Quantitative measures of sexual selection reveal no evidence for sex-role reversal in a sea spider with prolonged paternal care

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Taxa in which males alone invest in postzygotic care of offspring are often considered good models for investigating the proffered relationships between sexual selection and mating systems. In the pycnogonid sea spider *Pycnogonum stearnsi*, males carry large egg masses on their bodies for several weeks, so this species is a plausible candidate for sex-role reversal (greater intensity of sexual selection on females than on males). Here, we couple a microsatellite-based assessment of the mating system in a natural population with formal quantitative measures of genetic fitness to investigate the direction of sexual selection in *P. stearnsi*. Both sexes proved to be highly polygamous and showed similar standardized variances in reproductive and mating successes. Moreover, the fertility (number of progeny) of males and females appeared to be equally and highly dependent on mate access, as shown by similar Bateman gradients for the two sexes. The absence of sex-role reversal in this population of *P. stearnsi* is probably attributable to the fact that males are not limited by brooding space but have evolved an ability to carry large numbers of progeny. Body length was not a good predictor of male mating or reproductive success, so the aim of future studies should be to determine what traits are the targets of sexual selection in this species.

Keywords: opportunity for selection; Bateman gradient; paternity analysis; maternity analysis; pycnogonida

1. INTRODUCTION

Sex-role reversal in animals is defined primarily by the presence of more intense competition for access to mates among females than among males, resulting in stronger sexual selection on females (Vincent *et al.* 1992; Andersson 1994). This departure from conventional sex roles is thought to be due directly to a female-biased operational sex ratio (OSR; Emlen & Oring 1977), which in turn is influenced ultimately by a higher potential reproductive rate (PRR) in females than in males (Clutton-Brock & Vincent 1991). In addition to a higher expected variance in female mating success (Oring *et al.* 1991; Butchart 2000), tendencies for female-biased sexual dimorphism, and greater male choosiness (Berglund *et al.* 1986, 1989), many sex-role-reversed species also exhibit exclusive paternal care of progeny (Clutton-Brock 1991; Andersson 1994).

The presence of paternal care alone should not be used as a predictor of sex-role reversal, since most species with this mode of care (found mostly in fishes and amphibians) also display intrasexual selection on males (Breder & Rosen 1966; Blumer 1979). Paternal care may, however, promote female–female competition in cases where the level of care restricts male mate acquisition, and hence reduces the PRR of males relative to that of females (Berglund *et al.* 1989; Clutton-Brock & Vincent 1991;

Vincent 1994). For instance, in several pipefish species (family Syngnathidae), female reproductive success is limited by the available amount of male brooding space rather than by egg production itself (Berglund *et al.* 1989; Berglund & Rosenqvist 2003).

Paternal care of progeny has been observed in nearly all of the 1200+ species of the Class Pycnogonida (Arthropoda: Chelicerata), commonly known as sea spiders. After mating, male pycnogonids hold fertilized eggs in masses glued either to a pair of specialized legs (ovigers) or to the ventral surface of their bodies (King 1973; Bain & Govedich 2004a). Eggs are carried until hatching, which may take up to three months in some species (Tomaschko *et al.* 1997). For these reasons, pycnogonids have been suggested as candidates for sex-role reversal (Shuster & Wade 2003), and intense female competition for a mate has been observed in at least one species (*Propallene saengeri*; Bain & Govedich 2004b). However, pycnogonid mating systems remain poorly known.

In the absence of direct observations of female competition for mates, quantitative measures based on selection theory (Crow 1958; Wade 1979; Lande & Arnold 1983; Shuster & Wade 2003) and Bateman's principles (Bateman 1948; Arnold & Duvall 1994) can be used to assess the direction and intensity of sexual selection in a mating system (Kraaijeveld-Smit *et al.* 2003; Gopurenko *et al.* 2007; Broquet *et al.* 2009). Specifically, the opportunity for selection (I), the opportunity for sexual selection (I_s) and the Bateman gradient (or sexual selection gradient) have all been shown to respond well to variations in the mating system associated with changes in gamete investment (Bjork & Pitnick 2006), geography

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Figure 1. Male *P. stearnsi* carrying three overlapping egg masses. This individual was found attached to the base of an anemone (*Anthopleura xanthogrammica*) in Laguna Beach, California. Photograph by P. Bryant. Scale bar, 1 mm.

(Mobley & Jones 2007), OSR (Jones *et al.* 2002, 2004) and sex roles (Jones *et al.* 2000). Quantifying these metrics for both sexes using samples from nature, however, is often very difficult because both the sire and dam must be determined for each offspring.

In the current study, we employ DNA microsatellite markers to investigate the genetic mating system in a natural population of an intertidal pycnogonid, *Pycnogonum stearnsi*, for which little was known regarding mating behaviours and reproduction (reviewed in Bain & Govedich 2004a). Our study was motivated in part by previous observations on *P. littorale*, a related species that displays several features suggestive of sex-role reversal owing to limited male brooding space: males are markedly smaller than females (Tomaschko *et al.* 1997)—each male carries one large egg mass that he receives from a single female (Jarvis & King 1972)—and a male does not remate until all the eggs have hatched, which can take up to ten weeks (Jarvis & King 1972; Tomaschko *et al.* 1997). *P. littorale* and *P. stearnsi* have similar morphologies and egg-mass configurations. Here, we exhaustively sample a small breeding population of *P. stearnsi* to characterize genetic parentage and the fitness of individual males and females, and then use these data to interpret the population's mating system in the light of sexual selection theory.

2. MATERIAL AND METHODS

(a) Collection of samples

Most adult *P. stearnsi* were found attached to the base of *Anthopleura xanthogrammica*, a common anemone on rocky shores of the American Pacific coast. During three periods of low tide in January 2009, a total of 150 individuals was collected from anemones within a 5 m × 10 m rocky patch in Laguna Beach, CA (33°32' N, 117°48' W). Fifty-one males carried egg masses, and these were brought live to the laboratory; all other individuals were preserved in 95 per cent EtOH. Most males carried a single large egg mass, but 23 males each carried two or more distinct but adjoining egg masses (figure 1). Each egg mass was carefully separated from the guardian male and kept separately in a 1.5 ml centrifuge tube containing room temperature-filtered sea water. Males were then preserved in 95 per cent EtOH.

Egg masses were inspected every 2–3 days for newly hatched larvae, and sea water was replaced each time. Free-swimming larvae from the same egg mass were collectively preserved in 95 per cent EtOH until genetic assays, when they were transferred individually to the bottom of a PCR plate. We continued collecting larvae until all had hatched from the egg mass, or until no further development was observed for greater than 10 days.

(b) Genetic analysis

Isolation of microsatellite loci for *P. stearnsi* followed the enrichment protocol of Hamilton *et al.* (1999; Hauswaldt & Glenn 2003). Genomic DNA extraction and PCR conditions (reagent concentrations and thermal profiles) were performed for adults and larvae as described for another pycnogonid (Barreto & Avise 2008). Three highly polymorphic loci were chosen for this study after showing no significant deviations from Hardy–Weinberg equilibrium, no linkage disequilibrium and high parentage exclusion probabilities (electronic supplementary material, table S1). From the 87 egg masses collected, 15–75 progeny per egg mass (for a total of 1547 offspring) were genotyped. Maternal genotypes were deduced by exclusion after accounting for the guardian male's alleles in each of the progeny.

(c) Selection and Bateman gradient analyses

Under a dissecting microscope (model Leica 2000), digital images were taken of the dorsal full-trunk view of each collected adult, as well as of what remained of each egg mass. ImageJ software (NIH) was used to analyse these images. For each collected specimen, trunk length was measured as the distance from the eye tubercle to the base of the abdomen. Trunk length was first used as a conservative guide to identify mature individuals for which no matings were evident. For each sex, individuals were sorted by size and the smallest specimen that was unambiguously mature (i.e. had mated in our sample) was earmarked. The trunk length of the focal individual henceforth was considered the lowest size at maturity in our sample, and all specimens below this threshold were discarded from subsequent analyses. Trunk lengths were then used in selection analyses (below) and to determine the degree of sexual size dimorphism among mature individuals.

Reproductive success was measured as the number of offspring per mated individual, calculated as the total number of hatched larvae plus remaining eggs from each egg mass. Larvae still stored in EtOH were counted under a microscope by sampling small volumes of known proportion from each 1.5 ml tube. The number of unhatched eggs remaining in each mass was estimated from the digital photographs by measuring the two-dimensional area of the mass, dividing by the circular area of one of its respective eggs, and multiplying by the thickness of the mass (measured in the number of eggs).

Using the methods developed by Wade (1979) and Arnold & Duvall (1994), we calculated the opportunities for selection and for sexual selection (I and I_s , respectively) and the Bateman gradient. We compared the magnitude of these indices between the sexes by means of an ANCOVA (for the Bateman gradients) and Levene's test (for I and I_s), with standard errors for the latter measures estimated by bootstrap re-sampling of the data. These analyses allowed us to also estimate ΔI , the sex difference in the opportunity for selection (Shuster & Wade 2003; Shuster 2009). When

the sex ratio is unity, $\Delta I = I_{\sigma} - I_{\phi}$. The sign and magnitude of ΔI hence provide, respectively, an estimate of the direction and intensity of sexual selection.

We examined the possible influence of body size on reproductive success by calculating the standardized selection differential on trunk length (s' , Lande & Arnold 1983). We also report on two new related metrics: m' (the standardized mating differential on trunk length); and $s'_{\max}$ (the maximum standardized sexual selection differential). These latter metrics were proposed by Jones (2009), and they estimate, respectively, the covariance between phenotypic traits and mating success, and the upper limit of the intensity of pre-copulatory sexual selection on a trait.

Prior to these analyses, data were standardized following suggestions by Jones (2009). Individuals with no detected matings were included in these analyses, since these individuals represent a relevant component of fitness variance (Shuster & Wade 2003). All statistical analyses were performed in R 2.62 (R Development Core Team).

3. RESULTS

Larvae began to hatch 14–29 days from the time of collection. This is a minimum estimate of the gestation period, because we do not know when the eggs were oviposited. Enough larvae hatched to allow genetic analyses of each egg mass, but no egg mass hatched to completion in our laboratory, probably owing to inadequate rearing conditions. Thus, we were unable to estimate the total time a male is 'pregnant'. However, based on the comparable time to first hatches and size of mass, a full egg mass probably takes one to two months after oviposition to hatch completely, as suggested by observations in *P. littoralis* (Tomaschko *et al.* 1997). In the current study, 28 males carried one egg mass, 12 carried two, 10 carried three and one carried four egg masses. The mean number of progeny per egg mass was 5846 (range: 1855–10 800). Multiple egg masses carried by the same male did not differ significantly in the number of progeny (paired *t*-test, $t = 0.34$, $p = 0.28$), and these also did not differ from those found singly in males ($F_{1,41} = 0.009$, $p = 0.93$).

The adult sex ratio in our sample—61 males and 62 females—did not depart significantly from equality ($p > 0.95$, exact binomial test). From each progeny array and its known sire, we deduced the dam's multi-locus genotype for each egg mass, and then compared these with the genotypes of collected females. We found 79 matches, meaning that 90 per cent of the 87 masses could be assigned to one of the collected females. The expected probability of multi-locus identity of these matches ranged from 10^{-8} to 10^{-4} , and all females implicated as dams were found on the same anemone as the egg mass to which they matched. Thus, we were confident that the genotypic matches revealed actual mating events. As expected, no male was genetically excluded as the sire of all progeny he carried, and all progeny sampled from the same egg mass shared the same mother.

Adjacent egg masses on the same male were sometimes laid by the same female ($n = 8$), but more often were the result of a mate switch (exact binomial test, $p = 0.003$). Females that laid more than one egg mass probably did so as different mating events, since these masses were at different developmental stages more often than expected

Figure 2. Distribution of mating success in males (black bars) and females (white bars) of *P. stearnsi*.

by chance (exact binomial test, $p = 0.027$). The adult females were, on average, 30 per cent longer in body size than adult males ($\bar{x}_{\text{males}} = 2.69$ mm, $\bar{x}_{\text{females}} = 3.48$ mm; $F_{1,109} = 243$, $p < 0.0001$).

Males and females showed similar mating success patterns: both sexes had mated with multiple partners (figure 2). The standardized variances of selection and sexual selection (table 1) did not differ between the sexes according to Levene's test (I : $F_{1,121} = 0.27$, $p = 0.61$; I_s : $F_{1,121} = 0.06$, $p = 0.80$). Hence, $\Delta I = -0.05$, but it is not significantly different from zero. Estimates of m' and s' in males were each more than twice as high as those in females, but neither was significantly positive (table 1; male m' : $F_{1,51} = 1.14$, $p = 0.29$; male s' : $F_{1,51} = 1.88$, $p = 0.18$; female m' : $F_{1,56} = 0.31$, $p = 0.58$; female s' : $F_{1,56} = 0.35$, $p = 0.56$). The Bateman gradient for each sex (figure 3 and table 1), estimated by the least-squares regression of relative reproductive success on relative mating success, was significantly positive (male β_{ss} : $F_{1,59} = 220$, $p < 0.0001$; female β_{ss} : $F_{1,60} = 222$, $p < 0.0001$). However, as shown in figure 3, the Bateman gradients did not differ between the sexes (ANCOVA, $F_{3,119} = 0.86$, $p = 0.67$). Data used for the opportunity for selection and Bateman gradient analyses can be found in electronic supplementary material, table S2.

4. DISCUSSION

Pycnogonids have been considered as ideal candidates for studying the evolution of male parental care (Tallamy 2001; Bain & Govedich 2004a), but *P. stearnsi* is only the second species for which the genetic mating system has been elucidated (the first was *Ammonothea hilgendorfi*; Barreto & Avise 2008). Behavioural observations of a morphologically similar congener (Jarvis & King 1972) led us to hypothesize that male *P. stearnsi*, owing to the substantial egg masses they carry, would show limited capacity for multiple mating. However, our genetic appraisals demonstrate unambiguously that males routinely acquire multiple mates by adding additional egg masses to their bodies, even before previous ones have finished developing. Similarly, females mate with as many as three different males within the same window of time, as demonstrated by the fact that different egg masses from a single dam were often simultaneously present on multiple

Table 1. Quantitative measures of sexual selection in a natural population of the pycnogonid *P. stearnsi*. (The opportunities for selection (I) and for sexual selection (I_s), as well as the Bateman gradient (β_{ss}), were calculated according to Wade (1979) and Arnold & Duvall (1994). The standardized selection gradient (s') was estimated following the method of Lande & Arnold (1983), while the new metrics (m' , the standardized mating differential, and $s'_{\max}$, the maximum expected value for m' or s') followed Jones (2009). Standard errors of the estimates (in parentheses) were estimated from a normal distribution for $\bar{X}_{ms}$ (mean mating success), $\bar{X}_{rs}$ (mean reproductive success), and β_{ss} , or from bootstrap resampling of the data (for I and I_s) in R 2.62. (R Development Core Team).)

sex	$\bar{X}_{ms}$ (s.e.)	I_s (s.e.)	$\bar{X}_{rs}$ (s.e.)	I (s.e.)	β_{ss} (s.e.)	m'	s'	$s'_{\max}$
male	1.28 (0.1)	0.53 (0.11)	8429 (850)	0.62 (0.11)	0.96 (0.07)	0.11	0.14	0.68
female	1.16 (0.1)	0.52 (0.08)	7281 (755)	0.67 (0.11)	0.99 (0.07)	0.05	0.06	0.72

Figure 3. Relationships between mating success and reproductive success (Bateman gradients) in *P. stearnsi*. The data used for this analysis were standardized to a mean of unity for each sex, such that the axes are scaled to relative mating success and relative reproductive success. See text and table 1 for formal statistical descriptions. Filled circles and continuous lines, males; open circles and dashed lines, females.

males. Thus, *P. stearnsi* in nature exhibits a polygamous mating system.

Our analysis of the mating system via formal sexual selection metrics provides, for the first time in this taxonomic class of arthropods, quantitative evidence on which sex should be under stronger sexual selection. Our nearly exhaustive characterization of mating events in *P. stearnsi* in a small rocky patch, in one sampling period, shows that the sexes experience similar variances in fitness (i.e. $\Delta I = 0$), and similar, positive Bateman gradients. Furthermore, the ratio I_s/I is high (greater than 78%) for both sexes, suggesting that most of the variance in reproductive success within each sex is attributable to sexual selection (i.e. variance in mating success), rather than to variances in fertility or brooding limitations.

In sex-role-reversed species, $\Delta I < 0$ (Shuster & Wade 2003) and females are expected to show a significantly steeper Bateman gradient than males (Jones *et al.* 2000), meaning that sexual selection acts stronger on females. This condition can occur, for instance, when males provide extensive parental care and hence have reduced capacity for multiple mating. Females in turn exhibit higher variance in mate acquisition than males (Clutton-Brock 1991; Butchart 2000; Berglund & Rosenqvist 2003), as seen in many syngnathid fishes

(Vincent *et al.* 1992). In *P. stearnsi*, by contrast, our genotypic assays revealed that even though females can produce new clutches before their previous ones have hatched, pregnant males can continue to add clutches of eggs to their bodies (figure 1). Our estimates of ΔI and Bateman gradients strongly suggest that sexual selection in *P. stearnsi* seems to be equally important in males and females in generating variance in fitness. This condition has the potential to generate divergent selective pressures on males and females, increasing the degree of sexual dimorphism (Shuster & Wade 2003). Finally, we predict that competition for mates occurs in both sexes.

Selection analyses uncovered no evidence that trunk length influences mating success or reproductive success, as evidenced by the non-significant mating differentials (m') and selection differentials (s'). Since these differentials comprise only a small fraction of the upper bound of selection intensity ($s'_{\max}$) in both sexes, other traits probably are responsible for generating the observed variances. Male mating success, for instance, could be based on female preference for superior paternal abilities (Tallamy 2001), which may be unrelated or even inversely related to body size (Forsgren 1997; Wong 2004). Variation in male parental success may also explain why female *P. stearnsi* often distribute their successive clutches among different males. Moreover, strong female preference for larger males has been documented in mate choice experiments in some fishes that display paternal care of progeny (Berglund *et al.* 1986; Lehtonen *et al.* 2007), even when a relationship between male body size and mating success was not detected in nature (Jones *et al.* 1999; Mobley *et al.* 2009). Hence, an effect of body size on mating success cannot be ruled out completely for our samples.

Similarly, we cannot definitively conclude that female fecundity is unrelated to body size in *P. stearnsi*. Our study showed that this species is strongly female-biased size-dimorphic, a pattern that is conventionally explained by fecundity selection (Andersson 1994). Given that male *P. stearnsi* are able to brood multiple large egg masses simultaneously (and hence have a positive Bateman gradient), females may be selected to take advantage of these available 'oviposition sites'. Indeed, for populations of a pipefish species, mean number of mates per male appeared to be related inversely to the degree of female-biased size dimorphism (Rispoli & Wilson 2008). A more rigorous test of fecundity selection in females will require estimates of egg production by the same females over multiple mating episodes or, ideally, over their lifetime (Preziosi *et al.* 1996).

Our use of current theory and tools to quantify the relationship between sexual selection and mating systems revealed that access to mates is the largest single contributor to reproductive success in both male and female *P. stearnsi*. We emphasize, however, that these quantifications refer to one sampling episode. A complete measurement of selection intensities will require information on lifetime mating and fitness parameters (Arnold & Duvall 1994), which may also vary geographically (Mobley & Jones 2007; Rispoli & Wilson 2008). Such studies could provide the much needed insight into the temporal and spatial components of fitness variance within and among the sexes (Shuster & Wade 2003). Furthermore, selection at other levels of the fitness continuum should be examined, especially in cases where postzygotic care of offspring is extensive. For example, whereas male *P. stearnsi* clearly are able to collect additional egg masses, it is unknown whether they do so at the expense of offspring survivorship. These shortcomings merely highlight the potential of pycnogonid sea spiders for testing additional hypotheses within the theoretical framework of sexual selection and parental investment.

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