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Biological effects of contaminants: Use of liver pathology of the European flatfish dab (*Limanda limanda* L.) and flounder (*Platichthys flesus* L.) for monitoring

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Abstract

This publication provides quality assurance guidelines for the use of liver pathology of flatfish in biological effects of contaminants monitoring programmes. Information on the sampling procedures, including macroscopic examination, and minimum numbers and size categories of fish required to provide statistical rigour, is included. Details for laboratory processing and staining methods for histological assessment are also given, including protocols for resin embedding. Criteria for the histopathological diagnosis of liver sections are provided under categories on "early non-neoplastic toxicopathic lesions", "foci of cellular alteration", "benign neoplasms", and "malignant neoplasms", and descriptions of the normal appearance of liver tissue from dab (*Limanda limanda*) and flounder (*Platichthys flesus*) are included. More specific criteria are given for the different lesion types occurring in each of these categories. Each lesion type is represented by one or more colour micrographs taken from haematoxylin and eosin-stained sections to depict the key features. A diagnostic key for nodular hepatocellular lesions is included and sections on quality assurance, data treatment, and data submission are also provided.

Keywords: biological effects, liver, flatfish, dab (*Limanda limanda*), flounder (*Platichthys flesus*), histopathology, diagnostic criteria, quality assurance.

1 INTRODUCTION

Biological effects monitoring programmes incorporating externally visible fish diseases have been undertaken by a number of ICES Member Countries for many years (Lang, 2002a). Standardized methodologies for sampling, external disease evaluation, and data recording and analysis have been established by international collaboration and through various ICES activities, mainly initiated and coordinated by the Working Group on Pathology and Diseases of Marine Organisms (WGPDMO). These activities included sea-going workshops for the intercalibration of methodologies for fish diseases surveys (Dethlefsen *et al*., 1986; ICES, 1989; Lang and Mellergaard, 1999) and the establishment of a fish disease data bank as part of the ICES Marine Data Centre (Wosniok *et al*., 1999). Detailed guidelines for standardized studies on grossly visible fish diseases have been published (Bucke *et al*., 1996), which are now utilized by most fish disease monitoring programmes in the ICES area, especially for the target fish species, the common dab (*Limanda limanda*) and the European flounder (*Platichthys flesus*).

Whilst early efforts in fish disease monitoring focused on externally visible diseases, most programmes in the ICES area now also include studies on liver lesions. A wide range of toxicopathic neoplastic, putative pre-neoplastic, and non-neoplastic lesions have been recorded and the use of histological and histochemical biomarkers of toxic injury, dysfunction, and carcinogenesis is now well established as a powerful tool to detect and characterize biological endpoints of toxicant and carcinogen exposure. The utility of these lesions as sensitive and reliable indicators for the health assessment of wild fish populations has been demonstrated in several European and North American studies (Bucke *et al*., 1984; Kranz and Dethlefsen, 1990; Myers *et al*., 1990, 1991, 1992, 1994, 1998b; Köhler, 1991; Köhler *et al*., 1992, 2002; Bucke and Feist, 1993; Moore and Stegeman, 1994; Vethaak and Wester, 1996; Bogovski *et al*., 1999; Lang *et al*., 1999; Lang 2002b; Stentiford *et al*., 2003). In addition, several laboratory and mesocosm studies have demonstrated causal links between exposure to xenobiotics and the development of toxicopathic hepatic lesions (Malins *et al*., 1985a, 1985b; Varanasi *et al*., 1987; Stein *et al*., 1990, 1992; Moore and Myers, 1994; Vethaak *et al*., 1996).

Owing to increasing evidence of the existence of a cause-effect relationship between environmental contaminants and the occurrence of toxicopathic liver lesions in fish, studies on histopathological liver lesions in flatfish have been recommended repeatedly as one of the techniques to be used for contaminant-specific and general biological effects monitoring, for example, under the OSPAR Joint Assessment and Monitoring Programme (JAMP) (Stagg, 1998). However, in order to achieve quality assurance of data obtained in such programmes, there is a need for international standardization and intercalibration in the collection, processing, examination, and reporting of histopathological findings.

This publication provides guidelines for these aspects of fish disease monitoring and particularly for the diagnosis of hepatic lesions in dab and flounder. It follows similar publications by Boorman *et al*. (1997) and Myers *et al*. (1987) on the medaka (*Oryzias latipes*) and English sole (*Parophrys vetulus*), respectively. Descriptions and representative micrographs of pathological lesions both commonly and rarely encountered in the liver of dab and flounder are presented. However, where appropriate, lesions from other fish species are included for completeness. The focus of the present contribution is on routine histological techniques, involving paraffin-embedded material, staining with hematoxylin/eosin, and examination by means of light microscopy. However, due to the increasing availability of other techniques, brief information is also provided for other methodologies such as histology using methacrylate resin and cryotomy.

Parts of this publication are based on the results and recommendations of the ICES Special Meeting on the Use of Liver Pathology of Flatfish for Monitoring Biological Effects of

Contaminants (ICES, 1997) and those of the Biological Effects Quality Assurance in Monitoring Programmes (BEQUALM) Workshop on External Fish Diseases and Liver Histopathology held at the CEFAS Weymouth laboratory in 2001.

2 FISH SPECIES

Following the ICES guidelines (Bucke *et al*., 1996), dab and flounder are the main target species for monitoring purposes in the North Sea and adjacent areas, including the Baltic Sea. These species are benthic, abundant, known to be susceptible to externally visible diseases, and exhibit a range of pathological changes in the liver. Plaice (*Pleuronectes platessa*) and other flatfish species may also exhibit toxicopathic liver lesions, but few baseline data are available (Simpson *et al*., 2002). Similarly, very few pre-neoplastic or neoplastic hepatic lesions have been reported from gadoid species such as cod (*Gadus morhua*) and whiting (*Merlangius merlangus*) in European waters. However, it should be noted that these species are not regularly examined for liver pathology in routine monitoring programmes. Consequently, it is proposed that liver pathology information from these fish species should be improved before they are incorporated into biological effects monitoring programmes.

In North American monitoring programmes, flatfish species such as winter flounder (*Pleuronectes americanus*), English sole (*Pleuronectes vetulus*), starry flounder (*Platichthys stellatus*), and rock sole (*Lepidopsetta bilineata*) have been widely used for biological effects studies due to their susceptibility to contaminants and their propensity to develop toxicopathic liver lesions (Myers *et al*., 1998b).

3 SAMPLING PROCEDURES

Sampling procedures should follow the guidelines set out by Bucke (1994) and Bucke *et al*. (1996) for the gross examination of liver lesions. It is recommended that samples for histological assessment should be collected from fish samples which have also been assessed for externally visible diseases. Fish should be captured by trawling, using appropriate fishing gear. The duration of individual trawls should be minimized in order to reduce the effects of stress and the possible mechanical damage that results from net abrasion. As soon as possible after landing, the fish should be transferred directly to aerated flow-through seawater tanks. For necropsy, only live fish exhibiting normal opercular movement should be selected.

At present, two strategies are widely applied for the monitoring of liver lesions in flatfish. The first is based on the ICES guidelines for fish disease surveys and comprises a macroscopic examination of 50 fish per station/area for the presence of all liver nodules over 2 mm in diameter. For dab, the size of the fish should ideally be above 25 cm in total length and flounder should be larger than 30 cm in total length. (ICES, 1989; Bucke *et al*., 1996). All liver nodules detected should be resected and fixed for subsequent histological confirmation of the lesion type. The objective of this strategy is to detect the prevalence of neoplastic lesions in fish captured from a given station/area. However, a disadvantage of this method is that it probably underestimates the "true" prevalence of neoplastic lesions because it does not incorporate microscopic lesions that are not visible by macroscopic examination. In addition, it does not allow for the recording of other types of lesions (including early putative pre-neoplastic and non-neoplastic lesions) that may also be relevant as markers of the effects of contaminant exposure.

Thus, another strategy has been introduced in a number of monitoring programmes, which is based on the sampling of fish of a certain size group for routine liver histopathology. For dab, the size of the fish should preferably be 20–24 cm in total length and for flounder, between 25 cm and 35 cm in total length, since for these smaller fish, there is less variability in age than for

larger fish. A minimum of 30 fish should be sampled in order to provide a 95% confidence of detection of 10% disease prevalence in the population. Larger numbers are required to detect disease at lower prevalence rates, i.e., for 5% disease prevalence (n = 60) and for 2% disease prevalence (n = 150) (Ossiander and Wedermeyer, 1973). Both of these strategies can be applied in parallel, e.g., when there is reason to continue a long-term series on the prevalence and spatial distribution of macroscopic liver neoplasms.

3.1 Macroscopic Examination

The total length of each fish should be measured prior to examination for external signs of disease and parasitic infections. Fish should be sacrificed by a blow to the head and by severing the spinal cord just posterior to the brain. The sex of the fish should also be noted (in dab, this is usually possible by external assessment, while for flounder, internal assessment is usually required) and the otoliths removed for subsequent age determination. Once the body cavity has been opened and the viscera exposed, the liver should first be examined *in situ* and a careful note taken of its size and colour. Next, the presence of any macroscopically visible nodules, parasites or other lesions should be recorded. The liver should then be dissected away from the remaining viscera and removed from the fish for examination. If data on hepato-somatic indices are required, both the fish (before dissection) and the liver have to be weighed. The maximum diameter of any macroscopic nodules should be recorded with notes on its general appearance, including texture, degree of surface vascularization, and colour, since the latter can occasionally give an indication of the histological nature of the nodule. White and opaque nodules normally correspond to clear cell and acidophilic hepatocytes, while yellowish coloration often corresponds to basophilic hepatocellular lesions, and green nodules may be associated with benign and malignant tumours of the bile ducts (cholangioma and cholangiocarcinoma). Dark red staining nodules may indicate neoplasms involving vascular structures (such as hemangioma and hemangiosarcoma). It should be noted, however, that macroscopic appearance alone cannot be used for diagnosis.

3.2 Histological Sampling

In order to prevent the appearance of *post mortem* artefacts, it is essential that the specimen be handled with extreme care and that all tissues be placed into histological fixative or frozen in liquid nitrogen immediately after killing the fish. For the collection of multiple samples, the livers of individual fish may be divided into four segments (or more, if appropriate) for analytical chemistry, biochemistry, histochemistry, and histopathology (Figure 1). Accordingly, samples can also be collected at this time for other biomarkers (such as EROD, metallothioneins, etc.). In cases where there are no grossly visible lesions, a 3 mm slice is cut longitudinally through the central axis of the liver using a sharp blade (e.g., scalpel blade No. 24). If visible anomalies are present, a section should also be taken through the entire depth of the affected area(s), including, where possible, adjacent normal tissue. At this juncture, it is important that accurate notes are taken to describe the gross features of the lesion so that it may be confidently related to its microscopic appearance after the sample has been processed in the laboratory. Relevant photographs may also be taken to assist with the identification of small lesions during embedding (see Section 3.3). In all cases, it is essential that care be taken to avoid crushing or ripping the tissue with forceps or other dissection instruments. Two fixatives are recommended for histopathology, namely, 10% neutral buffered formalin (NBF) and Dietrich's fixative (see Annex 1). Bouin's fluid may also be appropriate, but is less practical for usage on board research vessels due to its toxicity. Tissues may be placed directly into individual pre-labelled containers of fixative or into pre-labelled histological processing cassettes which allow for placing tissues from a number of fish into a common container of fixative. Fixation is allowed to proceed for 12–24 hours with occasional agitation to ensure the

even fixation of samples. Samples may then be transferred to 70% ethanol for transportation and long-term storage. For possible application of samples to immunohistochemical techniques, fixation for more than 12–24 hours should be avoided. For some specimens, it may be desirable to embed the sample in glycol methacrylate. This allows for the improved resolution in histopathology possible with thinner sections. For this process, tissue samples of up to 10 mm × 5 mm are fixed in 4% Baker's formal saline for a maximum of 24 hours. If required, tissues can then be transferred to gum sucrose for indefinite storage at 4 °C.

For enzyme histochemistry, immunohistochemistry, lysosomal latency tests, and other relevant techniques, frozen liver tissue may be required. Tissue samples should be quenched (supercooled) in *n*-hexane to −70 °C in order to avoid uneven freezing and ice crystal damage, and stored under optimal temperature conditions (either in liquid nitrogen or at –70/80 °C). A practical way to prepare the liver for such techniques is to place samples (up to 1 cm^3) (see Figure 1) on a cooled, coded cryostat chuck and to proceed as above (see Köhler *et al*., 1992, 2002).

3.3 Processing Liver Samples for Embedding

For conventional histology, fixed tissue samples are dehydrated in alcohol, cleared, and embedded in paraffin wax prior to their placement into wax blocks. Tissues should preferably be processed using commercially available automatic tissue processors, such as "carousel-type" tissue processors or vacuum infiltration processors. Typical processing schedules are provided in Annex 2. After their transfer to paraffin wax (the last step in automatic processing), tissues are embedded in wax using appropriate moulds. Increasingly, plastic embedding media (e.g., glycol methacrylate) are being used for forming these blocks. They have the advantage that thinner (1–2 μm) sections can be obtained, providing better resolution of histopathological features. The protocol for embedding in glycol methacrylate is provided in Annex 3.

3.4 Sectioning and Staining

For conventional histology, sections are cut at 4 μm to 5 μm using a suitable microtome (a modern rotary microtome is ideal). Some modern microtomes are motorized, allowing for greater consistency in section thickness and overall quality of the histological section. It is important to ensure that the tissues are trimmed sufficiently to allow for sectioning of the complete area of interest within the sample. Care must also be taken not to trim through any lesions that were recognized at *post mortem*. In some cases, it may be necessary to take serial sections of the sample. For general monitoring purposes, and where multiple specimens will be collected, a single section of the sample will generally suffice. However, where resources allow, multiple sections taken throughout the tissue block will provide greater precision in detecting focal or multifocal lesions which could be missed in a single section. Sections are floated out in a water bath, which should be maintained at a temperature that is a few degrees below the melting point of the wax used to embed the specimen. Sections are then picked up on clean glass slides, which can, if necessary, be pre-coated with an adhesive (Polysine-VWR – Sigma Diagnostics) to avoid the loss of sections during the staining process. Drying of mounted sections should be carried out in a dust-free environment on a hotplate, following which sections are stained with haematoxylin and eosin (H&E), dehydrated, cleared, and mounted (see Annex 4). A variety of other staining techniques can be applied for specific purposes (Table 1). Protocols for all of these techniques can be found in standard texts on histotechniques (Bancroft and Stevens, 1996; Bancroft and Cook, 1994; Drury and Wallington, 1973; Romeis, 1989). In this paper, emphasis is given to the diagnostic features of toxicopathic lesions based on paraffin wax-embedded material stained with H&E. Additional techniques are suggested, as appropriate, where they may assist in a definitive diagnosis of lesion type.

Frozen tissue samples are fixed to metal chucks using cryo-embedding compounds and are cut in a cryostat, in which the ambient cabinet temperature can be adjusted to suit the particular structure and composition of the liver tissue (e.g., its lipid content) or the histochemical method to be applied. Usually, the temperature should be between –20 °C and –30 °C. Sections are generally prepared at 8–10 μm and are placed on glass slides, which may be pre-coated with a suitable adhesive (see above). As above, multiple sections are preferred where resources permit them to be taken. Slides can be stored in the cryostat cabinet until use, but preferably for no longer than 24 hours. Depending on the histochemical technique to be applied, fixation of the sections with paraformaldehyde is recommended.

A large variety of cytochemical, enzyme, and immunohistochemical techniques for the detection of pathological liver changes have been developed which can be applied directly to cryo-sections. However, it is not within the scope of the present publication to provide detailed guidelines for these techniques. The reader is, therefore, referred to other textbooks and literature published in scientific journals that provide information on theoretical considerations and preparation techniques (e.g., Hinton *et al*., 1988; Köhler, 1991; Van Noorden and Frederiks, 1992; Köhler *et al*., 1992; Köhler and Van Noorden, 1998).

Table 1. Special histological staining techniques for the diagnosis of liver histopathology.

1. Perls Prussian Blue for ferric iron – for the detection of resistance of iron uptake and deposition in foci of cellular alteration (FCA) and neoplasms.
2. Van Gieson – for the demonstration of collagen in cases of fibrosis, fibroplasia or cirrhosis.
3. Periodic acid Schiff (PAS) – for the demonstration of hepatocellular glycogen, some pigments, and fungal hyphae.
4. Gomori methenamine silver – for the demonstration of reticular fibres.
5. Feulgen – for the specific demonstration of DNA in normal and altered nuclei.
6. Giemsa – for the demonstration of protozoan and bacterial infections.
7. Gram stain – for the demonstration of bacterial infections.

4 HISTOPATHOLOGICAL DIAGNOSIS

The examination of each section should take into account any gross observations made at *post mortem* (or necropsy). It is also important that the reading of slides is conducted in a consistent manner and blindly, without knowing the origin of the sample. Individual pathologists will have their own preferences in approach for this, but as a general rule, it is better to examine slides using the lowest power objective lens first. It may even be useful to examine the slide by eye before placing it on the microscope in order to check for any large but indistinct lesions, since these can be difficult to discern at higher magnifications. In any case of doubt, it is strongly advised that additional sections be prepared and examined carefully in order to establish the correct diagnosis.

4.1 Histological Appearance of Normal Liver

The histological structure of the normal flounder (*P. flesus*) liver has been well described (Vethaak and Wester, 1996) and corresponds with the morphology of other fish species (Hinton

and Lauren, 1990; Hinton, 1994). The basic structural unit is the liver tubule or trabecula, which has a central bile canaliculus or ductule, and is separated from other tubules by sinusoids (Figures 2a and 2b). The tubules form muralial plates between larger vessels. The liver in flounder is characterized by the presence of exocrine pancreatic tissue and the organ is often referred to as "hepatopancreas". Other tissue elements include bile ducts that are lined with a cuboidal epithelium and surrounded by connective tissue and larger blood vessels, which may also be sheathed in connective tissue. Clusters of pigmented cells, termed "macrophage aggregates" (MAs) or "melanomacrophage centres" (MMCs), may also be present.

In dab (*L. limanda* L.) liver, exocrine pancreas is usually absent. However, small clusters of pancreatic acinar cells may occasionally be seen within the liver tissue. The architecture of the liver is similar to that of flounder liver, although in some livers, the tubule structure can be indistinct because of the wide variation in the cellular morphology of the hepatocytes, depending on the amount of storage products (glycogen and lipid) present (Bucke and Feist, 1993) (Figure 3). In both fish species, the degree of hepatocyte vacuolation is highly dependent on the stage in the reproductive cycle and the availability of an adequate food supply.

4.2 Histopathological Diagnosis of Liver Lesions

There are a number of non-specific lesions, which will be encountered during the assessment of liver pathology. These lesions include inflammatory changes following viral, bacterial or parasitic infections (infiltration and granulomatosis), atrophy, coagulative and liquefactive necrosis, increased apoptosis, and the presence of increased numbers and size of MAs. These lesions are regarded as of lesser importance for environmental monitoring purposes, but should nonetheless be recorded (ICES, 1998).

For monitoring purposes, the main lesion categories are as follows:

1) Early non-neoplastic toxicopathic lesions;

2) Foci of cellular alteration (FCA);

3) Benign neoplasms;

4) Malignant neoplasms.

Examples of these are provided in the following sections and in specimens deposited in the Registry of Aquatic Pathology (RAP) held at the CEFAS Weymouth Laboratory. Accession numbers for each specimen are indicated in the respective figure legends.

4.2.1 Non-neoplastic toxicopathic lesions

These include the following non-nodular lesion types:

- Hepatocellular nuclear pleomorphism;
- Hydropic vacuolation of biliary epithelial cells and/or hepatocytes;
- Phospholipidosis;
- Fibrillar inclusions;
- Peliosis and spongiosis hepatis.

Hepatocellular pleomorphism is characterized by the presence of hepatocytes exhibiting differences in morphological appearance from neighbouring hepatocytes. Most frequently, enlarged or hypertrophied cells, usually with a pale-staining eosinophilic cytoplasm and hypertrophied nuclei with clumped and vesicular chromatin, are present in a focal to diffuse distribution (Figure 4). It should be noted, however, that a similar cytopathology may be apparent in cases of hepatocellular adenoma and carcinoma, although in these cases, cells will be present within a nodular lesion (see Sections 4.2.4 and 4.2.5, below).

Hydropic vacuolation of hepatocytes can be difficult to differentiate from the normal accumulation of lipid within the cytoplasm. However, affected cells usually contain a few large vacuoles (macrovesicular steatosis) (Figure 5) or numerous small vesicles (microvesicular steatosis). Excessive lipid accumulation or steatosis of hepatocytes is generally regarded as a pre-neoplastic toxicopathic change in mammalian studies, but its significance in fish is less certain. It should be noted that excessive fat storage in mammalian livers is also often found in animals of good nutritional status that go through a phase of negative energy balance. This is not related to tumour development in the liver of these animals. In dab liver, the initial toxic lipidosis is difficult to diagnose but, in advanced stages, it can be clearly identified by large lipid droplets, especially during the spawning period when lipid is degraded during vitellogenesis. While being a fairly common lesion in flounder, hydropic vacuolation of the biliary epithelial cells (Figure 6) is rarely seen in dab or in plaice. Hydropic vacuolation of bile ductule cells and hepatocytes has been reported from several flatfish species, including the European flounder (Vethaak and Wester, 1996; Moore *et al*., 1997; Stehr *et al*., 1998; Simpson *et al*., 2000). Its occurrence in certain species has been associated with exposure to hydrocarbons and its utility as a histological biomarker for xenobiotic exposure, at least for those species, is established.

Peliosis hepatis lesions may be encountered in the liver of dab and flounder from the North Sea; this lesion type is distinct from spongiosis hepatis (or cystic parenchymal degeneration, see Myers *et al*., 1987) as described in experimental carcinogenesis in fish and rats (Hinton *et al*., 1988; Couch, 1991; Bannasch *et al*., 1981). The distinction is based upon the presence of blood within the large cystic structures (Figure 7). In diagnosis of this lesion type, care should be taken to ensure that the cystic blood-filled structures are not constituents of a hepatocellular carcinoma (see Section 4.2.4, criteria for the diagnosis of malignant neoplasms). Although peliosis hepatis has been included here for completeness, this lesion type (and that of spongiosis hepatis) is rarely encountered and is known to occur in fish from relatively uncontaminated reference areas (Dr Mark Myers, pers. comm.). At present, their value as a histopathological biomarker of contaminant exposure is uncertain. Phospholipidosis is a lysosomal storage disease caused by exposure to xenobiotics and has been recorded in flounder from the southern North Sea (Köhler *et al*., 2002). Hepatocytes are usually enlarged and have a foamy cytoplasm characterized by the presence of numerous intracytoplasmic eosinophilic granules. These granules are enlarged lysosomes filled with phospholipid aggregates and lamellated inclusions which can be visualized by electron microscopy (Lüllmann-Rauch, 1979).

Parallel cytoplasmic fibrillar inclusions in perisinusoidal hepatocytes have been recorded from flounder (Köhler, 1990; Vethaak and Wester, 1996), but only rarely from dab. The inclusions can have variable staining characteristics ranging from eosinophilic to basophilic and appear as bundles at various orientations within the cells. In some samples, inclusions are seen in almost all of the hepatocytes within a section (Figure 8). Ultrastructurally, they have been identified as enlarged microtubules (Köhler, 1990) and, although they appear more prevalent at sites impacted by contamination (Stentiford *et al*., 2003), their exact aetiology is currently unknown.

4.2.2 Hepatocellular nodules

For the purposes of this document, hepatocellular nodules may be defined as discrete lesions viewed in histological sections that are composed of ten or more cells, increasing in size to macroscopic nodules visible to the naked eye at *post mortem*. Morphological criteria for the different categories of liver nodules (foci of cellular alteration (FCA), adenoma, and adenocarcinoma) are set out in Sections 4.2.3, 4.2.4, and 4.2.5, below. A diagnostic key for the discrimination of FCA, hepatocellular adenoma, and hepatocellular carcinoma is provided in Chart 1.

4.2.3 Foci of cellular alteration

Foci of cellular alteration (FCA) appear as discrete aggregations of hepatocytes, the morphology and staining characteristics of which differentiate them from the surrounding hepatic parenchyma. All categories can be recognized using the following general morphological criteria:

1) A discrete focal lesion of ten or more cells in diameter with no upper size limits;
2) No evidence of compression of the surrounding tissue, with continuity of the trabecular structure with the surrounding hepatic parenchyma (this may be difficult to ascertain with clear cell and vacuolated foci; refer to the relevant sections below);
3) The normal tubular architecture and thickness should be maintained (again, this may be difficult to ascertain with clear cell and vacuolated foci; refer to the relevant sections below);
4) Mitotic figures are rare or absent;
5) Relative absence of MAs, exocrine pancreatic tissue, and bile ducts. On rare occasions, one or more of these components may be present and hence their absence cannot be used as a diagnostic criterion for FCA in isolation.

Lesions fulfilling the above morphological criteria can be further distinguished by variations in their cytological appearance and staining properties:

a) Clear cell foci can be recognized by the “ground glass” appearance of the cytoplasm of their constituent hepatocytes. This is indicative of increased glycogen storage within these cells. Hepatocyte nuclei in these foci are characteristically centrally placed (Figure 9). In some lesions, the cytoplasm may be slightly eosinophilic, making it difficult to distinguish them from an eosinophilic focus in an H&E section. Application of the Periodic acid Schiff (PAS) technique that demonstrates glycogen and other mucopolysaccharides may be useful for the correct diagnosis of the clear cell foci. However, it should be remembered that glycogen may be lost through the normal histological preparation process. Clear continuity of the foci cells with the surrounding parenchyma is often difficult to visualize in this lesion type due to the constituent cells being frequently enlarged with cytoplasmic vacuoles. Clear cell foci are relatively rare in dab and flounder.
b) Vacuolated foci contain hepatocytes that are characterized by the presence of several medium to large lipid vacuoles within the cytoplasm (Figures 10 and 11). As described above for glycogen, the lipid is lost during routine histological processing but can be demonstrated using frozen sections stained with Oil Red O. Unlike hepatocytes in clear cell foci, the nuclei of hepatocytes in vacuolated foci tend to be displaced to the cell margins. As in clear cell foci, the continuity of vacuolated foci with the surrounding liver parenchyma may be difficult to discern. Vacuolated foci are one of the most common toxicopathic lesions encountered in dab and flounder.

c) Eosinophilic foci, as the name suggests, have an affinity for eosin, with their constituent hepatocytes appearing pale to dark pink in H&E stained sections. This staining depends on the quality of the fixation of the tissue and the degree of differentiation used during the staining procedure (Figures 12 and 13). The cytoplasmic eosinophilia is indicative of the proliferation of smooth endoplasmic reticulum within affected cells (Bannasch *et al.*, 1997). Although the degree of eosinophilia can vary, the foci should always appear relatively more eosinophilic than the surrounding tissue. There is little or no variation from normal cytomorphology and there is an absence of basophilic cytoplasmic fibrils. This lesion type is seen occasionally in dab and flounder.

d) Basophilic foci are amongst the most commonly occurring pre-neoplastic lesions found in dab and flounder in the North Sea. The basophilia of the altered cells is due to the proliferation of rough endoplasmic reticulum and is usually more noticeable as basophilic fibrillar inclusions because cells usually contain little storage material (Figures 14, 15, and 16). Although the degree of basophilia can vary, as for the eosinophilic foci, the hepatocytes of the basophilic foci should always appear relatively more basophilic than the surrounding tissue.

e) Mixed cell foci may occasionally be encountered in the liver of dab and flounder. These should be placed into the category of the predominant cell staining type. Amphophilic foci containing hepatocytes with both eosinophilic and basophilic components in the cytoplasm are rare in flounder and dab.

4.2.4 Benign neoplasms

These lesions may be recognized by a set of morphological criteria that apply to all variants:

1) Clear and distinct separation of the tubules of the tumour from the surrounding tissues;

2) In addition to separation from the surrounding parenchyma, compression of the surrounding parenchymal cells and vessels may be evident;

3) Hepatocellular tubular thickening (greater than three cell layers) will be evident with mild architectural disorganization;

4) Relative absence of macrophage aggregates, exocrine pancreatic tissue, and bile ducts;

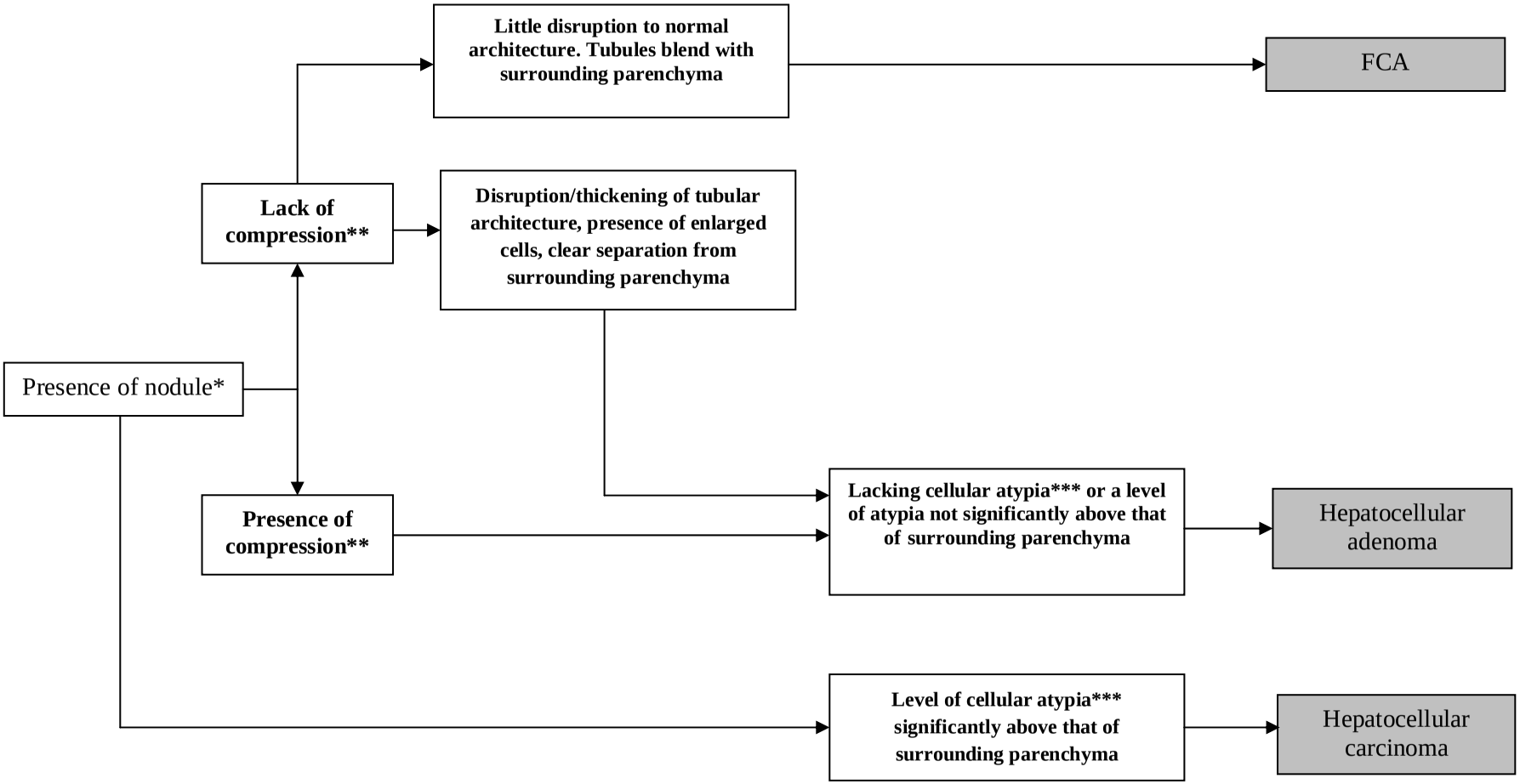
5) Increased cellular density;

6) Generalized cellular and nuclear atypia within the lesion will be absent or at a degree not significantly above that of the surrounding liver parenchyma.

Lesions fulfilling these criteria can be further categorized using differences in the staining properties for variants of hepatocellular adenoma (e.g., clear, vacuolated, eosinophilic, basophilic, and mixed cell) and by involvement of different constituent cell types (e.g., cholangioma, hemangioma, and pancreatic acinar cell adenoma in relation to bile ducts, blood vessels, and pancreatic tissues, respectively).

Hepatocellular adenomas can be recognized using a combination of the basic morphological criteria indicated above as well as the staining properties of the constituent cells. Hence, basophilic, eosinophilic, clear cell, and vacuolated hepatocellular adenomas can be distinguished (see Figures 17 to 23). Multiple adenomas may occur in the same liver and, in some cases, an inflammatory response consisting predominantly of an infiltration of lymphocytic cells can be seen at the periphery of the lesion. It should be noted that compression per se may not always be required for the diagnosis of hepatocellular adenoma. In cases of non-compressed adenoma, there will be a clear separation of tumour cells from the surrounding parenchyma.

Chart 1. Diagnostic key: Hepatocellular nodules



*** Nodule:** A discrete accumulation of hepatocytes (more than ten cells). These lesions usually have a decreased concentration of pigmented macrophage aggregates relative to the surrounding parenchyma.

** **Compression**: Complete compression around the circumference of a lesion is rare. More commonly, compression is seen at certain sections of the lesion periphery. Adjacent parenchyma will appear relatively compressed. The lesion periphery may be marked by the presence of MAs and flattened blood vessels.

*** **Atypia**: Most livers will contain a certain degree of nuclear and cellular atypia (though proportions of atypical nuclei and cells will be very small in most cases). In terms of lesion diagnostics, it is important to consider the degree of nuclear (size, shape, chromatin pattern) and cellular (size, shape, cytoplasmic content) atypia relative to the surrounding parenchyma. For the diagnosis of carcinoma *in situ*, the degree of cellular and nuclear atypia within the suspect carcinoma should be greater than that of the surrounding nodule parenchyma.

Cholangiomas are rarely found in sections of dab liver, but have been recorded in several other flatfish species. The lesion is composed of bile duct cells which have proliferated and which are generally well encapsulated by fibrous tissue (Figures 24 and 25). Histologically, the presence of glandular structures reminiscent of small bile ducts is a typical feature of cholangioma.

Hemangiomas are rare benign tumours arising from endothelial cells in blood vessels and capillaries in particular. They may be cystic in appearance but do not contain large amounts of blood in dab.

Pancreatic acinar cell adenomas have only rarely been recorded in European flounder and in certain North American pleuronectid species.

4.2.5 Malignant neoplasms

Malignant neoplasms are characterized by several key morphological and cellular features that generally relate to their invasiveness and their lack of clear differentiation with regard to the surrounding parenchyma. The main criteria of use for the diagnosis of malignant neoplasms are as follows:

1) A relative level of cellular atypia, including nuclear and cellular pleomorphism, significantly above that of the surrounding tissue parenchyma. A loss of cellular polarity may occur.

2) Invasion of adjacent parenchymal tissue, irregular borders to the lesion, and obvious "satellite" lesions with variable shape and histological characteristics.

3) Cystic structures, enlarged blood vessels, and necrosis (the latter may also occur in benign neoplasms, but less frequently) may be present within the main lesion.

4) Normal tissue architecture is lost.

5) The frequency of mitotically active cells is significantly higher than that of the surrounding parenchyma.

6) Relative absence of macrophage aggregates, exocrine pancreatic tissue, and bile ducts.

A number of malignant neoplasms have been recorded in marine flatfish species, though most of these occur only rarely and particularly in older fish. The following lesions may be encountered in dab and flounder:

- Hepatocellular carcinoma (Figures 26 and 27);
- Cholangiocarcinoma (Figures 30 and 31);
- Pancreatic acinar cell carcinoma (Figures 32 and 33);
- Mixed hepatobiliary cell carcinoma;
- Mixed angiosarcoma/hepatocellular carcinoma (Figures 34 and 35);
- Hemangiosarcoma;
- Hemangiopericytic sarcoma;
- Other.

It is important to note that carcinomatous foci or so-called "carcinoma *in situ*" may occur within some lesions diagnosed as hepatocellular adenoma (Figures 28 and 29). In such cases, the convention is to classify the lesion according to the most advanced stage of the neoplasm present; therefore, a diagnosis of hepatocellular carcinoma should be made. Unless it is clearly possible to establish the main cell type involved in malignant lesions, it is recommended that representative slides are prepared for an authoritative opinion to be sought from other pathologists involved in the assessment of flatfish liver pathology.

5 QUALITY ASSURANCE

An increasing number of countries are conducting studies on the biological effects of contaminants as part of their national monitoring programmes in order to assess the quality of the marine environment. Most of these national activities are coordinated in international regional monitoring programmes such as those organized by MEDPOL, the OSPAR Commission and HELCOM, which cover large areas of the seas surrounding Europe. Historically, most of these programmes have been based on measurements of contaminant residues in biota, sediments or water. However, it has been realized that a thorough assessment of the state of the marine environment requires an integrated monitoring programme, incorporating both chemical and biological effects measurements. Chemical data alone can only be compared to background values or against ecotoxicological reference criteria, the reliability and applicability of which have often been disputed. Biological effects monitoring has therefore been given high priority, particularly in newer monitoring programmes such as the OSPAR Joint Assessment and Monitoring Programme (JAMP).

In order to ensure that the institutes collaborating in international biological effects monitoring programmes produce comparable data, there is a need to develop and establish appropriate quality assurance procedures. For quality assurance of chemical monitoring, the QUASIMEME programme was a milestone. This programme was implemented in 1993 as an EU-funded project and thereafter was continued as a self-financing programme with contributions from participating laboratories. For biological effects monitoring, a similar EU-funded programme entitled BEQUALM was initiated in 1998, with funding ending in 2001. As part of the move to enhance quality assurance (QA) in biological effects techniques, BEQUALM is developing QA procedures for fish liver histopathology, based on the recommendations made by the ICES Special Meeting on the Use of Flatfish Liver Pathology for Monitoring Biological Effects of Contaminants (ICES, 1997).

The following components have been developed:

1) An agreed set of approved methods and standard operating procedures for:

   a) The sampling of fish;

   b) The dissection and macroscopic examination of the liver;

   c) The fixation and preservation of the liver;

   d) The histological processing and staining of the liver;

   e) The diagnosis and categorization of lesion types within the liver;

   f) The quantification of histopathological/histochemical changes occurring within the liver;

   g) The archiving and reporting of data;

   h) The statistical analysis of data.

2) A system for training and intercalibration in order to improve individual laboratory performance and to achieve good laboratory practice and a comparability of data, in particular with regard to disease diagnosis, through:

   a) Intercalibration and training workshops;

   b) The preparation and distribution of histological reference slides with characteristic histopathological liver lesions;

   c) The organization of ring test.

3) A system for monitoring the output of participating laboratories and assessing their compliance with agreed quality standards.

Because studies on fish liver histopathology are among the techniques applied within the biological effects components of international monitoring programmes such as the OSPAR Joint Assessment and Monitoring Programme (JAMP), it is essential that all collaborating institutes apply the quality assurance parameters detailed under the BEQUALM programme. It is the intention of this TIMES publication to provide guidelines for some of the components to be used as a basis for such a quality assurance programme.

6 DATA TREATMENT AND SUBMISSION

A prerequisite for a thorough quantitative study on the occurrence of contaminant-induced pathological liver conditions and, subsequent to this, an appropriate treatment of the data generated, is the development of a suitable study protocol which reflects the objectives of the study in question. The sampling strategy should be designed according to statistical requirements, particularly in terms of the sample size (see Section 3, above). For example, in a regional assessment where the expected prevalence of a particular lesion type is low, it does not make sense to use multiple sampling sites with a small number of fish sampled from each of these sites. The statistical probability of detecting such lesions in these samples would be low. Instead, it is more appropriate to reduce the number of sampling sites and to increase the sample sizes.

A number of epidemiological studies on diseases of wild fish have provided evidence for a multifactorial aetiology involving a large number of endogenous and exogenous factors. These factors affect an organism's susceptibility to disease and/or the presence and virulence of the pathogenic agents. This is not only true for infectious pathogens and parasites, but also for non-infectious diseases such as the neoplastic and non-neoplastic lesions suspected to be associated with contaminant exposure. It is therefore important for studies on the aetiology of putative toxicopathic liver lesions not only to measure contaminant residues in fish and their habitat, but also to record other factors which could potentially explain the presence or absence of these lesion types. These can either be host-specific (e.g., age, size, gender, stage of sexual maturation, condition, population density, occurrence of other diseases, etc.) or site-specific (e.g., water depth, temperature, salinity, oxygen content, etc.). Measurements of potentially explanatory factors involved in disease aetiology should as much as possible be integrated into each disease study, both spatially and temporally. If this is not the case, interpolation and extrapolation techniques have to be applied in a multivariate analysis on cause-effect relationships, which will introduce a certain degree of data bias (Lang and Wosniok, 2000; Wosniok *et al.*, 2000).

Data on liver lesions and associated factors obtained in either field or experimental studies should be recorded using an appropriate paper template or by direct entry into a computer using appropriate database software. All raw data should be transferred to a computer data bank which

should be designed to allow for linkage to other data banks containing other relevant data types. In such a way, statistical and graphical data treatment is facilitated. All data should be recorded and stored on an individual fish basis.

There are a number of statistical procedures commonly applied in the analysis of fish disease prevalence data. For a simple regional comparison, observed prevalences together with a calculated 95% confidence interval provides a first overview of the occurrence of statistically significant differences between sampling sites. In order to avoid data bias due to different population structures in terms of sex ratio and length/age composition, a common technique used in regional assessments is to restrict the comparison to one sex and one particular size group. For example, for the analysis of dab disease data in the ICES data bank, females within the size group 20–24 cm total length have frequently been used (Wosniok *et al*., 1999). However, in most cases this method does not make use of all data available and neglects other components of the population, which might contribute considerably to overall disease prevalence. One approach to overcoming this shortcoming is to define a standard fish population for which a predicted prevalence can be calculated following the principle of direct standardization used in human epidemiology (Lang *et al*., 1999).

For a multifactorial statistical analysis of disease data, multivariate tests based on logistic models (McCullagh and Nelder, 1989) have been applied successfully and are therefore recommended. In addition to enabling the identification of single host-specific and site-specific factors and their interaction with a significant relationship to the disease prevalence, these tests also allow for a quantification of their effects, thereby providing useful information on possible cause-effect relationships. Thorough descriptions for the design and application of such models are given in numerous studies (e.g., by Vethaak and Jol, 1996; Lang *et al*., 1999; Lang and Wosniok, 2000; Wosniok *et al*., 1999, 2000).

As indicated above, the ICES Marine Data Centre contains a fish disease database as part of its environmental databases. ICES Member Countries conducting regular fish disease monitoring programmes according to standardized methodologies submit their data using standardized ICES reporting formats which are available from the ICES Secretariat on request or from the ICES website (www.ices.dk/env/index.htm). Procedures for validating and the further processing of incoming data have been established. At present, the database contains information on grossly visible diseases of dab and European flounder. However, it is envisaged that, in the future, data obtained from studies on contaminant-associated histopathological liver lesions will also be incorporated, using the categories detailed in Section 4.2, above. The importance of the database will increase in the future since ICES also serves as the data centre for results arising from the biological effects measurements carried out under the OSPAR JAMP.

7 FINAL REMARKS

Toxicopathic liver lesions in fish are playing an increasingly important role as biomarkers of contaminant effects in marine biological effects monitoring programmes. The use of histological assessment of gross lesions is essential in order to eliminate the inclusion of non-target pathologies such as inflammatory foci and parasitic nodules. In addition, because there is evidence that there is a general decline in the overall prevalence of macroscopically visible hepatic neoplasms in dab from several regions in the North Sea (Lang, 2002b), it is likely that greater attention will be given to the prevalence and extent of microscopic lesions in livers exhibiting no evidence of gross lesions. For this, there is a continuing need for the standardization of diagnostic criteria applied to histological lesions. In addition, the application of specific enzyme biomarkers and the use of genomic and proteomic techniques are likely to

play an increasingly important role in understanding the histogenesis of hepatic neoplasia in fish and for the detection of inconspicuous lesions.

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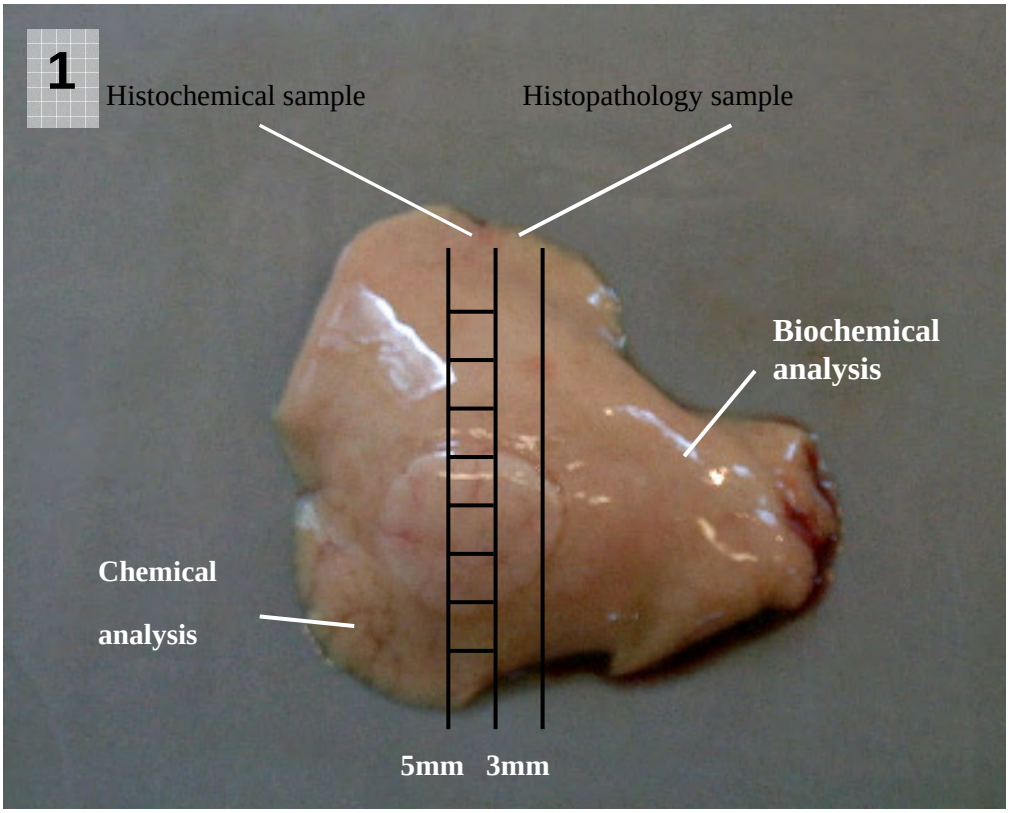
Figure 1. Diagram showing sampling procedure for dissecting multiple samples for analysis.



Figures 2a to 35 show micrographs of haematoxylin and eosin-stained sections of approximately 5 µm thickness unless otherwise stated. Registry of Aquatic Pathology (RAP) accessions held at the CEFAS Weymouth laboratory.

Figure 2a. Section of normal flounder liver showing exocrine pancreatic component (P) and bile duct (arrow). Bar = 100 µm.

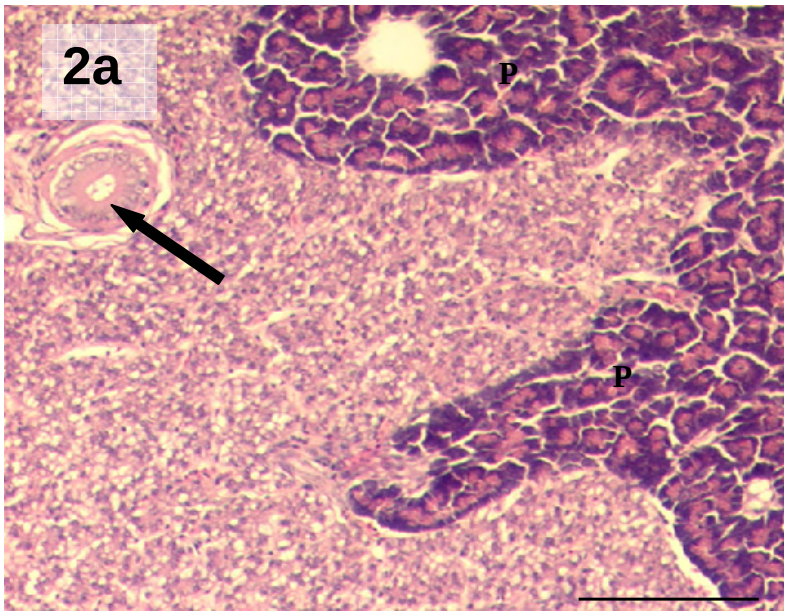


Figure 2b. Section of normal flounder liver showing a regular network of tubules separated by sinusoids (arrows). In this micrograph, the hepatocytes show little cytoplasmic vacuolation, indicating the depletion of storage materials (lipid and glycogen). Bar = 100 µm.

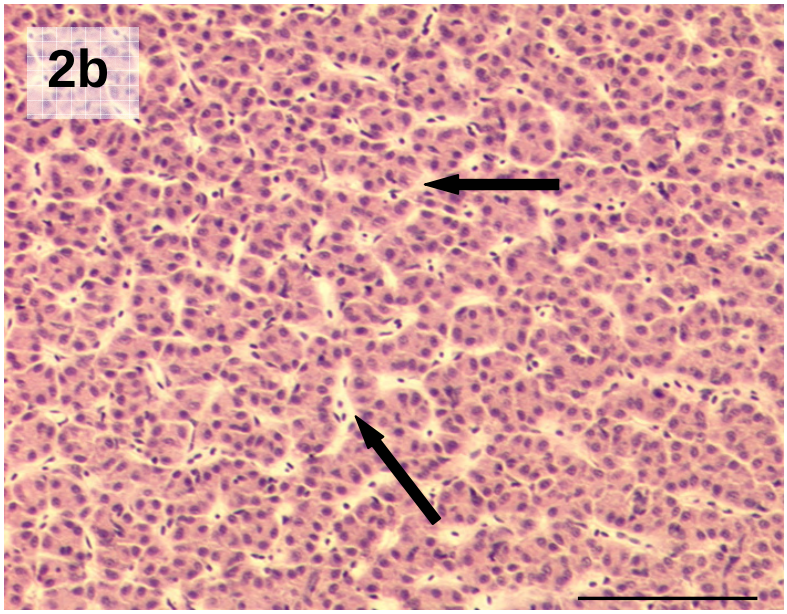


Figure 3. Section of normal dab liver with hepatocytes containing only few lipid vacuoles. Tubules are separated by sinusoids (arrow). Larger blood vessels (bv) and a bile duct (bd) are also present. Bar = 100 µm.

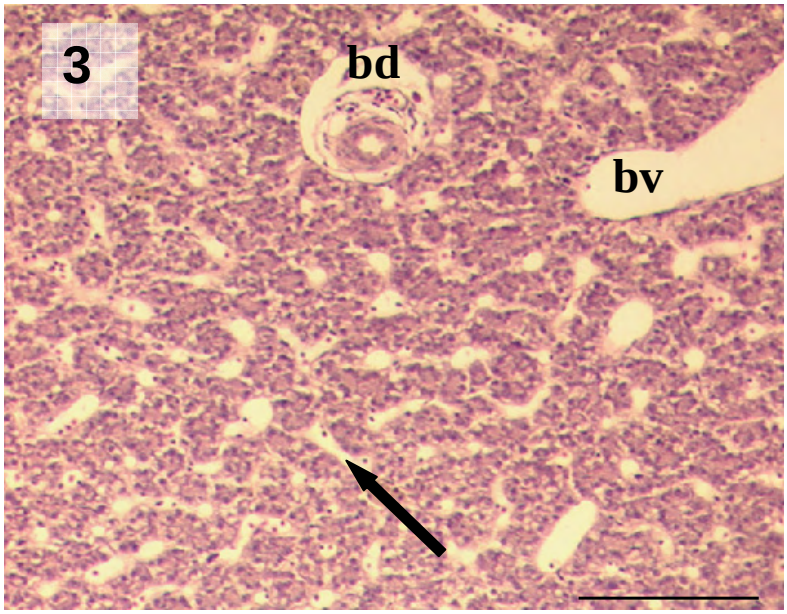


Figure 4. Hepatocellular pleomorphism in dab liver. Hepatocytes display variation in both cellular and nuclear profiles. Occasional binucleate hepatocytes may be seen (arrow). RAP Accession 4-D45. Bar = 25 µm.

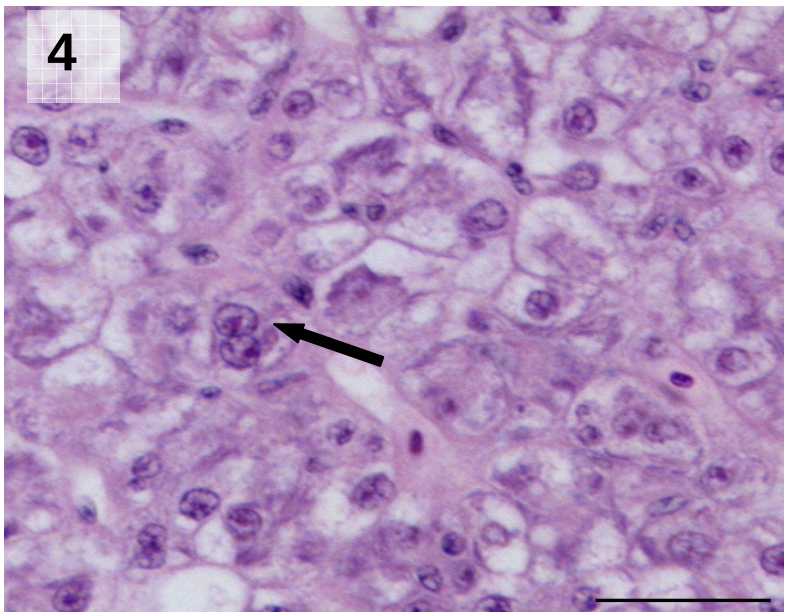


Figure 5. Focal region of hydropic vacuolation of hepatocytes in dab liver. Several hepatocytes containing few large vacuoles with nuclei displaced towards the cell membrane can be seen. RAP Accession 4-D46. Bar = 50 µm.

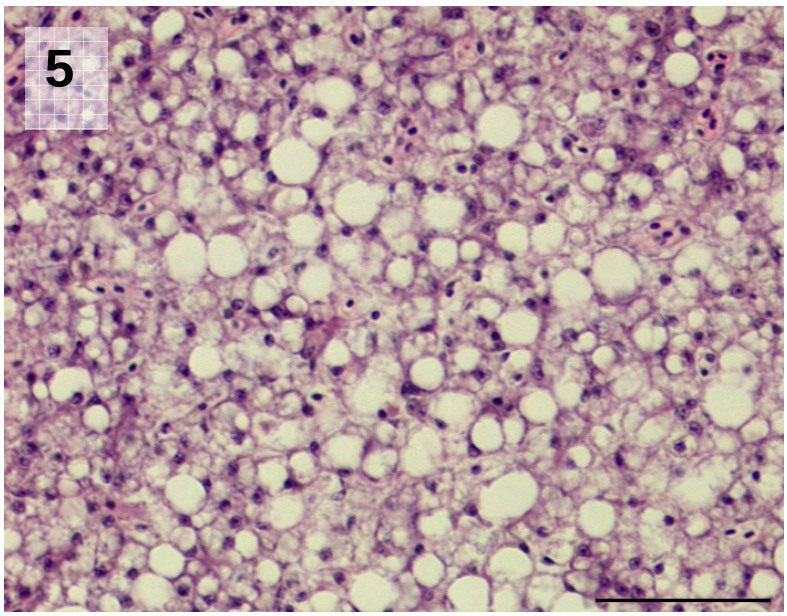


Figure 6. A bile ductule in dab liver exhibiting hydropic vacuolation of the epithelial cells (arrow). Hepatocytes appear normal. RAP Accession 4-D47. Bar = 50 µm.

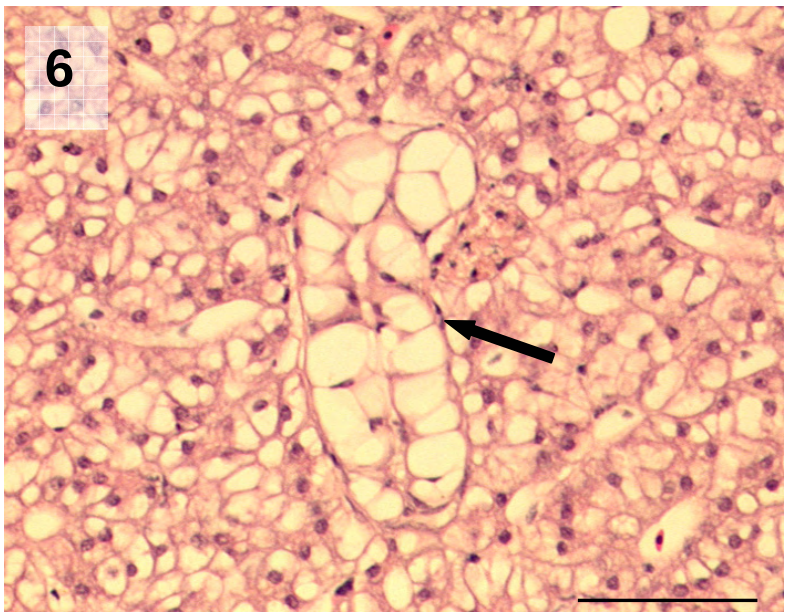


Figure 7. Peliosis hepatis in dab liver, characterized by the presence of large "blood lakes" containing intact erythrocytes and strongly eosinophilic serous material (S). RAP Accession 4-D48. Bar = 200 µm.

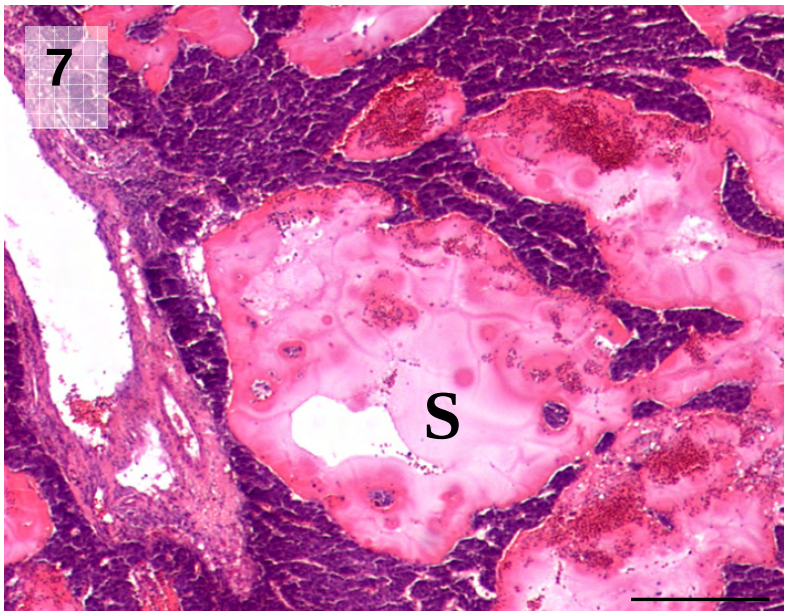


Figure 8. Hepatocellular fibrillar inclusions in flounder liver. Note the appearance of parallel stacks of cytoplasmic "fibres" at various orientations (arrow). RAP Accession 4-F12. Bar = 50 µm. (From Stentiford *et al*., 2003.)

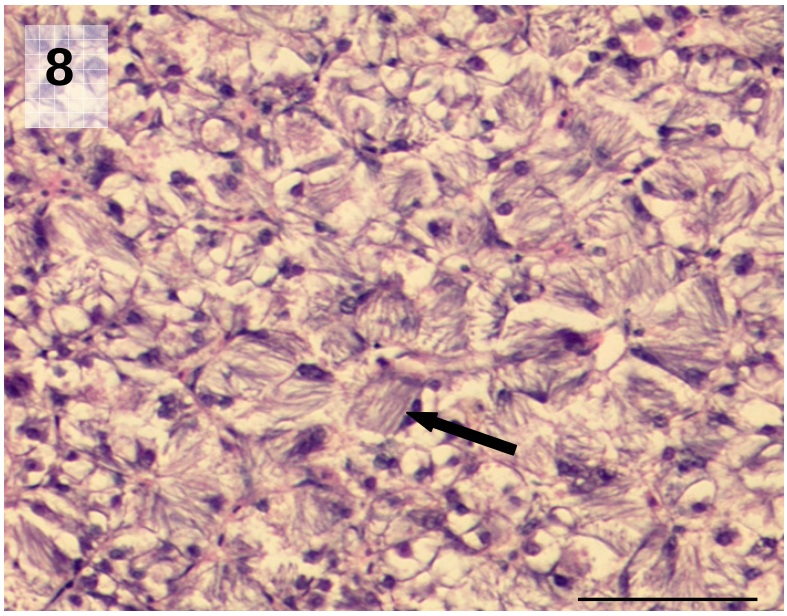


Figure 9. Clear cell focus of cellular alteration (FCA) in dab liver (arrows). Clear cell hepatocytes display pale eosinophilia with generally centrally placed nuclei. RAP Accession 4-D49. Bar = 50 µm.

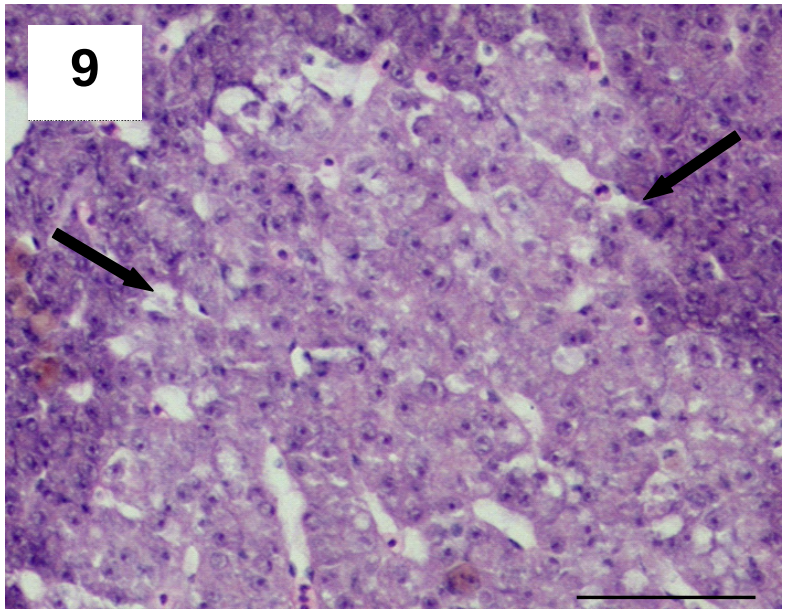


Figure 10. Low power view of a vacuolated FCA in flounder liver (arrows). Note the presence of pancreatic acinar tissue attached to the liver and in contact with the focus (P) with another small group of pancreatic acinar cells within the liver adjacent to a blood vessel (asterisk). RAP Accession 4-F13. Bar = 200 µm.

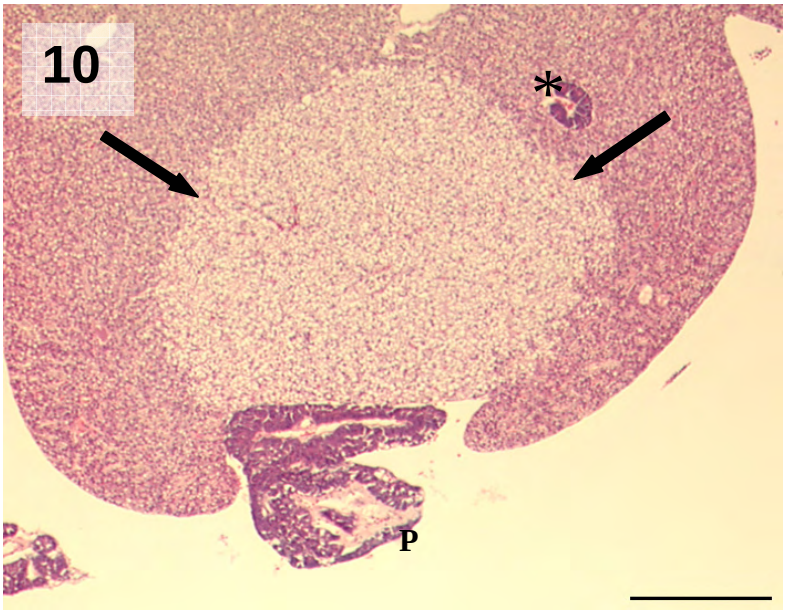


Figure 11. Vacuolated focus in flounder liver (arrows) containing hepatocytes characterized by the presence of several medium to large lipid vacuoles within the cytoplasm. Nuclei tend to be displaced towards the cell margins. Bar = 50 µm.

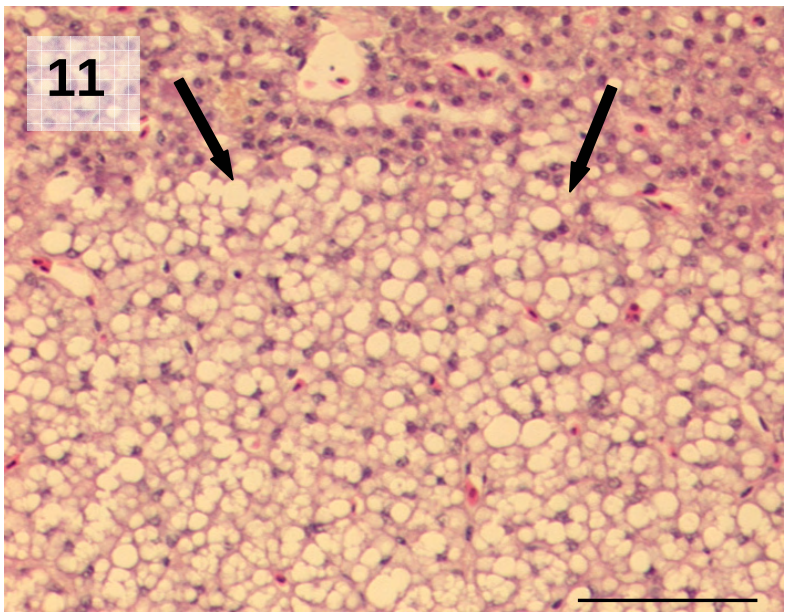


Figure 12. Section of dab liver containing an eosinophilic FCA (arrows). Note the relative increase in cytoplasmic eosinophilia of the focus compared with the surrounding hepatocytes. Bar = 100 µm.

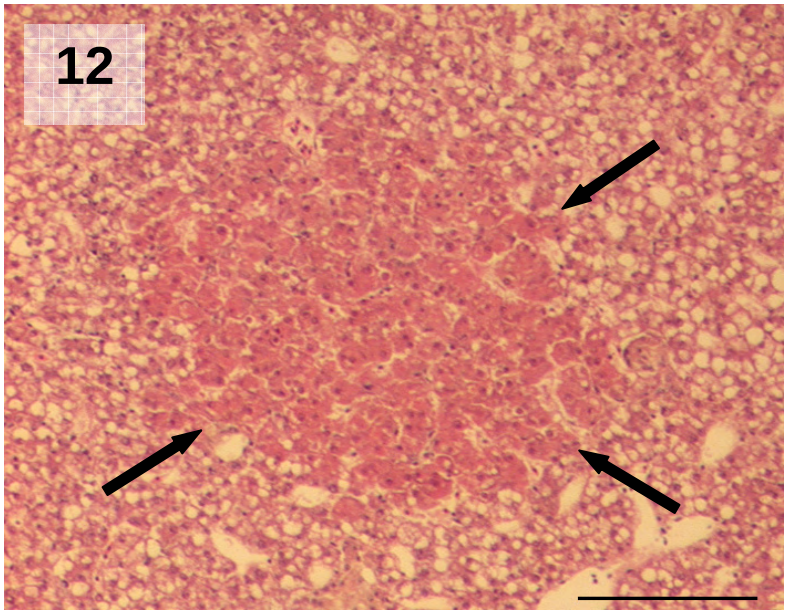


Figure 13. High power view of an eosinophilic FCA in dab liver showing a lack of cytoplasmic vacuolation and marked hypertrophy and eosinophilia of hepatocytes within the focus (arrow). RAP Accession 4-D50. Bar = 50 µm.

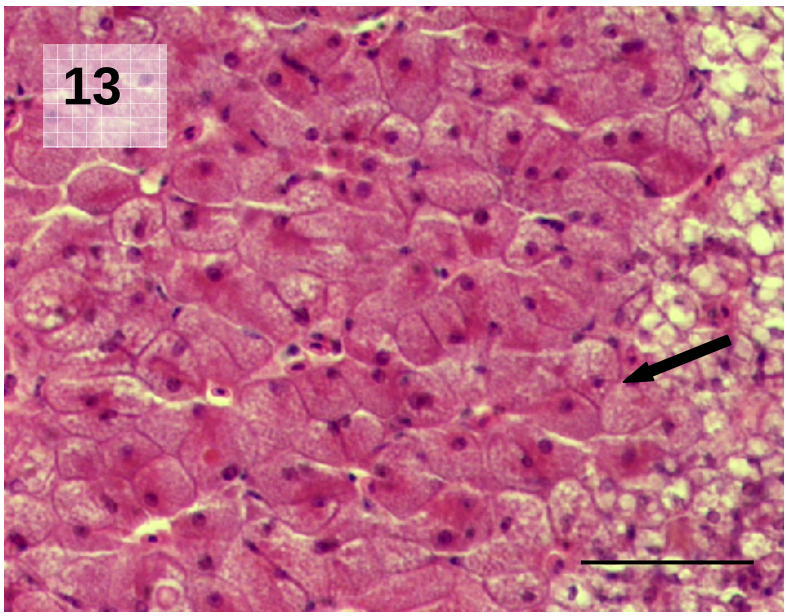


Figure 14. Low power view of dab liver containing a basophilic FCA (arrows). Small blood vessels (bv) can be seen within the focus. In this example, the hepatocytes of the FCA are particularly prominent against the vacuolated paler staining surrounding hepatocytes. RAP Accession 4-D50. Bar = 100 µm.

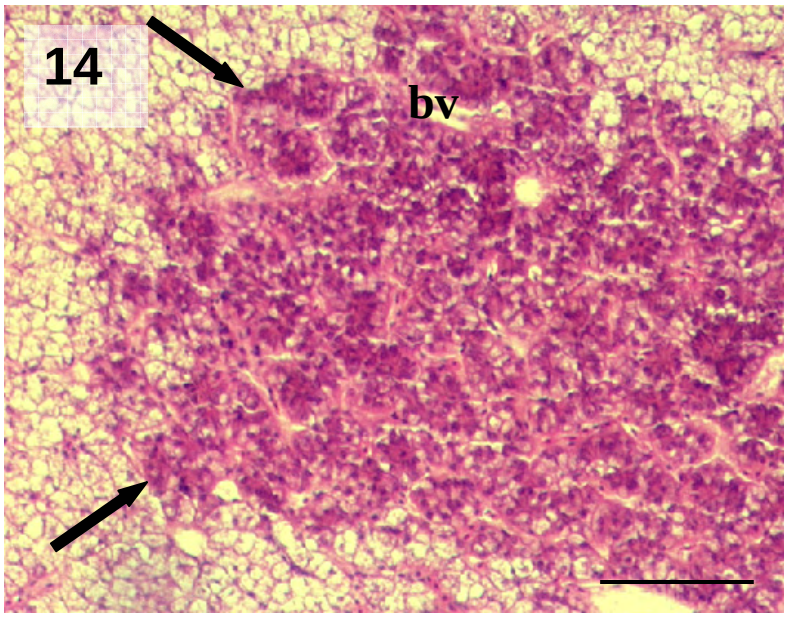


Figure 15. A relatively inconspicuous basophilic FCA in dab liver with a clear trabecular pattern of the tubules (arrow) compared to the less distinct structure of the surrounding liver tissue. Note the presence of macrophage aggregates (ma), a blood vessel (bv), and a bile duct (bd) at the periphery of the lesion. Bar = 100 µm.

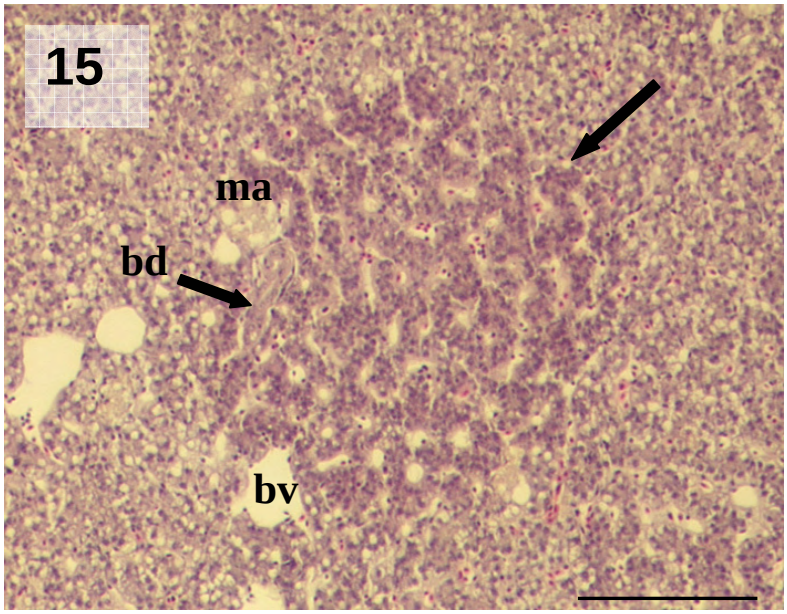


Figure 16. High power view of a FCA in dab liver showing the irregular border between the normal hepatocytes and those of the focus. Sinusoids (S) contain erythrocytes and are continuous between the normal liver and the focus. Tubules are typically two cells thick (asterisk). RAP Accession 4-D51. Bar = 50 µm.

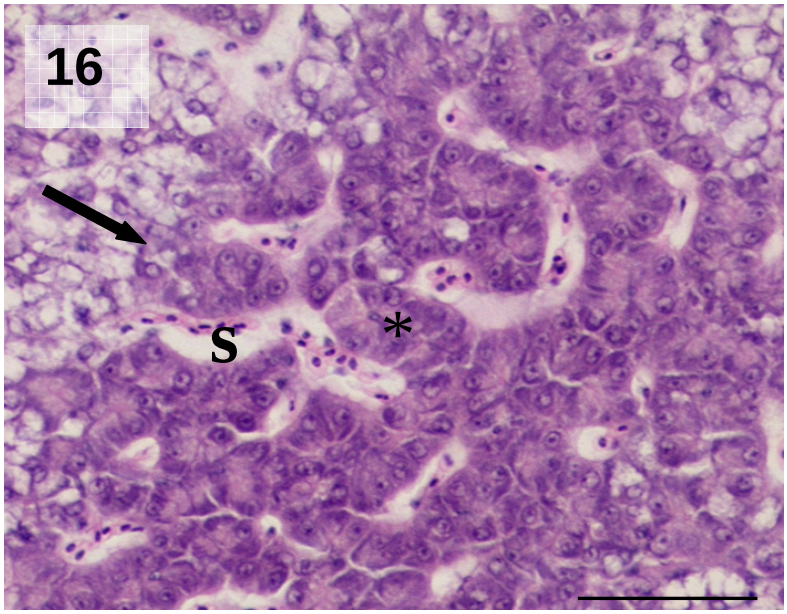


Figure 17. Low power view of a basophilic hepatocellular adenoma in dab liver. Clear compression of adjacent hepatocytes is evident around most of the lesion (arrows). RAP Accession 4-D52. Bar = 200 µm.

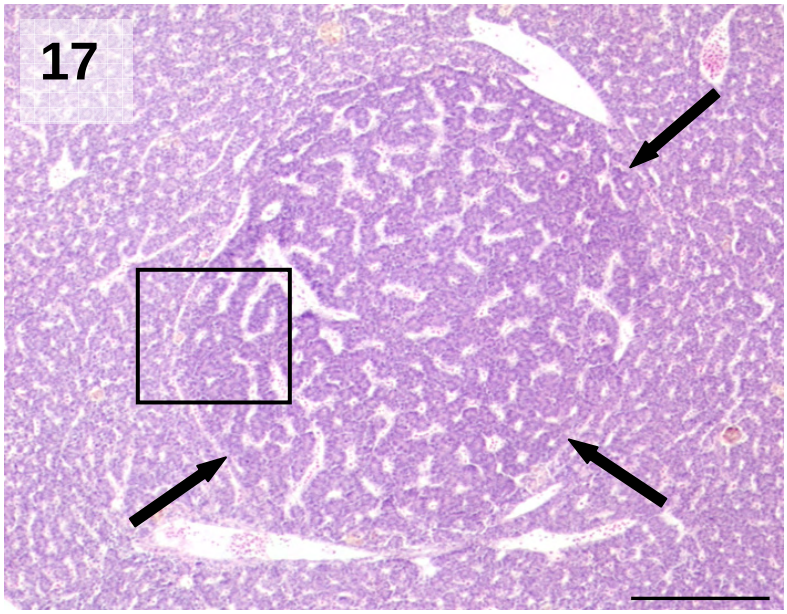


Figure 18. Detail from Figure 17 showing the increased cellular basophilia of the lesion compared to the surrounding liver tissue (arrows). Tubules of the adenoma are typically greater than two cells thick (compare with Figure 16). A small macrophage aggregate (ma) is also present at the periphery of the lesion. Bar = 50 µm.

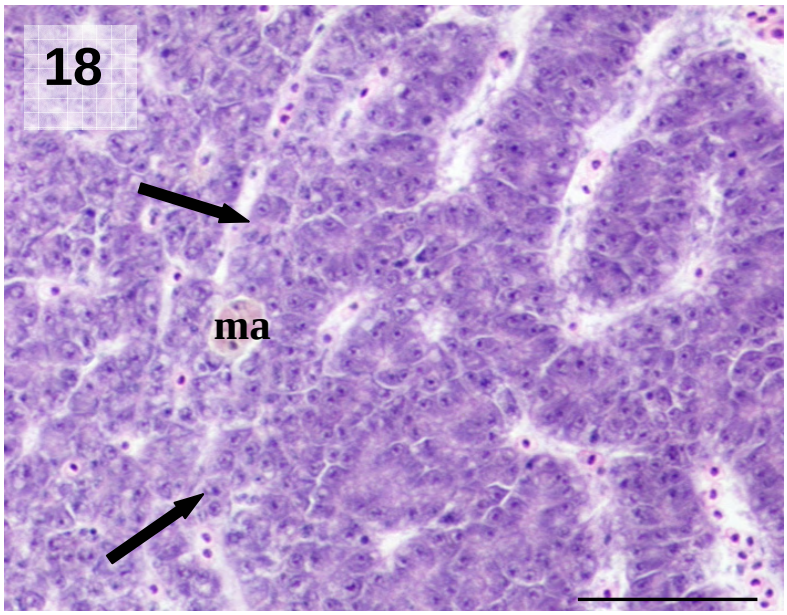


Figure 19. Eosinophilic hepatocellular adenoma in dab liver. The border of the lesion is arrowed. Tubules composed of eosinophilic hepatocytes are typically more than two cells thick and separated by prominent sinusoids. Compression of adjacent tissue is not apparent in this section. Bar = 50 µm.

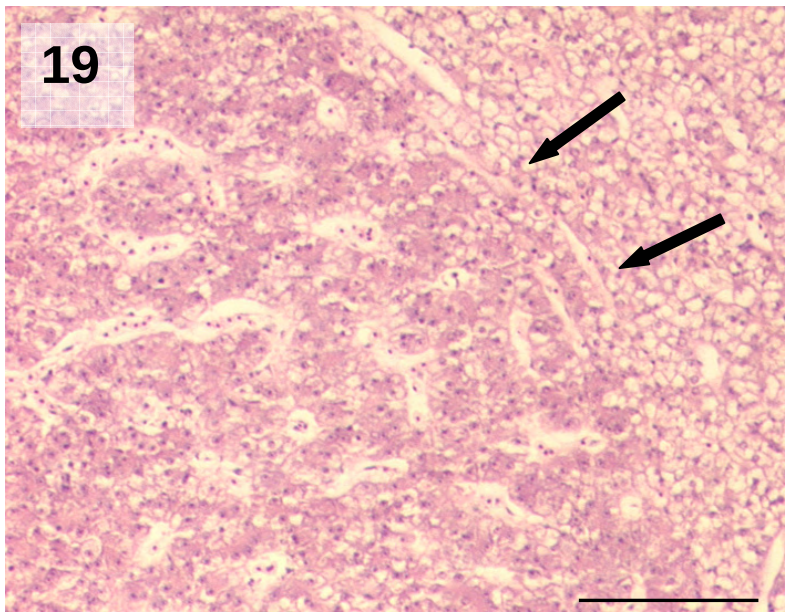


Figure 20. Low power view of a clear cell hepatocellular adenoma (arrows) in dab liver showing compression of the surrounding liver tissue. A small macrophage aggregate (ma) is visible at the periphery of the lesion. Bar = 200 µm.

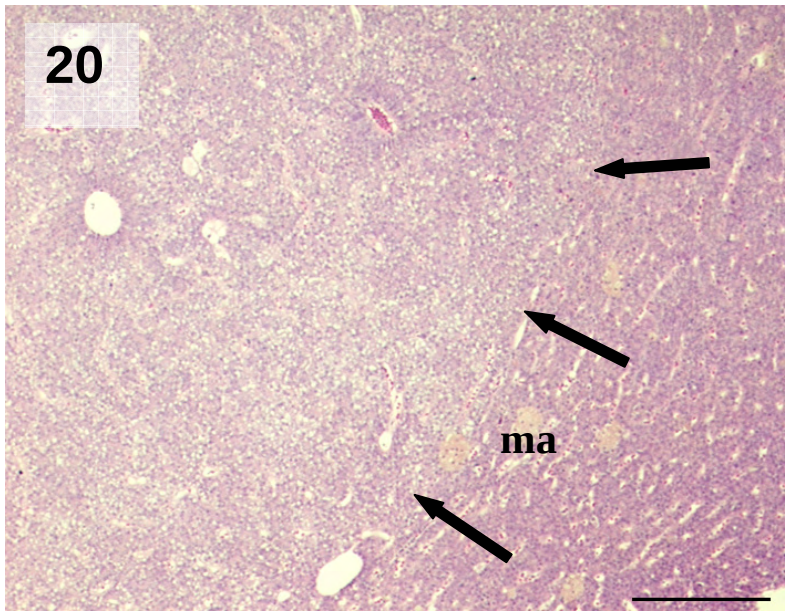


Figure 21. Low power view of a vacuolated hepatocellular adenoma in dab liver (arrows) showing compression of the surrounding liver tissue. RAP Accession 4-D53. Bar = 200 µm.

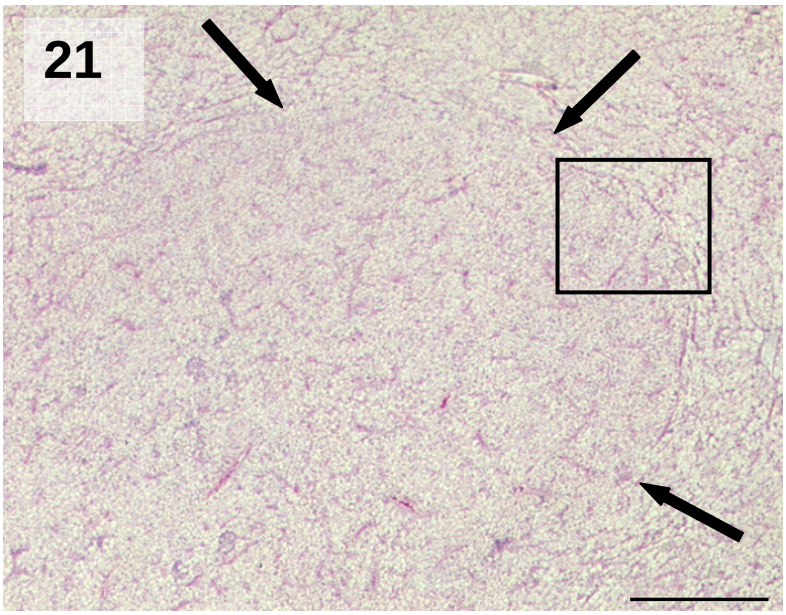


Figure 22. Detail from Figure 21 showing the border of the adenoma (arrows). In this example, the hepatocytes within the adenoma contain predominantly centrally located nuclei. Bar = 50 µm.

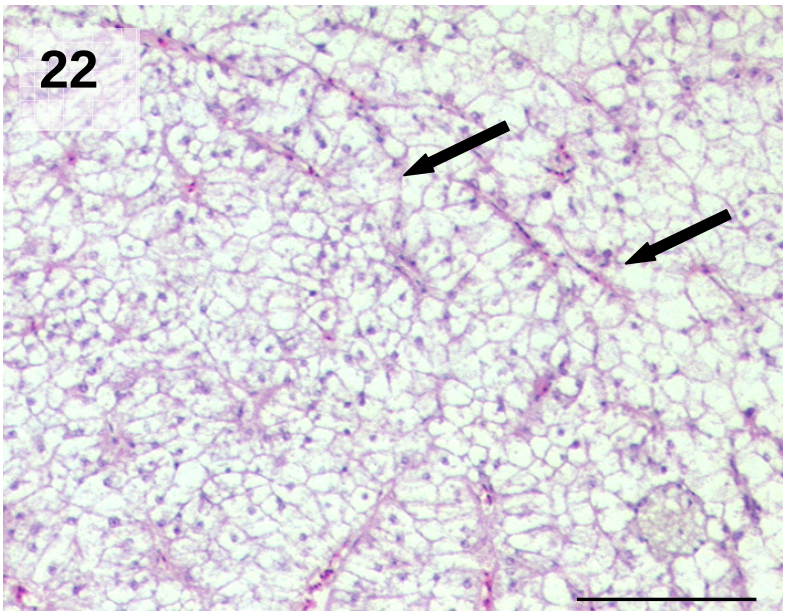


Figure 23. Localized inflammation at the junction of a basophilic hepatocellular adenoma in dab liver. Note the presence of foci of inflammatory cells within the adenoma (arrow). RAP Accession 4-D54. Bar = 50 µm.

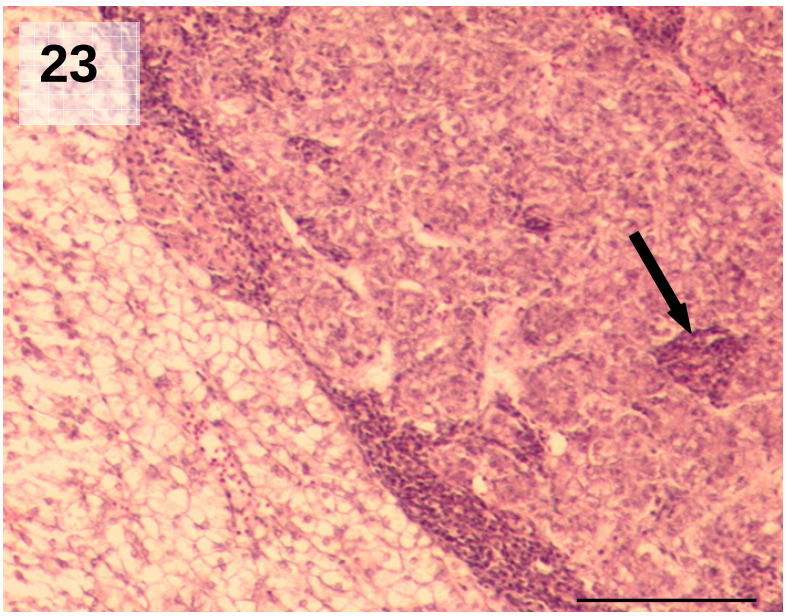


Figure 24. Low-power view of a cholangioma in dab liver. The lesion is characterized by the presence of numerous glandular structures (arrows), many of which are lined with a single layer of flattened or squamous to cuboidal epithelial cells. The outer margins of the lesion display oedematous change. Bar = 200 µm.

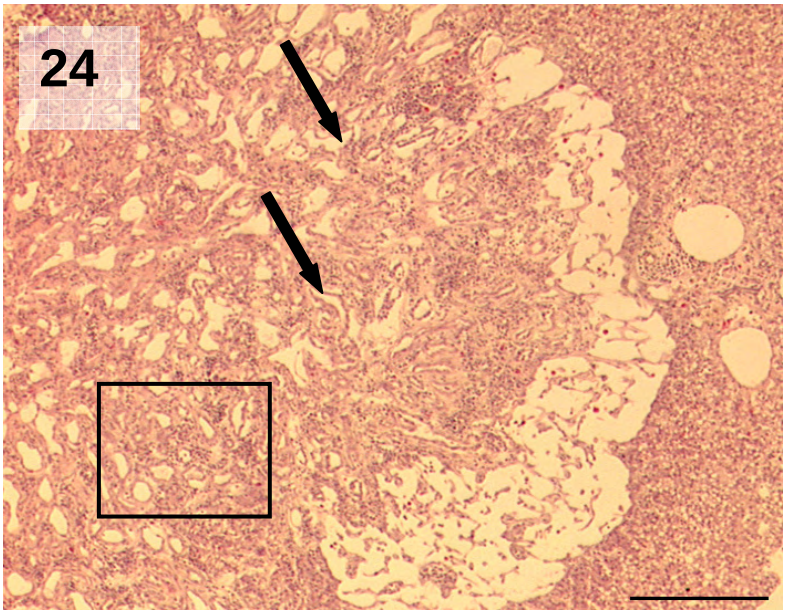


Figure 25. Detail from Figure 24 showing proliferation of bile ductules lined with a single layer of flattened epithelial cells (bd). Interstitial components of the lesion consist of inflammatory cells, capillaries, and fibrous connective tissue. Bar = 50 µm.

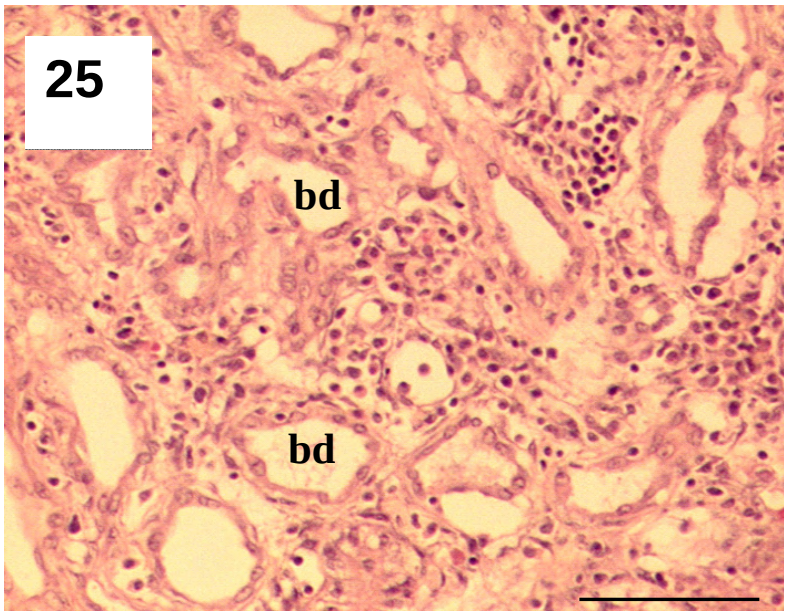


Figure 26. Low-power view of hepatocellular carcinoma in dab liver. The lesion consists of hepatocytes with mainly eosinophilic cytoplasm. It has irregular borders (arrows), disorganization of tubules, and a central region of fibrosis with necrosis present (F). Islands of more basophilic hepatocytes are also present (bh). RAP Accession 4-D55. Bar = 200 µm.

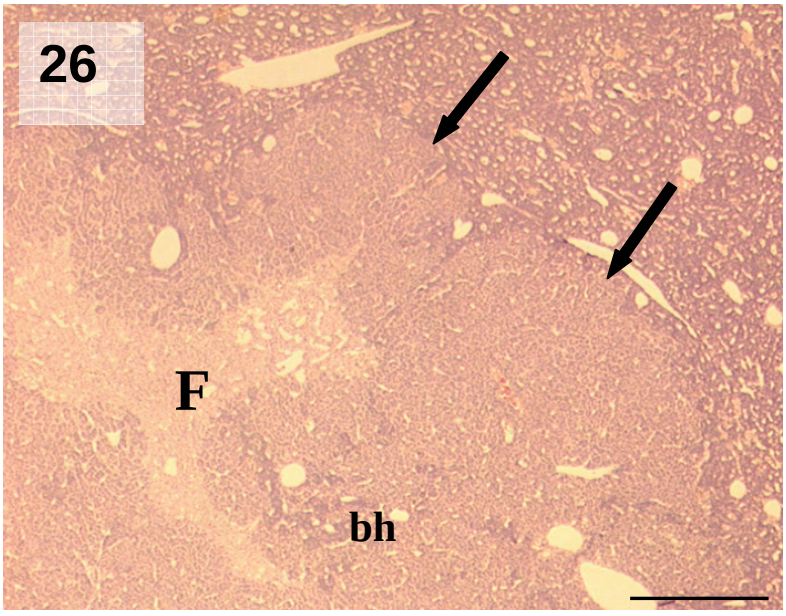


Figure 27. Hepatocellular carcinoma in dab liver. Tubules are thickened and constituent hepatocytes contain enlarged polymorphic nuclei (arrow). RAP Accession 4-D55. Bar = 25 µm.

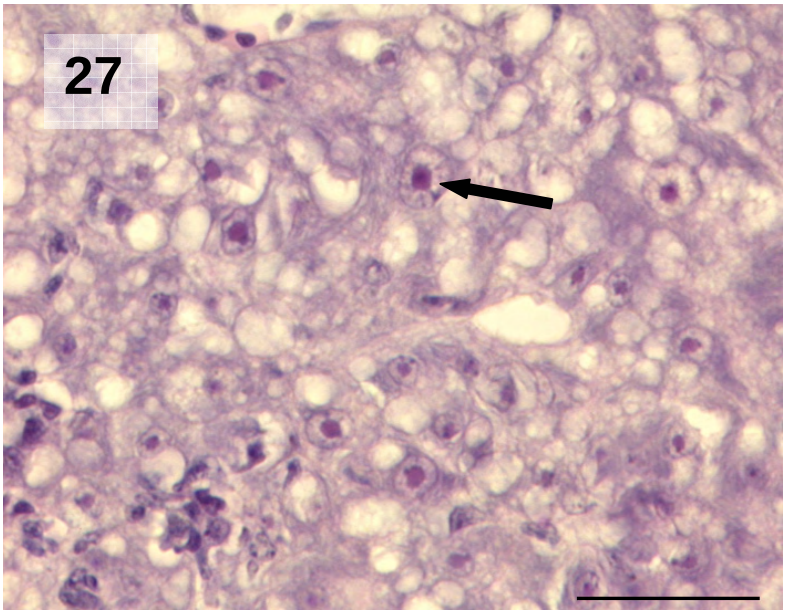


Figure 28. Section through an adenoma (A) in dab liver with a carcinomatous focus present (arrows). The focus contains swollen hepatocytes with slightly eosinophilic cytoplasm and enlarged nuclei. The diagnosis is hepatocellular carcinoma. RAP Accession 4-D56. Bar = 100 µm.

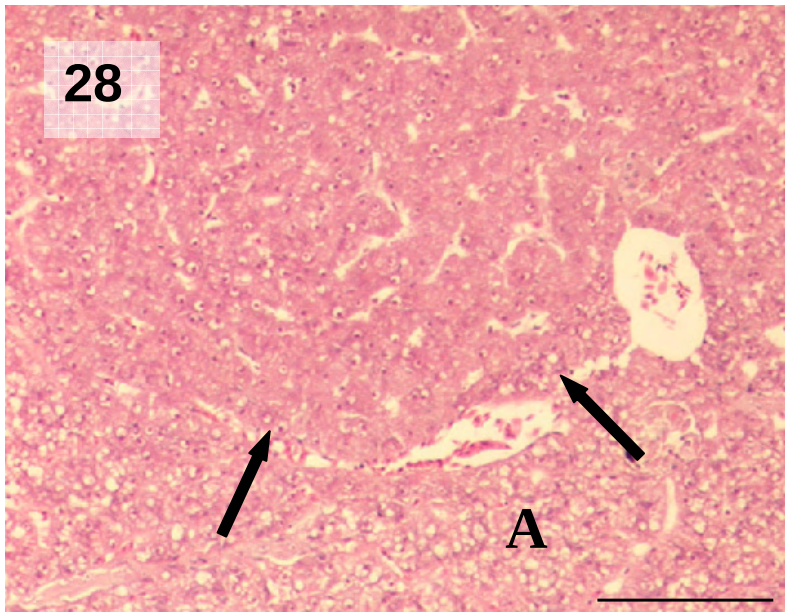


Figure 29. High power view of the carcinomatous focus seen in Figure 28. Several hepatocytes contain enlarged polymorphic nuclei (arrow). Binucleate hepatocytes are also present (asterisk). Bar = 25 µm.

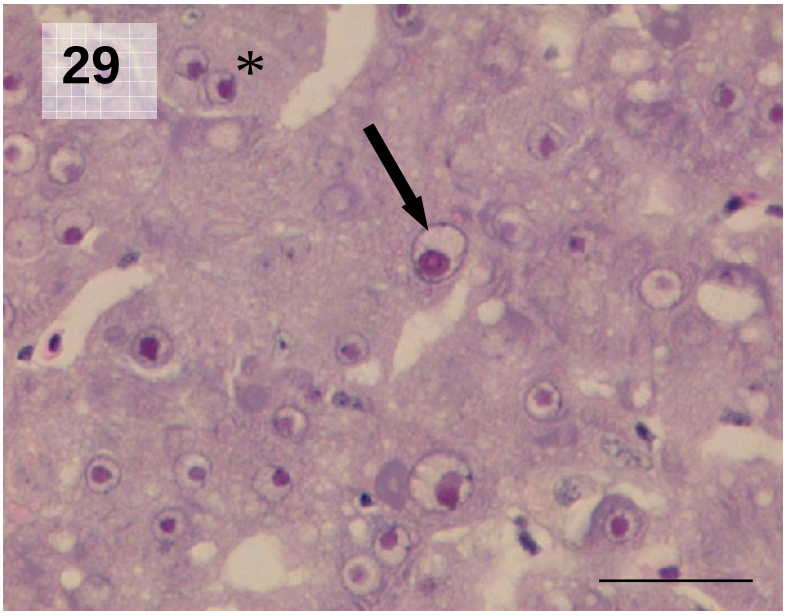


Figure 30. Low power view of a cholangiocarcinoma in dab liver. General features are shown including proliferation of ducts, a conspicuous connective tissue element and invasion of surrounding parenchyma. Unaffected liver tissue can be seen at the top right of the micrograph. RAP Accession 4-D57. Bar = 200 µm.

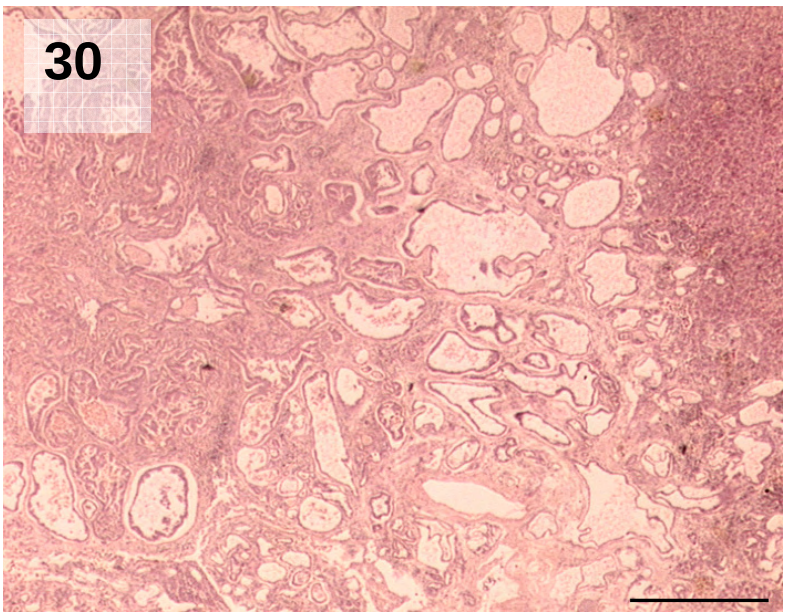


Figure 31. Detail of Figure 30 showing enlarged biliary ductules lined by an irregular epithelial layer. Epithelial cells exhibit crowding and form papillomatous ingrowths into the lumen (arrow). Necrotic debris can also be seen in the lumen of the ductule (asterisk). Bar = 50 µm.

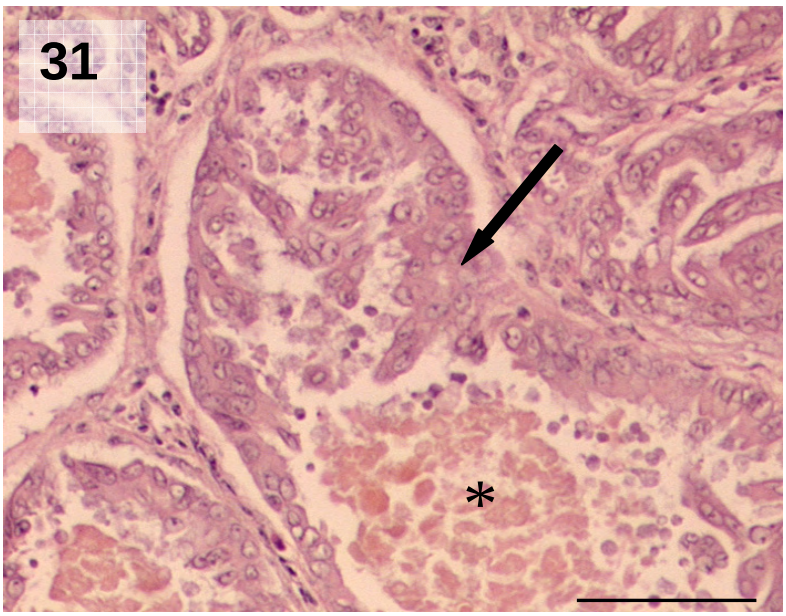


Figure 32. Low-power view of a pancreatic acinar cell carcinoma (arrows) in flounder liver. The tumour is clearly separated from the adjacent tissue. The tumour appears disorganised with whorls of densely stained acinar cells present. Bar = 50 µm. (Section provided by Dr G. Bylund.)

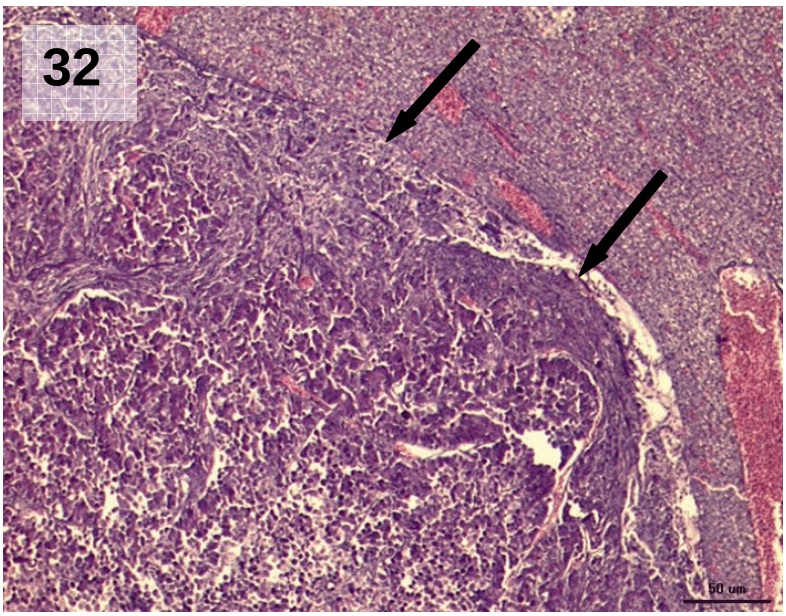


Figure 33. High-power view of the tumour shown in Figure 32. In a few areas, recognizable acinar cells containing strongly eosinophilic zymogen granules can be seen (arrow). Bar = 20 µm. (Section provided by Dr G. Bylund.)

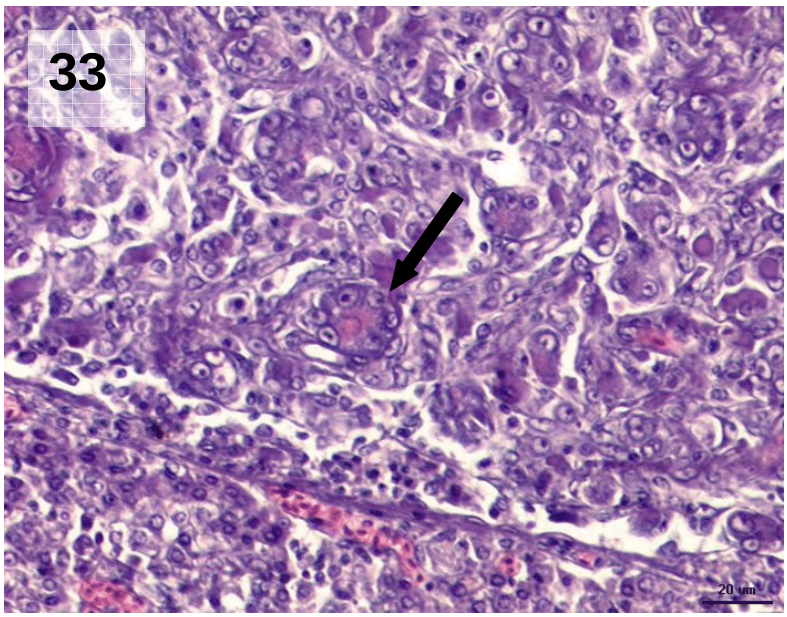


Figure 34. Mixed angiosarcoma/hepatocellular carcinoma from flounder. Islands of carcinomatous hepatocytes (CA) are surrounded by proliferating endothelial cells forming anaplastic vascular structures containing erythrocytes (arrow). Bar = 100 µm. H&E stained resin section.

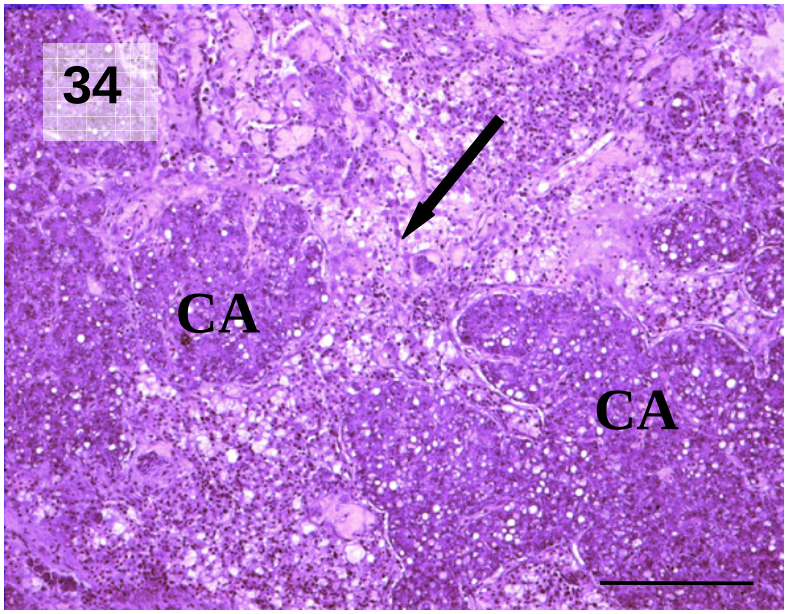
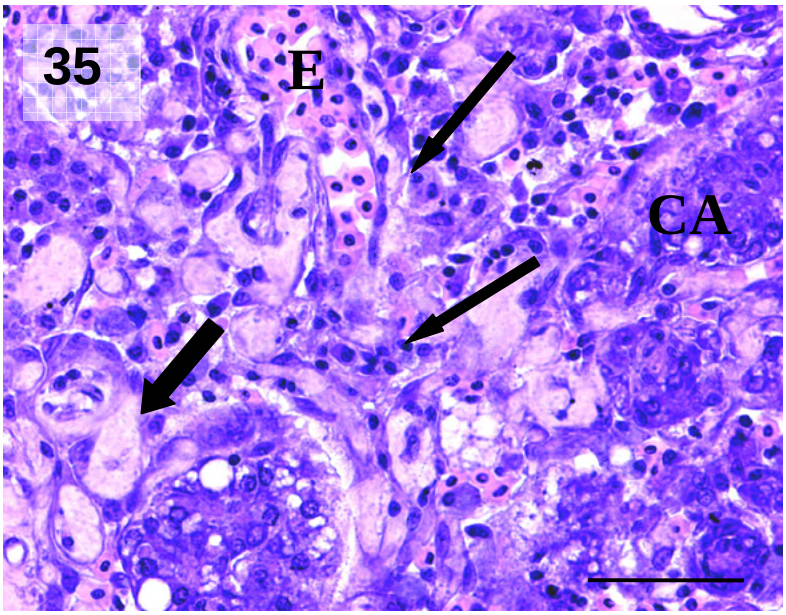


Figure 35. High power view of Figure 34. Carcinomatous foci (CA) separated by connective tissue (thick arrow) and several anaplastic vascular structures (arrows). Erythrocytes can be clearly seen within these (E). Bar = 100 µm. H&E stained resin section.



ANNEX 1

FORMULAE FOR HISTOLOGICAL FIXATIVES FOR USE IN DIAGNOSIS OF TOXICOPATHIC LESIONS IN FISH LIVER

Requirements for 10% Neutral buffered formalin (NBF) (10 litres):	
Formaldehyde solution - 37% w/v	1000 ml
Sodium di-hydrogen orthophosphate	40.0 g
Di-sodium hydrogen orthophosphate	65.0 g
Distilled water	9000 ml

Requirements for Dietrich's fixative (5 litres):	
Formaldehyde solution – 37% w/v	500 ml
Glacial acetic acid	100 ml
95% Industrial Methylated Spirit (I.M.S.)	1500 ml
Distilled water	2900 ml

NBF is the standard general purpose fixative and is suitable for all tissues and for the subsequent application of immunohistochemical techniques and certain histochemical procedures, e.g., demonstration of lipids. Dietrich's fixative has been found to give good results for liver histopathology and, in particular, it provides good nuclear fixation. Its inherent decalcifying properties are useful if other tissues are to be examined.

ANNEX 2

HISTOLOGICAL PROCESSING SCHEDULES

a) Processing schedule for conventional "carousel"-type processor

Station No.	Solution
1)	70% Industrial Methylated Spirit (I.M.S.)
2)	90% I.M.S.
3)	90% I.M.S.
4)	100% I.M.S.
5)	100% I.M.S.
6)	100% I.M.S.
7)	100% I.M.S./ Clearene or equivalent solvent (50:50)
8)	Clearene or equivalent solvent
9)	Clearene or equivalent solvent
10)	Paraffin wax (at 60 °C)
11)	Paraffin wax (at 60 °C)
12)	Paraffin wax (at 60 °C)

b) Processing schedule for vacuum infiltration processor – overnight processing.

Station No.	Solution
1)	70% Industrial Methylated Spirit (I.M.S.)
2)	90% I.M.S.
3)	100% I.M.S.
4)	100% I.M.S.
5)	100% I.M.S.
6)	100% I.M.S.
7)	Clearene solvent or equivalent
8)	Clearene solvent or equivalent
9)	Clearene solvent or equivalent
10)	Clearene solvent or equivalent
11)	Paraffin wax (at 60 °C)
12)	Paraffin wax (at 60 °C)
13)	Paraffin wax (at 60 °C)
14)	Paraffin wax (at 60 °C)

NB: Samples can be placed in 70% I.M.S. or Gum sucrose solution before processing begins. All stations are routinely set for one-hour duration, but this period could be reduced if necessary. Very rapid processing can be achieved if small samples are used and it is possible to complete processing within a working day, if desired.

ANNEX 3

PROCESSING SCHEDULE FOR EMBEDDING TISSUES IN GLYCOL METHACRYLATE

Monomer-Solution:	2-Hydroxyethylmethacrylate (Merck – stabilized with 200 ppm Hydroquinone)	80 ml
	2-Butoxyethanol (Ethyleneglycolmonobutylether)	12 ml
	Benzoylperoxide (with 25% H_2O)	0.27 g
Activator:	Polyethylene glycol 200 (Merck)	10 ml
	N,N-Dimethylaniline	1 ml

Fixation: Tissue blocks (ca. 5 mm × 5 mm × 5 mm) for 24 hours (4 °C) in Baker's Formol saline, blocks are then stored in Gum Sucrose solution at 4 °C.

Dehydration: All steps with continuous agitation.

1) Rinse twice for 15 min in distilled water or 0.1 M phosphate buffer, pH 7.4.

2) Rinse once for 30 min in distilled water or 0.1 M phosphate buffer, pH 7.4.

3) Rinse twice for 15 min in 70% acetone.

4) Rinse twice for 15 min in 100% acetone.

5) Rinse overnight in monomer solution.

Embedding: For each 5 ml Monomer add 0.13 ml Activator

1) Fill the embedding moulds.

2) Put in the tissue blocks.

3) Set the blockholder.

Polymerize for 4 hours (or overnight) in the refrigerator at 4 °C.

ANNEX 4

STAINING SCHEDULE FOR HAEMATOXYLIN AND EOSIN (H&E)

Procedure for Haematoxylin and Eosin Stain (H&E)

Solutions required:

- Clearing agent (e.g., "Clearene" (Surgipath, UK) or xylene)
- Graded alcohols
- Acid/alcohol (1% hydrochloric acid in 70% alcohol)
- 1% aqueous Eosin Y or alcoholic eosin (5% I.M.S.)
- Haematoxylin (Gill 3 formula) (Surgipath, UK) or equivalent.

Method:

Part 1: Taking slides to water

1) Place slides in Clearene to remove wax for a **minimum** of 2 minutes.
2) Repeat step 1 in fresh Clearene.
3) Place in 100% alcohol to remove Clearene for a **minimum** of 2 minutes.
4) Repeat step 3 in fresh 100% alcohol.
5) Wash slides in running tap water for 2–5 minutes, slides should be **clear**, not cloudy.

Part 2: Staining

1) Place in haematoxylin for 3 to 4 minutes.
2) Blue in running tap water for up to 10 minutes (It is not possible to "over blue").
3) Differentiate in acid/alcohol for a **maximum** of 10 seconds.
4) Rinse in running tap water until blue.
5) Microscope check for clear cytoplasm and blue nuclei.
6) Place in eosin solution for 3 minutes.
7) If necessary, wash for up to 1 minute in running tap water to differentiate eosin (take care not to over differentiate).

Part 3: Dehydration, clearing, and mounting;

1) Rinse well in 70% alcohol for 30 seconds.
2) Place in 100% alcohol for 1–2 minutes.
3) Repeat, using fresh alcohol.
4) Place in 50/50 alcohol/Clearene for 1–2 minutes.
5) Place in Clearene for 2 minutes.
6) Repeat, using fresh Clearene.
7) Mount in a synthetic mountant such as D. P. X. and leave to dry.

Results:

Nuclei:	blue
Muscle fibre:	red
Red blood cells:	bright red (depending on fixative)
Collagen:	pink

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