APPENDIX A. PROCEDURE FOR SAMPLING FISH, COLLECTING TISSUES, AND CONDUCTING AN EXTERNAL FISH HEALTH ASSESSMENT

OFWO SOP:

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Procedure for Sampling Fish, Collecting Tissues, and Conducting an External Fish Health Assessment

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1.0. BACKGROUND.

This Standard Operating Procedure (SOP) was developed for use by the Oregon Fish and Wildlife Office to facilitate conducting a fish health assessment on fish collected during investigations conducted by the Division of Contaminants at the Oregon Fish and Wildlife Office. This SOP was adapted from procedures derived by the U.S. Geological Survey (USGS), Biomonitoring of Environmental Status and Trends (BEST) program during their contaminant investigations in large river basins, as described in USGS (1999) and Smith et al. (2002). The purpose of this SOP is to provide direction to field crews on how to gather consistent data when conducting a fish health exam and collecting tissues for histology and quantification of chemical analytes.

2.0. TARGET SPECIES

The SOP describes procedures used to assess health and collect tissues specifically for white sturgeon.

3.0. EQUIPMENT AND SUPPLIES

Personal Safety Equipment

Safety glasses (1 pr)
Long sleeved shirts or coveralls
Latex gloves and work gloves
Aprons or lab coat

Sampling Equipment
110V AC power source (inverter, generator, or outlet) to run centrifuge if working outside
125-mL polyethylene bottles (for histopathology samples)
10% buffered formalin solution (for histopathology samples)
Acetone (0.5 L, for rinsing instruments)
Acetone squirt bottle w/ extra 28-mm lid (1)
Aluminum foil (300ft, heavy duty)
Ballpoint pens or Space pens (waterproof ink)
Calibration weights for balances
Cellular telephone (optional, but recommended)
Centrifuge (1)
Centrifuge tube rack (1, plastic or styrofoam)
Chain-of-custody forms
Clipboards
Cloth or other labeling tags (for fish carcasses)
Copy of USGS publication *Illustrated Field Guide for Assessing External and Internal Anomalies in Fish* –Smith et al. (2002) on waterproof paper
Cryovial tubes (for plasma after centrifuging) 2 ml, 4 vials per fish
Cryovial tube holder (if freezing in liquid nitrogen)
De-ionized water (4 L, for rinsing instruments)

Digital Camera and extra batteries
Dissecting tools (2 kits) – with plenty spare scalpel blades
Dry ice (4-5 lb for freezing & 10-15 lb for shipping) if collecting liver or plasma in the field
Duct tape (1 roll, for securing sharps containers, etc.)
Electronic balances (3 for measuring small and large tissue masses)
Field notebook
Field guides or other references for identifying fish
First-aid kit
Fish Health Assessment Data Sheets
Flashlights and other lights (optional)
GPS unit (1)
Ground-Fault interrupting (GFI) extension cord (if running centrifuge in the field)
Hand-lens
Hanging balance for large fish
Kitchen shears, large knife, and sharp, strong scissors
Large coolers for fish carcass transport
Large metal pipe (e.g., 1" X 18' 316 SS pipe) or blunt instrument for subduing fish
Latex or nitrile gloves (40 pr)
Measuring board, mm
Needles and syringes (or Vacutainer multi-sample needle, 1.5" long needle, 21 gauge)
Paper coin envelopes (for spines)
Paper towels -6 rolls Bounty paper towels-or cloth diapers to hold fish
Plastic trash bags for waste disposal
Pliers (1 pr)
Portable table (optional)
Rigid-walled container with lid (i.e., coffee can, milk jug) for sharp wastes (1)
Sharpies or other waterproof marking pens (3)
Small cooler (for plasma), ethanol and dry ice if making an ultra-cold dry-ice slurry in field SOP
Transfer pipettes (for separating plasma from spun whole blood cell pack)
Tubs or live boxes (if keeping fish alive prior to processing) (e.g. 128-162 qt igloo icechests)
Vacutainers, 10 ml, Heparinized
Vacutainer holder (to connect Vacutainer and needle)
Wet or dry ice and container (if taking samples in the field)
Weighing pans
Waterproof paper for labels (2 sheets)

4.0. RECORD KEEPING

- Make all data entries in ink on waterproof paper ('Rite in the rain' stock or equivalent).
- Each specimen is assigned unique identification number that can be traced to any sample collected from the specimen.
- Prepare a separate Fish Health Examination Sheet for each specimen.
- Leave no blank spaces on the Fish Health Examination Sheet.
- Have one person do the examination and another record data. Make a single line through any corrections and initial all changes.
- Check each fish data sheet carefully to be certain that all observations have been made and recorded before processing the next fish.
- Record by GPS the location of capture for each specimen.
- Record in a logbook the activities of the field crew.

5.0. PRE-TRIP PLANNING

- Pre-label tags, labels, and formalin containers if time permits. Pre-fill 125 –ml bottles with about 85 ml fixative (10% buffered formalin). Although the formalin is below levels considered hazardous (5%), fill containers under a hood or outdoors wearing latex gloves and safety glasses.
- Check equipment lists to be certain all materials needed are present.
- Fill dry-ice shipper or fill liquid nitrogen dry shipper if needed.

6.0 PROCEDURES

6.1. *FISH PROCESSING AND BLOOD SAMPLING*

Note: Wear gloves when handling and dissecting fish, and keep the heparin solution chilled.

a. Prepare the work surface by covering it with a piece of stripped foil (dull side up/shiny side down) large enough to wrap the fish you will be processing.

b. Assemble the needle and syringe and heparinize the needle and syringe by uncapping the needle and drawing a few milliliters of the heparin solution provided into the syringe, inverting it (i.e., needle up), and withdrawing the plunger to its full extent. Dispense the heparin back into its container. The small amount remaining on the walls of the syringe and in the hub of the needle will prevent clotting for most fishes. **Note: Heparin is powerful anti-coagulant -- be careful!**

Note: **For large fish such as sturgeon**, use needles that attach directly to heparinized Vacutainers. Place needle into Vacutainer holder and secure Vacutainer on opposite site. There is no need to use heparin as it is already in the Vacutainer. You will be exchanging Vacutainers with the needle still in the fish, as described below. **Use caution when inserting needle into Vacutainer holder to prevent stabbing yourself with the needle.**

c. Secure specimen, make positive identification, and initiate a fish health examination sheet. *Note: The authority for fish nomenclature is Robins et al. (1991).*

d. Record species and the station, date, and specimen I.D. number on the examination sheet.

e. To collect blood, secure the fish. It is helpful to hold the fish securely with a towel or diaper, leaving the caudal peduncle exposed (note: do not hold sturgeon by the tail). The preferred blood sampling location is the posterior caudal artery and vein, which lie together just ventral to the vertebral column. These vessels can be reached with a needle and syringe inserted between the hemal arches and spines from either of two directions:

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Lateral approach: Lay the fish on its (right) side, head elevated. Remove a few scales (if present) from the lateral line near the anal fin. Insert the needle from the side, just below the lateral line, to the approximate mid-sagittal depth.

Ventral approach (this is the preferred approach for sturgeon): Hold the fish on its side, head elevated, with the ventral surface facing you. Use two people to hold larger fish and do not hold the tail. Locate a depression directly midline behind the anal fin; your needle must be inserted exactly midline to find the caudal vasculature (see Photo 1). Push the needle toward the vertebrae (dorsally), angled anteriorly (Appendix 8-A and 8-B). *Note: This method does not work for all species. Be careful using a needle and syringe with a live, un-anesthetized fish. Don't stab yourself or a co-worker.*

Exert **slight** suction. When the needle contacts the base of the vertebrae (if drawing ventrally), withdraw the needle slightly. Too much suction will collapse the blood vessels and other tissues around the needle, restricting blood flow. It may be necessary to try more than once; if so, move anteriorly with each new attempt. Note, however, that repeatedly withdrawing the needle increases the likelihood of a clot forming in the needle. If a clot does form, withdraw the needle from the fish and expel the clot with slight pressure on the plunger of the syringe.

For sturgeon, fill at least four 10 ml Vacutainers will blood. Draw blood directly into Vacutainer until 80% full (roughly 7-8 ml), then place a new Vacutainer into holder and repeat the process until a sufficient number of Vacutainers are full (at least 4 for sturgeon). The first three Vacutainers will be capped and placed directly into a freezer or dry ice (*not slush!*). The last Vacutainer will be used for plasma, as described later in Section 6.6. Record the volume of blood obtained, as well as any problems encountered, on the fish heath datasheet.

Photo 1. Collecting blood from sturgeon by inserting needle exactly midline to find the caudal vasculature on the ventral side of the fish.

f. Using your pliers, crush the needle and put it in the sharps container for later disposal.

g. Remove the cap from a labeled, heparinized Vacutainer and gently dispense the blood from the needle-less syringe into the Vacutainer (unless blood is already in Vacutainer). Place the Vacutainer in the tube rack cradled in ice (wet) and chill until processed.

h. With the pliers, crush the syringe (if used) and put it in the sharps container for later disposal.

i. Expose the anterior dorsal surface of the specimen and, with the blunt instrument (1" X 18' 316 SS pipe) of your choice, subdue the fish with a sharp blow to the rear of its head.

j. Measure the weight of the fish and record on the fish health data sheet.

k. Measure the fish on the measuring board (total length, in millimeters; squeeze the caudal fins together – see Appendix 9-A and 9-B).

6.2. *EXTERNAL EXAMINATION*

Work quickly, as any tissues collected need to be frozen or preserved as rapidly as possible to avoid tissue necrosis.

Note: When photographing lesions or anomalies, use the previously prepared tag or scale envelope s a specimen identifier in each frame. Record the number of photos taken in each section of the fish data sheet.

Record general remarks about fins, skin, and other external features in the general comment field on page 2 of the fish health data sheet before you begin the specific observations of particular organs, tissues, structures, and systems. Important conditions to note are deformities, scale loss, external parasites, etc.

Record any capture markings on the fish on page 2 of the fish health data sheet. Examine fish for evidence of lateral line scute removal, fin clips, or PIT tags which are indicative of previous captures of an individual sturgeon. One of more scutes may be removed from various locations along the lateral line, and the fish may also be marked with a fin clip, PIT tag, or other mark indicating previous capture and release events. Identify and record scars from scute removal. Examine both sides of the fish for evidence of marking. Take photos of any capture markers (including the sample number in the photo) and note the number of photos on the data sheet. Use a scanner (if available) to locate any PIT tags in the fish, and remove any PIT tags found on the fish and place them in a labeled coin envelope used for spines.

Begin the observations in the order outlined on the fish data sheet (see *Appendix 9 as a guide to external fish anatomy)*. Be sure to record all observations by marking (x) in all boxes on the data sheet that apply, and collect and preserve any tissues that seem abnormal. It is helpful for the histopathologist to collect some normal tissue of the same type as the normal tissue. A clear, precise mark is necessary for subsequent data entry. If the observation does not seem to fit any of the listed categories, check "other" and describe your observations in the indicated areas.

6.2.1. **Body Surface** (Smith et al. 2002, Section 6.2.1, Figs. 8 to 18)

Examine all surfaces for any Tumors, Lesions, or Parasites. Remove them and place them in the labeled bottle of fixative.

Record lesion types found by checking the appropriate blocks on the fish health data sheet. Note their general location in the "other" field and record number of (pieces) in fixative.

Check around mouth and tongue for lesions and anomalies (Smith et al. 2002, Figs. 19 to 23). On sturgeon, place gloved finger on upper roof of mouth and pull downwards until a “tube” is observed. Check outside of tube for lesions or anomalies and record observations. Take photos of anything appearing abnormal along the mouthparts.

6.2.2. Eyes (Smith et al. 2002, Figs. 24 to 29). The left and right eyes should be examined and scored separately, as follows:

Normal—No aberrations evident; eyes "clear," not protruding, milky, opaque, or bloody.

Exopthalmic—Swollen, protruding eye. Commonly referred to as "popeye" or "goggle-eye."

Hemorrhagic—Bleeding in the eye.

Opaque—This is a very graphic category and you need not know whether the eye is functionally blind. It generally refers to opaque or cloudy eyes, or the appearance of cataracts. The nature of the opacity is not important here.

Missing—An eye appears to be missing from the fish (Note: it may *actually be scarred over).*

Emboli—Gas bubbles visible.

Other—Describe **a**ny manifestations that do not "fit" the above (i.e., parasites, spots, cuts, abrasions)

6.2.3. Opercles—(Smith et al. 2002, Figs. 30 to 33). It is necessary only to observe the degree of shortening of the opercular flaps. Score the opercles according to the following criteria (check all that apply):

Normal—No shortening, bills completely covered.

Slight shortening—Slight shortening of the opercle with a very small portion of the gill exposed.

Severe shortening—Severe shortening of the opercles with a considerable portion of the gill exposed.

Other—Describe any observation that does not fit above.

6.2.4 Gills (Smith et al. 2002, Figs. 34 to 37). The gills are examined and evaluated separately. Evaluate the gills as follows:

Normal— No apparent aberration. Be very careful in this observation. The gill can easily be affected by the manner in which the fish is handled during and after collecting.

Frayed— This generally refers to actual erosion of tips of gill lamellae resulting in "ragged appearing gills. Mere separation of gill lamellae can be construed as "frayed" but the condition may have been caused by something as simple as the manner in which the gill was exposed by the investigator.

Clubbed—This refers to welling of the tips of the gill lamellae. They can often appear bulbous or “club”-like.

***Marginate*—**A graphic description of a gill with a light discolored margin along the distal ends or tips of the lamellae or filaments. Margination can be and often is associated with "clubbing." If both seem to apply, check both.

Pale—This refers to gills that are very light in color. Severe anemia can result in gills that are discolored to the point of being white. Severe bleeding induced during blood sampling can also result in somewhat pale gills. Gills also begin to pale somewhat after death, which is common in fish taken from nets. All of this should be considered in making the observation.

Other—Describe any observation that does not fit the above.

6.2.5 Fins (Smith et al. 2002, Figs. 38 to 34). Eroded or "ragged" fins are a departure from normal condition and health. Previously eroded fins that are healed over and show no evidence of the active erosion are considered normal in this assessment. The evaluation of fins is relative to the degree of active erosion process in evidence. For the purposes of this procedure, the number and fin location involved is NOT significant. If only one fin is displaying active erosion, record the observation. If several fins are displaying erosion with unequal severity, check all that apply and note in the comments which fins are involved.

Note: Erosion usually involves both fin membrane and rays, whereas fraying involves only the membrane.

***Normal*—**No active erosion, fraying, or hemorrhage. *This includes previously eroded fins that are completely healed over.*

***Mild erosion*—**Active erosion process but no hemorrhage or secondary infection in evidence.

***Severe erosion*—**Active erosion with hemorrhage and/or evidence of secondary infection.

***Frayed*—**Margins of fins ragged or torn.

***Hemorrhagic*—**Reddened (i.e., bloody) areas visible with the intact fin.

***Emboli*—**Gas bubbles visible within the fin.

***Other*—**Describe **a**ny observation that does not fit above; collect aid preserve a sample if possible.

6.3 *INTERNAL EXAMINATION AND SAMPLE COLLECTION*

Note: This SOP describes a limited internal examination to identify sex, remove and collect stomach contents, examine the liver for lesions, removal and collection of entire liver, and collection of muscle tissue. Descriptions of a full internal examination are provided elsewhere.

6.3.1 Stomach contents removal (Smith et al. 2002, Fig. 45). Lay the subdued fish on the foil and open the abdominal cavity with a cut from the vent forward to the pectoral girdle; cut through or closely to one side of the pelvic girdle. Do **not** insert the scissors so far that the internal organs are damaged.

Reach into the anterior end of the abdominal cavity with one gloved finger and find the esophagus. Cut the esophagus with the scissors, and remove the entire viscera except for the kidneys, which will remain in the carcass. Use only gloved fingers and a blunt probe to free the internal organs from the carcass. Put the carcass aside (on the foil) while working with the excised internal organs. Use appendices 9-c and 9-d as a guide to internal anatomy.

Holding the viscera, make a cut along the stomach and push contents out onto a separate piece of clean aluminum foil (tare foil on balance prior to placing contents on it). Cut the remainder of the viscera and squeeze gut contents onto the aluminum foil. Briefly identify any remains if possible and note on health data sheet. Weigh contents on balance and record weight on stomach contents data sheet. Place stomach contents into separate chemically clean jar, label jar, and place in wet or dry ice; or, if stomach contents contain larger fish that exceed the size of sample jar, wrap all material in the aluminum foil. Place a label on the sample and wrap the sample again in another layer of aluminum foil. Place the sample in two plastic bags with a second label in between the bags. Place sample on ice immediately, and transfer to a freezer within 24 hours.

6.3.2. Liver Observation (Smith et al. 2002, Figs. 46 to 50). The appearance of the liver may be an artifact of the sampling; the observer should take that into consideration. Note that the appearance of the intact liver and gall bladder may, for example, vary with the length of time from collection to observation. It also depends on the extent of blood loss during sampling.

Dark- to light-red color—Dark red is the normal color. However, the liver is a blood storage organ, and it may be a lighter red color after bleeding, but not so pale as to be classified as general discoloration or as tan.

Tan or "coffee with cream color"--"Fatty" liver (i.e., more or less uniformly light tan color).

General discoloration— Uniform color other than the above (gray is common); describe.

Focal Discoloration—Color change in part of liver, giving it a mottled appearance; describe.

Nodules in Liver—Nodules in the liver, i.e., white mycobacterial cysts and incipient nodules, such as those in hepatoma (dark blotches) or cholangioma ("popcorn" look); swollen areas.

Other—Describe any aberration or deviation in the liver that does not fit into above scheme.

6.3.3 Liver Histopathology Sample Collection (if needed). Using acetone-rinsed scissors cut any grossly observable foci or lesions from the liver and put them into the fixative. *Try not to puncture the gall bladder* (see Smith et al. 2002, Figs 51 to 52, for pictures of gall bladder). Record any collections of the health data sheet. For fish without a discrete liver, such as common carp, goldfish, etc., the procedure is analogous, but the liver cannot be removed and weighed. Upon completion of the visual observation, locate and inspect as many hepatic nodules as possible. *(Note: Here, a nodule refers to the dispersed liver organs, not to a pathological condition).*

6.3.4. Liver removal for chemical analysis. Locate the gall bladder (Smith et al. 2002, Figs 51 to 52) and be sure not to break the bladder while removal or handling the liver. Locate the attachment points for the two lobes of the liver and gently isolate each lobe from the rest of the body, using clean probes or other clean device to break connective tissue. Cut the attachment points with scissors or scalpel; again, avoiding contact with the gall bladder. Carefully detach the gall bladder from the liver if needed and leave the bladder in the body cavity. Place the liver in a weight boat or suitable clean surface and record the weight of the entire liver (both lobes). Wrap the sample in aluminum foil. Place a label on the sample and wrap the sample again in another layer of aluminum foil. Place the sample in two plastic bags with a second label in between the bags. Place sample on ice immediately, and transfer to a freezer within 24 hours.

6.3.5. Muscle Tissue Fillet Sample with Skin (note: this procedure may be conducted at the field laboratory or at the laboratory conducting the homogenization of the whole carcass). Collect a 40-gram fillet sample of muscle tissue and associated skin. Place the fish on its ventral surface. Using a clean sharp knife, make an incision in the dorso-ventral plane along the vertebral column from the posterior base of dorsal fin to the base of the tail, inserting the knife to the vertebrae (where the ribs are joined with their respective vertebrae). Make a second incision perpendicular to the first incision along the vertebral column just above where the ribs join the vertebral column, thereby separating an approximately 90° wedge of muscle tissue and associated skin away from the sturgeon except at its anterior end. Make a third incision perpendicular to first two incisions at the anterior end of the muscle sample (i.e., at the posterior end of the dorsal fin) in order to separate the muscle sample from the sturgeon. Place the sample on a clean foil-covered work table. Place a piece of aluminum foil on an electronic balance, and tare the balance. Place the muscle sample on the foil on the balance and record mass. If the mass of the original sample is less than 40 grams, then additional tissue mass must be collected from the sturgeon in a similar fashion to that described above by proceeding anteriorly on the sturgeon. If the mass exceeds 40 grams, remove the sample from the balance and place it back on the work table. Carefully remove a mass of tissue approximating, but not exceeding, the mass by which the sample exceeds 40 grams, and reweigh the muscle sample. Repeat the process, if necessary until the muscle sample weighs approximately 40 grams. Replace the excess tissue back with the whole-body sturgeon sample. Wrap the sample in aluminum foil. Place a label on the sample and wrap the sample again in another layer of aluminum foil. Place the sample in two plastic bags with a second label in between the bags. Place sample on ice immediately, and transfer to a freezer within 24 hours.

6.4. SCALES OR SPINES

Collect a scale or spine sample from the **left** side of the fish (the fish's left side) unless the scales are damaged. For sturgeon, collect the spine from the area of the appressed pectoral fin (Appendix 9-A and 9-B). Place scales or spine in labeled envelope, record the side that the sample was collected and the side of the fish from which it was collected on the fish data sheet.

6.5. PREPARING CARCASS SAMPLES

Prepare the carcass for chemical analysis by placing the viscera back into the abdominal cavity of the fish. To avoid fins poking through the aluminum foil, cut fins at base. Wrap the carcass securely in the aluminum foil upon which the examination was made (still dull side in / shiny side out). Large fish may need to be double-wrapped. Tie the already prepared label securely to the caudal peduncle (outside of the foil), and place the carcass sample in a large plastic bag. All sturgeon samples will be wrapped and stored individually. Place sample on wet ice in a cooler and store in a freezer within 24 hours.

6.6. PROCESSING PLASMA SAMPLES

When an even number of specimens has been processed, you should: Place the chilled Vacutainers in the centrifuge *without their caps.* ***Note: Be sure to balance the rotor by spacing the tubes evenly!*** Centrifuge the tubes for 10 min, and check the samples; the plasma should be transparent and straw-colored. If necessary, spin the samples for another 10 min. To avoid warming, don't go beyond 20 min total without re-chilling the sample(s). Aspirate the plasma with a transfer pipette; divide each sample equally between labeled *Cryovials* with screw caps (fill at least 4 *Cryovials*). NOTE: Use a cryopen to mark *Cryovials* if freezing at ultracold temperatures. Please don't try to get every drop of plasma. It is better to leave a little in the *Cryovials* to contaminate the plasma with debris. If you disturb the sediment in the bottom of the tube, recentrifuge the sample for a few minutes. *Note: This procedure can be initiated at any time that an even number of samples has been obtained. Again, be sure to balance the centrifuge head by spacing the tubes evenly if fewer than six samples are being centrifuged.* Securely cap the Cryovials, then freeze them in dry ice (not slush!) or immediately in a freezer. Discard the excess plasma, and check the **number** of plasma samples collected on the fish data sheet (in FIRST BLOCK OF FIRST PAGE).

7. REFERENCES CITED

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