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## **Deliverable 2.3**

# **e-book with quality indicators and a photo-atlas**

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## Contributors

Participant Organisation Name	Short name	Team Members
Centro de Ciencias do Mar do Algarve	CCMAR	Deborah Power*, Babak Najafpour (PhD student), Soraia Santos (MSc student).
Panepistimio Thessalias (P1)	UTH	Katerina Moutou*, Andreas Tsipourlianos, Alexia Fytsili
Panepistimio Kritis	UoC	Giorgos Koumoundouros*, Stephanos Fragkoulis (PhD student), Chara Kourkouta (PhD student)
Universita degli Studi di Udine	UNIUD	Marco Galiotti*, Valentina Pacorig (PhD student), Paola Beraldo, Donatella Volpatti

\*Team leader

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## 1 Introduction

The shape, appearance and smell of fish is a well established way for consumers to choose the fish they want to purchase. The check-list includes shiny eyes and skin, clearly defined fins, moist, shiny and pink gills and a light pleasant odour. In fish hatcheries, managers and workers that produce the larvae to supply juvenile fish for the grow-out stage, also use external observations to assess the quality of eggs, larvae and juveniles. This capacity to judge by observation may be complemented by some simple laboratory tests but observation is a very important component. Factors taken into consideration include, the behaviour of the larvae, their size and their general appearance and represents a unique skill that is built-up over many years of experience and becomes an intrinsic part of being a good hatchery manager or technician. Scientists have also become engaged in characterising the hatchery production stage as it is a crucial element of the production pyramid for industry. Over the last decade or more, both European and National projects have been implemented with the aim of building a knowledge base for common aquaculture species for industry to use. Non-destructive external observations and destructive, more refined microscopic observation involving sacrifice of specimens and fixation and histological processing, form the basis of examining in detail the internal and external structures and establishing when possible the “healthy and normal development” from the “unhealthy and abnormal” development.

A range of textbooks are available about fish pathology but they tend to be mainly about disease-related pathologies and directed at training veterinary and animal health-related professionals. Books or an atlas with images of external and organ/tissue and cell specific developmental trajectories linked to age and culture conditions for use in MMFF hatcheries and producers are unavailable. Even though for the salmonids there exists a greater number of bibliographic resources, when the dimension, maturity and economic importance of the sector is considered the available resources are surprisingly limited. In fact, a detailed description of the developmental ontogeny and trajectory in images is extremely difficult to find. Interesting recent initiatives that are based on creating on-line resources/atlas include a digital fish library developed for museum fish collections (Berquist et al., 2012) using MRI data (<http://www.digitalfishlibrary.org>), a small collection of information and articles about malformations in fish aquaculture coming from a Collective Research Project in FP6 (FINE FISH, n° 12451) and “fishes of the wild” (<https://swfscdata.nmfs.noaa.gov/labeled-fishes-in-the-wild>), “*Illustrated field guide...*” (Smith et al., 2002) which are images of wild fish for training ecologists for field identification.

Transfer of knowledge within the hatchery is frequently acquired by “praxis” and hands-on experience and a “feeling for the organism” are key factors in becoming a good hatchery manager or technician. This means that hatchery managers and technicians possess untapped knowledge with a high potential and relevance for transfer. WP2 offers a unique opportunity to develop a resource for the MMFF sector that engages with end users to build something of practical use. As part of the objectives of WP2 a vast ontogenetic collection of larvae of the gilthead sea bream and the European sea bass has been acquired and built during the first two and a half years of PerformFISH. The egg and larval collection represents the production from five different companies across Europe and corresponds to populations/batches from different seasons and years. The collected larvae have been defined as “good” or “bad” performers based on the incidence of skeletal malformations and also their growth as represented by weight and length at a given ontogenetic stage. The collected material is being used to analyse external

morphology, histology and molecular markers that can be linked to the “good” or “bad” classification of individuals from specific production populations/batches. A vast library of images has been collected and samples analysed in detail to provide a multi-parameter classification of quality. The biological resources that the collection of eggs and larvae represent is very valuable but the photographs, mamography and associated outcomes that resulted from the sample analysis represent another very valuable and unique asset of PerformFISH. This resource will become open access in a way that makes it applicable by the end-users that can most benefit from it, the MMFF. The aim is to “free” all the relevant and high quality, checked and validated images that form the background to thesis and publications so they can become an e-atlas resource.

The PerformFISH e-Atlas is being developed as a spin-off from the extensive analysis that has been performed on the samples in **D2.2**. This e-Atlas represents a collaborative effort by industry and scientists to develop a “living” atlas that can be developed and upgraded as the project develops. The objective of the e-atlas is to develop an on-line image-based resource (histology, whole mount staining and mamography) that can be used by hatchery managers and trainees to assess production quality and obtain information about the morphological and histological characteristics of “good” and “bad” larvae.

The present version of the e-Atlas integrates the **sampling manual, molecular biology procedures and gene primer collection** (an extensive resource of primers optimised for gilthead sea bream and European sea bass, CCMAR and UTH), **skeletal morphology** section with methods and illustrations of normal and abnormal skeletal development (University of Crete), and also a **histopathology** section providing a tissue specific description of normal and modified tissue morphology (University of Udine). It is hoped this e-Atlas will become a resource that can be used by all hatcheries of the aquaculture sector involved in production of sea bream and sea bass juveniles. The e-Atlas is part of the PerformFISH legacy and will be a resource that remains alive for several years after completion of the project. The e-Atlas will become live on the PerformFISH website (<http://performfish.eu/>) at the end of October 2022.

## 2 Sampling Manual

### 2.1 Things to remember:

1. Several different analyses may be carried out and for this reason a number of different sampling procedures and preservations steps are reported and should be used each time you sample a production batch.
2. Recording uniform KPIs for the production batch you are sampling is essential. Traceability of production batches within a cycle are crucial. A template for such consistent recording has been created by PerforFISH partner UoC and you can find it at <https://doi.org/10.5281/zenodo.7271931>.
3. A big challenge during the sampling will be to obtain representative samples since you will be dealing with hundreds of thousands of eggs and larvae in a tank. To overcome this, make sure to sample a sufficiently large number of individuals and subsequent analyses of larval production is representative.
4. The procedures described in this manual are:
  - a) chemical fixation, this will allow analysis of animals whole mount (to look for malformation) and also to carry analysis on histological sections,
  - b) sampling into RNA later, this will allow analysis by molecular biology,
  - c) sampling into methanol, this will allow analysis of DNA or other whole body analysis.

#### **IMPORTANT**

##### **Safety**

Buffered formalin, buffered paraformaldehyde, Bouin and RNA later are toxic and should be manipulated with sterile gloves. When sampling, washing or handling tubes wear gloves as a safety precaution. Use the reagents in a fume hood or in a ventilated room to avoid a build-up of fumes.

##### **Waste disposal**

Do not discard the used reagents down the drain.

Do not mix the reagents after use in the same waste bottle keep separate bottles for each waste solutions (eg. 1 bottle for paraformaldehyde, 1 for formalin, 1 for Bouin and 1 for RNA later), discard waste liquid reagent via specialized waste disposal service.

Gloves and empty plastic tubes can be eliminated via normal garbage disposal.

### ARTIFACTS

1. Poor anesthesia, fixation or sample storage can give rise to a range of artifacts which severely reduce the usefulness of the analysis.
2. If larvae are anesthetized with inappropriate concentrations of anesthesia they may gasp or contort resulting in specimens with the mouth strained open or with damaged vertebral columns and makes analysis difficult.
3. Excessive time in anesthesia before transfer to RNA later can also lead to poor sample stabilization giving poor quality samples that cannot be used.
4. Poor fixation eg. Caused by putting too many larvae in fixative bottles, or by inadequate agitation during fixation can lead to sample deterioration, which will compromise analysis.
5. Remember procedures involving anesthesia of larvae should be supervised by a qualified veterinary surgeon or someone with a valid license for animal experimentation to ensure pain and suffering of animals is avoided.

## 2.2 Sampling eggs and larvae and anaesthetization

The general steps for sample preparation are the same for all the samples we (RD&I teams) require you to collect and starts with collection of a representative sample from the tank, anaesthetic, rinsing with clean sea water and then placing in fixative, RNA later or freezing. Wear gloves (eg. Latex) to ensure safety and also to maximize sample quality.

### 2.2.1 Sampling

- a) Net eggs or larvae by gently moving a hand-held net through the tank water. It is better to repeat this procedure a couple of times to harvest sufficient larvae than to try and capture lots of larvae in one go;
- b) Blot the net on a clean paper towel;
- c) Wash the captured eggs or larvae still in the net by submerging the net and larvae in a beaker of water containing clean, filtered, sterile seawater (if available);
- d) Then place the eggs and larvae in a beaker containing clean, filtered, sterile sea water and anaesthetic.
- e) Collect the larvae either by netting, filtering or using a plastic Pasteur pipette, wash in clean seawater to remove anaesthetic and then place larvae into fixative, RNA later or freeze.

Alternative to collecting eggs/larvae with a hand-held net

- a) An alternative to collecting eggs and larvae with a hand-held net is to siphon the eggs/larvae from the tank into a net in a beaker containing sea water and then proceed as above;

### **Making sure you have a representative sample**

When you sample make sure you are taking a representative sample by observing the position of the majority of eggs or larvae in the tank water and sampling from the same location. For example, if the majority of the larvae are at the top of the tank then collect them from there.

### 2.2.2 Anaesthetization

Fish should be anaesthetized by using a solution of Ethylenglycol-monophenylether (2-phenoxyethanol, Merck) in sea water ( $0.1\text{--}0.5\text{ ml l}^{-1}$ ) or if you prefer MS222 (tricaine methanesulfonate, Sigma-Aldrich) in sea water ( $50\text{--}100\text{mg/L}$ ). There is not a standard concentration of anaesthetic because its action is strongly affected by the age/size and the physiological condition of the larvae. In general, fish must be anaesthetized (stop swimming) 10–30 sec after they are introduced into the solution.

### 2.2.3 Anaesthetization, and preservation methods

#### 2.2.3.1 *Anaesthetization and preservation of the juveniles (ca 1.5 g W) for X-ray determination of malformations.*

Juveniles should be anaesthetized, straight positioned on a flat background and frozen at  $-20^{\circ}\text{C}$ . After they are frozen, they can be stored in plastic bags with their code written in pencil (on a rice paper). A second label should be added on the bag with permanent marker.

#### 2.2.3.2 *Fixation in formalin of the larvae for analysis of malformations*

Fixation should be performed immediately after larvae sink to the bottom of the beaker of anaesthetic. If the fish body becomes opaque or curved the density of the solution is very high and should be lowered.

Larvae should be transferred to the fixation vial by means of a plastic pipette with a broad opening. Do not worry about diluting the fixative with water of the anaesthetization beaker, because after the transfer of the larvae, diluted fixative solution has to be replaced. **Fixative solution has to have at least 10 times the volume of sample.** Store in the dark, and at room temperature.

Falcon vials of 50 ml are strongly recommended for these samples. Vials should be labeled with permanent markers. A transparent tape should be used to protect the label. A second sticking label should also be used and written with pencil.

#### 2.2.3.3 *Preparation of the formalin fixative (5% buffered formalin – for skeletal analysis*

Phosphate buffer (PBS) is a general buffer for fixation; it has the advantage that the stock solutions keep indefinitely in the fridge at  $4^{\circ}\text{C}$

##### PBS (X1) solution A

For 1 litre you need:

800ml deionized water (DI water)  
 8 g NaCl (137mM NaCl)  
 0.2 g KCl (27 mM KCl)  
 1.44 g  $\text{Na}_2\text{HPO}_4$  (100mM  $\text{Na}_2\text{HPO}_4$ )  
 0.24 g  $\text{KH}_2\text{PO}_4$  (2mM  $\text{KH}_2\text{PO}_4$ ).

After the dilution of the salts, add DI water to a total volume of 1 litre (Solution A)

**Alternatively, PBS (pH 7) tablets** can be purchased and used.

### Fixative solution

To prepare the fixative, remove 50 ml from the 1 litre of PBS prepared (so you have 950 ml) and then add 50 ml Formalin (formaldehyde 37%) to give a final volume of 1 litre at 5% formalin. Remember to mix well before use.

(TIPS: - You can store the fixative in a flask at room temperature in the dark for one week. - If you do not use the fixative within a week then you should throw it out.

- You can prepare a large volume and then freeze it in Falcon vials and thaw it out as need it).

#### 2.2.3.4 Fixation of larvae in Bouin for histology

Larvae to be fixed in the Bouin solution should be transferred by means of a plastic pipette with a broad opening. Do not worry about diluting the fixative with the anesthetic, because the fixative larvae are collected into has to be replaced with fresh fixative. The ratio between sample volume and fixative volume has to be 1:10 (eg. 1g larvae and 10 ml Bouin).

Fix the larvae in Bouin for 12 h at 4 °C.

Transfer the larvae from the fixative into ethanol 70 %, using a plastic pipette with a broad opening. The fixed larvae in ethanol can be stored at room temperature.

Falcon vials of 50 ml are recommended for these samples. Vials should be labeled with a permanent marker. A transparent tape should be used to protect the label.

Note: Purchase Bouin fixative (composed of picric acid, acetic acid and formaldehyde in an aqueous solution) ready-made from a laboratory supplier.

You need to have secure conditions (eg. laboratory and fume hood) for manipulation of Bouin and the waste solution needs to be collected and removed by a waste disposal expert. Do not allow Bouin solutions to dry out they become explosive and do not tip it down the sink it is very toxic.

#### Procedures recommended for collection and storage of samples in Bouin

STAGE	Larvae Number	type and N. tubes*	fixation time in Bouin at+4°C	washing in 70% ethanol	storage
<b>First Feeding</b>	100 or more	1 x 15 ml tube	1 hour	3 washing in 70% alcohol to remove Bouin	preservation in 70% ethanol at room temperature
<b>Flexion</b>	50	1 x 15 ml tube	3 hours	"	"
<b>End Larval Rearing (10-14 mm TL)*</b>	30	1 x 50 ml tube	12 hours	"	"
<b>Mid-Metamorphosis (15-20 mm TL)</b>	30	2 x 50 ml tubes	12 hours	"	"
<b>Juvenile 0.8 – 1.5 g</b>	20	2 x 50 ml tubes	12-15 hours	"	"

*\* Use tubes like Falcon which should be completely filled with liquid before shipping and sealed with parafilm.*

*\*\* The size of vial is just for guidance and you can increase the volume or number of vials depending on the size of the larvae. The approximate number of larvae required per stage and per sample are indicated.*

## 2.3 Collection of larvae for molecular biology

Sample collection and anaesthesia as indicated above in 4.2.1 and 4.2.2.

After anaesthesia, and once the larvae are settled at the bottom of the beaker, Larvae should be rinsed in sterile, filtered seawater and then preserved immediately in RNA later. A plankton filter is particularly useful for this task. Subsequently, larvae should be transferred to a 15 or 50ml Falcon vial by means of a plastic pipette with a broad opening after rinsing the larvae in clean, sterile seawater. Try to avoid diluting the RNA later with water, if dilution occurs then pass the larvae to a new 15 or 50ml Falcon containing new RNA later. **RNA later should be present in at least 5 times the volume of sample (eg. 1g sample + 5 ml RNA later).**

### 2.3.1 Processing of larvae sampled into RNA later

RNAlater™ Storage Solution (Sigma-Aldrich now MERCK). Larvae collected under as clean as possible conditions Collected larvae are submerged in approximately 10 volumes of RNAlater® solution at room temperature (as described above). Most samples in RNA later® Solution can be stored at room temperature for 1 week without compromising sample quality, or at –20° C (the tissue does not freeze). Do not immediately freeze the samples collected into RNA later® solution; store the samples at 4° C overnight (to allow the solution to thoroughly penetrate the larvae), then put the tube containing the larvae to –20° C for longer-term storage.

Falcon vials of 15 or 50ml should be used for the samples. Vials should be labeled with permanent markers. A transparent tape should be used to protect the label. A second sticking label can also be used and written with pencil to increase security if one label is lost. You can use 15 ml vials for Eggs, First Feeding and Flexion and use 50ml vials for End Larval Rearing (10-14 mm TL) and Mid-Metamorphosis (15-20 mm TL) samples.

For good fixation and storage, it is best to lay the tubes on their side so the samples have a bigger surface area or contact with the fixing solution. Make sure tubes are sufficiently full of RNA later to ensure permanent sample immersion.

### 2.3.2 Sampling of larvae into methanol

Sample collection and anaesthesia of larvae as indicated above in 4.2.1 and 4.2.2.

Immediately after larvae sink to the bottom of the beaker after anaesthesia, they should be placed in methanol (100%) in 50 ml falcons. Replace with new methanol when sampling is complete for a given time point and then put in a freezer at -20°C.

Screw cap vials of 50ml should be used for the samples. Vials should be labeled with permanent markers. A transparent tape should be used to protect the label. A second sticking label should also be used and written with pencil.

## 3 Molecular Biology Procedures

### 3.1 Sample reception

Samples of larvae at different stages fixed in Methanol and in RNA later (first feed, flexion, end of larvae rearing and mid metamorphosis stages) can be used for molecular biology procedures. After sample collection the next challenge is sample transport from the site of collection to the site of analysis. Best sample preservation is secured by transport in polystyrene boxes that maintain the thermal conditions. Dry ice is often difficult to obtain in remote sites and not handled by all couriers for this reason RNA later can be used as samples can then be transported in chiller boxes lined with cooling blocks and packed with ice. Samples should be in 15 ml or 50 ml screw cap vials, with the lid sealed with parafilm and the label on tubes written with indelible ink and covered with Sellotape to avoid loss of labels if tubes burst.

On arrival samples should be inspected, and reagent substituted and refilled if leakage or other changes have occurred to the preserving reagent before or during transport. Samples can then be given a unique handling code to identify origin, type of sample and date of sampling.

### 3.2 RNA extraction from egg and larval samples

For quality checking of the samples received (QC2) and for subsequence analysis of batches by molecular analysis RNA can be extracted from RNA later or methanol preserved gilthead sea bream and European sea bass. The RNA extracted from samples should be quantified and RNA quality be determined using spectroscopy (nanodrop) and by analysing samples using 0.8% agarose gel electrophoresis. Good integrity of 28S and 18S ribosomal RNA (with a more intense 28S rRNA) observed after agarose gel electrophoresis is an indicator of a high level of mRNA integrity.

#### 3.2.1 RNA extraction method

See the summary overview of the procedure in Figure 3.

An E.Z.N.A.<sup>®</sup> RNA extraction Kit (Omega Bio-Tek) is used for the extraction of RNA from egg and larvae samples. A range of kits are available commercially but this kit is cost effective, quick and generally gave high quality extracts when used in PerformFISH. Preliminary optimization was carried out to adjust quantities of sample to buffer, sample disruption etc and this is a crucial first step for any kit you choose to use. It is recommended that before running large scale extraction with a commercial kit you confirm suitability for the matrix you are working with in your study. The E.Z.N.A.<sup>®</sup> RNA extraction Kit has a column containing a HiBind<sup>®</sup> matrix and so processing is rapid and there is no need for phenol/chloroform extractions and time-consuming steps such as precipitation with isopropanol. The RNA purified using the E.Z.N.A.<sup>®</sup> RNA Purification System can be used after removal of any contaminating genomic DNA in RT-PCR, Northern blotting, and poly A+ RNA (mRNA) purification.

**Protocol:** optimized E.Z.N.A.<sup>®</sup> Total RNA Kit I, R6834-02, OMEGA used for extraction of eggs and larvae of gilthead sea bream and European sea bass.

##### 3.2.1.1 E.Z.N.A.<sup>®</sup> Total RNA Kit I Protocol - Animal Tissue Protocol

All centrifugation steps used are performed at room temperature. After elution put samples on ice immediately, the rest of the procedure should be carried out at room temperature.

Materials and equipment you need to have available before starting the RNA extractions:

- Microcentrifuge capable of at least 14,000 x g
- RNase-free pipet tips and 1.5 mL and 2mL o-ringer tubes
- 100% ethanol
- 70% ethanol in sterile DEPC-treated water
- TissueLyser equipment
- Vortex
- Pippetes and tips autoclaved
- Boxes to save samples
- E.Z.N.A.<sup>®</sup> DNase I Digestion Buffer
- RNase-free DNase I (20 Kunitz/ $\mu$ L) (-20°C)
- Dissection material
- 14.3M 2-mercaptoethanol ( $\beta$ -mercaptoethanol)
- RNaseZAP
- MQ Water
- Beads inox 5mm

Before Starting:

- Prepare RNA Wash Buffer II, KIT R6834-02, add 200ml of 100% ethanol and store at room temperature.
- Add 20  $\mu$ L 2-mercaptoethanol per 1 mL TRK Lysis Buffer.
- Clean dissection material and homogenization equipment with RNaseZap, 5min.
- Prepare all tubes and label

**Note:** Do steps 1 to 17 in a fume cupboard and according to experience you can run 6 – 24 samples at a time.

1. Determine the appropriate amount of starting material.

**Note:** The maximum binding capacity of the HiBind<sup>®</sup> RNA Mini Column is 100  $\mu$ g. The maximum amount of tissue that TRK Lysis Buffer can lyse in this protocol is 30 mg.

2. Add the beads for sample disruption and 700ul of Lysis buffer in a 2mL tube.

3. Weigh the tissue (20-30mg), and transfer to TRK Lysis Buffer with 2-mercaptoethanol (fume cupboard). Approximate numbers of larvae per stage are suggested: first feeding - 5 larvae; flexion - 3 larvae; end of larvae rearing - 1 larvae and mid metamorphosis - 1 larvae.

**Amount of TRK Lysis Buffer per Tissue Sample**

Amount of Tissue	Amount of TRK Lysis Buffer ( $\mu$ L)
$\leq 15$ mg	350 $\mu$ L
20-30 mg	700 $\mu$ L

**Note:** For samples stored in RNALater<sup>®</sup> use 700  $\mu$ L TRK Lysis Buffer.

**Figure 1. The proposed tissue: lysis buffer ration.** The working ratio of tissue to lysis buffer is recommended by the manufacturer. However, we have found that this may vary by sample type and the composition of fish larvae means that you should establish the best ration sample: lysis buffer for different larval stage (sizes).

4. Homogenize and disrupt the tissue, with TissueLyser, until everything is disrupted, for all larvae independent of age, use 3x 30sec at 30s.

**Note:** Incomplete homogenization of the sample may cause the column to clog resulting in decreased yield. If it needed repeat one more cycle of homogenization

5. Transfer the homogenate to new 1.5ml tube to take out the beads before centrifuge. This is a convenient point to stop, you can store homogenates in a -80°C freezer for several weeks to months without negative consequences (this was evaluated in a well controlled experiment).

6. Centrifuge at 12.000 rpm for 10 minutes at 4°C (if you are using frozen samples allow them to fully defrost over ice and then proceed as normal).

7. Transfer the cleared supernatant (~550ul) to a clean 1.5 mL microcentrifuge tube. **Note:** In some preparations, a fatty upper layer will form after centrifugation. Transfer of any of the fatty upper layer may reduce RNA yield or clog the column.

8. Add 1 volume (~550ul) 70% ethanol. Vortex strongly to mix thoroughly (10sec). Do not centrifuge.

**Note:** A precipitate may form at this point. This will not interfere with the RNA purification. If any sample lost volume during homogenization, adjust the volume of ethanol accordingly.

9. Insert a HiBind® RNA Mini Column into a 2 mL Collection Tube.

10. Transfer 550 µL sample (including any precipitate that may have formed) to the HiBind® RNA Mini Column.

11. Centrifuge at Max Speed for 1 minute, at RT.

12. Discard the filtrate and reuse the Collection Tube.

13. Repeat Steps 8-10 until all of the sample has been transferred to the column.

14. Add 250 µL RNA Wash Buffer I to the HiBind® RNA Mini Column and centrifuge at 12.000 rpm for 1 minute, at room temperature.

15. Discard the filtrate and reuse the collection tube and proceed to the DNase treatment procedure (5.2.2).

### 3.2.2 DNase I Digestion Protocol

The E.N.Z.A RNA purification kit eliminates most of the genomic DNA during extraction. Nonetheless, you should always treat RNA extracts with DNase to remove residual genomic DNA contamination that can create artifacts during gene amplification by PCR or sequencing. An easy and quick solution is to use the DNase digestion protocol during the RNA purification as recommended by the manufacturer. Prepare the mix as indicated below:

- DNase I Digestion Set (E1091)

1. For each HiBind® RNA Mini Column, prepare the DNase I stock solution as follows:

Buffer	Volume per Prep
E.Z.N.A.® DNase I Digestion Buffer	73.5 µL
RNase-free DNase I (20 Kunitz/µL)	1.5 µL
Total Volume	75 µL

**Important Notes:**

- DNase I is very sensitive and prone to physical denaturing. Do not vortex the DNase I mixture. Mix gently by inverting the tube.
- Freshly prepare DNase I stock solution right before RNA isolation.
- Standard DNase buffers are not compatible with on-membrane DNase I digestion. The use of other buffers may affect the binding of RNA to the HiBind® matrix and may reduce RNA yields and purity.
- All steps must be carried out at room temperature. Work quickly, but carefully.

**Figure 2. On-column DNase treatment of RNA.**

1. Add 75 µL DNase I digestion mixture directly onto the surface of the membrane of the HiBind® RNA Mini Column. Note: Pipette the DNase I directly onto the membrane. DNA digestion will not be complete if some of the mixture is retained on the wall of the HiBind® RNA Mini Column.
  2. Let sit at 25°C (dry bath) for 20 minutes.
  3. Add 250 µL RNA Wash Buffer I to the HiBind® RNA Mini Column and leave for 2 minutes at room temperature (approx 24 °C).
  4. Centrifuge at 10,000 – 12,000 rpm in a benchtop centrifuge for 1 minute, at room temperature and then discard the filtrate and reuse the Collection Tube.
  5. Add 500 µL RNA Wash Buffer II. Note: RNA Wash Buffer II must be diluted with ethanol (100%) before use as instructed in the kit.
  8. Centrifuge at 10,000 – 12,000 rpm in a benchtop centrifuge for 1 minute, at room temperature. Discard the filtrate and reuse the Collection Tube (do this step quickly) and repeat the wash with RNA Wash Buffer II two more times.
  9. Centrifuge the column with the DNase treated RNA at maximum speed for 3 minutes to completely dry the HiBind® RNA Mini Column matrix.
- Note:** It is important to dry the HiBind® RNA Mini Column matrix before elution. Residual ethanol will interfere with downstream applications.
10. Place the column in a clean 1.5 mL microcentrifuge tube and elute the RNA using 40 µL DEPC Water.

**Note:** Make sure to add the water directly onto the HiBind® RNA Mini Column matrix. After adding the water to the column, leave it for 1 minute at room temperature and then centrifuge the column at 10,000 – 12,000 rpm in a benchtop centrifuge for 2 minutes, at RT.

11. Store eluted RNA at -80°C, or keep on ice before quality checking (QC3) by spectroscopy and agarose gel electrophoresis.

### 3.2.3 Quantification and quality of extracted RNA

#### 3.2.3.1 Spectrophotometer (NanoDrop, Thermofisher, USA)

1. Use 1.5µL of the extracted RNA to quantify and assess quality in a NanoDrop spectrophotometer. Note the ng/ul and the value of the ratios 260/280 and 260/230.

**Note:**

Ratio 260/280 → the ideal value for RNA is >1.8 and values diverging from this are indicative of contamination with protein, salts etc.

Ratio 260/230 → the ideal value for RNA is >1.8 and never < 0.8 and deviation are indicative of contamination with ethanol.

#### 3.2.3.2 Agarose gel electrophoresis

1. For determination of RNA integrity, you can also use electrophoresis, but it is important to ensure all material is clean and free of RNase. Wash all material for electrophoresis (combs, supports, tanks, measurable items, etc.) in household detergent in sterile water and then wash with sterile water and dry off excess water with absorbent paper.

2. Prepare a 0.8% agarose gel with 1x TAE buffer (Annex I) by heating in a microwave until molten (approximately 3 -4 minutes), allow to cool for 3 – 5 minutes with gentle mixing, you should be able to handle the Erlenmeyer containing the agarose without discomfort. If you pour the agarose into the mould while it is still molten it will cause the acrylic mould to crack.

3. Add 10µL from 1/10 dilution of GreenSafe (NZYTech, Portugal) /50mL of agarose gel and pour the gel into the acrylic mould and place in the sample well combs. Leave for 20 min or until the agarose solidifies.

4. Prepare the samples for electrophoresis by mixing 1ul of sample + 2ul of loading buffer (6x concentrated, Annex I) and make up to 10 ul with sterile water.

5. When agarose gel has solidified, remove the combs and transfer the agarose gel in the acrylic mould into the electrophoresis tank filled with running buffer (TAE 1X) and ensure the gel is totally immersed.

6. Load the samples into the well in the agarose gel, and include in one well a DNA ladder (DNA ladder V/III) to provide molecular markers of known size.

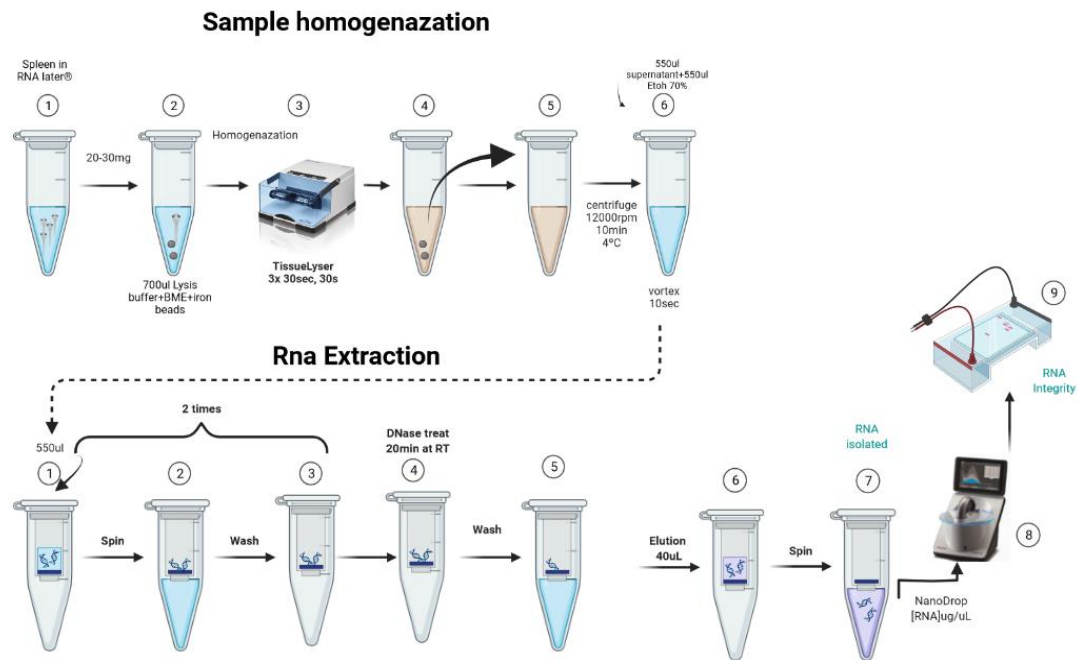
7. Close the lid, link the electrodes to electrical resource, switch ON adjust to 90 volts and run for, 20/30min. Observe the gel under UV light using a gel documentation system (e.g. Chemidoc XRS Chemiluminescent Gel by, Biorad) and verify the characteristics of the pattern of RNA bands visible.

**Note:** Material contaminated with green safe should be discarded through a specialised refuse collection company.

### Elimination of genomic contamination

Confirm that genomic contamination has been eliminated by running a PCR using a reference gene 18S using the extracted total RNA (QC4) as the template using a 1/40 diluted (same dilution as cDNA sample) in the PCR reaction (see 3.3 point 7). Analyse the PCR products by 1.5 % agarose gel electrophoresis in 1x TAE buffer. Load 10 µl of the PCR product onto the gel and run the electrophoresis at 120 volts for 20 min.

EXTRA STEP IN CASE OF NEED: In case of consistent genomic contamination, use an alternative post-purification approach. We have found the Precision™ DNase kit (Primer Design, VWR) to be very effective.



**Figure 3. Overview of RNA extraction and quality assessment procedure**

### 3.3 Synthesis of cDNA

The extracted RNA needs to convert to cDNA for subsequent quantification by PCR. For this reason, after confirming integrity and quantity of RNA extracted those that passed QC4 (checking for the absence of genomic contamination) can be then used for cDNA synthesis. An optimized synthesis method was used, common for all samples, and efficiency was tested by running RT-PCR to amplify 18S rRNA with all the cDNA synthesized (QC5). Since the same quantity of RNA is used for cDNA synthesis then for each sample of a given stage a similar yield of cDNA and quantity of 18S rRNA template would be expected. This means that after RT-PCR amplification the yield of 18S rRNA should be approximately the same. A summary figure of the main steps for cDNA synthesis and QC5 are shown in Figure 4.

1. Dilute RNA (500ng) extracted from each sample in nuclease-free water. During cDNA synthesis for batches of sample always include a –RT control sample (omit the reverse transcriptase from the synthesis reaction).
2. Add 1ul of Random Hexamers (100uM/200ng) to each sample.
3. Denature the RNA at 65°C for 5 min, in a thermocycler (T100 Thermal Cycler, BioRad) and place on ice for 5 min before centrifugation at 500 rpm at room temperature for 30 seconds.
4. To each cDNA synthesis reaction, add 26 µl of reaction mix composed of nuclease-free water (14,75 µl); 5x reaction buffer (8 µl); dNTPs 10mM (2 µl); 0.25 µl of RiboLock™ RNase Inhibitor (40U/µl) and 1 µl of the enzyme reverse transcriptase (200U/µl) (RevertAid™ Reverse Transcriptase ThermoFisher). If you have many samples prepare a master mix that contains all the reagents and pipette the appropriate volume of mix into each tube. Remember Reverse Transcriptase is extremely unstable so always hold it on ice and work quickly.
5. Carry out the cDNA synthesis reaction in a Thermocycler using the following program: 10 min at 25°C; 60 min at 42°C; 10min at 70°C and then hold at 8°C if you are not free to immediately place in a freezer -20°C for storage.
6. Confirm the efficiency of the cDNA synthesis by running a reverse-transcriptase polymerase chain reaction (RT-PCR) with each sample using the reference gene 18S rRNA. If using the same sample for cDNA synthesis, then you should expect all samples to give a similar intensity/quantity of the amplified gene.
7. The following reaction is used for the RT-PCR. 1ul of cDNA (1/10 dilution) and add to 9 ul of the PCR reaction mix composed of, 7.36 µl of sterile water, 1 µl of 10x reaction buffer (supplied with the Taq polymerase), 0.2 µl dNTPs (10mM), 0.2 µl of the forward (Fw) primer (10mM), 0.2 µl of the reverse (Rv) primer (10mM) and 0.04 µl of DreamTaq (DreamTaq™ Green DNA Polymerase (5 U/µL), Thermo Scientific).
8. Amplification of the reference gene should be carried out with the following thermocycle: 2 min at 95 °C, 17X (20 sec at 95 °C, 20 sec at 60°C, and 30 sec at 72°C); 5 min at 72°C and ∞ at 8°C. Confirm cDNA synthesis and quality by running 10 µl of the PCR product on a 1x TAE electrophoresis agarose gel 2% about 20min, 120V.

### 3.4 Quantitative polymerase chain reaction (qPCR) analysis

Quantitative PCR is a modern technology used to study gene expression, in which PCR products amplified are detected in real-time using oligonucleotides labelled with fluorochromes. The method is highly sensitive and has a large dynamic range and can be used to accurately quantify gene expression in samples. When looking at gene transcription then RNA is extracted from the tissue of interest, complementary DNA (cDNA) is synthesised using the enzyme reverse transcriptase and then used as a template for the PCR. Using specific primers designed against the gene of interest it is possible to quantify its expression level.

1. Prepare an appropriate volume of cDNA in the case of European sea bass and gilthead sea bream larval samples a dilution of 1/10 can be used in qPCR analysis. Although depending on the abundance of genes it may be necessary to increase or decrease the dilution. This is part of the optimization process when developing primers for qPCR.

2. Prepare a master mix of all reagents required for the qPCR and to give a volume that is sufficient for the samples to be analysed. Here it is important to take into account the replicates to be run for each sample (2- 3 replicates are recommended). The reagents required per sample are: 3µL of Forget-Me-Not™ EvaGreen® Master Mix (Biotium, USA), 0.76 µL of sterile water, 0.12 µL of the forward and reverse primer.

3. Add 4ul of master mix plus 2ul of the template into a 384 well plate. For the negative control use sterile water instead of the template. For the positive control you can use the amplicon used to confirm the specificity of the primers. If using several plates on different days or weeks then an internal standard can be used to correct for any minor drift between plates that may occur.

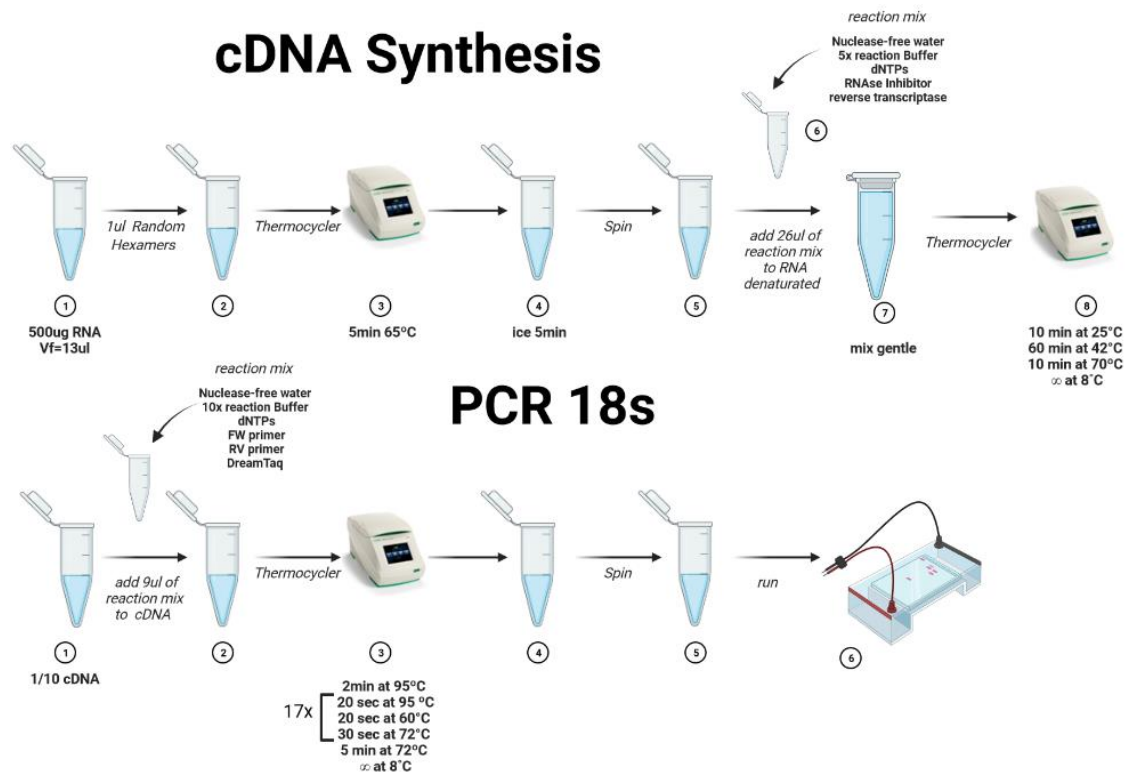
4. The thermocycling conditions will vary with the primers. To optimise qPCR the best strategy is to optimise all primers for a similar annealing temperature (we opt for 58, 60 and 62 °C) and thermocycle. The thermocycle conditions we used are: 95°C for 2 min; 40 cycles of 95°C for 5 s at the optimal annealing temperatures for 10 s (depending on the primers); 95°C for 10 s; melt curve detection at 60°C for 10 s to 95°C increment 0.5°C. RNA expression levels can be calculated after normalizing with the reference genes (we tend to use, EF1- $\alpha$ , RPS18, 18S rRNA,  $\beta$ -actin). It is important to always verify for the samples being analysed what are the reference genes that have the most stable expression and use them.

### 3.4.1 Primers

A primer databank was generated using de novo designed primers coming from the transcriptomes generated during PerformFISH and bibliographic searches ([DOI: 10.5281/zenodo.7271303](https://doi.org/10.5281/zenodo.7271303)). The primer list presents primers that were optimized and specificity for the target gene confirmed by sequencing the amplicon; primers that were optimized but specificity for the target gene not confirmed by sequencing the amplicon and primers reported in the literature for, which confirmation of specificity was first checked by database searches to confirm using bioinformatics that primers anneal to the expected candidate genes and those that we were unable to confirm.

#### Optimization of primers

Primers were purchased and used to test what genes were expressed in larvae, using as the template a cDNA generated from a pool of larvae at the four development stages used for the study (RNA extracted from the samples were pooled in equivalent concentrations and used for cDNA synthesis). Candidate genes were analysed by RT-PCR, those that were only amplified after 32 cycles were not included in the study. Primer optimization can involve varying the concentration of the primers used, varying the annealing temperature and varying the thermocycle. It is advisable to sequence the amplicon to confirm the specificity of primers for the candidate gene of interest.



**Figure 4. Overview of the main step for cDNA synthesis and 18S PCR to check the quality of cDNA synthesis reactions**

## 4 Skeletal abnormalities

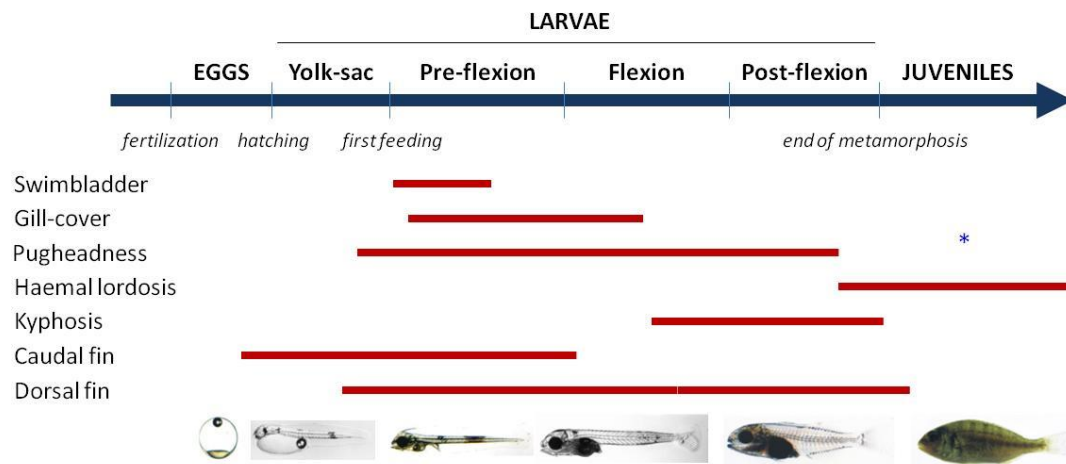
Skeletal abnormalities are characterized by a remarkable variability in respect to their phenotype, degree of severity and ontogenetic window during which they form (Figure 5). As it was suggested for saddleback syndrome (SBS) in sea bass (Fragkoulis et al. 2017), the wide ontogenetic period during which different skeletal parts develop and causative factors may act, is a major source of phenotypic variation (anatomical area affected, severity degree, correlation between different abnormalities).

In the Mediterranean finfish mariculture, most skeletal abnormality types develop up to the post-flexion stage (ca 35-45 days post-hatching, dph), whereas haemal lordosis continues developing during the following ontogenetic period (Koumoundouros et al. 2010, Figure 5). On the basis of this ontogenetic discrimination of skeletal abnormalities, a two-step quality control can be applied in the commercial hatcheries to estimate as early as possible the rate of abnormal fish. The earliest abnormality control can be performed at 35-45 dph, following the double staining (Alizarin red S, Alcian Blue) of formalin fixed specimens. The second abnormality control is performed during the pre-growing period by visual macroscopic examination (gross) of anaesthetised specimens, and by radiography (aiming to haemal lordosis and other vertebral defects).

Following the latest study of Kourkouta et al. (under review) and the associated results of WP2, the inward folding of gill cover (Figure 6B - 6C) is the most important abnormality in commercial sea bream hatcheries. It is followed by the compression of the ethmoid area and upper jaws (pugheadedness, Figure 6D - 6I), which may be associated with abnormalities of the maxillaries and pre-maxillaries (Figure 6D - 6F), and to a lesser extent with fracture of the parasphenoid-vomer bar (Figure 6H, 6I), or with fused maxillaries and pre-maxillaries (Figure 6G). Rest, less frequent abnormalities include shortening, stricture or duplication of the caudal fin (Figure 7), pterygiophore abnormalities and saddleback syndrome (Figure 8A, 8B), as well as kyphosis, lordosis and abnormalities of single vertebral centra (Figure 8C, 8D). A rare but new abnormality type, ray-resorption syndrome (RRS), has lately been recorded in seabream larval samples. The RRS may be macroscopically evident on the spines and lepidotrichia of all the fins, in the form of irregular, mosaic ossification pattern (Figure 9A - 9B).

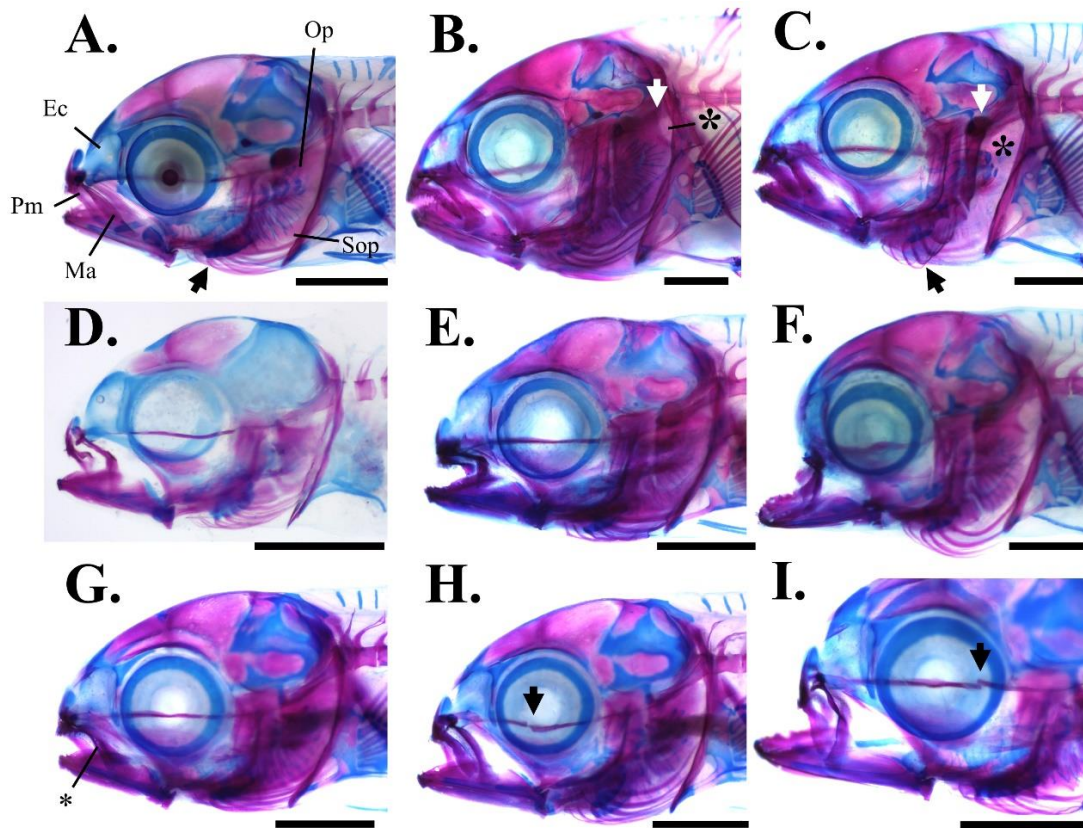
Haemal lordosis is the only abnormality that develops on an initially normal skeleton, due to the action of swimming intensity, as well as other causative factors (recently reviewed by Fragkoulis et al. 2019, Printzi et al. 2021). The abnormality can be examined by radiographies of fish juveniles and presents a continuous severity range (Figure 10, Figure 11). Fragkoulis et al. (2019, 2022) demonstrated that this abnormality may continue evolving its phenotype during the grow-out period, by following a significant recovery process.

Saddleback syndrome is nowadays the most important abnormality of sea bass larvae. It may be expressed with a wide range of severity degrees, from disarrangement and abnormalities of dorsal fin pterygiophores to lack of fin spines and lepidotrichia (Figure 12A - 12B, see Fragkoulis et al. 2017 for a full ontogenetic and anatomical description). Rest abnormality types that may nowadays appear in sea bass larvae include the ray resorption syndrome (Figure 12C), prognathism of the lower jaw (Figure 12D) and miscellaneous vertebral defects (Figure 12E).



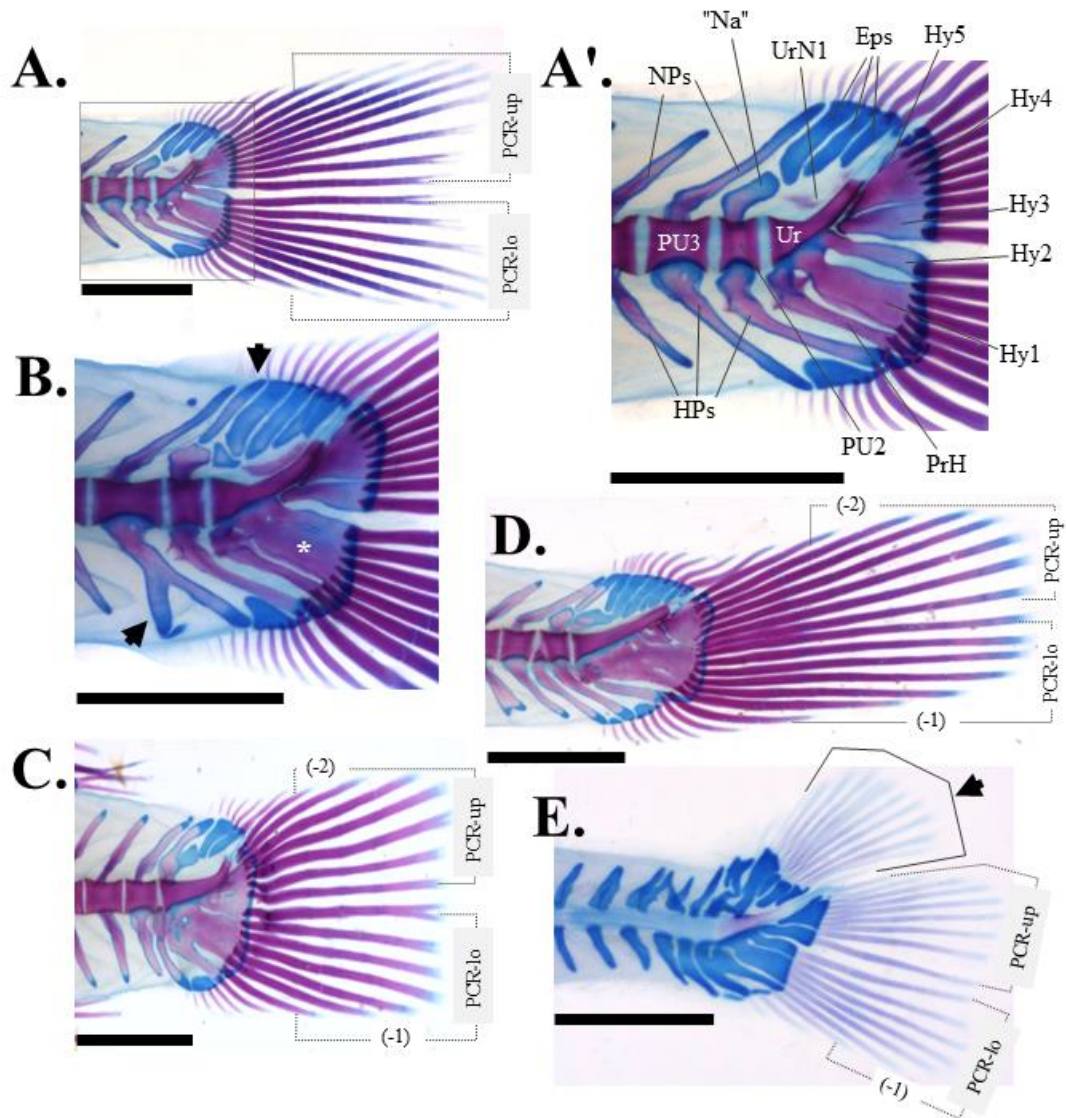
**Figure 5. Critical ontogenetic windows of the main types of skeletal abnormalities in Mediterranean aquaculture** (Koumoundouros 2010, updated with data from Fragkoulis et al. 2017, 2019). \* indicates the recovery potential of haemal lordosis.

#### 4.1 Common cranial abnormalities – gilthead sea bream



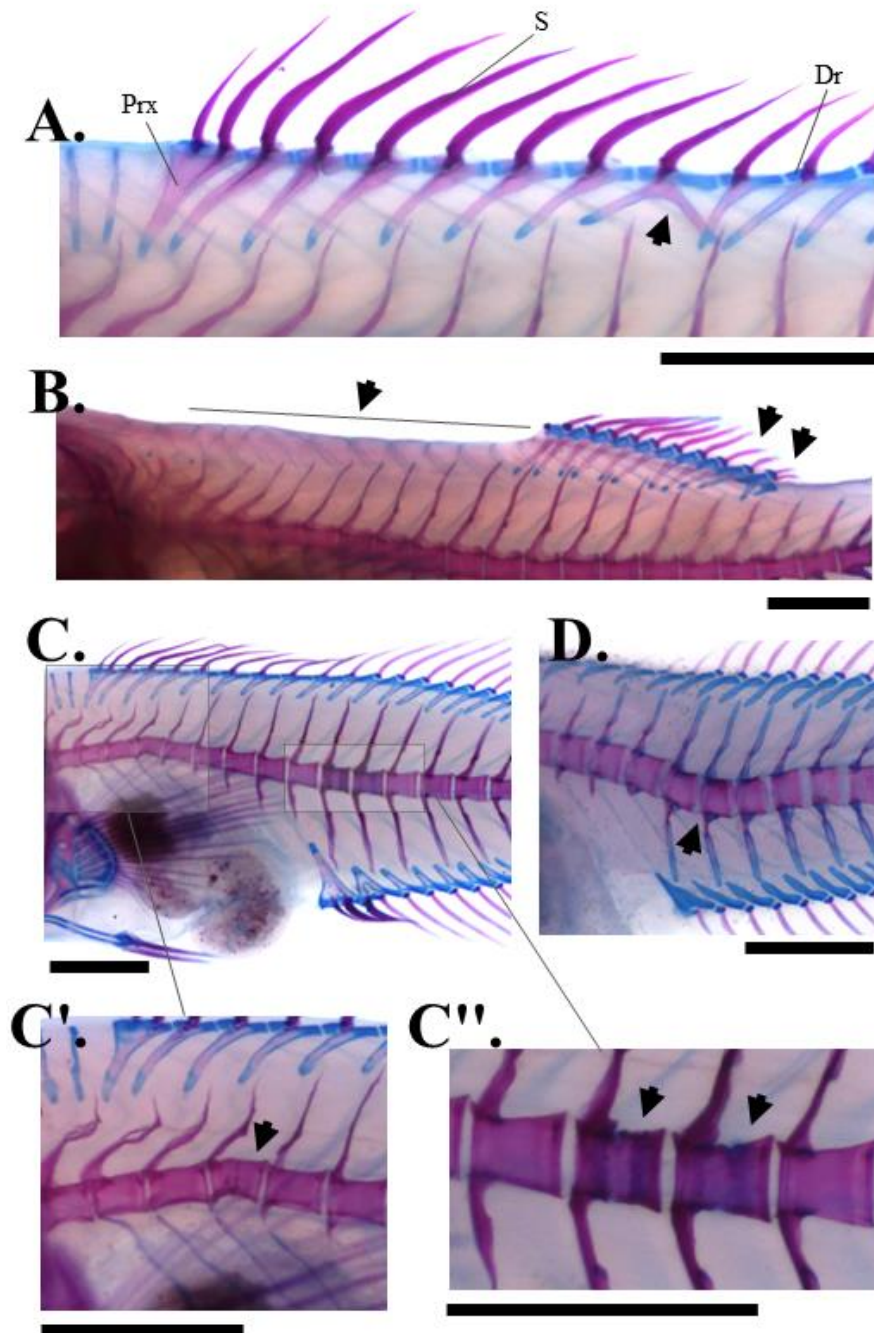
**Figure 6. Variability of cranial abnormalities in hatchery reared sea bream samples.** **A.** Normal. **B-C.** gill-cover abnormality, light (B) and severe (C), with inward folding of the operculum (white arrow), sub-operculum and branchiostegal rays (C, black arrow). Asterisks indicate the gill-chamber. **D-E.** light (D) and severe (E) pugheadedness, associated with twisted maxillaries and pre-maxillaries. **F.** Severe pugheadedness, with lack of pre-maxillaries, malformed maxillaries, ethmoid cartilage and lower jaw. **G.** Fusion (\*) between the maxillary and pre-maxillary bones. **H-I.** Pugheadedness, associated with the fracture of the parasphenoid-vomer bar (arrows). Ec, ethmoid cartilage. Ma, maxillary. Op, operculum. Pm, pre-maxillary. Sop, sub-operculum. Scale bars equal to 1 mm. **from Kourkouta et al., under review in Sci. Rep.).** Photos: University of Crete.

## 4.2 Common caudal-fin abnormalities – gilthead sea bream



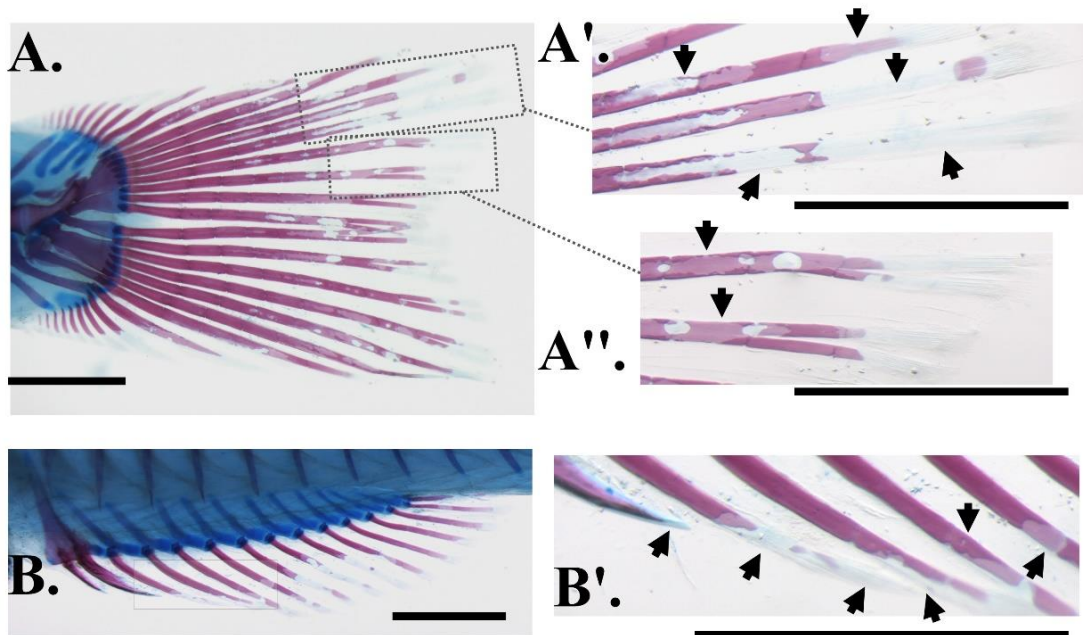
**Figure 7. Variability of caudal-fin abnormalities in hatchery reared sea bream samples. A.** Normal. **A'.** Inset of figure A, showing in detail the internal anatomy of the caudal-fin. **B.** Light abnormalities (arrows) of the caudal fin. Asterisk indicates the abnormal fusion of hypurals 1 and 2. **C-E.** Severe abnormalities of the caudal-fin (PCRs shortening, C; fin stricture, D; fin duplication, E), associated with multiple abnormalities of the fin supporting elements. Eps, epurals. HPs, haemal processes. Arrow in E indicated the extra-numerous PCRs. Hy1-Hy5, hypurals 1-5. "Na", modified neural arch. NPs, neural processes. PCR-lo, lower principal caudal-fin rays. PCR-up, upper principal caudal-fin rays. PrH, parhypural. PU2 and PU3, pre-ural centrum 2 and 3 respectively. Ur, urostyle. UrN1, uroneural 1. Numbers in brackets indicate the difference of ray number from the normal (9 PCR-up and 8 PCR-lo). Scale bars equal to 1 mm. (from Kourkouta et al., under review in *Sci. Rep.*). Photos: University of Crete.

### 4.3 Common dorsal-fin and vertebral abnormalities – gilthead sea bream



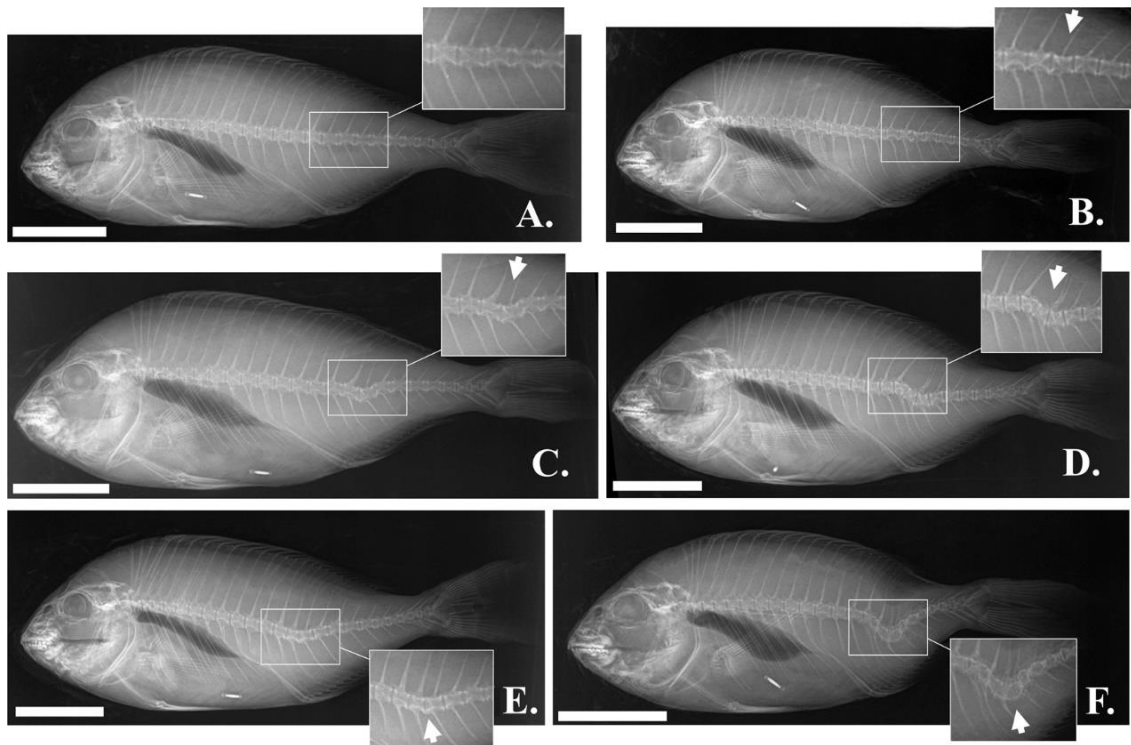
**Figure 8. Variability of dorsal-fin and vertebral abnormalities in hatchery reared sea bream samples.** **A.** Light abnormality (arrow) of the dorsal fin. **B.** Saddleback syndrome. Arrows indicate the missing pterygiophores and spines (anterior) or abnormal rays (posterior). **C-C''.** Light kyphosis (C') and abnormalities of centra without associated axis deviations (C''). **D.** Haemal lordosis (arrow). Scale bars equal to 1 mm from Kourkouta et al., under review in Sci. Rep.). Photos: University of Crete.

#### 4.4 New abnormality - ray-resorption syndrome in gilthead sea bream



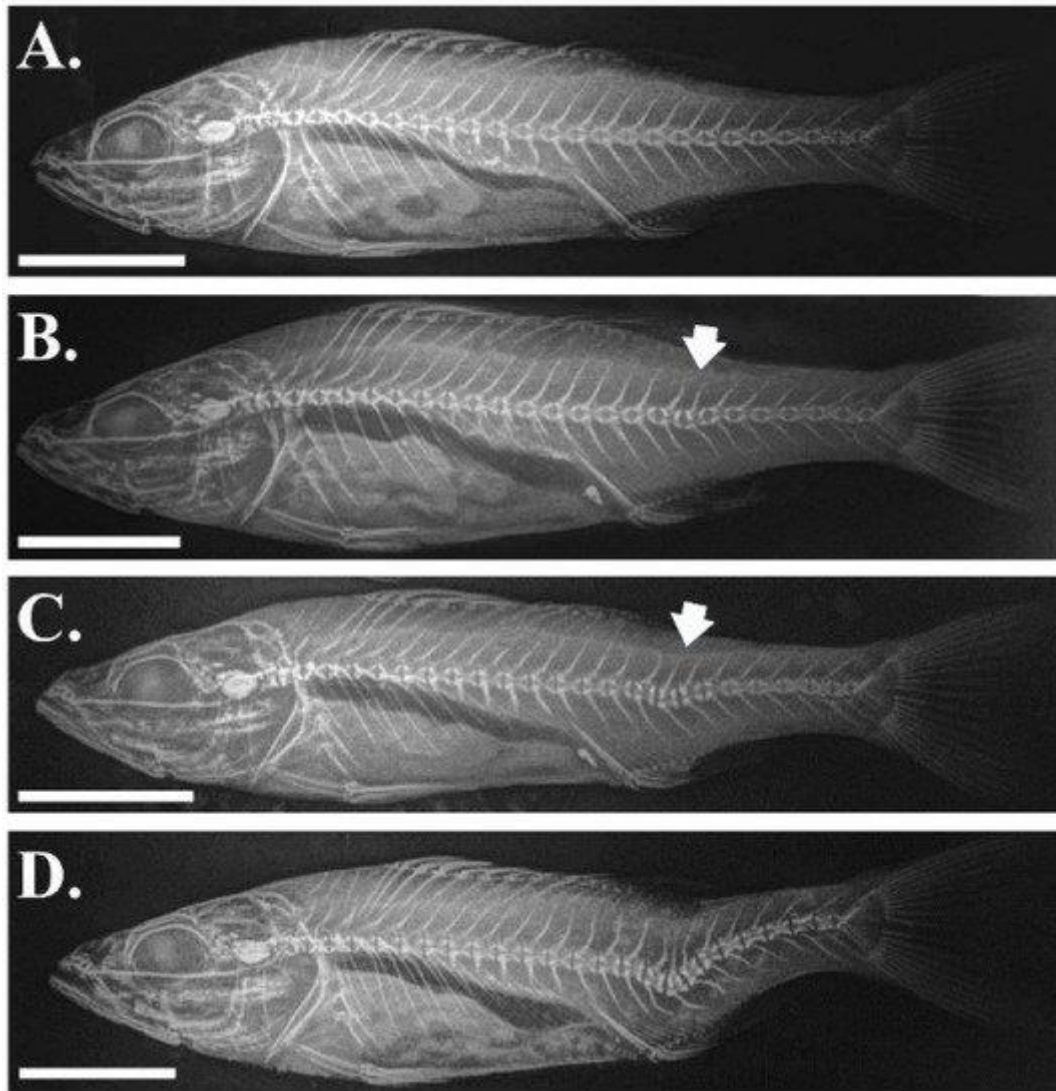
**Figure 9. Representative examples of ray-resorption syndrome in hatchery reared sea bream samples.** A. Caudal fin. B. Anal fin. A'-A'' and B'. Insets of figure A and B, showing in detail the non-mineralized areas, resembling typical resorption lacunae (arrows). Scale bars equal to 1 mm. from Kourkouta et al., under review in *Sci. Rep.*). Photos: University of Crete.

#### 4.5 X-ray - Haemal lordosis in juvenile gilthead sea bream



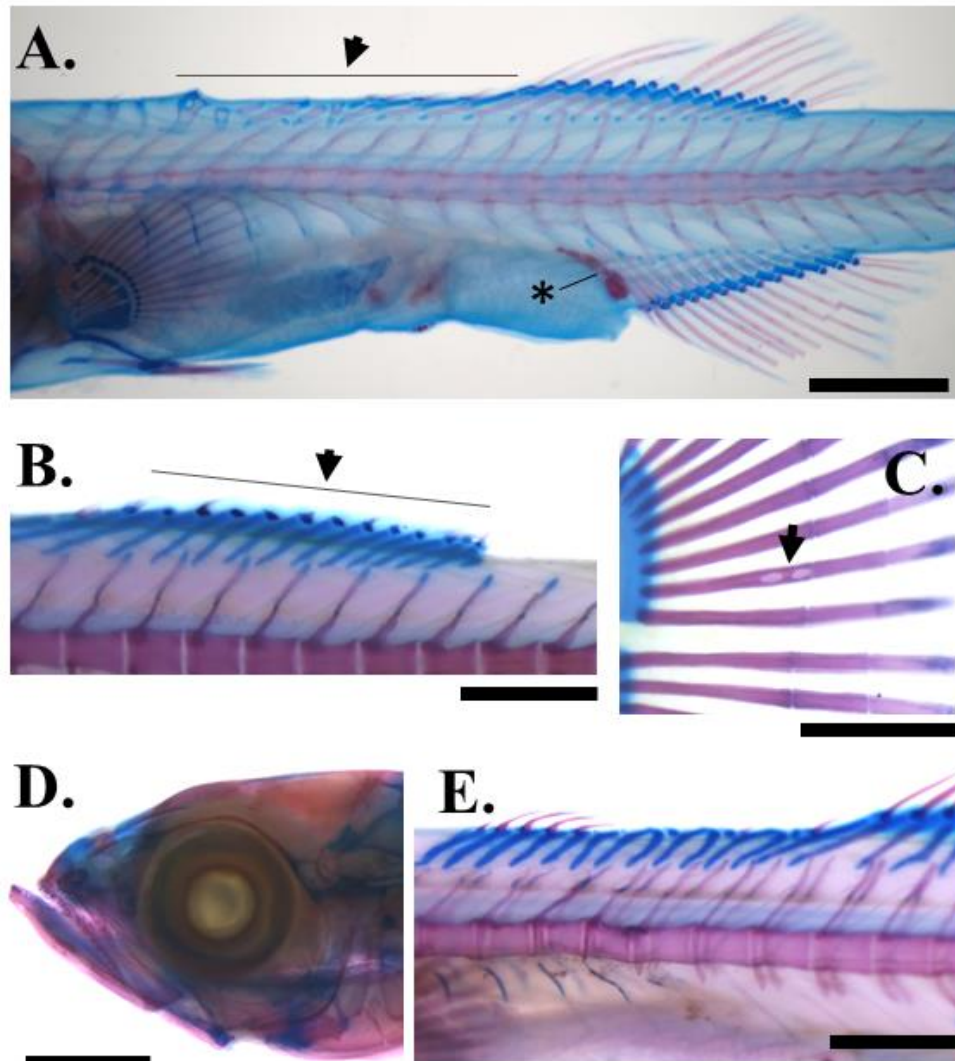
**Figure 10. Haemal lordosis (C–F) of variant severity in seabream at the end of the on-growing period (434 days post-tagging).** (A) Normal fish. (B) Normal fish with minor abnormalities of individual centra (arrow). (C) Fish with light internal lordosis and a normal external phenotype. (D) Fish with a kyphosis anterior to lordosis. (E) Fish with an uncertain external morphology and a severe internal lordosis. (F) Fish with an abnormal external morphology and a severe internal lordosis. Scale bars are equal to 5 cm. (from Fragkoulis et al., Sci Rep 9, 9832 (2019). <https://doi.org/10.1038/s41598-019-46334-1>). Photos: University of Crete.

#### 4.6 X-ray - Haemal lordosis in European sea bass juveniles



**Figure 11. Haemal lordosis in sea bass juveniles at 111 dph.** (A) juvenile with normal external morphology and normal vertebral column; (B,C) juveniles with normal external morphology and minor vertebral abnormalities and light lordosis (arrows); (D) juvenile with lordotic external morphology and severe lordosis. Scale bars are equal to 1 cm. (from Fragkoulis et al., *Aquac. J.* 2022, 2, 1-12. <https://doi.org/10.3390/aquacj2010001>). Photos: University of Crete.

#### 4.7 Cranial, fin and vertebral abnormalities – European sea bass



**Figure 12. Variability of cranial, fin and vertebral abnormalities in hatchery reared sea bass larval samples.** **A.** abnormalities of dorsal-fin pterygiophores with missing rays. Notice the urinary calculus (\*). **B.** missing dorsal-fin rays with normally developed pterygiophores. **C.** ray-resorption syndrome on caudal-fin. **D.** prognathism of the lower jaw. **E.** abnormalities of the vertebral centra and processes. Scale bars equal to 1 (A, B, D, E) or 0.25 (C) mm. Photos: University of Crete.

## 5 Histology and Histopathology

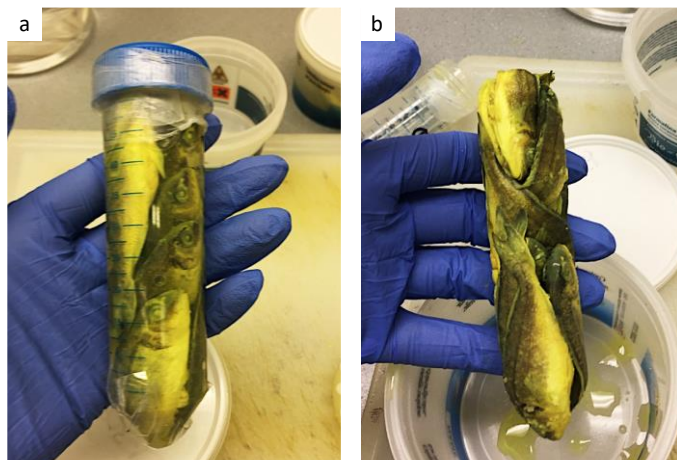
Histology is the branch of biology, which studies the normal microscopic anatomy of cells, tissues, and organs, looking at structures visible only with a microscope and is the counterpart of the gross anatomy, i.e. the study of anatomy at the visible or macroscopic level. Histopathology, instead, is a branch of the anatomopathology that studies the changes in cells, tissues and organs as a result of disease processes or insults (for example the effects of pathogens or chemical injuries) applying histological methods.

### 5.1 Preparation of a histological slide for light microscopy

The preparation of a long-term permanent slide for histological observation under an optical microscope requires a series of procedures that make the sample so thin to be crossed by the light sources. The optimal preparation for morphological analysis consists of a section (thickness of 4-10µm) which has the same structure and organization present in the living specimen. Therefore, the approach is conservative and includes the following procedures:

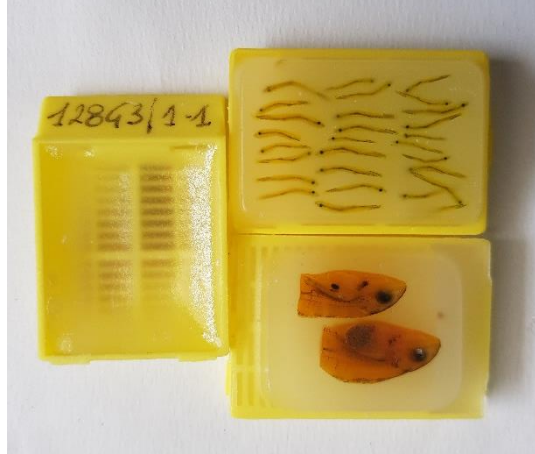
**Sampling.** In order to obtain good preparations, the material collected must be very fresh. In fact, after specimen collection, the fixation phase must take place in the shortest time possible to avoid autolytic alterations. Standardized sampling procedures were developed by the PerformFISH partners and they are presented in the PerformFISH Sampling Manual “Juvenile Quality and Growth Potential Standard Operating Procedures for Sampling and Data Recording”.

**Fixation.** This is the most important step in producing good histologic slides: a proper histological evaluation depends absolutely on an excellent tissue fixation. This procedure stops cell autolysis, thus preserving the morphology of the tissue or organ under study over time. Different fixative solutions can be used according to the study (4% buffered formaldehyde, Bouin’s solution and others) and for adequate penetration of the fixative, the sample volume should not exceed 1/10 of the volume of fixative within the sample tube. Poor tissue fixation (Figure 13) can cause artifacts or even the impossibility of histological evaluation of samples under the microscope. After fixation, the sample prepared for histology using a modern enclosed automatic tissue processor which includes melted paraffin for inclusion.



**Figure 13.** Example of inadequate fixation of gilthead seabream juveniles due to a volumetrically inadequate container to guarantee a proper sample-to-fixative solution ratio (1:10).

When the specimen is completely impregnated with molten paraffin, it must be oriented in a paraffin block mould and allowed to harden (Figure 14), taking care to orientate the specimen carefully in the mold because its placement will determine the "plane of section," which is crucial in both diagnostic and research histology. If tissue processing is properly carried out, the paraffin blocks containing the tissue specimens are very stable and represent an important source of archival material.



**Figure 14. Paraffin blocks for which it can see the laboratory number code on the cassette and the embedded specimen cutting plan.**

The histological sections are obtained by means of a microtome (manual or automated); the paraffin block is secured to it and oriented appropriately with respect to the knife. With each revolution of the microtome handle, the specimen moves across the blade and a section of the desired thickness (4-7µm) is cut. Sections are collected and transferred to glass slides. After drying at 37°C, the sections are ready to be stained. The purpose of staining is to highlight important features of the tissue as well as to enhance the tissue contrast, in order to illustrate different cell structures, detect tissue infiltrations or deposits and detect pathogens. The most commonly used staining technique in both the histological and pathological fields is Hematoxylin and Eosin (H&E). Hematoxylin is a basic dye that stains the nuclei giving them a bluish colour, while eosin (another stain dye) stains the cell's cytoplasm giving it a pinkish colour. However, there are other staining techniques used to identify particular cells and components. After staining, a coverslip is placed on top of the sections to protect the sample. Now the histological slide (Figure 15) is observable under the microscope and can be preserved over time.



**Figure 15. Histological slide of gilthead seabream juvenile stained with H&E.**

## 5.2 The use of histology in studies of fish

Fish histology has become an important tool exploited in fields such as basic biomedical research, ecotoxicology, environmental resource management and aquaculture (Wolf et al. 2015). Aquaculture studies include a wide range of experimental topics, requiring the control and modulation of different rearing parameters (genetics of individuals, water parameters, nutrition) in order to evaluate their effects. Histological investigative methods are potentially exploitable to all of these scientific settings and, therefore, paraphysiological adaptations, pathological findings and changes can be compared (Sirri et al., 2018). Histology is largely used in toxicological studies, as it has been known for long time that the exposure of fish to contaminants can induce lesions in different organs (Bernet et al., 1999; Mahboob et al., 2020). Histology represents a rapid method to detect effects of irritants, especially chronic ones (Johnson et al., 1993). In the field of nutrition, histological evaluation of digestive organs is a good instrument for the evaluation of the fish nutritional status: additional and effective information on mechanisms involved in digestion, feed utilization and metabolism can be obtained to explain fish zootechnical performances (Sirri et al., 2018). Fish histology is also helpful to assess the effects of rearing parameters such as, for example, temperature (Puvanendran et al., 2015). Moreover, according to several authors (Zimmerli et al., 2007; Saraiva et al., 2015) histology is also useful to unravel the health status of fish because the overall effects of all the farming factors are displayed by the organ condition (Saraiva et al., 2015). Finally, obviously, histology plays a role of great importance in the diagnosis and the study of histopathological changes caused by pathogens (Toffan et al., 2017; Elgendy et al., 2015).

Scientists can employ different histological evaluation methods, which can be used individually or in combination according to the purpose of the study:

1. descriptive/qualitative, this method can be implemented with the simple use of a light microscope by an operator and it is based on the description of structures and lesions; it provides purely qualitative data and does not allow a comparison between different groups or statistical analysis of the results;
2. semi-quantitative tool to attribute a score to the studied phenomenon and allows to derive data from biological systems (eg, tissues) for analysis and group comparisons (Gibson-Corley et al., 2013);
3. quantitative approach is based on a measurement, biological data are acquired from morphometry that produce quantitative data, relevant examples include length or area (Gibson-Corley et al., 2013); this method requires the use of image analysis software;
4. fractal dimension analysis is a mathematical concept where a multi-scale set exhibits the same repeating pattern at every scale; fractal parameters can be viewed as a measure of irregularity or heterogeneity of spatial arrangements and, in recent years, there has been growing interest in the application of fractal geometry to observe spatial complexity of natural features at different scales (Di Cataldo and Ficarra, 2017).

## 5.3 Marine larvae/juvenile quality assessment

It is largely recognized that the success of marine aquaculture strictly depends on the production of good quality larvae/juveniles, nevertheless this phase remains one of the main bottlenecks. Although current management practices should guarantee the maximum zootechnical

performances (growth rate, feed conversion efficiency, and survival) associated with a low health risk (Plumb and Hanson, 2011 quoted by Saraiva et al., 2015), pathological events (malformations or decrease in survival) and poor growth of fish are often observed (Valdstein et al., 2004; Støttrup, 1993 quoted by Valdstein et al., 2013). Some of the problems associated with juvenile quality are visible only in later stages (Logue et al. 2000 quoted by Valdstein et al., 2013), so that it is important to have tools allowing precocious identification of pathological changes so that early action can be taken to modify factors that could negatively affect production.

Within the framework of the H2020 project PerformFISH, UNIUD set up an innovative Multiparametric Semi-quantitative Scoring System-MSSS-(scoring range 1–5) for larval/juvenile histological evaluation and it includes 18 descriptors related to 6 organ districts. The values of each descriptor can be summarized in two indexes: the CHI (Cumulative Histological Index), giving general information about the quality of a fish batch in that precise moment and the OCV (Organ condition value) showing the general condition of each organ and by the individual descriptors. The MSSS tool is fully described in the article by Pacorig, Galeotti and Beraldo (2022); this tool promptly allows problems to be highlighted in marine fish larval batches because of managerial practices, suggesting to fish farmers which direction could be taken to resolve them.

Therefore, this chapter will summarize UNIUD's work through pictures illustrating the development of gilthead sea bream and European sea bass at the different ages: first feeding, flexion, end of the larval rearing, mid-metamorphosis and juveniles. The following section reports some examples of the histological score adopted in the application of MSSS, the microscopic anatomy at different ages and frequent pathological alterations found in the organ districts will be described.

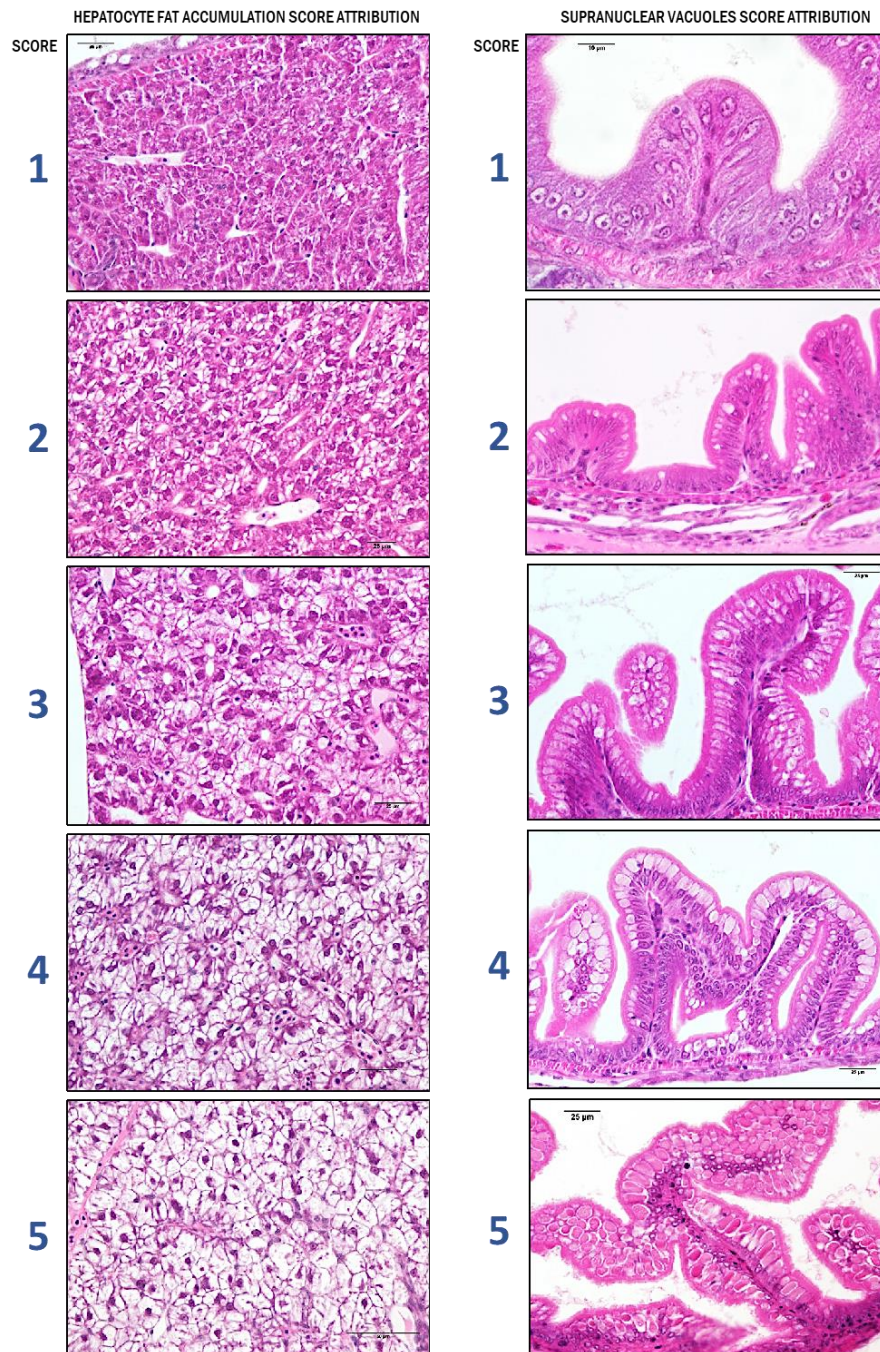
## 6 Histological Photo-Atlas

The following sections consist of single or composite images of histological specimens of gilthead sea bream and European sea bass. All the samples were sacrificed with an overdose of MS-222 and immediately fixed in Bouin's solution (Bio-Optica, Mila), processed according to the UNIUD lab histological techniques and stained with Hematoxylin and Eosin (H&E) or other histochemical protocols.

The histological evaluation was performed using a Nikon optical microscope (Eclipse Ni) equipped with a Nikon camera (DSFi3) and the NIS Elements BR software was used to take the pictures.

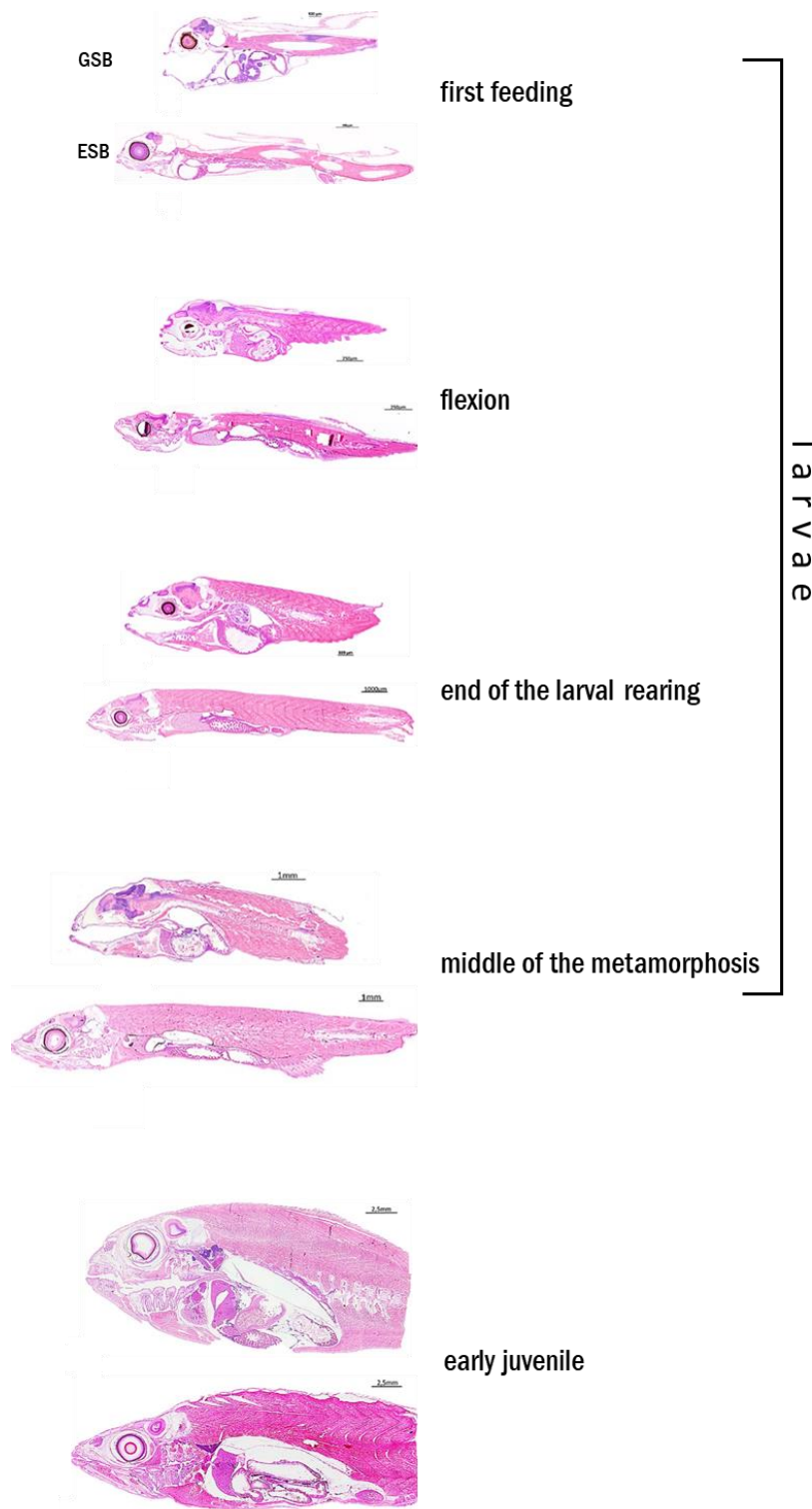
## 6.1 Representative example of MSSS grading

In the section, we have provided examples of scoring attribution visualized by images. Due to the enormous number of pictures available (about 1000), for descriptors of each organ, stage and species (Pacorig, Galeotti and Beraldo, 2022), only the most common pathologies will be considered, liver lipid accumulation and the presence of sopranuclear vacuoles in the posterior intestine (Figure 16). For some of the chosen descriptors, 5 pictures represent the scores from 1 to 5.



**Figure 16.** liver lipid accumulation and the presence of sopranuclear vacuoles in the posterior intestine

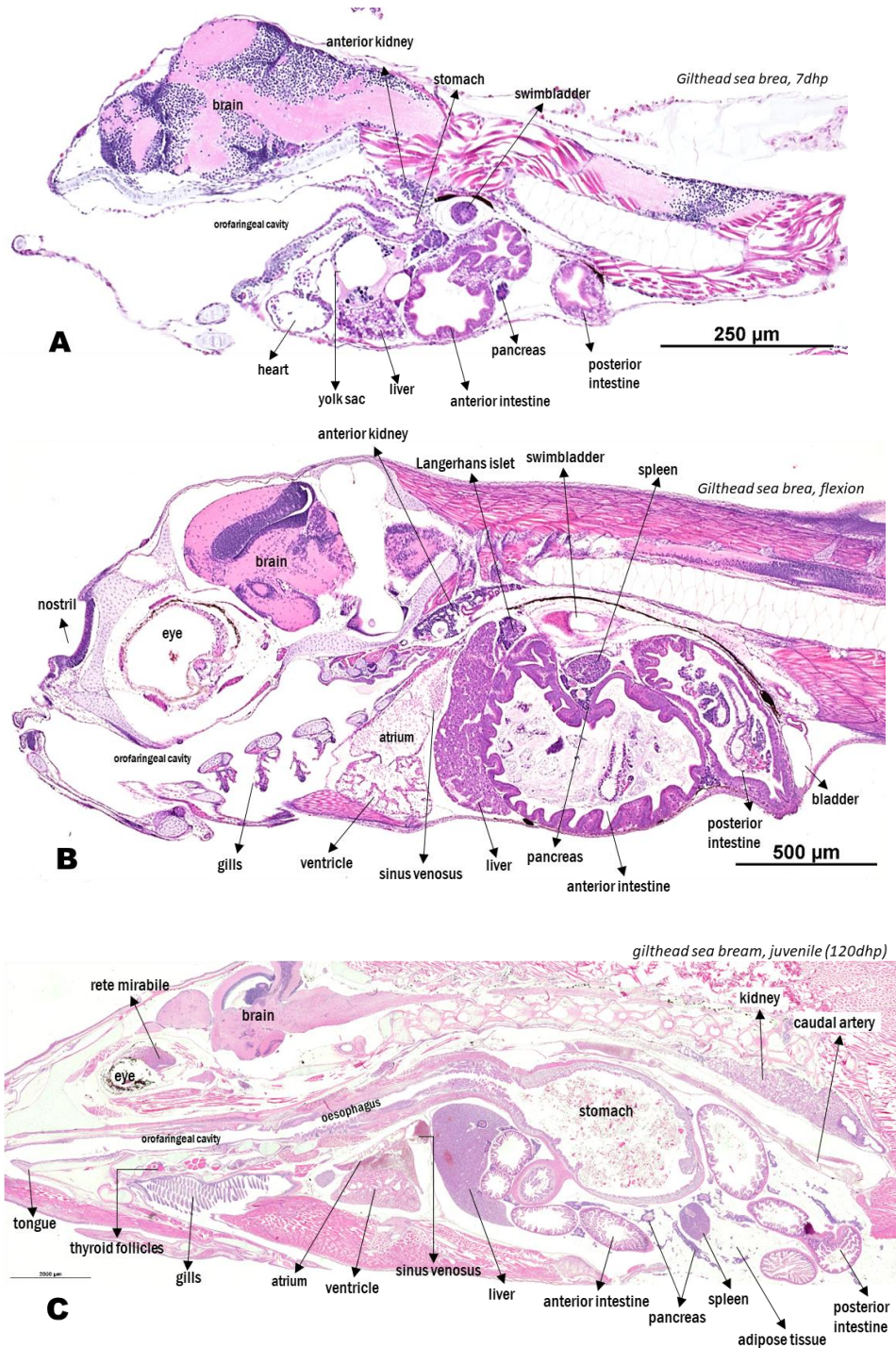
## 6.2 Gilthead sea bream and European sea bass stages



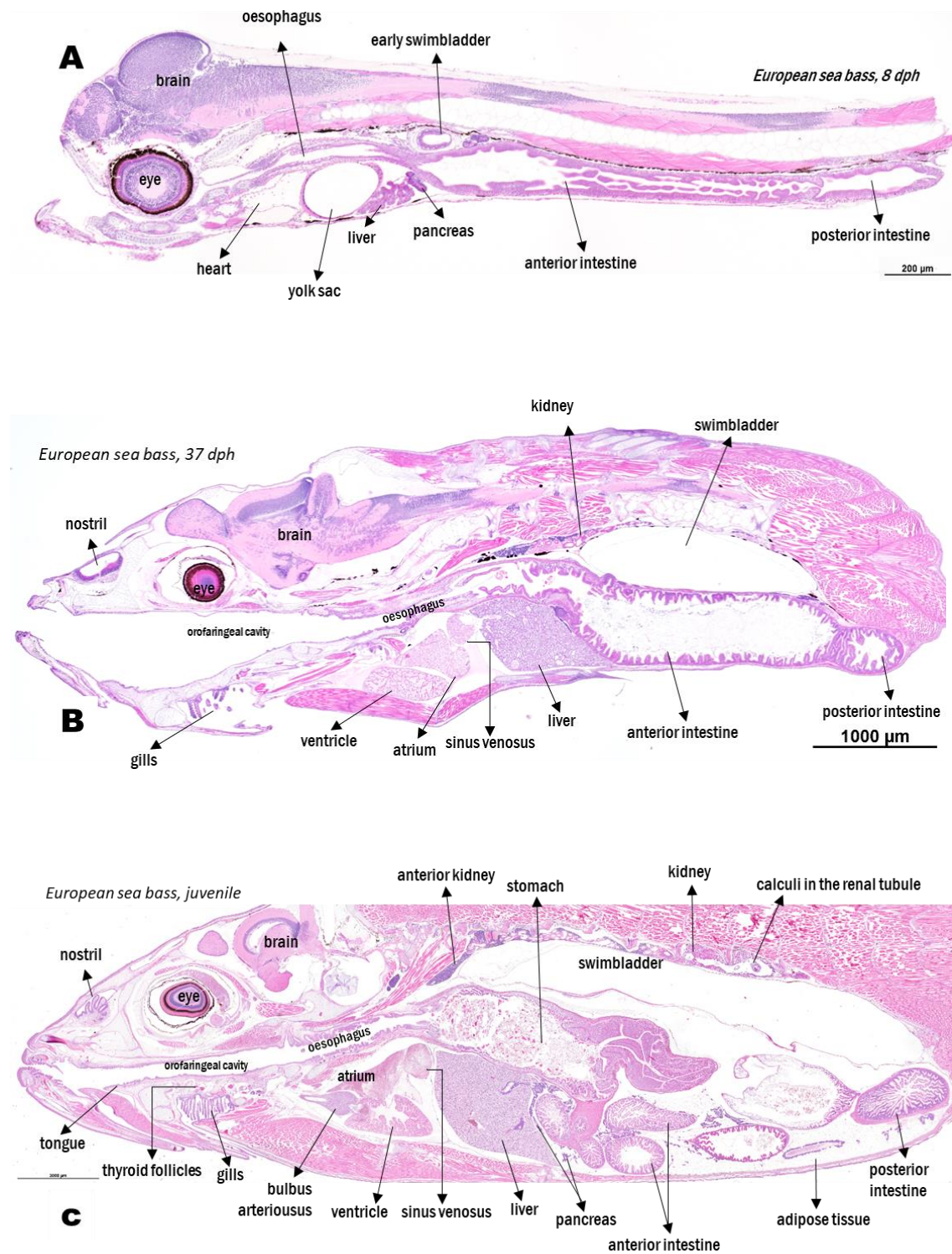
**Figure 17.** Histological diagram of gilthead sea bream (GSB) and European sea bass (ESB) stages analysed: First feeding, flexion, end of the larval rearing (10-14 mm TL), middle of the metamorphosis (15-20 mm TL) and early juvenile (0.8-1.5 g).

### 6.3 Parasagittal sections of gilthead sea bream and european sea bass at different stages

The following images represent gross views of parasagittal sections of whole larva/juvenile stage stained with Hematoxylin and Eosin (Figure 18, Figure 19). They allow a better understanding of the location of organs and their development over the time.



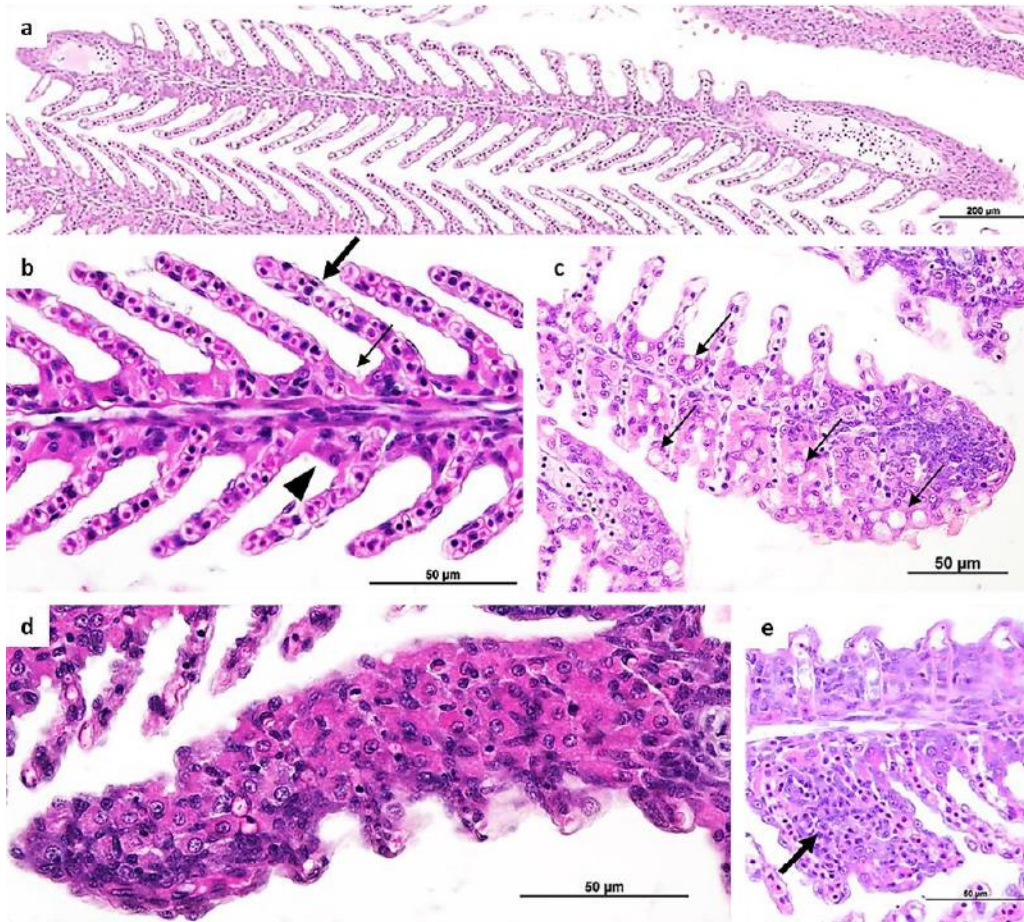
**Figure 18. GILTHEAD SEA BREAM. A) First feeding, B) flexion and C) juvenile.**



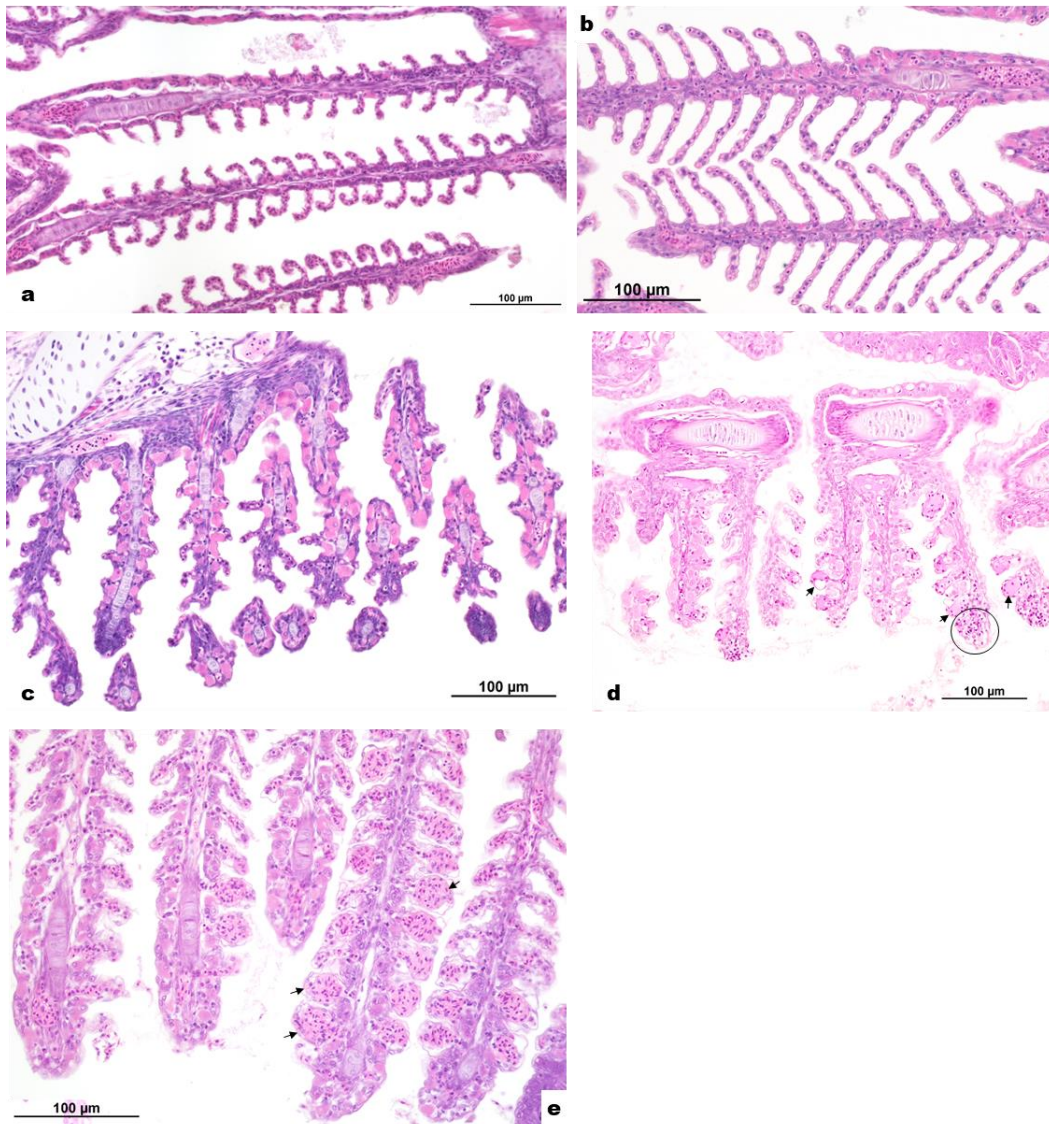
**Figure 19. EUROPEAN SEA BASS. A) First feeding, B) flexion and C) juvenile.**

## 6.4 Gills

The gills evaluation was performed only in the ELR, MM, JU stages for both species, since in the two initial stages gills are structurally poorly differentiated into primary and secondary gill filaments (Figure 20, Figure 21).



**Figure 20. Gilthead sea bream gills.** a) juvenile (102dph), normal primary gill filament, H&E; b) juvenile (102 dph), secondary lamellae structure: epithelial cells (thick arrow), chloride cells (arrow head) and mucous cells (thin arrow) located within interlamellar sulci, H&E; c) juvenile (85dph), mucous cells (arrows) are well detectable in secondary lamellae; chloride cells hypertrophy and epithelial cell hyperplasia are also observable, H&E; d) middle metamorphosis (53dph), severe hyperplasia and mild hypertrophy of chloride cells, H&E; e) juvenile (92dph), secondary epithelial fusion with inflammatory infiltration (arrow), H&E.

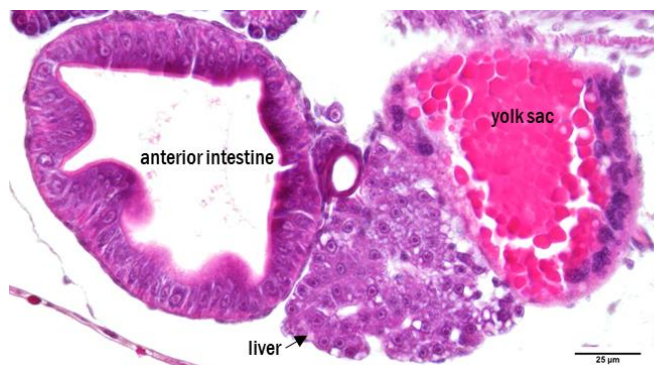


**Figure 21. European sea bass gills.** a) and b) quite normal gill filaments respectively of middle metamorphosis (45 dph) and juvenile (95 dph), H&E; c) End of larval rearing (37 dph), marked hypertrophy of chloride cells that appear distinctly light pink in color, H&E; d) flexion (33dph), hypertrophy of chloride cells, pathological multifocal lamellar oedema (arrows) and mild mononuclear cell infiltration at the top of the primary lamellae (circle), H&E; juvenile (97 dph), aneurysms of secondary lamellae (telangiectasia) caused by breakdown of vascular integrity due to rupture of the pillar cells and a pooling of blood, H&E.

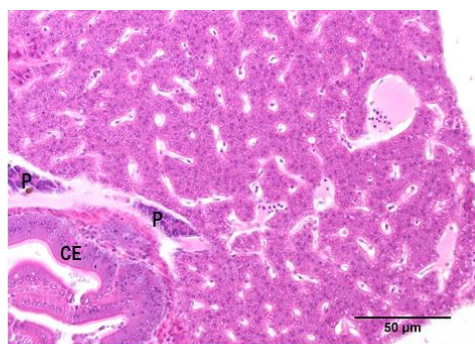
## 6.5 Liver

The main histopathological lesions found in liver at the different stages were ceroid accumulation, nuclear atypia (macrocariosis and polycariosis), presence of pseudogranulomas and necrosis. The hepatocyte fat accumulation was scored as described in a previous paragraph (Figures 22-26).

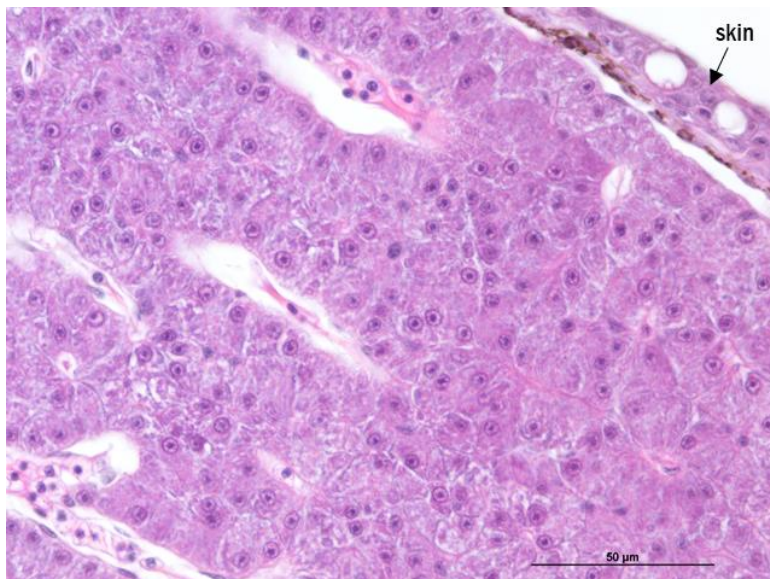
### Normal architecture of the liver



**Figure 22.** *Gilthead sea bream*. First feeding larva (7 dph), normal condition of liver without lipid accumulation; still visible the yolk sac in resorption, H&E.

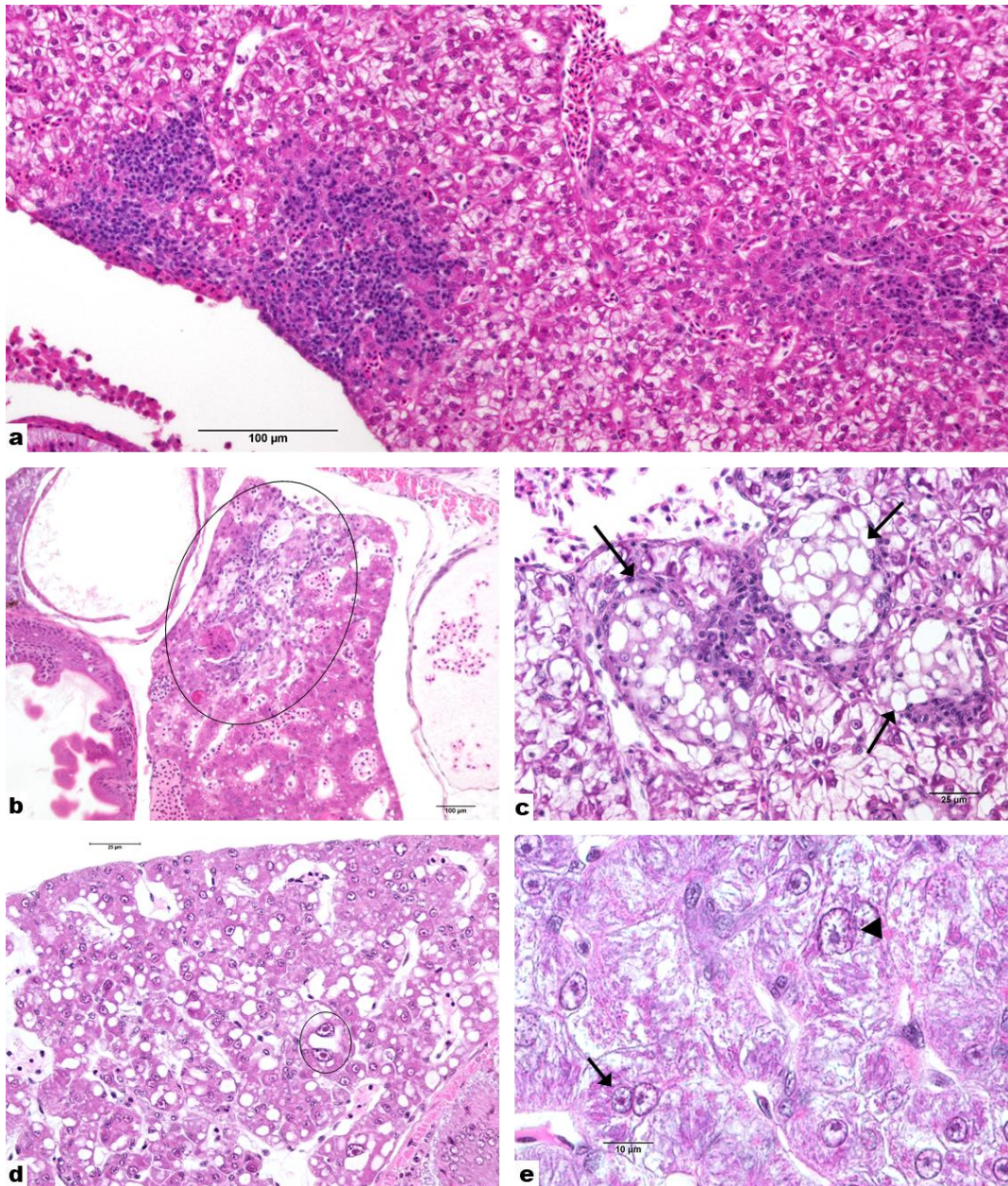


**Figure 23.** *Gilthead sea bream*. End of the larval rearing (33 dph), normal architecture of liver. P, pancreas; CE, epithelium of caecum. H&E

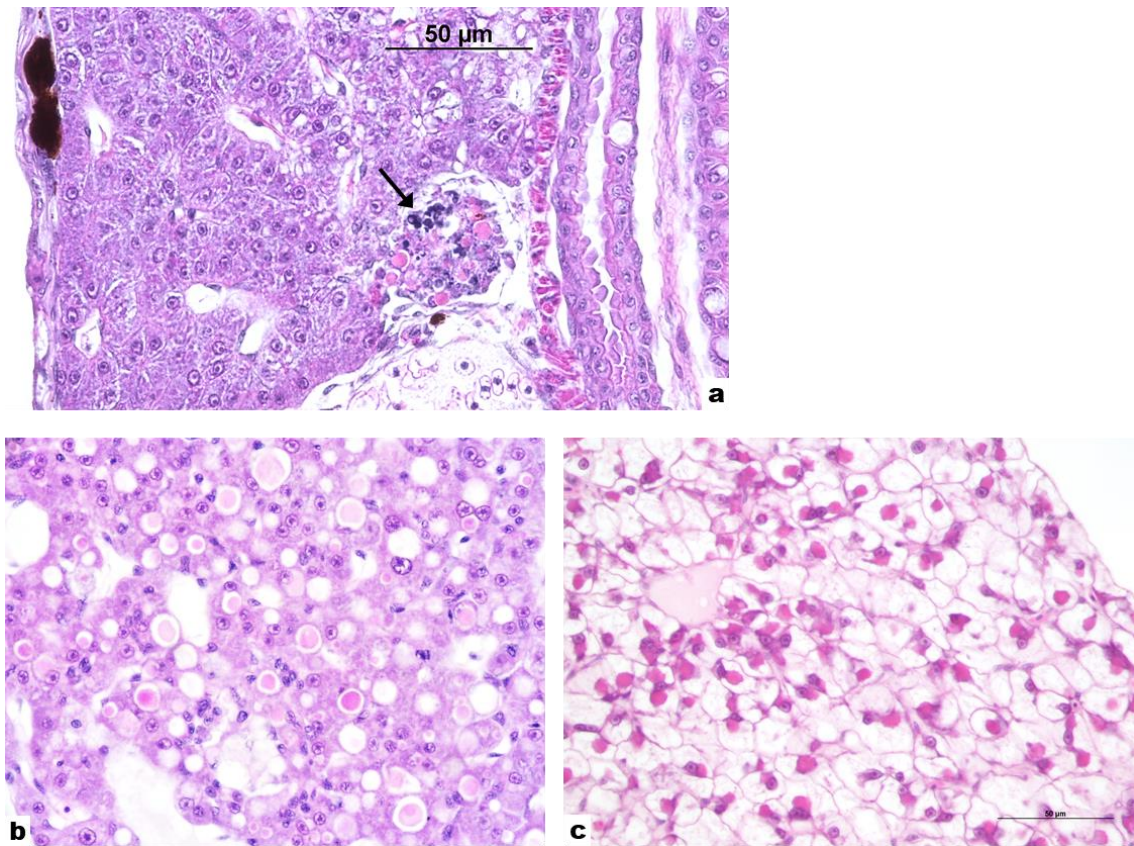


**Figure 24.** *European sea bass liver*. Mid-metamorphosis (53 dph), normal architecture of liver. H&E

### Pathological alterations of the liver



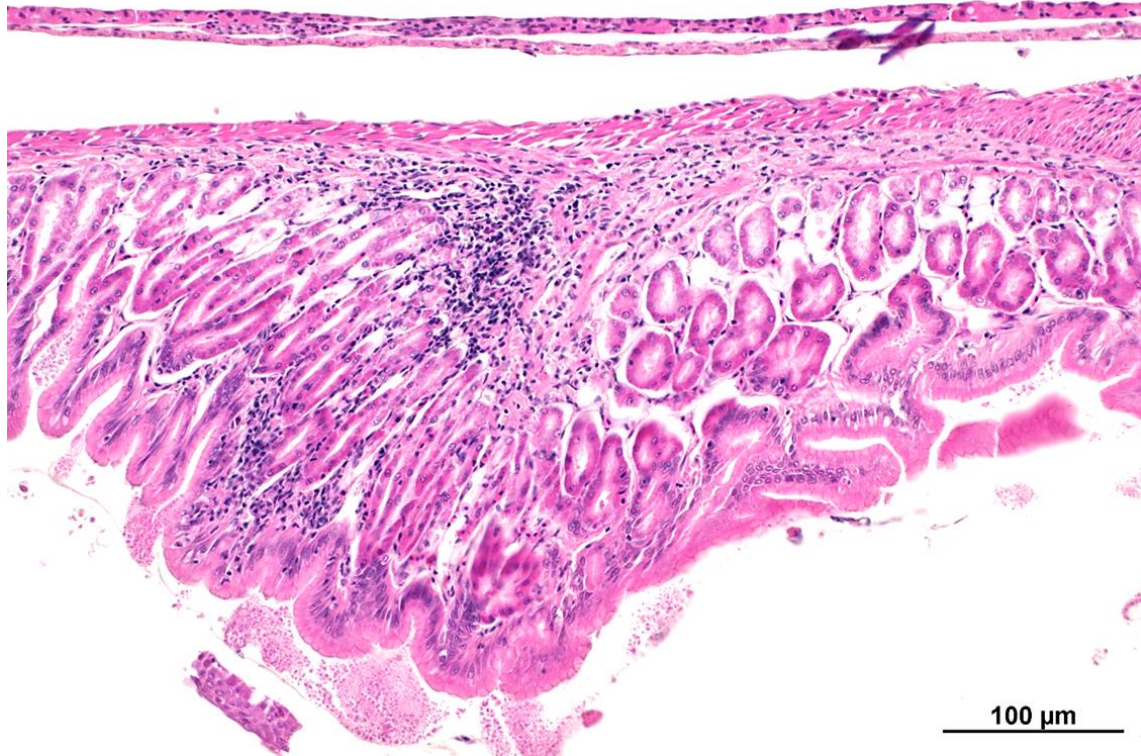
**Figure 25. Gilthead sea bream liver.** a) juvenile (112dph), multifocal inflammatory cells are observed, including mainly lymphocytes but also acidophilic granulocytes (G7+ and GM-CSFR $\alpha$ +, data not shown) and eosinophilic granular cells (EGCs); the distribution of cellular infiltrate is often periductal, H&E; b) middle metamorphosis (59dph), large area of hepatocellular necrosis (circle), H&E; c) juvenile (94dph), abundant and diffuse fat accumulation with pseudogranulomatous lesions (arrows) (lipogranuloma), probably caused by necrotic hepatocytes surrounded by lymphocytes, H&E; d) end of larval rearing (38 dph), macro- and microvacuolization of hepatocytes, similar to those caused by toxic noxa; it is possible to note also nuclear atypia (macrocariosis) (arrows), H&E; e) flexion (23 dph), high magnification of liver parenchyma where it easy noted macrocariosis (arrow head) and bi-nucleated cell (arrow), H&E.



**Figure 26. European sea bass liver.** a) flexion (20 dph), focal necrosis in the liver, H&E; b) and c) middle metamorphosis (50 dph), diffuse presence of spherical acidophilic intracytoplasmic inclusions in hepatocytes, that is ceroid as demonstrated by Schmorl staining (not shown), H&E.

## 6.6 Stomach

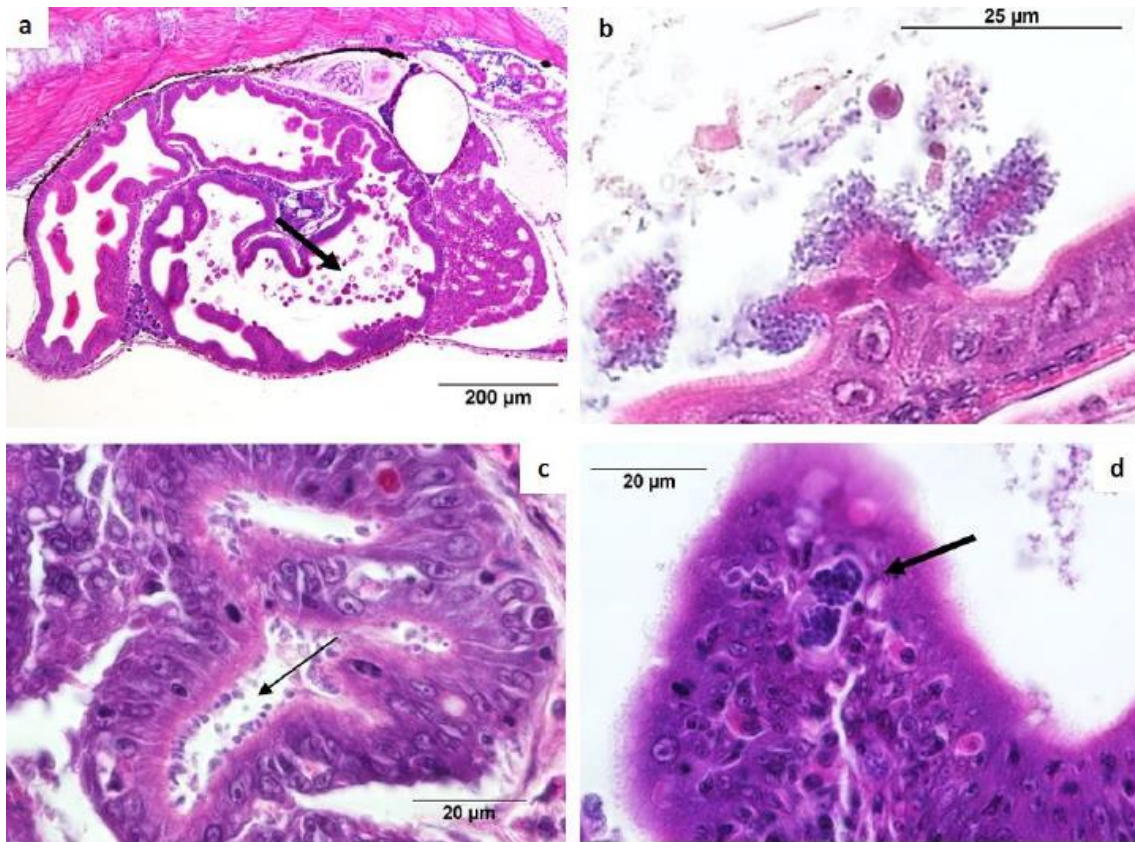
Within the histological study for the PerformFISH project, alterations in the gastric district were rarely observed for both sea bream and sea bass; chronic gastritis was observed only in very few subjects (Figure 27).



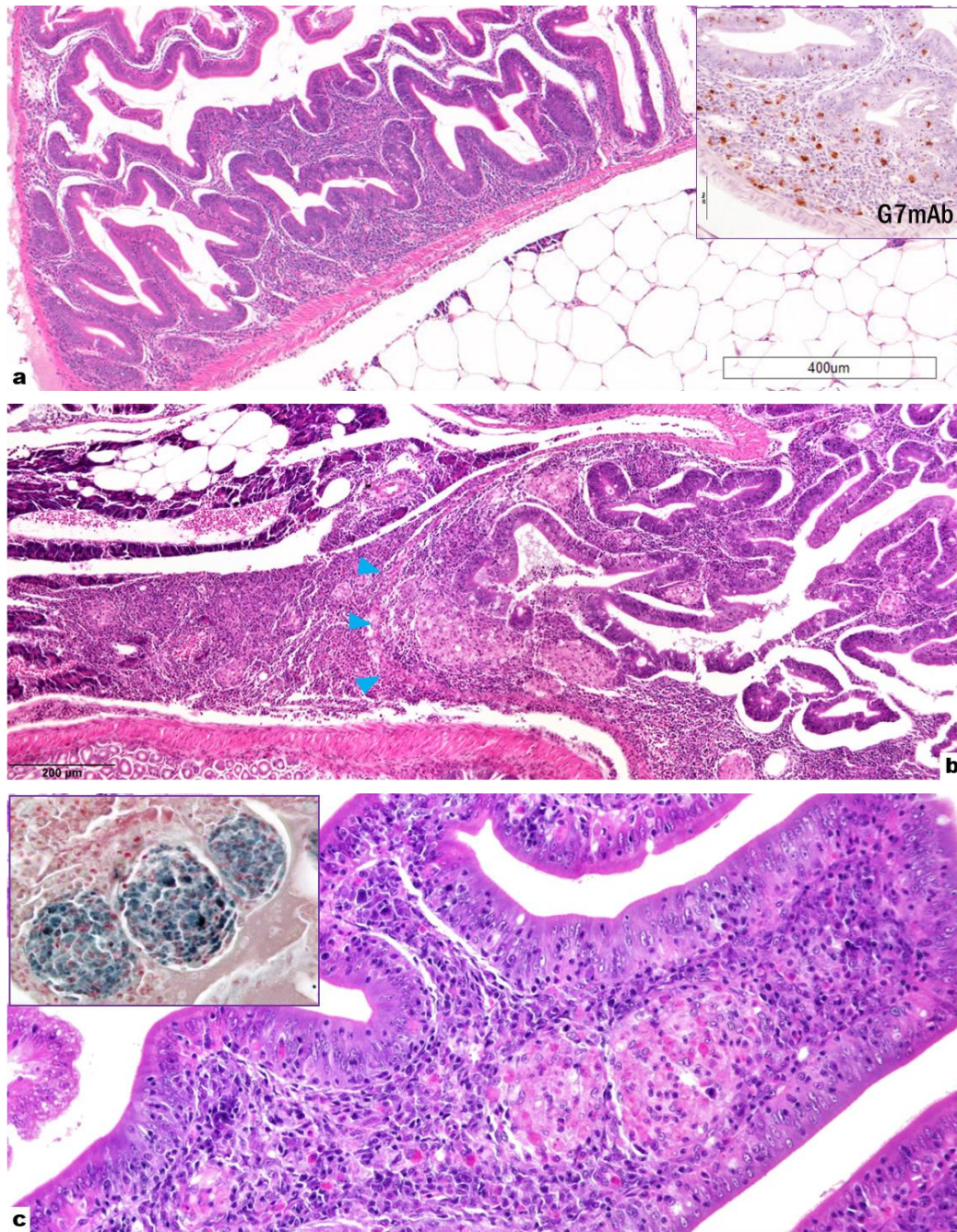
**Figure 27. Gilthead sea bream stomach.** Juvenile (85 dph), superficial lymphocytic infiltration is observed, but also in the deep layer of the lamina propria of the gastric wall, H&E.

## 6.7 Anterior Intestine

### Pathological alterations of anterior intestine



**Figure 28. Gilthead sea bream anterior intestine.** a) Flexion (25 dph), abundant desquamated cells in the gut lumen (arrow), H&E; b) flexion (21 dph), bacteria attached to the intestinal epithelium where some cells are in necrosis, H&E; c) juvenile (100 dph), presence of round bodies suggestive of the developmental stages of *Cryptosporidium*, probably *C. molnari*, was observed in the apical position (arrows) within the intestinal epithelial cells (occasionally present), H&E; d) juvenile (100 dph), intraepithelial agglomerates of round-shaped bodies (arrow), probably of parasitic origin (occasionally present), H&E.

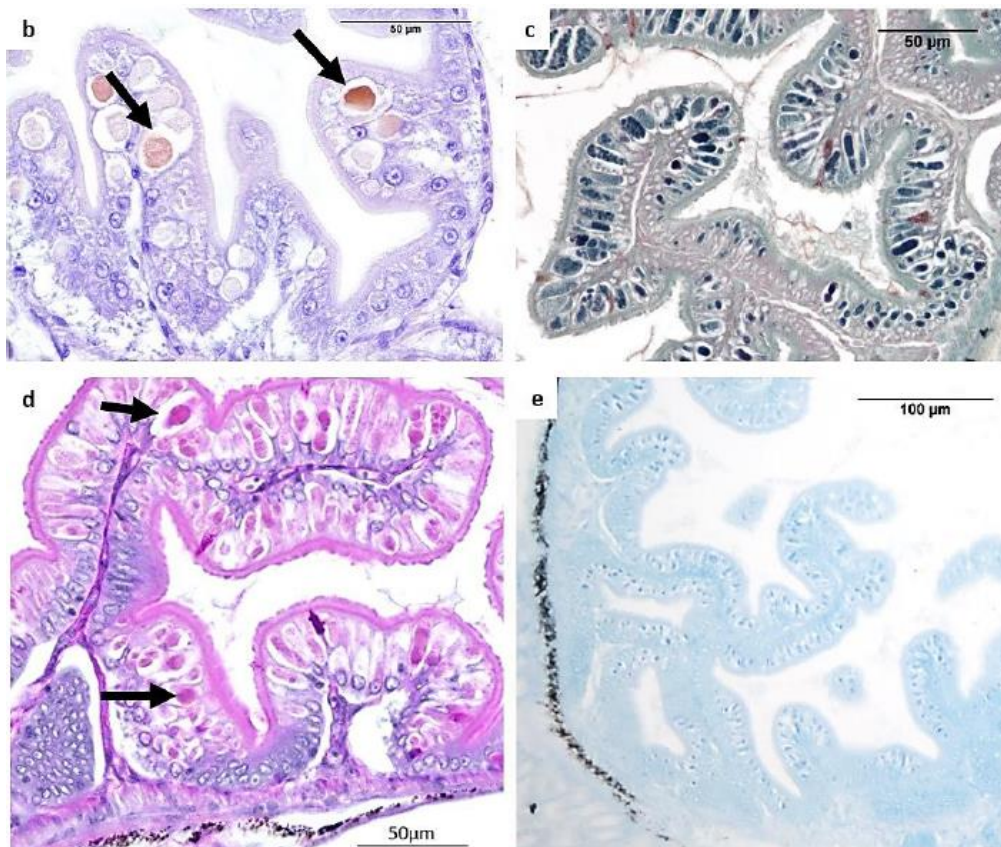
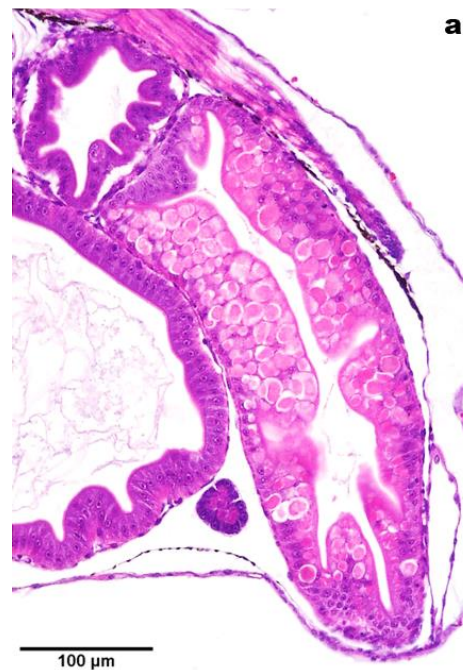


**Figure 29. 90-112 dph gilthead sea bream, chronic enteritis.** Recurrently a diffuse lymphocytic infiltration of the lamina propria of intestinal folds is detected (a), also associated to the presence of acidophilic granulocytes (inset: positivity for G7: mAb specific for gilthead sea bream acidophilic granulocytes) and EGCs. Sometimes lipogranuloma-like lesions are observable in lamina propria (c), composed of aggregated macrophages engulfed of oxidized lipids (inset: Schmorl's stain). Rarely abundant lymphocytes also infiltrate the epithelium. Figure b) shows a severe and diffuse pancreatic inflammation where evident intra-abdominal adhesions have affected the intestinal wall (arrowheads); furthermore, the chronic inflammatory pattern is characterised by granulomas; enteritis is also evident.

## 6.8 Posterior Intestine

During the transition from endogenous to exogenous feeding, the posterior intestine has a basic nutritional role in absorbing protein macromolecules by pinocytosis as an alternative pathway until the development of the stomach and acid digestion takes place. Thus, the presence of supranuclear vacuoles with acidophilic inclusions is a typical feature of the posterior intestine of fish larvae during early life stages. Although supranuclear vacuoles are normal, their overdevelopment is considered in larval European sea bass and gilthead sea bream as a pathological condition, potentially resulting in cytological deterioration combined with cell degeneration and pronounced epithelial abrasion.

This condition (Figure 30) was the main alteration detected in the posterior intestine and one of the scored descriptor evaluated (as described above).

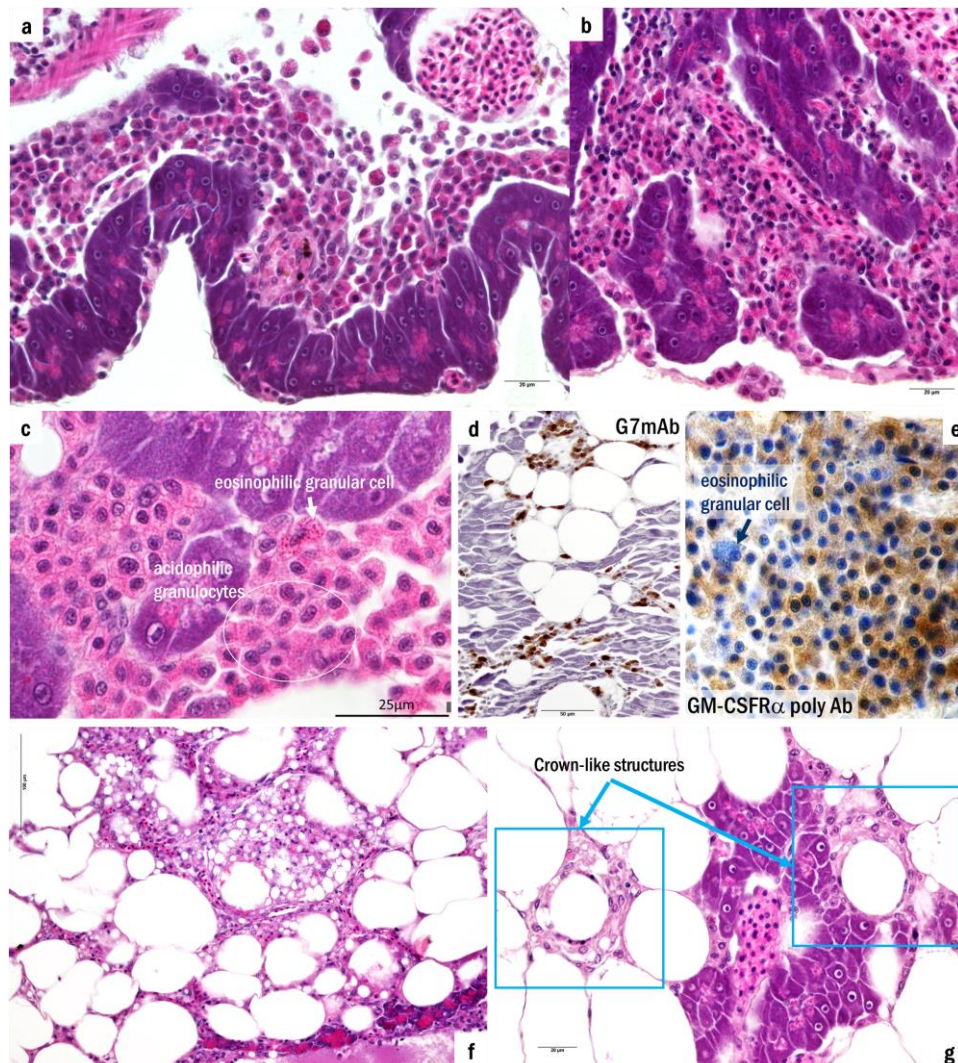


**Figure 30. Gilthead sea bream posterior intestine.** a) first feeding (10dph), posterior intestine where the sopranuclear vacuoles (SNVs) are very prominent, strongly acidophilic and causing the thickness of the wall, H&E. Middle metamorphosis (50dph), b) vacuole content showing natural brownish pigmentation (arrow), light Hematoxylin; c) green-blue staining demonstrating

positivity to Schmorl's stain; d) SNV content present mild to strong PAS positivity; e) dark blue staining of SNVs to Lillie's Nile Blue.

## 6.9 Pancreas and Adipose Tissue

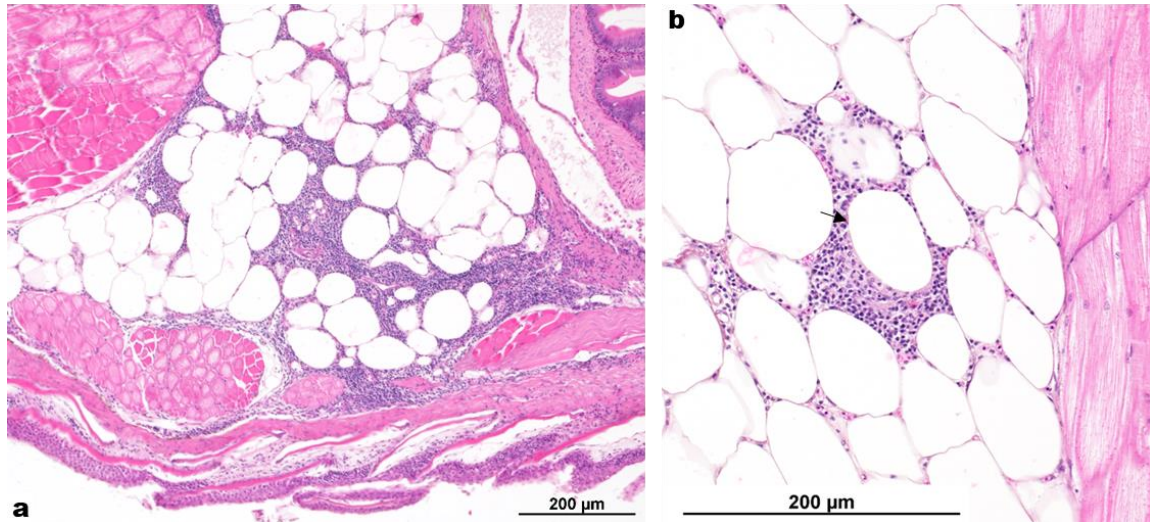
Pancreas and Visceral Adipose Tissue often had signs of chronic inflammation (Figure 31), most of all in gilthead sea bream. Some current aquaculture practices increase fat deposition in storage tissues, which may compromise fish health condition: vegetable oils frequently used for fish feed, induce more hypertrophic adipose tissue growth, lipolysis and lipid accumulation than fish oils, provoking an excess of fat deposition that decreases the product quality and could lead to a lower insulin sensitivity; juvenile gilthead sea bream visceral fat depots are particularly influenced by this replacement. The severe presence of Inflammatory Infiltrate can be due to these causes too.



**Figure 31.** 50-112 dph gilthead sea bream. Mild to abundant diffuse pancreatic cellular infiltrate (a-c) mainly composed by acidophilic granulocytes (G7+ and GM-CSFR $\alpha$ +, d-e), EGCs and lymphocytes is frequently observable, associated to focal necrosis and moderate

*pancreatic atrophy. In general abundant adipose tissue accumulation (most of all in gilthead sea bream juveniles) is detected and the pancreatic associated adipose tissue is also affected by the same inflammatory infiltrate. In the worst cases the chronic pancreatitis is associated to steatonecrosis and lipogranulomatosis (f); crown-like structures are easy noticeable (g), characterised by indistinct cell borders surrounded by foamy macrophages.*

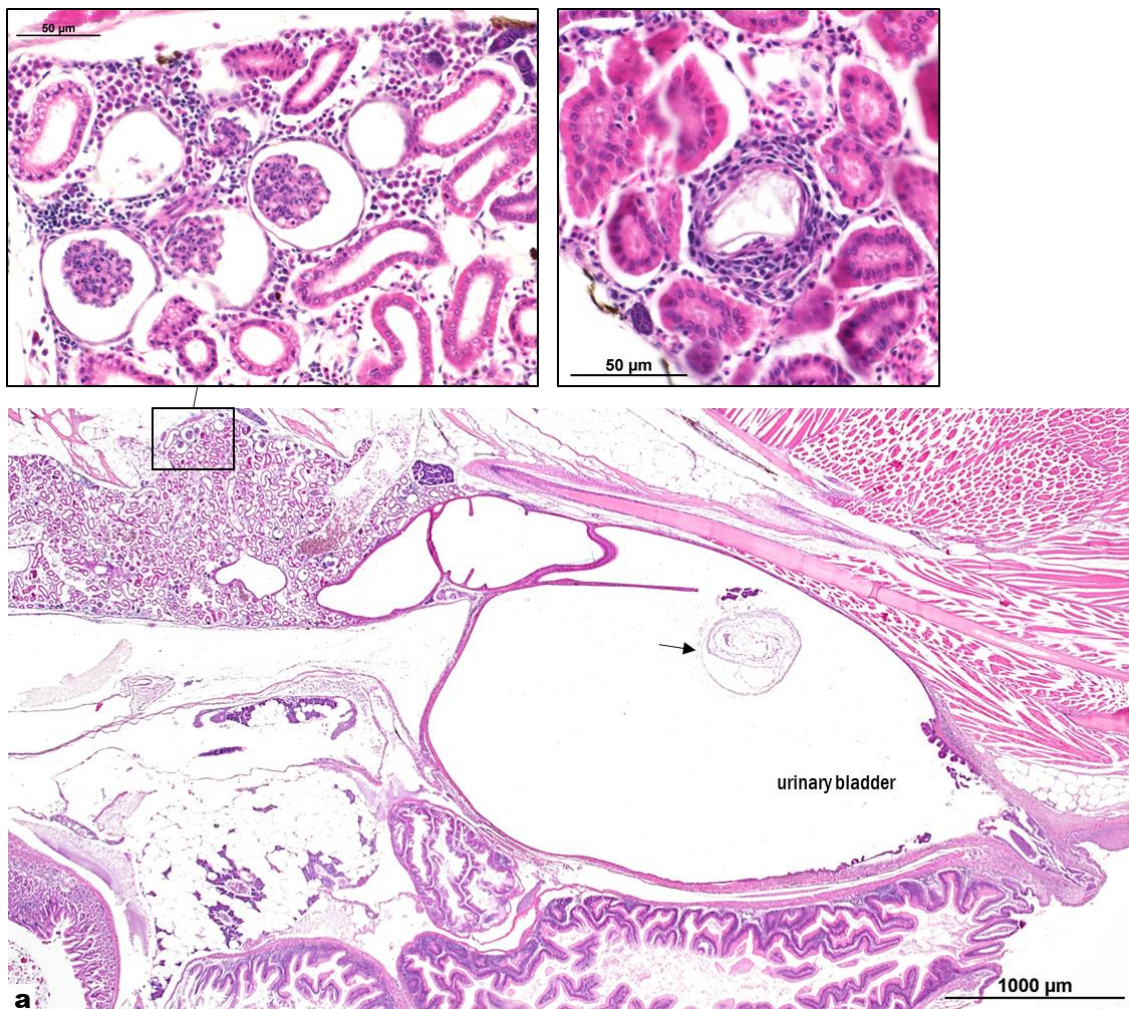
## 6.10 Subcutaneous Adipose Tissue



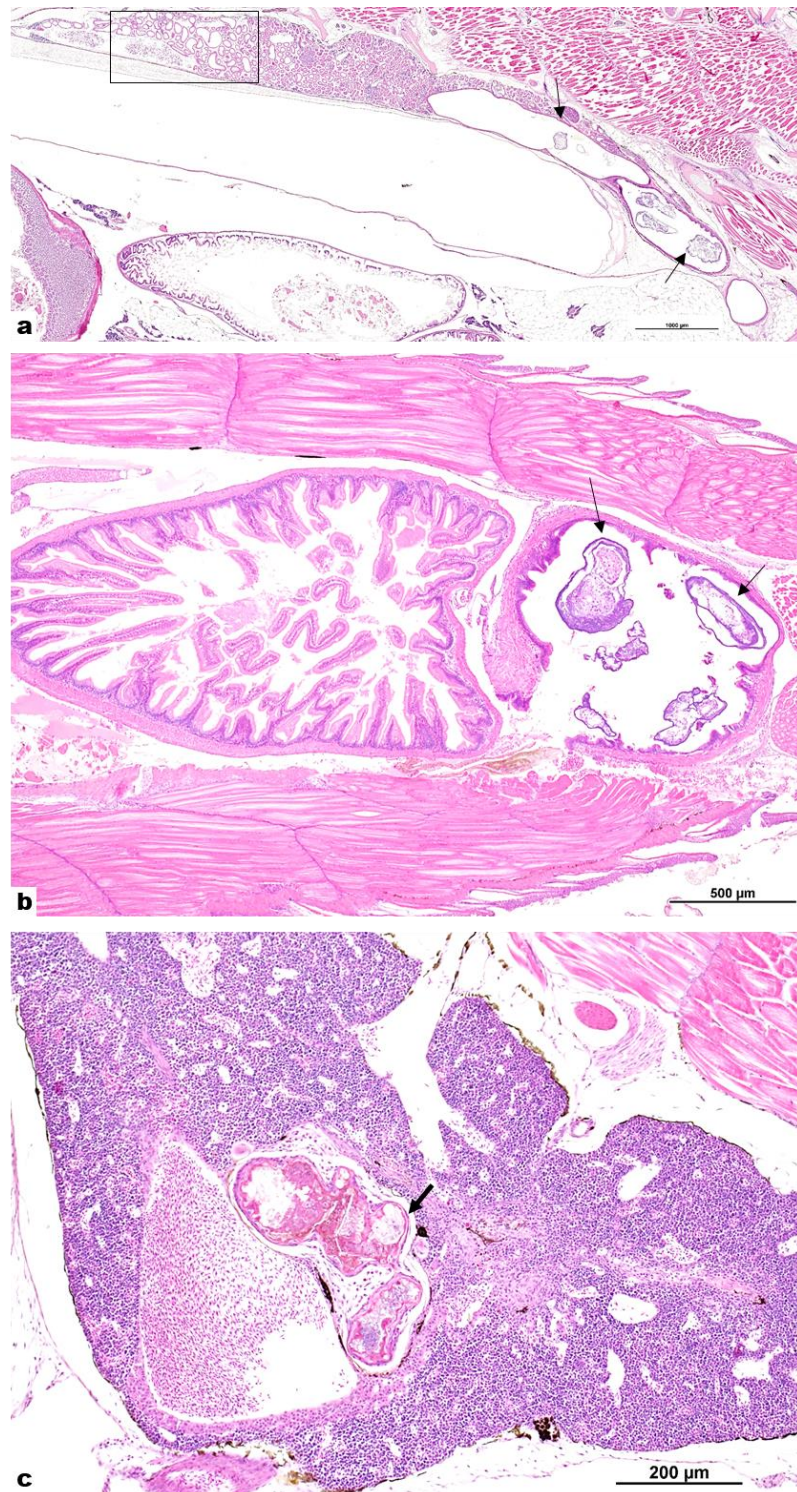
**Figure 32. Juvenile (97 dph) gilthead sea bream subcutaneous adipose tissue.** a) Diffuse and abundant mononuclear cell infiltrate (mainly lymphocytes) in subcutaneous adipose tissue, H&E; b) crown-like structures are detectable also in subcutaneous adipose tissue, H&E.

## 6.11 Kidney

The kidney of teleost fishes is a mixed organ that includes hematopoietic, endocrine, and excretory elements, contributing to body fluid homeostasis, therefore its correct functionality is vital for the fish. Nephrocalcinosis is a frequent pathology, characterised by ectasia of ducts and bladder, glomerulopathy, epithelia desquamation, necrosis, presence of granulomas, Bowman capsule hyperplasia and, of course, presence of calculi (Figure 33, 34). Nephrocalcinosis is a chronic condition affecting the kidney, characterized by the presence of mineral deposits within the renal tissue. This pathological alteration is usually associated with increased levels of free CO<sub>2</sub> in the water and coexisting conditions, such as decreased levels of dissolved oxygen and reduced pH. A high prevalence of calcinosis (more than 40 percent of the fish population) can be interpreted as a sign of poor environmental and feeding rearing conditions, which generally results in low survival rate or poor performance. The frequent observation of this pathological alteration in larvae and juveniles of sea bream and sea bass is probably attributable to the rearing system (RAS) or water quality used (high CO<sub>2</sub> concentration).



**Figure 33. Kidney of gilthead sea bream juveniles (97 dph).** a) Marked ectasia of the ureter and urinary bladder in which lumen is observable a part of a calculi; marked ectasia of renal tubules with vacuolization of epithelial cells and necrosis, glomerulopathy and granulomas presence are observable (see the insets).



**Figure 34. Kidney of European sea bass juveniles (120 dph).** a) Severe ectasia of the ureter in which lumen is observable some calculi (arrows); ectasia of renal tubules is appreciable (frame), H&E; b) frontal section at the ventral level where the presence of large calculi in the lumen of the ureter can be easily appreciated (arrows), H&E; c) anterior kidney where prominent granulomatous formations (arrow) derived from early nephrocalcinosis are observed, H&E

## 7 Annex I – buffers and reagents for agarose electrophoresis

### ***TAE 1x buffer (1L), used for agarose gel electrophoresis***

20ml de TAE 50x + 980 ml of sterile water.

### ***1L 50X stock of TAE Buffer***

Tris-base: 242 g

Acetate (100% acetic acid): 57.1 ml

EDTA: 100 ml 0.5M sodium EDTA

Add sterile water to make up to one litre.

To make 1x TAE from 50X TAE stock, dilute 20ml of stock into 980 ml of sterile water

### ***6X Loading Dye – 10 ml, used to prepare RNA samples for agarose gel electrophoresis***

0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water

Reagents needed:

25 mg bromophenol blue

25 mg xylene cyanol FF

3.3 ml Glycerol

6.7 ml ddH<sub>2</sub>O

### **Directions:**

1. Add 25 mg of bromophenol blue to 6.7 ml of sterile water and mix.
2. Add 25 mg of xylene cyanol FF and mix.
3. Add 3.3 ml of glycerol and mix.
4. Aliquot and freeze at -20 °C for long-term storage.

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