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GENETIC ASSESSMENT OF PARENTAGE IN THE CARIDEAN ROCK SHRIMP *RHYNCHOCINETES TYPUS* BASED ON MICROSATELLITE MARKERS

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ABSTRACT

Over the past decade, the common rock shrimp, *Rhynchocinetes typus* H. Milne Edwards, 1837, has been the focus of extensive investigations on mating behaviour. The species is now perceived as a model system for the study of reproductive strategies and sexual conflict in crustaceans displaying external fertilization. Using molecular markers, the current study assesses whether social mating behaviour in common rock shrimp translates into true genetic parentage. In a large mesocosm tank with >200 individuals of both sexes, the analysis of 15 families (22 eggs per female) for three informative microsatellites unambiguously confirmed multiple paternity in 11 instances (73%) involving, in each case, two to four males. Where more than one male was identified siring a particular brood, reproductive skew was apparent towards a single individual. Results suggest that multiple paternity in this species results from subordinate male coercive behaviour, female solicitation of multiple male matings or a combination of both.

KEY WORDS: convenience polyandry, cryptic female choice, multiple paternity

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INTRODUCTION

In recent years, molecular parentage studies have been revolutionising our understanding of mating behaviour. While studies based on the direct observation of individuals' interactions may provide relevant information about mating behaviour and/or strategies, only genetic based investigations can establish actual biological relationships of individuals. Thus, genetic data are particularly suited to investigate the evolution of such behaviour and/or strategies. Substantial variation in mating strategies has been reported in crustaceans, which have been linked to their high level of morphological diversity and extensive habitat ranges (Martin and Davies, 2001; Thiel and Duffy, 2007). In general, studying mating behaviour in crustaceans in their natural environments is difficult due to logistic constraints imposed by several factors including nocturnal behaviour and extreme habitat types. Species that are amenable to laboratory conditions are particularly useful as model systems by which observed mating behaviours can be compared with genetic parentage inference to enrich mating theories.

Over the past decade, the common caridean rock shrimp *Rhynchocinetes typus* H. Milne Edwards, 1837 has been the focus of detailed behavioural studies. The species is also particularly amenable to laboratory experimentation making it a strong candidate for a model system for the study of reproductive strategies and sexual conflict. *R. typus* is a gonochoric shrimp, endemic from the south east Pacific along northern and central Chile, commonly found in subtidal habitats (Caillaux and Stotz, 2003; Correa and Thiel, 2003). The species displays nocturnal behaviour and is found across a wide range of subtidal communities, from caves and crevices to stony barren grounds (Caillaux and Stotz, 2003; Ory et al., 2012).

As in some other shrimp species (Bauer, 1986a), mating in this species occurs after the female's molt and appears coercive in nature (Thiel and Hinojosa, 2003). High levels of male competition have resulted in females evolving opposing behaviours for the intersexual conflict of mating including, for instance, avoidance and/or delayed spawning mating tactics, presumably enabling them to select the fittest males (Diaz and Thiel, 2003). In addition, females have also developed pre-copulatory behaviour of tolerating advances by subordinate males of different ontogenetic stages. Females may allow these males to transfer spermatophores to their pleon to avoid injury by harassing males, a process described as convenience polyandry (Thiel and Hinojosa, 2003). Following the initial acceptance of spermatophores, females have been observed removing the spermatophores of subordinate males before receiving spermatophores from dominant males which have been linked to the commencing of egg extrusion (Thiel and Hinojosa, 2003). Since females seem to have evolved efficient behaviours to discriminate against egg fertilization by subordinate, less desired males, it has even been suggested that females induce mating con-

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tests in order to become guarded and mated by the strongest, dominant male (Thiel and Correa, 2004).

An increasing number of genetic based studies have reported on the incidence of multiple paternity in crustacean species (Walker et al., 2002; Streiff et al., 2004; Toonen, 2004; Bilodeau, 2005; Gosselin et al., 2005; Yue et al., 2010; Bailie et al., 2011). Genetic parentage analyses on broods of this species, however, have not yet been undertaken. In this study, we test the hypotheses that female behaviour of *R. typus* (handling multiple spermatophores) is conducive to (1) multiple paternity, and (2) cryptic female choice, which likely results in highly skewed male success. To test these hypotheses we generated and analysed microsatellite multi locus genotypic data for females and their broods to infer the paternal contribution.

Material and Methods

Rhynchocinetes were collected in the winter of 2008 from the Bahia La Herradura in Coquimbo (29°58′S, 71°21.2′W), located on the coastline of northern-central Chile. Specimens were collected from hard substrata at a depth range of 4-7 m. The populations of *R. typus* (200+ shrimps) were maintained in a large semi-natural mesocosm (167 cm × 227 cm in surface area and 30 cm in depth) in a research laboratory at the University Católica del Norte in Coquimbo, Chile, between October and December 2008. Morphological inspection of the specimens of *R. typus* indicated that females and males were present in equal numbers. The number of shrimps in the mesocosm (200+) reflects the high densities occasionally observed in natural populations (Ory et al., 2012). Since sample collection ended in mid-November, and ovigerous females were sampled at the end of December, the broods of all females had been fertilized within the mesocosm tank. This assumption can be confidently made given that embryonic development for *R. typus* takes 30 days in water temperatures above 15°C (Dupré et al., 1992), which has been the case in the experimental tank, and consequently, all females must have liberated previous broods and produced new broods while being maintained in the tank.

A subset of the population sample comprising 15 ovigerous females was removed in December 2008 for parentage analysis. Females of this species carry their eggs externally on their pleon; therefore, biopsy tissue samples from females and their eggs can be effortlessly preserved in 99% molecular grade ethanol for subsequent genetic testing. On average, 22 fertilized eggs (1-3% of the brood) were randomly sampled from each of the ovigerous females' abdominal region in three cross sections to ensure good coverage of the brood (i.e., random representation of eggs). Genomic DNA was extracted from the abdominal tissue of all females and eggs using the Promega Wizard DNA Purification system (http://www.promega.com).

Microsatellite marker development followed the protocol described by Fitzpatrick et al. (2011). Polymerase chain reaction (PCR) primers were designed (PRIMER SELECT, DNASTAR) for those sequences containing microsatellites with unique flanking regions of adequate length and features, e.g., no repetitive elements identified in local BLAST analysis (Bioedit). Following initial marker characterisation (Fitzpatrick et al., 2011), three polymorphic microsatellite markers were selected for this parentage study as follows: RS64 (forward primer 5′-AAT TGC GAT TGC GAA GTA AAG A-3′; reverse primer 5′-TTA GGC AGC TTA AAT GTT GAA TCA-3′), RS93 (forward primer 5′-AGC CTG CCA ACT ATA CTA AAA GAA-3′; reverse primer 5′-AAG AGT AGT GGC AAG CTG TGT CAG-3′), and RS186 (forward primer 5′-CTT TCG GAT ATG AAG CAT GAT AAC-3′; reverse primer 5′-AAC ATC GGG TAC AAA ATT CTC TTT-3′). These markers were used to produce the genotypes from genomic DNA extracted from all adults and eggs through a multiplex PCR developed and optimised for an ABI genotyping platform to reduce screening costs.

Multiplex PCR reactions were carried out in 3.5 μl volumes consisting of 1 μl of template genomic DNA (5-12 ng), 1.75 μl of plain PP mastermix (http://www.top-bio.com), 0.1 pM of each primer with double-distilled water used to complete the volume. For each of the primer pair combinations the forward primer was labelled with a fluorescent dye as follows: VIC-RS64, FAM-RS93 and NED-RS186. PCR thermo-cycling consisted of 1 cycle at 95°C for 5 min followed by 35 cycles of 95°C for 1 min, 57°C for 30 s and 72°C for 90 s, and 1 final cycle at 60°C for 30 min. PCR products were diluted 1:2 with ddH2O and 1.5 μl of this dilution transferred to a new 96-well microtitre PCR plate containing 9 μl of a Hi-Di formamide solution containing GeneScan™-600LIZ size standard (stock comprised of 5 μl of GeneScan™-600LIZ size standard and 900 μl of Hi-Di formamide; Life Technologies). PCR products were subsequently denatured at 95°C for 3 min, directly followed by 5 min incubation on ice. Plates were assembled into septa plates and run on a 96 capillary 3730XL DNA Analyzer (Applied Biosystems). Genotype calls from raw fragment size profiles for each individual were obtained using GENEMAPPER v4.1 (Applied Biosystems).

The power of the three microsatellite markers for detecting multiple paternity was assessed through simulation analysis implemented in the software PrDM (Neff and Pitcher, 2002). The simulations take into consideration varying numbers of males (two to four males based on other crustacean parentage genetic studies) and varying reproductive skew of the males' contribution (equal, moderately or highly skewed). Summary population statistics were determined using diveRsity (Keenan et al., 2013). The parentage reconstruction software GERUD version 2.0 (Jones, 2005) was used to determine the minimum number of males contributing to a brood. Multiple paternities were concluded for a brood when more than two paternal alleles were identified and this pattern was consistent across more than a single locus to rule out mutation as an underlying factor.

Results

The three microsatellite markers utilised in the current study were found to be sufficiently informative to address the hypotheses of multiple parentage. No significant departures from Hardy Weinberg equilibrium or genotypic disequilibrium were observed in the three loci (Table 1). Expected heterozygosity ranged from 0.30 to 0.91 (average 0.69), allelic diversity ranged from five to 14 (average 9.7), allelic richness across all three loci was 8.12 and null alleles were not detected in the current study (Table 1). Given the average number of eggs screened per family in the current dataset ($N = 22$), the power provided by the three microsatellite markers utilised ranges from 84 to 100% (Table 2).

Results from GERUD analysis indicate that multiple paternities were evident in 11 of the 15 female broods examined (73%). Further scrutiny of the paternal allelic contribution in each case indicates that for 9 of these 11 families (60%), multiple male alleles are present across two or more loci (see Table S1 in the Supplementary material in the online version of this journal, which can be accessed via http://booksandjournals.brillonline.com/content/journals/1937240x). For the two remaining families, where additional paternal alleles were only observed at one single locus, further examination of results from GERUD analyses indicates that the allelic segregation of the brood for the other two loci deviates from that expected under Mendelian inheritance of a 1:1 ratio, therefore confirming multiple male

Table 1. Summary statistics for the sample of *R. typus*. N = total number of individuals, A = number of alleles and AR = allelic richness per locus and overall. Observed and expected heterozygosities (Ho and He, respectively) including statistical test for departure from Hardy Weinberg Equilibrium (HWE).

	RS64	RS93	RS186	Overall
N	15	15	15	15
A	14	5	10	29
AR	11.60	3.82	8.95	8.12
Ho	1.00	0.33	0.93	0.76
He	0.91	0.30	0.88	0.69
HWE	$p > 0.05$	$p > 0.05$	$p > 0.05$	$p > 0.05$

Table 2. The probability of detecting multiple mating scenarios determined by PrDM simulations based upon the three microsatellite markers used in this study, demonstrating the likelihood of detecting multiple mating scenarios: (1) equal contribution, (2) slightly skewed contribution and (3) highly skewed contribution. The simulations take into consideration the possibility of varying numbers of males ranging from two to four. Numbers in italics are discussed throughout the text.

Mating scenarios and ratio of male contributions		Brood size (N)				
		10	20	30	40	50
2 males	(50:50)	0.98	1.00	1.00	1.00	1.00
	(75:25)	0.91	0.99	0.99	1.00	1.00
	(90:10)	0.61	*0.84*	0.93	0.97	0.98
3 males	(33:33:34)	1.00	1.00	1.00	1.00	1.00
	(50:25:25)	1.00	1.00	1.00	1.00	1.00
	(45:45:10)	0.99	1.00	1.00	1.00	1.00
	(75:15:10)	0.92	0.99	1.00	1.00	1.00
4 males	(25:25:25:25)	1.00	1.00	1.00	1.00	1.00
	(30:30:30:10)	1.00	1.00	1.00	1.00	1.00
	(50:20:20:10)	1.00	1.00	1.00	1.00	1.00
	(50:25:13:12)	1.00	*1.00*	1.00	1.00	1.00

contributions. Only four families (27%) were exclusively sired by one male. On instances where multiple paternity was observed, in each case the number of males siring the females' brood was found to range from two to four as follows: five families (33%) involved multiple mating with two males, two families (13%) can be explained by the contribution of three males, and in the remaining four families (27%), four males were involved (Fig. 1).

For the 11 polyandrous families identified (Fig. 1), the relative contribution of each male, i.e., male skew, is of particular interest to address the hypothesis of female cryptic choice. On the five instances where two males were identified, for four of them, a significant skew (90% success) was observed for one of the males. For the remaining case, while a skew in contribution was also apparent, it was of a more moderate level of approx. 75% (Fig. 1). It is important to note that the power to detect multiple paternity in those instances involving two males and disproportionate contributions to the brood was very high, ranging from 84 to 98.5% (Table 3). For four of the six remaining cases involving more than two males, while two (out of three) or three (out of four) males appear to have been equally successful in terms of contribution to the brood, in all instances one male is highly skewed against (Fig. 1). Power estimates to detect multiple paternity under these scenarios ranged from 99 to 100% (Table 3). For the other two families involving four males, one of them contributes to 50% of the brood, two with an equal 20% contribution, while the fourth male is highly skewed against (Fig. 1). The power to detect multiple paternity in these particular cases was 99.6% (Table 3). To summarise, in all detected incidents of multiple paternities, a single male was strongly discriminated against, whilst in nine families (82%) a significant skew in contribution was observed in favour of a single male (responsible for siring 50-90% of the brood).

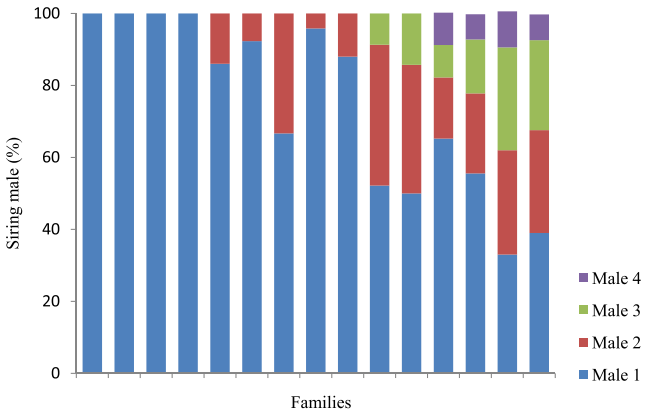

Fig. 1. Bar graphs produced from GERUD analyses that represent each of the 15 *R. typus* families analysed. The colours within each bar depict the contribution from individual sire to each brood.

Discussion

Coercive mating by males is common in many crustacean species that display external fertilisation (Ra'anan and Sagi, 1985). Common rock shrimp male mating behaviour has been the focus of many studies; it can vary greatly depending on ontogenetic developmental stage, male competition and recent mating history (Van Son and Thiel, 2006). As females moult asynchronously, this results in a skewed operational sex ratio (OSR) which invariably leads to a higher level of male competition (Correa and Thiel, 2003). During mating interactions, dominant males, i.e., robustus males, tend to adopt a mate and guarding strategy whereas subordinate males, i.e., smaller typus and intermedius males, usually adopt a sneaking mating tactic (Correa et al., 2003). Male mating behaviour is also related to their recent mating history. Thus recently mated dominant males have been reported to be able to conserve their large sperm reserves in an attempt to increase mate quality in subsequent encounters (Van Son and Thiel, 2006). This behaviour is not as common in subordinate males as they have significantly smaller sperm reserves in comparison (Hinojosa and Thiel, 2003). Given that intersexual conflict favours males up until the point of copulation, females have evolved a repertoire of strategies to ensure their influence over the mating outcome.

Table 3. Summary of the frequency of observed patterns of paternal skew within the 11 polyandrous families identified. Five scenarios were categorised and the probability of detecting these mating scenario in the rock shrimp broods were simulated using PrDM software.

	No. of sires				
	2 males		3 males	4 males	
Skew detected	(90:10)	(75:25)	(45:45:10)	(30:30:30:10)	(50:20:20:10)
No. of families	4	1	2	2	2
Probability of detecting skew pattern (PrDM)	0.841	0.985	0.999	1.000	0.996

Coercive mating by males is counteracted by females by avoidance and/or delayed spawning mating tactics, ultimately enabling them to select the fittest males (Diaz and Thiel, 2003; Thiel and Hinojosa, 2003; Thiel and Correa, 2004). Females accept multiple spermatophores to avoid injury by harassing males during the mating process, which is known as convenience polyandry (Thiel and Hinojosa, 2003). Based on such behavioural observations, the hypotheses would be that multiple males contribute to the females' brood and hence multiple paternity is the norm in this species. However, this hypothesis underestimates the impact of female behaviour once she has been coerced to accept spermatophores from multiple males.

Despite male mate guarding, female manipulation of spermatophores is known to occur in *R. typus* (Thiel and Hinojosa, 2003). Notwithstanding the role that sperm competition, i.e., spermatophore from multiple males, may play during the mating process for *R. typus*, spermatophore manipulation by females has been observed, and is likely to be related to male ontogenetic stage, i.e., females showing preference. It appears that females tend to remove the spermatophores of smaller (typus or intermedius), subordinate males, but retain those of large (robustus), dominant males, which they received later during the mating interactions (Diaz and Thiel, 2003; Thiel and Hinojosa, 2003). This strategy is known as cryptic female choice. In addition to these strategies, females have been suggested to search for higher quality males (Diaz and Thiel, 2004). This behaviour by females also appears to counteract coercive male mating and guarding strategies raising additional questions over the true genetic outcome (in terms of parentage) of all these mating tactics.

In this preliminary study, genetic data confirm that the broods of *R. typus* are often sired by multiple males. Coupled with known mating behaviour (Thiel and Correa, 2004), results suggest that mating with multiple males is a common mating strategy in the common rock shrimp. The frequency of multiple paternities is analogous to what has been reported for other crustacean parentage studies (66.7% in Bailie et al., 2011; 54.6% in Streiff et al., 2004; 60% in Walker et al., 2002). The estimates of the minimum numbers of males identified are also comparable to numbers previously published for other polyandrous crustacean species (Walker et al., 2002; Streiff et al., 2004; Toonen, 2004; Bilodeau et al., 2005; Gosselin et al., 2005; Yue et al., 2010; Bailie et al., 2011). Again in concordance with previous studies (Walker et al., 2002; Gosselin et al., 2005; Bailie et al., 2011), for all polyandrous *R. typus* broods analysed, reproductive skew was evident. In the majority of these instances, this skew meant that one male was invariably more successful siring offspring than others.

Despite the results, demonstrating for the first time in *R. typus*, evidence for both multiple paternities and skewed reproductive male success, there are still a number of unanswered questions. For instance, it is not known whether multiple paternity is the outcome of coercive behaviours by subordinate males as suggested by Correa et al. (2003) or whether it is a consequence of females actively inducing mating contests among multiple males as suggested in previous studies (Thiel and Hinojosa, 2003; Thiel and Correa, 2004) or a combination of both. It is also unknown whether/how the reproductive history of both males and females may influence the outcome of mating interactions (Van Son and Thiel, 2006).

To summarise, our results from this preliminary study suggest that multiple paternity in *R. typus*, is likely to be the result of a combination of factors involving subordinate male coercive behaviour and female solicitation of multiple male matings. It is still not known, however, which intrinsic or extrinsic factors are responsible for generating the observed variation in paternity. To address these questions, in addition to further studies looking into intersexual conflict over mating success, ecological factors will also have to be considered including, for instance, demography, e.g., population density and distribution, and/or environmental dynamics, for instance, habitat and predation pressures (Correa and Thiel, 2003; Van Son and Thiel, 2006; Ory et al., 2012). A genetic approach, as the one employed in here, in combination with well designed breeding experiments and future field studies could help to address these questions.

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SUPPLEMENTARY MATERIAL

Table S1. Allelic data from the three microsatellite marker loci for 15 families of *R. typus*. In each instance the maternal genotype (recorded) and paternal alleles (inferred from offspring by subtraction) are reported (alleles and genotypes expressed as size in base pairs). The minimum number of males that the female must have mated with to explain the observed offspring allelic combinations is also reported. An asterisk (*) following the family ID indicates that multiple males are evident across multiple loci and, therefore, ruling out mutation as a factor. The hashtag (#) suggests multiple males at a single locus but segregation of alleles within the brood deviate from that expected by Mendelian inheritance and, therefore, confirming multiple male contributions. Four families were found to have contribution from a single male only.

Family ID	Microsatellite locus						Min. no. of males
	RS64		*RS93*		*RS186*		
	Female genotype	Paternal alleles	Female genotype	Paternal alleles	Female genotype	Paternal alleles	
Family 1#	*136*, *142*	*128*, *134*	*371*, *371*	*371*	*222*, *226*	*224*, *226*, *228*	2
Family 2	*128*, *134*	*140*, *148*	*371*, *371*	*352*, *371*	*226*, *236*	*224*, *228*	1
Family 3*	*138*, *146*	*122*, *130*, *136*, *138*, *140*, *142*, *144*	*365*, *371*	*341*, *352*, *371*, *378*	*222*, *234*	*220*, *228*, *234*, *236*, *238*	4
Family 4	*136*, *144*	*130*, *146*	*349*, *371*	*371*	*226*, *232*	*224*, *228*	1
Family 5*	*138*, *150*	*128*, *130*, *134*	*352*, *371*	*365*, *371*	*232*, *236*	*224*, *230*, *232*	2
Family 6*	*130*, *146*	*122*, *136*, *138*, *142*	*371*, *371*	*371*	*218*, *234*	*214*, *220*, *230*	2
Family 7	*142*, *146*	*124*, *144*	*352*, *371*	*371*	*224*, *236*	*202*, *238*	1
Family 8#	*130*, *140*	*146*, *148*, *156*	*371*, *371*	*371*	*228*, *232*	*228*, *234*	2
Family 9*	*140*, *154*	*124*, *130*, *132*, *138*, *142*, *144*	*368*, *371*	*371*, *374*	*234*, *234*	*202*, *218*, *220*, *234*, *236*, *238*	3
Family 10	*142*, *148*	*138*, *142*	*371*, *371*	*371*	*228*, *230*	*214*, *230*	1
Family 11*	*140*, *142*	*130*, *140*, *142*, *150*	*371*, *371*	*360*, *371*	*220*, *226*	*228*, *234*, *236*	2
Family 12*	*120*, *132*	*122*, *130*, *136*, *140*, *144*, *150*	*371*, *371*	*363*, *365*, *371*	*224*, *232*	*218*, *220*, *224*, *226*, *228*, *236*	4
Family 13*	*144*, *154*	*128*, *132*, *146*, *154*, *156*	*371*, *371*	*371*, *374*	*230*, *236*	*218*, *224*, *226*, *228*, *232*	4
Family 14*	*144*, *148*	*124*, *130*, *138*, *142*, *144*, *146*	*371*, *371*	*371*	*226*, *228*	*202*, *214*, *226*, *228*, *234*, *236*, *238*	4
Family 15*	*138*, *142*	*138*, *142*, *146*, *148*, *152*	*371*, *371*	*352*, *371*	*224*, *226*	*224*, *226*, *234*, *236*, *243*	3