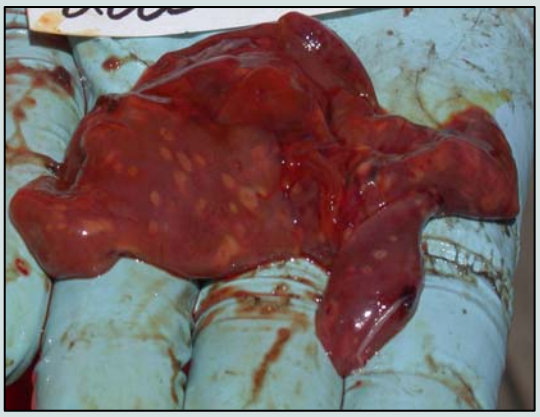
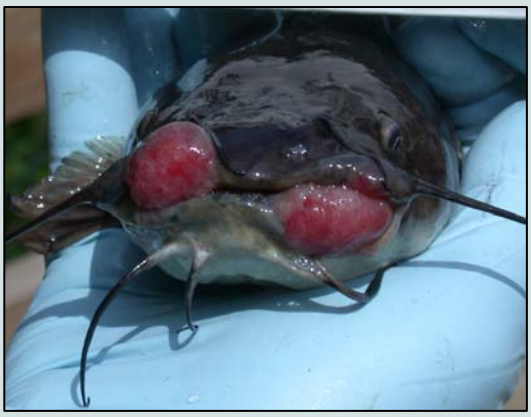
Manual for the Microscopic Diagnosis of Proliferative Liver and Skin Lesions in the Brown Bullhead (*Ameiurus nebulosus*)





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1.0 INTRODUCTION

1.1 Background Information

The Great Lakes Water Quality Agreement, in which the United States and Canada agreed to restore and preserve the biological, physical and chemical integrity of the Great Lakes Basin Ecosystem, was first signed in 1972. In 1987, a Protocol was signed by both governments which defined Great Lakes Areas of Concern (AOC) as "geographic areas that fail to meet the general or specific objectives of the agreement where such failure has caused or is likely to cause impairment of beneficial use of the area's ability to support aquatic life." The U.S. and Canadian governments identified 43 such areas; 26 in the U.S., 12 in Canada and five shared between the U.S. and Canada on connecting river systems. The Great Lakes Water Quality Agreement, as amended via the 1987 protocol, directs the two federal governments to cooperate with state and provincial governments to develop and implement Remedial Action Plans (RAPs) for each AOC. The Protocol also called for reports on restorative progress and for the International Joint Commission (IJC) to review RAPs proposed by the 43 Areas of Concern (IJC, 1987). Three AOCs (Collingwood Harbor, Severn Sound and Oswego River) have been delisted and Presque Isle Bay has been designated an Area of Recovery.

Fourteen beneficial use impairments were identified and the various AOCs have different combinations of these impairments. One impairment, listed as “fish tumors or other deformities” is defined as occurring when “the incidence rate of fish tumors or other deformities exceeds rates at unimpacted or control sites or when survey data confirm the presence of neoplastic or preneoplastic liver tumors in bullhead or suckers” (IJC, 1989; www.epa.gov/lakeerie/buia/reports). Currently, 16 of the 40 AOCs have the presence of tumors or other deformities listed as one of their beneficial use impairments. It is important to recognize that although neoplasia has been linked with specific contaminants very little “cause and effect” evidence, particularly well designed exposure studies, exists for brown bullhead.

1.2 Purpose of the Manual

Over the years numerous surveys have been undertaken to assess the incidence of skin and liver tumors, as well as other contaminant-associated microscopic lesions at AOCs. However, a major problem is a lack of consistent criteria for evaluating microscopic (histological) changes in bullhead liver and skin. For instance, in some studies there is no distinction between preneoplastic (altered foci) and neoplastic lesions, or no good descriptions of individual lesions (Black 1983; Pyron et al. 2001). Some studies included only carcinomas as neoplasia, while in others adenomas, carcinomas, and cholangiomas were included (Baumann et al. 2000) and some included carcinomas, adenomas and altered foci (Maccubbin and Ersing 1991). Skin tumors are often diagnosed visually without the supporting histological verification. Hence, standardization of the criteria being used to evaluate the impairment is necessary, so that data from different years, reference sites and Areas of Concern can be adequately compared.

Pennsylvania Department of Environmental Protection, U.S. Environmental Protection Agency Great Lakes National Program Office and Pennsylvania Sea Grant sponsored a number of conferences related to Fish Tumors in Great Lakes Areas of Concern. At the initial meeting, committees were formed to work on 1) standardized monitoring protocols and 2) criteria for standardized assessment of skin and liver lesions. This manual is the product of the Histopathology Subcommittee and the purpose is to describe neoplastic and non-neoplastic proliferative lesions of the liver and skin of the brown bullhead and suggest terminology that can be consistently used at AOCs throughout the Great Lakes and other areas.

2.0 SPECIMEN PREPARATION

2.1 Field Collection

A companion manual "Field Manual for Assessing Internal and External Anomalies in Brown Bullhead (*Ameiurus nebulosus*)" by Rafferty and Grazio (2007) provides guidance for the identification of lesions and deformities observed during a gross examination and recommends standard operating procedures for the collection, necropsy, and preservation of tissue collected in the field. Briefly, external lesions, primarily raised lesions of any color and consistency should be sampled. It is best to remove the whole lesion plus some normal surrounding tissue, if possible. If the lesion is large, a subsample should be removed. For the identification of preneoplastic or neoplastic liver lesions it is necessary to sample all livers as most of the lesions will not be observed grossly. It is suggested that at least five pieces of liver be placed in fixative in the field (Figure 1). Pieces of skin and liver are generally fixed in 10% buffered formalin, Davidson's (Luna 1992), or Z-Fix (Anatech LTD, Battle Creek, MI).

2.2 Specimen Processing

There have been no studies to statistically validate the number of sections that need to be examined from an individual bullhead liver to have a given confidence of diagnosing a particular lesion if present. To our knowledge the only morphometric study of liver neoplasms and altered foci currently available was conducted on mummichogs *Fundulus heteroclitus*, a much smaller fish species (Stine et al. 2004). In that study six livers (five of which had visible lesions) were serially sectioned. It was determined that from 3-15 (mean of 7) sections were required to diagnose neoplasia or altered foci from each liver with 95% accuracy. It is likely that for fish with larger livers more sections will be required. Hence, it is suggested that from the five or more pieces collected in the field, six to ten pieces should be placed in cassettes and routinely processed for embedding in paraffin, sectioned at a thickness of 4-6 µm and stained with hematoxylin and eosin (Luna 1992; Profet et al. 1992).

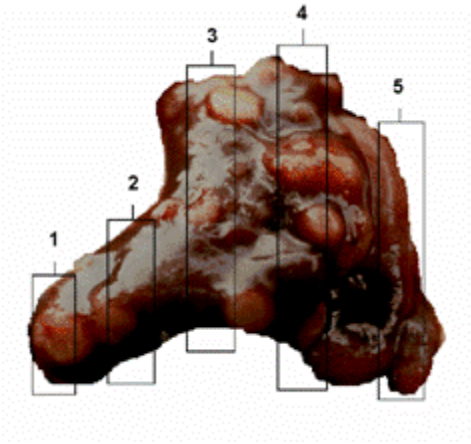


Figure 1. Illustration of liver pieces to be sampled and placed in fixative in the field.

3.0 LIVER

Part of the definition of the beneficial use impairment is "when survey data confirm the presence of neoplastic or preneoplastic liver tumors in bullhead or suckers". Liver neoplasms and other microscopic liver lesions have been associated with PAH and other contaminant exposure in brown bullheads (Baumann 1992; Baumann and Harshbarger 1995; Baumann and Harshbarger 1998; Pinkney et al. 2001, 2004) and other fish species, including English sole *Parophyrs vetulus* (Myers et al. 1990), mummichog *Fundulus heteroclitus* (Vogelbein et al.1990), and winter flounder *Pseudopleuronectes americanus* (Gardner et al. 1989). Although it is easy to define and describe neoplastic lesions, those to be considered preneoplastic are more difficult as there have been no good exposure studies to document the progression of liver neoplasia in bullhead. While both bile duct proliferation and foci of cellular alteration may be associated with chemical exposures and are considered preneoplastic in some fish species, bile duct proliferation may also be associated with myxozoan parasites and is often reactive in bullhead. Hence, although bile duct proliferation should be documented, we do not suggest it be considered a chemically-induced change at AOCs. In addition, there is insufficient evidence to indicate that it is a preneoplastic lesion. Conversely, foci of cellular alteration are almost always associated with toxicopathic effects. In wild fish surveys their presence has been highly correlated with exposure to PAHs, PCBs, DDTs, chlordanes, dieldrin, mercury, pulp mill effluent and aromatic hydrocarbon metabolites in English sole, white croaker *Genyonemus lineatus*, Pacific staghorn sculpin *Leptocottus armatus*, starry flounder *Platichthys stellatus*, dab *Limanda limanda*, flounder *Platichthys*

flesus, rock sole *Lepidopsetta bilineata* and Dover sole *Microstomus pacificus* (reviewed by Au 2004; Feist et al. 2004; Koehler 2004) and are recommended for assessing chronic toxicity (Hinton 1994). Laboratory exposures of a number of fish species have indicated altered foci to be preneoplastic, particularly basophilic foci (Hendrick et al. 1984; Grizzle and Thiyagarajah 1988; Hinton et al. 1988; Hawkins et al. 1990; Law et al. 1994). Hence, we recommend that these foci of cellular alteration be considered preneoplastic.

3.1 Normal Liver Histology

Bullhead livers, as in most other teleosts, are composed of hepatic tubules. In liver sections from bullheads collected at reference sites observable bile ducts are often sparse and not prominent in sections examined by light microscopy (Figure 2A and B). Portal veins are commonly surrounded by pancreatic tissue and bile ducts may be observed within this tissue (Figure 2B). Normal hepatocyte appearance can vary from having little to no observable vacuolization, lipid or glycogen storage (Figure 2A and C), to having extensive vacuolization (Figure 2B and D). Macrophage aggregates are also observed within pancreatic and hepatic tissue (Figure 2A and C). More detailed descriptions on normal piscine hepatic histology and hepatotoxicity can be found in (Boorman et al. 1997; Metcalfe 1998; Hinton et al. 2001; Wolf and Wolfe 2005).

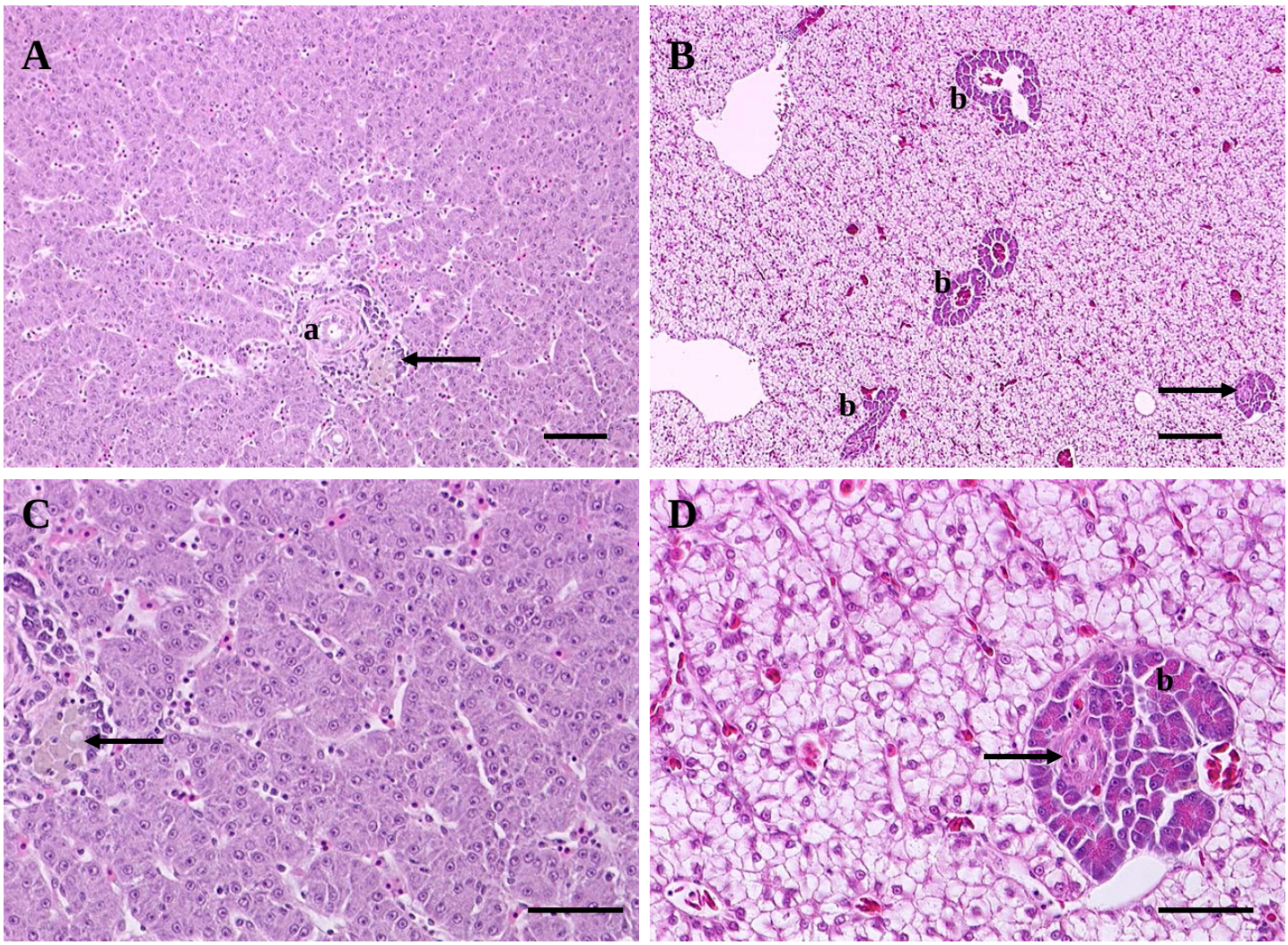


Figure 2. Microscopic appearance of normal bullhead liver. A. Section of normal liver illustrating the tubular structure, a bile duct (a) and macrophage aggregate (arrow) within the exocrine pancreatic tissue. Bar = 150 µm. B. Normal liver with highly vacuolated hepatocytes. Blood vessels are often surrounded by pancreatic tissue (b) which occasionally contain bile ducts (arrow). Bar = 150 µm. C. Higher magnification of A illustrating the tubular structure and a macrophage aggregate (arrow). Bar = 50 µm. D. Higher magnification of B illustrating highly vacuolated hepatocytes, pancreatic tissue (b) and a bile duct (arrow). Bar = 50 µm. H&E stain.

3.2 Non-Neoplastic Hepatic Lesions

3.2.1 Proliferation of Macrophage Aggregates

Macrophage aggregates are structures found in the spleen, kidney and liver of fish (reviewed by Aguis and Roberts 2003; Wolke 1992). They have been shown to increase with age (Blazer et al. 1987) and in response to environmental stress (Fournie et al. 2001). Hence, in young fish, and/or fish from reference sites, macrophage aggregates may not be observable (Figure 2B) or they may be present at a low density within the liver or hepatic pancreatic tissue (Figure 2A and 3A), while at impacted sites a higher density may be observed (Figure 3B). Three pigments - melanin, hemosiderin and the lipopigments, lipofuscin/ceroid, can be observed within these structures using the Perl's Prussian Blue method which stains hemosiderin blue (Luna 1992). In bullheads the lipopigments and hemosiderin are most commonly observed. Some have a higher proportion of hemosiderin, an iron-containing pigment, which stains blue (Figure 3B and D), while others contain more of the lipofuscin/ceroid pigments, formed by oxidation of lipid and retaining a yellowish-brown appearance (Figure 3A and C).

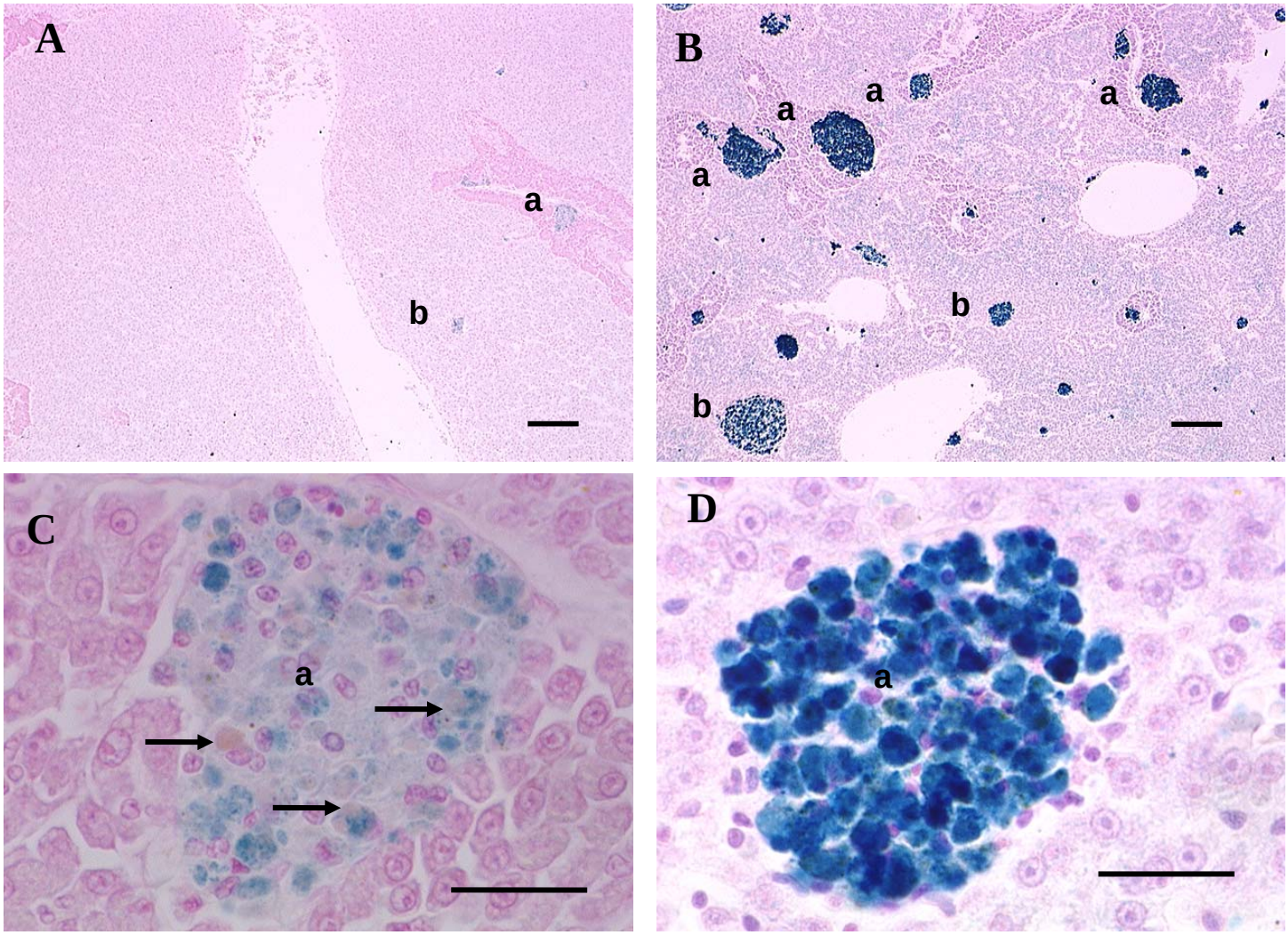


Figure 3. Macrophage aggregates in liver sections of brown bullhead stained by the Perl's Prussian Blue method. A. Liver section from a bullhead collected at a reference site illustrating an aggregate in the hepatopancreatic tissue (a) and another within hepatic tissue (b). Bar = 100 µm. B. A liver section from a bullhead collected at an AOC illustrating numerous aggregates within hepatopancreatic (a) and hepatic (b) tissue. Bar = 100 µm. C. Macrophage aggregate (a) with a moderate amount of hemosiderin (blue) and ceroid/lipofuscin (arrows). Bar = 20 µm. D. Macrophage aggregate (a) with high levels of hemosiderin (blue). Bar = 20 µm.

3.2.2 Proliferative Responses to Parasites

Helminth parasites are commonly observed within liver tissue of bullhead collected in some areas. They can include trematodes, nematodes, acanthocephalans and cestodes. These may be the most commonly observed, grossly visible lesions (Figure 4A). They may appear as irregularly-shaped, elongate or rounded, pale, raised areas. However, raised pale areas may also be tumors (Figure 4B).

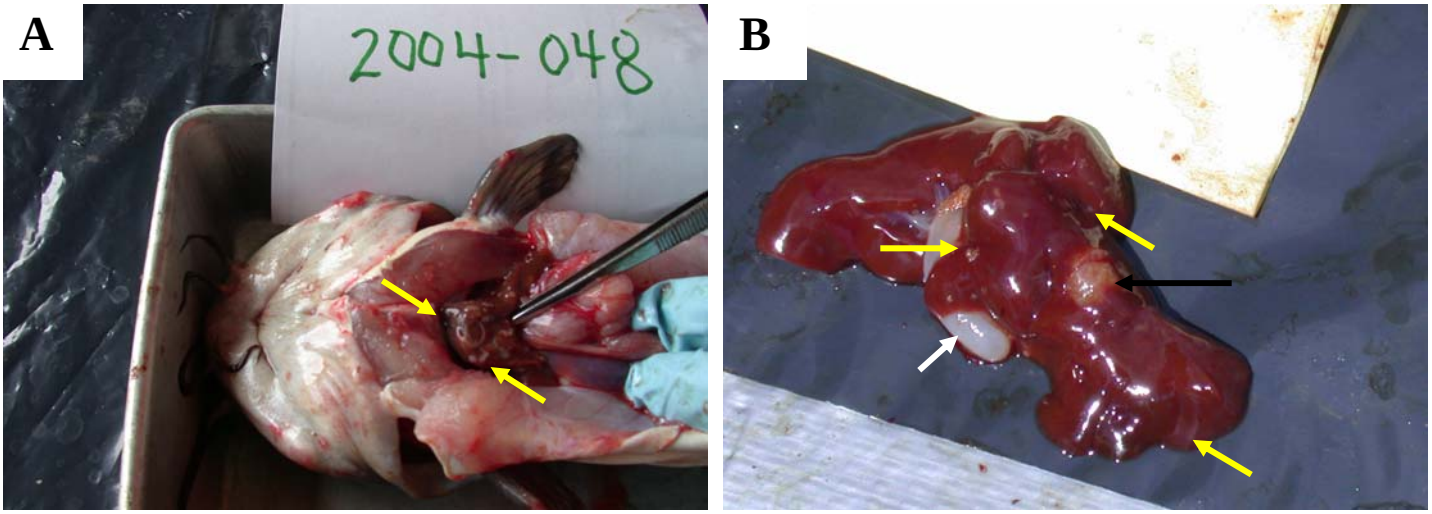


Figure 4. Grossly visible lesions in brown bullhead liver. A. Numerous pale irregularly-shaped areas, some on the surface of the liver and some within the parenchyma (yellow arrows). B. Pale discolored areas of variable size and shape on the surface and within the parenchyma of the liver. These included parasites and the proliferative inflammatory response to them (yellow arrows), illustrated in Figure 5, and a cholangiocarcinoma (black arrow), illustrated in Figure 16. Gallbladder is present (white arrow).

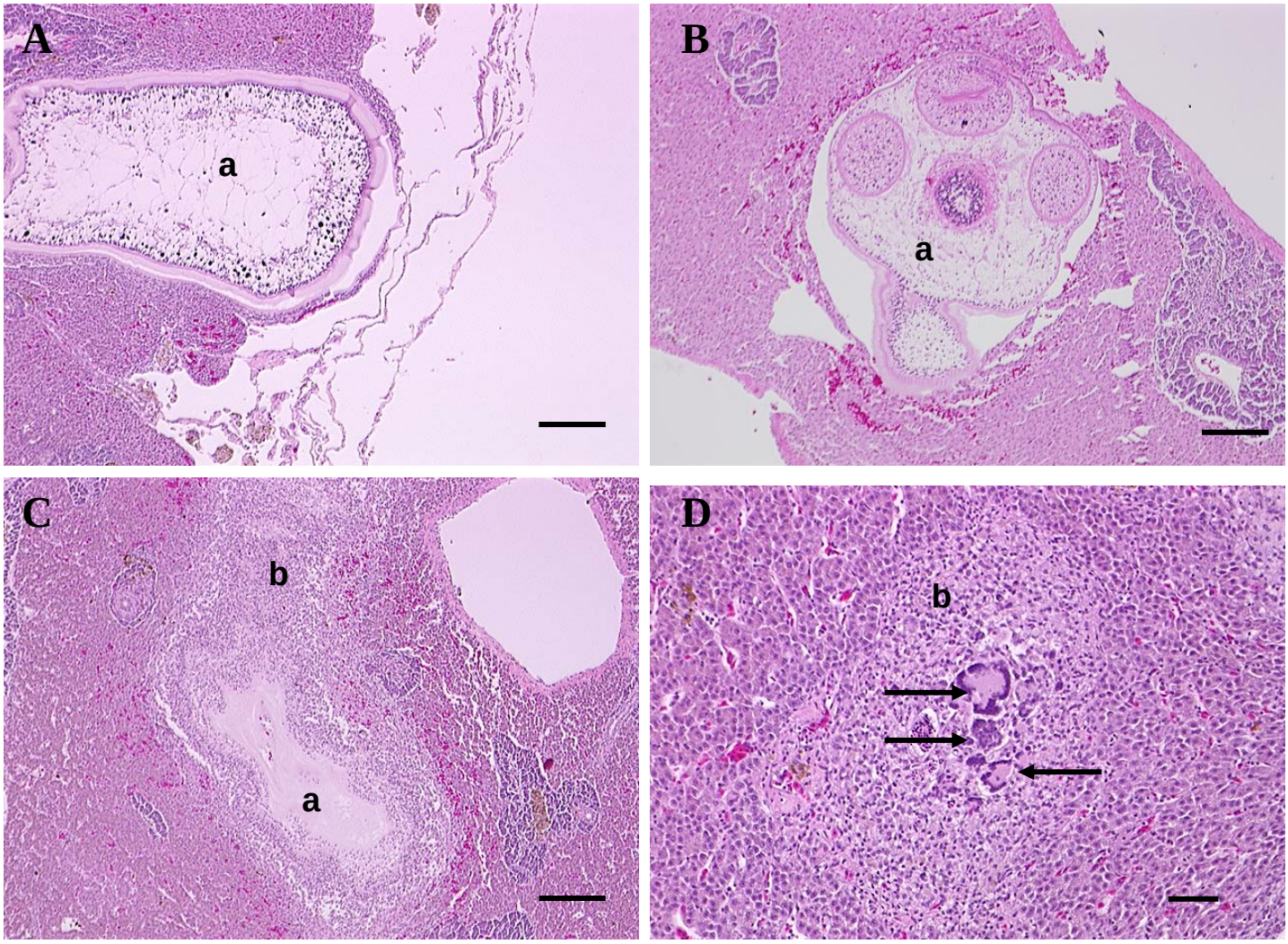


Figure 5. Helminth parasites and the accompanying inflammatory responses in brown bullhead liver. A and B. Cestode parasite (a) on the surface and extending into the parenchyma of the liver. Bar = 150 µm. C. Remnants of a helminth parasite (a) and extensive inflammatory response (b) within the liver. Bar = 150 µm. D. Chronic granulomatous inflammatory response (b) and giant cells (arrows) within the liver of brown bullhead. Bar = 50 µm. H&E stain.

3.3 Putatively Preneoplastic Lesions - Foci of Cellular Alteration

Four categories of foci of cellular alteration are recognized in fish livers stained with hematoxylin and eosin, based on the tinctorial characteristics of the hepatocyte cytoplasm. In rats, it has been documented that these differences in staining are due to accumulations of specific components within the cells. Cells within eosinophilic foci are rich in smooth endoplasmic reticulum, while cells within basophilic foci are rich in RNA. Clear cell foci contain abundant glycogen. Foci of vacuolated cells contain lipid in some fish species and a mixture of lipid and glycogen in others (reviewed by Bunton 1996). The margins of these foci are generally distinct but the hepatic tubules are arranged in a relatively normal pattern, merge imperceptibly with the surrounding parenchyma, and little to no compression is observed.

3.3.1 Basophilic Foci

Basophilic foci are round to irregular clusters of hepatocytes rich in rough endoplasmic reticulum and increased basophilic staining compared to adjacent cells (Figure 6A). The cells may be smaller than adjacent hepatocytes, but cellular atypia and mitotic figures are generally not present (Figure 6B).

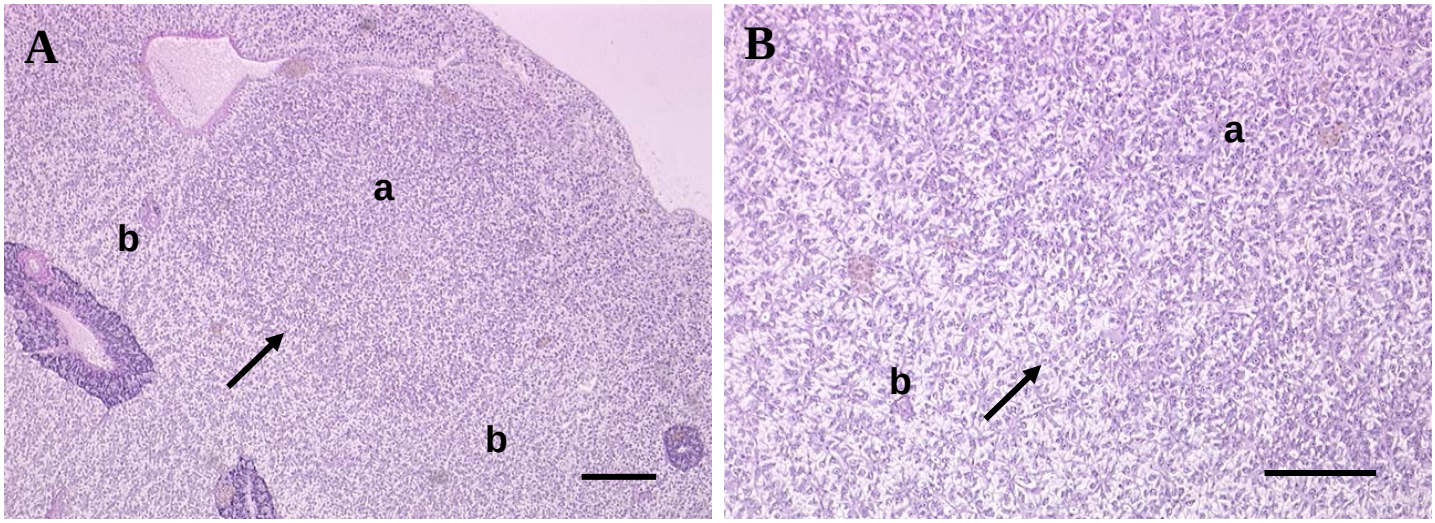


Figure 6. Basophilic focus in the liver of brown bullhead. A. Discrete focus of hepatocytes (a) staining more basophilic than surrounding (b) hepatocytes. Bar = 150 µm. B. Higher magnification of a basophilic focus (a), illustrating the margin of the focus (arrow), which merges into the adjacent hepatocytes (b). Bar = 100 µm. H&E stain.

3.3.2 Eosinophilic Focus

Eosinophilic foci are round to irregular areas of hepatocytes with increased eosinophilia compared to adjacent cells (Figure 7A). The cells may be slightly enlarged with a granular eosinophilic cytoplasm (Figure 7B).

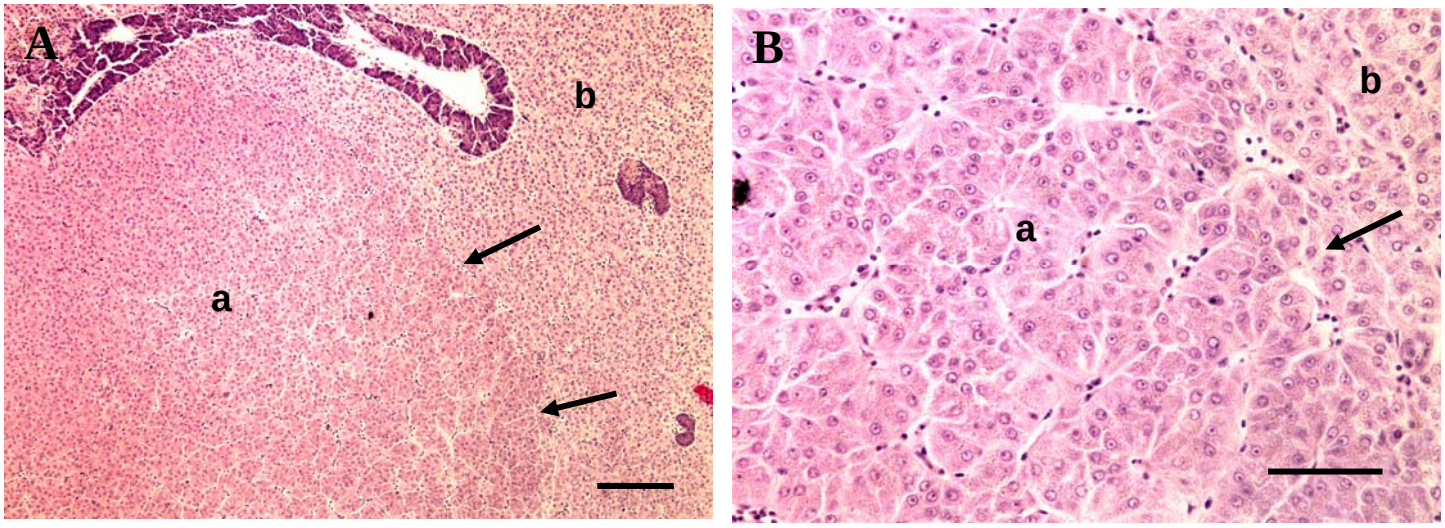


Figure 7. Eosinophilic focus in brown bullhead liver. A. Area of hepatocytes with increased eosinophilia (a) when compared to surrounding hepatocytes (b). Arrows indicate the border of the lesion. Bar = 150 µm. B. Hepatocytes within the eosinophilic focus (a) have a finely granular cytoplasm and are slightly larger than surrounding hepatocytes (b). Bar = 50 µm. H&E stain.

3.3.3 Vacuolated and Clear Cell Foci

Vacuolated foci are described as round to irregular, containing hepatocytes with medium to large, clear, lipid-containing, cytoplasmic vacuoles. The nuclei are often displaced to the periphery of these cells (Figure 8). Clear cell foci, as described in other fish species, are characterized by enlarged hepatocytes with a "ground glass" appearance to the cytoplasm and the nuclei are generally centrally located within the cells. The clear cytoplasm is the result of glycogen accumulation which is dissolved out in aqueous fixatives. Clear cell foci are less commonly observed than vacuolated foci (Boorman et al. 1997; Feist et al. 2004). In bullhead, these foci often appear to be mixed foci with cells characteristic of both clear cell and vacuolated foci (Figure 9). Cells within the focus may have both centrally-located and eccentric nuclei and the ground glass appearance is not as evident.

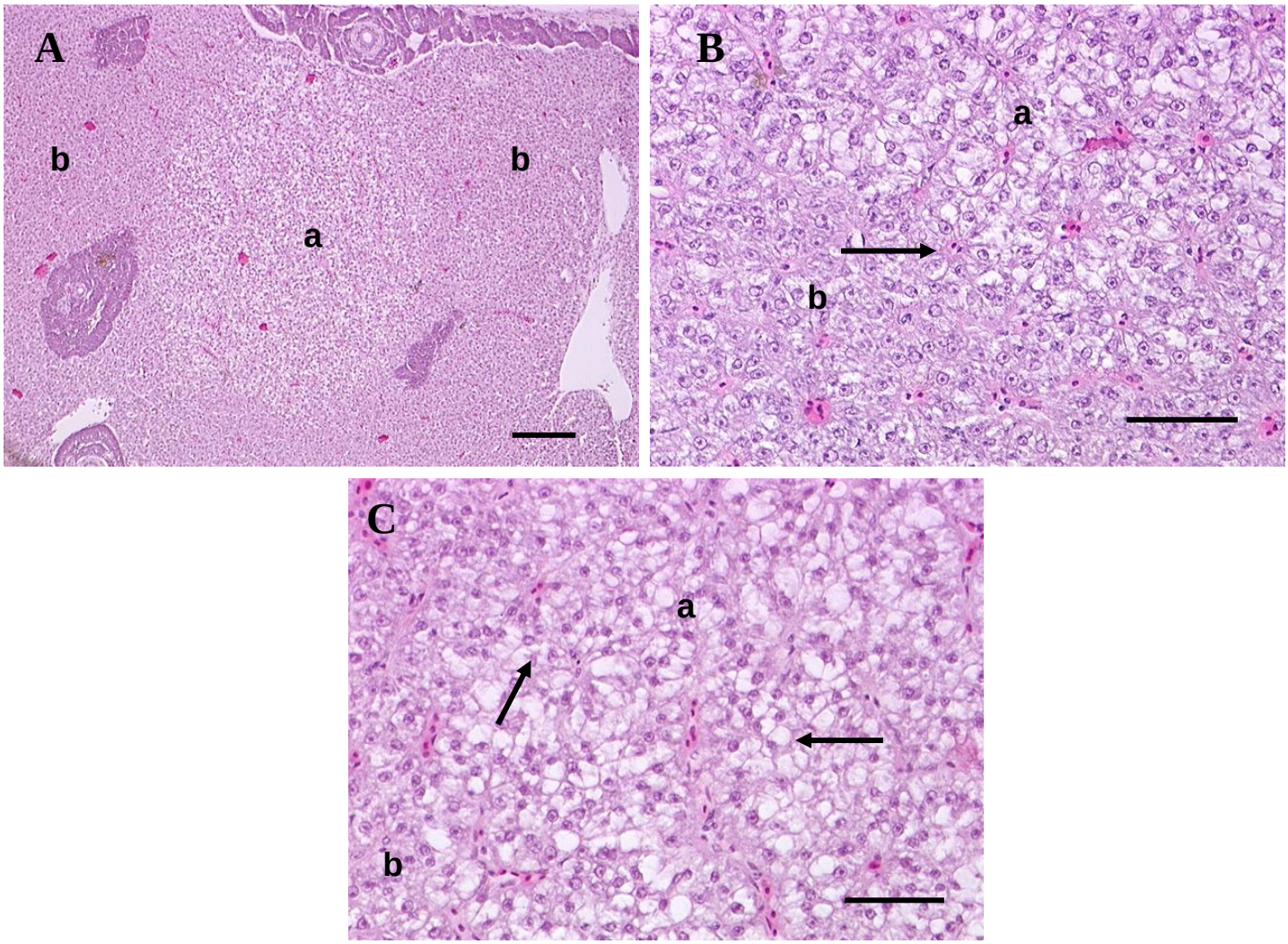


Figure 8. Vacuolated Cell Foci. A. Focus (a) with border blending into surrounding hepatocytes (b). Bar = 150 µm. B. Higher magnification illustrating the lack of compression or encapsulation between the border (arrow) of the focus (a) and surrounding hepatocytes (b). Bar = 100 µm. C. Cells within the focus have variably-sized clear vacuoles and eccentric nuclei (arrows). Bar = 50 µm. H&E stain.

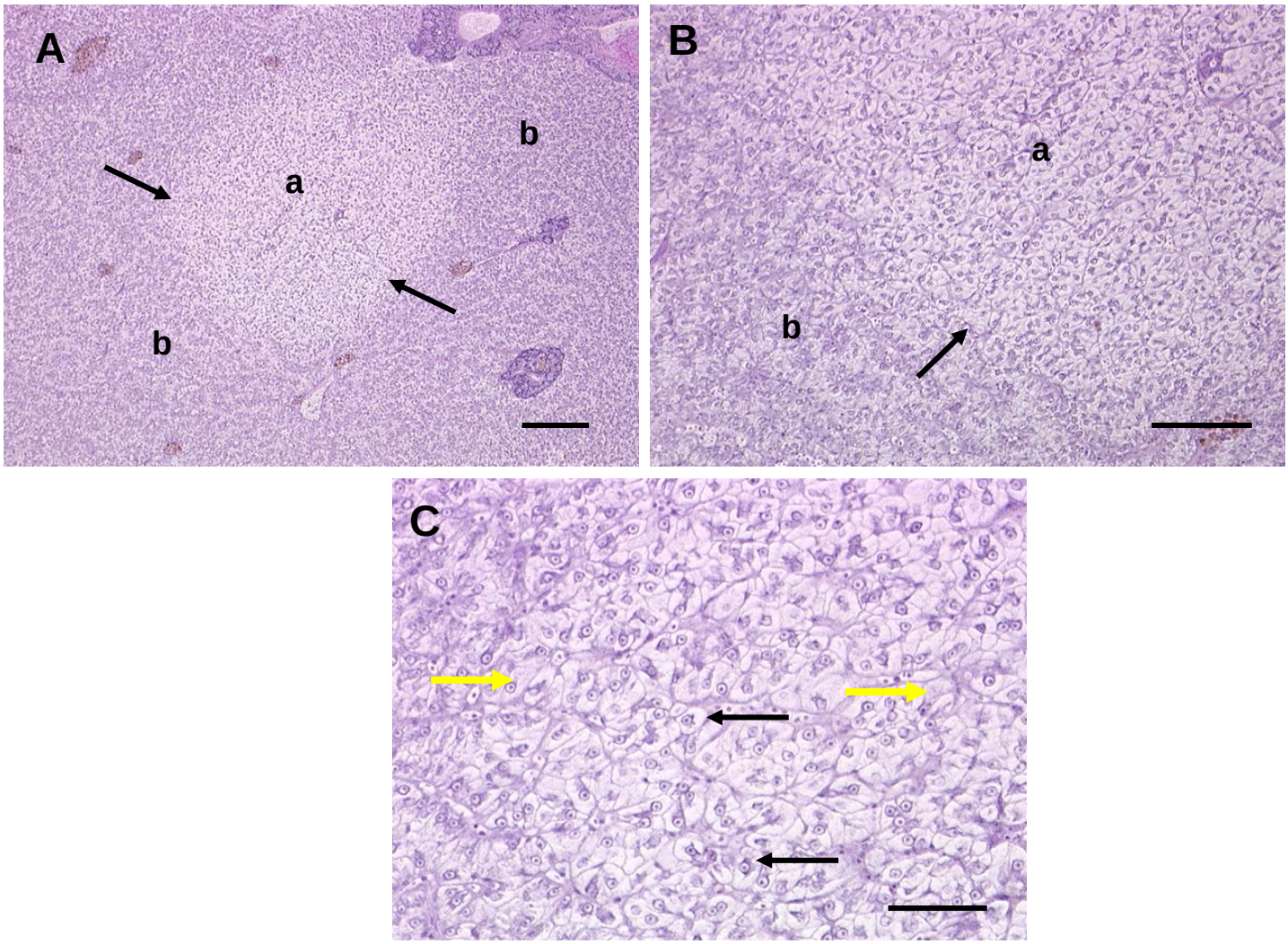


Figure 9. Clear or Mixed Cell Focus. A. Focus (a) with border blending into surrounding hepatocytes (b). Bar = 150 µm. B. Higher magnification illustrating the lack of compression or encapsulation between the border (arrow) of the focus (a) and surrounding hepatocytes (b). Bar = 100 µm. C. Some cells within the focus have centrally-located nuclei (arrows), while others are eccentric. Some cells have cytoplasm with a ground glass appearance (yellow arrows). Bar = 50 µm. H&E stain.

3.4 Neoplastic Hepatocellular Lesions

3.4.1 Hepatocellular Adenoma

Adenomas are discrete lesions which generally have a distinct border. The cells may exhibit altered staining properties and hence appear more eosinophilic, basophilic or vacuolated compared to the surrounding tissue. Cells are usually not arranged in normal growth patterns and usually there is very little pleomorphism. Mitotic figures are rarely observed. Macrophage aggregates, pancreatic tissue and other structures are often missing or sparse within the neoplastic lesion. Adenomas may be demarcated by compression of the adjacent parenchymal cells (Figure 10B), however in some cases this is not as obvious (Figure 10A).

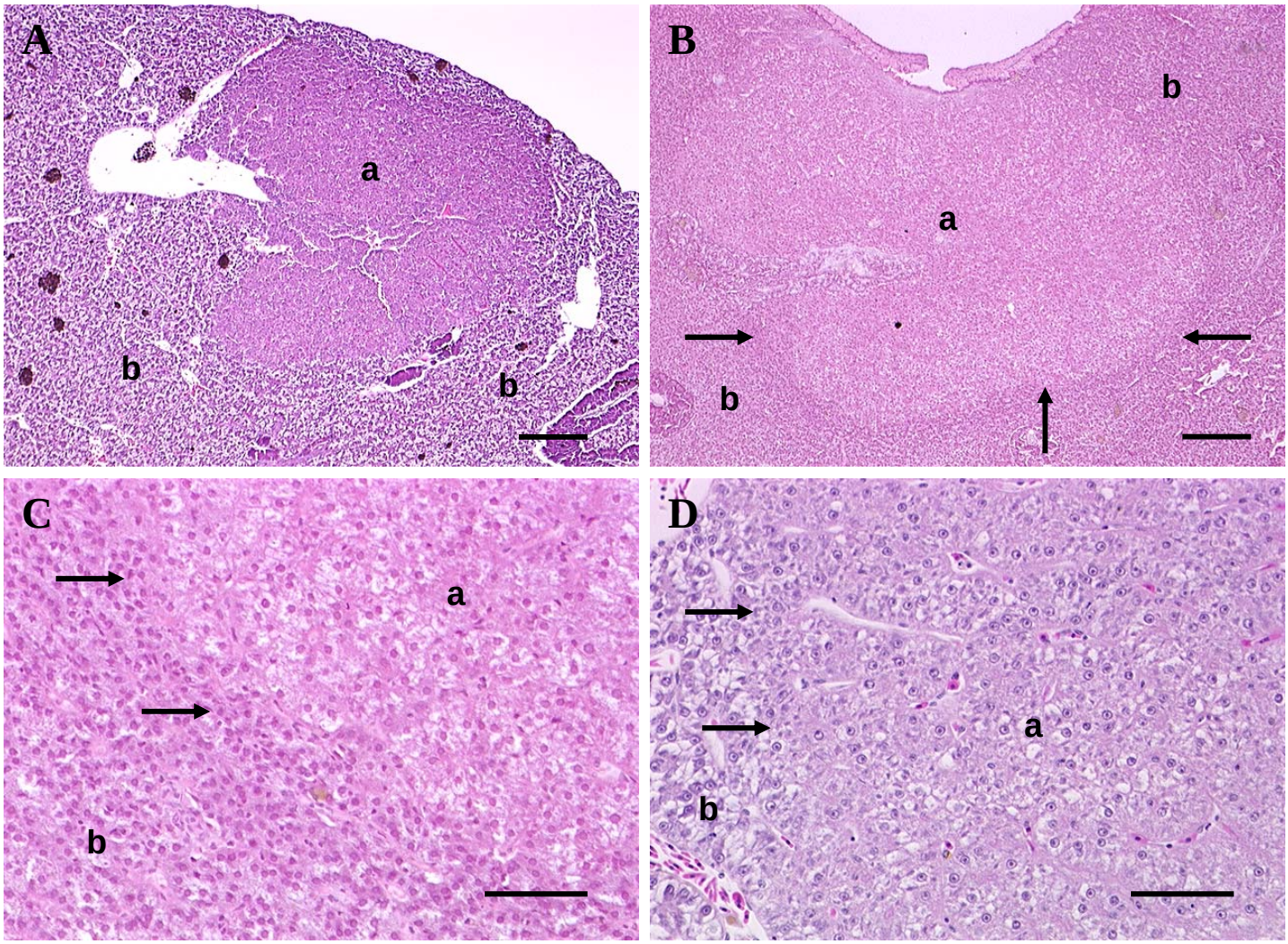


Figure 10. Hepatocellular adenoma in the liver of brown bullhead. A. Neoplastic cells within the adenoma (a) stain eosinophilic compared to surrounding hepatocytes (b). Bar = 150 µm. B. Adenoma (a) showing obvious compression (arrows) of surrounding tissue. Bar = 150 µm. C and D. Higher magnification of the periphery of two adenomas (a) which have some cellular atypia and moderate compression (arrows) of surrounding tissue. Bar = 50 µm. H&E stain.

3.4.2 Hepatocellular Carcinoma

Hepatocellular carcinomas, malignant hepatic neoplasms, are often diffusely spread throughout the hepatic parenchyma (Figure 11A and B) in brown bullhead, but may be distinct foci with irregular borders (Figure 11C and E). Neoplastic cells invade the adjacent parenchyma (Figure 11D). Cellular pleomorphism and nuclear atypia are key features (Figure 11F) and there is often an increase in the number of mitotic figures. There can be some tumor giant cell formation and these may be small or large lesions.

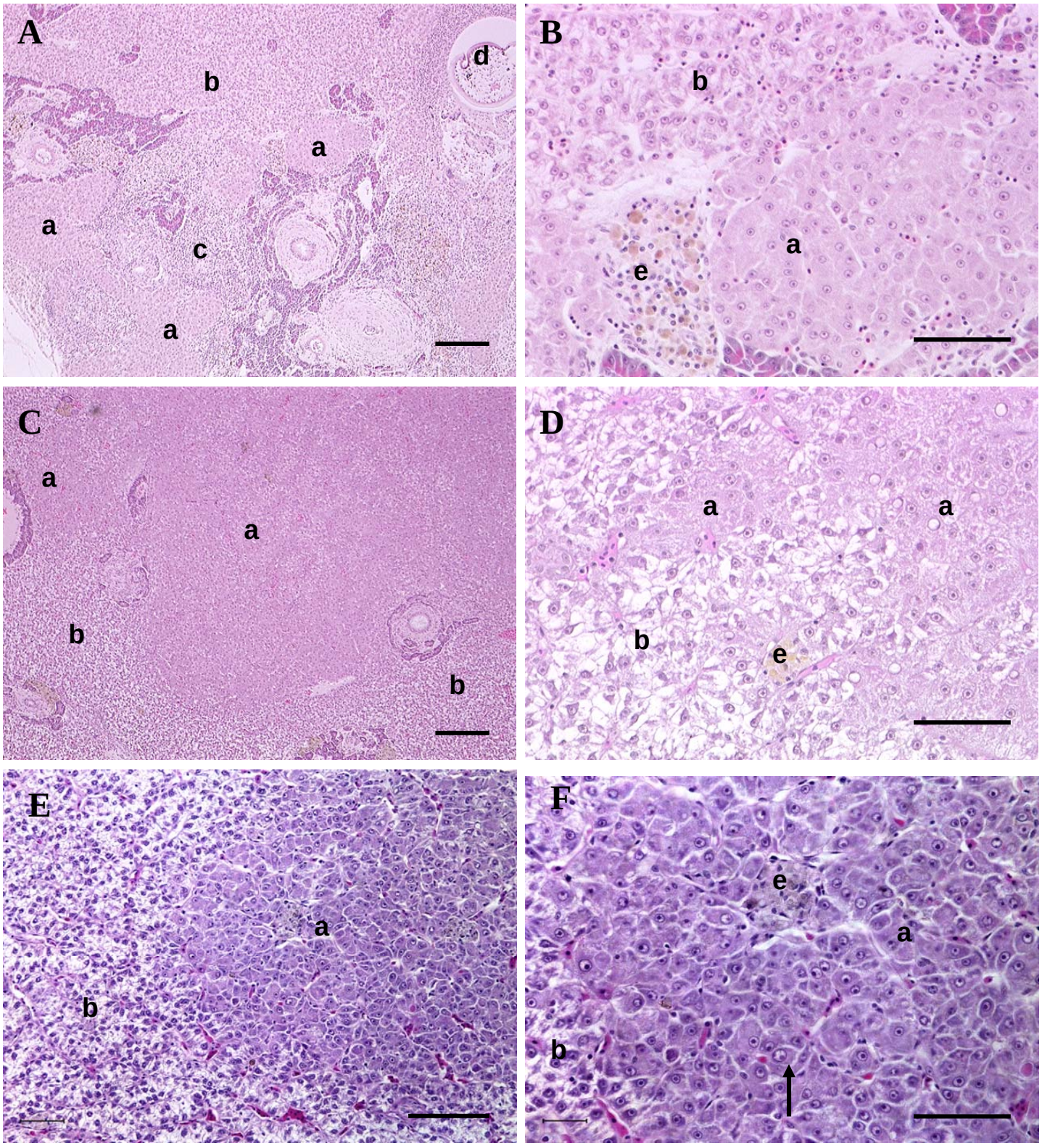


Figure 11. Hepatocellular carcinoma in the liver of brown bullhead. A. Foci of neoplastic cells (a) are observed throughout the normal hepatic parenchyma (b). Inflammation (c), probably in response to helminth parasites (d) is also observed. Bar = 150 µm. B. Higher magnification of A, indicating focus of neoplastic cells (a), normal liver (b) and a macrophage aggregate (e). Bar = 50 µm. C. Larger, more invasive carcinoma (a) infiltrating normal liver (b). Bar = 150 µm. D. Higher magnification of C illustrating the invasiveness of the neoplastic cells (a). Bar = 50 µm. E. Well-differentiated hepatocellular carcinoma (a). Bar = 100 µm. F. Higher magnification of E illustrating neoplastic cells which vary in size and shape and may have multiple nucleoli within the nucleus (arrow). A macrophage aggregate is evident (e). Bar = 50 µm. H&E stain.

3.5 Non-Neoplastic Biliary Lesions

3.5.1 Bile Duct Proliferation and Fibrosis

Normal small and intermediate bile ductules/ducts have columnar epithelium and a thin band of connective tissue (Figure 12A). Large bile ducts are surrounded by connective tissue and are prominent near the common bile duct (Figure 12B). Bile duct hyperplasia consists of an increased number of variably-shaped bile ducts, when compared to the normal appearance (Figure 2A and B). Bile ducts are often scattered throughout the liver and do not form discrete masses (Figure 13A), although occasionally small clusters are observed (Figure 13B). The biliary epithelium is always well-differentiated (normal-appearing), however there may be moderate fibrosis associated with proliferating bile ducts (Figure 13C). Cholangiofibrosis consists of bile ducts or ductules with extensive periductal fibrosis (Figure 13D), however the biliary epithelium is still normal.

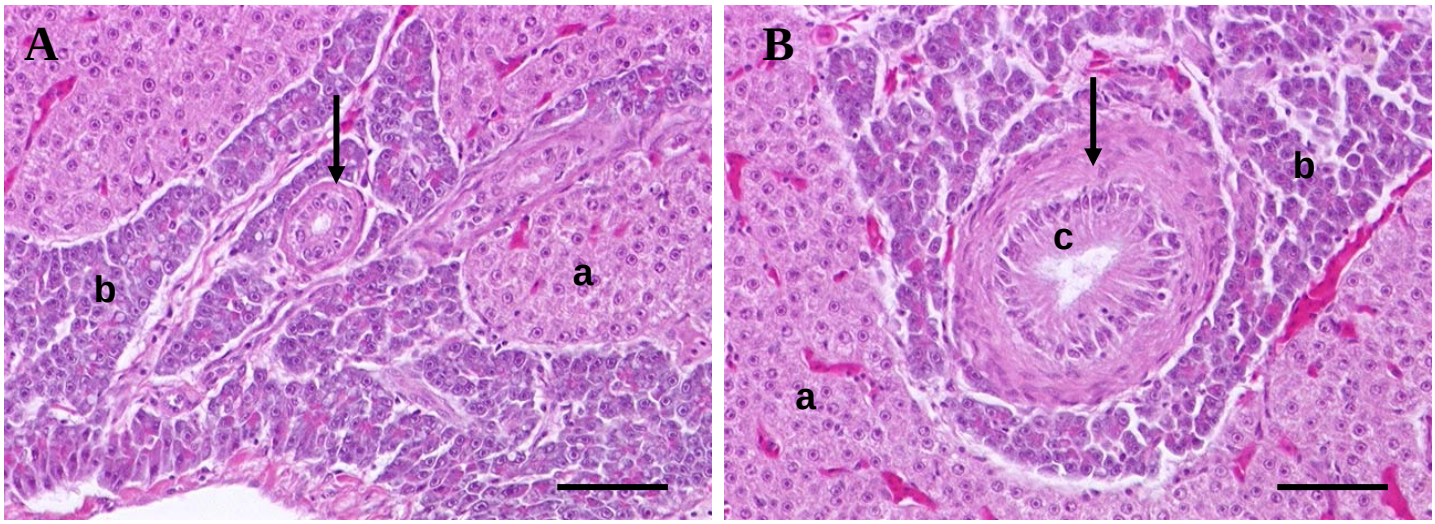


Figure 12. Bullhead liver showing normal bile ductule and duct. A. Within the hepatic tissue (a) bile ductules (arrow) are commonly located within the pancreatic tissue (b) and are bounded by a thin band of connective tissue. Bar = 50 µm. B. Larger ducts have a thicker layer of connective tissue (arrow) and columnar epithelium. (c). Bar = 50 µm. H&E stain.

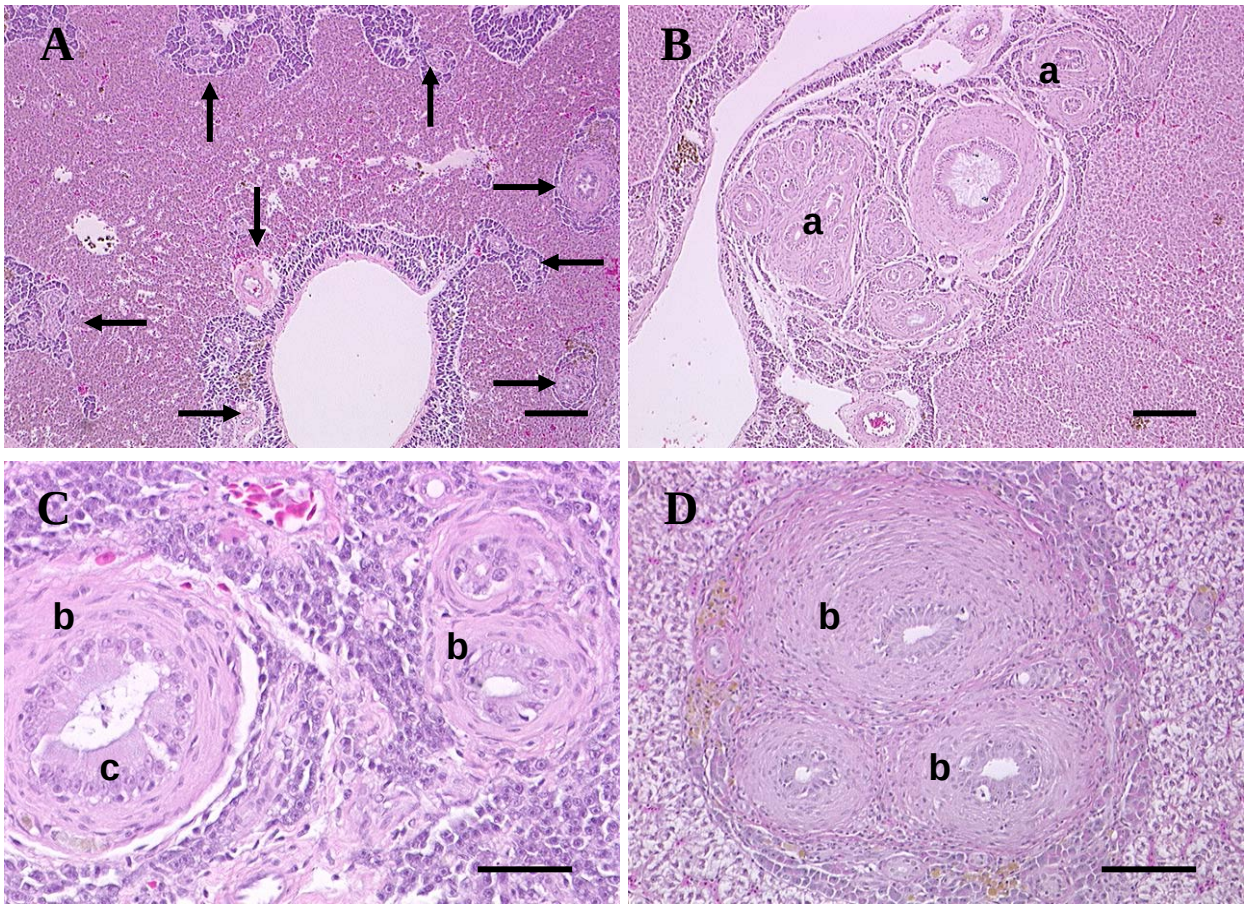
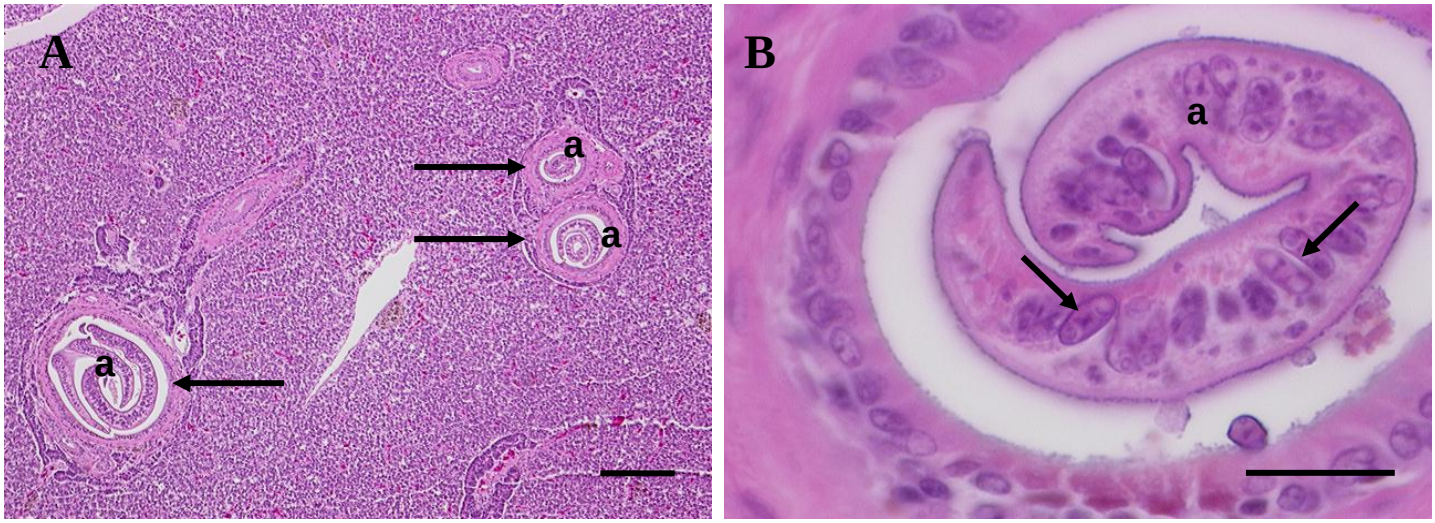


Figure 13. Bile duct proliferation and fibrosis. A. Proliferation of individual bile ductules and ducts (arrows) throughout the liver. Bar = 150 µm. B. Focal proliferation of bile ducts (a) with a moderate amount of fibrosis around the ducts. Bar = 150 µm. C. Higher magnification illustrating increased fibrous tissue (b) and normal epithelium (c). Bar = 100 µm. D. Extensive fibrosis (b) around a focal area of bile duct proliferation. Bar = 50 µm. H&E stain.

3.5.2 Bile Duct Parasitism

Sporoplasms of a myxozoan parasite have been observed within the bile ducts of brown bullhead from numerous areas. The affected bile ducts are often thickened and in some areas there is evidence of hyperplasia of the epithelium or necrosis (Figure 14). It is currently not known what association this parasite may have on bile duct proliferation or biliary neoplasia.



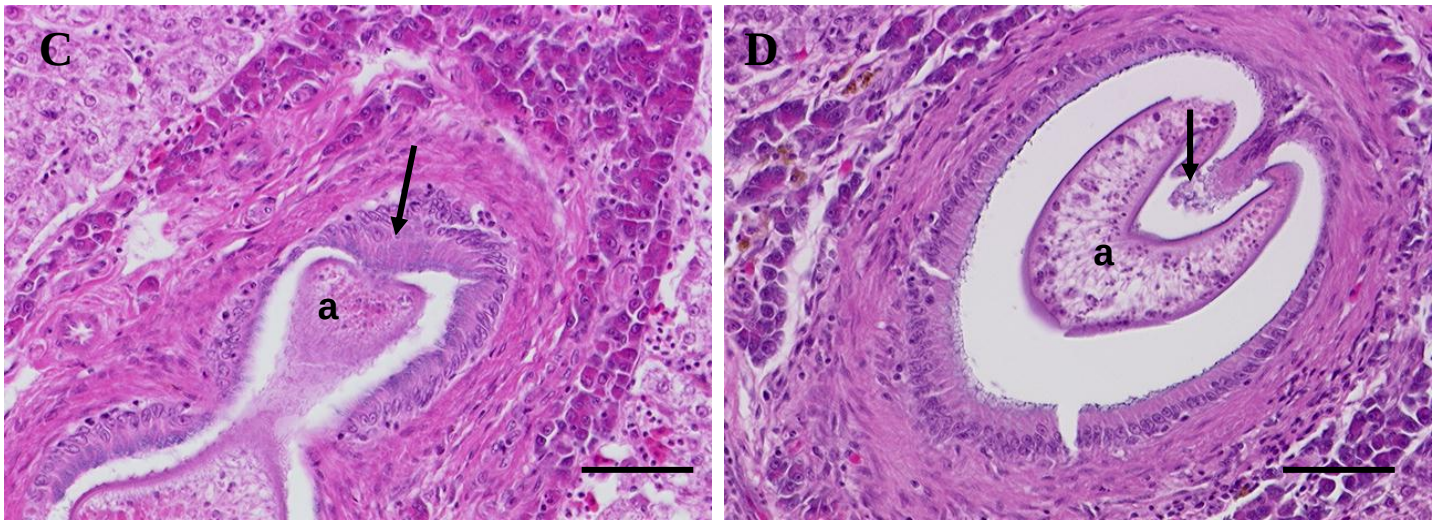


Figure 14. Bile duct myxozoan parasites of brown bullhead. A. Sporoplasms (a) of a myxozoan parasite within the bile ducts (arrows). Bar = 150 µm. B. Higher magnification of the sporoplasm (a) containing mature spores (arrows) as well as immature stages. Spores are elongate with polar capsules on either end. Bar = 20 µm. C. Affected bile ducts may show signs of epithelial hyperplasia (arrow) associated with the presence of the sporoplasm (a). Bar = 50 µm. D. Some affected bile ducts show signs of epithelial necrosis (arrow) associated with the parasite (a). Bar = 50 µm. H&E stain.

3.6 Neoplastic Biliary Lesions

3.6.1 Cholangioma

Cholangiomas are benign tumors of bile ducts within the liver. These are clusters of bile ducts which are well-differentiated and often have a discrete border between the nodule and surrounding hepatic parenchyma (Figure 15). Many of the bile ducts may be irregularly-shaped and dilated.

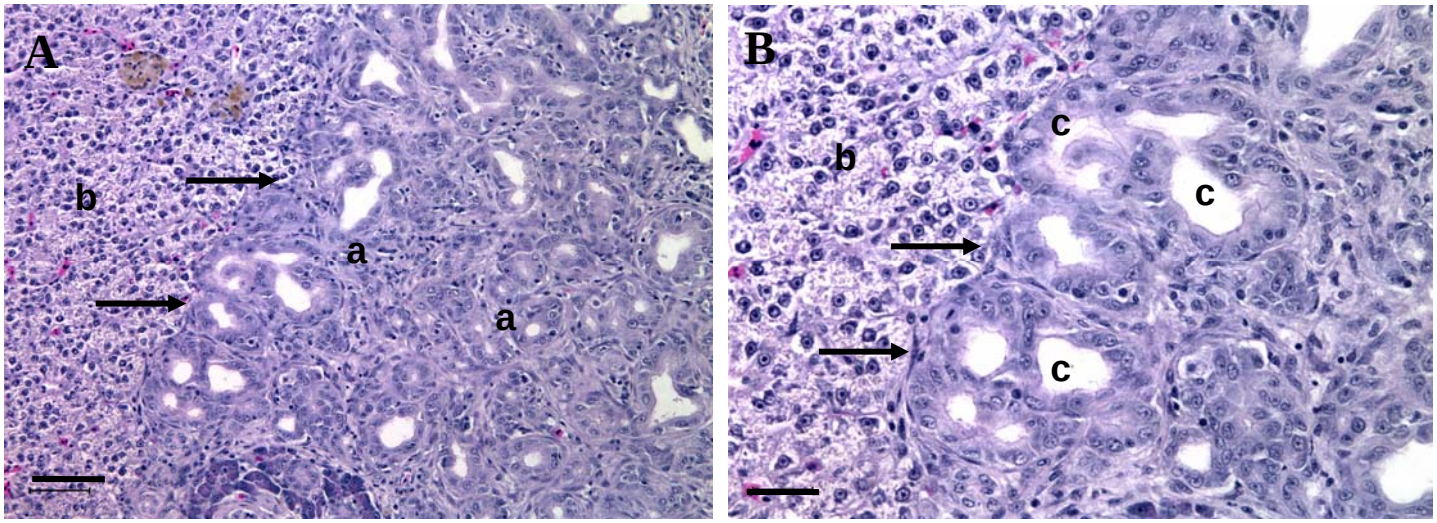


Figure 15. Cholangioma within the liver of a brown bullhead. A. Nodule of neoplastic bile ducts (a) is separated from the normal hepatic tissue (b) by a well-defined border (arrows). Bar = 50 µm. B. Higher magnification illustrating the well-defined border (arrows) and neoplastic bile ducts (c) which are irregularly shaped and dilated. B. Bar = 25 µm. H&E stain.

3.6.2 Cholangiocarcinoma

Cholangiocarcinomas are malignant tumors of bile ducts. Some tumors contain moderately to well-differentiated bile ducts, which vary greatly in size and shape and invade into the surrounding parenchyma (Figure 16A and B). The proliferating bile ducts may be associated with proliferating stroma and inflammation (Figure 16C and D). In some tumors, there is very little normal duct formation and the neoplastic epithelial cells are pleomorphic with mitotic figures and are surrounded by a proliferating spindle cell stroma (Figure 16E and F).

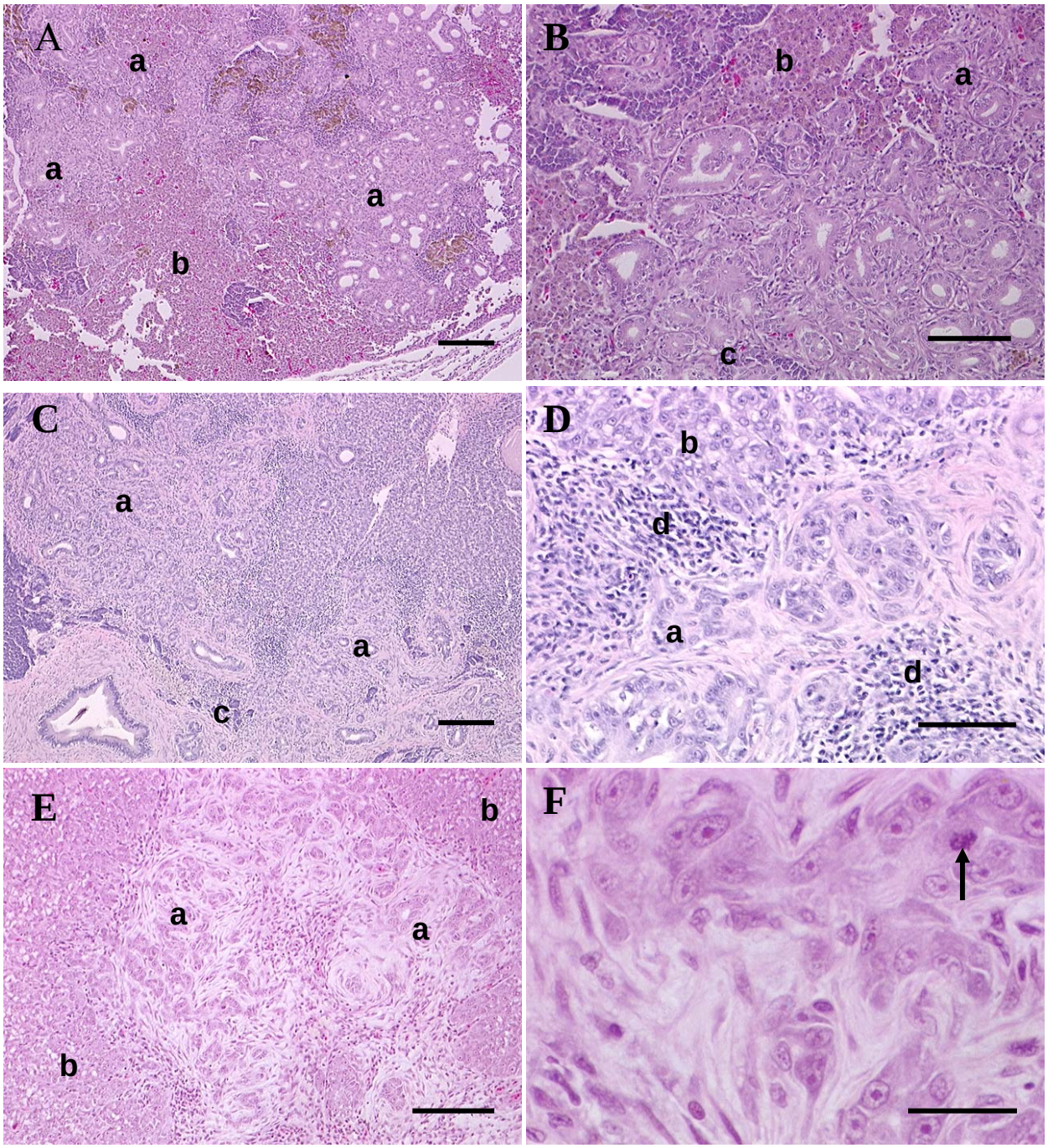


Figure 16. Cholangiocarcinoma in brown bullhead liver. A. Proliferating neoplastic bile ducts (a) invade into the surrounding hepatic parenchyma (b). Bar = 150 µm. B. Higher magnification of invading neoplastic bile ducts (a). Bar = 100 µm. C. A cholangiocarcinoma in which the proliferating neoplastic bile ducts surround pancreatic tissue (c). Bar = 150 µm. D. Higher magnification illustrating inflammation (d) around the proliferating bile ducts (a). Bar = 50 µm. E. Small nodule of undifferentiated bile ducts (a) separated from normal liver (b) by inflammation. Bar = 100 µm. F. Higher magnification illustrating pleomorphic cells with mitotic figure (arrow). Bar = 20 µm. H&E stain.

3.7 Metastatic Neoplasia

Metastasis of malignant neoplasms to the liver is rare in fish. However, metastases of dermal melanomas to the liver have been observed in brown bullhead (M. Wolfe, personal observation).

4.0 SKIN

The presence of external neoplasms, in bullhead and white sucker *Catostomus commersoni* in the Great Lakes region and elsewhere, has been used as an indicator of environmental health (Baumann 1992). Skin tumors reported in brown bullhead include epidermal papillomas, squamous cell carcinomas and melanomas (Sonstegard 1977; Kim et al. 1989; Bowser et al. 1991). Henry David Thoreau may have been the first to document a raised growth in bullhead. A study written by Thoreau and reported by Osburn (1925) of bullheads in a pond on Cape Cod, described lesions varying from melanistic spots to large raised tumors, attributed to "black coccoid bacteria", which were probably melanosomes (organelles responsible for melanin production). Elevated prevalences of raised skin lesions have been reported at contaminated and/or industrialized sites in numerous fish species (Smith et al. 1989; Hayes et al. 1990; Kinae et al. 1990; Okihiro et al. 1993; Mikaelian et al. 2000), although, the association with chemical exposure in bullheads is less conclusive than with liver neoplasia (Poulet et al. 1994; Pinkney et al. 2001). Papillomas are the most frequently reported cutaneous neoplasms and affect a wide variety of freshwater and saltwater fishes. Some laboratory studies have indicated a chemical etiology for bullhead papillomas (Black 1983; Grizzle et al. 1984), while others have suggested a viral etiology based on the observation of viral particles (Edwards and Samsonoff 1977). A number of studies have looked for, but not confirmed, the presence of viral particles (Bowser et al. 1991; Poulet et al. 1996) and attempts to transmit the orocutaneous neoplasms have not been successful, suggesting a multifactorial etiology (Poulet et al. 1993). Viruses have been implicated in the induction of tumors or tumor-like growths in a number of other fish species (Yamamoto et al. 1985; Lee and Whitfield 1992; Anders and Yoshimizu 1994; Quackenbush et al. 2001). It has been suggested a higher prevalence of skin tumors in fish collected at contaminated areas may be explained by a lower resistance or immunosuppression (Au et al. 2004). These raised growths are most commonly observed on the lips and barbels, however can be found anywhere on the body surface and fins.

4.1 Normal Skin, Barbel, and Oral Cavity Microscopic Appearance

The histologic appearance of bullhead skin and other tissues is similar to that described for channel catfish *Ictalurus punctatus* (Grizzle and Rogers 1976). Bullhead skin, like that of other catfishes, does not contain scales. The skin is composed of the epidermis and underlying dermis and hypodermis and varies in thickness in different areas of the body. The epidermis is composed of columnar basal cells which become squamous in the outer layer. Goblet or mucous cells are present in all areas but vary in abundance. Alarm substance, club or fright cells are also common, while melanophores and taste buds are found in some areas (Figure 17A and B). The dermis is composed of a compact layer of fibrous connective tissue below the epidermis. In most areas there is a thin layer of melanophores immediately beneath the epidermis.

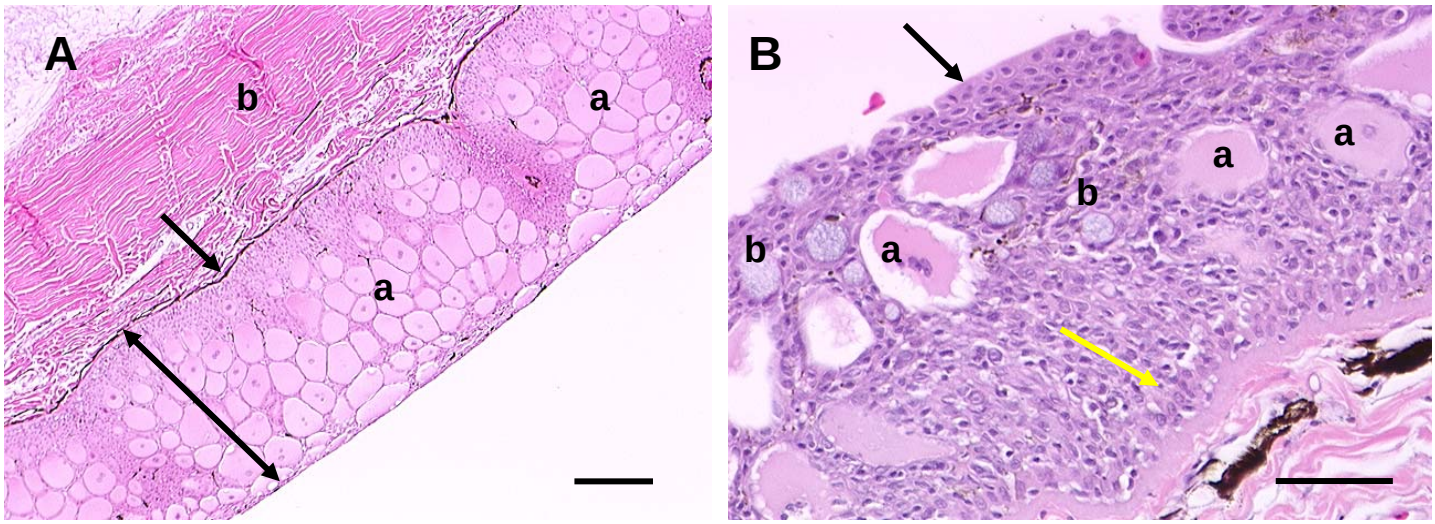


Figure 17. Microscopic appearance of normal brown bullhead skin. A. Skin from the lateral surface with numerous alarm substance cells (a) in the epidermis (between double-headed arrow). The dermis (b) immediately below the epidermis contains a thin line of melanophores (arrow). Bar = 150 µm. B. An area of skin with fewer alarm substance cells (a) and more mucous or goblet cells (b). The epithelial cells at the base of the epidermis (yellow arrow) are columnar cells, becoming more squamous (black arrow) at the surface. Bar = 50 µm. H&E stain.

The barbels are composed of a central core of pseudocartilage and nervous tissue, covered with a dermis and epidermis, both generally containing melanophores. Taste buds are very numerous in the epidermis (Figure 18A and B) of the barbels.

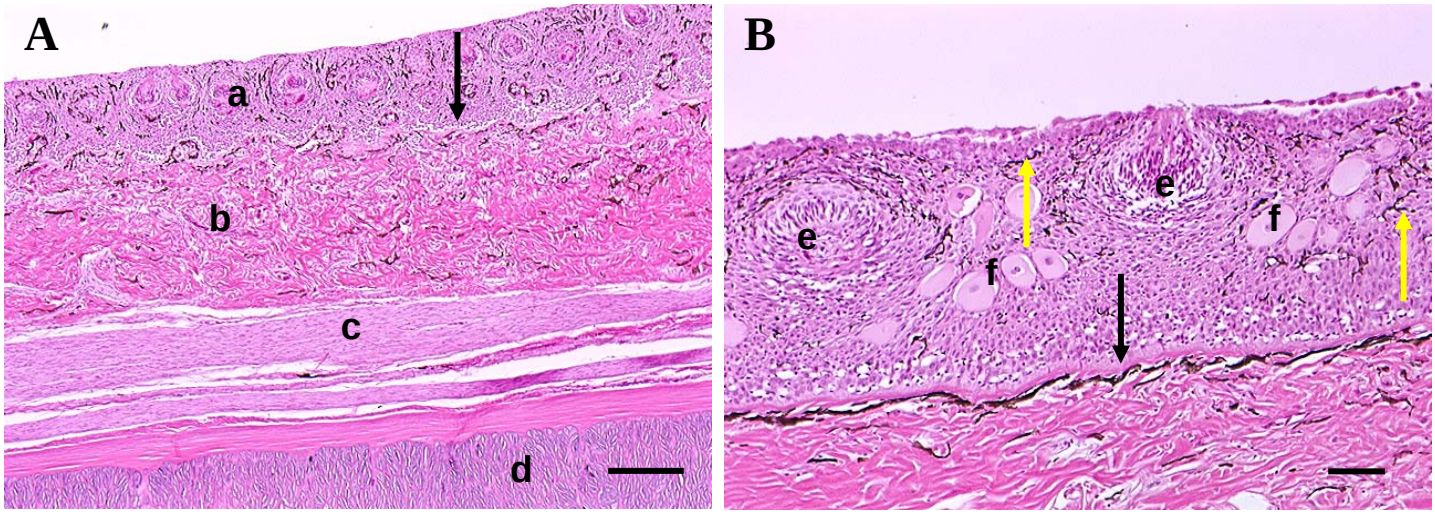


Figure 18. Microscopic appearance of brown bullhead barbels. A. Barbels consist of an epidermis (a), dermis (b) with a layer of melanophores (arrow), a central portion of nervous tissue (c) and pseudocartilage (d). Bar = 150 µm. B. The epidermis of the barbel has abundant taste buds (e), some alarm substance cells (f) and melanocytes (yellow arrows). Bar = 50 µm. H&E stain.

The oral cavity has an epithelium similar to the outer skin, however it has a more folded appearance (Figure 19A). Most areas have fewer alarm substance cells, while taste buds are abundant. Teeth are ankylosed to the premaxilla and dentary bones of the mouth (Figure 19B). The teeth are covered with a thin enamel-like material and formed of a dentine-like material which surrounds a pulp cavity that contains nerves, blood vessels and fibrous connective tissue. Modified epithelial cells surround the teeth.

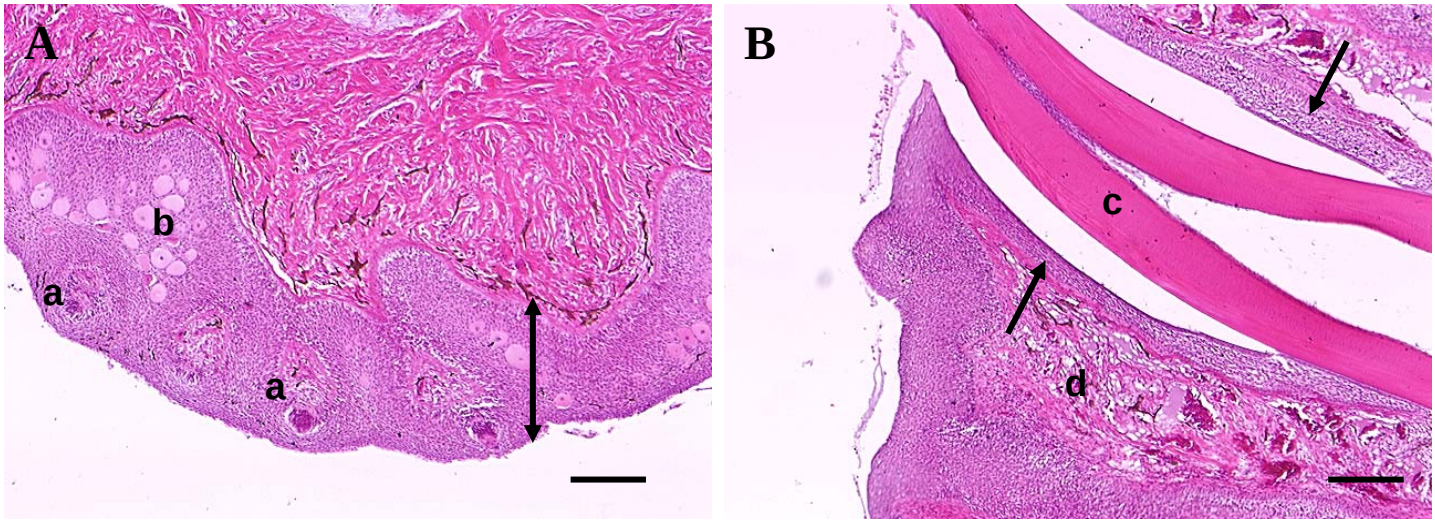


Figure 19. Microscopic appearance of the normal oral cavity. A. Mucosal epithelium (between double headed arrow) contains taste buds (a) and alarm substance cells (b). Bar = 150 µm. B. Teeth (c) extend from the bone through the propia mucosa and mucosa, and are surrounded by modified epithelial cells (arrows). The subdermal area (d) consists of highly vascularized loose connective tissue. Bar = 150 µm. H&E stain.

4.2 Black Pigmented Skin Lesions

Black pigmented lesions in brown bullhead range from non-raised melanistic areas to large raised black areas. In bullheads, there have been no studies that address the progression of pigmented lesions. It is unknown whether the non-raised melanistic areas (melanotic hyperpigmentation) are precancerous lesions that progress to melanomas. We have observed examples of all in wild-caught bullheads (Figures 20-26). The *Xiphophorus* model has been used extensively to study molecular and genetic mechanisms involved in melanoma induction (Setlow et al. 1989; Schartl 1995; Nairn et al. 1996). In this fish species, a classification scheme for spontaneous tumors and neoplastic lesions induced by UV radiation and exposure to the carcinogen *N*-methyl-*N*-nitrosourea has been described. These lesions include nonneoplastic melanotic hyperpigmentation, preneoplastic melanosis and melanomas (Gimenez-Conti et al. 2001). A correlation between exposure to environmental chemicals and pigment cell neoplasia has been suggested in some fish species (Kimura et al. 1984; Kinae et al. 1990; Okihiro et al. 1993).

4.2.1 Non-Raised Melanistic Spots

Melanistic areas are non-raised, black pigmented spots on the head, body surface or fins. They can vary greatly in size and shape (Figure 20A and B). Histologically, these melanistic lesions consist of increased numbers of melanocytes primarily within the epidermis, but sometimes the dermis as well. The melanocyte may have extensive cytoplasmic extensions or be in large groups (Figure 20 C and D).

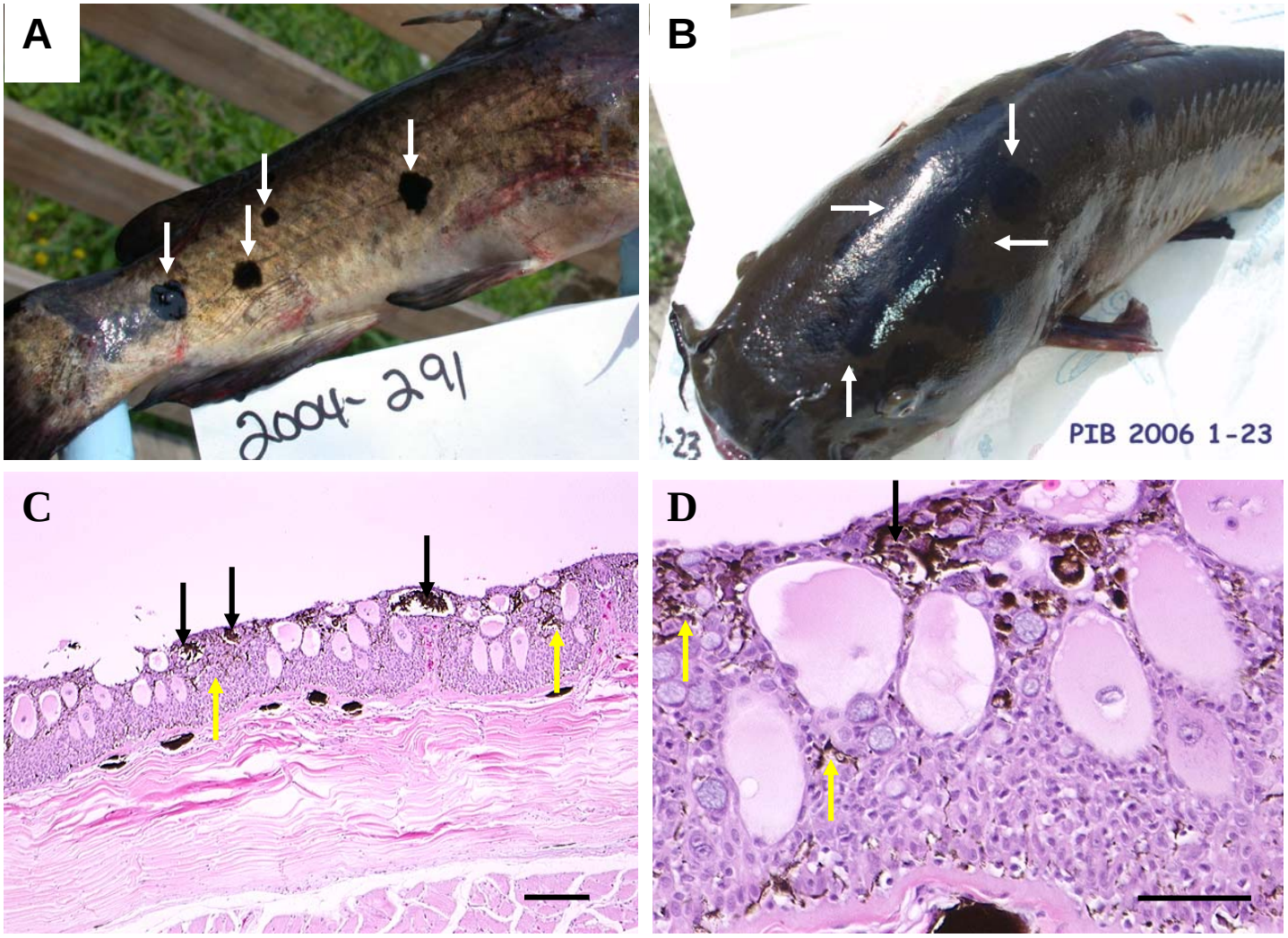


Figure 20. Melanistic spots. A. Multiple, nonraised, black spots on the lateral body surface. B. A large, irregular, non-raised black area on the head and body surface. C. Microscopic appearance of a melanistic spot. Epidermis (between double-headed arrow) contains groups of melanocytes (black arrow) as well as individual cells with extensive cytoplasmic extensions (yellow arrows). Bar = 150 µm. D. Higher magnification illustrating groups of melanocytes of varying sizes (black arrow) and individual melanocytes with extensive cytoplasmic extensions (yellow arrows). Bar = 50 µm. H&E stain.

4.2.2 Raised Black Skin Lesions

These areas can vary from small raised areas to larger raised areas on fins, body surface, lips and barbels. It is necessary to examine these lesions histologically to determine whether they are parasite-associated raised lesions (Figure 21), melanotic hyperplasia (Figure 22), melanomas (Figures 23-25), or papillomas with increased melanocytes within the proliferating epidermis (Figure 26). Grossly, these may all look very similar.

4.2.2.1 Parasite-Associated Black, Raised Lesions

Some of the raised black areas observed on barbels and the head region are found to contain trematode metacercariae. These parasites appear primarily around and within the nerves of the barbels, and are associated with a proliferation of dermis, underlying connective tissue, dermal melanocytes and in some cases a proliferation of the overlying epidermis (Figure 21). Similar raised black lesions are known to occur in blue catfish (*Ictalurus furcatus*), associated with *Ichthyophonus* infections (Fournie, personal observations).

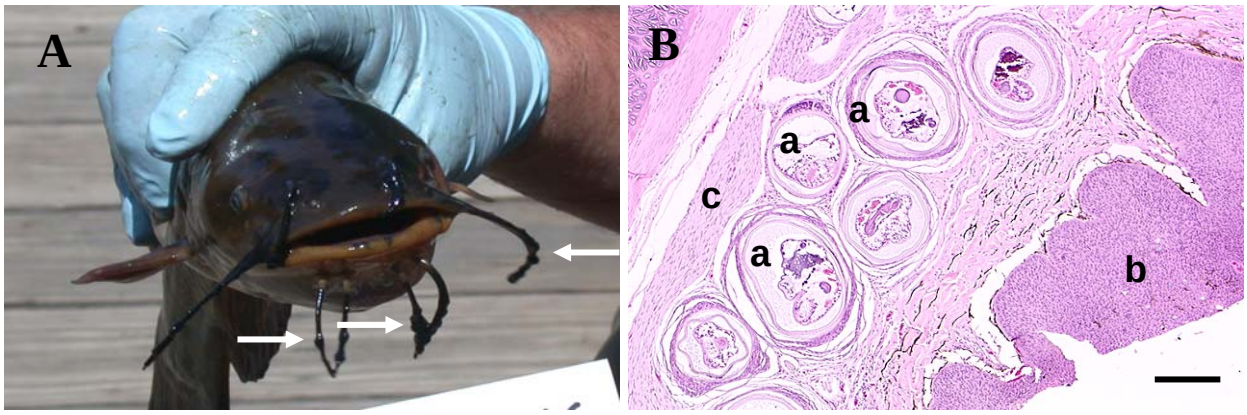


Figure 21. A. Small raised, black areas (white arrows) on the barbels of a brown bullhead. B. Microscopic appearance of the barbels illustrating parasitic cysts (a) within the nerves (c) and dense connective tissue. In some areas this response is associated with a proliferation of the epidermis (b). Bar = 150 µm. H&E stain

4.2.2.2 Melanotic Hyperplasia

Slightly raised and irregular pigmented areas have been noted in some bullhead, which microscopically exhibited epidermal hyperplasia and abnormal-appearing melanin accumulations, and are possibly preneoplastic (Figure 22).

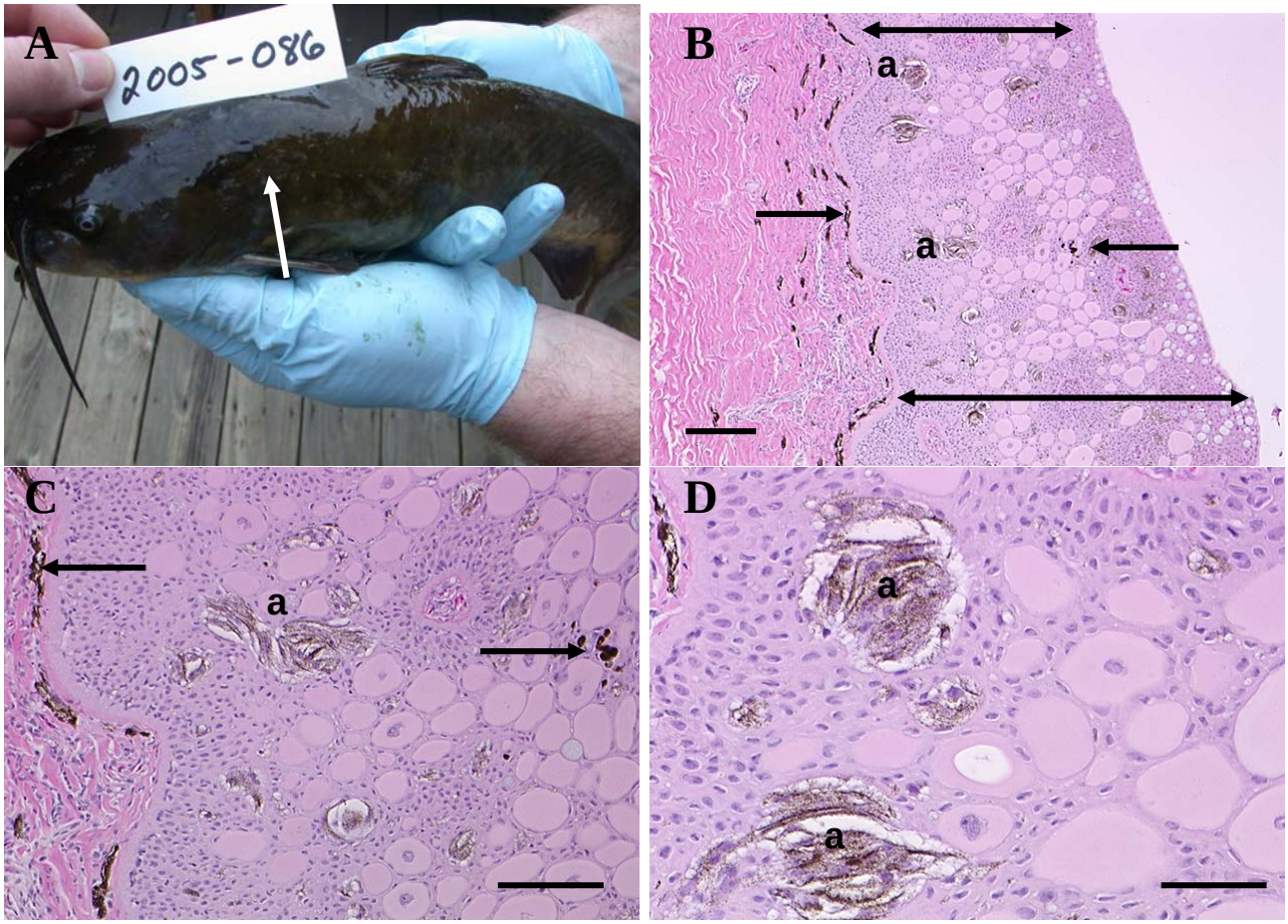


Figure 22. A. Slightly raised melanistic area on the body surface of a brown bullhead. B. Microscopic appearance of a section of the area. The epidermis (between the double-headed arrows) is of varying thickness and contains groups of abnormal, spindle-shaped cells with melanin (a). Normal melanocytes are present in the epidermis and dermis (arrows). Bar = 150 µm. C. Higher magnification showing normal melanocytes in dermis and epidermis (arrows) and groups of abnormal melanocytes in epidermis (a). Bar = 100 µm. D. Abnormal melanocytes are spindle-shaped and vacuolated (a). Bar = 50 µm. H&E stain.

4.2.2.3 Melanoma

Lesions diagnosed as melanomas range from very small raised areas on the skin or barbels (Figure 23 and 24) to very large raised areas (Figure 25). A number of types of melanomas similar to those described by Gimenez-Conti et al. (2001) have been observed in brown bullhead and are illustrated in the following figures. These include the melanocytic melanomas (Figure 23) which are characterized by a proliferation of melanocytes that are dendritic in shape and contain variable amounts of pigment.

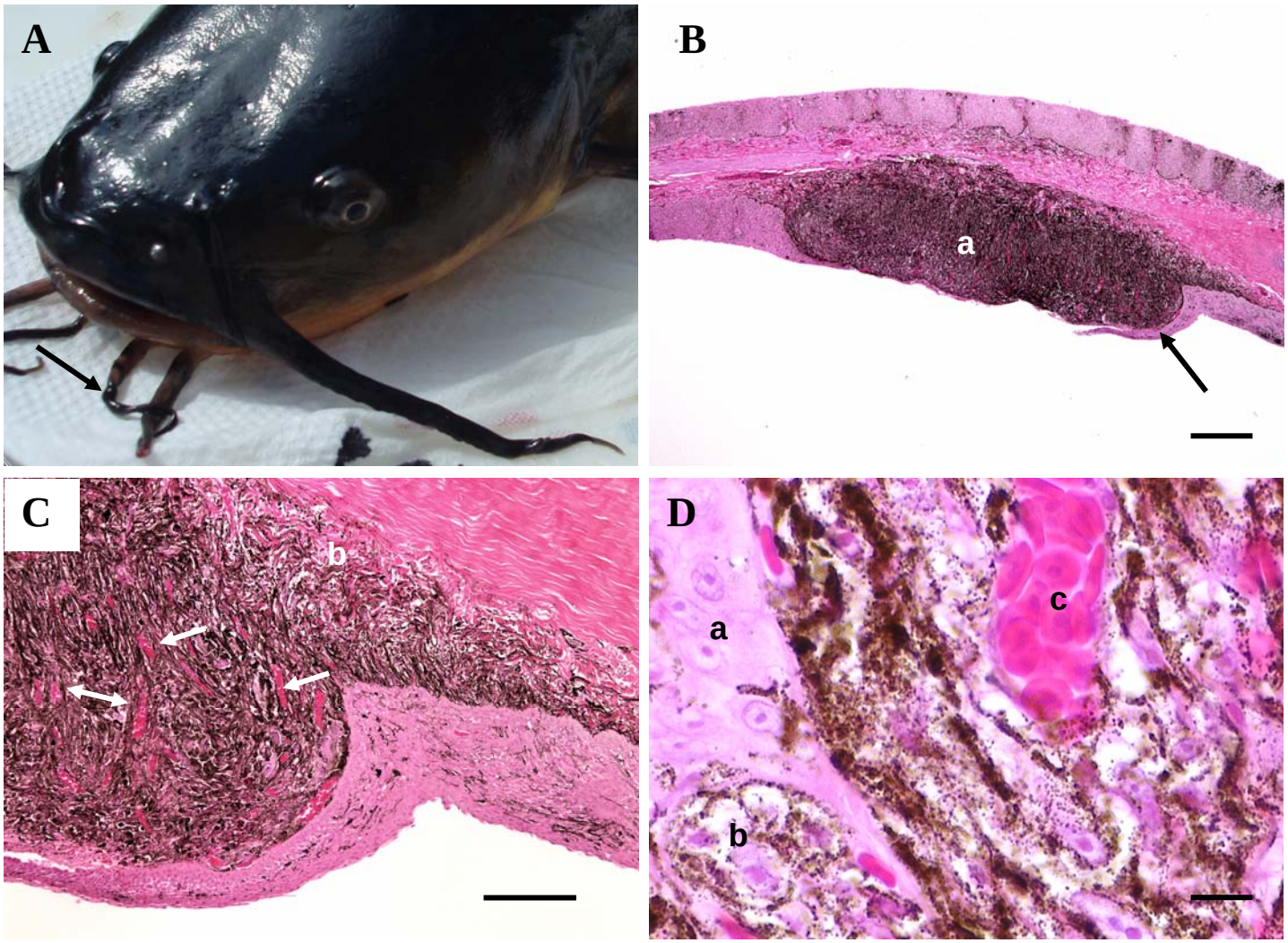


Figure 23. Melanoma on the barbel of a brown bullhead. A. Small raised, black area on the barbel. B - D. Histologic appearance of barbel melanoma. B. Epidermis (arrow) is intact over most of the melanoma (a). Bar = 300 µm. C. The proliferating neoplastic cells extend into the dense connective tissue of the dermis (b). There are numerous blood vessels throughout the tumor (arrows). Bar = 50 µm. D. In areas there are foci of neoplastic cells (b) within the epidermis (a). The neoplastic cells surround blood vessels (c), are dendritic and contain varying amounts of pigment. Bar = 10 µm. H&E stain.

The melanoma on the body surface (Figure 24) is usually a polymorphic melanoma. These tumors are heterogenous with both heavily and lightly pigmented areas. Cell types within the tumor include melanocytes, epithelioid cells and macromelanophores.

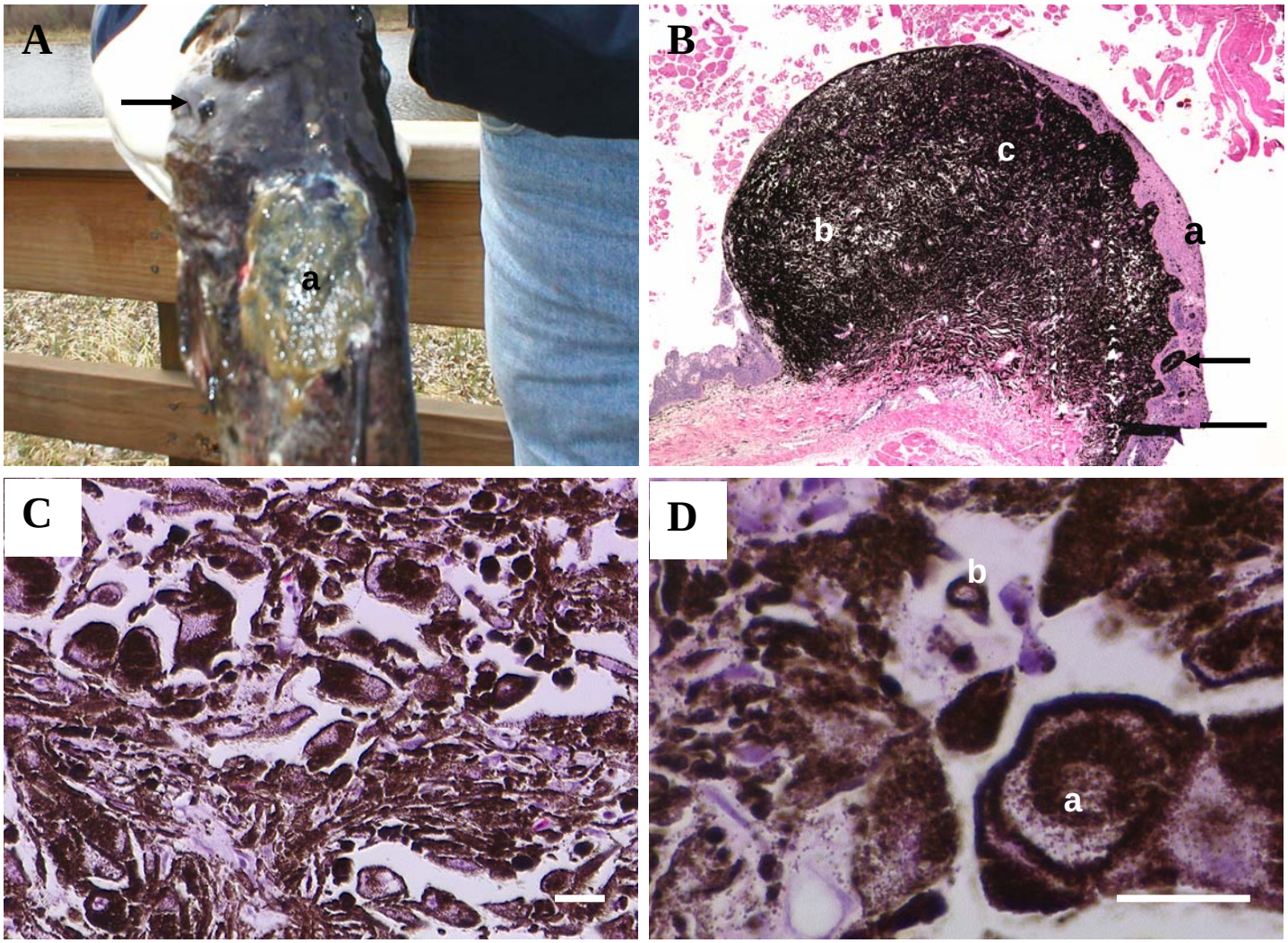


Figure 24. Melanoma on the body surface of a brown bullhead. A. Grossly visible, raised, black lesion (arrow) on the lateral surface of a brown bullhead which also has a large discolored lesion (a), covered by fungal growth. B. Microscopic appearance of the melanoma. In some areas the epidermis covering the tumor is thickened (a) and contains pegs of the neoplastic cells (arrow). The tumor contains some areas that are less densely stained (b) than others (c). Bar = 300 µm. C. Higher magnification of the tumor illustrating the variably sized and shaped cells and bizarre growth pattern. Bar = 50 µm. D. Neoplastic cells include very large macromelanophore cells (a) and cells of relatively normal size (b). Bar = 20 µm. H&E stain.

The large, black raised lesion on the body surface (Figure 25) resembles a spindle cell melanoma histologically, and is very similar to a dermal melanoma previously described from brown bullhead as having schwannoma-like differentiation (Sakamoto and White 2002).

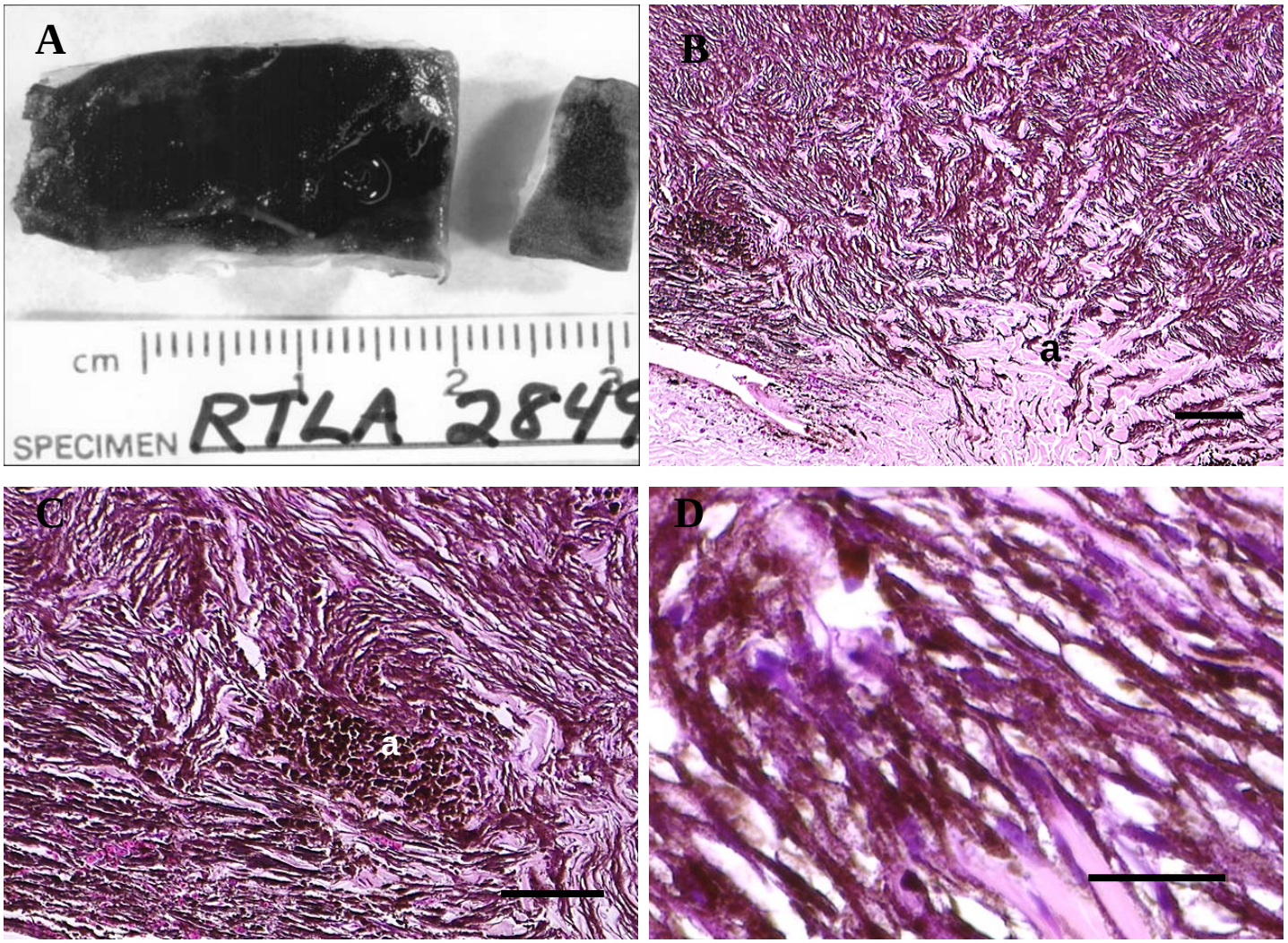


Figure 25. Large melanoma on the body surface of a brown bullhead. A. Irregular raised black area on the skin. B. Microscopic appearance illustrating a "herringbone" pattern with a connective tissue stroma (a). Bar = 150 µm. C. Occasional clusters of rounded, densely pigmented melanophores (a) were observed. Bar = 100 µm. D. The majority of neoplastic cells were spindle-shaped with varying amounts of melanin. Bar = 20 µm. H&E stain.

4.2.2.4 Melanotic Papilloma

Some raised black areas are papillomas (see 4.3.2.1) that are characterized by a proliferation of melanocytes within the epidermis and along the connective tissue trabeculae (Figure 26).

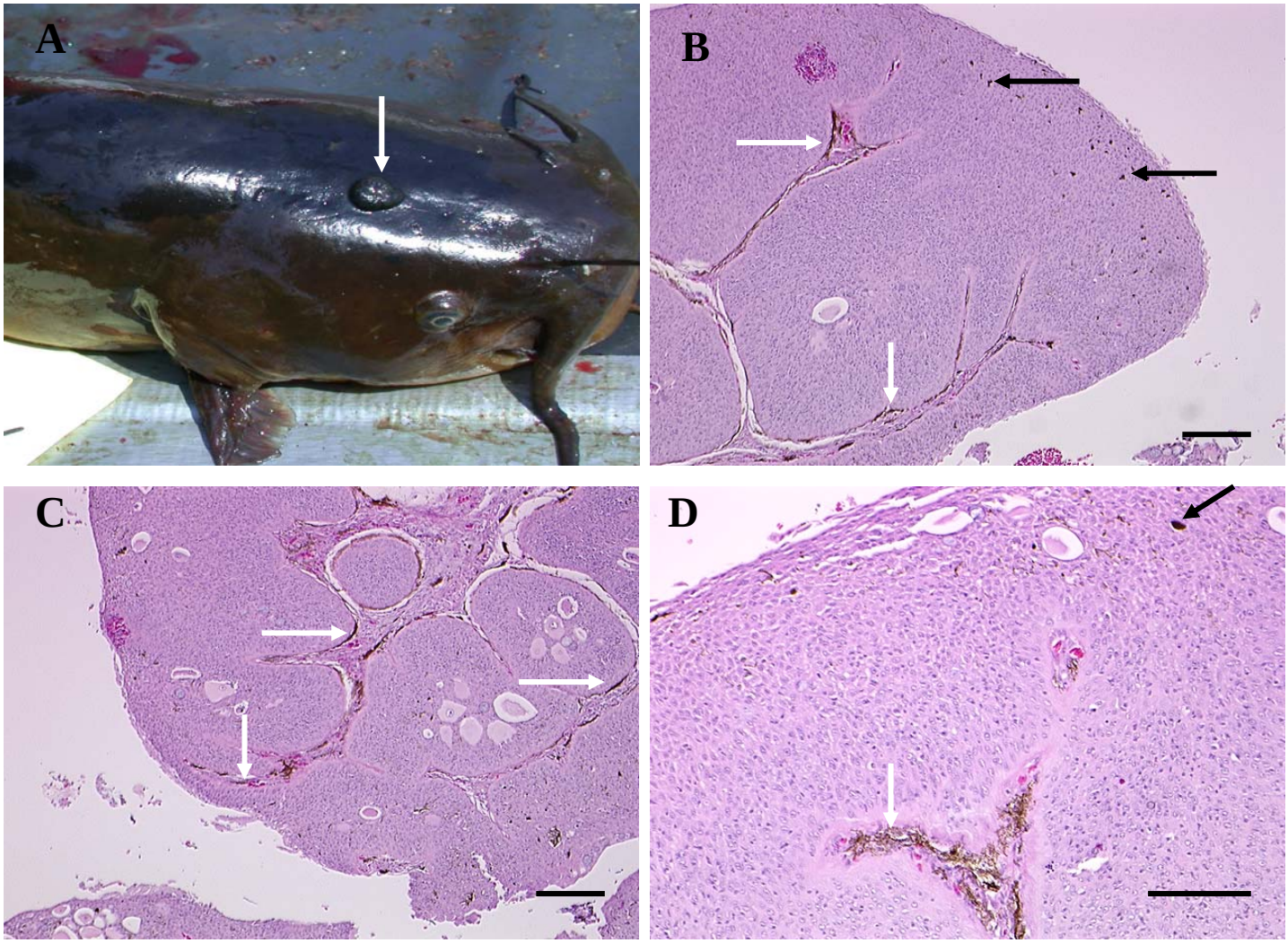


Figure 26. A. Sessile papilloma (arrow) on the head of a brown bullhead. B. Microscopically, the lesion was composed of proliferating epithelial cells with melanocytes within these proliferating areas (black arrows). Bar = 150 µm. C. These proliferating epithelial cells form the typical papillomatous folds supported by the connective tissue stroma lined by melanocytes (white arrows). Bar = 150 µm. D. Higher magnification illustrating accumulations of melanocytes in the stroma (white arrow) and individual melanin-containing cells in the proliferating epidermis (black arrow). Bar = 100 µm. H&E stain.

4.3 Pale Areas of Discoloration

Areas of discoloration, generally white to yellow, range from small, non-raised to large, raised, multicolored and multilobed lesions. Microscopically, these areas show epidermal hyperplasia of one or more cell types, papillomas or squamous cell carcinomas. A number of studies have suggested a progression from epidermal hyperplasia to papilloma and occasionally to carcinoma in brown bullhead (Lucké and Schlumberger 1941; Smith et al. 1989; Poulet et al. 1994; Bunton 2000).

4.3.1 Non-Raised Areas of Discoloration

These areas on bullhead are generally gray or yellow areas such as illustrated in Figure 27A. Microscopically, they appear to be due to proliferation of certain cell types. The pale gray areas contain increased epithelial and mucous cells, while the yellow areas contain increased mucous and alarm substance cells.

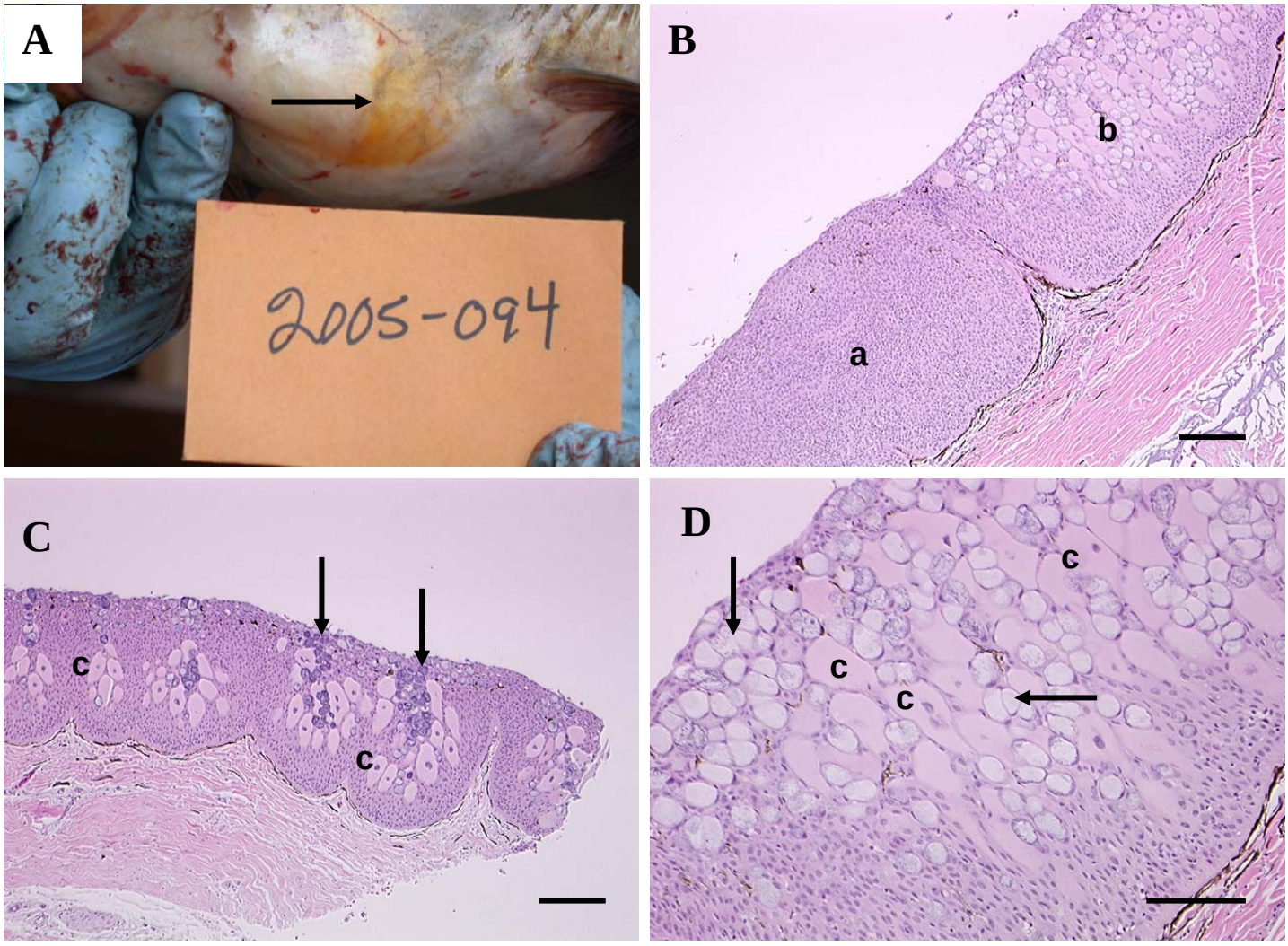


Figure 27. A. Non-raised yellow spot on the ventral surface of a brown bullhead. B. Microscopic appearance at the periphery of the discolored area showing the difference between the normal epithelium (a) and the discolored area (b). Bar = 150 µm. C. Microscopically, the yellow lesions contain areas with increased mucous cells (arrows) and alarm substance cells (c). Bar = 150 µm. D. Higher magnification of the affected area in B, illustrating the proliferation of mucous cells (arrows) and alarm substance cells (c). Bar = 100 µm. H&E stain.

4.3.2 Raised Pale Areas

Raised, pale growths can be observed on the lips, fins and body surface and can vary from small, slightly raised, plaque-like areas to very large multiple, coalescing nodules. In bullheads they are most common in the oral cavity and on the lips. Most often these growths are papillomas or squamous cell carcinomas.

4.3.2.1 Papilloma

Papillomas are considered benign growths and can range from slightly raised, homogeneous-appearing areas (Figure 28A) to small raised, slightly eroded and reddened plaques (Figure 28B) to larger, often multiple nodules (Figure 28C and D) which may vary in color from pale to black (Figure 28C).

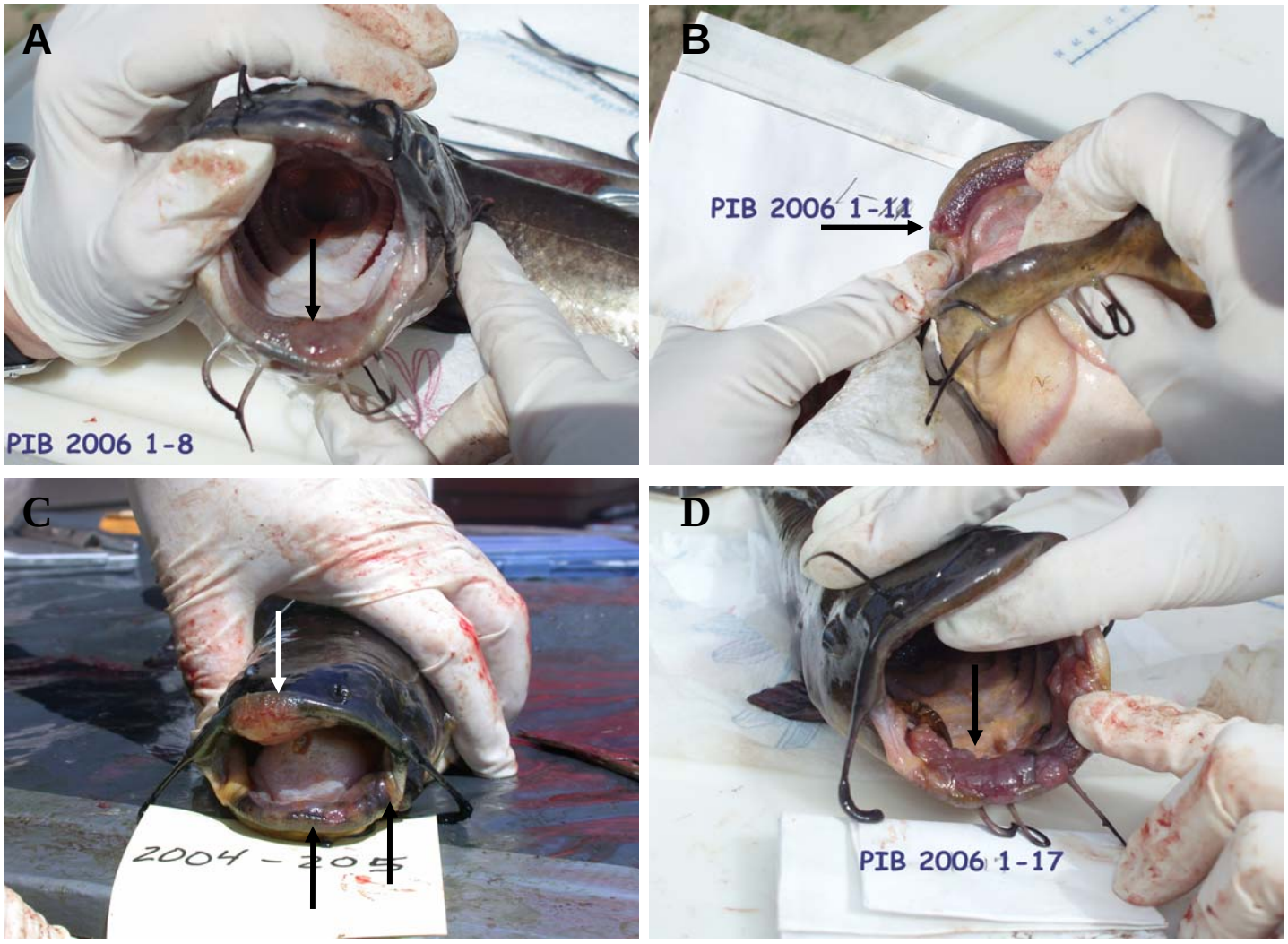


Figure 28. Raised growth on the mouth of brown bullhead. All were determined to be papillomas by microscopic evaluation. A. A slightly raised, plaque-like, pale growth on the lower lip. B. A small, slightly raised, reddened and eroded growth on the upper lip. C. Larger, multiple raised growths on the lips, some dark (black arrows) and some very pale (white arrows). D. Multiple, raised growths on the lower lip.

Histologically, the epithelial proliferation causes papillary folds to form which are supported by connective tissue cords. These folds extend above the normal skin surface and extend into the underlying dermis. Proliferation is primarily of the epidermal malpighian cells. However, in some areas there may be proliferation of mucous or alarm substance cells causing the area to appear pale, while in others there may be a proliferation of melanocytes giving the area a dark appearance.

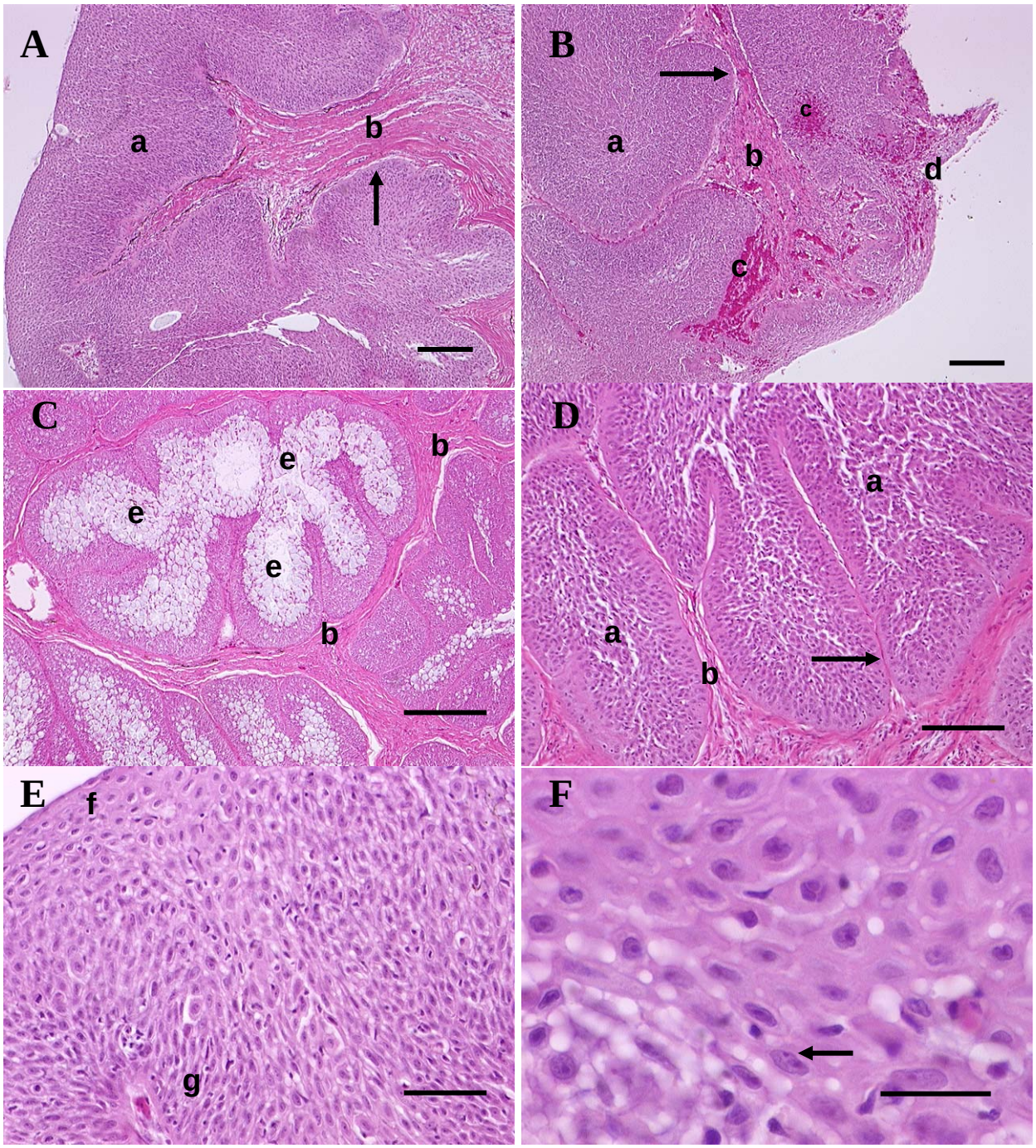


Figure 29. Microscopic appearance of the papillomas illustrated in Fig. 28. A. Proliferating epidermal (malpighian) cells (a) form folds or papillae supported by connective tissue (b). Neoplastic cells do not infiltrate below the basement membrane (arrow). Bar = 150 µm. B. Raised growths that appear reddened and/or abraded (Figure 28B) contain areas of hemorrhage or congestion (c) and the outer surfaces may be necrotic (d). Bar = 150 µm. C. Individual growths may appear very pale and translucent (Figure 28C) due to a proliferation of mucous cells (e). Bar = 100 µm. D. Higher magnification illustrating the well-defined folds of the epidermis (a) within the connective tissue support (b). Bar = 100 µm. E. The proliferating cells are squamous (f) in the outer regions and columnar to cuboidal (g) toward the dermis. Bar = 50 µm. F. Proliferating cells vary in size and shape, nuclei are enlarged and may contain multiple nucleoli (arrow). Bar = 20 µm. H&E stain.

4.3.2.2 Squamous Cell Carcinoma

Squamous cell carcinomas have been described in a number of fish species (Fournie et al. 1987; Harshbarger and Clark 1990; Hanjavanit and Mulcahy 2004) including brown bullhead (Bunton 2000; Pinkney et al. 2001). In a study of bullhead from the Back River, MD, an area with elevated PAH levels, a progression from hyperplasia to

papilloma to carcinoma was suggested. Local invasion through the basal lamina occurs as the lesion progresses to carcinoma (Bunton 2000). Some areas are masses of neoplastic cells in which small islands of supportive tissue, mucous or alarm substance cells may become entrapped (Figure 30 B). Other areas may contain cords or nests of neoplastic cells interspersed with fibrous connective tissue (Figure 30 C and D). Carcinomas are characterized by pleomorphism of the epithelial cells. Large, often polygonal cells, with enlarged nuclei, are observed with a loss of normal orientation (Figure 30 E and F). They are also characterized by extension through the basement membrane (Figure 30 F).

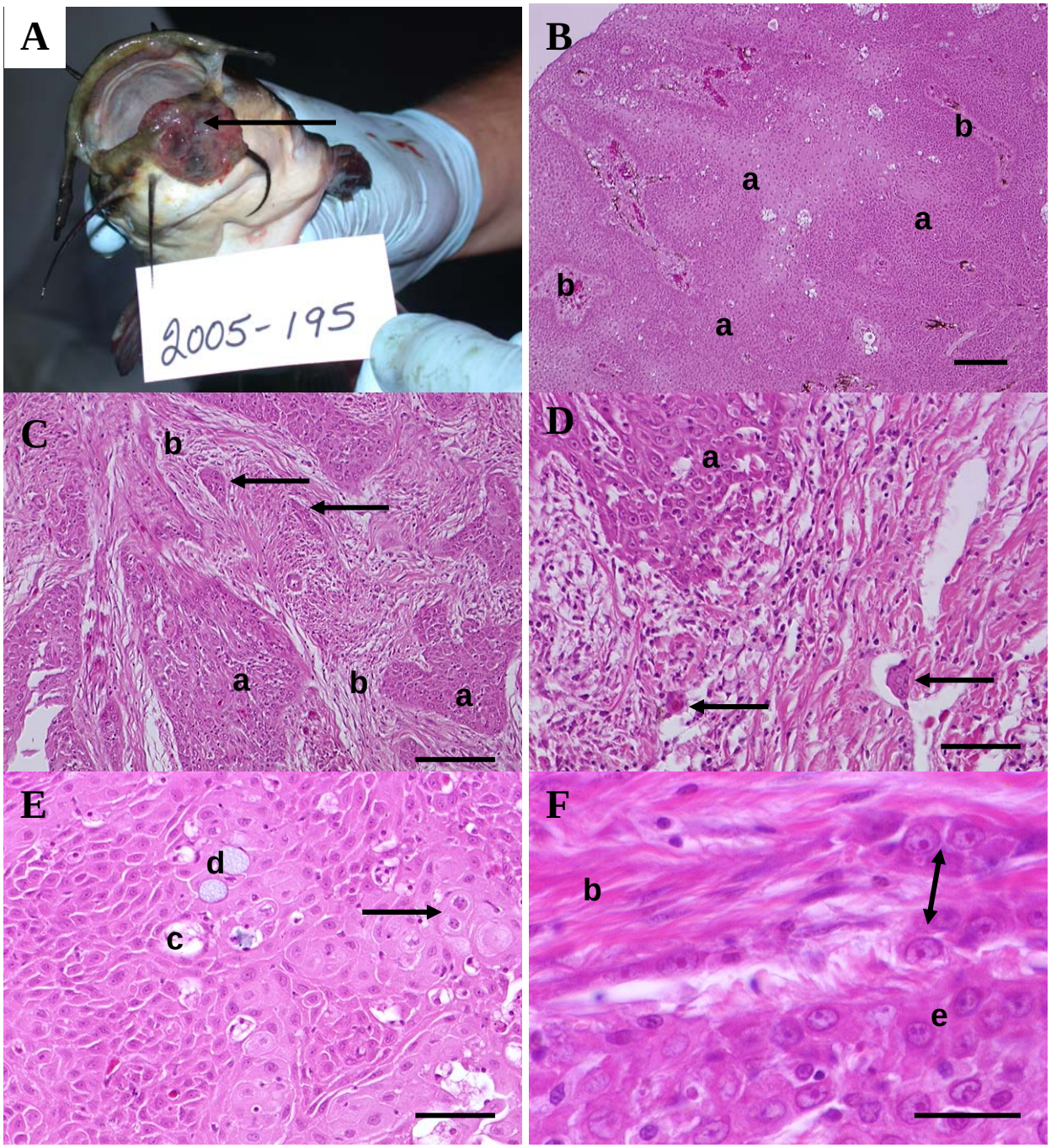


Figure 30. Squamous cell carcinoma in brown bullhead. A. Large growth on the lower lip and jaw of a brown bullhead. B. Microscopic appearance of the neoplastic growth illustrating the lack of well-defined folds (a) and islands of supporting tissue (b). Bar = 150 µm. C. In some areas, cords (a) or nests (arrows) of neoplastic cells are found within fibrous tissue (b). Bar = 100 µm. D. Higher magnification of neoplastic cells (a) that have extended through the basal lamina and small nests of neoplastic cells (arrows) in the dermis and hypodermis. Bar = 50 µm. E. Area of the carcinoma illustrating the lack of normal orientation of the large, often polygonal cells. Necrotic areas (c), mucous cells (d) and mitotic figure (arrow) may be observed. Bar = 50 µm. F. Invasion of the neoplastic cells (e) through the basement membrane (double arrow) and into the underlying tissues (b). Bar = 20 µm. H&E stain.

4.3.2.3 Miscellaneous Raised Growths

Occasionally, other tumor types are found to be responsible for the raised growths observed on bullhead fins, body surface or mouth. An osteoma and osteosarcoma were both diagnosed from the same bullhead. These lesions were described grossly as a firm, raised mass on the pectoral fin and a hyperplastic lesion on the upper lip, respectively. The fin lesion was diagnosed microscopically as an osteoma (Figure 31A and B) while the jaw lesion was an osteosarcoma (Figure 31C and D). To our knowledge, there are no other reports of bony tumors in bullhead, however individual cases have been described in a few other fish species (Grizzle et al. 1995; Manera and Biavati 1999; Pereira and Peleteiro 2002). The osteoma is comprised of trabeculae of mature bone, generally with a well-defined border (Figure 31 A and B). The osteosarcoma contained less mature bone and the periphery was omposed of irregularly-shaped extensions of osteoid surrounded by pleomorphic, basophilic osteoblasts (Figure 31 C and D).

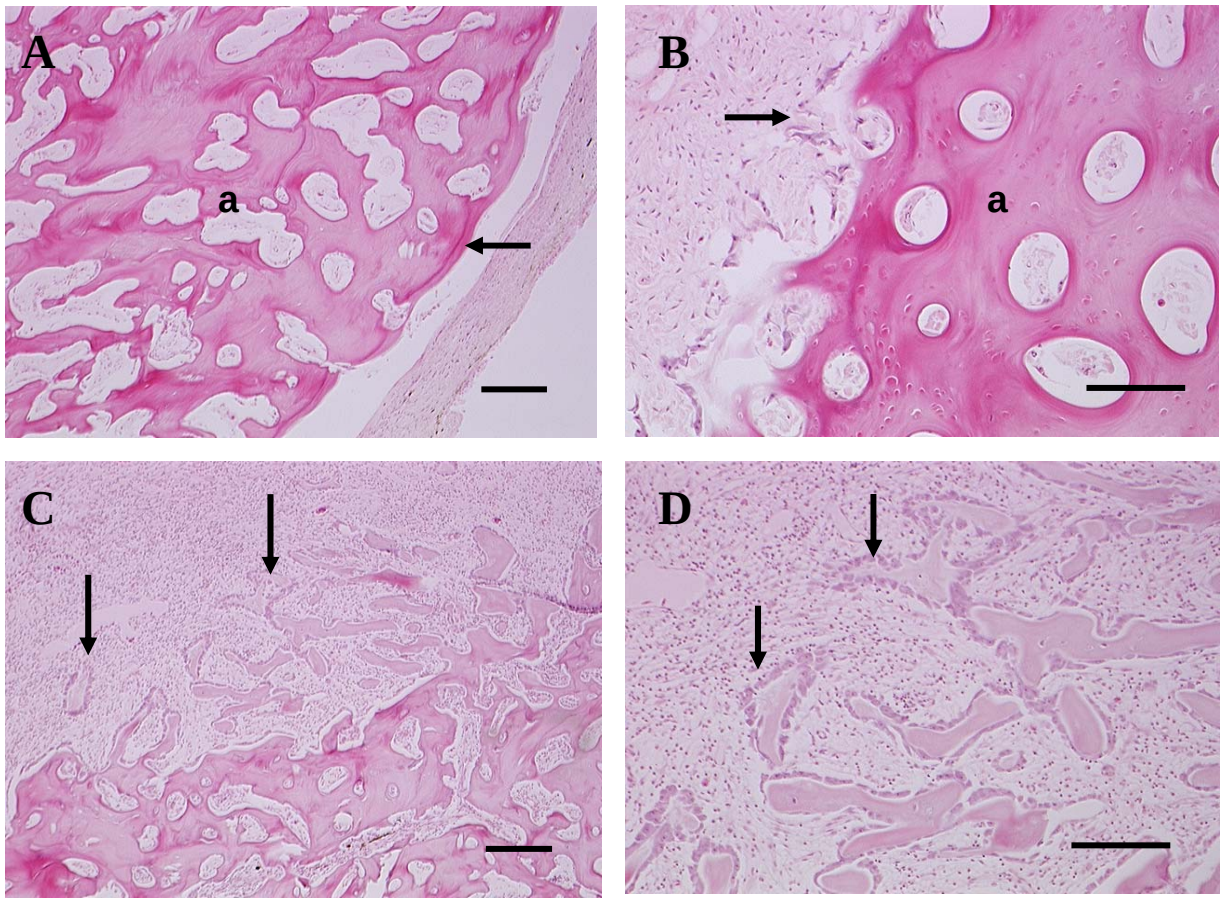


Figure 31. Raised bony tumors on the fin and jaw of a brown bullhead. A. Microscopic appearance of the osteoma on the fin. Trabeculae of mature bone (a) with a well-defined border (arrow) in most areas. Bar = 150 µm. B. In some areas of the tumor (a) there is osteoblastic activity (arrow) on the periphery. Bar = 50 µm. C. Microscopic appearance of an osteosarcoma on the jaw of a brown bullhead. Periphery is composed of irregularly-shaped extensions of osteoid (arrows). Bar = 150 µm. D. Higher magnification illustrating the pleomorphic, basophilic osteoblasts (arrows) surrounding the osteoid branches. Bar = 50 µm. H&E stain.

5.0 ACKNOWLEDGEMENTS

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