Evidence for Multiple Maternal Contributors in Nests of Kelp Greenling (Hexagrammos decagrammus, Hexagrammidae)

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Twenty-three nests of kelp greenling (Hexagrammos decagrammus) were examined from British Columbia and central California. The average nest had four clutches of eggs that were associated with rock or rock and a biological substrate, encompassed an area of 1.7 m², and was guarded by a male averaging 31 cm in length. Frequency distributions of egg-size classes were examined for 13 gravid females. Females were batch spawners capable of producing at least three clutches of eggs per spawning season. Variation in mitochondrial DNA among 107 individuals from 83 clutches representing 20 nests sampled from British Columbia and central California was analyzed. Different maternal contributors were found among clutches in 27% of nests from British Columbia and 55% of nests from California.

Parental care in fishes requires an expensive energy investment, yet it increases survivorship of young and occurs in 21% of teleost families (Gross and Sargent, 1985). Guarding is the most common form of care and is usually done by the male (Potts, 1984; Gross and Sargent, 1985); therefore, males have the potential to strongly influence survival of the young. Like other members of the family Hexagrammidae (Gobunova, 1970), kelp greenling produce demersal eggs that are attached to a substrate and then guarded by the male parent. Ranging from the Aleutian Islands to La Jolla, California, kelp greenling utilize rocky reefs and kelp forests for habitat and reproduction. Kelp greenling are sexually dimorphic, a phenomenon that usually indicates different sexual roles in nearshore demersal egg layers (Potts, 1984). In kelp greenling, males are territorial during the reproductive season, from late fall to early winter. Males guard nests with several discrete clutches of eggs at various stages of development (DeMartini, 1986). A nest is the area containing clutches defended by a male. Clutches are golf ball–to tennis ball–sized masses of benthic adhesive eggs. In Puget Sound, male kelp greenling guarded one to 10 clutches per nest (mean = 4.5) that were adjacent or up to 1.9 m apart (DeMartini, 1986). In kelp greenling nests, the guardian male is the putative father, but sneak spawning also has been observed (J. Heine, pers. comm.)

Much less is known about the reproductive biology of female kelp greenling. Spawned eggs are 2.2–2.5 mm in diameter (mean = 2.5 mm), and in Puget Sound, clutches consisted of an average of 4340 eggs (SE = 311; DeMartini, 1986). Females may be batch spawners, producing several clutches of eggs per spawning season. If so, it is not known whether females deposit clutches in one nest or among several nests. Multiple spawning has been reported in six other hexagrammids and is suspected in Hexagrammos decagrammus (Kurita et al., 1995). This study describes spatial and temporal patterns of maternal contributions to kelp greenling nests. We described and compared nests from two distinct parts of the kelp greenling range, determined whether females are batch spawners capable of contributing several clutches per spawning season, and determined whether maternal differences exist among clutches within nests by comparing mitochondrial DNA (mtDNA) of eggs from all clutches within 20 nests. Maternally inherited, mtDNA is an ideal marker for determining maternity of clutches within nests, because different mtDNA haplotypes among clutches in a nest would indicate that clutches were contributed by different females. Identical haplotypes, however, would not necessarily imply a single female contributor because different females may have identical DNA sequences for the region of mtDNA compared.

Materials and Methods

Sampling.—In the field, we characterized kelp greenling nests, collected adult females for ovary and mtDNA analysis, and sampled egg clutches within nests for mtDNA analysis. Fieldwork was conducted using SCUBA off Vancouver Island, Canada, and near Monterey, California. To sample nests, we first identified guarding males on the basis of their behavior. Males returning to an area in the presence of a diver were considered guarding. The area within a 1-m radius around a guardian male was searched intensively. Once identified, a clutch was flagged, and the area within 1 m of it was searched intensively until all clutches within the nest were found. A sample of each clutch was
taken and stored at −20°C for mtDNA analyses. Every nest discovered was sampled (i.e., nests were not sampled randomly). The number of clutches, approximate size of nest, and size of guardian male was recorded. We also recorded the length, width, associated substrate, color, and development stage of each clutch. Clutch volume was not calculated because of inconsistencies in clutch dimensions. Size of each nest was calculated as the area of a circle, with a radius equal to the distance from the approximate center of the nest to the farthest clutch. To determine whether they could be combined, data on nest characteristics from Canada and California were compared with the tTEST or, alternatively, the Mann-Whitney test when variances associated with mean nest size were unequal.

The Canadian site was near Bamfield Marine Station (BMS), on the west coast of Vancouver Island, British Columbia. The subtidal environment consisted of granite ledges and channels surrounded by sand. The predominant benthic flora was encrusting coralline algae. Kelp greenling were abundant in this habitat, establishing and defending spawning nests on the rocky substrate. Gonads of 13 females, taken from Aguilar Point near BMS (48°50.75′N, 125°08′03″W), were excised and preserved in 70% ethanol. Liver tissue from these 13 adults was preserved at −20°C for mtDNA sequence analysis. In November 1993, 49 clutches from 11 nests were sampled near Taylor Rock, a small islet located approximately 3 km from BMS (48°49′6″N, 125°11′8″W).

The subtidal habitat in central California sampling sites differed from Canada in that Macrocystis pyrifera was the dominant kelp. The substrate had greater spatial heterogeneity because of the abundance of articulated coralline and fleshy red algae. Kelp greenling were not abundant, and considerably more effort was spent searching for nests in California than in Canada. In January 1994, 34 clutches from nine nests were sampled at three sites off the Monterey Peninsula: four nests from Stillwater Cove, three off Otter Point, and two off Monastery Beach. Seven adults from central California were obtained from sport fishers for mtDNA analysis of liver tissue.

Ovary analysis—Diameters of eggs present in ovaries of adult females were measured with a dissecting microscope and an image-analysis system. Two hundred eggs were measured from each of six subsamples: anterior, middle, and posterior of the right and left ovaries of four fish. This analysis indicated that egg-size classes were not partitioned within ovaries of kelp greenling (data not shown) and that frequency distributions of each subsample were representative of the whole ovary. Approximately 800 eggs were measured from all subsequent ovaries.

Mitochondrial DNA analysis.—DNA was extracted from approximately 100 mg of liver tissue from adults and from one egg in every clutch sampled. Additionally, DNA was extracted and sequenced from each of several eggs in each of six clutches from Canada to confirm homogeneity of clutches. Tissue was powdered in liquid nitrogen and extracted following the protocol of Hoss and Pääbo (1993). A 354-base segment of the mitochondrial D-Loop region was PCR amplified and sequenced using the primers H16498 (Meyer et al., 1990) and Pro-L (ctc cca act ccc aaa gc). Amplifications were performed in a reaction mixture composed of 0.5 μl template DNA preparation, buffer (0.67 M Tris-HCL, pH 8.8, 40 mM MgCl₂, 0.16 M (NH₄)₂SO₄, 0.1 M β-mercaptoethanol, 0.1 mg/ml BSA), 0.2 mM each of dGTP, dATP, dTTP, and dCTP in tetralithium salt solution; pH 7.0, 0.5 mM of each primer, two units Taq DNA polymerase and deionized, sterile water for a final volume of 30 μl. The thermal profile used for amplification began with 2 min at 94 C for complete denaturation of double-stranded DNA followed by 30 cycles of 45 sec at 94°C denaturation, 45 sec at 50°C annealing, and 90 sec at 72°C for extension. Products were visualized by electrophoresis on a 1.5% agarose gel along with and appropriate molecular size marker. Product bands were gel purified, following the protocol of Mukhopadhyay and Roth (1991), and used as sequencing template. Double-stranded sequencing reactions were performed according to manufacturers recommendations from the fmol DNA Sequencing Kit (Promega). Primers were end labeled with γ ATP. Twelve samples were sequenced on both strands with the PCR primers; remaining samples were sequenced with primer H16498. The thermal profile used for the sequencing reaction began with 2 min at 94°C followed by 30 cycles of 40 sec at 94°C denaturation, 40 sec at 50°C annealing, and 60 sec at 72°C for extension. Sequences were analyzed with the program Geneworks 2.1.1 (IntelliGenetics, Mountain View, CA). Homogeneity of mtDNA haplotypes between clutches, adults from Canada and California, and all samples pooled at both sites was tested using the Monte Carlo approach of Roff and Bentzen (1989) with 1000 randomizations.
TABLE 1. DISTRIBUTION OF HAPLOTYPES AND DEVELOPMENTAL STAGES OF CLUTCHES WITHIN NESTS. Developmental stage of each clutch is indicated by column, and haplotypes are indicated by A, B, C, D, or E. Nests with more than one haplotype are denoted by *. Total is number of clutches in each nest.

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RESULTS

Nest descriptions.—In Canada, 14 nests were described, at depths ranging from 5–16 m. Nests contained 1–8 clutches (mean ± SE = 4.2 ± 0.5). The size of each nest ranged from 0.5–7.0 m², with an average nest encompassing an area of 2.69 m² (SE = 0.74, n = 11). The average standard length (SL) of guardian males was 31 cm (SE = 1.9, n = 10, range 23–43 cm). In California, nine nests were described at depths of 8–17 m. Nests contained 2–11 clutches (mean ± SE = 4.4 ± 0.9). Size of nests was 0.01–2 m², with an average nest area of 0.92 m² (SE = 0.28, n = 7). The average guardian male was 31.0 cm SL (SE = 1.5, n = 8, range 26–40 cm). There were no significant differences between Canada and California for number of clutches (t = 0.058, P = 0.81), length of guardian male (t = 0.2, P = 0.82), and area of nest (U = 56, P > 0.10).

In Canada, 50% of all clutches sampled occurred on rock or in rock crevices; 30% were associated with rock and a biological substrate such as a barnacle test, scallop shell, worm tubes, freshy red algae, *Heteropora*, or *Balanophylia*; and 20% were associated solely with a biological substrate such as a barnacle test, scallop shell, articulated coralline (*Calliasthrhon*), or freshy red algae. Length and width dimensions for 51 clutches indicated an average surface area of 1788 mm² (SE = 131, n = 51). In California, only 20% of clutches sampled occurred on rock or in rock crevices; 68% were associated with rock and a biological substrate such as *Het- eropora*, *Calliasthrhon*, worm tubes, or *Phidolopora*; and 12% were found in *Heteropora* or worm tubes. Average surface area of clutches was 2670 mm² (SE = 365, n = 34).

Developmental stages of clutches within nests were not distributed evenly. Ninety percent of nests sampled in Canada and California contained all or several (≥ 3) clutches at the same stage of embryonic development (Table 1). New clutches were grey, pink, or purple and were uniform in color. More developed clutches were brown or brownish-grey, exhibited a brown dia- tom epiflora, and had several white opaque eggs throughout. Mature clutches were metallic silver, because of pigmentation of larvae, and were uniform in color. Most of these larvae hatched in the sample bottles during transport.

Egg size frequencies.—The presence of several, distinct egg-size classes, including both hydrated and nonhydrated eggs, indicates the ability to spawn multiple batches (Conover, 1985). Ova- ries of 13 females contained three distinct egg-size classes with diameters in the categories 0.1–
0.3 mm, 0.7-1.0 mm, and a transitional size class, approaching maturity, from the 1 mm to the 2.5 mm size class (Fig. 1). Eggs ranging from 2.2-2.5 mm in diameter were hydrated and ready to be spawned. Ovaries with this mature size class of eggs had a transitional size class of small eggs (i.e., 1.1-1.5 mm); whereas ovaries lacking the mature size class had a transitional size class of larger eggs (diameter = 1.5-2.3 mm). These data, from ovaries sampled on different dates in November 1993, indicate that female kelp greenling are multibatch spawners and that they evidently maintain three distinct size classes of eggs during spawning season (Fig. 1).

There were no clear patterns of partitioning among subsamples of ovaries (data not shown). However, in one sample, there was a distinct group of eggs congregated posteriorly, in the center of the ovary, adjacent to the urogenital pore. These eggs were 2.5 mm in diameter, hydrated, and ready to be spawned. Apparently, these mature oocytes were ovulated from follicles throughout the ovary and migrated freely through the ovarian lumen to congregate near the urogenital pore before spawning.

Each clutch of eggs is spawned in a cohesive stroma. Adhesion to the substrate is aided by the viscid stroma and by the spawn mass conforming to the substrate structure while it is laid by the female in a semifluid state.

Mitochondrial DNA haplotypes in kelp greenling nests.—In Canadian samples, two of 350 bases of the 5' D-Loop region of kelp greenling mtDNA were polymorphic. Transitions between T and C were observed at positions 186 and 258 (Fig. 2). All four possible combinations of these transitions were found and described as haplotypes A(TC), B(CC), C(CT), and D(TT). A length polymorphism, consisting of a 9-base duplication inserted between bases 18 and 19 of haplotype A (Fig. 2), was observed in one clutch from nest 7 and called haplotype E. No variation of mtDNA haplotypes was detected within clutches. Nests with different maternal haplotypes among clutches occurred in three of 11 nests in Canada (Table 1). Sequences of mtDNA from 12 adults sampled off Aguilar Point in Canada revealed 11 to possess haplotype A and one to possess haplotype B (Table 2). There was no significant difference in the frequency of haplotypes of clutches (n = 49) and adults (n = 12) in Canada ($\chi^2 = 1.6181, P = 0.914$).

In samples from California, kelp greenling mtDNA was polymorphic at position 186 only; therefore, only haplotypes A and B were observed (Table 2). Even so, five of nine nests contained clutches with different maternal haplotypes (Table 1). Length polymorphism was not observed in California samples. Sequences of mtDNA from seven adults off California revealed all seven to possess haplotype A (Table 2). Frequency of haplotypes of clutches (n = 34) and adults (n = 7) off California also did not differ significantly ($\chi^2 = 1.4471, P = 0.364$). Finally, although no significant difference in
haplotype distributions between pooled samples from Canada (n = 61) and pooled samples from California (n = 41; χ² = 5.9906, P = 0.138) was found, the ability to detect population structure was low because of the overwhelming predominance of the common haplotype A.

**DISCUSSION**

**Spatial and temporal patterns of reproduction in kelp greenling nests.**—Greenling nests occurred at a range of depths in which suitable habitat, consisting of structurally complex rocky ledges and channels, was available. Kelp greenling appear to be opportunistic when selecting nest and spawning sites, utilizing the heterogeneity of the rocky substrate and biological structures for shelter. The spatial distribution of clutches in nests may indicate the number of clutches and area that a male can guard effectively. The average kelp greenling nest in Canada and California encompassed a 2.5-m² area (SE = 0.5, n = 18), had 4.0 clutches (SE = 0.4, n = 29) up to 1.3 m apart, and was guarded by a male of 31 cm standard length (SE = 1.17, n = 18). There was no evident correlation between length of the guardian male and size of the nest or number of clutches. In *Hexagrammos agrammus*, however, only males over one year of age (corresponding to 119 mm SL) guarded nests (Kurita et al., 1995). Maximum size or number of clutches for kelp greenling nests cannot be inferred from data presented here because observations were made only during a one-month window in the reproductive season. Nest characteristics are likely to change throughout the reproductive season as clutches mature and new clutches are laid. Although duration of spawning for kelp greenling is not known, the reproductive season for two other hexagrammids (*Pleuragrammus azorus* and *H. agrammus*) spans a period of three months (Gorbunova, 1970; Kurita et al., 1995).

**Size frequencies of eggs within ovaries suggested each female can contribute a minimum of three clutches of eggs per spawning season. Similarly, other hexagrammids appear to spawn**
multiple batches, as indicated by empty follicles (Kurita et al., 1995) and the occurrence of three distinct size classes of eggs in ovaries (Gorbunova, 1970; Kurita et al., 1995). In kelp greenling, each clutch within a nest was assumed to be deposited by a single female because clutches were discrete and bound together by a hardened cohesive stroma; all eggs within a clutch were at the same stage of embryonic development; and no differences were found in mtDNA sequences from several eggs from each of six clutches.

Presence of a mature egg-size class, growth rate of the largest egg-size class in ovaries, and the clumped distribution of developmental stages of eggs suggest synchronicity of spawning in kelp greenling. Females sampled between 6 and 19 November had mature eggs. Ovaries sampled on or after 25 November lacked mature eggs but had a transitional size class approaching maturity. These data suggest that a spawning event may have occurred between 19 and 25 November and that female kelp greenling can hold mature eggs until suitable conditions occur.

Females can maximize their reproductive potential and fitness by selecting nests that result in reduced offspring mortality. Female selection of males and nest characteristics, including number and age of clutches present in the nest, is associated with increased offspring survival (Gross and Sargent, 1985). In three species of pomacentrids and threespine stickleback, females select nests containing early stage clutches (Sikkel, 1988; Jamieson and Colgan, 1989) and selectively deposit clutches in proximity to early stage eggs within the nest (Knapp et al., 1995). Kelp greenling may exhibit a similar pattern based on the temporal schedule suggested by the largest egg-size class within ovaries and our observation that kelp greenling nests contained all or several clutches at the same stage of development. Several causes for this behavior have been proposed: (1) a male guarding a nest with existing clutches may already have proven its abilities for parental care (Ridley and Rechten, 1981); (2) an overall dilution effect reduces risk of predation to eggs (Ridley and Rechten, 1981; DeMartini, 1987); (3) male parental care may increase with increasing brood size (Coleman et al., 1985); (4) females may have a search image for early stage eggs (Knapp et al., 1995); and (5) males may give preferential care to early stage clutches (Knapp et al., 1995). In painted greenling, Oxylebius jactus, somatic condition of the male and quality of care declined in proportion to time spent guarding (DeMartini, 1987). Female reproductive success was greatest for clutches contributed earliest to a nest. DeMartini (1987) found that male painted greenling partially cannibalized their brood to compensate for lost energy resulting from guarding from conspecifics and other fishes. However, polygamous mating in painted greenling, resulting in nests with more clutches, remained advantageous for both males and females because there was a greater dilution of predation (DeMartini, 1987). Indeed, the guts of several female kelp greenling sampled contained kelp greenling eggs (males were not sampled).

Different maternal contributors to kelp greenling nests.—Although it is possible that one female could contribute several clutches to a nest if she partitions one clutch into several or contributes clutches to the same nest on different occasions, at least 27% of nests sampled off Canada and 55% of nests sampled off California contained clutches from more than one mother. In H. agracnum, females evidently do not partition clutches because the number of mature, ovulated eggs in ovaries was equivalent to the number of fertilized eggs in clutches (Kurita et al., 1995). If we assume female kelp greenling do not partition clutches, the clumped distribution of developmental stages of clutches within nests also suggests multiple maternal contributors. Because female kelp greenling are multibatch spawners, they could maximize reproductive potential by distributing clutches among several nests. Thus, it would be beneficial for females to deposit clutches with more than one male if it resulted in reduced mortality of young due to stochastic events or if one male could not give adequate care to all clutches. Polyandry also may increase genetic robustness of a female’s progeny and the population.

Both males and females contribute to spatial and temporal patterns of clutch distribution in kelp greenling nests. Males establish nest sites and compete for clutches. Paternity of eggs within clutches and clutches within nests of kelp greenling should be evaluated. In threespine stickleback, Rico et al. (1992) found that 23 of 170 fry, from 17 nests, had been fertilized by a male other than the guardian. Male bluegill, Lepomis macrochirus, guard colonies of eggs with paternity as low as 41% resulting from sneak spawning (Philipp and Gross, 1994). Sneak spawning has been observed in kelp greenling and other hexagrammids (Z. Kamamoto, pers. comm.). Paternity of eggs should be quantified to better understand benefits of the male’s reproductive effort and to evaluate whether sneak spawning results in reduced care and increased
mortality of young. Females select spawning sites and choose nests with desirable males and nest characteristics. It would be interesting to investigate further the possibility of synchrony spawning, spawning frequency, and environmental or ethological cues associated with spawning in kelp greenling. Definition of the reproductive season is also necessary to evaluate fecundity of this species.

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LITERATURE CITED


