Genetic Analysis of Klamath River Green Sturgeon

(Acipensar medirostris)

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June 2000

Funded By:

Klamath River Basin Conservation Area Fishery Restoration Program

Abstract

The utility of using isozyme analysis to study the stock structure among West Coast populations of green sturgeon (*Acipenser medirostris*) was assessed. Isozyme analysis was not determined to be an adequate method for assessing stock structure in green sturgeon for the following reasons: 1) the complex nature of isozyme expression 2) the relatively poor resolution of enzyme systems for our samples 3) the invasive nature of collecting tissue samples for isozyme analysis, which necessitates sacrificing the fish, precludes collection of samples from some rivers.

The stomach contents of 23 green sturgeon collected from fish harvested in the Klamath River were analyzed. Only four stomachs contained identifiable food items, with the others containing food far too digested for identification, gravel, or no contents. Two stomachs contained the small gastropod, *Olivella pyna*, one stomach contained the carapace remains of a female Dungeness crab, *Cancer magister*, and one stomach contained the posterior portions of three ammocoetes, *Lampetra tridentata.*

Preliminary analysis was conducted using mtDNA to assess the potential of using this technique for assessing the stock structure of green sturgeon. Although the Klamath River Fishery Restoration Program did not fund this analysis, the results are presented because this work was designed to yield complementary information to the isozyme study. No diagnostic differences were detected between samples collected from the Klamath River and the Columbia River estuary using Restriction Fragment Length Polymorphism analysis. Study results indicated that microsatellite DNA analysis might be the most appropriate current genetic technique for assessing the population structure of green sturgeon.

Introduction.

Quantitative assessments regarding abundance trends for green sturgeon within the Klamath River, and along the Pacific Coast, are not available. Harvest estimates along the Pacific Coast have generally declined over the past 15 years (Appendix A), however changes in fisheries management (such as reduced seasons, bag limits, and size limit ranges) confound the use of this harvest information as an indication of population status. Concern regarding the status of green sturgeon prompted the U.S. Fish and Wildlife Service (USFWS) and the Klamath River Fisheries Assessment Program to designate it as a high priority species for restoration efforts in the late 1980s (USFWS 1989). More recently, due to concern that green sturgeon abundance has decreased and the continued degradation of freshwater habitat, it has been recommended that green sturgeon be formally listed as a threatened species (Moyle et al. 1992). In spite of these concerns, green sturgeon have been poorly studied and little is known about the habitat requirements, life history, and stock structure of this species.

Fisheries that harvests green sturgeon along the Pacific Coast include the following: sport and commercial fisheries in the Columbia River, Willapa Bay, and Grays Harbor; a sport fishery along the Oregon Coast; trawl fisheries along the Oregon and Washington coasts, and Tribal fishereis in Grays Harbor and the Klamath River. The largest fisheries for green sturgeon in North America consist of commercial fisheries in the Columbia River region (Columbia River estuary, Grays Harbor, Willapa Bay) where an average of 4216 fish were taken annually from 1985 to 1999 (Appendix A). Within the Klamath-Trinity River Basin, green sturgeon are harvested by Indian gill net fishereis, with the Yurok and Hoopa Valley Tribes harvesting an annual average of 265 and 16 respectively from 1985 to 1999 (Appendix A).

Green sturgeon taken in fisheries along the Pacific coast are managed on a regional basis with no understanding of their population structure. This is of major concern to fisheries scientists because, in theory, managing a fish population for optimum yield requires that each stock be identified and managed separately. Of special concern is that no spawning adults have been documented from the Columbia River system, suggesting that sturgeon harvested in this area may be originating in the Klamath or Sacramento Rivers of California (Moyle et al. 1992). We initiated a genetic study, using standard protein electrophoretic techniques and mitochondrial DNA (mtDNA) analysis to study the stock structure of green sturgeon from the Sacramento, Klamath, and Columbia river systems. Genetic information on the stock structure of green sturgeon is critical for the successful management of this species.

Methods

Stomach Content Analysis

While monitoring the Yurok Tribal spring fishery, tribal technicians collected stomachs from green sturgeon that had been harvested by tribal fishers between May and July 1995. These stomachs were subsequently frozen and transferred to the Humboldt State University Marine Laboratory for analysis of contents. Prey items identified from the stomach contents were identified to species.

Genetic Studies –

Tissue Sample Collection

Eye, liver, muscle, and heart tissue samples were collected from green sturgeon from the Columbia River estuary, lower 44 miles of the Klamath River, and the Sacramento River. As soon as samples were removed from the specimens, they were frozen with dry ice and transferred to the Humboldt State University, where they were stored in an ultrafreezer (-80^{0}C).

Yurok Tribal Fisheries Program (YTFP) staff collected tissue samples from green sturgeon that were harvested as bycatch during a commercial fishery that targets white sturgeon in the Columbia River estuary during August, 1995. YTFP staff also collected tissue samples from green sturgeon that were harvested by tribal members on the Yurok Reservation during the spring and early summer of 1995.

In March 1998, tissue samples were obtained from 11 green sturgeon of Sacramento River origin from the University of California at Davis aquaculture facility. These fish were collected in 1995 as juveniles from an entrainment screen on the Sacramento River and were subsequently maintained in a culture facility at the University of California at Davis. The fish from which the tissue samples were collected were mortalities at the aquaculture facility. These fish had been dead for an uncertain period of time prior to collection, and had been frozen for an extended period of time in a –20^{0}C freezer prior to being transferred to Humboldt State University.

Isozyme Analysis

The screening phase of the isozyme analysis was carried out using the standard methodology of horizontal starch gel electrophoresis (Abersold et al. 1987, Morizot and Schmidt 1990). Following electrophoresis the gels were sliced and enzymes were visualized using histochemical staining. All stains in this study were applied to the gel as agar overlays. After incubation, staining patterns were scored by hand and in some instances photographed using a Polaroid FCR10 gel visualization system.

Preliminary analysis involved screening small numbers of specimens from the Columbia River and Klamath River samples. During this phase of the study enzymes and buffer systems that potentially had discriminatory value were identified. During the preliminary analysis 28 enzymes were screened on three buffer systems (Table 1). These buffer systems included: I. Amine citrate, (pH 6.1) (modified from Clayton and Tretiak 1972); II. Lithium hydroxide, Borate (pH 8.0)/Tris, Citrate (pH 8.2) (modified from Ridgeway et al. 1970); and III. Tris Citrate (pH 7.0) (Shaw and Prasad 1970). Enzymes that were not resolved adequately using these three buffers were tested on one or more additional buffer systems. These additional buffers included: IV. Amine citrate (pH 6.8) (modified from Clayton and Tretiak 1972); ; V. Tris EDTA borate (pH 8.6) (Markert and Faulhaber 1965); and VI. Tris EDTA Citrate Borate (pH 8.7) (Shaklee and Tamaru 1981).

The second phase of the isozyme analysis was carried out on an increased number of specimens from the Columbia River and Klamath River. An abbreviated screening was carried out on a small number of fish of Sacramento River origin.

Results and Discussion

Stomach Content Analysis

A total of 23 green sturgeon stomachs were collected from fish harvested within lower 44 miles of the Klamath River between May and July 1995. Only four stomachs contained food items. The other stomachs contained food far too digested for identification, gravel, or were completely empty. Stomachs of two males from the mid-Klamath (Highway 101 Bridge to Surpur Creek), one 159 cm FL, and one, 142 cm FL, contained three and six individuals respectively of the small gastropod, *Olivella pyna*. The stomach from a 168 cm male from the upper Klamath (upstream of Surpur Creek) contained the carapace remains of a female Dungeness crab, *Cancer magister*. Finally an individual from the mid-Klamath, 181 cm FL, had the posterior portions of three ammocoetes, presumably larvae of the Pacific lamprey, *Lampetra tridentata*.

Genetic Studies - Isozyme Analysis

The results of preliminary screening indicated that fifteen enzymes had potential for use in the isozyme analysis (Table 1). Two of these enzymes (MDH and AK) were tentatively identified as potential markers for discriminating between Klamath River and Columbia River fish. Several difficulties that were encountered during this phase of the analysis are discussed below. The second phase of the isozyme analysis presented additional problems that are also discussed below.

There were two general areas of concern with regard to this project: (1) complexities of the actual isozyme analysis, and (2) difficulties in acquiring adequate samples from all populations of interest. The following discussion pertains to each of these areas of concern, explaining how

we attempted to resolve these problems. In addition, we summarize the preliminary green sturgeon mtDNA work that we carried out in conjunction with this isozyme study, and suggest possibilities for future genetic work with green sturgeon.

(1) **Analysis of tissue samples**

Green sturgeon isozymes proved to be much more difficult to successfully resolve than other fish that we processed during this same time period such as rock sole, *Pleuronectes bilineatus*, California halibut, *Paralichthys californicus*, and rainbow trout, *Oncorhynchus mykiss*. This difficulty results from a variety of factors. Overall, isozymes from our green sturgeon samples were characterized by major imperfect resolution subsequent to staining as well as extensive sub-banding (Richardson et al., 1986). Strength of staining also varied dramatically in some instances, both between individual specimens and between river systems. It is possible that these problems were due to improper collection or handling of the samples. This especially might be expected in the Klamath River samples because the samples were taken and transported (on dry ice) to final storage in the ultrafreezer in a discontinuous fashion, under difficult field conditions, and over a long period of time. For example, alcohol dehydrogenase, an enzyme noted to be particularly susceptible to age/treatment related modifications, including decreased staining intensity (Richardson, et al., 1986), was never visualized in any specimen from any sampling site, even though a variety of staining methods were used. Although this factor probably explains some of the variation in expression and resolution that was noted, it is unlikely that it explains many of the problems. For example, inter-specimen variation did not appear consistent across enzyme systems as would be expected (Richardson, et al., 1986) if samples had been improperly handled. Warping, which can be an indication of improper sample handling, was not a

problem. Implementation of a pre-slicing methodology (modified from Murphy et al. (1996)) toward the end of the period of preliminary analysis was the most successful procedural modification and resulted in improved staining of some previously poorly resolved systems.

Another problem was the high lipid content of many of the tissue samples. This can cause smearing and poor resolution of many enzymes. Sample loading was therefore carried out using a hypodermic to draw up the sample supernatant, rather than dipping the wick in the sample. This procedure appeared to overcome the lipid problem, because the same degree of resolution was achieved in both Klamath River (extremely high lipid content) and Columbia River (extremely low lipid content) heart and liver samples. Centrifugation would not adequately separate the lipid in muscle samples. Very poor resolution was characteristic of almost all muscle enzymes. However, this problem occurred in both the high lipid (Klamath River) and low lipid (Columbia River) muscle samples and was probably not totally attributable to lipid contamination. Chloroform extraction was not attempted.

Essentially all of the enzyme systems that were tested appeared to be comprised of multiple loci in our green sturgeon samples. It is common for many loci to be duplicated in fishes. However, this tendency is expected to be magnified in the green sturgeon, presumably because sturgeons are polyploid (Buth 1983). Multiple loci are potentially an advantage in isozyme analysis because the potential for variable loci is increased, however it also greatly increases the level of difficulty of zymogram interpretation. While this latter factor was anticipated, the complexity of gene expression coupled with the above-mentioned resolution difficulties necessitated a much more extensive screening process than is normally required and in the end resulted in an inability to resolve a sufficient number of loci.

(2) **Sample availability**

Sufficient samples of green sturgeon that included all tissue types necessary for the isozyme analysis were obtained only from the Klamath (38 specimens) and Columbia (39 specimens) Rivers. Ideally, in order to identify the relationship of the Columbia River fish to sturgeon spawning in other rivers along the West Coast, samples are needed from all river systems where green sturgeon spawn. In March1998, we obtained tissue samples from 11 green sturgeon of Sacramento River origin from the University of California at Davis aquaculture facility. These fish were collected in 1995 as juveniles from an entrainment screen on the Sacramento River and were subsequently maintained in a culture facility at the University of California at Davis. The fish from which we were able to collect tissue samples were mortalities at the aquaculture facility, had been dead for an uncertain period of time at collection, and had been frozen for an extended period of time in a -20°C freezer. UC Davis personnel were unwilling to have tissue samples taken from the remaining live fish and, in any case, we could only have collected muscle plugs from living specimens. As mentioned above, muscle enzymes resolved very poorly in our green sturgeon samples. Limited analysis of the samples taken from the mortalities indicated that the samples were not of sufficient quality for electrophoretic analysis. We also obtained muscle plugs from 30 green sturgeon from Humboldt Bay. We did not use these samples for isozyme analysis because no loci were clearly resolved in muscle tissue from the Klamath and Columbia River samples.

Genetic Studies - Mitochondrial DNA (mtDNA) Analysis

Although the mtDNA analysis was not part of the work funded by the grant from the Klamath River Fishery Restoration Program, we are reporting the results of this work because the two studies are so closely linked and because they were designed to yield complementary information.

In order to identify stocks of west coast green sturgeon, we used the Polymerase Chain Reaction (PCR) followed by Restriction Fragment Length Polymorphism (RFLP) analysis and/or direct sequencing to examine the D-loop region of the green sturgeon mtDNA molecule. The D-loop or control region of the vertebrate mtDNA molecule is the most variable region of that molecule and is generally thought to be a suitable region for studies of intraspecific genetic variation (Avise 1994). PCR is a technique that allows amplification of picogram amounts of DNA in order to generate relatively large quantities of DNA that can then be subjected to discriminatory procedures such as RFLP analysis. The primary advantage of this process relative to isozyme analysis is that minimal amounts of tissue are needed, thereby avoiding invasive sampling techniques and the need to sacrifice animals. Given the concern regarding the present status of sturgeon populations world wide, this may be a decisive advantage. However, PCR based analyses have the disadvantage of surveying a much smaller portion of the genome than isozyme analysis and therefore may yield a lesser amount of genetic information.

We used primers based on white sturgeon, *A.transmontanus*, D-loop mtDNA sequences (Brown et al. 1993) to amplify a 597 base pair (bp) fragment from the D-loop region of 35 Klamath River and 25 Columbia River green sturgeon. We performed a RFLP analysis on these samples using nine restriction endonucleases: AluI, AseI, AvaII, BsrI, MspI, PflMI, RsaI, XbaI,

and XmnI. Based on the sole published sequence of this region obtained from a single green sturgeon (Brown et al. 1993), we assumed all of these enzymes should have a restriction site within the D-loop region in question and this proved to be true. This suite of restriction enzymes allowed us to directly survey about 10% of the target sequence. The RFLP analysis identified only one potential polymorphism (an additional RsaI site) detected in a single Klamath River green sturgeon. No diagnostic differences between rivers were detected.

In May, 1997 we also initiated sequencing of this same region of the D-loop in a subsample of fish from the Columbia and Klamath Rivers. Our initial attempts to sequence this region did not yield good results. This was not particularly surprising because protocols had not previously been worked out in our lab for this species. The funding for this DNA work was only for preliminary analyses, designed to determine if pursuing further funding was desirable. We decided not to attempt to pursue further funding to extend this work for reasons discussed in the following summary.

Genetic Studies - Summary and Recommendations for Future Investigations

Isozyme analysis, restriction analysis, sequencing in conjunction with some type of fragment analysis, and single locus mini- or microsatellites analysis are the only methods designated as appropriate and effective methods of studying genetic variation at the level of population subdivision and geographic variation within a species by Hillis et al.(1996). However, based on our work, we do not recommend isozyme analysis as a feasible method for addressing stock structure questions in green sturgeon. This decision is based on the two areas of concern discussed above. Our work indicated that the complex nature of isozyme expression combined with the relatively poor resolution of enzyme systems from our samples make protein electrophoresis a poor method to use with this species. Perhaps most importantly, isozyme studies involve invasive tissue sampling that necessitates the sacrifice of adult or juvenile green sturgeon. Our initial goal was to obtain samples of 50 specimens from each river of interest. However, we were unable to obtain adequate samples from either the Rogue or the Sacramento Rivers. Our samples from the Columbia (39 specimens) and the Klamath River (38 specimens) were available only as bycatch from a white sturgeon commercial fishery and a Native American subsistence fishery, respectively. Concern regarding the present status of these fish may preclude obtaining more samples for continued isozyme analysis in the future.

Although we do not recommend isozyme analysis as a feasible method for addressing stock structure questions in green sturgeon, we are seeking a small amount of inhouse money to continue a limited isozyme analysis on a limited number of loci that appeared to be of interest, in particular MDH, AK, and CK. This may be unsuccessful, especially given the present age of the samples, and, in any case would be an attempt to answer questions about the nature of the

particular loci and almost certainly would not be of use in answering the questions about green sturgeon stock structure that are the goal of this project.

These points indicate that some form of DNA analysis would be a preferable method. When using PCR in conjunction with the chosen method of DNA analysis, adequate samples can be obtained in a noninvasive manner that does not require sacrificing the animals. The three most appropriate methods of DNA analysis for the level of population structure that we are concerned with are RFLP analysis and direct DNA sequencing, which we used in our DNA work, and mini- or microsatellite analysis. We detected extremely low variability in our RFLP analysis. Continued sequencing would probably detect more variation. Although sequencing is considerably more expensive than the RFLP analysis, it has a higher probability for detecting population structure, as it directly surveys 100% of the target sequence. Direct sequencing of the D-loop of white sturgeon from the Columbia and Fraser Rivers indicated some degree of population subdivision existed (Brown et al. 1993), while restriction enzyme analyses of the entire mtDNA molecule showed little geographic subdivision (Brown et al. 1992).

Our DNA work was very limited and designed only to indicate the potential value of applying these techniques to the analysis of green sturgeon stock structure. While they appear to be viable techniques, we decided not to pursue further funding to expand this work. Our suggestion as to the most promising technique to use for future investigations of this species would be the last of the above mentioned non-invasive DNA techniques, microsatellite analysis. Tremendous advances have been made in the application of this technique in the last few years. Identifying microsatellite loci and designing primers are the most difficult portion of this technique (Strassman et al. 1996). May et al., (1997) identified 11 microsatellite loci in lake sturgeon (*Ascipenser fulvescens)*, 10 of which produced electrophoretically resolvable products in green

sturgeon. This groundwork, coupled with the advantages of noninvasive sample collection, cost effectiveness, portion of the genome surveyed, and the applicability of the this method to population level genetic analysis, lead us to suggest that microsatellite analysis is the most appropriate technique to use at this time in further genetic analysis of green sturgeon.

Table 1. Enzyme systems utilized in isozyme analysis of green sturgeon, *Acipenser medirostris*, from the Klamath and Columbia Rivers. Buffer systems (I - IV) are those that give successful resolution of at least one locus of a given enzyme, - indicates insufficient resolution. Buffer systems are defined in text.

Enzyme		EC Number	Buffer System
Aconitate hydratase	AH	4.2.1.3	-
Adenosine deaminase	ADA	3.5.4.4	-
Adenylate kinase	AK	2.7.4.3	I
Alcohol dehydrogenase	ADH	1.1.1.1	-
Aspartate aminotransferase	AAT	2.6.1.1	-
Creatine Kinase	CK	2.7.3.2	II
Fructose-bisphosphate aldolase	FBALD	4.1.2.13	-
Fumarate hydratase	FH	4.2.1.2	-
Glucoseposphate isomerase	GPI	5.3.1.9	II
Glutathione reductase	GR	1.6.4.2	-
Glyceraldehyde-3-phosphate dehydrogenase	GAPD	1.2.1.12	IV
Glycerol-3-phosphate dehydrogenase	GPD	1.1.1.8	III
Hexokinase	HK	2.7.1.1	-
Isocitrate dehydrogenase	IDH	1.1.1.42	I/III
L-iditol dehydrogenase	IDDH	1.1.1.14	-
Lactate dehydrogenase	LDH	1.1.1.27	III
Malate dehydrogenase	MDH	1.1.1.37	IV
Malic Enzyme ($NADP^+$ dependent MDH)	ME	1.1.1.40	IV
Mannose 1 phosphate isomerase	MPI	5.3.1.8	-
Nucleoside phosphorylase	NP	2.4.2.1	-
Peptidase D	PEPD		-
Peptidase A	PEPA		-
Peptidase B	PEPB	3.4.	II
Phosphoglucomutase	PGM	5.4.2.2	IV/III
Phosphogluconate dehydrogenase	PGD	1.1.1.44	IV/III
Phosphoglycerate kinase	PGK	2.7.2.3	-
Superoxide dismutase	SOD	1.15.1.1	I
Triosephosphate isomerase	TPI	5.3.1.1	IV

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Appendix A. Green sturgeon harvest along the Pacific Coast (distributed at a green sturgeon workshop held in Weitchpec, CA, March 2000.

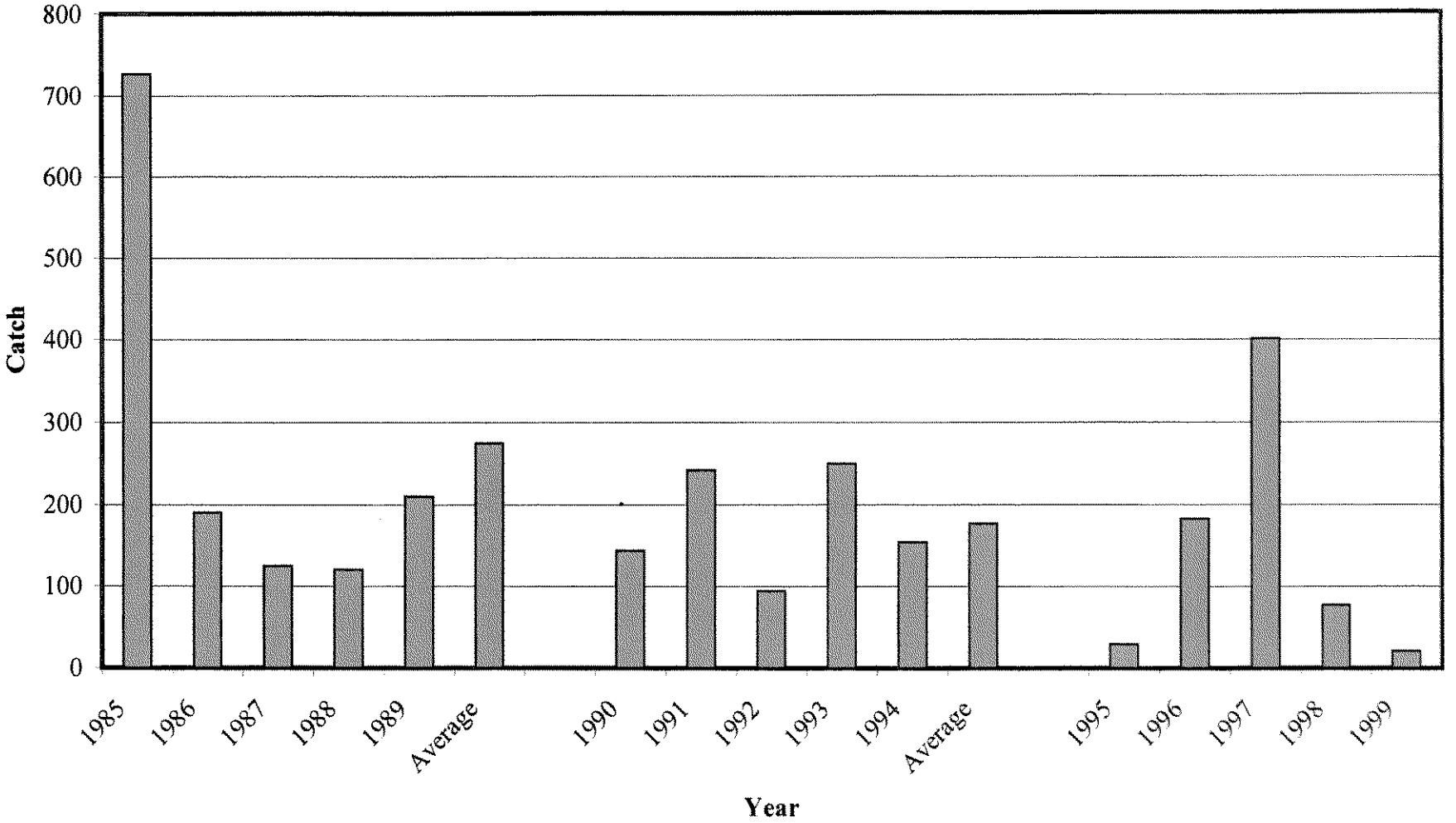
Green Sturgeon Catches[1]

1985-1999

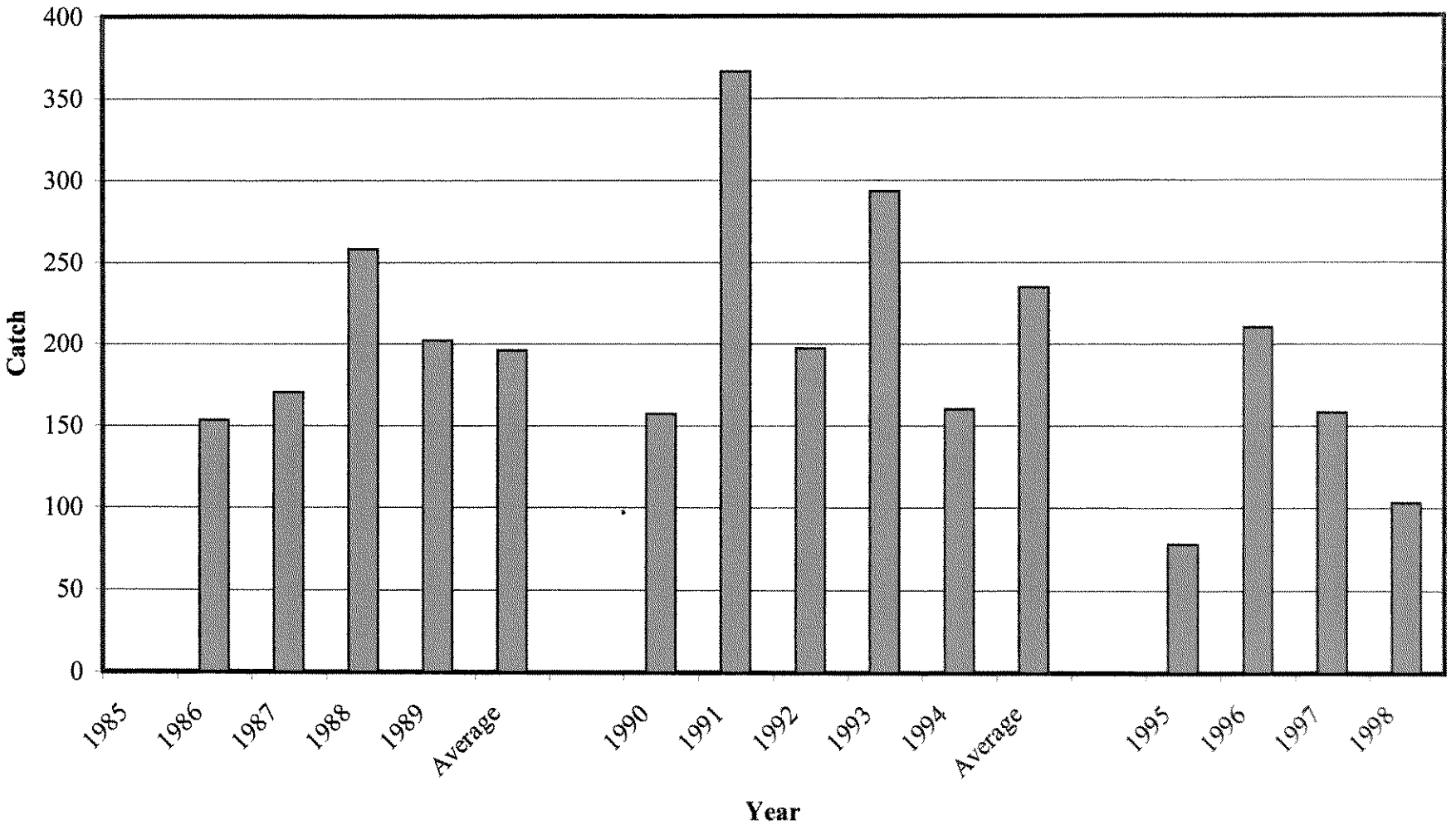
	Oregon Coastal		Columbia River OR and WA		Willapa Bay		Grays Harbor/Chehalis River			Wash. Ocean	Klamath River			SF Bay/ Sac/SJ River	
Year	Sport	Trawl	Sport	Comm.	Sport	Comm.	Sport	Comm.	Tribal	Trawl	Yurok	Hoopa	Sport	Sport	Total
1985	N/A	726	533	1,600	N/A	1,289	N/A	236	5	359	351	10	Few	Few	5,109 +
1986	153	190	407	6,000	N/A	921	N/A	635	3	123	421	30	Few	Few	8,883 +
1987	170	124	228	4,900	N/A	877	N/A	781	5	65	171	20	Few	Few	7,341 +
1988	258	120	141	3,300	1	1,599	4	610	1	37	212	20	Few	Few	6,303
1989	202	210	84	1,700	4	465	12	872	2	128	268	30	Few	Few	3,977
Average	196	274	279	3,500	3	1,030	8	627	3	142	285	22	N/A	N/A	6,323 +
1990	157	143	86	2,200	2	954	4	743	9	61	242	20	Few	Few	4,621
1991	366	242	22	3,190	0	922	0	1,530	3	16	312	13	Few	Few	6,616
1992	197	94	73	2,160	0	1,002	0	740	3	2	212	3	Closed	Few	4,486
1993	293	250	15	2,220	32	320	112	545	3	2	417	10	Closed	Few	4,219
1994	160	154	132	240	13	300	25	39	22	1	293	14	Closed	Few	1,393
Average	235	176	66	2,002	9	700	28	719	8	16	295	12	N/A	N/A	4,267
1995	78	29	21	390	12	302	92	559	185	2	131	2	Closed	Few	1,803
1996	210	182	63	610	24	129	71	290	153	1	119	17	Closed	Few	1,869
1997	158	400	41	1,614	4	16	117	316	197	5	306	7	Closed	Few	3,181
1998	103	77	73	894	12	65	29	25	53	0	335	10	Closed	Few	1,676
1999	N/A	21	93	1,362	N/A	9	N/A	14	56	3	184	27	Closed	Few	1,769 +
Average	137	142	58	974	13	104	77	241	129	2	215	13	N/A	N/A	2,060 +
Grand Average	193	197	134	2,159	9	611	42	529	47	54	265	16	N/A	N/A	4,216 +

[1] Catch estimates are generally not good indicators of abundance or population status trends because of changes in management practices over time. Most green sturgeon catch occurs incidentally to other species and is subject to fishery constraints associated with abundance and allocation issues for the target species.

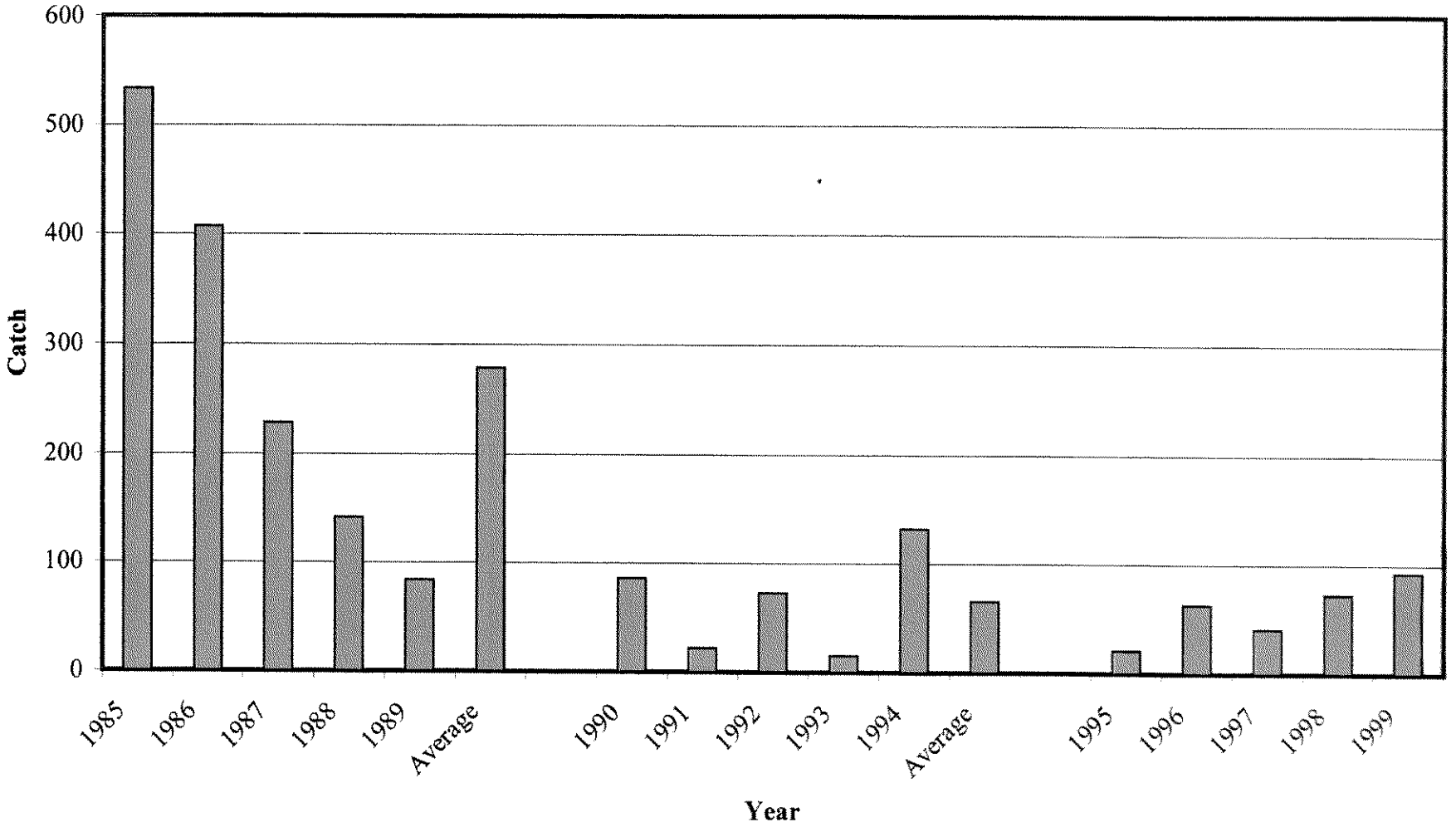
Green Sturgeon Catch
Oregon Coast Trawl



Green Sturgeon Catch
Oregon Coast Sport



Green Sturgeon Catch
Columbia River Sport



Green Sturgeon Catch
Klamath River Yurok Tribal