copula, the precopulatory-like interactions with antennae and mouth resume, and her genital orifice begins to close. Postcopulatory attendance lasts about a minute (Cooper 2010). The male eventually dismounts and leaves, although cues from the female for doing so are not apparent. Males are polygynous, and females appear to be monandrous under many conditions (Cooper 2010; Kuban 2012).

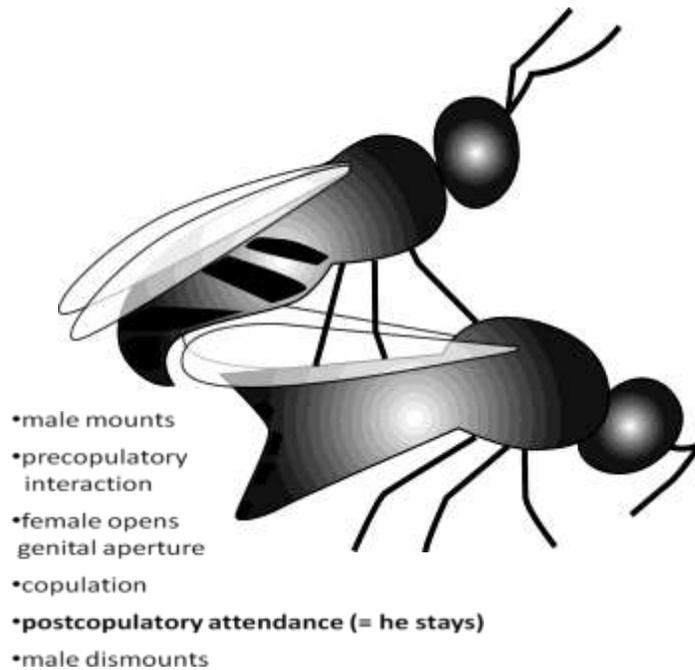


Fig. 1 Mating behavior sequence in *Urolepis rufipes*

In the present study, a male's attendance of a female was experimentally prevented or allowed in order to test the function of the attendance. The guarding now hypothesis was addressed by asking whether attended (mounted) females are less readily mounted by other males and whether males that mount an already mounted female then copulate. The guarding in absentia hypothesis was addressed by asking whether females that are prevented from receiving postcopulatory attendance remain attractive and receptive to subsequently encountered males. Finally, the sex ratio hypothesis was addressed by asking whether postcopulatory attendance increases daughter production.

Methods

The *U. rufipes* was a strain originally collected from cattle feedlots in southern Alberta (Floate personal communication). Wasps were reared at approximately 25°C with a photoperiod of 12 h light: 12 h dark. *Musca domestica* pupae were used as hosts and were reared following Nichols et al. (2010). Parasitized host pupae were individually isolated in glass test tubes prior to the wasps' emergence in order to obtain virgin wasps. Wasps were 1 d old or less at testing. Females and males were assigned to treatments at random. Mated wasps were produced by placing a virgin male and a virgin female into a test tube and watching for copulation. Wasps were never tested with a wasp with which they had previously mated.

Behavioral tests were performed in sand dishes, except where noted otherwise. The dish (5.7 cm diameter, 1 cm height) was half full of damp sand to keep humidity high, which reduces static. Clean containers and sand were used for each test. Durations of behaviors were determined with a stopwatch.

Statistical tests were two-tailed. Nonparametric tests were employed when the assumptions of normality were not met. Tests of independence of categorical data were by G tests, which are also called likelihood ratio chi square tests.

Guarding now hypothesis

This hypothesis predicts that 1) attended (mounted) females will not be as readily mounted by other males or 2) being attended will interfere with a second male that mounts being able to copulate. These

predictions were tested in two separate experiments.

Quickness to mount an unattended versus attended female. This experiment examined whether a male being mounted on a female deters other males from mounting. The two treatments were attended ($n=15$) and unattended females ($n=17$). The female was dead so this experiment examines male behavior in the absence of female behavioral responses. She had been killed by freezing and was presented dorsal side up. Males readily attempt to mate with such females and seem to stay mounted for longer than with live females (personal observation; van den Assem and Jachmann 1982). The dead female was placed near the edge in a small dish of damp sand. A virgin male was introduced at the opposite edge. Duration until the male mounted was recorded for up to 10 min. In the attended treatment, the female had just been mounted by a virgin male prior to the “intruding” test male’s introduction to the dish.

Duration until mounting was compared between treatment and control by survival analysis, specifically Cox’s regression, with males that failed to mount within 10 min as uncensored events. Survival analysis accounts for the possibility that if allowed a longer testing period, some males that did not mount within the 10 min test period eventually might (review of survival analysis in van Alphen et al. 2003).

Second mounting male’s ability to copulate. This experiment examined whether the presence of the first mounting male prevents the second mounting male from copulating with the female. A virgin female was placed into a glass test tube (1.2 cm diameter, 7.5 cm length) containing two virgin males, and they were observed for 10 min ($n=37$). Two responses were recorded: which male first copulated with the female and whether the other male copulated with the female before she closed her genital orifice.

This experiment was only intended to address whether second males are completely prevented from copulating by another male’s postcopulatory attendance, not whether the attendance makes the second male merely less likely to copulate. The reason is that the second male, by definition, was less eager/quick to mate than the first male. Thus fewer copulations by second males than by first males could have resulted from either this reduced eagerness or from the first male’s attendance on the female.

Guarding in absentia hypothesis

This hypothesis predicts that postcopulatory attendance will make females unreceptive. These two experiments tested the role of copulation and postcopulatory attendance in turning off female receptivity by examining the receptivity of females that had been mounted and received precopulatory courtship but whose mate had been removed experimentally prior to copulation.

Interruption prior to copulation. Preliminary observations suggested that having simply opened her genital orifice for copulation might be sufficient to turn off a female’s receptivity. Thus, in this experiment, we compared the response of each test male to either an: 1) I=interrupted female, which had been courted to the point of opening but had not copulated or received postcopulatory attendance ($n=21$), 2) M=fully mated female ($n=21$) or 3) V=virgin female ($n=21$). If opening and closing is sufficient even in the absence of copulation and postcopulatory attendance, interrupted females should be as resistant to remating as mated females, and both should be more resistant than virgin females. In the interruption treatment, as soon as the female opened her genital aperture and the male began to back up to copulate, he was removed with clean metal forceps before he could copulate. In the fully mated treatment, the male was removed immediately upon dismounting to control for any disruption caused by the removal of the male in the interruption treatment.

Females were tested for receptivity immediately after treatment. The test male was virgin. He was tapped into a clean dish, followed by a female from one of the treatments. The duration until mounting was recorded. All males mounted within 10 min. After he mounted, the following observations were recorded: whether the female opened her genital aperture, whether copulation occurred, the duration from

mounting until copulation or dismount (whichever came first), the duration of copulation and the duration of postcopulatory attendance. In performing this experiment, we soon realized that interrupted females varied in whether their genital orifices had closed by the time that the test male mounted. Thus, whether it would be possible for the test male to simply mount and copulate without providing courtship varied. (If she was already open, courtship by him was unnecessary for copulation.) Virgin females and fully mated females were never observed to be open when the test male mounted.

To improve normality, the duration until mounting and the duration of the mount were log transformed prior to statistical analyses. Whether more interrupted females were receptive than either virgin females or mated females were each examined with a likelihood ratio test of independence. These analyses were first done for all females and then for just closed females (i.e., females that were known to be closed by the time that the test male mounted).

Interruption plus delay. In this experiment, interrupted-treatment females ($n=12$) were not presented with a test male until 3 min after they became receptive for the first male, by which time all females were completely closed. Fully mated females ($n=12$) were also tested for comparison.

Sex ratio hypothesis

This hypothesis predicts that postcopulatory attendance will increase daughter production. This experiment tested that by experimentally manipulating whether females received postcopulatory attendance, and then testing their subsequent production of daughters. There were two treatments, females that received a complete mating ($n=12$) and females whose mate had been removed immediately after copulation in order to prevent postcopulatory attendance ($n=12$).

About 10 min after mating, each female was given 30 hosts per day for 10 d. The hosts were presented in glass vials (7 cm high, 2 cm diameter) plugged with cotton, with a drop of honey on the side as food. Ten days was considered sufficient because a previous study found that females produce 88% of their offspring within their first 6 d (Matthews and Petersen 1990). Hosts were 3 d old or less, and the age of hosts that females received within a given replicate (a replicate being one of each treatment) was matched to the nearest day. Adult offspring were allowed to complete development and emerge and then were counted. Number of daughters provides an estimate of male fitness, and total number of offspring provides an estimate of female fitness. Both were compared between treatments by t-tests. Assumptions of normality were met at $P > 0.05$; in addition, t-tests are very robust to deviations from normality, particularly when sample sizes are equal (Zar 1999), as they were.

Results

Guarding now hypothesis

Quickness to mount an unattended versus attended female. Only one of the 15 attending males dismounted before the intruding male had mounted; that attending male dismounted to chase away the intruder; that female was still considered attended. A female's being attended did not reduce how quickly a second male mounted her (Cox's regression: $\beta=0.13\pm 0.41$; $\chi^2=0.10$, $P=0.76$; 71% of unattended females and 87% of attended females were mounted within 10 min: $G_1=1.24$, $P=0.27$).

Second mounting male's ability to copulate. Second males often copulated. In 29 of the 37 replicates, both males had the opportunity to copulate with the female. Both males mounted the female; and at least one male copulated, indicating that she was receptive. Among these 29 replicates, usually either both males copulated or the male that first mounted the female was the only male to copulate (Fig. 2). There

were only two instances in which the male that mounted the female second was the only male to copulate with the female.

Despite being aggressive toward each other prior to mounting, during postcopulatory attendance, males did not defend their mate from another male mounting. This may explain why postcopulatory attendance was ineffective at preventing another male from mounting and sometimes even copulating.

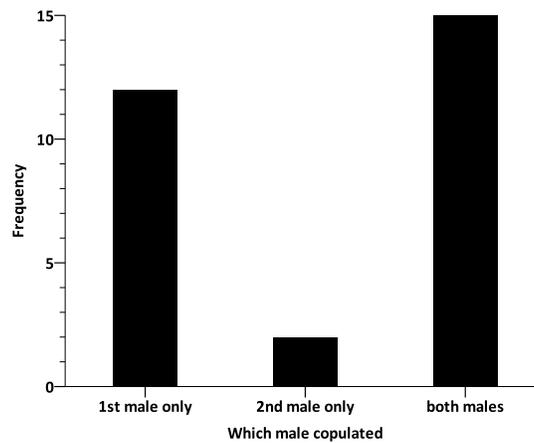


Fig. 2 Frequency of second-to-mount males that copulated with a virgin female when both males had mounted the female (n=37)

Guarding in absentia hypothesis

Copulation and the postcopulatory attendance that follows were not necessary for a reduction in female receptivity. Instead, these two interruption experiments suggest that in order for her to become as unreceptive as a mated female, some aspect of precopulatory courtship, and perhaps the opening in response to this courtship, needs to happen and then some amount of time needs to pass. She normally closes as this time passes.

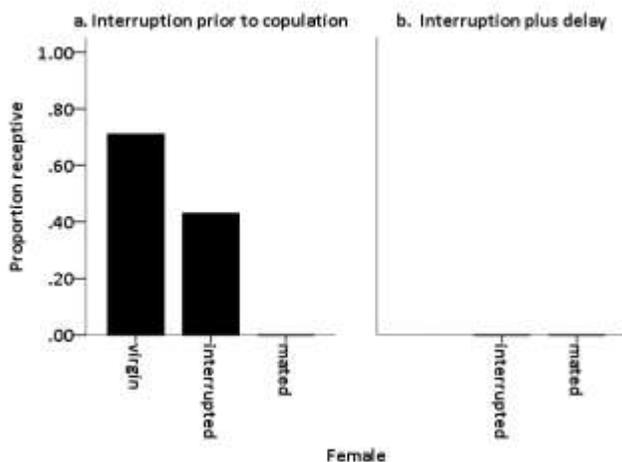


Fig. 3 Proportion of females that were receptive in a) the Interruption-prior-to-copulation experiment (where whether an interrupted female had closed her opened genital orifice was unknown) and b) the Interruption-plus-delay experiment (where testing was 3 min after interruption by which time all females had closed their genital orifice)

Interruption prior to copulation. Interrupted females tended to be less receptive than virgin females

(Fig. 3a; $G_1=3.56$, $P=0.059$), and the difference became significant when the analysis was restricted to interrupted females that we knew to be closed ($G_1=6.85$, $P=0.009$). Even fewer mated females than interrupted females were receptive (Fig. 3a; $G_1=14.96$, $P<0.001$), even when the analysis was restricted to females known to be closed ($G_1=4.85$, $P=0.028$).

Besides addressing the guarding in absentia hypothesis, this experiment also provided information on other aspects of male response to receptivity. When females were receptive, copulation always followed. However, there was no evidence that males recognized female receptivity prior to mounting. Test males did not mount virgin females sooner than interrupted females or mated females ($F_{2,59}=0.36$, $P=0.70$). Likewise, when males mounted virgin females, they did so just as quickly in replicates where the female subsequently became receptive as in replicates where the female did not become receptive ($t_{19}=0.96$, $P=0.35$).

Copulation quickness and duration were not affected by treatment among copulating pairs. That is, among females that copulated with the test male, interrupted females did so as quickly after being mounted as virgin females (Mann-Whitney $U=58.0$, $n=8, 15$, $P=0.93$), and copulated for equally long ($t_{22}=0.09$, $P=0.93$). (Fully mounted until copulation took 6-53 s, $n=23$, median of 9 s; and copulation lasted 12-32 s, $n=24$, mean \pm SE duration of 22 ± 1). However, after copulation, males remained mounted almost twice as long on virgins as on interrupted females; i.e., postcopulatory courtship was 60 ± 5 s, $n=14$, 25-84 versus 37 ± 4 s, $n=9$, 25-53 ($t_{21}=3.35$, $P=0.003$).

Giving up times on unreceptive females did not differ depending on female mating status. Specifically, a male invested an average of 49 ± 9 s, 10-147 s, $n=21$ total time mounted on a mated female (such females are always unreceptive), which was not significantly different from what he invested on an unreceptive virgin female ($t_{25}=0.36$, $P=0.72$).

On unreceptive females, males did not give up and dismount right when a receptive female would typically become receptive. Specifically, the 49 s spent mounted on mated (unreceptive) females was about four times as long as time mounted prior to copulation with a receptive virgin (12 ± 2 , 6-37 s, $n=15$; Mann-Whitney $U=23.0$, $P<0.001$). Nevertheless, the 49 s mounted on an unreceptive female was still significantly less than the 94 ± 5 , 55 -117 s, $n=14$ that it took to complete a mating on a receptive virgin female ($t_{33}=3.85$, $P<0.001$).

Interruption plus delay. If the stimuli that females receive from copulation and postcopulatory attendance contribute to their ongoing postmating loss of receptivity, interrupted females would have been more receptive than mated females, but they were not. There was no difference between the interruption treatment and the mated treatment in terms of receptivity or the percent of males that mounted; males fanned their wings at all females and mounted all females, yet no females became receptive (Fig. 3b).

Despite the lack of female receptivity, about half of all males attempted copulation (54%, $n=24$). While mounted, the female still hit the male in the face with her antennae, as is seen in subsequently successful matings.

Sex ratio hypothesis

Postcopulatory attendance increased a female's 10 d production of daughters and her total number of offspring but not her production of sons (Table 1; Fig. 4). During uninterrupted (normal) matings, by the time the male dismounted, females ranged from barely beginning to close their genital aperture to it being completely closed.

Table 1. Mean \pm SE (minimum – maximum) number of sons, daughters, and total offspring produced by fully mated females ($n=12$) and by interrupted females (no postcopulatory attendance) ($n=12$)

Mothers:	Sons	Daughters	Total Offspring
Fully mated	18.50 ± 2.16 (8 - 28)	64.75 ± 5.52 (35 - 98)	83.25 ± 5.84 (58 - 123)
Interrupted	18.33 ± 2.22 (9 - 32)	48.25 ± 5.12 (11 - 69)	66.58 ± 5.46 (25 - 96)
	$t_{22}=0.054$ $P=0.96$	$t_{22}=2.19$ $P=0.039$	$t_{22}=2.08$ $P=0.049$

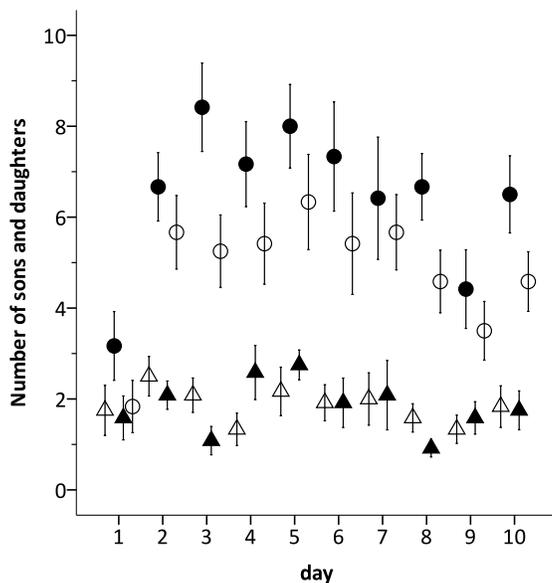


Fig. 4 Postcopulatory attendance (solid symbols: completed; empty symbols: prevented) increased a female's 10 d production of daughters (circles, $P=0.04$), but not sons (triangles, $P=0.96$)

Discussion

Under the conditions tested here, *U. rufipes* males should stay and should not go after copulation. Staying appears to be beneficial through increased daughter production. Thus, the present study adds to a limited number of examples of males influencing offspring sex ratio, particularly in an apparently adaptive manner (Shuker et al. 2009; Saragusty et al. 2012). In contrast, support for the guarding now hypothesis was limited. Males readily mounted females that were already mounted, and second males were frequently able to copulate with females despite attendance. The guarding in absentia hypothesis was not supported because females became unreceptive sometime within 3 min after closing their genital orifice even when they had not received either copulation or postcopulatory attendance. Longer term effects on receptivity cannot be completely ruled out; and if females become receptive later in life, attendance might influence sperm precedence. However, postmating loss of receptivity is not regained later in life in two confamilials of *U. rufipes*, *Lariophagus distinguendus* (Steiner et al. 2008) and *Spalangia endius* (King and Bressac 2010; King and Fischer 2010).

The sex ratio hypothesis was supported for *U. rufipes* in that allowing postcopulatory attendance resulted in more daughters. Theoretical discussions of male influence on offspring sex ratio in haplodiploids are often framed in terms of genomic conflict, specifically sexual conflict

(Hawkes 1992; Kraaijeveld 2009; Shuker et al. 2009). However, like males, females also had greater reproductive success when postcopulatory attendance was allowed; daughter production increased without a concurrent reduction in son production. Thus if long term fitness effects parallel the effects on reproductive success that were seen in the present study, there is not currently sexual conflict. However, many factors influence sex ratio selection, making long term, i.e., multigenerational, fitness effects difficult to estimate (Charnov 1982; West 2009). One factor known to reduce sexual conflict over sex ratio is LMC (local mate competition) (Shuker et al. 2009; West 2009), and partial LMC almost certainly applies to *U. rufipes* given what is known about other parasitoid wasps (e.g., Hardy 1994; Burton-Chellew et al. 2008; Grillenberger et al. 2008; Somjee et al. 2011).

Several questions remain to be addressed in *U. rufipes* in relation to postcopulatory attendance. Which aspects of attendance cause increased daughter production? What are the opportunity costs? The present study focused on benefits of staying, but staying means not doing other things, lost opportunities for males to search for females and for females to search for hosts. Is attendance shortened when opportunity costs are greater, as when additional females and hosts are present? The duration of postcopulatory attendance is clearly not fixed in *U. rufipes*, as evidenced by it being almost twice as long on virgin females as on interrupted females. How the duration of postcopulatory attendance is affected by the presence of other males (Bretman et al. 2011) also remains to be seen. Do *U. rufipes* males dismount sooner when another male mounts, allowing the second male's courtship to replace his own attendance (Field and Keller 1993)? The sex ratio benefit of staying could explain why males are not usually aggressive to other males once mounted, whereas males are aggressive in other situations. Finally, what stimuli cause males to stop postcopulatory attendance, i.e., to dismount? In the confamilial *S. endius*, as well as in a biting midge and a bean weevil, females use their hind legs to brush the male off their back and somewhat effectively (Linley and Mook 1975; Crudgington and Siva-Jothy 2000; King 2010), but brush offs were not observed in *U. rufipes*.

As noted earlier, in contrast to the guarding hypotheses, the sex ratio hypothesis for postcopulatory attendance has been tested in relatively few species. The sex ratio hypothesis is supported in two aphelinid parasitoids (Kajita 1986). In these parasitoids, if males are prevented from providing postcopulatory courtship, a greater proportion of their mates fail to oviposit daughters (80% or 90% of females versus 30%). How the female's reproductive success was affected was not presented. In contrast, the sex ratio hypothesis is not supported in two other parasitoid wasps, the confamilial *S. endius* (King and Bressac 2010) and the encyrtid *Ooencyrtus kuvanae* (Ablard et al. 2011). Also in contrast to *U. rufipes*, in both *S. endius* and *O. kuvanae*, the guarding in absentia hypothesis is supported.

That male – female conflicts over sex ratio have received relatively little attention among parasitic hymenopterans (Shuker et al. 2009; West 2009) contrasts with the large attention given to other genomic conflicts over sex ratio in the last few decades (e.g., Shuker et al. 2009; Wild and West 2009; Ross et al. 2010). The limited attention to male influence may be because of early and frequent studies on the influence of mothers (e.g., Flanders 1939, 1946, 1956). The present study adds to the few studies that suggest that males can also influence sex ratio even among virgin males (Kajita 1986; Henter 2004; Shuker et al. 2006, 2007). We specify virgin males to differentiate from cases where multiply mated males have simply run out of sperm. Such sperm depletion has been viewed as a constraint, rather than as an adaptation. Another mechanism by which males might influence their mate's sex ratio is with seminal fluid proteins, but whether they do so remains to be seen (Shuker et al. 2009). The present study instead indicates a postcopulatory mechanism. How postcopulatory attendance affects the female physiologically is unknown for *U. rufipes*. However, the mechanism may be similar to one of the mechanisms seen in cryptic (postcopulatory) female choice, where the issue is which of multiple mates' sperm is used (Eberhard 1996; Fedina 2007). For example, perhaps attendance increases daughter production by increasing the female's uptake of sperm from her bursa

copulatrix into her spermatheca (Bloch Qazi et al. 1998) or by preventing her from removing or ejecting sperm from her body (Cordoba-Aguilar 2006; Xu and Wang 2010).

Another way that a male may influence his mate's sex ratio behaviorally was suggested for another haplodiploid species, the mud-daubing wasp *Trypoxylon politum* (Brockmann and Grafen 1989). When a male guards a nest as a female provisions it, she provisions more; and more provisions is associated with her being more likely to produce a daughter (Brockmann and Grafen 1989).

Examples of male influence on offspring sex ratios are also relatively scarce among vertebrates. Male pygmy hippopotamuses are an exception (Saragusty et al. 2011). They influence offspring sex ratio by differential ejaculation of X-chromosome bearing spermatozoa. The resulting female-bias may reduce local resource competition between fathers and sons.

Mate guarding is clearly important in many species (reviewed in Arnqvist and Rowe 2005). However, the present study provides a reminder that attending a female can have other fitness consequences and can have effects beyond the time of the male's physical presence. More generally, this study provides another reminder that caution should be exercised with terms that imply function, but are also used descriptively (p. 83, Lehner 1998), not only "mate guarding", but also, for example, "cooperative breeding" and "helpers at the nest". The originally posited function may turn out not to be the only one or even to be absent (Leonard et al. 1989; Eguchi et al. 2002).

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