Temporal dynamics of mating and paternity in the squid *Loligo pealeii*

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ABSTRACT: We investigated short-term changes in relative paternity among a female's offspring in the long-finned squid *Loligo pealeii*. We conducted mating trials in the laboratory in which a single female mated with 2 males over the course of a few hours while laying successive egg capsules. Through microsatellite typing, we determined the males' relative paternities for the first and second egg capsules laid during each of 10 mating trials. Relative paternity of the first egg capsule was typically in favor of the first male to mate in the trial, although this was the only male to mate before the the first egg capsule was laid. When the female mated with an additional male before the second egg capsule was laid (all 10 trials), the first male to mate typically continued to achieve high relative paternity in the second capsule when the interval between first mating and second egg laying was relatively brief (i.e. 40 min or less). Dramatic differences in relative paternity were observed in the second capsule when the interval between the first mating and the laying of the second egg capsule was longer than 140 min. The present study argues against routine second and later-male sperm precedence in loliginid squids, pointing to other influences on paternity, such as the interval between insemination and egg laying.

KEY WORDS: *Loligo pealeii* · Squid · Sexual selection · Multiple paternity · Female choice · Polyandry · Sperm competition

INTRODUCTION

Sperm competition, which occurs in many animals, is a result of a female mating with 2 or more males (Parker 1970, Birkhead & Moller 1998, Jennions & Petrie 2000, Shuster & Wade 2003). Within a species, multiple matings by females may reflect selective pressure on males to maximize the number of fertilizations, as well as possible direct and genetic benefits accrued by females (Eberhard 1996, Arnqvist & Nilsson 2000, Jennions & Petrie 2000, Simmons 2001a,b, 2005 Arnqvist & Rowe 2005). Females, for example, might directly benefit from multiple matings by replenishing sperm supply. Via multiple mating females might also garner genetic benefits, which include increasing the diversity of the female's offspring, 'trading up' in mate quality, selecting for 'sexy sperm' that are heritably superior in sperm competition and avoiding genetic incompatibility between the

female and any one mate (Keller & Reeve 1995, Eberhard 1996, Zeh & Zeh 1996, 1997, Jennions & Petrie 2000, Simmons 2005).

Regardless of the reasons for a female's polyandry, multiple matings can lead to multiple paternity among a female's offspring. Studies on sperm competition typically involve collecting a female's reproductive output at a fixed point in time after some period of multiple mating (Birkhead & Moller 1998, Simmons 2001a). The paternity shares of competing males are then determined, with each male being assigned a certain proportion of paternity. Such a study design obscures the temporal patterning of paternity among a female's offspring. In some species, females may intersperse matings between laying successive clutches of eggs over a relatively brief period of time (i.e. within a few hours), as in frogs (Pyburn 1970, Halliday 1998) and cephalopods (Arnold 1962, Hanlon & Messenger 1996, Hanlon et al. 1997). In the present study, we examined such

short-term changes in relative paternity in the long-finned squid *Loligo pealeii*.

Loligo pealeii migrates in spring to inshore spawning sites in the northeastern USA (Summers 1983, Black et al. 1987). At spawning sites, squids form mating aggregations where females lay eggs communally. Large males fight to form consortships with females, while small males use several sneaking tactics to inseminate females (Arnold 1962, Hanlon 1996). Large males typically mate in a 'parallel' position and deposit spermatophores in the female's mantle cavity near the opening of the oviduct (Drew 1911, Arnold 1962, Hanlon 1996). Small males typically mate in a head-to-head position and place spermatophores near the female's seminal receptacle in her buccal area. Fertilization within the mantle cavity is technically external; free-swimming sperm have immediate access to egg capsules as they are extruded from the oviduct into the mantle cavity. After passing through the mantle cavity, the egg capsule is extruded out of the funnel and between the 4th pair of arms into the female's buccal area. Females may release stored sperm from their seminal receptacle at this time (as suggested by Hanlon et al. 1997).

On the spawning ground, an individual female can lay multiple egg capsules over the course of a few hours, with each capsule containing from 50 to 400 eggs (Maxwell & Hanlon 2000). Females have been observed to mate with multiple males during this period of egg laying, both in nature and in captivity (Arnold 1990, Hanlon et al. 1997, Hanlon 1998). Genetic analysis of egg capsules collected from the field demonstrate multiple paternity within egg capsules in this species (Buresch et al. 2001), as well as in several other cephalopods including 2 loliginid squids, *Loligo forbesi* (Shaw & Boyle 1997) and *L. vulgaris reynaudii* (Shaw & Sauer 2004), and in the eggs of the Australian giant cuttlefish *Sepia apama* (Naud et al. 2004). While these paternity studies suggest complex fertilization dynamics in loliginids and other cephalopods, changes in paternity over a relatively short period of time have been examined in only one trial for multiple clutch breeders (*L. bleekeri*, Iwata et al. 2005) or in species that lay single eggs successively (*S. apama*, Naud et al. 2005).

In the present study, we examine the temporal dynamics of mating and paternity in *Loligo pealeii*. Because of the complexities of this mating system and the elusiveness of squid in the field, we conducted this study in the laboratory. In captivity, we were able to (1) identify the females and the mating males, (2) directly observe and record individual matings and the time elapsed from mating to egg laying, and (3) collect egg capsules just after laying. We used microsatellite typing of egg capsules to determine paternity of competing males and to document how paternity between egg capsules changed over the short term.

MATERIALS AND METHODS

Animal collection and care. Squids were caught from Vineyard and Nantucket sounds offshore from Cape Cod, Massachusetts, USA (41° 30' N, 70° 20' W), using trawls or squid jigs, during May to September 1997 through 2002. Individuals were maintained in large tanks (2 to 4 m diameter, ca. 1 m water level) in the Marine Resources Center of the Marine Biological Laboratory (Woods Hole, Massachusetts). The tanks had a gravel/sand substrate and were supplied with flow-through natural seawater at ambient temperature of 12 to 22°C and salinity of 30 to 32. Females and males were kept in separate tanks. The tanks were exposed to the ambient light cycle (ca. 14 h light:10 h dark). Individuals were fed 2 to 4 live mummichog *Fundulus heteroclitus* per day. Healthy squids, with little or no skin damage, were selected for mating trials.

Mating trial protocols. Mating trials were performed by placing 2 males of similar size (within 2 cm mantle length [ML]) simultaneously with a single female into a large round tank (366 cm diameter, 91 cm water level). Different squids were used for each mating trial. Females were isolated for 3 d before the beginning of the trial. A previously laid bundle of egg capsules was added to stimulate mating and provide a substrate for the female to lay new egg capsules. Newly laid egg capsules, which were smooth and slippery, were easily distinguishable from the older egg capsules placed in the tank, which were bumpy and discolored from exposure to seawater. Egg capsules were collected for paternity analysis 1 to 10 min after being deposited by the female. The removal of newly laid egg capsules neither prevented a female from laying more egg capsules nor prevented additional matings. Two observers recorded the following data: squid identities and times of copulations, position of copulations (parallel or head-to-head), duration of copulations and times of egg laying. In addition, male and female behaviors were recorded throughout each trial: consortship formation by males, male–male agonistic interactions (raised arms, chasing, grappling), male–female signaling (testis flashing, oviducal flashing, all arms curled and turned upward) and female rejections (including aversion and spermatophore ejection). Ten mating trials were analyzed for relative paternity, as these trials involved mating by a female with 2 males, one before the first egg-laying bout and one before the second egg-laying bout. Other trials were conducted (n = 31 additional trials), but were excluded from analysis for 1 of 4 reasons: (1) no mating occurred, (2)

the female mated with only one male, (3) the female mated with multiple males before the first egg-laying bout or (4) the embryos did not develop far enough to allow for genotyping.

Tissue collection and incubation of egg capsules. Tissue samples (gills and arm tips) were collected from all adults in the mating trials and stored at –20°C until DNA extraction (10 females, 20 males). The first egg laid after the female's first mating and the first egg laid after the second mating were collected for paternity analysis, for a total of 20 egg capsules. After each laying, the egg capsule was transferred to an aerated mesh container within a large tank containing seawater at ambient temperature. Each capsule was incubated for ca. 20 d until the embryos were near hatching (i.e. with very little yolk sac remaining). We then placed each egg capsule in a Petri dish with sea-water, and gently pricked it along its surface. This tactile stimulation induced hatching along the egg capsule. We randomly sampled 50 to 150 hatchlings by finger-pipetting them from the seawater dish. The number of hatchlings taken per egg capsule varied with the size of the egg capsule and the condition of the embryos. For every egg capsule, our random sub-sample represented 40 to 99% of the progeny within. We stored the hatchlings at –20°C until DNA extraction. We extracted DNA from the hatchlings' whole bodies.

Paternity analysis. Methods for genomic DNA ex-traction, microsatellite screening methods, primer se-quences and DNA amplification via PCR are described elsewhere (Maxwell et al. 2000, Buresch et al. 2001). Samples representing offspring within a given egg capsule were run on the same gel with those of the ob-served mother and potential sires to verify allele matches. Adults and offspring were genotyped at 3 loci developed specifically for *Loligo pealeii* ($Lp1$, $Lp4$ and $Lp5$; GeneBank accession numbers AF165913, AF169208 and AF 169209) and one locus adapted from a congener species, *L. forbesi* ($Lfor3$; Shaw 1997, Gene-Bank accession no. U66149). Population allele frequen-cies and heterozygosity values for these loci appear elsewhere (Maxwell et al. 2000, Buresch et al. 2001).

Parentage was assessed using the maximum likeli-hood ratio program CERVUS v. 2.0 (Marshall et al. 1998, Slate et al. 2000). This program computes likeli-hood ratios (likelihood of parentage of a candidate par-ent relative to the likelihood of parentage of an arbi-trary unrelated candidate parent) to distinguish statistically between non-excluded candidates. In our case, maternal genotypes were known, so the program was used to determine candidate fathers. CERVUS takes into account typing errors arising from null alle-les and inaccurate allele sizing. Putative fathers were determined at a 95% confidence level.

We defined a male's relative paternity as the propor-tion of sampled offspring that were sired by the male. To assess the change in relative paternity between the first and second egg capsules within each trial, we identify the most successful male in terms of relative paternity for the trial's first egg capsule ('fittest male'). We then calculate r_p, which is the ratio of the male's relative paternity for the second egg capsule to that of the first capsule, as follows: $r_p = p_2 / p_1$, where p_2 = rel-ative paternity of fittest male for second egg capsule, and p_1 = relative paternity of fittest male for first egg capsule. Thus, $r_p = 1$ signifies that the fittest male has the same relative paternity in the first and second egg capsules.

RESULTS

Mating trials are summarized in Fig. 1, with attention to sequence of mating males before the laying of the first and second egg capsules. In the 10 trials, the mean (±SE) ML of males was 20.6 ± 0.9 cm (n = 20), whereas that of females was 14.7 ± 1.3 cm (n = 10). In 9 of 10 trials, a large male (19.3 to 29.9 cm ML) formed a consortship with the female within 10 min after intro-ducing the squid to the tank. The consort male typi-cally guarded the female by spreading out his arms and chasing another male from her; grappling was rarely observed. Most matings were in the parallel position (90%, 26 of 29 matings from 10 trials), with the males depositing spermatophores within the females' mantle cavities. The remaining 3 matings were in the head-to-head position (Trials 1 and 3), performed by 3 of the smaller males in these trials (12.1 to 14.1 cm ML). Females typically indicated their readiness to mate by remaining near the consort male and by flashing their white oviducal gland to him. In 2 of 10 trials, females rejected matings by attempting to dart away (Trials 1 and 10). In Trial 10, Male T (see Fig. 1b) forced copula-tions after grappling with the female, and the female subsequently ejected spermatophores from her mantle cavity. Attempts to genotype these spermatophores were unsuccessful.

The first egg capsule was laid within 20 min after the first mating of the trial in 9 of 10 trials (mean ± SE inter-val = 16 ± 11 min). Paternity of the first egg capsule was dominated by the first male to mate in the trial (Fig. 1). The first male to mate was determined to be the 'fittest male' in all trials, typically siring all of the sampled offspring in the first egg capsule (mean rela-tive paternity of first-mating male = 96 ± 4.5%, n = 10; Fig. 1). Interestingly, 45% of the offspring in the first egg capsule in Trial 3 were sired by an unknown male, despite only one male (E) observed to mate with the female before the first egg capsule (Fig. 1b). This was
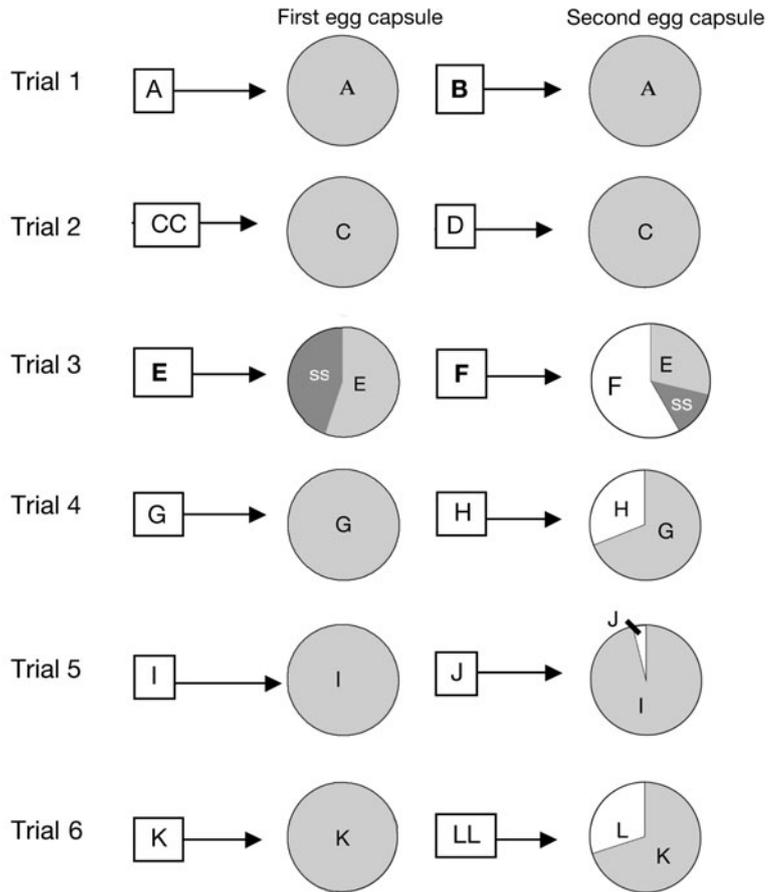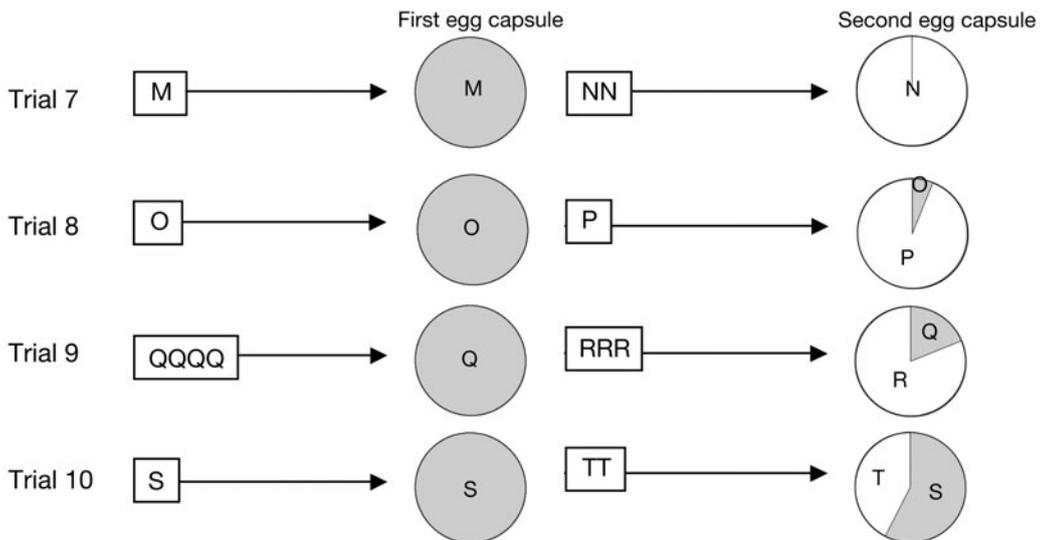
Fig. 1. *Loligo pealeii*. Mating trial scenarios and resultant paternity of egg capsules. Each box represents a mating or series of matings that occurred before an egg capsule was laid. Each letter represents a different male. Letters in **bold** indicate head-to-head matings; all other matings were in the parallel position. Trials are separated into (a) those with a short time interval (<40 min) from the first mating to the laying of the second egg capsule and (b) those with a long time interval (>140 min) from the first mating to the laying of the second egg capsule

also the only trial in which the first mating was in the head-to-head position.

The second egg capsules revealed fertilization success for second-mating males in 8 of 10 trials (Fig. 1). Some second-mating males dominated the paternity of the second egg capsule (e.g. Trials 3, 7, 8 and 9), while others achieved very little to no fertilization success (e.g. Trials 1, 2 and 5). The time interval between the first mating and laying of the second capsule varied markedly between trials, spanning 15 to 303 min (mean interval = 105 ± 35 min, n =10). The interval followed a strongly bimodal distribution. In 6 of the trials, the interval was less than 40 min ('short' interval), while the interval was 140 min or longer in 4 of the trials ('long' interval). When the interval was shorter than 40 min, the average r_p value (i.e. ratio of the first capsule's 'fittest' male's relative paternity for the second egg capsule to that of the first capsule) was high, meaning that the most successful sire for the first capsule was also the most successful sire for the second capsule (mean r_p = 0.81 ± 0.08, n = 6). When the interval was 140 min or more, however, the average r_p value was low (mean r_p = 0.27 ± 0.11, n = 4), reflecting lowered relative paternity over time for the initially most successful sire (Mann-Whitney *U*-test: *U* = 23, p = 0.02).

DISCUSSION

The present study indicates the importance of the timing of insemination and egg laying to male fertilization success in the context of sperm competition. Relative paternity of the first egg capsule was in favor of the first male to mate in the trial. When the female mated with a second male before the laying of a second egg capsule, the first male to mate typically continued to achieve high relative paternity in the second capsule when the interval between first mating and second egg laying was relatively brief (i.e. 40 min or less). Dramatic differences in relative paternity were observed in the second capsule when the interval between first mating and second capsule was longer than 140 min. These results point to the need for subsequent experimentation, involving controlled access to females by males, to examine the effect of timed intervals between sperm transfer and egg laying.

The present study argues against routine second and later-male sperm precedence in the short term (i.e. over a few hours), as suggested by other studies on paternity in loliginid squids. In *Loligo vulgaris reynaudii* in South Africa, a male that had previously guarded a female showed high relative paternity rate for a field-collected egg capsule (Shaw & Sauer 2004). In mating trials with *L. bleekeri* in Japan, the last male

to mate sired the majority of offspring, but in all cases this male had also mated with the female more frequently (Iwata et al. 2005). These 2 studies, along with the present study, indicate that other factors besides mating order determine relative paternity within and between egg capsules. Those factors include the interval between mating and egg laying, the mechanics of insemination and fertilization, female choice and the number of inseminations by a given male.

The interval between mating and egg laying is an important factor for paternity in another cephalopod, *Sepia apama*. In this species, Naud et al. (2004) reported a paternity advantage to males when eggs were laid 20 to 40 min after insemination. They hypothesized that less than 20 min may be too soon for sperm to be released adequately from the newly placed spermatophore, while greater than 40 min may involve significant sperm loss during the process of incorporation into the female's buccal receptacle. It is possible that a similar process of sperm loss over time occurs in *Loligo pealeii*, especially with regard to copulations in the parallel position, wherein spermatophores are placed near the oviduct within the mantle cavity. Sperm cells begin to escape from the spermatophores almost immediately (Drew 1911), so that many sperm may be lost from the female's mantle cavity after 140 min due to normal ventilations, which in squid involve the entire water volume in the mantle cavity. In *L. pealeii*, sperm loss from the mantle cavity need not be a purely passive process, as females appear capable of manipulating or even ejecting spermatophores. In Trial 10, the female visibly ejected spermatophores, flushing them from her mantle cavity after 2 forced parallel copulations with a large male (Male T); Male T fertilized the fewest eggs of the second-mating males in the 'long' interval trials.

The importance of mating interval to a given male's relative paternity has long been appreciated in the literature (Boorman & Parker 1976, Griswold & Prezioso 1981, Gromko et al. 1984, Simmons 2001a). The interaction between mating interval and fertilization physiology has ramifications for both male and female fitness (Arnqvist & Rowe 2005). Typically, fertilization occurring shortly after insemination benefits the first copulating male, while slower fertilization rates benefit the later males (Birkhead & Moller 1998, Simmons 2001a). Slower fertilization rates may also benefit the female, affording her the opportunity for polyandry and its potential direct and indirect benefits (Eberhard 1996, Jennions & Petrie 2000, Simmons 2005). Females in other taxa have several post-copulatory ways of affecting the paternity of the offspring (Walker 1980, Eberhard 1996). It is possible that female *Loligo pealeii* can delay fertilization by delaying the extrusion of eggs from the oviduct after mating with a certain male.

In situ observations of the congener *L. opalescens* suggest that females may control the timing of egg laying, since egg-laying females appear to increase the interval between successive egg capsules after a new mating (N. Kangas and R. T. Hanlon pers. comm.). In these cases, the females might delay egg laying to bias paternity towards the new mate. More directly, females can probably control the timing of the release of sperm stored in the seminal receptacle onto the extruded egg capsule. As mentioned above, females are also capable of ejecting spermatophores from the mantle cavity.

The present study has implications for male mating tactics in natural populations of squid. In *Loligo pealeii,* large males form consortships with receptive females at the communal spawning beds, typically mating in the parallel position (Arnold 1962, Hanlon 1996). A male that mates with and continues to guard a female over the subsequent ca. 40 min would appear to have a paternity advantage over rival males. Yet paternity data suggest that it is difficult for a male to maintain a monopoly on paternity. Second-mating males achieved some fertilization success in all but 2 trials in the present study, especially when long intervals occurred between first mating and second egg capsule. We acknowledge that the present study involved fewer mates than *L. pealeii* females have been observed to mate with in nature (Hanlon et al. 1997, Hanlon 1998), yet we observed multiple paternity within egg capsules in our controlled setting. Field-collected egg capsules suggest that multiple paternity is frequent within an egg capsule in *L. pealeii* (Buresch et al. 2001). Multiple paternity within egg capsules appears to be a common feature in other loliginids, including *L. forbesi* (Shaw & Boyle 1997) and *L. vulgaris reynaudii* (Shaw & Sauer 2004). Within-capsule multiple paternity can come about through various ways, including sperm mixing from sequential matings by multiple males, rival males displacing or removing sperm from the female's mantle cavity, sufficient passage of time between mating and the laying of egg capsules or the female's preferential use of recently received sperm or stored sperm. With regard to sperm removal by rival males, we did not observe males removing sperm from the females' mantle cavities or buccal areas in the present study. With regard to the female's use of stored sperm, some offspring were sired by males that were unaccounted for in the present study (e.g. Trial 3), suggesting that females used sperm received from matings before the actual mating trials and stored in the seminal receptacle.

In head-to-head matings by loliginid squid, sperm are presumably deposited in or near the female's buccal seminal receptacle (Hanlon & Messenger 1996, Hanlon et al. 1997). This mating position is typically used by non-consort males, especially small 'sneakers.' Although only 3 of the 20 males in this study mated in the head-to-head position, this tactic is common in the field in this species and *Loligo vulgaris reynaudii* (Hanlon & Messenger 1996, Hanlon et al. 1997, 2002). This position afforded some fertilization success to 2 males in the present study. Although the low number of head-to-head matings precludes a comparison of this position to the parallel position in terms of fertilization success, our data suggest variability in fertilization success for head-to-head matings (e.g. Trial 1 versus 3). In cuttlefish *Sepia* spp., males have been observed to mate exclusively in the head-to-head position, depositing sperm in or near a receptacle in the buccal area (Hanlon et al. 1999, Hall & Hanlon 2002, Naud et al. 2004, 2005). A field study on paternity in *S. agama* showed that, although the volumes of sperm present in the buccal receptacle and on the female's buccal surface (i.e. sperm forming the 'spermatangia' mass) were comparable, the majority of the fertilizing sperm came from the buccal surface rather than the buccal receptacle (Naud et al. 2005).

Use of stored sperm represents a form of cryptic female choice (Eberhard 1996), and one that may lie outside of the consort male's control. While a consort male can actively shield the female and physically repel rival males, he appears incapable of removing sperm stored in the female's seminal receptacle or preventing her from releasing stored sperm onto the egg capsule. In *Loligo pealeii,* a female's probable postcopulatory influence on the paternity of her offspring enriches our understanding of sperm competition, particularly in this mating system where competition among males is so conspicuous in the form of skin displays and physical fights (Arnold 1962, Hanlon 1996, Hanlon & Messenger 1996, DiMarco & Hanlon 1997, Hanlon et al. 1999). The integration of female reproductive interests with sperm competition among males will shed further light on the factors that determine reproductive success in promiscuous mating systems.

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