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RESEARCH ARTICLE

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Multiple paternity in the thalassinidean ghost shrimp, *Callichirus islagrande* (Crustacea: Decapoda: Callianassidae)

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Abstract Adult ghost shrimp, *Callichirus islagrande* (Schmitt, 1935), are obligate inhabitants of burrow systems that they excavate deeply into beachfront sediments in the northern Gulf of Mexico. Mating presumably occurs in these burrows but has never been directly observed in *C. islagrande* or any other thalassinidean. A variety of possible mating systems is suggested by those of other decapods, among which are examples of both internal and external sperm deposition, sperm competition among males, and male adaptations for paternity assurance. We used genetic markers to determine if clutches brooded by individual female ghost shrimp had been fertilized by multiple males. The two microsatellite loci we employed were sufficiently polymorphic to detect 95% of the occurrences of fertilization by two males. Among 40 ovigerous females collected from the Louisiana Isles Dernieres barrier island, eight (20%) carried egg masses for which more than two paternal alleles were detected, indicating fertilization by multiple males. In two cases of multiple paternity, alleles from one of the males were detected in only a portion of an egg mass. These observations suggest several, possibly novel characteristics of the mating system that can be further investigated.

Introduction

Diverse mating systems are known from the crustacean order Decapoda although they have been well characterized for only a few groups. In all decapod species that have been examined sperm are non-motile (Felgenhauer and Abele 1991; Jamiesen and Tudge 2000) and are deposited by males in packets (spermatophores) either externally on the female's exoskeleton or internally in special receptacles (spermathecae; Bauer 1986; Subramoniam 1993). In some taxa (e.g., advanced brachyurans) sperm stored in spermathecae are retained through molts and can fertilize eggs months after mating has occurred (Subramoniam 1993). Sperm storage also allows females to mate sequentially and store sperm from multiple males, which in turn has promoted adaptations for males either to displace the sperm of a female's previous mates or to block the deposition of sperm by subsequent mates (e.g. Diesel 1991).

For those decapod taxa in which spermatophores are externally deposited, females do not usually store sperm (Subramoniam 1993). Without sperm storage females can collect sperm from multiple males only by mating with them within a brief period of time, either simultaneously or in succession. Studies of crustacean mating systems have revealed several mechanisms that should increase paternity assurance and decrease the incidence of multiple paternity (Diesel 1991; Koga et al. 1993). These include precopulatory and postcopulatory mate guarding (Hinsch 1968; Watson 1972; Hooper 1986; Murai et al. 1987; Caldwell 1991; Durbaum 1995; Jormalainen 1998; Minouchi and Goshima 1998). However mate guarding is not universal, and in some taxa multiple mating could be the norm. For example, in a mole crab reported as *E. asiatica* H. Milne Edwards, junior synonym of *E. emeritus* (Linnaeus, 1767), up to five males have been observed simultaneously depositing spermatophores on a single female (Subramoniam 1977, 1979). Other mechanisms of paternity assurance include repetitive copulation (Bauer 1992), sperm plugs (Elner et al. 1985; Bauer and Min 1993), and increased ejaculate size (Diesel 1991). However it is difficult to determine the effectiveness of these mechanisms without examination of the actual paternity of egg clutches in natural populations.

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The application of genetic markers to determine paternity in other taxa (e.g., mammals and birds) has revealed many surprising examples of polyandry in species that had been thought to be monogamous (Morell 1998). Although studies of paternity in crustaceans are relatively limited, multiple paternity of crustacean egg clutches has been demonstrated with allozyme markers for lobsters (Nelson and Hedgecock 1977), isopods (Heath et al. 1990), and crabs (Burfitt 1980; Sevigny and Sainte-Marie 1996) and more recently, with microsatellite markers for crayfish (Walker et al. 2002), lobsters (Jones et al. 2003), and crabs (Urbani et al. 1998a, 1998b; Sainte-Marie et al. 1999).

Thalassinidea is recognized as an infraorder of decapod crustaceans with uncertain and possibly paraphyletic relationships to other Decapoda (Tudge and Cunningham 2002). As adults, thalassinideans are fossorial, either burrowing in marine sediments or occupying mud-lined cavities within rocky or coralline substrates. Because mating occurs in these cryptic locations our limited knowledge of their mating systems is based on indirect lines of evidence. Spermathecae have not been described for any thalassinidean despite attempts to find them. Experiments with the callianassid *Nihonotrypaea harmandi* (Bouvier) also suggest that sperm are not stored; when males were physically excluded from post-molt females fertilization did not occur (Tamaki et al. 1996,1999). Externally deposited spermatophores have yet to be reported for any thalassinidean, so their presence, morphology, and means of placement remain a mystery. Threadlike strands of spermatophores have been observed in the lower vas deferens of *Callichirus islagrande* (Schmitt), but no observations of mating or deposition of spermatophores have been recorded to date, despite concerted attempts to do so (L. Leblanc and D. Felder, unpublished observations).

C. islagrande constructs deep burrows in intertidal and shallow subtidal beachfront quartzite sands of the northern Gulf of Mexico (Felder 2001). Adult burrows can extend 2 m or more below the surface and interconnect through horizontal passageways. Burrow diameters appear to fit the individuals that occupy them too closely to allow adults to move past each other, although there are larger chambers that could accommodate multiple individuals and the horizontally connected portions of the burrow matrix are larger in diameter than vertically oriented burrows. Females brood several thousand eggs on their pleopods until newly hatched larvae are released into the burrow waters. After a planktonic phase of approximately 2 weeks, the decapodid larvae of *C. islagrande* settle and metamorphose into burrowing juveniles (Strasser and Felder 2000). It is believed that mating occurs within the burrows because adults have not been observed outside them and appear helpless if forcibly removed. However essentially nothing is known about the mating behavior of *C. islagrande*. Males possess an enlarged chela, although its function in either mating or intrasexual competition is unknown. It is not known how many potential mates are typically encountered by females, although multiple encounters seem likely with burrow densities in the range of 5 to 100 m^{-2}. The lack of evidence for sperm storage and the confinement of adults within a system of narrow burrows suggest the possibilities of mate guarding, pair formation, or other mechanisms of paternity assurance. To investigate these possibilities, we used microsatellite markers to determine if egg clutches brooded by individual females had been fertilized by multiples males.

Materials and methods

Study sites and collections

Ovigerous females ($n = 40$) were collected from a 20-m section of beach at Isles Dernieres, Louisiana, between May and August 1998 with a hand-operated vacuum suction device (yabby pump). The density of burrows at this site varied from 15 to 50 m^{-2} with a mean of 24 m^{-2}. Ovigerous females were maintained in seawater with a salinity of 35‰ until tissue sample collection. Genomic extracts of ovigerous females and their egg clutches were prepared from fresh muscle tissue of the minor chela and two samples of each egg clutch: 150 to 225 eggs from the right first pleopod, and 34 to 65 eggs from the left fourth pleopod. Because there were numerous eggs in each sample we determined the total set of alleles represented in each sample of eggs but not the genotypes of individual eggs. For all samples, genomic DNA was extracted and purified following PureGene (Gentra Systems) and Prep-A-Gene (Bio-Rad) protocols for animal tissue and quantified on a Hoefer TKO100 fluorometer.

Microsatellite detection and characterization

Microsatellite sequences were isolated from a genomic library of *C. islagrande* in the plasmid vector pZERO-2.1 (Invitrogen). Miniprep DNA samples from 428 recombinant clones were UV-crosslinked to (Magnagraph) nylon membranes (Micron Separations, Inc.) and sequentially probed with $(GA)_n$, $(CA)_n$, and $(AAT)_n$ digoxigenin-labeled oligonucleotides (Boehringer Mannheim). Positive clones were sequenced with Big Dye Terminator Sequencing chemistry on an ABI Prism 310 genetic analyzer (Applied Biosystems).

PCR primers were designed for those microsatellite sequences that had a minimum of 24 repeats and suitable flanking sequences. For this study we utilized two microsatellite loci (*1–3* and *1–10*) to detect multiple paternity. The primer sequences for these loci were:

1–3: TGAAGACAACCAGAAGTGAAGA, CGACGACAATACACATACCTCG

1–10: ATGATAAAAAGGAAAGATGACA, GTAAGACTAACGACGCCGAAC

Amplification conditions were optimized on a Stratagene Robo-Cycler and were used for all subsequent reactions. The optimized amplification profile was 10 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 51°C, 59°C (for *1–3* and *1–10*, respectively), and 1 min at 72°C, and ended with 10 min at 72°C. Each 25-μl reaction included 10 pmol of each primer; 2 m *M* of each dNTP; 1× PCR buffer (10 m *M* Tris-HCl, pH 8.3, 50 m *M* KCl, 1.5 m *M* $MgCl_2$, 0.01% (w/v) gelatin, PE Applied Biosystems); 1.25 U Taq polymerase (PE Applied Biosystems); and template DNA (10–25 ng). Amplification products were analyzed with an ABI Prism Genetic Analyzer and GeneScan 4.0 software (Perkin Elmer Applied Biosystems). For cases in which the multiple peaks of allele traces overlapped, we compared these traces with those from synthetic mixtures of the alleles to verify our scoring interpretation.

An egg clutch was considered a product of multiple paternity if three or more non-maternal alleles were detected at one or both microsatellite loci. The probability of detecting multiple paternity was estimated from observed allele frequencies among ovigerous females. A computer program (available on request from JN) was used to consider the single-locus parental genotypes of every possible mating that would involve one female and two males. The probability of each type of mating was calculated as the product of the frequencies of the six alleles represented by the three parental genotypes. Cases in which multiple paternity could be detected were defined as those in which the two males' genotypes considered together included more than two alleles not found in the female's genotype (more than two non-maternal alleles in eggs would imply fertilization by more than one diploid male). The probabilities of all cases in which multiple paternity could be detected were summed to give the overall probability of detection for each locus. The probability of detection for both loci, *P*(detection), was calculated with the assumption of no linkage as

$$P(\text{detection}) = 1 - P(\text{not detected with 1 - 3}) \times P(\text{not detected with 1 - 10})$$

Results

Both microsatellite loci were highly polymorphic. Among the genotypes of the 40 ovigerous females, 29 alleles were found at locus *1–3* and 31 alleles at locus *1–10*. In no cases did there appear to be more than two alleles amplified from a single female. This lack of artifactual products has also been confirmed with amplifications from an additional 513 adult and juvenile specimens of *C. islagrande* (unpublished data). There was no evidence for null alleles at *1–3* (every female was heterozygous). However, amplification of locus *1–10* was unsuccessful for seven females, which could indicate they were homozygous for null alleles. An additional 10 females were either null allele heterozygotes or true homozygotes for other alleles. Null alleles could alter the probability of specific instances of multiple paternity being detected, but they should not result in "false positives." Based on observed allele frequencies at both loci, the probability that an egg clutch fertilized by two males would be detected as a product of multiple paternity was 0.90 for locus *1–3* and 0.52 for locus *1–10*. In combination, the probability of multiple paternity detection was 0.95.

All egg clutches that were analyzed contained developing embryos. Multiple paternity was detected in 8 of the 40 egg clutches (Table 1). In clutch 44, five non-maternal alleles were detected for locus *1–3*, which indicated fertilization by three males. In two of the clutches classified as multiple paternity, alleles from one of the male parents were detected in the eggs sampled from the fourth pleopod but not the first pleopod. This observation suggests that the sperm from these males was distributed to only a portion of the egg mass.

Discussion and conclusions

Unlike most investigations of paternity with genetic markers, the present study does not extend prior observations of mating behavior. In *Callichirus islagrande* and other species for which direct observations of mating are impractical, genetic analysis of paternity is one of the few means available to learn anything about mating under natural conditions. Along with other indirect evidence, genetic data can suggest hypotheses about mating systems that can be tested by subsequent experiments or morphological investigations. Our genetic data indicate that egg clutches of some females (20% in our sample) are fertilized by two or more males. There is no evidence for spermathecae in *C. islagrande* and experiments with *Nihonotrypaea harmandi* by Tamakai et al. (1996) suggest that sperm storage is not a characteristic of the Thalassinidea. A lack of sperm storage in *C. islagrande* would imply that these females mated with multiple males either simultaneously or over a short period of time. Thus, it does not appear that mating pairs of *C. islagrande* are isolated from other adults within the system of burrows, but rather that interconnections between burrows allow females to encounter multiple males. In two cases alleles from one male were restricted to a portion of the egg mass. This indicates that sperm are not completely mixed with eggs during oviposition, and perhaps that the location or timing of contact with sperm from any one male could be suboptimal.

No observation of deposition of spermatophores has been recorded for any thalassinidean. Spermatophoric strands observed in the lower vas deferens of *C. islagrande* could assume a shape similar to those observed in other decapods (e.g., anomurans) upon extrusion and be

Table 1 Genotypes of females and alleles detected in their egg clutches. Alleles are designated by length in base pairs (bp). *Parentheses* indicate possible null alleles. For alleles found in egg clutches, maternal alleles are in *boldface*and *asterisks* indicate alleles detected in eggs from the left fourth pleopod but not the right first pleopod

Female	Female genotype	Egg alleles	
		Right first pleopod	Left fourth pleopod
Female 5			
Locus 1–3	306 318	268 282 **306 318** 330	268 282 **306 318** 330
Locus 1–10	256 264	216 254 **256 264**	216 254 **256 264**
Female 13			
Locus 1–3	280 314	270 **280** 300 304**314**	270 **280** 300 304**314**
Locus 1–10	214 232	**214 232** 252 265	188* **214 232** 252 265
Female 15			
Locus 1–3	292 306	278 **292 306** 324 334	278 **292 306** 324 334
Locus 1–10	200 (200)	**200** 250 254 266	**200** 250 254 266
Female 23			
Locus 1–3	310 316	296 298 **310 316**	296 298 **310 316**
Locus 1–10	256 (256)	178 192 200 216 **256**	178 192 200 216 **256**
Female 25			
Locus 1–3	322 328	292 294 **322 328** 332	292 294 **322 328** 332
Locus 1–10	258 266	220 224 **258 266**	220 224 **258 266**
Female 34			
Locus 1–3	290 304	272 **290 304** 312 318	272 **290 304** 312 318
Locus 1–10	258 268	198 224 252 **258 268**	198 224 252 **258 268**
Female 42			
Locus 1–3	284 302	270 278 **284** 288**302** 306	270 278 **284** 288**302** 306
Locus 1–10	236 (236)	208 220 **236** 254 258	208 220 **236** 254 258
Female 44			
Locus 1–3	300 322	268 274 282 292 **300** 314**322**	268 274 282 292 **300** 314**322**
Locus 1–10	264 266	214 **264 266**	190* 214 **264 266**

deposited externally on ventral surfaces of the female, but no such spermatophores have been detected on several thousand wild-captured females examined over many years of study. We thus cannot exclude the possibility of a novel thalassinidean mating system, perhaps one that would favor multiple paternity of a female's egg mass. The obligate fossorial habitat of thalassinideans could restrict mating opportunities to burrows that are periodically interconnected by water flow. The burrow system of *C. islagrande* is a matrix of interconnected burrows; this structure could favor a novel system of sperm transfer in thalassinideans, just as fossorial adaptations are also reflected in behaviors and structures for burrow ventilation, respiration, and feeding (Coelho et al. 2000; Coelho and Rodrigues 2001; Felder 2001).

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