

AN ABSTRACT OF THE THESIS OF

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Title: EPIDERMAL TUMORS ON JUVENILE ENGLISH SOLE  
(PAROPHRYS VETULUS) FROM YAQUINA BAY, OREGON

Abstract approved: \_\_\_\_\_ Dr. Robert E. Olson

Juvenile English sole (Parophrys vetulus) were collected in Yaquina Bay, Oregon from January, 1986 to March, 1987. Recruitment of young-of-the-year sole occurred from January to June, 1986 and again from December, 1986 to February, 1987. Emigration from Yaquina Bay started in September, 1986, but a small portion of the English sole population over-wintered in the estuary.

Angioepithelial nodules, epidermal papillomas and plaque-like, spreading lesions were identified by their gross morphology.

Epidermal tumors were present on 18.7 percent of the 1612 English sole captured and affected fish averaged 2.8 tumors each. Tumorous fish ranged in length from 28 to 130 mm and were collected each month. The larger, over-wintering sole had a higher tumor prevalence than the bulk of the population.

In histologic preparations, typical X-cells were present in all tumors examined. When present in angioepithelial nodules, X-cells were smaller than those found in epidermal papillomas. In 73 percent of the epidermal papillomas sectioned, numerous X-cells contained a single, basophilic, spherical nuclear inclusion. No virus particles were seen in X-cells, nuclear inclusions, or in other tissue components of epidermal papillomas when viewed with an electron microscope.

Attempts to culture X-cells using primary tissue culture techniques or by inoculating malt-yeast-agar were unsuccessful.

X-cell mitotic figures were not found in tissue cultures, histologic sections or tissue imprints.

Laboratory observations of tumorous fish suggest a variable rate of tumor growth and some angioepithelial nodules develop into epidermal papillomas, while others disappear. The epidermis at the site where angioepithelial nodules were lost, healed completely and tumorous tissue did not reappear. Epidermal papillomas did not regress under laboratory conditions.

Neither the etiology of epidermal tumors on English sole nor the nature of X-cells was determined in this study.

EPIDERMAL TUMORS ON JUVENILE ENGLISH SOLE  
(PAROPHRYS VETULUS) FROM YAQUINA BAY, OREGON

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EPIDERMAL TUMORS ON JUVENILE ENGLISH SOLE  
(PAROPHRYS VETULUS) FROM YAQUINA BAY, OREGON

I. INTRODUCTION

Neoplasms of fishes have long stimulated the interest of the scientific community (Mawdesley-Thomas, 1969, 1975; Dawe et al., 1976; and Peters, 1984). Skin tumors of pleuronectid flatfishes have been among the most studied fish neoplasms (Mix, 1986). One tumor type, the epidermal papilloma, has been documented on 20 species of the family Pleuronectidae (Table 1).

Early reports of skin tumors on flatfishes were based on observations of single fish. Sandemann (1893) reported neoplastic growths on plaice (Pleuronectes platessas) and Atlantic flounder (Platichthys flesus) from European waters. Later, single cases of tumorous Atlantic halibut (Hippoglossus vulgaris), dab (Limanda limanda) and plaice were noted (Johnstone 1925A, 1925B).

The earliest reference to epidermal tumors on flatfish from the west coast of North America was that of Carl Hubbs who observed tumorous English sole (Parophrys vetulus) in San Francisco Bay in 1922 (field notes by Herald and Innes, personal communication; cited by Nigrelli et al., [1965]). Five years later, similar tumors were observed on English

Table 1. Species of the family Pleuronectidae with reported epidermal papillomas.

<u>Species</u>	<u>Location</u>
<u>Platichthys flesus</u> <sup>a</sup>	North Sea, Europe
<u>Pleuronectes platessa</u> <sup>a</sup>	North Sea, Europe
<u>Hippoglossus vulgaris</u> <sup>b</sup>	European waters
<u>Limanda limanda</u> <sup>c</sup>	European waters
<u>Parophrys vetulus</u> <sup>d</sup>	San Francisco Bay
<u>Psettichthys melanostictus</u> <sup>a</sup>	British Columbia
<u>Hippoglossoides elassodon</u> <sup>e</sup>	San Juan Islands, WA.
<u>Microstomus pacificus</u> <sup>f</sup>	Santa Monica Bay, CA.
<u>Lepidopsetta bilineata</u> <sup>d</sup>	British Columbia
<u>Glyptocephalus zachirus</u> <sup>g</sup>	Orcas Island, WA.
<u>Platichthys stellatus</u> <sup>h</sup>	Puget Sound, WA.
<u>Pseudopleuronectes herzensteiri</u> <sup>i</sup>	Wakasa Bay, Japan
<u>Limanda herzensteiri</u> <sup>i</sup>	Wakasa Bay, Japan
<u>Hippoglossus dubius</u> <sup>i</sup>	Wakasa Bay, Japan
<u>Glyptocephalus stelleri</u> <sup>j</sup>	Sea of Japan
<u>Glyptocephalus cynoglossus</u> <sup>j</sup>	Sea of Japan
<u>Isopsetta isolepis</u> <sup>k</sup>	Washington waters
<u>Limanda schrenki</u> <sup>l</sup>	Hokkaido, Japan
<u>Verasper moseri</u> <sup>l</sup>	Hokkaido, Japan
<u>Kareius bicoloratus</u> <sup>m</sup>	Unknown

from: a) Sandeman, 1893; b) Johnstone, 1912;  
 c) Johnstone, 1925B; d) Nigrelli et al., 1965;  
 e) Wellings et al., 1964; f) Young, 1964;  
 g) Wellings et al., 1965; h) Wellings et al., 1966;  
 i) Kimura et al., 1967; j) Honma and Kon, 1977;  
 k) McArn et al., 1968; l) Oishi et al., 1976;  
 m) Yamazaki et al., 1978.

sole from Puget Sound, Washington by Dr. E. V. Smith of the University of Washington (Pacis, 1932; and Good, 1940).

English sole spawn offshore (Budd, 1940; Harry, 1959; Jow, 1969; Kruse and Tyler, 1983; and Mundy, 1984) and the pelagic eggs hatch within 12 days (Alderdice and Forrester, 1968; and Orsi, 1968). The bilaterally symmetrical larvae are pelagic for up to 120 days prior to metamorphosis to an asymmetrical fish (Ketchen, 1956; Laroche et al., 1982; and Rosenberg and Laroche, 1982). At this time, the fish assumes a benthic life style and measures between 18 and 22 mm in standard length (Laroche et al., 1982).

Tumorous English sole populations have been studied in San Francisco Bay and Humboldt Bay, California and Puget Sound, Washington. Tumor rates have ranged from approximately 2% to 58% (Pacis, 1932; Good, 1940; McArn et al., 1968; Cooper and Keller, 1969; Kelly, 1971; McArn and Wellings, 1971; Angell et al., 1975; Stich and Acton, 1976; Foster, 1987; and Olson, unpublished data). Table 2 reports the prevalence of tumors on English sole in various geographic locations.

In Oregon, the English sole fishery ranks among the top four of the 13 harvested flatfishes in terms of poundage and ex vessel price (Jackson, 1981; and Lukas and Carter, 1988).

Juvenile English sole enter bays and estuaries shortly after metamorphosis (Westrheim, 1955) and open ocean

Table 2. Prevalence of tumorous English sole in various geographic locations.

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Investigators	Location	Prevalence
Herald and Innes (1922)	San Francisco Bay	noted
Cooper and Keller (1969)	So. San Francisco Bay	8.9
Cooper and Keller (1969)	No. San Francisco Bay	15.5
Kelly (1971)	So. San Francisco Bay	9.6
Kelly (1971)	No. San Francisco Bay	1.7
Foster (1987)	Humboldt Bay, CA.	9.3
Pacis (1932)	Seattle, WA.	4.8
Good (1940)	Seattle, WA.	4.5
Angell <i>et al.</i> (1975)	Seattle, WA.	18.7
Wellings <i>et al.</i> (1964)	San Juan Islands, WA.	noted
McArn <i>et al.</i> (1968)	Puget Sound, WA.	5.2
McArn and Wellings (1971)	Bellingham Bay, WA.	4.8
Nigrelli <i>et al.</i> (1965)	No. Hecate Strait, B. C.	0
Stich and Acton (1975)	Vancouver, B. C.	58
Chung (pers. comm.)	Netarts Bay, OR.	noted
Williams (1975)	Yaquina Bay, OR.	noted
Olson (pers. comm.)	Yaquina Bay, OR.	22

nursery grounds have also been reported (Laroche and Holton, 1979)

Wellings et al. (1964, 1965), McArn et al. (1968), Brooks et al. (1969), and Yamazaki et al. (1978) provided detailed descriptions of the morphology and histology of epidermal tumors on several flatfish species. Three distinct tumor morphologies were identified by these researchers, the angioepithelial nodule, the angioepithelial polyp, and the epidermal papilloma.

The smallest tumor, the angioepithelial nodule, is raised, has a smooth surface, measures between 1 and 4 mm and may be pigmented, unpigmented or reddish in coloration (Wellings et al., 1964, 1965; McArn et al., 1968; McArn and Wellings, 1971; and Miller and Wellings, 1971). The angioepithelial nodules consist mainly of hypertrophied dermis covered with slightly hypertrophied epidermis and are present on fish younger than 14 months old (Miller and Wellings, 1971).

The second tumor type, the epidermal papilloma, measures from 1 to 6 cm in diameter, has a convoluted surface and may be either pigmented or unpigmented (Wellings et al., 1964; 1965; McArn et al., 1968; McArn and Wellings, 1971; and Miller and Wellings, 1971). Epidermal papillomas are composed mainly of folded, hypertrophied epidermis which is supported by thin strands of dermal connective tissue (Wellings and Chuinard, 1964).

Flatfishes between 10 months and two years old were reported to have epidermal papillomas (Wellings et al., 1964; and Miller and Wellings, 1971).

Miller and Wellings (1971) identified a transitional form between the angioepithelial nodule and the epidermal papilloma on fish 8 to 14 months old. This stage involves a spreading, slightly hypertrophied edge around the raised angioepithelial nodule. Progression from angioepithelial nodules to epidermal papillomas has been observed in the laboratory (Wellings et al., 1964, 1965; Kelly, 1971; McArn and Wellings, 1971; Miller and Wellings, 1971; Angell et al., 1975; Stich et al., 1977B; and Campana, 1983).

The third tumor type, the angioepithelial polyp, was first observed by Wellings et al. (1965) on sand sole (Psettichthys melanostictus) and later identified on English sole and starry flounder (Platichthys stellatus) (McArn et al., 1968). These tumors are abruptly raised above the surrounding epidermis and convoluted. In contrast to the epidermal papilloma, angioepithelial polyps are composed mostly of vascular connective tissue with only slightly hypertrophied convoluted epidermis (Wellings et al., 1965; McArn et al., 1968; and McArn and Wellings, 1971).

Brooks et al. (1969) studied these epidermal tumors ultrastructurally and described a cell morphologically distinct from any known teleost cell; these were termed



"X-cells". X-cells, were found in the epidermis and dermis of all three tumor types on flathead sole, sand sole, and starry flounder and were distinguished by their large oval shape, large nucleus and prominent nucleolus (Brooks et al., 1969). X-cells had mitochondria that possessed only a few short rounded cristae and an endoplasmic reticulum that occasionally lacked ribosomes. The condensed cytoplasm appeared vacuolated and contained membrane bound granules and lipid concentrations. An extracellular coat between 250 and 500 A was observed around many X-cells. In addition, neighboring X-cells were separated by, but were unattached to envelope cells which were connected to each other by desmosomes (Brooks et al., 1969).

When examined with the light microscope, X-cells have a pale staining cytoplasm and nucleus and an eosinophilic nucleolus (Wellings et al., 1976). Peters et al. (1983) observed a nuclear inclusion within the X-cells of 20 to 30 percent of the epidermal papillomas examined.

X-cells have also been observed in tumors of Atlantic cod, Gadus morhua; yellowfin goby, Acanthogobius flavimanus; Pacific cod, Gadus macrocephalus; walleye pollock, Theregra chalcogramma; Pacific Ocean perch, Sebastes alutus; Laval's eelpout, Lycodes lavalaei; and black croaker, Cheilotrema saturnum (Morrison et al., 1982; Ito et al., 1976; Alpers et al., 1977; McCain et al., 1979;

Myers, 1981; Dresser and Kahn, 1982; and Kent et al., 1988).

The etiology of epidermal tumors on pleuronectid flatfishes has been the subject of numerous studies.

Wellings and Chuinard (1964) observed two putative viruses, a 45 nm particle and a 160 to 200 nm granular body enclosed in a membranous sac within the cytoplasm of neoplastic cells. A third possible viral particle (160 to 200 nm) had an electron lucent core with radiating rods, possibly capsomeres (Wellings et al., 1976).

Although these virus particles have been observed within affected cells by electron microscopy, no cytopathic effect or cell transformation was observed following inoculation of flatfish primary tissue cultures with papilloma homogenates (Wellings et al., 1976).

Although environmental pollutants have been suggested as factors in epidermal tumor etiology (Young, 1964; Cooper and Keller, 1969; Stich et al., 1976; and Cross, 1984), no causal relationship between pollutants and epidermal papillomas has been established (Mearns and Sherwood, 1974). In addition, tumorous fish have been collected from waters apparently free of domestic pollutants (Nigrelli et al., 1965; Levings, 1967; and Oishi et al., 1976).

Stich et al. (1976) and Peters and Watermann (1979) suggested that a synergistic relationship between a virus and environmental factors may contribute to tumor growth.

McArn et al. (1968) noted that the presence of erythrocytes, lymphocytes and plasma cells in the angioepithelial nodule resembled an inflammatory response rather than a true neoplasm. Brooks et al. (1969) reported the engulfment of X-cells by macrophages and suggested that X-cells could be a parasitic, unicellular organism.

Dawe (1981) and Dawe et al. (1979) presented evidence supporting the hypothesis that X-cells are parasitic protozoans. Through the use of DNA binding, Dawe et al. (1979) determined that the amount of nuclear DNA in X-cells from pseudobranch tumors of Pacific cod was about one third the amount found in non-tumorous cod blood cells. Some X-cells were multinucleate and others exhibited a type of mitosis similar to that described in some species of amoebae Bishop and Tate (1939) and reviewed by Singh (1975). Daughter X-cells also had minute masses of chromatin within the nucleus. The analysis of 26 enzyme systems revealed four to six extra isozymes in the pseudobranch tumor that were not present in 12 different normal tissues including normal pseudobranch. Dawe (1981) concluded that X-cells were not fish cells but were protistan in nature and possibly members of the amoeba family Hartmannellidae.

The presence of morphologically identical X-cells in pseudobranch tumors of Pacific cod and epidermal tumors of

pleuronectid flatfish may link these two conditions to a common etiology.

Many questions concerning epidermal tumors of pleuronectids remain to be answered in spite of research that intensified in the 1960's. Tumorous pleuronectid flatfishes occur in Puget Sound, Washington and in San Francisco Bay, California, but little is known about the prevalence and intensity of tumorous sole in Yaquina Bay, Oregon. Likewise, the etiology of the epidermal tumors on flatfishes and the nature of X-cells remains uncertain.

In the context of this thesis, the word "tumor" is defined as a tissue swelling and does not necessarily imply neoplasia.

This study had three specific objectives. The first was to determine the prevalence and intensity of epidermal tumors on English sole in Yaquina Bay, Oregon. The second objective was to document the rate of tumor development by observing individual tumorous fish in the laboratory for an extended period of time. The final objective was to attempt to culture X-cells on artificial media and as primary tissue cultures in an effort to determine whether X-cells were protozoans.

## II. MATERIAL AND METHODS

Juvenile English sole were collected in Yaquina Bay monthly during 1986 and early 1987 with a 5 meter, semi-balloon trawl towed from the R/V Sacajawea. The sampling site was approximately one km from the mouth of Yaquina Bay (Fig. 1) and was characterized by a sand/silt substrate, considerable tidal exchange, and seasonal salinity fluctuation (Klum and Byrne, 1966).

Tows were limited to 10 minutes each and the sampling continued until 40 or more English sole were captured. All English sole were transported live to the Hatfield Marine Science Center for examination.

In the laboratory, the standard length of each fish was recorded before examination for tumors. The location of each tumor was recorded and a random group of tumors was measured and categorized as angioepithelial nodules, transitional, or epidermal papillomas according to their gross morphology.

Adult English and petrale sole (Eopsetta jordani) with epidermal papillomas were obtained from the Oregon Department of Fish and Wildlife and Oregon State University researchers. These fish were examined, tumorous tissues fixed in Bouin's solution and otoliths were removed for age determination.

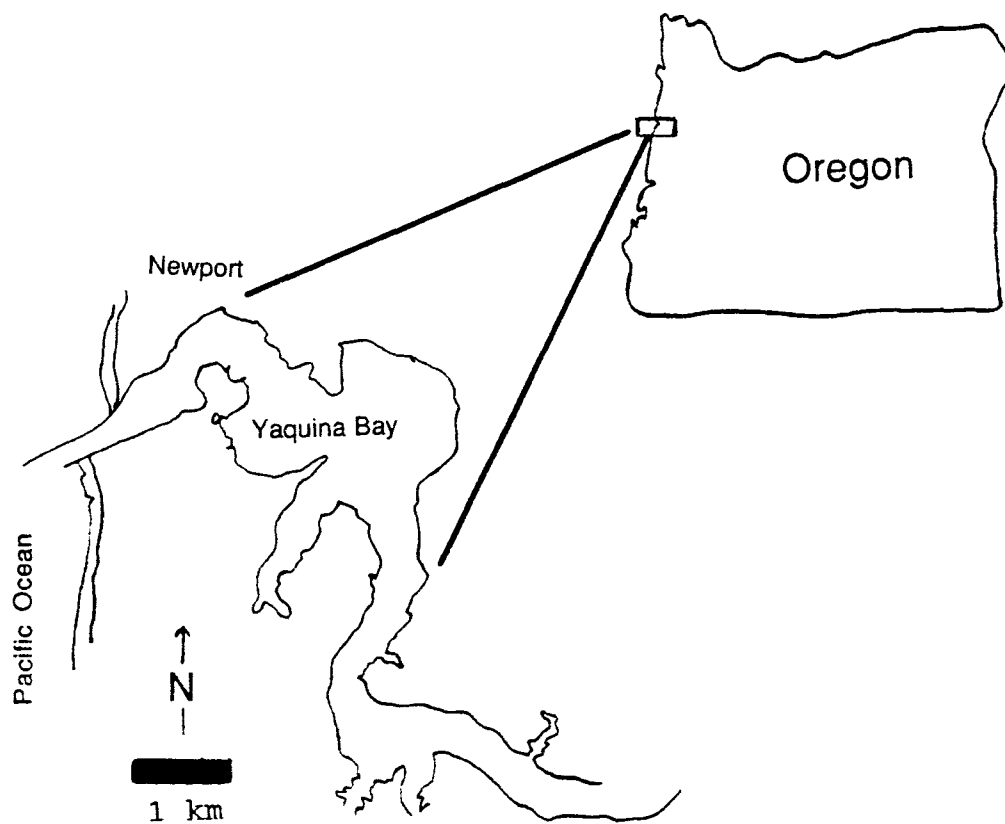


Figure 1. Map of collection site, Yaquina Bay, Oregon.

Selected tumorous English sole were held in individual compartments (25 cm long, 22 cm wide, 9 cm high) within a fiberglass tank to observe any change in the size and appearance of individual tumors. Individual compartments were constructed of plexiglass drilled with holes to allow water flow throughout the tank. Pathogen-free water entered the tank in five compartments and exited at the opposite end of the tank. Gravel was placed in each compartment to prevent fish from escaping into adjacent compartments.

Tumorous English sole between 18 and 30 mm that were selected for tumor observations were first fed brine shrimp nauplii (Artemia sp.) and ground up clams. Once these fish had begun to feed actively, they were maintained on a gelatin based diet modified from Peterson et al. (1967). Larger fish were initially fed fresh clams prior to being fed the gelatin based diet (Appendix 1).

Approximately every two weeks, the fish under observation were placed on a photographic copy stand and photographed with a 35 mm camera equipped with a macro lens. At this time both the fish and tumors were measured and changes in tumor appearance recorded.

Twice during the observation period, the removal of the monogenetic trematode, Gyrodactylus sp. was necessary when numerous flukes were observed on several fish. This

ectoparasite was eliminated by immersing infected fish in a 1:6000 formalin solution for half hour.

Normal epidermal tissue and tumors at various stages of development were prepared for histologic examination. All tissues were fixed in Bouin's solution for at least 24 hours prior to dehydration in ethyl alcohol. Dehydrated tissues were held in toluene overnight before infiltration with paraffin, then embedded in paraffin blocks using a Tissue Tek II, Tissue Embedding Center. Tissue blocks were sectioned from 5 to 7 microns on an American Optical, Spencer 820 microtome. Sections were affixed to the slide with Mayer's albumen affixative and subsequently stained with either Harris hematoxylin and eosin, iron hematoxylin, Giemsa, or Feulgen's nuclear stain. Sections were studied and photographed with an Olympus BH2 microscope fitted with an Olympus C35AD camera.

To prepare tissue imprints, the cut surface of the epidermal papilloma was dabbed onto a microscope slide, allowed to air dry, then floated face down for 5 minutes on Bouin's solution for fixation. The slide was then rinsed with distilled water and stained with either Giemsa, iron hematoxylin or Feulgen's nuclear stain.

Culture experiments were conducted using a malt-yeast-seawater agar known to support the growth of Paramoeba, a marine amoeba (Jones and Scheibling, 1985). Epidermal papillomas were swabbed with iodophor, minced with scalpels



and incubated for an hour in an antibiotic incubation mix of penicillin-streptomycin (Sigma), Fungizone (Sigma) and Gentamicin (see Appendix 2) before being placed in a petri dish of sterile malt-yeast-agar (see Appendix 3) and overlaid with sterile seawater or fish saline. A portion of the fluid overlay was removed daily for 2 weeks and examined for amoeboid protozoans.

For preparation of primary cell cultures, epidermal papillomas were disinfected with iodophor, minced with scalpels and incubated in the same antibiotic incubation mix for one hour. Minced tissues were placed in a 25 ml tissue culture flask and associated fluids drained for 30 minutes to promote adherence of explants to the flask. Five ml of medium 199 (Sigma), supplemented with 20% fetal bovine serum (FBS), penicillin and streptomycin (see Appendix 4) was added to each flask. Cultures were incubated at 18° C and inspected daily with a Wild M40 inverted microscope. After numerous cells had accumulated in the medium, the culture fluid was removed and centrifuged at 1500 rpm for 5 minutes to concentrate cellular debris. Equal parts of fresh growth medium and clarified medium totaling 5 ml was returned to the culture flask and incubation continued. Cells in one primary culture were fixed with cold absolute methanol and stained with May-Grünwald-Giemsa. Cells in this culture were

examined with a compound microscope for amoebas and evidence of cell division.

Several epidermal papillomas were prepared for examination by electron microscopy. Epidermal papillomas were cut into 1 mm<sup>3</sup> pieces and fixed in 10% gluteraldehyde for 24 hours. Tissues were then rinsed and stored in Hank's balanced salt solution (BSS) until further processing at the Oregon State University, Electron Microscope Service Laboratory. Stored tissues were post-fixed in 1% osmium tetroxide for 1 hour prior to dehydration in acetone. Dehydrated tissues were then infiltrated and embedded in Spurr's plastic resin. Samples were sectioned between 800 and 1100 Å on a Porter-Blume ultramicrotome. All sections were stained with lead citrate and uranyl acetate, placed on copper grids, and examined with a Phillips 300 transmission electron microscope.

### III. RESULTS

#### Fish Collection

Both normal and tumor-bearing juvenile English sole were captured in Yaquina Bay each month from January, 1986 to March, 1987. The catch-per-unit-effort (CPUE) was low from January to April, 1986 and ranged from 7.5 to 23.2 sole per 5 minute trawl (Fig. 2). At this time of year, newly metamorphosed fish (18-22 mm) are known to enter the estuary (Laroche et al., 1982; and Rosenberg and Laroche, 1982). Fish up to 30 mm in length were considered to be relatively recent arrivals into the estuary. Fish of this size group were collected through June but were most numerous in May when 52.7 percent of the fish captured were smaller than 31 mm. The CPUE was highest from May through August when it varied from 92 to 127 fish per trawl. The CPUE then declined from September through December when juvenile English sole are known to emigrate from Yaquina Bay (Olson and Pratt, 1973). Emigration from the bay was nearly complete by November although a few over-wintering sole were collected through March, 1987 (Fig. 2 and 3). Length frequency distribution data revealed that the bulk of the fish left the bay prior to reaching 110 mm in length (Fig. 3). Only 1.7 percent of all sole collected were larger than 110 mm.

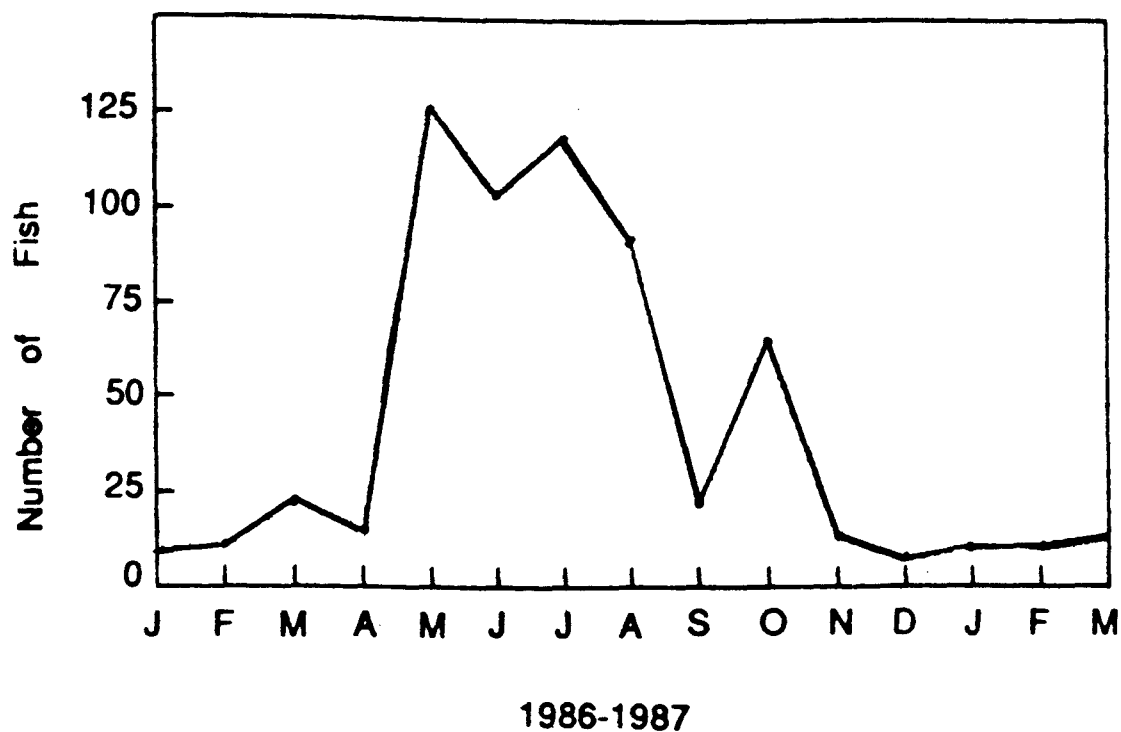


Figure 2. Catch-Per-Unit-Effort for juvenile English sole from Yaquina Bay, Oregon.

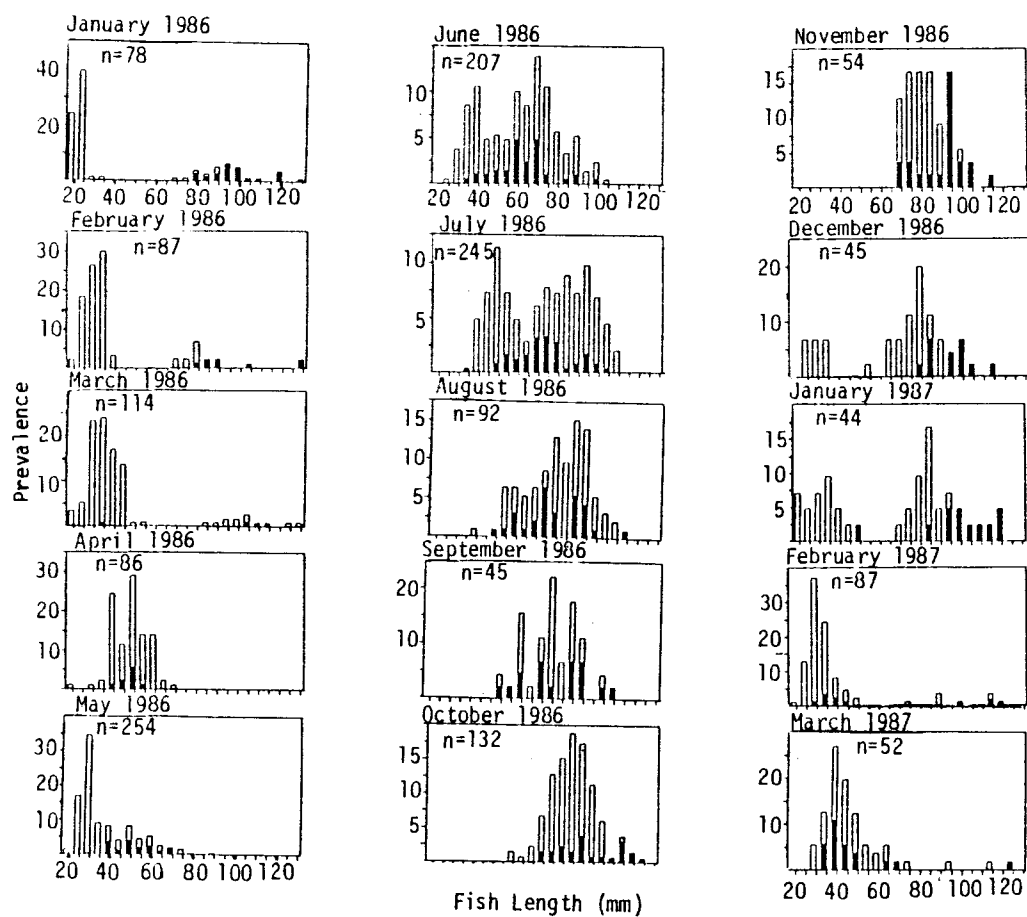


Figure 3. Length frequency distribution of juvenile English sole.  
 ■ Tumorous fish      □ Non-tumorous fish

The average length of all juvenile English sole by month of collection was estimated (Figure 4). The absence of over-wintering sole and a large influx of young-of-the-year fish in May resulted in the lowest average length observed (36.1 mm). The average length peaked at 88.5 mm in October then declined as a result of emigration by larger fish.

Juvenile English sole captured from January to March, 1986 and from December to March, 1987 consisted of two size groups. The smaller fish (<110 mm) represented the young-of-the-year sole while larger individuals were fish that did not emigrate in the fall.

#### Gross Morphology of Tumors

Macroscopic examination of epidermal tumors revealed two distinct morphologic types, the angioepithelial nodule and epidermal papilloma. Also observed was a spreading, plaque-like edge (Wellings et al., 1965) surrounding some angioepithelial nodules and epidermal papillomas.

Angioepithelial nodules were smooth surfaced, abruptly raised above the surrounding epidermis and located on either side of the fish (Fig. 5). Nodules were generally pigmented when present on the dextral side and unpigmented on the sinistral side. Other tumors that also had characteristics of angioepithelial nodules were reddish in

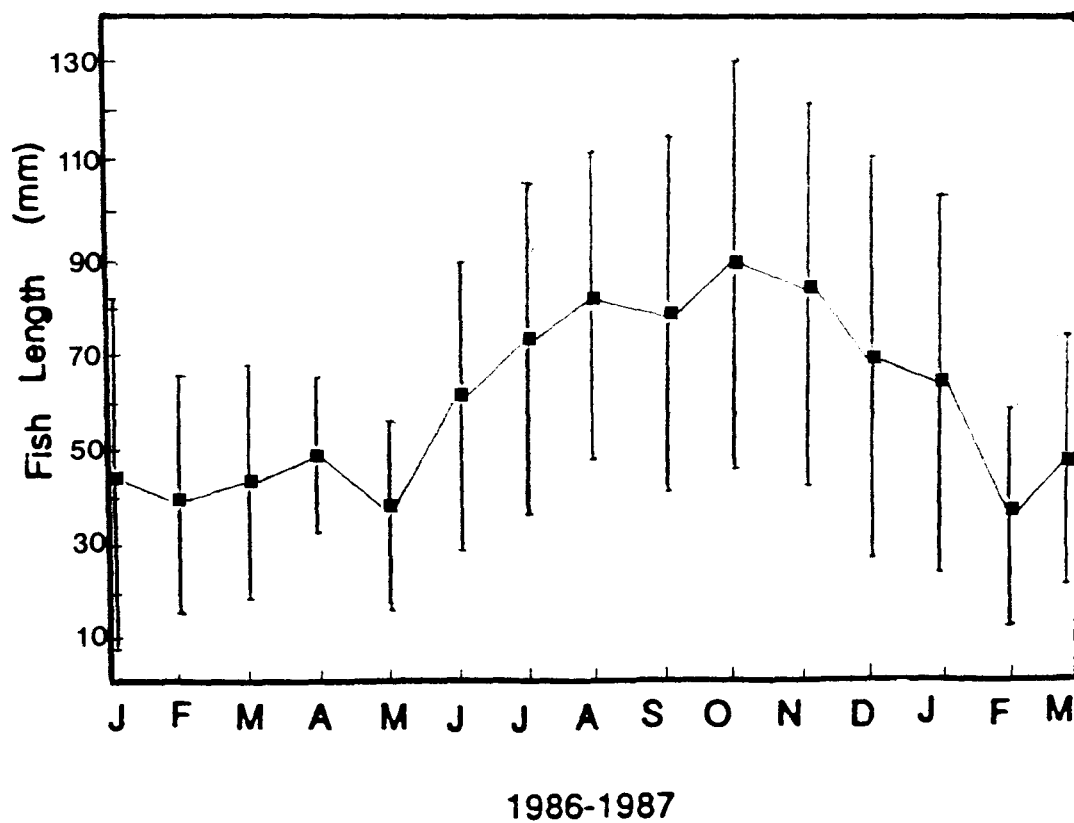


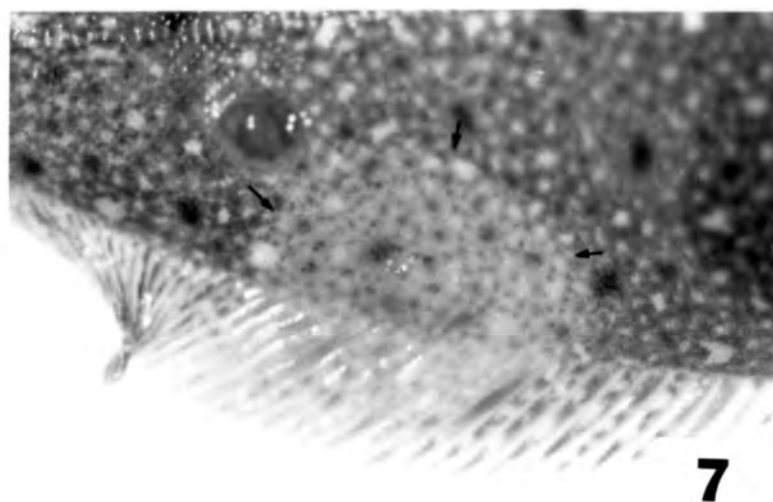
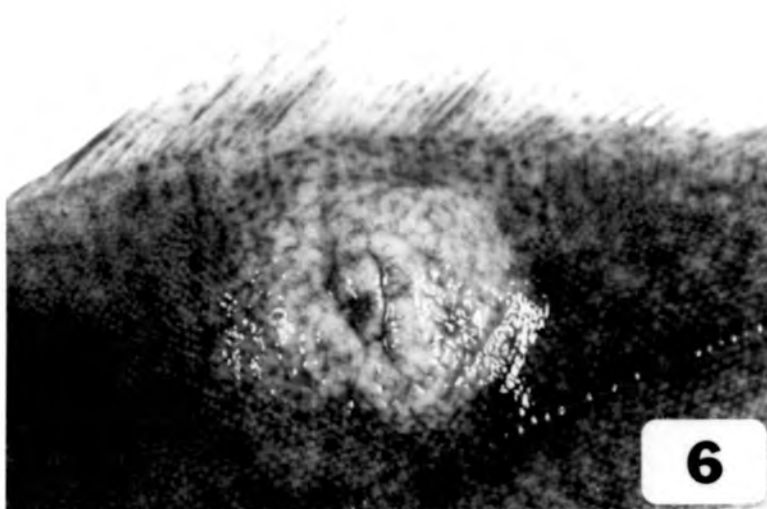
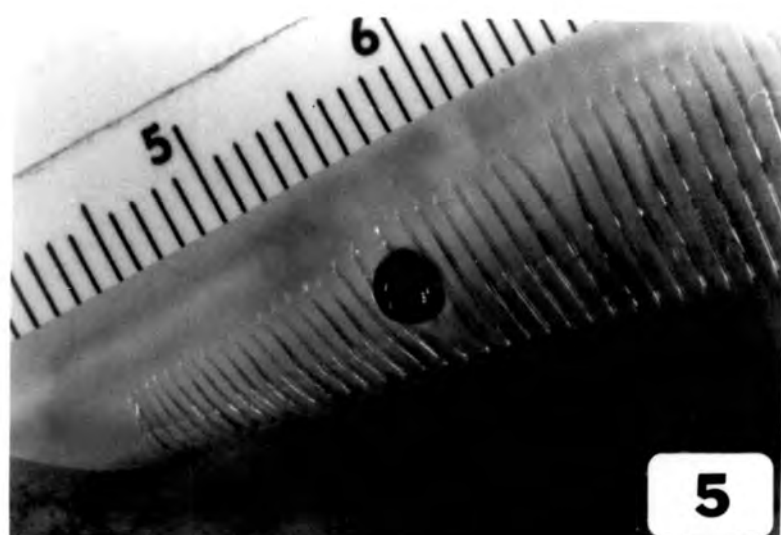
Figure 4. Monthly average length of all English sole.

Figure 5. An angioepithelial nodule on the anal fin of a 97 mm English sole (Parophrys vetulus).

Figure 6. An epidermal papilloma on an 87 mm English sole (Parophrys vetulus).

Figure 7. A plaque-like, spreading area on a 77 mm English sole (Parophrys vetulus). (Arrows indicate the edge of the plaque-like area).





coloration due to intense vascularization of the tissue. Nodules up to 8 mm in diameter were observed.

The second morphologically distinct tumor, the epidermal papilloma, was characterized by a raised convoluted surface of hyperplastic epidermis (Fig. 6). These tumors, which were found on both sides of the fish, usually had pigmentation similar to the surrounding tissue. Papillomas ranged in size from 4 to 45 mm and extended up to 15 mm above the adjacent, unaffected tissue.

A slightly raised, spreading plaque-like edge was also observed around both angioepithelial nodules and epidermal papillomas on either side of the fish (Fig. 7). The coloration of this area was slightly lighter than the unaffected tissue but corresponded to the gross pigmentation of the surrounding epidermis.

#### Tumor Occurrence on English Sole From Yaquina Bay

A total of 18.7 percent (302/1612) of the English sole captured during this study had angioepithelial nodules and/or epidermal papillomas.

Tumorous fish averaged 2.8 tumors each with a mode of one tumor (Fig. 8). The maximum number of epidermal tumors observed was 19 on a 98 mm sole collected January, 1987.

Approximately 50 percent of the tumorous fish had only angioepithelial nodules, while fish with only epidermal papillomas comprised 27 percent of the tumorous fish

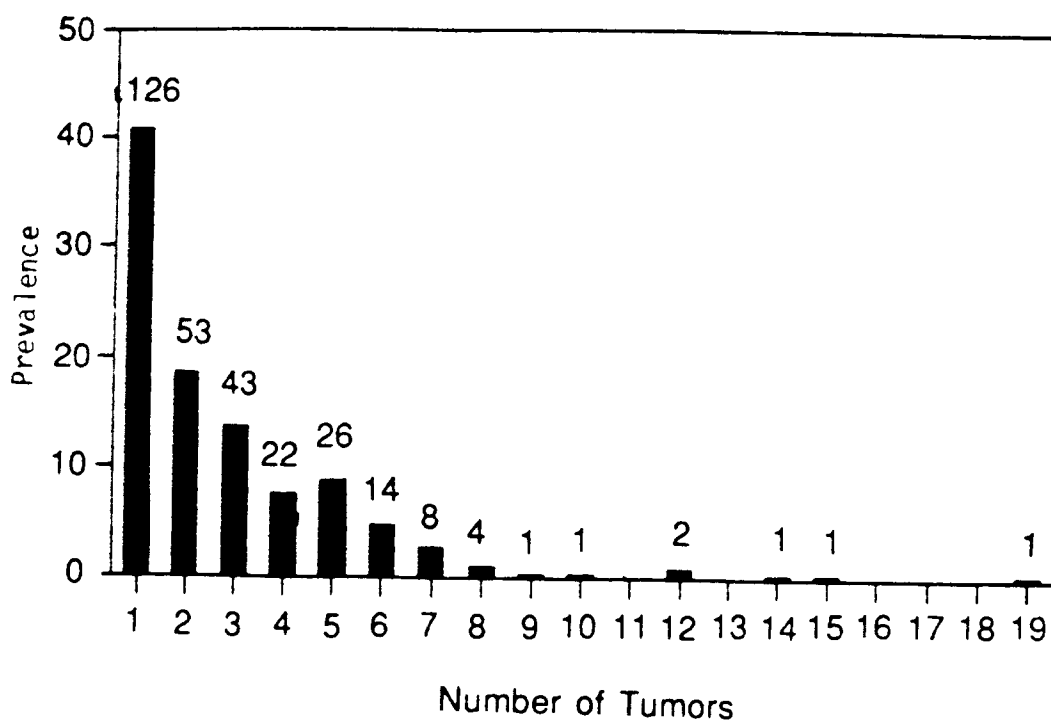


Figure 8. Frequency distribution of the number of tumors per affected English sole. Number above each bar represents the number of tumorous fish with that number of epidermal tumors.

collected. Twenty-three percent of the tumor-bearing fish had both angioepithelial nodules and epidermal papillomas.

Fish with angioepithelial nodules averaged 59 mm in length, ranged in size from 28 to 126 mm and were captured throughout the year.

Sole that had epidermal papillomas averaged 87.9 mm in length (range 52-130 mm). Young-of-the-year fish with epidermal papillomas were first observed in June and then were found on fish collected each month thereafter.

The plaque-like, spreading lesions ranged in diameter from 3 to 33 mm and were present on fish from 39 to 126 mm in length. These lesions were not observed on age-0 fish prior to May but were noted on fish captured each succeeding month.

Fish that had both angioepithelial nodules and epidermal papillomas averaged 90.5 mm in length and ranged in size from 57 to 130 mm.

Chi-square analysis was conducted to determine if tumor location significantly differed with respect to the dextral (eye side) and sinistral (eyeless) side of the fish. Both tumor types were pooled for this analysis. A significantly greater number of tumors were present on the dextral side than on the sinistral side. The results of this analysis are given in Table 3.

Table 3. Chi-square analysis for the significance of difference in tumor occurrence on the dextral and sinistral side. Expected values in parenthesis.

	Dextral side	Sinistral side	Total	$\chi^2$
total	319 (285)	251 (285)	570	8.11

$\chi^2_{.05} = 3.84$ , one degree of freedom.

### Relationship Between Tumor Occurrence and Fish Length

The average length of tumorous and normal fish by month was calculated (Figure 9). Tumorous and non-tumorous fish collected between April and October, were similar in length. During this period, the average length of unaffected fish increased from 47 to 77.7 mm while tumor-bearing fish increased in length from 47.6 to 80.1 mm. The average length of juvenile English sole peaked during October and December at 88.5 and 100.7 mm for non-tumorous and tumorous fish respectively.

Major differences between the average length of fish with and without tumors occurred between January and March, 1986 and December to February, 1987 due to the presence of two size groups of English sole in Yaquina Bay. During these periods, the monthly average length of tumorous fish declined as the larger, age-0+ fish emigrated from the bay. The average length of unaffected fish captured from November to February decreased due to the immigration of smaller, young-of-the-year fish and emigration by large non-tumorous fish.

Epidermal tumors were infrequent on small English sole (16-30 mm). Examination of 149 fish between the lengths of 16 and 25 mm captured from January to June 1986, and December, 1986 and February, 1987, revealed no epidermal tumors. During the same period only 2 of the 191 fish

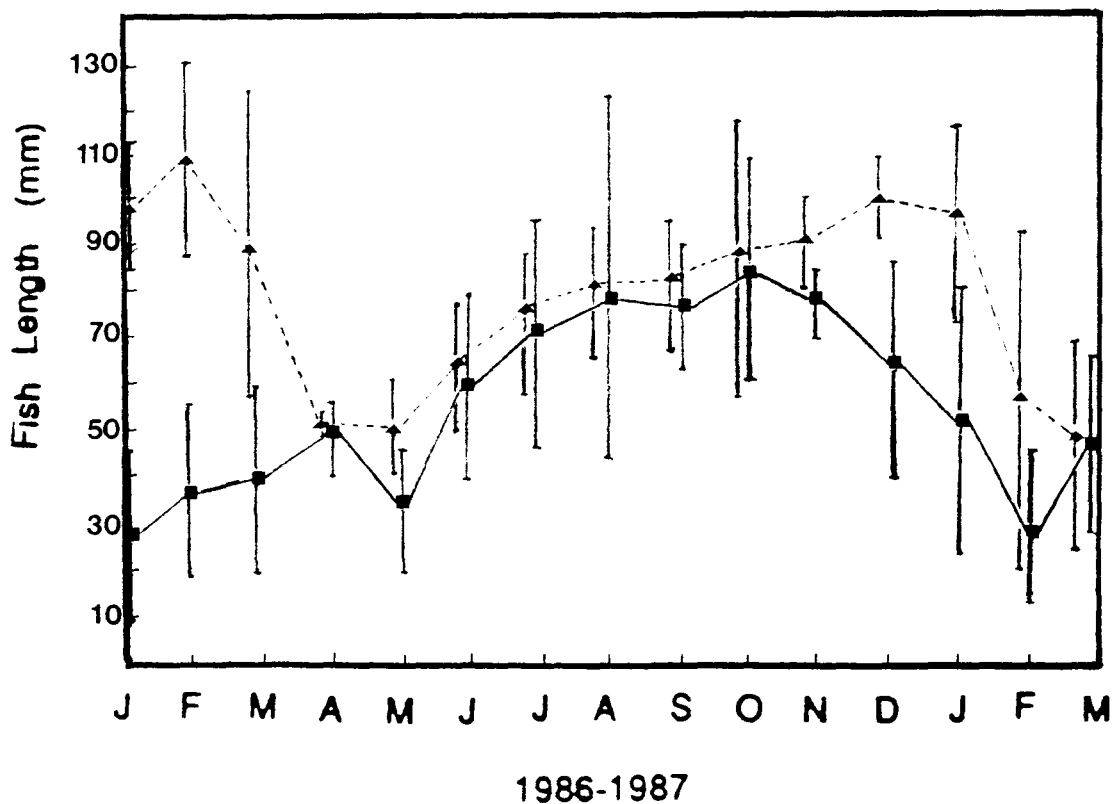


Figure 9. Monthly average length of tumorous and non-tumorous juvenile English sole.  
---▲ Tumorous fish    ■—■ Non-tumorous fish.

between 26 and 30 mm had tumors. The prevalence of tumorous fish increased to 6.9 percent for sole between 31 and 35 mm in length and varied from 12.6 to 100 percent in larger size intervals (Fig. 10). Seventy-one percent of the fish over 110 mm in length (n=42) had epidermal tumors.

Variation in tumor intensity with fish length is shown in Figure 11. Tumor intensity was generally higher for fish between 80 and 110 mm in length (2.7 to 5.5 tumors per fish) than for other length intervals that varied between 1.0 and 3.0 tumors per fish.

#### Relationship Between Tumor Occurrence and Season

The tumor prevalence was lowest during February and March, 1986 (4.6 and 5.3 percent) when only larger, over-wintering English sole possessed tumors. The prevalence of tumorous fish generally increased monthly until September when 35.6 percent of fish captured possessed epidermal tumors (Fig. 12).

The monthly tumor intensity for fish in the 1986 year class is shown in Figure 13. Tumorous young-of-the-year sole were not observed until March, 1986 and tumor intensity generally increased through the year and reached a maximum intensity of 5.7 tumors per fish in over-wintering sole collected in February, 1987.



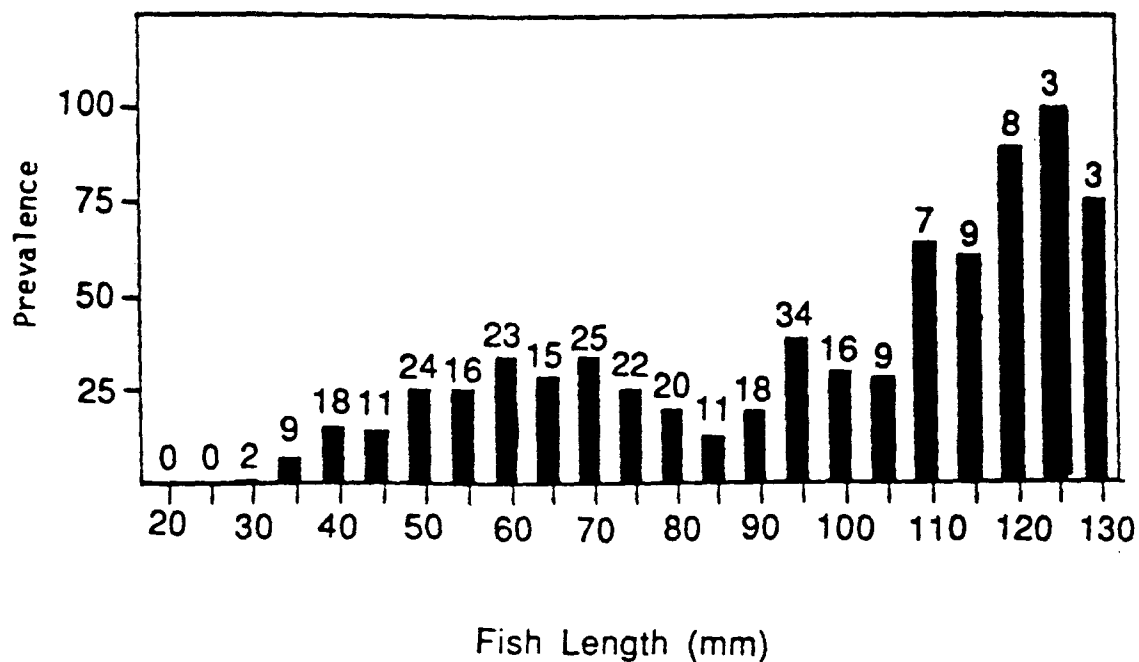


Figure 10. Relationship between tumor prevalence and fish length. Number above each bar represents tumorous fish in the interval.

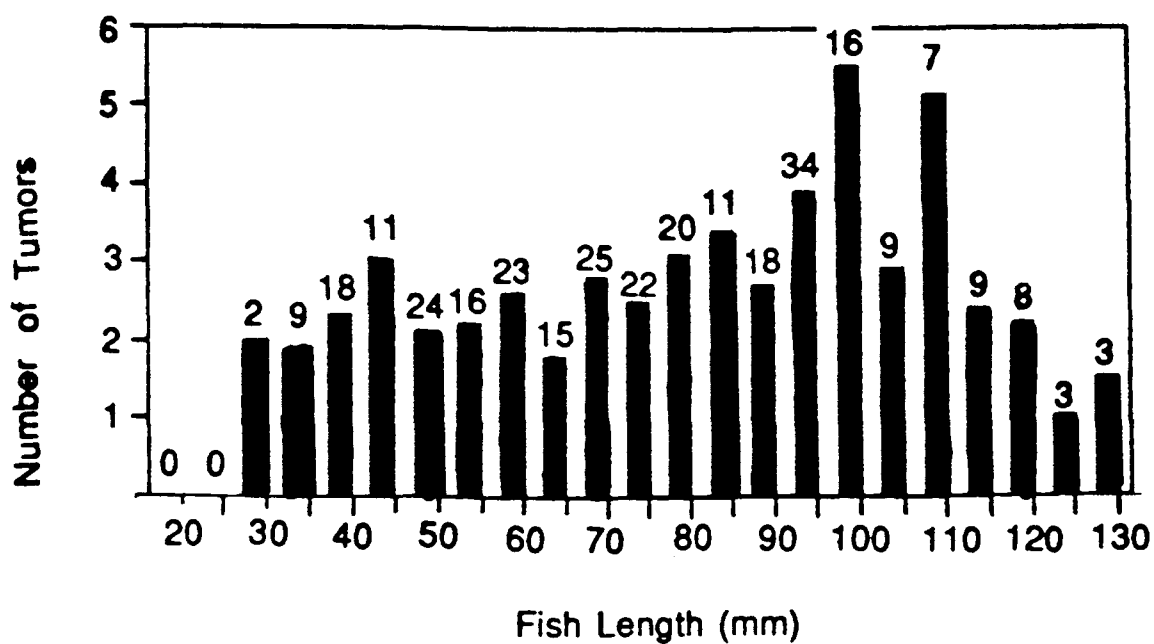


Figure 11. Relationship between tumor intensity and fish length. Number above each bar represents tumorous fish in the interval.

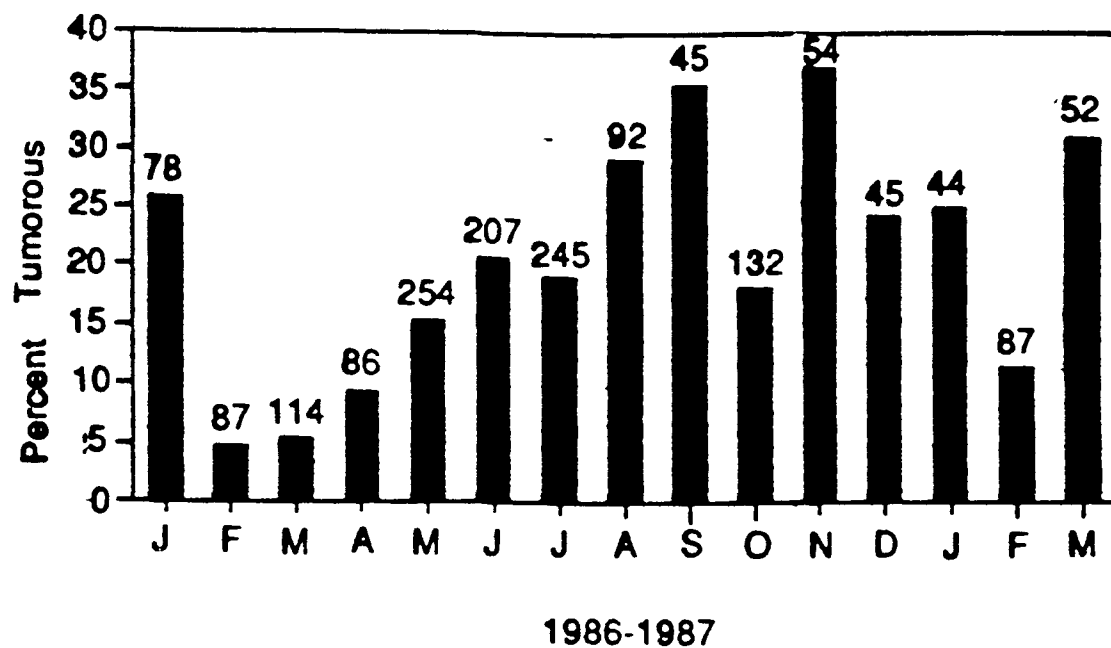


Figure 12. Relationship between tumor prevalence and month of collection. Number above each bar represents fish collected that month.

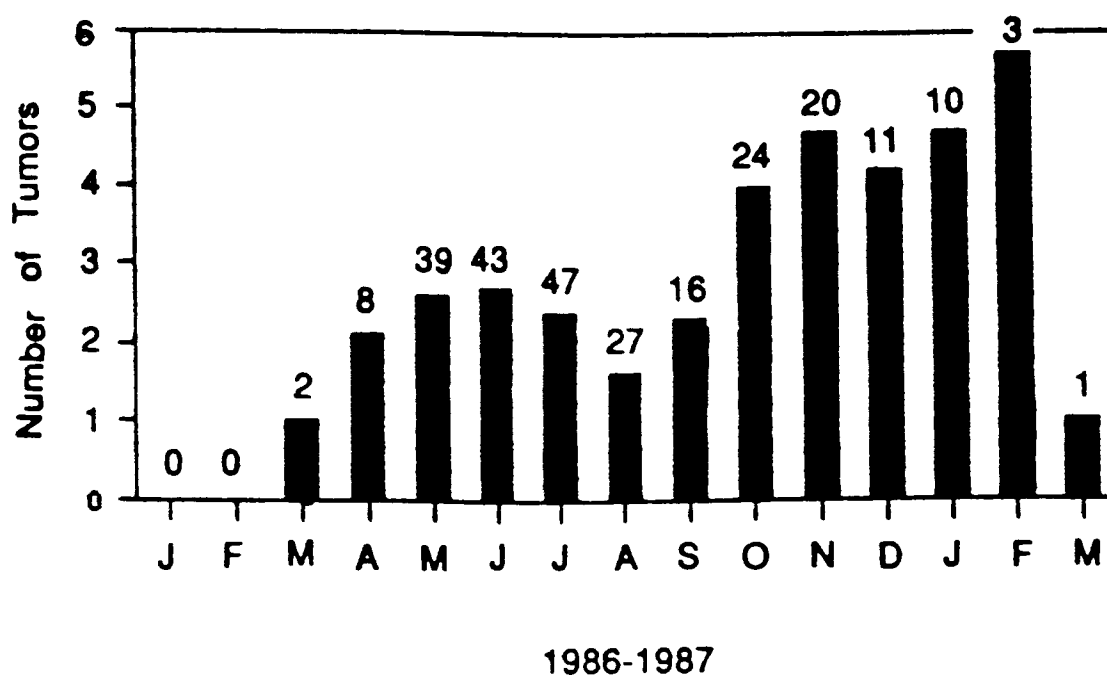


Figure 13. Relationship between tumor intensity and month of collection. Number above each bar represents tumorous fish collected that month.

### Tumor Occurrence on Adult English and Petrale Sole

Two adult English and four petrale sole with epidermal tumors that were grossly similar to those on juvenile English sole were caught off the Oregon coast by commercial and recreational fishermen.

The two English sole measured 20.2 and 27.2 cm and were estimated to be 2 and 3 years old based on the number of otolith annuli (Dan Nichol, personal communication). The smaller fish of the two had 5 epidermal tumors that measured from 5 to 30 mm in diameter. The larger sole had a single tumor 80 mm in diameter that extended onto both sides of the fish.

The four petrale sole each had 1 or 2 epidermal tumors that ranged in size from 9 to 70 mm in diameter. These fish measured between 23.2 to 34.1 cm in length and were estimated to be between 3 and 8 years old (Dan Nichol, personal communication).

### Observation of Tumors on Laboratory Held English Sole

Selected tumorous fish were maintained in the laboratory for observations of the morphological changes in epidermal tumors with time. Young-of-the-year fish without tumors were also held in the laboratory to determine if and when tumors would develop.

The first group comprised twenty-two juvenile sole each with one to eight epidermal tumors. These fish were

collected from February to September, 1986 and maintained in the laboratory from 42 to 349 days. Tumors that were initially categorized as angioepithelial nodules either regressed in size, developed into epidermal papillomas or showed little change in gross morphology. Tumors that were considered to be epidermal papillomas at the start of the observation period showed little morphological change. Both the regression of angioepithelial nodules or their progression into epidermal papillomas occurred on the same fish.

Examples of both gradual and abrupt disappearance of angioepithelial nodules and the development of angioepithelial nodules into epidermal papillomas are described in Cases I-III.

#### Case I

Twelve angioepithelial nodules on 7 fish gradually regressed during a 101 day period in the laboratory. An example of this type of change occurred on a 67 mm English sole that had a single angioepithelial nodule. This fish was collected on July 17, 1986 and observed for 143 days. Initially the nodule measured 7 mm, was reddish in color, smooth surfaced and abruptly raised above the surrounding tissue (Fig. 14A).

Figure 14. Case I. The regression of one angioepithelial nodule on a 67 mm English sole (Parophrys vetulus). A) Day 1; B) Day 18; C) Day 31.





Thirteen days after capture, the nodule measured only 4 mm but otherwise appeared unchanged. Five days later, the tumor remained 4 mm in diameter but was reduced in height. An edge of this angioepithelial nodule graded gradually into the adjacent tissue and several capillaries were visible within the clear tumorous tissue (Fig. 14B). By the 31st day, the nodule was gone and only a slightly depressed 2 mm area remained where the tumor had been (Fig. 14C). The tissue in this area appeared normally pigmented 82 days after tumor loss. No recurrence of tumor growth was observed.

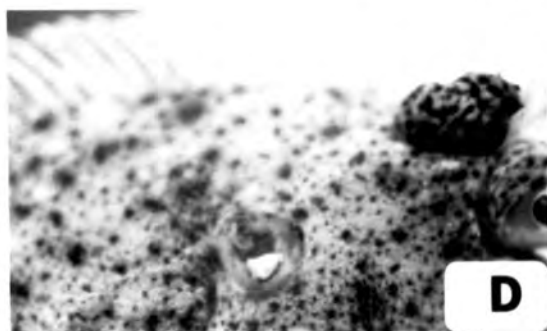
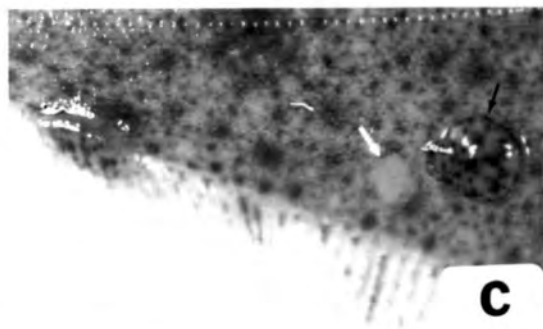
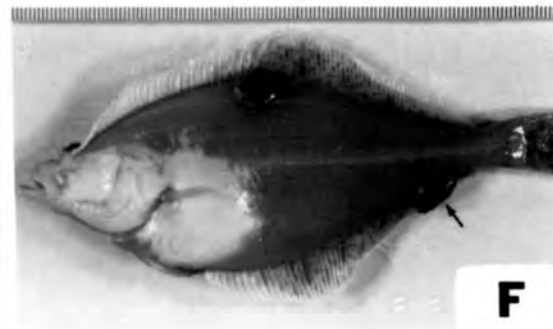
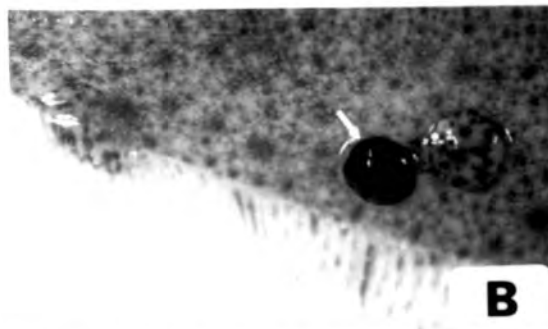
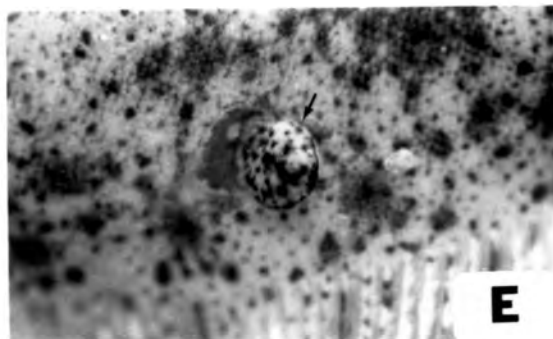
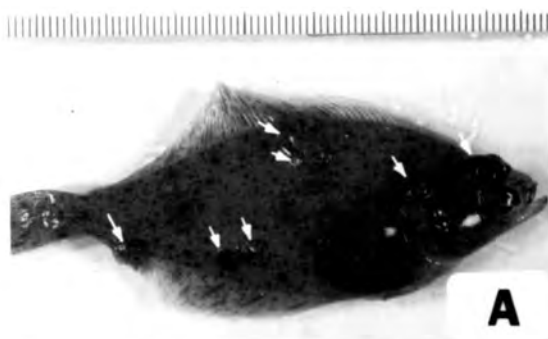
## Case II

The abrupt disappearance of 13 angioepithelial nodules occurred on 9 fish. One of these fish was typified by a 71 mm English sole with 5 pigmented tumors on the dextral side, one unpigmented tumor on the sinistral side and one anal fin tumor (Fig. 15A). This fish was collected on September 10, 1986 and observed for 120 days. Tumors initially measured from 3 mm to 6 mm in diameter, were round, smooth surfaced and abruptly raised above the surrounding tissue.

One angioepithelial nodule completely regressed between the 21st and 31st day in the laboratory. This 4 mm nodule became reddish in color prior to its disappearance

Figure 15. Case II. Eight angioepithelial nodules on a 71 mm English sole (Parophrys vetulus).

A) Day 1, arrows indicate nodule location in addition to one on the sinistral side; B) Day 21, one nodule reddish in color (white arrow); C) Day 31, nodule missing; D) Day 62, nodule on head region missing; E) Day 62, same nodule as in figure 15C (black arrow); F) Day 112; G) Day 120, compare with figure 15F; H) Day 120, two nodules developed into epidermal papillomas (arrow).



(Fig. 15B). The tissue remaining at the site of the tumor was less pigmented than was the adjacent epidermis (Fig. 15C).

Another 4 mm angioepithelial nodule disappeared between the 53rd and 62nd day of observation (Fig. 15D). By the 82nd day in the laboratory, a cavity initially visible in the epidermis where the tumor had been was no longer visible.

On the 62nd day of observation a 5 mm tumor was partially detached from the trunk of the fish (Fig. 15E). Two days later, the tumor was absent and a shallow cavity with lightly pigmented tissue remained at the site (Fig. 15D). This area was no longer distinguishable 18 days later.

By the 64th day in the laboratory, a 3 mm nodule above the left eye increased in pigmentation and no longer had an entirely smooth surface (Fig. 15D). Five days later, this tumor had disappeared and only typically pigmented tissue was evident.

During the first 112 days, the tumor on the anal fin increased in size from 6 to 15 mm in diameter, had become reddish in color and its surface changed from smooth to slightly convoluted (Fig. 15F). Four days later, the tumor was reduced to 10 mm but other features of the tumor were unchanged. On the 120th day of observation, the tumor was reduced in diameter to 8 mm, exposing several fin rays and

was unpigmented (Fig. 15G). This fish died when the water flow to the tank was inadvertently left off.

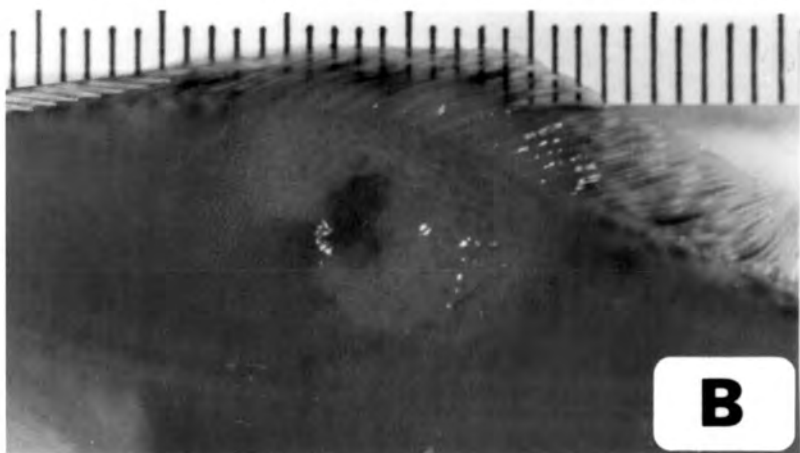
In summary, the complete regression of 4 tumors and the partial regression of a fifth was observed while two angioepithelial nodules developed into epidermal papillomas by the 53rd day after capture and the angioepithelial nodule on the sinistral side increased in size but did not change in gross morphology (Fig. 15H).

### Case III

A total of 13 angioepithelial nodules on 8 fish developed into epidermal papillomas. A typical example of this change occurred on a 59 mm English sole collected on February 5, 1986. This fish developed a single angioepithelial nodule on the sinistral side 48 days after capture.

This nodule, which initially measured 1 mm, increased to 4 mm in diameter after 51 days (Fig. 16A). Despite the increase in diameter, the general appearance of the angioepithelial nodule remained unchanged. After 94 days, the tumor had a convoluted, raised 4 mm center, typical characteristics of an epidermal papilloma, and had spread over a 12 mm area that extended onto the dorsal fin (Fig. 16B). On the 162nd day of observation, the tumor involved a 34 x 27 mm area and was partially pigmented (Fig. 16C). By the 234th day of observation, the tumor

Figure 16. Case III. The development of an angioepithelial nodule into an epidermal papilloma on a 59 mm English sole (Parophrys vetulus). A) Day 51; B) Day 94, epidermis is slightly convoluted; C) Day 162; D) Day 349.



covered a 52 x 36 mm area (Fig. 16D). At final observation after 349 days, the papilloma was slightly raised above the adjacent epidermis, measured 55 x 50 mm sinistral side and extended 9 mm onto the dextral side.

#### Laboratory Observations of Normal Young-of-the-Year Sole

The second group of fish under observation consisted of 72 young-of-the-year sole (20-49 mm) without tumors that were captured on February, 1987. These fish were examined each week for 2.5 months and 21 angioepithelial nodules developed on 18 fish between 24 and 56 days after capture. No new angioepithelial nodules developed on fish without tumors after the 56th day and no further observations were made after the 70th day.

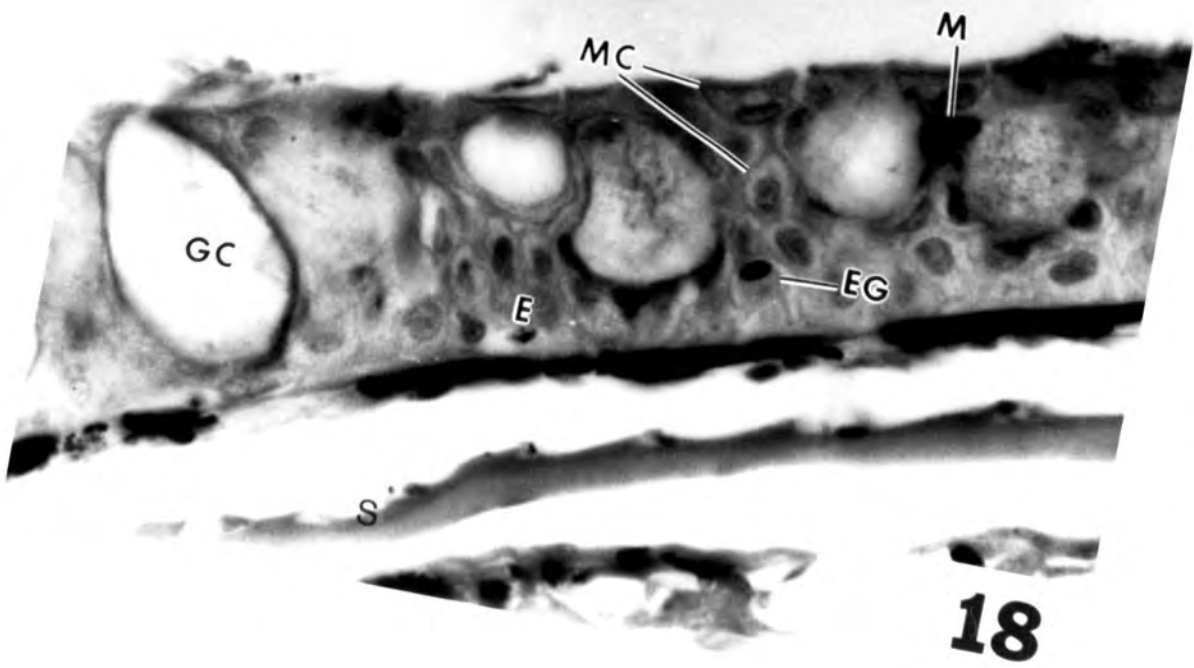
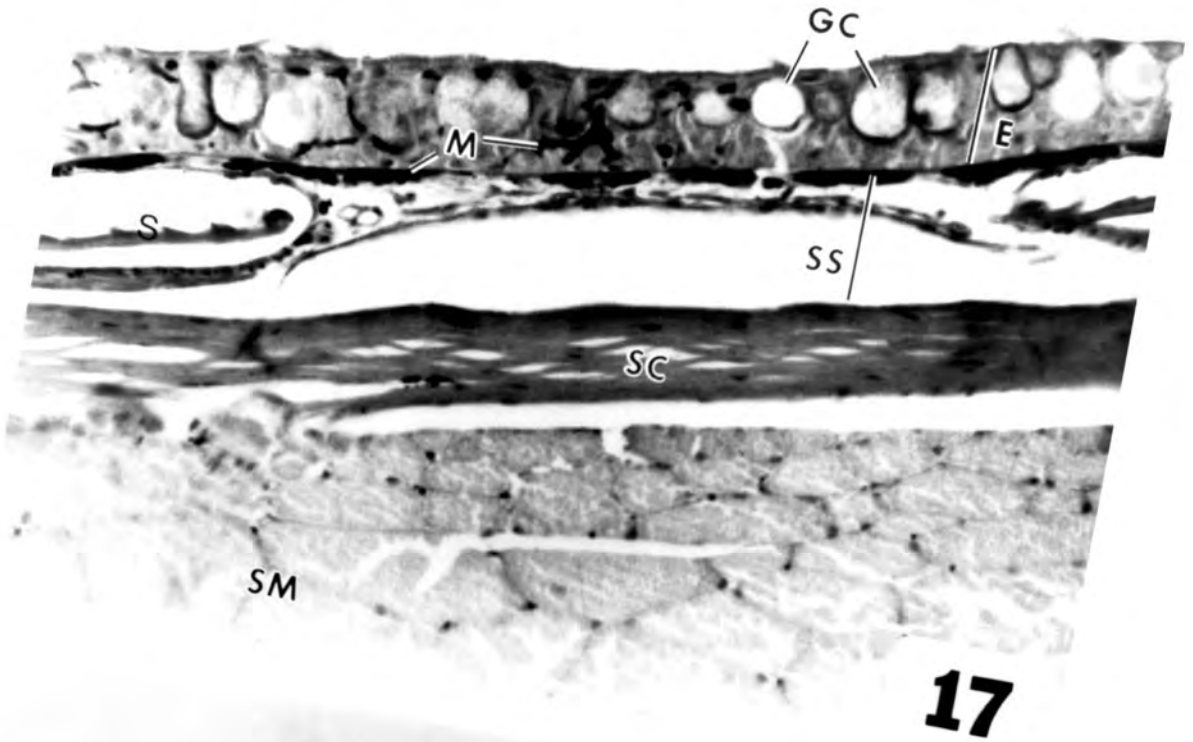
#### Normal Tissue Histology

The epidermis of normal juvenile English sole averaged 30 micrometers in thickness (24 to 41  $\mu$ m) and was from 4 to 7 cells deep (Fig. 17). The major cellular component of the epidermis was Malpighian cells (Bullock and Roberts, 1974; and Yasutake and Wales, 1983). These were pleomorphic, with a lightly eosinophilic cytoplasm and basophilic, granular nucleus. Mucous cells were numerous and often clustered near the surface of the epidermis (Fig. 18). Eosinophilic granule cells (Roberts *et al.*, 1971; and Bullock and Roberts, 1974), were present near the basal



Figure 17 Section of normal integument of English sole (Parophrys vetulus). (E = epidermis; SC = stratum compactum; SS = stratum spongiosum; S = scale; GC = goblet cells; M = melanophores; SM = skeletal muscle) (hematoxylin and eosin) (400 x).

Figure 18 Section of normal integument of English sole (Parophrys vetulus). (E = epidermis; GC = goblet cells; MC = Malpighian cells; EG = eosinophilic granule cells; M = melanophores; S = scale) (hematoxylin and eosin) (1000 x).



layer of the epidermis and branching melanophores were common among the Malpighian cells (Fig. 17 and 18). The basal lamina of the epidermis was intensely eosinophilic. Beneath this epidermal/dermal junction, a layer of melanophores often formed a band parallel to the epidermis.

Directly beneath the epidermal/dermal junction was the stratum spongiosum, a layer of loosely arranged fibroblasts and collagen fibers in which scales were embedded (Fig. 17). Beneath the stratum spongiosum was the stratum compactum which consisted of a dense band of collagen fibers and fibroblasts (Fig. 17).

#### Angioepithelial Nodule Histology

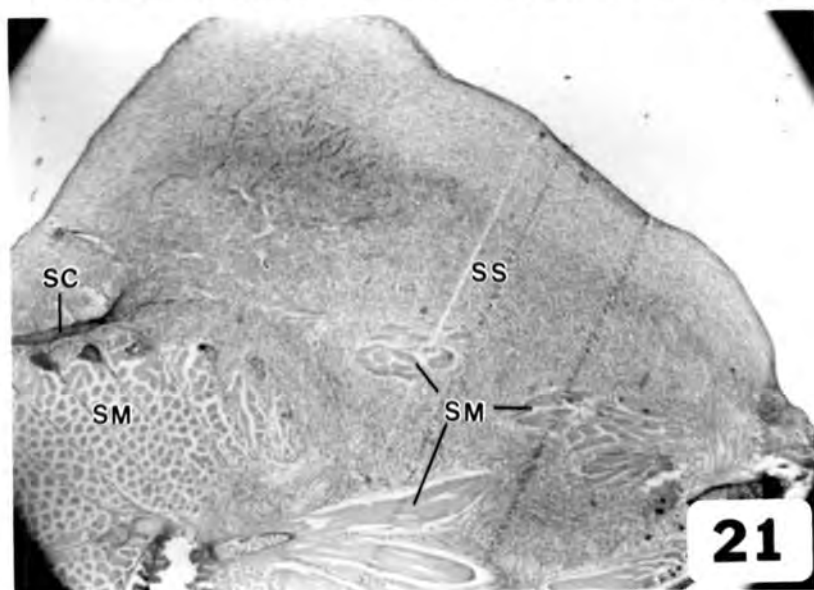
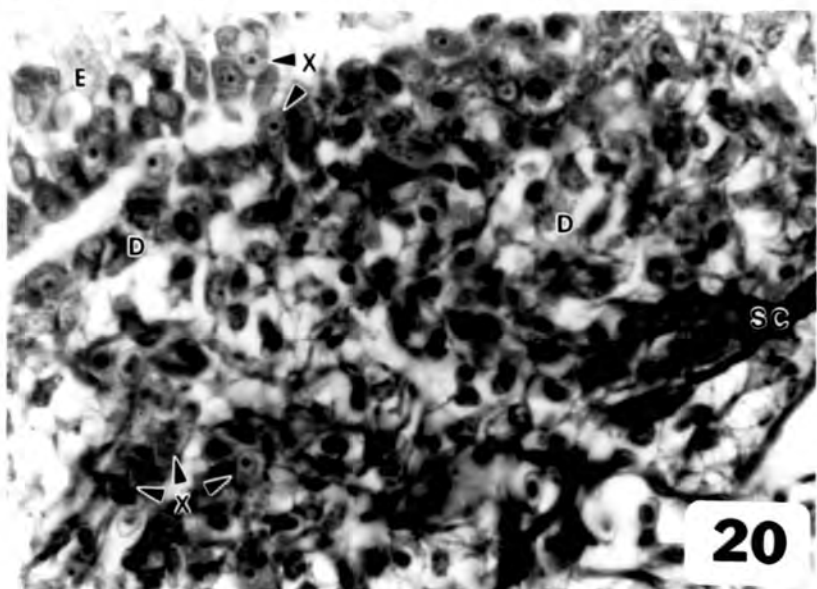
Angioepithelial nodules consisted mostly of a highly vascularized, hyperplastic stratum spongiosum covered by a thickened epidermis (Fig. 19).

The hyperplastic epidermis of an angioepithelial nodule averaged 73 micrometers in thickness, (27-164  $\mu$ m) and consisted mainly of Malpighian cells. Mucous cells were either absent or were less abundant than in normal integument while eosinophilic granule cells were common. X-cells were present but uncommon in the epidermis and averaged 7.7 micrometers in width and 9.6 micrometers in length (6.5 to 10.0  $\mu$ m wide and 7.1 to 13.5  $\mu$ m long). These X-cells had an eosinophilic cytoplasm, a large, pale staining nucleus and an eosinophilic nucleolus.

Figure 19. Section of an angioepithelial nodule on English sole (Parophrys vetulus).  
(E = epidermis; SC = stratum compactum; SS = stratum spongiosum; GC = goblet cells; R = fin rays) (hematoxylin and eosin) (100 x).

Figure 20. Section of an angioepithelial nodule on English sole (Parophrys vetulus).  
(E = epidermis; D = dermis; SC = stratum compactum; X = X-cells) (hematoxylin and eosin) (1000 x).

Figure 21. Section of an angioepithelial nodule on English sole (Parophrys vetulus) that has infiltrated skeletal muscle. (SM = skeletal muscle; SC = stratum compactum; SS = stratum spongiosum) (hematoxylin and eosin) (1000 x).



Mitotic figures were not observed within these cells. The epidermal basal lamina was usually visible but on occasion was indistinct.

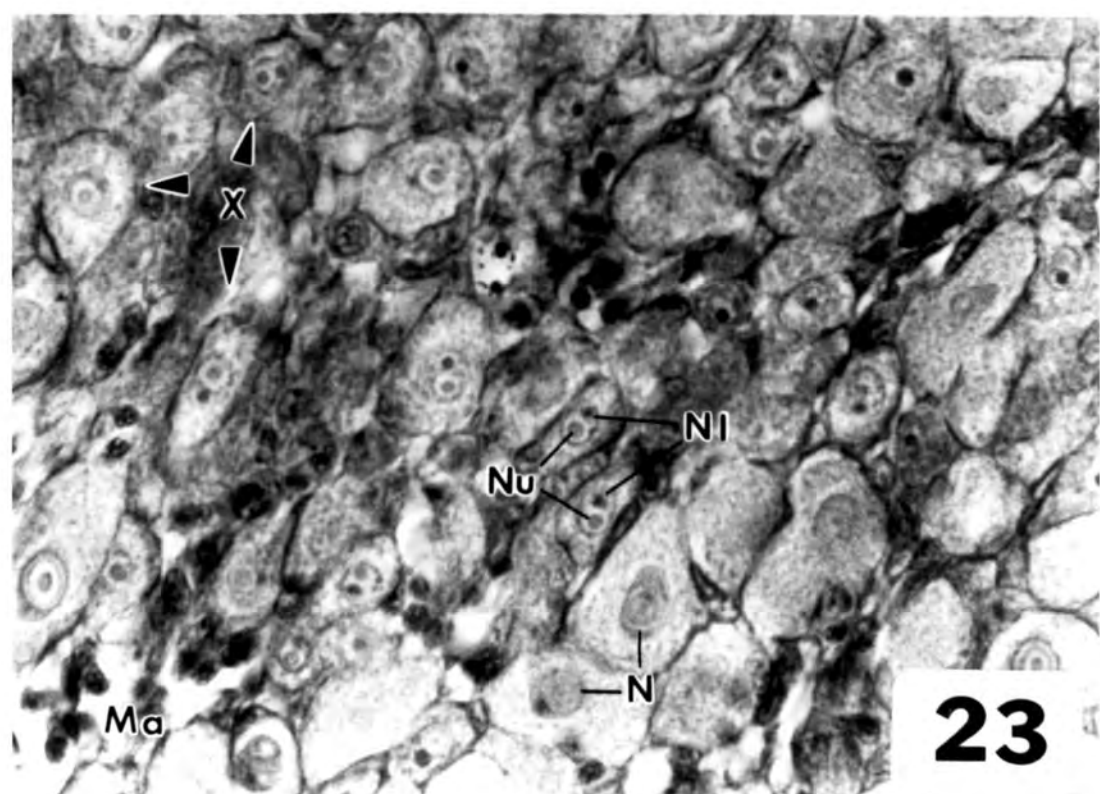
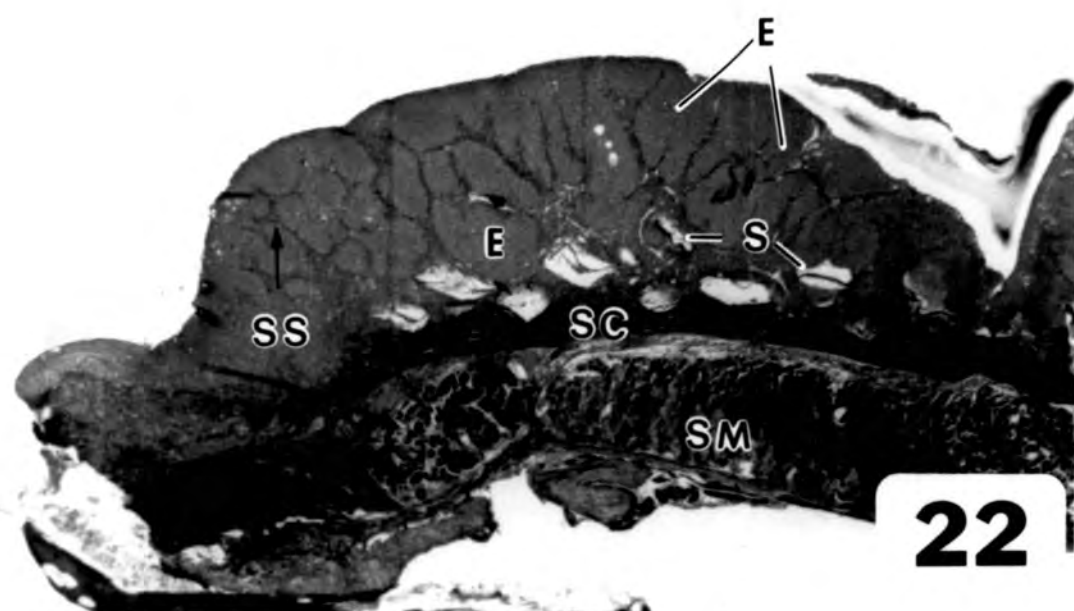
X-cells in the stratum spongiosum were abundant and smaller than those in the epidermis (Fig. 20). The stratum spongiosum of some nodules infiltrated the stratum compactum and in these tumors the stratum compactum was not distinct (Fig. 19). Skeletal muscle bundles beneath several angioepithelial nodules were infiltrated by stratum spongiosum (Fig. 21).

#### Epidermal Papilloma Histology

Epidermal papillomas were composed of convoluted, hypertrophied epidermis that extended deep into the dermal layer and was supported by thin strands of dermal connective tissue in which distinct layers of stratum spongiosum and stratum compactum were not seen (Fig. 22). The surface of the papilloma was typically covered by 2 to 3 layers of flattened cells resembling Malpighian cells. The convoluted epidermal layer contained many X-cells that were similar in morphology to those in angioepithelial nodules but were larger and averaged 15.5 micrometers in width and 22.4 micrometers in length (8.8 to 15.9  $\mu$  wide and 10 to 25.3  $\mu$  long). Interspersed between X-cells were envelope cells (Wellings et al., 1976) (Fig. 23).

Figure 22. Section of an epidermal papilloma on English sole (Parophrys vetulus). (E = epidermal folds; SS = thin strands of stratum spongiosum; SC = stratum compactum; S = scales; SM = skeletal muscle) (iron hematoxylin) (40 x).

Figure 23. Section of epidermal folds of an epidermal papilloma on English sole (Parophrys vetulus). (X = X-cells; N = nucleus; Nu = nucleolus; NI = nuclear inclusion; Ma = macrophages) (hematoxylin and eosin) (1000 x).





A major proportion of X-cells in 73 percent of the epidermal papillomas examined contained a round, basophilic, nuclear inclusion that measured between 1 to 3 micrometers (Fig. 23).

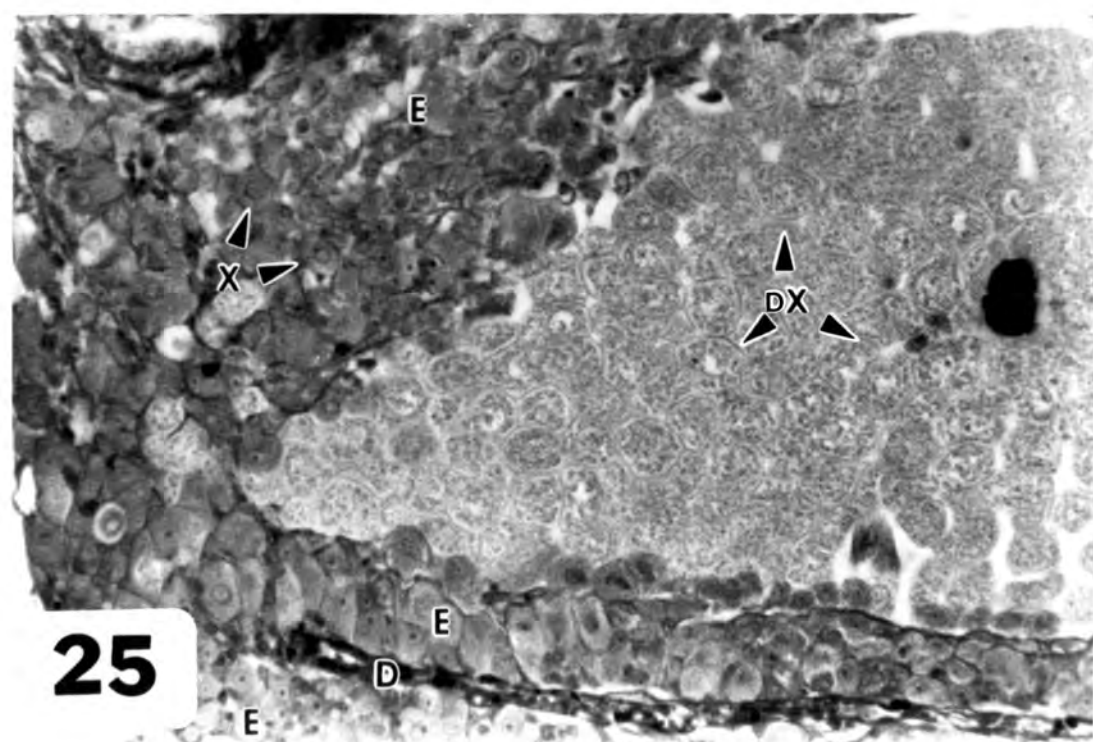
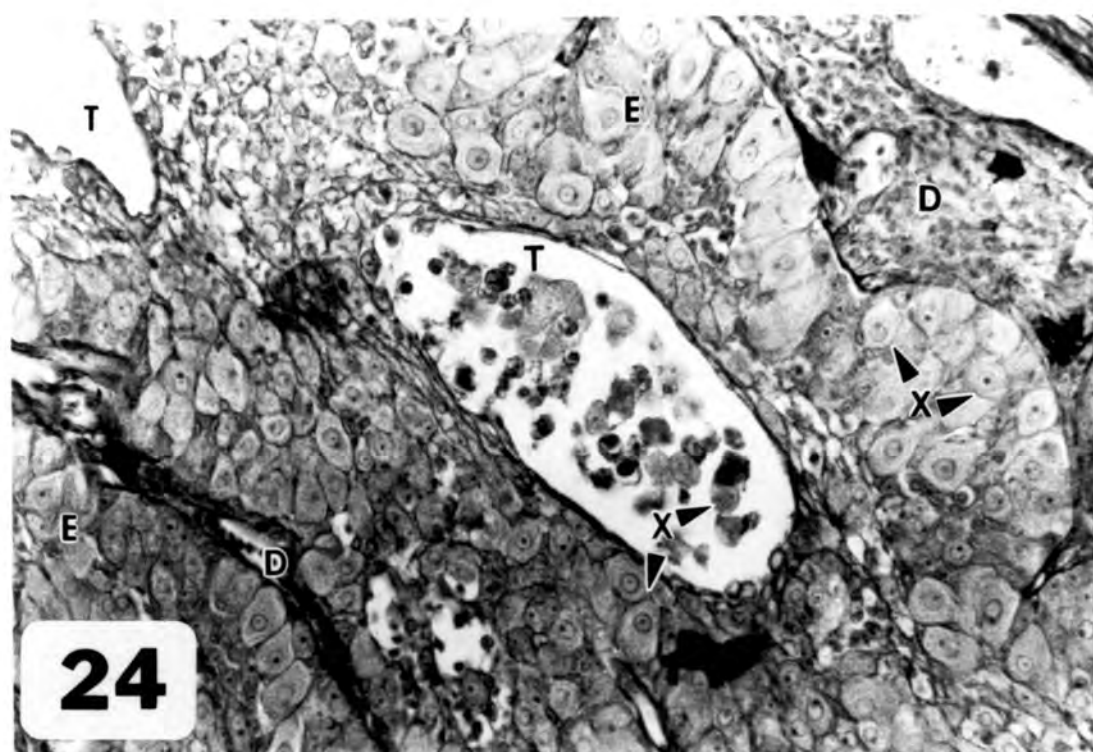
Mucous cells and eosinophilic granule cells, numerous in normal epidermis, were uncommon in the epidermal folds and melanophores were present at the epidermal/dermal junction of the papilloma. Macrophages were often clustered in the epidermis and were recognized by their irregular shape, pale staining cytoplasm and basophilic nucleus (Ellis, 1976; and Ganz et al., 1985) (Fig. 23).

Tissue spaces were present in the epidermis of about one-half of the epidermal papillomas examined and were either empty or contained X-cells and cellular debris (Fig. 24). Clusters of X-cells in the epidermis of several papillomas had a granular, eosinophilic cytoplasm and nucleus, lacked a nucleolus and may have been necrotic (Fig. 25). Some X-cells had angular eosinophilic nuclei rather than pale, oval nuclei but otherwise appeared morphologically similar to typical X-cells.

X-cells in the strands of dermal connective tissue between epidermal folds were numerous and smaller than those in the epidermis. These X-cells averaged 5.1 micrometers in width and 7.4 micrometers in length (2.9 to 8.8  $\mu$  wide and 5.3 to 11.2  $\mu$  long).

Figure 24. Section of tissue spaces in an epidermal papilloma on English sole (Parophrys vetulus). (E = epidermis; D = dermis; T = tissue space; X = X-cells) (hematoxylin and eosin) (400 x).

Figure 25. Section of an epidermal papilloma on English sole (Parophrys vetulus) with possible degenerate X-cells. (E = epidermis; D = dermis; X = X-cells; DX = degenerate X-cells) (hematoxylin and eosin) (400 x).



Evidence of mitosis was not observed in X-cells present in either the epidermal or the dermal layers.

#### Angioepithelial Polyp Histology

Angioepithelial polyps were identified only by histology and not by gross morphology. These tumors were composed primarily of dermis covered by a convoluted hyperplastic epidermis that extended less than half way into the dermal layer (Fig. 26). The epidermal layer appeared similar to that of an epidermal papilloma and included Malpighian cells, X-cells, and melanophores but lacked mucous cells and eosinophilic granule cells. The dermal portion of the polyp was vascular, had X-cells, collagen, melanophores and fibroblasts and appeared similar to the dermis of angioepithelial nodules.

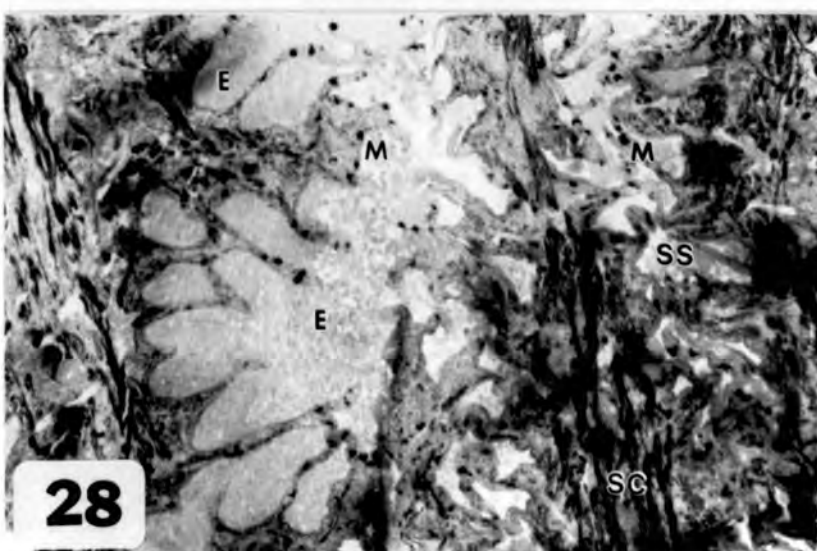
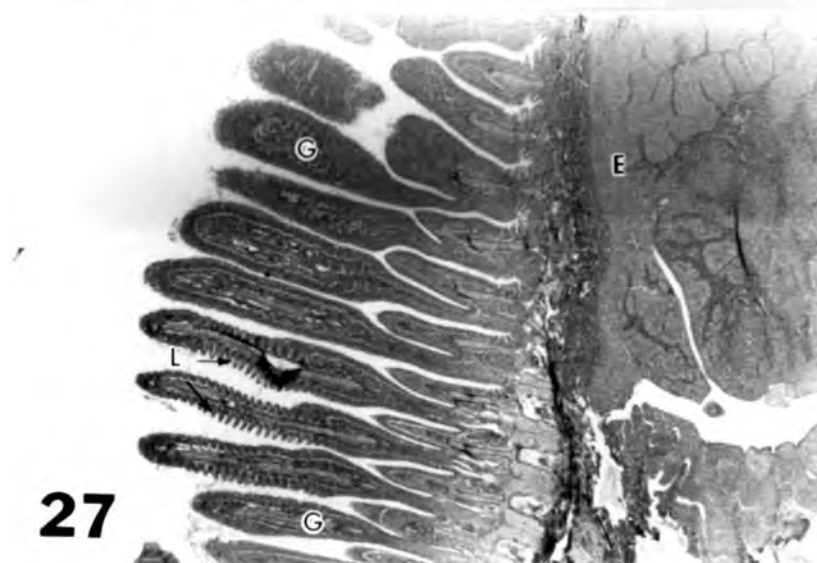
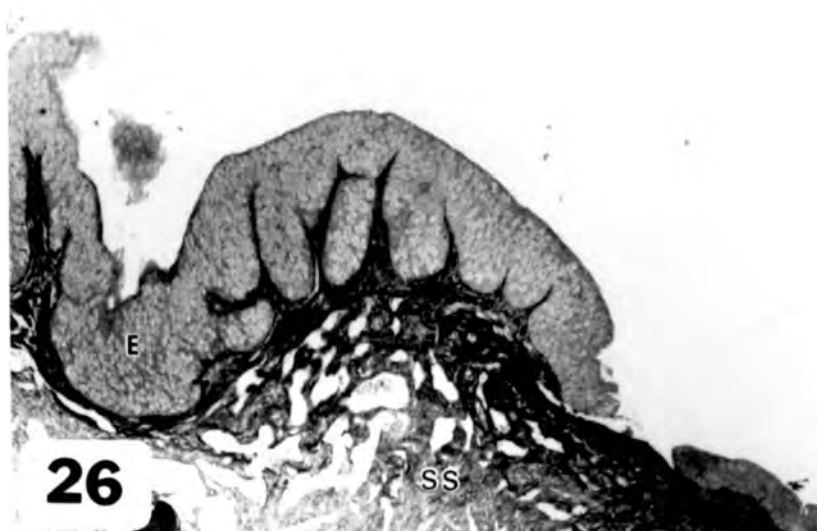
#### Gill Epidermal Papilloma Histology

Histologic examination of tumors on the gills of two English sole revealed typical epidermal papilloma tissue on the anterior portion of the gill arch. The convoluted epidermis contained X-cells, macrophages and cells resembling Malpighian cells and was supported by thin strands of dermal connective tissue (Fig. 27). Highly affected gill filaments were thickened, had numerous X-cells in the epithelium and had indistinct secondary lamellae (Fig. 27). The proliferation of gill epithelium

Figure 26. Section of an angioepithelial polyp on English sole (Parophrys vetulus). (E = epidermal folds with numerous X-cells; SS = stratum spongiosum) (hematoxylin and eosin) (100 x).

Figure 27. Section of gill papilloma from English sole (Parophrys vetulus). (E = convoluted epidermis with numerous X-cells; G = gill filaments that contain X-cells; L = secondary lamellae). Note that some gill filaments lack secondary lamellae. (hematoxylin and eosin) (40 x).

Figure 28. Section of an epidermal papilloma on an adult English sole (Parophrys vetulus). (E = epidermal folds; SC = stratum compactum; SS = stratum spongiosum; M = melanophores) (hematoxylin and eosin) (100 x).



resulted in the reduction of space between adjacent lamellae.

#### Histology of Epidermal Papillomas on Adult Fish

The epidermal papillomas found on adult English and petrale sole were similar microscopically to the epidermal papilloma of juvenile English sole. Because tissues were not fixed shortly after collection, necrotic changes made histologic description difficult. The convoluted epidermis extended deep into the dermis and was supported by a relatively thick layer of dermal connective tissue (Fig. 28). Although X-cells could not be positively identified due to tissue necrosis, there were in the epidermal folds, numerous cells that were approximately the size of X-cells and were eosinophilic with a large nucleus.

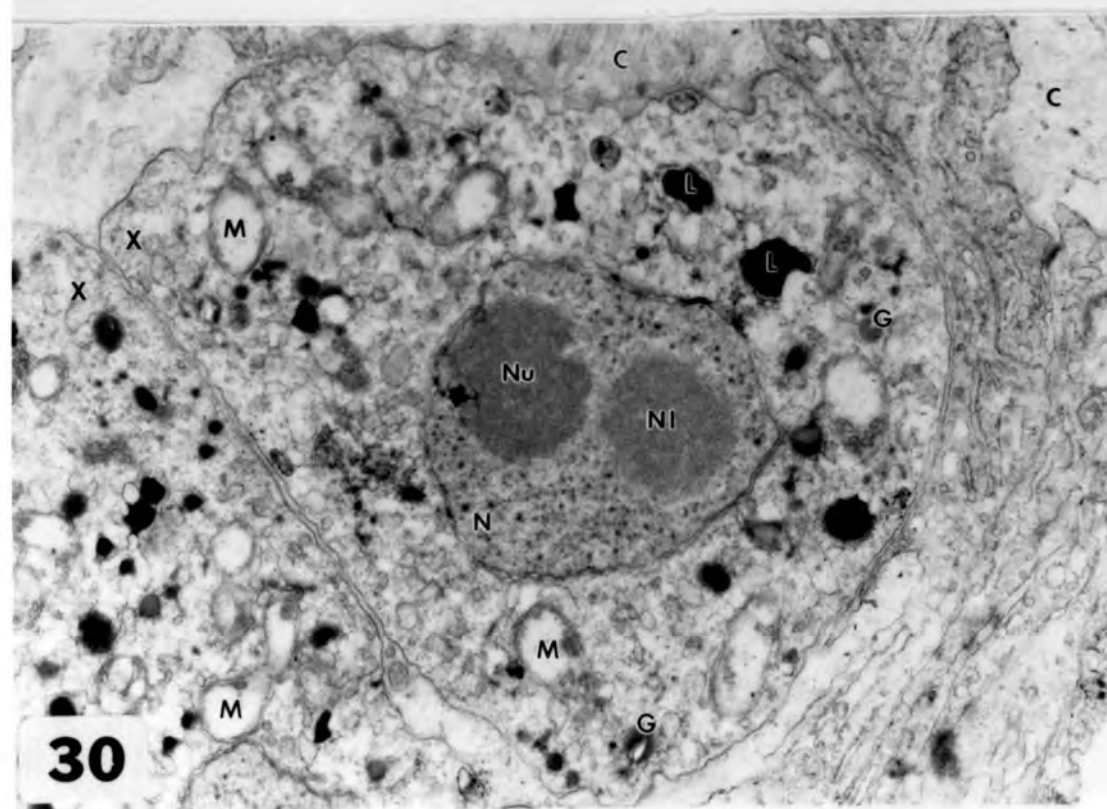
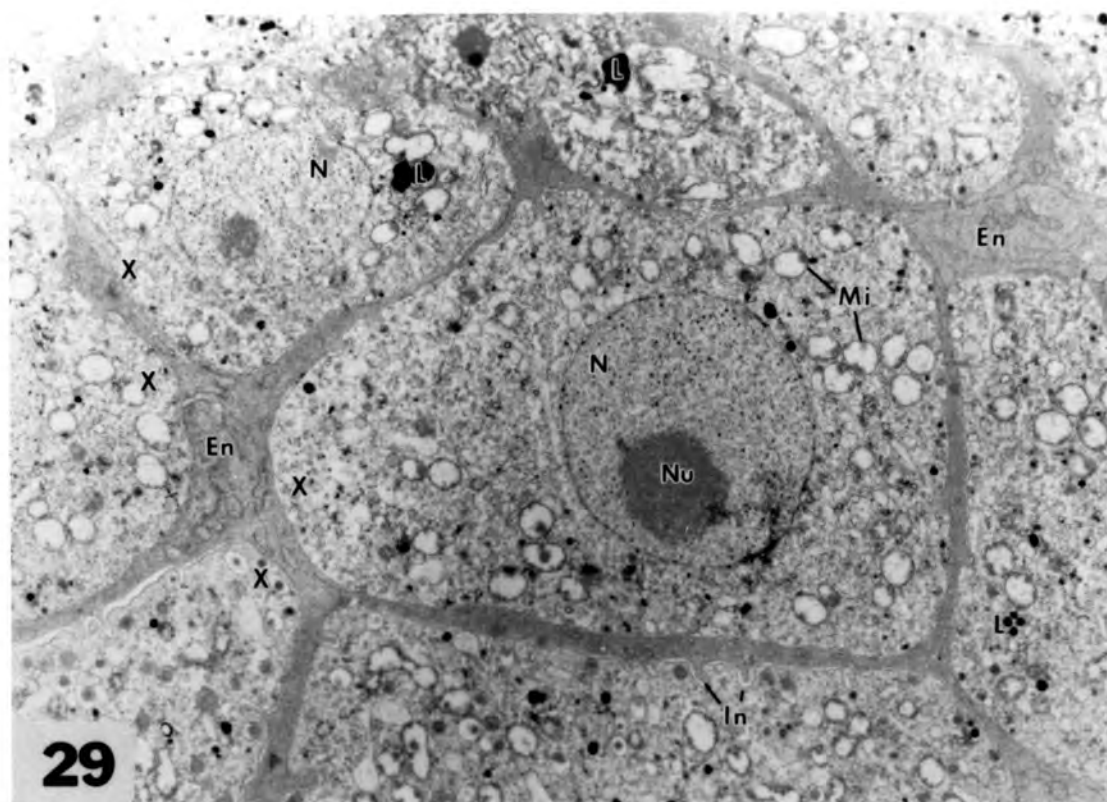
#### Electron Microscopy of Epidermal Papillomas

Observation of papilloma tissue by transmission electron microscopy revealed X-cells with a large nucleus with nuclear pores and an electron dense nucleolus (Fig. 29). X-cells were surrounded by either collagen fibers or envelope cells. The thin processes of envelope cells prevented adjacent X-cells from having direct contact with each other and also occupied invaginations in the X-cell outer membrane (Fig. 29). Mitochondria were numerous, rounded in appearance and either lacked, or had few rounded cristae. Occasional unbound granules, possibly

Figure 29. Electron micrograph of X-cells from an epidermal papilloma on English sole (Parophrys vetulus). (X = X-cell; En = envelope cells; In = invaginations; N = nucleus; Nu = nucleolus; Mi = mitochondria; L = possible lipid concentrations) (lead citrate and uranyl acetate) (4500 x).

Figure 30. Electron micrograph of an X-cell with a nuclear inclusion from an epidermal papilloma on English sole (Parophrys vetulus). (X = X-cells; N = nucleus; Nu = nucleolus; NI = nuclear inclusion; M = mitochondria; L = possible lipid concentrations; G = unbound granular bodies; C = collagen) (lead citrate and uranyl acetate) (1200 x).





lipid concentrations and electron dense membrane bound granular bodies were in the X-cell cytoplasm. Nuclear inclusions were unbound and less electron dense than the nucleolus (Fig. 30). No virus-like particles were seen in the X-cells or in the nuclear inclusions.

#### Tissue Imprints of Epidermal Papillomas

No evidence of mitosis was seen in imprint preparations of X-cells from epidermal papillomas. The nucleus, nucleolus, and nuclear inclusion were observed within Feulgen, Giemsa, and iron hematoxylin stained X-cells (Fig. 31).

#### X-cell Cultures

The use of malt-yeast-seawater agar (Page, 1973; and Jones and Scheibling, 1985) or malt-yeast-distilled water agar with a fluid overlay of either saltwater (32.5 ppt) or fish saline (6.5 ppt) failed to support the growth of X-cells. After three days in culture, the X-cells ruptured. Amoeba-like movement or cell division were never observed under these culture conditions.

Primary cultures of epidermal papilloma tissue resulted in an extensive X-cell containing monolayer after 8 days (Fig. 32). X-cells were observed individually or in clusters of up to 50 cells and were recognized by their

Figure 31. Tissue imprint of X-cells. (X = X-cells;  
N = nucleus; Nu = nucleolus; NI = nuclear  
inclusion; RBC = red blood cell) (Giemsa)  
(1000x).

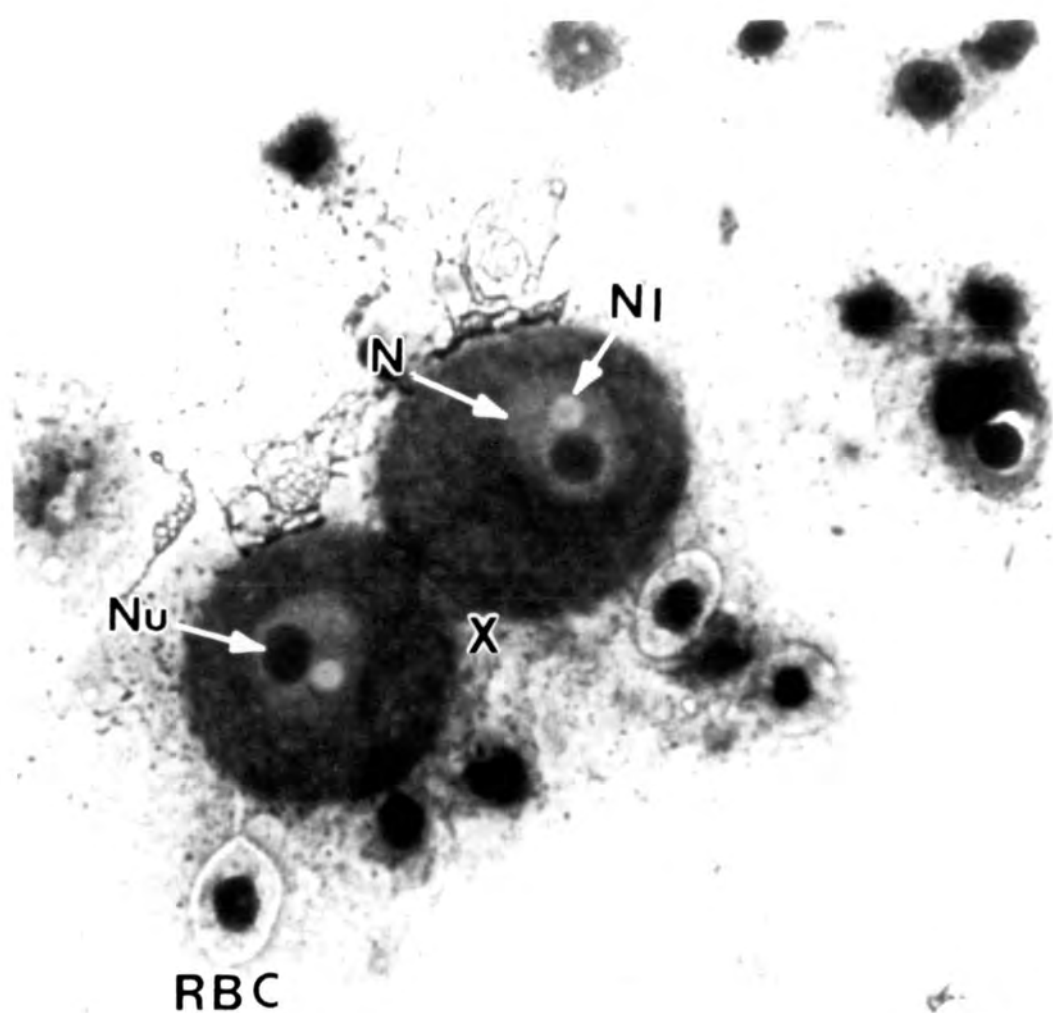
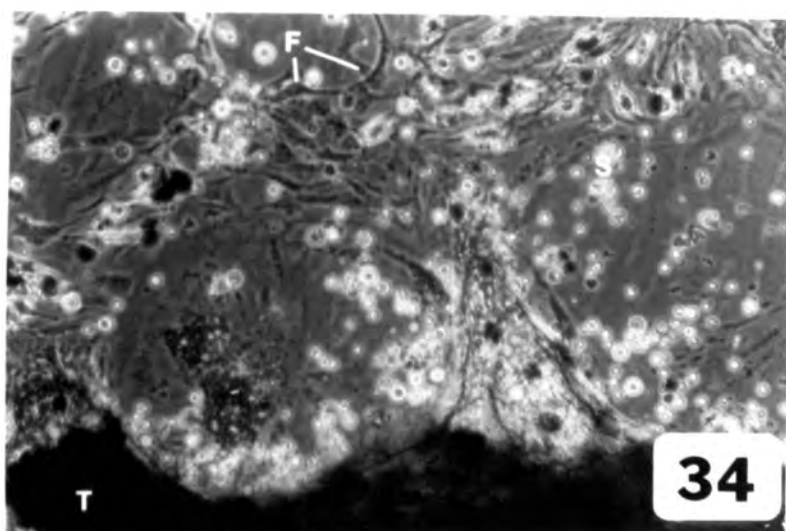
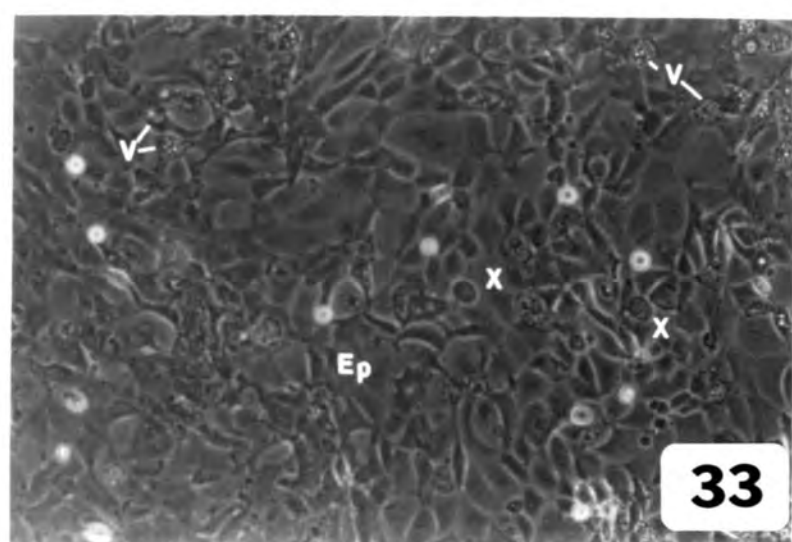
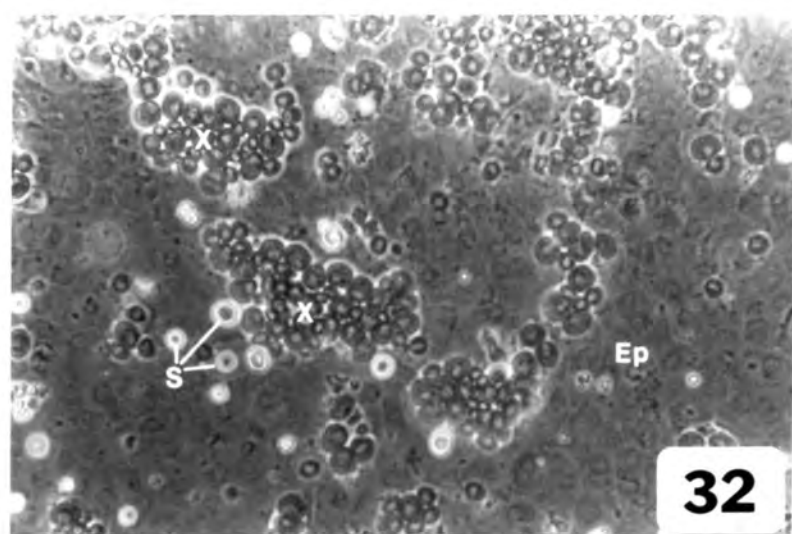
**31**

Figure 32. Primary culture of X-cells after 8 days.  
(X = X-cells; Ep = epitheloid cells;  
S = cells suspended in medium) (Inverted  
microscope) (100 x).

Figure 33. Primary culture of X-cells after 12 days.  
(X = X-cell; Ep = epitheloid cells;  
V = vacuolated cells; S = cells suspended in  
medium) (Inverted microscope) (100 x).

Figure 34. Primary culture of X-cells after 18 days.  
(T = tissue explant; F = fibroblastic cells;  
S = cells suspended in medium) (Inverted  
microscope) (100 x).



shape and large nucleus. After 11 days, epitheloid cells were vacuolated and the cell sheet pulled back as both X-cells and epitheloid cells detached from the flask surface (Fig. 33). After 18 to 21 days of culture, only tissue explants, small clusters of epitheloid cells, fibroblastic cells and melanophores adhered to the flask surface (Fig. 34).

Although X-cells varied in size and frequently appeared in direct contact with other X-cells, no mitotic figures or dividing cells were observed. X-cell movement was never detected although the position of X-cells changed as the cell sheet expanded.

Both X-cells and epithelial cells stained poorly with May-Grünwald-Giemsa. Examination of fixed and stained X-cells revealed no evidence of mitotic figures or cell division.

## IV. DISCUSSION

English sole were present in Yaquina Bay each month with recruitment of newly metamorphosed individuals occurring from January to June, 1986 and from December, 1986 to February, 1987. This extended period of immigration is probably due to a prolonged spawning period (Budd, 1940; Harry, 1959; Jow, 1969; and Kruse and Tyler, 1983) that can vary from year to year (Laroche and Richardson, 1979; and Hayman and Tyler, 1980). In this study, English sole as small as 16 mm in length were collected; this is reported to be the size of metamorphosis to a benthic form (Ahlstrom and Moser, 1975; Misitano, 1976; and Laroche and Richardson, 1979). Most English sole remained in the bay until September when CPUE indicated that fish had begun to emigrate to the open ocean. English sole have been reported to emigrate from the estuary nursery grounds after reaching 120 to 140 mm (SL) (Westrheim, 1955; Ketchen, 1956; Olson and Pratt, 1973; and Misitano, 1976) but only 1.7 percent of the fish collected in this study were over 110 mm in length. Westrheim (1955) reported that less than 5 percent of the population remained in the Yaquina Bay estuary during their first winter before emigrating in the following spring.

The gross morphology and size of angioepithelial nodules and epidermal papillomas conformed to previous



descriptions (Wellings et al., 1964, 1965; McArn and Wellings, 1971; and Miller and Wellings, 1971).

Angioepithelial polyps were not recognized by gross examination and only a few were identified by histologic characteristics. Wellings et al. (1976) reported that only one percent of the pleuronectid epidermal tumors examined were angioepithelial polyps.

Both normal and tumor-bearing age-0 English sole were collected each month except in January and February, 1986 when the only tumorous fish were larger, over-wintering individuals. Tumorous young-of-the-year fish were first collected in March although recruitment into the bay had begun by January.

The number of tumors per fish has been reported to be greatest on age-0 individuals (Wellings et al., 1964, 1965; McArn and Wellings, 1971; Miller and Wellings, 1971; and Angell et al., 1975) and a decrease in tumor prevalence with increased age has also been reported (Nigrelli et al., 1965; Miller and Wellings, 1971; Angell et al., 1975; Oishi et al., 1976; Stich et al., 1977B; Campana, 1983; and Cross, 1984). In this study, a comparison between age-0 and age-1 fish was not possible due to the absence of older fish in Yaquina Bay.

A significantly greater number of tumors occurred on the dextral or pigmented side of the English sole.

Other studies have noted similar results (Wellings et al., 1964; McArn et al., 1968; Miller and Wellings, 1971; and Foster, 1987), opposite results (Oishi et al., 1976), or have found no significant difference (Cooper and Keller, 1969; Kelly, 1971; Campana, 1983; and Cross, 1984).

McArn and Wellings (1971) collected English sole and starry flounder at the same time from Bellingham Bay, Washington and reported that a greater number of tumors occurred on the dextral side of English sole, while no significant difference occurred on starry flounder. Angell (1972) sampled English sole from the same area during three years and found no significant differences in tumor location for the first two years but during the final year noted a significantly greater number of tumors on the sinistral side.

The relationship between fish length and tumor prevalence revealed that only 0.6 percent (2/340) of the fish under 30 mm in length were tumorous. The general absence of tumors on young fish suggests there is either an interval of time prior to exposure to the etiologic agent(s) or an incubation period is required prior to development of tumors on susceptible fish.

Relatively few fish over 110 mm in length were collected and those that were, were considered overwintering individuals that did not emigrate in the fall with the bulk of the English sole population. Of these 42

over-wintering fish, 71 percent had tumors. The reason for the high tumor prevalence in over-wintering English sole is not known, but Angell, et al. (1975) suggested that emigration may be delayed in tumorous fish.

Tumor intensity was highest in fish 80 to 110 mm in length, averaged 3.8 tumors per fish while fish larger than 110 mm averaged 2.5 tumors. This is consistent with other reports that larger and therefore older individuals have fewer tumors than juveniles (Wellings et al., 1964, 1965; McArn and Wellings 1971; Miller and Wellings, 1971; and Angell et al., 1975).

Tumor prevalence was lowest during February and March, 1986 when only over-wintering fish were tumorous. The prevalence peaked in September and fluctuated irregularly through March, 1987. A similar seasonal pattern of tumor prevalence has been observed in San Francisco Bay, Puget Sound, and Humboldt Bay (Cooper and Keller, 1969; Angell, 1972; and Foster, 1987).

The presence of tumorous adult English sole and petrale sole in this study as well as tumorous adult flathead sole (Hippoglossoides elassodon), rex sole (Glyptocephalus zachirus), and sand sole (Wellings et al., 1965; and Nigrelli et al., 1965) indicates that some affected fish reached maturity and that epidermal tumors may not be invariably fatal. Nevertheless, if the tumor were located on the eye, snout, or mouth, ability of the

affected fish to obtain food could be hindered. Likewise, tumor location may decrease the ability of tumor bearing fish to detect or escape predators.

The rate of tumor development and fate of epidermal tumors on fish held in the laboratory varied. The development of angioepithelial nodules into epidermal papillomas was observed on 8 fish and was consistent with previous observations (Wellings et al., 1965; Kelly, 1971; Miller and Wellings, 1971; Angell et al., 1975; and Campana, 1983). Angioepithelial nodules took an average of 94 days to develop into epidermal papillomas. Miller and Wellings (1971) found that the youngest flathead sole with epidermal papillomas were early in their second year of life.

Regression of epidermal tumors on pleuronectid flatfishes held in the laboratory has been observed by several (Nigrelli et al., 1965; Kelly, 1971; and Miller and Wellings, et al., 1971), but not all, investigators (Wellings et al., 1964; and Angell et al., 1975). In this study, the complete regression of angioepithelial nodules was observed on 9 of 22 fish held in the laboratory. Tumor regression took place between 31 and 120 days, but averaged 70 days.

No epidermal papillomas were found to regress in this study although 10 fish with a total of 15 papillomas were observed for periods up to 349 days. Kelly (1971) reported

the regression of epidermal papillomas in 49 of 66 English sole held in the laboratory . Miller and Wellings (1971) reported regression of the plaque-like edge of a transitional stage between the angioepithelial nodule and the epidermal papilloma and suggested that starvation may have been a factor in this regression.

Regression of angioepithelial nodules in this study left a characteristic scar at the tumor site (Fig. 15C, white arrow). Several newly collected juvenile English sole showed similar scars. This suggested that angioepithelial nodules may regress under natural conditions as well as in the laboratory. In the absence of knowledge of the etiological agent(s) of epidermal tumors, it is difficult to speculate about the mechanism for tumor regression in some individuals. The increased vascularization of angioepithelial nodules during the regression process suggests possible involvement of some facet of the immune system. However other factors such as nutrition and laboratory holding conditions may be involved.

The development of angioepithelial nodules in the laboratory occurred only on fish less than 50 mm in length. This observation is consistent with the reports that only fish less than 50 mm developed tumors in the laboratory (Cooper and Keller, 1969; and Kelly, 1971).

Miller and Wellings (1971) and Campana (1983) found that the length and weight of age-0 tumorous and non-tumorous flatfish did not differ significantly, but that in age-1 and older fish, normal fish were significantly larger and heavier than were tumorous individuals.

Campana (1983) reported a significantly higher mortality in tumorous starry flounder than in normal flounder after exposure to salinity and temperature changes in laboratory experiments. The same result was obtained when tumorous and normal flounder were transported together at different temperatures and densities (Campana, 1983).

Histologically, the epidermis of normal juvenile English sole was the same as that described for various other flatfish species (Wellings et al., 1967; Brown and Wellings, 1969; Roberts et al., 1971; Bullock and Roberts, 1974; and Roberts and Bullock, 1976). The angioepithelial nodules examined in this study were identical to earlier descriptions (Wellings et al., 1964) and appeared to be more like an inflammatory response than a neoplasm, because of the presence of blood vessels, macrophages and lymphocytes in the dermal layer (McArn et al., 1968; Brooks et al., 1969; Miller and Wellings, 1971; McArn and Wellings, 1971; Angell et al., 1975; and Peters et al., 1978). Although the stratum spongiosum of several angioepithelial nodules had infiltrated the stratum

compactum and skeletal muscle bundles, Wellings et al. (1964) and Peters et al. (1978) did not interpret this condition as a metastasis and did not consider those tumors as malignant.

There were no histological differences between the epidermal papillomas of this study and those previously described (Wellings et al., 1964). Light microscopy revealed that several epidermal papillomas contained portions of epidermis that appeared necrotic. Cellular debris and individual X-cells occupied tissue spaces in the epidermis while large aggregates of degenerative X-cells were present in the epidermal folds of several epidermal papillomas examined. These degenerate X-cells were strongly eosinophilic, had a faint nucleus and granular cytoplasm but lacked a nucleolus. The presence of necrotic tissue in the advanced tumor may indicate that the disease is regressing as suggested by Nigrelli et al. (1965). McVicar et al. (1987) suggested that possibly the degenerate condition of tissue rather than the presence of an exogenous agent stimulates macrophage migration into the affected area.

Viral particles have occasionally been observed in tumorous tissue (Wellings and Chuinard, 1964; Wellings et al., 1965, 1967, 1976; McArn et al., 1968; and Diamant et al., 1988) but this has not been a consistent finding and attempts to isolate viruses in cell culture have been

unsuccessful (Wellings et al. 1976). Diamant et al. (1988) isolated infectious pancreatic necrosis virus (IPNV) in dab with epidermal papillomas but were unable to induce X-cell lesions by exposing dab to the isolated virus and suggested that there was no relationship between IPNV and X-cell lesions. In this study and in Peters et al. (1983) nuclear inclusions were observed within numerous X-cells both by light and electron microscopy. Although such inclusions in cells of other organisms contain virus particles (Reno et al., 1978; Lightner and Redman, 1981; and Lightner et al., 1983), no virus-like particles were found in nuclear inclusions, X-cells, or in other epidermal papilloma tissue components examined in this study or in Peters et al. (1983).

Environmental pollutants have been proposed as causes of tumorigenesis (Stich et al., 1976, 1977B) although no consistent correlation between water quality and the presence of tumorous flatfish has been found. Mearns and Sherwood (1974) found no relationship between tumor bearing fish and wastewater discharge in southern California, while tumorous fish have also been collected from pristine waters (Levings, 1967) although the migration of tumorous fish into these locations could explain this finding. Stich et al. (1977A) proposed the possibility that a virus could render fish more susceptible to chemical carcinogens and lead to tumor formation.



The origin and nature of the X-cell has been questioned ever since it was first described. X-cell lesions have been found in the integument, gills, pseudobranch and intestine of fishes in the family Pleuronectidae, Gadidae, Gobidae, Scorpaenidae, Zoarcidae, Notothenioidae and Scianidae (Johnstone, 1912; Morrison et al., 1982; Ito et al., 1976; Myers, 1981; Dresser and Kahn, 1982; Franklin and Davison, 1988; and Kent et al., 1988). If X-cell lesions are caused by the same etiologic agent(s) then a wide spectrum of fish and tissues are susceptible. There also remains the possibility that different etiologic agents could be manifested in the form of X-cell tumors. A similar situation exists in ulcerative lesions of salmonids. Although these appear identical, different species of gram negative bacteria can be the agent responsible for the lesion.

Although angioepithelial nodules and epidermal papillomas were prepared using standard histological techniques, X-cell mitotic figures were not recognized. This agrees with the observations of Cooper and Keller (1969); Peters et al. (1978); and Peters and Watermann (1979) although X-cell mitotic figures have been observed in tumorous Pacific cod, Pacific Ocean perch and starry flounder (Dawe, 1981; Myers, 1981; and Kent, personal communication).

The transformation of host tissues such as Malpighian cells, pigment cells, lymphocytes, histiocytes, wandering cells, fibroblasts or undifferentiated small epithelial cells have all been mentioned as the potential precursor of X-cells (Brooks et al., 1969; Peters et al., 1978, 1983; Yamazaki et al., 1978).

X-cells have been interpreted by some investigators (Yamazaki et al., 1978; Peters and Watermann, 1979; Kranz et al., 1980; and Peters et al., 1983) as being degenerate due to foamy cytoplasm, vacuolization of the mitochondria and endoplasmic reticulum, but these characteristics were not seen in my samples.

In this study, X-cells could not be maintained in viable condition on either malt-yeast-agar or in primary tissue cultures. Under these culture conditions no amoeba-like morphology, movement, mitosis or cell division was observed in X-cells.

Brooks et al. (1969) suggested that X-cells could be parasitic, unicellular organisms. Dawe et al. (1979) hypothesized that X-cells were protozoans (specifically amoebae) and presented three pieces of evidence in support of this proposal. First, the tissue lesions contained isozymes that were not found in normal tissue. In addition, quantitative analysis of DNA revealed that X-cells had only one third to one quarter the amount of DNA found in envelope cells and other cod cells. Finally,

X-cell mitotic figures were considered to be similar to those characteristic of amoebae in the family Hartmanellidae.

Morphologically similar X-cells are present in both the pseudobranch tumors of cod and epidermal tumors of English sole but several differences exist between the two pathologic conditions. Embryologically, the pseudobranch is derived from the pharyngeal endoderm while ectoderm gives rise to the epidermis. Multinucleated X-cells have been observed in pseudobranch tumors so these apparently undergo nuclear division without cellular division (Dawe, 1981). This feature of pseudobranch tumors has not been reported in studies of pleuronectid epidermal tumors.

Future research involving isozyme analysis of tumorous tissue and quantitative analysis of X-cell DNA from English sole tumors may clarify whether or not conclusions concerning pseudobranch tumors of Pacific cod can be applied to epidermal tumors of pleuronectid flatfish. X-cells could be considered non-host cells if there are isozymes present in tumorous tissue that are absent normal tissue. The same conclusion might be suggested if the quantity of DNA in X-cells differs significantly from that of normal cells. Investigation of the DNA homology between X-cells and normal fish cells may also provide some insight as to the nature of X-cells. If a substantial degree of homology exists between X-cells and normal cells, then

X-cells might be considered to be transformed fish cells.

Unequivocal evidence concerning the identity of X-cells remains to be demonstrated. Future research could address the development of a culture medium and protocol that would support the growth and multiplication of X-cells. Success in this area would aid in the identification of the nature of X-cells.

The inability to culture viable X-cells in this study does not support or disprove any hypothesis concerning the cause(s) of X-cell lesions. The isolation of an infectious agent, followed by the initiation of identical tumors in susceptible fish exposed to the agent, and re-isolation of the agent from symptomatic fish would be required to demonstrate an infectious etiology for epidermal tumors in English sole.

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## APPENDICES

## APPENDIX 1

## GELATIN BASED DIET FOR MARINE FISHES

Commercial salmon food	980 g
Squid	280 g
Herring	560 g
Frozen spinach	280 g
Frozen carrots	280 g
Paprika	10 g
Amijex Twelve Fifty (nutritional supplement)	4 cc
Hot water	3200 ml

1. Completely thaw all ingredients.
2. Thoroughly rinse krill with tap water.
3. Place all ingredients but the gelatin into blender.
4. Blend until homogenized.
5. Add gelatin while blending.
6. Pour into containers and place in refrigerator overnight.
7. After mixture has set, freeze for storage.

## APPENDIX 2

## ANTIBIOTIC INCUBATION MIX

Penicillin Streptomycin	10.0 ml of 10,000 U/10,000 ug/ml
Fungizone	1.0 ml of 500 ug/ml
Gentamicin	0.5 ml of 50 mg/ml
MEM-O	88.5 ml

(All components obtainable from Sigma)

Store frozen

MEM-O (Eagles's Minimum Essential Medium with Earle's Salts)

1x Eagle's Minimum Essential Medium	
with Earle's Salts (Sigma)	450 ml
L-Glutamine (200 mM) (Sigma)	5 ml

Adjust pH to 7.2-7.4 with sterile 7.5% NaHCO<sub>3</sub> (~5 ml)

Store refrigerated



## APPENDIX 3

## MALT-YEAST AGAR MEDIUM

Malt Extract (Difco)	0.1 g
Yeast (Difco)	0.1 g
Bacto-Agar (Difco)	15 g
Distilled water or seawater	1.0 l

Dissolve all ingredients in the fluid base.

Autoclave

Cool to 40-45° C.

Pour 5 ml per petri dish.

Store refrigerated.

## APPENDIX 4

## MEDIUM 199 FOR PRIMARY CULTURE

10x Filtered sterilized Medium 199	
with Hank's Salts (Sigma)	10 ml
Fetal Bovine Serum (Hyclone)	20 ml
Penicillin/Streptomycin	
(10,000U/10,000ug/ml) (Sigma)	2 ml
L-Glutamine (200mM) (Sigma)	5 ml

Bring to 100 ml with sterile tissue culture grade water.

Adjust pH to 7.2-7.4 with sterile 7.5%  $\text{NaHCO}_3$

Store refrigerated

## L-GLUTAMINE (200 mM)

L-Glutamine	2.92 g
Distilled water	100 ml

Filter sterilize

Aliquot in 5.0 ml amounts

Store frozen