Female caridean shrimps with egg-filled ovaries are receptive to mating and evoke copulatory behavior from males just after their prespawning (partmolt) molt (Bauer, 2004). Upon physical contact of any female surface with an antennal flagellum, the male typically grasps and copulates with the female, depositing spermatophores on the underside of the female (qualitative observations in Höglund, 1943; Burkenroad, 1947; Bauer, 1976; Berg and Sandifer, 1984; Correa et al., 2000). Burkenroad (1947) concluded that, in Palaemonetes vulgaris (Say, 1818), a male recognises a receptive (postmolt parturial) female on the basis of a “non-diffusible coating of the integument of the female” perceived by the male’s antennal flagella. Burkenroad’s conclusion, perceptive as it may turn out to be, was based solely on anecdotal behavioral observations.

Sex pheromones are chemical compounds that transmit information about the opposite sex and that stimulate sexual activity. Olfactory sex pheromones are soluble (dissolved) in the surrounding medium, usually quite low in concentration, and are perceived via olfaction by males at a distance from an emitting female (Bauer, 2004). Gustatory or taste (contact) sex pheromones are relatively insoluble and relatively high in concentration, and physical contact must be made between male chemoreceptors and female pheromones for perception to take place. Olfactory sex pheromones have been indicated or demonstrated in decapod crustaceans by a variety of observational and experimental studies on behavior (e.g., Ryan, 1966; Atema, 1986; Dunham, 1988; Kamio et al., 2002). However, unlike insect olfactory pheromones, chemical identification of these compounds in crustaceans has remained elusive (Gleeson, 1991; but see Asai et al., 2000). Although contact sex pheromones are well known and have been identified in a variety of insect species (e.g., Ferveur et al., 1996; Bray and Amrein, 2003; Ginzel et al., 2003; Simmons et al., 2003), they have not been well investigated in decapod crustaceans.

Behavioral studies have indicated in some caridean species that olfactory sex pheromones play a role in sex attraction of males to recently molted parturial females (Kamiguchi, 1972; Bauer, 1979; Seibt and Wickler, 1979). In the hippolytid Heptacarpus paludicola, males are attracted to upstream receptive females without evoking an obvious change in behavior. In H. paludicola, male recognition of a receptive female was apparent only upon physical contact of the male with the female, usually with a male antennal flagellum. In another caridean, Rhynchocinetes typus (H. Milne Edwards, 1837), males can only recognize the reproductive status of females upon physical contact using the antennal flagella; however, females use olfactory cues (distance chemoreception) to recognize dominant males (Diaz and Thiel, 2004).

Palaemonetes pugio Holthuis, 1949, is a common estuarine “grass shrimp” of eastern North America and the Gulf of Mexico whose basic reproductive biology has been well studied (Williams, 1984; Berg and Sandifer, 1984; Bauer and Abdalla, 2000, 2001). Females with ovaries full of vitellogenic oocytes are attractive to males and receptive to mating just after a molt, termed the parturial molt, which precedes spawning. Males are attracted to postmolt parturial females after touching them with the antennal flagella or pereopods. After contact, a male grasps the female, presses his posterior thoracic sterna against hers, and deposits a pair
of sperm cords (spermatophores) to her external surface. The pair immediately separates, and the female usually resists any further mating attempts from the same or other males (Bauer and Abdalla, 2001). Spawning and attachment of fertilized embryos takes place within 2–3 hours of copulation. Multiple broods are produced during the breeding season in the southerly part of species range and can be induced in the laboratory by higher water temperatures. Ovarian development may occur during embryo incubation so that another parturial molt occurs within two days after hatching of the incubated brood ("fast successive parturials;"") interspawning interval of 14 days at 27–28°C). Alternately, ovarian development may not occur again until after hatching of embryos, sometimes preceded by a nonparturial molt, with a subsequent increase in the interspawning interval to 23 days (at 27–28°C).

In experiments with *Palaemonetes pugio*, Bauer and Abdalla (2001) showed that males had greater numbers of brief contacts with a prespawning female nearing the molt than with an intermolt female. However, the increased number of contacts was only significant in the hour before the parturial female’s molt, and no copulatory behaviors or other apparent sign of sex recognition were observed. Bauer (2004) hypothesized that in this highly aggregated species, females may not advertise their upcoming sexual receptivity with an olfactory pheromone to avoid harassment from males. Females may be unable to hide their sexual condition until the molt because of the increased presence of molting-related metabolites in the urine as the molt draws near. After the molt, male antennal contact with the newly molted female strongly evokes male grasping of and copulation with the female (qualitative behavioral observations in Berg and Sandifer, 1984; Bauer and Abdalla, 2001).

Although qualitative or anecdotal observations about males being attracted to newly molted parturial females of *Palaemonetes pugio* are abundant in the literature, formal experimental studies on the stimuli involved are few and incomplete, as noted above. In this investigation, we performed behavioral experiments to test hypotheses about the stimuli (visual, tactile, chemical) used by males of *Palaemonetes pugio* in sex attraction to and recognition of recently molted (postmolt) parturial females.

**Materials and Methods**

Live specimens were collected at Cypremort Point (Vermilion Bay, Louisiana, U.S.A.) between March 2002 and June 2003 as needed for experiments. Mature females (5–7 mm carapace length (CL), Bauer and Abdalla, 2001) were maintained on a water table at a salinity of 6 ppt, temperature of 24–28°C (postmolt) parturial females (receptive to mating) and intermolt females (at least 48 h postmolt, no embryos nor ovarian development) in a separate aquarium with the female (qualitative behavioral observations in Berg and Sandifer, 1984; Bauer and Abdalla, 2001).

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a postmolt parturial female, the other without contact with shrimp exoskeleton ("blank"). In this experiment, only parturial females that had molted in the previous four hours or less were used. The time of molting was determined, as described above, by viewing recorded time-lapse video observations on females with vitellogenic ovaries. In each of 20 replicates, two males were acclimated in a testing arena 16.5 cm long, 11.4 cm wide, and 10 cm depth, and then test sponges were lowered and placed 8 cm apart in the test arena just above the substratum. In this bioassay, two males instead of one, as in the first sponge bioassay, were used in an attempt to increase the probability that a physiological healthy male was being tested (not too near, not just after the molt). The order of presentation (right or left side of the arena) of blank and treatment sponges was done at random (determined by a coin toss). During a 20 min observation period recorded by video, contacts of males with the sponges were defined as (a) direct forward movements into the sponge with the anterior part of the body, (b) grasping of the sponge with chelipeds, or (c) climbing onto the sponge.

**RESULTS**

**Visual Attractiveness of Females**

Males did not appear to visually recognize sexually receptive females (recently molted, prespawning = postmolt parturial). In 68% of replicates of both treatments, males made no contact with the glass jar containing a postmolt parturial (sexually receptive) or an intermolt (not receptive) female ($n = 25$ replicates/treatment).

**Sexual Attractiveness of Individuals in Different Molt/Reproductive Stages**

Males were primarily attracted to recently molted females with ovaries full of vitellogenic oocytes. Copulatory responses were limited to “following” only or to the complete copulatory sequence (grasping, mounting, copulation). No other partial copulatory responses (e.g., grasping only) were observed. Males copulated with postmolt parturial females in 14 of 25 replicates (Fig. 2). In the other 11 replicates of this treatment, males showed no copulatory response or signs of interest in the females. In two of 25 replicates involving postmolt females with immature ovaries, males responded with following behavior. Newly molted males, premolt females without embryos but with prespawning ovaries, and intermolt females with embryos elicited no copulatory responses from males (Fig. 2). The null hypothesis of no difference among treatments was tested and rejected (Fisher exact test, $P < 0.001$).

**Duration of Sexual Attractiveness of Postmolt Parturial Females**

There was a decrease in female attractiveness to males with passing of time from the parturial molt (Fig. 3). The time of molting in isolated prespawning females with mature ovaries was determined with time-lapse video observation. Copulation occurred in 16 of 20 replicates when the male...
was exposed to the female within 2 h of her parturial molt, but this declined to a minimum of 6 of 20 replicates when the female was 6–8 h postmolt (Fig. 3). Other possible copulatory responses, such as following only or partial copulation (i.e., grasping and mounting) were not observed in this experiment. Longer postmolt time periods could not be tested because isolated females always spawned after 8 h from the parturial molt. The hypothesis of no difference among time periods from the parturial molt was tested and rejected (CMH Row Mean Scores Test, \( P = 0.903 \)).

**Sponge Bioassays for Contact Sex Pheromones**

In bioassay 1, males exposed to sponges wiped on the carapace of postmolt parturial females did not respond more than those exposed to sponges rubbed on the carapace of intermolt females or sponges treated with only seawater (Fig. 4). The hypothesis of no difference in male contacts with sponges among treatments was tested and accepted (CMH Row Mean Scores, \( P = 0.148 \)). Likewise, in bioassay 2, male contacts with the sponges were infrequent (Fig. 5) and there was no difference between the number of contacts with blank sponges with those that had been rubbed on postmolt parturial females (Wilcoxon test, \( P = 0.903 \)).

**DISCUSSION**

As Kamiguchi (1972) found with the shrimp *Palaemon paracaudens* De Haan, 1844, males of *Palaemonetes pugio* were not sexually attracted to postmolt parturial females in sealed glass vials. Although this negative evidence does not completely rule out visual stimuli, the absence of visual cues in sex recognition in this species would not be unexpected. The water of the marshy environment in which *P. pugio* lives is turbid, and perhaps even more importantly, molting and subsequent mating of prespawning females occur mainly at night (personal observations from time-lapse video recordings in this study and from Bauer and Abdalla, 2001).

Our results indicate that a possible cue used by males to detect prespawning, receptive (postmolt parturial) females in *Palaemonetes pugio* is an insoluble substance on the surface of the female exoskeleton, as suggested by anecdotal observations by Burkenroad (1947). The soft texture of the newly molted exoskeleton of a parturial female might serve as the stimulus detected by the male’s long chemotactile antennal flagella, usually the first appendages with which a shrimp contacts a nearby object. However, males showed no response to newly molted (soft) males and little response to newly molted females that were not parturial, i.e., without vitellogenic ovaries and not sexually receptive. Males only responded with obvious and intense copulatory behavior (grasping, mounting, spermatophore transfer) when touching a postmolt parturial female. Incomplete male copulatory responses were limited to a following behavior in two of the replicates with postmolt nonparturial females. Given that putative chemoreceptors on the male antennal flagella must make physical contact with the female exoskeleton to perceive the insoluble chemical stimuli, it is appropriate to term the substances involved “contact sex pheromones,” terminology commonly used in the insect literature (e.g., Ginzel et al., 2003).

In time-lapse video observations in which males were maintained with parturial females (Bauer and Abdalla, 2001), copulation always occurred within minutes of the female molt. In the present study, in which postmolt parturial females were presented to males some time (unknown exactly but less than one day) after their molt, but prior to spontaneous spawning, the percentage of pairings in which copulation occurred was much less (only 14 of 25 replicates). The lack of copulatory response or other signs of interest in the postmolt parturial female by the male in the other 11 replicates was initially surprising. These results suggested to us that the duration of attractiveness of parturial females after the molt was more limited in time than that documented for another caridean, the hippolytid *Heptacarpus paludicola* (see Bauer, 1979). The lack of male response in 11 replicates might have been caused by an extended period between the female molt and subsequent pairing of male and female. When pairings were then arranged in which the time of the female molt was exactly known from time-lapse video
observations, it was found that female attractiveness declined steadily over an eight hour period after the female molt. After eight hours, isolated females spawned without mating, and such females were not attractive to males. The rapid gradual decline in female attractiveness suggests that the contact pheromone may be a substance involved with the chemical modifications going on in the exoskeleton with time after the molt. On the other hand, Bauer and Abdalla (2001) found that females became unattractive within 30 minutes after copulation, which occurred within minutes of molting when males were present. This would suggest that the contact sex pheromone is secreted onto the exoskeleton by the female, rather than occurring as an integral chemical component of the exoskeleton. In the former case, the female would stop secreting it after successful copulation. Alternately, females may have rejected males after successful copulation with subtle behavioral signals that were not obvious in observations taken in Bauer and Abdalla (2001).

An alternate explanation suggested to us for the apparent decline in attractiveness of postmolt parturial females with the passage of time after the molt is that newly molted females are unable, perhaps because of the physiological shock of molting or the very soft cuticle, to resist copulation by males. With increasing time, females might be able to escape more readily from males without completion of copulation, thus giving rise to an appearance of unattractiveness to males. However, we observed no partial copulations nor pre-copulatory escapes of females from males in our observations. Furthermore, newly molted females of _Palaemonetes pugio_ and other carideans (e.g., _Lysmata wurdemanni_ (Gibbes, 1850), personal observation) are fully capable of escaping from pursuing males using the retrograde escape response. In Bauer and Abdalla (2001), parturial females maintained with two males mated within minutes of the parturial molt, immediately resisting further copulatory attempts from the same or the other male in the test arena.

The period of attractiveness of unmated postmolt parturial females to males appears to vary greatly among caridean species studied, although reports from previous studies are qualitative and not derived from formal experiments. In the palaemonids _Palaemonetes pugio_ (this study), _P. vulgaris_ (anecdotal observations, Burkenroad, 1947), and _Palaemon paucicentis_ (see Kamiguchi, 1972), the duration of attractiveness was no more than eight hours. In contrast, unmated postmolt parturial females are attractive to males and receptive to males up to 24 hours after the molt in the hippolytid _Heptacaropus paludicola_ (see Bauer, 1979) and _Rhyncocinetes typus_ (see Diaz and Thié, 2004). Given that all the above species occur at rather high densities, the period of attractiveness of the latter two species does not seem related to low frequency of contact with males.

The sponge bioassays were planned as first attempts to mechanically remove a possible contact sex pheromone from the cuticular surface of sexually attractive females. However, in both sponge bioassays, one in which the time from the molt of sample female was precisely known and less than four hours, males were not stimulated to copulatory behavior by sponges that had been rubbed over postmolt parturial (sexually attractive and receptive) females. The lack of male response in these bioassays might argue against a secreted contact pheromone, which should be removable, in favor of a substance, modified with time after the molt, which is an integral part of the exoskeleton. However, the null results are not strongly conclusive. Even if a putative contact pheromone were removed by the sponge, the male might require other female stimuli simultaneously, e.g., structural or behavioral, in order to respond with copulatory behavior. Behavioral stimuli are not always necessary to stimulate male copulatory behaviors, however, as shown by positive male responses to postmolt parturial females in amphipods which had been rendered immobile by freezing (Borowsky, 1991). Likewise, in a variety of insect species, in which a mix of cuticular hydrocarbons serve as contact sex pheromones, males will mate with freeze-killed females in receptive condition prior to death (e.g., Ginzel _et al._, 2003; Stoffolano _et al._, 1997). The intent in our sponge bioassay was to go one step further and remove the putative contact pheromone mechanically; however, the bioassays were negative. Various explanations are possible: a contact sex pheromone might not be present; the pheromone is present but not removable; or it was transferred to the test sponge but other stimuli, structural or behavioral, are necessary to evoke a mating response in male _P. pugio_. Mechanical removal of contact pheromone is a possibility, as shown by the study of Ginzel _et al._ (2003) on the coleopteran _Megacyllene robiniae_ ( Förster), in which wipe samples were used to collect pheromone (cuticular hydrocarbons) for gas chromatography–mass spectroscopy (GC–MS) analysis (but not bioassay).

Cuticular hydrocarbons, which prevent desiccation in insects (Simmons _et al._, 2003) but also serve as contact pheromones (Howard and Blomquist, 2004) are also present in the crustacean epicuticle (Dennell, 1963; Moore and Francis, 1985). Another alternative is that males might detect, using contact chemoreception, changes in the newly molted exoskeleton of a postmolt parturial female, such as quinone tanning (sclerotization) (Stevenson, 1985) and calcification (Roer and Dillaman, 1984). Glycoproteins and/or small proteins have been indicated as contact sex pheromones on the surface of female copepods, perceived by the males with the antennules (not antennae, as in _P. pugio_) upon physical contact (Ting and Snell, 2003). In many insects, the male antennae perceive the female contact sex pheromone, but taste receptors on the forelegs and mouthparts are used in male _Drosophila melanogaster_ (Bray and Amrein, 2003). Likewise, not only the long antennal flagella, but also antennular flagella of similar length and morphology in many caridean species, as well as thoracic appendages, may bear taste receptors (contact chemoreceptors) that detect contact sex pheromine in carideans shrimps or other crustaceans.

If a contact sex pheromone does exist for _Palaemonetes pugio_ and other carideans, what might be its source? One possibility, suggested above, is that the compounds associated with the newly molted exoskeleton might serve as a signal. Sex-specific cuticular proteins on or near the surface of the newly molted female might become less attractive to males as they become cross-linked by quinones during sclerotization ("quinone tanning," Stevenson, 1985), thus explaining the decrease in attractiveness of postmolt parturial females to males with time from the molt. Cuticular hydrocarbons, known contact sex pheromines in insects; surface glycoproteins,
implicated as contact sex pheromones in copepods (Kelly and Snell, 1998; Ting et al., 2000); or phenoloxidases involved in sclerotization might serve as contact sex pheromones in crustaceans. Such substances might be secreted onto the surface of the female from hypodermal (epidermal) cells via pore canals (Halcrow and Bousfield, 1987; Borowsky, 1991; Roer and Dillaman, 1984) or tegumental glands (Telbat and Demers, 1993; Bushman and Atema, 1996) that open onto the cuticular surface. The chemical nature and timing of secretions by these structures could be varied, which would make them useful sources of sex pheromones indicating receptivity of post molt parturial females.

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