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Polygynandry and sexual size dimorphism in the sea spider *Ammothea hilgendorfi* (Pycnogonida: Ammotheidae), a marine arthropod with brood-carrying males

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Abstract

Species that exhibit uniparental postzygotic investment by males are potentially good systems for investigating the interplay of sexual selection, parental care and mating systems. In all species of sea spiders (Class Pycnogonida), males exclusively provide postzygotic care by carrying fertilized eggs until they hatch. However, the mating systems of sea spiders are poorly known. Here we describe the genetic mating system of the sea spider *Ammothea hilgendorfi* by assaying nearly 1400 embryos from a total of 13 egg-carrying males across four microsatellite markers. We also determine the extent of sexual dimorphism in trunk and leg size, and assess how reproductive success in males varies with these morphological traits. We detected instances of multiple mating by both sexes, indicating that this species has a polygynandrous mating system. Genotypic assays also showed that: males do not mix eggs from different females in the same clusters; eggs from the same female are often partitioned into several clusters along a male's oviger; and clusters are laid chronologically from proximal to distal along ovigers. Females were on average larger than males with respect to leg length and width and trunk length, whereas males had wider trunks. Among the genotyped egg-carrying males, neither the number of eggs carried nor the number of mates was correlated with body-size traits. Nevertheless, the high variance in mating success, genetically documented, suggests that males differ in their ability to acquire mates, so future studies are needed to determine what traits are the targets of sexual selection in this species. In addition to providing the first description of the mating system in a sea spider, our study illustrates the potential uses of this group for testing hypotheses from parental investment and sexual selection theories.

Keywords: multiple mating, parentage analysis, paternal care, paternity assurance, pycnogonida, sexual dimorphism

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Introduction

Due ultimately to anisogamy, exclusive male parental care of progeny is the most rare form of postzygotic investment in animals (Ridley 1978; Clutton-Brock 1991). Sperm can be produced in prodigious quantity, so males (typically more so than females) tend to be under selection for promiscuity and fertilization success but less often for extensive offspring care (Trivers 1972; Clutton-Brock 1991; Andersson 1994). Exceptions to this rule offer special opportunities to test sexual selection hypotheses (Darwin 1871; Williams 1975; Andersson 1994).

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For example, exclusive paternal care characterizes all taxa known to be sex-role reversed (Andersson 1994). In such species, paternal care is associated with higher potential reproductive rates in females than males (Clutton-Brock & Vincent 1991) and a female-biased operational sex ratio (Emlen & Oring 1977) that results in stronger competition by females for mates, higher variance among females in mating success (Oring *et al*. 1991; Butchart 2000), the evolution of secondary sex characters in females, and male mate choice (Berglund *et al*. 1986, 1989). Even in species with otherwise 'conventional' sex roles, male parental behaviour is sometimes viewed as a sexually selected means to attract mates (Trivers 1972; Tallamy 2000, 2001). For example, nest-tending males in some fish species have evolved striking morphological traits (such as body parts that mimic eggs; Page

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& Swofford 1984; Goldschmidt 1991; Porter *et al*. 2002) or behaviours (such as nest piracy and egg thievery; Constantz 1985; Unger & Sargent 1988; Rico *et al*. 1992; Jones *et al*. 1998) that appear in effect to advertise a male's parenting 'skills' to potential mates.

Most empirical studies on the evolution of paternal care in the context of sexual selection have focused on vertebrates, but paternal care also has evolved independently in at least nine invertebrate taxa (reviewed in Clutton-Brock 1991; Tallamy 2000, 2001; Shuster & Wade 2003). Notable among these is the Class Pycnogonida (Arthropoda: Chelicerata) or sea spiders, in which uniparental care by males is widespread. More than 1200 species of these marine organisms are distributed from tropical to polar regions, on rocky shores, reefs, soft bottom interstices, and abyssal trenches (King 1973; Arnaud & Bamber 1987). Egg-carrying males have been found in nearly all pycnogonid species, and for this reason sea spiders have been deemed plausible candidates for sex-role reversal (Shuster & Wade 2003) and proffered as potential model organisms for studying the evolution of paternal care (Tallamy 2000, 2001; Bain & Govedich 2004a). However, the mating systems of pycnogonids remain poorly known; most empirical studies have focused instead on life history (Tomaschko *et al*. 1997; Wilhelm *et al*. 1997; Lovely 2005), morphology (Miyazaki & Bilinski 2006; Fahrenbach & Arango 2007), ontogeny (Jarvis & King 1972; Maxmen *et al*. 2005; Jager *et al*. 2006), or phylogeny (Arango & Wheeler 2007; Nakamura *et al*. 2007).

What little is documented about mating behaviour in pycnogonids is based mostly on visual observations on several species in the laboratory (Bain & Govedich 2004a). During mating, a female transfers her eggs to a male who holds them with a specialized pair of legs (ovigers) and fertilizes them externally. The male then glues the eggs into clusters and carries them on his ovigers (for more than 3 months in some species) until hatching (Tomaschko *et al*. 1997). Individual males are known to carry as many as 14 distinct egg clusters simultaneously (King 1973). Multiple mating by males has been observed in the laboratory (Nakamura & Sekiguchi 1980), but the number of genetic dams for a male's brood has not been estimated in any setting, nor has multiple mating by females been critically addressed (Bain & Govedich 2004a).

Here we present the first investigation of genetic mating system in any sea spider species. We focus on *Ammothea hilgendorfi* (Ammotheidae; Fig. 1), a relatively large species found on the West Coast of North America. We have characterized and employed DNA microsatellite markers in a natural population of *A. hilgendorfi* to address the following: (i) the number of females that contribute to each male's egg clusters; (ii) whether individual males partition eggs from different dams into distinct clusters; (iii) whether individual females mate with more than one male; and (iv) whether males sometimes are cuckolded. We also describe the extent of sexual dimorphism with respect to body size characters. Sexual size dimorphism is widespread among animals (Slatkin 1984; Hedrick & Temeles 1989) and is often germane to the direction and intensity of sexual selection (Darwin 1871; Andersson 1994). Our findings provide the first description of a pycnogonid mating system, and highlight the usefulness of this taxonomic group for studies of paternal care and sexual selection.

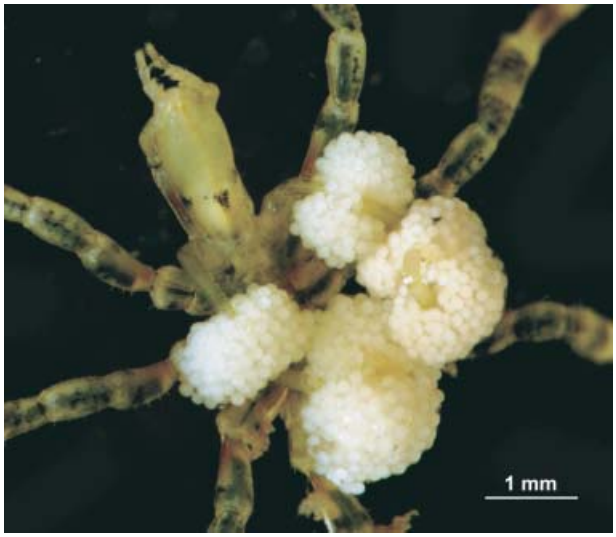

Fig. 1 Ventral view of a live male *Ammothea hilgendorfi* carrying five egg clusters. Photo by P.J. Bryant.

Materials and methods

Sample collections and incubation of eggs

A total of 138 individuals (69 males, 50 females, and 19 juveniles) were collected from under small boulders (~75 cm in diameter) in rocky intertidal areas between Newport Beach and Laguna Beach (33°32′–35′N, 117°48′–52′W), California, during periods of extremely low tides in January and February 2006, and April 2007. Twenty-five of the males carried egg clusters. We define an egg cluster as a physically distinct (often spherical) batch of eggs on a male's oviger, a clutch as any set of full-sib embryos on a male regardless of the number of clusters, and a brood as the entire collection of clusters carried by an individual male. Thirteen males (with numbers of egg clusters ranging from one to seven) were kept alive in 1.5-mL microcentrifuge tubes with seawater; other individuals were preserved immediately in 95% ethanol. Under a dissecting microscope, the position of each egg cluster on each live male's ovigers was recorded. Each cluster was then removed from its guardian and placed in a separate 1.5-mL tube containing filtered room-temperature seawater. Males then were preserved in ethanol.

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Live egg clusters were kept at room temperature and the contents of each tube were inspected every 2–3 days for newly hatched larvae (with seawater replaced each time). Hatched larvae were individually picked with a micropipettor containing 0.5 μL of deionized water and each was transferred to the bottom of a 0.2-mL polymerase chain reaction (PCR) tube. All larval samples were stored at – 80 °C until used in genetic assays. The collections were continued until all larvae had hatched from an egg cluster, or until no further egg development was observed for more than 10 days.

Since we kept each egg cluster unattended by the male custodian, the hatching patterns we observed may not accurately represent those under natural conditions. However, in another pycnogonid species, the order at which larvae hatched among clusters was not different between attended and unattended eggs (Tomaschko *et al*. 1997). Thus, for our males that carried multiple egg clusters, we estimated the order at which each cluster was laid by recording when larvae were first observed at the free-swimming stage.

Morphometric analysis

Individuals were sexed based on the shape and length of ovigers, and on the size and number of gonopores. Males of *Ammothea hilgendorfi* have ovigers with longer segments, the last of which is modified into a club-shaped structure (Bain & Govedich 2004a). The less-developed ovigers of juvenile males could be mistaken with those of females, so females were identified by the presence of visible gonopores on the second coxal segment of all four pairs of ambulatory legs.

Under a dissecting microscope (model Leica 2000), digital photographs were taken of the second ambulatory leg and dorsal full-trunk view of every undamaged adult specimen. Using ImageJ (NIH), linear measurements were made (to the nearest 0.01 mm) of seven traits: femur length, mid-femur width, tibia 1 length, tibia 2 length, mid-tibia 2 width, trunk length (from base of chelifores to the posterior edge of the second trunk segment), and trunk width (distance between the edges of the lateral processes on the second trunk segment).

No specific morphological trait has yet been identified as a reliable indicator of sexual maturity in this species, so we used the presence of eggs in the femora (in females) or egg clusters on the ovigers (in males) and relative size to separate individuals according to maturity status. Within each sex, individuals first were sorted by femur length, and then the individual with the shortest femur *and* that was unambiguously mature (i.e. female with visible eggs in the femora or male carrying at least one egg cluster) was identified. All individuals larger than the noted specimen were assumed to be mature (regardless of presence/absence of eggs or clusters), whereas smaller specimens were removed from the morphological analyses. Log-transformed measurements were checked for normality (Shapiro–Wilk's test, $P > 0.05$) and subjected to a principal component analysis in Statistica 6.0 (StatSoft). Absolute size values were compared using *t*-tests and analysis of variances (ANOVA); relative differences in shape were subjected to analysis of covariance (ANCOVA).

Microsatellite marker development

Isolation of microsatellites followed a modified version of the enrichment protocol of Hamilton *et al*. (1999) (Hauswaldt & Glenn 2003). Briefly, 3.5 μg of genomic DNA from a single adult was digested with *Bst*UI and *Rsa*I restriction enzymes (New England Biolabs). Fragments were ligated to double-stranded SuperSNX-24 linkers (forward: 5′-GTTTAGGCCTAGCTAGCAGAATC-3′; reverse: 5′-GATTCT-GCTAGCTAGGCCTTAAACAAAA-3′) and then hybridized to a mixture of biotinylated oligonucleotide probes containing the repeats $(GT)_{12}$, $(CT)_{12}$, $(GATC)_6$, $(GATA)_6$, and $(GACA)_6$. Probes were captured on streptavidin-coated magnetic beads (Dynal) and beads were removed from solution using a magnetic block. Probes were recovered from the beads by heating and ethanol precipitation. Procedures for probe hybridization, bead capture, and fragment recovery were repeated to promote microsatellite enrichment.

Recovered fragments were PCR amplified using primers designed to complement the ligated SuperSNX linkers. PCR products were cloned using materials supplied in the TOPO TA cloning kit (Invitrogen), and transformed bacteria were grown on Luria-Bertani plates containing S-Gal (Sigma). Positive clones were picked and lysed in 20 μL of deionized water at 100 °C for 5 min. One microlitre of bacterial lysate was used in PCR with M13-forward (–20) and M13-reverse (–29) primers. PCR products were next electrophoresed on 1% agarose gels to determine size of the cloned inserts. Fragments of size between 500 bp and 1000 bp were purified using ExoSAP-IT (USB) and sequenced with BigDye Terminator Cycle Sequencing Kit (version 3.1, Applied Biosystems) on an ABI PRISM 3100 genetic analyser. Sequences were edited in EditSeq (DNA*), and primers flanking the microsatellite repeat region were designed using the PrimerQuest software (Integrated DNA Technologies). Primer pairs that amplified fragments of the appropriate size were further optimized and checked for polymorphism in a panel of 16 adult individuals. A total of four highly polymorphic loci were chosen for the current study.

Genotypic assays and parentage analysis

Genomic DNA from adults was extracted from three legs using a standard phenol–chloroform–isoamyl protocol, with proteinase K digestion and ethanol precipitation (Milligan 1998). Before PCR amplification, aliquots of adults' genomic extracts were diluted 10-fold in deionized water. To tubes containing larvae, 12 μL of lysis buffer (10 mM Tris-HCl

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pH 8.3, 50 mM KCl, 0.5% Tween-20, 250 μg/mL proteinase K) were added. Tubes were incubated at 55 °C for 3.5 h, followed by a proteinase inactivation period of 15 min at 95 °C. Samples were then centrifuged at full speed for 2 min to pellet cellular debris.

Microsatellite amplifications were performed in 12.5-μL reactions containing 1× PCR buffer (Promega), 0.2 mM of each dNTP, 0.4 μM of each primer, 1.5 mM $MgCl_2$, 0.5 U of *Taq* DNA polymerase (Promega), and 1 μL genomic DNA. PCR cycling parameters were identical for all loci, and consisted of an initial denaturation at 95 °C for 4 min, followed by 34 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min, with a final extension step at 72 °C for 4 min. Before electrophoresis, PCR products were pooled at the following proportions: 2:4:1:1, for loci AhC-9, AhC-102, AhC-140, and AhC-181, respectively, followed by an additional 2 μL of deionized water. One microlitre of the pooled mixture was combined with 9.55 μL of Hi-Di formamide, and 0.45 μL of GeneScan 500 size standard (ROX-labelled, Applied Biosystems), and samples were denatured at 95 °C for 5 min and then chilled on ice until electrophoresis. Fragments were resolved on an ABI PRISM 3100 genetic analyser and scored for size with GeneMapper software version 4.0 (Applied Biosystems).

Using the genotypes from all 138 adults, the program GenePop (Raymond & Rousset 1995) was used to estimate population-wide allele frequencies and to test for Hardy–Weinberg equilibrium (Guo & Thompson 1992) and linkage disequilibrium. Probabilities of genetic exclusion were calculated under the assumption that one parent was known (Jamieson & Taylor 1997). By comparing single-locus genotypes of each progeny to those of its guardian male, maternally derived alleles were deduced by subtraction. The minimum number of dams contributing to each egg cluster was estimated by dividing the number of maternal alleles by two and rounding-up to the nearest integer. Finally, a chi-squared test was used to assess whether within-cluster genotypic proportions conformed to expected Mendelian ratios. Type I errors from multiple testing were addressed with sequential Dunn–Sidák's adjustments of α-values (Sokal & Rohlf 1995).

Results

Sexual size dimorphism

Sixty-two males and 44 females were provisionally deemed sexually mature based on relative femur length and reproductive status, but fewer individuals (57 males, 42 females) were sufficiently undamaged to permit measurements of all seven linear traits. In the principal component (PC) analysis, the first principal component (PC1) accounted for 57.5% of the total variation, and it received high loadings (0.82–0.92) in all five leg traits. The second principal component (PC2), which basically described trunk shape (because trunk length and trunk width had loadings of 0.71 and 0.95 along this axis), accounted for an additional 27% of morphological variation. PC1 and PC2 clearly separated males and females (Fig. 2), and mean PC scores differed significantly between the sexes (*t*-tests, d.f. = 97, PC1: $\bar{x}_{males} = -0.66$, $\bar{x}_{females} = 0.89$, $P < 0.0001$; PC2: $\bar{x}_{males} = 0.37$, $\bar{x}_{females} = -0.50$, $P < 0.0001$).

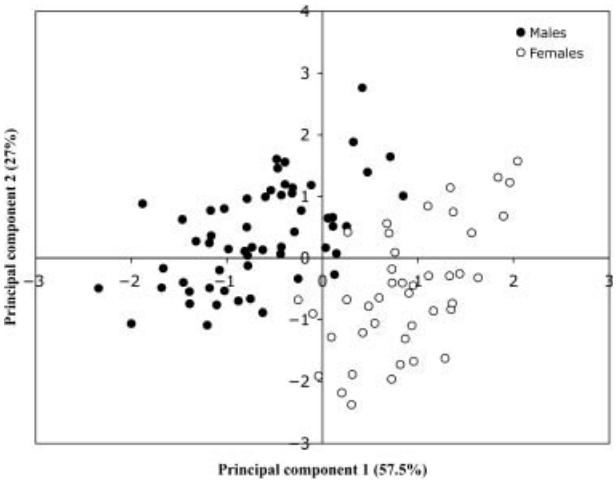

Fig. 2 Principal component scores of *Ammothea hilgendorfi* males ($N = 57$) and females ($N = 42$) based on seven linear morphological variables. The sexes scored significantly different on average along both axes (*t*-tests, $P < 0.0001$).

Because length of the tibia segments was highly correlated with that of femur ($r = 0.95$–0.96, $P < 0.001$), only the latter was used to represent leg length in further univariate analyses. Male trunks were on average 7% wider than those of females ($F_{1,97} = 17.6$, $P < 0.0001$), whereas females had longer (15%; $F_{1,97} = 35.5$, $P < 0.0001$) and wider (30%; $F_{1,97} = 138.7$, $P < 0.0001$) femora (Table 1). Tibia 2 was also significantly wider in females ($F_{1,97} = 37.0$, $P < 0.0001$), but less so than in the comparisons of femora (15%; Table 1). The trunks of females also averaged 4.5% longer than those of males ($F_{1,97} = 4.7$, $P = 0.034$).

Sexes were also size-dimorphic according to shape. When trunk length was adjusted between the sexes (i.e. used as the covariate), females had longer femora (ANCOVA, $F_{1,96} = 31.4$, $P < 0.0001$) whereas males had wider trunks (ANCOVA, $F_{1,96} = 75.4$ $P < 0.0001$). Finally, females had significantly wider femora than males when femur length was the covariate (ANCOVA, $F_{1,96} = 81.3$, $P < 0.0001$).

Genetic markers

The four cloned microsatellite loci (the first such markers reported for any pycnogonid) were highly polymorphic and permitted unambiguous assignments of paternity for all surveyed progeny (Table 2). No pair of adults shared an

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identical multilocus genotype. After Dunn–Sidák's adjustments for multiple comparisons, no linkage disequilibrium was detected among pairs of loci, genotypic counts within progeny arrays showed no deviation from expected Mendelian ratios, and none of the loci showed departure from Hardy–Weinberg equilibrium.

Locus AhC-102 exhibited a null (nonamplifying) allele, which was clearly manifested in the brood of male M107. This male carried four egg clusters from a total of three females (see beyond), and he was originally genotyped as homozygous for allele 237. Within each of his clusters, however, only about 50% of his progeny displayed that allele (plus one of two different maternally derived alleles). The other 50% of his progeny appeared to be homozygous for one of the maternal alleles. While cuckoldry could in principle account for these mismatches, the presence of a null allele is a more likely explanation. The inheritance pattern at the other three loci was consistent with M107 being the sire, and to exclude M107 as the sire of these clutches would require that he was carrying offspring of the mating between another male (the cuckolder) who happened to share his three-locus genotype (with random probability of 2.98×10^{-6}) and three females. Moreover, after assigning M107's genotype as being heterozygous (237/null), chi-squared tests detected no departure from Mendelian genotypic ratios ($P > 0.29$) in any of the four egg clusters. Null alleles at this deduced frequency (0.018) can complicate parentage analyses (Brookfield 1996; Dakin & Avise 2004), but are often accounted for when progeny arrays and their putative parents are assayed at multiple loci (as in the current study).

De novo mutations, if unrecognized as such, are another phenomenon that can complicate microsatellite-based parentage assays, since they otherwise might be interpreted as evidence of cuckoldry (when originating in the paternal germ-line) or multiple mating (when originating in the maternal germ-line). We detected five cases of progeny–parent mismatch — each involving one allele only — that likely resulted from *de novo* mutation. In two of these cases, involving separate broods, a single offspring differed from its guardian male by one paternal-origin allele (at locus AhC-9). The other three *de novo* mutations (one each at loci AhC-102, AhC-140, and AhC-181) were of maternal origin, since the implicated offspring in each case had a multilocus genotype consistent with that of the guardian male but that differed from that of the deduced dam by one allele. All five of the cases involved a single progeny among the 40 or more assayed embryos from the respective egg clusters, and each allelic mismatch differed by one dinucleotide repeat from its nearest-sized parental allele. In each case, genotypes at the other three loci were consistent with the respective putative parent, and the probabilities of spurious identity for these three-locus genotypes were low (8.9×10^{-8} to 1.9×10^{-5}), thus making new germ-line mutations the most likely explanation for these findings.

Table 1 Mean (and 95% confidence intervals) of trunk and leg measurements for male and female *Ammothea hilgendorfi*. SDI [size-dimorphism index, calculated as $(\bar{x}_{females}/\bar{x}_{males}) - 1$] describes the proportion by which females are larger (positive SDI) or smaller (negative SDI) than males

	Mean (95% CI)		
	Males (mm)	Females (mm)	SDI
Femur length**	1.96 (1.91–2.02)	2.25 (2.18–2.33)	0.148
Femur width**	0.42 (0.41–0.43)	0.55 (0.53–0.57)	0.310
Tibia 2 width**	0.34 (0.33–0.35)	0.39 (0.37–0.40)	0.147
Trunk length*†	1.12 (1.10–1.15)	1.17 (1.14–1.20)	0.045
Trunk width**	1.49 (1.46–1.52)	1.39 (1.35–1.42)	–0.067

*$P < 0.05$; **$P < 0.0001$; ANOVA. †In this study, trunk length was measured from morphological landmarks that could be conveniently seen in the digital photographs, so these values do not depict actual full trunk length.

Table 2 Microsatellite loci characterized for the pycnogonid *Ammothea hilgendorfi*. Population-level parameters (number of alleles, observed heterozygosity, and exclusion probabilities) were estimated from a sample of 138 adults

Locus	Primer sequences (5′–3′)	Repeat motif*	No. of alleles	H_O	Excl. Prob.†
AhC-9	F:/NED/CACAGAATCTCACCATACACCG R: ATAGCGGCAGGATTTGAACG	$(GA)_{21}$	9	0.791	0.639
AhC-102	F: GCCGCCACACCATGACAAATTA R:/NED/AGATATGAGCTGCTTTGCGTGC	$(CT)_{28}$	36	0.884	0.831
AhC-140	F:/HEX/CTTCATTTAGCCACGTAACTTC R: CTTCAGACGTATCCTTCCAC	$(GA)_{23}$	14	0.849	0.777
AhC-181	F: GGCTATTTGTCGCGTGAACGAT R:/FAM/AGTGCAGTAATCGAGTTGGAGC	$(GA)_{16}$	14	0.855	0.720

*Number and sequence of microsatellite repeat found on original clones. †Probability of genetic exclusion under the assumption that one parent was known. Combined probability is 0.996, based on Jamieson & Taylor (1997).

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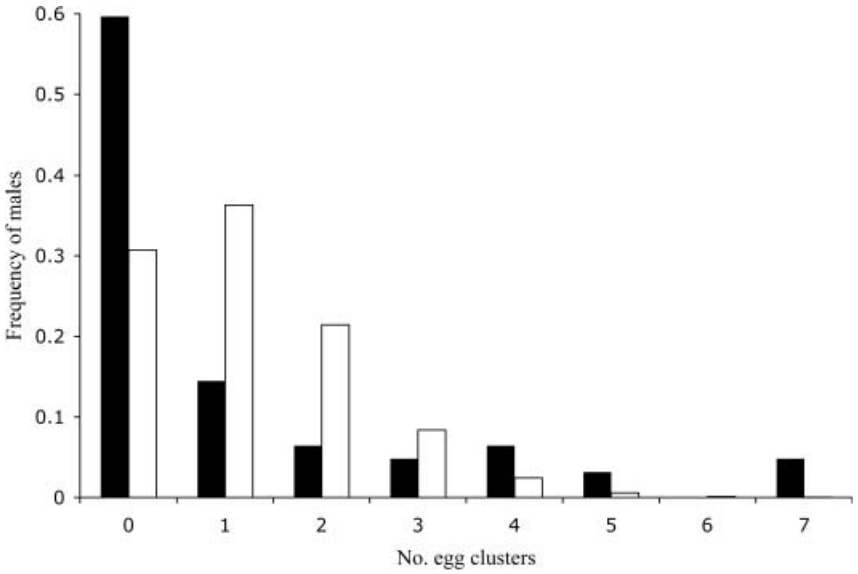

Fig. 3 Frequency of male *Ammothea hilgendorfi* with respect to the number of egg clusters carried. Solid bars show the observed distribution ($N = 62$) whereas open bars show the expected frequencies under a Poisson distribution. The distributions were significantly different ($G = 46.9$, d.f. = 3, $P < 0.0001$).

Given the number of progeny genotyped (1394), our empirical estimates of *de novo* mutation rate are thus 7.2×10^{-4} for locus AhC-9, and 3.6×10^{-4} for each of the other three microsatellite loci. These estimates are comparable to those reported for microsatellites in other species (Jones *et al*. 1999; Walker *et al*. 2002; Tatarenkov *et al*. 2006).

Pattern of paternity and maternity

Sea spider densities on the particular boulders under which specimens were found ranged from 2 to 41 individuals/m^2 (mean: 9.5/m^2). Most mature males (60%) carried no egg clusters at the time of capture, while others carried as many as seven. The number of eggs and the number of egg clusters carried per male were highly correlated ($r^2 = 0.65$, $N = 13$, $P < 0.001$). The frequency distribution of egg clusters among males (mean: 1.2 per male; variance: 3.7) did not fit a Poisson distribution ($G = 46.9$, d.f. = 3, $N = 62$, $P < 0.0001$; Fig. 3), showing an excess of males with no clusters and of males with many clusters when compared to random expectations. With respect to leg and trunk measurements, males that carried egg clusters were not significantly different from males with no clusters ($N = 57$, $F_{1,55} = 0.16$, $P = 0.69$; and $N = 57$, $F_{1,55} = 0.09$, $P = 0.77$, respectively). Similarly, the morphological traits examined were not good predictors of the number of egg clusters ($N = 24$; PC1: $r^2 = 0.034$, $P = 0.39$; PC2: $r^2 = 0.051$, $P = 0.29$) or of the number of eggs (Fig. 4) carried by males.

Larvae from broods of 13 males were successfully hatched under laboratory conditions, and a total of 1394 progeny were genotyped at all four loci (Table 3). After accounting for the aforementioned *de novo* mutations and null allele, no male could be excluded as the sire of the embryos he carried, since every progeny assayed shared

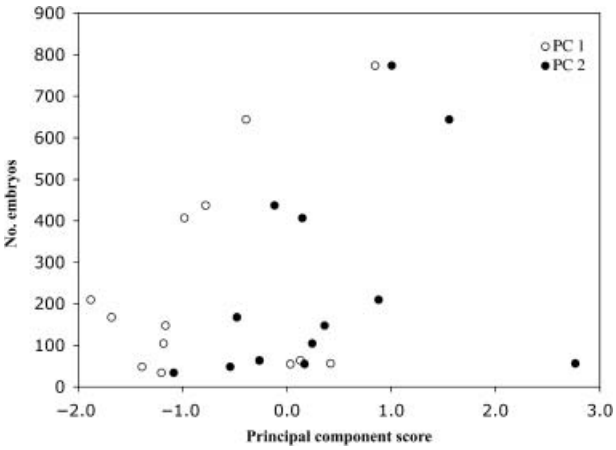

Fig. 4 Relationship between male body size and number of embryos carried. Body size is represented by scores on PC1 (leg length and width) and PC2 (trunk length and width). Neither regression is significant ($N = 13$; PC1: $r^2 = 0.13$, $P = 0.25$; PC2: $r^2 = 0.11$, $P = 0.3$).

one allele at each locus with its guardian. This allowed us to identify, unambiguously, the maternally derived alleles and to estimate the minimum number of dams contributing to each male's progeny. All surveyed males with multiple egg clusters proved to have mated with at least two dams, but all embryos in each egg cluster invariably shared the same mother (Table 3). The number of egg clusters carried by a male was not a reliable predictor of mate number (for example, no male had mated with more than three females despite carrying up to seven egg clusters; Table 3). In other words, progeny in different clusters often shared the same dam (examples in Fig. 5). Among the 13 males, six had one mate, four had two mates, and three had three mates

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Table 3 Summary of deduced genetic parentage for embryos carried by 13 male *Ammothea hilgendorfi* based on four polymorphic microsatellite loci. Egg clusters are labelled according to their relative position along each oviger [e.g. L1 is the most proximal cluster on the left (L) oviger]. For males with multiple mates, egg clusters are listed in the approximate order in which they were laid. Bold font indicates dams whose deduced multi-locus genotypes (with designation 'D') perfectly match those from collected individuals (in parentheses); symbols * and † indicate the two females each of whom mated with two males. Also shown are probabilities of spurious identity for deduced dam genotypes that matched those of collected females or that were detected in multiple clusters

Male ID	Egg cluster	No. of progeny assayed	Total progeny in cluster	Deduced dam I.D.	Probability of identity
Males with single cluster					
M100	R1	61	168	**D1 (F101)**	4.3×10^{-8}
M67	L1	24	57	D2	
M68	L1	34	64	D3	
M99	L1	14	49	D4	
M79	L1	20	35	D5	
M90	L1	40	56	**D6 (F94)**	3.0×10^{-6}
Males with multiple clusters					
M107	L1	49	106	D7	
″	R1	46	149	D8	
″	L2	68	200	**D9 (F103)**	2.1×10^{-8}
″	R2	68	189	″	
M106	L1	41	119	D10	3.4×10^{-10}
″	R1	40	127	″	
″	R2	61	270	D11	4.0×10^{-8}
″	R3	63	160	″	
″	L2	24	98	″	
M31	L1	20	52	D12	
″	R2	18	68	D13	5.7×10^{-9}
″	R3	58	90	″	
M53	R1	46	72	D14	
″	L1	15	33	D15	
M80	R1	33	57	**D16 (F87)***	1.4×10^{-7}
″	L1	43	63	**D17 (F86)†**	1.1×10^{-6}
″	L2	47	60	″	
″	R2	53	82	″	
″	L3	70	87	″	
″	R3	14	29	D18	3.4×10^{-7}
″	L4	8	29	″	
M81	R1	46	75	**D16 (F87)***	
″	L1	45	73	**D17 (F86)†**	
M89	L1	34	100	D19	1.6×10^{-8}
″	R1	74	95	″	
″	R2	39	67	**D20 (F92)**	1.2×10^{-6}
″	L2	8	59	D21	5.5×10^{-8}
″	R3	70	116	″	

(Table 3). The number of mates per male was not correlated with body-size variables (PC1: $r^2 = 0.001$, $P = 0.88$; PC2: $r^2 = 0.11$, $P = 0.22$).

Six of the 50 females displayed identical multilocus genotypes to those we deduced for the respective dams of twelve egg clusters. That these matches are spurious (not indicative of maternity) is highly unlikely given the low expected frequencies of the deduced genotypes, which ranged from 1.1×10^{-6} to 4.3×10^{-8} (Table 3). In all six cases, the dam was collected under the same boulder as the sire that carried her clutch.

Multiple mating by females was also uncovered in the genetic analyses. Female D17 deposited more than 290 eggs (split into four clusters) on male M80 and 73 eggs (in a single cluster) on male M81; and female D16 deposited a single cluster of almost 60 eggs on male M80 and another cluster of 75 eggs on male M81 (Table 3; Fig. 5). The genotypic matches by which maternity was deduced are again supported by the extremely low probabilities of spurious genetic identity. All four of the individuals involved in these matings were found under the same boulder (albeit not in close association at the time of capture).

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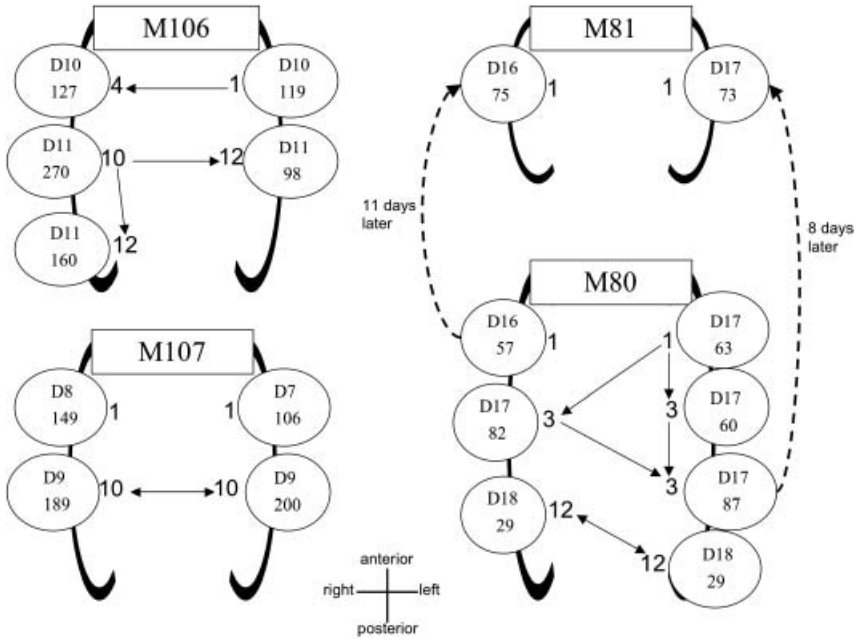

Fig. 5 Diagrammatic representation of egg clusters (each indicated by a circle) along the ovigers (*curved black bands*) of four male *Ammothea hilgendorfi*. The upper number within each circle shows the deduced dam I.D. (same labelling as in Table 3) and the lower value indicates the number of eggs deposited. The number next to each cluster indicates the day on which its larvae first began to hatch, starting with day 1 for the first observed hatchings within a male's collection of clusters. Arrows connect clusters contributed by the same female; dashed arrows depict events of multiple mating by females. Ovigers are not drawn to scale.

Mating timing and order

Observation of larvae hatching order, coupled with the deduced genetic identity of each cluster's dam, revealed several behavioural patterns. Invariably, clusters were laid chronologically from proximal to distal along a male's oviger ($N = 11$, unassayed broods included; Table 3; Fig. 5), with a significant tendency for the first cluster to be placed on the left oviger (exact binomial test, $P = 0.039$, $N = 12$, males with single clusters included). Multiple clusters from the same dam were adjacent to each other, and were laid within a short time span (~1–3 days; we did not sample hatchlings every day, so exact time differences cannot be determined). It also appears that males sometimes switched mates in a short period, perhaps in the same day. For instance, M81 carried two clusters (each from a different dam) that started to hatch on the same day. A similar pattern was observed for M80 and M107 (Fig. 5). Finally, the two multiple-mating females (D16 and D17) both first mated with M80 within a 1–2-day period, and then, several days later, mated with M81 also within 1–2 days (Fig. 5).

Discussion

Molecular parentage analyses in numerous taxa have highlighted the need to distinguish between the social mating system and the genetic mating system; only the latter assuredly reflects parental contributions and reproductive successes of breeding adults (Birkhead & Møller 1992; Avise *et al*. 2002). Here we have developed and utilized microsatellite markers to assess, for the first time, the genetic mating system in a pycnogonid species.

Paternity assurance

In general, the evolution and maintenance of intensive postzygotic investment by males are likely to be facilitated when the caregivers have high assurance of genetic paternity (Clutton-Brock 1991). On the other hand, external fertilization and high densities of breeding individuals open windows of opportunity for cuckoldry that frequently are realized, such as in many fish species in which males build and tend nests (review in Avise *et al*. 2002). In our current study of *Ammothea hilgendorfi*, no instances of cuckoldry (which would have been evidenced by foster embryos) were detected for any of the nearly 1400 genotyped progeny from 13 embryo-carrying males. The outcome is perhaps not too surprising, given how sea spiders mate: the gonopores generally are in close proximity while the male grasps the female, and the eggs are fertilized either while the female still holds them (Nakamura & Sekiguchi 1980) or immediately after they have been deposited directly onto a male's ovigers (Wilhelm *et al*. 1997).

The personal cost to males of providing prolonged care for young has not been quantified in pycnogonids, but it is an important parameter if paternal care is to be viewed as a significant parental investment (Trivers 1972) or as a sexually selected 'handicap' (Townsend 1986; Zahavi 1997). Because paternity assurance is thought to be a common prerequisite for the evolution of substantial postzygotic investment by males (Trivers 1972; Clutton-Brock 1991), the high level of paternity assurance observed in *A. hilgendorfi* suggests that at least some costs to males (such as reduced foraging ability, increased predation risk, lower mobility) may well exist.

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Male mating behaviour: multiple mating

Our genetic assessments show, unambiguously, that individual males of *A. hilgendorfi* routinely mate with multiple females in nature. Furthermore, because each egg cluster on a male invariably consisted of full siblings, males clearly do not mix egg batches from different dams. In another sea spider species—*Propallene longiceps*—Nakamura & Sekiguchi (1980) had demonstrated multiple mating in the laboratory by observing that males ended up with egg clusters of different colour after being placed in containers with gravid females that had been experimentally stained with red and blue dyes. That study involved one male and two females per trial, whereas our sampling revealed that males in nature may carry embryos from at least three mates. Other reports of multiple mating by pycnogonids have been based on relative developmental stages of different egg clusters on a male (Bain & Govedich 2004a). Our genotyping results for *A. hilgendorfi*, coupled with the timing of larval hatching we observed in our sample, showed that a male often mates with different females within a short time span, such that the resulting egg clusters can be close in their stage of embryonic development. This means that developmental stage alone is not a reliable indicator (i.e. will often underestimate) the true number of mates in this species.

Male mating success parameters can also be examined from our data. The mean and variance in the number of mates per male were 0.71 and 1.01. Much of the variance is due to the large proportion (60%) of potentially breeding males collected with no egg clusters. These males might represent immature individuals mistakenly deemed as adult (this would artificially inflate the variance in mating success), but this possibility seems unlikely because mated and unmated males were indistinguishable based on leg and trunk size measurements. Perhaps males without clusters had not yet encountered gravid females, but this explanation also appears unlikely because most (81%) of the adult females in our collection were gravid. Further developmental and behavioural studies will be needed to critically address these possible sources of variation in male mating success in *A. hilgendorfi*.

Alternatively, the variance in male mating success may reflect biological differences among males in their abilities to acquire mates (Darwin 1871; Andersson 1994). We do not know what factors might be involved, but measures of male body size were not correlated with reproductive or mating success in our data (e.g. Figure 4). However, a relationship with body size cannot be ruled out conclusively; females of the pipefish *Syngnathus typhle* prefer larger males (Berglund *et al*. 1986) even though male body length and mate number were not correlated in natural populations (Jones *et al*. 1999). Additionally, sexual selection on male *A. hilgendorfi* may be related to morphological or behavioural traits not examined in this study.

Male mating behaviour: brood partitioning

When a male carried multiple egg clusters, two or more (and as many as four) of his clusters typically had come from the same female (Table 3). Whether this reflects multiple mating events or the partitioning of eggs from a single mating is unclear. The absolute and relative numbers of eggs in such clusters varied both within and among males. For instance, male M107 carried roughly equal numbers of eggs (189 and 200) in each of his two clusters from dam D9; male M80 carried 29 eggs in each of his two clusters from dam D18; and male M106 carried 270, 160, and 98 eggs in his three clusters from dam D11 (Table 3). The number of eggs in a male's cluster may in part be a function of egg availability in a female's femora; Nakamura & Sekiguchi (1980) observed in *Propallene longiceps* that eggs from each of the female's legs ended up in a distinct cluster on the male's ovigers. Egg-space limitations in males, and/or early terminations of a mating event by either individual, might also account for the considerable disparities in egg numbers among the clusters carried by males.

Eleven males of *A. hilgendorfi* carried two or more egg clusters on a given oviger, and in every case, the most advanced (fully developed) cluster was most proximal to the trunk. This suggests that after the most proximal cluster hatches, the remaining clusters are moved up towards the body as the male places newer ones distally. Exactly the reverse spatial pattern on the ovigers was reported in two other sea spider species: *Nymphon aequidigitatum* (Bain & Govedich 2004a) and *Propallene longiceps* (Nakamura & Sekiguchi 1980). Direct observations of mating behaviour would likely confirm qualitative differences in the mechanics of egg transfer and partitioning between *A. hilgendorfi* and these other two species.

Female mating behaviour

Prior speculation about the possibility of multiple mating by female pycnogonids was limited to the observation that a recently mated female often retains unused mature eggs in one or more femora, giving her the potential to mate with additional partners. In our genetic analyses of 34 egg clusters, we deduced multiple mating by two females (Fig. 5). Such documentations are fortuitous in the sense that they require that the males with whom a female mated happen to have been included in the original sample, which seems rather unlikely when natural populations are large. Although our data probably underestimate the true frequency of multiple mating by female sea spiders, they do provide the first conclusive evidence for this behaviour in nature.

The advent of genetic analysis in animal mating systems has revealed that female multiple mating is taxonomically widespread (Westneat *et al*. 1990; Andersson 1994; Birkhead & Møller 1998). In species with external fertilization and

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male parental care, the advantages of multiple mating to females likely involve 'bet-hedging' (Berglund *et al*. 1988; Schneider & Elgar 1998). For example, by distributing her clutch among different males, a female increases the likelihood that at least some of her offspring may receive indirect genetic benefits (Watson 1991; Yasui 1998) and/or paternal care from a quality provider (Berglund *et al*. 1988). Multiple mating may also allow a female to choose mates sequentially when she is not able to assess the quality of many males simultaneously (Walker 1980; Møller 1992; Yasui 1998).

Sexual size dimorphism

Females are the larger sex in most poikilotherms, especially among invertebrates (Arak 1988; Hedrick & Temeles 1989; Andersson 1994), and pycnogonids generally are thought to conform to this pattern (Hedgpeth 1941; Arnaud & Bamber 1987; Tomaschko *et al*. 1997; Bain & Govedich 2004a; but see Bamber 2002). Female-biased size dimorphism is conventionally explained by fecundity selection in which egg production continually increases with body size (Darwin 1871; Williams 1966).

We found significant trunk size dimorphism in *A. hilgendorfi*, but in opposite directions for length (females were 4% longer) and width (females were 7% narrower). Dimorphism in leg dimensions was more conspicuous and also consistent in favour of larger size for females (Table 1). Among the examined traits, the greatest size difference between the sexes involved the femur, a result that may not be surprising because vitellogenesis (yolk deposition) is believed to occur primarily in that segment of the leg (King & Jarvis 1970; Arnaud & Bamber 1987).

Although these findings are consistent with the action of fecundity selection on female-biased size dimorphism, a rigorous test of this possibility must account for (i) instantaneous and lifetime estimates of egg production in relation to size (Shine 1988; Preziosi *et al*. 1996), and (ii) how differences in net selective pressures might cause optimum body size to be smaller in males than females (Arak 1988; Hedrick & Temeles 1989). Moreover, the presence of fecundity selection does not exclude sexually selected advantages of large size, such as higher competitive ability or greater attractiveness to males (Andersson 1994; Berglund & Rosenqvist 2003).

The mating system

Our genetic analysis revealed that both males and females in *A. hilgendorfi* have multiple mates, indicating this species is polygynandrous in nature (Andersson 1994). Resolving whether this species has conventional or reversed sex roles requires in addition that we determine which sex exhibits stronger competition for mates, and is hence under more intense sexual selection (Berglund & Rosenqvist 2003). A key determinant of the direction of sexual selection is the potential reproductive rate of each sex (i.e. the sex-specific number of offspring per unit time; Clutton-Brock & Vincent 1991), which in turn influences the operational sex ratio (OSR, the ratio of sexually active males to receptive females; Emlen & Oring 1977).

In pycnogonids, estimating these parameters would involve determining, among other variables, the number of carried embryos (or egg clusters) at which a male is unable or unwilling to mate. Although empirical studies are needed to address these issues, some predictions can be made with our sample. If we assume that each of the three captured males with seven egg clusters had reached his storage capacity, then in our sample 59 males and 36 females (those with visible eggs in the femora) were available to mate at the time of capture. The OSR was thus significantly male-biased (exact binomial test, $P = 0.023$), despite an approximately even adult (mature) sex ratio ($P = 0.098$). Coupled with the significant increase ($r^2 = 0.53$, $N = 13$, $P < 0.005$) in reproductive success (number of offspring) with increased mating success (number of mates), an excess of available males suggests that male reproductive success is limited by mate acquisition more so than by fertility or brooding space (Arnold & Duvall 1994; Arnold 1994). The high variance that we observed in male mating and reproductive success also hints that males compete for mate acquisition.

We cannot with current data ascertain the direction of sexual selection in this population because this would entail comparisons of the above parameters to those of females (Berglund *et al*. 2005). Female courtship and mate competition have been observed in one pycnogonid (*Propallene saengeri*; Bain & Govedich 2004b), so sex-role reversal in *A. hilgendorfi* cannot be ruled out based on current data. The microsatellite markers we developed, coupled next with laboratory studies of mating, should prove useful in addressing these topics.

While our paper highlights the usefulness of sea spiders for studying the role of sexual selection on the evolution of paternal care, a recent model (Manica & Johnstone 2004) suggests male care is favoured under certain conditions (e.g. high population densities and short female time to remating) even if no mate choice is invoked. A prerequisite for this model is that males have overlapping broods that permit remating while guarding, such as shown here for *A. hilgendorfi*. Since the number of broods guarded simultaneously, population densities, and reproductive cycles vary widely among pycnogonid species (King 1973; Arnaud & Bamber 1987), this group should also prove ideal for estimating the relative role of natural selection in the evolution of uniparental male care.

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Felipe Barreto is a PhD student in John Avise's laboratory, and this study is part of his dissertation research, which is aimed at assessing the role of sexual selection in the mating system of sea spiders. Felipe Barreto's research interests also include speciation, phylogeography, and reproductive behaviour in marine animals. Research in the Avise laboratory involves the use of molecular markers in studies of animal behaviour, ecology, and evolution.

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