THE REPRODUCTIVE BIOLOGY OF KELP GREENLING HEXAGRAMMOS DECAGRAMMUS

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CERTIFICATION OF APPROVAL

I certify that I have read The Reproductive Biology of Kelp Greenling, *Hexagrammos decagrammus* by Karen Dorine Crow, and that in my opinion this work meets the criteria for approving a thesis submitted in partial fulfillment of the requirements for the degree: Master of Science in Marine Science at San Francisco State University.

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Male kelp greenling (*Hexagrammos decagrammus*, Hexagrammidae) guard nests with several discrete clutches of eggs. Twentythree nests were described from British Columbia and central California. The average nest had 4 clutches associated with rock or rock and a biological substrate, encompassed an area of 1.7m^2 and was guarded by a male 31cm 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. DNA of 107 individuals was sequenced from 83 clutches from 20 nests from British Columbia and central California. Different maternal contributors were found among clutches in 27% of the nests from British Columbia and 55% of the nests from California, based on mtDNA haplotypes.

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Introduction

Parental care in fishes can be costly yet it increases survivorship of the young and occurs in 21% of teleost families (Gross & Sargent 1985). Because greatest mortality occurs during embryonic and larval stages of fishes, egg guarding has the potential to greatly increase reproductive success (Balon 1975). Guarding is the most common form of care and is usually given by the male (Potts 1984; Gross & Sargent 1985). Males have the potential to strongly influence egg survival (Trivers 1972). Parental care and larval development of demersal eggs are affected by predation and oxygen availability (Balon 1975; Potts 1984). Males can reduce predation by defending eggs and fan eggs to increase oxygenation. Nests often are established on exposed rocky surfaces with increased water circulation and complex substrates to minimize visibility by predators (Potts 1984).

The reproductive strategy of kelp greenling (*Hexagrammos decagrammus*, Hexagrammidae) is exemplary of the most common form of parental care exhibited by fishes. Like most members of its family (Gorbunova 1970), the kelp greenling produces demersal eggs which are attached to a substrate and guarded by the male parent. Ranging from the Aleutian Islands to La Jolla, California (Miller & Lea 1972), kelp greenling utilize rocky reefs and kelp forests for habitat and reproduction. Kelp greenling are sexually dimorphic. In fact, the genders were considered different species for over 70 years. Sexual

dimorphism usually indicates differentiation in sexual roles in nearshore demersal egg layers (Potts 1984). In kelp greenling, the 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 defended by a male containing clutches. Clutches are golf ball to tennis ball sized masses of benthic adhesive eggs. In Puget Sound, male kelp greenling guarded one to ten clutches per nest (mean=4.5) which were adjacent to 1.9 m apart (DeMartini 1986). In kelp greenling nests, the guardian male is the putative father, but sneak spawning (i.e. when a male, other than the guardian, enters the nest and fertilizes eggs) also has been observed (J. Heine personal communication).

Much less is known about the reproductive biology of female kelp greenling. Spawned eggs are 2.2-2.5 mm in diameter (mean=2.3 mm) and in Puget Sound clutches consisted of an average of 4,340 eggs (SE=311; DeMartini 1986). Females may be batch spawners, producing several clutches of eggs per spawning season. If so, it is not known if 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). The purpose of this study was to describe the spatial and temporal patterns of maternal contributions of kelp greenling nests. I described and compared nests from 2 distinct parts of the kelp greenling range, determined whether females are

batch spawners capable of contributing several clutches per spawning season, and determined maternal differences among clutches within nests by sequencing and comparing mitochondrial DNA (mtDNA).

Inherited maternally, mitochondrial DNA is an ideal marker for determining maternity of clutches within nests. Differences in mtDNA haplotypes among clutches in a nest would indicate clutches were contributed by different females. Similar haplotypes, however, would not imply a single female contributor since different females could possess identical haplotypes for the region of mtDNA analyzed.

Materials and Methods

Sampling

In the field I characterized kelp greenling nests, collected adult females for ovary and mtDNA analysis, and sampled egg clutches within nests for mtDNA analysis. Field work was conducted, using scuba off Vancouver Island, Canada and near Monterey, California. To sample nests, I first identified guarding males on the basis of their behavior in the presence of a diver. The area within a 1 m radius around a guardian male was searched intensively. When a clutch was identified, it was flagged and the area within 1 m of it was searched intensively until all clutches within the nest were identified. A portion of each clutch was taken and stored at -20°C

for mtDNA analysis. Every nest discovered was sampled (i.e. nests were not sampled randomly). The number of clutches, approximate size of nest, size of guardian male, clutch substrate, color and developmental stage of each clutch was recorded. Size of nests 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 if data could be combined, data on nest characteristics from the Canada and California sites were compared using a t-test, or Mann-Whitney U when the assumptions for parametric statistics were not met.

The Canadian site was located near Bamfield Marine Station (BMS), on the west coast of Vancouver Island, British Columbia, Canada. The substrate consisted of granite ledges and channels surrounded by sand. The predominant benthic flora was encrusting coralline algae with minor amounts of fleshy red algae. *Macrocystis integrifolia* and *Nereocystis sp.* were common at this site in depths <3 m. Grazers, such as urchins (*Stronglyocentrotus purpuratus* and *S. franciscanus*) and northern abalone (*Haliotis kamtschatkana*), were abundant below this depth. The kelp greenling was 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 and 125°08.03'W), were excised and preserved in 70% ethanol. Liver tissue from these thirteen adults was preserved at -20°C for mtDNA sequence analysis. In November 1993, 48 clutches

from 11 nests were sampled from Taylor Rock, a small islet located approximately 3 km from BMS (48°49.6'N and 125°11.8'W).

The substrate in central California sampling sites differed from Canada in that *Macrocystis pyrifera* was the dominant kelp in this region and the substrate had greater spatial heterogeneity due to the abundance of articulated corallinè and fleshy red algae. Kelp greenling were not abundant and considerably more effort was spent searching in California compared with Canada. In January 1994, 35 clutches from 9 nests were sampled at 3 sites off the Monterey Peninsula: 4 nests from Stillwater Cove, 3 off Otter Point, and 2 off Monastery Beach. Liver tissue for DNA analysis from seven adults was obtained from central California sport fishermen.

Ovary analysis

The diameters of eggs present in the ovaries of adult females were measured using 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 ovary of four fish. This analysis indicated that egg size classes were not partitioned within the ovaries of kelp greenling (i.e. frequency distributions of each subsample were representative of the whole ovary). Therefore, approximately 800 eggs were measured from all subsequent ovaries.

mtDNA Analysis

DNA was extracted from approximately 100 mg of liver tissue from adults and from one egg from every clutch sampled. Additionally, DNA was extracted and sequenced from each of several eggs from six clutches, and from two tissues (liver and gonad) from two adult fish, to rule out the possibility of heteroplasmic variation within clutches or individuals. Tissue was powdered in liquid nitrogen and extracted following the protocol of Hoss and Paabo (1993). A 354 base segment of the mitochondrial D-Loop region was PCR amplified and sequenced with the following primers: H16498 (Meyer *et al.* 1990) and Pro-L (Palumbi *et al.* 1991). Amplifications were performed in a reaction mixture composed of 0.5 μl template DNA preparation, buffer (0.67M Tris-HCl, pH 8.8, 40 mM $MgCl_2$, 0.16 M $(NH_4)_2SO_4$, 0.1 M *B*-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, 2 units *Taq* polymerase and deionized, sterile water for a final volume of 30μl. 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 s at 94°C denaturation, 45 s at 50°C annealing, and 90 s at 72°C for extension. Products were visualized by electrophoresis on a 1.5% agarose gel along with an appropriate size marker. Product bands were gel purified and used as a sequencing template. Double strand sequencing reactions were performed according to manufacturers recommendations from the

fmol™ DNA Sequencing Kit (Promega). Primers were end labeled with $ATP^{33}\gamma$. Twelve samples were sequenced on both strands using the PCR primers, the remaining samples were sequenced using primer H16498. The thermal profile used for the sequencing reaction began with 2 min. at 94°C followed by 30 cycles of 40 s at 94°C denaturation, 40 s at 50°C annealing, and 60 s at 72°C for extension. Sequences were analyzed with the program Geneworks 2.1.1 (IntelliGenetics, Mountain View, CA).

Results

Nest descriptions

In Canada, 14 nests were described, at depths ranging from 5 to 16m. Nests contained 1 to 8 clutches, averaging 4.2 (SE=0.5). The size of each nest ranged from 0.5 to $7m^2$ with an average nest encompassing an area of 2.69 m^2 (SE=0.74, n=11). The average standard length (SL) of the guardian male was 31 cm (SE=1.87, n=10) but ranged from 23 to 43 cm. In California, nine nests were described at depths of 8-17m. Nests contained 2 to 11 clutches with an average of 4.4 (SE=0.91) Size of nests was 0.01 m^2 to 2 m^2, with an average nest area of 0.92 m^2 (SE=0.28, n=7). The average guardian male was 31 cm SL (SE=1.46, n=8) but ranged from 26 to 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 ($f=12$, $P<0.01$; $U=56$, $0.10<P<0.20$).

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, fleshy red algae, *Heteropora*, or *Balanophyllia*.; and 20% were associated solely with a biological substrate such as a barnacle test, scallop shell, articulated coralline (*Calliarthron*), or fleshy red algae. 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 *Heteropora, Calliarthron*, worm tubes, or *Phidolopora*; and 12% were found in *Heteropora* or worm tubes.

The developmental stages of clutches within nests had a clumped distribution. Ninety percent of nests sampled in Canada and California contained all or several (3 or more) clutches at the same stage of embryonic development (Table 1). New clutches were grey, pink, or purple and uniform in color. More developed clutches were brown or brownish-grey, exhibited a brown diatom epiflora and had several white opaque eggs throughout the clutch. Mature clutches were silver, due to pigmentation of the larvae, and were uniform in color. Most of these larvae hatched in the sample bottle during transport.

Egg size frequencies

The presence of several distinct size classes of eggs including both hydrated and non-hydrated eggs indicates the ability to spawn multiple batches (Conover 1985; DeMartini 1987a). The ovaries of 13 females contained three distinct size classes of eggs 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 2.3 size class (Fig. 1). Eggs ranging from 2.2 to 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 females maintain three distinct size classes of eggs during spawning season (Fig. 1) and that female kelp greenling are multibatch spawners.

There were no clear patterns of partitioning among subsamples of these ovaries (Fig. 2). Subsamples (n=200) were representative of the combined data (Fig. 2c). In one sample there was a distinct size class of eggs congregated posteriorly, in the center of the ovary, adjacent to the urogenital pore (Fig. 2d). These eggs were 2.3 mm in diameter, hydrated, and ready to be spawned. These mature oocytes were apparently 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 the spawn mass conforming to the substrate structure while it is laid by the female in a semi-fluid state.

mtDNA haplotypes in kelp greenling nests

In Canadian samples, 2 of 350 bases of the 5' D-Loop region of kelp greenling mtDNA were polymorphic. Positions 186 and 258 were both transitions between T and C. All four possible combinations of these transitions were observed and described as Haplotypes A (TC), B (CC), C (CT), and D (TT); (Fig. 3). A length polymorphism, consisting of a 19 base duplication inserted between bases 18 and 19 of haplotype A (Fig. 3), was observed in one clutch from nest 7, which I called haplotype E. Clutches with different maternal haplotypes occurred in three of eleven nests in Canada indicating different mothers contributed clutches to these nests (Table 2).

DNA extracted from 12 adults (liver tissue) from Aguilar Point in Canada was PCR amplified and sequenced. Eleven were haplotype A and one was haplotype B. The frequency of haplotypes between clutches and adults in Canada were not different significantly (χ^2=1.747, 0.5>P>0.25, rare haplotypes C, D, and E were pooled; Table 3).

In California samples, kelp greenling mtDNA was polymorphic at position 186 only. Therefore only 2 haplotypes were detectable:

A (TC) and B (CC); (Table 2). Even so, five of nine nests contained clutches with different maternal haplotypes (Table 2). Length polymorphism was not observed in the California samples. DNA extracted from liver tissue of seven adults off California also was analyzed. All seven were haplotype A. Frequency of haplotypes between clutches and adults off California were not different significantly (χ^2=2.492, 0.25>P>0.1; Table 3). The difference in haplotype distributions between Canada and California (i.e. two polymorphisms vs. one polymorphism) may indicate population structure.

Discussion

Spatial and temporal patterns of reproduction in kelp greenling nests

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, whereas females select spawning sites and choose nests with desirable males and nest characteristics. Kelp greenling are opportunistic when selecting nest and spawning sites, utilizing the heterogeneity of the rocky substrate and biological structures for shelter. Greenling nests occurred at a range of depths, wherever suitable habitat, consisting of structurally complex rocky ledges and channels, was available. The spatial distribution of clutches in nests may indicate the number of clutches and area that

can be guarded effectively from predators and competing males. The average kelp greenling nest in Canada and California encompassed a 2.35 m^2 area (SE=0.5, n=18), had four clutches (SE=0.45, n=23) ranging from 0.01 to 1.3 m apart, and was guarded by a male 31 cm in length (SL, SE=1.17, n=18). There was no correlation between the length of the guardian male and the size of the nest or number of clutches for these data. However in *Hexagrammos agrammus*, only males greater than one year of age (corresponding to 119 cm SL) guarded nests (Kurita *et al.* 1995). The maximum size or number of clutches for kelp greenling nests cannot be inferred from the data presented here because these data only characterize a one month window of the reproductive season for kelp greenling. Nest characteristics are likely to change throughout the reproductive season as clutches mature and new clutches are laid. The reproductive season for two other hexagrammids (*Pleurogrammos azonus* and *Hexagrammos agrammus*) has been determined to span a period of 3 months (Gorbunova 1970; Kurita *et al.* 1995).

Although it is not known precisely when spawning season begins and ends for kelp greenling, the size frequency of eggs suggests that each female can contribute a minimum of three clutches of eggs per spawning season (Fig. 1). Similarly, other hexagrammids have been determined to spawn multiple batches, indicated by the presence of empty follicles (Kurita *et al.* 1995) and the presence of three distinct size classes of eggs in the ovaries

(Gorbunova 1970; Kurita *et al.* 1995). In addition, each clutch within a nest was assumed to be deposited by one female because: 1) clutches were discrete and bound together by a hardened cohesive stroma, 2) all eggs within a clutch were at the same stage of embryonic development, and 3) DNA sequences from several eggs from each of six clutches had identical haplotypes.

The presence of a mature size class of eggs and the growth rate of the largest size class in the ovaries of kelp greenlings may suggest periodicity in spawning. Females sampled between the 6th and 19th of November had a mature size class of eggs. Ovaries sampled on or after the 25th lacked the mature size class, but had a transitional size class approaching maturity. These data suggest that a spawning event occurred between the 19th and 25th of November, and that female kelp greenling may hold a mature size class of eggs until suitable conditions occur for spawning.

The female's reproductive potential is limited by the ability to produce eggs (Blumer 1979). According to Blumer (1979), it is beneficial for females to desert the nest following oviposition to resume feeding and produce more eggs . There is no evolutionary advantage in both parents caring for eggs or defending territory in demersal egg layers (Potts 1984). Female selection for male characteristics and nest characteristics, including the number and age of clutches present in the nest, is associated with reduced offspring mortality and increased female fitness (Gross & Sargent 1985; Knapp *et al.* 1995). Selection of spawning sites then, by females laying

demersal eggs, should increase offspring survival (Gross & Sargent 1985). Among nest guarding fishes, females of three species of pomacentrids and threespine sticklebacks select nests containing early stage clutches (Sikkel 1988; Jamieson & Colgan 1989) and selectively deposit clutches in close proximity to early stage eggs within the nest (Knapp *et al.* 1995). Kelp greenling may exhibit a similar pattern. Most kelp greenling nests contained all or several clutches at the same stage of development (Table 1). Several causes for this behavior have been suggested: 1) a male guarding a nest with existing clutches may already have proven its attractiveness and abilities for parental care (Ridley & Rechten 1981); 2) an overall dilution effect, reducing the risk of predation to eggs (Ridley & Rechten 1981); 3) male parental care may increase with increasing brood size (Coleman *et al.* 1985); 4) males may give preferential care to early stage clutches (Knapp *et al.* 1995), and 5) females may have a search image for early stage eggs (Knapp *et al.* 1995). If kelp greenling respond to this behavioral cue then synchronous spawning events may occur.

Multiple spawning is important to males because a male's reproductive output is a function of the number of clutches he obtains (Blumer 1979). Guarding multiple clutches maximizes the male's genetic contribution to the population and territorialism exhibited by nest guarding fish increases the probability of fertilization (Potts 1984). Defending a territory may have little reproductive cost to males in lost matings, future fertility, or future

reproductive success (Gross & Sargent 1985). Additional factors, however, such as male condition, may affect the mating success of males. In the painted greenling, *Oxylebius pictus*, the somatic condition of the male and the quality of care declined in proportion to the time spent guarding (DeMartini 1987b). Among nest guarders, the mating success of the male is strongly dependent on the female's choice of mates (Rico *et al.* 1992). The male's reproductive potential is limited by the ability to attract females, and males that remain at the oviposition site may be more successful in obtaining mates and fostering the survival of the offspring (Blumer 1979).

Different maternal contributors to kelp greenling nests

Although it is possible that one female could contribute several clutches to a nest, at least 27% of the nests off Canada and 55% of the nests off California contained clutches from different mothers. Clutches with similar haplotypes could be deposited by the same mother if mature eggs were partitioned into several clutches or if a female contributed clutches to the same nest on different occasions within a spawning season. Because female kelp greenling are multi-batch spawners, they could maximize their reproductive potential by distributing clutches among several nests. It may be beneficial for females to deposit clutches with more than one male if it results in reduced mortality of their young due to stochastic events or if the male cannot give adequate care to all clutches. Blumer (1979)

suggested that an increased number of clutches within a nest may be more subject to predation. Also, polyandry may increase the genetic robustness of a female's progeny, and the population, by increasing heterozygosity.

The possibility that differences observed among clutches within a nest could be due to heteroplasmic mothers laying clutches with different haplotypes should be considered. Heteroplasmy is the presence of multiple forms of mtDNA within an individual (Buroker et. al. 1990). Heteroplasmy has been reported for several organisms across taxa including the following fishes: bowfin, *Amia calva* (Bermingham *et al.* 1986); American shad, *Alosa sapidissima* (Bentzen *et al.* 1988); white sturgeon, *Acipenser transmontanus* (Buroker *et al.* 1990); Atlantic cod, *Gadus morhua* (Arnason & Rand 1992); and the gulf sturgeon, *Acipenser oxyrhynchus desotoi* (Miracle & Campton 1995). Heteroplasmy is usually characterized by length polymorphisms due to variable numbers of tandem repeats (Brown *et al.* 1992), whereas heteroplasmy due to nucleotide substitution is rare (Hale & Singh 1986). For example, white sturgeon and cod, had length variation heteroplasmy, but none due to nucleotide substitution (Brown *et al.* 1992; Arnason & Rand 1992). The length variation in haplotype E is typical of heteroplasmy but the transitions seen in haplotypes A-D are not.

To determine if differences in clutch haplotypes were due to heteroplasmy, DNA from several eggs was sequenced from six clutches in Canada: three eggs from each of four clutches in nest BC 7

including the clutches with haplotypes C and E, five eggs from one clutch in nest BC11, and 10 eggs from one clutch in nest BC 9. In addition, individual heteroplasmicity was tested by extracting DNA from liver and ovary tissue and sequenced in two adults from Canada, including the adult with haplotype B. Indeed, Casane *et al.* (1994) found that the distribution of haplotypes due to length variation heteroplasmy varied in liver and gonad in rabbit. In no case was heteroplasmy detected among eggs from the same clutch, or from different tissues within the same individual. Based on these results, differences in haplotypes were attributed to differences in maternal contributors.

Molecular mechanisms indicated by the D-loop sequence of kelp greenling

Heritable mutations in mtDNA originate in heteroplasmic individuals (Arnason & Rand 1992), and are conserved through maternal lineages (Brown *et al.* 1992). Interestingly, the length polymorphism observed in haplotype E, and the repetitive unit from which it is formed, are capable of forming secondary structures (Fig. 4). The presence of secondary structure has been suggested as the mechanism causing length polymorphism heteroplasmy in cod, white sturgeon, and gulf sturgeon (Buroker *et al.* 1990; Arnason & Rand 1992; Miracle & Campton 1995). Buroker's model of this mechanism would explain the insertion and deletion of the repeat unit observed

in haplotype E. However, the occurrence of a terminal associated sequence (TAS) in the repeat unit, necessary for D-loop termination, may explain why the repeat sequence is conserved (Brown *et al.* 1992). Multiple TAS segments in tandem repeats could also cause heteroplasmy for length variation (Buroker *et al.* 1990). The potential for secondary structure and the presence of two TAS segments seen in haplotype E is typical of heteroplasmy but again, sequences from several eggs within that clutch did not vary. This suggests that a maternal ancestor of the kelp greenling with haplotype E exhibited length polymorphism heteroplasmy and it would be interesting to investigate the existence of heteroplasmy in modern kelp greenling.

Conclusions and suggestions for future study

Both males and females contribute to nest structure in kelp greenling. Males select nest sites and may compete for clutches until the reduction in male fitness, from inability to guard clutches adequately, outweighs the benefit of fertilizing more clutches. Kelp greenling nest characteristics describing the limits of a male's reproductive potential could not be determined, however, future studies should characterize kelp greenling nests throughout the reproductive period. Sneak spawning has been observed in kelp greenling and other hexagrammids (Dr. Kanamoto, personal communication). In the

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. The male bluegill, *Lepomis macrochirus*, guards colonies of eggs with paternity as low as 41% due to sneak spawning (Philipp & Gross 1994). Paternity of eggs in kelp greenling nests should be quantified to better understand the benefits of the male's reproductive effort and to evaluate if sneak spawning results in reduced care, therefore, increased mortality of the young.

Female kelp greenling may benefit from investing their reproductive effort with several different males to reduce offspring mortality. Females are most likely to select the most appropriate nest for each clutch individually, throughout spawning season. There may be synchronicity in spawning events for kelp greenling that is cued by the presence of early stage clutches within nests. It is also possible that females may be responding to some external or environmental cue resulting in periodicity as well. The possibility of periodicity in spawning in kelp greenlings should be further investigated. Several females contribute clutches to kelp greenling nests based on differences in maternal haplotypes and the fact that all or several clutches were at similar stages of development. Each clutch of eggs is deposited by one female, but the number of eggs per size class within ovaries clutch should be compared to the number of eggs within clutches to determine if females

partition one mature size class of eggs into several clutches. In *Hexagrammos agrammus*, the number of mature, ovulated eggs in the ovaries was equivalent to the number of fertilized eggs in clutches (Kurita *et al.* 1995). Finally, the reproductive potential of females would be more clearly understood if the spawning period for kelp greenling were defined and fecundity was determined.

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TABLE 1 Distribution of developmental stages of clutches within nests. The total number of clutches in each nest is shown. The developmental stage of each clutch within the nest is indicated by an X in the appropriate column. New clutches were grey, pink or purple, later stage clutches exhibited a brown diatom epiflora, and mature clutches were metallic silver.

		Clutch Stages				
	NEST	NEW	LATER	MATURE	NO DATA	TOTAL
CANADA	BC 1		XXX			3
	BC 2	XXX				3
	BC 3	X	XXXXX		OO	8
	BC 4	XXX	XXX			6
	BC 5	XX		XXX		5
	BC 6	XXX				3
	BC 7		XXXX	XX		6
	BC 8	XX	X			3
	BC 9			XXXX		4
	BC 10			XXX		3
	BC 11	XXXXX				5
CALIFORNIA	CA 1		X	XXX		4
	CA 2			XXXX		4
	CA 3	X	XX	X		4
	CA 4			XXXX		4
	CA 5			XXX		3
	CA 6		XX			2
	CA 7	X		XXXX		5
	CA 8	XX				2
	CA 9			XXXXXX		6

TABLE 2. Summary of haplotypes in kelp greenling nests. H=number of Haplotypes found in nest. N=number of nests sampled. Heterogeneity reflects the number of nests with different maternal haplotypes among clutches. Canada nests were sampled near Bamfield, British Columbia. California nests were sampled from three sites off the Monterey Peninsula.

				Haplotypes				
	NEST	#CLUTCHES	H	A	B	C	D	E
CANADA	BC 1	3	1	3				
	BC 2	3	1	3				
	BC 3	8	1	8				
	BC 4	6	1	6				
	BC 5	5	3	2		2	1	
	BC 6	3	1	3				
	BC 7	6	3	4		1		1
	BC 8	3	1	3				
	BC 9	4	2	2	2			
	BC 10	3	1	3				
	BC 11	5	1	5				
Total	N=11	49	5	42	2	3	1	1
Heterogeneity	3							
CALIFORNIA	CA 1	4	2	3	1			
	CA 2	4	2	3	1			
	CA 3	4	1	4				
	CA 4	4	2	2	2			
	CA 5	3	1	3				
	CA 6	2	1	2				
	CA 7	5	2	4	1			
	CA 8	2	2	1	1			
	CA 9	6	1	6				
Total	N=9	34	2	28	6			
Heterogeneity	5							

TABLE 3 Frequency of haplotypes among clutches and adults sampled in Canada and California. The genotype for haplotype A was T C for the two polymorphic positions: 186 and 258. The genotypes for haplotypes B-D are indicated. Haplotype E was identical to haplotype A with a length polymorphism (LP). Comparisons of distributions of haplotypes between clutches and adults were not different significantly in Canada (χ^2=1.747, 0.5>P>0.25, rare haplotypes C, D, and E were pooled) and California (χ^2=2.492, 0.25>P>0.1, using Yates correction for v=1).

			HAPLOTYPES				
			A	B	C	D	E
			T C	C C	C T	T T	L P
Population	Sample	n					
Canada	Clutches	49	.857	.041	.061	.02	.02
	Adults	12	.916	.083	0	0	0
California	Clutches	34	.824	.176	0	0	0
	Adults	7	1.0	0	0	0	0

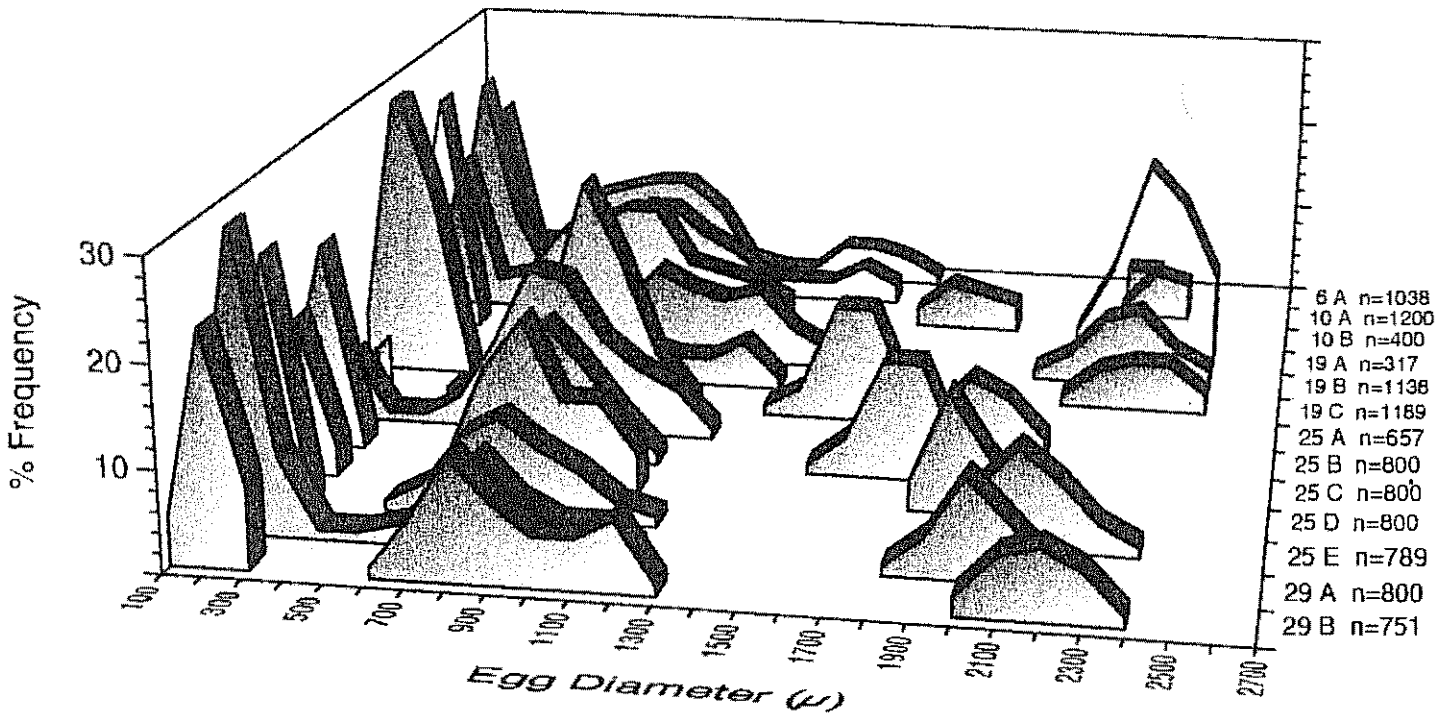
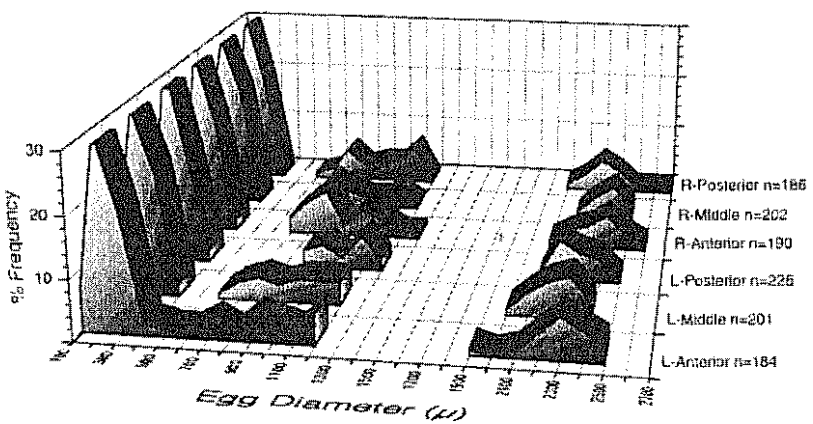
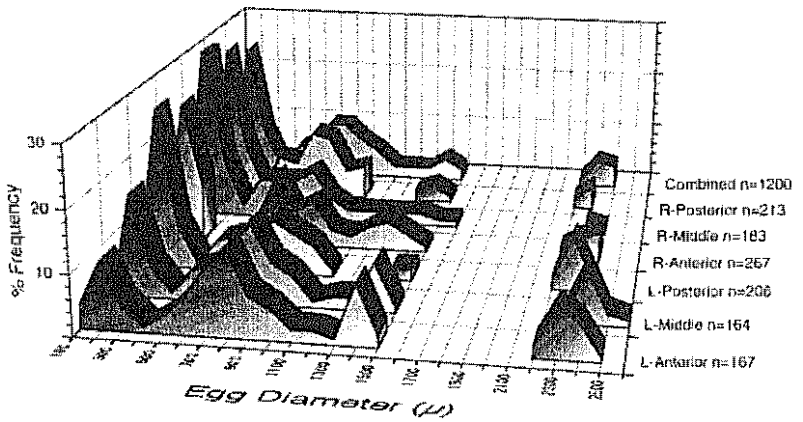


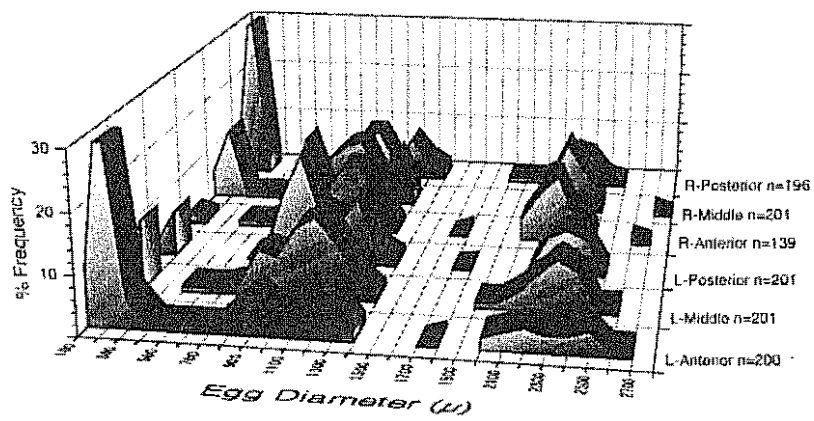
FIGURE 1. Percent frequency distributions of egg diameter in the ovaries of 13 kelp greenling from British Columbia, Canada. Distributions are shown in order of sampling date in November, 1993, with letter designations for multiple fish sampled on the same date (i.e. ovary 10 B was from the second fish sampled on November 10). n is the number of eggs measured from each ovary.



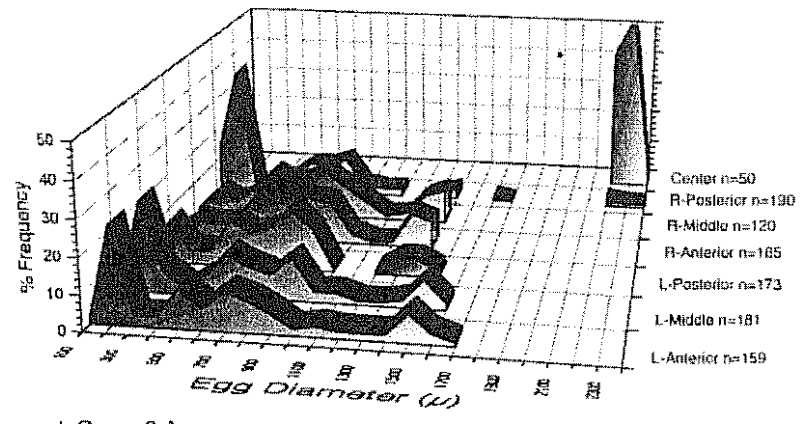
a. Ovary 19 C



c. Ovary 10 A



b. Ovary 19 B



d. Ovary 6 A

FIGURE 2. Egg size frequencies of subsamples from the ovaries of four kelp greenling sampled in November, 1993. Number designation refers to the date sampled and the letter identifies the individual from several sampled on the same date. n is the number of eggs measured from subsamples taken from each fish: anterior, middle and posterior from the right and left ovaries. Fig. 2c. compares combined data with subsamples. Fig. 2d. shows a 7th subsample from the posterior center ovary.

TTAAAAATACATATATGTATT

TACACGAATA	TATGTATTTT	TAATACATAT	ATGTATTAAC	ACCATTAATT	50
TATATTAACC	AAATCAATAG	TATTTCAGTA	CATATATGTA	TAATAACCAT	100
ATATAGGTGT	TAACCATTCA	TACAACAGCA	TAAATTCAAG	ATAAACATTA	150
AGCATGTAGA	GTTTTAGGTA	ACATTTAATT	AAATC[T]AGGA	TAGGCGAAAT	200
TTAAGATCGA	ACACTTCTAC	CCATTTGTTA	AGTTATACGT	TTTCCAACAT	250
TGTATAC[C]AC	AAAACATCCG	ATGTAGTAAG	AACCTACCAT	CAGTTGATTT	300
CTTAATGCCA	ACGGTTATTG	AAGGTGAGGG	ACAAGTATTC	GTGGGGGTTT	350
CACA					354

FIGURE 3. Kelp greenling D-Loop sequence (5'end). Position 1 refers to the first base after the Proline tRNA. Positions 186 and 258 (in boxes) were polymorphic. Haplotype A (T186, C258) is shown. Haplotypes B, C and D correspond to C186, C258; C186,T258; and T186, T258 respectively. Haplotype E had a 19 base pair insertion shown here between positions 18 and 19. Terminal associated sequence (TAS) is underlined.

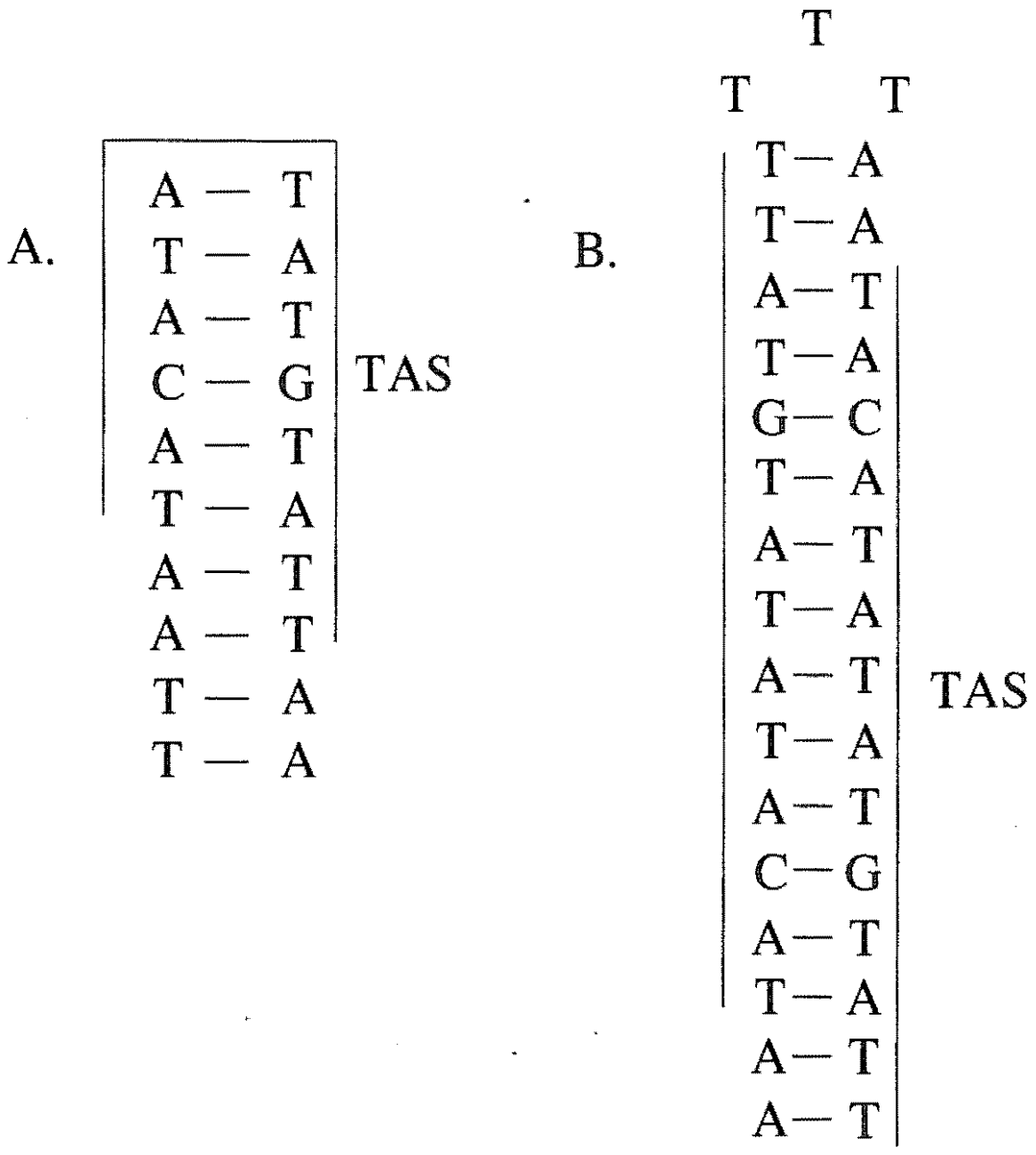


FIGURE 4. Potential secondary structure in kelp greenling D-Loop. A. Positions 20-39 in haplotypes A, B, C, & D. B. Positions 24-58 in haplotype E with length polymorphism. Terminal associated sequence (TAS) is indicated by the line.