Dissection protocol

NMBU 23-06-2020

Check list

- ☐ One polystyrene box with ice, one with dry ice
- ☐ Labeled cryotubes or Eppendorf tubes
- ☐ Balance and weigh boats
- ☐ Scalpel and blades
- ☐ Tweezers and scissors
- ☐ Labelled Petri dishes
- ☐ 1 litre of cold PBS
- ☐ Ethanol and distilled water to clean the tools
- ☐ Aluminium foil, kimwipes and absorbent paper
- ☐ Marker, pen and notepad
- ☐ Dietrich solution
- ☐ Air-tight containers (histology)

Fish dissection (*Salmo salar*)

Euthanasia

1. Fish should be starved for 24 hours prior to sampling.

2. Euthanize the fish with an overdose of anesthetic or a with a strong concussive blow to the head (be careful to not damage the skull and destroy the brain).

3. Two persons to sample each fish immediately after death; one to dissect out tissues, the other to prepare and document tissues for freezing (e.g. cuts in smaller pieces of the recommend weight, weights the pieces and snap-freezes on dry ice).

Dissection of the tissues

Preparation

1. Make a copy of the AQUA-FAANG metadata standards file (link) and record all relevant metadata as required, for example: standard length, fork length, total length, weight, water salinity, age, photoperiod, etc.

2. *(optional)* Take a picture for external anatomy registration purpose.

3. We recommend that after excision all organs/tissues are washed/pre-cleaned in a PBS filled "Collection" petri dish then transferred into a second "Sample" petri dish containing fresh PBS buffer. We also recommend labeling a petri dish for gonad samples collected for histology. The following table suggests how you can label your Petri dishes:

Collection dish ("C") *(contents)*	**Sample dish ("S")** *(contents)*	**Sample dish** *(histology)*
C-Brain *(full tissue)*	**S-Brain** *(after removal of pituitary and brain stem)*	-
C-Liver *(full tissue)*	**S-Liver** *(after separation from vascular network)*	-
C-Gonads *(full tissue)*	**S-Gonads** *(after removal of spermatozoa / eggs)*	**H-Gonad** (*1 or 3 samples)*
C-Distal Intestine *(full tissue)*	**S-Distal intestine** *(after removing feces and mucus)*	-
-	**S-Head Kidney** *(full tissue)*	-
C-Gill *(full tissue)*	**S-Gill** *(after removing rakers and blood)*	-

C-Muscle *(full tissue)*	**S-Muscle** *(after removal of skin)*	-

Sampling

4. To access the brain, make a transverse cut across the skull and lateral cuts (posterior to anterior) to create a flap which you can remove along with any skin, cartilage, or fat. Identify the brain.

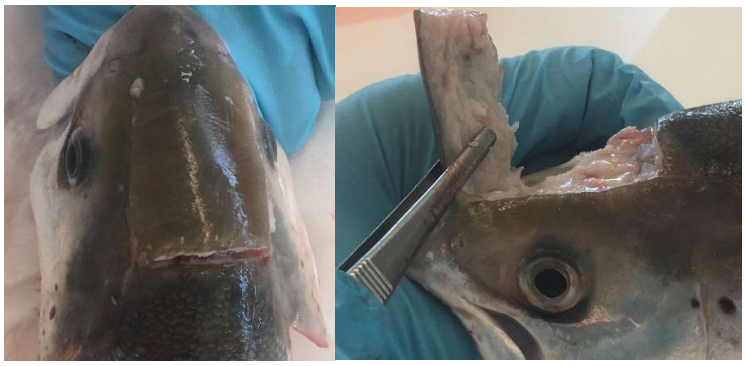

Figure 1. Accessing the fish brain

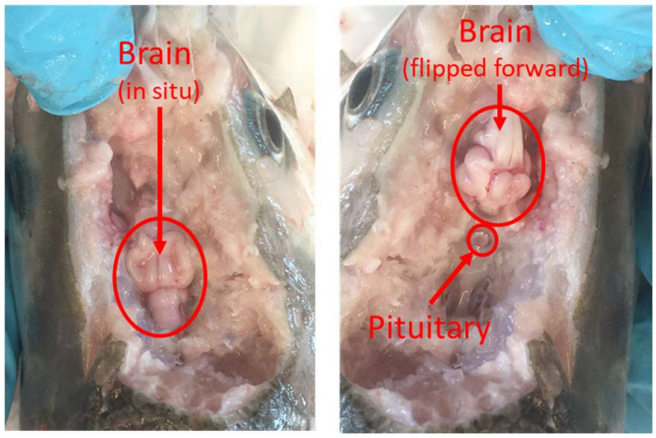

Figure 2. Exposing the brain

5. Carefully cut across the brain stem and transfer the **brain** to a Collection petri dish placed on ice and containing a volume of PBS sufficient to wash the tissue. Remove the brain stem and pituitary and any extra contaminating tissue, and transfer to Sample petri dish.

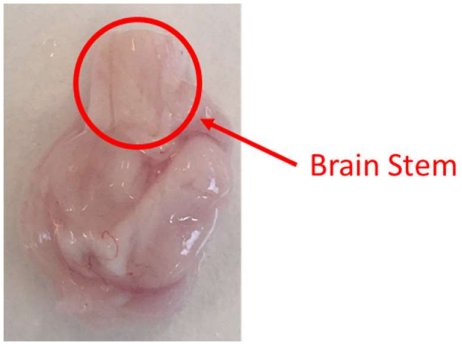

Figure 3. Illustration indicating brain stem

6. Open the fish by making a shallow horizontal cut 5mm below the lateral line from the gill to the anal fin, and two vertical cuts from the lateral incision to the belly. Cut through the skin and muscle but not so deep that you damage the internal organs.

Figure 4. Immature male fish

7. Open the fish by flipping muscle and skin layer down and stretching it to expose the internal organs.

8. Remove fat (if necessary) expose the digestive organs and prepare to extract six organs in the following order: liver, gonad, distal intestine, head kidney, gill and muscle.

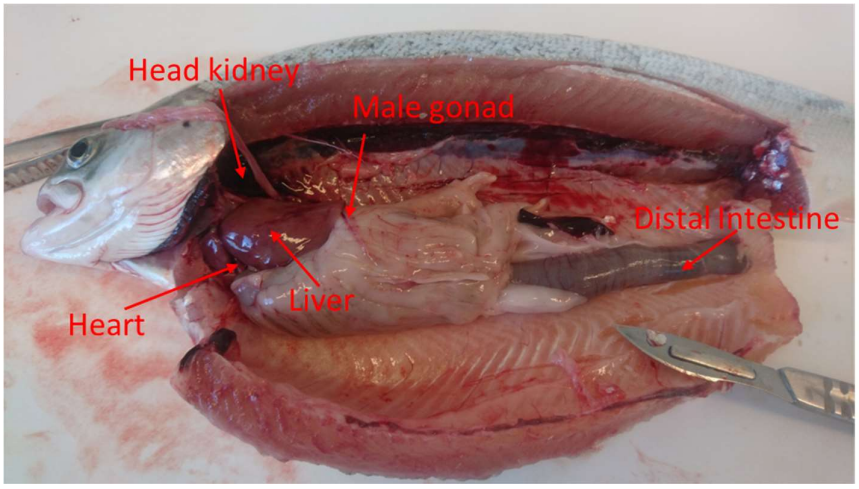

Figure 5. Immature male fish exposed

9. Remove the intact **liver** after cutting the hepatic vein, and transfer to a Collection petri dish on ice containing PBS. If nessesary to subsample this potentially large organ, remove a 1cm wide strip from middle of the tissue (in an anterior → posterior direction). Avoid or remove the vascular network and transfer the final trimmed sample to its Sample petri dish.

   NOTE : Because this is an easy tissue to handle and relatively large, we will use liver as a source of DNA for whole genome sequencing of each individual. Please ensure that you collect at least 2gm total mass.

10. Gonads
    - In <u>immature male</u> fish, **testes** are similar in appearance to the bladder, both are fine and delicate and look like sausage skins.

    a. The bladder lies over the testes and can be lifted to expose the underlying testes, take a photo of the gonads *in situ*.
    b. Gently release the testes by cutting at the anterior end.

c. See Appendix 1 for details. Remove a sample(s) for histological analysis and place in appropriate Histology petri dish ("H"), then add PBS to remaining whole tissue (in collection dish; "C") AND to histology sub-samples to prevent the tissue from drying out.

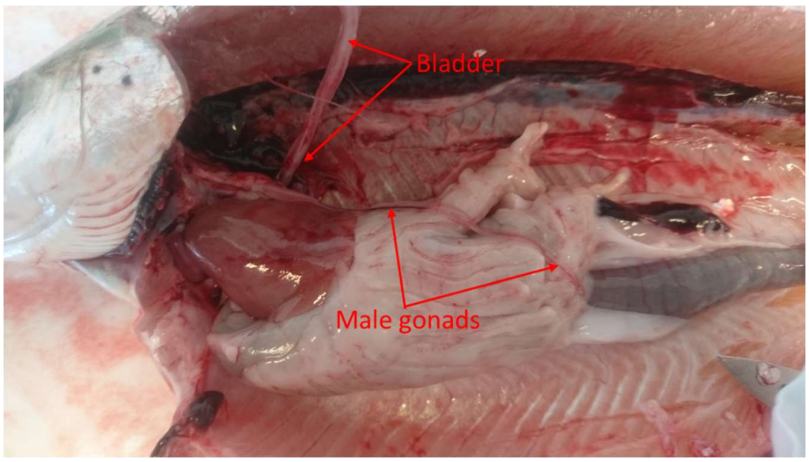

Figure 6. Immature male gonads

- In mature male fish, **testes** appear as very large white sacks.

a. Take a photo of the gonads *in situ*, then release the testes by cutting at the anterior end.
b. See Appendix 1 for details. Remove a sample(s) for histological analysis and place in appropriate Histology petri dish ("H"), then add PBS to remaining whole tissue (in collection dish; "C") AND histology sub-samples to prevent the tissue from drying out

- In immature female fish, the **ovaries** are found under the digestive organs and appear as pinkish round sacks.

a. Take a photo of the gonads *in situ*, then using tweezers, pull these away from the body cavity and cut at the point of attachment to liberate the organ.
b. See Appendix 1 for details. Remove a sample(s) for histological analysis and place in appropriate Histology petri dish ("H"), then add PBS to remaining whole tissue (in collection dish; "C") AND histology sub-samples to prevent the tissue from drying out.

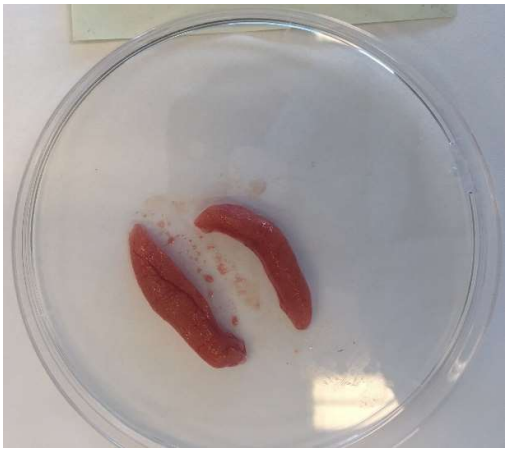

Figure 7. Immature female gonads

- In mature females fish, the **ovaries** are large pink sacks and possibly contain eggs.

a. Take a photo of the gonads *in situ*, then using tweezers, pull these away from the body cavity and cut at the point of attachment to liberate the organ.
b. See Appendix 1 for details. Remove a sample(s) for histological analysis and place in appropriate histology petri dish ("H"), then add PBS to remaining whole tissue (in collection dish; "C") AND histology sub-samples to prevent the tissue from drying out.
c. If possible, attempt to wash away or press out as many eggs as possible and discard.

11. Make a cut in the anterior part of the main digestive link (at the esophagus/pharynx level) to release the beginning of the digestive tract.

12. Lift the intestines out and locate the most terminal (distal) region proximal to the anus, cut to release the tract completely.

13. Locate and isolate the **distal intestine**. If feces are present, place the DI in a clean petri dish and gently use your finger to press them out. Transfer the DI to the Collection petri dish (containing PBS) and cut it open along its length to expose its interior, then gently scrape with the back of a scalpel or spatula to remove mucus. Wash thoroughly in PBS, and transfer to the Sample petri dish.

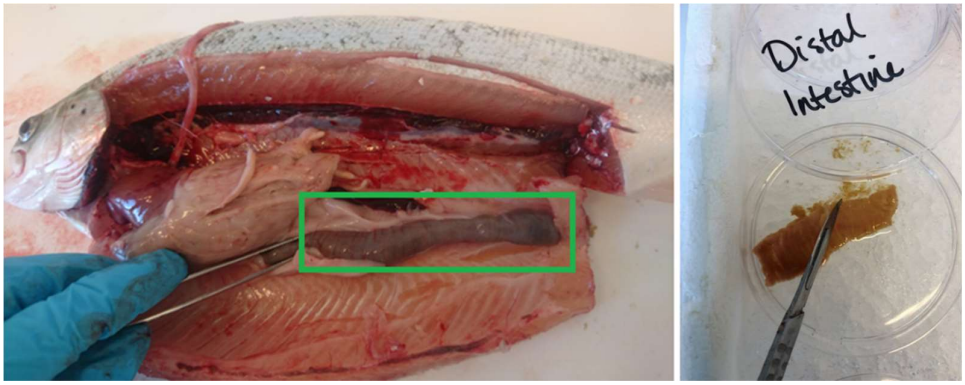

Figure 8. Immature fish distal intestine

14. Remove and discard the heart to give better access to the **head kidney**, which is the dark red tissue found under vertebral line and behind the head, it is a bit larger than the kidney that follows the vertebral line all along the body. The head kidney is very fragile and extra care must be taken when extracting it. A small plastic spoon or spatula may be appropriate to use. Place directly into Sampling petri dish (do not use a Collection dish) containing PBS.

15. Cut the **gill** at its root and immerse in a Collection petri dish filled with PBS and scrape the surface to remove blood. Cut out and discard cartilaginous tissues (rakers) but retain the gill filaments and transfer these to a fresh Sample petri dish with PBS.

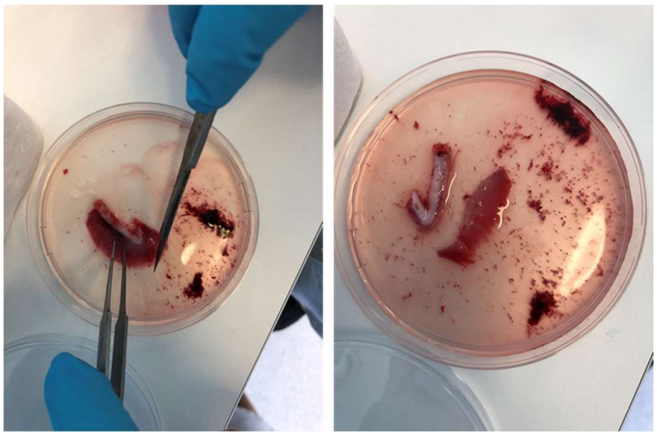

Figure 9. Immature fish gill dissection

16. Sample a generous amount of **skeletal muscle** from the region anterior to the dorsal fin (see Fig 10). Place into the corresponding Collection petri dish and remove skin and fat. Transfer cleaned sample to Sample petri dish and immerse in PBS.

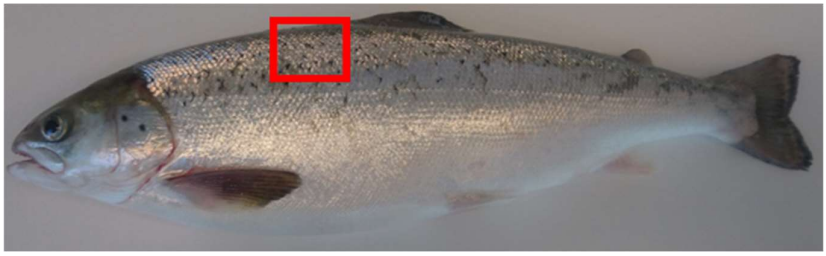

Figure 10. Region to sample skeletal muscle.

NOTE : Skeletal muscle is a relatively poor source of nuclei and the library protocols involve extensive filtering which will return low yeilds of nuclei. For this reason we recommend sampling at least 3gm (preferably 6) to ensure sufficient material.

RNA preservation and Freezing tissue

If possible, these steps should be performed in a cold room

1. Place an unfolded piece of aluminum foil (not less than 10 x 10cm) or disposable weigh-boat on a flat layer of dry ice ensuring good contact. At the same time, place two pairs of tweezers in dry ice so that the ends of them (bottom half) can become very cold and place pre-labeled cryotubes into dry ice so that they are cold to receive sample.

2. Transfer tissue from sampling petri dishes ("S") to a new and dry petri dish, make an effort to use Kimwipes to remove excess PBS and record the sample weight (subtracting the weight of the petri dish). This weight can be used to see if there is enough sample for all assays.

3. With a clean scalpel, cut tissue samples into small pieces (approximately 2-3mm^3).

4. Using room temperature tweezers and following the guidelines in the table below*, drop tissue pieces into 5-10 volumes RNAlater (or equivalent). See recommendations from the manufacturer, but we recommend storing the sample at 4°C overnight before transferring to -20°C for long-term storage.

Tissue	µg total RNA per mg (v. approx.)	mg TO PRESERVE
Brain	1	15
Distal Intestine	1	15
Gill	1	15
Gonad	1	15
Muscle	0.25	100
Liver	3	15
Headkidney	2	15

* *These are very rough estimates, if you have experience with RNA extraction from these tissues that predicts higher or lower yields please adjust your sample and let other partners know. We expect that **10ug total RNA will be needed to prepare mRNA and sRNA libraries**, assuming some loss during RNA extraction and quality checking, we think is it wise to collect an amount of tissue containing at least 15ug DNA. For larger organs (liver, muscle etc) please sample more mass, but take these masses as the minimum to collect for limited organs like brain and immature gonads.*

5. Next, using room temperature tweezers, drop all remaining tissue pieces onto the foil (if the tissue is sticky, this may require some aggressive flicking, or the use of a second pair of tweezers).

6. Tissue will freeze rapidly, try to place pieces separately on the foil and avoid clumping.

7. After freezing the last tissue piece, use the chilled tweezers from step 2 to pluck each tissue piece from the foil and place in a pre-labeled cryotube. If everything is kept cold, the small frozen pieces should not stick to each other or to the cryotube. (NOTE: the cold tweezers will warm up once removed from dry ice, you should swap between your two pairs of tweezers several times while collecting frozen tissue fragments to always ensure they are very cold).

8. Next, you can place the tube in -80°C. This will allow you to extract later a desired amount of tissue without thawing the whole sample. Later, when removing a desired amount of tissue chunks, always work on dry ice and with cold tweezers.

Appendix: Gonadal sampling

Background

Reproductive strategies of fish are extremely varied and to assess correctly the gonadal stage, one important aspect is the number, size and location of the pieces fixed for histology. Some protocols suggest taking up to five 1-cm pieces from the posterior end (bottom tips) of the gonads if large, the whole gonad if small. In only a few species has there been any attempt to validate that endpoints such as stage, intersex, atresia and other findings are randomly distributed throughout the gonad. Some authors examined sections from three parts of the ovary – anterior, middle and posterior – to determine if oocyte development was consistent along the length of the ovary and in all the studies, stages were randomly distributed throughout the ovary so we could assess that a portion from the middle of the gonad is sufficient for the evaluation.

Preparation

1. Prepare 1020ml Dietrich's solution (sufficient for approx. 50 samples) as a fixative by combining:
    - 600ml ddH_2O
    - 300ml 95% Ethanol
    - 100ml 40% formaldehyde
    - 20ml Acetic acid

NOTE: Bouins solution has also been recommended for use, but may be problematic to purchase, store and use because it contains picric acid. At NMBU, we have chosen NOT to use Bouins and therefore do not provide a working protocol for it here. If you choose to use Bouins, please ensure you first check with Daniela with regards shipping conditions.

2. Purchase small volume (15-20ml) containers/tubes for storage of histological samples (see photos below for examples, or here https://www.fishersci.ca/shop/products/simport-scientific-securtainer-ii-tamper-evident-specimen-containers-5/p-4121042). These must be leak proof. Histological cassettes are not required.

Sampling

1. After excision, take a photo and weigh the entire gonads. If the gonad is large enough, prepare to sample 3 portions (anterior, middle and posterior), otherwise sample just the middle portion. Take pictures before and after sampling. See as example the sea bass pictures below.

2. For histological sampling, make a clean cut using a sharp blade to ensure the integrity of the tissues and remove a section(s) not larger than $1cm^3$.

3. Place the samples in 10 volumes (at least) of Dietrich's solution for 10 days in sample containers. During this time, the samples should be kept cool and dark.

4. After 10 days, transfer the sample into new sample containers, fill these with 50% Ethanol (at least 10 volumes) in preparation for shipping. Avoid having a large volume of air in the container. Label samples with clear text and using unambiguous naming.

What happens next?...(Inclusion, cutting and staining)

After fixation in Dietrich solution and preservation in ethanol, samples will be dehydrated through a graded series of ethanol and embedded in paraffin. Sections will be cut at a thickness of 4µm using a microtome, mounted on slides, stained with haematoxylin and eosin and examined with an Olympus Vanox photomicroscope (New York Microscope Company, Hicksville, NY, USA) to evaluate the general morphology and stage.