In polyandrous species, females may influence paternity by biasing sperm usage in favour of particular males. In the guppy, *Poecilia reticulata*, the number of sperm inseminated in a copulation depends on the female's perception of male attractiveness. We videorecorded copulations in the laboratory to test the hypothesis that there is a positive correlation between the number of sperm inseminated and the duration of the copulation. Duration was positively correlated with the number of sperm retrieved from the female's gonoduct. Once copulation duration was statistically controlled for, more sperm were retrieved from the gonoduct in courtship copulations than in coercive copulations, and in postpartum females than in virgin females. Copulation duration was positively correlated with the degree of carotenoid coloration of the male, suggesting that copulation duration might be the proximate mechanism of cryptic female choice in this species. The intensity of ‘jerking’, a postcopulatory behaviour of male guppies, was positively correlated with the number of sperm inseminated, suggesting a possible signalling function of this display.

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Keywords: carotenoids; cryptic female choice; ejaculate size; *Poecilia reticulata*; postcopulatory sexual selection; sperm competition; Trinidadian guppy

One typical consequence of polyandry is sperm competition, that is, the sperm of two or more males compete to fertilize the same set of ova (Parker 1970). Female sexual promiscuity, however, also creates the opportunity for the females to bias sperm usage in favour of a particular male, so-called cryptic female choice (Thornhill 1983; Eberhard 1996). Whereas sperm competition has been shown to be a pervasive evolutionary phenomenon (Birkhead & Møller 1998), the importance of cryptic female choice remains controversial (Telford & Jennions 1998; Birkhead & Pizzari 2002). One problem with demonstrating cryptic female choice unambiguously is that it can be influenced by male behaviour (e.g. Simmons et al. 1996; Edvardsson & Arnqvist 2000; Tallamy et al. 2002), and the direct manipulation of male traits makes it difficult to determine which sex ultimately controls fertilization (see discussion in Birkhead & Pizzari 2002). Despite these difficulties, experimental evidence of cryptic female choice has been rapidly accumulating in recent years (e.g. Edvardsson & Arnqvist 2000; Elgar et al. 2000; Pizzari & Birkhead 2000; Ward 2000; Miller & Pitnick 2002; Tallamy et al. 2002; Qazi 2003; Calsbeek & Sinervo 2004; Pilastro et al. 2004).

Demonstrating cryptic female choice, however, also requires the identification of the mechanisms that females use to affect their cryptic choice (Pitnick & Brown 2000). At least 20 different mechanisms of cryptic female choice have been proposed (Eberhard 1996). One simple way for females to bias paternity in favour of a particular male is to control the number of sperm that are retained in their genital tract after copulation. This number may be controlled by expelling a portion of the entire ejaculate after copulation. For example, female feral fowl, *Gallus gallus domestics*, often eject the sperm from matings with subordinate males, whereas the semen of dominant males is usually retained (Pizzari & Birkhead 2000). Alternatively, in species in which the sperm is transferred gradually
during the copulation, females may influence the number of sperm inseminated by controlling the duration of the copulation. If so, copulation duration and number of sperm inseminated should be positively correlated. This seems to be the case in insects, in which copulation duration is usually positively correlated with the number of sperm inseminated (Thornhill 1976; Simmons & Siva-Jothy 1998; Simmons 2001). Indeed, in damselflies copulation duration is under female control (Andres & Cordero Rivera 2000), suggesting that controlling the duration of the copulation may be a mechanism of cryptic female choice in insects.

Among vertebrates, copulation duration does not usually correlate with the number of sperm transferred (e.g. Birnhead & Muller 1998), with the possible exception of reptiles (Olsson & Madsen 1998; Olsson 2001; but see Tokarz 1999; Moreira & Birnhead 2004). In mammals, polyandrous species, which have larger testes and larger ejaculates, have shorter copulations than monandrous species (Stockley & Preston 2004). In other cases copulation is prolonged after ejaculation, but is considered to be a form of mate guarding (Gomendio et al. 1998; Stockley & Preston 2004). Collectively, there is no evidence that controlling copulation duration is a potential mechanism of cryptic female choice in vertebrates.

We focused on the copulatory behaviour of the guppy, Poecilia reticulata, an internally fertilizing species of freshwater fish with a polyandrous, nonresource-based mating system (Houde 1997). During precopulatory mate choice, females prefer relatively colourful males with high rates of courtship. In particular, the area of carotenoid coloration (including orange, red and yellow) consistently influences female mating decisions (Endler & Houde 1995; Houde 1997; Evans et al. 2004). Outside periods of female sexual receptivity, males have the potential to undermine precopulatory female choice by using sneaky matings, a form of forced copulation called ‘gonopodial thrusts’ (Liley 1966). This alternative mating strategy has, on average, a low insemination success (Matthews & Magurran 2000; Russell et al. 2006), although sometimes large ejaculates are delivered during sneaky matings (Pilastro & Bisazza 1999; Evans et al. 2003a). Phenotypically attractive males produce more sperm (Matthews et al. 1997; Pilastro & Bisazza 1999; Pitcher & Evans 2001) faster and more viable sperm (Locatello et al. 2006) and inseminate more sperm than their less ornamented counterparts (Pilastro et al. 2002). As a result, attractive males have higher success in sperm competition, both in natural copulations (Evans & Magurran 2001; Pitcher et al. 2003; but see Becher & Magurran 2004) and when sperm numbers are held constant (Evans et al. 2003b). Pilastro et al. (2004) showed that the number of sperm inseminated during solicited copulations is influenced by the female’s perception of male attractiveness, independently of any direct manipulation of the focal males themselves. However, the mechanism by which females are able to control the sperm transfer is unknown. In this study we tested whether copulation duration (as estimated from the duration of the physical contact between male and female during matings) varies between matings and correlates with the number of sperm retrieved from females after copulation.

**METHODS**

**Experimental Fish**

The guppies used in this experiment were descendents of fish caught in the Tacarigua river, Trinidad (10°40.736’N, 61°19.168’W) in 2002. The males were reared in several mixed-sex 150-litre aquaria (ca. 1:1 sex ratio) until required. Water temperature was maintained between 25 and 27°C and illumination was set on a 12:12 h light:dark cycle (Philips TLD 36 W fluorescent lamps). All fish were fed a mixed diet of brine shrimp nauplii and commercially prepared flake food. Before mating, males were isolated from females for 3–5 days to facilitate full replenishment of their sperm reserves (Kuckuck & Greven 1997; Pilastro et al. 2004).

**Mating Trials**

The experimental apparatus consisted of a tank (29 × 32 cm and 40 cm deep) containing a smaller ‘mating arena’ (20 × 20 cm) where copulations were videorecorded. On one side of the partition delimiting the mating arena we fixed a black plastic ‘start box’ with a trap door leading into this arena. The door was connected to a monofilament line on a pulley, which made it possible for an observer to raise and lower it from a remote location. The tank was filled to a depth of 22 cm. Aeration and water filtration were provided by a small water filter placed in the smaller compartment (i.e. not the mating arena) of the experimental tank. The bottom of the mating arena was covered with a green plastic panel and the back with a black plastic panel, to provide a homogeneous background for recording the fish behaviour. Matings were videorecorded with two digital video cameras, one in front (Sony DCR-TRV33E) and one above (Sony CCD-TR1718E) the mating arena. The front video camera was set up close to the bottom of the tank to record the fish from below.

A sexually receptive female was placed in the mating arena and allowed to settle for 10 min. We used either virgin females or postpartum females that were isolated from males for at least 2 weeks before being used in the trials. Both types of females are sexually receptive and postpartum females isolated for 2 weeks are devoid of recoverable sperm from previous matings (Pilastro & Bisazza 1999). At this point, the male’s partition was raised, allowing the male to enter the mating arena and copulate once with the female. Copulations were considered successful only if they were followed by a series of postcopulatory jerks, which signal sperm transfer (Liley 1966). After a copulation, males jerk the body up and forwards, several times at first rapidly and then with decreasing frequency. We noted the time (min) for the pair to copulate once the frequency of courtship display and copulatory thrusts before the copulation and the number and duration of jerks after copulation. These two measures of postcopulatory male display are significantly correlated (Pearson correlation: $r_{19} = 0.89, P < 0.001$) and for brevity we present only one measure (number of jerks). The male was allowed to remain in the mating arena for 10 min after copulation.
so that we could record his jerking behaviour. After this each male was removed from the tank and anaesthetized in a water bath containing MS222 for sperm stripping and phenotype measures (see below).

Sperm Extraction

The female was removed after the mating and placed in a bottle for 30 min, after which she was anaesthetized in a water bath containing MS222. We followed established protocols to extract sperm from the female’s gonoduct (Clark & Aronson 1951; Pilastro et al. 1997; Pilastro & Bisazza 1999). Briefly, with a Drummond micropipette, 3 μl of physiological solution (NaCl 0.9%) were injected into the female’s gonoduct and then retrieved and placed in a sample tube. We carried out this operation five times to ensure the recovery of all sperm. The sperm contained in the solution drained from the female (15 μl) were counted with an ‘improved Neubauer chamber’ haemacytometer (Pilastro et al. 2002, 2004).

To test whether the number of sperm retrieved from the female is significantly correlated with the number of sperm inseminated, we conducted a control experiment. A group of nine gravid females, isolated from males for 15 days after parturition, were artificially inseminated with 5 (2 females), 10, 15, 20, 30, 45, 60 and 75 sperm bundles, which were stripped from nine different males, following established protocols (Evans et al. 2003b). For each male, we determined the number of sperm per bundle on a subsample of 10 bundles obtained from the same stripped sperm, to estimate the actual number of sperm artificially inseminated for each female. We extracted the sperm from the gonoduct, 30 min after the artificial insemination, according to the previous protocol, the only difference being that the gonoduct was flushed with 3 μl 10 consecutive times (instead of five) and we counted the sperm retrieved from each flushing. We compared the number of sperm retrieved after five and 10 flushings and the number of sperm originally inseminated to measure the efficiency of the sperm retrieval method. The sum of the number of sperm retrieved after five flushings represented a mean ± SE of 88.6 ± 5.9% of the sperm retrieved after 10 flushings. The number of sperm retrieved declined rapidly and the 10th flushing allowed us to retrieve on average 2.3 ± 1.6% of the total sperm retrieved. The number of sperm retrieved after five and 10 flushings was highly correlated with the number of sperm inseminated (sperm retrieved after 10 flushings: \( r_s = 0.90, P = 0.001 \); sperm retrieved after five flushings: \( r_s = 0.82, P = 0.007 \)). The number of sperm retrieved from the females represented on average ± SE 74.7 ± 18.2% (five flushings) and 84.2 ± 19.5% (10 flushings) of the sperm inseminated.

Male Phenotype Measurement

After mating, each male was photographed on millimetre paper for reference, with a digital camera (Nikon CoolPix 4500). The standard length of each male (distance from the snout to the base of the tail fin, SL), total area of the body (including head and caudal fin) and the areas of colour spots were estimated from the digital images obtained with Image Tool software (available at http://ddsdx.uthscsa.edu/dig/download.html). We considered three components of these patterns: (1) the area of carotenoid pigmentation (including orange, red and yellow), (2) melanistic black spots, and (3) the iridescent structural colours, which include blue and green. We measured all colour components because black and iridescent colours can also influence female choice, at least in some populations (e.g. Brooks 1996; Kodric-Brown & Nicoletto 1996). To control for differences in body size we expressed body ornamentation as the proportion of total body area covered by carotenoid, melanistic and iridescent spots. To collect sperm, we placed each male on a petri dish under a low-power dissection microscope. The gonopodium was swung forward and gentle pressure was applied to the side of the abdomen, just anterior to the base of the gonopodium. This action released sperm in the form of a number of spermatozeugmata (sperm bundles). We repeated this procedure to ensure all sperm bundles were removed.

Copulation Duration

The video recordings were analysed frame by frame (25 frames/s) with the software Zoomplayer 2.60 (VirtualMedia, www.inmatrix.com/files/zoomplayer_downloads.html). The duration of the copulation was estimated as the number of video frames (subsequently converted into seconds) that the male and the female remained in physical contact (i.e. the two bodies were in side-by-side contact) during the copulation. Two observers (M.M. and M.D.), independently and blind of male phenotype and of the number of sperm retrieved from the female, estimated the number of frames in which male and female bodies were in contact in a subsample of 22 matings. We compared the estimated duration of the copulation obtained from upper and frontal video recordings (within scorer) and between scorers (within recording). Repeatability scores were calculated following Lessells & Boag (1987). In all cases repeatability was >0.96. For the following analyses we used the estimated copulation duration as scored from the frontal recordings and by one observer (M.M.). Sperm counts and copulation duration were log transformed, whereas the proportion of carotenoids was arcsine transformed.

Ethical Note

This experiment was approved by the Department of Biology, University of Padova. The fish were fully anaesthetized before sperm extraction and phenotypic measurement. Manipulation, which was conducted by an expert operator following established procedures (Pilastro et al. 1997, 2004; Evans & Magurran 1999; Pilastro & Bisazza 1999; Bozynski & Liley 2003), was minimized and was usually completed in less than 5 min. For the same reasons, we used the lowest number of individuals necessary to achieve the aims of the experiment. All individuals recovered fully from anaesthesia and were
returned to postexperimental tanks. Mortality rate in post-experimental tanks was similar to that observed in the other stock tanks, suggesting that manipulation had no negative effect on the subsequent survival of experimental fish.

**RESULTS**

We obtained 31 matings, 21 with virgin females and 10 with postpartum females. Table 1 gives the descriptive statistics of the matings. Twenty-six of the matings were courtship copulations and five were sneaky copulations (i.e. the male approached the unaware female from behind and thrust his gonopodium towards her genital pore). Sneaky copulations were significantly shorter (X ± SD = 0.72 ± 0.11 s; Student’s t-test: t24 = 1.63, P = 0.04; Fig. 1). In contrast, the relative area of melanistic and iridescent colour spots was not significantly associated with copulation duration (all matings: melanistic: r20 = 0.46, P = 0.006; iridescent: r20 = 0.46, P = 0.26). Considering only cooperative copulations, copulation duration did not differ significantly between virgin and postpartum females (virgin: 0.81 ± 0.44 s; postpartum: 0.52 ± 0.26 s; t24 = 1.63, P = 0.12), and the difference remained nonsignificant after we controlled for the relative area of male carotenoid spots (ANCOVA with copulation duration as the dependent variable: model: F2,23 = 6.50, P = 0.006; factor: female status: F1,23 = 2.73, P = 0.11; covariate: carotenoid spots: F1,23 = 9.41, P = 0.005). The mean number of sperm retrieved from the female’s gonoduct after courtship copulations ±SD was 556,810 ± 787,838 (N = 26). In three of the five observed sneaky matings we did not retrieve any sperm from the female’s gonoduct, whereas in the other two matings we retrieved 8000 and 95,750 sperm. The number of sperm inseminated did not differ according to female status (i.e. whether the female was virgin or postpartum; only courtship copulations: virgin: 463,400 ± 833,012; postpartum: 766,970 ± 677,221; t24 = 0.82, P = 0.42), but was significantly and positively correlated with the duration of

<table>
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<tr>
<th>Table 1. Summary statistics of male and female phenotype, copulatory behaviour and ejaculation size from courtship copulation</th>
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<tr>
<td>Mean ± SD</td>
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<tr>
<td><strong>Virgin females (N=18)</strong></td>
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<tr>
<td>Sperm retrieved from the female (×1000)</td>
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<tr>
<td>Male body length (SL, mm)</td>
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<td>Female body length (SL, mm)</td>
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<tr>
<td>Male carotenoid spot area (% of total body area)</td>
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<td>Copulation duration (s)</td>
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<td>No. of postcopulatory jerks</td>
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<td><strong>Postpartum females (N=8)</strong></td>
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<td>Sperm retrieved from the female (×1000)</td>
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<td>Male body length (SL, mm)</td>
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<td>Female body length (SL, mm)</td>
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<td>Male carotenoid spot area (% of total body area)</td>
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<td>Copulation duration (s)</td>
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<td><strong>Total</strong></td>
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<td>Sperm retrieved from the female (×1000)</td>
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<td>Male body length (SL, mm)</td>
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SL: standard length.
the copulation (Pearson correlation: all matings: $r_{29} = 0.73$, $P < 0.001$; only copulations resulting in sperm transfer: $r_{36} = 0.56$, $P = 0.002$; only courtship copulations: $r_{24} = 0.42$, $P = 0.033$; Fig. 2). To test simultaneously for the effect of copulation duration, copulation type, female status and male phenotype (including the sperm reserves as estimated from the number of sperm reserves after copulation plus the number of sperm inseminated) we did a stepwise generalized linear model in which the number of sperm retrieved from the female was the dependent variable, copulation type and female status were entered as factors, and copulation duration, male phenotype (SL and size of colour spots, sperm reserves) were entered as covariates. The final model ($F_{3,30} = 19.28$, $P < 0.001$) included copulation duration ($t_{27} = 2.97$, $P = 0.006$), copulation type ($t_{27} = 2.54$, $P = 0.017$) and female status ($t_{27} = 2.11$, $P = 0.045$) as significant predictors of the number of sperm inseminated (all other variables: $P > 0.61$). In particular, more sperm were retrieved from the gonoduct in courtship copulations than in coercive copulations, and in postpartum females than in virgin females.

The postcopulatory behaviour of the male was correlated with the number of sperm inseminated. Specifically, the number of postcopulatory jerks was positively correlated with the number of sperm inseminated (Pearson correlation: all matings: $r_{29} = 0.68$, $P < 0.0001$; only copulations resulting in sperm transfer: $r_{36} = 0.57$, $P = 0.002$; only courtship copulations: $r_{24} = 0.51$, $P = 0.008$; Fig. 3).

**DISCUSSION**

Pilastro et al. (2004) showed that the number of sperm transferred during copulation in guppies depends on the female’s perception of mate attractiveness, suggesting that sperm transfer is partially under female control. The results of the present study show that the number of sperm found in the female’s gonoduct after copulation depends on copulation duration, suggesting that copula interruption may be the mechanism used by female guppies to control the number of sperm inseminated. This conclusion is supported by our finding that coercive copulations, where females tend to avoid insemination by fleeing from the male (Liley 1966), were significantly shorter than cooperative copulations. Copulation duration explained nearly half of the variance (48%) in the number of sperm inseminated, even though the estimation of intromission duration from the duration of the side-by-side body contact between male and female is likely to have some error. Other nonmutually exclusive mechanisms to control the number of inseminated sperm, such as sperm ejection (Pizzari & Birkhead 2000), cannot be excluded. Indeed, it is sometimes possible to observe intact sperm bundles lost into the water after copulation (A. Pilastro, personal observations), as a result of either interrupted copulations or active sperm ejection by females, as suggested by early observations (Liley 1966, page 42). Indeed, the observation that the insemination rate of coercive copulation is lower after statistically controlling for the duration of the copulation may suggest that females adopt active sperm ejection when forcibly inseminated, although the small sample size of coercive copulations prevents us from drawing firm conclusions about the role of this mechanism. Distinguishing between these two possibilities (active female ejection or interrupted copulation), however, may not be easy even with a larger sample size.

The ability of the females to control the number of sperm transferred during copulation represents an obvious counterstrategy to forced copulations, to which females are continuously exposed (Magurran 1998): by
manipulating the duration of copulation, females can mitigate the paternity cost of mating with a nonpreferred male. However, females make use of this ability also during courtship copulations, when females mate cooperatively with two or more males during the same receptive phase. A paternity study in which ejaculate size and female behaviour were controlled through artificial insemination of equal sperm numbers from two males yielded a uniform distribution of paternity share (Evans et al. 2003), contrasting with the bimodal paternity distribution observed when two males can mate naturally with the same female (Evans & Magurran 2001; Pitcher et al. 2003) and when the number of sperm inseminated by each male is likely to be different (Pilastro et al. 2002). This suggests that the success in sperm competition is likely to be influenced by differences in sperm quality (Locatello et al. 2006), sperm–egg interaction/compatibility effects (Zeh & Zeh 1997; Karr & Pittnick 1999; Alipaz et al. 2001) and sperm numbers. By controlling the number of sperm inseminated during a copulation, a female copulating with a suboptimal mate ensures that she has sufficient sperm to fertilize her eggs, but can bias the paternity towards the best-quality male (by accepting a large number of his sperm) among those she has mated during her sexually receptive phase (Pitcher et al. 2003), regardless of mating order (Evans & Magurran 2001). A sperm competition study using artificial insemination of different sperm numbers would allow the relative importance of sperm number in determining fertilization success to be measured (Snook 2005).

A further finding from our study was that postcopulatory male behaviour was correlated with the ejaculate size. We found that the intensity of the male postcopulatory ‘jerking’ was positively correlated with the number of sperm retrieved from the female. Jerking has been a puzzling behaviour for poeciliid biologists, because it is a very obvious display which follows courtship and copulation and is likely to increase the conspicuousness (and therefore susceptibility) of males to predators (Pocklington & Dill 1995). Several functions have been proposed to explain postcopulatory jerking. For example, it may function to replenish the gonopodium with sperm (Constantz 1989). Alternatively, Houde (1997) suggested that it may serve to dislodge external parasites from the gonopodium or as a signal to other males or females. It has long been known that postcopulatory jerking is linked to sperm transfer, because mating not followed by jerking never leads to pregnancy, whereas nearly all copulations followed by jerking do lead to pregnancy (Liley 1966). Our observation that jerking intensity and ejaculate size are positively correlated suggests that, whatever the physiological or anatomical mechanism linking them, there may be a reason for the maintenance of such an honest signal of ejaculate size, especially as this behaviour is not observed in other poeciliids. It may be advantageous for the male to signal honestly to the female the number of sperm inseminated. For example, in situations where males transfer relatively few sperm it may pay the male to signal ejaculate size to the female, because she may be willing to copulate with him again. Indeed, repeated copulations with the same male occur frequently (Houde 1997). In the opposite situation, the male may signal a large ejaculate because the female may then be less willing to mate with a second male since the possibility of biasing paternity in favour of the second male is reduced.

Sperm are costly (Dewsbury 1982; Wedell et al. 2002), and males could cheat by inseminating few sperm and then jerking intensely, thus fooling the female into not mating with a different male. However, since sperm from a single copulation can be stored for months in the female’s ovary (Constantz 1984), opportunities for cooperative copulations are rare because females are sexually receptive for only a few days per month (Houde 1997), and males can replenish their sperm stores in a few days (Kuckuck & Greven 1997), it is reasonable to expect males to inseminate as many sperm as possible. Furthermore, after a first successful copulation, male guppies try to copulate immediately with the same female and the intensity of these attempts is inversely correlated with the number of sperm inseminated during the first copulation (Pilastro & Bisazza 1999), indirectly suggesting that males always attempt to deliver large ejaculates.

The function of jerking as a signal to the female is supported by the fact that this behaviour is performed in front of her (Liley 1966). It would be interesting to investigate experimentally the function of jerking during male–female interactions, especially since third parties of both sexes may eavesdrop and acquire information about ejaculate size which they may use in their future mating decisions.

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