ORIGINAL PAPER

Multiple paternity and extra-group fertilizations in a natural population of California grunion (*Leuresthes tenuis*), a beach-spawning marine fish

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Abstract Although individuals in many fish species move to shallow waters to spawn, the California grunion (*Leuresthes tenuis)* is almost unique in its constitutive display of synchronous full-emergence beach spawning. During a spawning event, fish ride large waves onshore to spawn on beach land, where their eggs incubate terrestrially. Here, we employ molecular markers to ascertain how this unusual reproductive behavior impacts genetic parentage. We developed and utilized four highly polymorphic microsatellite markers to assess maternal and paternal contributions in a total of 682 progeny from 17 nests of a natural population of *L. tenuis*. Alleles deduced to be of paternal origin in progeny were used to determine the minimum number of sires per nest and to estimate the true number of sires per nest via Bayesian analysis. We document the following: (a) no instances of multiple maternity for progeny within a nest; (b) a high frequency of nests (88%) with multiple paternity; and (c) an appreciable fraction of nests (18%) in which the estimated number of genetic sires (as many as nine) proved to be greater than the observed number of male attendants, thus implicating occasional extra-group fertilization events. From these and other observations, we also conclude that spawning behavior in grunions may involve site choice but not explicit mate choice. In addition to providing the first analysis of molecular parentage in a beach-spawning fish, we compare our findings to those reported previously for a beach-spawning arthropod, and we discuss the forces that may be maintaining this peculiar reproductive behavior.

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Introduction

Terrestrial spawning may afford several special advantages for a marine fish. Egg development can be accelerated by the increased availability of oxygen (Strathmann and Hess 1999) and by elevated temperatures in a terrestrial environment (Seymour and Bradford 1995; Smyder and Martin 2002); and, the lack of aquatic predators on a beach, and the synchronous release (when present) of hatched offspring, may increase egg (Middaugh 1981) and hatchling survival, respectively. However, potential disadvantages of terrestrial spawning include the risk of egg desiccation (Strathmann and Hess 1999), and depredation on spawning adults, eggs, or hatchlings by birds (Middaugh et al. 1983) or other beach predators. In addition, beached fish risk physical injury from wave action, exposure to infection from incurred wounds, and asphyxiation.

Spawning in many fish species entails movements of adults into shallow waters (Clark 1925; Middaugh 1981; Leggett and Frank 1990; Yamahira 1996; Martin et al. 2004), where individuals may experience, to a partial degree, some of the reproductive risks and benefits of terrestrial spawning. However, few fish species engage in either limited or full-emergence beach spawning. Indeed, the California grunion (*Leuresthes tenuis*) and its congener (*L. sardina*) are the only known fish species with synchronous and constitutive full-emergence beach spawning, during which the adults are completely out of the water for up to several minutes and the developing embryos are strictly land-based during early development (Walker 1952).

Nearly a century ago Thompson and Thompson (1919) and Clark (1925) detailed spawning events in *L. tenuis*. Briefly, during a mass spawning "run" (which lasts up to several hours), grunions ride large waves high up onto the beach. The event takes place during the first 3–4 nights following full-moon or new-moon tides, which coincide with the most extreme high tides of the year. Each female twists her body and uses her tail to bury herself—up to her head—in the sand, where she will deposit many eggs. Males likewise surf ashore but remain on the sand surface, releasing milt to the eggs buried below. Some males wrap their bodies around the partially exposed females, but all males quickly wriggle back to the sea. Females then work themselves out of the holes they have dug by twisting their bodies back and forth, and they too then make their way back across the beach to the water.

The deposited eggs incubate terrestrially in the moist sand for about 2 weeks until the next semilunar tide, at which time high waves once again reach the level of oviposition and hatching is initiated by agitation of the eggs (Walker 1952). The young are released synchronously to the water. Although the physical and chemical aspects of egg development associated with beach spawning have been well detailed (David 1939; Smyder and Martin 2002), aspects of the grunion mating system have not been addressed to date.

Full-emergence beach spawning by a marine fish is a highly unusual reproductive behavior that is likely to include impacts on patterns of genetic parentage. For example, empirical work by Levitan (1991) has shown that the diffusive properties of water rapidly (i.e., within a short distance) dilute sperm that have been released from a point source, an effect that is likely to be especially pronounced in open-water spawners. For the beach-spawning grunion, however, such diffusive effects might be somewhat diminished in the low-water-volume sand environment, perhaps leading to a greater probability of multiple paternity (by nearby sperm-releasing males) within a female's clutch. Alternatively, a female's oviposition site (the pit that she has dug in the sand) may restrict the number of males whose sperm have access to her nest. In another synchronous beach-spawner, the horseshoe crab *Limulus polyphemus*, paternity analyses have shown that males who are in direct physical contact with the female sire almost all of the young (Brockmann et al. 2000).

Behavioral observations suggest that as many as eight grunion males may fertilize the eggs in a given nest (Walker 1952), but this claim has not been verified genetically. With regard to paternity in any grunion nest, three distinct outcomes seem plausible: (a) one male (perhaps the first to reach the female, or the specimen who physically embraces the female or otherwise out-competes his rivals) fertilizes all of the eggs; (b) two or more males who are in direct behavioral consort with the focal female contribute to her clutch of offspring; or, (c) males that are not near the focal female at the time of her spawn also make a genetic contribution to her clutch via sand-stored pools of sperm (either deposited on-site from an earlier spawn, or diffused from nearby locations across the light film of water on the sand).

In this study, we test these possibilities by employing microsatellite markers to assess genetic parentage (paternity and maternity) in a natural population of California grunion. We determine the percentage of nests with multiple sires, the number and source of males that contributed to each nest, and we also address whether female body size influences sire numbers. We compare our genetic findings to those for a previously studied beach-spawning marine invertebrate, and interpret results in the context of sexual selection theory.

Materials and methods

Study site and sampling

The mating behavior of the California grunion may be observed along the species' range from San Francisco to Magdalena Bay, Baja California (Miller and Lea 1972), during the spawning months of March to August. For this study, all observations and sampling of *Leuresthes tenuis* occurred at Laguna Beach (Orange County, 33°32′N, 117°47′W), California, during spawning runs on 13 and 14 June 2006 and 4 April 2007. In total, 17 females and their eggs were collected for parentage analysis. We identified each nesting female as an individual positioned vertically in the sand, and with at least one male attendant wrapped around her. Previous research has shown that this behavior indicates that the female is depositing eggs in the sand, at a depth of about 5–15 cm, while the male(s) release milt into the hole she has dug (Thompson and Thompson 1919). In addition, we collected 50 random adults (gender unknown) to generate population genetic data.

During initial reconnaissance, we noticed that males quickly departed if a human observer approached within a few feet during spawning activity. Thus, to minimize any effect we might otherwise have on the behavior of our study samples, we stood at least several meters away while the fish were in consort, and we also waited until the attendant males departed of their own accord (presumably after releasing milt) and the female had begun to twist her way back out of the sand. At that point we quickly captured the female and then used a small hand shovel to collect the sand with eggs directly under her. Females were immediately stored on ice and then frozen at −80°C in the laboratory. Eggs from each nest were placed in a plastic container

of sand kept at ambient temperature, and brought to the laboratory for incubation. No males from study nests were collected, as it did not seem possible to do so without disrupting mating behavior. We did observe, however, that the nests we collected had as few as one to as many as six male attendants each.

Incubation and hatching of eggs

Holes were cut in the bottom of the plastic containers holding the eggs to allow for water drainage, and the containers then were suspended over a large tray. Each container was loosely covered to allow ventilation, and deionized water was added periodically to prevent egg desiccation. Eggs were incubated at 22°C for 8 days, at which time the sand–egg mixture was briefly mixed and about one quarter to one-third of the mixture was transferred to a large jar of seawater and agitated for 1 min to initiate hatching. Forty or more hatchlings from each nest were collected at random and stored in lysis buffer at −20°C for subsequent analysis.

Microsatellite isolation

Genomic DNA was extracted from individual hatchlings via a proteinase K lysis procedure (Hoelzel and Green 1998), and from each of 17 nesting mothers and 50 random adults using a Qiagen DNeasy kit (animal tissue protocol). To isolate microsatellite markers, we followed a protocol of Hamilton et al. (1999) as modified by Hauswaldt and Glenn (2003). Briefly, about 5 μg of genomic DNA was isolated from one of the 50 random individuals and was digested with the restriction enzyme BstU I (New England Biolabs). Double-stranded SuperSNX24 linkers were then ligated to the resulting fragments. To isolate fragments containing microsatellite sequences, we hybridized biotinylated microsatellite oligonucleotides (GT, GACA, GATC, GATA, and CT repeats) to the fragments with SuperSNX24 linkers,

incubated the product with magnetic beads (Dynabeads, Dyna1) that bind to the probes, and then captured and washed these fragments using a magnetic particle collecting unit. Next, the microsatellite-containing fragments were amplified via PCR with the SuperSNX24 linkers utilized as priming sites, and the enriched DNA was cloned with a TOPO TA cloning kit (Invitrogen). The transformed bacteria were grown on LB plates and colonies were incubated overnight in LB broth and amplified via PCR with M13 primers (F: GTAAAACGACGGCCAGT, R: CAGGAAAG AGCTATGAC). These PCR products were then run on agarose gels, and the products with inserts ranging in size from 500–1,000 bp were sequenced on an ABI 3130xl Genetic Analyzer. Primers were designed for desirable sequences and these primer sets were tested on a select set of population DNA samples to estimate site variability. In all, four polymorphic loci (B18, B19, B39, B82) were selected. Two of the primer sets (B18 and B82) were fluorescently labeled with FAM and two (B19 and B39) were labeled with HEX.

Genetic analyses

PCR amplifications of hatchling and adult DNA were performed in a 15-μL mix composed of the following: 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM each of forward and reverse primer, and 0.75 units GoTaq DNA Polymerase in a buffer supplied by the manufacturer (Promega). The amplifications began with a 2-min denaturation at 95°C, followed by 35 cycles of 95º for 30 s, 55 or 56° (see Table 1) for 1 min, and 72° for 1 min, and a final extension at 72° for 5 min. One μL of diluted PCR product was then mixed with 0.45 μL of GeneScan-ROX 500 size standard (Applied Biosystems) and 9.55 μL Hi-Di Formamide (Applied Biosystems), denatured at 95° for 3 min, and electrophoresed on an ABI 3100 Genetic Analyzer in two multiplex loading groups (B18 and B19; B39 and B82). Alleles were sized

Table 1 Characterization of developed microsatellite loci in the California grunion, *Leuresthes tenuis*

Locus	Primer sequence	Repeat motif[a]	Size range (bp)	N_A	T_A(°C)
B18	F: GCTGCTGAAAATCATCATGTC	$(GT)_{12}$	231–263	11	55
	R: FAM-CAGATTGTATGTAACAGGC				
B19	F: GCGTGTGTATTCTCATGTGTGC	$(GT)_{15}$	203–241	16	55
	R: HEX-CAATGCGTTAATGATATGC				
B39	F: GTGAAATGATCCCAGGCAGC	$(GT)_{24}$	233–311	30	56
	R: HEX-CAAGGGATGATGCACAGAAC				
B82	F: FAM-ATGTGAAAGAGGTGTGTTC	$(GT)_{23}$	239–309	24	56
	R: GTGTCAGGTTAGCGCATACAG				

Values are based on a random sample of 50 individuals from the Laguna Beach, California, population

N_A number of observed alleles, T_A annealing temperature

[a] Results from one sequenced individual

using GeneMapper 4.0 software with verification done by eye.

Fifty random individuals were used to estimate population allelic diversity at our four loci. We used the program GENEPOP (Raymond and Rousset 1995) to estimate allele frequencies, to test for Hardy–Weinberg equilibrium at each locus, and to test for linkage disequilibrium between pairs of loci. Bonferroni corrections were applied for multiple tests where applicable, and probabilities of exclusion were calculated following Jamieson and Taylor (1997). The estimated allele frequencies were then used in the simulation program BROOD (DeWoody et al. 2000) to estimate the mean number of hatchlings per nest sufficient to detect the total number of sires. The program output suggests that a mean of 41 hatchlings per nest probably would elucidate all of the sires given approximately equal contributions among males. We genotyped a mean of 40.1 young per nest (SD = 2.52).

Finally, the number of sires per nest was assessed in two ways: (a) as the *minimum* number of sires, calculated as one-half the number of paternal alleles observed at the locus with the greatest allelic richness for that nest (rounded up for odd-numbered values); and (b) as a statistically adjusted estimate of the true number of sires per nest, calculated using the program PARENTAGE (Emery et al. 2001). This program uses Bayesian inference and Markov chain Monte-Carlo to sample from the posterior distributions of interest, and it attempts to account for multi-locus parental genotypes, mutation, and mis-scoring of alleles. We input allele frequencies of the parental population estimated from 50 random adult individuals, and the Markov chains were carried out with 5,000 iterations for each nest

following a burn-in of 5,000 iterations and a thinning interval of 400. A prior for the mutation rate was set using a gamma distribution with a shape parameter of 2 and a mean of 0.001. This allowed for 95% of the mutation rates to lie between 0.00014 and 0.0028 mutations per generation, which is in accordance with observed mutation rates for microsatellite loci (Weber and Wong 1993). To address the potentiality that eggs from one "nest" might actually contain fertilized eggs from two or more females (either from a neighboring nest of the same run, or from a nest deposited during a previous run), the genotype of each focal female was input into PARENTAGE as a potential maternal genotype without restricting the possibility of additional dams.

Physical measurements

To address whether the number of sires per nest correlates with female body size, we measured body weight and length (fork length or FL, from the tip of the snout to the fork of the tail) of all nest females. Data then were visualized with regression analysis.

Results

Microsatellite markers and genetic population summary

In our genetic analysis of 50 random adults, allelic diversities were high at all four microsatellite loci. The mean number of alleles per locus was 20.25 (range 11–30) (Table 1; Fig. 1), and each surveyed fish displayed a unique multi-locus

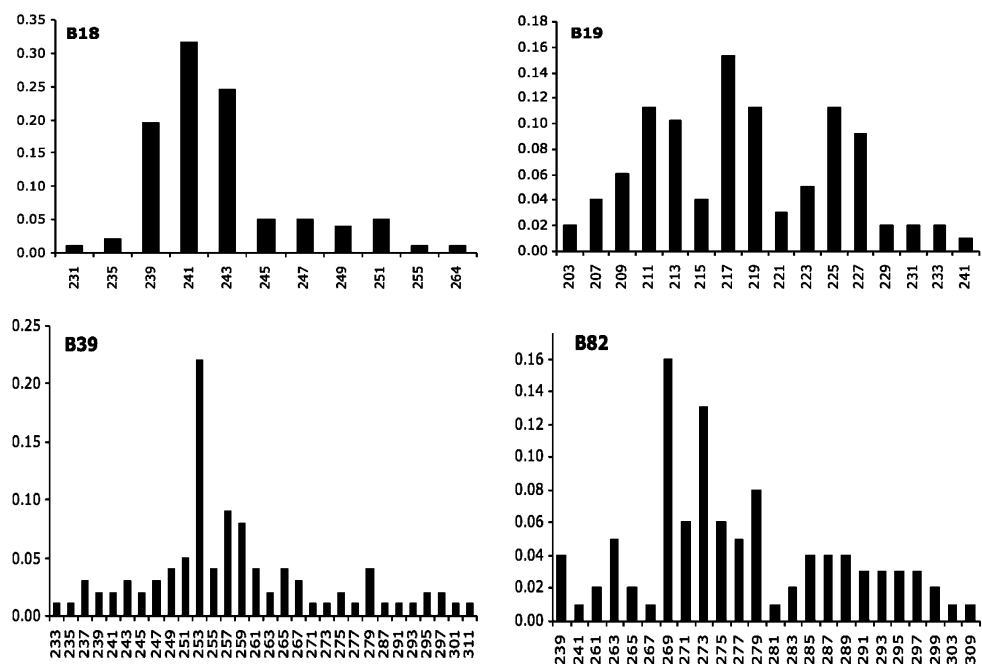**Fig. 1** Allele frequencies for four microsatellite loci in the California grunion, *Leuresthes tenuis*

Table 2 Characteristics of microsatellite loci estimated from a sample of 50 adults from the Laguna Beach, California, population

Locus	Heterozygosity		P value for HWE	Exclusion probabilities
	Observed	Expected		
B18	0.84	0.80	0.98	0.43
B18	0.90	0.92	0.16	0.68
B39	0.92	0.93	0.06	0.73
B82	0.88	0.94	0.08	0.75
Combined			0.07	0.99

genotype. Mean overall heterozygosity was 0.89, and at no locus did the observed heterozygosity depart from the Hardy–Weinberg expectation. The four loci provided a combined exclusion probability of 0.99 (Table 2), and in pairwise combinations showed no indication of linkage disequilibrium ($P > 0.05$ after Bonferroni correction). These attributes confirm that the four loci are powerful and independent markers for parentage analyses.

De novo mutation and null alleles

Nonamplifying (null) alleles tend to be common in microsatellite data and must be accounted for in parentage studies (Pemberton et al. 1995; Dakin and Avise 2004). We concluded that null alleles were not of significant concern in our combined four-locus assessments for two reasons. First, all hatchling fish shared an allele with their suspected mother at each of the four assayed loci, indicating Mendelian inheritance of alleles (and also confirming the status of each nest-attendant female as the genuine dam). Second, we tested for a heterozygote deficiency using our random population data following a method by Brookfield (1996), from which we estimated a mean frequency of null alleles of 0.01. Such a low frequency of null alleles would not materially affect our assessments of genetic parentage in the current multi-locus characterizations.

De novo mutations, when unrecognized as such, can potentially confound parentage analysis by inflating the inferred number of sires or dams. The program PARENTAGE identified 16 new mutations among the 682 young surveyed at four loci (5,456 alleles in total), thus yielding an estimated mutation rate of 2.9×10^{-3}. This rate is similar to those detected in various other microsatellite studies (Weber and Wong 1993; Hancock 1999; Mackiewicz et al. 2002). It should be also noted that this estimate encompasses any mis-scoring of alleles (i.e., we did not distinguish de novo mutation from possible mis-scoring for the aberrant alleles in our analysis). Thus, the true de novo mutation rate may be lower than the value reported here.

Genetic assessment of parentage

Hatchling genotypes invariably were consistent with the expectation of one dam per nest. In other words, no hatchling lacked an allele from its presumed mother at any locus. Likewise, statistical analysis of nest maternity with the program PARENTAGE supported a single-mother model for each nest.

On the other hand, 88% of the nests clearly displayed multiple paternity. Indeed, from the genetic evidence, only two among the 17 nests had a single contributing sire each (Fig. 2). For the remaining nests, hatchling genotypes identified 2–5 sires per nest as a documented minimum (mean = 3.18, SD = 1.42), or a mode of 2–9 sires per nest as calculated in the statistically adjusted estimate in PARENTAGE (mean = 4.30, SD = 2.54). P_{mode} values reported by PARENTAGE indicate the observed proportion of the modal value based on 5,000 simulations. In those nests with a modal number of sires greater than 6 (nests 4, 9, and 16), P_{mode} values were much lower (mean = 0.45) than those values for nests with a modal number of sires less than or equal to 6 (mean = 0.83; Table 3), indicating that precision of analysis was reduced in these nests and we may have less confidence in the modal number observed. However, $P_{>6}$ (the sum of observed proportions for values greater than 6) for each of the three nests was 1.00 (Table 3), confirming that, at the least, we have confidence that the true number of sires at nests 4, 9, and 16 is greater than 6. Neither body weight nor body length of the nesting females correlated with sire numbers per nest ($r^2 = 0.006$ and 0.004, respectively; Fig. 3).
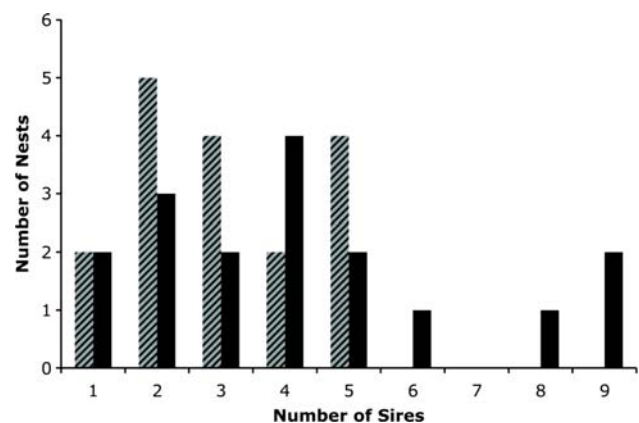

Fig. 2 Distribution of the number of sires per grunion nest ($n = 17$). *Bars* with hatching are the minimum number of sires per nest; *black bars* represent the estimated number of sires per nest based on 5,000 simulations in the program PARENTAGE

Table 3 Summary of results regarding paternity at each *Leuresthes tenuis* nest, as estimated by allele counting (Minimum No. Sires) and inferred via the program PARENTAGE

| Nest | No. offspring analyzed | Minimum no. sires | PARENTAGE | | | |
			Range	Mean (±SE)	Mode	P_{mode}
1	40	3	2–5	3.21 (0.004)	3	0.79
2	38	5	5–9	6.39 (0.006)	6	0.60
3	40	2	2–3	2.02 (0.002)	2	0.98
4	40	5	7–12	8.68 (0.009)	9	0.39*
5	40	1	1	1.00 (0.000)	1	1.00
6	40	3	5–7	5.04 (0.002)	5	0.96
7	40	3	3–5	3.64 (0.005)	4	0.63
8	40	3	3–5	3.85 (0.004)	4	0.84
9	40	5	7–12	8.85 (0.008)	9	0.49*
10	49	2	2	2.00 (0.000)	2	1.00
11	40	2	3–5	3.37 (0.005)	3	0.64
12	40	3	4–7	4.19 (0.004)	4	0.82
13	40	4	4–7	5.01 (0.007)	5	0.54
14	39	5	4–6	4.95 (0.004)	5	0.84
15	40	2	2–3	2.00 (0.000)	2	1.00
16	36	5	6–11	7.64 (0.007)	8	0.48*
17	40	1	1–2	1.00 (0.007)	1	1.00

P_{mode} indicates the observed proportion of samples with the modal value based on 5,000 simulations

* $P_{>6} = 1.00$

Fig. 3 Correlation between **a** female fork length and estimated number of sires ($r^2 = 0.006$, $n = 17$), and **b** female weight and estimated number of sires ($r^2 = 0.004$, $n = 17$)

Discussion

Genetic paternity

In this study we provide the first information on genetic parentage in a beach-spawning fish. As previously suspected through behavioral observations (Thompson and Thompson 1919; Walker 1952), multiple paternity proved to be common within grunion nests (characterizing 15 of the 17 progeny cohorts we genetically analyzed). This finding might seem to have been foregone given the dense spawning aggregations in these fishes and the synchrony of gamete release, but only detailed genetic analyses could rule out the possibility that the first male to reach a female's nest is rewarded with most or all of the fertilizations. In many animal species, similar kinds of genetic parentage analyses have revealed that the social mating system is not always a reliable guide to the actual genetic contributions of adults to the next generation (Avise 2004).

Perhaps more surprising were our documentations of high numbers of sires (estimated mean = 4.3) per nest, and the fact that for three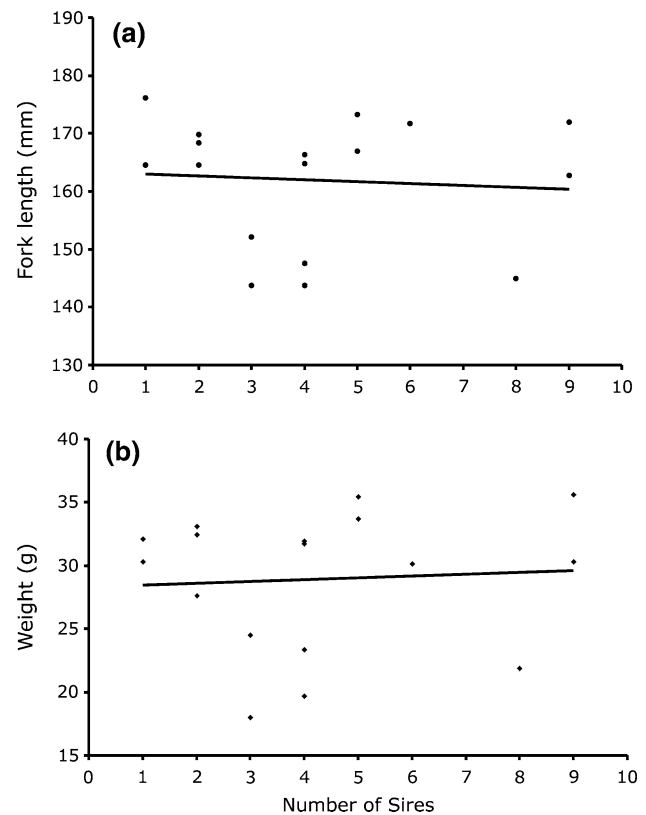 of the 17 surveyed nests (18%), the genetically estimated number of sires (8–9, or more conservatively, number of sires >6; see Table 3) was greater than the observed number of male attendants at the nest (6 at most). This latter finding suggests that extra-group fertilizations also take place on occasion, a phenomenon that presumably requires an available "background pool" of male gametes onshore. Such a pool of gametes could in principle represent sperm that were deposited earlier at a different but proximate nest, concurrently released at a nearby nest and passively carried to the focal nest by water action, or perhaps "shotgun-released" by non-attendant males who are not situated with any particular female. We cannot decide among these possibilities with available genetic evidence, but addressing this issue could make for interesting future research on mating behaviors and sexual selection in this species.

To our knowledge, the only other beach-spawning marine animal whose mating system has been genetically analyzed to date is the horseshoe crab, *Limulus polyphemus*. Using microsatellite markers as applied to dams and their offspring, plus attendant males, Brockmann et al. (1994) documented multiple paternity within many *Limulus* nests, and also determined that only a small fraction of the fertilization events was attributable to non-attendant males.

Brockmann et al. (1994) posited that the success of attendant males may help to make beach spawning advantageous despite the risks.

In our current study, the mean number of grunion offspring genotyped per nest (40.1) was well below the total progeny count, which can approach 3,000 per nest (Thompson and Thompson 1919). Thus, our current estimates of sire numbers per nest are conservative and may underestimate the true count. It should also be noted that our samples were taken during mass spawning runs with dense aggregations of individuals. The number and spatial distribution of grunions on a beach during any given spawning run varies naturally with time of year and location of the event. We would expect the level of multiple paternity to vary accordingly. Finally, we took care when collecting the nests to keep the eggs and the sand above it undisturbed to the greatest extent possible. However, it is possible that our collection method influenced the number and/or source of sperm with access to the eggs, and therefore influenced our sire estimates. In such an event, it would seem most likely that in removing the nest from the surrounding sand we would have excluded potential sources of sperm and we would therefore have underestimated the total number of sires per nest.

Mating behavior

Levitan (1998) posits that for external fertilizers, sexual selection is influenced both by sperm competition (where two or more males compete to fertilize one female's eggs; Parker 1970) and by sperm limitation (where dilution of male gametes results in many fewer zygotes than available eggs; Pennington 1985). In light of these forces, members of a species may engage in a variety of alternative mating tactics and spawning behaviors that should increase their own fitness. Fish species, in particular, display a wide array of reproductive tactics and mating behaviors (Avise et al. 2002), with male reproductive behavior being especially diverse (Breder and Rosen 1966; Gross and Sargent 1985; Taborsky 1994). The following illustrate just a few such mating behaviors: nest-tending male damselfish (Gronell 1989) may adopt eggs from other species to appear more desirable to conspecific females; similarly, female striped darters preferentially mate with males tending larger nests (even though the eggs may be adopted) or with those displaying egg mimicking pigmentation on their fins (Porter et al. 2002); plainfin midshipman fish (Brantley and Bass 1994) use their pectoral fins to fan their sperm towards a nest guarded by another male; some "bourgeois" bluegill sunfish males provide offspring care yet frequently are cuckolded by sneaker or satellite males (Neff 2001); and some Atlantic salmon spawn either as socially dominant males after returning from the sea or as smaller, socially inferior

parr that have remained in freshwater (Thomaz et al. 1997). In general, such alternative mating tactics by male fish fall within one of four strategies: be quicker than rival males in "scramble competition"; cooperate or trade with resource holders for access to mates; exploit the monopolization of resources or mates via reproductive parasitism; or monopolize resources or mates themselves (Taborsky 2001).

The latter tactic seems to be employed by particular grunion males who, during the spawn, curl around a female and place the vent close to the female's body. Such behavior suggests that these males in effect are trying to ensure their own reproductive success by depositing milt directly on top of the eggs while physically blocking sperm from nearby males from entering the female's nest that he surrounds. If so, this attempt to monopolize the mate in grunions is likely an evolved behavior in response to sperm competition.

Strong levels of sperm competition have been documented in other externally fertilizing fish species, especially when spawning densities are high (Parker 1990; Petersen and Warner 1998). Given that spawning individuals form dense aggregations and that the high beach is a low-water-volume environment, intense sperm competition seems likely in grunions as well, a prediction that gains additional support from the aforementioned mate-guarding behavior as well as from the high frequency of multiple paternity that we detected in the current study (Fig. 2).

Previous studies have reported high fertilization success rates in grunion (Thompson and Thompson 1919; Walker 1949). Similarly, we found no obvious evidence for sperm limitation in this study: no unfertilized eggs were noted in our collected nests (as determined by observations of embryonic development including a change from many small orange oil globules in each egg to one large clear one; an increase in size of the egg; and the appearance of eyes, tails, and/or circulation of the embryo through the semi-transparent membrane) and hatching rates in the lab were high. Such observations support the notion that sperm availability is not typically a serious limitation in grunion reproduction. However, we did not measure fertilization success directly and cannot discount the possibility that sperm is sometimes a limiting factor during the spawning episodes, especially along the outer reaches of the spawning aggregations or during runs of fewer individuals.

Female grunion appear to lack overt behaviors that might influence (positively or negatively) the number of males given access to their eggs. Many males arrive on the beach well after a female has dug her way into the sand and committed her eggs to a nest location; and once vertical in the sand, a female would not seem to be in a position where she could exert great control over the sources of milt that flow to her eggs (unless perhaps some undetected chemical signaling is somehow involved; see Hassler and

Brockmann 2001). This raises a possible conundrum: if a female cannot exercise choice amongst her suitors, what if any benefits might she nonetheless receive from multiple paternity? One possibility is that multi-sire clutches are of no direct mean benefit to females, but rather they are a by-product of male–male (sperm) competition (Halliday and Arnold 1987). If so, it could be that during the evolutionary transition to beach spawning, females have forgone active mate choice in return for the aforementioned benefits of terrestrial egg incubation.

Alternatively, perhaps females do gain a direct fitness benefit from multi-sire clutches via increased quality or quantity of offspring. This might happen in any of several ways: if "better" males produce more or better sperm, and such qualities are heritable (Gomendio et al. 1998); if greater genetic diversity in multi-sire clutches (Sugg and Chesser 1994; Chesser and Baker 1996) is adaptive in a variable habitat; if multiple paternity is used as a bet-hedging tactic either because a female is unable to gauge a male's fitness (Jennions and Petrie 2000), or genetic incompatibilities exist between particular spawning pairs (Tregenza and Wedell 2002), or because multiple males might provide fertilization insurance; or, finally, if a female welcomes fertilizations by multiple males in an attempt to avoid unwanted mates (Alcock et al. 1977). This latter possibility might have added significance for grunions because high-beach egg-laying means that precious little time is available to spawn.

Spawning site choice

Although a male undoubtedly is under selection pressure to monopolize mating at a given nest, his choice of nest may be random with respect to his fitness. Previous work has shown that larger, more mature female grunion produce more eggs (up to ~3,000; Thompson and Thompson 1919; Walker 1952). Thus, if all else were equal, a male would fare better by choosing a larger female with whom to spawn (Halliday 1983). However, the lack of any correlation between female size and number of sires per nest (Fig. 3), plus the general setup of spawning in the turbulent beach environment, suggest that the pairing of grunion males with females is probably quite random at a beach site.

In contrast to the California grunion, mate choice in beach-spawning horseshoe crabs does appear to be affected by female size. Brockmann (1996) reported that the number of males in consort with a female increases with female weight and carapace width. In addition, mate choice by horseshoe crabs probably entails chemical cues (Hassler and Brockmann 2001; Schwab and Brockmann 2007). These evolutionary outcomes are likely influenced by the fact that these organisms, unlike the grunions, are able to pair in the water, actively crawl onshore, and are not

dependent on tides for their terrestrial mobility. Thus, wave action and water velocity on the beach undoubtedly limit any opportunities for mate choice in grunions far more so than in horseshoe crabs.

The high average number of grunion sires per nest (4.3), a female's apparent lack of control over which males fertilize her eggs, the lack of correlation between female size and mate number, and the dramatic environmental differences between terrestrial and submerged sites, all indicate that grunion mating behavior probably reflects choice of spawning site far more so than choice of mate. If a female's oviposition site affects the quality of the abiotic environment in which her eggs mature, including the level of predation on her eggs, then grunion will have been under strong selection pressure for proper habitat choice (Rausher 1983). Such selection for oviposition site has been well documented in insects (Rausher 1983), amphibians (Rieger et al. 2004), and fish (Jones 1981; Warner 1990). Although the choice of general spawning location (i.e., a given stretch of beach) might in principle be based on either genetic instincts or culturally transmitted traditions (Warner 1988) in grunion, the specific micro-site of each nest, and who fertilizes its eggs, probably has a large stochastic component due to the second-by-second idiosyncrasies of tide and wave action as each female and each male surfs ashore during its brief terrestrial sojourn. The observed mating system of the grunion probably registers how males and females have made the best of this peculiar ecological situation.

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