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Dr. C.N.Shuster Jr.
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Dear Dr. Shuster:

As promised, I am sending herewith the revised manuscript of our paper on L. polyphemus. You will note that several portions have been deleted in accordance with the journal review -- but these sections may someday re-appear elsewhere! Your comments and papers were extremely helpful to us in this revision and we have cited you in several places as "personal communication", as you suggested. We would appreciate hearing from you as to whether you approve of these citations.

Once again, many thanks for your assistance.

Best regards,

A handwritten signature in cursive script, reading "Jim Cohen".

James A. Cohen

Encl. Revised Limulus ms.

For C.N. Shuster
With Thanks.
Jim Cohen

Revised

BREEDING ACTIVITY AND MATE SELECTION IN THE
HORSESHOE CRAB, *LIMULUS POLYPHEMUS*

James A. Cohen and H. Jane Brockmann

Running Head: Cohen and Brockmann: Mating in *Limulus*

ABSTRACT

The breeding behavior of a living fossil, the horseshoe crab (*Limulus polyphemus*), was studied at Seahorse Key, Florida. This *Limulus* population differs from others that have been studied in lunar and diel rhythmicity, and in the location of nests. Large numbers of females nest at the tide line each spring and summer during high tides associated with full and new moons. Males arrive on the beach either accompanying the females, clasping their carapaces, or alone, joining other nonclasping males in breeding groups around pairs. Recapture data show that nonclasping males return to the beach with a much higher frequency than do females and clasping males. Moreover, circumstantial evidence suggests that these nonclasping males may also shed sperm into nests, thereby achieving some fertilizations. The strongly male-biased sex ratio on the breeding beach would suggest strong competition for access to females. Despite this expectation, we found no displacements of clasping by nonclasping males, no evidence of assortative mating for body size and no differences between clasping and nonclasping males in body or clasper size. Seven nests were excavated and five clutches were counted, ranging from about 2,000 to 30,000 eggs. This suggests substantial differences in fecundity among females. However, we have no clear evidence that males use such differences in choosing mates.

The American horseshoe crab, *Limulus polyphemus*, is widely regarded as a phylogenetic relic or "living fossil" (Selander et al., 1970; Riska, 1981). Despite its abundance and ease of observation, surprisingly little is known about the behavior of *Limulus*. Natural history studies by Shuster (1955, 1979) have helped establish the broad outlines of *Limulus* ecology, but only Rudloe (1978) has directly investigated behavioral questions in any depth, stressing population-level studies of orientational mechanisms (Rudloe and Herrnkind, 1976), movements and activity patterns (Rudloe, 1978, 1980). We studied mating and nesting behavior of individually marked *Limulus* in an effort to assess the factors influencing reproductive success in this species. In addition, we describe tidal and diel rhythms in a population which shows interesting differences from those studied by other workers.

Breeding behavior of *Limulus* is readily observed on sandy beaches along the Atlantic and Gulf coasts of North America. In Florida, nesting is restricted to spring tide periods and then only for a few hours around the time of each high tide (Rudloe, 1978, 1980; personal observations). Females are 35 to 50% larger than males (Shuster, 1955). When approaching the beach to nest, a female is almost always accompanied by a male clasping her with his modified claws. They may be met by one or more unattached (here called "satellite") males who follow them closely until the female has selected a nest site at the high tide line. She then arches her prosomal-opisthosomal joint (the "hinge"; Shuster, 1955), thereby forcing her prosoma slightly downward into the sand. By

pushing backwards with her modified sixth pair of legs, she advances forward as she extends the hinge. She continues in this manner, until she is buried completely or, more commonly, to the level of the lateral eyes. She may remain in this position, with occasional digging movements and postural adjustments, for up to one hour until egg-laying and external fertilization occur. During the entire nesting process, the satellite males (if present) move around the pair, sometimes apparently attempting to wedge the leading or lateral edges of their prosomas underneath the body of the clasping male. The satellite males appear to be vying for "preferred" locations around the female and attempting to dislodge the clasping male (which we have never witnessed). In this paper, we examine more closely the nature of this apparent reproductive competition.

METHODS

The study was conducted at the University of Florida Marine Laboratory, located on Seahorse Key (29°06' N, 83°04' W), an island in the Gulf of Mexico, approximately 4 km SW of Cedar Key, Levy County, Fl. The south side of the island has a long, sandy, low-energy beach. Immediately offshore there is a 10 to 15 m muddy sand flat, followed by a 30 to 50 m zone of the seagrass *Halodule beaudettei*. This is replaced abruptly by a 100 to 150 m zone of the sea grass *Thalassia testudinum*. *Limulus* can often be found at low tides, partially buried in the intertidal grass and mud flats.

Initial observations were made on 12 and 13 April 1980 when, with the aid of 20 undergraduate students, we marked (with temporary tags) and measured nearly 500 *Limulus*. During this time we noted that most nesting occurred during a period of 1.5 h prior to, until 0.5 h after, the predicted time of the high tide, and was largely restricted to the central 700 m stretch of the south beach. Thus, all later observations of breeding behavior were confined to this location and time.

Regular mark-and-recapture studies were then conducted by one of us (JAC), usually for 2 days and 2 nights (spanning 3-5 high tides; = one "tidal series") centered around each spring tide for the period 12 July to 10 September 1980. The observer continually walked along the 700 m transect line, marking each animal encountered. Plastic tags with embossed numbers (Dymo Visual Systems, Inc., Augusta, Ga.) were affixed with thumb tacks to the animal's prosoma, antero-lateral to the right compound eye. This method can be used with a minimum of handling of animals, caused no apparent discomfort, and rarely interrupted ongoing activity. Tags of individuals recaptured up to four months after marking showed no indications of loosening. Thumb tacks used by Sokoloff (1978) were still firmly in place in *Limulus* recovered 2 years after marking. One possible source of tag loss might be molting, but most molting occurs in winter (Rudloe, 1978). Hence we assume tag loss to be negligible throughout our study.

Males were scored at first sighting for one of three mutually-exclusive mating status categories:

--*Clasping*: the male is positioned directly posterior to the female, grasping the posterior margin of her opisthosoma with one or both clasps.

--*Satellite*: the male is not clasping a female but is within 10 cm of her, or of a male clasping her, or of another satellite male associated with her. There are normally 0-6 satellite males associated with a female at any one time.

--*Solitary*: the male is neither clasping nor satellite. For analysis we will sometimes combine solitary and satellite males into a category called "Nonclasping".

Upon sighting each female, we noted the total number and identity (tag numbers) of any clasping and satellite males associated with her.

We measured the body size of all individuals as the interocular (IO) width, which is the linear distance between the opthalmic spines dorsal to the lateral eyes. This measure is highly and significantly correlated with the more traditional index of body size, ventral prosomal width ($r = 0.84$, Pomerat, 1933; $r = 0.85$, Cohen and Brockmann, unpublished data), but is preferable for our studies since it does not usually require handling and disturbing the animals. For animals resighted on subsequent tides, we again noted the tag number, mating status category (if male), and number of associated males (if female).

We returned to Seahorse Key with another group of 20 students on 3 May 1981 to repeat some of our measurements. In addition, we separated 24 pairs in order to measure the tibial lengths of the males' right claspers. These were compared with the tibial lengths of the satellite or solitary male nearest each pair. In order to obtain egg clutches for counting, we placed stakes beside 7 nesting females and excavated their nests several hours later during low tide. To our knowledge, these are among the first actual counts of *Limulus* clutch sizes.

RESULTS

Activity Patterns

The numbers of *Limulus* adults on or visible from the breeding beach are shown in Fig. 1. In general, more adults appeared on the beach during high tides of the full moon than those of the new moon of the same month (although the predicted heights of the full and new moon tides were similar; U. S. Dept. Commerce, 1979).. Although breeding occurred during both daytime and nighttime high tides throughout the summer, daytime numbers consistently exceeded nighttime numbers ($P < 0.05$; Sign Test). Moreover, the predicted heights of day tides were always higher than those of night tides of the same date ($P < 0.05$; Sign Test; Fig. 1).

Males and females were recaptured in about the same proportions as they were originally marked. Thus, of a sample of 267 tagged *Limulus*, 73% were males, compared with 78% of the 95 recaptured

animals ($\chi^2 = 0.73$; $P > 0.05$). This result pools recaptures within, as well as among, tidal series (but not within a single tide). However, when we compare the numbers of animals seen only during a single tidal series with those encountered in more than one series (Table 1), we find that nonclasping males were more likely than either females ($\chi^2 = 12.2$; $P < 0.001$) or clasping males ($\chi^2 = 16.7$; $P < 0.001$) to be seen again sometime during the breeding season. Only two pairs returned to the beach together after a two-week period, presumably having remained clasped for that length of time. Four other females were resighted with new males.

Sex Ratios and Mate Selection

Sex ratios in the breeding population normally ranged from 2 to 6 males per female (Fig. 2). On two high tides (8 Sept: day; 9 Sept: night), no females were found but male numbers on those occasions were also very low. The numbers of both sexes were reduced at night (Sign Test; $P < 0.05$), but the percent change between night and day was more pronounced for females than for males, and this resulted in higher nighttime sex ratios (Sign Test; $P < 0.05$). There was an inverse relationship between sex ratio and the predicted height of the tide (Sign Test; $P < 0.05$; Fig. 2).

We compared both the interocular (IO) widths and right tibial lengths of clasping and nonclasping males, finding them, in each case, not to be significantly different (Table 2).

About half of the pairs were accompanied by at least one satellite male. About 2% had more than 5 (max = 9) such males,

suggesting that certain females may be preferred over others. In order to test whether males prefer larger females as mates, we compared the number of clasping plus satellite males associated with each of 225 females of 3 different IO size classes: 100-120, 121-140, 141-160 mm. No effect of female body size on the number of males present was detected ($\chi^2 = 6.4$; $P > 0.05$). To test for assortative mating for body size, we compared the IO widths of females with those of their clasping males. These correlations were not significant ($P > 0.05$) for either the 1980 ($r = 0.18$; $N = 96$ pairs) or 1981 ($r = 0.19$; $N = 95$ pairs) sample.

Table 3 presents what we believe are the first systematic counts of natural clutch sizes in *Limulus*. As can be seen, these vary from about 2,000 to 30,000 eggs per nest. Female number 347 laid her 29,165 eggs in what appeared to be 8 distinct "subclutches" (range: 1199 to 9610 eggs), all at approximately the same depth and within a few centimeters of one another. No general relationships are evident between clutch size and either female body size or the number of attending males, but it may be noteworthy that the female with the largest (by far) clutch was surrounded by the largest (also by far) number of males. We do not know which, if any, of these females may have bred previously, a factor that could affect observed clutch sizes.

DISCUSSION

Our results differ from those of Rudloe (1978, 1980) with respect to activity patterns. We found that during the summer on

Seahorse Key, *Limulus* breed during both daytime and nighttime high tides, with a slight preference for the former (see Fig. 1). However, the explanation for this is not clear. It may be that the animals prefer nesting during the day or that they have a preference for nesting on the highest possible tides, which were daytime tides during this period. In contrast, Rudloe's mainland population, only some 150 km away, along the Florida panhandle, bred almost exclusively at night during the summer months, despite lower nighttime tidal heights (Rudloe, 1980). The factors determining these population differences are not known but may include differential risks from diurnal and nocturnal predators at different sites.

The male-biased sex ratios on the breeding beach apparently do not reflect an overall skew in the population, since Rudloe (1980) reported female-biases of up to 2.5 females per male in offshore trawling samples. Also, Koons (1883) found a sex ratio of unity among a sample of subadult *Limulus* in New England. These results suggest that only a fraction of the adult females in the population come to the beach to breed at any given time. In contrast to a female, a male might produce gametes more or less continuously and increase his fitness by more frequent appearances on the breeding beach, as found by Rudloe (1978, 1980). We have now refined this result (Table 1) by showing that nonclasping males are 4 to 6 times more likely than females or clasping males to return to the same breeding beach. Since females contain an estimated 88,000 eggs (Shuster, personal communication), only a small proportion of which will be laid in a single nesting (see

Table 3), we think it likely that the pairs leave Seahorse Key to breed again elsewhere.

Assortative mating for body size has been reported for a number of externally fertilizing animals (see, e.g., Willoughby and Pomerat, 1932; Davies and Halliday, 1977). Pomerat (1933), however, found no such correlation in *Limulus* mate sizes (Ridley and Thompson's [1979:380] statement to the contrary notwithstanding). Shuster (personal communication) reports the presence of assortative mating for five of ten measured body characters, including prosomal width but excluding interocular (IO) width. Our data on the latter character also do not demonstrate assortative mating. Since we found a high correlation between prosomal width and IO width (see earlier), there would likewise be no significant assortative mating for prosomal width in our population. It would therefore be interesting to know whether these two characters are not correlated in Shuster's population on Delaware Bay. Such a difference in body proportions among sites could produce the reported differences in assortative mating.

Since the sex ratios on the beach were male biased, we expected competition for females to be severe. Under similar conditions for many other arthropods (see, e.g., Blum and Blum, 1979), male body size correlates with success in gaining mates. Thus, we were surprised to find that this was not the case in our *Limulus* population. Faced with this result, we hypothesized that sexual selection might instead act on other secondary sexual characteristics, such as clasper size. However, Table 2 shows that clasper tibial lengths of clasping and nonclasping males do

not differ significantly. Other possible determinants of male mating success (e.g. speed, agility, clasper strength, hormonal levels, general "pugnacity", etc.) remain to be studied.

We hasten to add that the significance of clasping itself in terms of paternity is not at all clear, for Shuster (personal communication) has observed satellite males milting on the beach. It therefore seems possible that these males might well shed sperm into open nests. After the female leaves her nest together with her clasping male (pairs never separated after spawning; cf Barnes, 1980:595), the satellite males present during nesting often remain behind, circling around and walking slowly over the nest site. If they are indeed shedding sperm and gaining fertilizations at this time, then the real advantage accruing to a clasping male may be simply a mechanical one: he has a greater chance of being present at the time of egg-laying than do nonclasping males who are frequently dislodged by waves and conspecifics. On the other hand, despite hundreds of observations of males apparently attempting to dislodge a clasping male, we have never witnessed a single such displacement. Sperm competition studies (see, e.g., Parker, 1970) would be valuable in determining the relative paternity-advantages of clasping and nonclasping.

Our results on clutch sizes (Table 3) contradict numerous statements in both the popular (e.g., Teale, 1957:365) and professional (e.g., Barnes, 1980:595; Shuster, 1950) literature that females lay only 200 to 300 eggs per nest. More recently, Botton and Shuster (personal communication) have estimated that clutches contain a mean

of 3,650 eggs. Sokoloff (1978:226) relates that egg masses "may contain some 1,000 eggs", but undisturbed females may deposit over 10,000 eggs per nest. One female we observed (#347; Table 3) laid over 29,000 eggs in a single nest. These were distributed among 8 "subclutches" with a mean of 3,646 eggs per subclutch. Interestingly, Schrank et al. (1967) report that electrical stimulation of females results in the release of 2,000 to 3,000 eggs. Further stimulation of the same female within 15 minutes yields yet another "subclutch". It is possible that the occasional "postural adjustments" of nesting females (see earlier) are associated with the phenomenon of subclutching.

It is intriguing that female #347, who produced an exceedingly large clutch, had been surrounded by an unusually large number of males (8). Indeed, this is one of the highest breeding group sex ratios we have witnessed at Seahorse Key, and occurred on a day when 90% of all nesting females were surrounded by three or fewer males. While this may be a chance result, it might instead suggest that males can somehow assess the differential fecundity of females and are attracted to those who will lay the largest clutches. Alternatively, it is possible that the stimulation of a large breeding group in some way induces a female to lay more eggs than she otherwise would. Finally, it is possible that the longer a female remains in a nest, the more eggs she lays and the more males she accumulates through random processes. We are continuing investigation of this issue.

Finally, we can identify three aspects of the breeding behavior of *Limulus* that illustrate considerable diversity among

populations. (1) Lunar rhythmicity: In Florida (Rudloe, 1978, 1980; this study) and some northern areas (Shuster, personal communication), *Limulus* are known to breed mainly during spring tide periods, whereas breeding activity of some other northern populations (e.g. Cavanaugh, 1975; Riska, personal communication) is apparently not dependent upon the lunar cycle. (2) Nest location: Florida *Limulus* usually nest at the high tide line, whereas some populations in Massachusetts and South Carolina apparently nest subtidally, in up to 1 m of water (Riska, personal communication; see also references in Rudloe, 1980). Botton and Shuster (personal communication) found most nests in Delaware Bay in the intertidal zone, rather than at the high-water mark. These differences may be related to the relative risks of predation by land-based and aquatic egg predators at different sites. At Seahorse Key, we have found schools of *Fundulus majalis* and *F. similis* burrowing into the sand to feed on *Limulus* eggs in those few nests that were intertidally located. These fish also swarm around stationary *Limulus* in the water. The choice by females of high daytime tides for nesting (Fig. 1), places eggs high up on the beach where they will be protected from such egg predators. While there is, therefore, selection against intertidal nesting due to *Fundulus* at Seahorse Key, it is probably counteracted to some extent by predation from shoreline-wading birds, of which there are large numbers. The balance of such selective forces may vary among sites. (3) Diel rhythmicity: Both Rudloe (1980) and Cavanaugh (1975) report night-only breeding during at least part of the breeding

season, whereas we have found *Limulus* breeding during both daytime and nighttime high tides. Further investigation of such differences may well reveal a degree of local, behavioral adaptation comparable to that reported for morphological (Riska, 1981) and genetical (Selander et al., 1978; Shuster, 1955, 1979) traits.

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Table 1. The numbers of females, clasping males, and nonclasping males observed during only one tidal series versus more than one tidal series.

Status when originally marked	Observed		Totals
	Once	More than once	
Female	65 (92%)	6 (8%)*	71
Clasping male	63 (95%)	3 (5%) ⁺	66
Nonclasping male	89 (70%)	38 (30%) [§]	127 ^Φ

* Includes 4 resighted with new males.

⁺ Includes 1 resighted as nonclasping.

[§] Includes 6 resighted as clasping.

^Φ Three nonclasping males omitted due to incomplete data.

Table 2. Interocular widths and tibial (clasper) lengths of
clasping and nonclasping male *Limulus*.

		1980 Sample		1981 Sample	
		Clasping	Nonclasping	Clasping	Nonclasping
Interocular width (mm)	\bar{X}	92.7	93.1	94.8	96.6
	S^2	61.3	71.1	132.6	58.3
	N	203	318	96	62
		t = 0.55		t = 1.18	
Tibial length (mm)	\bar{X}			11.3	11.8
	S^2			4.9	4.7
	N			24	24
		t = 1.03, matched pairs			

P>0.05 for each comparison (t-test)

Table 3. Female interocular widths (IO), clutch sizes, and the number of males (clasped plus satellite) at 7 nests, 3 May 1981.

<u>♀ Tag #</u>	<u>IO (mm)</u>	<u># Eggs/nest</u>	<u># Males present</u>
427	132	4,782	2
1094	135	8,511	1
537	144	0	2
1083	150	2,615	1
431	152	8,187	2
347	*	29,165 ⁺	8
115	*	0	2

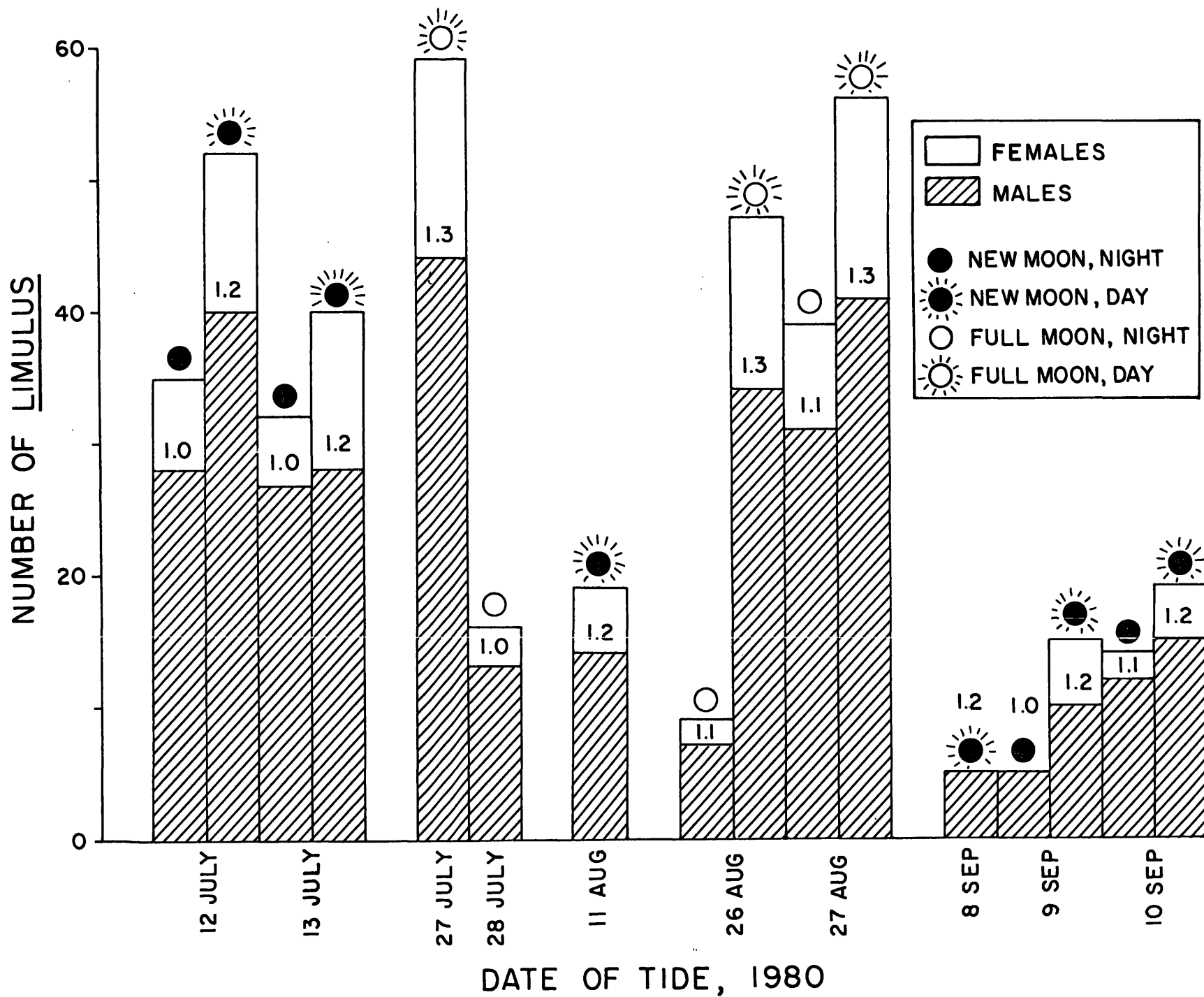
* Not measured.

⁺ Eggs laid in 8 subclutches.

FIGURE LEGENDS

Fig. 1: Numbers of adult *Limulus* on or near the breeding beach, 12 July-10 September, 1980. Numbers inside bars indicate the predicted height of the tide in meters.

Fig. 2: Sex ratios of adult *Limulus* on or near the breeding beach. These ratios are consistently higher during night tides than day tides. Numbers above bars indicate the predicted height of the tide in meters.



NUMBER OF MALES PER FEMALE

