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Paternity in horseshoe crabs when spawning in multiple-male groups

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Unpaired or satellite male horseshoe crabs, *Limulus polyphemus*, are attracted to and often form a group around a pair (a female with an attached male) that is nesting in the high intertidal zone. These males are engaged in sperm competition. We observed nesting pairs and their associated satellites in the wild, collected and reared their eggs and used genetic markers to examine paternity. We found that the unpaired, satellite males are highly successful at fertilizing eggs; two satellites can leave the attached male with few fertilizations. Two satellites together are each as successful as one spawning with a pair. A satellite's location around the female greatly affects his success, and males compete for access to a position over the dorsal canal between the prosoma and opisthosoma of the female and under the front margin of the paired male where they are most likely to fertilize eggs. Although eggs and sperm retain their viability for some time after spawning, nearly all eggs are fertilized by the satellites that are around the nesting pair at the time of egg laying and by the attached male. A number of factors including beach current, female size and male behaviour affect the outcome of sperm competition in this externally fertilizing species.

Multiple mating by females sets the stage for a complex set of events that includes both male–male competition in the form of sperm competition (Parker 1970; Birkhead & Møller 1992; Birkhead & Parker 1997) and female choice in the form of sperm manipulation and cryptic female choice (Thornhill 1983; Eberhard 1996). The events associated with mating can be broken down into stages: those that occur before sperm are released, after sperm are released but before the egg has been contacted, and those associated with fertilization. Processes operating at each of these stages may result in single-male or multiple-male paternity of a clutch of eggs and differential success of some males (and genotypes) over others. The constraints, selective pressures and mechanisms that are operating during each of these stages may differ markedly among species. For internally fertilizing species, adaptations by which males achieve greater success are well known and include behaviour such as extended copulation, postcopulatory guarding and displays; morphology such as plugs, sperm removal structures and specialized genitalia; and physiology such as chemicals and nutrients that alter the probability of fertilization (Christy 1987; Birkhead & Møller 1992; Eberhard 1996; Birkhead & Parker 1997). Much less is known about the behavioural adaptations for sperm competition in externally fertilizing species, which is the focus of this study.

In some externally fertilizing species such as corals (Levitan & Petersen 1995), 'sperm limitation' is a factor, in that not all eggs are fertilized apparently due to a lack of sperm availability (Pennington 1985; Levitan 1993). This dramatically alters the face of sexual selection in these species because it means selection favours adaptations by females to attract males to ensure fertilization rather than to exert female choice. But in externally fertilizing species such as fish (Shapiro et al. 1994; Stockley 1997), frogs (Coe 1967; Halliday & Verrell 1984; Kusano et al. 1991; Feng & Narins 1992; Kasuya 1992; d'Orgeix & Turner 1995; Halliday 1998) and horseshoe crabs (Brockmann 1990), all eggs are fertilized and male–male competition for mating exists. Clear evidence for sperm competition also exists in two invertebrates that broadcast sperm but retain eggs inside the female (Yund & McCartney 1994). Adaptations that are thought to play a role in sperm competition in externally fertilizing species include increasing sperm number, investment in male function and testis size per body weight (Kusano et al. 1991; Yund & McCartney 1994; Stockley et al. 1997), synchronizing sperm release (Levitan 1998), changing positions which alters the male's proximity to spawning females (Levitan 1998) and the deployment of sperm in

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water currents (Pyburn 1970; Feng 1991; Feng & Narrins 1992; Jennions & Passmore 1993; Kaminsky 1997), concentrating dilute sperm from sea water and storing sperm (Bishop 1998) and increasing the amount of sperm released during group- as compared with single-male spawning (Shapiro et al. 1994). In this study we identify a number of behavioural adaptations associated with sperm competition in multiple-male groups of horseshoe crabs, *Limulus polyphemus*.

Horseshoe crabs are the only arthropods with true external fertilization, one of several primitive traits retained in this lineage (Fahrenbach 1973; Fisher 1984). They also retain a primitive spermatozoon structure more like that of annelids and molluscs than of any living arthropod (Fahrenbach 1973). The opportunities for sperm competition are legion: highly synchronized spawning and strongly male-biased operational sex ratios often lead to many satellite males pushing against and crawling over the spawning pairs (Rudloe 1980; Shuster & Botton 1985; Barlow et al. 1986; Brockmann 1996). In a previous study, we demonstrated that satellite males fertilize eggs but we only observed pairs with one satellite male (Brockmann et al. 1994). In this study we examine patterns of paternity in larger groups of two to four satellites that were found in association with nesting couples. One result that is immediately apparent (and also found in other species) is that substantial variation exists among individuals in their success in sperm competition. This variation may be due to differences in physical conditions (such as water currents) during spawning, differences among satellites in their ability to encroach on attached males, differences between attached males in their ability to stave off the advances of satellites, and differences in the ability of females to manipulate water currents or control the number of satellites that fertilize their eggs or other unidentified stochastic factors. In this study we describe the patterns and evaluate possible explanations for variation in fertilization success.

Every spring millions of horseshoe crabs crawl out of the sea to breed on the beaches and sandbars of the eastern U.S. (Shuster 1982). Spawning is highly synchronous with the highest tides available in the springtime (i.e. the high tides associated with the new and full moons; Rudloe 1980; Barlow et al. 1986). Females come ashore already paired with what we refer to as an ‘attached’ male, which grabs hold of the female with a pair of modified claws and clings tenaciously to her terminal spines in a sort of amplexus (Botton et al. 1996). The female crawls up the beach, digs into the sand and lays her eggs in a cluster that we call a ‘clutch.’ The female then plows forward in the sand about 10 cm and lays the next clutch of eggs (a nest is made up of 1–10 clutches). Unattached males are attracted to nesting couples (Brockmann 1996), move into contact and may remain with them throughout a nesting. One clutch takes 3–10 min to lay and may contain as few as a hundred eggs or as many as 8000, but normally a clutch contains approximately 2000 eggs (Brockmann 1990). As they are laid, the male(s) fertilizes the eggs externally with aquatic, free-swimming sperm. If the female gets too far out of the water during nesting due to the receding tide, she pulls herself out of the sand, swims out to sea and then nests again at a lower level on the beach a few minutes later with the same attached male but different satellites. When the tide recedes from its high level, the pair goes back to sea still attached and usually returns on the next high tide or on a tide 24 h later. After several days of nesting, the female then returns to sea until the next season, whereas many males return during the next tidal cycle either attached to another female or as a satellite, which explains the male-biased operational sex ratio on the beach (Botton & Loveland 1992; Brockmann & Penn 1992).

Good evidence exists that female horseshoe crabs choose nesting sites (Barlow et al. 1986; Botton et al. 1988; Penn & Brockmann 1994). By nesting only in the high intertidal zone on the extra high spring tides, females nest in an area of the beach where their eggs develop quickly: above this level the sand is too dry and the eggs desiccate and below this level the sand has a lower oxygen content and horseshoe crab eggs will not develop under anaerobic conditions (Penn & Brockmann 1994). With each wave and as the tide recedes, fresh sea water is drawn through and oxygenates the sand in this intertidal region. The eggs hatch into trilobite larvae, which live in the sand for a couple of weeks, then swim to the surface on a high tide and are washed into the subtidal region. Trilobite larvae do not feed and quickly moult into juvenile horseshoe crabs that feed inshore for years. Horseshoe crabs remain as juveniles for about 9–10 years (Shuster 1982) and have a terminal moult into the adult form (Sekiguchi et al. 1982). Adult horseshoe crabs feed on molluscs offshore and may live 5–10 years as an adult (Botton & Ropes 1988). Satellite males tend to be among the older males in the population whereas attached males tend to be younger males that are in better condition (Brockmann & Penn 1992).

How does fertilization take place? Eggs are ejected by the female into the sand from paired gonopores located posterior to the flabellum, just anterior to the gills (Fig. 1b). Sperm are released from paired gonopores just posterior to the flabellum and move in water currents until they encounter the eggs. Respiratory water currents travel from anterior to posterior over the female’s gills; water enters the canal between the prosoma and opisthosoma and exits below the terminal spines near the telson (Fig. 1; Barthel 1974). When spawning, however, these water currents are reversed through the action of the female and male and water exits the canal between the prosoma and opisthosoma (Sekiguchi 1988; personal observation). Newly released sperm are not capacitated and move passively in these water currents until they are within 0.5 cm of an egg (Brown 1976). At this distance the sperm capacitate when they contact a sperm motility initiating peptide released from the egg or from oviducal fluids (Shoger & Bishop 1967). They swim up this chemical gradient (Brown 1976) and undergo an acrosomal reaction when they contact the egg a few seconds later. The acrosomal filament penetrates the basement lamina and inner vitelline envelope of the egg (Shoger & Brown 1970), rotating as it elongates, thus screwing through the egg jelly (Tilney 1975). Within 3 min of the first

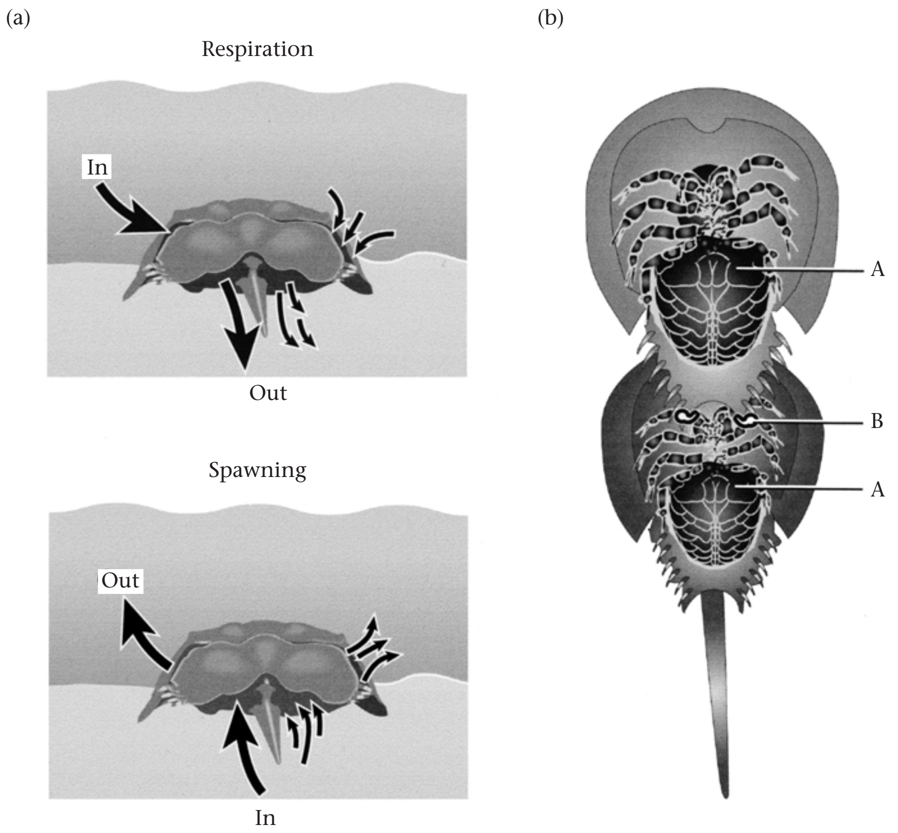

Figure 1. (a) Posterior view of a horseshoe crab showing the respiratory currents that run anterior to posterior and the spawning currents that run in the reverse direction (picture modified from Barthel 1974). (b) Ventral view of a pair of horseshoe crabs (female in front of male) showing the male holding onto the terminal spines of the female (indicated by 'B'). The female's telson and some legs are not shown for clarity. Eggs and sperm are released from paired gonopores just underneath and posterior to the flabellum (indicated by 'A').

acrosomal reaction, the egg has undergone a number of changes that visibly alter its appearance. Pits appear on the surface caused by the fusion of small vesicles with the overlying plasmalemma and the expansion of the perivitelline space, which apparently prevents further sperm attachments (Bannon & Brown 1980; Brown & Clapper 1980; Brown & Barnum 1983). Nevertheless, up to a million sperm may undergo acrosomal reactions with a single egg (Shoger & Brown 1970; Brown & Humphreys 1971). Nothing seems to be known about the process by which the DNA of a particular sperm moves to the egg nucleus, but Brown & Knouse (1973) suggest that a selective egg-envelope process may exist by which only some sperm nuclei are allowed to contact the egg cell membrane, but no further work has been done.

Given this fertilization process, anything that a male does to alter water currents to his advantage or to get his sperm into a current that has already been initiated or closer to the site of egg release should increase his chances of fertilizing eggs. Differences may exist between males in their ability to compete with other satellites or the attached male in this process. It may also mean that various environmental factors such as waves and tidal currents could influence the fertilization process. Females may also manipulate water currents or satellite positions during spawning, which could alter fertilization to their advantage. In this study we use genetic markers to examine paternity patterns during multiple-male spawning. We also evaluate the effect of physical conditions and the characteristics and behaviour of individuals that make them more or less likely to fertilize eggs.

METHODS

Observations

We conducted observations on the south-facing beach of Seahorse Key, a small Gulf coast, offshore island that is part of the Suwannee River National Wildlife Refuge near Cedar Key, Florida (March and April 1995). We located nesting pairs of horseshoe crabs with at least two satellite males present and observed their behaviour closely, making video records of five groups. Any satellite male that joined the group was recorded, as were his movements around the group. If a satellite left the group he was collected and placed in a wading pool. Nesting females push into the sand and then remain quiet for several minutes before pushing forward again. Previous research (Brockmann 1990) has shown that a clutch of eggs is deposited during these quiet periods, so when the female ceased forward movement, we placed wire flags in the sand on either side of the female's prosomal–opisthosomal hinge (the gonopores are located below this point). In this way a pair of flags marked out the position of each clutch. After three to five clutches were completed, we individually marked and placed each member of the group in a wading pool. We positioned 20 plastic, wading pools (diameter 1.2 m) at intervals along the shoreline and filled them with 15 cm of fresh sea water so that the crabs were covered. We held the horseshoe crabs in these wading pools after the tide so that measurements and tissue samples could be taken during daylight hours and so that we could complete observations during the high tide period (held 4–6 h after nighttime tides and 1–3 h after daytime tides). We released all animals back to the sea (no mortality occurred) and we saw most individuals nesting again on the following day.

Measurements

After the tide, we took measurements on all individuals, using the same measures as in previous studies (Brockmann & Penn 1992; Brockmann 1996). We measured crab size as carapace width (CW, measured on the ventral surface at its widest point) and weight (placed in a Styrofoam minnow bucket and weighed with a hand-held Pesola scale). We also measured the length and height (at its highest point near the base) of the telson with a caliper. We estimated the surface condition of the animal using five measures: (1) whether the carapace showed any erosion by chitinoclastic bacteria or blue-green algae, (2) by the condition of the eyes (whether soft or covered with epibiotic organisms), (3) by the condition of the terminal spines (whether worn or broken), (4) by the percentage of the dorsal surface that was covered with epibionts, such as barnacles, slipper shells and algae, and (5) by the number of *Bdelloura candida* egg cases found on 10 leaves of the crab's book gills (measured on the first three sections of the gills). *Bdelloura candida* is a triclad turbellarid flatworm that is found on most horseshoe crabs. As adults, the worms feed on scraps of food left around the crab's mouth (leg bases), but they also lay their eggs in the leaves of the book gills where they cause considerable damage (Huggins & Waite 1993) including degeneration and destruction of the gill cuticle (Groff & Leibovitz 1982). We combined these five measures of condition into a simple index: if the animal showed the poorest condition in all five categories, it received a rank of 5; if it showed the highest rank in all five categories it received a score of 25 (a middle rank in all five categories received a score of 15).

Egg Rearing

After the tide had receded, we dug up the clutches from the observed groups by hand. We measured the depth of the eggs and the distance between the clutches with a tape measure. Using a spoon we collected the clusters of eggs along with the sand in which they were buried. We washed this sand away by using a sieve and counted the eggs by funneling them into a graduated cylinder (1 ml=88 eggs). We placed the eggs for rearing (100 from each clutch) in 11.5 × 3.5 cm plastic dishes (five replicates for each clutch). These eggs were then held at 28°C in a Forma Scientific Incubator under a 14:10 h light:dark cycle. Every 2–3 days we removed the old water and replaced it with fresh sea water (following Brown & Clapper 1981; Brockmann et al. 1994). Nearly all eggs developed and hatched into trilobite larvae. These larvae were then placed in eppendorf tubes and frozen (−80°C) for paternity analysis.

Paternity Analysis

Immediately after the tide and before the measurements were taken, we removed one leg (fourth right walking leg taken at the joint) from each participant in the nesting group (i.e. the female, the attached male and all satellites). The legs were frozen immediately in dry ice or liquid nitrogen and transported from Seahorse Key to the University of Florida where they were held at −80°C for later genomic DNA extraction. We allowed each crab to recover in the wading pool for at least 1 h prior to release (horseshoe crabs have a rapid clotting response and many crabs have legs missing naturally). We saw most nesting again on the following day (no mortality occurred).

We evaluated paternity for each clutch using the methods described in Brockmann et al. (1994) (conducted by C.N. and W.P.). Briefly, for adults, we ground a 1-cm^3 sample of muscle tissue from each leg in a mortar and pestle precooled with liquid nitrogen. For 16–24 larvae from each clutch, we ground the entire larva in a 1.5-ml microcentrifuge tube with a tube-shaped metal pestle. We extracted DNA from these ground tissues using standard phenol: chloroform procedures. We used each genomic DNA as a template in a polymerase chain reaction (PCR) to amplify a single microsatellite locus developed previously. PCR products were resolved on ethidium-stained, 7% polyacrylamide gels. We determined paternity by comparing electrophoretic bands of each larva with the mother, attached male and all satellites that participated in the nesting.

Paternity analysis was possible with a single locus for two reasons. First, the locus is extremely polymorphic, with 22 alleles observed in 33 adults from our previous study (Brockmann et al. 1994). Although we did not make a precise assessment of the allelic frequencies in the population of this study, inspection of the gels indicated a genetic diversity similar to the previous study. That study documented that all alleles had relatively low allelic frequencies, with the most common allele having a frequency of 0.14. So, even in the worst-case scenario where the female, the attached male and one satellite male had the six most common alleles, there was still a 52% chance that any randomly drawn sperm from the population would be recognized. The mean and median allele frequency was 0.05, so the power to recognize extragroup fertilizations was approximately 0.7, considerably greater than this worst-case example. In our previous study, fertilization by sperm with an allele not present in the putative parents was only 3% (11 of 324). Even if all of these were due to sperm left in the sand by other males (some could be due to mutation, misgenotyping and mishandling of samples), the rate is sufficiently low not to affect the general trends and conclusions of either our previous or present findings. Furthermore, in the current data set, fertilization by sperm with an allele not present in the putative parents was 0.2% (2 of 846), providing additional evidence that extragroup fertilizations were rare and inconsequential in this study.

Second, maternity was known, and the number of possible fathers was known and small, ranging from two to five. We only analysed clutches from mating groups where there was no allele sharing among the putative fathers, which occurred in 15 of 19 mating groups. Consequently, for each offspring, we were able to assign paternity explicitly to one male from the set of putative fathers.

Sperm and Egg Survival

As in internally fertilizing species, external fertilizers may sometimes have the opportunity to store sperm. Given the concave shape of the female's carapace and the fact that sperm can survive for some time after spawning, we thought this possible. Brown & Knouse (1973) showed that sperm are capable of surviving for 96 h but their study was conducted at 4–5°C, a temperature at which horseshoe crabs are not active and do not spawn. For this reason H.J.B. conducted a study to evaluate the survivorship of eggs and sperm in sea water prior to fertilization at normal nesting temperatures of 28°C. Eggs and sperm were obtained using the methods of Brown & Clapper (1981). Twenty-one males were electro-ejaculated by stimulating (3–4 V, 0.5–1.0 mA) below and slightly lateral to the gonopore and the ejected semen was collected with a clean pipette. The sperm were diluted in sea water (1 ml semen: 9 ml sea water) and held for 5 min to 53 h prior to fertilization (roughly corresponding to tidal cycles). Eggs from 13 females were electro-ovulated by giving the females 1-s pulses of current (3–4 V, 0.5–1.0 mA) 2 cm below the gonopore (10–50 eggs were ejected at a time from each gonopore) and the eggs were collected with a plastic spoon. The eggs were held in plastic containers for 1–40 min prior to fertilization; eggs were not held longer because Brown & Clapper (1980) stated that eggs must be fertilized within 5–10 min or the proportion developing is greatly reduced. Eggs were fertilized by squirting 10 ml of the sperm suspension over the eggs then changing the water 30 min later to remove excess semen. Eggs that were less than 10 min old were fertilized with sperm that was 0–2 h (17 sets), 5–15 h (18 sets), 20–26 h (six sets) and 40–53 h (11 sets) old; eggs that were 10, 20 and 40 min old were fertilized with sperm that was 0–2 h (eight sets each), 5–15 h (one set each) and 20–26 h (one set each) old. The eggs were kept for 1 month as described under egg rearing above. The number of eggs developing after 2 weeks were counted, that is, the number that had been fertilized in each treatment group (no additional eggs showed development after 2 weeks).

Tidal Currents

Fertilization success of some species is known to be affected by current speed (Pennington 1985; Yund & McCartney 1994). In 1997 we placed a General Oceanics mechanical flow meter (model 2030 with low-speed rotor) 2–3 m from shore in 0.5 m water and measured the direction and velocity of the current (number of revolutions/10 min) for 37 tides. We measured the current 1 h before the tide, at the time of the predicted maximum high tide and 1 h after the maximum.

Data Analysis

This study was based on 15 nesting pairs of horseshoe crabs and their associated satellite males that spawned along the beach at Seahorse Key on 31 March and 14–18 April 1995 on both night and day (17–18 April) tides. We observed, marked and measured a total of 26 mating groups on these dates, but seven were excluded because no eggs were laid or the eggs were mixed with those of a female that nested earlier in the season. An additional four were excluded because paternity was ambiguous due to allele sharing in two or more of the putative sires. We do not know of any specific bias that was introduced by the exclusion of these four mating groups. These 15 pairs and their 106 associated satellites completed 40 clutches (two to five clutches from each pair) with two to four satellites each. (One clutch in the sample was completed with one satellite when a male left after completing two clutches with that nesting pair). Because satellites were free to move among nests, and because there were no differences attributable to clutch number, we treated these 40 clutches as independent data points for analyses of satellites. However, to avoid problems of pseudoreplication, when the analyses involved the female or the attached male (such as whether the size of the female affected satellite male paternity), then we used data from the first clutch of each nest ($N=15$). Descriptive data are reported as means ± SE for continuous data and as proportions for categorical data. We used a repeated measures analysis of variance (ANOVA) on ranked data within the general linear model procedure (GLM, SAS Institute 1990) to evaluate the combined effects of variables hypothesized to affect paternity (Agresti 1996).

RESULTS

Description of Paternity

Satellite males were highly successful at fertilizing eggs. Overall, 624 of the 846 (74%) larvae in this study were fathered by satellite males. On average satellite males could expect equal paternity (32 ± 3.1%) with that of attached males (25.5 ± 5.0%) when in a group of two to four satellites (Wilcoxon signed-ranks test: $Z=-1.3$, $N=40$, $P=0.18$), but wide variation existed: both satellites and attached males fathered 0–100% of the eggs that were laid in a clutch (Fig. 2). The average success of attached males when spawning with two satellites (23.3 ± 6.8%, $N=23$ clutches) was much lower than when spawning with one satellite (51%) or no satellites (100%) (from Brockmann et al. 1994). However, an attached male's

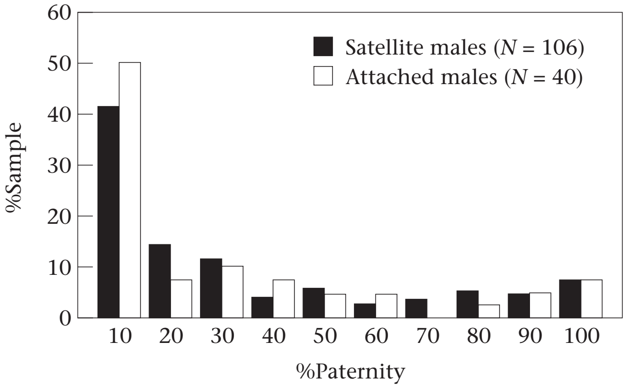

Figure 2. Frequency distribution of paternity of clutches for satellite and attached male horseshoe crabs (percentage of clutch fathered by male) when the mating group around the nesting pair contained two to four satellites.

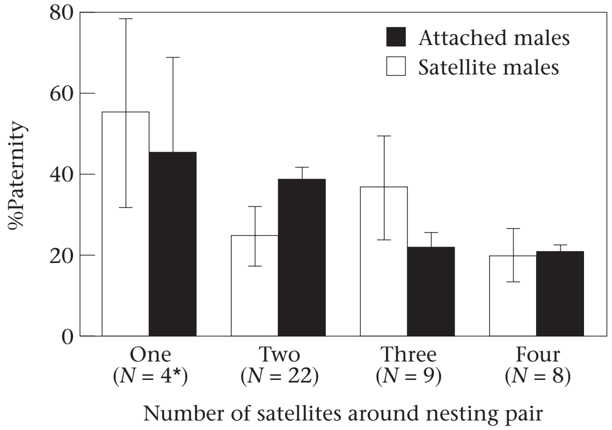

Figure 3. The effect of the number of satellites on the mean percentage of paternity per male for attached and satellite males. Sample sizes are number of nests. *Includes three data points taken from Brockmann et al. (1994) collected from the same site in Florida and one data point from the present data set.

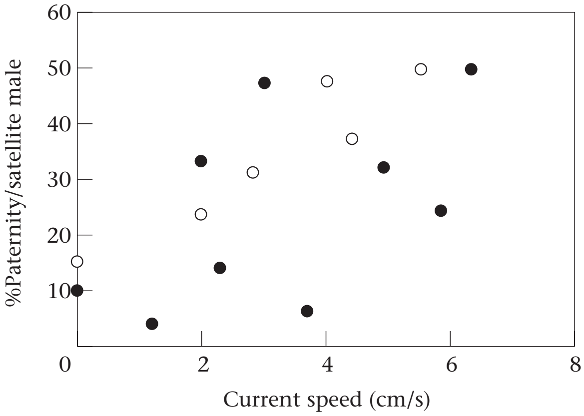

Figure 4. Correlation between current speed and the mean percentage of paternity by satellite males (r_S=0.62; N=15 clutches, P=0.02). Each data point refers to the first clutch from each nest. ○: Incoming tide (r_S=0.94, N=6, P=0.03); ●: outgoing tide (r_S=0.43, N=9, P=0.22).

success did not differ significantly when spawning with two or more satellites (three satellites: 36.4 ± 12.8%, N=9 clutches; four satellites: 19.5 ± 6.8%, N=8 clutches; for two to four satellites: Kruskal–Wallis test: H_{23}=1.5, P=0.46). In contrast, the average paternity of satellites was affected by the number of other satellites present (Kruskal–Wallis test: H_{23}=12.3, P=0.0021; Fig. 3); the mean success of two satellites spawning with a pair (40.5 ± 4.3%, N=23 clutches) was higher than the mean success of three (21.2 ± 4.3%, N=9) or four satellites (20.1 ± 1.7%, N=8). Therefore, when two satellites were present, most of their effect on paternity occurred by reducing the success of the attached male, whereas when three or more satellites were present, they reduced the success of other satellites.

Effect of Sperm and Egg Survival on Paternity

Unfertilized eggs retained their ability to be fertilized for at least 40 min at 28°C (i.e. there was no association between egg age and the percentage of eggs developing within the range tested). Sperm could fertilize eggs up to 20 h after ejaculation but sperm that was less than 2 h old was significantly more likely to fertilize eggs than sperm that was more than 5 h old (chi-square test: χ^2_3=23.5, N=83, P<0.0001). Sperm had the potential, then, to fertilize eggs well after they were spawned.

Despite this potential, extragroup fertilization rarely occurred: in 39 of 40 clutches, paternity of all eggs was consistent with the males present when the clutch was laid. No eggs were fertilized by males that had departed after completing the preceding clutch (five cases). In the single clutch where offspring genotypes were inconsistent with any putative father, only two of 24 eggs were inconsistent. These two cases could be due to mutation, misgenotyping or fertilization by extragroup sperm. As discussed above, the probability of detecting an extragroup fertilization was above 50%. Because only two of 846 eggs were inconsistent with any of the putative males, we conclude that fertilization by extragroup sperm was rare and can be ignored.

Effect of Tidal Conditions on Paternity

Horseshoe crabs nest in a narrow strip at the top of the high tide line (Penn & Brockmann 1994) and at Seahorse Key they nest along a south-facing beach. Flow meter measurements made on this beach in 1997 showed that the current runs west–east along the beach on the incoming tide (average velocity 4.4 ± 0.3 cm/s) and reverses direction and flows slightly faster (5.5 ± 0.4 cm/s) on the outgoing tide. The current was strongest 1–2 h before and after the maximum high tide and declined to almost no current around the time (± 30 min) of the maximum high tide (1.6 ± 0.2 cm/s). The average fertilization success of the satellite males was strongly affected by this tidal cycle (incoming tide: 47 ± 2.4%, N=5; high tide: 24.6 ± 3.2%, N=22; outgoing: 38.9 ± 6.6%, N=13; Kruskal–Wallis test, H_{23}=9.6, P=0.008) and by the current: the faster the current the higher the average paternity for satellites (r_S=0.62, N=15, P=0.02; Fig. 4).

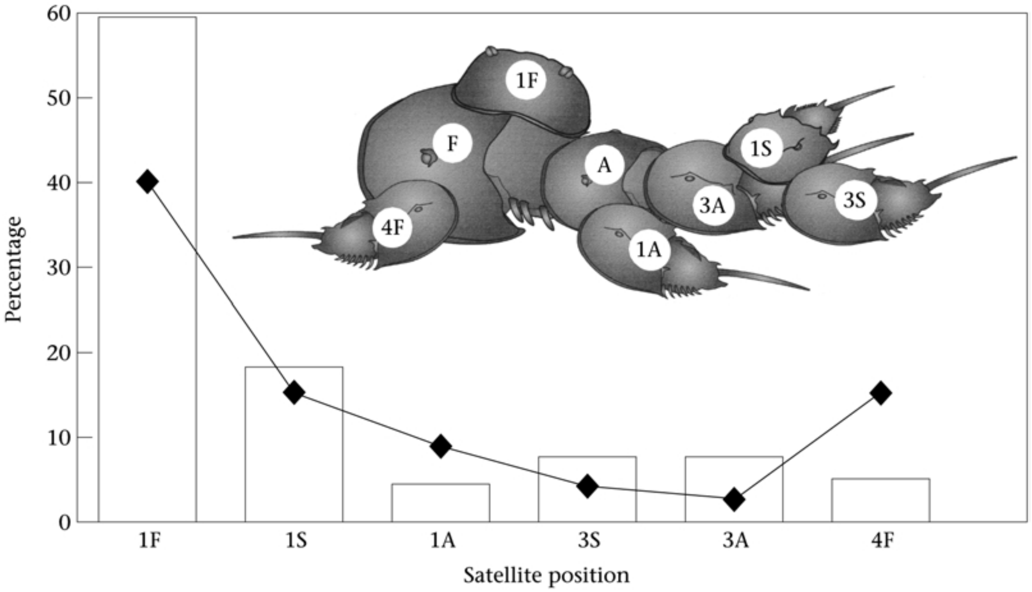

Figure 5. Frequency distribution of positions in which satellite male horseshoe crabs were found in the field at Seahorse Key (☐) and their associated average paternity per male (◆) when in those positions (N=106 satellite males), both expressed as a percentage (drawing modified from Brockmann 1990). F: female; A: attached male; S: satellite male. Position 1 is over the canal between the prosoma and opisthosoma and may be on either the right or left sides of the female (1F), attached male (1A) or satellite (1S). Position 3 is over the terminal spines and tail of the attached male (3A) or a satellite (3S) and position 4 refers to all other positions around the female (4F), attached male (4A) or satellites (4S).

The effect of current was particularly strong on the incoming tide (r_S=0.94, N=6, P=0.03; Fig. 4). Attached male paternity was lower, on average, on the incoming tides (5.9 ± 4.8%, N=5) than on the outgoing tides (25.4 ± 8.8%, N=13) and highest at the time of the maximum high tide (30.0 ± 7.3%, N=22) when there was little current, but the effect was not significant (Kruskal–Wallis test: H_{23}=3.4, P=0.18).

Effect of Satellite Behaviour on Paternity

Satellites had strong preferences for particular positions around the female. The position over the female's incurrent canal (Fig. 5, position 1F) was occupied far more frequently than any other. There was a large difference in success depending on whether the satellite was in position 1F or not (Kruskal–Wallis test: H_{34}=19.2, P=0.0002; Fig. 5): satellites in position 1F had, on average, 40% (± 4.5%, N=62) paternity, overwhelmingly more than in other positions. Males that were satellites on the attached male (rather than the female) fertilized on average 7.5 ± 3.0% (N=13) of the eggs in a clutch and those that were satellites on other satellites fertilized 12.2 ± 3.9% (N=25), while attached males fathered 26 ± 5.0% (N=40). Although males did better when they were in particular positions around the female, if a satellite changed position during a clutch, he was much less likely to father the eggs of that clutch (mean success for animals that moved: 10 ± 3.7%, N=22; did not move: 33 ± 3.7%, N=84; Mann–Whitney U test: U=481, P=0.0005).

When in position 1F, satellite males pushed and shoved on the attached male to get under the front margin of his carapace ('under' position) and once in this position, the satellite male usually remained for the entire nesting. Overall, the under position did not have a significant effect on paternity: satellites that were over the attached male's carapace ('over' position) fathered the same proportion of the eggs in a clutch (29 ± 9.2%, N_1=14) as those that were in the under position (42.8 ± 4.9%, N_2=50) (Mann–Whitney U test: U=261, P=0.15). However, when two satellite males were present and both were in position 1F, then the under position affected paternity. When both satellite males were in the over position, the attached male was as successful as when only one satellite was present (45.4 ± 18.7%, N=5), but the attached male's success dropped precipitously when one (34.3 ± 8.0%, N=19) or particularly both (9.5 ± 3.3%, N=15) satellites held the under position (Kruskal–Wallis test: H_{23}=7.8, P=0.02; Fig. 6). This meant that when both satellites were under the attached male's carapace their average success (43 ± 2.7% each, N=39) did not differ from the average success of a single satellite spawning in the 1F position (45%; see Fig. 3). The success of other satellites in the group was not affected by the number of satellite males that were in a position under the attached male's carapace (Kruskal–Wallis test: H_{23}=2.2, N=19, P=0.29).

Variation in Paternity among Satellite Males

A major source of variation in success among satellite males occurred because one of the two males in position 1F usually achieved far greater paternity than the others, even when both were in the under position. The average paternity for the more successful of the two satellites,

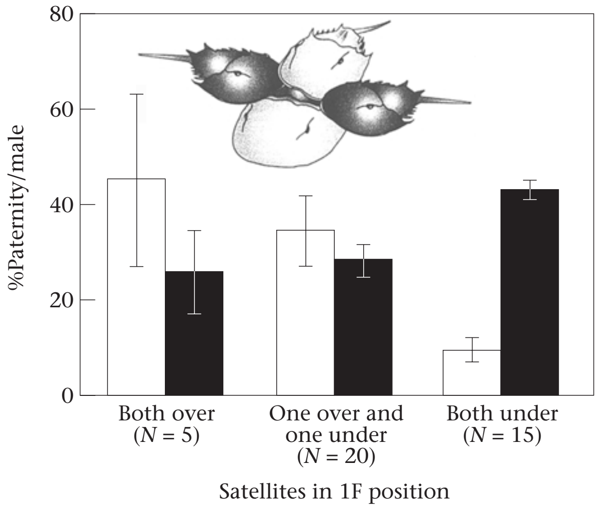

Figure 6. The average percentage of paternity of the attached male (□) and of two satellite (■) males when both satellites occupied the 1F position (see Fig. 5) and both satellite males were in the over position, one was under and one over, or both males were under the attached male's carapace. Sample sizes are number of clutches. The drawing (modified from Brockmann et al. 1994) illustrates the case of one satellite under (left) and one satellite over (right) the front margin of the attached male's carapace.

called the S1 male, was 56.4 ± 5.2% (N=39), which was significantly greater than for the less successful male in position 1F, called the S2 male, which averaged 11.9 ± 2.0% (N=39) of the eggs fertilized (Wilcoxon signed-ranks test: $Z=-5.170$, $P<0.0001$). The S1 male was significantly more successful than the attached male (Wilcoxon signed-ranks test: $Z=-2.782$, N=40, P=0.0054), whereas the S2 did not differ in success from attached males (Wilcoxon signed-ranks test: $Z=-1.77$, N=39, P=0.075) or from males that were not in position 1F, called S3 males ($Z=-0.094$, N=14, P=0.92). What might explain these high levels of variation in male success?

Effect of tide and current

Horseshoe crabs nested at the top of the high tide line (in Florida but not in Delaware; Penn & Brockmann 1994) on both the incoming and the outgoing tides, which meant that nesting animals faced different tidal currents during one tide. If sperm were being washed over the female's eggs by water currents, it seemed possible that tidal currents could affect satellite paternity. However, S1 males that were upcurrent were no more successful on average (43 ± 5.7%, N_1=33) than those that were downcurrent (36.4 ± 6.6%, N_2=31; Mann–Whitney U test: U=440, P=0.33) and the same held for S2 males (upcurrent 12.4 ± 3%, N_1=16; downcurrent 10.8 ± 2.8%, N_2=19; Mann–Whitney U test: U=136, P=0.6) regardless of whether they were nesting before or after the tide. The current speed did not differentially affect the paternity of either S1 (r_S=0.26, N=40, P=0.11) or S2 (r_S=0.1, N=39, P=0.62) males.

Effect of clutch characteristics on paternity

Although clutch completion time varied (time to complete one clutch: 5.3 ± 0.38 min, range 2–13 min, N=40), paternity was not affected by this factor (attached male: r_S=0.01, N=40, P=00.94; S1 male: r_S =0.015, N=40, P=0.93; S2 male: r_S=0.14, N=39, P=0.38) and there was no correlation between female carapace width and clutch completion time (r_S=0.14, N=15, P=0.62). Clutch depth also varied (12 ± 0.39 cm, range 7–17 cm, N=40), but paternity was not correlated with egg depth and there was no correlation between female size and egg depth (r_S=0.09, N=15, P=0.72). Although our sample consisted of two to five clutches for each group, paternity of the attached males and satellite males did not change with clutch number (Kruskal–Wallis test: H_{34}=2.1, P=0.54). Clutch size differed markedly between females (range 250–2650 eggs), but these differences were not associated with female size (r_S=0.10, N=15, P=0.26), female condition or male paternity for attached or satellite males.

Effect of male size and condition

Attached males did not differ in size from satellite males but they were, on average, in somewhat better condition (chi-square test: χ^2_1=5.3, N=137, P=0.02). Fertilization success of attached males was not correlated with their size or condition, but was correlated with female size (CW: r_S=0.65, N=15, P=0.015; Fig. 7) but not with female condition. Because of the many variables interacting in this data set, we conducted an ANOVA for categorical data (Agresti 1996). Two variables were strongly associated with increased attached male success when combinations of variables were examined together (GLM: $F_{16,23}$=9.93, r^2=0.87, $P<0.0001$): larger female size (CW: $F_{5,15}$=9.5, P=0.0001) and better female condition ($F_{7,15}$=4.26, P=0.0038). Since the number of satellites in the under position was associated with male success, we also examined the factors that affected this variable: poorer attached male condition ($F_{7,22}$=15.3, P=0.0001) and higher weight of the S1 male ($F_{15,22}$=5.6, P=0.0006) showed the strongest association (GLM: $F_{22,17}$=9.63, r^2=0.93, $P<0.0001$) with being in the under position.

S1 and S2 males did not differ in size or any measure of condition and furthermore their paternity was not associated with their size or condition. Female size did not affect the success of S1 (Fig. 7) or other satellite males. No measure of female condition was correlated with the success of either the S1 or S2 males. Taken together, two variables were associated with S1 male paternity: the condition of the attached male ($F_{7,17}$=4.77, P=0.004) and the weight of the S1 male ($F_{15,17}$=5.25, P=0.0008) (GLM: $F_{16,23}$=8.15, r^2=0.92, P=0.001). We found no significant relationships explaining S2 male paternity.

DISCUSSION

Satellite males nesting in groups of two to four are highly successful at fertilizing eggs in competition with attached males: 74% of the eggs were fathered by satellites. This means that satellites in this study had a per capita success

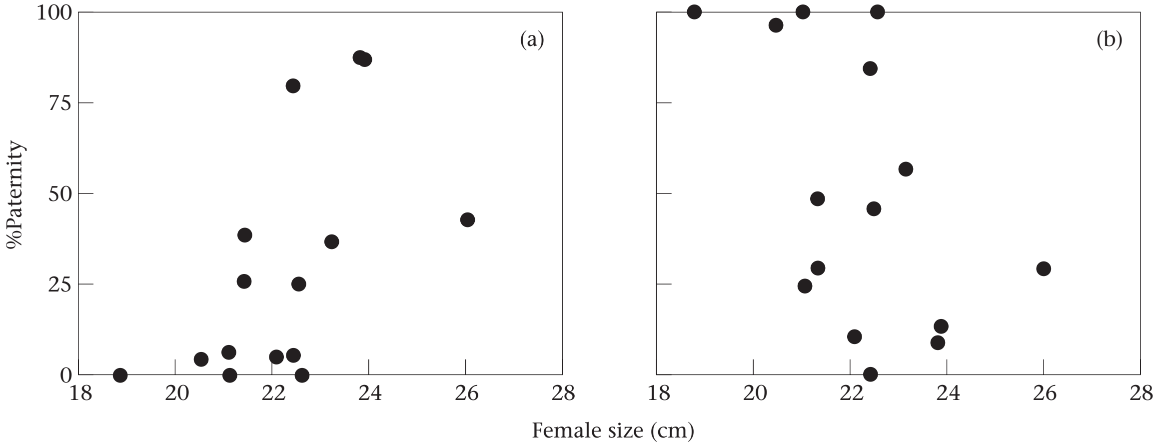

Figure 7. The correlation between female size (carapace width) and paternity of (a) attached males r_S=0.65, P=0.015, and (b) satellite males (S1: r_S=−0.4, P=0.12) for the first clutch of 15 nests.

rate (28.4 ± 3.2%, N_1=104) comparable to that of attached males (25.5 ± 5.0%, N_2=40) (Mann–Whitney U test: U=1957, P=0.58). This high success rate of satellites may explain one surprising feature of horseshoe crab behaviour: despite intense male–male competition, few take-overs of the attached male's position occur (Brockmann 1990; Brockmann & Penn 1992). The results of this study suggest that satellites have little to gain in an immediate sense from the intense, long struggle that precedes a take-over.

Although sperm can easily survive at least one tidal cycle (12 h) at natural beach temperatures and unfertilized eggs continue to remain viable for at least 40 min (and probably longer), the vast majority of eggs were fertilized by the males that we observed in the nesting group. In this study only two eggs (0.2%) were fertilized by unknown males. We had similar results in our previous study (when there was one satellite per group; Brockmann et al. 1994) with only 11 of 324 eggs (3%) being fertilized by unknown males. This means that males may be able to fertilize eggs when not directly involved in a nesting due to sperm that the female is carrying under her carapace from previous nesting or due to sperm that are present in the sand from previous spawning, but the effect is very small.

Horseshoe crabs at Seahorse Key approach the beach 1–2 h before the maximum high tide and stay at the beach 1–2 h afterward (Penn & Brockmann 1994). Satellites arrive at the same time as the paired animals and remain on the beach throughout the tide, clustered around the nesting couples. Our results show that satellite male success is highest on the incoming and outgoing tides and lowest during the slack tide, the hour around the maximum high tide. Because sperm are travelling in water currents to the female's eggs, this result is not surprising, but we do not yet understand the mechanics of water flow around a nesting couple nor why tidal currents interfere with fertilization by the attached male (or enhance the satellite male's ability to fertilize eggs).

As with other externally fertilizing species with sperm competition (Thomaz et al. 1997; Mjølnerød et al. 1998), satellite male position affects success. When satellite male horseshoe crabs are in position 1F, which is directly over the female's incurrent canal (Fig. 5), they reduce the paternity achieved by the attached male. Satellites are dumping sperm into the canal or into currents that bring the sperm into contact with the eggs. When there is only one satellite, he often moves around the female until he gets into the favoured position (Brockmann 1990). When there are several males, they compete to get into position 1F on either side of the female (Brockmann et al. 1994). Satellites repeatedly push on and undermine the anterior margin of the attached male until they are securely underneath. The attached male responds by bucking his carapace, lashing his spiny tail from side to side, and holding onto the female very tightly. Attached males are extremely difficult to dislodge and might lose a claw rather than let go. Once in position 1F, the satellites usually remain there through many clutches, unless knocked away by waves or another male. It is now clear why males fight for this position: they achieve far higher success than in any other position. A satellite that changes position during a clutch has greatly reduced paternity, but this may occur because he moved or he may have moved because he was not achieving high fertilization success in his previous location; this study will not allow us to distinguish these two possibilities.

The most striking result of this study is that when two satellite males are in position 1F, both under the front margin of the attached male, their average success is the same as when they are nesting alone with a nesting couple. Larger group sizes, however, result in reduced paternity for satellites. Put another way, when two satellites are in the 1F under position, the attached male is particularly unsuccessful. This would suggest that there should be more groups than expected on the beach with one or two males and fewer with more. Brockmann (1996) shows this result from data collected in Delaware where the density of nesting horseshoe crabs is much higher than in Florida, but unfortunately her study also shows that there are more groups without satellites and more groups with large numbers of satellites (more than five) than expected. Clearly, more work is needed to explain these results.

An alternative interpretation exists for the high success of two satellites in competition with attached males. Gage et al. (1995) and Parker et al. (1996) modelled a situation quite like that of group-spawning horseshoe crabs where males gather around a female and ejaculate when the female sheds her eggs. In this model sperm are expensive and success is determined by the relative numbers of sperm present in the 'raffle' (Parker 1990). The model predicts that ejaculate effort (number of sperm released) should increase with the number of competitors (Stockley et al. 1997). Because satellites always face sperm competition whereas attached males often nest alone with a female, we can expect that, on average, satellites will invest relatively more in sperm than attached males. Furthermore, Parker et al.'s model predicts that there should be a level of sperm competition at which a male should choose not to join in spawning (Parker 1998). This suggests that attached males might cut back on sperm when in particularly competitive situations and what we are seeing as differences in success may be due in part to differences in investment by attached males (known to occur in an externally fertilizing fish; Shapiro et al. 1994). The only way to know which interpretation is correct is to find a way to measure the relative quantities of sperm released by males under different conditions (Petersen & Warner 1998).

Brockmann & Penn (1992) describe profound behavioural and physical differences between males. Attached and satellite males do not differ in size, but satellite males are, on average, in worse condition and they are probably older than attached males (also found in this study). Satellite males also are less likely to find a female, and when they do, they pair more slowly, are more likely to let go of the female, are less likely to return with the female on the following day and are less active than the younger, attached males, which are often in better condition. Satellite males are also less likely to right themselves if turned over (Penn & Brockmann 1995). We do not know the underlying causes of these differences between attached and satellite males. They may be due to the accumulated effects of years of wear and tear, ultraviolet damage, parasitism and settlement by benthic organisms or they may reflect differences among males in their resistance to parasitism, settlement and immune function. Despite all these differences between satellite males and attached males, we were not able to find any differences in paternity between them. Paternity was also not associated with differences in condition either for satellite males or attached males.

One of the most puzzling features of our data is the high variability in paternity under seemingly similar conditions, a result shared by others studying alternative male strategies (Phillip & Gross 1994; Thomaz et al. 1997; Mjølnerød et al. 1998). When two males are in the same 1F position on either side of the female, usually one male achieves far higher success than the other. This poses the question of whether there are differences between males in their ability to compete for paternity. Our results suggest that the condition of the attached male and the size of the S1 male may be associated with differential success between S1 and S2 males, but a study specifically designed to evaluate this question is needed. We suspect that the two males differ either in the quantity of sperm released, in the timing of release, or in their ability to set up currents that bring sperm in contact with eggs. In some species males differ in their investment in testis per body weight (Kusano et al. 1991; Bass 1996; Halliday 1998; Petersen & Warner 1998). The reason that we have not evaluated this possibility in *Limulus* is that the testis is a network of anastomosing tubules throughout the opisthosoma and prosoma where it mingles with the hepatopancreas (Fahrenbach 1973) making it impossible to measure.

It is not easy, either in a conceptual or a practical sense, to separate the influence of the male and the female on sperm competition (Knowlton & Greenwell 1984). For example, males or females may be affecting the flow of water around the nesting couple, which may give the advantage to one male over another. Nesting females clearly have some control when they leave the beach, shed their satellites and return a few minutes later to continue nesting with the same attached male and new satellites (Brockmann 1996). One consistent result in this data set is that attached males are more successful when nesting with larger females. We do not know the reason for this but it may be that attached males are relatively closer to the eggs or are able to set up more favourable currents when nesting with larger females.

Like our previous paternity analysis for groups with one satellite (Brockmann et al. 1994), the present study reveals that satellites are unusually successful. In other externally fertilizing species, the average success rate is 5–17% (Maekawa & Onozato 1986; Hutchings & Myers 1988; Jordan & Youngson 1992; although some studies have found levels of 26–33%: Philip & Gross 1994; Thomaz et al. 1997) rather than 40% paternity for one satellite with a pair (Brockmann et al. 1994) or 74% paternity for two to four satellites with a pair. In fact, this study shows that attached and satellite behaviour have about equal success under the highly competitive conditions of this study. But paternity is not the only factor in the equation. The success of attached males also depends on the probability of finding a female, the risks associated with getting to the beach with a female and the risks associated with nesting (such as ultraviolet exposure, increased exposure to settlement between tides) and remaining attached (such as the inability to feed while attached). The success of satellite males depends on the probability of locating a group and attaining the 1F position and the risks associated with joining multimale groups, such as being overturned in waves. This study has shown that satellite behaviour has considerable benefits but it may also have greater costs than attached male behaviour (Botton & Loveland 1989; Penn & Brockmann 1995). Complete time budgets of satellite and attached males are needed to evaluate the relative success of the two patterns of behaviour.

Levitan (1998) argues that in externally fertilizing invertebrates, sexual selection is driven by both sperm limitation and sperm competition. The results of this study clearly demonstrate sperm competition in an externally fertilizing invertebrate. Male horseshoe crabs have evolved specific behavioural adaptations for sperm competition such as positioning themselves over the female's incurrent canal, fighting with the attached male until the 1F under position is attained, and responding to satellites with specific behaviour patterns (Brockmann 1990). Female adaptations to enhance fertilization success also exist. Brockmann (1990) observed that buried (i.e. apparently nesting) females with no attached males, but with males in the satellite position, do not lay (these are egg-laden females with broken terminal spines, which makes it difficult for males to remain attached). This study also showed that when attached-male function was disrupted, satellites did not end up fertilizing all the eggs, which may explain the behaviour of the unattached females. Such disruption may occur under natural conditions with a take-over when an attached male may literally be lifted above the female by the interloper (Brockmann 1990); this may explain why females usually leave the beach if a prolonged take-over occurs. Under most normal conditions, however, there is little evidence of sperm limitation in the sense that almost all eggs are fertilized (1.2 ± 1.2% undeveloped, Brockmann 1990). With a strongly synchronized pattern of mating and a male-biased operational sex ratio, with highly mobile males that attach and travel with females as they move among nesting sites and with the frequent occurrence of multimale groups spawning with one female, it is clear that sperm competition is an important component of the reproductive behaviour of this species. The research presented here was evaluated and approved by the Animal Behavior Society's Animal Care Committee on 27 April 2000.

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