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The relative roles of predation and sperm competition on the duration of the post-copulatory association between the sexes in the blue crab, *Callinectes sapidus*

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Abstract In many species, post-copulatory mate guarding prevents other males from mating with the guarded female. In crabs, males stay with their mates to protect the female from predators because, in some species, mating occurs when she is soft and vulnerable after molting. I tested the relative roles of sperm competition and predation on the duration of the post-copulatory association in the blue crab, *Callinectes sapidus*. Unpaired females suffered greater predation mortality than paired females and males stayed with the female longer in the presence of predators than in their absence, suggesting that the post-copulatory association protects females during their vulnerable period. However, the association may also occur in blue crabs because of sperm competition since spermathecal contents of females in the field indicate that 12.4% mated twice. Females experimentally mated with two males contained both males ejaculates and each ejaculate had access to the unfertilized eggs, suggesting that the size of a male's ejaculate influences his fertilization rate in a multiply-mated female. Males stayed longest in response to a high risk of sperm competition. Longer post-copulatory associations allowed the first male's ejaculate to harden into a type of sperm plug, which limited the size of a second inseminator's ejaculate in a non-virgin female as compared with a virgin. Males passed larger ejaculates in the presence of rivals and when previous ejaculates were in the female spermathecae, another response to sperm competition. Larger ejaculates may need longer post-copulatory associations before a more effective sperm plug forms. Large males stayed with the female longer, which is consistent with their ability to pass larger ejaculates than small males and suggests that there may be costs to minimizing the duration of the post-copulatory association. In the field, associations last long enough to protect the female during her vulnerable phase and may ensure that the guarding male fertilizes the most eggs in the female, even if she remates. Thus, the post-copulatory association protects female blue crabs from additional inseminators as well as from predators.

Key words Sperm competition · Predation · Post-copulatory mate association · Blue crab

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Introduction

Darwin (1871) defined the two main forces shaping the evolution of male secondary sexual characteristics as female choice and male-male competition. Male-male competition takes many forms, although previous research has focused mainly on aggressive interactions among males (LeBoeuf 1974; Eberhard 1979; Watson 1990; Madsen et al. 1993). However, males also compete via sperm competition, which occurs when the sperm from multiple males compete within the female for a limited number of egg fertilizations (Parker 1970a; Smith 1984). The way in which the different inseminations are used, or the sperm precedence pattern, can influence which inseminator of a multiply-mated female fertilizes the most eggs. When the sperm from the last male to mate has precedence in fertilizing the female's eggs, or if sperm from the different inseminators mix within the female reproductive tract, traits that prevent subsequent insemination of the female may have a selective advantage (Parker 1970a, 1984). Sperm compet-

tion can provide strong selection for the evolution of male adaptations designed to limit the number of eggs any subsequent male's sperm will fertilize; these adaptations include sperm plugs that block the female reproductive tract (Parker 1970a; Diesel 1990; Barker 1994), sperm displacement (Waage 1986; Arnqvist 1988; Eady 1994), and post-copulatory mate guarding (Smith 1984).

Post-copulatory mate guarding is widespread (Smith 1984) and can be an effective way for a male to insure that he is the primary inseminator of a particular female (Parker 1970b; Smith 1984). The duration of guarding will be a balance between the eggs gained from guarding versus those that could be received from a similar amount of searching for a new mate (Parker 1978). Post-copulatory mate guarding occurs in many crabs but its role in sperm competition has rarely been examined, despite evidence that sperm competition may occur in crabs (Salmon 1983; Christy 1987; Diesel 1991; Koga et al. 1993). In some species, females mate with multiple partners (Murai et al. 1987; Brockmann 1990; Diesel 1991; Henmi et al. 1993), females store sperm for extended periods of time (Paul 1984; Bauer 1986; Diesel 1988; Sainte-Marie 1993), and male ejaculates harden to form what may be a sperm plug (Bigford 1979; Elner et al. 1980; Bawab and El-Sherief 1989; Diesel 1990). If the hardened ejaculate prevents other males from mating with the female, the duration of guarding may coincide with sperm plug formation.

Post-copulatory mate guarding has been assumed to protect female crabs from predation because guarding is common in species that mate shortly after the female molts (Hartnoll 1969; Salmon 1983), when her exoskeleton is soft and she is vulnerable to predators (Ryer et al. 1990; Shirley et al. 1990) and/or cannibals (Laughlin 1982; Hines et al. 1987; Mansour 1992). The costs and benefits of guarding against predation are influenced by the probability of predation on the female, how long the female remains vulnerable, and the male's expected egg gain, which may be influenced by the size of his ejaculate and/or his mate (Sivinski 1983). Few predators may successfully prey on a fully hardened, post-molt female blue crab, thus reducing the male's gain from guarding after the female's carapace hardens. On the other hand, it may take longer for the new exoskeleton of large post-molt females to harden, requiring longer post-copulatory mate guarding times. Mate guarding may protect the female against predation; however, post-copulatory mate guarding has come to mean guarding against sperm competition. Therefore, the term "post-copulatory association" will be used to describe the association between a male and his mate after copulation and two possible functions for the behavior of the male will be examined: protection against predators and protection against sperm competition.

The blue crab, *Callinectes sapidus*, offers the opportunity to test the relative roles of predation and sperm competition on the duration of the post-copulatory association between the sexes. Female blue crabs mate after their final molt to maturity, suggesting that there are benefits to protecting the female from predation. In Chesapeake Bay, molting crabs and juveniles are vulnerable to fish (e.g., striped bass, American eel) and especially intermolt crabs (Hines et al. 1987; Mansour 1992; Dittel et al. 1995; Hines and Ruiz 1995). Molting crabs may occupy refuge habitats that effectively prevent predation by fish but cannot eliminate the threat of cannibalism (Hines et al. 1987). It has been assumed that the association lasts until the female is no longer vulnerable to predation (Van Engel 1958). Alternatively, the duration of the association may coincide with the female's susceptibility to remating. The seminal fluid portion of a male's ejaculate hardens over time and may form a sperm plug (Wenner 1989), suggesting a role for sperm competition in the post-copulatory association of males and females. However, it is not known whether female blue crabs can remate while their exoskeleton is hardening or whether post-molt females continue to attract males with a urine-borne pheromone (Gleeson 1980).

This paper tests the predation and sperm competition hypotheses for the duration of the post-copulatory association between the sexes in blue crabs. If predation on females during their soft, post-molt phase favors males that guard females while their exoskeleton hardens, then we can predict that (1) females should be particularly vulnerable during their post-molt phase and (2) that a greater risk of predation will result in longer associations. To test these predictions, the mortality and injury rates of paired females was compared with that of unpaired females in the presence of potential predators. The duration of the association in the presence and absence of potential predators was also compared.

Sperm competition favors males that limit the number of eggs subsequent inseminators fertilize. The sperm competition hypothesis predicts (1) that females will sometimes mate with more than one male, (2) that each inseminator's sperm will gain access (not necessarily equal) to the unfertilized eggs, and (3) that the post-copulatory association will last longer when the risk of sperm competition is higher. To test these predictions, the incidence of female multiple-mating was determined, the fate of both male's ejaculates in females that mated twice was assessed, and the duration of the post-copulatory association in the presence and absence of other males was compared.

If sperm from the different inseminators mix in the female reproductive tract, then the male with the largest sperm contribution will fertilize more eggs. This type of sperm competition predicts that the duration of the association should limit the size of a subsequent male's ejaculate and that males should pass larger ejaculates when the risk of sperm competition is increased. The effect of the duration of the association on the amount of ejaculate males pass to females was determined and compared with estimates of the duration of the association in the field. The size of male ejaculates in the presence and absence of rivals was also compared

Methods

This research was carried out at the Smithsonian Environmental Research Center (SERC) on the Rhode River, a sub-estuary of Chesapeake Bay, in Maryland (38° 51′N, 76° 32′W) from mid-June through late September 1991–1994. All crabs used in experiments were collected in the field. Seines and trawls were sometimes used but most specimens were taken with a dip net, two or three times per week, from the sides of 150- to 200-m-long commercial pound nets stretched between vertical posts near the mouth of the Rhode River. Crabs were transported to SERC, measured, separated by sex, maintained in floating field cages in the Rhode River and fed fish daily until used in experiments.

Data collected from field captured crabs included sex, paired status (pre-copulatory, post-copulatory, copulating, or unpaired), molt stage (see below), sexual maturity (juvenile, pre-pubertal or pre-molt, mature), carapace width (distance, in millimeters, between the tips of the lateral spines), and the number and identity of autotomized limbs. Molt stage was determined by examining the propodus on the fifth appendage for evidence of epidermal retraction and color variation (Van Engel 1958). Pre-molt females were designated: early/D_0 (9–10 days pre-molt); early-mid/D_1 (7–8 days pre-molt); mid/D_2 (5–6 days pre-molt); mid-late/D_3 (3–4 days pre-molt) and late/D_4 (1–2 days pre-molt) (Drach 1939). The time required to harden the newly formed carapace after molting was used to designate the molt stages of post-molt crabs as follows: early (< 24 h post-molt), mid (> 48 h post-molt), late (> 72 h post-molt) and intermolt (> 96 h post-molt). Pre-molt females have a triangular, darkened abdomen while adults have a semi-circular abdomen. Males were designated sexually mature if the second pleopods lay within the first pleopods (intromittent organs), if the penes were inserted into the second pleopods and if the abdomen easily pulled away from the sternum (Van Engel 1990). I used only mature, intermolt males that possessed both chelae and that were missing not more than one walking leg, a condition that does not affect mating behavior and/or mating success (Smith 1992). Crabs in experiments were never held in field cages for more than 1 week or reused.

Statistical procedures

The field and experimental data were analyzed using Systat (SYSTAT 1992). In all instances, data were tested for normality using the Kolmogorov-Smirnov test and for homogeneous variances using Bartlett's test (Sokal and Rohlf 1981). When transformed data failed to meet the assumptions of parametric tests, the appropriate non-parametric test was used. Tests include one-way and two-way ANOVA with Tukey multiple comparisons test to detect differences between treatments, the *t*-test, paired *t*-test and *G*-test with William's correction. The William's correction was used because it improves the approximation of *G* to chi square especially when sample sizes are low (Sokal and Rohlf 1981). In the text, means are presented with their standard error (± SE).

Pool experiments

All of the experiments described below were performed in plastic pools (2 m in diameter and 0.3 m deep). Each pool contained approximately 10 cm depth of sand and was filled with water from the Rhode River. The pools were constantly aerated and the water was completely changed every 1–2 days. A 0.5-m-high "fence" of hardware cloth was placed around the inside perimeter of each pool and covered with a piece of plywood. The cover protected crabs from terrestrial predators (e.g., raccoons), direct sunlight, and elevated water temperatures. The water salinities matched that of the Rhode River (5–10 ppt) and water temperatures were approximately 5° C below that of the Rhode River, varying little among pools (22–27° C). Crabs were exposed to the ambient light:dark cycle (14h : 10h). Pools were monitored five to ten times daily for the presence of courtship, copulation, and pre- or post-copulatory association. The post-copulatory association begins directly after copulation, which may last 5–12 h (Van Engel 1958). When the end of copulation was not observed, I assumed copulation lasted 12 h. By over-estimating the duration of copulation when it was not directly observed, the estimates of the duration of the post-copulatory association were conservative. All pools were equally monitored, hence there were no observation biases towards any treatment in any of the experiments. Crabs in pools were fed mussels (*Mytilus* sp.) daily.

Measuring ejaculate size: the pleopod removal technique

In the experiments described below, ejaculate weight was used as a measure of the amount of sperm males pass to females. The number of spermatophores (NS) increases with ejaculate weight (EW) ($NS = 0.613EW + 2.49$, $r^2 = 0.332$, $n = 29$, $P = 0.001$). However, the average size of spermatophores (which may influence the number of sperm they contain) does not vary significantly with either ejaculate weight ($n = 29$, $P = 0.592$) or male size ($n = 29$, $P = 0.446$) (Jivoff 1995). Male blue crabs store spermatophores and seminal fluid in paired vas deferentia. Each vas deferens is connected to an external pleopod, through which seminal fluid and spermatophores are passed to one of the female's two spermathecae (Van Engel 1958). I found no difference in the weight of material males store in their vas deferentia (paired $t = 0.306$, $df = 10$, $P = 0.766$) or pass through each pleopod (paired $t = 0.276$, $df = 158$, $P = 0.783$) (Jivoff 1995). Therefore, the following protocol was used as an unbiased measure of the weight of male ejaculates.

One (randomly assigned) pleopod from each male was removed with dissecting scissors at least 24 h prior to mating. After one copulation, each vas deferens was weighed (to the nearest 0.01 g). The intact vas deferens transferred the normal amount of ejaculate while the vas deferens lacking a pleopod ("unmated") transferred none. As a result, the weight of the unmated and intact vas deferentia represented the amount of seminal fluid and spermatophores stored before and after mating, respectively. Ejaculate weight was calculated by the difference between the weights of the intact and unmated vas deferentia. The proportion of available material passed was also calculated by dividing the ejaculate weight by that of the unmated vas deferens. The surgical procedure proved effective because neither spermatophores or seminal fluid were lost after pleopod removal, and no difference was found between the calculated weight of ejaculate passed and the measured weight of spermathecae contents (paired $t = 0.704$, $df = 42$, $P = 0.485$). Pleopod removal did not adversely affect the functioning of the intact vas deferentia since no difference was found between the amount of material passed by the remaining pleopod, either left (Mann-Whitney $U = 120$, $P > 0.05$) or right (Mann-Whitney $U = 128$, $P > 0.05$), and that of a corresponding pleopod from intact males.

Post-copulatory associations: duration in the field

The duration of post-copulatory associations in the field were estimated using observations of the amount of time required to harden the newly formed carapace of females that molted in experimental pools and holding cages. A post-copulatory association begins immediately after copulation, which is coupled with molting. Therefore, an estimate of the amount of time that has elapsed since a guarded female molted (carapace formation) is a good approximation for the duration of a post-copulatory association. Only singly-mated females were included in this sample.

Protective value of the post-copulatory association

I examined whether the post-copulatory association protected molting females against the risk of predation. Adult females in the intermolt stage were used as potential predators on molting pre-

molt females. Adult females were used rather than large fish predators because (1) gut contents (Laughlin 1982; Hines et al. 1987; Mansour 1992) and my own observations of adult blue crabs indicate they are cannibalistic, and therefore a realistic threat, (2) adult females are more readily available than other predators, and (3) their behavior under laboratory conditions is more predictable than that of large fish predators. One male, two size-matched, pre-molt females (late molt stage) and one adult female were placed in each pool. Male blue crabs have never been observed in association with two females simultaneously. Therefore, I assumed only one of the pre-molt females would be associated with the male. The pools were monitored daily for evidence of cannibalism on either pre-molt female. After both pre-molt females molted, they were scored for mortality, limb loss and body damage. Only those cases in which the pre-molt females molted simultaneously and thus equally susceptible to cannibalism during their soft phase were used. The *G*-test was used to test if mortality, limb loss and body damage were associated with paired status. The numbers of limbs lost by paired and unpaired females were compared with a *t*-test.

The effect of sex ratio and male size on post-copulatory associations

I manipulated sex ratios and male size to determine if the potential for predation and sperm competition influence (1) the duration of the post-copulatory association, (2) the size of male ejaculates, and (3) if male size influences the ejaculate or association response to sperm competition or predation. The experiment was a 3×3 factorial design, with sex ratio and male size as the independent variables. The operational sex ratio was used to manipulate the risk of sperm competition and males were divided into three size categories: small (120–135 mm), medium (135–145 mm) and large (> 145 mm). Two sex ratios (number of males:number of pre-molt females) were used: 3:1 and 1:1. The third treatment ("cannibal treatment") consisted of an operational sex ratio of 1:1 plus two intermolt adult females, and thus had the same density of crabs as the 3:1 treatment without the risk of sperm competition (Fig. 1). The males in the 3:1 treatment were well fed, and therefore I assumed the risk of sperm competition was greater than that of cannibalism.

The pleopod removal technique was used to facilitate the measurement of ejaculates. The males in the same pool had a common (i.e., left or right) pleopod removed to compare the ejaculates of similar sized first and second inseminators. Males that mated or that were associated with a post-molt female were sacrificed at the end of the experiment. The female's spermathecae were also weighed (to the nearest 0.01 g). Two-way ANOVA (with Tukey multiple comparisons test) was used to compare the durations of the post-copulatory associations and percent of material passed among the treatments.

Female multiple mating in the field

Once an ejaculate is passed to the female, the pink seminal fluid hardens over time and most of the spermatophores accumulate in a single, large mass at the distal end of the spermathecae (Johnson 1980). The spermathecal contents from field collected females were visually compared with those from females that mated with two or three males in experimental pools. I noted the number of spermatophore masses and their position within the spermathecae, and the color (dark versus light) and texture (hard versus soft) of the seminal fluid. The spermathecae of the adult females from each collection date were weighed (to the nearest 0.01 g). The weight of the spermatheca itself was subtracted using the linear regression equation obtained between adult female width (AFW) and empty spermathecae weight (ESW) ($n = 40$, ESW = 0.003AFW–0.230, $r^2 = 0.214$, $P = 0.003$) (Jivoff 1995). I compared the sum of the weights of both spermathecae contents between singly- and doubly-mated females from the field using the *t*-test.

The duration of the post-copulatory association and the size of subsequent ejaculates

To test the effect of the duration of the post-copulatory association on the ejaculate size of future inseminators, non-virgin females were remated after different association durations and the ejaculate weight of each inseminator was compared. Females were mated with two males in succession: the first had one pleopod removed, while the second was normal (both pleopods intact) and size-matched with the first inseminator.

After the first mating, each female contained one mated and one unmated spermatheca (Fig. 2). The mated females were subsequently isolated for one of four periods (24, 48, 72 or 96 h), and then remated with an intact male. Each period simulated a different duration of the post-copulatory association. If the second male copulated successfully, then each female contained one spermatheca inseminated by both males (a doubly-mated spermatheca) and one spermatheca inseminated only by the second male (a singly-mated spermatheca) (Fig. 2). Both spermathecae were weighed (to the nearest 0.01 g). The difference between the weights of the first male's unmated and intact vas deferentia was his contribution to the doubly-mated spermatheca. The second male's contribution to the doubly-mated spermatheca was the weight of the spermatheca minus the first male's contribution. Finally, the weight of the singly-mated spermatheca was the second male's contribution to that spermatheca. The weight of the spermatheca itself was subtracted from the above weights using the linear regression equation obtained between pre-molt female width (PFW) and empty spermathecae weight (ESW) (ESW = 0.009PFW–0.458, $r^2 = 0.363$, $n = 28$, $P = 0.001$) (Jivoff 1995). At each time interval, paired *t*-tests were used to compare: (1) the first versus second male's ejaculate in the doubly-mated spermatheca, and (2) the second male's ejaculate in the doubly-mated versus the singly-mated spermatheca. I tested the effect of association time on the second male's ejaculate in the doubly-mated spermatheca using one-way ANOVA.

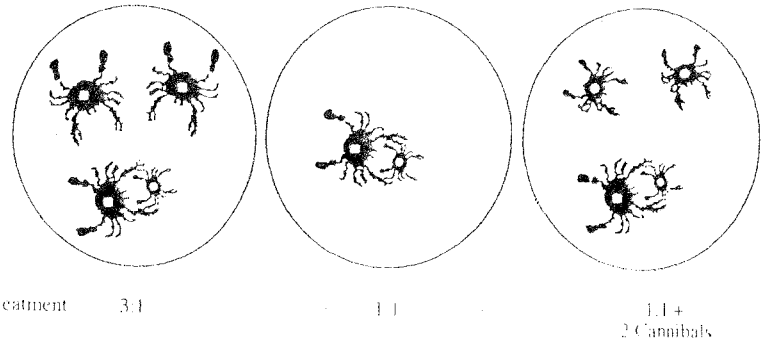

Fig. 1 Sex ratio experimental design. Three sex ratio treatments are shown and the sexes are labeled as follows: males *open squares*, pre-molt females *open circles*, and adult females *dotted circles*. Each sex ratio treatment is replicated using three size classes of males: small (120–135 mm), medium (135–145 mm) and large (> 145 mm)

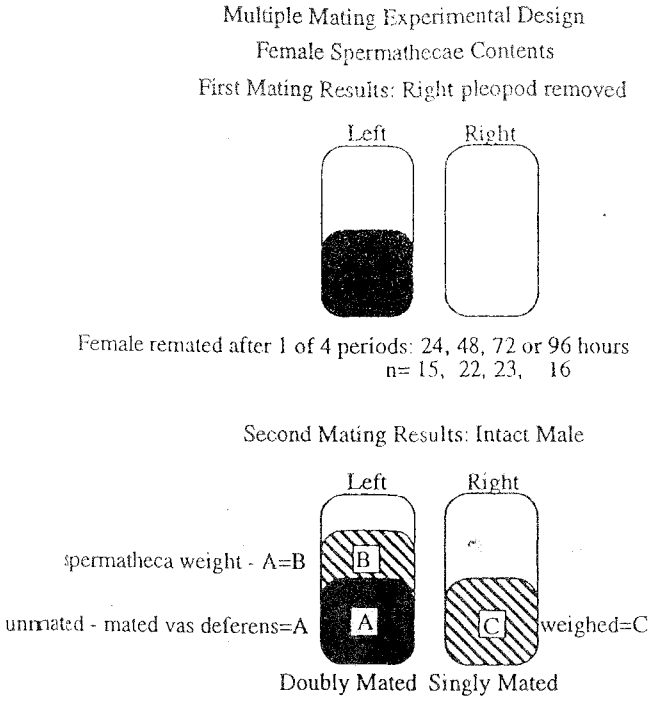

Fig. 2 Multiple mating experimental design. This shows the results of ablating the right pleopod of the first male to mate. Each male's contribution to each spermatheca is shown

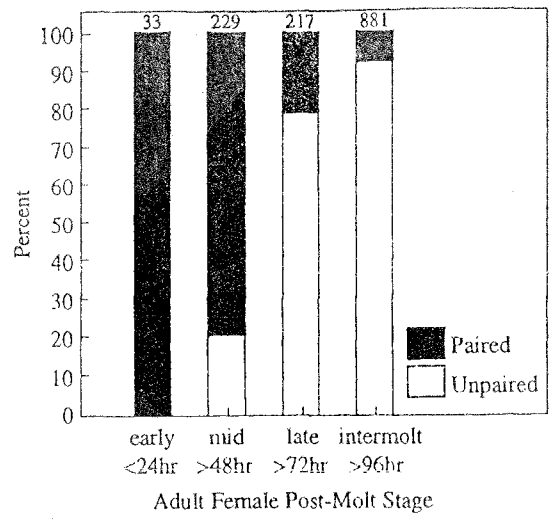

Fig. 3 Percentage of paired and unpaired adult females captured in the field during four post-molt stages. *Percent* is the number of paired or unpaired females in each molt stage, divided by the total number of females in that stage. The number of hours since the females molted is shown for each molt stage. The *numbers* above the bars are sample sizes. Data are pooled from all years (1991–1994) of the study

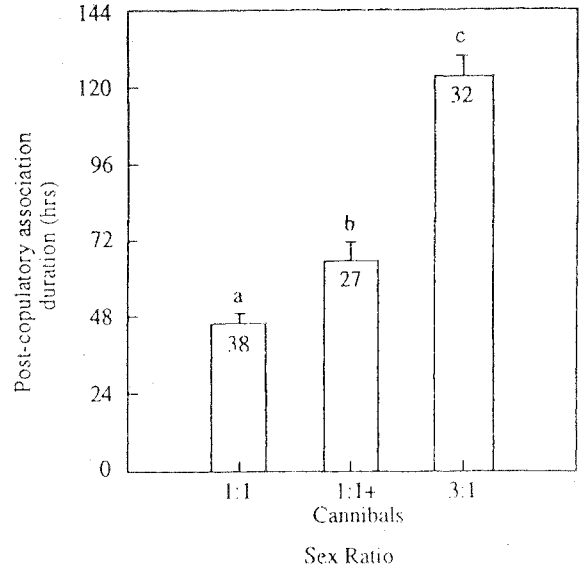

Fig. 4 Differences in the duration of the post-copulatory association among the sex ratio treatments. The *numbers* in the bars are sample sizes. The *letters* above the bars are the results of a Tukey multiple comparisons test. Bars sharing the same *letter* are not significantly different (all $P < 0.001$). The *vertical bars* are 1 SE

Results

The duration of post-copulatory associations in the field

All adult females less than 24 hours post-molt were paired. The majority (79.5 %) of adult females 48 h post-molt were also paired. However, adult females 72 and 96 hours post-molt were paired only 21.2 % and 7.9% , respectively (Fig. 3). These results indicate that post-copulatory associations often last longer than 48 h and may continue for over 96 h.

The post-copulatory association: protection against predators

There was a greater incidence of mortality ($G = 16.62$, $df = 1$, $P < 0.001$) and body damage ($G = 14.45$, $df = 1$, $P = 0.001$) among unpaired females than paired females. Although the incidence of limb loss was not statistically significant ($G = 1.15$, $df = 1$, $P > 0.05$), the number of limbs lost by unpaired females was significantly greater than that of paired females ($t = 2.31$, $df = 36$, $P = 0.027$). In addition, males guarded significantly longer in the presence of potential cannibals than in their absence ($F_{5,97} = 65.03$, $P < 0.001$; Tukey HSD, $P < 0.001$) (Fig. 4).

The post-copulatory association: the sperm competition hypothesis

The sperm competition hypothesis requires that at least some females mate with more than one male. Based on

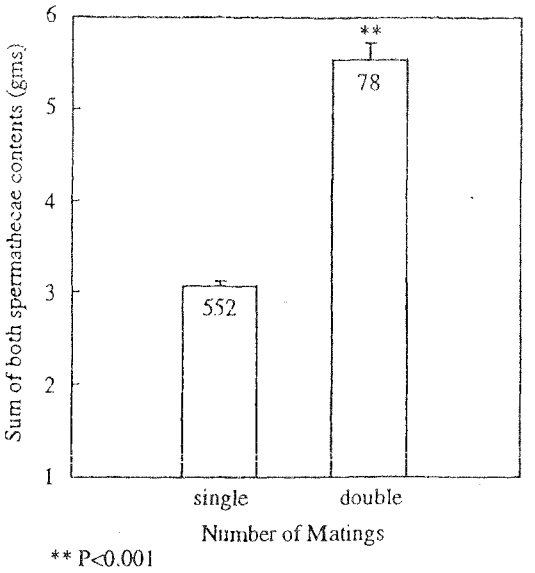

Fig. 5 Sum of both spermathecal contents from adult females captured in the field identified as either mating once or twice. The weights of the empty spermathecae have been removed. The *numbers* in the bars are sample sizes. The *vertical bars* are 1 SE. Note that the incidence of double mating is 12.4%

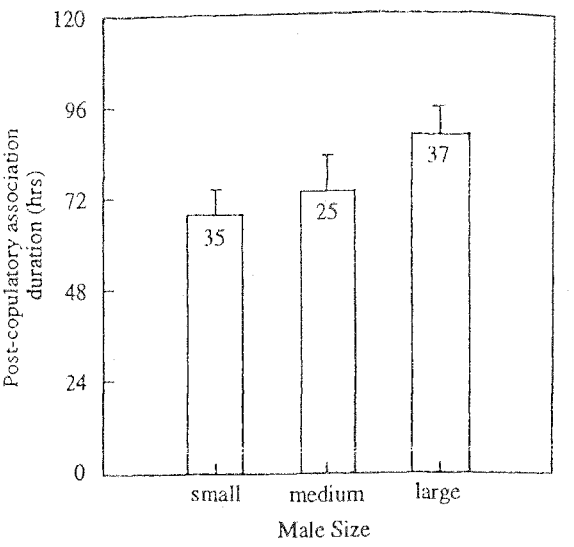

Fig. 6 Differences in the duration of the post-copulatory association among the male size classes. The size ranges (in mm) for each category are as follows: small (120–135), medium (135–145) and large (> 145). The *numbers* in the bars are sample sizes. Large males guarded longer than small males (Tukey HSD $P < 0.05$). The *vertical bars* are 1 SE.

visual comparisons of spermathecae from females that were known to mate with two or three males, female spermathecal contents from field collected crabs indicate that 12.4% mated twice. There were no cases of females that mated more than twice. In females that mated once, there was one spermatophore mass at the distal end of the spermathecae, whereas double matings resulted in one spermatophore mass at each end of the spermathecae. The first insemination was dark and completely hardened whereas the second was light and still soft. Doubly-mated females had nearly twice the amount of spermatophores and seminal fluid than singly-mated females (Fig. 5).

Sperm competition and male size effects on post-copulatory association duration and ejaculate size

Sex ratio ($F_{2,92} = 65.03$, $P < 0.001$) (Fig. 4) and male size ($F_{2,92} = 3.64$, $P = 0.03$) (Fig. 6) had significant effects on the duration of mate guarding. The interaction between sex ratio and male size was not significant ($F_{4,88} = 0.106$, $P = 0.98$). Males stayed with females significantly longer when the sex ratio was male biased than under the equal sex ratio treatment (Tukey HSD, $P < 0.001$) or the cannibal treatment (Tukey HSD, $P = 0.001$). Large males stayed with females significantly longer than small males (Tukey HSD, $P < 0.05$). There were significant differences in the proportion of ejaculate passed to females among the sex ratio treatments ($F_{2,101} = 3.67$, $P = 0.03$) and among the male size categories ($F_{2,101} = 4.19$, $P = 0.02$). Males passed larger proportions of their available material under the male biased sex ratio than the equal sex ratio without cannibals (Tukey HSD, $P = 0.015$) (Fig. 7). Small males passed a larger percentage of material than medium sized males (Tukey HSD, $P = 0.020$). Neither male size ($F_{2,101} = 2.50$, $P = 0.09$) nor sex ratio ($F_{2,101} = 2.16$, $P = 0.12$) had a significant effect on the percentage of seminal fluid passed to females. The percentage of spermatophores passed to females was also not influenced by male size ($F_{2,101} = 1.06$, $P = 0.35$) or sex ratio ($F_{2,101} = 0.07$, $P = 0.94$).

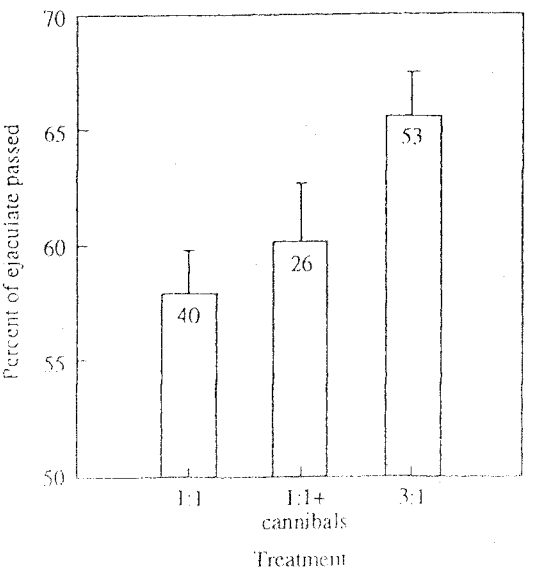

Fig. 7 Differences in the percent of ejaculate passed by males among the sex ratio treatments. *Percent* is the amount passed divided by the total amount of material available in the male reproductive tract. The numbers in the bars are sample sizes. The 3:1 treatment is significantly larger than the 1:1 treatment ($P < 0.05$). The vertical bars are 1 SE.

I observed 12 double matings and 3 triple matings during this experiment. In the double matings, I found no difference in the proportion of ejaculate second males passed compared with that of the first inseminator ($t = 0.820$, $df = 21$, $P = 0.42$). The average duration of the post-copulatory association by first males was 53.3 h ($n = 12$, SE ± 12).

The duration of the post-copulatory association and the size of subsequent ejaculates

Female blue crabs remated even 96 hours after their first insemination. When the time between matings was 24 h, I found no difference in the ejaculate that second males passed to the doubly-mated spermatheca as compared with the singly-mated spermatheca (paired $t = 0.24$, $df = 14$, $P = 0.82$) (Fig. 8). When the time between matings was 48 h (paired $t = 2.92$, $df = 21$, $P = 0.01$), 72 h (paired $t = 5.73$, $df = 22$, $P < 0.001$) and 96 h (paired $t = 8.14$, $df = 15$, $P < 0.001$), the second male passed significantly less to the doubly-mated spermatheca than to the singly-mated spermatheca (Fig. 8). While there was a trend for second males to pass less material to the doubly-mated spermatheca with increasing time between matings, the differences among doubly-mated spermathecae over time were not significant ($F_{3,64} = 0.96$, $P > 0.05$).

Compared to the ejaculates that first males passed to the doubly-mated spermatheca, second males passed significantly more material after 24 h (paired $t = 6.27$, $df = 13$, $P < 0.001$), 48 h (paired $t = 8.47$, $df = 19$, $P < 0.001$), and 72 h (paired $t = 4.09$, $df = 18$, $P < 0.001$) (Fig. 8). I found no difference in the ejaculates of first and second males after 96 h (paired $t = 0.74$, $df = 14$, $P = 0.47$). Furthermore, second males passed significantly more to a singly-mated spermatheca than did the first male after all times between matings (24 h, paired $t = 3.14$, $df = 13$, $P = 0.008$; 48 h, paired $t = 4.97$, $df = 19$, $P < 0.001$; 72 h, paired $t = 6.51$, $df = 18$, $P < 0.001$; 96 h, paired $t = 6.53$, $df = 14$, $P < 0.001$) (Fig. 8). Overall, second males passed more to the singly than to the doubly mated spermatheca and passed more than first males.

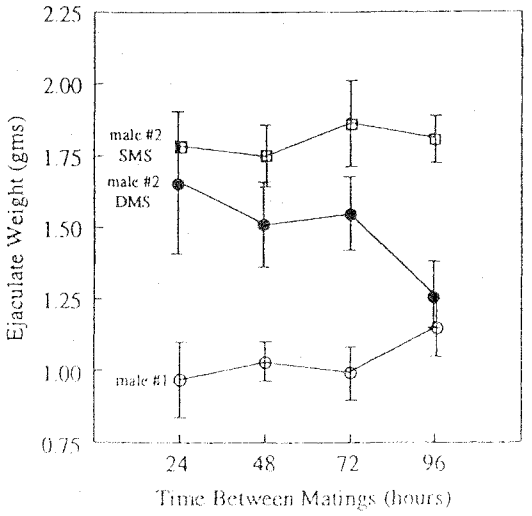

Fig. 8. Ejaculate weight of first male (○), second male to singly-mated spermatheca (*SMS*) (□), and second male to doubly-mated spermatheca (*DMS*) (●). The significant differences are as follows: male 2 SMS > Male 2 DMS, 48 h ($P < 0.05$), 72 h ($P < 0.001$) and 96 h ($P < 0.001$); male 2 DMS > Male 1, 24 h ($P < 0.001$), 48 h ($P < 0.001$), 72 h ($P < 0.001$), and male 2 SMS > Male 1, 24 h ($P < 0.008$), 48 h ($P < 0.001$), 72 h ($P < 0.001$) and 96 h ($P < 0.001$). The *vertical bars* are ± 1 SE

Discussion

Predation and the duration of the post-copulatory association

It has long been assumed that the post-copulatory association in crabs functions to protect the recently molted female from predation (Van Engel 1958; Hartnoll 1969). The coupling of molting and mating is unique in crabs such that females are most vulnerable at the time of mating. My experimental data show that unpaired females are more vulnerable to attack from cannibals and have higher mortality rates during molting than paired females, which is consistent with field experiments done on blue crabs (Shirley et al. 1990). In other taxa, the act of mating makes females (or pairs) more vulnerable to predation and typically reproductive activities (e.g., guarding or copulation times) are shortened (Gwynne 1989; Magnhagen 1991). However, male blue crabs stay longer in the presence of predators than in their absence, which is also true for the stone crab, *Menippe mercenaria*, another species that mates when the female is soft and vulnerable(Wilber 1989).

Post-copulatory associations should continue until the benefits from protecting the female from predators and/or other males are outweighed by those of finding a new mate. In the blue crab, the hardening of the new exoskeleton reaches an ionic equilibrium after 48 h (Vigh and Dendinger 1982), suggesting that post-molt females remain vulnerable for at least 48 h. Therefore, if the post-copulatory association functions to protect the female from predators, it should last at least 48 h. In the absence of cannibals, males stayed with females for about 48 h, and in the field, 79.5% of females post-molt for more than 48 h were paired; both findings indicate that females are protected while they are vulnerable. Males stayed with females almost 24 h longer in the presence of cannibals than in their absence, suggesting that males will stay with females for 72 h in order to protect them from predation. However, in the field, 29.1% of females post-molt for more than 72 h were still paired, suggesting that males benefit from association times that exceed those needed to protect the female from predation.

In the field, I found evidence that female blue crabs had remated and that each inseminator's ejaculate has access to the unfertilized eggs, indicating that sperm competition may occur in blue crabs. However, the incidence of female multiple-mating was only 12.4% , suggesting that the male's presence effectively deters female remating and that sperm competition is not common. In contrast, most female insects mate with at least two males (Parker 1970a; Ridley 1988). The level of sperm competition may not be as high in blue crabs, because non-virgin females may be more difficult to find and mate with than virgins. Pre-molt females attract males with a pheromone; however, post-molt females may be unable to produce the pheromone as they harden (Gleeson 1980). As a result, search times for non-virgins may be high and/or encounter rates may be low. Non-virgins may seek out additional males to mate with if they are released while still vulnerable to predation or if, as in a variety of other species, they receive a small amount of sperm (Gromko et al. 1984; Simmons 1988; Waddy and Aiken 1990; Sainte-Marie 1993). Otherwise, non-virgins may resist the mating advances of males because paired females miss feeding opportunities necessary for growth after molting. Adult female blue crabs may succeed in resisting males because they are usually larger (Jivoff 1995).

Sperm competition and the duration of the post-copulatory association

Even if sperm competition occurs rarely in blue crabs, when I experimentally created conditions that may lead to high levels of sperm competition, males altered their behavior in a way that was consistent with increasing their egg gain within the female. The experimental results indicate that the increased possibility of sperm competition prolonged the post-copulatory association by more than 48 h beyond the time needed to protect females against predators. At high male sex ratios, males stayed with females for approximately 125 h, which, according to the multiple mating experiment, may ensure that the first male's ejaculate is larger than another inseminator's. Large males stayed with females longer than small males perhaps because large males pass larger ejaculates (Jivoff 1995) and thus may receive greater egg gains. Large males may also have assessed a greater risk of female remating than small males. Paired males may estimate the risk of female remating by the frequency and/or intensity of aggressive encounters with competitors. As males get larger, they may have more frequent and/or more intense aggressive interactions with similar sized rivals (Smith et al. 1994; Huntingford et al. 1995). In the experiment, the males were size-matched; hence large paired males may have received more frequent and/or more intense takeover attempts than small males.

Takeovers and the subsequent amount of ejaculate a second male can pass also help explain the occurrence of multiple mating in the experiment. When multiple mating occurred, the second male obtained the female, on average, 53 h after the first male copulated. If the second male successfully displaced the paired male, he did so in time to pass a larger ejaculate than the first male because, according to the multiple mating experiment, second inseminators pass larger ejaculates than the first male when they mate with a non-virgin less than 72 h after her first mating.

When females mate multiply, males that prevent competitors from mating with their female or that limit the size of a competitor's ejaculate within their female can fertilize more of her eggs (Parker 1970a). My experimental results suggest that post-copulatory associations of greater than 24 h significantly limit the ejaculate that second males pass to non-virgins as compared to virgins. Specifically, 48-h associations reduce another inseminator's ejaculate by 20% compared to what the second male will pass to a virgin. In the field, 79.5% of females post-molt for more than 48-h were paired, suggesting that males remain long enough to limit the ejaculate of competitors by 20% . The largest reductions in the ejaculates passed to non-virgins, as compared with those passed to virgins, occurred after associations of 96 h. However, in the field, most males remain for less than 96 h, suggesting that the first male's ejaculate continues to limit the ejaculate of rival males after the female has been released and thus may act as a type of sperm plug. In some insects the sperm plug completely prevents rival males from inseminating non-virgin females (Parker 1970a); however, in blue crabs, second inseminators can pass larger ejaculates than the first male to mate. Therefore, the first male's ejaculate may simply be a "space occupier", which reduces the volume of spermathecae available in a non-virgin female as compared with a similar sized virgin. As a result, males pass smaller ejaculates to non-virgins than to virgins.

In some crabs, females may be unable to mate when their exoskeleton is hard (Hartnoll 1968, 1969), suggesting that the exoskeleton of female blue crabs may also limit the ejaculates that males pass to non-virgins. Furthermore, if the ejaculate functions as a type of sperm plug, then second males may have an incentive to pass smaller ejaculates as the female hardens because there is less risk of female remating. However, the ejaculates that second males passed to virgins did not decrease over time, indicating that the female's exoskeleton did not limit the ability of males to pass ejaculates or that males passed smaller ejaculates based on the female's opportunity to remate.

Sperm competition and ejaculate size

In a multiply-mated female, a male's egg gain may be influenced by his ejaculate size and/or the order in which he mated (Parker 1970a). In other species, the last male has precedence in fertilizing eggs if (1) he removes previous ejaculates from the female sperm storage organ

(Waage 1986), (2) his ejaculate displaces previous ejaculates from the sperm storage organ (Parker 1970a), or (3) his ejaculate prevents previous ejaculates from accessing unfertilized eggs (Diesel 1990). My observations of doubly mated females suggest that in blue crabs each inseminator's ejaculate is present and will have access to some unfertilized eggs. In female blue crabs that are preparing to spawn, the seminal fluid softens and gradually disappears, allowing the spermatophores to mix in the spermathecae (Wenner 1989 and personal observation). Therefore, the size of a male's ejaculate may influence his egg gain in a non-virgin more than the order in which he mated.

The experimental results suggest that second inseminators increase the size of their ejaculate, which may raise their fertilization rate above that of the first male. Second inseminators passed larger ejaculates than the first male when the post-copulatory association lasted less than 96 h. However, first and second inseminators passed similar sized ejaculates if the association lasted more than 96 h. In the field, 7.9% of females post-molt for 96 h were still paired, suggesting that some males that remain with the female prevent rivals from passing ejaculates larger than their own but that staying longer may limit additional mating opportunities. Second inseminators also passed significantly more to a virgin spermatheca than first males, suggesting that the presence of the first male's ejaculate in the other (non-virgin) spermatheca may have provided a cue for second males to alter their ejaculate size while first males had no such cue (e.g., the presence of competitors or previous ejaculates).

In some species, males have the ability to manipulate their ejaculate according to the risk of sperm competition (Baker and Bellis 1989, 1993; Svard and Wiklund 1989; Baker et al. 1990; Gage 1991). My results suggest that male blue crabs respond to the increased risk of sperm competition (e.g., the presence of competitors during mating or previous ejaculates) by passing larger ejaculates but that they do not manipulate the individual components (spermatophores and seminal fluid) of ejaculates. No difference was found in male ejaculate size between the cannibal and high sex ratio treatments, indicating that males did not pass larger ejaculates simply in response to a higher density of crabs. Larger ejaculates may be more effective at preventing other males from mating with a non-virgin and/or limiting the ejaculate size of rivals. In blue crabs, if the first male's ejaculate limits the ejaculate that rival males pass to non-virgins as compared with virgins by occupying space within the spermathecae, then a larger ejaculate would be more effective. However, larger ejaculates may require longer post-copulatory associations before they harden. Male blue crabs also stayed longer when the risk of sperm competition was higher, suggesting that larger ejaculates do indeed require more time to become an effective sperm plug.

This paper is the first to show that sperm competition may occur in blue crabs. The results suggest that females can remate, that the size of each male's ejaculate in the female may influence the number of eggs each male fertilizes and that males remain with females longer and pass larger ejaculates under greater risk of sperm competition. The post-copulatory association protects females from both predators and other males and the experiments identified how long of an association may be needed for each function. Association durations of 72 h or less protect the female from predators and other males but longer associations function primarily to prevent sperm competition.

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