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Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams

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Abstract

Intersex white suckers (*Catostomus commersoni*) were collected in Boulder Creek and the South Platte River downstream of wastewater treatment plant (WWTP) effluent but not at reference sites. Eighty-three percent of the 60 white suckers collected downstream of the Boulder WWTP in spring and fall were female compared to 45% upstream ($n=33$). Only female ($n=16$) and intersex white suckers ($n=4$) were collected in the South Platte River downstream of the Denver WWTP in the spring. Gonad deformities and delayed follicular maturation were noted in some white suckers in Boulder Creek and the South Platte River downstream of the WWTP effluents. Asynchronous ovarian development was found in some female white suckers downstream of the WWTP effluents, but not upstream. The types and extent of sexual disruption differed in each river studied.

Keywords: Intersex; Fishes; Reproductive disruption; White sucker

1. Introduction

Intersex fishes have been documented downstream of wastewater treatment plant (WWTP) effluents in a number of European countries, including the United Kingdom (Jobling et al., 1998, 2002; Nolan et al., 2001; Van Aerle et al., 2001), Italy (Viganò et al., 2001), and Spain (Solé et al., 2003). The gonads of intersex fishes in Europe exhibited feminization downstream of WWTP effluents ranging from the presence of only a few oocytes among normal testicular tissue (Nolan et al., 2001) to a level where gonads were an approximately equal mix of ovarian and testicular tissue (Van Aerle et al., 2001). In addition, ovarian-like cavities developed in the gonads of one species (Nolan et al., 2001).

WWTP effluents contain steroidal estrogens, estrogenic alkylphenols, and other compounds known to induce intersex in some fish (Länge et al., 2001; Murphy et al., 2003; Jobling et al., 2002). Flows in some rivers on the eastern plains of Colorado are dominated by WWTP effluent discharges. Streams and rivers in this relatively arid region are small and widely separated. The effluents from WWTP serving large metropolitan areas make up a substantial proportion of stream flow downstream of the various effluents. For example, Boulder Creek streamflow, just downstream of the Boulder WWTP effluent, ranges from 2 m^3/s during low flow conditions to 8 m^3/s during high flow conditions. During low flow conditions, effluent can contribute up to 77% of total streamflow (Murphy et al., 2003). The average daily flow of the mainstem South Platte River is 15.8 m^3/s downstream of the Denver Metropolitan Sewage District WWTP effluent with an average effluent flow of 6.5 m^3/s, or 41% of total streamflow; during low flow conditions effluent can comprise 90% of total streamflow (T. Harris, Denver Metro Sanitation District, pers. comm.). Compounds known to induce an intersex condition in fish have been measured in Boulder Creek (Barber L. pers. comm.; Murphy et al., 2003) and the South Platte River in Colorado, USA (Kolpin et al., 2002), and other waters throughout the USA. Thus, the conditions needed to induce intersex fishes or other forms of reproductive disruption exist in some Colorado

Abbreviations: mm, millimeters; n, number; m^3/s, cubic meters per second; km, kilometers; µm, micrometers.

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waters. The presence of the intersex condition could influence the reproduction, distribution, and abundance of Colorado native fishes as described for wild fish populations in the United Kingdom (Jobling et al., 2002; Kime, 1998).

The primary objective of this study was to determine if reproductive disruption was present in waters of the Central Shortgrass Prairie Ecoregion in Colorado, USA in effluent dominated stream segments where a large proportion of flow is composed of domestic WWTP effluent. A secondary objective was to determine if other forms of reproductive disruption were present such as a skewed sex ratio, a change in ovarian development in female fish or an altered growth rate.

2. Materials and methods

We sampled white suckers (*Catostomus commersoni*) in two waters on the eastern plains of Colorado at locations downstream of WWTP effluents, Boulder Creek downstream of the city of Boulder WWTP, and the South Platte River downstream of the Denver Metropolitan Sewage District WWTP. The white sucker is a long-lived species that widely inhabits stream reaches upstream and downstream of WWTP effluents throughout Colorado.

Treated WWTP effluent contributes to a relatively high proportion of stream flow in the chosen stream reaches. Individual sample sites were selected based on legal access and the presence of heterogeneous habitat. Boulder Creek was sampled at a point approximately 50 m downstream of the Boulder WWTP. The mainstem South Platte was sampled 3 km downstream of the Denver Metropolitan effluent. Selection of reference sites was complicated by two factors. Pristine comparison sites were not available upstream of the two WWTPs at locations inhabited by the target species. Both waters receive nonpoint urban stormwater runoff and agricultural return flows upstream of the WWTPs chosen for study. In addition, several WWTPs discharge to the mainstem South Platte at points upstream of the Denver Metropolitan WWTP. Reference sites were selected based on a comparatively close proximity to study sites and locations where the proportion of WWTP effluent flows made up a lower proportion of stream flow than the study sites. A stream reach beginning 2 km upstream of the Boulder, Colorado WWTP was used as a Boulder Creek reference site. Clear Creek enters the mainstem South Platte River about 100 m downstream of the sampling location where fish were collected for the current study. A site on Clear Creek 6.5 km upstream of the confluence with the South Platte was chosen as a reference site for the Denver Metropolitan WWTP.

Fish were collected using electrofishing equipment with a pulsed DC current in March and May of 2002. A second set of samples was collected from Boulder Creek in October and November 2002. Fish collection and handling procedures were approved by the IACUC of the University of Colorado. All macrohabitats were sampled in the South Platte River and Boulder Creek including riffles, runs, pools, eddies and backwaters to eliminate the possibility of sampling error. All sizes of white suckers were collected. Fish were anesthetized with MS 222, weighed to the nearest 1 g and measured for total length to the nearest mm. Gonads were removed and preserved in 10% buffered formalin. Relative size and color of gonads were noted and any abnormalities described. Scales were taken from below the dorsal fin at most sample sites to get an approximate estimate of the age of smaller white suckers. Scales can be used to age white suckers less than 5 years of age with a high degree of accuracy but the age of white suckers older than 5 years may be underestimated by this technique (Beamish, 1973).

Small portions from the head, middle and caudal sections of large gonads were embedded in paraffin for histological analysis. When gonads were small enough, the entire organ was mounted and sectioned laterally. Five-micrometer sections were mounted on microscope slides and stained using hematoxylin and eosin (Presnell and Schreibman, 1997). Gonads were examined under a light microscope to determine the sex, stage, and pattern (synchronous or asynchronous), of sexual maturation of each individual. Assessment of gonadal development was based on Blazer (2002) using the mounted sections of gonads. Group synchronous ovarian development is characteristic of many seasonal breeders whereas asynchronous development is observed in fishes that are multiple spawners (Blazer, 2002). Six stages of ovarian development were identified including three pre-vitellogenic stages (1. Chromatin nucleolar, 2. Chromatin perinucleolar, and 3. Corpora alveolar) and three vitellogenic stages (4. Early vitellogenic, 5. Mid-vitellogenic, and 6. Mature). Ovarian development was classified as group synchronous if only two types of oocytes were observed in one gonad, including a pre-vitellogenic stage. Ovarian development was classified as asynchronous if three, or more, oocyte stages were observed in each ovary. Testis development was evaluated based on relative proportion of spermatocytes, spermatids and spermatozoa using the mounted gonad sections. The four stages of testis development identified were: 1. Pre-spermatogenic, 2. Early spermatogenetic, 3. Mid-spermatogenetic, and 4. Late spermatogenetic. A standard weight (W_s) equation (Bister et al., 2000) was used to calculate the relative weight (W_r) for all white suckers greater than 100 mm total length. The mean W_r for each site and appropriate reference site was compared using a *t* test for unequal *n*'s (Steel and Torrie, 1980).

3. Results

3.1. Boulder Creek, spring

The spring 2002 Boulder Creek sampling resulted in the collection of 21 white suckers downstream of the Boulder WWTP (Table 1). The sex ratio was skewed strongly towards females ($n=20$) with only one male collected. The mean total length of the female white suckers was 292 mm (range 237 mm to 334 mm). The only male white sucker collected was smaller (total length 225 mm). The six smallest fish, including the male, were more than three years old.

The testes of the single male white sucker were fibrotic with only small areas of spermatogenetic activity. Twelve female white suckers exhibited group synchronous ovarian development, with follicles in the pre-vitellogenic and mid-vitellogenic stages. Seven female white suckers exhibited asynchronous ovarian development. The ovaries of one female white sucker contained only malformed pre-vitellogenic oocytes. Two females of the

Table 1
Number of fish collected at sites and numbers of intersex fish

Municipal WWTP	River and sampling site	Sample date	Number of fish	Females	Males	Intersex fish	Unknown
City of Boulder WWTP	Boulder Creek Upstream of Boulder WWTP*	3/12/02	13	8	5	0	0
		11/04/02	20	10	9	0	1
	Downstream of Boulder WWTP	3/12/02	21	20	1	0	0
		10/17/02	39	29	4	4***	1
Denver Metro WWTP	Clear Creek Upstream of South Platte River*	5/10/02	6	4	2	0	0
	South Platte River Downstream of Denver Metro WWTP	5/07/02	20	16	0	4**	0

* = Reference site. ** = appear to be testes containing ovarian tissue. *** = may be ovaries containing testicular tissue or an incompletely transformed testis.

asynchronous ovarian development group also displayed asynchronous nodular maturation, where each ovary was composed of a strand of connective tissue with two or three isolated nodes of ovarian tissue in the middle of the organ.

Five male white suckers (38%) and seven female white suckers (62%) were collected in Boulder Creek at the reference site upstream of the Boulder, Colorado WWTP in April 2002 ($n=12$). The mean total length of the female white suckers was 347 mm (range 227 mm to 449 mm). Males were of a similar size, mean length 336 mm (range 265 mm to 387 mm). The mean white sucker relative weight (W_r) upstream of the Boulder WWTP effluent was similar to that of fish collected downstream in March and April 2002, 1.00 and 1.00, respectively ($p=0.98$).

Intersex fish were not found and gonad deformities were not observed in any of the white suckers collected upstream of the Boulder WWTP. Group synchronous ovarian development was observed in all seven female white suckers (Table 2). All males were stage 4, late spermatogenetic.

3.2. Boulder Creek, fall

Fall sampling of Boulder Creek resulted in the collection of 39 white suckers downstream of the Boulder WWTP. The sex ratio was again skewed towards females ($n=30$) with four males and four intersex fish. No identifiable gonad tissue was recovered from one fish. The mean total length of the female white suckers was 232 mm (range 127 mm to 367 mm). The males were smaller with a mean total length of 168 mm (range 145 mm to 230 mm).

Gonadal sex of three of the male fish was visually determined at time of dissection. The gonads of these three males were white in color and composed of a series of ovate lobes. Testes of the fourth fish were thin clear gelatinous tubes and were identified as male only after microscopic examination. The sexual maturation of the four males was mixed (two at stage 1 and two at stage 4). Three of the four males were one-and-a-half years of age based on analysis of scale annuli, while the age of the other male could not be determined.

Gonads from 15 female white suckers from the downstream site were randomly chosen for histological analyses. Group synchronous ovarian development was observed in five female white suckers (Fig. 2A, Table 2). Follicles were in an early vitellogenic stage of development in four of these five females and at a mid-vitellogenic level in the fifth female. Asynchronous ovarian development was observed in seven female white suckers (Fig. 2B). Various combinations of three or more stages of follicular growth were observed in the seven females that exhibited asynchronous ovarian development. The ovaries of these seven females were also disorganized and highly fibrotic. The three smallest females were one-and-a-half years of age with a mean total length of 133 mm (range 127 mm to 148 mm). The ovaries of the three smallest females appeared normal in appearance, all pre-vitellogenic.

Four of the white suckers were designated as intersex, with small numbers of spermatogenetic cysts scattered in disorganized, highly fibrotic tissue contained oogonia and malformed pre-vitellogenic oocytes (Fig. 1). These four intersex fish were two years of age, or older, ranging from 216 mm TL to 230 mm TL (mean = 225 mm).

Twenty white suckers were collected from the reference site upstream of the Boulder WWTP in November 2002, nine males and ten females. No gonadal tissue was recovered from one fish.

Table 2
Maturation stage white suckers in Boulder Creek, Clear Creek and South Platte River

Municipal WWTP	River and Sampling Site	Sample date	Maturation stage of females				Maturation stage of males			
			1	2	3	4	1	2	3	4
City of Boulder WWTP	Boulder Creek, Upstream of Boulder WWTP*	3/20/02	0	1	1	5	0	3	2	0
		11/04/02	2	0	7	1	0	4	6	9
	Boulder Creek, Downstream of Boulder WWTP	3/15/02	1	8	8	3	1	0	0	0
		10/17/02	8	7	4	0	2	0	0	2
Denver Metro WWTP	Clear Creek, Upstream of South Platte River*	5/10/02	0	0	0	4	0	0	2	0
	South Platte River, Downstream of Denver Metro WWTP	5/07/02	5	9	2	0	0	0	2	0

* = Reference site.

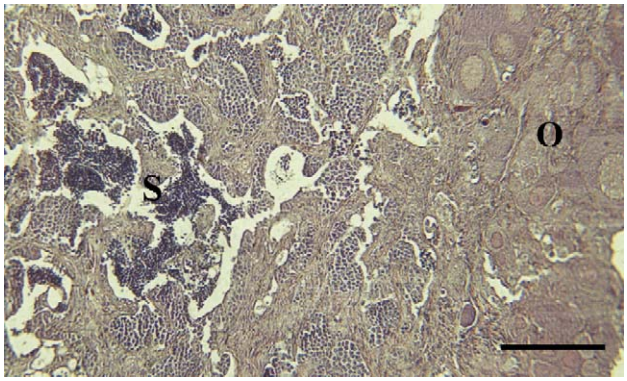

Fig. 1. Intersex gonad (25×) from below WWTP includes oocytes (O) and spermatogenetic tissue (S). Scale bar indicates 50 μm.

The mean total length of the females was 225 mm (range 200 mm to 253 mm). The males were of a similar size 215 mm (range 204 mm to 258 mm). The mean white sucker relative weight (W_r) upstream of the Boulder WWTP effluent was similar to that of fish collected downstream in November 2002, 0.83 and 0.80, respectively ($p = 0.244$).

No intersex fish were found upstream of the WWTP effluent in November 2002. Gonads of all fish were normal in appearance based on visual observations and histologic examination. The follicles of ten of the females were either early or mid-vitellogenic and all exhibited group synchronous ovarian development. Ovaries of one fish were undeveloped. The gonads of all nine males were stage 4, late spermatogenetic.

3.3. South Platte River

Twenty white suckers were collected in the mainstem South Platte River downstream of the Denver Metropolitan District WWTP effluent in May 2002 (Table 1). Four of these fish were intersex and the other 16 were female. The mean total length of the female South Platte white suckers was 192 mm (range 149 mm to 253 mm). The intersex fish were smaller with a mean total length of 157 mm (range 152 mm to 205 mm). Three of the intersex white suckers were about one-and-a-half years of age based on analysis of scale annuli. The other intersex fish was greater than 2 years of age. The age of the seven smallest females ranged from about one-and-a-half years of age to more than 3 years of age.

The gonads of all four intersex white suckers were thin clear gelatinous tubes less than 4 cm in length that were macroscopically identified as immature ovaries when the fish were first dissected. The extent of the intersex condition varied. In two fish the gonads were fibrotic with scattered oocytes in tissue that otherwise appeared as a regressed male gonad. In the two intersex fish, oocytes comprised 30% to 50% of the gonadal tissue examined while the remainder appeared to be testicular in various stages of spermatogenetic development. Group synchronous ovarian development was observed in eight female white suckers (Fig. 2A, Table 2). Follicles were pre-vitellogenic (chromatin nucleolar and perinucleolar) in seven of these females, while the follicles were pre- and mid-vitellogenic in the eighth female. Asynchronous ovarian development was present in seven females (Fig. 2B) and various combinations of three or more stages of developing oocytes were observed. The ovaries of one female appeared to be unstimulated and contained malformed oocytes and increased levels of connective tissue.

Six white suckers were collected at Clear Creek, the reference site for the South Platte River (2 males and 4 females). Intersex fish were not found, nor were any gonadal deformities noted. Group synchronous ovarian development was observed in all four females. The follicles of all females were classified as pre-vitellogenic and mid-vitellogenic in contrast to fish from the South Platte River, and the two males were likewise at a last stage of maturation (stage 3). The total lengths of the two male fish were 153 mm and 279 mm. The mean total length of the females was 337 mm (range 271 mm–434 mm). All of these fish were more than three years of age except the 153 mm male which was one-and-a-half years old.

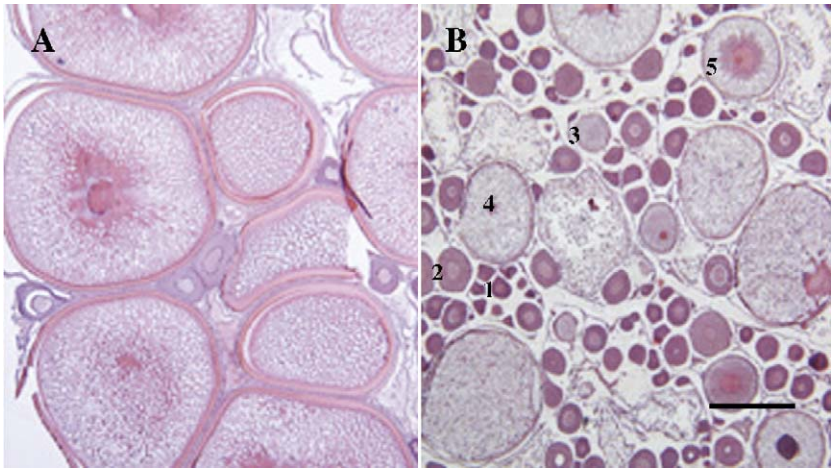

Fig. 2. Representative cross sections through white sucker ovaries. A. Reference ovary (4×). B. Asynchronously developing ovary from below WWTP (B-4×). Note multiple stages of oocyte development (1–5, see text). Scale bar indicates 200 μm.

4. Discussion

An initial survey of white sucker in Boulder Creek and the South Platte River on the eastern plains of Colorado identified intersex fish, female-biased sex ratios, delayed gonadal development, and other gonadal abnormalities downstream of WWTP effluents.

Intersexes are rare among adult gonochoristic teleost fishes (Strüssman and Nakamura, 2002) and only one instance has been reported previously in white suckers (Sikstrom et al., 1975). No intersex white sucker were found among the 33 fish from Boulder Creek reference sites, or among the six fish from the Clear Creek reference. Subsequent collections on Boulder Creek (2003, 2004) have yielded intersex fish only at the downstream site (Vajda et al., 2004). Additional sampling is required to verify the absence of intersex fish from Clear Creek. The gonads from the four intersex white suckers collected in Boulder Creek appeared to contain primarily ovarian tissue with scattered spermatogenetic cysts. The gonads from the four intersex white suckers in the South Platte River appeared to be testis–ova, with primary oocytes interspersed in an otherwise normal testis. Additional sampling is required to determine whether the prevalence of ovarian or testicular tissue within the gonads of intersex fish is site-specific.

Intersex fishes have been reported elsewhere in the United States from waters contaminated by mixed municipal (Harshbarger et al., 2000) and industrial (Mikaelian et al., 2002; Van Eenennamm and Doroshov, 1998) effluents, but have not previously been reported downstream of a WWTP effluent. Cause of the intersex condition remains speculative but appears to be associated with endocrine-active contaminants, including 17β-estradiol and 4-nonylphenol, identified in the WWTP effluent of Boulder (Gray et al., 2005; Murphy et al., 2003) and other municipalities (Länge et al., 2001; Jobling et al., 2002).

Endocrine disruption of sexual differentiation may also contribute to the low numbers of male white suckers downstream of the WWTP effluents. Alternatively, female biased sex ratios could be attributed to differential migration of males, differential mortality of males, or sampling error. Sampling error was probably not responsible for the skewed sex ratio as relatively high percentages of males were found at reference sites on Boulder Creek. Although heat shock has been employed in artificial sex reversals (Feist et al., 1996) mean temperature differences between sites are small (mean difference <5 °C, Murphy et al., 2003) and are unlikely to be responsible for the sex ratios and frequency of intersex observed. Furthermore, in those teleosts that exhibit temperature sensitive sex differentiation, higher temperatures are associated with a lower proportion of females; intermediate temperatures yield balanced sex ratios, not intersexes (Strüssman and Patiño, 1995). Although hormonally-induced sex reversal has not been reported for this genus, sex reversal of white suckers from male to female cannot be discounted as a possibility downstream of the WWTP effluent.

The cause or causes of the reproductive disruption found in this study are not known. Further sampling of Boulder Creek and the South Platte River is underway to determine whether the reproductive disruption noted in this initial survey was a seasonal or episodic event. Reproductive disruption may be a cause of at least localized population reduction of some fish species. Additional sampling downstream of WWTP effluents is warranted in regions where effluent discharges routinely comprise a large proportion of the total river flow to determine extent and magnitude of reproductive disruption in other streams and rivers.

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