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To cite this article: Kevin S. Williamson & Bernie May (2002) Incidence of Phenotypic Female Chinook Salmon Positive for the Male Y-Chromosome-Specific Marker *OtY1* in the Central Valley, California, Journal of Aquatic Animal Health, 14:3, 176-183, DOI: [10.1577/1548-8667\(2002\)014<0176:IOPFCS>2.0.CO;2](https://doi.org/10.1577/1548-8667(2002)014<0176:IOPFCS>2.0.CO;2)

To link to this article: [https://doi.org/10.1577/1548-8667\(2002\)014<0176:IOPFCS>2.0.CO;2](https://doi.org/10.1577/1548-8667(2002)014<0176:IOPFCS>2.0.CO;2)



Published online: 09 Jan 2011.



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Incidence of Phenotypic Female Chinook Salmon Positive for the Male Y-Chromosome-Specific Marker *OtY1* in the Central Valley, California

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Abstract.—Fall-run chinook salmon *Oncorhynchus tshawytscha* collected during 1999 carcass surveys of seven locations within the Sacramento River basin and six locations within the San Joaquin River basin, California, were screened using the Y-chromosome-specific marker *OtY1* to evaluate inconsistencies between sexual genotype and phenotype. Of 287 phenotypic females screened, 46 (16% overall) tested positive for the Y-chromosome marker. Stream populations had higher frequencies of sex-reversed males (American River, 20%; Battle Creek, 35%; Feather River, 20%; Merced River, 24%; Mokelumne River, 38%; and Yuba River, 25%) than hatchery populations (Feather River Hatchery, 0%; Nimbus Hatchery, 12%; Merced River Hatchery, 14%; and Mokelumne River Hatchery, 4%), and several other streams had intermediate frequencies. All 150 male fall-run chinook salmon from the sampling locations tested positive for the Y-chromosome marker. These results present evidence that some genetic males have been sex reversed and have the appearance of females. The negative impacts that successful breeding by sex-reversed individuals may have on reproduction and population genetics, thereby hindering population persistence, are discussed.

Inconsistencies between sexual genotype and phenotype have been recently documented in wild populations of chinook salmon *Oncorhynchus tshawytscha* (Nagler et al. 2001). That study reported a high frequency of fish that are genetically male but phenotypically female. Hypothesized causal factors of this phenomenon include temperature fluctuations and environmental contaminants such as xenoendocrine disruptors (Sumpter 1998) in the aquatic environment. If sex-reversed males (XY females) are reproductively successful, the resulting sex ratio of the progeny becomes skewed toward males. Successful reproduction by sex-reversed genetic males may negatively impact the reproduction and population genetics of fish and thus population persistence. In this article we report preliminary evidence of phenotypic female chinook salmon that test positive for a Y-chromosome-specific marker (*OtY1*) in the waters of two river drainages in the Central Valley of California. The potential impact of successful breeding by sex-reversed individuals on the genetic health of chinook salmon populations is discussed. We then suggest future research that would aid resource management decisions re-

garding chinook salmon populations experiencing sex reversal.

The DNA probe (*OtY1*) developed from and specific to male chinook salmon (Devlin et al. 1991) segregates in a pattern consistent with its being tightly linked to male-determining genes on the Y chromosome (Devlin et al. 1994). The *OtY1* locus does not produce an exclusively male signal when amplified by polymerase chain reaction (PCR). Amplification of the *OtY1* locus yields a 209-base-pair (bp), male-specific DNA fragment and several larger (214-, 230-, 259-, and 360-bp), non-sex-specific fragments. It is presumed that when male-specific sequences are present they are more efficiently amplified than the non-sex-specific sequences. The regions on the autosomal and/or X chromosomes of chinook salmon are sufficiently similar to those of the male-specific sequences on the Y chromosome to permit the larger, non-sex-specific fragments to be amplified in the absence of the smaller male-specific sequences. Hence, the 209-bp Y-chromosome marker is amplified in genetic males but not in genetic females.

Methods

Fin clips were obtained during 1999 carcass surveys of seven locations within the Sacramento River basin (American River, Battle and Clear creeks, Feather River and Feather River Hatchery, Nimbus Hatchery, and the Yuba River) and six

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Received October 22, 2001; accepted March 30, 2002

locations within the San Joaquin River basin (Merced River and Merced River Hatchery, Mokelumne River and Mokelumne River Hatchery, and Stanislaus and Tuolumne rivers). The fin clips were cut from carcasses of fish that had already spawned and were stored individually on ice in 10 mM tris buffer (pH 8). The razor blades used for sampling fish were mechanically cleaned and rinsed between uses. Samples were then transferred to the California Department of Fish and Game's (CDFG) Rancho Cordova Salmonid Tissue Archive for long-term storage at -80°C . The fish were phenotypically sexed by internal examination of gonads (James Navicky, CDFG, personal communication). Genomic DNA was extracted from fin clips using a QIAgen DNA tissue extraction kit and quantified using a Molecular Dynamics 595 fluorimeter (Sunnyvale, California). Polymerase chain reaction (PCR) assays were carried out using 40 ng of genomic DNA, 1.25 mM MgCl_2 , 0.2 mM each deoxynucleotide triphosphate, 0.2 μM of each PCR primer, 0.25 units of *Taq* DNA polymerase, 20 mM tris (pH 8.5), and 50 mM KCl in 10- μL volumes. Amplifications were performed in PTC100 thermal cyclers (MJ Research, San Francisco, California) by means of the following process: one denaturation cycle at 95°C for 210 s; 35 amplification cycles at 95°C for 60 s, 55°C for 60 s, and 72°C for 60 s; and a final extension cycle at 72°C for 30 s. The PCR primers used were those developed from chinook salmon (Devlin et al. 1994). The PCR products were resolved by electrophoresis through a gel consisting of 5% polyacrylamide and 7 M urea at 1500 W (40 mA) for 1 h. The resolved products were stained with SYBR-green (BioWhittaker Molecular Applications, Inc., Rockland, Maine) using an agarose overlay (Rodzen et al. 1998) and visualized by means of a Molecular Dynamics 595 fluorimeter. Fish that tested positive for a 209-bp PCR fragment and that did not produce a series of larger PCR products characteristic of the *OtY1* locus in females (Devlin et al. 1994) were scored as positive for the Y-chromosome marker (genetic males). When a fish with ovaries produced a robust 209-bp PCR fragment, it was scored as a female positive for the male-specific *OtY1* PCR fragment. Such fish were considered to be sex-reversed males (XY females). When the larger PCR fragments characteristic of females were present and the 209-bp PCR fragment was either not present or very faint, the fish was scored as a genetic female.

Results

The number of female and male chinook salmon evaluated from each site is given in Table 1. Of 287 females screened, 46 (16% overall) tested positive for the Y-chromosome marker. Several stream populations had higher frequencies of sex reversal (American River, 20%; Battle Creek, 35%; Feather River, 20%; Merced River, 24%; Mokelumne River, 38%; and Yuba River, 25%) than the hatchery populations (Feather River Hatchery, 0%; Mokelumne River Hatchery, 4%; and Merced River Hatchery, 14%). The difference in the frequency of sex reversal between the stream populations and hatchery fish is even more apparent when populations from the same river are compared. For instance, the frequencies of XY females from the American River and Nimbus Hatchery were 20% and 12%, respectively, those from the Feather River and Feather River Hatchery 20% and 0%, those from the Merced River and Merced River Hatchery 24% and 14%, and those from the Mokelumne River and Mokelumne River Hatchery 38% and 4%. The remaining streams, Clear Creek and the Stanislaus and Tuolumne rivers, had intermediate frequencies of 6, 12, and 12% of sex-reversed males, respectively. All 150 male fall-run chinook salmon from the sites in the screening tested positive for the male-specific 209-bp fragment (Table 1).

Fish that had testes and produced a robust 209-bp PCR fragment (Figure 1, lanes 3 and 4) were scored as males. Fish that had ovaries and produced a robust 209-bp PCR fragment (Figure 1, lanes 5 and 6) were scored as sex-reversed males (XY females). The patterns of PCR fragments characteristic of female fish were similar to those seen previously by Devlin et al. (1994). Here, females produced various combinations of 214-, 226-, 230-, 236-, 351-, and 355-bp PCR fragments. Two classes of female fall-run chinook salmon were observed. The first class comprised fish that had ovaries and produced only the PCR fragments characteristic of females (Figure 1, lanes 8 and 9). The second class comprised fish that had ovaries and produced both the PCR fragments characteristic of females and a very faint 209-bp fragment (Figure 1, lanes 10 and 11). Both classes were pooled to give the number of female fish that were negative for the male-specific marker shown in Table 1.

Discussion

These results present evidence that genetic males have been sex reversed and have the ap-

TABLE 1.—Fall-run chinook salmon from 1999 carcass surveys tested for the presence of the *OtYI* male-specific 209-base-pair polymerase chain reaction fragment.

Sampling location	Phenotypic sex	Fish positive for male-specific fragment	Fish negative for male-specific fragment
Sacramento River basin			
American River	Male	2	0
	Female	1	4
Battle Creek	Male	7	0
	Female	6	11
Clear Creek	Male	12	0
	Female	1	17
Feather River	Male	12	0
	Female	1	4
Feather River Hatchery	Male	12	0
	Female	0	31
Nimbus Hatchery	Male	12	0
	Female	3	22
Yuba River	Male	7	0
	Female	2	6
Total	Male	64	0
	Female	14	95
San Joaquin River basin			
Merced River	Male	9	0
	Female	9	29
Merced River Hatchery	Male	29	0
	Female	4	25
Mokelumne River	Male	10	0
	Female	11	18
Mokelumne River Hatchery	Male	25	0
	Female	1	23
Stanislaus River	Male	6	0
	Female	3	21
Tuolumne River	Male	7	0
	Female	4	30
Total	Male	86	0
	Female	32	146

pearance of phenotypic females. As in the study by Nagler et al. (2001), none of females from the Feather River Hatchery were positive for the *OtYI* marker. However, unlike in that study, small numbers of female fish from the Nimbus Hatchery and Merced and Mokelumne River hatcheries carried the Y-chromosome-specific marker. These fish may actually have been progeny from naturally spawning fish in their respective rivers. The two smallest numbers of XY females occurred in the Feather and Mokelumne River hatcheries (Table 1).

If the incidence of sex reversal in chinook salmon is due to endocrine-disrupting contaminants, the differences between hatchery and naturally spawning fish may lie in the water supplies to the hatcheries. For instance, if a hatchery is supplied with well water rather than river water containing estrogenic contaminants, sex reversal may occur less frequently in the hatchery fish. Another possibility is that there is a natural, constituent level of sex reversal within wild chinook salmon pop-

ulations that has been eliminated from hatchery populations through selection. During carcass surveys performed by CDFG personnel, the gonads of collected fish were inspected to verify sex (Navy, personal communication). Thus, it is highly unlikely that the sex of sampled fish was misidentified due to ambiguities in external secondary sexual characteristics.

The female chinook salmon that exhibit a very faint 209-bp *OtYI* PCR fragment (Figure 1, lanes 10 and 11) may be sex-reversed males. It is possible that a polymorphism occurs within one of the *OtYI* PCR primer annealing sites on the Y chromosome. Such a change in the Y-chromosome-specific target sequence could reduce its amplification to the point where the larger, non-sex-specific sequences are capable of being amplified despite the presence of the Y chromosome. If this were the case, the incidence of sex-reversed males in both stream and hatchery populations would be much greater than indicated in Table 1.

The ability to identify the source population of

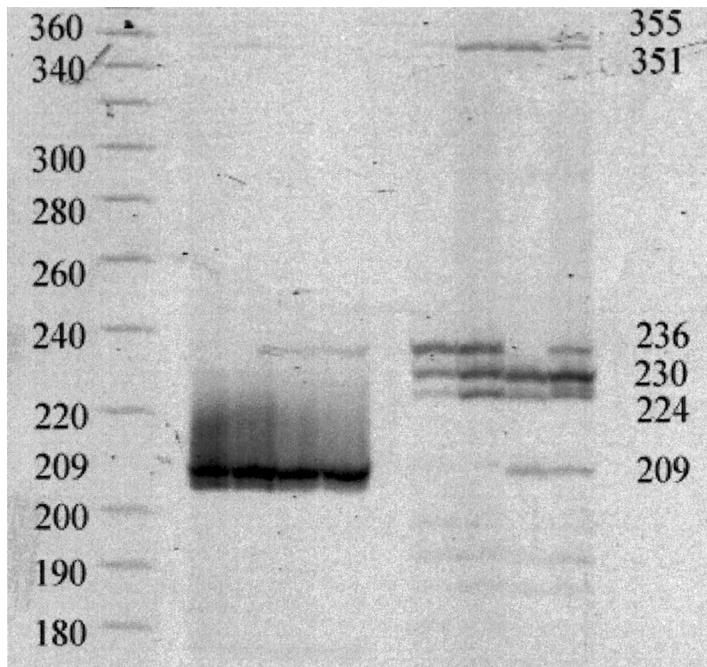


FIGURE 1.—Polyacrylamide gel of *OtY1* polymerase chain reaction (PCR) amplification products from genomic DNA of fall-run chinook salmon collected during 1999 carcass surveys in the Central Valley, California. Males (lanes 3 and 4), sex-reversed males (lanes 5 and 6), females negative for the male-specific band (lanes 8 and 9), females positive for the male-specific band (lanes 10 and 11), DNA size standards (lane 1), blank well (lane 2), and negative PCR control (lane 7), are shown. The robust 209-base-pair (bp) PCR fragment indicative of males and the PCR fragments (226, 230, 236, 351, and 355 bp) indicative of females are shown.

individual sex-reversed males would provide a basis for testing hypotheses regarding the causes of sex reversal. As yet, no method is available to adequately differentiate between unmarked hatchery-reared and naturally produced fall-run chinook salmon in the Central Valley. Our laboratory is currently developing a baseline of genetic variation in alleged wild and hatchery fall-run chinook salmon populations in the San Joaquin River basin. The genetic baseline is being developed using microsatellites; these hypervariable genetic markers are ideal for describing the population genetic structure of recently diverged populations (Sunucks 2000). While discriminating between hatchery-reared and naturally produced fish is not the overall aim for which the genetic baseline is being developed, microsatellites may reveal such population substructure if it exists and the loci examined have diverged sufficiently. Previous studies (Banks et al. 1996, 2000; Scribner et al. 1996; Nelson et al. 2000) have successfully resolved genetic differences between populations of chinook salmon. However, C. Heberer (1999 memorandum to C. Wingert, National Marine Fisheries Ser-

vice, Southwest Region) estimated that 20–40% of the returning naturally spawning fish in California rivers are of hatchery origin. Under this scenario, one could argue that generations of introgression between hatchery and naturally spawning fish has resulted in the genetic homogenization of populations. Establishing the source population of sex-reversed fish will be the focus of future work.

Successful breeding by sex-reversed individuals may negatively impact reproduction and population genetics, thereby hindering population persistence. In coho salmon *O. kisutch*, mating between normal males and females results in a progeny sex ratio of one female to one male, whereas mating between a normal male and a sex-reversed male results in a sex ratio skewed toward males. A ratio of one female to three males occurred in approximately one-half of the coho salmon families produced when normal and sex-reversed males were crossed, and a ratio of one female to two males occurred in the remaining families (Hunter et al. 1982). This suggests that there is variability in YY viability in coho salmon. In this first gen-

eration, there is an overabundance of male progeny. Furthermore, in crosses that produce a 3:1 male : female sex ratio, one-third of the male progeny bear the YY genotype. These supermale progeny were viable to 9 months of age (Hunter et al. 1982). Since the progeny were sacrificed in order to inspect their gonads, the study did not address whether the YY fish were fertile. A similar study of rainbow trout *O. mykiss* by Scheerer et al. (1991) showed that YY males are viable and capable of reproducing. To the best of our knowledge, it has not been reported that YY chinook salmon produced from the cross between a normal male and a sex-reversed male are viable and fertile. Given the close phylogenetic relationship between chinook and coho salmon (Utter and Allendorf 1994), it is possible that such fish are indeed both viable and fertile.

Since supermale individuals pass on only Y-chromosome-bearing gametes, any mating they engage in will result entirely in male progeny in the second generation. If the YY male, however, mates with a sex-reversed male, all of the progeny will be male and 50% will have the YY genotype. In a scenario in which the sex reversal of male fish is chronic and supermales are reproductively functional, the likelihood that the two will interbreed increases as the frequency of either increases. Nonetheless, the population sex ratio may not be driven far from natural levels since the sexual development of male fish in the population will be altered even though the frequency of the Y chromosome in the population is increasing with each successive generation. As long as the level of sex reversal approximately compensates for the excess of male progeny produced within the population, it is unlikely that any decrease in effective population size would result from a skewed sex ratio. However, once the population is no longer influenced by the causal factor(s) behind sex reversal, breeding by the sex-reversed males remaining in the reproductive segment of the population may skew the population sex ratio of subsequent cohorts. This may result in a decrease in the effective population size and accelerate the effect that genetic drift has in reducing the genetic variation within the population. While loss of genetic variation may result in a concomitant decrease in a population's ability to cope with new environmental challenges, such as disease and changing climatic conditions, demographic factors have a more immediate impact on population persistence (Lacy 1988; Lande 1988). A highly skewed population sex ratio may hinder reproduction by af-

fecting mating behavior or decreasing the probability of encountering a suitable mate. This, in turn, would impact the population's ability to persist, especially when it experiences large fluctuations in size (as do many species of Pacific salmon). A reduction in the relative number of true genetic females, which may have a greater reproductive value than sex-reversed males (Pandian and Sheela 1995), will further diminish the ability of the population to maintain itself.

Known or suspected endocrine-disrupting compounds have been detected in a variety of taxa and their environments. Definitive etiological links, however, have yet to be established for more than a few cases. Studies on American alligators *Alligator mississippiensis* in Lake Apopka, Florida, have provided some of the more convincing evidence that endocrine disruption in wildlife has resulted from exposure to hormone-disrupting mimics (Tyler et al. 1998). Alligators exposed to the organochlorine dicofol and its metabolites (DDD, DDE, and chloro-DDT) experienced reproductive disorders and altered reproductive development. Females had double the plasma concentration of estradiol found in normal females in the control lake, Lake Woodruff, as well as abnormal ovarian morphology (Guillette et al. 1994). Males had poorly organized testes, abnormally small phalli, and greatly reduced serum testosterone levels compared with males from Lake Woodruff (Guillette et al. 1996). The finding that the concentration of *p, p'*-DDE in the eggs of demasculinized alligators was 90-fold that required to produce antiandrogenic activity in vitro provides strong support for the hypothesis that the gonads of alligators had been permanently modified in ovo by DDE, altering steroidogenesis and inhibiting normal sexual maturation. The reproductive impacts of endocrine disruption led to the decline of the Lake Apopka population for several years (Jennings et al. 1989; Guillette et al. 1994).

Imposex—the development of male characteristics, including a penis and a vas deferens, in females—has been found in marine mollusks (Tenhallers-Tjabbes et al. 1994; Oehlmann et al. 1996b). This form of reproductive impairment has been linked to tributyltin (TBT)-based antifouling paints on the hulls of ships, and it has decimated marine mollusk populations (Mattheissen and Gibbs 1998). It is believed that TBT interferes with testosterone metabolism by competitively inhibiting the aromatization of androgens to estrogens (Betten et al. 1996) and inhibiting the conjugation of testosterone (Ronis and Mason 1996). This would

result in the accumulation of androgens within tissues and, in turn, stimulate the development of a penis and/or vas deferens and prostate tissue in females (Oehlmann et al. 1991, 1992, 1996a). Where the environmental concentration of TBT has reached 6–8 ng/L, complete reproductive failure and local extinction due to the sterility of females has resulted (Bryan et al. 1986).

Rainbow trout experience adverse health and reproductive effects from exposure to estrogenic pollutants. Work by Harries et al. (1997, 1999) has demonstrated that excessive concentrations of the egg yolk protein precursor vitellogenin (VTG) are produced in the bloodstream of male rainbow trout in a dose-dependent manner when they are exposed to estrogenic sewage effluent. Only miniscule amounts of VTG are normally detected in the blood of male rainbow trout, since its production is estrogen dependent (Copeland et al. 1986). Herman and Kincaid (1988) have suggested that the prolonged and excessive synthesis of VTG under the influence of orally administered estradiol probably results in high metabolic stress to both male and female fish, leading to damage and necrosis of the kidney and liver and possibly to death. Caragher and Sumpter (1991) have suggested that the high constitutive synthesis of VTG in rainbow trout may result in the diversion of vital proteins or lipids and the decalcification of the scales. Both the physiological stress of continued VTG synthesis and the loss of integrity of the scales through decalcification have the potential to predispose fish to disease.

Adverse reproductive effects in juvenile rainbow trout from exposure to incompletely biodegraded alkylphenol ethoxylates (APEOs) may impact the viability of their populations. Ashfield et al. (1998) have shown that laboratory exposure of female juvenile rainbow trout to environmentally relevant concentrations of APEOs resulted in marked and sustained decreases in growth and modification of the ovosomatic index. Jobling et al. (1995) showed that the exposure of male juvenile rainbow trout to a variety of APEOs caused not only vitellogenin synthesis but also inhibition of testicular growth and spermatogenesis in a manner that correlated with the estrogenic potency of the compounds. A study by Gimeno et al. (1998) of the effects of sublethal concentrations of 4-tert-pentylphenol and 17 β -estradiol on common carp *Cyprinus carpio* produced similar results. The exact mechanisms by which APEOs alter the growth rate and testicular development of fish are currently unknown.

The sex ratio of the progeny of viviparous eelpout *Zoarces viviparus* exhibit a substantial bias toward males when adults are exposed to pulp mill effluent. Larsson et al. (2000) suggest that endocrine disrupters may interfere with sexual differentiation and thereby affect the reproductive potential of fish populations.

As pointed out by Sumpter (1998), the consequences of endocrine disruption at the population level are unknown. Direct evidence of the impact that endocrine-disrupting compounds have on the health and reproduction of wildlife populations is scarce. To date, most studies of endocrine disruption in wildlife have implicated these compounds in the impairment of homeostatic or reproductive function in individuals rather than showing a direct linkage between sex reversal and health or disease in a population. This is particularly problematic for a taxon such as fish, which exhibit remarkable plasticity in phenotype and reproductive output in response to changes in their aquatic environments. Most of the evidence of endocrine disruption in fish points to an intersex condition rather than to sex reversal per se. Until we know whether intersex fish can produce gametes, whether those gametes can be released, and whether they are viable, the population-level impact of intersexuality cannot be quantified (Sumpter 1998). In addition to the physiological and reproductive impacts of endocrine-disrupting compounds, there could be disturbances to the secondary sexual characteristics of fish that could have repercussions on mating efficiency or sexual selection and thus on the evolution of populations (Jalabert et al. 2000).

Resource management decisions regarding chinook salmon require knowledge of the numbers of males and females returning to spawn each season. Combined with other information, these data are used to make predictions of future population abundance, which influences commercial and sportfishing regulations. A high frequency of sex-reversed males prevents accurate appraisal of future abundance, especially if their external secondary sexual characteristics and gonads are visually indistinguishable from those of genetic female fish. A systematic survey to monitor genotypes and gonad phenotypes in both wild and hatchery populations over time may shed light on whether this phenomenon is being perpetuated or selected against in hatchery populations of chinook salmon in the Central Valley. Accurate appraisal of its geographic scope within the Central Valley would assist in identifying the degree to which populations are impacted by sex reversal

and provide a means of narrowing the list of compounds that are potential endocrine disruptors. Such mapping would also identify activities designed to increase the abundance of chinook salmon in individual tributaries that may be hindered by this reproductive and genetic stressor. Similarly, if there are differences in the frequency of sex reversal between the seasonal runs of chinook salmon in the Central Valley, studying them may aid in identifying the factors contributing to such reversal.

Acknowledgments

This study was made possible by a scholarship from the Marin Rod and Gun Club. We thank J. Navicky and associates of the California Department of Fish and Game's Rancho Cordova Salmonid Tissue Archive for making samples available and J. Cordes for editorial review.

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