Notes

Persistence of Triploid Grass Carp in Devils Lake, Oregon

Benjamin J. Clemens,* John J. Spangler, Paul L. Robertson, Gary M. Galovich, Craig R. Banner, Stephanie L. Gunckel, Lindsay L. Ketchum, R. Kanani Bowden, Sean T. Spagnoli, Michael L. Kent, James P. Kirk

B.J. Clemens
Oregon Department of Fish and Wildlife, 28655 Highway 34, Corvallis, Oregon 97333

J.J. Spangler
Oregon Department of Fish and Wildlife, 810 SW Alder St., Unit C, Newport, Oregon 97365

P.L. Robertson
Devils Lake Water Improvement District, P.O. Box 974, Lincoln City, Oregon 97367

G.M. Galovich
Oregon Department of Fish and Wildlife, 4034 Fairview Industrial Drive SE, Salem, Oregon 97302

C.R. Banner
Oregon Department of Fish and Wildlife and Department of Microbiology, Oregon State University, Corvallis, Oregon 97331

S.L. Gunckel, L.L. Ketchum, R.K. Bowden
Oregon Department of Fish and Wildlife, 28655 Highway 34, Corvallis, Oregon 97333

S.T. Spagnoli, M.L. Kent
Department of Microbiology, Oregon State University, Corvallis, Oregon 97331

J.P. Kirk
Engineer Research and Development Center, Environmental Laboratory, 3909 Halls Ferry Road, Vicksburg, Mississippi 39180

Abstract

Grass carp *Ctenopharyngodon idella* are sometimes used as a biological tool for managing aquatic vegetation in reservoirs. Sterile, triploid fish were stocked in Devils Lake, Oregon, during 1986, 1987, and 1993 to control aquatic vegetation. We present a case study for using multiple measures on the same fish to determine whether illegal stocking of fertile, diploid grass carp occurred. An investigation into the estimated age of a dead grass carp found in Devils Lake suggested that it was significantly younger than would otherwise be expected, given the only stocking events occurred during 1986, 1987, and 1993. To determine whether illegal stocking or reproduction by presumed sterile grass carp had occurred in Devils Lake, we conducted a study that balanced the needs of lethally sampling grass carp for biological measures with the socially and politically sensitive sentiment of the pro–grass carp citizenry of Devils Lake. These considerations, in combination with a low catch per-unit effort, resulted in a modest sample size for grass carp. We sampled grass carp and recorded multiple measures for each fish. Ploidy testing of blood samples indicated the grass carp were all triploid. Based on gonadal histopathology, six fish were male, two were female, and two were sex-indeterminate with severe gonadal dysgenesis. Age estimates from lapillus otoliths were consistent with fish originating from the legal stocking events in Devils Lake. The grass carp were 21–30 y old, and we were unable to find published reports of grass carp anywhere else in the world that are older. The grass carp were significantly smaller than much younger fish from other regions. The small size of these grass carp relative to their age in Devils Lake suggests food limitations that stunted growth. The dead grass carp that was the impetus for this study was aged by anatomical structures that we have since found to be unreliable. This suggests that the dead grass carp was probably in fact older and originated from the legal stockings. The use of multiple biological measurements on a modest sample size of grass carp, combined with the knowledge that no juvenile grass carp have been observed since legal stocking occurred, lead us to conclude that the grass carp in Devils Lake are sterile fish that originated from legal stocking events.

Keywords: Asian carp; gonadal histology; intersex; maximum age; sterile

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* Corresponding author: Ben.Clemens@oregonstate.edu

Introduction

Grass carp *Ctenopharyngodon idella* were introduced from Malaysia into the United States in 1963 as a biological control of noxious aquatic vegetation; soon after, more were received from Taiwan (Mitchell and Kelly 2006). Grass carp were used as a replacement for expensive and potentially ecologically damaging herbicides or costly and inefficient mechanical removal (Allen and Wattendorf 1987; Bonar et al. 1987; Mitchell and Kelly 2006). By 1972 grass carp were widely distributed in the United States (Mitchell and Kelly 2006). By 1983 triploid (sterile) grass carp were produced as a means of controlling noxious aquatic vegetation while preventing the establishment of self-sustaining populations of grass carp that might affect native flora and fauna (Mitchell and Kelly 2006). In 1985 the U.S. Fish and Wildlife Service (USFWS) set forth a biological opinion that triploid grass carp are an environmentally safe means of controlling aquatic weeds, and in that same year established an inspection program to test and certify ploidy on these fish (Mitchell and Kelly 2006).

The first known stockings of grass carp in the Pacific Northwest occurred in Devils Lake, Oregon (Bonar et al. 1987; Pauley et al. 1987). Sterile (triploid) grass carp, ranging in age from 1 to 2 y old, were stocked into Devils Lake during 1986, 1987, and 1993 (Table 1) to reduce aquatic vegetation, which covered most of the lake surface by late summer and severely limited recreational activities in the lake. By 1994 essentially all large aquatic plants were eliminated from the lake and this vegetation has not re-established since that time. Population estimates suggest that, of the original releases of $>$32,000 individuals, $\sim$1,000 grass carp currently remain in Devils Lake (Table 1; Eilers 2015).

Administrative rules for the state of Oregon prohibit and control the stocking of grass carp in the state. These rules were written to help control against unwanted escapees of grass carp outside of their targeted water body and invasion into other watersheds. Only in particular circumstances meeting a number of strict criteria are allowances made for stocking triploid grass carp in Oregon. Illegal releases of diploid grass carp or escaped diploid grass carp thought to be sterile (improperly screened for ploidy) are a concern because they can lead to self-sustaining populations (Schultz et al. 2001; Papoulias et al. 2011; Chapman et al. 2014; Wittmann et al. 2014).

A dead grass carp was recovered from Devils Lake in 2014; it was estimated to be 8–15 y old based on examination of its scales, subopercles, and opercles, suggesting that the fish may have originated from an illegal stocking event. Therefore, we initiated a survey effort to determine if the grass carp in Devils Lake were triploid individuals from the original, sanctioned stocking events or if the population consisted of younger, diploid fish originating from subsequent illegal stocking. Our four objectives specific to addressing this goal were 1) assess the ploidy of each fish to determine if they were sterile (triploid) or fertile (diploid); 2) assess fish stages of gonad development for any evidence of extensive meiotic development indicative of sexual maturation; 3) determine which anatomical structure(s) provided the greatest precision for age estimates among readers and the greatest logical accuracy of the estimated ages in consideration of other evidence; and 4) estimate the age of these fish with the intent of providing a definitive age range that could be used to infer the likelihood that the dead grass carp that we estimated at 8–15 y old originated from legal stockings in 1986, 1987, or in 1993 (Table 1).

Methods

Study site

Devils Lake is located in Lincoln City, Oregon (44°58′42.59″N, 123°59′28.60″W). The 31.1-km^2 watershed drains predominately forest with some agricultural lands, whereas the 14.7-km shoreline has been highly developed (Kramer et al. 1983). With a surface area of 277.2 ha, a volume of $7.092 \times 10^6 \cdot m^3$, and a mean depth of 2.56 m, Devils Lake is a large and shallow, highly eutrophic lake (Eilers et al. 2005).

Fish sampling

We targeted grass carp for capture using a boat-mounted electrofisher. We sampled grass carp in Devils

Table 1. Year and expected age range of grass carp *Ctenopharyngodon idella* stocked into Devils Lake (Lincoln City, Oregon). All fish were cultured in Arkansas. Fish from each source of stocking were screened for pathology.

Stocking	Year of stocking	Expected age in July 2014	Body size at stocking (mm)	No. stocked	Stocking density (fish/ha)[a]
1st	1986[b]	28–30[c]	203–279	10,000	36.1
2nd	1987[d]	27–29[c]	203–279	17,090	61.7
3rd	1993[d]	21–23[c]	203–279	5,000	18.0

[a] Devils Lake has a surface area of 277.2 ha.
[b] A random subsample of 240 grass carp from this lot were tested prior to stocking by the U.S. Fish and Wildlife Service for ploidy with a Coulter counter, and all were found to be triploid.
[c] Fish age at stocking ranged from 10 to 24 months (Hopper-Stephens Hatcheries, Inc., Lonoke, AK, personal communication).
[d] A random subsample of 120 grass carp from this lot were tested prior to stocking by the U.S. Fish and Wildlife Service for ploidy with a Coulter counter, and all were found to be triploid.

Lake on two occasions in 2014: 1) on 8 July to determine the most efficient electrofisher settings and lake locations for collecting grass carp, and 2) on 22 July, when we sampled grass carp. On July 22 we electrofished for 8,056 s at a 30-Hz pulse rate, <6-ms pulse width, and 500-V peak voltage. We sampled grass carp along the shoreline of the lake, where we temporarily immobilized them with electricity and then landed them with dip nets. We euthanized the fish by blunt-force trauma to the head (Leary et al. 2013). We recorded several metrics for each fish, including fork lengths (to the nearest 12 mm), ploidy testing to infer sterility or fertility, gonadal histology for identification of sex and maturity, and age estimates from anatomical structures.

Ploidy testing

We sampled blood from grass carp with heparinized needles via cardiac puncture, and placed samples on ice. We preserved blood samples in Alsevere's solution. We sent the blood samples to the USFWS (Warm Springs Fish Health Center, 5151 Spring St., Warm Springs, GA 31830) for ploidy testing. The protocols described by Wattendorf (1986) were followed with some modification. Briefly, a Coulter Counter Multisizer 3 particle sizer (Beckman Coulter, Inc., Miami, FL) was used to measure the diameter of the erythrocyte nuclei after lysis of the cellular membrane. One μL of blood was placed in 10 mL of isoton solution with 50 μL of coulter zapaglobin for control samples. The volume of each blood sample was titered to allow ≤10% occupancy of the aperature tube orifice during screening in the Coulter counter. Samples were run for 20 s to produce a clear modal peak. Sample counts of erythrocytes averaged 13,633 ± 8,598 standard deviation (SD). The blood samples from the grass carp from Devils Lake were compared with freshly collected blood from known diploid ($N = 2$) and triploid ($N = 2$) grass carp held in captivity at the Warm Springs Fish Health Center. A 2.8-μ polystyrene bead was used as a standard cut-off size for triploids in the USFWS Triploid Certification program (USFWS 2015).. This represents the mean nuclear erythrocyte diameter of diploid grass carp modal peaks, plus two standard deviations of the mean established through program testing (B. Hickson, USFWS, Warm Springs Fish Health Center, personal communication). The modal erythrocyte nuclei diameter was used as the metric for distinguishing ploidy (Wattendorf 1986; B. Hickson, personal communication).

Gonad sampling for sex identification and maturity status

We sampled anterior and posterior sections of the gonads of each grass carp and placed them in 10% buffered formalin in the field. We later prepared the gonad samples for histological examination, including embedding, sectioning, and staining with hematoxylin and eosin. We mounted gonad sections on slides and viewed them under a microscope to determine sex and categorize maturation status and morbidity. We identified fish gonads as one of three categories of sex and morbidity (Luo et al. 2011): 1) males, 2) females, and 3) sex-indeterminate: severe gondadal dysgenesis (SISGD). Based on gonadal histology, we defined as males the fish with testes containing all stages of spermatogenesis, and as females the fish with oocytes and no obvious spermatogenesis occurring. Fish identified as SISGD had gonads consisting only of stroma containing degenerate gametogenic precursor cells and no spermatids, spermatozoa, or identifiable oocytes. These fish may be intersex or females without maturing ova because, microscopically, a definitive determination of gonadal sex cannot be made.

Age estimates

We sampled carcasses for scales, pectoral fin rays, opercles, subopercles, and lapillus otoliths. We sampled scales, pectoral fin rays, opercles, and subopercles from the left side of the fish, except when we observed damage, in which case we sampled anatomical structures from the right side of the fish. We processed the anatomical structures and read them to estimate age because different structures can provide various estimates of age precision (Nuevo et al. 2004; Phelps et al. 2007). At least three, and in some cases five readers (e.g., lapillus otoliths), estimated ages from each structure on an initial read, and then differences in age estimations were resolved through a second, combined read (Borgerson et al. 2014).

We sampled scales dorsal to the tip of the pectoral fin. We allowed the scales to dry and viewed them with microfiche readers. We removed the flesh from the

Table 2. Body size (FL = fork length in mm), sex, and age estimates for grass carp *Ctenopharyngodon idella* sampled from Devils Lake (Lincoln City, Oregon) during July 2014. Sex was identified by gonadal histology (Table 3). The evidence suggests that all of these fish originated from one of the three legal stockings (Table 1).

Fish no.	FL	Sex	Age estimate[a]	Likely stocking year[b]
1	800	Male	18–22	1993
2	762	SISGD[c]	20–22	1993
3	864	SISGD[c]	25+	1986 or 1987
4	648	Male	24+	1986 or 1987
5	775	Female	23	1993
6	838	Male	25+	1986 or 1987
7	775	Female	25+	1986 or 1987
8	686	Male	27–30	1986 or 1987
9	775	Male	20–23	1993
10	660	Male	22–25	1993

[a] From examination of lapillus otoliths.
[b] As calculated from the age estimate, and in relation to Table 1.
[c] Sex indeterminate: severe gonadal dysgenesis (SISGD).

pectoral fin rays by placing them in boiling water. We then sectioned the pectoral fin rays with a low-speed saw (TechCut 4™ by Allied High Tech Products, Inc., Compton, CA) at the broad base of the fin ray, about 5 mm from the base of the spine, as per Nuevo et al. (2004). We transferred sections to microscope slides, which we viewed under a dissecting microscope and a compound light microscope at a maximum magnification of 100–200×. We placed the opercular apparatus in boiling water to remove the flesh and separate the bones, as per Phelps et al. (2007). We then viewed the opercle and subopercle structures with the naked eye and with a dissecting microscope. We harvested lapillus otoliths by severing the head of each fish behind the opercles by the "guillotine method" (Secor et al. 1992); we then extracted the otoliths with forceps from the back of the cranial cavity. We placed otoliths in 95% ethanol for up to 3 d to clean them, and then allowed them to air dry. We then embedded otoliths into epoxy (Epo-kwick™ by Buehler, Lake Bluff, IL) and sectioned them with a low-speed saw (TechCut 4™ by Allied High Tech Products, Inc.). The section made on each otolith was frontal (per Secor et al. 1992). We transferred the sections to microscope slides, and then polished them to accentuate annular marks. We viewed the sections with a compound light microscope at a maximum magnification of 100–200×.

Results

The 8,056 s of electrofishing yielded 10 grass carp, for a catch per-unit effort of 4.5 grass carp/h. Based on body sizes and estimated ages, no juvenile grass carp were observed or collected. The 10 grass carp we sampled were 648-864 mm in fork length (Table 2). The results of ploidy testing by the USFWS indicated that all of the grass carp ($N = 10$) were triploids, when compared with laboratory standards. Mean modal diameter of the erythrocytes of the sampled grass carp was 3.129 ± 0.027 SD μ. One fish exhibited a second peak at ~2.1 μ in diameter, which is smaller than the typical peak at 2.6–2.7 μ. Gonadal histology of the grass carp is shown in Figure 1 and described Table 3. Six of the fish were true males, two were females, and two were SISGD (Figure 1; Tables 2 and 3). Various levels of gonad deterioration existed among several fish (Figure 1; Table 3). Of the five structures examined, lapillus otoliths had the lowest coefficient of variation among readers and the highest age estimates (Figure 2). The lapillus otolith sections were challenging to read because of the close imposition of the annular rings, particularly after about the seventh annular ring (Figure 3). The age estimates we have generated place the grass carp within the timeframe of when stocking occurred (Tables 1 and 2). These data suggest that the individual ages of grass carp ranged between 21 and 30 y old.

Discussion

An investigation into the estimated age of a dead grass carp found in Devils Lake suggested that it was significantly younger than would otherwise be expected from the three legal stocking events. We therefore sampled additional grass carp currently in Devils Lake and measured several different biological characteristics on the same fish to determine if they were the same sterile fish from the original stockings. Ploidy testing of blood samples indicated these grass carp were all triploid (sterile). Based on histopathology, gonadal dysgenesis was fairly common. Age estimates from lapillus otoliths of grass carp in Devils Lake indicate an age range consistent with the fish originating from legal stocking events in 1986, 1987, and 1993. What about the dead grass carp that precipitated this study? Because we used anatomical structures to estimate the age of the dead grass carp that we have since found to underestimate age, we now hypothesize that this fish was probably in fact older, originating from one of the three legal stockings.

Maximum ages of grass carp have been reported at 21 y of age (Gorbach 1961—cited in Kirk and Socha 2003; Fish Base 2015; Kirk et al. 2014) and above 21 y ("21+" per Shireman and Smith 1983). We were unable to find published accounts of grass carp anywhere else in the world that are older than the 21–30-y-old grass carp currently in Devils Lake, Oregon. Despite these old ages, the grass carp we sampled had body lengths that overlapped with much younger grass carp elsewhere. For example, in North and South Carolina, grass carp total length was within the size range for Devils Lake fish, but for ages two through seven (Kirk et al. 2014), and about ages two through five for grass carp from Alabama (Morrow and Kirk 1997). The size range for the grass carp from Devils Lake, when compared with the size at stocking for the releases into the lake (Table 1) suggests a growth range of 381–661 mm over 21–30 y, equating to a growth rate of 13–31 mm/y, compared with larger mean growth rates of 58–138 mm/y over 5–9 y elsewhere (Morrow and Kirk 1997; Kirk et al. 2014). The

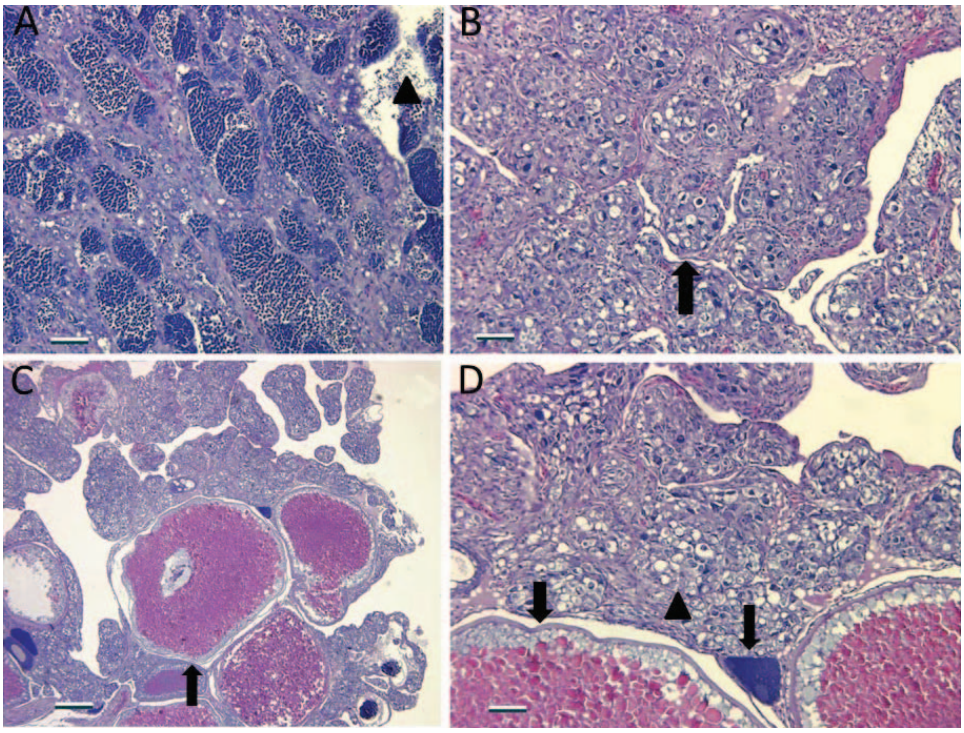

Figure 1. Gonadal histology of grass carp *Ctenopharyngodon idella* from Devils Lake (Lincoln City, Oregon) during July 2014. The gonads were stained with hemotoxyline and eosin. **(A)** Normal male testes. All stages of spermatogenesis are present, including flagellated spermatozoa (arrowhead, top right of panel). Bar = 50 micrometers. **(B)** Sex-indeterminate: severe gonadal dysgenesis. Normal sperm and ova are absent from these gonads. Presumptive gametogenic precursor cells are severely dysplastic. Bar = 50 micrometers. **(C)** Female. Note the prominent vitellogenic ova (black arrow). Bar = 200 micrometers. **(D)** Magnified view of plate C—female with severe gonadal dysgenesis. This gonad contains both vitellogenic ova (leftmost arrow) and previtellogenic ova (rightmost arrow) embedded in a stroma identical to the male gonad with severe gonadal dysgenesis (arrowhead). Bar = 50 micrometers.

Table 3. Results of assessments of gonadal histology for sex, maturation status, and morbidity of grass carp *Ctenopharyngodon idella* from Devils Lake (Lincoln City, Oregon) during July 2014.

Fish no.	Assessment
1	**Male**. All developmental stages up to spermatids present. Mild necrosis/apoptosis throughout gonad.
2	**Sex indeterminate: Severe gonadal dysgenesis**. Lack of identifiable male or female gametes with only degenerate gamete precursors observed. Consistent with gonadal dysgenesis.
3	**Sex indeterminate: Severe gonadal dysgenesis**.
4	**Male** Similar to fish number 1. Appears the same as normal testis.
5	**Female with severe gonadal dysgenesis**. Most stages of oocyte development present, including vitellogenic follicles.
6	**Male**. Similar to fish 1.
7	**Female.** Early primordial oocytes present in stroma with gonadal dysgenesis.
8	**Male**. All stages of testicular development, including spermatozoa, present.
9	**Male**. Similar to fish 1.
10	**Male**. All stages of testicular development, including spermatozoa, present.

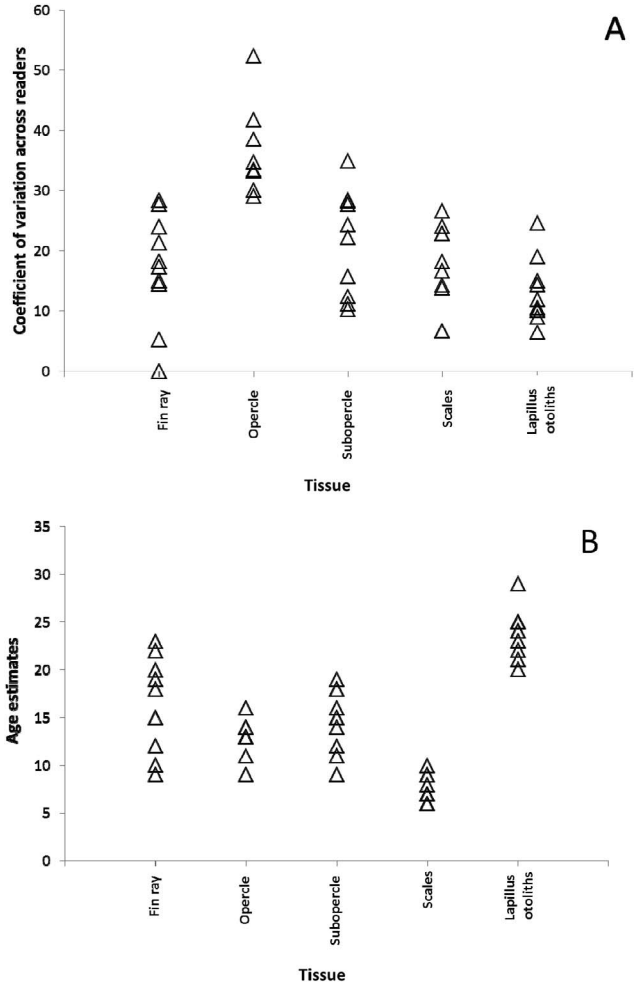

Figure 2. Data pertaining to age estimates from grass carp *Ctenopharyngodon idella* sampled from Devils Lake (Lincoln City, Oregon) during July 2014. **(A)** Coefficient of variation for 3 readers on 5 different tissues from each of 10 grass carp. **(B)** Reconciled age estimates from 5 tissues from each of 10 grass carp. Actual age estimates for lapillus otoliths, including ranges, can be found in Table 2. In cases where a range of age estimates was provided for a particular fish, a median age was used in graph B. In cases where a "+" was provided in Table 2 for an age estimate for a particular fish (e.g., "25+"), the age alone was used in graph B (i.e.,"25"). These qualifiers indicate the uncertainty around the actual age, given the tendency for annuli to become superimposed at the periphery of the lapillus otoliths (Figure 3).

small size of grass carp relative to their age in Devils Lake suggests food limitations that stunted growth.

Our examinations of the gonads of grass carp from Devils Lake yielded results on sexual maturity that were similar to what others have reported for grass carp, with males apparently generating functional sperm and females producing a few vitellogenic oocytes (Papoulias et al. 2011). Gonadal dysgenesis was common in the two sex-indeterminate fish (SISGD) and two female grass carp from Devils Lake. The fish with gonads assessed as SISGD may be intersex or females without maturing ova. Cyprinids such as grass carp are gonochoristic, and the

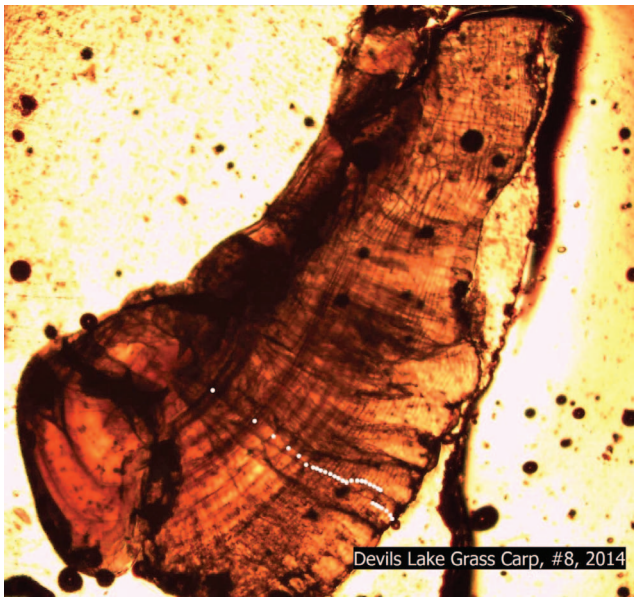

Figure 3. Cross-section of a lapillus otolith from a grass carp *Ctenopharyngodon idella* sampled from Devils Lake (Lincoln City, Oregon) during July 2014 (magnification = 25×). This fish was estimated to be 27–30 y old (fish number 8; see Table 3). The dots indicate the annular rings. The range of ages in this and other lapillus otoliths is a reflection of the uncertainty about annular rings on the periphery.

inclusion of male and female sex cells (also known as "intersex") in the gonad is not normal. Intersex is "…the co-occurrence of cells of both sexes in the gonad, with one sex cell type predominating in a species that is otherwise gonochoristic." (Clemens et al. 2012:1202). Papoulias et al. (2006) reported on the incidence of intersex in other Asian carps (bighead carp *Hypophthalmichthys nobilis* and silver carp *H. molitrix*) in the Missouri River, and they were not able to link the incidence of intersex to a specific cause. Endocrine-disrupting compounds such as mercury, PCB, DDT and its derivatives, sex hormones, and other chemicals from municipal wastewater treatment have been linked to incidence of intersex in fish (Mills and Chichester 2005; Hinck et al. 2009; Iwanowicz et al. 2009; Schwindt et al. 2009). However, it is not clear whether the procedure used to induce triploidy creates intersex in our grass carp.

We have presented a case study for using multiple biological measures on the same grass carp to determine if they were fertile, diploid fish, which could suggest illegal stocking had occurred. Our study balanced the needs of lethally sampling grass carp for biological measures with the socially and politically sensitive sentiment of the pro–grass carp citizenry of Devils Lake. These considerations, in combination with a low catch per-unit effort, resulted in a modest sample size for grass carp. However, the multiple biological measures we used on each fish enabled us to arrive at our conclusion that the grass carp currently in Devils Lake originated from legal stocking events. In support of this conclusion, very small, presumably juvenile-sized grass carp have not

been observed or reported by the public or by biologists since the legal stocking events.

Supplemental Material

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Any use of trade, product, or firm names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

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