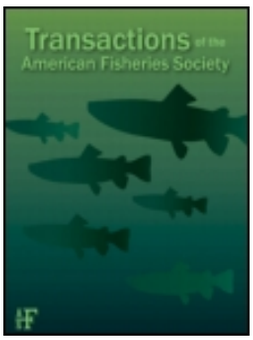

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[Article]

Inheritance Studies Implicate a Genetic Mechanism for Apparent Sex Reversal in Chinook Salmon

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Abstract.—The apparent increase in altered sexual differentiation in Pacific salmon *Oncorhynchus* spp. is a growing concern. Previous studies suggest that incongruence between genetic and phenotypic sex in Chinook salmon *O. tshawytscha* may be a result of altered sexual differentiation through exposure to endocrine-disrupting chemicals (EDCs). Artificial crosses between genotypically normal Chinook salmon, or between genotypically normal males and apparent sex-reversed males (XY females), were performed to test the validity of *OtY1* and growth hormone pseudogene (*GH*Ψ) genetic markers as indicators of phenotypic sex in fall-run Chinook salmon. The offspring produced were genotyped with the Y-chromosome-specific markers and were dissected to observe gonad morphology. The results of the breeding experiments indicate that approximately half of the phenotypic female offspring of XY females have a male genotype according to both Y-chromosome markers. These results refute an earlier hypothesis that phenotypic female Chinook salmon with a male genotype (XY females) are the result of altered sexual differentiation caused by EDC exposure. Instead, either the *OtY1* and *GH*Ψ markers have recombined between the Y and X chromosomes or an autosome, or a mutation has inactivated the sex-determining region of the Y chromosome. In none of the 2,384 Chinook salmon evaluated did the genetic markers contradict one another in a single individual. These results present evidence that both *OtY1* and the *GH*Ψ genetic markers appear to not be diagnostic for sex in fall-run Chinook salmon in the Central Valley of California.

Recent data for Chinook salmon *Oncorhynchus tshawytscha* in the Pacific Northwest (Nagler et al. 2001; Chowen and Nagler 2004) and in California (Williamson and May 2002) suggest that the observed incongruence between genetic and phenotypic sex may be a result of altered sexual differentiation. The potential for this phenomenon to adversely impact salmon population persistence is a growing concern. Phenotypic female Chinook salmon that have a male genotype are fertile and cannot be visually distinguished from genetically normal females (Williamson and May 2002). These fish may be inadvertently incorporated into artificial propagation programs of hatcheries that reside in watersheds and have populations with a high frequency of sex reversal. This could exacerbate population-level genetic effects posed by sex-reversed fish (Williamson and May 2002). Mating between a sex-reversed male (XY female) and a genetically normal male would result in a 3:1 (male : female) genotypic and phenotypic sex ratio in the offspring. One-third of the male offspring produced from this cross would be YY. Subsequent reproduction by YY males produces all-male offspring, regardless of whether they mate with genetically normal males or XY females (Williamson and May 2002).

Petit et al. (1997) identified a number of pollutants that have estrogenic properties in salmonid bioassays. Since exposure of developing salmonids to hormones (Baker et al. 1988; Chevassus et al. 1988; Devlin et al. 1994a) or pollutants (Jobling et al. 1998; Larsson et al. 2000; Afonso et al. 2002) can alter gonadal differentiation (sex-reversed fish have been observed in watersheds heavily impacted by pollution), hypotheses regarding Chinook salmon with altered sexual differentiation caused by exposure to endocrine disrupting chemicals (EDCs) have been put forward by previous researchers (Nagler et al. 2001; Williamson and May 2002). However, other possibilities exist. Nagler et al. (2001) suggested that translocation of a

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region of the Y chromosome containing the *OtY1* sequence to another chromosome may have occurred. Similarly, Chowen and Nagler (2004) suggested that female Chinook salmon that test positive for *OtY1* are not phenotypically sex-reversed males but are, in fact, genetic females, and that the observed incongruence between sexual genotype and phenotype is evidence of past genetic rearrangement involving the Y chromosome. Water temperature fluctuations during early development have been hypothesized as another possible cause of altered sexual differentiation. However, Nagler et al. (2003) showed no significant sex ratio differences in spring-run Chinook salmon as a result of daily temperature changes that occur during the embryonic period, which is the time when the gonad differentiates sexually.

The organization of *OtY1* on the Y chromosome increases its chance of being involved in a genetic rearrangement. The *OtY1* male sex-specific fragment is part of an 8.0-kilobase pair (kb) repeat sequence that occurs approximately 300 times as a head-to-tail tandem array comprising 2.4 mega–base pairs (Mb) of the Y chromosome (Devlin et al. 1991, 1994a, 1998). Recombination between the sex chromosomes (or the Y chromosome and an autosome) that occurs within the repetitive region could carry copies of *OtY1* away from the Y chromosome and has a greater chance of being involved in a recombinatorial event by virtue of its high copy number (relative to a single-copy locus. The tandem arrays containing *OtY1* are localized on the distal end of an acrocentric chromosome considered to be the Y chromosome in Chinook salmon (Stein et al. 2001). If a translocation occurs near or within this region, many copies of *OtY1* as well as the *GHΨ* locus could be moved from one chromosome to another.

Salmonid sex chromosomes are in an early state of differentiation. Previous studies of Chinook salmon (Devlin et al. 2001) and other salmonids (Hunter et al. 1982; Chevassus et al. 1988) have shown that YY individuals are viable and fertile, suggesting that the Y chromosome has not degenerated to the point that it lacks vital genes present on the X chromosome. Differentiation between the salmonid sex chromosomes is probably limited to the region immediately adjacent to the sex-determining region, and the remainder of these chromosomes retain sufficient homology so that genetic exchange can still occur (May et al. 1989; Allendorf et al. 1994).

Controlled breeding experiments to evaluate the inheritance pattern of sex-specific markers on fish with discrepant sexual genotype and phenotype have not been performed in any previous study. Original analysis of the inheritance pattern of *OtY1* involved controlled crosses of Chinook salmon obtained from British Columbia populations (Devlin et al. 1991, 2001). Moreover, a survey of regional variation of Y-chromosomal DNA markers in Chinook salmon populations across the Pacific Northwest (Devlin et al. 2005) revealed a north-to-south cline of increasing incidence of phenotypic males and females that had an incongruent genotype at one or both markers. Chowen and Nagler (2004) stated that the potential exists that *OtY1* is an inconsistent genetic marker for sex in the more southern populations of Chinook salmon. By evaluating the pattern of inheritance of the markers in controlled breeding experiments with phenotypic female Chinook salmon that have a male genotype (XY females), one would be able to discern whether or not sex-reversed Chinook salmon are a result of EDCs in the environment or are the product of a genetic rearrangement involving the Y chromosome.

In this study, we use two previously developed Y-chromosome markers, *OtY1* (Devlin et al. 1991, 1994b) and the growth hormone pseudogene (*GHΨ*; Du et al. 1993), in conjunction with controlled breeding experiments to test the hypothesis that there is no difference between a 1:1 (male : female) genotypic and phenotypic sex ratio in offspring produced between genotypically normal Chinook salmon, or between genotypically normal males and apparent sex-reversed males (XY females).

Methods

Sample collection for artificial crosses.—Fin clips for genetic analysis and gametes from fall-run Chinook salmon returning to the Merced River Fish Hatchery (MRH) were collected with the assistance of California Department of Fish and Game (CDFG) personnel between November and December 2003. Approximately 2 cm^2 of caudal fin tissue near the caudal peduncle was excised with scissors from each fish sampled and placed into individually labeled coin envelopes. Between samples, the scissors were mechanically cleaned and rinsed in clean running water to prevent cross contamination between the DNA of individual fish. Eggs from phenotypic females were expressed into prelabeled plastic urine analysis cups, sealed, and immediately placed on a raised platform within an ice chest. Milt from phenotypic males was expressed into labeled ziploc bags and similarly

stored. Tissue samples and gametes were stored between 5°C and 8°C while transported to the University of California, Davis, Genomic Variation Laboratory (GVL), for genetic analysis (tissue samples), and for use in controlled breeding experiments (gametes) at the Center for Aquatic Biodiversity and Aquaculture (CABA).

Genetic screening to detect apparent sex-reversed male (XY female) fish.—The selection criterion for sets of gametes to be used in artificial crosses was based on the sexual genotype at the growth hormone pseudogene and *OtY1* loci of putative parents. Genomic DNA from fin clips was extracted with a DNA extraction kit (QIAGEN, Inc., Valencia, California). Unused portions of fin clip samples were oven dried at 32°C for 15–18 h and then placed in the GVL sample repository. All phenotypic female and male fall-run Chinook salmon sampled were screened by polymerase chain reaction (PCR) assays with the *OtY1* primers developed from Chinook salmon by Devlin et al. (1991, 1994b) and an alternate version (E. LaHood, National Marine Fisheries Service, personal communication) of growth hormone pseudogene (*GH*Ψ) primers developed by Du et al. (1993). The alternate primer set contains the primers originally developed for *GH*Ψ and includes a control primer (5′-GTT CCT CCT GAC GTT GCC GTC G-3′), which produces an 84-base pair (bp) control band in Chinook salmon when used in conjunction with the forward primer. The control band alleviates the potential of obtaining a false negative signal caused by the chance failure of an individual PCR reaction. Both the *OtY1* and *GH*Ψ forward primers were 5-end labeled with the fluorophores FAM and TET, respectively. Assays for each genetic marker were carried out separately with 20 ng of genomic DNA, 1.25 mM $MgCl_2$ for *OtY1* and 1.75 mM $MgCl_2$ for *GH*-Ψ, 0.2 mM each deoxynucleotide triphosphate, 0.1 μM of each PCR primer, and 0.25 units of *Taq* DNA polymerase, 20 mM tris (pH 8.5), and 50 mM KCl in 10-μL volumes. Amplification of the *OtY1* locus was performed in a PTC100 thermal cycler (MJ Research, San Francisco, California) under the following conditions: one denaturation cycle at 95°C for 210 s, 35 amplification cycles at 95°C for 60 s, at 55°C for 30 s, at 72°C for 60 s, and a final extension cycle at 72°C for 30 s. Amplification of the *GH*Ψ locus was performed under the following conditions: one denaturation cycle at 95°C for 210 s and 35 amplification cycles at 95°C for 60 s, at 60°C for 60 s, and at 72°C for 120 s. The DNA fragments amplified by PCR were resolved on a 5.5% acrylamide–7 M Urea gel and imaged by a BaseStation (MJ Research). Individual genotypes were scored with software developed by researchers at North Carolina State University (QTL Cartographer, version 1.2.3, available at statgen.ncsu.edu/qtlcart/WQTLCart.htm) as well as manually verified for every individual genotyped.

Chinook salmon that tested positive for a 209-bp PCR fragment, did not produce a series of larger PCR products characteristic of the *OtY1* locus in females (Devlin et al. 1994), and tested positive for a 276-bp band indicative of the *GH*Ψ (Du et al. 1993) were scored as being positive for having the Y-chromosome markers (genetic males). In the case where a fish that had ovaries produced both a robust 209-bp PCR fragment (*OtY1*) and a 276-bp fragment (*GH*Ψ), that fish was scored as a XY female. When the larger PCR fragments characteristic of females were present, the 209-bp PCR fragment was not present, and the 276-bp band indicative of the *GH* pseudogene was also absent, the fish was scored as being negative for having the Y-chromosome markers (genetic female).

Breeding experiments.—The eggs from each single phenotypic female fish selected were split into roughly two equal portions and placed into separate Styrofoam containers. Aliquots of eggs were separately fertilized with milt from separate, single, genetically normal males. In this manner, each family of fish had only two parents. Artificial crosses were performed by transferring approximately 0.2 mL of milt to the eggs with a sterile pipette. Eggs were gently swirled with a clean, dry, latex-gloved finger to coat them evenly in milt. Enough 12°C water was added to just cover the eggs and activate the sperm. The gametes were gently swirled for a few seconds to ensure adequate mixing and were allowed to incubate 30–45 s. More 12°C water was added to allow for swelling of fertilized eggs. Eggs were incubated for approximately 15 min to allow them to begin water-hardening. During this incubation, the water was changed once to keep the eggs cold and oxygenated. An equal volume of a 1:100 dilution of Argentyne iodofore disinfectant (Scubla Aquaculture, Udine, Italy) in 12°C water was added to the eggs for 10 min as a prophylaxis against possible contamination with infectious viruses (i.e., infectious hematopoietic necrosis virus). Fluid was decanted from the eggs and eggs were rinsed twice with 12°C water. Each ''family'' was transferred to a labeled hatch-out (Heath) tray, each supplied with 12°C running water. No more than 30 h

elapsed between the harvest of eggs at the MRH and their fertilization at CABA.

Dead eggs, developing embryos, and alevins were removed on a daily basis to prevent development of bacterial or fungal growth in the incubation trays. Specimens were stored in 100% ethanol at 4°C until processed for genotyping. Although the gonad morphology of these specimens could not be ascertained since the gonads had not yet developed to a point where they could be visually differentiated between the sexes, these specimens were genotyped with both the *OtY1* and *GHΨ* Y-chromosome markers. Hatchlings from individual families were incubated at 12°C for approximately 45 d (just before swim-up stage) in the Heath trays before being transferred to separate, larger rearing tanks. The tanks were equipped with screens to prevent inadvertent cross contamination of families by escaped individuals from adjacent tanks. During the rearing period, juvenile fish were fed a commercially available diet twice daily. Any dead parr were removed and stored in 100% ethanol at 4°C until processed for genotyping. Juvenile fish were raised to a fork length of approximately 160 mm (approximately 120–140 d postfertilization) before euthanized for dissection. Juveniles were euthanized by anesthetic overdose via immersion in 12°C water containing 500 mg/L tricaine methanesulphonate (MS-222; Argent Laboratories, Redmond, Washington). Euthanized specimens were stored on ice until they could be dissected the same day.

Observation of gonad gross morphology in sampled juvenile Chinook salmon.—The gonads of juvenile fish were visualized with a dissection microscope (60× magnification). Two incisions were made to allow access into the body cavity. The first ventral incision extended from the gill isthmus to the vent and a second lateral incision started immediately posterior of the dorsal fin into the dorsal musculature. As was noted by Jensen and Hyde (1971), great care must be exercised to not perforate the swim bladder, since detection of the gonads is very difficult once the swim bladder has been deflated. In juvenile phenotypic males, the immature testes appear as two long, flattened, smooth, approximately 1-mm-wide, translucent white tubular organs that run along the ventral surface of and are closely associated with the swim bladder. The immature ovaries of juvenile phenotypic females appear as two long, roughly triangular, opaque yellowish-white tubular organs that are granular in appearance and texture. The ovaries are approximately 2–3 mm wide at the apical end and quickly taper to ribbon-like structures that run along the ventral surface of and are closely associated with the swim bladder. The granular appearance of the immature ovaries is caused by the presence of developing ova. Gross morphology of both gonads was verified by necropsy for all individuals, except in those families where all individuals had died before gonad development had progressed to a point at which phenotypic sex could be ascertained.

Statistical analysis.—Genotyping data were examined for consistency of Y-chromosome marker scores within individuals and were statistically tested to evaluate the offspring genotypic sex ratio in each family. The offspring phenotypic sex ratio of each family (based on internal observation of gonad gross morphology) was evaluated in the same manner. The consistency of sex marker scores was evaluated by merely observing whether or not the genetic markers corroborated one another in each individual tested. A chi-square goodness-of -fit analysis, utilizing a Yates (1934) correction for continuity to prevent inflating the probability of committing a type I error, was used to determine whether the observed genotypic and phenotypic sex ratios from individual families deviated significantly ($\alpha = 0.05$) from what is expected under a null hypothesis. For control crosses between genotypically normal Chinook salmon, or experimental crosses between genotypically normal males and apparent sex-reversed males (XY females), the null hypothesis of no difference between a one male: one female sex ratio in the sampled progeny was tested. The chi-square goodness-of-fit analyses of offspring sexual genotype were performed on families where the phenotypic sex of offspring could be ascertained as well as those families whose offspring had died before developing to a point where gonad gross morphology could be evaluated with certainty.

Results

In none of the 2,384 adults and progeny evaluated did the *GHΨ* and *OtY1* Y-chromosome-specific markers contradict one another. The tight linkage observed between these two markers in Chinook salmon is similar to that observed by Devlin et al. (2001). A total of 156 adult fish (135 phenotypic females and 21 phenotypic males) collected from the MRH were genotyped. Genetic analysis revealed 8 out of the 135 (~6%) phenotypic females examined had a male genotype according to the *GHΨ* and *OtY1* genetic markers. The remaining 127 phenotypic females screened

TABLE 1.—Observed genotypic and phenotypic sex ratios of progeny in individual families of fall-run Chinook salmon produced from breeding experiments. Phenotypic female parents that were genotypically female or male at both Y-chromosome markers are designated as normal or XY-female (XYF), respectively. Gross morphology of gonads was verified by necropsy for all individuals except in those families where all individuals had died before gonad development had proceeded to a point at which phenotypic sex could be ascertained. Chi-square test results for 1:1 and 3:1 (male [M] : female [F]) genotypic sex ratios are shown in the seventh and eighth columns, respectively. Counts of phenotypic males (PM) with male genotype (GM), phenotypic females (PF) with male genotype, and phenotypic females with female genotype (GF) are shown in the last three columns. Statistical significance is as follows: $P < 0.05$*; $P < 0.001$**.

Family	Phenotypic female parent	*N*	Observed phenotypic ratio (M:F)	Phenotypic chi-square value	Observed genotypic ratio (M:F)	Genotypic chi-square value (1M:1F)	Genotypic chi-square value (3M:1F)	Observed counts		
								PM–GM	PF–GM	PF–GF
84 × B	Normal	92	1.14:1	0.27	1.14:1	0.27		49	0	43
85 × B	Normal	82	1.41:1	0.99	1.41:1	0.99		48	0	34
85 × D	Normal	69	1.09:1	0.38	1.09:1	0.38		36	0	33
105 × A	Normal	96	1.18:1	0.51	1.18:1	0.51		52	0	44
105 × B	Normal	96	0.62:1	4.59*	0.62:1	4.59*		37	0	59
112 × D	Normal	96	0.83:1	0.51	0.83:1	0.51		44	0	52
128 × D	Normal	90	0.83:1	0.27	0.83:1	0.27		41	0	49
87 × B[a]	XYF	40			3.44:1	11.02**	0.01			
87 × D[a]	XYF	32			2.55:1	5.28*	0.04			
93 × A	XYF	106	1.35:1	5.63*	4.30:1	39.86**	1.81	61	25	20
93 × B[a]	XYF	16			3.00:1	3.06	0.08			
118 × C	XYF	83	1.68:1	4.89*	4.93:1	35.57**	2.51	52	17	14
118 × D	XYF	144	1.18:1	0.45	3.50:1	43.34**	1.25	78	34	32
126 × C	XYF	154	1.26:1	0.94	4.31:1	58.60**	2.80	86	39	29
126 × D	XYF	142	1.09:1	0.17	4.26:1	53.30**	2.40	74	41	27
130 × A	XYF	152	0.91:1	0.32	2.62:1	29.53**	0.43	72	38	42
130 × D	XYF	116	1.23:1	1.04	4.04:1	41.04**	1.39	64	29	23
Grand total: 1,606										

[a] Gonads not verified.

were genetic females, and all 21 phenotypic males screened were genetic males according to both genetic markers.

Using both genetic markers, we genotyped a total of 622 dead embryos, alevins, and parr (<40 mm fork length) whose phenotypic sex had not been evaluated since gonadal development and for which development had not proceeded to a point that it could be ascertained. The mortalities had occurred in all families before commencing dissections of the offspring (that had developed for 120–140 d). The two genetic markers corroborated one another in all individuals tested.

Families 87xB and 87xD had offspring genetic sex ratios that deviated significantly from a 1:1 (male : female) ratio (Table 1). These families, originating from XY female #87 mated with males B and D, respectively, suffered very high early mortality within 72 h of fertilization. All of the individuals analyzed from the two families were either dead embryos or alevins that had developed for a sufficient period of time to permit the harvest of an adequate amount of tissue for genetic analysis of sexual genotype. The 16 offspring analyzed from family 93xB were also either dead embryos or alevins. High mortality before hatch was also observed for families from genetically normal females collected the same day as XY female #87. The MRH manager observed high mortality of eggs that were collected on the same day as female #87 (M. Kozart, CDFG, personal communication). It is possible that the elevated mortality observed in these families was caused by a spike in water temperature that had occurred just before and during the collection of these fish (M. Kozart, personal communication). The water source of the MRH is the Merced River.

A total of 1,606 individuals from 14 out of 17 artificial crosses successfully reared for 120–140 d were genotyped and dissected to verify gonad gross morphology. No statistically significant deviations from 1:1 (male : female) phenotypic or genotypic sex ratios were observed in 6 out of 7 families (Table 1) or in all families combined ($\chi^2 = 0.03$, df = 1, $P = 0.8500$) from genetically normal phenotypic female parents. All phenotypic male offspring from these families were positive for both Y-chromosome markers and all phenotypic female offspring were negative for both markers. Family 105xB, the sole exception, had a significantly higher ($P < 0.0500$) number of phenotypic female offspring than would be expected

by chance alone (Table 1). Male and female offspring of this family did not suffer mortality differentially, and the reason for the significantly different sex ratio observed is unknown. Here, too, sexual genotype of both markers was in concordance with sexual phenotype in all offspring.

Seven families produced by XY females had enough offspring survive so that both sexual genotype and phenotype could be ascertained. Observed offspring phenotypic sex ratios of the 7 families combined differed significantly (χ^2 = 6.61, df = 1, P = 0.0100) from a 1:1 (male : female) sex ratio. The significant difference from a 1:1 phenotypic sex ratio in offspring produced by XY females was a result of two families, 93xA and 118xC. Both had significantly higher ($P <$ 0.0500) numbers of phenotypic male offspring and each exhibited some of the most highly skewed genotypic sex ratios (Table 1). Neither of these two families suffered early mortality differentially between the sexes. No significant deviation from a 1:1 (male : female) offspring phenotypic sex ratio was observed when the 5 remaining families produced by XY females were combined (χ^2 = 2.26, df = 1, P = 0.1300) or assessed individually (Table 1).

Unlike the families produced by genetically normal phenotypic females, discrepancies between sexual genotype and phenotype were observed in the offspring of XY females. Statistically significant deviations ($P <$ 0.0500 or 0.0010) from a 1:1 (male : female) genotypic sex ratio (Table 1) were observed in 9 out of 10 families produced by XY females. Since the sample size of 93xB was small (N = 16), the power to detect a significant deviation from the expected 1:1 (male : female) genotypic sex ratio was low. Hence, family 93xB lacked statistical significance (0.1000 $> P >$ 0.0500), despite having a 3:1 (male : female) genotypic sex ratio (Table 1). The observed offspring genotypic sex ratio of the combined 7 XY-female families (nonasterisked in Table 1) was significantly different (χ^2 = 327.94, df = 1, $P <$ 0.0001) from 1:1 (males : females), and it was significantly different from the offspring genotypic sex ratio observed in the combined families produced by genetically normal phenotypic females, which did not differ significantly (χ^2 = 0.03, df = 1, P = 0.8800) from 1:1 (males : females). All phenotypic male offspring produced by apparent XY females were genotypically male at both Y-chromosome markers. Roughly one-half of the phenotypic female offspring produced by apparent XY-female parents were genotypically male at both markers. The remaining phenotypic female offspring were genotypically female at both markers. In short, the excess of male genotypes at both *GHΨ* and *OtY1* in offspring produced by apparent XY-female fish is caused by roughly half of the phenotypic female offspring of these crosses being scored as males.

The genotypic sex ratios of offspring produced by ''apparent'' XY females ranged from 2.55:1 to 4.93:1 (average, 3.7:1; males : females). The observed genotypic sex ratio of offspring produced by XY-female families did not significantly differ from 3:1 (males: females; Table 1). However, when the XY-female families (nonasterisked in Table 1) were combined, a significantly higher number of male genotypes (χ^2 = 9.06, df = 1, P = 0.0020) were observed than would be expected by chance alone from a 3:1 (male : female) sex ratio. The detected deviation from a 3:1 genotypic sex ratio for the combined XY-female families is caused, in part, by increased power via an increase in sample size.

Discussion

The observed incongruence between sexual genotype and phenotype in half of the phenotypic female progeny of apparent XY females strongly suggests that a genetic rearrangement or mutation rather than altered sexual development caused by endocrine disruption is responsible for the XY-female fall-run Chinook salmon in the Central Valley. Accordingly, neither of the two Y-chromosome markers used in this study are 100% diagnostic of female sex in Central Valley fall-run Chinook salmon. The observed 1:1 (male : female) offspring phenotypic sex ratio was not the expected 3:1 ratio that would be obtained from the cross between a true sex-reversed male (XY female) and a normal (XY) male (Figure 1C). If, as was hypothesized by Williamson and May (2002), apparent XY females were the result of altered sexual differentiation as a result of exposure to environmental EDCs, one would not expect to observe incongruent sexual genotype and phenotype within individual progeny of these fish. If the developmental pathway(s) controlling sexual phenotype was altered by exposure to EDCs at a critical period of development, such changes may occur without necessarily also eliciting a heritable change in the germ cells of exposed individuals. Hence, previous reports (Nagler et al. 2001; Williamson and May 2002) regarding altered sexual differentiation in Chinook salmon as a result of endocrine disruption were incorrectly interpreted.

Several genetic rearrangements are possible that

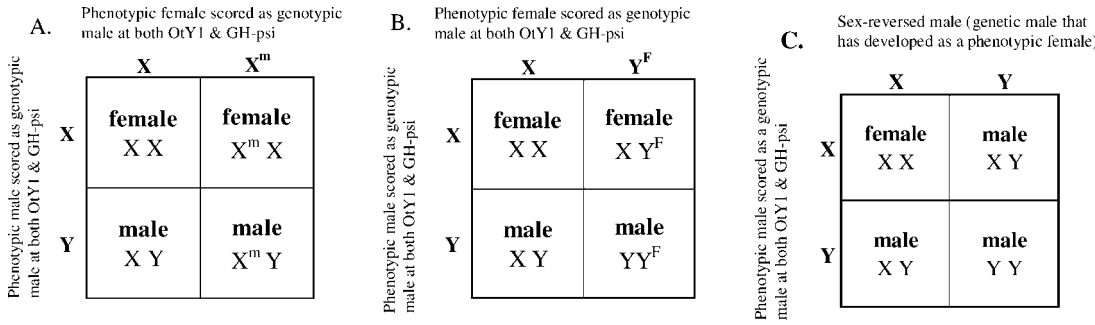

FIGURE 1.—Alternate models to explain the incongruence of genotypic and phenotypic sex in half of the phenotypic female offspring produced by apparent XY-female fall-run Chinook salmon from the Central Valley, California. The first two panels are Punnett squares depicting the possible offspring produced between a normal male and **(A)** a phenotypic female parent carrying an X chromosome with a translocated portion of the Y chromosome designated by X^m and **(B)** a dysfunctional Y chromosome designated by Y^F. Both models predict the phenotypic and genotypic sex ratios (1:1 and 3:1 [male : female], respectively) observed in offspring from XY females used in breeding experiments. A mating between a true sex-reversed male and a normal male, as shown in panel **(C),** would produce 3:1 (male : female) genotypic and phenotypic sex ratios in the offspring. Wild-type X and Y chromosomes are designated by X and Y, respectively; the sexual phenotype of offspring is in bold.

would explain the observed pattern of inheritance. First, there may have been a recombination event (or translocation) between the Y and X chromosomes or an autosome that carried along copies of both the *OtY1* and *GHΨ* loci. Phenotypic female fish carrying an autosome or X chromosome to which a portion of the Y chromosome bearing the markers has translocated, or carrying a Y chromosome bearing a mutation or deletion that functionally inactivates the sex-determining region (Figures 1A and 1B, respectively) would, when mated to a normal male, produce offspring with a 3:1 (male : female) genotypic sex ratio while maintaining a 1:1 phenotypic sex ratio. In either scenario, half of the phenotypic female offspring would bear a male genotype since they have a 50% chance of inheriting the recombinant chromosome or translocation from the phenotypic female parent. Inactivation of the sex-determining region on the Y chromosome can occur from a frame shift, a nonsense mutation as a result of an insertion or deletion mutation, or both. Matsuda et al. (2002) described a nonsense mutation in Japanese medaka *Oryzias laptipes*. The Awara mutation is a single nucleotide insertion in exon 3 of *DMY** that causes a frame shift and subsequent truncation of *DMY**. All offspring that inherited the Awara mutant allele of *DMY** were female (Matsuda et al. 2002). Alternatively, decreased expression of a gene that plays a role in differentiation of the bipotential gonad may be responsible. The Shirone mutation in Japanese medaka (Matsuda et al. 2002) leads to a very low expression of *DMY**, resulting in a high proportion of XY females in fish that carry the mutation.

The genetic rearrangement or mutation responsible for producing apparent XY-female fall-run Chinook salmon in the Central Valley has probably arisen independently of any such genetic changes that have occurred in other more northern populations of Chinook salmon. In this study, all phenotypic males and apparent XY females had a clearly defined male genotype according to both *OtY1* and *GHΨ* markers. All phenotypic females categorized as normal were negative for both male markers. In contrast, Devlin et al. (2005) observed low numbers of phenotypic females and males that were positive for only either *OtY1* or *GHΨ* and phenotypic males negative and phenotypic females positive for both markers. Devlin et al. (2005) described weakly amplifying allelic variants for both loci in males and females and suggested recombination, deletion, and PCR-priming site sequence variation (in the case of allelic variation) of *OtY1* copies and the *GHΨ* locus as possible explanations for the observed variation. The far lesser degree of sex marker variation observed in California Chinook salmon (Williamson and May 2002) and the fact that *GHΨ* is a single-copy marker (Du et al. 1993) suggests that a single genetic change different from those in more northern populations has occurred. If a translocation of markers from the Y to X chromosome occurred, it probably happened only once, since translocations are rare events.

Only when all XY-female families are combined is there a higher-than-expected number of male

genotypes (relative to a 3:1 [male : female] sex ratio). An explanation might be that chromosomes carrying the genetic alteration have a slight fitness advantage relative to their homologous, wild-type counterparts. The delay of the second meiotic division in females, coupled with partitioning of chromatids to the polar bodies, may play a role in the process. If the genetic alteration does confer a chromosomal fitness advantage (or at least a relatively lower probability of being shunted to a polar body), then its frequency within a population will probably increase in subsequent generations. This may explain, in part, how an alleged single mutation could spread throughout the Central Valley.

Current data from the breeding experiments do not indicate which of the alternate models (Figures 1A and 1B) is more probable. Fluorescent in situ hybridization (FISH) assays performed with *OtY1* (Devlin et al. 1991, 1994b) and *GHΨ*(Du et al. 1993) can be used to probe lymphocyte chromosome spreads obtained from the offspring of phenotypic females positive for both Y-chromosome markers and genetically normal phenotypic females. Comparison of chromosome staining patterns obtained from the offspring of normal and apparent XY females may provide a way to differentiate which chromosomal mechanism is responsible for producing apparent XY-female fall-run Chinook salmon in the Central Valley. This assumes, however, that the FISH assay can provide sufficient resolution to differentiate between chromosome staining patterns produced by a Y to X chromosome or autosome translocation and anormal, intact Y chromosome.

The FISH methodology would not differentiate between a wild-type Y chromosome (one lacking a functional sex-determining locus caused by a small heterozygous insertion or deletion mutation) or a chromosome translocation, particularly if the translocated region is large. In this case, other molecular genetic techniques may provide a means to differentiate between the proposed alternate models. Differences in the DNA strand may be observed by performing chromosome walks on *OtY1*- and *GHΨ*-positive clones of bacterial artificial chromosome libraries created from a phenotypic female offspring positive for the markers and a phenotypic male offspring from a control cross. Alternatively, suppressive subtractive hybridization (SSH) performed on genomic DNA isolated from phenotypic female offspring that do and do not carry the rearrangement or mutation can be used to create a DNA library enriched for recombinant sequences (in the case of a recombinant or translocated chromosome) or the nonwild-type Y chromosome (in the case of a mutation on the Y chromosome). Sequence data obtained from SSH-enriched library clones positive for *OtY1* and *GHΨ* can then be compared with similarly positive clones from a library created from a phenotypic male offspring that does not carry the rearrangement or mutation. The DNA sequences that differ between subtracted and nonsubtracted library clones can be evaluated by testing their segregation patterns in offspring of normal and apparent XY females. Resolution of the genetic mechanism responsible for the two types of phenotypic females should help us understand sex determination in salmonid fishes.

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