Studies on perispawning mortalities in brown trout (Salmo trutta L.) from Loch Leven, Kinross, Scotland.

by

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Dedicated to my wife, whose constant encouragement and help eventually allowed the completion of this project.

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ABSTRACT

Investigations into perispawning mortalities in the brown trout (Salmo trutta L) population of Loch Leven, Kinross, revealed that death occurred as a result of infection with a particular species of Saprolegnia diclina Humphrey type 1. Increased surface area of infection was correlated with a decrease in ion and protein content of the blood and was further reflected by changes in the electrocardiogram pattern. These changes were essentially a widening of the QRS complex with inflection of the RS component, changes consistent with a decrease in certain ions in mammals. Histological changes associated with sexual maturity and fungal infection are described and compared with changes resulting from the administration of androgens to brown trout.

I GENERAL INTRODUCTION

Loch Leven, Kinross, Scotland is a freshwater loch lying about 40km north of Edinburgh at a latitude of 56° 10' N, a longitude of 3° 30' W and an altitude of 107m above sea level. Its area is 13.3 km^2 and it has a mean depth of just under 4m. The catchment area of the loch is drained by four streams shown in Figure 1, the North and South Queich, the Gairney Water and the Pow Burn. The outlet from the Loch is to the River Leven by means of sluices. A partial drainage of the Loch for land reclamation purposes occurred between 1830 and 1832, lowering the water level by about 1.4m to leave a shallow water margin with two deep areas in the middle part of the loch which are "kettle-holes", marking the site of large, detached ice blocks buried in sand and gravel deposits (Kirby, 1974). The loch itself is eutrophic with a considerable nutrient input from adjoining agricultural land and the effluent from a local woollen mill. In 1966, the International Biological Programme on Freshwater Productivity was initiated at Loch Leven and since then extensive research in many areas of interest has been carried out.

The loch has a long-established natural population of brown trout (Salmo trutta L) which is exploited as a sport fishery. Replenishment of the fish stock is by natural spawning in the tributary streams and the stimulus to the research work presented herein was the investigation

of Thorpe & Roberts (1972) into mortalities in the brown trout in their perispawning period. The major conclusion of the above work was that a considerable number of fish (7,700) died of Aeromonas spp. infection during the spawning season of 1970/1971. Fungal skin infection, though present in some fish, was not thought to have been a major cause of death. Mortalities in subsequent spawning seasons have been much reduced and death from bacterial infection appears to have been replaced with fungal-related mortality.

Integumentary fungal infections of spawning salmonids have been described over many years. Major mortalities from Saprolegnia infection occurred in spawning Atlantic salmon (Salmo salar L) in Britain during the late nine-teenth century, first reports of the disease apparently coming from the Solway Firth area in 1877 followed by a rapid spread to the rest of Scotland, England and Wales (Smith 1878; Stirling 1878; Brook 1879; Walpole & Huxley 1882). Sporadic outbreaks of this disease had been noted in the years preceding 1877 in the Reports of the Inspector of Salmon Fisheries for England and Wales for 1881 and 1882 (1882; 1883) but losses of epidemic proportions occurred in Britain in the period 1887-1881. The disease was also noted at this time in France (Vion 1879) but accounts of the disease become fewer through the 1890's and it seems likely that the disease disappeared completely by the early 1900's. Although members of the Saprolegniaceae were considered responsible for the

disease at that time, reports of concomitant bacterial infection were published (e.g. Hume Patterson 1903).

An outbreak of a similar condition, which has since become known as ulcerative dermal necrosis or U.D.N. was detected in the Waterville River system in south-west Ireland in 1964 (Brown & Collins 1966). From that date spread to the rest of Britain was very rapid and large numbers of fish were affected (Strickland & Carbery 1967; Carbery 1968; Carbery & Strickland 1968; Elson 1968; Munro 1970). Several workers note a pre-fungal stage in U.D.N. which takes the form of skin ulcerations in the head area (Carbery & Strickland 1968; Roberts, Shearer, Munro & Elson 1970a, b), but Saprolegnia infection rapidly occurs in fresh water and fish may be treated with antifungal agents to affect a complete cure (Roberts, Ball, Munro & Shearer 1971). It has been, without doubt, the public and governmental interest in this condition that has led to the expansion of interest in fish pathology in Britain in recent years. Fungal infection in spawning salmonids, which does not necessarily start in the head area, is common (Richards 1978) and it is into the latter grouping that the disease of fish in Loch Leven falls.

Investigations were first carried out to determine the relative importance of bacterial and fungal pathogens in the condition in Loch Leven. Because a specific type of Saprolegnia has usually been associated with infection in salmonids, details of the fungal species concerned were also elaborated.

It was also considered important to establish the nature of the changes occurring in the fish during the peri-spawning period which may have encouraged infection with bacteria or fungi. A natural consequence of such work was to determine the manner in which fish eventually died. Saprolegnia almost always occurs as a skin infection and pronounced changes in the skin and internal organs occur during the spawning season in salmonids (Robertson & Wexler 1957, 1960; Oguri 1960). Such changes are essentially a skin hyperplasia and variation in mucus production and a series of degenerative changes in internal organs with the exception of the interrenal tissue which first becomes hyperplastic and then shows evidence of degeneration (McBride & van Overbeeke 1969b). Such degenerative changes are more pronounced in those species such as the Pacific salmon, which exhibit a high mortality at spawning and it has been shown that the degenerative changes are probably a result of high circulating corticosteroids (Robertson & Wexler 1959; Heyl & Carpenter 1972; Idler & Truscott 1972). Elevated androgen levels present in sexually mature male and female fish have been shown to cause skin thickening and also to lead to the degenerative changes associated with sexual maturity (McBride & van Overbeeke 1971) and it has been suggested that this occurs as a result of the direct effect of sex steroids on the interrenal gland (Hane, Robertson, Wexler & Krupp 1966). Such work is lacking in the brown trout and the effect of androgens on intact and gonadectomised fish was investigated in

order to gain a better understanding of the mechanisms of androgen action in this species.

The skin in salmonids is important in maintaining the osmotic "milieu interieure" and extensive skin loss might be expected to result in osmotic defects in the fish. Such an effect of integumentary fungal infection was suggested by Tiffney (1939) and Richards & Pickering (1978) and haematological, histological and bacteriological studies were carried out on the brown trout from Loch Leven in order to determine whether death was occurring as a result of bacterial septicaemia, as suggested by the work of Thorpe & Roberts (1972) or from direct osmotic loss through the skin or perhaps through some other agency. Once the likelihood of osmotic loss was established as a major result of fungal skin infection, the question still remained as to how death eventually occurred in such fish. As ionic defects in other animals and man regularly lead to defects in conductance in the heart (Fisch 1973, Rollason 1975) and eventually heart failure, an attempt was also made to relate such changes to electrocardiographic changes in fungal-infected fish.

Specific areas of discussion of a comparative nature in this text have been dealt with in detail in the relevant chapter in an attempt to compartmentalise the available information.

II SEASONAL HISTOLOGICAL CHANGES

II. 1. Introduction

The histological changes that accompany sexual maturation and spawning have been described in some detail in a number of salmonids, including the Pacific salmon, Genus Oncorhynchus, (Robertson & Wexler 1957, 1959, 1960, 1961, 1962b; Hane & Robertson 1959; Oguri 1960, and Colgrove 1966) the Atlantic salmon, Salmo salar L. (Chester-Jones 1957; Heyl & Carpenter 1972) and the migratory (steelhead) and non-migratory rainbow trout, Salmogairdneri Richardson (Robertson & Wexler, 1959, 1962a; Robertson, Krupp, Thomas, Favour, Hane & Wexler, 1961b).

Although starvation was suggested as a cause of many of the changes occurring in the spawning salmonid (McBride 1967) it is now generally accepted that degenera-tive changes result from hyperadrenocorticism induced by high levels of gonadal steroids acting directly on the interrenal gland rather than via the pituitary gland (McBride & van Overbeeke 1969b). Robertson & Wexler (1962c) further suggested that such changes may represent an acceleration of the normal ageing process. The extent of degenerative change has been associated with variations in levels of circulating corticosteroids and with variations in mortality after spawning, the most extensive changes occurring in anadromous species, especially the Pacific salmon (Robertson & Wexler 1959; Robertson et al 1961b; Heyl & Carpenter 1972).

Work on the brown trout is notably lacking and it was thus decided to examine such fish from Loch Leven to determine whether similar changes occurred in this species.

II. 2. Materials and Methods

A total of 109 fish were sampled between 1973 and 1975. During the spawning season, fish migrating along the burns supplying the Loch (see map, figure 1) were obtained using box traps previously described by Thorpe & Roberts (1972). Occasional fish were caught with landing nets from the burns during this period. Samples of fish were also obtained throughout the year in the main body of the Loch by beach seining. Fish were anaesthetised in MS222 (Sandoz Pharmaceuticals Basle) at a concentration of approximately 1 : 10,000, weighed, measured and a scale sample removed. Assessment of maturity was also made at this stage according to the scheme of Kesteven (1960). In immature fish, if sex was not apparent, small pieces of gonad were removed and fixed in 10% neutral buffered formalin prior to processing for histology.

Blood samples were removed from the caudal vein and processed as described in the section on haematology. Fish were then killed by spinal section and samples of a variety of organs taken and fixed in 10% neutral buffered formalin, processed for routine histology and stained by routine methods (see appendix 1). Organs removed included gill, stomach, lower intestine, spleen, liver, heart, brain (including pituitary), head kidney (to include interrenals), mid-kidney and posterior kidney, pyloric caecae (to include pancreas)

and skin. Skin sections were taken from a point mid-way between the lateral line and the dorsal fin, measuring approximately 1 cm x ½ cm x ½ cm. Samples were removed from both sides of the fish.

Serial sectioning of organs such as the pituitary gland and anterior kidney was often necessary to determine morphological variations.

Skin Measurements

Parameters measured were epidermis thickness, stratum compactum thickness, mucous cell diameter, mucous cell number per unit area and mucous cell number at the epidermal surface. Epidermis and S. compactum thickness was estimated as the mean of 20 random measurements of histological sections. A squared graticule was used to measure mucous cell numbers. Full epidermal thickness was examined on each occasion and the number of mucous cells in a 325μ square area counted. The mean of ten such measurements was estimated. Similarly surface cells were counted along a 325μ length of epidermal surface and a mean of ten measurements was used. Mucous cell diameter was estimated from an average of 50 random cell measurements, the largest diameter being measured in each case.

Statistical values were then computed with the SPSS Fortran package program for the CDC 7600 computer (Nie, Hull, Jenkins, Steinbrenner & Bent, 1975) or the conversion of SPSSG for the ICL 4130 computer (Bland, Lloyd-Jones & McGoldrick 1976).

II. 3. Results

Histological changes associated with sexual maturity

a) Skin

Immature fish

There was no obvious sexual difference in skin structure in immature fish except that the thickness of the epidermis was slightly greater in male fish (see tables 1, 2 and 3).

The epidermis consisted of a so-called stratum germinativum or basal layer above which was a layer of malpighian and mucous cells of between 2 and 10 layers thickness. Cell division occurs throughout all epidermal layers.The basal layer was composed of a regular single layer of cells which were cuboidal to low columnar in appearance containing a prominent nucleus, usually in the upper two-thirds of the cell and being about half the total cell volume (figure 2). This cell layer rested on a basement membrane of flat appearance with very few undulations or foldings at the light microscopic level and which stained distinctly with the P.A.S. technique.

The malpighian cells are the major component of the epidermis and vary considerably in size and shape and in nuclear outline but bizarrely-shaped nuclei and cells were commonly seen in all fish. Mucous cells were a prominent feature of the skin of immature fish, occupying

up to about half the total surface area of any section examined. They appeared to be formed in the immediately suprabasal layers of the malpighian cells and gradually increase in size towards the skin surface.

Cells at the skin surface measured an average of 24 μ diameter. Nuclei of mucous cells were usually flattened and situated basically in mature cells. The contents of the mucous or goblet cells stained positively with alcian blue at pH 2.5 and with P.A.S., but did not stain with H & E; at the surface of the skin, mucous cells appeared to open to the exterior through a pore through which the cell contents were released (figure 3).

Another cell of the epidermis present in variable numbers in any one section, but occurring to some extent in most immature fish was the so-called "albumen" cell (figure 4). This cell stained intensely eosinophilically with H & E and positively with Millon's reagent, suggesting a proteinaceous content. Such cells were usually present near the epidermal surface and were of approximately the same diameter as the mucous cells but some shrinkage of the eosinophilic cytoplasmic contents usually occurred with fixation in neutral buffered formalin. Flattened basal nuclei were present but difficult to visualise.

Scattered through the epidermal surface and especially in the area immediately above the basal layer were small rounded cells which resemble lymphocytes with prominent nuclei and a small quantity of light staining cytoplasm (figure 2).

Such cells were usually only present in small numbers but were a prominent feature of the mature fish (see later).

Immediately beneath the basement membrane is the stratum spongiosum, the most superficial part of the dermis. In immature fish this consisted of a loose meshwork of collagen and reticulin fibres containing chromatophores in superficial areas. The melanocytes were evident as large cells with pale-staining round nuclei (figure 5) and with variable amounts of melanin in the cytoplasm. The melanocytes usually formed a thick continuous line of pigment in the superficial stratum spongiosum. Also in the stratum spongiosum were the scales and scale- beds and the melanin layer was often reflected along the lower scale surface also. Spaces around the scales were often seen as a cutting artefact. In some sections scales were seen protruding into the epidermis; the epidermis in these cases invested the protruding part of the scale and extended downwards some small distance into the dermis. The rest of the stratum spongiosum was composed of collagen and reticulin fibres, neural elements and blood and lymphatic vessels and was often very thin in the immature fish, usually only about 70 μ in diameter.

Beneath the stratum spongiosum is the stratum compactum of the dermis, a layer of dense collagen bundles together with occasional fibroblasts (figure 6). The stratum compactum varied in thickness between individual fish and

with age, being thicker in older fish, but generally measured between 300μ and 550μ in immature fish. Between the stratum compactum and underlying muscle is the hypodermis, well demarcated by another layer of chromatophores. Melanophores were usually larger and more densely-staining than their counterparts in the stratum spongiosum.

Mature Male Fish

The major difference between this group of fish and the immature fish was a pronounced thickening of both epidermis and dermis. (see tables 3 and 9).

The basement membrane of the epidermis showed a series of fluctuations forming ridges or rugae at the epidermis/dermis junction (figure 7). This was most marked in spawning and spent fish. Because of the rugose nature of the basement membrane in these fish, the tip of a dermal villus was frequently seen in the epidermis in section. The basal layer of the epidermis became tall columnar, cells measuring 40μ in height and showing increased mitotic activity. The nuclei were oval in shape and found in the upper half of the cell. The malpighian cells were formed in a variety of bizarre shapes and had a relatively greater cytoplasmic volume than in immature fish. Occasional macrophages containing melanin were seen at this stage in the epidermis.

In maturing fish (Kesteven groups 3,4 & 5) mucous cells were plentiful in the epidermis and usually concentrated in the top ½ - ¾ of any section - no cells recognisable as

maturing mucous cells were evident in the lower parts of the section by light microscopy. In mature and spent fish (Kesteven groups 6 & 7) mucous cells were reduced to zero in some fish (figure 8), although others showed increased numbers of mucous cells. In mature males with reduced or negligible numbers of mucous cells, surface malpighian cells were usually very much flattened and squamous in nature. Such cells were commonly seen sloughing off at the skin surface (figure 9). "Albumen" cells were very much reduced or absent in these fish.

The scales were generally completely enclosed in the depths of the dermis (figure 10) but when an occasional scale was seen near the epidermis/dermis junction, an invagination of epidermis usually partly invested the lower surface as well as the upper surface of the scale. Such epidermal areas under the scale usually contained a number of mucous cells (figure 11).

Increased numbers of lymphocyte-like cells were frequently seen in spawning and spent fish, especially in the immediately suprabasal layers of the epidermis. Local areas of cell swelling and loss of cohesion were also seen in such fish, with or without concomitant fungal infection - these changes are described as Type 1 & 2 in the section on histopathology of fungal infections.

In spawning and spent fish, the basal layer of the epidermis was often irregular with numbers of necrotic cells present (figure 12). Remains of cells were evident as bizarrely-shaped condensed packages of nuclear material surrounded by a clear space following nuclear pyknosis and karyolysis. Cells of this type were present in large numbers through the malpighian cell layers; quantities of melanin were seen throughout the thickness of the epidermis with melano-macrophages apparently migrating through the basal epidermal layer (figure 13).

The stratum spongiosum of the dermis was thicker in adult males and also more cellular (figure 14). Increased quantities of collagen were present, though both this layer and the stratum compactum often appeared oedematous. Melanocytes were scattered through the surface layers of the stratum spongiosum and were seen in increased numbers around vessels penetrating the stratum compactum. The total dermis thickness was much greater than in immature fish.

Mature Female Fish

The skin in this group of fish is also markedly thickened (both dermis and epidermis) in comparison with immature fish, (see tables 3 and 9).

In the epidermis, the basal epidermal layer was low columnar but not so tall as the equivalent in the mature male fish (figure 15). This layer was not so distinct as in

the mature males, but generally stained a little more intensely than the rest of the malpighian cells. No rugae were present in the basement membrane area and the general epidermal structure was very similar in morphology to the immature fish, though type 1 & 2 changes described in the fungal histopathology section did occur. Mucous cells were very numerous, especially in the upper epidermis and many cells were seen apparently discharging their contents to the surface through a pore. A squamous surface layer was not present.

Scales remained superficial in the surface layers of the stratum spongiosum and invested with the lower layers of the epidermis over much of their surface. The layer of melanophores beneath the basement membrane was very pronounced and often formed a continous band of melanin with occasional chromatophores visible (figure 16). In other fish, more densely staining isolated melanin areas were evident in the form of melanophores and melanocytes. The stratum spongiosum remained a thin layer in these fish but the stratum compactum was usually of increased thickness.

Results of Statistical analysis on skin structure

Tables 1 and 2 give epidermal thickness in μm for the four sex groupings used in this study. The results of the t-tests comparing these values show highly significant ($p < 0.001$) differences between immature fish (both sexes pooled) and mature fish (both sexes pooled). Similar results are obtained for individual sexes. Furthermore there is a significant ($p < 0.001$) difference between the mature males and females, males having a significantly thicker epidermis. When immature males are compared with immature females, the results are less significant, though the male fish do show a thicker skin than the females. The above results apply to all ages pooled. To determine whether age differences were present, fish were grouped into age-classes of 1 + 2, 3 + 4 and 5 + 6 and t-tests carried out in individual sex classes between these age groupings (Tables 4 and 5). The results show that there is no significant effect of age with the exception of the immature male grouping, when 1 and 2 year old fish had significantly ($p < 0.05$) thicker skins than 3 and 4 year old fish, though comparison with 5 and 6 year old fish showed no significance. Unfortunately the absence of 1 and 2 year old mature fish precluded a complete comparison of groupings. To further check the effect of age, t-tests were carried out between sex groupings for 3 and 4 year old fish, when very similar results to the pooled age groups were obtained (Table 6).

Tables 7, 8 & 9 compare stratum compactum thickness

between groups. Mature male fish show significantly thicker stratum compactum than immature males and mature female fish, but mature female fish did not show a significantly thicker stratum compactum when compared with immature female fish. The effect of the mature male fish is so great that when mature fish (sexes pooled) are compared with immature fish (sexes pooled) there is a highly significant difference.

Tables 12 - 14 give the values for mucous cell parameters in the four sex classes and t-tests between classes. There are significantly fewer mucous cells at the skin surface in immature males than in mature males but the mean number of mucous cells per unit area gives the reverse result though the mucous cells present are larger in the mature males. A comparison of female fish gives a similar result for surface cells and for cell diameters but there is no significant difference between numbers present per unit area. A comparison of immature fish of either sex shows no significant difference whilst the only parameter that is significantly different in mature fish is that of mucous cell numbers which are significantly reduced in mature male fish.

When sexes are grouped there are significant differences between immature and mature fish with respect to surface cell number and mucous cell diameter.

Pearson correlation coefficients were also calculated for each parameter measured within each of the four sex classes in order to determine whether changes in the

various parameters were related in individual fish. The only regular correlation ($p < 0.05$) was found between number of mucous cells and number of surface cells. This correlation applied to all but the group of immature male fish. A significant correlation was also found between epidermal thickness and stratum compactum thickness ($p < 0.05$) in the immature male fish and between mucous cell diameter and stratum compactum thickness in the mature female group ($p < .005$) (Tables 15 - 18).

TABLE 1

Epidermal thickness (μ) for Male Loch Leven fish. In each case, column (1) denotes fish identification number, column (2) denotes fish age and column (3) gives the epidermal thickness.

Immature Males (Kesteven groups I & II)			Mature Males (Kesteven groups III - VIII)		
(1)	(2)	(3)	(1)	(2)	(3)
1	3	68.06	27	4	251.37
2	3	96.79	28	5	317.21
3	4	66.35	29	3	337.73
4	4	81.74	30	6	284.54
5	3	75.24	31	5	221.62
6	3	57.11	32	6	327.12
7	4	46.51	33	6	237.52
8	4	57.80	34	5	282.15
9	4	24.62	35	5	190.84
10	5	49.93	36	6	259.75
11	2	31.12	37	6	241.28
12	5	32.15	38	6	240.94
13	1	34.20	39	6	212.72
14	5	47.88	40	5	214.61
15	1	34.20	41	5	152.87
16	4	123.12	42	4	201.44
17	3	76.61	43	5	256.84
18	5	123.80	44	5	277.53
19	4	50.27	45	6	252.05
20	4	108.76	46	4	220.25
21	3	102.60	47	6	205.54
22	4	61.22	48	4	208.45
23	4	94.73	49	4	215.46
24	3	71.82	50	3	202.29
25	4	95.08	51	5	164.16
26	5	100.55	52	5	166.04
			53	6	239.57
			54	5	240.94
			55	5	265.22
			56	5	216.49
			57	2	267.79
			58	6	226.40
			59	5	194.26
Mean		69.7022	Mean		236.151
S.D.		29.114	S.D.		44.024
S.E.		5.71	S.E.		7.664

TABLE 2

Epidermal thickness (μ) for Female Loch Leven fish. In each case, column (1) denotes fish identification number, column (2) denotes fish age and column (3) gives the epidermal thickness.

Immature Females (Kesteven groups I & II)			Mature Females (Kesteven groups III - VIII)		
(1)	(2)	(3)	(1)	(2)	(3)
60	3	52.33	91	4	100.55
61	6	79.69	92	4	129.28
62	4	78.32	93	4	191.52
63	4	90.97	94	6	166.21
64	2	41.04	95	5	102.60
65	4	73.87	96	5	137.83
66	3	80.71	97	5	151.51
67	3	44.46	98	5	124.15
68	4	35.91	99	5	143.98
69	3	66.69	100	4	145.69
70	4	112.86	101	5	126.20
71	4	47.88	102	5	77.63
72	4	38.30	103	4	136.80
73	1	40.01	104	4	128.59
74	1	34.20	105	3	107.39
75	4	31.81	106	4	116.62
76	4	29.41	107	6	212.72
77	4	31.81	108	4	244.53
78	1	32.83	109	4	201.10
79	3	42.41			
80	3	39.67			
81	4	62.59			
82	3	27.02			
83	3	49.93			
84	5	63.95			
85	4	25.65			
86	5	48.22			
87	4	79.34			
88	4	79.34			
89	4	76.61			
90	4	69.08			
Mean		55.062	Mean		144.468
S.D.		22.311	S.D.		42.326
S.E.		4.007	S.E.		9.71

TABLE 3

Results of t-tests for pooled age groups comparing epidermal thickness between the four sex classes.

Group 1 = Immature Males

Group 2 = Mature Males

Group 3 = Immature Females

Group 4 = Mature Females

Group Comparison	T value	p
1 v 2	16.61	<.001
1 v 3	2.1	<.05
1 v 4	7.03	<.001
2 v 3	20.55	<.001
2 v 4	7.41	<.001
3 v 4	9.79	<.001
1 + 3 v 2 + 4	15.69	<.001

Table 4

Results of T-test comparing age classes in individual sex classes (Epidermal thickness)

a) Immature males

	T-value	p.
3 & 4 v 5 & 6	.25	.406
1 & 2 v 5 & 6	1.61	.079
1 & 2 v 3 & 4	2.89	.0045

b) Mature Males

3 & 4 v 5 & 6	.08	.468
1 & 2 v 5 & 6	NOT COMPUTABLE	
1 & 2 v 3 & 4	NOT COMPUTABLE	

c) Immature Females

3 & 4 v 5 & 6	.68	.272
1 & 2 v 5 & 6	2.89	.051
1 & 2 v 3 & 4	1.66	.054

d) Mature Females

3 & 4 v 5 & 6	.62	.272
1 & 2 v 5 & 6	NOT COMPUTABLE	
1 & 2 v 3 & 4	NOT COMPUTABLE	

Table 5

Listing of data used in T-tests comparing age classes in individual sex classes. (Epidermal thickness)

a) Immature Males

Age Class	No. of Cases	Mean	S.D.	S.E.
1 & 2	3	33.174	1.78	1.03
3 & 4	18	75.468	24.77	5.84
5 & 6	5	70.862	39.21	17.53

b) Mature Males

1 & 2	1	267.786	0.00	0.00
3 & 4	7	233.855	48.84	18.46
5 & 6	25	235.529	44.09	8.82

c) Immature Females

1 & 2	4	37.021	4.11	2.05
3 & 4	24	56.957	23.56	4.81
5 & 6	3	63.954	15.73	9.08

d) Mature Females

1 & 2	0	0	0	0
3 & 4	10	150.206	46.82	14.80
5 & 6	9	138.092	38.45	12.82

Table 6

Results of T-tests for 3 & 4 year old fish,comparing epidermal thickness between the four sex classes.

Group	Class	No.of cases	Mean	S.D.	S.E.
1	Immature Males	18	75.468	24.767	5.838
2	Mature Males	7	233.855	48.839	18.46
3	Immature Females	24	56.957	23.556	4.808
4	Mature Females	10	150.206	46.817	14.805

Group Comparison	T value	p.
1 v 2	10.84	<.001
1 v 3	2.45	.0095
1 v 4	5.56	<.001
2 v 3	13.48	<.001
2 v 4	3.53	.002
3 v 4	7.78	<.001
1 + 3 v 2 + 4	10.51	<.001

TABLE 7

S. Compactum thickness (μ) for Male Loch Leven Fish.

Immature Males		Mature Males	
Fish Number	Thickness	Fish Number	Thickness
1	434.70	11	585.12
2	518.88	12	935.64
3	394.68	13	738.30
4	298.08	14	597.54
5	469.20	15	427.80
6	407.10	16	621.00
7	579.60	17	772.80
8	434.70	18	833.52
9	524.40	19	513.36
10	247.02	20	873.54
Mean	430.836		689.862
S.D.	101.883		166.023
S.E.	32.218		52.501

TABLE 8

S. Compactum thickness (μ) for Female Loch Leven Fish.

Immature Females		Mature Females	
Fish Number	Thickness	Fish Number	Thickness
21	340.86	31	1046.04
22	553.38	32	487.14
23	445.74	33	263.58
24	322.92	34	505.08
25	427.80	35	401.58
26	299.46	36	422.28
27	303.60	37	397.44
28	259.44	38	437.46
29	408.48	39	397.44
30	390.54	40	293.94
Mean	375.222		465.198
S.D.	87.566		217.376
S.E.	27.691		68.74

TABLE 9

Results of T-tests comparing stratum compactum thickness between the four sex classes.

Group 1 = Immature males

Group 2 = Mature males

Group 3 = Immature females

Group 4 = Mature females

Group comparison	T value	p.
1 v 2	4.21	<.001
1 v 3	1.31	0.103
2 v 4	2.6	.0095
3 v 4	1.21	0.12
1 + 3 v 2 + 4	3.24	.0015

TABLE 10

Mucous cell parameters for Male Loch Leven Fish.

Column 1 = Fish identification number; Column 2 = Mucous cell number per unit area; Column 3 = Surface cell number and Column 4 = Mean mucous cell diameter.

Immature Males				Mature Males			
1	5.46	12.00	17.10	11	2.37	12.00	23.09
2	3.11	1.00	15.05	12	3.41	12.00	21.03
3	3.82	3.00	17.44	13	1.38	11.00	20.52
4	3.60	0.00	18.13	14	2.37	20.00	24.97
5	3.55	3.00	15.05	15	1.29	0.00	26.33
6	3.11	10.00	15.39	16	1.13	10.00	25.99
7	2.24	6.00	18.47	17	2.59	14.00	24.62
8	2.02	0.00	17.78	18	0.00	0.00	34.20
9	2.20	3.00	19.67	19	1.44	12.00	22.91
10	3.62	5.00	14.02	20	1.92	16.00	30.10
Mean	3.273	4.3	16.8093		1.79	10.7	24.396
S.D.	1.013	4.057	1.828		0.951	6.325	2.946
S.E.	0.32	1.283	0.578		0.301	2	0.982

TABLE 11

Mucous cell parameters for Female Loch Leven Fish.

Column 1 = Fish identification number; Column 2 = Mucous cell number per unit area; Column 3 = Surface cell number and Column 4 = Mean mucous cell diameter.

Immature Females				Mature Females			
21	6.13	11.00	15.05	31	3.43	9.00	30.10
22	3.50	5.00	14.71	32	2.97	15.00	22.91
23	2.79	2.00	10.94	33	3.21	4.00	18.47
24	5.34	6.00	16.42	34	4.49	8.00	23.26
25	5.41	4.00	17.78	35	5.04	18.00	19.15
26	2.47	0.00	18.47	36	4.77	19.00	25.65
27	3.25	3.00	15.73	37	3.99	19.00	20.18
28	4.42	3.00	13.68	38	6.35	14.00	20.52
29	4.08	2.00	17.44	39	5.79	21.00	26.33
30	-	-	13.68	40	5.73	29.00	20.52
Mean	4.1544	4	15.39		4.577	15.6	22.7088
S.D.	1.27	3.162	2.28		1.17	7.276	3.702
S.E.	0.423	1.054	0.721		0.37	2.301	1.171

TABLE 12

Results of T-tests comparing mucous cell numbers per unit area between the four sex classes.

Group 1 = Immature males

Group 2 = Mature males

Group 3 = Immature females

Group 4 = Mature females

Group comparison	T value	p.
1 v 2	3.38	.0015
1 v 3	1.66	.059
2 v 4	5.85	<.001
3 v 4	0.75	.231
1 + 3 v 2 + 4	1.05	.149

TABLE 13

Results of T-tests comparing numbers of surface mucous cells between the four sex classes.

Group 1 = Immature males

Group 2 = Mature males

Group 3 = Immature females

Group 4 = Mature females

Group comparison	T value	p.
1 v 2	2.69	.0085
1 v 3	0.18	.429
2 v 4	1.61	.062
3 v 4	4.41	< .001
1 + 3 v 2 + 4	4.96	< .001

TABLE 14

Results of T-tests comparing mucous cell diameters between the four sex classes.

Group 1 = Immature males

Group 2 = Mature males

Group 3 = Immature females

Group 4 = Mature females

Group comparison	T value	p.
1 v 2	6.66	<.001
1 v 3	1.54	.072
2 v 4	1.1	.142
3 v 4	5.32	<.001
1 + 3 v 2 + 4	8.12	<.001

TABLE 15

Correlation between skin parameters measured in Immature Male Fish

r = Correlation coefficient

p = Probability value

1 = Epidermal thickness

2 = Mucous cell number per unit area

3 = Surface mucous cells

4 = Stratum compactum thickness

5 = Mean mucous cell diameter

		1	2	3	4	5
1	r		- .2002	.4282	.6452	.2855
	p		.290	.108	.022	.212
2	r			.5205	- .4063	- .3316
	p			.061	.122	.175
3	r				.0424	- .1690
	p				.454	.320
4	r					.3849
	p					.136
5	r					
	p					

TABLE 16

Correlation between skin parameters measured in Mature Male Fish

r = Correlation coefficient

p = Probability value

1 = Epidermal thickness

2 = Mucous cell number per unit area

3 = Surface mucous cells

4 = Stratum compactum thickness

5 = Mean mucous cell diameter

		1	2	3	4	5
1	r	.	.5077	.5176	.1044	.5296
	p		.067	.063	.387	.071
2	r		.	.6646	.2361	- .2730
	p			.018	.256	.239
3	r			.	.1769	.0419
	p				.312	.457
4	r				.	- .0555
	p					.444
5	r					.
	p					

TABLE 17

Correlation between skin parameters measured in Immature Female Fish

r = Correlation coefficient

p = Probability value

1 = Epidermal thickness

2 = Mucous cell number per unit area

3 = Surface mucous cells

4 = Stratum compactum thickness

5 = Mean mucous cell diameter

		1	2	3	4	5
1	r		.3982	.0043	.2995	- .0801
	p		.144	.496	.200	.413
2	r			.8029	- .0917	.2211
	p			.005	.407	.284
3	r				.0013	- .0574
	p				.499	.442
4	r					- .2054
	p					.285
5	r					
	p					

TABLE 18

Correlation between skin parameters measured in Mature Female Fish

r = Correlation coefficient

p = Probability value

1 = Epidermal thickness

2 = Mucous cell number per unit area

3 = Surface mucous cells

4 = Stratum compactum thickness

5 = Mean mucous cell diameter

		1	2	3	4	5
1	r		- .3405	- .0493	.2687	.1462
	p		.168	.446	.226	.343
2	r		.5743	- .3304	- .1196	
	p		.041	.176	.371	
3	r			- .3482	- .0715	
	p			.162	.422	
4	r				.7782	
	p				.004	
5	r					
	p					

b) Heart

The major pathological finding in the hearts of Loch Leven brown trout was a pericardial infection with metacercariae of Cotylurus spp. There was no particular sex or age incidence of severity of infection but infection was rare in 1+ fish. Infections were not linked with sexual maturity or spawning. Metacercariae were most commonly seen either at the apex of the ventricle or at the junction of the bulbus arteriosus and the ventricle. The presence of metacercariae was confined to the pericardial sac where they elicited a marked inflammatory response and the development of pericarditis and pericardial and epicardial adhesions.

The inflammatory response often extended over large areas of the bulbus and ventricle and usually consisted of a mass of monocytes and lymphocytes with large numbers of eosinophilic granular cells.(figure 17). The inflammatory response usually involved only the most superficial layers of the epicardium (figure 18), although in one or two cases more extensive areas involving the superficial myocardium were affected (figure 19).

In the most seriously affected fish, local areas of myofibrillar necrosis were evident in the myocardium, usually associated with a marked infiltration with eosinophilic granular cells (figs 20 & 21) and oedema of the outer muscle layers. The parasites themselves were usually contained in thick walled cysts consisting of a dense layer of fibrous tissue and epithelioid cells surrounded by a zone of eosinophilic granular cells (figure 22).

Fibrous adhesions were common in the pericardial cavity, but affected fish appeared in good condition and such lesions had no apparent effect on the electrocardiogram (see section on electrocardiogram changes).

Aggregations of eosinophilic granule cells were found in the wall of the bulbus arteriosus in virtually all fish examined, but no apparent pathological change was associated with such findings.

Changes commonly found in sexually mature fish examined were areas of localised cytoplasmic degeneration and occasional focal necrosis of myofibrils with an associated localised inflammatory response. The ventricle was the most frequent site of such lesions. Three fish also showed areas of haemorrhage into the epicardium and myocardium with necrosis of affected myofibrils, but these were fish which may have been present in traps for several days and such damage may have arisen from efforts to escape.

Despite extensive examination, no lesions were seen in blood vessel walls which may have represented any condition similar to arteriosclerosis. Occasional outgrowths from coronary artery walls (figure 23), although initially causing some confusion, have now been confirmed as vessel junctions (Professor G.A.Gresham pers. comm. 1977).

c) Liver

The livers of immature fish usually showed appreciable quantities of fat in the cytoplasm of hepatocytes. This was much decreased, though variable, in mature fish (figure 24).

Degenerative changes were rare, but occasional mature fish showed haemorrhage into the substance of the liver and areas of focal cytoplasmic change and cell necrosis (figure 25).

In one mature male fish a hepatoma was present. Hepatoma tissue was infiltrating large areas of liver tissue and elicited a marked E.G.C. response (figure 26). Centres of some neoplastic areas became necrotic and melanin deposits were evident in the E.G.C. zone.

Another change variably present in both immature and mature fish was a marked macrophage infiltration around blood-vessels and bile ducts in the liver parenchyma. Melanin deposition was variable in such areas, but was increased in older fish. Cells of Ito were generally more prominent in spawning fish (figure 27).

d) Gill

Gill lesions were variably present in association with a variety of ectoparasite infections and included telangiectasis (figure 28) and fusion and overgrowth of gill epithelia (figure 29). No sexual differences or effects of maturation were seen, but eosinophilic granule cells were especially common in the epidermis of gill skin in all fish at all times of the year. So-called albumen cells were also present, and their incidence paralleled that in the skin.

(e) Gonads

Testis

The immature testis was composed of a number of lobules containing large primordial germ cells separated by indistinct connective tissue septa containing blood capillaries. The primary germ cells contain a nucleus occupying about half the cell diameter with a single nucleolus. Sertoli cells were also seen in small numbers within the lobules and were of irregular shape and with a dense nucleus and prominent nucleolus. Lobule boundary cells were also seen in small numbers and were flattened cells with elongated nuclei (Figure 30).

With development, larger numbers of primary spermatogonia were formed around narrow lumina and with cell division, secondary spermatogonia were also seen within the lobules (Figure 31). Further division led to the production of primary and secondary spermatocytes (Figs. 31 & 32), development being asynchronous with a variety of stages present in any single section.

At full maturation, spermatids were formed which themselves formed spermatozoa so that in a fully mature testis, spermatozoa and spermatids were by far the predominant cell-types within the lobules. At this stage the lobule boundary cells were present in greater quantities, (Figure 33).

In the spent fish, a few spermatozoa remained, but the testis consisted essentially of thick septa of

connective tissue containing numerous lobule boundary cells and occasional spermatogonia (Figure 34).

Ovary

The ovary in the immature fish contained developing oocytes of stages II, III and IV in varying numbers (Figure 35). Stage II oocytes typically contained a central nucleus surrounded by a compact layer of cytoplasm. Stage III oocytes were similar but larger in size and with a distinctly vacuolated periphery (Figure 36).

Stage IV oocytes were at the vitellogenic stage and were large cells with numerous oil droplets in the cytoplasm (Figure 37) and at this stage chorion development was evident and there was a well-formed nutritive follicle cell layer. In spawning fish many cells were at this latter stage. In spent fish, a few such oocytes remained but there was a much greater proportion of connective tissue : oocytes, a few stage I and II oocytes were present and corpora atretica were evident, often infiltrated with melanomacrophages (Figure 38).

f) Kidney

A variety of degenerative changes were found in both immature and mature fish. None of the changes found was consistently present or absent in either group of fish, but the incidence of all changes was greater in the mature group. No sex differences were noted.

Cytoplasmic vacuolation and degeneration was commonly present in tubule cells especially in the distal segment (figure 39). Hyaline eosinophilic droplet formation in tubule cells especially in the proximal segment were commonly present in both immature and mature fish (figure 40 Fine glomerular and tubular eosinophilic deposits were also commonly found in both groups of fish; such deposits in glomeruli were usually associated with shrunken glomerular tufts and slight capillary sclerosis and this seemed most marked in mature fish. Slight thickening of Bowman's capsule was variably present in association with the above glomerular change (figure 41). In no case was glomerular damage found in all glomeruli in any particular section examined. Congestion of glomerular tufts was occasionally seen at all stages of maturity and there was not evident change in the structure of the corpuscles of Stannius.

Immature fish often showed an infiltration of larger collecting duct and ureter walls with E.G.C's and occasional very large cells (10 - 12 μ) with bright red-staining cytoplasm were found amongst haemopoietic tissue of immature fish (figure 42).

In one immature fish, haemorrhage associated with infection with an unidentified septate fungus was noted, but was not extensive.

Basophilic tubular elements of reduced size were commonly seen but there was no relation of their presence to any particular state of maturity.

g) Adrenal Tissue & Haemopoietic Tissue

The adrenal cortical tissue in the brown trout is situated in the "head kidney", a cruciform area of predominantly haemopoietic tissue found dorsal to the heart (figure 43). The adrenal cortical cells, in the immature fish, were found in small clusters surrounding the cardinal veins and their branches or as more discrete islets of tissue. Most frequently an aggregation of cells up to four cells' thickness was found surrounding a vein or venule, individual cells possessing a round nucleus with a distinct nucleolus and fine chromatin network and a cytoplasm with finely dispersed small granules. The amount of adrenal tissue in individual immature fish and sections of the head kidney from any one fish was very variable, but on average was considerably less than that found in mature fish. Cells varied in size in immature fish from 8 μ to 12 μ with nuclei ranging between 5 μ and 7 μ. Nuclei generally stained quite distinctly with H & E but cytoplasmic staining was variable and often indistinct with variable granulation. In a few immature fish the adrenal cells were arranged into "ducts", cells being circularly arranged around a central channel (figure 44). The haemopoietic tissue was generally very densely-packed

with a range of nuclear size and shape though cells usually showed small cytoplasmic volume (<50%). Many of the nuclei of individual haemopoietic cells were horse-shoe or sausage-shaped (figure 45) and fine blood sinusoids were seen permeating the entire organ. In immature fish, "nests" of cells with densely-staining nuclei and very little cytoplasm (figure 46) were occasionally seen in the haemopoietic areas.

Melanin deposits were very variable in quantity and staining properties, though older fish generally exhibited increased quantities of dense-staining melanin. No correlation of quantity of melanin with state of maturity was present.

Mature fish showed a marked variation in adrenal tissue morphology from the immature pattern. Hyperplasia of adrenal tissue was virtually always present, cells being arranged in large masses, usually around a vein or venule, with a well-developed network of blood sinusoids infiltrating the area (figure 47). Individual cells were usually slightly hypertrophied (approx. 14μ with nuclei measuring 8μ) and showed less dense nuclear staining in combination with a distinctly granular cytoplasm.(figure 48). Mitoses were rarely seen at either stage.

Degenerative changes in adrenal cortical cells were very variable but were present to some extent in the majority of mature fish. Early signs of degeneration consisted of loss of cytoplasmic detail and cellular outline with occasional vacuolation and loss of granulation of the cytoplasm (figure 49). Actual adrenal cell necrosis and bulla formation occurred in

worst-affected fish (figure 50). Nuclei were generally only affected in the terminal stages when pyknosis and karyorrhexis were seen. Sinusoids were generally much more distinct when degenerative change was present.

Changes were also seen in the haemopoietic tissue in mature fish. A more pronounced sinusoidal development was obvious and cells were usually easily separated into two distinct size ranges. Small germinal-type cells, approximately 4 μ in diameter with very little cytoplasm and larger 11 μ diameter cells with faint-staining nuclei were seen. The larger cells were especially common along sinusoids together with an increased connective tissue element and these cells did show cytoplasm degenerative changes in some fish.

h) Gut and Pancreas

Pancreatic tissue in the brown trout is found in the fat surrounding the gut and pyloric caecae and consists of granular exocrine cells and discrete islets of Langerhans containing the endocrine tissue.

There is generally a considerable amount of fat present around the intestinal loops of immature fish (figure 51). It is usually present in greater quantity in summer than winter. Pancreatic tissue was dispersed amongst the fat cells with relatively few islets in the immature fish. Islets were usually small 75μ to 200μ on average, but islets up to 350μ were seen in immature fish. The number and size varied considerably depending on the area examined in any particular fish.

Mature fish showed a dramatic reduction in the amount of abdominal fat. Individual fat cells were usually shrunken and of decreased cytoplasmic volume. Considerable quantities of connective and fibrous tissue were usually present amongst the fat cells in the mature fish (figure 52). The quantity of exocrine tissue present was difficult to assess, but allowing for the decrease in fat, was probably similar in both immature and mature fish. Islet tissue however did seem increased in quantity and generally in individual islet size (250μ to 400μ on average and islets up to 770μ .diameter were seen) in mature fish, though considerable variation was present. α/β cell differentiation showed a distinct increase in β cells in the mature fish.

Degenerative changes were evident in three mature fish and consisted of cytoplasmic vacuolation and breakdown with fibrosis and prominent melanin accumulation.
Such fish were maturing and numbers of fat cells were still intact amongst the damaged tissue. In approximately one-third of the mature fish examined, islet degeneration was evident. This consisted of varying degrees of cytoplasmic vacuolation and degeneration eventually leading to nuclear damage and cellular destruction so that often, only "ghosts" of cells remained in parts of the islet (figure 53).
There was no associated inflammatory infiltrate.

There were also changes in the gut and pyloric caecae in mature fish. The submucosa and lamina propria were

generally much more cellular and usually increased in volume compared with the other gut layers. An inflammatory cell infiltrate, especially with eosinophilic granular cells (E.G.C.'s) was common (figure 54), in association with a general increase in E.G.C.'s at the level of the stratum granulosum. The mucosal surface showed no change from that in the immature fish.

Oedema was commonly seen in the stomach wall especially at the junction of stratum compactum and circular muscle in the submucosa (figure 55). Local muscle necrosis associated with inflammatory cell infiltrates was occasionally seen in both muscle layers (figure 56). There was an increase in the amount of nervous tissue (Auerbach's plexus) and blood vessels in the loose connective tissue between circular and longitudinal muscle coats.

Early intimal hyperplasia was seen in 3 fish in the larger vessels passing through the fat surrounding the gut and was associated with reduplication and splitting of the internal elastic membrane (figure 57).

Cestode infection was commonly found in the gut lumen of both immature and mature fish, but appeared to cause no damage to the gut wall (figure 58). Fish with cestode infections appeared in good condition.

Occasional encysted larval cestodes were seen on two occasions in mature male fish in the abdominal fat and outer

surface of the gut wall (figure 59). The parasites were walled off by an intense local inflammatory response in which the E.G.C. was a prominent cell.

i) Spleen

Spleen in immature fish showed a distinct differentiation into areas containing red blood cells and those producing white blood cells. Germinal areas of white blood cell formation were extensive and the commonest cell-type was a small basophilic (4 µ) round or oval cell with a distinct nucleus and very little cytoplasmic volume (figure 60). Cells were aggregated around the connective tissue of an ellipsoid which generally contained a few melanomacrophages. The amount of melanin present was very variable and bore no relation to sexual maturity. Melanomacrophage centres were variable in presence and size.

The major change in mature fish was a marked depletion of apparent leukopoietic tissue and congestion of the organ. Depletion was frequently associated with depletion of melanomacrophages in the melanomacrophage centres and melanomacrophages were seen in large numbers in the lumina of splenic blood vessels at this stage (figure 61).

Melanin deposition throughout the splenic tissue was very variable in quantity and type. In older fish, large quantities of very dark staining melanin was deposited throughout the organ (figure 62) whereas in younger fish complete absence of any melanin or its precursors was often found.

Other fish possessed large numbers of cells containing lighter-staining melanin precursors (figure 63).

No cellular degenerative change was seen in the spleen of any group of fish.

j) Pituitary Gland

Serial sectioning was found necessary to determine relative sizes of different lobes and numbers of cell-types in the pituitary gland. Even then, only a qualitative estimate of the changes present was made.

Immature fish

Following the terminology of Robertson & Wexler (1962) the pituitary gland was seen to consist of four lobes, the anterior, dorsal, ventral and neural lobes. The mammalian equivalents and synonyms used in other fish work are considered in the discussion on this section together with a functional consideration of the different cell-types.

Using mammalian techniques, it was found that one tinctorial abnormality was present in the brown trout - acidophils in the anterior lobe which should stain red by Barrett's method and orange-yellow by Slidder's technique in fact stain magenta by Slidder's technique.

The ventral lobe contained groups of loosely-arranged cells separated by small quantities of connective

tissue and sinusoids. Neural elements were seen within this glial tissue originating from the neural lobe. The majority of cells were cuboidal or columnar in shape with large nuclei and acidophilic cytoplasm containing small granules. In immature fish this was the largest lobe, occupying between 1/2 and 2/3 of total pituitary volume.

The neural lobe consisted of glial tissue and neural elements. No colloid was formed.

The dorsal lobe consisted of columns of cells with varying staining affinities (figure 64), separated by small quantities of connective tissue. The principal cell-type in the immature fish was the acidophil, a cell with a densely-staining nucleus situated basally and a large number of densely-staining coarse cytoplasmic granules. This tissue was found especially at the lateral areas of the lobe.

Basophils were also found at this stage but were less numerous than the acidophils. The nucleus was less deeply-staining and situated centrally and the cytoplasm was finely granular.

Chromophobe cells were only seen very occasionally at this stage.

Neural elements from the neural lobe also permeated this lobe.

The anterior lobe was formed anteriorly, surrounding the dorsal lobe and cells were cuboidal or columnar and arranged in follicles with spherical lumina. Cells stained acidophilically with granules present in the parts of the cell distal to the lumina

Maturing Fish

The major change in maturing fish was a decrease in the relative volume of the ventral lobe and a relative increase of the anterior and dorsal lobes, especially the latter. This increased size of the dorsal and anterior lobes was accompanied by an increased vascularity. Both basophils and acidophils were increased in number in the dorsal lobe but the increase was most marked in the basophils. Chromophobe cells were rarely seen. This increased number of cells was accompanied by an increased granulation and staining affinity. The follicles of the anterior lobe also increased in quantity and colloid was often found in the lumina. (figure 65).

The columnar arrangement of the ventral lobe was replaced with a rather folliculoid appearance and an increase in connective tissue was evident.

Spawning and Spent Fish

An even greater increase in the size of the dorsal lobe was evident with basophils now by far the most prominent cell. The cells were arranged in a columnar fashion and

staining intensity of individual basophils at this stage was very variable. Chromophobes were found in increased numbers (figure 66).

Anterior lobe follicles were also further increased in size and cells were more elongated.

The ventral lobe contained increased quantities of connective tissue and degenerative change was present in all lobes to a small degree and consisted of vacuolation with some pyknosis.

II. 4. Discussion

Skin

The detailed structure of normal teleost skin has been reviewed by Bullock & Roberts (1975) and although only marine teleosts were discussed in detail, anadromous salmonids were included in this work and cell-types similar to those found in the present study were described.

The principal changes occurring in the skin of spawning brown trout found in the present study were an increased thickness in the skin of both sexes at sexual maturity. This was greatest in the male. Similar sex and maturity-related changes have been described for three varieties of Pacific salmon (Robertson & Wexler 1960), and for the brown trout and the char (Pickering 1977). An increase in thickness of skin at spawning time with no reference to sexual variation has been noted in salmonid fish by Roberts, Shearer, Elson & Munro (1970) and by Travis-Jenkins (1942) - quoted by Stoklosowa (1970). Stoklosowa (1966) also noted a sexual dimorphism in the skin of the spawning sea trout S. trutta L. but he described a thin epidermis in the male and a thick epidermis in the female. He later showed (Stoklosowa 1970) that there was no dimorphism in immature fish and no dimorphism was noted in spawning lake trout. Only small numbers of fish were however examined in his study and he does suggest that thinness of male epidermis may be due to sloughing, which was readily observed.

The increased skin thickness in the present study involves both dermis and epidermis in the male, confirming the findings of Robertson & Wexler (1960) and the dermal increase noted by Stoklosowa (1966 & 1970). However, the mature females did not show an increased stratum compactum thickness over the immature females. Measurements of stratum compactum were made rather than total dermis thickness because the stratum spongiosum thickness was very variable over any one section and in some cases cutting artefacts artificially increased its thickness. This may explain the difference in findings of the present work with those other workers mentioned previously. It is also perhaps surprising that there was no correlation between stratum compactum thickness and epidermal thickness with the exception of the immature fish though this may be due to the small number of samples and large standard errors. Nevertheless thickening of the two structures could occur at slightly differing times or in response to different stimuli.

Variation in mucous cell numbers has been investigated in detail by Pickering (1974). He described increased numbers in male and female fish in pre-spawning and post-spawning periods, but a massive decrease in males as opposed to a peak in number in females at spawning. Such findings were similar in the char. In these studies, Pickering counted the numbers of cells open to the surface of the skin by surface-staining with alcian blue. In the present investigation attempts were made to count cells histologically. Decreased mucous

cells were also noted in spawning male salmonid fish by Stoklosowa (1966 & 1970). This was not noted by Robertson & Wexler (1960). Roberts et al (1970b) noted increased mucous cell numbers in both sexes.

From the present work, surface opening cells are dramatically increased in both sexes with maturity but cells per unit area are decreased in the male and remain unchanged in the female. This is equivalent to a dramatic rise in total number in mature female fish as the epidermis is markedly thicker. There was considerable variation in the numbers of cells present in mature male fish - in some fish, numbers similar to that found in mature female fish were found whilst in others the number of cells was reduced to zero. In the present work, prespawning and spawning fish were not subdivided but further examination showed that the spawning males had markedly reduced numbers of mucous cells, supporting Pickering's findings. No such fall occurred in the mature female fish. Mucous cell diameter was also increased in mature fish in the present work but the eventual mucous production at the skin surface depends also on the cell kinetics of the skin - an increased turnover of epidermis would further increase mucous production and it is interesting to speculate that if epidermis kinetics are increased in spawning fish, the reason for the drop of mucous cells to zero in spawning fish may be a further increase in cell turnover so that sufficient time is not available for mucous cell differentiation. It was noted

that in many mature fish, mucous cells were only found in the upper half of the epidermis, and particularly in mature male fish with reduced or absent mucous cell numbers, mitotic activity in the epidermis was much increased. Handling has also been shown to lead first to an increase in mucous cell production and then to an apparent exhaustion of production before eventual recovery. These factors would seem to lend further support to the suggestion that an increased rate of cell turnover is involved but detailed work in cell kinetics is necessary to adequately test this hypothesis.

"Resorption" of scales into the epidermis in mature male salmonids is well-known to fishery workers and makes difficult the taking of scale-samples for age determinations. This scale resorption has been noted by Robertson & Wexler (1960) and by Yamazaki (1972) and was again confirmed in the present study.

The layer of lymphocyte-like cells present in the suprabasal areas of the epidermis has been noted in a number of salmonids (Pickering & Macey 1977, Roberts, Shearer, Munro & Elson 1969).

A cellular response in the suprabasal epidermal layers similar to that seen in the present study has been described and shown to be a result of cell-mediated immunity (Carlisle 1977). It has also been suggested (Roberts 1978, pers. comm.) that these lymphocytes present in the epidermis may represent the fish equivalent of

the Bursa of Fabricius in avian species, lymphocytes being formed in skin without the necessity of transfer of cells from the blood. Certainly, no evidence of lymphocyte migration was seen through the dermis at any stage in the normal skin in the present study and intense infiltration described in the section on fungal histopathology did appear to lead to skin sloughing, also noted as a result of cell damage in the suprabasal layers by Roberts, Shearer, Munro & Elson (1970b) in the Atlantic salmon. This may also explain the findings of Stoklosowa (1966 & 1970) in which he describes sloughing of mature male skin leading to thinness of the epidermis.

There also appears to be a decrease in the number of albumen cells in mature male fish in the present study. These cells were described as albumen cells by Ashley (1972) and similar cells were described by Pickering & Macey (1977). Blackstock & Pickering (pers. comm. 1979) have examined their structure in detail and the protein content of these cells suggests that they may possibly be involved in the fish immune system. A similar drop in albumen cells in mature male fish has also been noted in brown trout in Windermere (Pickering 1979, pers. comm.).

Another very marked change in mature male fish noted in the present study was the formation of rugae at the epidermis/dermis junction. This has also been noted in the brown trout in Windermere (Pickering 1979 pers. comm.) and a similar change has been noted in healing lesions of

UDN (Roberts, Ball, Munro & Shearer 1971). The rugae are reminiscent of the ridges found in normal human skin and their presence in healing conditions suggests that their formation might be due to the need for an increased surface area at the basement membrane surface either to improve the mechanical adhesion of the two layers or to decrease the distance over which nutrients must pass from the dermis. The latter suggestion seems rather unlikely however, as degenerative changes usually originate in the suprabasal layers of the epidermis, not in areas distant from the blood supply. The investment of more superficial scales with epidermis in both immature fish and mature fish might serve a similar mechanical function.

The presence of melanin-containing cells in the epidermis of mature male fish is also unusual. Although a normal feature of certain teleosts (Bullock & Roberts 1975) such as the plaice Pleuronectes platessa L, such cells have not previously been described as a normal component of salmonid epidermis, but they may represent removal of degenerative elements from the dermis by macrophages moving through the epidermis (Roberts, McQueen, Shearer & Young 1973; Laird, Roberts, Shearer & McArdle, 1975).

Heart

The present survey has indicated that sexual maturation in the Loch Leven brown trout has little effect on the heart with the exception of some mild myofibrillar cytoplasmic degeneration and necrosis. The effects of an intercurrent Cotylurus infection do not appear to be related to sexual maturity. Wootten (1973) has discussed similar parasitic infections in brown and rainbow trout in Essex and described a life-cycle involving molluscs and gulls. He also described pericardial infections, primarily involving the ventricle, but not affecting the heart muscle itself. Infection of fish was thought to take place primarily in the summer and the parasite appears well-adapted to its host, often surviving for more than a year as an encysted metacercaria in the fish.

No arterial changes in the coronary arteries or their branches or indeed in any other blood vessels in other organs were found in the present study with the exception of mild intimal hyperplasia in gut vessels. The only report of heart disease in brown trout is that of calcific heart disease in New Zealand brown trout described by Prior, Webber, Alexander & Barclay (1968). The bulbus arteriosus was affected in 20% of fish examined and was grossly dilated with calcium deposits present in the trabecular layer. The initial lesion was thought to be a vasculitis leading to an area of avascular necrosis which ultimately became calcified. Coronary arteries

were not affected and there was no apparent sex incidence or relation to sexual maturity, but the disease was more prevalent in older fish.

Degenerative changes in the cardiovascular system associated with sexual maturation and spawning have been noted for a number of salmonids including the steelhead and rainbow trout (Robertson, Krupp, Thomas, Favour, Hane & Wexler, 1961b; Van Citters & Watson, 1968; McKenzie, House, McWilliam & Johnson, 1978), Atlantic salmon Salmo salar (Manèche, Woodhouse, Elson & Klassen 1972) and the Pacific salmon (Robertson, Wexler & Miller 1961 ; Moore, Mayr & Hougie 1976). Lesions have been described in the ventral aorta of the carp Cyprinus carpio (Eastman 1969), although these changes were linked to age rather than sexual maturation. Vastesaeger, Delcourt & Gillot (1965) have also described arterial lesions found in 18 out of 101 fish examined from a wide range of families, although none of the salmonids examined showed lesions; lesions which may be described as atherosclerotic, i.e. containing lipid deposits, were only found in the bluefin tuna Thunnus thynnus L (originally described by Vastesaeger, Gillot & Vastesaeger 1962), lesions in other fishes being small focal fibroelastic intimal proliferations, together with large numbers of lipid-laden histiocytes in early stages.

The work on the three species of salmonids mentioned above showed lesions of arteriosclerosis of varying degree linked with sexual maturation and spawing in each

case. No fat involvement or typical atherosclerotic lesions were found in any of this work. Lesions in all three species consisted of intimal proliferation with disruption or proliferation of the internal elastic membrane immediately beneath lesions. Staining methods used showed increased mucopolysaccharide deposits within such lesions. Partial vessel occlusion was also frequently seen and resulted either from the lesions described above or from thickening of vessel walls. Such changes in the vessel walls were not seen by Maneche et al (1972) in the Atlantic salmon, but Van Citters & Watson (1968) reported fibrous replacement of the media in more advanced cases in the steelhead trout. Robertson et al (1961a) reported some medial thinning and disruption of muscle fibres, but the most marked change in the Pacific salmon was increased thickness of the adventitia due to collagen deposition, which often resulted in partial or complete occlusion of vessels in heart and other organs. This change was not noted in the more recent study of Moore et al (1976). Thick adventitia and narrow lumina were frequently seen in Loch Leven brown trout in a variety of organs and at varying age and stages of maturity, but in no case did this appear pathological or linked to any one group of fish.

Robertson et al (1961a) described a much increased lesion incidence as maturity developed, but did not study post-spawning fish. The work of Van Citters & Watson (1968) & Maneche et al (1972) indicates that regression of lesions may take place after spawning and that lesions

do not appear to be cumulative from one season to the next. (An almost universal death occurs after spawning in Pacific salmon and so post-spawning fish would be difficult to obtain and presumably could not be investigated by Robertson et al 1961a.) Such lesion regression is unusual, but may occasionally occur. Starvation, wasting disease and malnutrition may lead to regression of coronary artery lesions in man (Strom & Jensen 1950; Wilens 1947; Tzinzerling, Zakhar'evskaia & Volkova 1962) and diet-induced lesions may regress on alteration of diet or manipulation of hormone levels (Anitschkow, 1928; Horlick & Katz 1949; Pick, Stamler, Rodbard & Katz 1952). Marked changes in adrenocorticosteroids occur in the spawning salmonid, in particular the Pacific salmon (see section on hormone manipulations and references by Hane & Robertson 1959, Idler et al 1959, Robertson et al 1961b; and Schmidt & Idler 1962) and this may in part explain the genesis of lesions. Post-spawning fish are exposed to prolonged starvation and this may again in part explain possible lesion regression.

Robertson et al (1961a) also describe changes in the heart muscle of spawning Pacific salmon consisting of vacuolation and loss of fibrils with degeneration of nuclei and enlargement of individual fibres. Cartilaginous metaplasia and actual cartilage formation was also seen, especially at the aortic valve ring and in the valve cusps.

Though no cartilaginous metaplasia was found in the

Loch Leven fish, some vacuolation and myocardial necrosis were seen in occasional fish. Similar changes have been described following the feeding of diets deficient in potassium to rats (Schrader, Prickett & Salmon 1937; Thomas, Mylan & Winternitz 1940, Follis, Orent-Keiles and McCollum 1942). Adrenal corticosteroid administration also causes similar effects in a variety of animals (Darrow & Miller 1942; Kochakian 1943). Extensive potassium depletion in spawning Pacific salmon has been demonstrated by Robertson et al (1961a), but this only occurs in Loch Leven trout with extensive fungal lesions and has not been reported in other salmonids.

Such changes as intimal hyperplasia and splitting of the internal elastic membrane appear to be generally more advanced in spawning fish and to have most serious effects in anadromous species such as the varieties of salmon and the steelhead trout, being most serious in thosespecies suffering massive mortalities at spawning-time, i.e. the Pacific salmon. Robertson et al 1961a report only mild lesions in non-migrating rainbow trout and these findings are paralleled by a lack of degenerative change in brown trout from Loch Leven.

Interrenal tissue

Changes in interrenal cell morphology at varying stages of sexual maturity have been described in a number of species of Pacific salmon (Robertson & Wexler 1957, 1959, 1960, 1961; Hane & Robertson 1959, Robertson et al 1961b, Nandi 1962, Colgrove 1966) and Atlantic salmon

(Giacommini 1911, Chester Jones 1957, Heyl & Carpenter 1972). Measurement of plasma 17α hydroxycorticosteroid levels at various stages of the reproductive cycle was also undertaken (Fontaine & Leloup-Hatey 1954; Hane & Robertson 1959; Idler Ronald & Schmidt 1959; Robertson et al 1961b; Schmidt & Idler 1962; Donaldson & Fagerlund 1968, Fagerlund & Donaldson 1970; Heyl & Carpenter 1972). Levels were comparable in immature fish of both genuses e.g. 25.5 µg/100 ml in immature Atlantic salmon compared with 11.8 µg/100 ml for Pacific salmon (Hane & Robertson, 1959). Proliferation of interrenal tissue became so extensive in association with sexual maturation in Pacific salmon that such tissue was grossly visible in cross-sections of anterior kidney (Robertson & Wexler 1959). Although interrenal proliferation was marked in Atlantic salmon (Heyl & Carpenter 1972) it was seldom of the same magnitude as that observed in the Pacific salmon. In Atlantic salmon, plasma 17α hydroxycorticosteroid levels rise as high as 42 µg/100 ml but regress again to normal immature levels following spawning (Heyl & Carpenter 1972). In the Pacific salmon, levels rise much higher (60-100 µg/ml - Robertson & Wexler 1959) and do not regress, and fish ultimately die. Idler & Truscott (1963) have shown that cortisone metabolism is more effective in the Atlantic than the Pacific salmon and suggest this as a cause of the continued elevated levels in the Pacific salmon.

Differences are also evident between migratory and non-migratory rainbow trout (Robertson & Wexler 1959;

Robertson et al 1961b). Many migratory trout apparently die after spawning but the majority of non-migratory rainbow trout survive to spawn again (Meigs & Pautzkee, 1941; Robertson & Wexler 1962b). The degree of hyperplasia of adrenocortical tissue in spawning Pacific salmon and steelhead trout was shown by Robertson & Wexler (1959) to be extensive but equivalent in both species, but much less developed in non-migratory rainbow trout. Degenerative changes at, and following, spawning were most marked in the Pacific salmon and least evident in the non-migratory rainbow trout and consisted of a variety of nuclear and cytoplasmic changes and haemorrhage. Plasma 17α-hydroxycorticosteroid levels paralleled these changes. The work of Robertson et al (1961b) in fact, produced lower average figures for 17α-hydroxycorticosteroids in spawning steelhead trout (39.6 µg/ml) than in Pacific salmon (63.9 µg/100 ml) and may partially explain the difference in spawning mortality rate. Plasma levels for immature non-migratory rainbow trout (2.7 µg/100 ml - Hane & Robertson, 1959) compared with higher levels of 10.6 µg/100 ml for wild fish (Robertson et al 1961b). Similarly, levels in spawning hatchery-reared fish only rose to 10 µg/100 ml compared with 43 µg/100 ml in wild fish. Plasma levels for spawning steelhead trout were similar to those of wild non-migratory trout (Robertson et al 1961b). Although blood levels of corticosteroids were not measured in the brown trout in the present study, hyperplastic and degenerative changes were very similar to those described in non-migratory

rainbow trout (Robertson & Wexler 1959, Robertson et al 1961b) and far less frequent than those in the various species of Pacific salmon.

Robertson & Wexler (1961) described two distinct cell-types in the maturing salmon adrenal - only one type of cell was evident in maturing brown trout in the present study. They also suggest vascularisation as the initial stage in degenerative change and this would also seem to be the case in the brown trout from Loch Leven, increased sinusoidal development preceding actual degenerative cell changes. Mitoses were prevalent at certain stages in both salmon and steelheads, but were not obvious at any stage in brown trout maturation.

McBride & van Overbeeke (1969b) showed that the interrenal hypertrophy was only partially prevented by feeding, but that gonadectomy of sexually mature fish led to a rapid involution of the hyperplastic interrenal tissue. When gonadectomy was performed prior to sexual maturation, there was no development of hypertrophic change. Donaldson & Fagerlund (1970) and Fagerlund & Donaldson (1970) also showed that gonadectomy reversed the increased secretion rate of cortisol and cortisone accompanying sexual maturation. McBride & van Overbeeke (1969b) showed there was no change in ACTH cells of the pituitary during interrenal hyperplasia, suggesting that the effect of the gonodal steroids on the interrenal gland was direct rather than mediated via the pituitary gland.

The effects of hypophysectomy on the rainbow trout (Donaldson & McBride, 1967) do suggest that in the normal immature animal, there is a negative feedback mechanism between the interrenal and the pituitary. Administration of ACTH to gonadectomised sockeye salmon also results in an increase in plasma cortisol and cortisone levels and stimulates the interrenal tissue (Donaldson & McBride 1974). In the same work, gonadotrophins were shown to have no direct effects on the interrenal gland, unlike the catfish Heteropneustes fossilis, in which species gonadotrophins are thought to act directly on the interrenal to produce corticosteroids which then stimulate ovulation (Sundararaj & Goswami 1969).

Work carried out on mammalian species indicates that the gonads have a considerable but variable influence on adrenal activity (see review by Kitay, 1968). Adrenal changes have also been associated with gonadal changes during the breeding cycle of birds (Riddle 1923; Burger 1938; Hohn 1947; Hohn, Sarker & Dzubin 1965) and several studies utilised gonadectomy and replacement therapy (Breneman 1942, Chan & Phillips 1973). Kar (1947) noted that adrenal cortical hypertrophy took place in male castrated chickens and was prevented by androgen replacement therapy and Chan & Phillips (1973) noted a similar inhibitory effect of androgens on in vitro adrenal corticosteroid production in the male domestic duck Anas platyrhynchos. Such an effect is the opposite of that occurring in salmonids.

Early work in mammals (Chester Jones 1955) suggested that the inhibitory effect of androgens on the adrenals was mediated via the pituitary by increasing the storage of FSH and diminishing ACTH secretion. It is now thought however, that much of the effect is a direct one by inhibiting 11β-hydroxylase and thereby reducing the ability of the adrenal cells to metabolise progesterone to corticosterone and resulting in an accumulation of deoxycorticosterone (Sharman, Forchielli & Dorfman 1963; Brownie, Colby, Galliault & Skelton, 1970). It has also been suggested that testosterone can block the stimulatory effect of ACTH in the adrenal and inhibit adrenal protein synthesis (Roy & Mahesh 1964). Whether the direct effect of androgens on interrenal tissue in fishes is a result of interference with enzyme systems such as 11β-hydroxylase, a result of an effect on protein synthesis, or whether some other mechanism may be involved, has not yet been established.

Gonads

The sequential changes in the morphology of the testis were very similar to those described for rainbow trout by Weisel 1943; Robertson 1958; and Drance, Hollenberg, Smith & Wylie 1976. The appearance of lobule boundary cells correlated well with the appearance of organ changes thought to result from elevated androgen levels. These cells are thought to be the equivalent of the Leydig cells of mammals (Marshall & Lofts 1956; Yaron 1966).

Pancreas

In the brown trout in the present study, the major changes associated with sexual maturity were a hyperplasia of islet tissue with degenerative change in occasional fish and a decrease in the quantity of peripancreatic fat. Such changes were also reported in the Pacific salmon at sexual maturity by Robertson & Wexler 1960 and Colgrove 1966 and in migratory and non-migratory rainbow trout by Robertson et al 1961b.

Similar changes were also found in senile castrated kokanee salmon by Robertson & Wexler (1962c) and were readily reproduced in castrated Pacific salmon by administering androgens or cortisol (McBride & van Overbeeke 1971). A preponderance of β cells in the islets of mature fish was also noted by the above workers and was thought due to hyperglycaemia induced by high levels of circulating corticosteroids (Robertson et al 1961a, 1963). A decrease in fat surrounding pancreatic tissue in the present study was also associated with an inflammatory cell infiltrate. No effects on the acinar tissue were seen and in this respect the tissue resembled the peripancreatic fat in Pansteatitis, a nutritional disease of salmonid fish (Roberts & Richards 1978, 1979).

Gut

Changes in the stomach and intestine of brown trout in the present study were markedly different to those reported in the Pacific salmon. In the Pacific salmon, stomach changes at sexual maturity consist of a loss of villous structure, a change in the nature of the stomach epithelium and an atrophy of the circular muscular layer of the gut, with an associated increased thickness of the longitudinal muscle (Greene 1913; Robertson & Wexler 1960). Such degenerative changes were not found in the non-migratory rainbow trout at spawning (Robertson et al 1961b) although there was a diminution in the size and number of villi in the migratory rainbow trout. Such changes were found in the senile castrated kokanee salmon (Robertson & Wexler 1962c). Such changes have been linked to starvation and the feeding of sockeye salmon during maturation and spawning in captivity largely prevented these changes (McBride et al 1965). The brown trout in Loch Leven do feed during maturation and this may explain the differences found between this species and the Pacific salmon.

Some degenerative changes were found in the present study and consisted of an increased cellularity and inflammatory change in the submucosa and lamina propria. Eosinophilic granule cells were prominent in this infiltrate and the stratum granulosum itself was increased in size, a change also noted in the Pacific salmon (Greene 1913; Robertson & Wexler 1960).

Kidney

Changes in kidney histology in spawning brown trout from Loch Leven were similar to those found in the Pacific salmon, but less marked in extent (cf. Robertson & Wexler 1960). Colgrove (1966) described a range of changes including marked hyaline degeneration of glomeruli and tubules, tubules being extensively affected. This has frequently been seen in routine histological examination of apparently healthy brown and rainbow trout and is thought to be a result of aberrant protein metabolism which often occurs during starvation. In the liver and kidney of mice and rats endocytosis of "foreign" or denatured proteins occurs in association with lysosome action and lysosomal proteases then degrade such proteins into their constituent amino-acids, presumably for further utilisation (Mego 1973). Dannevig & Berg (1978) have shown that in the char (Salmo alpinus L) human serum albumen is taken up almost exclusively by the kidneys and that the kidney contains a high activity of lysosomal proteases. Restriction of this action to the kidney is possibly explained by a lack of Kupffer cells in fish liver (Ferguson 1974). Unfortunately Dannevig & Berg do not discuss histological changes in such kidneys nor do they state which area of the kidney is principally involved, but it is tempting to speculate that the hyaline changes seen in salmonid kidneys may well represent protein endocytosis.

Thickening of Bowman's capsulewas occasionally seen

in the present study but this is thought to be a normal feature of older fish (Roberts, pers. comm. 1977) and was described as an ageing process in kokanee salmon (Robertson & Wexler 1962c). Very few degenerative changes were seen in the kidneys of migratory and non-migratory rainbow trout.

The results of histological examination of various stages in the reproductive cycle of brown trout from Loch Leven has demonstrated that the major changes occurring are similar to those in other salmonid species. Though degenerative changes were not so extensive as those occurring in the anadromous species in which post-spawning mortality is high, the changes were qualitatively similar and have been shown in other species to be a result of hyperadrenocorticism, probably induced by elevated levels of gonadal steroids. Although these degenerative changes have been reproduced with cortico-steroids, the pronounced changes in the skin have only been reproduced with gonadal steroids. It was consequently decided to examine the effects of androgens on the brown trout.

Pituitary Gland

Extensive changes occurred in the pituitary gland of the brown trout in the present study during sexual maturation, and principally involved the dorsal lobe. Similar changes were seen in the sockeye salmon (Colgrove 1966, van Overbeeke & McBride 1967), chum salmon (Nagahama & Yamamoto 1970a, b), king salmon (O. tschawytscha) blueback salmon (O. nerka nerka) and kokanee salmon (Robertson & Wexler, 1957, 1962a) and Atlantic salmon (Olivereau 1954; Kawashima, Ichikawa, Mori, Ueda & Shirahata 1976), but evidence of pronounced degenerative change was also present. Such changes were also noted in the rainbow trout (Robertson & Wexler 1957, 1959, 1962b; Boddingius 1975), but degenerative changes present in spawning and spent fish were developed to a far greater extent in the steelhead trout than in the non-migratory rainbow trout. It has been suggested that the lesser degenerative changes of focal vacuolation, degranulation and pycnosis probably result from excessive stimulation and exhaustion of cell function and may be reversed (Robertson & Wexler 1962a). Although pronounced changes occurred in the pituitary glands of other species during the sexual maturation cycle, degenerative changes were not noted in, for example, the killifish Fundulus heteroclitus (Mathews 1936) the bitterling Rhodeus amarus (Bretschneider and Duyvene de Wit 1941), the carp Cyprinus carpio (Scruggs 1951) or the blind cave fish Astyanax mexicanus (Atz 1953). This may bear some relation to post-spawning survival and be related to high circulating 17-hydroxycorticosteroid

levels in the blood at this time (Robertson & Wexler 1962b; Robertson et al 1961b, 1963; van Overbeeke & McBride 1967).

Considerable confusion has arisen in the literature on fish pituitary morphology because several systems of nomenclature have been used (Gorbman, 1965; van Oordt, 1965). The anterior lobe has been variously termed the follicular lobe, proadenohypophysis, or rostral pars distalis; the dorsal lobe has been called the mesoadenohypophysis or proximal pars distalis and the ventral lobe has been termed the meta-adenohypophysis or pars intermedia. These three lobes are together equivalent to the mammalian adenohypophysis. The neural lobe (neurohypophysis or pars nervosa) is the equivalent of the mammalia neurohypophysis, but differs in that it ramifies into the ventral lobe in fish. Van Overbeeke & McBride (1967) described nine different cell types in the sockeye pituitary gland and suggest that six functionally different cell types occur. Cell types 2 and 6 were thought to be gonadotrophs (basophils in the anterior and dorsal lobes), whilst cell type 7 (chromophobes), thought by Olivereau (1954) and Robertson & Wexler (1962b) to be an exhausted basophil (gonadotroph) was uncertain in terms of classification by van Overbeeke & McBride (1967) but was somehow linked to sexual maturation.

One of the principal problems associated with the characterisation of various cell types in the pituitary gland is the difficulty in distinguishing between

thyrotrophs and gonadotrophs in the dorsal lobe, and one or two gonadotroph types may be present dependent on species (Ball & Baker 1969). Up to the present time, though two distinct types of gonadotroph have been postulated for both Pacific and Atlantic salmon (Olivereau, 1961, 1963a, b, 1967, 1977; Olivereau & Ridgway, 1962; van Overbeeke & McBride 1967 & Kawashima et al 1976) only one gonadotropin has been isolated (Yamazaki & Donaldson 1967, 1968; Donaldson, Yamazaki, Dye & Philleo 1972; Olivereau 1977). Similarly only a single gonadotropin has been isolated from rainbow trout (Breton, Jalabert & Reinaud 1976). McBride & van Overbeeke (1969a) on the basis of response to castration, were only able to distinguish one gonadotroph in Oncorhynchus nerka.

Yamazaki & Donaldson (1968) isolated a single salmon gonadotropin which produced spermatogenesis and spermiation in male goldfish and vitellogenesis and ovulation in female goldfish and thus suggested that only one gonadotropin may be produced in the fish pituitary gland. Otsuka (1956) had previously tested two different proteins isolated from salmon pituitary glands on newts and mice and suggested that two different gonadotrophic hormones were present with different physiological effects. The situation in salmonids is thus far from clear.

Robertson & Wexler (1962b) and van Overbeeke & McBride (1967) suggested that in the mature Pacific salmon, the PAS positive cells in the dorsal lobe were gonadotrophs. Further evidence for this conclusion was provided by the

rapid degranulation of these cells following gonadectomy (McBride & van Overbeeke 1969a) and the binding by these cells of fluorescent anti-ovine L.H. but not anti-bovine thyrotrophin (McKeown & van Overbeeke 1971). It does however seem that certain of these cells may be thyrotrophs. Chestnut (1970) demonstrated some thyrotrophic potency in spawning Coho salmon in PAS positive pituitary cells and McBride & van Overbeeke did note that certain PAS positive cells did not degranulate after gonadectomy. Cook & van Overbeeke (1972) further showed in an electron microscopic study that thyrotrophs were present but possessed relatively few granules and might thus have appeared as agranular weakly-staining cells with the light microscope. Cells have also been recognised as thyrotrophs in immature salmonids either by radio-thyroidectomy or by treatment with the antithyroid agent thioureawhich presumably interferes with a negative feedback mechanism and leads to visible depletion of thyrotrophic cells (Olivereau, La Roche & Woodall 1964; Olivereau & La Roche 1965; Norris 1966; Chestnut 1970; and Olivereau 1972). The effects of thiourea on intact sexually maturing sockeye salmon were difficult to ascertain as an effect on gonadtroph differentiation was seen but in gonadectomised fish, characteristic thyrotrophs were demonstrated (McBride & van Overbeeke 1975).

Differentiation between thyrotrophs and gonadotrophs was not undertaken in the present study and a range of basophil cells with differences in intensity of staining, cell size, granule size and frequency was seen.

III BACTERIOLOGICAL STUDIES

III 1. Introduction

A study of the normal commensal flora of the gut and skin of fish was originally carried out in order to learn more of the spoilage processes occurring in food fish (Hunter 1920; Fellers 1926; Schönberg 1930; Snow & Beard 1939; Wood 1940; Thjøtta & Sømme 1943; Dyer 1947; Shewan 1949; and Tarr 1954). It was later suggested (Evelyn & McDermott 1961; Shewan 1961; Shewan & Hobbs 1967; Horsley 1973) that the bacterial flora of fish was simply a reflection of the environment, though quantitative variation did occur.

Bacterial disease in wild salmonids is of frequent occurrence (Richards & Roberts 1978) and is especially associated with stressful conditions such as low oxygen levels, poor water quality or the stress of spawning. The investigation by Thorpe & Roberts (1972) into peri-spawning mortalities in brown trout (Salmo trutta L) from Loch Leven, Kinross, suggested that extensive losses had occurred from a septicaemia with Aeromonas hydrophila.

Seven thousand, seven hundred sexually mature adult brown trout were estimated to have died during the spawning season of 1970/1971 and though fungal skin infection was occasionally seen on moribund and dead fish, the majority of fish showed no evidence of such fungal infection but did show histological evidence of bacterial damage. It was therefore decided to examine such peri-spawning mortalities in more detail and during the spawning season of 1973/74 and 1974/75 detailed bacteriology was carried out.

Aeromonas hydrophila, originally described as Bacillus hydrophilus fuscus by Sanarelli (1891) is a common organism in freshwater, especially if organic content is high and it may also be a normal part of the resident gut flora of fish (Shotts, quoted by Richards & Roberts, 1978). It is commonly associated with haemorrhagic septicaemia in fish under stress, but is especially associated with disease in cyprinids. It has also been associated with disease in amphibians and reptiles (Cooper, Needham & Griffin, 1978), and mammals, including man (Caselitz 1966). Whilst A. hydrophila is frequently associated with such conditions in wild salmonids, another Aeromonad species, A. salmonicida is more frequently associated with disease of farmed salmonids.

III 2. Materials and Methods

Thirty fish, both fungal-infected and "clean", were obtained from the streams supplying Loch Leven and from the outlet to the river Leven, using box traps (see Figure 1 for sites) during the spawning season of 1973/1974 and 30 fish were similarly obtained during the 1974/1975 spawning season. Eight clean fish were also seine-netted from the Loch at monthly intervals and all fish were sampled bacteriologically and histologically.

Fish were anaesthetised in MS222 at 1:10,000 concentration, the ventral mid-line was swabbed with alcohol and the fish were opened by ventral mid-line incision with a sterile scalpel. Internal organs were then exposed by pinning aside the abdominal body wall. Bacteriological samples were taken using aseptic techniques from spleen, liver and kidney by searing the organ surface with a hot spatula and then removing an inoculum from the depths of the tissue by passing a sterile pasteur pipette through the seared surface. Inocula were then plated out onto nutrient agar (Difco) and blood agar plates and subsequently incubated at 20°C. Isolates were then purified by subculture and identified biochemically according to Bergey's Manual of Determinative Bacteriology, Eighth Edition (1974).

Samples of all organs were also taken and fixed in 10% neutral buffered formalin for embedding in paraffin wax and sectioning at 5μ. These sections were subsequently stained by haematoxylin and eosin and by the Gram Humberstone technique (Humberstone 1963).

III 3. Results

Massive bacterial infection of all organs sampled was only found in four fish out of thirty sampled from the Loch Leven streams during the 1973/1974 spawning season and in three fish out of thirty in 1974/1975. In all these cases, pure cultures of Aeromonas hydrophila were obtained, which possessed the biochemical characteristics expressed in Table 19. All fish with massive bacterial infection were also suffering from extensive Saprolegnia skin infection. In a further ten fish in 1973/1974 and twelve fish in 1974/1975 isolated colonies of a variety of bacteria were found (see Tables 20 and 21) but again, the predominant organism was Aeromonas hydrophila.

The results of the bacteriological examination of clean fish from the loch are represented in Table 22. In no instance was massive infection found and again Aeromonas hydrophila was the predominant organism. Water temperatures at the time of sampling and mean monthly water temperatures are also given in Table 22.

Although histological evidence of the changes associated with sexual maturity was found in the sexually mature fish, evidence of organ damage due to bacterial infection was only found in those fish suffering massive infection with Aeromonas hydrophila. Histological changes consisted of inflammatory cell infiltration and necrosis of the skin associated with mixed fungal and bacterial infection described in Section IV. The kidney

TABLE 19

Biochemical characteristics of Aeromonas hydrophila

Motility	+	Polar flagella
Oxidase	+	
Glucose	AG	
O-F media	F	
Pigment	-	
O/129 sensitivity	-	
Novobiocin sensitivity	-	
Sewater required	-	
Salt not tolerated	-	
Growth at 5°C	+	
Growth at 37°C	+	
Indol production	+	
Voges-Proskauer test	+	
Methyl Red test		
2:3 butanediol production	+	
Ammonia from peptone	+	
Nitrate utilisation	+	to NO_2
Citrate utilisation	+	
Urease	-	
Catalase	+	
Starch hydrolysis	+	
Casein hydrolysis	+	
β-haemolysis	+	
Gelatin liquefaction	+	
Arginine dehydrolase	+	
Lysine decarboxylase	-	
Ornithine decarboxylase	-	
Sugar Fructose	AG	
Lactose	-	
Sucrose	AG or -	
Maltose	AG	
Mannitol	AG	
Glycerol	AG or -	
β-galactosidase (ONPG)	+	

A = Acid G = Gas

TABLE 20

The occurrence of bacteria in spawning trout taken by box trap from the streams supplying Loch Leven during 1973/1974

	Disease Status	Fish Ident. No.	Organ	Isolate
1973/74	I	1	Kidney	A. hydrophila
			Liver	A. hydrophila
			Spleen	A. hydrophila
	C	4	Kidney	A. hydrophila
	I	5	Kidney	Alcaligenes/ Achromobacter
			Liver	Pseudomonas
	I	7	Kidney	A. hydrophila
	I	8	Kidney	A. hydrophila
			Liver	A. hydrophila
			Spleen	A. hydrophila
	C	9	Liver	Alcaligenes/ Achromobacter
			Spleen	A. hydrophila
	I	10	Kidney	Alcaligenes/ Achromobacter
	I	15	Kidney	Coryneform
	I	16	Kidney	A. hydrophila
			Spleen	A. hydrophila
			Liver	A. hydrophila
	C	23	Kidney	Coryneform
	I	25	Spleen	A. hydrophila
			Kidney	A. hydrophila
	C	28	Liver	A. hydrophila
			Kidney	A. hydrophila
	C	29	Kidney	A. hydrophila
	I	30	Kidney	A. hydrophila
			Spleen	A. hydrophila
			Liver	A. hydrophila

C = clean I = infected

TABLE 21

The occurrence of bacteria in spawning trout taken by box trap from the streams supplying Loch Leven during 1974/1975

Disease Status	Fish Identification Number	Organ	Isolate
C	1	Kidney	Acinetobacter spp.
C	3	Kidney	Moraxella spp.
C	4	Kidney	A. hydrophila
		Liver	Pseudomonas fluorescens
I	6	Kidney	A. hydrophila
		Spleen	A. hydrophila
		Liver	A. hydrophila
I	7	Liver	Pseudomonas fluorescens
C	8	Spleen	Nocardia-Rhodocrous complex
		Liver	A. hydrophila
I	11	Kidney	A. hydrophila
I	12	Kidney	A. hydrophila
		Spleen	A. hydrophila
		Liver	A. hydrophila
I	16	Liver	A. hydrophila
		Kidney	Coryneform
C	23	Spleen	Achromobacter/ Alcaligenes
I	25	Kidney	Pseudomonas fluorescens
I	26	Kidney	A. hydrophila
		Spleen	A. hydrophila
		Liver	A. hydrophila
I	27	Kidney	Acinetobacter spp.
I	28	Kidney	A. hydrophila
C	30	Liver	Coryneform
		Kidney	Moraxella spp.

I = infected C = clean

TABLE 22

The occurrence of occasional bacteria in apparently healthy brown trout taken by seine net from Loch Leven during 1974

Month	Mean Monthly T°	T° at time of sampling	Fish Ident. Number	Organ	Bacterium
Jan.	3.2	3	2	Kidney	A. hydrophila
Feb.	4.9	5	1	Spleen	A. hydrophila
			5	Kidney	A. hydrophila
				Liver	A. hydrophila
March	4.6	5	5	Liver	Pseudomonas
			6	Liver	Achromobacter/Alcaligenes
				Kidney	Pseudomonas fluorescens
April	10.4	8	1	Kidney	A. hydrophila
May	11.7	12			
June	15.7	16			
July	15.8	16			
Aug.	15.9	16			
Sept.	14.2	14	4	Liver	Moraxella spp.
			6	Spleen	Acinetobacter spp.
			7	Spleen	Achromobacter/Alcaligenes
Oct.	9.7	10	1	Kidney	Pseudomonas fluorescens
			2	Kidney	A. hydrophila
			8	Liver	Pseudomonas
Nov.	6.6	6	1	Kidney	Coryneform
				Spleen	A. hydrophila
			5	Kidney	Coryneform
			6	Kidney	Arthrobacter
				Spleen	A. hydrophila
Dec.	5.0	5	2	Kidney	A. hydrophila
			7	Kidney	Arthrobacter

often showed sloughing of tubular epithelium into the lumina and excessive quantities of eosinophilic amorphous luminal deposits (Figure 67). Interstitial haemopoietic tissue was necrotic and on occasion, large colonies of gram-negative bacteria could be seen in such areas, particularly when Gram Humberstone staining was employed (Figure 68). Shrinkage of glomerular capillary tufts was also seen in some fish. The spleen was usually congested and areas of focal necrosis of melanomacrophage and lymphopoietic tissue were evident. Some fish exhibited spread of bacteria through all organs associated with focal necrosis but little inflammatory response. Other organ changes were minimal.

In those fish from which occasional bacteria were isolated, no histological evidence of organ damage was present.

III 4. Discussion

Although a large number of spawning fish died in 1970/1971 as a result of Aeromonas hydrophila infection, perispawning mortalities in subsequent years have been very much reduced. In no instance in the present study did fish suffer from extensive bacterial infection without concomitant fungal infection, though moribund fish were occasionally found suffering from extensive fungal infection with no associated bacterial infection. Peri-mortem invasion of fish tissues with a variety of bacteria commonly occurs (Horne, pers. comm. 1971) but the presence of bacteria in small numbers in the tissues of apparently healthy fish in the colder months confirmed the findings of Thorpe & Roberts (1972). Bissett (1947, 1948) suggested that the bacteria present were a reflection of the fish skin or intestine flora at the time of sampling and that further, at low temperatures, there exists a balance between the body defences of poikilotherms and the pathogenic power of their bacterial parasites. Cell-mediated immunity, antibody production and the inflammatory response vary considerably with temperature in fish (Cushing 1970; Finn & Nielsen 1971; Anderson & Roberts 1975; Ellis, Munroe & Roberts 1975) as do other bodily processes such as serum enzyme activity (Sauer & Haider 1977). Clearance of bacteria from tissues of rainbow trout has been shown to be temperature-dependent and the time taken for clearance is much slower at lower temperatures - the presence of bacteria in the organs of brown trout from Loch Leven at lower temperatures may possibly be explained in a similar manner.

IV INTEGUMENTARY FUNGAL CONDITION

IV. 1. Introduction

Although a wide variety of fungal pathogens may affect fish (Wolke 1975, Richards, 1978) surface lesions in salmonid fish are commonly associated with infection with species of the family Saprolegniaceae. Reports of such conditions in the literature are numerous but often confused because of difficulties with taxonomy. Infections may arise as a result of physical trauma and are thought to be often secondary to bacterial, viral or parasitic disease (Roberts & Shepherd 1974), although a small body of experimental evidence exists which suggests that, under certain conditions, Saprolegnia spp. may act as primary pathogens in a variety of fish species (Tiffney 1939; Vishniac & Nigrelli 1957; Hoshino, Sano & Sunayama 1960; Scott & Warren 1964; Egusa 1965; McKay 1967; Nolard-Tintigner 1970, 1971, 1973 and 1974; and Neish 1977).

Saprolegnia spp. infection is particularly common in salmonids in the perispawning periods (Baudouy & Tuffery 1973; Roberts & Shepherd 1974; Wood 1974; White 1975; Neish 1977) and is often associated with significant mortalities at this time. Other species of fish also succumb to fungal infections at this stage in their life history (Baudouy & Tuffery, 1973). An epizootic of spawning Atlantic salmon (Salmo salar L) and brown trout (Salmo trutta L) known as Ulcerative Dermal Necrosis (U.D.N.)

is also closely associated with Saprolegnia spp. infection, especially in the more chronic stages, and it is generally accepted that fungal infection in this condition is the most frequent cause of death (Carbery & Strickland 1968). The cause of the condition is still unknown, although pre-mycotic stages have been described (Roberts, Shearer, Munro & Elson 1970) and a filtrable agent has been suggested as the cause on the basis of a limited transmission study (O'Brien 1974). The later stages of U.D.N. are impossible to differentiate from saprolegniasis per se but in the earlier stages, U.D.N. may be differentiated as lesions are confined to the head (Carbery 1968, Roberts et al 1969, 1970, Gerard 1971).

In Saprolegnia spp. infections associated with salmonid sexual maturity a single fungal strain (or group of closely-related strains) with a low degree of homothallic sexuality is often isolated. (Neish 1977, Pickering & Willoughby 1977, Willoughby & Pickering 1977). Earlier work refers to such strains as Saprolegnia parasitica (Coker 1923, Kannuse 1932), Saprolegnia Type 1 (Willoughby 1969, 1971) and more recently Saprolegnia diclina Humphrey Type 1 (Willoughby 1978). Similar sterile isolates were obtained from infected eel Anguilla japonica Temminck & Schlegel (Hoshina, Sano & Sunayama 1960) and the rudd Scardinius erythrophthalmus L. (Nolard-Tintigner 1973).

A sexual difference in patterns of Saprolegnia infection in mature fish has been shown by White (1975) and by Richards & Pickering (1978). Differences in skin structure described in section II between sexes and

different states of maturity have also been found in the sea trout, Salmo trutta L (Stoklosowa 1966 , 1970) and in hatchery-reared brown trout (Pickering 1977) and the relationship of such changes to patterns of fungal infections is discussed later.

Seasonal variation in the frequency of isolation of Saprolegniaceae from natural bodies of water have also been described (Coker 1923, Lund 1934, Waterhouse 1942, Ziegler 1958, Hughes 1962, Alabi 1971) and may be of significance when considering epizootics in wild fish populations.

The histopathology of Saprolegnia spp. infections has been documented by a number of workers. Infections of fairly large salmonids generally resulted only in superficial infections of skin and perhaps dorsal musculature (Roberts et al 1969, 1970), whereas infections of smaller fish species often resulted in extensive spread to internal organs such as the liver and spinal cord (Bootsma 1973, Nolard-Tintigner 1973).

Saprolegnia spp. infections are generally thought to cause death in spawning salmonids either by allowing secondary bacterial infection (Thorpe & Roberts 1972) or through osmoregulatory failure (Tiffney 1939, Richards & Pickering 1978).

IV. 2. Materials and Methods

a) Fish.

30 brown trout were obtained from the streams supplying Loch Leven, Kinross and from the outlet to the River Leven during the spawning periods of 1973/1974 & 1974/75 using box traps in the trapping sites shown in figure 1. Live fungal-infected fish were removed from the traps, anaesthetised in MS222 (1:10,000), weighed, measured and a scale sample taken. State of maturity was assessed on a scale of I - VIII using the method of Kesteven (1960). Fish were sampled bacteriologically as described in Section III.

b) Fungal isolation.

Primary fungal isolates were made by removing a small tuft of fungus from an infected area of skin with a pair of forceps, washing the sample several times in distilled water and then placing it on the centre of a Corn-meal agar plate (Difco). Growth in such primary cultures was then encouraged by incubation at 18°C. Radial mycelial growth was generally apparent in the culture medium within 24-48 hrs. of incubation. At this stage, single hyphal tips were then removed from the extreme edge of the fungal growth in a small block of agar and transferred to a fresh plate. Single manipulations of this kind were generally sufficient to obtain cultures free of bacterial contaminants. Additional transfers were used as required until bacteria-free cultures were obtained. The use of "Raper's ring" (Raper 1937), ultra-violet irradiation methods (Blank & Tiffney 1936).

or antibiotic-enriched media (Hoffman 1949, Seymour 1970) were never required during the course of this study to obtain bacteria-free isolates. Single-spore isolates were then made using the method described by Seymour (1970) - spores were obtained from a discharging sporangium with a pipette and streaked onto a fresh corn-meal agar plate. After about six hours incubation, single sporelings were then readily isolated and re-plated onto fresh media and the resultant single-spore isolate used to characterise the species of the fungus. Sterilised hempseed halves were then placed, cut surface down, onto developing isolates and after two days incubation at 18°C, seeds with their fungal growth were removed aseptically and transferred to sterile loch water at 7° and 23°C.

For maintenance of isolates over extended periods, autoclaved cress seeds were used in place of hemp seeds. When the mycelial growth had invested seeds placed on the agar plates, the seeds were removed aseptically and placed in bottles of sterile distilled water and sealed. Isolates treated in this way have remained viable for more than five years. Mineral oil preservation was not used (Buell & Weston 1947).

c) Incidence and frequency of infections.

The positions and shape of any fungal lesions were carefully mapped out onto a standard chart (see figure 69) detailing body and fins on both sides so that two charts were obtained per fish. Any deep lesions were noted at this time. When individual colonies could be clearly distinguished, the

number of fungal colonies per side was also noted. Each standard chart illustrating the distribution pattern of fungal infection was divided into 391 squares by means of superimposed graph paper with numbered divisions (figure 69) and a record was made of the presence or absence of fungus within each square. Fish were then pooled according to sex and maturity (Groups III - VIII (Kesteven 1960) were considered mature and groups I & II immature) and the incidence of infection for each of the 391 areas on the body and fins was obtained for each group. The incidence of infection for each square was then compared by χ^2 analysis with the mean incidence of infection for all the squares. Thus, a statistical comparison was made for each of the 391 areas on the body and fins between the observed incidence of infection and the expected incidence of infection if no significant pattern existed. The levels of significance for each square were then re-plotted on a standard chart and significant patterns of infection were obtained. Using similar techniques it was also possible to determine the areas of the fish significantly uninfected.

A further χ^2 analysis was made between the patterns of infection obtained from different groups of fish. In this case the observed incidence of infection within each of the 391 squares for a particular group was compared with the incidence of infection for the corresponding squares of the other group of fish. The numbers of data were standardized by recalculating the incidence of infection of the larger number in terms of the smaller number. For

example, if the comparison was between seventeen incidences out of a possible forty for one group of fish and ten incidences out of a possible fifty for the second group, this second figure was recalculated as 10 x (40/50)=8. Thus, the χ^2 analysis would be between the observed figure of 17 incidences out of 40 and the expected figure of 8 incidences out of 40 if no difference existed between the patterns of infection of the two groups of fish. In this way it was possible to compare patterns of infection statistically and determine where significant differences between patterns occurred.

χ^2 analysis of the total numbers of infected and non-infected fish from different groups were performed using 2 x 2 contingency tables (Elliott 1971).

d) Spore-counting in Water Samples

An attempt was made to estimate the monthly variation in spore number of the Saprolegniales in Loch Leven using the method first described by Willoughby (1962). Three different sampling sites were used on each occasion:-

1. Approximately one hundred yards upstream of the fish trap in the S. Queich.
2. Approximately ten yards offshore at a fixed point in the bay to the left of the fishing boat harbour and
3. Ten yards offshore at a fixed point in the boat bay (see figure 1). 500ml samples were taken at each site from the water surface and placed into sterile flasks and sealed. On return to the laboratory the water samples were vigorously shaken and a sample of 4 ml was incorporated into 30 ml of cornmeal agar (Difco) and poured into a sterile Petri dish. When cool, the agar in the plate was then cut using aseptic precautions into eight equal sectors. Each of these sectors was then placed in another sterile petri dish, covered with sterile water and incubated at 18°C. Samples were taken five times from each water sample giving 40 sectors from each sample (replicated for each of the three sampling sites). The water in the dishes was changed for fresh water after seven days to reduce bacterial contamination (see Willoughby 1962) and sectors were finally examined after 9 days of incubation for mycelial growth fringes and those resulting from growth of Saprolegnia species were counted. In early attempts at this work,

distilled water from the laboratory was used to cover the agar sectors - very little growth into this water occurred however, and it was noted that the pH of the water used was 3.7. Consequently in the sampling work noted in the results section, sterilised loch water was used to cover the plates.

Willoughby's (1962) method was used to estimate the actual number of propagules/litre. For each estimation, the total water sample used was 20 ml and the number of sector dishes was 40. If the observed number of positive sectors was x, the observed mean number of positive sectors was $\frac{x}{40}$.

Using a correction curve (Figure 70) which utilises Poisson distribution variation, the true mean may then be read off (say, y). The corrected number of propagules would then be $y \times 40$ and the number of propagules/litre equal to

$$y \times 40 \times \frac{1000}{20}$$

Samples were taken monthly over the period November 1974 to March 1975.

e) Histology

Small blocks of tissue, approximately ½-1 cm^3 were removed from all organs including fungal-infected and non-infected skin, fixed in 10% neutral buffered formalin and processed routinely as in Appendix 1. Sections were then stained with Haematoxylin and Eosin (H & E), Gram-Humberstone (Humberstone 1963), Weigert's Haematoxylin and Eosin, Per-iodic-acid-Schiff's technique (PAS), Grocott's modification of Gomori's methenamine silver technique for fungi, and Martius scarlet blue technique for fibrin (see Appendix 1 for details).

f) Electron-microscopy of spore-cases :-

Six isolates from the present study supplied to Dr. Willoughby of the Freshwater Biological Association, Windermere (FBA) were used in the study of the fine-structure of spore-cases described by Pickering, Willoughby & McGrory (1979). Electron-microscopic observations were made with an AEI EM6B electron microscope on both primary and secondary zoospore cysts (see Pickering et al 1979 for details of production techniques) after fixation with OsO4 vapour on carbon/formvar coated copper grids, and subsequent shadowing with gold-palladium.

IV 3. Results

A total of 30 fungal isolates was examined. All were identified as members of the Genus Saprolegnia, but it was impossible to classify further 18 of these isolates as they failed to form sexual organs. Of the remaining 12 isolates, 11 conformed to the grouping of Saprolegnia diclina Humphrey Type 1 (Willoughby 1978) and 1 fitted into S. diclina Humphrey Type 2. Four of these isolates were sent on to Dr. Willoughby who confirmed these findings.

The Saprolegnia isolates all possessed the following characteristics:

All were classified as of the Division Eumycota, sub-division Mastigomycotina, class Oomycetes, order Saprolegniales, family Saprolegniaceae.

The mycelium consisted of stout branched tubular multinucleate hyphae, aseptate and not constricted except where vegetative or sexual structures were formed and at hyphal tips.

Asexual reproduction took place with the formation of biflagellate zoospores in long cylindrical or clavate zoosporangia, which were terminal, multinucleate hyphal swellings measuring between 142μ-198μ × 26μ-43μ (figure 71). Spore discharge occurred through an exit pore at the apex and secondary zoosporangia were frequently formed by internal proliferation (within a previously emptied primary sporangial case). The secondary sporangium grew through the primary one, maturing either within it or beyond it.

Frequently several sporangia were successively formed in this way. Empty sporangial cases thus remain attached to the somatic hyphae. In some cases, several sporangia appeared to be produced simultaneously at the apex of a mycelial branch giving rise to the appearance of a long single beaded sporangium. Both primary and secondary zoospores were formed. Both types were biflagellate with tinsel and whiplash flagellae. The primary zoospores were pyriform with two subapical flagellae and the secondary were reniform with laterally-arranged flagellae. Zoospores were thus dimorphic and also were diplanetic, there being two motile swarming periods separated by a period of encystment.

In old cultures, aplanoid zoospore discharge was frequently seen, in which zoospores were seen to germinate *in situ* laterally through the sporangial walls by means of short germ tubes (figure 72) (also noted by Huxley 1882, Coker 1923, McKay 1967, Willoughby 1968, Seymour 1970 and Neish 1977). Less frequently in old cultures, secondary zoosporangia were formed by basipetalous or cymose branching (as commonly found in *Achlya* spp.), each new sporangium developing at the apex of a branch which originates laterally from below the basal septum of the previous sporangium in the succession.

Initially none of the 30 isolates could be induced to form sexual structures at 20°C. In view of this they were sub-cultured onto sterile mustard seeds. Several months later, when a comparison of 20°C and 7°C culture

was used, sexual organs were induced in some, but 18 of the cultures remained sexually sterile at both temperatures and were eventually classified as Saprolegnia spp. Only 12 cultures did form sexual organs at 7°C. On this basis it was possible to assign 11 of these isolates to S. diclina Type 1 and 1 isolate to S. diclina Type 2 (Willoughby 1978). The Saprolegnia diclina Type I isolates possessed the following characters.

Diclinous antheridia and oogonia were eventually formed after a minimum of 23 days at 7°C. Several months incubation was often necessary for the development of sexual structures. No sexual organs were formed at 20°C. Oogonia were typically elongated, terminal and their walls were unpitted, thin and smooth (figure 73). The form of the oospores varied between subcentric and centric, even from the same culture and there was often a typical "bird-nest" type extensive investment of the oogonium (figure 74). Oospore diameter varied between 18µ and 26µ but mean diameters were closely similar in individual isolates. The number of oospores within an oogonium varied between 4 and 22 and in some cases, oospore "ghosts" appeared to be present. The antheridia were simple and delicate in form and between 1 and 5 were present on a single oogonium. Elongated incipient sporangia also appeared to develop into oogonia occasionally.

The single isolate which appeared to fall into the S. diclina Type 2 grouping differed in that numerous oogonia were formed after 9 days at 7°C. There were few

oospores per oogonium (between 2 and 5) and these were relatively large (21μ - 25μ). Oogonia infrequently showed delicate pitting but antheridial investment was intense, as in Type 1 isolates.

Fungal Mapping

The severity of the Saprolegnia infection (% area infected and no. of colonies) of sexually mature brown trout is shown below (figures given are mean ± S.E.M (n)).

	Sex	% Area infected	Number of separate fungal colonies per side
Brown trout 1973 - 1975	Male	26.7± 1.43(50)	4.3± 0.25 (50)
	Female	22.9± 1.69(44)	3.4± 0.25 (44)

There was little difference between sexes.

Tables 23 to 26 give an analysis of total fish trapped at Loch Leven during the spawning seasons of 1973/1974 and 1974/1975. Individual figures for the different trapping sites and sex differences between clean, diseased and dead fish are provided. There was no apparent sexual difference in incidence of infection in either season.

When the distribution patterns of infection of male and female brown trout are compared (see figures 75 and 76), it becomes evident that there are marked differences between the sexes. In the male, significantly infected areas occur along the dorsal surface of the body and extend ventrally towards the lateral line. In the female fish, the tail and peduncle region are more frequently affected. In both sexes, significantly non-infected areas occur around the lower jaw, operculum and pectoral region (excluding the pectoral fin). Mature males also show significant freedom from infection along the ventral surface.

TABLE 23

Male brown trout trapped at Loch Leven during the spawning season of 1973/1974

Location	Disease Status	State of Maturity						Total
		I-III	IV	V	VI	VII	VIII	(IV-VIII)
South Queich	Live Clean	0	0	0	0	2	0	2
	Live Diseased	3	3	0	3	45	28	79
	Dead Diseased	0	0	0	0	0	7	7
Sluices	Dead Clean	1	2	0	0	0	0	2
	Live Clean	0	0	0	0	12	3	15
	Live Diseased	0	0	0	2	13	12	27
	Dead Diseased	0	3	0	0	1	4	8
Gairney	Live Clean	0	0	0	0	0	0	0
	Live Diseased	1	0	0	2	24	14	40
	Dead Diseased	0	0	0	0	0	3	3
North Queich	Live Clean	0	0	1	0	0	0	1
	Live Diseased	1	1	0	0	1	4	6
	Dead Diseased	0	0	0	0	0	0	0

		Overall Total
Male Fish	Live Diseased	152
	Dead Diseased	18
	Live Clean	2
	Dead Clean	18
Immature Fish (sex undetermined)	Live Diseased	5
	Dead Diseased	0
	Live Clean	0
	Dead Clean	1

TABLE 24

Male brown trout trapped at Loch Leven during the spawning season of 1974/1975

Location	Disease Status	State of Maturity						Total
		I-III	IV	V	VI	VII	VIII	(IV-VIII)
South Queich	Live Clean	3	0	0	0	2	0	2
	Live Diseased	2	0	1	2	50	18	71
	Dead Diseased	0	2	0	1	1	7	11
Sluices	Live Clean	2	0	1	3	9	0	13
	Live Diseased	1	1	0	4	12	0	17
	Dead Diseased	0	0	0	2	1	0	3
Gairney	Live Clean	1	0	0	0	0	0	0
	Live Diseased	2	0	0	6	57	27	90
	Dead Diseased	1	3	0	3	6	3	15
North Queich	Live Clean	0	0	0	0	0	0	0
	Live Diseased	1	2	1	0	6	2	11
	Dead Diseased	0	0	0	0	0	0	0

		Overall Total
Male Fish	Live Diseased	189
	Dead Diseased	29
	Live Clean	15
Immature Fish (sex undetermined)	Live Diseased	6
	Dead Diseased	1
	Live Clean	6

Table 25

Female brown trout trapped at Loch Leven during the spawning season of 1973/1974.

Location	Disease Status	State of Maturity					Total
		IV	V	VI	VII	VIII	(IV-VIII)
South Queich	Live Clean	0	0	1	0	1	2
	Live Diseased	2	0	4	24	60	90
	Dead Diseased	0	0	0	3	4	7
Sluices	Dead Clean	1	0	0	0	2	3
	Live Clean	0	0	0	11	3	14
	Live Diseased	0	0	1	8	19	28
	Dead Diseased	3	0	0	2	1	6
Gairney	Live Clean	0	0	0	2	1	3
	Live Diseased	0	0	3	22	35	60
	Dead Diseased	0	0	0	1	7	8
North Queich	Live Clean	0	0	0	1	0	1
	Live Diseased	0	0	3	5	0	8
	Dead Diseased	1	0	0	0	1	2

Female Fish	Overall Total
Live Diseased	186
Dead Diseased	23
Live Clean	20
Dead Clean	3

Table 26

Female brown trout trapped at Loch Leven during the spawning season of 1974/1975.

Location	Disease Status	State of Maturity					Total
		IV	V	VI	VII	VIII	(IV-VIII)
South Queich	Live Clean	0	0	0	1	2	3
	Live Diseased	0	4	10	42	29	85
	Dead Diseased	4	0	0	1	12	17
Sluices	Live Clean	0	0	1	3	0	4
	Live Diseased	0	0	0	5	0	5
	Dead Diseased	0	0	0	2	0	2
Gairney	Live Clean	0	0	0	0	2	2
	Live Diseased	0	0	4	35	29	68
	Dead Diseased	3	0	0	6	2	11
North Queich	Live Clean	0	0	0	0	0	0
	Live Diseased	0	0	1	9	6	16
	Dead Diseased	0	0	0	3	10	13

Female Fish	Overall Total
Live Diseased	174
Dead Diseased	43
Live Clean	9

Spore-counting

The results of monthly estimations of spore-counts of Saprolegniaceae at the three sampling sites in Loch Leven are given in Table 28. Although there is general agreement in figures between sampling sites, there are some variations which have become magnified by the method used for propagule estimation. There is however, a trend of low propagule numbers in spring and summer with high numbers in autumn and winter.

TABLE 28

Estimates of propagule numbers of Saprolegniaceae per litre of water

Date	Corrected Estimates Site 1	Site 2	Site 3	Mean Value
Jan 1973	2300	1100	1390	1597
Feb	<50	560	260	273
Mar	<50	100	1200	433
Apr	50	50	<50	<50
May	<50	50	<50	<50
June	50	100	50	67
July	<50	<50	<50	<50
Aug	<50	<50	<50	<50
Sept	<50	150	1390	513
Oct	3920	1600	1840	2453
Nov	3200	150	780	1377
Dec	3920	2980	2980	3293
Jan 1974	1840	2140	1390	1790
Feb	1200	780	100	693
Mar	50	50	320	123

Note: Numbers of <50 are denoted as 0 for calculations of mean values

Results

Gross Lesions

Fungal lesions were evident on the skin surface of affected brown trout as grey-white circular or oval patches of varying size, raised from the skin surface as a cotton-wool like growth. The colour of the fungal mat varied from white to dark brown depending on the amount of silt trapped in the mycelia and the presence of haemorrhage. Lesions varied considerably in diameter but there was no correlation between diameter of lesion and depth of penetration. Many lesions were so superficial that apparently normal intact skin could be visualised beneath a fungal area and the fungal mycelium was easily pulled away from the surface skin. Other lesions extended deeply into the musculature and were associated with extensive haemorrhage, (figures 77 and 78).

Where lesions involved the gill, death often occurred without major skin infection. Although individual fungal patches on fish were relatively few in the majority of fishes, radial spread of fungal growth occasionally resulted in coalescence of such areas of infection and the involvement of a considerable surface area of the fish skin.

Histological Findings

a) Skin

As the time-course of infections in the wild fish population at the time of sampling individual fish was unknown, it is impossible to classify lesions absolutely into primary, secondary etc. stages but lesions were seen which correspond well with those described by Roberts, Shearer, Munro & Elson (1970) for fungal infection in U.D.N. in the wild Atlantic Salmon (Salmo salar L.) and sea-trout (Salmo trutta L.) and by Roberts & Hill (1975) in the brown trout (Salmo trutta L.).

Skin lesions were seen without associated fungal hyphae in fish suffering fungal infection but in sites distant from any evident infection. Such lesions were epidermal in origin and at the earliest stage consisted of swelling and degeneration of isolated nuclei throughout the epidermal layers (figure 79). Such changes were also found frequently in mature male fish which showed no gross or histological evidence of Saprolegnia infection. Difficulty was often experienced in demonstrating the cytoplasm of affected cells. Cells resembling lymphocytes were occasionally seen in small numbers in the epidermis of apparently uninfected fish, but such cells were particularly prominent in fish infected with Saprolegnia. These cells were seen at all levels of the epidermis but were commonest in the cell layers immediately above the so-called stratum germinativum.

In some fish focal areas of cell necrosis with nuclear pyknosis and karyorrhexis were frequently found in the 2 - 3

cell layers immediately above the stratum germinativum (figure 80). In others this change was widespread, though focal, throughout the epidermal layers (figure 81). Where more extensive, this resulted in bulla formation (figure 82)and even areas of complete epidermal separation just above the stratum germinativum.

Such changes were found in both sexes at varying stages of maturity, but were most frequent in mature males in which mucous cell counts were also markedly reduced. In the thickened skins of male and female fish, whether showing such necrotic changes or not, mitotic figures were frequent (figure 83). The above changes are hereafter referred to as "Type 1".

Another common finding in the epidermis of mature fish of both sexes was a pronounced swelling of surface epithelial cells and loss of cohesion with neighbouring cells. (Type 2 lesion). Such cells usually showed more intense staining of both cytoplasm and nucleus and nuclei were frequently pyknotic and surrounded with a clear "halo" (figure 84) and often densely-stained nucleoli. Type 1 changes were variably present in such sections. Sloughing of areas of superficial epidermis was commonly found in association with such swollen surface cells.

In many cases, individual fungal hyphae were seen in close association with an area of Type 2 change. Local necrosis of both malpighian and mucous cells was evident immediately adjacent to the fungus (figures 85 & 86) with an area of cell swelling surrounding it. Such fungal elements were often difficult to visualise and serial sectioning together with specific fungal staining such as P.A.S. or Gomori's

Methenamine Silver (figure 87) were often necessary to confirm the presence of fungal material.

The appearance of dermis in both the Type 1 and Type 2 lesions was usually normal. Changes in the number and appearance of dermal melanocytes were frequently seen in fish with no apparent epidermal lesions at varying stages of sexual maturity.

Lesions classified as Type 3 consisted of large areas of epidermal sloughing, usually, but not always, associated with surface fungal infection. The stratum germinativum was often the only epidermal structure remaining, sometimes covered by a layer of swollen malpighian cells. Spongiosis or intercellular oedema in the epidermis was commonly observed (figure 88) in skin areas surrounding areas of sloughing at this stage. Dermal changes at this time often involved either dense clumping of melanin just below the epidermis in the outer layers in the stratum spongiosum (figure 89) or else a complete dispersal of melanin in such areas with only a few light brown granules remaining at this site. Haemorrhagic areas were occasionally found in the S. spongiosum of the dermis at this stage.

When extensive fungal infection occurred, spread often developed through the basement membrane of the epidermis into the S. spongiosum of the dermis. Melanin clumping was again common at this stage in the dermis and dermal oedema in both the S. compactum and S. spongiosum was a frequent finding (figure 90). Haemorrhage in the S. spongiosum and also the hypodermis (figure 91)was usually found at this stage. Concomitant fungal invasion into the dermis (Type 4 lesion) resulted in local necrosis of dermal elements,

but inflammatory response was generally absent unless a similar dermal bacterial infection also developed (Figure 92). The inflammatory response in bacterially infected dermal lesions was variable in degree and type, but usually involved large numbers of neutrophils together with a few macrophages.

Muscular lesions (Type 5) were uncommon, but occasionally developed when fungal and bacterial penetration to surface muscle occurred. Bacterial infection was almost always present and associated with prominent myophagia and muscle oedema and spread of fungus and bacteria along fascial planes (Figure 93). Inflammatory response at this stage was usually extensive.

b) Fungal spread to other internal organs was never seen and histological changes in other organs were those associated with sexual maturity as described in Section II.

Results

Electron microscopy of spore-cases.

In all isolates the primary cyst case bore a tuft of tripartite flagellar hairs, each hair comprising a base, shaft and two terminal fibrils. The primary cyst case was also covered with single unbranched hairs of approximately 1.5μm length.

On secondary cyst cases the hooked hairs were long and aggregated into bundles (figure 94). The isolate previously classified as Type 2 from oogonial characteristics showed very similar secondary cyst case structure. Figure 95 shows the structure of a secondary cyst case from a typical Type 2 isolate which shows single short hooked hairs. Further details are given by Pickering et al 1979.

IV. 4. Discussion

There has been a considerable body of research directed towards indentifying fungal parasites of fish. A large number of species of a wide variety of families of the Oomycetes have been shown capable of parasitism when inoculated experimentally, though primary traumatic damage was often necessary to initiate infection (Murray 1885, Monsma 1936, Tiffney 1939, Hoffman 1949, Vishniac & Nigrelli 1957, Scott & O'Bier 1962, Scott 1964).

Natural outbreaks of fungal skin infection have occurred in a number of fish species in which a number of species of Saprolegniaceous fungus have been involved (Tiffney 1939, Scott & O'Bier 1962, Scott 1964, Wilson 1976, Pickering & Willoughby 1977) though in many such outbreaks it was not possible to encourage the formation of sexual structures in isolates and such sexually sterile forms were classed together as Saprolegnia parasitica, first named by Coker in 1923. In 1932, Kanouse described sexual structures in S. parasitica after applying the Klebsian principle of the gradual reduction of food supply as a means of control of vegetative and reproductive manifestations in fungi. Sexual structures were eventually formed after long periods of incubation on hemp seeds at 10°C., or with specific mixtures of peptone with leucin, glucose or maltose at 10°C. Since 1932, S. parasitica has ceased to become a repository for all those Saprolegnia

species not forming sexual organs, although unfortunately certain workers did persist in calling sexually sterile forms S. parasitica (Tiffney 1939, Hoshina & Ōokubo 1956). Many workers (Pieters 1915, Tiffney 1939, Scott & O'Bier 1962, Scott 1964) however have appreciated that sexual sterility may simply be linked to inadequate conditions of culture and that some isolates only produce sexual organs after considerable periods of time in culture. (Pieters 1915, Scott 1964, Neish 1977).

Indeed there are occasional heterothallic species such as Dictyuchus spp. (Couch 1926) and Achlya bisexualis Coker (Johnson 1956) and heterothallism was suggested as a possible explanation of sexually sterile Saprolegnia species (Stuart & Fuller 1968, Willoughby 1970).

Willoughby found that Saprolegnia species causing skin lesions in salmonids, including U.D.N., appeared to be of a particular type which is usually sexually sterile and which he named Saprolegnia Type I (Willoughby 1968, 1969, 1971). Other workers also regularly isolated sexually sterile Saprolegnia spp. from diseased salmonids (Scott & O'Bier 1962, Stuart & Fuller 1968, Roberts, Shearer, Munro & Elson 1970, White 1975, Neish 1977). Willoughby (1971) found that temperature was important in the production of sexual organs - strains of S. type I sterile at room temperature produced sparse oogonia between 5 - 10°C.

The fungi isolated in the present study did not produce sexual organs at 20°C. but some did at 7°C. It has been pointed out by numerous workers that this is a common feature of many Saprolegnia isolates, especially those in the S. parasitica - S. diclina complex (Coker 1923; Kanouse 1932; O'Bier 1960; Willoughby 1971; Neish 1977).

The above authors pointed out that close similarities existed between S. parasitica and S. diclina and much recent work has involved attempts at separation of the two species.

Kanouse (1932) defined S. parasitica as having oospores in the range 17-22μ, whereas Seymour (1970) defined the size of S. diclina oospores as 23-28μ. Neish (1977) divided isolates into four categories on the basis of zoosporangium and oogonium production (see Table 27). Neish (1977) found it impossible to type groups A & C further than Saprolegnia spp. because of the lack of oogonium formation. Category C is otherwise similar to D and B remains distinct. Category B isolates produced abundant gemmae, oogonia and antheridia at 10°C and oospores measure 24-29μm. Oogonial walls were thin-walled and occasionally pitted and mature centric to sub-centric oospores were infrequently produced.

TABLE 27

Categorisation of Saprolegnia spp. isolates from sockeye salmon (Neish 1977)

Category	Zoosporangium production (14 ± 1°C)		Oogonium production (10 ± 1°C)
	Hemp Seeds	Salmon Tissue	Hemp Seeds
A	Poor	Good	One oogonium observed
B	Poor	Good	Consistent, abundant
C	Good	-	None observed
D	Good	-	Sporadic, not abundant

Category D isolates produced only sparse oogonia after considerable culture periods and were thin-walled and unpitted and oospores were 18 - 22μm in diameter and sub-centric and similar to those described as S. parasitica Coker by Kanouse (1932).

Type D isolates are also closely similar to Saprolegnia Type I described by Willoughby (1968, 1969, 1971).

Willoughby (1978) in a more recent appraisal of such salmonid isolates separates his isolates into two main groupings - Saprolegnia diclina Type I and Saprolegnia diclina Type 2. Saprolegnia diclina Type I is that most frequently isolated from salmonids and corresponds closely with Neish Type D. These isolates again form no oogonia at 20^{o}C. but do so at 7^{o}C. The percentage of isolates with a length: breadth ratio of the oogonia greater than 2:1 is greater than 13% and oogonial investment is intense. Oospore diameter varies between 17.9 - 27.4μm, but individual isolates form oospores in either the lower or higher part of the range. This was also found in S. parasitica isolates by Monsma (1936), and falls into the oospore size groupings noted for S. parasitica (17 - 22μ) by Kanouse (1932) and S. diclina (13 - 28μ) by Seymour (1970). The typical "birdnest" investment of the oogonia is thought due to both antheridial hyphae and attached and germinated zoospores and oospore non-development is thought to result from destruction by invasive hyphae. Neish (1976), quoted in Willoughby (1978) also describes antheridia covering the entire surface of the oogonium, presumably equivalent to Willoughby's birdnest investment. The eleven isolates

described in the present study would appear to fall into the same grouping.

Willoughby (1978) suggests that the formation of elongated oogonia is due to late development in these isolates. It is thought that antheridial contact transforms hyphae tips or even sporangial cases into oogonia and elongated incipient sporangia which apparently develop into oogonia are frequently seen. The more rapid development of Type 2 isolates apparently allows more synchronous induction of sexual organs and results in spherical oogonia.

Fungal Mapping

The results of mapping areas of fungal infection in Loch Leven fish described in this study have been compared with similar fish from Lake Windermere, Cumbria, England and with a hatchery-reared group of brown trout (Richards & Pickering 1978). It was shown that the hatchery-reared fish were more severely infected than the wild fish from both localities in terms of the % area of the body covered with fungus, and the fins of hatchery-reared fish were particulary prone to Saprolegnia infection regardless of sex. This difference in severity of infection is thought due to the increased number of separate fungal colonies per fish and is probably a direct result of the increased fungal spore-counts in hatchery water (maximum counts of over 20,000 spores/l compared with 5,000 spores/l in Windermere and 4,000 spores/l in Loch Leven). A predominant fin infection in hatchery fish might be expected due to trauma and cannibalism, and the concentration of mucous cells on the fins is significantly lower than on the rest of the body (Pickering 1974).

A sexual difference in the pattern of infection was also shown for hatchery as well as wild fish, the flanks of the male fish appearing particularly susceptible to infection as opposed to the caudal and ventral fins of the females. These findings compare well with those of White (1975) who investigated infection of wild brown trout with a sexually sterile form of Saprolegnia. He carried out a χ^2 test of lesion distribution amongst five body areas and again found

that females had significantly more lesions on the caudal peduncle area and males on the anterior part of the body. White (1975) suggests that this pattern may be due to wounding of fish associated with spawning, the females using the caudal peduncle for redd-digging and the male exposing the anterior aspect of the body in territorial defence. Such conclusions were partially supported by the findings of Richards & Pickering (1978) - incidence of infection in wild female brown trout appears to increase after spawning. However, hatchery-reared fish were also heavily infected and in this case no substrate was available for redd-building - it is postulated that perhaps attempts at redd-building were nevertheless made. It must, however, be stated that in both Windermere and Loch Leven, fungal infection is evident on fish of both sexes prior to their entering the spawning streams.

Male aggression may again partially explain lesions in the male. It is well known among fish-farmers that male fish may cause considerable skin damage to one another when mature and for this reason, as well as to ease the manage-ment of hand-stripping ,, fish are generally segregated into monosex populations some time prior to spawning in fish-farms (Shorthouse 1977, pers. comm.).

These sexual differences in infection may also be partially explained by differences in skin structure at maturation. Richards & Pickering (1978) suggest that the drop in mucous cell concentration in males at spawning may

explain increased susceptibility due to the loss of the protective mucous coat. (It has been shown that mucus may protect fish by removing viable spores from the body surface - Willoughby & Pickering (1977)). However, a more detailed analysis of differences in skin structure does indicate that at the time of infection in many male fish, the mucous cell number is in fact increased (see results for skin structure - section II.

Richards & Pickering (1978) further showed that mature fish were far more frequently infected than immature fish. A difference was shown in sexual frequency of infection between Loch Leven and Windermere. Although there was no apparent difference in frequency between sexes in Loch Leven fish, there was an increased incidence in male fish in Windermere prior to spawning. The reduction in infection noted in male spent fish in Windermere may be due to increased mortalities in affected male fish after spawning. An increased mortality amongst male salmonids has been noted by other workers (Lamond 1916, Hutton 1925, Le Cren & Kipling 1963, Netboy 1968), but may not necessarily be due to an increase in fungal infection. Degenerative changes in the organs of mature salmonid fish have been regularly demonstrated ,

and these changes are often more marked in male fish. This is thought to be connected with a much elevated plasma testosterone level in male fish leading to degenerative changes in a variety of organs (see section VI and references by Hane & Robertson 1959, and Robertson & Wexler 1959, 1960, 1961.)

The sex difference in distribution pattern noted in the present work was reflected in the wild Windermere fish, but hatchery fish did not show so marked a variation. All groups showed an area free from infection around the lower jaw and operculum, and indeed in mature male Loch Leven fish this uninfected area extends along the ventral surface. Richards & Pickering (1978) have suggested that the bony areas of the undamaged head may provide a less suitable substrate for fungal development than the softer tissues elsewhere. This may partly be due to the increased concentration of mucous cells at the anterior end of the body (Pickering 1974), but this is in marked contrast to the findings in U.D.N. in which the head area is almost exclusively affected, and it seems likely that in this instance there may be some primary agent such as a virus (O'Brien 1974) or parasitic attack, although positive proof of any of these is lacking.

It is difficult to estimate the actual percentage of spawning fish affected with fungal infection. The traps supplying the spawning streams would certainly be selective for infected fish though all fish entering the

River Leven (Sluices) should have been caught in the trap there. When one examines the figures of fish trapped there between 1973/1975, 55 out of 85 fish were diseased. Because traps were not emptied on a daily basis it could be that some of the 55 fish actually developed fungal infection in the trap - such fish would be prone to damage and adjacent to a high fungal zoospore concentration in the water. Delays in emptying the trap would seem to explain the two fish found dead, but not fungal-infected. However, the possibly high figure of 55 out of 85 fish or 65% does compare well with the figure of 66% quoted by White (1975) as a percentage of sexually mature wild fish suffering fungal infection.

Spore-counting

The results of the work on counting of propagules of Saprolegniaceae in loch water did show a marked seasonal variation in the number of propagules present. Certainly numbers of propagules were considerably higher at the times of year when temperature was low, rainfall was high and the brown trout suffered extensive fungal infection. Such work does however, suffer from two major drawbacks; samples were only taken monthly and climactic or other possibly important factors relating to total propagule numbers will vary considerably over such long periods and increased frequency of sampling would certainly give more meaningful results. Secondly, it would be preferable to type each growth fringe. Only aseptate fungi belonging to the Saprolegniaceae were counted but these were not identified further; identification as to the species depends on the formation of oogonia and antheridia, as noted in the section on typing isolates from fish. There is no proof from this survey that types specific to trout were being counted, although it seems reasonable to speculate that this may be the case. It certainly seems possible that fungal-infected live and dead fish could well contribute considerably to the overall increase in propagules found during the spawning season. No evidence is presented to suggest whether the large rise in propagule numbers occurs prior to fish infection or as a result of it. Clearly, the establishment of such facts would involve considerable time and materials.

The results noted from Loch Leven are similar to those of Willoughby (1962) for Lake Windermere, but markedly different to the findings for Wraymires hatchery. Willoughby noted a maximum propagule number of 5,200/ litre during the winter months of 1959 and very low values in the summer for Lake Windermere. Values for the hatchery were much more variable throughout the year and were never less than 400/litre and it was suggested that fungal contaminated uneaten food and dead fish were probably contributing to this latter situation. If this was the case, then the fungal-infested mature fish at Loch Leven might correspondingly have been making a major contribution to the total counts. Willoughby (1962) also found markedly elevated propagule counts at the outlets of ponds containing fungal-infected fish when compared with inlet water estimations. The later work of Willoughby & Pickering (1977) also showed that infection of fish with Saprolegnia type I led to a massive increase in the spore-count of the hatchery water which was solely due to this specific type of fungus. They further suggest that infection of fish first occurs from a low background spore count in incoming hatchery water and fungal infection of fish then leads to a secondary rise in spore numbers.

The secondary zoospore would appear to be the infective part of the fungal life-cycle in fish (Willoughby & Pickering 1977) and was also the stage which germinated from lake water by Willoughby's method (Willoughby 1962).

Secondary zoospore formation was first noted in the Saprolegniaceae by Weston (1919) and Salvin (1941) showed that the secondary zoospores were much more efficient than the primary zoospore in survival and distribution of fungus because of their increased resistance to temperature changes and extremes and their ability to swarm more readily, at a faster rate, and over a greater period of time. Such may be the explanation of their being the infective stages in fish.

Seasonal periodicity in the Saprolegniaceae has been noted by a number of workers (Coker 1923; Forbes 1935; Waterhouse 1942; Naumur 1954 (cited by Hughes 1962); Perrot 1960; Hughes 1962; Alabi 1971). It has been suggested that temperature and pH changes generally lead to such variations with maximal counts occurring in spring and autumn (Coker 1923; Lund 1934; Forbes 1935; Perrot 1960; Roberts 1963) and the results of spore-counting in Loch Le-ven confirms these findings.

Histological Changes in Fungal Infection

Histological findings in fungal-infected fish were variable but are of considerable interest because of the close similarity to the earlier work on U.D.N. (Roberts, Shearer, Munro & Elson 1969, 1970). The condition described here in Loch Leven brown trout would not however appear to be that of U.D.N. Although the "premycotic" stage of U.D.N. (Roberts *et al* 1970b) is similar in many ways to that described here, the typical pattern of infection (Roberts *et al* 1970b, De Kinkelin & Le Turdu 1971) in U.D.N. consists of early lesions around the head area, or occasionally the adipose fin. The "syndrome mycosique", on the other hand, shows simultaneous development of fungal lesions on many parts of the body, but also affects salmonids during the peri-spawning period (Gerard 1971, Baudouy & Tuffery 1973). The gross lesions presented here do not therefore classify the condition as U.D.N.

By analogy with the work of Roberts *et al* (1970b), the earliest lesions are those described here as "Type I" with lesions in the depths of the epidermis with no associated fungal involvement (described as Grade 1 by Roberts *et al* 1970b). Roberts *et al* (1970b) note that mucous cells were frequently deficient at lesion sites; they do not however distinguish between sexes and previous findings (section II) show that a decrease in

mucous cell concentration is a regular finding in the epidermis of normal "clean" mature male fish. Changes in mucous cell numbers, except those normally expected in fish of a particular sex and state of maturation, were not noted in this study and it is considered that previous findings may have been a result of normal sex variation. Similarly the elongation of cells in the S. germinativum is a normal finding in mature fish of both sexes, but especially in males. Roberts (pers comm) states that the incidence of U.D.N. in males and females is approximately equal and that a reduction of mucous cell numbers occurs in both sexes.

Bulla formation, noted by Roberts et al (1970b) in Grade 1 lesions is also a frequent finding in Type 1 lesions in this study. Lymphocyte migration was also found at this stage in the epidermis.

The rounding of the surface cells described by Roberts et al (1970b) in Grade 1 lesions is presumably equivalent to the acantholysis described as Type 2 lesions in this work. Careful study of Type 2 lesions revealed that early fungal infection was almost always involved. Such findings are reported by Mulcahy(1969). As serial sections were often required to show this, it is possible that such early infections might not have been noticed in earlier work. The cuticle of the skin, a structure formed of mucus and disintegrating surface cells, is seldom seen in routine histology (Whitear 1970)

and the present study is no exception. This cuticular layer is easily removed and removal during spawning migration and trapping as in the present work might lead to the loss of the primary osmotic barrier protecting surface epidermal cells. Resultant osmotic imbalance in surface cells might easily lead to influx of water and ballooning of cells giving a histological picture very similar to that seen in type 2 lesions. Fungal infections would presumably readily develop in such areas of cuticular loss. Such a possibility was previously suggested by Willoughby (1969), who also pointed out (1971) that, although mucus was a good growth medium for Saprolegnia species, continual sloughing of mucus from the body did in itself tend to remove germinating zoospores and thus prevent infection. Similarly, primary fungal growth through the cuticle into surface epidermal layers might lead to similar local effects on damaged cells adjacent to the fungus. Recent work has suggested that the surface damage caused by Saprolegnia infection may be related to a chymotrypsin-like enzymatic activity (Peduzzi, Nolard-Tintigner & Bizzozero 1976; Peduzzi & Bizzozero 1977). Such enzymatic activity has been found in a variety of fungal species affecting a range of vertebrate hosts (Biguet, Tran Van Ky, Fruit & Andrieu 1967; Tran Van Ky, Vancelle, Andrieu, Torck, Floc'h & Quesnoit 1969;Nicolet, Bannerman & Krawinkler 1974; Soderhall & Unestam 1975). This may explain the local acantholysis occurring around fungal hyphae in the early stages of Type 2 lesions.

It is difficult to explain the lymphocyte infiltrations in Type 1 lesions. If the response was merely to damaged epidermal tissue, then one might rather expect a macrophage response. The lymphocyte response could be explained as an immune response to fungal infections, and indeed the possibility of a cell-mediated immune response occurring in the skin has been suggested by several groups of workers (e.g. Carlisle 1977; Carlisle & Roberts 1977; Timur, Roberts & McQueen 1977; Roberts 1978). but from the histological evidence, such infiltration is not always associated with fungal infection and may occasionally be found in apparently healthy uninfected fish. Roberts et al (1970b) suggested auto-immune disease as another possibility but later work (Roberts, Shearer & Munro 1972) would appear to disprove this theory. Another interesting finding is that such a response is never seen in the dermis, either as an increased percentage of lymphocytes in vessels in dermal areas or as an actual dermal infiltration. The source of such lymphocytes has thus not been visualised, though we must assume that they are introduced via the dermal blood-supply. A blood-mediated precipitating antibody response has been shown to be produced in naturally-occurring fungal disease and after inoculation of fungal extracts into fish (Hodkinson & Hunter 1970, Peduzzi 1975, Peduzzi & Bizzozero 1977).

Hodkinson & Hunter (1970) remark that such antibody responses were found in non-fungal infected fish and may represent prior contact with Saprolegnia antigens, either in the skin (Stuart & Fuller 1968) or through the digestive

tract as noted with bacterial antigens (Duff 1942; Krantz, Reddecliff & Heist 1964). Infections of the digestive tract with Saprolegnia spp. have been described (Agersborg 1933; Davis & Lazar 1941) and so the possibility of contact with digestive epithelia has been established.

Primary immune responses have been described for a number of teleosts (Ridgway Hodgins & Klontz 1966) and may persist for considerable lengths of time (Ridgway 1962). It has also long been recognised that such responses are temperature-dependent (Cushing 1942; Bisset 1947; Nybelin 1968) and certainly would seem to explain the bacteriological findings in section III. Teleosts also produce an anamnestic response (Uhr, Finkelstein & Franklin 1962; Sigel & Clem 1965) and this may partly explain individual variation in response to infection.

Although separation of the epidermis with incipient sloughing was seen in the suprabasal layers, actual ulcers with only S. germinativum remaining were not seen unless fungal infection was also present.

The Grade 3 lesions of Roberts et al 1970b were not noted in the present study.

The inflammatory response seen in Grade 4 lesions of Roberts et al 1970b was not seen in the Loch Leven fish unless bacterial involvement was present. Fungus alone seemed to elicit little response. The finding of an inflammatory response to bacteria at low temperature contradicts the

findings earlier (section III) of low circulating levels of bacteria with no apparent clinical effect.

Although dermal effects seen in the healing response to U.D.N. (Roberts, Ball, Munro & Shearer 1971) would appear to be a definite dermal healing response, the appearance of peg-like dermal papillae described in that work could easily be interpreted as normal male mature salmonid skin structure. Lack of details of sex in this former work preclude further assessment of this finding though Roberts (pers comm) suggests that this change occurs in both sexes. Lesions never developed more deeply than surface musculature. Such findings were also reported by Roberts et al (1969,1970b)

Nolard-Tintigner (1973) suggests that the growth of Saprolegnia in tissues is dependent on time. Thus infections in small fish with Saprolegnia spp. rapidly led to invasion of internal organs such as brain and liver (Nolard-Tintigner 1973; Bootsma 1973). Nolard-Tintigner (1973) suggested that such invasion of internal organs was the most frequent cause of death. Certainly, Saprolegnia infection would not appear to be tissue-specific but as initial Saprolegnia infection usually begins at the skin surface (with the exception of recorded outbreaks of gut infection (Agersborg 1933; Davis & Lazar 1941) further organ involvement would seem to be precluded by a rapid death due to osmoregulatory disturbance.

Electron-Microscopy of spore cases

Tiffney (1939) first suggested that the secondary zoospore was the infective stage in members of the Saprolegniaceae, but his evidence was not conclusive. The work of Nolard-Tintigner (1973) supports his claim. Willoughby & Pickering (1977) noted that attachment of germlings was by means of the secondary cyst case and the first portion of the germ tube, but not the developing mycelium. Manton, Clarke and Greenwood (1951) described delicate double-headed hooks on long slender stalks on the zoospore cases of S. ferax (Gruith). Thuret, and Meier & Webster (1954) and Nagai & Takahashi (1962) noted that the secondary cyst cases, but not the primary ones of a range of Saprolegnia spp. bore long double-headed hooks.

Pickering et al (1979) have confirmed the findings of Meier & Webster (1954), but have also shown that salmonid isolates classified as either Saprolegnia diclina Humphrey Type 1 or as sexually sterile Saprolegnia spp. all show secondary cyst cases with bundles of long hooked hairs. Type 2 isolates and other saprophytic Saprolegnia spp. show short, single hooked hairs.

There was no statistical difference in these structures between Loch Leven Type 1 isolates and Windermere Type 1 isolates. The isolate previously referred to as Type 2 because of its oospore characteristics falls into the typical Type 1 grouping according to secondary cyst structure.

Variation occurred in the cyst morphology of Type 1 and sterile isolates but all fell within a similar range, suggesting that the sterile isolates examined were probably closely related to Type 1 strains. This corresponds well with a range of growth characters described by Tiffney (1939) for S. parasitica and supports the suggestion that increased genotypic diversity is more likely in the diclinous species of the Saprolegniaceae.

Several workers have suggested that the hooks on cyst cases are somehow concerned with cyst attachment (Manton et al 1951; Meier & Webster 1954; Pickering et al 1979), and the increased length and grouping of these hairs may represent a parasitic adaptation (Meier & Webster 1954; Pickering et al 1979). It has also been suggested that such long-haired varieties are more buoyant and perhaps remain in the water-body for long periods of time, thus increasing the chances of fish contact. Other short-haired varieties are more adapted to a saprophytic mode of existence on the lake bottom. This does not, however, give a suitable explanation to the apparent species-specificity of Type 1 isolates.

V EFFECTS ON THE FISH HOST

V. 1. SERUM LEVELS IN RELATION TO INFECTED AREA OF SURFACE

V.1.1 Introduction

It is well known that fish skin serves as an efficient barrier to osmotic change and damage to the skin may lead to a rapid uncontrolled flow of water and salts across the skin, resulting in profound changes in the "milieus interieure" and an osmotic imbalance within the animal (Parry 1966). The type of skin damage may be very variable, but the result is still osmotic imbalance. Trauma through handling fish, as occurs in several stages of the production cycle of farmed fish, regularly leads to death and this has often been linked to osmotic disturbance (Parrish, Blaxter & Holliday, 1958; Holliday & Blaxter, 1961; Potts & Parry, 1964; Lewis, 1971; Hickey, 1978). Death from Saprolegnia infection has similarly been attributed to osmotic imbalance (Gardner 1974; Mulcahy 1975).

It has been shown that survival of fish with skin damage was improved if they were maintained in water which was more or less isosmotic with blood (Parrish et al 1958; Collins & Hulsey 1963; Lewis 1971; Wedemeyer 1972; Hickey 1978).

Fortunately, adult teleosts possess the ability to rapidly cover an integumentary defect with epidermis, an obviously important factor in the survival of species living in water (Harabath 1928; Arey 1932, 1936; Berlin

1951; Mittal & Munshi 1974; Anderson & Roberts 1975; Laird, Roberts, Shearer & McArdle 1974; Phromsuthirak 1977; Bullock, Marks & Roberts, 1978), but this ability is temperature-dependent. Wound closure occurs through migration of epidermal cells in the early stages rather than cell division.

The presence of persistent fungal growth in such damaged skin areas will obviously retard healing and it was decided to see whether any correlation existed between changes in serum parameters in relation to the extent of fungal infection.

V 1.2 Materials and Methods

Fifty three, sexually mature, brown trout (mean weight 1074g.) were taken by traps and net from the streams supplying Loch Leven and from the outlet to the River Leven during the periods October - December, 1973 - 1975. Twenty "clean" fish were also taken by beach seine during March - June, 1973 - 1975. Each fish was anaesthetised in MS222 (Sandoz pharmaceuticals, Basle) at approximately 1 : 10,000 concentration, weighed and measured and the distribution of any fungal infection was mapped as described in the fungal section and the percentage area of body surface infected was calculated. A blood sample of approximately 5 ml. was then removed from the caudal vein and the sex and stage of maturity determined. Fish were then either killed by spinal section to confirm maturity determination or, following recovery in fresh water, fish were returned to the burn to spawn. Blood samples were then allowed to clot in centrifuge tubes at ambient temperature for thirty minutes and then spun down at 2,000 r.p.m. and the serum removed. When a delay of more than thirty minutes between sampling and centrifugation was inevitable, as often occurred during seine-netting, samples were stored on ice. Sera were frozen at -20°C. until required for further analysis.

Sodium and potassium were later measured with a

Corning Eel Flame Photometer (Model 430) and calcium and magnesium with a Unicam SP90A Atomic Absorption Spectrophotometer. Total protein concentration was determined using the Biuret method and serum albumen levels were measured with an autoanalyser using the bromocresol green technique (Bartholemew & Delaney, 1966).

Polyacrylamide gel electrophoresis was carried out according to the method described as System 1 by Norris & Ribbons (1971), but it was found necessary to add 30µl of temed to each 32 ml. gel mixture to aid gelling. Tris-glycine buffer at pH 8.3 was used as tank buffer. Serum samples were diluted 1 : 10 with distilled water and 5µl. samples together with 5µl. of tracking dye (0.05% Bromophenol Blue) were layered in a sucrose solution (80 gm./100 ml.) on to the gel and a current of 4 milliamps/tube passed for 1½ hrs. Gels were stained with Coomassie Brilliant Blue R250 and destained in 10% methanol/10% acetic acid, (see Appendix 2).

A semi-quantitative estimate of the protein composition of the serum was achieved with a Unicam SP1800 Densitometer at 544nm. From the traces obtained, the relative movement of each peak was calculated and an assessment of the relative area of each protein peak was obtained by multiplying peak height by width at half height. The relative peak area of individual peaks in individual fish was then calculated by multiplying the percentage of total area for the peak by the total protein concentration.

Means of peak areas for individual peaks in particular groups of fish were then calculated.

V 1.3 Results

Saprolegnia infection of sexually mature brown trout from Loch Leven resulted in a significant reduction in all serum parameters measured, potassium levels showing the least significant fall (see table 29). A direct, and highly significant correlation was also found between the serum sodium concentration and the degree of infection of the fish when expressed as the percentage of the body surface colonised by Saprolegnia (see figure 96). There were no apparent differences in response to Saprolegnia infection between male and female fish, but the mean proportional reduction in concentration of each ion as a result of infection varied between 20% for sodium and 40% for potassium.

A significant decrease in albumen/globulin ratio associated with fungal infection also suggests variation in proportional reduction of individual serum proteins. As no consistent differences were noted between the electrophoretic patterns of male and female fish at either time of year (March - June and October - December) data from both sexes were pooled for further analysis. Individual protein peaks were identified by their mobilities relative to bromophenol blue and a total of 18 separate peaks was observed. However, there was considerable variation between individual fish (see table 30) and no attempt was therefore made to identify individual peaks as particular serum proteins. Peak 1 was the only peak

present in all the fish examined. The mean number of protein peaks from the serum of non-infected fish (8.95 $\pm$ 0.30 (21), range 7 - 11) was not significantly different to that of infected fish (9.05 $\pm$ 0.28 (21), range 7 - 11). In addition, X^2 analysis failed to show any significant difference in the frequency of occurrence of any single protein peak between infected and non-infected fish (Table 30). When estimates of the areas of individual peaks were made it was found that 10 out of the 18 peaks were significantly reduced in the infected fish. Moreover, if the peaks are pooled into 4 groups (Group 1 = peaks 1,2,3,4,5; Group 2 = peaks 6,7,8,9; Group 3 = peaks 10,11,12,13; Group 4 = peaks 14,15,16,17,18) in order of decreasing electrophoretic mobility, it can be shown that there are significant decreases in the mean area of the first 3 groups ($p<0.001$, Student's 't' test) but not in group 4, the group of slowest moving proteins (table 32). Figure 97 is a diagrammatic representation of the mean electrophoretogram results expressed in Table 30.

Table 29

Composition of the blood serum of Saprolegnia-infected and non-infected brown trout from Loch Leven. Results expressed as mean ± S.E.M. (n). A 't' test comparison was made between infected and non-infected male and female fish sampled in the period October - December.

Sample	Sex	Mean ± S.E.M. Na	K	Ca	Mg	Total Protein	Albumen	A.G. ratio	% Infection
		mM/l	mM/l	mM/l	mM/l	g/100ml	g/100ml		
Non-infected brown trout March - June	M	164.4 ±1.65(10)	1.8 ±0.06(10)	2.49 ±0.03(10)	0.84 ±0.007(10)	5.8 ±0.21(10)	2.90 ±0.11(10)	0.48 ±0.009(10)	
	F	165.3 ±1.43(10)	1.8 ±0.05(10)	2.52 ±0.027(10)	0.84 ±0.007(10)	5.4 ±0.28(10)	2.71 ±0.13(10)	0.50 ±0.013(10)	
Non-infected brown trout October - December	M	162.4 ±1.46(11)	2.1 ±0.09(11)	2.2 ±0.10(11)	0.8 ±0.01(11)	7.4 ±0.48(11)	3.6 ±0.21(11)	0.49 ±0.01(11)	
	F	166.0 ±1.05(9)	2.1 ±0.07(9)	2.3 ±0.06(9)	0.8 ±0.01(9)	6.0 ±0.24(9)	2.9 ±0.13(9)	0.47 ±0.01(9)	
Infected brown trout October - December	M	130.2 ±4.65(18)	1.3 ±0.22(18)	1.51 ±0.13(12)	0.49 ±0.07(14)	3.3 ±0.38	1.04 ±0.26(18)	0.25 ±0.04(18)	32.9 ±4.00(18)
	F	131.1 ±4.7(15)	1.2 ±0.26(15)	1.61 ±0.14(12)	0.50 ±0.07(12)	3.4 ±0.55(15)	1.3 ±0.4(15)	0.28 ±0.05(15)	30.1 ±4.8(15)

Table 29 contd.

Positive Results of T tests. (Infected vs. non-infected - Winter only)

(1) Males - Inf. vs. non-inf. Winter

Analysis	P<	% reduction
Na	0.001	19.9
K	0.05	39.1
Ca	0.001	31.4
Mg	0.001	39.7
Protein	0.001	55.5
Albumen	0.001	71.1
A:G Ratio	0.001	49.0

(2) Females - Inf. vs. non-inf. Winter

Analysis	P<	% reduction
Na	0.001	21.1
K	0.05	42.9
Ca	0.001	30.0
Mg	0.01	37.5
Protein	0.01	43.3
Albumen	0.01	55.2
A:G Ratio	0.01	40.4

All other comparisons of the data on serum analysis were not significantly different.

TABLE 30(a) Mean value for areas of peaks 1-18 in uninfected fish

Peak No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Mean % Movement (relative to Bromophenol blue)	59.8	52.4	50.0	47.3	44.0	41.4	38.6	34.3	31.5	28.6	25.9	23.1	19.2	15.0	11.9	8.1	4.5	2.6
Range	59.0 60.2	51.6 53.4	49.3 51.0	46.0 48.7	43.0 44.9	40.2 42.6	36.9 39.7	32.3 35.7	30.6 32.8	27.2 29.5	24.9 26.9	21.2 24.5	17.1 20.8	13.7 15.6	10.0 13.9	7.2 9.3	3.0 6.5	2.5 2.7
S.E.	0.13	0.13	0.27	0.28	0.37	0.33	0.19	0.35	0.22	0.29	0.20	0.31	0.32	0.29	0.31	0.24	0.77	0.10
Frequency	21	18	6	13	7	8	14	10	11	7	11	12	15	9	14	8	5	2
Area	59.4	61.4	65.4	59.4	93.36	43.5	142.7	125.3	79.4	76.6	78.1	68.3	66.9	34.3	41.9	72.5	44.1	50.2
S.E.M.	5.68	7.42	8.82	14.89	29.36	10.23	14.33	20.86	24.01	14.10	13.30	12.56	14.34	12.62	7.17	19.59	18.71	2.85
n	21	17	6	13	7	8	14	10	11	7	11	12	15	9	14	8	5	2

TABLE 30(b) Mean values for areas of peaks 1-18 in infected fish

Peak No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Mean % Movement (relative to Bromophenol blue)	59.9	52.7	50.6	47.8	44.3	40.7	38.1	34.6	30.7	28.5	25.6	23.0	18.1	14.7	11.7	7.8	4.1	1.3
Range	59.0 62.0	51.2 53.5	50.4 50.8	46.2 48.4	43.0 45.8	40.0 41.3	36.9 39.0	33.2 35.9	29.8 32.7	27.7 29.4	24.7 26.5	22.0 24.3	16.8 19.8	14.1 15.4	9.9 13.6	7.0 8.5	2.9 6.3	0.4 2.4
S.E.M.	0.14	0.20	0.12	0.22	0.32	0.65	0.20	0.58	0.33	0.49	0.16	0.26	0.32	0.38	0.36	0.14	0.34	0.40
Frequency	18	15	3	10	10	2	13	5	10	3	13	9	12	3	11	12	8	6
Area	30.5	29.9	66.3	30.3	34.3	24.6	61.2	52.5	43.4	13.5	62.8	38.5	27.9	26.4	19.9	19.3	36.0	2.63
S.E.	5.83	4.57	19.78	3.06	6.10	13.45	12.67	2.52	9.11	1.85	13.95	13.45	5.92	19.62	6.11	4.41	10.04	5.25
n	15	12	3	8	9	2	10	5	8	3	10	8	10	2	9	10	6	6
% reduction in area	48.7	51.3	+1.4	-4.90	63.3	43.4	57.1	58.1	45.3	82.4	19.6	43.6	58.3	33.0	52.5	73.4	19.4	47.6
t test	<0.005	<0.005	NSD	NSD	<0.05	NSD	<0.001	<0.05	NSD	<0.05	NSD	NSD	<0.05	NSD	<0.05	<0.01	NSD	<0.05

TABLE 30(c)

Results of x^2 test between individual serum protein peaks for infected and diseased fish

Peak number	1	2	3	4	5	6
x^2	0	0.07	0.12	0.01	0.48	1.73
	NSD	NSD	NSD	NSD	NSD	NSD
Peak number	7	8	9	10	11	12
x^2	0.03	0.03	0.03	0.02	0.05	0.48
	NSD	NSD	NSD	NSD	NSD	NSD
Peak number	13	14	15	16	17	18
x^2	0.05	1.27	0.0006	1.24	1.92	1.92
	NSD	NSD	NSD	NSD	NSD	NSD

TABLE 31(a)

Relative peak areas (grouped) for individual clean fish

Peaks	1,2,3,4,5	6,7,8,9	10,11,12,13	14,15,16,17,18
2	150.7	241.5	116.7	21.0
3	56.7	106.0	37.1	10.3
35	269.0	231.6	62.1	7.4
38	207.8	234.2	106.8	65.7
27	171.8	305.5	157.7	9.8
25	160.6	309.2	149.8	32.3
4	213.6	227.9	18.5	22.3
1	168.8	237.7	130.6	42.6
26	150.9	301.0	146.2	53.8
37	233.5	184.3	132.7	97.2
36	128.5	172.7	91.6	244.9
13	173.4	231.8	175.0	161.5
17	349.7	174.0	273.1	48.5
18	327.6	233.9	236.7	201.8
15	292.0	285.5	241.1	181.0
16	213.8	244.4	173.7	149.7
29	217.0	211.6	243.6	37.6
14	141.0	232.1	125.4	123.8
31	173.5	195.2	154.2	121.6
30	300.8	86.2	217.0	72.3
28	119.0	170.4	190.2	90.1
Mean	200.9	219.8	151.4	85.5
SEM	16.1	12.7	14.8	15.1
n	21	21	21	21

TABLE 31(b)

Relative peak areas (grouped) for individual infected fish

Peaks	1,2,3,4,5	6,7,8,9	10,11,12,13	14,15,16,17,18
7	106.2	60.1	36.9	7.7
5	248.5	268.4	209.5	56.3
11	196.1	67.6	150.1	68.8
23	63.9	49.3	50.5	65.9
21	93.3	38.4	69.3	86.8
8	80.3	60.0	33.7	15.5
32	83.9	58.7	106.7	60.6
22	161.0	66.8	34.7	137.1
24	162.9	106.4	74.8	110.3
20	122.5	55.4	79.8	68.9
40	26.0	42.6	69.1	14.2
10	76.4	102.1	102.2	20.9
9	44.6	88.5	72.5	15.1
6	38.5	69.0	88.0	4.3
19	44.1	38.9	77.7	67.8
Mean	103.2	84.8	81.7	53.3
SEM	16.5	15.7	12.1	10.3
n	15	15	15	15

Table 31(c)

Relative peak areas (grouped) for clean and infected fish.

		Peaks 1,2,3,4,5.	Peaks 6,7,8,9.	Peaks 10,11,12,13.	Peaks 14,15,16,17,18.
Clean fish	Mean	200.9	219.8	151.4	85.5
	S.E.M.	16.1	12.7	14.8	15.1
	n	21	21	21	21
In-fected fish	Mean	103.2	84.8	81.7	53.3
	S.E.M.	16.5	15.7	12.1	10.3
	n	15	15	15	15
% reduction		48.6	61.4	46.0	37.7
p		< 0.001	< 0.001	< 0.005	NSD

V. 1.4 Discussion

The normal sodium concentration (164 mM/l) measured was slightly higher than some reported values (Phillips & Brockway, 1958; Gordon, 1959), but Oduleye (1976) has shown that the plasma sodium concentration may vary between 110 and 187 mM/l depending upon the water temperature, calcium concentration and the time of year. Serum potassium values were somewhat lower than previously reported (Phillips & Brockway, 1958; Oduleye, 1976), but are within the range determined by Gordon (1959). Serum calcium and magnesium values for uninfected brown trout in freshwater were similar to those of Phillips & Brockway (1958).

A highly significant decrease in the serum concentration of all ions measured was produced by Saprolegnia infection and a significant correlation was demonstrated between the degree of infection (expressed as the percentage body surface area covered by the fungus) and the sodium concentration. A similar correlation between serum osmotic pressure and degree of infection was shown in Saprolegnia-infected brown trout in Windermere, England by Richards & Pickering (1979). When the two correlations were standardised by expressing the serum osmotic pressure and sodium concentration as a function of the values for uninfected fish, a comparison of the two regression lines by analysis of variance showed that there was no significant difference between either the slopes of the two lines or their intercepts on the y axis. This suggests that the

drop in serum osmotic pressure is predominantly caused by reductions in sodium and chloride concentrations. In severe cases sodium concentration was reduced to less than 100 mM/l and it has been shown (Leivestad, Hendrey, Muniz & Snekvik, 1976) that a similar drop in serum sodium concentration associated with acid water-induced osmoregulatory failure is fatal to brown trout.

Unfortunately the animals used in this study were wild fish suffering Saprolegnia infection at the time of capture and sampling. There was no way of determining the length of time that fish had been suffering fungal infection. This is obviously of importance as extent of electrolyte loss will depend on both area of infection and duration of infection. Similarly, though the majority of fish were only suffering superficial infections, the occasional fish did have deep lesions with exposure of muscle tissue to the water and frequently haemorrhage, which would further complicate electrolyte loss. However, from fish farm experience and occasional problems with experimental fish, it is evident that fish can die from fungal infection with no secondary infection in as little as two or three days after initial infection. From the above work, the primary cause of death would appear to be haemodilution. This conclusion supports the work of Gardner (1974) who related changes in the blood serum of Atlantic salmon Salmo salar L. to U.D.N. and Saprolegnia infection.

However, the changes in electrolyte composition that occur cannot be totally accounted for by a simple haemodilution due to an increased osmotic influx of water as all ions were not reduced by the same proportion, a finding also noted by Gardner (1974).

Serum potassium reduction is a characteristic stress response in certain teleost fish (Oikari & Soivio, 1975) and there can be little doubt that fungal infection in salmonid fish is a severe stress factor (Mazeaud, Mazeaud & Donaldson, 1977). Calcium and magnesium were also reduced by a greater proportion than sodium in the infected fish.

The average protein concentration in the serum of teleosts is quoted by Alexander (1977) as between 4 - 5g/100ml, but in salmonids values between 2.9 - 12g/100ml have been noted (Meisner & Hickman, 1962; Booke, 1964 a,b; Sanders, 1964; Snieszko, Miller & Atherton, 1966; Poston, 1966; Mulcahy, 1969, 1971, 1975; Davis, 1975; Denton & Yousef, 1975). Sampling in the above studies was generally taken at only one particular time of year, which varied from one study to another and sex differentiation was not always carried out. Sex differences have been noted in brook trout, Salvelinus fontinalis (Booke, 1964a), rainbow trout Salmo gairdneri (Snieszko, Miller & Atherton, 1966) and brown trout Salmo trutta (Ingram, 1974; Ingram & Alexander, 1977), and the Atlantic salmon Salmo salar (Alexander, 1977). Protein

concentration was higher in female brook and rainbow trout, whilst the converse was true in brown trout and Atlantic salmon. Ingram, quoted by Alexander (1977), found no consistent sex difference in protein levels in brown trout and such was the finding in the present survey, and quoted by Richards & Pickering (1979).

A variety of viral and bacterial infections in salmonid fish leads to serum protein depletion (Hunn,1964; Klontz, Yasutake & Parisot, 1965; Cardwell & Smith, 1971; Shieh & MacLean, 1976).

The severe hypoproteinemia associated with Saprolegnia infection in the brown trout is similar to that noted by Mulcahy (1969, 1975), Wilkins (1972) and Gardner (1974) for U.D.N. infected salmon and by Carbery (1970) and Mulcahy (1971) for U.D.N. infected brown trout. In the present work, this serum protein depletion was proportionally greater than any of the ionic changes so far discussed. Gardner (1974) found a dissociation between ionic and protein (measured as total nitrogen) regulation in U.D.N. infected salmon as indicated by their ability to maintain a normal serum ionic composition but not a normal protein level, when held in brackish water.

Protein concentration in the extravascular fluid has been shown to be similar to the plasma protein concentration in the marine teleosts Gadus morhua L. and Pleuronectes platessa L. and is thought to be due to high

capillary permeability (Hargens, Millard & Johansen, 1974). If such is the case in the brown trout, any loss of protein from the extravascular fluid at sites of fungal damage may be expected to be reflected by a similar rapid decrease in plasma proteins and may explain the rapidity with which the fungal-infected fish can die.

A major factor contributing to the hypoproteinemia is the relative reduction in the serum albumen levels as shown by the decrease in A/G ratio. Serum albumen was determined by autoanalyser using bromocresol green (BCG) after the method of Bartholomew & Delaney,(1966). This method was initially used because of its increased accuracy when used in the autoanalyser instead of other standard dye methods such as 2 - (4' hydroxybenzeneazo) - benzoic acid (HBABA). However, it has been found that if BCG results are compared with those obtained by a cellulose acetate method, a considerable discrepancy exists between the two methods at low albumen levels (Webster, 1974; Webster, Bignell & Attwood, 1974). Similarly, it has been shown that electrophoretically isolated globulin fractions may react with BCG to form a false increase in albumen levels, which will be especially noticeable at low albumen levels (Webster, 1974). Cellulose acetate electrophoretic methods were shown in both the above studies to give good correlation with immunoprecipitation techniques. This may explain the apparent variation seen in some samples between albumen measured by BCG and relative concentrations of peaks

thought by analogy with human results to be albumen in the electrophoretic studies. Consequently the A/G ratios obtained during the present investigation must be treated with some reserve. Nevertheless, a decrease in the serum A/G ratio is also characteristic of U.D.N. infected brown trout (Carbery, 1970) and of Sacramento River chinook disease virus (SRCDV) infection in rainbow trout, Salmo gairdneri irideus Gibbons (Klontz et al, 1965). Thus it seems likely that a reduction in the A/G ratio may be a characteristic response of fish to many different diseases.

Electrophoretic analysis of the serum proteins of uninfected and Saprolegnia-infected brown trout failed to reveal any consistent qualitative changes associated with infection. This agrees with the observations of Mulcahy (1971) for U.D.N. infected brown trout and contrasts sharply with her work on U.D.N. infected salmon (Mulcahy, 1969) in which the disease was associated with a specific electrophoretic pattern. Some electrophoretic evidence is presented to show that the drop in serum total protein concentration is associated with the faster migrating proteins and that the slower moving bands are not reduced. Although no attempt was made to characterize and identify individual protein peaks, by analogy with other work both on fish and mammals, it would seem likely that the fastest moving proteins include the albumens and that the slower moving proteins are predominantly globulins. Thus this general change in electrophoretic pattern supports the observation that the A/G ratio is decreased in Saprolegnia-infected fish.

It has been shown (Post, 1963; Klontz et al, 1965) that the immune globulins of rainbow trout are found in a B_2 fraction. This fraction is relatively slow-moving and it may be that the relative increase of slower-moving globulins in the present work is related to immunoglobulin production by the fish in response to the fungus. Further work on characterisation of the serum proteins from these fish is necessary before this hypothesis may be adequately tested.

V. 2. Electrocardiogram Studies

V. 2.1 Introduction

The electrocardiogram has been widely used in fish physiological studies, primarily as a measure of heart rate (Marvin & Heath, 1968; Sutterlin, 1969; Nomura, Ibaraki, Hirose & Shirahata, 1972; Priede, 1974; Priede & Tytler, 1977). Several workers have utilised the electrocardiogram to study the effects of anaesthetic agents on heart rate (McFarland, 1959; Houston, Madden, Woods & Miles, 1971; Houston, Czerwinski & Woods, 1973) whilst others have used the cardioinhibitory reflexes measured by the electrocardiogram in classical conditioning experiments (Chapman & Hawkins, 1973; Facey, McCleave & Doyon 1977).

Only very few reports have examined the waveform of the electrocardiogram in any detail and notable amongst these are the works of Kisch, 1948; Oets, 1950; Tebecis, 1967; Nanba, Murachi, Kawamoto & Nakano, 1973 and Wardle & Kanwisher, 1974. Oswald (1978) has examined waveform changes following administration of certain injectable anaesthetic agents to fish.

The waveform of the electrocardiogram is notoriously variable with even minor variations in electrode placement and with size and species of fish. Nanba et al (1973) have suggested that certain stable electrode placements may be usefully employed for pathophysiological analysis in fish.

The effects of variation in plasma electrolytes upon the electrocardiogram are well known in man and other animals and may be used clinically (Fisch, Knoebel, Feigenbaum & Greenspan 1966; Katz & Bigger 1970; Surawicz & Gettes 1971; Fisch 1973; Rollason, 1975).

The ECG has been especially useful in the detection of changes in blood K+ levels which may reflect renal and endocrinological function.

The present study is apparently the first of its kind in fish.

Anamnesis

The trout heart consists of four chambers in series (figure 98), the sinus venosus, atrium, ventricle and bulbus arteriosus. All chambers except the bulbus contain typical cardiac muscle fibres and are contractile and this property, together with a system of valves between chambers, maintains a unidirectional flow of blood through the heart. Individual cardiac muscle fibres are generally smaller in fishes than in mammals and the rate of rise of the cardiac muscle action potential is very low in fish e.g. 9.5 ± 0.7 v/sec. in the skate as opposed to 560 v/sec. for the Purkinje fibres of a sheep (Satchell, 1971) and this is thought due to the relatively high concentration of intracellular sodium ions. Conduction velocities are also slower in fish (Bennion, quoted by Randall, 1968).

Initiation of the heartbeat usually occurs in the sinoatrial node (Mott, 1957) but the site and extent of pacemaker tissue is very variable between fish species and in some species potential pacemaker areas are found scattered in islets of tissue throughout the myocardium (Kisch, 1948). Conduction velocities from the pacemaker areas are slower than in mammalian hearts and there are fast-conducting pathways in the ventricle of the rainbow trout which lead to its apex, causing it to contract before the basal ventricular layers around the atrioventricular junction (Randall, 1968). Chiesa, Noseda & Marchetti (1962)

have also shown that the left apical region is activated prior to the right.

All fish hearts with the exception of Hagfish are innervated by a branch of the vagus nerve (Mott, 1957; Randall, 1968), stimulation of which causes bradycardia which can be blocked by atropine. Application of acetylcholine also causes bradycardia (Johansen, Franklin & van Citters, 1966), all evidence suggesting that the vagus contains cholinergic fibres. However, some investigators have produced cardiac acceleration by vagal stimulation (Campbell, 1970) and adrenergic fibres have also been found to innervate the trout heart (Yamauchi & Burnstock, 1968; Gannon & Burnstock, 1969). Blood pressure is maintained relatively constant and it is thought that chemoreceptors and baro-receptors in the pseudobranch act via the vagus to maintain this. The trout heart also obeys Starling's Law (Bennion, 1968).

The sinus venosus is generally only weakly contractile in fish, the main contractile effort arising from atrium and ventricle. Blood flow into the ventral aorta is maintained by elastic recoil of the bulbus during ventricular relaxation. Heart-beat is also co-ordinated with breathing so that blood remains in the gills during periods of maximum water-flow (Satchell,1961; Hughes & Shelton, 1962; Hughes, 1964 .). The major response to exercise is a large increase in stroke volume with little effect on heart rate

(Stevens & Randall, 1967 a,b) - this effect is thought due to increased venous return rather than an effect on the vagus (Randall, 1968). The blood supply to the trout heart is from coronary arteries which are branches of the anterior hypobranchial system.

The ECG is similar to that of mammals and has a typical P wave, QRS complex and T wave. The P wave represents depolarisation of the atrium, and atrial systole follows the P wave. The PR interval represents the delay in passage of the impulse from atrium to ventricle and the QRS complex represents ventricular depolarisation and the onset of ventricular systole. Increase in ventricular pressure at this stage closes the a - v valves and when this pressure exceeds that in the bulbus and ventral aorta the valves between bulbus and ventricle open to allow passage of blood into the bulbus. The QT interval is longer than in mammals and represents the time interval during which the ventricle remains depolarised and the T wave represents repolarisation of the ventricle and relaxation of the ventricular muscle.

All time intervals become decreased with decreasing temperatures, as in hibernation and myxoedema in homeothermic animals.

V. 2.2 Materials and Methods

Nineteen fish, 11 "clean" fish and eight fungal infected fish were obtained by trap and seine-net between December and February, 1973. Fish weighed between 480gm. and 1990gm. They were maintained in 250 litre tanks in a constant temperature room with aeration and a continual flow of freshwater. For electrocardiographic measurements anaesthesia was induced by placing fish in a tank containing benzocaine solution (50mg/l). Solutions used were freshly constituted at the ambient temperature of the freshwater supply to the holding facilities. After induction of anaesthesia fish were transferred to an anaesthetic system and operating table similar to that employed by Smith & Bell (1967) (see figure 99). Fish were held in dorsal recumbency in a sponge fish-holder lined with disposable wet tissues and gills were perfused with benzocaine solution (30mg/l) via a mouthpiece for maintenance of anaesthesia. The anaesthetic system incorporated recirculation, reaeration and temperature control of the anaesthetic solution. Anaesthetic flow-rate was measured with a Rotameter (G.A. Platen Ltd.) and fish could rapidly be switched over to an alternative freshwater supply as necessary. The room housing the anaesthetic apparatus was temperature controlled at approximately 10°C. and the fish were frequently sprayed with water to maintain the integrity of the skin and cuticle.

Electrocardiograms were detected with two punctate electrodes made of 200µm insulated stainless steel (Dimel-Johnson, Matthey) bored for 2mm at the tip and inserted subcutaneously into right and left pectoral axillae. Electrocardiographic potentials were amplified and displayed on a "CEPTU" unit (Epil Products Ltd.) and recorded on Devices M X 212 or George Washington 400 MD/2 penrecorders. Recordings were made at a bandwidth of 1 KHZ and a time constant of 1S. Amplification was differential with the non-inverting input connected to the left axillary electrode. A needle electrode inserted through the caudal peduncle served to ground the fish.

Following recording of electrocardiograms, 2ml blood samples were removed from the caudal vein and serum parameters measured as on page 141.

Statistical analysis of data was carried out with the SPSS Fortran package program for the CDC 7600 computer (Nie, Hull, Jenkins, Steinbrenner & Bent 1975) or the conversion of SPSSG for the ICL 4130 computer (Bland, Lloyd-Jones & McGoldrick 1976).

V. 2.3 Results

ECG Changes

An example of a normal brown trout ECG is shown in Figure 100 and the parts of the ECG waveform labelled to indicate the portions measured in this investigation. Figure 101 gives a selection of ECG's recorded from uninfected brown trout (a,b,c) and, additionally, ECG's recorded from other species as a comparison. Figure 102 shows examples of ECG's recorded from brown trout infected with varying degrees of Saprolegniasis.

From inspection of the ECG records, it is evident that there are two obvious differences in the appearance of the ECG in infected fish compared to control fish. First, there is a marked increase in the duration of the QRS complex, (see figure 102). Second, there is often evident a secondary inflexion or notching of the RS section of the QRS complex (see figure 102a, b, c). No other changes were noticeable except that fish with heavy infections sometimes exhibited low heart-rates under anaesthesia.

Statistical Analysis

Tables 33 (a)(b) and (c) show the mean values for each of the parameters measured in control and infected fish. In addition, Table 33 (c) shows the percentage change recorded in the infected fish compared to the mean values recorded for the control fish. It can be seen that for

ECG parameters the greatest changes occur in mean QRS, T and TP durations. All plasma components have decreased and there is a notable decrease in magnesium levels, (less than 35% of control values) and all other plasma components are reduced to 80% or less of the control levels. There is a large element of variability in the ECG parameters as evidenced by their large S.E.'s; the plasma components in infected fish on the other hand, have low S.E.'s.

Table 34(a) shows the result of t-tests comparing the means between the infected and control fish. It can be seen that there are highly significant differences between the two groups for QRS wave durations and all serum parameters, particularly magnesium and sodium levels. No significant differences were found (Tables 34(b) and (c) between control males and females, or infected males and females.

In order to see whether any relationship existed between changes in ECG parameters and plasma component levels, regressions were performed and Pearson correlation coefficients calculated for each component/ECG parameter combination. The resultant correlation matrix is set out in Table 35. The pooled values for all fish (Table 35a) show strong negative correlations for QRS duration with magnesium, potassium and sodium, QRS duration increasing as the ion levels decrease. Similarly strong negative correlations are found of T with magnesium and sodium and TP with sodium. In the control fish (Table 35)

there are significant positive correlations of sodium and calcium with QRS, PR, ST, QT and CT. There are also positive correlations of TP with sodium and CT with magnesium and negative correlations of TP with potassium and HR with magnesium, calcium and sodium. This means that for positive correlations, the ECG duration decreases with increase in ionic values. An examination of values for infected fish (Table 35) only shows significant positive correlations of T and CT with calcium, total protein and albumen and negative correlations of HR with calcium and albumen.

Tables 36a and b show the result of t-tests between the data from this investigation and that found in fish analysed 1973/1974 (see also Richards & Pickering 1979) in order to show the validity of the plasma analyses. There are no marked differences except for total proteins and albumen in the control fish and a significant difference in the magnesium levels between the two groups of infected fish.

TABLE 33(a) Control fish plasma and ECG parameters. Values for plasma ion components are given in millimoles/litre, values for total proteins and albumen are in grams/litre and all ECG values are in milliseconds except for heart-rate (HR), measured in beats/min.

Fish	Sex	QRS	PR	ST	T	TP	CT	HR	QT	MG	CA	K	NA	Tot. Prot.	Alb.
1	M	62.67	227.33	367.33	335.00	100.00	961.00	56.55	430.00	0.80	2.30	2.10	160	48.0	24.0
2	M	64.67	208.33	306.67	316.00	83.30	863.33	63.38	371.33	0.82	2.28	2.00	158	44.0	21.0
3	M	37.33	167.33	371.67	283.30	67.00	840.97	66.08	409.00	0.68	2.21	2.30	153	37.0	20.0
4	F	72.67	274.67	505.33	366.66	96.60	1182.99	46.89	578.00	0.78	2.22	2.60	156	87.0	18.0
5	F	86.00	365.33	426.00	333.33	0.00	1167.66	51.38	512.00	0.82	2.35	2.20	160	41.0	20.0
6	F	84.67	287.33	420.00	116.67	100.00	866.34	62.09	504.67	0.71	2.32	2.30	160	40.0	21.0
7	M	46.67	266.00	484.67	273.33	383.30	1047.33	41.94	531.33	0.81	2.33	1.80	161	46.0	23.0
8	F	102.40	349.60	530.00	260.00	240.00	1190.80	41.93	632.40	0.85	2.64	1.70	165	44.0	24.0
9	F	100.0	304.80	656.00	340.00	220.00	1350.80	38.20	756.00	0.81	2.47	1.90	165	45.0	22.0
10	M	93.60	821.60	587.20	320.00	280.00	1275.60	38.57	680.80	0.82	2.60	2.00	165	52.0	27.0
11	F	132.00	353.67	576.00	380.00	300.00	1375.67	35.81	708.00	0.85	2.44	1.80	166	47.0	23.0
mean		80.242	284.182	475.533	302.21	170.02	1102.04	49.34	555.78	0.795	2.378	2.064	160.8	43.727	22.0
S.E.		8.185	19.209	32.420	21.66	36.19	59.482	3.351	38.35	0.016	0.043	0.081	1.256	1.402	0.74

TABLE 33(b) Infected fish plasma and ECG parameters. Values for plasma ion components are given in millimoles/litre, values for total proteins and albumen are in grams/litre and all ECG values are in milliseconds, except for heart-rate (HR), measured in beats/mins.

Fish	Sex	QRS	PR	ST	T	TP	CT	HR	QT	MG	CA	K	NA	Tot. Prot.	Alb.
12	F	174.00	459.67	703.33	533.33	2166.60	1783.33	15.19	877.33	0.31	0.16	0.90	116	12.0	4.0
13	F	200.67	174.67	201.333	383.33	30.00	859.66	67.44	402.00	0.18	1.32	0.80	125	16.0	6.0
14	M	131.33	294.00	366.67	599.98	799.90	1326.31	28.22	498.00	0.56	2.23	0.80	124	38.0	20.0
15	M	261.33	318.07	492.83	600.00	34.00	1541.57	38.08	754.17	0.23	2.25	0.80	143	44.0	18.0
16	M	259.33	439.33	274.67	933.30	1250.00	1776.97	19.82	534.00	0.18	2.31	2.40	117	43.0	22.0
17	F	109.33	228.00	483.33	333.33	300.00	1099.33	42.88	592.67	0.15	1.25	1.50	156	10.0	6.0
18	M	240.00	357.67	228.80	400.00	300.00	1106.47	42.66	468.80	0.20	1.71	1.10	130	21.0	12.0
19	F	124.67	271.33	290.00	466.66	333.00	1090.33	42.15	414.67	0.41	1.70	1.20	123	22.0	13.0
mean		187.583	317.842	380.121	531.24	651.69	1322.99	37.05	567.70	0.278	1.616	1.187	129.250	25.750	12.625
S.E.		21.932	34.786	60.076	67.28	260.22	121.85	5.77	59.45	0.050	0.254	0.194	4.847	4.909	2.442

TABLE 33(c) Fish plasma and ECG parameters of infected fish expressed as a percentage of the mean values of control fish in Table 1(a)

Fish	Sex	QRS	PR	ST	T	TP	CT	HR	QT	MG	CA	K	NA	Tot. Prot.	Alb.
12	F	216.84	161.75	147.90	176.48	1274.32	161.82	30.79	157.86	38.99	6.73	43.60	72.13	27.44	18.11
13	F	250.08	61.46	42.34	126.84	17.64	78.01	136.68	73.33	22.64	55.51	38.76	77.73	36.59	27.16
14	M	163.67	103.45	77.11	198.53	470.47	120.35	57.19	89.6	70.44	93.78	38.76	77.11	86.90	90.53
15	M	325.68	111.92	103.64	198.54	20.00	139.88	77.18	135.70	28.93	94.62	38.76	88.92	100.62	81.48
16	M	323.19	154.60	57.76	308.82	735.21	161.24	40.17	96.08	22.64	97.14	116.28	72.75	98.34	99.59
17	F	136.25	80.23	101.64	110.30	176.45	99.75	86.91	106.64	18.87	52.57	72.67	97.00	22.87	27.16
18	M	299.10	125.86	48.11	132.36	176.45	100.40	86.46	84.35	25.16	71.91	53.29	80.84	48.03	54.32
19	F	155.36	95.48	60.98	154.42	195.86	98.94	85.43	74.61	51.57	71.49	58.14	76.48	50.31	58.85
mean		233.771	111.844	79.935	175.79	383.30	120.05	75.09	102.15	34.97	67.96	57.51	80.37	58.89	57.15
S.E.		21.932	34.786	60.076	22.265	153.05	11.06	11.71	10.70	0.05	0.25	0.19	4.85	4.91	2.44

TABLE 34(a) Comparison between infected and control fish (sexes pooled) ECG and plasma components. Significant differences are denoted: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

	QRS	PR	ST	QT	T	TP	CT	HR	MG	CA	NA	K	Alb.	Tot. Prot.
t value	4.59	0.85	1.4	0.17	3.43	1.72	1.3	1.37	9.79	3.76	7.26	4.16	3.71	3.52
p	***	*			**				***	**	***	***	**	**
Mean (Control fish)	80.24	284.18	475.53	555.78	302.21	187.02	1102.04	49.35	0.8	2.38	160.82	2.06	22.09	43.73
S.E.	8.19	19.21	32.42	38.35	21.65	35.33	59.48	3.35	0.02	0.04	1.26	0.08	0.74	1.4
Mean (Infected fish)	187.58	317.84	380.12	567.7	530.94	435.27	1256.33	41.16	0.28	1.8	129.25	1.19	12.63	25.75
S.E.	21.93	34.79	60.08	59.45	77.7	166.84	102.59	4.96	0.05	0.15	4.85	0.19	2.44	4.91

Number of Cases (Control fish) = 11 except for Total Protein where (Control fish) = 10

(infected fish) = 8 " " (Infected fish) = 7

Table 34(b)

Comparison between male and female control fish : ECG and plasma components.

Significant differences are denoted :- * p <0.05, ** p < 0.01, *** p < 0.001.

	QRS	PR	ST	QT	T	TP	CT	HR	MG	Ca	NA	K	ALB	TP
t	2.76	2.76	1.54	1.87	0.14	0.30	1.77	1.06	0.50	0.70	1.02	0.27	1.10	1.06
Mean M	60.*99	238.*12	423.51	484.49	305.53	182.72	997.65	53.31	0.79	2.34	159.40	2.04	23.00	45.40
S.E. M	9.60	26.23	50.02	55.77	11.66	63.17	78.63	5.58	0.03	0.07	1.97	0.08	1.23	2.48
Mean F	96.29	322.57	518.89	615.18	299.44	159.43	1189.05	46.05	0.80	2.41	162.00	2.08	21.33	42.33
S.E. F	8.42	15.68	36.88	42.03	40.32	45.76	74.30	3.96	0.22	0.06	1.61	0.14	0.88	1.50

Number of Cases (Male fish) = 5, (Female fish) = 6 except for TP where (Male fish) = 5, (Female fish) = 5

Table 34(c)

Comparison between male and female infected fish : ECG and plasma components.

Significant differences are denoted :- * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

		QRS	PR	ST	QT	T	TP	CT	HR	MG	Ca	NA	K	ALB	TP
t		1.89	0.99	0.63	0.06	2.24 *	1.52	2.20 *	2.29 *	0.28	3.73 **	0.14	0.42	3.67 **	3.61 **
Mean	M	222.99	352.27	340.74	563.74	633.32	595.97	1437.82	32.19	0.29	2.13	128.50	1.27	18.00	36.50
S.E	M	30.93	31.85	58.24	64.86	110.55	269.68	143.76	5.10	0.09	0.14	5.52	0.38	2.16	5.33
Mean	F	152.17	283.42	419.49	571.66	295.83	165.75	1074.83	50.11	0.26	1.47	130.00	1.10	7.25	15.00
S.E.	F	21.25	61.98	111.43	110.79	102.37	87.51	80.53	5.92	0.06	0.11	8.88	0.16	1.97	2.65

Number of Cases (Male fish) = 4, (Female fish) = 4 except for TP & T where (Male fish) = 4, (Female fish) = 3

Table 35(a)

Correlation between ECG parameters and plasma components.

Pooled values for all fish.

r = Correlation coefficient
p = Probability value

		MG	CA	K	TP	ALB	NA
QRS	(r)	-0.794	-0.343	-0.554	-0.324	-0.398	-0.687
	(p)	0.001	0.075	0.007	0.086	0.046	0.001
PR	(r)	-0.142	0.197	-0.069	-0.039	-0.396	-0.289
	(p)	0.281	0.210	0.389	0.436	0.465	0.115
ST	(r)	-0.377	0.326	-0.088	-0.199	-0.143	-0.423
	(p)	0.056	0.087	0.359	0.207	0.279	0.035
QT	(r)	-0.015	0.156	-0.185	0.039	-0.053	0.084
	(p)	0.476	0.262	0.224	0.437	0.415	0.366
T	(r)	-0.561	-0.064	-0.225	-0.011	-0.059	-0.723
	(p)	0.008	0.400	0.185	0.483	0.408	0.001
TP	(r)	-0.286	0.101	0.028	0.076	0.154	-0.529
	(p)	0.133	0.349	0.457	0.386	0.278	0.015
CT	(r)	-0.238	0.275	-0.080	0.238	0.183	-0.255
	(p)	0.163	0.127	0.372	0.163	0.226	0.146
HR	(r)	0.178	-0.234	0.131	-0.191	-0.213	0.225
	(p)	0.233	0.168	0.297	0.217	0.191	0.177

TABLE 35(b)

Correlation between ECG parameters and plasma components (Control fish)

		MG	CA	K	TP	ALB	NA
QRS	(r)	0.583	0.676	-0.396	0.367	0.313	0.809
	(p)	0.030	0.011	0.114	0.134	0.174	0.001
PR	(r)	0.627	0.690	-0.357	0.302	0.278	0.762
	(p)	0.019	0.009	0.141	0.184	0.204	0.003
ST	(r)	0.420	0.673	-0.369	0.370	0.367	0.734
	(p)	0.099	0.012	0.132	0.131	0.134	0.005
QT	(r)	0.480	0.713	-0.397	0.391	0.377	0.793
	(p)	0.068	0.007	0.113	0.117	0.127	0.002
T	(r)	0.537	0.015	-0.073	0.245	0.005	0.117
	(p)	0.044	0.482	0.416	0.234	0.494	0.365
TP	(r)	0.609	0.604	-0.732	0.617	0.597	0.721
	(p)	0.031	0.032	0.008	0.029	0.034	0.009
CT	(r)	0.667	0.642	-0.370	0.413	0.313	0.745
	(p)	0.012	0.017	0.131	0.103	0.174	0.004
HR	(r)	-0.706	-0.690	0.535	-0.544	-0.473	-0.801
	(p)	0.008	0.009	0.045	0.042	0.071	0.002

TABLE 35(c)

Correlation between ECG parameters and plasma components (Infected fish)

		MG	CA	K	TP	ALB	NA
QRS	(r)	-0.494	0.474	0.207	0.526	0.382	-0.185
	(p)	0.107	0.117	0.312	0.090	0.175	0.330
PR	(r)	0.022	0.492	0.368	0.278	0.261	0.499
	(p)	0.479	0.108	0.185	0.253	0.266	0.104
ST	(r)	0.093	-0.044	-0.223	-0.194	-0.330	0.160
	(p)	0.414	0.459	0.298	0.323	0.213	0.353
QT	(r)	-0.089	0.131	-0.149	-0.001	-0.192	0.093
	(p)	0.417	0.379	0.362	0.499	0.324	0.414
T	(r)	0.096	0.843	0.616	0.853	0.881	-0.517
	(p)	0.419	0.009	0.070	0.007	0.004	0.117
TP	(r)	0.237	0.578	0.735	0.503	0.692	-0.520
	(p)	0.304	0.087	0.030	0.125	0.043	0.116
CT	(r)	0.012	0.857	0.548	0.809	0.762	-0.155
	(p)	0.488	0.003	0.080	0.008	0.014	0.357
HR	(r)	-0.284	-0.799	-0.583	-0.713	-0.822	0.093
	(p)	0.248	0.009	0.065	0.024	0.006	0.413

Table 36 (a)

Comparison of blood values (ECG fish) with data from blood value work (section VI). Significant differences are denoted :- *p<0.05, ** p<0.01, *** p<0.001.

Comparison of control fish (both sexes)

	MG	CA	NA	K	ALB	TOT. PROT.
Mean (ECG fish)	0.80	2.38	160.82	2.06	22.09	43.73
S.E. (ECG fish)	0.02	0.04	1.26	0.08	0.75	1.40
Mean (Blood fish)	0.81	2.28	164.00	2.06	33.05	68.03
S.E. (Blood fish)	0.01	0.06	0.99	0.06	1.55	3.21
t-value	0.90	1.24	1.99	.090	6.38	5.43
p					***	***

Table 36(b)

Comparison of blood values (ECG fish) with data from blood value work (section VI).
Significant differences are denoted :- *p<0.05, **p<0.01, ***p<0.001.

Comparison of infected fish (both sexes).

	MG	CA	NA	K	ALB	TOT. PROT.
Mean (ECG fish)	0.28	1.80	129.25	1.19	12.63	25.75
S.E. (ECG fish)	0.05	0.15	4.85	0.19	2.44	4.90
Mean (Blood fish)	0.49	1.56	133.04	1.20	12.60	34.69
S.E. (Blood fish)	0.05	0.09	3.80	0.17	2.90	4.05
t-value	2.97	1.32	0.62	0.03	0.01	1.40
p	**					

V 2.4 Discussion

The results show that decreasing ionic and protein levels in the blood of Saprolegnia-infected brown trout (which have been shown to correspond with the percentage of skin surface area affected) are related to alterations in electrocardiogram patterns. These changes in gross ECG morphology are first, a marked increase in QRS duration and second, a notching of the RS component. An increase in duration of the QRS component represents an increased ventricular depolarisation time and is found in a variety of conditions in man and animals. Widening of the QRS complex occurs in hyperkalaemia in man as a result of slowed conduction caused by both decreased rate of rise and decreased amplitude of the cardiac muscle action potential. QRS widening also occurs in hypokalaemia, but in this case it occurs because of decremental conduction, the rate of repolarisation also being slowed. Hypocalcaemia increases the duration of the whole QT component because of increased duration of the plateau phase of the cardiac muscle action potential.

Barnes, Davis & McKay (1938) and Sykes & Alfredson (1940) have also showed that a doubling of QRS duration occurs in calves fed on low potassium rations and exhibiting a resultant decreased serum potassium level. Sykes & Alfredson (1940) noted that these changes were very similar to those in men and dogs suffering left bundle-branch block, but they showed that section of the left bundle-branch in calves did not

produce similar effects. Thomas, Mylon and Winternitz (1940) also related a variety of changes in the ECG to diets deficient in potassium linked with a parallel B_6 deficiency in pigs. Myocardial infarction changes led to T wave reversal and an upward displacement and convexity of the RST component, but the QRS complex was widened and also notched suggestive of intraventricular block.

Magnesium deficiency in animals leads to a variety of heart changes. Moore, Sholl & Hallman (1936) and Greenberg, Anderson & Tufts (1936, cited by Moore, Hallman & Sholl (1938)) working on calves and rats respectively, showed that myocardial necrosis and the development of cellular infiltrations in the myocardium was a common effect of dietary magnesium deficiency. Moore, et al (1938) showed that the primary effect of dietary magnesium deficiency on the circulatory system of calves was a calcification of certain elastic fibres and elastic membranes in vessel walls, leading to extensive calcium plaque formation in both heart and larger vessels, but ECG records were not done in this study. This work was, however, carried out on calves fed deficient rations over a five month period and so represents a chronic, rather than an acute, syndrome.

Tufts & Greenberg (1936) and Cowey, Knox, Adron, George & Pirie (1977) in rats and rainbow trout respectively have shown that diets low in magnesium and high in calcium lead to kidney calcification. The latter fish work did not discuss any heart changes and once more, both experiments were long-term dietary trials.

Exhaustion of the heart in long lasting experiments has also been shown to result in widening of the QRS complex (Kisch, 1948). The notching of the RS segment doubtless represents asynchrony of ventricular depolarisation. As fast conducting pathways in the trout heart lead to the apex of the ventricle from the atrium and the left side of the ventricle becomes depolarised just before the right (Randall, 1970) it is interesting to speculate that disturbance of conduction of the wave of depolarisation to different parts of the heart may occur in fish examined in the present study leading to notching of the RS segment.

Examination of the results of statistical analysis of ionic and electrocardiogram changes reveals several interesting points. In control fish, most ECG intervals are increased whilst heart-rate is reduced when decreases in ionic concentration occur. This is especially true of variations in sodium and calcium level. Such correlations are not so obvious in infected fish and the correlations which do occur seem particularly due to two heavily infected female fish (see below). Standard errors, especially for the ECG measurements are much larger than in the controls and this will obviously lead to a greater variance and thus less likelihood of significant correlations. This has probably occurred because although all fish were classified as "infected" the level of infection was very variable from light infection involving only a small percentage surface skin area to extensive infection with the fish appearing moribund.

A comparison between infected and control fish of ion and ECG shows highly significant differences in ion levels and in QRS and T intervals (Table 34a) and no sex difference was evident (Tables 34b and c) with the exception in particular of calcium, albumen and total protein levels in the infected fish. However if the original data are examined, it can be seen that this is particularly due to the contributions from fish 12 and 17, females with particularly low values for these parameters. The effect of these two fish is particularly noticeable because of the low number of cases in the female group (only 4). Otherwise there do not seem to be any major sexual differences, though continuation of this work with a much larger number of samples will be essential to prove this suggestion adequately.

There was little variation in ion values between control fish from the ECG work and those used for the work comparing ion and protein level with percentage area infected (Section V(1)). Fish caught during the same period of the year were used for the comparison, but the fish were actually caught in different years. There is however a highly significant variation in the serum protein levels. Changes of serum protein level in normal fish have previously been associated with age and season in the rainbow trout (Sano 1960, Haider 1971, Denton & Yousef 1975) though seasonal effects have been contradictory (cf. Haider 1971 with Denton & Yousef 1975). Alexander (1977) found a similar variation in protein

concentration in the Atlantic salmon (Salmo salar L) between 1967 and 1975. Although sex and seasonal variations were apparent, the mean protein concentration increased in both sexes over this period to a value of more than twice that of the earlier figures. Denton & Yousef (1975) suggested that diet, metabolic variation and activity may have been the causes of such variation. Sexes were pooled in the comparison carried out in the present work as no sex difference was evident in either group of fish.

A statistical difference between the infected ECG fish and the infected fish in Section V(1) was only evident in magnesium levels. This is perhaps surprising as one might have expected considerable variation in levels due to differences in the extent of infection. It is not easy to suggest a reason for variation only in magnesium levels, although magnesium and calcium levels in particular do fall after prolonged storage (Watanabe, Shimizu & Yamada 1978).

The major problems associated with the interpretation of the relationship of ionic changes to ECG parameters in fungal-infected fish are really twofold. First, variation is occurring in almost every blood parameter examined. Many of these variables do themselves interact ionically, e.g. sodium with potassium and calcium with magnesium and it is difficult to assess the contribution provided by any single parameter. The classic experimental work that has been carried out in animal electro-

cardiography has utilised changes in only one parameter at a time though clinical conditions in man, such as chronic diarrhoea, extensive burns or surgical shock, may lead to loss of varying quantities of different ions. Even in such cases, however, electrocardiographic variations can usually be linked to loss of particular ions. For instance surgical shock, dehydration, severe burns and low sodium diets all lead to tall peaked T waves in the electrocardiogram. These were thought originally to be due to high potassium levels. On the other hand, excessive diarrhoea and the action of certain diuretics often lead to a prolonged QT interval due to the production of low broad T waves, associated with hypokalaemia. The effects of variation in calcium level are seen in association with variation in parathyroid function in man (Rollason 1975). Hypoparathyroidism leads to hypocalcaemia and a prolonged QT interval while hyperparathyroidism results in hypercalcaemia and a shortened QT interval and as in the present work, QT interval varies inversely with calcium concentration. In order to study the precise effects of individual ion variation on the ECG of fish, perfusion studies would probably be necessary.

The second main problem in interpreting ion and ECG variations is that <u>serum</u> levels only are being measured. Changes in the cardiac cell are caused by differences in transmembrane voltage and this is maintained by ionic differences between the inside and outside of the cell. The most important ions involved in this mechanism have been shown to be potassium, sodium and calcium. With

the exception of potassium, this would seem to be the case in the present work. No measurement has been made of intracellular ion concentration and this would really be necessary to demonstrate the ionic fluxes occurring.

The work carried out has however, shown that particular changes in the electrocardiogram do occur in association with fungal skin infection and that these may be linked to loss of electrolytes and protein from the skin leading to depletion in the blood.

VI Androgen Studies

VI. 1 Introduction

Hyperadrenocorticism was considered by several workers to be the direct cause of many of the degenerative tissue changes occurring in spawning Pacific salmon (Hane & Robertson 1959; Robertson & Wexler 1959, 1960, 1961; Robertson, Krupp, Favour, Hane & Thomas 1961a; Robertson, Krupp, Thomas, Favour, Hane & Wexler 1961b; Robertson, Hane, Wexler & Rinfret 1963) which ultimately led to the death of the fish, following reproduction, but gonadectomy of sockeye salmon (O. nerka) at an advanced state of sexual maturation prolonged their life-span well beyond the time of natural reproduction and death (McBride, Fagerlund, Smith & Tomlinson 1963,1965;McBride & van Overbeeke 1969a, b). It led to the loss of secondary sexual characteristics and halted, or partially reversed, the degenerative tissue changes found in spawning fish. In particular, hypertrophy of interrenal tissue was prevented or reversed (McBride & van Overbeeke 1969b; van Overbeeke & McBride 1971) and the usual increase in volume of distribution and metabolic clearance rate of radioactivity after injection of a tracer dose of $4^{-14}C$ cortisol was prevented (Donaldson & Fagerlund 1968a, b;1970 1972). It was suggested that hypercorticism at sexual maturation in Pacific salmon was caused by gonadal hormones rather than excessive metabolic demands at this time (Hane, Robertson, Wexler & Krupp 1966). Indeed, administration of androgens to immature or gonadectomised fish resulted in increased physiological activity of andreno-

cortical hormones (Donaldson & Fagerlund 1969; Fagerlund & Donaldson 1969) as well as hyperplasia and hypertrophy of interrenal tissue (van Overbeeke & McBride 1971). The absence of a direct response of pituitary corticotrophs to gonadectomy (McBride & van Overbeeke 1969b) and the lack of response of these same cells to injection of gonadal steroids (van Overbeeke & McBride 1971) led these workers to believe that the effect was a direct one and not mediated via ACTH produced from the pituitary gland.

The hyperplasia occurring in the skin during the spawning period has been reproduced by injection of androgens and oestrogens, but not by cortisol (Idler, Bitners & Schmidt 1961; McBride & van Overbeeke 1971; Yamazaki 1972) or hydrocortisone (Robertson et al 1963). Most of the other hyperplastic or degenerative organ changes may be reproduced with either androgens, oestrogens or cortisol (McBride and van Overbeeke 1971; van Overbeeke & McBride 1971). The majority of work carried out in fish has utilised testosterone or 17α methyl testosterone on intact or gonadectomised fish in order to study the effect of androgens (e.g. Donaldson & Fagerlund 1969; van Overbeeke & McBride 1971; McBride & van Overbeeke 1971). Since the discovery that 11 keto testosterone is a potent fish androgen (Idler, Schmidt & Ronald 1960) work has also involved the use of this compound (van Overbeeke & McBride 1971; McBride & van Overbeeke 1971).

A large body of evidence, reviewed by King & Mainwaring (1974) suggested that in mammals, testosterone was converted

to 5α dihydrotestosterone in the skin and other target organs and that this was the biologically active metabolite. 5α reductase was thought to be the enzyme involved in this conversion and Hay, Hodgins & Roberts (1974 & 1976) have shown that this enzyme is present in appreciable quantities in fish skin.

As little work had previously been carried out on the brown trout (Salmo trutta L.) it was decided to investigate the effects of 17α methyltestosterone on intact and gonadectomised fish to confirm that similar changes occurred in this species as in other salmonids and to determine whether or not such changes were representative of those associated with sexual maturity in this species. As 11 keto-testosterone had been shown to be a potent androgen in a variety of salmonids this was also utilised.

Although evidence from enzyme studies had suggested that 5α dihydrotestosterone may have been the metabolically active metabolite in fish skin, the effects of injection of this compound directly into fish had not been investigated so this was therefore also examined.

VI. 2 Materials and Methods

A series of experiments was carried out in order to determine the effect of the androgens on fish organs, in particular the skin. Brown trout (Salmo trutta L.) weighing between 400g and 550g were used throughout these studies and were obtained from Howietoun & Northern Fisheries Ltd., Bannockburn. The original source of broodstock fish for the brown trout used in this work was Loch Leven, Kinross. The fish were maintained at Howietoun & Northern Fisheries in the University's Home Office licensed facility in six foot square tanks with continual river water flow and natural photoperiod (Fig.103).

Experiment 1

The first experiment investigated the effect of intramuscular injection of three different concentrations of 17α methyl testosterone over a period of four weeks between 1.5.73 and 29.5.73. It was not possible to distinguish between the sexes of the fish at the start of the experiment as fish were immature, but histological examination of gonad at the end of the experiment allowed sex determination to be carried out. One fish became mature and died during the course of the experiment and is noted as such in the results section. Nineteen fish were used in this experiment. Three fish were killed as controls at the start of the experiment and four fish (one fish from each experimental group) were then killed weekly. Three testosterone solutions were made up in arachis oil at concentrations of 1000 µg/ml oil, 400 µg/ml oil, 200 µg/ml oil and pure arachis oil was used as a

control. The fins of fish in respective groups were clipped for identification purposes. Fish were injected intramuscularly using a 23 gauge needle and syringe at a dose-rate of ½ cc solution/100 gm weight, following anaesthetisation of fish in a 1:15,000 solution of MS222 (tricaine methane sulphonate) - Sandoz pharmaceuticals. Fish were injected three times weekly, hormone suspensions being freshly made up on a daily basis. An ultrasonicator at 14 microns was used to provide a fine, even suspension of the hormone in the oil.

During the course of the experiment it was found that a certain amount of the oil suspension was squeezed out of the site by muscular contraction of the fish during swimming. In order to minimise hormone loss from this source, 0.25 cc of oil were drawn into the syringe prior to the hormone suspension so that when the total syringe volume was injected intramuscularly, the first part of the injection aliquot expressed would be oil only.

Because the amounts of oil lost in this way varied considerably, however, an experiment was designed to try out different methods of administration of the hormone (vide infra). Cutaneous fungal infections with Saprolegnia developed in a number of fish during the course of the experiment, particularly those on the highest hormone dosage levels and it was found necessary to treat the infection by a flush treatment with malachite green (Koch-Light labs) at a concentration of 2 ppm for one hour. All fish were treated in the same manner.

For sampling purposes, fish were killed by decapitation following anaesthetisation in MS222, and small blocks of tissue approximately 1cm × ½cm × ½cm were excised and fixed in 10% neutral buffered formalin, processed as in Appendix 1, paraffin wax imbedded and sections cut at 5μ, and stained by Haematoxylin and Eosin (Appendix 1).

Sections were taken from liver, spleen, mid-kidney, anterior kidney, gut, gill, pituitary gland, heart & skin. Skin sections were always taken from both sides of the fish at a point mid-way between the lateral line and the dorsal fin. Epidermal and dermal thickness, together with mucous cell parameters of skin sections were measured as for the normal Loch Leven fish.

Experiment 2

Brown trout were again obtained from Howietoun & Northern Fisheries. In this experiment three routes of administration of 17α methyl testosterone were used with two different dosage rates as in Table 39. The experiment was carried out between January and March 1976. Because of colder temperatures prevailing at that time, injections were made through a 19 gauge needle because of increased viscosity of the oil at low temperatures and difficulties in administration. Intraperitoneal injections were made at a point midway between the lateral line and the ventral mid-line and at a point just posterior to the insertion of the pelvic fins. Fish were injected twice weekly. Fish were again anaesthetised and weighed prior to injection.

In the feeding trial used, two levels of hormone were administered to the test fish. The required quantity of hormone was mixed into a small volume of slurried trout food (approximately 3 gm), the required amount being prepared fresh daily. This was then drawn into a length of 3 mm plastic tubing and attached to a syringe containing water. The fish were then anaesthetised and the food given by stomach tube, the water in the syringe flushing out any remaining food. The fish were observed during recovery to ensure that no regurgitation of food took place. Food was administered twice weekly in this way, fish being fed on normal pelletted food according to feeding tables (Table 37) during the remainder of the time. Fish were sacrificed according to the time-scale in the Tables and histological sections of major organs prepared as in Experiment 1.

During the period of the experiment, many fish suffered quite heavy fungal infections and malachite green treatment was required frequently. Whenever used, the treatment was applied equally to all groups. Many male fish also became mature during the course of the experiment (marked MM in the tables).

Experiment 3

(a) Gonadectomy

Because of the variable state of sex and maturity of experimental fish in Experiment 2, it was decided to carry out future experiments using castrated fish.

TABLE 37 Manufacturer's Feeding Tables for Experimental Fish

Number of trout per kilogram (2,2 lbs)										
5000	2000	713	211	89	50	27	17	11	7,5	< 5,5
2000	713	211	89	50	27	17	11	7,5	5,5	
Weight of trout in grams										
0,2-0,5	0,5-1,4	1,4-4,9	4,9-11,2	11,2-20	20 - 60		60 - 135		135 - 300	
Length in centimeters (0,4 inch)										
2,5	3,5	5	7,5	10	12,5	15	17,5	20	22,5	> 25
3,5	5	7,5	10	12,5	15	17,5	20	22,5	25	

Water		Daily quantity of food as a percentage of liveweight									
°C	°F										
4	39,2	2,6	2,4	2,1	1,8	1,5	1,3	1,1	0,9	0,8	0,7	0,6
6	42,8	3,0	2,8	2,5	2,1	1,7	1,5	1,3	1,1	1,0	0,9	0,8
8	46,4	3,5	3,3	2,9	2,4	1,9	1,7	1,5	1,3	1,2	1,1	1,0
10	50,0	4,1	3,9	3,4	2,8	2,2	2,0	1,7	1,5	1,4	1,3	1,2
12	53,6	4,8	4,6	4,0	3,2	2,5	2,3	2,0	1,7	1,6	1,5	1,4
14	57,2	5,6	5,4	4,6	3,7	2,9	2,6	2,3	2,0	1,8	1,7	1,6
16	60,8	6,5	6,3	5,3	4,3	3,5	3,0	2,6	2,3	2,1	2,0	1,9
18	64,4	4,6	4,4	3,7	3,0	2,4	2,2	1,9	1,6	1,5	1,4	1,3
20	68,0	2,8	2,6	2,3	2,0	1,6	1,4	1,2	1,0	0,9	0,8	0,7

Type of food	Starter Crumbs	Crumb No. 0 1,2 mm	Crumb No. 1 1,7 mm	Crumb No. II 2,75 mm	Pellet No. III 3,5 mm	Pellet No. IV 5 mm

The method of castration was similar to that described by McBride, Fagerlund, Smith & Tomlinson (1963) and is redescribed by Brown & Richards (1979). Fish were maintained under MS222 anaesthesia (1:10,000) during the course of the operation using the apparatus in Figure 99. The fish were placed in dorsal recumbency and when anaesthesia was complete, a ventral mid-line incision was made from a point anterior to the pelvic fins up to the insertion of the pectoral fins.

Retractors were used to hold open the abdomen and a pair of forceps used to remove gonadal elements by simple retraction at the origins and insertions. This method had been found previously to result in complete removal of gonadal elements (as evidenced by a complete lack of regeneration) and furthermore prevented all but a very minor blood loss (presumably by elastic recoil in blood vessels). Previous attempts at castration which used scissors or scalpel to remove gonadal elements resulted in excessive bleeding and attempts at tying off such vessels proved impractical in small fish of 150 gm.

The wounds in abdominal musculature were closed using a variety of stitching techniques - horizontal mattress or continuous lockstitch proved most effective. It was however found that a lockstitch gave the quickest result and was perfectly satisfactory. No. 3 catgut and a 15Δ half curved needle were found to give best results. Stitches were not removed manually, but disintegrated after a period of a week or so, at which time

healing was well advanced and complete by about four weeks post-operation (see Figures 104 and 105). A stock of gonadectomised fish were established in this way during Spring 1975. The original sex of the fish was determined by clipping the adipose fin in female fish and all fish were freeze-branded (Figure 105).

This stock of fish was then used for Experiment 3 in 1976.

Experiment 3

(b) Hormone Administration

This experiment was used to compare the effects of three androgens - (a) 17α cH_3 testosterone; (b) methyl dihydro testosterone; and (c) 4-androsten-17β-ol-3, 11-Dione (11 keto-testosterone).

Fish were freeze branded at least a week prior to the start of an experiment using liquid nitrogen and the apparatus in Figure 106 after the method of Laird, Roberts, Shearer & McArdle (1975). Brands were placed on the "shoulder" and lower abdominal areas and were easily visible at the start of the experiments (Figure 105). Thirty fish were used for the first part of the trial comparing (a) and (b), five groups of fish were used as in Table 40, with six fish in each group. The required dose of the hormone was administered intraperitoneally in oil twice weekly, and two fish of each group were sacrificed at one week, three weeks and six weeks and organs taken for histological work as in previous experiments. At time of sampling, confirmation of the absence of gonadal regeneration was made and the sex of the fish

(determined by adipose fin clipping) prior to castration was noted. This first part of the work was carried out between 18.1.76 and 8.3.76. On 15.11.76 the third hormone, 11 keto testosterone, of which only a small quantity was available, was tested similarly, but in this case only ten fish were used.

Table 40 shows the dosages used in individual fish.

This work was actually first carried out during the summer of 1975 on a larger number of fish, but no results were obtained, as the experimental fish were killed following pollution of the water source. The subsequent experiment reported here utilised the remaining stock of gonadectomised fish.

VI 3 Results

(a) Skin

The results of measurement of epidermal thickness of skin sections from experimental fish in Experiments 1, 2 and 3 are given in Tables 38, 39 and 40.

Experiment 1(a) examined the use of differing dose-rates of 17α CH_3 testosterone given by the intramuscular route. Fish of both sexes were used as it was not possible to distinguish between sexes at the start of the experiment. Sex was subsequently determined at the termination of each experimental treatment. Cutaneous fungal infection was a problem throughout the experiment and several malachite green treatments proved necessary to control fungal spread. Even with the use of such treatment, two fish died during the course of the experiment; both fish were sexually mature male fish and unfortunately it was not possible to carry out worthwhile histological examinations on the carcases as advanced post-mortem degeneration had occurred prior to the deaths being discovered.

Epidermal thickness in the control fish remained relatively constant over the four week experimental period. Skin thickness changes in the remaining fish were variable though distinct thickening occurred in the fish receiving the 200 µg/ml solution over three weeks and fish receiving the 400 µg/ml solution over two and three weeks. Little thickening occurred at the greatest hormone concentration used.

Experiment 2 examined three different routes of administration of 17α CH_3 testosterone and two differing dose-rates by the injectable routes. Control fish showed slight skin thickening, especially following prolonged treatment (7 weeks). Three male fish became mature during the course of the experiment and results from these fish have not been considered as representative of the non-mature fish. By the intramuscular route, little effect was apparent with the low dosage regime until the final sampling at seven weeks. Thickening was seen after only a week with the higher dosage used, though no further increase appeared to take place until the seven week sample. Both dose-rates evoked similar thickening after seven weeks.

Use of the intraperitoneal route gave similar results at both dose-rates, though the one week results are difficult to interpret. Thickening occurred at two weeks and was equivalent to that obtained by intramuscular injection by seven weeks.

No effect of administration by the oral route was seen until the seventh week.

Experiment 3 utilised gonadectomised fish. Survival following surgical removal of gonads proved to be 98%. The only real problem encountered was one of peritonitis in a small number of fish.

Because of losses encountered following silt damage in the original stock of gonadectomised fish, a time-

interval elapsed between administering the first two hormones and 11 keto testosterone. Also, because of the great difficulty in obtaining adequate supplies of 11 keto testosterone, only ten fish were used in testing the latter compound.

Intraperitoneal inoculation was utilised because from the previous trial (Experiment 2), it did appear to produce a standardised effect on the skin and it also seemed to be the most reliable method of administering a known quantity of hormone.

The larger of the two dose-rates was double that used in the previous experiment in order to determine whether the increase would lead to either more rapid onset of epidermal thickening or greater overall thickness of epidermis. Because of the small number of fish receiving 11 keto testosterone, only two samples were taken, after three and six weeks.

After only one week, skin thickening was apparent with both concentrations of 17α CH_3 testosterone and 17α CH_3 dihydrotestosterone and in each case, larger concentrations gave the most marked thickening. Fish receiving the 17α CH_3 dihydro testosterone showed the greatest thickness at this stage. Fish sampled at three weeks showed slight increases in skin thickness over the one week sample, most marked in the low-dose 17α CH_3 dihydro testosterone. At six weeks results were variable, all skins showing thickening when compared with controls, but with considerable individual variation between samples

TABLE 38

The Effects on Epidermal Thickness of the Administration of 17α CH_3 testosterone at three dose-rates by the intramuscular route.

Time of Sampling	Arachis Oil	100µg/100g fish	200µg/100g fish	500µg/100g fish
Day 1	94.4			
	119.7			
	77.6			
1 week	95.8	102.6	116.3	104.0
2 weeks	88.9	93.0	268.1	117.0
3 weeks	82.1	207.2	165.5	MM
4 weeks	102.6	131.3	MM	120.0

All measurements are in microns (µ)

MM = Mature male fish

TABLE 39 The effect on epidermal thickness of the administration of 17α CH_3 testosterone by three different routes.

Time of Sampling	1/m Oil	1/m 5	1/m 10	1/p Oil	1/p 5	1/p 10	Oral Oil	Oral 10
1 week	82.1	85.5	126.5	78.7	109.4	123.1	86.2	96.8
	88.9	53.0	MM	90.6	MM	95.8	81.4	MM
2 weeks	95.8	85.5	126.5	97.5	140.2	126.5	91.7	95.8
								78.7 (11 days)
	78.7	MM	123.1	99.2	128.2	119.7	100.5	92.3
7 weeks	119.7	249.7	229.1	130.0	225.7	218.9	136.8	225.7
								249.7
	123.1	236.0	236.0	109.4	246.2	232.6	132.0	208.6

All measurements are in microns (μ)

MM = Mature male fish

TABLE 40 The effects on epidermal thickness of the administration of three hormones by the intraperitoneal route

Time of Sampling	Arachis Oil (Jan 76)	17α CH_3 testosterone 5	17α CH_3 testosterone 20	17α CH_3 dihydrotestosterone 5	17α CH_3 dihydrotestosterone 20	Arachis Oil (Nov 76)	11 ketotestosterone 5	11 ketotestosterone 20
1 week	102.6	135.1	160.7	126.5	188.1			
	114.6	133.4	148.8	150.5	174.4			
3 weeks	95.8	147.7	162.4	177.8	198.4	95.8	136.8	188.1
	116.3	143.0	168.9	167.6	121.4	82.1		
6 weeks	106.0	195.0	147.1	229.1	171	88.9	273.6	273.6
	109.4	222.3	188.1	155.6	198.4	75.2	205.2	171.0
	116.3	205.2						
	126.5							

All measurements are in microns (μ)

on the same treatment regime. No marked differences were evident between fish of different sex prior to gonadectomy and little difference was ultimately evident at six weeks between the first two hormones or the two dose-rates.

Fish receiving 11 keto testosterone again showed epidermal thickening, and this ultimately (at 6 weeks) became greater in some individual samples than with the other two hormones. These fish were however, used at a different time of year when temperature, day-length etc. were different and so care must be taken in drawing comparisons, especially with such low numbers of fish.

Further analysis of skin parameters was carried out on samples from fish used in all three experiments. Stratum compactum thickness, mucous cell diameter and mucous cell number per unit area were measured and are represented in tables 41 to 49.

17α methyl testosterone administered by different routes produced little change in dermis thickness and individual differences were noticeable in samples under the same treatment regime. No attempt to statistically compare such values was made because of the small number of samples. Similarly a comparison of the three hormones did not show any constant change, although hormone-treated fish did show an increased dermis thickness compared to controls on certain occasions, especially after three and six weeks in Experiment 3. There was

TABLE 41

The effect on stratum compactum thickness of administration of 17α CH_3 testosterone at 3 dose-rates by the intramuscular route.

Time of Sampling	Arachis Oil	100µg/100g fish	200µg/100g fish	500µg/100g fish
Day 1	332.5			
	361.6			
	410.2			
1 week	356.6	443.2	341.9	382.9
2 weeks	386.5	298.4	306.8	369.4
3 weeks	292.4	364.8	299.1	284.2
4 weeks	342.3	344.4	382.4	264.6

All measurements are in microns (µ)

TABLE 42 The effect on stratum compactum thickness of administration of 17α CH_3 testosterone by three different routes

Time of Sampling	1/m Oil	1/m 5	1/m 10	1/p Oil	1/p 5	1/p 10	Oral Oil	Oral 10	
1 week	400.2	343.6	365.7	349.1	393.3	365.7	322.9	289.8	
	295.3	258.1	MM	418.1	MM	356.0	367.1	MM	
2 weeks	379.5	277.4	365.7	374.0	438.8	356.0	350.5	378.1	
								173.9	(11 days)
	338.1	MM	379.5	313.3	387.8	265.0	369.8	430.6	
7 weeks	356.0	389.2	362.9	458.2	419.5	437.5	353.3	443.0	
								470.6	
	310.5	369.8	383.6	368.4	455.4	270.5	342.2	310.5	

All measurements are in microns (μ)

TABLE 43 The effects on stratum compactum thickness of the administration of three hormones by the intraperitoneal route

Time of Sampling	Oil (Jan 76)	17α CH_3 testosterone 5	17α CH_3 testosterone 20	17α CH_3 dihydrotestosterone 5	17α CH_3 dihydrotestosterone 20	Oil (Nov 76)	11 ketotestosterone 5	11 ketotestosterone 20
1 week	343.6	288.4	296.7	313.3	400.2			
	321.5	278.8	368.5	285.6	404.3			
3 weeks	347.8	339.5	328.4	581.0	400.2	350.5	524.4	499.6
	362.9	383.6	357.4	509.2	306.4	361.6		
6 weeks	347.8	706.6	407.1	587.9	425.0	378.1	477.5	408.5
	382.3	520.3	502.3	527.2	447.1	346.4	447.1	426.4
	357.4		448.5					
	412.6							

All measurements are in microns (μ)

TABLE 44

The Effects on mucous cell counts of the administration of 17α CH_3 testosterone at 3 dose-rates by the intramuscular route.

Time of Sampling	Arachis Oil	100µg/100g fish	200µg/100g fish	500µg/100g fish
Day 1	2.61			
	3.12			
	2.80			
1 week	2.56	3.24	2.94	3.02
2 weeks	3.46	4.06	3.24	2.87
3 weeks	2.82	2.91	2.80	3.42
4 weeks	3.06	4.26	2.64	2.80

TABLE 45 The effect on mucous cell counts of administration of 17α CH_3 testosterone by three different routes

Time of Sampling	Arachis Oil 1/m	1/m 5	1/m 10	1/p 5	1/p 10	Arachis oil 1/p	Food 10	Food Oil
1 week	2.59	4.13	2.57	4.47	4.37	2.11	4.25	2.42
	3.6	6.25	MM	MM	4.28	3.2	MM	2.86
2 weeks	3.72	3.24	3.75	3.04	3.89	4.01	3.5 (11 days)	3.26
	2.82	MM	2.71	3.28	4.07	2.2	4.33	2.9
							5.55	
7 weeks	2.8	4.49	2.56	3.64	3.41	3.23	4.12	3.0
	2.46	2.62	3.25	2.91	1.91	3.91	3.26	2.86
							2.51	

1/m etc. = intramuscular, intraperitoneal

TABLE 46 The effect on mucous cell counts of administration of three androgens by the i/p route at two different concentrations

Time of Sampling	Arachis Oil	17αCH_3 Testosterone 5	17αCH_3 Testosterone 20	17α CH_3 dihydrotestosterone 5	17α CH_3 dihydrotestosterone 20	11 ketotestosterone 5	11 ketotestosterone 20	Oil
1 week	4.33	4.15	3.31	3.51	2.9			
	3.65	4.09	3.1	3.09	3.33			
3 weeks	3.82	3.96	3.25	2.47	3.15	2.04	2.88	3.62
	4.0	4.10	3.20	2.66	1.71			4.12
6 weeks	3.78	3.2						
	4.06							
	4.26	2.6	4.35	0.77	2.53	1.68	1.72	3.28
	4.34	5.78	3.62	2.55	1.76	0.27	2.26	3.60

TABLE 47

The effect on mucous cell diameter of administration of 17α CH_3 testosterone at 3 dose-rates by the intramuscular route.

Time of Sampling	Arachis Oil	100µg/100g fish	200µg/100g fish	500µg/100g fish
Day 1	15.2			
	17.3			
	14.8			
1 week	18.0	21.9	18.5	17.8
2 weeks	17.1	16.5	19.2	16.5
3 weeks	14.4	18.2	18.6	19.3
4 weeks	19.5	15.3	15.4	21.9

All measurements are in microns (µ)

TABLE 48 The effect on mucous cell diameter of administration of 17α CH_3 testosterone by three different routes

Time of Sampling	1/m Oil	1/m 5	1/m 10	1/p Oil	1/p 5	1/p 10	Oral Oil	Oral 10
1 week	13.7	14.7	12.6	17.1	18.1	12.6	15.7	13.3
	15.4	17.4	MM	15.0	MM	14.7	15.0	MM
2 weeks	15.0	14.7	15.4	18.5	20.5	15.4	15.4	19.5 (11 days)
								15.0
	15.7	MM	21.2	16.4	16.1	15.7	14.7	14.4
7 weeks	18.8	18.8	18.8	20.5	19.8	18.1	17.1	20.5
								21.9
	16.4	17.8	16.4	19.8	20.5	19.2	17.4	23.6

All measurements are in microns (μ)

TABLE 49 The effects on mucous cell diameter of the administration of three hormones by the intraperitoneal route

Time of Sampling	Oil (Jan 76)	17α CH_3 testosterone 5	17α CH_3 testosterone 20	17α CH_3 dihydrotestosterone 5	17α CH_3 dihydrotestosterone 20	Oil (Nov 76)	11 ketotestosterone 5	11 ketotestosterone 20
1 week	18.8	20.2	19.8	18.5	24.2			
	21.2	14.7	22.9	21.9	21.2			
3 weeks	18.5	18.5	19.2	20.5	17.4	19.8	20.2	20.9
	19.2	21.2	19.8	16.8	18.5	18.5		
6 weeks	22.2	23.9	21.2	17.8	20.2	24.2	21.2	18.5
	15.7							
	20.9	21.5						
	22.2	21.2	20.9	19.2	21.2	18.5	21.9	21.2

All measurements are in microns (μ)

some variation in size of individual fish in these experiments and this may have had some bearing on these results.

There was similarly little apparent change in mucous cell diameter in all experiments as a result of hormone administration and a lot of individual variation was present. There was also a lot of individual variation in Experiments 1 and 2 in mucous cell numbers, but in Experiment 3 certain fish receiving androgens did show a marked reduction in mucous cell numbers. This was especially noticeable in those receiving 11 ketotestosterone but was also present in certain fish receiving 17α CH_3 dihydrotestosterone, though this latter group did show variation in particular treatment groups.

Interrenal tissue

Control fish at the start of the experiments had interrenal histology comparable with immature fish, the only exceptions being those control fish in the first two experiments having maturing gonads. The extent of the interrenal tissue was variable, but most frequently only isolated groups of cells were seen surrounding a small vein (Fig.107). Cells were closely packed together and measured approximately 10-12μ diameter with spherical nuclei of approximately 6.5μ diameter. A fine chromatin network was evident in the nuclei which possessed inconspicuous nucleoli. The cytoplasm was finely granular and pink staining in H & E stained sections. Occasional cells were present with prominent nucleoli. In all cases, vascularisation of the tissue was poor.

In the first experiment marked changes in the interrenal gland had taken place after only one week, in the group receiving 200 µg/100g (Group 3). Hyperplasia and hypertrophy of the tissue had occurred, forming large islands of tissue with a marked increase in vascularity (Figure 108). Haemorrhages were present in some areas in association with focal areas of cell necrosis. There was considerable variation in nuclear and cell diameter. Cell diameters were difficult to visualise because of poor definition of cell limits, but nuclei up to 23µ were seen with a large number of nuclei averaging about 9-10µ. In many cells one or two prominent nucleoli were seen and mitotic figures were frequent (Figure 109). Similar but less marked changes were present in the 100 µg/100g group (Group 2), but there was marked increase in vascularity in both interrenal and haemopoietic tissue. Interrenal changes in the 500 µg/100g (Group 4) and control fish (Group 1) after one week appeared as in the control fish at the start of the experiment.

Changes after two, three and four weeks were essentially similar to those in Group 3 after one week, but present in varying degrees among individual treated fish. The control fish showed slight hypertrophy and hyperplasia, but this was not so pronounced as in the other groups and no marked increase in vascularity occurred. No precise pattern of change was evident under any of the dose regimes used.

The results of Experiment 2 showed a more orderly

development of changes, essentially of hypertrophy, hyperplasia and increased vascularity with some degenerative changes in the latter stages. In the intramuscular group little change in the interrenal cells was seen from the control fish after one week, but a definite hyperplasia and hypertrophy with increased vascularity was present after two and seven weeks. Similar changes were present after one and two weeks on both hormone levels, but after seven weeks on both treatments, hyperplasia, hypertrophy and increased vascularity were still present in the interrenal tissue. Cell arrangement however, had become less defined and there was an increase in connective tissue through the interrenal tissue. Cells were frequently arranged to form ducts surrounding central sinusoids and often formed columns (Fig. 110). At this stage also, degenerative changes were evident and these consisted of cytoplasmic vacuolation and occasional pyknosis or karyorrhexis of nuclei. Haemorrhages throughout the tissue were more frequent. The degenerative changes tended to be focal in distribution.

The effects of intraperitoneal administration were similar to those obtained by the intramuscular route. Adrenal hyperplasia was present in the group on oral administration after only one week, but later results were as found in the intramuscular group. In all experiments, increased vascularity of interrenal tissue was usually associated with a similar change in the haemopoietic tissue of the anterior kidney. Paranuclear bodies as described by Heyl & Carpenter (1972) were not seen.

Changes after seven weeks were similar in all hormone treatment groups by all routes. In control fish, interrenal changes were very variable and though extensive hypertrophy and hyperplasia did occur in occasional fish this was the exception rather than the rule.

Experiment 3 showed that all three hormones were equipotent in their effects on the interrenal. Little change occurred after one week, but hyperplasia, hypertrophy and increased vascularity were present after three weeks, which became marked by six weeks with some degenerative changes as in Experiment 2. Degenerative changes were most marked in those fish on 11-keto testosterone (see Figure 111). A massive increase in the melano-macrophage areas in the anterior kidney was present. The individual cells showed an aggregation of fine melanin granules and many showed evidence of necrotic change. There was also a marked increase in diffuse connective tissue spread through the haemopoietic tissue, and some of the latter tissue was also showing necrosis. Similar necrotic changes were also present in other organs (vide infra).

Liver

The essential liver pathology observed in all three experiments was the development of fatty change leading ultimately to lipoid liver degeneration with some focal necrosis. The extent of this change varied with the length of treatment but not with the class of hormone administered (with the exception of 11-keto testosterone).

When oil injection techniques were used, fatty change was evident after between two and three weeks and was prominent after six - eight weeks. Controls were affected in a similar manner. Fish in Experiment 2 on oral administration showed slight fatty change but no obvious degeneration.

11-keto testosterone was the only hormone to cause any other liver effect. After four weeks administration on both dose-rates, excessively fatty livers were present and on the low dose-rate, marked bile-duct proliferation and increased fibrous tissue in the duct walls were present (Fig. 112). After eight weeks, fatty degeneration was not prominent, but extensive focal liver necrosis was present (Fig. 113).

Spleen

There appeared to be no change in spleen histology following hormone administration.

Posterior Kidney

Degenerative changes in the excretory kidney tissue did occur following hormone administration, but these were never very extensive. Changes consisted of slight thickening of Bowman's capsule and some capillary sclerosis in the glomeruli with tuft shrinkage. Deposits were variably found in both glomeruli and tubules. Tubular damage, when present, consisted of luminal deposits and sloughed cellular debris in tubule lumina, with cytoplasmic and nuclear vacuolation and disintegration of tubule wall cells. This was never extensive.

Eosinophilic droplet formation was seen in tubule cells (Fig. 40), but was not associated with any particular treatment and was common also in control fish. In Experiments 1 and 2, degenerative changes were variably present at all dose-rates and routes used after two weeks, but more frequent at the end of hormone administration. At termination of these experiments, glomerular damage was most noticeable.

In the fish on 11-keto testosterone in Experiment 3 an increased cellularity of glomerular tufts was evident on both dose-rates after four weeks. By eight weeks, this was very marked and there was a marked increase in melanomacrophage area, as was the case in the anterior kidney. Haemopoietic necrosis was present. Glomerular and tubular degenerative changes were otherwise similar to those observed on other hormone administrations.

Heart

Very little change occurred in heart muscle or blood vessels as a result of hormone administration. Some isolated ventricular fibre vacuolation and necrosis was present in Experiment 2 in one fish after seven weeks on i/p administration at low dosage. Similar changes were present in Experiment 3 in all fish after six weeks' administration of 17α CH_3 testosterone. After six weeks' administration of 11-keto testosterone in one fish at each dose-rate, such degeneration was more extensive and associated with areas of cellular infiltration and myophagia.

Pancreas

Changes occurring in the pancreas during experimental hormone administration included variation in size and number of Islets of Langerhans and degenerative changes occurring therein; variation in amount of fat; vacuolar degeneration of acinar tissue and infiltrative changes associated with peritonitis. Although comments will be made on islet size and number, it became evident during the course of this work that great variation occurred with site sampled. Islets were particularly plentiful in the region around the pylorus of the stomach where the pyloric caecae originated. When sectioning gut and pancreas grossly, it is particularly difficult to ensure that a similar region is always removed and the only real answer would be to serially section several inches of gut and associated pancreas. Such a task was not performed. Only general conclusions on distribution and size of islets have therefore been made in the following account.

Experiment 1

The pancreas consists of both exocrine and endocrine portions scattered through the omental fat surrounding the pyloric caecae and stomach. In control fish, from half to as much as 9/10 of the tissue consisted of typical large fat cells with indistinct cell outline. The remainder of the tissue was composed mainly of exocrine acinar cells with a typical differentiation into an amorphous basophilic area together with a distinctly granular eosinophilic portion. Islets were scarce, often

only one or two islets per section being present. The average diameter of such islets was approximately 100-120μ although occasional islets were much larger, the largest measured in a control fish being 240μ in diameter. They were usually spherical in shape and consisted of a mixture of basophilic cells staining either lightly or densely (α and β cells) and most islets contained an occasional red blood cell. Cytoplasmic granulation was evident in both types of cell. Ducts, blood-vessels and nerves were also in evidence throughout the tissue.

After one week's treatment, the control fish injected with arachis oil alone had a pancreas of similar appearance to the non-injected controls above, with the exception of the acinar tissue. This showed a greater proportion of eosinophilic granular staining (up to 2/3 of the cell volume) and occasional cells showed marked vacuolation. Fat and islet tissue appeared unaffected. In the fish injected at 100 and 200 μg/100g, islet hyperplasia had taken place, several islets being present in each field examined. There appeared to be a preponderance of β cells grouped around central vascular channels in a duct-like formation (Fig. 114). No marked hypertrophy of islets was present. As in the control fish, increased eosinophilic granulation was present in the acinar cells and the cytoplasmic vacuolation was even more marked (Fig. 115). The fish receiving 500 μg/100g showed similar pancreatic morphology to uninjected control fish.

After two weeks treatment, control oil-injected fish had similar pancreas histology to the one week fish, except that vacuolation of acinar cells was absent. The 100 and 200 µg/100g fish showed islet hyperplasia, but this was less than in the previous one week group and acinar vcauolation was not as marked. Cytoplasmic vacuolation and disintegration within the islets was obvious at this stage. The 500 µg/100g fish again appeared as controls at the start of the experiment.

After three weeks, control fish appeared as the uninjected control. Fish receiving 100 and 200 µg/100g were similar and there was no 500 µg/100g fish sampled at this time.

After four weeks, fat was almost non-existent in the 100 and 500 µg/100g fish and was replaced with small quantities of connective tissue. Acinar tissue was compact and with occasional vacuolation. Islets showed similar hyperplasia to the 100 and 200 µg/100g fish after two weeks, but degenerative change was more pronounced. Evidence of possible early arteriosclerosis was present in the 500 µg/100g fish with intimal proliferation and splitting and reduplication of the internal elastic lamina (Fig. 116).

Experiment 2

Control fish at the start of the experiment appeared as in Experiment 1. After one week on i/m administration, the control oil-injected fish had a similar histological

appearance to the original controls, except that one very large islet measuring 640μ was present, other islets being small (up to 100μ) and few in number. In none of the fish in this trial was there any evidence of increased eosinophilia and granulation. Both fish receiving the lowest hormone concentration had pronounced vacuolation of the acinar cells, though this was not present in the fish receiving the high dose. No hyperplasia of islets was present in any of these fish and fat content was variable, though at least half of the area of the tissue in all cases. No degenerative changes were present in fat or islets.

After two weeks on i/m administration, the control fish showed increased eosinophilic granulation of acinar cells, but otherwise were as previous control fish. The fish in the low dose-rate appeared as controls with no islet hyperplasia or degenerative changes. Increased eosinophilic granulation was however present. The larger dose-rate again appeared as control fish with the exception of the acinar cells. These cells were densely basophilic and many had several small cytoplasmic vacuoles as opposed to the single large vacuole seen previously.

After seven weeks' administration by the i/m route, all fish showed a decreased fat content amongst the pancreatic tissue, and all fish showed some degree of cytoplasmic vacuolation in the islets. In one of the control fish, an early arteriosclerotic lesion was evident (Fig. 117). Hypertrophy of islets was present in all

hormone-treated fish, associated with a change of outline from spherical to more ellipsoidal or asteroid shaped (Fig. 118) and islets up to 700μ were regularly found, especially around the base of the pylorus. Hyperplasia of islet tissue was also present in one fish out of two at each dose-rate. Increased vascularity of hypertrophied islets was evident but cytoplasmic detail and cell outline became indistinct. Acinar tissue appeared to increase in direct contrast to the decreased fat tissue, but no degenerative changes in this tissue were seen. No replacement of fat cells with round cell infiltrates was seen.

After one week's administration by the intraperitoneal route, one of the control fish was as the uninjected control, while the other showed decreased quantities of fat, slight islet hypertrophy and hyperplasia and a focal round cell infiltration in certain areas (Fig. 119). Other hormone-injected fish at this stage showed no effect on islets, fat or acinar tissue with the exception of one fish at the higher dose-rate which again showed marked vascular change in acinar tissue.

After two weeks, no degenerative changes were seen. Slight hypertrophy and hyperplasia occurred in one of the two fish on each hormone concentration associated with a decreased fat content of the pancreas. The tissue was otherwise as in control fish.

After seven weeks, three fish (one from each group) showed lesions of chronic peritonitis with an invasion of

inflammatory cells and a fibrotic reaction around certain areas in the pancreas. Pancreatic tissue within such areas however appeared unaffected. Some hyperplasia and hypertrophy of islet tissue was present in one control, with decreased fat. Marked islet hypertrophy was present in one fish on the high dose, other hormone treated fish appearing to possess islet tissue similar to controls. In all hormone-injected fish, there was a degree of cytoplasmic islet degeneration, most marked in the fish showing hypertrophy (Fig. 120). In this fish considerable cell breakdown was present.

None of the fish receiving oral androgens without arachis oil showed any vacuolation in acinar tissue. Islet size and number were as in control fish until seven weeks, when both hypertrophy and hyperplasia were present. Islets up to 700μ were found and duct-like formations within the islet tissue were common.

Experiment 3

Experiment 3 compared three hormones by the intraperitoneal route. Fish on all three hormones showed a similar pancreatic response. Control fish in all cases remained much as previous controls. There was no acinar degeneration in any fish examined and pronounced hypertrophy and hyperplasia of islets did not occur until the six week sample and in all fish on all dose-regimes there was a variable but nevertheless definite increase in size and number of islets. Fat varied considerably from fish to fish, but was generally decreased after six weeks.

Gonads - Testis

The effects on the testis of administration of 17α methyl testosterone varied with the state of sexual maturity. Several fish (see Tables 38 and 39) became mature during the course of the experiment and contained fully developed sperm. These fish showed no degenerative changes and histological structure was that of a fully developed mature male fish. Other fish which possessed immature testes at the end of the experimental period exhibited a range of degenerative changes which appeared similar on all dose-rates. State of maturity of fish at the start of the experiment was unknown except that no fish showed any secondary sexual characteristics.

Figure 30 shows the structure of a normal immature testis at the start of experimentation. There are large numbers of primordial germ cells and spermatogonia with a number of associated Sertoli cells and lobule boundary cells. The testis is formed into a series of lobules with loose connective tissue septa and capillaries containing red blood cells. The primordial germ cells are large oval or spherical cells with light-staining nuclei containing a single large densely-staining nucleolus. Spermatogonia are distinguished by having spherical nuclei and multiple nucleoli. Sertoli cells are seen scattered through the tissue and are dark-staining with irregular nuclei. Lobule boundary cells possess elongated dark staining nuclei. After one week of treatment in the intramuscular and oral groups of fish and after

two weeks by intraperitoneal injection (Experiments 1 and 2) degenerative changes were evident in the primordial germ cells and spermatogonia. Nuclear and cytoplasmic breakdown had taken place in many cells resulting in the formation of cyst-like areas in the testis (Figure 121). Individual cells showed marked cytoplasmic vacuolation and nuclear shape became very variable and staining intensity increased.

After two weeks by i/m and oral routes, these cysts were much more obvious and increased in size up to 50μ (Figure 122). Cytoplasmic vacuolation increased at this stage and the numbers of germ cells and spermatogonia became reduced.

At the end of seven weeks' administration, degeneration had progressed much further. The cystic appearance of the testis was much increased, a marked reduction in spermatogonia and germ cells had taken place and there was a much increased quantity of connective tissue. A proliferation of both Sertoli cells and lobule boundary cells was also present (Figure 123). Large areas of cell necrosis were present and many cells with large bizarre nuclei and little cytoplasm were seen. Nuclear and cytoplasmic disintegration were widespread and nuclear aggregations (Figure 123) were often visualised though cell type was impossible to determine at this stage.

Ovary

Ovaries of control fish were in the vitellogenic phase at the start of the experiment (Figure 124). Degenerative changes again occurred after one week in the i/m and oral route groups, the i/p group showing much less severe changes. After one week oocytes at all stages showed some vacuolation around cell edges and in occasional yolk-laden oocytes there appeared to be an increase in the number of follicle cells lining the outside of the oocyte (Fig.125). Such peripheral vacuolation was not seen in any fish after the one week sample. Nuclear disintegration of yolk-laden oocytes appeared to be the earliest form of damage with densely-staining nuclear remnants present. Fish after two weeks' administration possessed normal pre-yolk oocytes but showed marked atresia of yolk-laden oocytes with degeneration of nucleus and cytoplasm, shrinkage of cell volume, a massive increase in the follicle-cell layers and a much reduced or absent chorion (Figure 126). After seven weeks, occasional "Corpora atretica" remained, but the mass of the ovary contained apparently normal pre-yolk oocytes (Figure 127). No female fish became sexually mature during the course of the experiment.

Pituitary Gland

The pituitary glands of control gonadectomised fish receiving arachis oil appeared as immature fish with numerous chromophobe cell types. Granulated gonadotrophs (basophils) were only very occasionally seen. Administration of all three androgens led to the development of increased numbers of basophils in the dorsal lobe of the gland and a corresponding reduction in chromophobes. There was no apparent difference between route of administration, dose-rate of hormone or type of hormone used in this study.

Stomach and Intestine

No regular changes in the histology of stomach or intestine occurred during the present study.

VI 4 Discussion

a) General comments on androgens

Although it appears that many hormones are not metabolised in their target organs, there is now a considerable body of evidence to suggest that metabolism of testosterone does take place in the target organs in a number of animals. With only a few exceptions, metabolism takes place outside the liver to form 5α reduced compounds. In general terms reduction processes in the liver tend to lower the biological activity of testosterone whereas reduction in the accessory sexual organs potentiates biological activity. Liver enzyme activity rapidly converts 5α dihydrotestosterone to its inactive end products, but such enzymatic activity is much lower in accessory sexual organs. 5α dihydrotestosterone also binds to receptor proteins (present in accessory sex glands but not liver) with a particularly high affinity, affording further protection from enzymatic degradation.

Classical studies have utilised accessory sex organs such as the rat ventral prostate and the chick comb for studying androgen metabolism (reviewed by King and Mainwaring 1974) and the majority of work has suggested that dihydrotestosterone is the active metabolite in a number of species (Bruchovsky & Wilson 1968a, b; Wilson & Gloyna 1970). 5α reductase is the enzyme involved in converting testosterone to 5α dihydrotestosterone prior to receptor binding and nuclear translocation (King & Mainwaring 1974) and Gloyna & Wilson (1969) showed that the specific activity of this enzyme is considerably higher in accessory

sexual organs than in other organs like the liver in a number of higher animals.

The activity of 5α reductase, in, for instance, the prostate gland, is controlled by levels of circulating testosterone, being lowered by castration and ageing (Shimazaki, Matsushita, Furuya, Yamaka & Shida 1969), but restored by testosterone treatment (Siiteri & Wilson 1970). These changes seem relatively specific since enzymes such as 17β OH steroid dehydrogenase do not change under the above conditions. It thus appears that testosterone stimulates 5α reductase in accessory sex organs (the reverse is true in liver (Bullock, Bardin, Gram, Schroeder & Gillete 1971)) and the balance between testosterone and enzyme levels controls biological activity. The reverse may be true in the adrenal (Kitay, Coyne & Swygert 1970).

In fact it has been suggested that the androgen-dependent conditions, acne vulgaris (Sansone & Reisner, 1971, Hay & Hodgins 1974) male-pattern alopecia (Bingham & Shaw, 1973) and idiopathic hirsutism (Kutten & Mauvais-Jarvis, 1975) may result from increased 5α reductase activity in androgen target cells of the skin, leading to a local increase in the rate of formation of 5α DHT. It is tempting to speculate that a similar situation may occur in the skin of salmonids at spawning time. The studies of Hay, Hodgins & Roberts (1976) showed that similar patterns of steroid metabolism were present in adult and immature trout, but degree of sexual maturity was not stated and so direct comparisons of quantities obtained are not valid.

Research on the control of sebaceous function and hair growth in human skin by testosterone suggested a conversion in the skin of testosterone to 5α dihydro-testosterone (Voigt & Hsia 1973; Imperato - McGinley, Guerrero, Gautier & Peterson, 1974). It has also been shown (Hay & Hodgins 1973; Sharp, Hay & Hodgins 1976) that other androgens such as dehydroepiandrosterone and androstenedione may have a similar function to testosterone and be converted in the skin to 5α dihydrotestosterone by a number of enzymatic reactions. Suggested pathways are shown in Figure 127.

Hay, Hodgins & Roberts (1974) showed from metabolism of steroids with skin samples of rainbow trout and an analysis of metabolic products that 5α reductase, 17β H.S.D. (hydroxysteroid dehydrogenase) 3α H.S.D. and 3β H.S.D. were all found in appreciable quantities, 5α reductase being particularly plentiful. 3β H.S.D. D^{4-5} isomerase activity was very low. Further work by Hay, Hodgins & Roberts (1976) showed that such activity was present in dorsal and ventral skin and in dorsal skin from male and female, adult and immature fish. 5α reductase activity was predominantly present in the dermis, only low activity being present in the epidermis, the reverse of the situation in human skin. Another difference between human and trout skin was that 5α reductase is present in much greater quantities than the other enzymes concerned in trout skin, whilst the reverse occurs in mammals, and 17β and 3α H.S.D. systems are thought to control the quantity of 5α D.H.T. in the mammal.

3α - H.S.D.$^{4-5}$ isomerase activity is concentrated in the pilo sebaceous units in mammalian skin, and the absence of such units in fish skin probably explains the much lower enzyme activity in the latter species.

Close parallels are present between 5α reductase site and quantity in fish skin and in animal prostate glands, particularly the dog.

It is of interest to note that with the exception of the rat, the highest levels of 5α reductase are found in the prostate gland of those animals which commonly develop prostatic hypertrophy e.g. man, dog and lion. It has been shown (Cowan, Cowan, Grant & Elder, 1977) that 5α reductase activity is found principally in the stroma of benign hyperplasic prostatic tissue, and not in the epithelium. Conversely dehydroepiandrosterone sulphate sulphatase was found principally in the epithelium. It has been shown that dehydroepiandrosterone (DHA), the product of sulphatase activity, can be converted to DHT, at least to some extent, by the prostate gland (Collins, Koullapis, Bridges & Sommerville 1970; Harper, Pike, Peeling & Griffiths 1974) and that the gland may utilise adrenal DHA sulphate, especially if plasma testosterone is low.

This would suggest that the stroma of the prostate was the androgen target tissue and is interesting in that certain workers believe that localised stromal proliferation is the first even in prostatic hyperplasia in man. Franks, Riddle, Corbonell & Gey (1970) showed that the adult human prostate epithelium lacks growth capacity if separated from its stroma and Cowan et al (1977) suggest

that this result, together with the effects of androgenic stimulation on epithelio-mesenchymal interactions in primordial gland structures described by Cunha (1972a, b), might indicate that the prostatic stroma in fact supplies androgens to the epithelium. Epithelial cells may then become completely dependent on stromal DHT in hyperplastic conditions and would be unable to proliferate in tissue culture in the absence of stroma.

Although in both man and dog increased levels of DHT are found in hyperplastic prostate tissue (Gloyna, Siiteri & Wilson 1970; Siiteri & Wilson 1970; Albert, Geller, Geller & Lopez, 1976; Habib, Lee, Stitch & Smith 1976) and increased stromal 5α reductase may have caused this increase in DHT and perhaps initiated the condition, the two conditions are different. In man, the hyperplasia is nodular, involving both stroma and epithelium, whereas in dog the hyperplasia is epithelial (Franks 1954; O'Shea 1962).

The results of the present study show that in both normal maturation and as a result of testosterone administration, an increased thickness of both dermis (stroma) and epithelium take place in the brown trout. The effect is most marked in the epithelium though the work of Hay et al (1976) showed that, in the rainbow trout at least, 5α reductase activity was found principally in the dermis, and in far greater quantities than in mammals. The biological activity of 17α CH_3 DHT has been shown to be equivalent to that of 17α CH_3 testosterone in the present work, adding further evidence that this compound may well be a biologically active metabolite in the brown trout.

11β hydroxytestosterone was found in incubates of rainbow trout testis (Arai & Tamaoki 1967a, b) and Idler, Truscott & Stewart (1969) and Idler & Macnab (1967) found this steroid in gonadal and interrenal tissue of Atlantic salmon. Idler, Horne & Sangalang (1971) isolated it in plasma of Atlantic salmon. In the latter work it was shown that 11-ketotestosterone and 11β OH testosterone increased in level as maturation proceeded but testosterone levels remained relatively constant. They explain these differences by the metabolism of testosterone to 11β OH testosterone and 11 ketotestosterone and perhaps an increased metabolic clearance rate of testosterone during maturation. Idler et al (1969) showed that 11β OH testosterone is converted only slowly to 11 ketotestosterone and this may explain the rise in level of 11β OH testostesterone that occurs during maturation. Other metabolic pathways for 11 ketotestosterone synthesis not involving 11β OH testosterone may also be involved (see Figure 128).

Kime (1978) has shown that the catabolism of cortisol by the liver leads to the production of cortisone, 11β hydroxyandrostenedione and androstenetrione. The latter two compounds have been suggested as precursors of 11β hydroxy and 11 oxo-testosterone (Idler & MacNab 1967). Although the extent of testicular production of 11 oxygenated androgens from hepatic precursors is not yet known, gonadal androgens do stimulate adrenal corticosteroid production and Kime suggests that an adrenal-hepatic-gonadal-adrenal positive feedback mechanism may exist

which, once initiated, may lead to increased sexual development and hyperadrenocorticism.

The androgenic potency of 11-ketotestosterone has been a subject for discussion ever since its finding in large quantities in salmon plasma in the 1960's.

Hay et al (1976) suggest that removal of the 11-ketotestosterone group for conversion into 5α DHT is unlikely but the possibility exists that 11-ketotestosterone could be converted into an 11-oxy-5α reduced metabolite in fish skin. Although 11-oxysteroids are apparently poor substrates for the 5α reductases of human skin (Voigt, Fernandez & Hsia 1970) and rat prostate gland (Frederiksen & Wilson 1971) 11-ketotestosterone is a potent androgenic hormone as determined by the chick-comb bio-assay (Idler, Schmidt & Biely, 1961) and acts as a potent androgen in salmonids by increasing skin thickness and causing the development of secondary sexual characters (Idler, Bitners & Schmidt 1961; McBride & van Overbeeke 1971). 11-ketotestosterone was also found to be seventeen times more potent than testosterone in the induction of male secondary sexual characteristics in the female medaka, Oryzias latipes (Hishida & Kawamoto 1970). The results of the present study confirm the biological androgenic potency of this hormone on fish skin.

In the blood, testosterone and derivatives are generally bound to proteins, the type of protein varying between species. For many animals, it is thought that

5α dihydrotestosterone has a particular affinity for such binding sites suggesting it cannot act except by conversion in accessory sex tissues. In the human, such binding is accomplished by a particular sex-steroid binding β globulin (SBG). Very high binding of testosterone has been shown in the Atlantic salmon and thorny skate by Idler & Freeman (1968) leading to high circulatory levels, but the binding protein involved was not equivalent to SBG. Human plasma concentrations of testosterone are usually about 500 ng/100 ml and similar values are obtained from rat, Rhesus monkey, guinea-pig and rabbit (King & Mainwaring 1974) but levels in fish such as the salmon and skate are much higher, between 4-20 µg/100 ml (Schmidt & Idler 1962; Idler & Truscott 1968).

Idler, Schmidt & Ronald (1960) isolated 11-ketotestosterone from the blood of spent male sockeye salmon at a level of 12 µg/100 ml and noted that previous workers such as Phillips, Holmes and Boudy (1959) had probably reported this substance as corticosterone. 11-ketotestosterone and other androgens were quantified in both sexes of sockeye salmon (Grajcer & Idler 1961) and Schmidt & Idler (1962) carried out further quantification work prior to and during the spawning migration of this species and showed 11-ketotestosterone to be a major androgen in this species. The latter workers also reported 11-ketotestosterone in spawned Atlantic salmon of both sexes. Idler, Freeman & Truscott (1964) showed

that this hormone was the principal androgen in spawned Atlantic salmon. The high levels of 11-keto testosterone and also adrenal corticosteroids reported in the blood of mature and spawned Pacific salmon (Idler, Ronald & Schmidt 1959; Hane & Robertson 1959) were suggested by Idler & Truscott (1963) to be due to impaired clearance from the blood. As such high levels were not found in other salmonid species which usually survive spawning, it was suggested that these levels were probably a sign of approaching death rather than a result of sexual maturation and spawning.

Pathways for 11-ketotestosterone synthesis were also given by Idler & Truscott (1963) and are shown in Figure 128.

Gonadectomy was carried out in order to remove endogenous androgen and oestrogen production. Of particular interest are the findings of Idler & Macnab (1967) in Atlantic salmon. They showed that in vitro slices of gonad and interrenal tissue synthesise 11-ketotestosterone and 11β-hydroxytestosterone from adrenosterone and testosterone. Adrenosterone was the major precursor of 11-ketotestosterone and testosterone was the major precursor of 11β-hydroxytestosterone. They conclude that the testis produces by far the greater quantity of 11-ketotestosterone in vivo but that a significant production of 11β-hydroxytestosterone may occur in vivo in the interrenal tissue, though again the testis forms the primary source. In the in vivo situation in salmonids, 11β-hydroxytestosterone is not found in blood, but Idler &

Macnab suggest the possibility that it may be rapidly converted to 11-ketotestosterone, either directly or via andrenosterone. If such interrenal production is significant in castrated fish, then this could seriously affect any results of androgen administration and must be borne in mind when considering the results of androgen administration. Shreck, Flickinger & Hopwood (1972) measured plasma androgens in intact and gonadectomised rainbow trout. Androgen levels in intact maturing male and female fish were found to be similar, as also noted previously in the sockeye salmon (Grajcer & Idler 1961; Schmidt & Idler 1962) but levels increased in fully mature males. In the gonadectomised fish, levels remained at a considerable level (6.8 µg/ml in males and 4.8 µg/ml in females) at least up to 42 days post-operation, suggesting that a significant contribution to plasma androgens may be produced extra-gonadally.

In mammals, 11β-hydroxylation usually occurs in the adrenal cortex but may be found in interstitial cell tumours of the testis (Dorfman & Unger 1965; Savad, Dorfman, Baggett, Fielding, Engel, McPherson, Lister, Johnson, Hamblen & Engel, 1960). It has been further suggested that in the normal mammal, probably all of the 11-oxygenated urinary steroids are of adrenal origin and in Cushing's syndrome due to bilateral adrenal hyperplasia, excretion of 11-oxygenated steroids in the urine is considerably increased, (Deane1962). An oxygen atom at C-11 is present in more than half of the adrenal steroids

and is less reactive at this position than at any other position in the steroid molecule.

11-keto derivatives of a variety of precursors have been produced by incubating bovine adrenal (Axelrod & Arroyave 1953; Jeanloz, Levy, Jacobsen, Hechter, Schenker & Pinius 1953; and Meyer, Hayano, Lindberg, Gut & Rodgers 1955) or human adrenal tissue slices (Chang, Mittleman & Dao 1963). The normal testis tissue of mammals, in contrast to that of salmonids, does not produce 11β-hydroxytestosterone.

b) Histology

Skin

The first trial involved the use of three different dose-rates of testosterone. Results were very variable and indeed the highest dose-rate caused the least effect on skin thickness. The most likely explanation of this anomaly is the observed loss of oil and hormone from the injection site with fish movement. The trial did, however, show that a marked thickening (up to 3x) could occur in as little as two weeks with a dose of 400 ug/ml of hormone.

The second trial showed that all three routes of administration were effective in causing epidermal thickening, but the intraperitoneal route was most rapid in effect, especially at the lower dose-rate. The end result after seven weeks was equivalent no matter what the dose-rate or route of administration.

During the course of the above two trials, certain male fish became sexually mature, and eventually died from fungal infection. When measurable, the epidermis in such fish was thickened, but such an effect may well have been due to endogenous hormone production by the mature testes. Consequently such results cannot be considered representative of the effects of hormone administration. Testosterone administration certainly did not prevent sexual maturation when administered at the dose-rates used at such a short time prior to maturation. Epidermal thickness of the control fish was also increased somewhat during the experiment; this may well

have been the result of excessive handling of the fish. Pickering & Macey (1977) showed that handling increased the concentration of superficial mucous cells in the char (Salvelinus alpinus) but had little effect on thickness under their experimental regimes. However skin thickening occurred in control fish following androgen administration to sockeye salmon (McBride & van Overbeeke 1971).

Similarly the regular application of malachite green may well have affected skin thickness. It certainly causes marked epithelial overgrowth in gill tissues when used too often or at too high a concentration (unpublished observations).

It is interesting to note that there was little difference in the response of the epidermis of immature fish of either sex. It has been shown that testosterone is produced in small quantities in maturing female fish as well as male fish and though oestrogens also cause skin thickening, the low level of testosterone may have an added effect. The intra-peritoneal route seemed to give the most reproducible results though the small numbers of fish used in these experiments make statistical analysis difficult. The intra-peritoneal route certainly seemed the most efficient in terms of administering exact doses. Though the results of intramuscular injection seemed more regular in the second experiment, perhaps because of the use of the extra arachis oil, loss of hormone from the injection site still remained a possibility as did loss of food and hormone by regurgitation when the

oral route was used. The method of oral administration did allow accurate administration of hormone, but involved considerable handling of the fish. Because of feeding variations in conscious active fish, administration of treated food without some form of stomach-tubing seemed too inaccurate.

The results of the third trial showed a remarkable similarity in the effects of both 17α CH_3 testosterone and 17α CH_3 dihydrotestosterone, though the latter seemed to act more rapidly. The results from the administration of 11 keto testosterone are very interesting, though care must be taken in assuming too much from so few figures, especially since the fish were treated almost 10 months after the first experiment at a slightly different time of year. The major difference is that skin thickening is more marked and changes occur in the structure of the epidermis which are much more similar to those occurring naturally in mature male fish.

There is no published account of such peg-like formation in salmonid skin following androgen administration but the present results suggest that such changes may be a specific effect of 11-ketotestosterone in salmonid fish. A more detailed examination of the effects of this hormone would be necessary to prove this hypothesis. This peg-like formation has previously been described as a healing response in salmonid skin (Roberts, Ball, Munro & Shearer 1971).

Published accounts of the administration of androgens to salmonids are limited. Idler, Bitners & Schmidt (1961) showed that 11 ketotestosterone was a major androgen in salmonid fish and caused a thickening of the skin which was more marked in male than female fish. The effects of this androgen seem to vary with the species and organ used. Arai (1967) showed that 11 ketotestosterone was ten times as active as testosterone in inducing the development of male secondary sexual characters in adult female medaka (Oryzias latipes) but the hormones were used in solution in the aquarium water and counting of leucophores and papillary processes on the anal fins were used to determine androgenic effects.

In 1971, McBride & van Overbeeke examined the effect of 11 keto-testosterone and 17α CH_3 testosterone on gonadectomised male sockeye salmon. The hormones were administered twice weekly at a dose-rate of approximately 2.5mg/kg/week. Large fish weighing approximately two kilos were used during the experiment and fish sacrificed after four and seven weeks. A six-fold increase in epidermal height occurred by the fourth week and did not increase further. In control fish a slight increase in thickness occurred after four weeks and was almost double after seven weeks. Both androgens evoked similar effects.

In the first trial the dose-rates of 17α CH_3 testosterone used were 3, 6 or 15mg/kg/week. In this trial, epidermal thickness only increased three-fold at most, though in this case the fish were not gonadectomised.

In the second trial 2 and 4mg/kg/week were used and in the third trial 2 and 8mg/kg/week were injected. Only double the normal epidermal thickness was achieved, though with 11 keto-testosterone a 3.5x increase in thickness did occur in two male gonadectomised fish.

17α CH_3 dihydrotestosterone and 17α CH_3 testosterone produced their maximum effects after one week. The maximum effect of 11 keto-testosterone did not appear until six weeks. The hormones were administered however at differing times of year.

In the second trial 17α CH_3 testosterone was administered in food. A dose-rate of 10mg/kg/week was used and resulted in a three fold increase yet again. Yamazaki (1972) fed 17α CH_3 testosterone at 50 and 100ug/g diet to pink salmon (O. gorbuscha) and chum salmon (O. keta) weighing approximately 80gm and achieved a four fold increase in skin thickening by three weeks. Fish were not individually fed and so intake must have varied but it was found that both diets gave the same response. No details of actual amount of hormone/kg fish/week were given.

In a similar experiment McBride & Fagerlund (1973) fed 17α CH_3 testosterone at 10 and 50mg/kg diet to coho salmon (O. kisutch) and chinook salmon (O. tschawytscha) weighing an average 3.79g and 0.78g respectively. Though there were no marked changes in the small chinook salmon increase in thickness up to six fold by six weeks occurred in the coho salmon. The thickness increased at each sampling and was greatest in the group on the highest concentration of dietary hormones.

Mucous cells

Yamazaki (1972) showed that methyltestosterone caused an increase in mucous cell size and number after a week's oral adminsitration. McBride & van Overbeeke also found mucous cells in increased numbers throughout the thickened epidermis following hormone administration, and further showed that the effects of 17α CH_3 testosterone and 11 ketotestosterone were equivalent. Yamazaki however utilised intact fish whilst McBride & van Overbeeke used gonadectomised fish. Brown trout were not used in either work.

The results of the examination of skin changes during sexual maturation reported in section II show a decreased number of mucous cells in mature male fish at spawning time, a finding also noted by Pickering (1974). Mature Loch Leven brown trout of both sexes also developed larger mucous cells than the immature fish, a finding not obvious as a result of hormone administration.

Though it is possible that androgens affect mucous cell size and number, either directly or indirectly, there are certainly other possible mechanisms for effects on mucous cells in fish. Prolactin certainly has been shown to affect mucous cells in a number of fish species (e.g. Egami & Ishii 1962; Mattheij & Stroband 1971) and seasonal variations in prolactin secretion could be the possible factor in varying mucous cell size and number, and may explain the findings of Donaldson & McBride (1967) who showed that hypophysectomy leads to a decrease in

mucous cell number. This may also be the explanation of the lack of a marked effect on androgen administration on mucous cells in the present study. It is however, interesting to note that a marked decrease in mucous cell number did occur in fish receiving 11 keto-testosterone and may represent a specific effect of this particular hormone. It is unfortunate that so few fish could be utilised in this study and these results again demonstrate the importance of carrying out further work on 11 keto-testosterone with statistically viable numbers of fish.

It is interesting to note the similarity in effect of 17α CH_3 dihydrotestosterone and 17α CH_3 testosterone, synthetic compounds with a relatively long half-life in the fish body whilst the natural products have a short half-life.

Although the use of these compounds may be equivalent to continual low-dose stimulation with the natural compound, the results must be treated with some reserve if considered as a true reflection of the natural situation. It would however seem that in fish, as in man, dihydro compounds may well be active metabolites. The application of 11 keto-dihydrotestosterone may further elucidate the biochemical pathways existent in salmonid fish.

Dermis

The effects of administration of androgens on stratum compactum thickness in the present work were not marked. Although there was some individual variation from fish to fish there was no evidence of any constant effect on thickness. Yamazaki (1972) found similar results when he administered methyltestosterone to salmonids. He did find however, that the stratum spongiosum did increase in thickness - accurate measurement of this parameter did not prove possible in the present work.

A thicker stratum compactum was seen in the mature male fish from Loch Leven.

Interrenal Gland

Androgens and oestrogens have been shown to cause interrenal hypertrophy and some degenerative changes in gonadectomised sockeye salmon (van Overbeeke & McBride 1971). The results showed that there was some variability in this effect but that 17α methyltestosterone was more marked in its effects than 11 ketotestosterone and in particular, induced more degenerative change. The administration of cortisol in the above work induced atrophy of this tissue and a similar result was obtained with hydrocortisone in rainbow trout (Robertson et al 1963). The present series of experiments showed that androgens administered by a variety of routes caused hypertrophic and hyperplastic changes in the interrenal

tissue of brown trout. In contrast to the findings of van Overbeeke & McBride, 11 ketotestosterone produced more degenerative change than the other androgens utilised.

Liver and Kidney

Degenerative changes in liver and kidney were induced in gonadectomised sockeye salmon by feeding androgens and cortisol (McBride & van Overbeeke 1971) and by administering hydrocortisone to rainbow trout (Robertson et al 1973). These changes consisted of cytoplasmic vacuolation and eventual degeneration of cytoplasm and nuclei of parenchymal cells in the liver and thickening of glomerular capillaries and Bowman's capsule in the kidney. Such changes were similar to those found in spawning Pacific salmon (Robertson & Wexler 1960; McBride et al 1968). Degenerative changes in the liver following androgen administration in the brown trout in the present study were limited to fatty liver degeneration and necrosis but were also present in control groups and may possibly have been due to either the diet used or the arachis oil utilised in the injection experiments. The effects of 11 ketotestosterone are difficult to interpret because of the small number of fish used.

Kidney changes were minor, but eosinophilic droplet formation was seen in tubule cells in both control and injected fish and was similar to that occurring in the natural brown trout population in Loch Leven.

Pancreas

The effects of 17α methyltestosterone and 11 keto-testosterone on the pancreas of gonadectomised sockeye salmon were shown to be equivalent (McBride & van Overbeeke 1971). The essential changes were a marked hypertrophy of islet tissue (α and β cells) and cytolysis of pancreatic acinar cells and these changes increased with time. The administration of oestrogens to such fish produced a similar, though lesser effect and cortisone produced changes of an even less developed nature. A decrease in the quantity of fat surrounding the pancreatic tissue also occurred during the experiment. Robertson, Hane, Wexler & Rinfret (1963) produced similar pancreatic changes by administering hydrocortisone to immature rainbow trout but the islet hyperplasia was particularly linked to β cell hyperplasia and Robertson et al (1961a, 1963) have suggested that this is connected with hyperglycaemia caused by elevated corticosteroid production.

In the present study, hyperplasia of islet tissue resulted from administration of all three androgens but considerable variation in number and size of islets was present due to difficulties in obtaining standard tissue samples. Degenerative changes were also seen in the islets of certain fish receiving androgen treatment, especially in the long-term treatments. The effects of androgen administration were essentially similar to those reported in earlier work but there was an interesting change in acinar structure during the course of the work;

vacuolation of acinar cells, similar to that described by McBride and van Overbeeke (1971) was seen in both control and androgen injected fish when arachis oil was used as injection medium. No such change was seen in fish receiving oral androgens without arachis oil, suggesting that such vacuolation was probably the result of using arachis oil. The development of this change merits further investigation as arachis oil is commonly used as the injection medium in a variety of hormone experiments.

Testis

There has been considerable discussion over the role of the pituitary gland in maintaining spermatogenesis in fishes, reviewed by Dodd (1972). It now seems certain that the lobule boundary and Sertoli cells are responsible for androgen secretion in many fish species.

The effects of hypophysectomy on spermatogenesis in seasonal breeders varies with time of year (Ahsan 1966a; Lofts, Pickford & Atz 1968). Work carried out on Fundulus heteroclitus quoted by Dodd (1972) suggests that spermatogonial division is reduced but does continue and post-spermatogonial stages are not formed. Spermatogonial division also continues in the catfish Heteropneustes fossilis (Sundararaj & Nayyar (1967)) though later stages are again blocked. In other species such as the guppy Poecilia reticulata (Pandey 1968 cited by Dodd 1972) and Carassius auratus (Yamazaki & Donaldson 1968) spermatogonial division is blocked. The fate of stages beyond spermatogonia depends on the state of testicular

maturity prior to hypophysectomy - in some instances normal transformation to sperm occurs (Barr 1963b, Ahsan 1966b) whilst in other cases necrosis ensues (Lofts et al 1966, 1968). In the guppy, pituitary hormones appear essential for spermatogonial mitosis and for transformation of spermatogonia into spermatocytes, but have no effect on transformation of the latter into spermatids and thence to spermatozoa and spermatophores. The use of chemical gonadotrophin inhibitors has produced similar results (Dodd 1972).

It is not certain whether the gonadotrophins act directly on the germinal epithelium or whether the effect is on lobule boundary cells or Sertoli cells causing release of androgens to mediate the effect.

Effects of androgen administration on male gonads appear to be quite varied. In hypophysectomised Fundulus (Lofts, et al 1966) and in the catfish (Sundararaj & Nayyar 1967) spermatogenesis was stimulated. Administration of methyltestosterone to pink salmon (O. gorbuscha) and chum salmon (Yamazaki 1972) resulted in suppression of spermatogenesis and atresia of spermatogonia. Some spermatogonia hypertrophied and became vacuolated prior to disintegration and a decrease in number of spermatogonia was present. Interstitial-like cells hypertrophied, as was also noted following testosterone propionate treatment in hypophysectomised Fundulus (Lofts et al 1966). Similar testicular effects have also been described following androgen administration (McBride & Fagerlund

1973) to coho salmon and chinook salmon and Hirose & Hibiya (1968a, b) noted similar changes in the rainbow trout and the goldfish.

The findings of Pandey (1968)(cited by Dodd 1972) are of particular significance to the present work. He found that although spermatogonial division was stimulated in hypophysectomised mature guppies, there was a lack of effect on juvenile individuals.

The results of the present study closely agree with the degenerative changes reported by certain of the aforementioned workers in immature fish and consist principally of damage to germ cells with a proliferation of Sertoli cells and lobule boundary cells. The lack of degenerative effects of gonads in a more advanced state of maturation might well indicate a possible stimulation of maturation as indicated by Lofts et al (1966) and Sundararaj & Nayyar (1967). Lofts et al (1966) do in fact suggest that androgens exert their stimulatory effect mainly through an acceleration of post-spermatogonial development.

The effects on lobule boundary cells and Sertoli cells are more difficult to explain. If one accepts a negative feed-back mechanism involving the pituitary gland, then administration of androgens should reduce the output of gonadotrophin from the pituitary, which in its turn should limit androgen secretion by the LBC's and Sertoli cells. Of course an increase in number of cells does not necessarily mean that an increasedoutput

from these cells is present and measurement of a variety of plasma hormone levels would be necessary to confirm or reject such a hypothesis.

Ovary

The effects of the pituitary on ovarian development are complex. There is evidence to suggest that the highest rate of oocyte production occurs when the gonadotrophin levels are lowest (Hann 1927; Bullough 1939, 1942; Lagios 1965; Wiebe 1969). Wiebe (1968) also showed that oocyte numbers increased following methallibure blockage of gonadotrophins in the goldfish. The pituitary is not apparently involved in growth of primary oocytes following transformation from oogonia and prior to the laying down of yolk (Vivien 1938, 1941; Barr 1963b; Yamazaki 1965; Dodd 1972).

However, hypophysectomy at the yolk stage in all species examined causes follicles containing yolk to become atretic and become gradually reabsorbed. Hypophysectomy also causes hypertrophy of follicle cells, which become phagocytic in atretic eggs. It is not known whether or not these corpora atretica have an endocrine function but the evidence suggests that this is not the case (Dodd 1972).

Pickford & Atz (1957) concluded that mammalian or synthetic androgens are almost entirely inhibitory on the fish ovary. Yamazaki (1972) produced rapid (one week) degeneration of ovarian tissue following methyl-

testosterone administration and concluded that the effect was direct and not mediated via the pituitary as all oocytes were affected. Hypophysectomy in other fish species induced atresia only of yolk-laden oocytes, small and yolkess oocytes being unaffected (Pickford & Atz 1957; Ball 1960; Yamazaki 1965). The time taken for these effects to occur was also considerably longer in the above work than in that by Yamazaki (1972).

Similar degeneration was found by Hirose and Hibiya (1968a, b) in goldfish and rainbow trout but McBride & Fagerlund (1973) fed 17α-methyltestosterone to coho salmon and chinook salmon and found no such degenerative effects though the ovaries of fish in the latter study were in the perinucleolar stage prior to the laying-down of yolk. As the teleost pituitary is not thought to be responsible for the development of oocytes prior to the yolk stage (Dodd 1972), this result suggests the androgen inhibitory effect on the ovary is mediated via the pituitary.

The results of the present work also suggest an effect principally on oocytes in the vitellogenic stage. The degenerative changes in primary oocytes described by Yamazaki (1972) were not found. Hypertrophy and hyperplasia of follicle cells were presumably involved in phagocytosis of the degenerating yolk-laden oocytes.

Pituitary Gland

The effect of gonadectomy on the pituitary gland in the brown trout in this study is similar to that described in the sockeye salmon by McBride & van Overbeeke (1969). The effects of androgen administration were also similar to those found by van Overbeeke & McBride (1971) in the sockeye salmon in that there was a development of granulated basophils in the dorsal lobe. Administration of androgens to intact guppies resulted in progressive atrophy and loss of stainability of the gonadotrophs (Sage & Bromage 1970a). Unfortunately pituitary glands were not sampled in the first hormone experiment in the present study and so a comparison of these findings cannot be made. As there is good evidence for the existence of a negative feed-back mechanism for control of gonadotrophic activity by gonadal steroids (Egami & Ishii 1962; Ball & Baker 1969; Sage & Bromage 1970b) one might expect an inhibitory effect on gonadotrophs and it may be that such granulation represents an inhibited release of hormone or alternatively, that such cells were in fact thyrotrophs although again Sage & Bromage (1970a) found that androgens were inhibitory on thyrotrophs. A more detailed study would be necessary to adequately resolve this point.

Stomach and Intestine

Androgen administration to gonadectomised sockeye salmon led to marked atrophy of gastric villi, degeneration of gastric glands, increase in the cells of the stratum granulosum and decrease in the thickness of stomach muscle layers (McBride & van Overbeeke 1971). The effects of 17α methyltestosterone and 11 ketotestosterone were equivalent and the effect increased with time. Similar changes were induced in the rainbow trout with hydrocortisone (Robertson et al 1963).

The brown trout in the present study showed little response in stomach and intestine to androgen administration and marked changes were not noted in the normal sexually-maturing fish in section II. This may represent a species difference from the changes occurring in Pacific salmon and may well be related to starvation rather than to a direct androgenic effect.

GENERAL DISCUSSION AND CONCLUSIONS

Histological changes accompanying sexual maturation in the brown trout of Loch Leven were found to be similar to those occurring in other salmonid species. Many changes, in particular those of a degenerative nature, were however less pronounced than those occurring in the Pacific salmonids and more akin to those found in spawning rainbow trout. This supports the conclusion of Robertson & Wexler (1959), Heyl & Carpenter (1972) and many other workers, who suggested that degenerative changes were more marked in anadromous fish and may perhaps be linked to a long period of starvation and high levels of circulating corticosteroid. Although statistical analysis of changes in many organs was not undertaken, hyperplasia and hypertrophy of the interrenal tissue was a regular finding in sexually mature fish and may well be linked to high corticosteroid levels in the blood. Statistical analysis of skin changes was undertaken and in general supported the findings of other workers noted in section II. A particular change which has not been frequently noted in the literature is that of a drastic reduction in the number of mucous cells in sexually mature male brown trout in association with a well-developed undulation of the epidermis-dermis junction. This change has also been noted by Pickering (pers. comm. 1979) in the brown trout of Windermere and may represent an acceleration of cell kinetics in such fish which does not allow adequate

time for differentiation of mucous cells. The possible relation of this change to susceptibility to fungal infection has not been investigated and it would certainly be of great interest to examine cell kinetics in such fish and to further elucidate the changes occurring with the electron microscope. Variation in mucous cell numbers on different areas of the body has been noted by Pickering (1974) and this may bear some relation to area of infection with Saprolegnia. It would be interesting to examine such surface differences in the sexually mature brown trout. These anatomical differences have not been noted in other salmonid species except as a healing reaction in U.D.N. in the salmon when sexual differentiation was not undertaken (Roberts, Ball, Munro & Shearer, 1971). Another possible explanation of this change could presumably be a healing response following fungal infection or other damage in a previous season, though for this hypothesis to be tenable, one would have to assume an extremely high incidence of fungal infection affecting only male fish and in the same area of body (skin samples were taken from a point midway between the lateral line and the dorsal fin), though this particular area was significantly more frequently infected in male fish in the study carried out on mapping areas of infection.

Another interesting finding in the epidermis which was particularly obvious in the sexually mature fish was the area of lymphocyte-like cells in the suprabasal area. Although fish skin does have immunological properties

and antibodies may be found in surface mucus to a variety of agents including Saprolegnia (Wilson, 1976) the source of this immunologically competent material is not known. One might expect transfer of lymphocytes to occur from the bloodstream, but this is not seen histologically in these fish and it is tempting to speculate on the possibility of the development of immunologically competent cells in situ in the epidermis as an analogue of the Bursa of Fabricius in birds. At the present time, however, this remains very much a matter of speculation. A detailed electronmicroscopical study may help to determine whether these cells are in fact lymphocytes.

Variations in mucous cell number are also seen at various stages of maturity. Although this study was a "static" examination and details of rate of production would be necessary to determine actual mucus production, changes do certainly seem to be present. It has been shown that the mucus may contain antibodies which may help prevent infection (Wilson 1976) but antibody production in fish is temperature-dependent and the development of fungal infections during the colder months of the year may in fact be promoted because increased quantities of mucus cause spores to adhere more readily to the skin. Increased turnover rate of mucous cells might also serve to slough off germinating sporelings from the skin, as suggested by Willoughby & Pickering (1977). The relationship between mucus and fungus germination is thus still a complex one.

A considerable body of evidence now exists to suggest that a particular species of Saprolegnia, namely Saprolegnia diclina Humphrey type 1, is commonly associated with fungal skin infection in salmonids (Willoughby 1978) and such was the finding of the present study. This does not appear to be the situation in other species (Willoughby 1970; Pickering & Willoughby, 1977) and the reason for this is not yet clear. The salmonid infections are particularly common in association with sexual maturity in the colder months of the year whilst many of the infections in other species occur in summer months. Although propagule numbers of the Saprolegniaceae have been shown to be far greater in the winter months, no further typing was carried out to show whether this represented increased numbers of Saprolegnia diclina type 1, although Willoughby & Pickering's work (1977) suggests that spores of this type are found in increased numbers during an outbreak of fungal skin disease in farmed salmonids and that a rise in numbers of this specific type occurs prior to the infection developing.

It is also possible that this particular fungus has a specific biochemical requirement found particularly in salmonid skin or that its particular penetration mechanisms act most efficiently on salmonid skin. Little work has been carried out on the mechanism of invasion of Saprolegnia into the skin although a chymotrypsin-like action has been suggested (Peduzzi et al 1976; Peduzzi & Bizzozero 1977). Sexual differences in the pattern of

infection were noted in the present study and though these may be linked to structural differences in the skin in different areas of the body, this was not examined and provides an area for future research. Another possibility, suggested by Richards & Pickering (1978) is that these differences in infection pattern may have a behavioural origin: for example, increased damage to the tail area might be expected in a female fish involved in redd-digging.

The histological features of Saprolegnia infection in the skin have been described and are essentially similar to those described in fungal infection in the skin by Roberts and his co-workers. Although the Loch Leven condition defies description as U.D.N., histological changes very similar to "pre-fungal" lesions of U.D.N. were seen in the Loch Leven fish, and this may suggest that these changes are not specific to U.D.N. Moreover, when serial sections of such areas were examined, a single fungal hypha could usually be observed penetrating the epidermis. This presumably is equivalent to the earliest stage in fungal development and localised spongiosis may represent an enzyme action of the invading fungus. More difficult to relate to fungal penetration is the development of bullae in the deeper layers of the epidermis. Fungal hyphae are not frequently found in such areas but this reaction may still possibly be a result of a fungal product affecting cells in the area and one may perhaps speculate on some form of immune reaction connected with the presence of lymphocyte-like cells in the area.

Fungal development seldom penetrated deeper than very superficial musculature and was not involved with the stimulation of a marked inflammatory response unless bacterial infection was also present. Although small numbers of circulating bacteria were found, even in apparently normal fish, particularly in the colder months, confirming the findings of Bisset (1947, 1948), Thorpe & Roberts (1972), histological evidence of bacterial lesions was lacking in the majority of fish. This suggested that the original work of Thorpe & Roberts (1972) was carried out in a year with a specific aeromonad problem which did not recur during the course of this investigation. This may perhaps be explained as an imbalance in the trout population of the Loch during 1970/71 which was naturally adjusted by a reduction in population from bacterial infection. In the spawning seasons examined during the present study, fewer fish died and very few died of bacterial infection. Examination of serum parameters showed that the fish were losing large quantities of ions and proteins, presumably through defects in the integument. The loss cannot however be explained by simple haemodilution and it is not known whether the particular haematological effects found in this study were specifically related to this type of fungal infection. In order to clarify this point, it would be necessary to carry out similar examinations after infecting brown trout with different species of fungus or to remove areas of skin and monitor the blood changes resulting from such interference. These changes in ionic and protein content

certainly seem linked to variations in electrocardiographic pattern as one might expect from a comparison of the effects of ion loss in other animals. The changes in electrocardiographic pattern are difficult to analyse in terms of specific ions because variation in many ion species is occurring simultaneously. It would be interesting to carry out perfusion experiments to determine the effects of particular ion deficiencies on heart function in fish. Even if this were carried out, a true picture of heart muscle action would not really be obtained as the electrocardiogram is measuring differences in ionic concentration both within and without cardiac muscle cells. Measurements with intracellular electrodes would add a great amount of detail to the general picture already obtained.

Changes in skin structure in particular and in other organs to a lesser extent during sexual maturation does seem linked to the development of fungal infection in salmonids. As these changes have been linked to high levels of circulating androgens at this time in other species (see Section VI) it seemed a logical step to investigate the effects of these compounds on the brown trout. In order to remove the effects of endogenous androgens, gonadectomised fish were used during most of this work. Unfortunately, because of losses through pollution during the course of this work and limited availability of 11-ketotestosterone results were only obtained from limited numbers of fish and detailed statis-

tical analysis of results did not prove possible. Nevertheless, epidermal thickening, interrenal hyperplasia and a variety of organ changes were obtained with all three hormones used confirming the findings of McBride & Van Overbeeke (1971) and others. 11-ketotestosterone was most interesting in its effects as it reproduced the epidermis-dermis rugae and the fall in mucous cell numbers seen in mature Loch Leven fish. Unfortunately only very few fish were used in this study and the work needs to be expanded considerably before any definite conclusions may be drawn. The activity of 11-ketotestosterone in fish presents a major difference to the mammalian situation. Considerable interest in mammalian endocrinology is now centred around the active metabolite of testosterone, dihydrotestosterone. This compound was shown in the present work to be an active androgen in fish. Perhaps the most interesting question yet to be answered in fish endocrinology is the nature of the active metabolite of 11-ketotestosterone in fish - does a dihydro form of 11-ketotestosterone exist and is it the potent active androgen in salmonids? Experiments utilising this compound would perhaps answer many questions on the basis of fish androgen activity. Enzyme studies similar to those carried out by Hay, Hodgins & Roberts (1976) may further elucidate the situation, particularly in terms of quantifying differences between sexually mature and immature fish.

Another problem which may significantly influence the choice of any method of future prevention of sexual

maturation is that of androgen biosynthesis by the adrenal gland. This certainly does occur to a limited extent in some fishes (Idler & MacNab 1967) but significant amounts of androgens are not thought to be formed. The subject certainly seems to merit further research.

As well as the high mortalities found in natural populations of spawning salmonids, there are likewise problems associated with sexual maturation amongst farmed salmonids. Mortality due to fungal and bacterial infections is common in broodstock salmonids in the peri-spawning period, particularly after stripping fish, when considerable traumatic damage occurs. Such fish respond poorly to antifungal agents and presumably either osmotic loss through the damaged integument or reinfection with new fungal spores ultimately leads to the death of such fish.

Another particular problem associated with the farming of salmonids is the development of precocious sexual maturity, particularly amongst male fish (Schmidt & House 1979). This situation also arises in wild fish populations and such fish are thought to make a significant contribution to total fecundity of the species. Precocious Pacific salmonids apparently generally survive spawning (Robertson 1957, Gebhards 1960, Lee & Power 1976). The incidence of precocious maturity has been reported as high as 64% in certain steelhead trout hatcheries (Schmidt & House 1979) and death from fungal infection

of precocious male fish is a major problem in many salmonid farms in Britain. At present there are a variety of approaches to the solution of this problem. Treatment with antifungal agents, in particular malachite green, has been a standard therapeutic measure to reduce losses from cutaneous fungal infection. The use of malachite green has been restricted, particularly in America, leaving no other available effective antifungal agent. Considerable research effort has correspondingly been directed towards the finding of new therapeutic agents. The alternative approach is one of prevention of sexual maturity. As the testis commonly matures in the second year of growth, whilst the ovary seldom develops till the third year, a method very suitable for prevention of maturity in table-size (approximately half pound) rainbow trout is sex reversal to produce all female stock (Johnstone, Simpson & Youngson 1978). This is effectively carried out by feeding 17β-oestradiol to juvenile salmonids during the first few weeks of life, and as the half-life of oestradiol in rainbow trout was shown by the above workers to be less than twelve hours, there are no problems of harmful tissue residues. Another possible method of producing all-female stocks is to mate masculinised animals of female genotype with normal females. However, though this has been reported in the medaka (Oryzias latipes) by Yamamoto (1969), similar work on salmonids has not yet proved successful.

The major drawback to producing feminised stock is

that these fish may still become sexually mature females at a later date and problems of skin infection, poor food conversion and loss of pigment from flesh may still occur if fish are raised for longer periods of time to a larger size. An approach which avoids this further complication is one of gonadectomy. A method currently being investigated at Stirling University is surgical castration, as described by Brown & Richards (1979). Up to the present time, however, gonad regeneration has been unacceptably high and modifications of the technique are being investigated in order to reduce this. Another method also being investigated at Aberdeen University is to immunologically castrate fish by inducing an auto-immunity to gonad tissue, but the results of this study are as yet inconclusive. Either method, if successful, would allow salmonid culture in salt water to expand considerably and involve the production of much larger fish.

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APPENDIX 1

Histological Methods

Processing Schedule

50% Methylated Spirits	1 hour
80% " "	2 hours
8% Phenol in methylated spirits	3 hours
8% " "	2 hours
8% " "	2 hours
Absolute alcohol	2 hours
" "	1 hour
Chloroform	1 hour
"	1 hour
Wax	2 hours
"	2 hours
"	1 hour

HAEMATOXYLIN AND EOSIN STAIN (H & E)

1.	Xylene	5 minutes
2.	Absolute Alcohol	2 "
3.	Meths	1½ "
	Wash	
4.	Mayer's Haematoxylin	10 "
	Wash	
5.	1% Acid Alcohol	1 - 3 dips
	Wash	
6.	Scott's Tap Water Substitute*	Till Blue
	Wash well	
7.	Eosin	5 minutes
	Wash	
8.	Meths	30 seconds
9.	Absolute Alcohol I	2 minutes
10.	Absolute Alcohol II	1 minute
11.	Xylene	5 minutes

* After Scott's Tap Water Substitute, sections are examined microscopically and

if too dark, differentiated again in 5.

if too light, process is repeated from 4.

P.A.S. Method (Per-iodic acid Schiff)

Reagents

A. 0.5 per cent aqueous per-iodic acid

B. Schiff's reagent

Basic fuchsin	1	g.
Sodium metabisulphite	1.9	g.
N/1 hydrochloric acid	15	ml.
Distilled water	85	ml.
Activated charcoal	0.5	g.

The basic fuchsin and metabisulphite are dissolved in the acid and water. The mixture is shaken frequently during two hours. The charcoal is added and the mixture is shaken well for 1-2 minutes followed by filtration and storage at 0-4°C. The solution should be colourless; when it becomes coloured it must be discarded. The reagent loses its properties in 6-8 weeks.

C. Sulphurous acid rinse

10% aqueous sodium metabisulphite	6 ml.
N/1 hydrochloric acid	5 ml.
Distilled water	to 100 ml.

This solution must be freshly prepared but the constituents may be kept as stock solutions, in which case the metabisulphite is stored at 0-4°C.

D. Carazzi's haematoxylin

E. Saturated solution (0.3 per cent) of tartrazine in cellosolve

Method

1. Sections to water
2. Per-iodic acid 2 minutes
3. Running tap water 5 minuts
4. Rinse in distilled water
5. Schiff's reagent 15-20 minutes in a Coplin jar

6. Sulphurous acid rinse 2 minutes
7. Sulphurous acid rinse 2 minutes
8. Sulphurous acid rinse 2 minutes
9. Running tap water 5-10 minutes
10. Carazzi's haematoxylin 1 minute
11. Running tap water 5-10 minutes
12. Rinse in alcohol
13. Tartrazine ½-1 minute
14. Rinse in alcohol
15. Clear, and mount

Results

Positive result: magenta
Nuclei: greyish-blue
Other structures: yellow

Martius-Scarlet-Blue (MSB)

Reagents

A. Weigert's haematoxylin

B. 1 per cent hydrochloric acid in 70 per cent alcohol

C. Yellow mixture

Martius yellow	0.5 g.
Phosphotungstic acid	2 g.
Water	5 ml.
Alcohol	95 ml.

D. Red mixture

Brilliant Crystal Scarlet 6R	1 g.
Glacial acetic acid	2.5 ml.
Distilled water	97.5 ml.

E. 1 per cent aqueous phosphotungstic acid

F. Blue mixture

Aniline blue	0.5 g.
Glacial acetic acid	1 ml.
Distilled water	99 ml.

Method

1. Sections to water
2. Weigert's haematoxylin 15-20 minutes
3. Running tap water 5 minutes
4. Acid alcohol 5-10 seconds
5. Running tap water 5-10 minutes
6. Rinse in alcohol
7. Yellow mixture 2 minutes
8. Rinse in tap water
9. Red mixture 10 minutes
10. Rinse in tap water
11. Phosphotungstic acid 5 minutes
12. Rinse in tap water
13. Blue mixture 10 minutes
14. Rinse in tap water
15. Dehydrate, clear, and mount

Results

Fibrin: bright red
Muscle: red
Red blood corpuscles: yellow
Nuclei: grey
Collagen; basement membranes; reticulin; elastic fibres: blue
Other structures: pale

Alcian blue for mucin (Lison's modification)

Reagents

A. Weigert's haematoxylin

B. 1 per cent hydrochloric acid in 70% alcohol

C. Alcian blue solution

1% aqueous alcian blue	100 ml.
1% aqueous acetic acid	100 ml.
Thymol	a few small crystals

D. Curtis's picro-ponceau mixture

1% aqueous solution of ponceau S	10 ml.
Saturated aqueous solution of picric acid	90 ml.
Glacial acetic acid	1-2 ml.

Method

1. Sections to water
2. Weigert's haematoxylin 20 minutes
3. Running tap water 5 minutes
4. Acid alcohol 10-20 seconds
5. Running tap water 5 minutes
6. Alcian blue solution 10 minutes
7. Rinse in tap water
8. Picro-ponceau mixture 1-2 minutes
9. Rinse briefly in water
10. Dehydrate rapidly, clear, and mount.

Result

Acid mucopolysaccharides: bluish-green
Nuclei: dark grey
Collagen: red
Other structures: yellow

Barrett's Method for Pituitary

Reagents

A. Weigert's haematoxylin

B. 1 per cent hydrochloric acid in 70 per cent alcohol

C. Red mixture

Acid fuchsin	2.5g
Ponceau 2R	2.5g
1% aqueous acetic acid	500ml

D. 2 per cent aqueous acetic acid

E. Blue mixture

Aniline blue	10g
Distilled water	400ml
Glacial acetic acid	10ml

F. Red Differentiator

Stock differentiator	160ml
20% alcoholic haematoxylin	80ml
Alcohol	80ml
Distilled water	80ml

Stock differentiator

Phosphotungstic acid	100g
Picric acid	11.6g
Alcohol	380ml
Distilled water	20ml

Method

1. Sections to water
2. Weigert's haematoxylin 15-20 minutes
3. Running tap water 5 minutes
4. Acid alcohol 5-10 seconds
5. Running tap water 5-10 minutes
6. Red mixture 5 minutes
7. Rinse in weak acetic acid
8. Blue mixture 3 minutes
9. Rinse in weak acetic acid
10. Red differentiator ½-2 minutes
11. Rinse in weak acetic acid
12. Dehydrate rapidly, clear, and mount

Results

Acidophil granules: red
Basophil granules: blue
Chromophobe cells: pale blue-grey
Nuclei: dark grey
Red blood corpuscles: orange-red

Note: Differentiation is controlled microscopically and must continue intil the acidophil cells are no longer purple but clear red.

Per-iodic acid Schiff and orange G

Reagents

A - D As for the per-iodic acid Schiff method

E Orange G mixture

Orange G	2g
Phosphotungstic acid	5g
Distilled water	100ml

The above are mixed well; allowed to stand for 24 hours and filtered before use.

Method

1-11 As in the per-iodic acid Schiff method
12. Orange G 10-20 seconds
13. Differentiate in running tap water, using microscopical control, until the acidophil cells stand out clearly (15-45 seconds).
14. Dehydrate, clear, and mount.

Results

Basophil granules and granules in some chromophobe cells: magenta

Acidophil granules: orange
Other chromophobe cells: unstained
Nuclei: dark grey
Red blood corpuscles: yellow

Slidders's Method

Reagents

A. Bromine water

10% aqueous hydrobromic acid	45ml
2.5% aqueous potassium permanganate	5ml

Mixed immediately before use.

B. Alcian blue mixture

Alcian blue 8GX	0.1g
Concentrated sulphuric acid	1ml
Glacial acetic acid	9ml
Distilled water	90ml

The dye and the sulphuric acid are mixed with a glass rod. The acetic acid is then cautiously added and mixed thoroughly. The distilled water is then added, the solution mixed and filtered before use.

C. Weigert's haematoxylin

D. 1 per cent hydrochloric acid in 70 per cent alcohol

E. Orange G. mixture

Orange G	0.5g
Phosphotungstic acid	2g
Alcohol	95ml
Distilled water	5ml

The mixture is heated in a water bath and then cooled and filtered before use.

F. Fuchsin mixture

Acid fuchsin	0.5g
Glacial acetic acid	0.5ml
Distilled water	99.5ml

G. 1 per cent aqueous phosphotungstic acid

H. Light green mixture

Light green	1.5g
Glacial acetic acid	1.5ml
Distilled water	98.5ml

Method

1. Sections to water
2. Rinse in distilled water
3. Bromine water 5 minutes
4. Running tap water 2-3 minutes
5. Rinse in distilled water
6. Alcian blue ½-1 hour
7. Running tap water 5 minutes
8. Weigert's haematoxylin 15-20 minutes
9. Running tap water 5 minutes
10. Acid alcohol 5-10 seconds
11. Running tap water 5-10 minutes
12. Rinse in alcohol
13. Orange G 2 minutes
14. Rinse in distilled water
15. Fuchsin 2-5 minutes
16. Rinse in distilled water
17. Phosphotungstic acid 5 minutes
18. Rinse in distilled water
19. Light green 1-2 minutes
20. Rinse in distilled water
21. Dehydrate, clear, and mount.

Results

S granules of basophil cells: dark green-blue
R granules of basophil cells: magenta red .
Acidophil granules: orange-yellow
Chromophobe cells: pale grey-green
Nuclei: dark grey
Red blood corpuscles: yellow
Connective tissue: pale green

Aldehyde fuchsin

Reagents

A. Acidified permanganate

1% aqueous potassium permanganate	50ml
Distilled water	50ml
Concentrated sulphuric acid	0.5ml

B. 2 per cent aqueous sodium metabisulphite

C. Gomori's aldehyde fuchsin

Basic fuchsin	1g
70% alcohol	200ml
Concentrated hydrochloric acid	2ml
Paraldehyde	2ml

The fuchsin is dissolved in the alcohol, the acid and paraldehyde are added, then left at room temperature for 48-72 hours, mixing occasionally.

D. Loffler's methylene blue

1% alcoholic solution of methylene blye	30ml
1% aqueous potassium hydroxide	1ml
Distilled water	90ml

Method

1. Sections to water
2. Permanganate 2 minutes
3. Rinse in distilled water
4. Sodium metabisulphite until decolorized (1-2 minutes)
5. Running tap water 2 minutes
6. Rinse in alcohol
7. Aldehyde fuchsin 2 minutes in a closed vessel
8. Rinse well three times with 95% alcohol
9. Wash well in running tap water
10. Loffler's methylene blue diluted with 9 volumes of distilled water 30-40 seconds
11. Rinse in tap water
12. Dehydrate, clear, and mount.

Results

Pancreatic beta cells; pituitary basophil cells; elastic fibres: purple.

Other structures: colourless to pale blue

Phloxine tartrazine

Reagents

A. Carazzi's haematoxylin

B. 1 per cent hydrochloric acid in 70 per cent alcohol

C. Phloxine solution

Phloxine	0.5g
Calcium chloride (anhydrous)	0.5g
Distilled water	100ml

This solution keeps well.

D. Saturated solution of tartrazine in cellosolve (0.3 per cent)

Method

1. Sections to water
2. Carazzi's haematoxylin 10 minutes
3. Running tap water 5 minutes
4. Acid alcohol 5-10 seconds
5. Running tap water 5-10 minutes
6. Phloxine 30 minutes
7. Rinse in tap water
8. Rinse in alcohol
9. Tartrazine until only the red blood corpuscles and pancreatic beta cells remain red (5-15 minutes)
10. Rinse in alcohol, clear, and mount.

Results

Pancreatic beta cells; red corpuscles; fibrin; red

Nuclei: greyish blue

Other structures: yellow

Grocott's method for fungi

Reagents

A. 5 per cent aqueous chromic acid

B. 1 per cent aqueous sodium bisulphite

C. Silver solution

Gomori's silver-methenamine solution	25ml
Distilled water	25ml
5% aqueous sodium tetraborate (borax)	2ml

This solution must be freshly prepared.

D. 0.1 per cent aqueous gold chloride

E. 2 per cent aqueous sodium thiosulphate

Method

1. Sections to water
2. Rinse in distilled water
3. Chromic acid 1 hour
4. Wash in tap water
5. Sodium bisulphite 1 minute
6. Running tap water 5 minutes
7. Rinse three times in distilled water
8. Silver solution 30 to 60 minutes at 50°C in the dark
9. Rinse three times in distilled water
10. Gold chloride 3 minutes
11. Rinse in distilled water
12. Sodium thiosulphate 2 minutes
13. Running tap water 5 minutes
14. Counterstain as required
15. Dehydrate, clear, and mount.

Results

Fungal cell wals: black
Inner parts of fungi; some mucin: dull purple-red

Weigert's elastic stain (Hart's modification)

Reagents

A. Weigert's stock solution

Resorcin-fuchsin	1.5g
95% alcohol	200ml
Concentrated hydrochloric acid	4ml

Ingredients are boiled for 20 minutes in a water-bath and then cooled.

B. 1 per cent hydrochloric acid in 70 per cent alcohol

C. Working solution

Weigert's stock solution	5-20ml
Acid alcohol (solution B)	45-30ml
Total	50ml

The exact proportion varies with each new batch of stock solution and must be discovered by trial and error. An ideal mixture will stain all elastic fibres, including fine ones, but no other tissue component. It must be replaced after 2-3 weeks, or sooner if much of it evaporates.

D. Harris's haematoxylin

E. Curtis's picro-ponceau mixture

1% aqueous solution of Ponceau S	10ml
Saturated aqueous solution of picric acid	90ml
Glacial acetic acid	1-2ml

Method

1. Sections to Water
2. Rinse in alcohol
3. Elastic stain (working solution) overnight.
   The container should be closed.
4. Rinse in acid alcohol
5. Running tap water at least 10 minutes
6. Harris's haematoxylin 10 minutes
7. Running tap water at least 5 minutes
8. Differentiate in acid alcohol 10-15 seconds
9. Running tap water at least 10 minutes
10. Curtis's picro-ponceau mixture 2-5 minutes
11. Rinse rapidly in running tap water
12. Dehydrate rapidly, clear, and mount.

Results

Elastic fibres: black
Collagen: red
Nuclei: grey
Other structures: yellow

Millon Reaction (Modified by Baker, 1956)

Reagents

A. Mercuric sulphate solution

Sulphuric acid (conc.)	10 ml
Distilled water	90 ml
Mercuric sulphate	10 g

The acid is added to the water and then the mercuric sulphate is dissolved in the solution by heating. When cool, the solution is made up to 200 ml.

B. 0.25% aqueous solution of sodium nitrate.

Method

1. Sections to water
2. Place sections in beaker containing 30 ml mercuric sulphate solution and 3 ml sodium nitrate solution. Heat gently until solution boils and leave sections for 30 seconds.
3. Remove sections and rinse in three changes of distilled water (1-2 minutes each).
4. Dehydrate, clear and mount.

Results

Tyrosine - containing proteins are stained red, pink or yellow-red.

Gram-Humberstone Technique

Solutions

A. Gallego's fuchsin

Carbol fuchsin	3 ml
Glacial acetic acid	0.5 ml
Distilled water	50 ml

B. Gallego differentiator

Distilled water	50 ml
40% formaldehyde	1 ml
Glacial acetic acid	0.5 ml

C. 1% aqueous crystal violet

D. Gram, 1884

Potassium iodide	2 g
Distilled water	300 ml

Dissolve the potassium iodide in the water; when it has dissolved, add 1 g. of iodine.

E. Tartrazine	0.1 g
Cellosolve	100 ml

Method

1. Remove wax from paraffin sections with xylene and bring section through graded alcohols to tap water.
2. Stain in solution A for 5 minutes
3. Rinse in tap water
4. Differentiate and fix basic fuchsin in solution B for 5 minutes
5. Rinse well in tap water
6. Stain in solution C for 2 minutes
7. Rinse in distilled water
8. Mordant in solution D for 5 minutes
9. Rinse in distilled water
10. Blot section dry
11. Differentiate in solution E until violet stain no longer streams away from section
12. Blot dry
13. Clear in xylene
14. Mount in any neutral mountant such as Xam

Results

Gram-positive organisms	-	blue-black
Gram-negative organisms	-	brick red
Nuclei	-	brick red
Background	-	bright yellow

APPENDIX 2

Polyacrylamide Gel Methodology

a) Solutions to give gel of pH 8.9 containing 7% w/v acrylamide.

	Reagent/100ml water		Ratio by volume
Solution A)	Acrylamide	28.0g)	
)	Bis	0.735g)	2
Solution B)	1N HCl	48 ml)	
)	Tris	36.6g)	
)	TEMED	0.75 ml)	1
Solution C	$(NH_4)_2S_2O_8$	0.14g	4
Solution D	Water		1

b) Method

One volume of B is added to two volumes of A and one volume of water in a buchner flask. Four volumes of C are then added and the mixture is degassed prompty using a vacuum pump.

Electrophoresis tubes are then filled about 3/4 full with the degassed mixture and distilled water is layered over the top of the gels, which are left for about half an hour to polymerise. When polymerised the water is shaken out and the tubes fitted into the electrophoresis apparatus, which contains a buffer at pH 8.3 (2.88g glycine + 0.6g tris/1 water).

The serum samples are then diluted 1:10 with distilled water and a 5µl sample together with 5µl 0.05% bromo-phenol blue as tracking dye placed onto the top of the

gel through a layer of sucrose solution (80gm/100ml water).

A current of 4 milliamps/tube is then passed for 1½ hours.

Staining of Gels

Gels are first fixed in 12.5% acetic acid for 30 minutes and then transferred to the dye solution for 30 minutes. The dye used in the present study was Coomassie Blue R250 (1% solution) diluted 1:20 with 12.5% acetic acid.

Excess stain is then removed in 10% methanol/10% acetic acid.

Samples are then read with a densitometer.

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Frequency and distribution patterns of *Saprolegnia* infection in wild and hatchery-reared brown trout *Salmo trutta* L. and char *Salvelinus alpinus* (L.)

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Abstract. Fungal infection of sexually mature brown trout and char was associated with a particular type of *Saprolegnia* exhibiting a low degree of homothallic sexuality. Hatchery-reared brown trout were more severely infected (in terms of the % area of the body covered with the fungus) than were wild fish. The fins of hatchery-reared fish were particularly prone to *Saprolegnia* infection regardless of sex. In wild brown trout, a sexual difference in the pattern of infection was demonstrated. The flanks of the male fish appeared to be more prone to infection when compared with the female and there was a greater susceptibility of the caudal and ventral fin of the female when compared with the male. Evidence is presented which suggests that the incidence of infection in mature male salmonid fish prior to spawning is significantly greater than in the females. This difference may not be apparent in spent fish after spawning. These findings are discussed in relation to the background concentration of fungal spores in the water, the behavioural characteristics of spawning fish and differences in epidermal structure.

Introduction

Fungal infection, or 'saprolegniasis', of teleost fish is usually considered to be secondary to bacterial or viral diseases (Wolke 1975; Richards 1977) or the consequence of physical damage to the surface of the fish (Roberts & Shepherd 1974). The condition may be caused by any one of a wide variety of aquatic fungi (Scott & O'Bier 1962) and on occasions several different fungi may occur together on a single fish (Willoughby 1970; Pickering & Willoughby 1977). However, there is a small body of experimental evidence which suggests that, under certain conditions, *Saprolegnia* spp. may act as primary pathogens (Tiffney 1939; Hoshina, Sano & Sunayama 1960; Neish 1977).

In salmonids, fungal infections are often associated with sexual maturity of the fish (Roberts & Shepherd 1974; White 1975; Neish 1977) and in this situation the infection often consists of a single fungal strain (or group of closely related strains), with a low degree of homothallic sexuality, belonging to the genus *Saprolegnia* (Neish 1977; Pickering & Willoughby 1977; Willoughby & Pickering 1977). These particular fungus strains have been variously referred to as *Saprolegnia parasitica* (Coker 1923; Kanouse 1932; see also Neish 1977), *Saprolegnia* Type 1 (Willoughby

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1969) and *Saprolegnia diclina* Humphrey Type 1 (Willoughby 1978). A report published whilst the present investigation was in progress indicates that a difference in the pattern of *Saprolegnia* infection may exist between sexually mature male and female brown trout, *Salmo trutta* L. (White 1975). It is known that a sexual dimorphism occurs in the structure of the epidermis of the sea trout, *Salmo trutta* L. (Stoklosowa 1966, 1970) and hatchery-reared, brown trout (Pickering 1977). This difference in epidermal structure consists, in part, of a reduction in the concentration of goblet cells, and since the continual secretion of mucus by the epidermis is believed to protect the fish by preventing potential pathogens from colonizing the body surface (Jakowska 1963) it was of interest to investigate the pattern and frequency of *Saprolegnia* infection in sexually mature salmonid fish in greater detail.

The present paper consists of an analysis of the frequency of occurrence of *Saprolegnia* and its regional distribution on the body and fins of both wild and hatchery-reared, brown trout and char *Salvelinus alpinus* (L.).

Materials and methods

Fish

Brown trout and char were reared at the hatchery of the Freshwater Biological Association (FBA) Laboratory in large (≈1500 litres), outdoor, fibreglass tanks supplied with a constant flow of Windermere lake water (normal temperature range 3–14°C). The fish were fed commercial trout food in the form of dry pellets. On sampling, the fish were sexed and classified as either immature or mature.

Wild brown trout were obtained from Windermere during the months October–January of the years 1967–76. The majority of the fish were caught in gill nets designed primarily to catch pike (Thompson & Bagenal 1973). Fish from Windermere were classified as immature (a group which includes 'resting' fish—see Frost & Brown 1967) or mature. Mature fish were further divided into ripe fish which had not yet migrated into the spawning streams and spent fish which had spawned and returned to the lake.

Wild brown trout were also obtained from the streams supplying Loch Leven, Kinross, Scotland, and from the outlet to the River Leven, using box traps throughout the years 1973–75 (for details of techniques and trapping sites see Thorpe & Roberts 1972). State of maturity was assessed on a scale of I–VIII using the method of Kesteven (1960). For the purposes of the present analysis fish from groups III–VIII were considered to be mature. Only mature fish were used to determine the pattern of fungal infection on the body and fins. Fish with no obvious signs of fungal infection were also obtained from Loch Leven by beach seine at monthly intervals and sampled bacteriologically and histologically.

A sample of wild char was obtained by means of gill nets set close to their spawning grounds in Windermere during January 1976. The majority of these fish were sexually mature and would have spawned on their spring spawning grounds

within a month (for details of the life history of the char in Windermere (previously referred to as *S. willoughbii* (Gunther)), see Frost 1965).

Each fish was anaesthetized with MS 222 (Sandoz), examined by eye for the presence of fungus on the body and fins and classified accordingly as clean or infected. The positions of any fungal patches were carefully mapped on a standard chart outlining the body and fins, a procedure which was adopted for both sides of the fish. Thus, two charts showing the distribution pattern of infection were normally obtained for each fish. The percentage area of the fish infected by fungus was calculated from these charts and the number of fungal colonies/side (when individual colonies could be clearly distinguished) was determined. The fish was then weighed and measured, killed by spinal section and the state of sexual maturity determined.

Microbiology

(a) *Mycology*. During the course of this investigation a random sample of thirteen fungal isolates from infected fish was kindly identified by Dr L. G. Willoughby (FBA). The sample consisted of seven isolates from wild brown trout in Windermere, four isolates from wild brown trout in Loch Leven, one isolate from hatchery-reared brown trout and one isolate from wild char in Windermere. The isolates were initially grown in glucose–yeast extract–penicillin–streptomycin (GY–PS) agar, transferred to sterile hempseeds and then incubated with sterile lake water at 7°C to encourage the formation of sexual structures (for details of techniques see Willoughby & Pickering 1977).

(b) *Bacteriology*. Samples were taken from both fungal infected and non-infected fish from Loch Leven between 1973 and 1975. Fish were anaesthetized in MS 222 and opened by a midline abdominal incision. Samples were taken aseptically from spleen, liver and kidney and plated out on to nutrient agar (Difco) and incubated at 20°C. Isolates were subsequently purified by subculture and identified biochemically according to *Bergey's Manual of Determinative Bacteriology*, 8th edition 1974.

Histology

Small samples (0·5 cm^3) of heart, spleen, liver and kidney were removed, fixed in 10% neutral buffered formalin, embedded in paraffin wax and sectioned at 5 μm. Sections were stained with Ehrlich's acid haematoxylin and eosin (H & E) and by the gram Humberstone technique (Humberstone 1963).

Statistics

Each standard chart illustrating the distribution pattern of fungal infection was divided into 391 squares by means of superimposed graph paper with numbered divisions and a record was made of the presence or absence of fungus within each square. This was performed on every chart from fish within a particular group and the incidence of infection for each of the 391 areas on the body and fins was obtained. The

incidence of infection for each square was then compared by χ^2 analysis with the mean incidence of infection for all the squares. Thus, a statistical comparison was made for each of the 391 areas on the body and fins between the observed incidence of infection and the expected incidence of infection if no significant pattern existed. The levels of significance for each square were then replotted on a standard chart and significant patterns of infection were obtained. Using similar techniques it was also possible to determine the areas of the fish significantly uninfected.

A further χ^2 analysis was made *between* the patterns of infection obtained from different groups of fish. In this case the observed incidence of infection within each of the 391 squares for a particular group was compared with the incidence of infection for the corresponding squares of another group of fish. The numbers of data were standardized by recalculating the incidence of infection of the larger number in terms of the smaller number. For example, if the comparison was between seventeen incidences out of a possible forty for one group of fish and ten incidences out of a possible fifty for the second group, this second figure was recalculated as $10 \times (40/50) = 8$. Thus, the χ^2 analysis would be between the observed figure of seventeen incidences out of forty and the expected figure of eight incidences out of forty if no difference existed between the patterns of infection of the two groups of fish. In this way it was possible to compare patterns of infection statistically and determine where significant differences between patterns occurred.

χ^2 analyses of the total numbers of infected and non-infected fish from different groups were performed using 2×2 contingency tables (Elliott 1971).

Results

Nature and severity of infection

Thirteen fungal isolates were identified according to the criteria of Willoughby (1978). All thirteen were determined as *Saprolegnia* spp. but three isolates (two from Windermere and one from Loch Leven) failed to produce the sexual stages at 7°C and 20°C and could not therefore be identified further. The remaining ten isolates all produced antheridia and oogonia at 7°C but not at 20°C. All showed the 'birdsnest' type of investment of the oogonium by the diclinous antheridia and attached zoospores. There was also appreciable non-development of oospores, apparently mediated by hyphal invasion of the oogonium. Oogonia were elongated in nine isolates, but in one case (an isolate from a brown trout in Loch Leven) the oogonia were spherical. All ten isolates were identified as *Saprolegnia diclina* Type 1 but the isolate with spherical oogonia appeared to have at least this one characteristic in common with *Saprolegnia diclina* Type 2 (Willoughby 1978).

Massive bacterial infection of all organs sampled was only found in four fish out of thirty samples from the streams supplying Loch Leven during the spawning season of 1973–74 and in three fish out of thirty in 1974–75. In all these cases pure cultures of *Aeromonas hydrophila* were obtained. In a further ten fish during the spawning season

of 1973–74 and in twelve fish during 1974–75 occasional colonies of a variety of bacteria (including *A. hydrophila*, *Acinetobacter* spp., *Moraxella* spp. and *Alcaligenes* spp.) and unidentified yeasts were isolated. The predominant organism within these fish was again *A. hydrophila*.

Table 1. The occurrence of occasional bacteria (predominantly *Aeromonas hydrophila*) in apparently healthy brown trout taken by seine net from Loch Leven during 1974

	Jan.	Feb.	March	April	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.
Mean monthly water temp. (°C)	3·2	4·9	4·6	10·4	11·7	15·7	15·8	15·9	14·2	9·7	6·6	5·0
No. fish with bacteria	2	1	2	2	1	0	0	0	0	2	3	3
No. fish in sample	8	8	8	8	8	8	8	8	8	8	8	8

The results of examination of ostensibly clean fish taken by seine net from Loch Leven are presented in Table 1. In no instance was massive bacterial infection found and of the bacteria isolated from apparently healthy fish, *A. hydrophila* again predominated.

Histological evidence of bacteraemic disease with areas of focal necrosis and cellular infiltration around concentrations of bacteria was only found in those seven fish showing massive bacterial infection on sampling. There was no evidence of any bacterial lesion in the fish from which only one or two colonies were isolated. Other histological changes were those associated with sexual maturity or with *Cotylurus* spp. metacercarial infection of heart and pericardium.

Table 2. Severity of *Saprolegnia* infection (as indicated by the % area infected and the number of fungal colonies) of sexually mature, diseased brown trout from three different populations. Mean ±s-e.m. (*n*)

	Sex	Area infected (%)	No. separate fungal colonies/side
Loch Leven, 1973–75	♂	26·7 ± 1·43 (50)	4·3 ± 0·25 (50)
	♀	22·9 ± 1·69 (44)	3·4 ± 0·25 (44)
Windermere, 1976	♂	17·9 ± 2·90 (13)	6·9 ± 1·31 (13)
Ferry House Hatchery, 1976	♂	51·0 ± 2·75 (86)	13·2 ± 1·13 (51)
	♀	38·6 ± 2·33 (46)	10·6 ± 1·03 (34)

The severity of the *Saprolegnia* infection (as indicated by the percentage area of the fish infected and the number of individual colonies) is presented in Table 2. It is clear that the hatchery-reared fish used in the present investigation had significantly

Table 3. Frequency of *Saprolegnia* infection in samples of wild and hatchery-reared brown trout and char

	Species	Sampling method	Date	Condition	Male Immature	Male Mature	Female Immature	Female Mature
Loch Leven	Brown trout	Traps	Oct.–Jan. 1973–75	Clean	6	33	0	29
				Infected	11	341	0	360
Windermere	Brown trout	Gill nets	Oct.–Jan. 1967–76	Clean	124	29	47	46
				Infected	13	40	1	24
Ferry House Hatchery	Brown trout	Total capture	Oct.–Dec. 1976	Clean	15	12	60	0
				Infected	0	43	6	2
Windermere	Char	Gill nets	Jan. 1976	Clean	4	21	7	28
				Infected	0	8	0	0
Ferry House Hatchery	Char	Total capture	Dec. 1975	Clean	0	13	0	22
				Infected	0	8	0	0

larger areas of the body and fins infected and significantly more fungal colonies than the wild fish from Loch Leven and Windermere.

Frequency of infection

The total numbers of clean and infected fish used during the course of this investigation are summarized in Table 3. χ^2 analyses of the data presented in this table reveal several interesting points. Sexually mature, hatchery-reared, male brown trout are more frequently infected with *Saprolegnia* than immature fish of either sex ($P<0{\cdot}001$). In view of the low number of sexually mature females within this group it is not clear whether this difference is due to the state of sexual maturity regardless of sex or a combination of both sex and state of maturity. The results from gill nets in Windermere indicate that mature brown trout of both sexes are more frequently infected than immature fish ($P<0{\cdot}001$ for both males and females) and therefore sexual maturation in both sexes appears to favour fungal infection. However, the data for both brown trout and char in Windermere, and for hatchery-reared char also, indicate that the incidence of infection in sexually mature male fish is significantly greater than in mature females ($P<0{\cdot}01$, $0{\cdot}01$, $0{\cdot}005$ respectively). This sexual difference in the frequency of infection of mature fish is not apparent in brown trout from Loch Leven. Furthermore, when the data for the brown trout in Windermere are analysed in more detail (Table 4) it becomes clear that the difference only occurs in the ripe fish prior to spawning ($P<0{\cdot}001$) and is not present in the spent fish.

Distribution patterns of Saprolegnia infection

Significantly infected areas on the body and fins of sexually mature brown trout from three different populations are illustrated in Fig. 1. It is clear that the patterns of infection for male and female fish from Loch Leven are totally different. In the male, the significantly infected areas occur along the dorsal surface of the body and extend ventrally towards the lateral line. By comparison, female fish from the same population are infected on the tail and peduncle region. It is interesting to note that the pattern of infection for mature, male, brown trout from Windermere is very similar to that for Loch Leven males. In mature, hatchery-reared, brown trout this sexual difference is not as apparent. In both sexes, the areas most vulnerable to *Saprolegnia* infection appear to be along the leading edges of the fins.

The most noticeable feature of the patterns illustrating the regions of the fish that are *not* infected by fungus (Fig. 2) is the area around the lower jaw, operculum and pectoral region (excluding the pectoral fin), an area which is significantly uninfected in all groups of fish. In mature males from Loch Leven, areas along the whole of the ventral surface of the fish appear to be free of the fungus as are large areas on the flanks of hatchery-reared, female brown trout.

When statistical comparisons are made between the patterns of infection in male and female fish a remarkable similarity can be seen between Loch Leven fish and hatchery-reared fish (Fig. 3). In both groups of fish there are large areas on the flanks

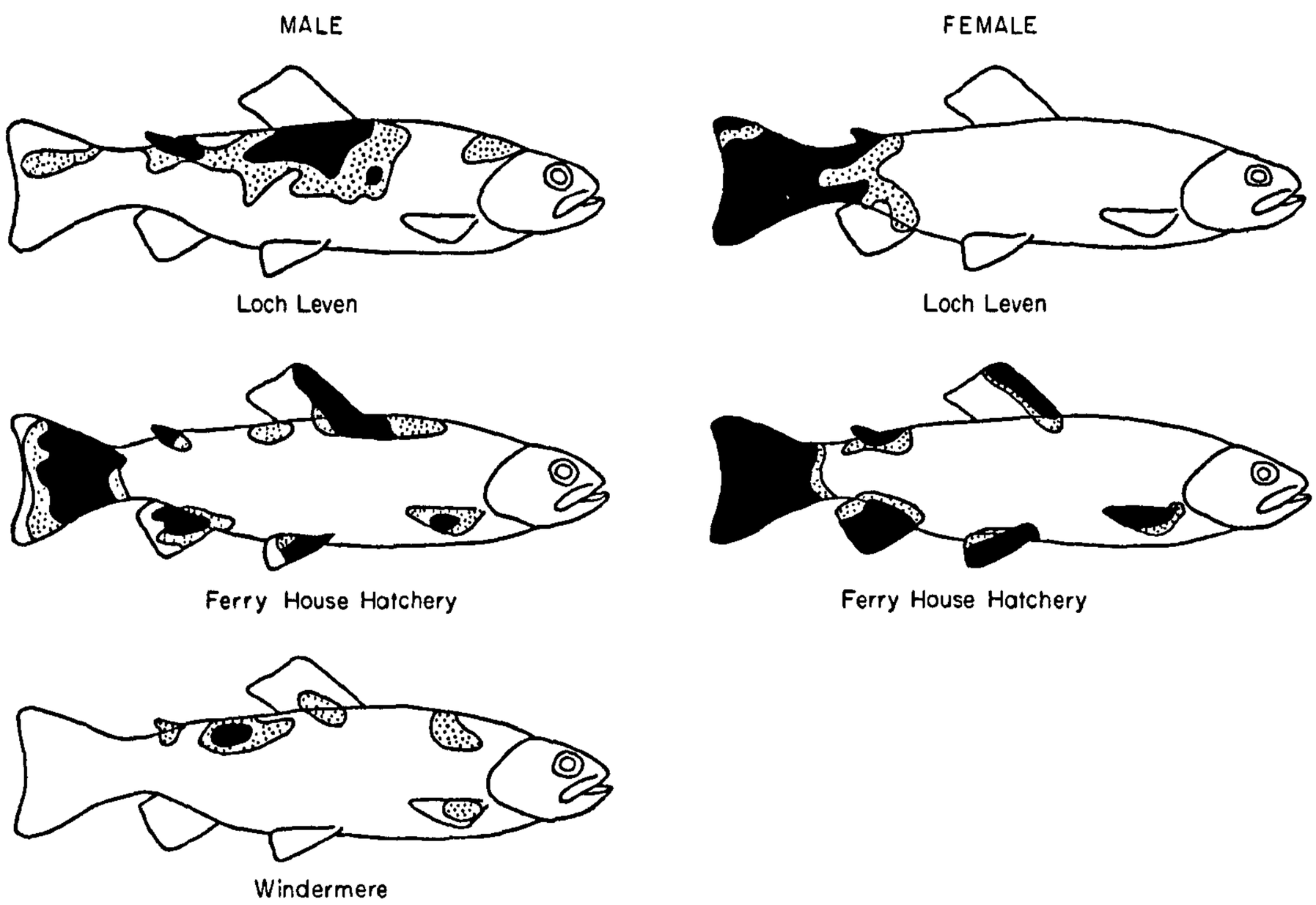

Figure 1. Patterns of infection of sexually mature brown trout from three different populations. Shaded areas are significantly more frequently infected than the rest of the body. Black: $P<0{\cdot}001$; stippled: $P<0{\cdot}05$.

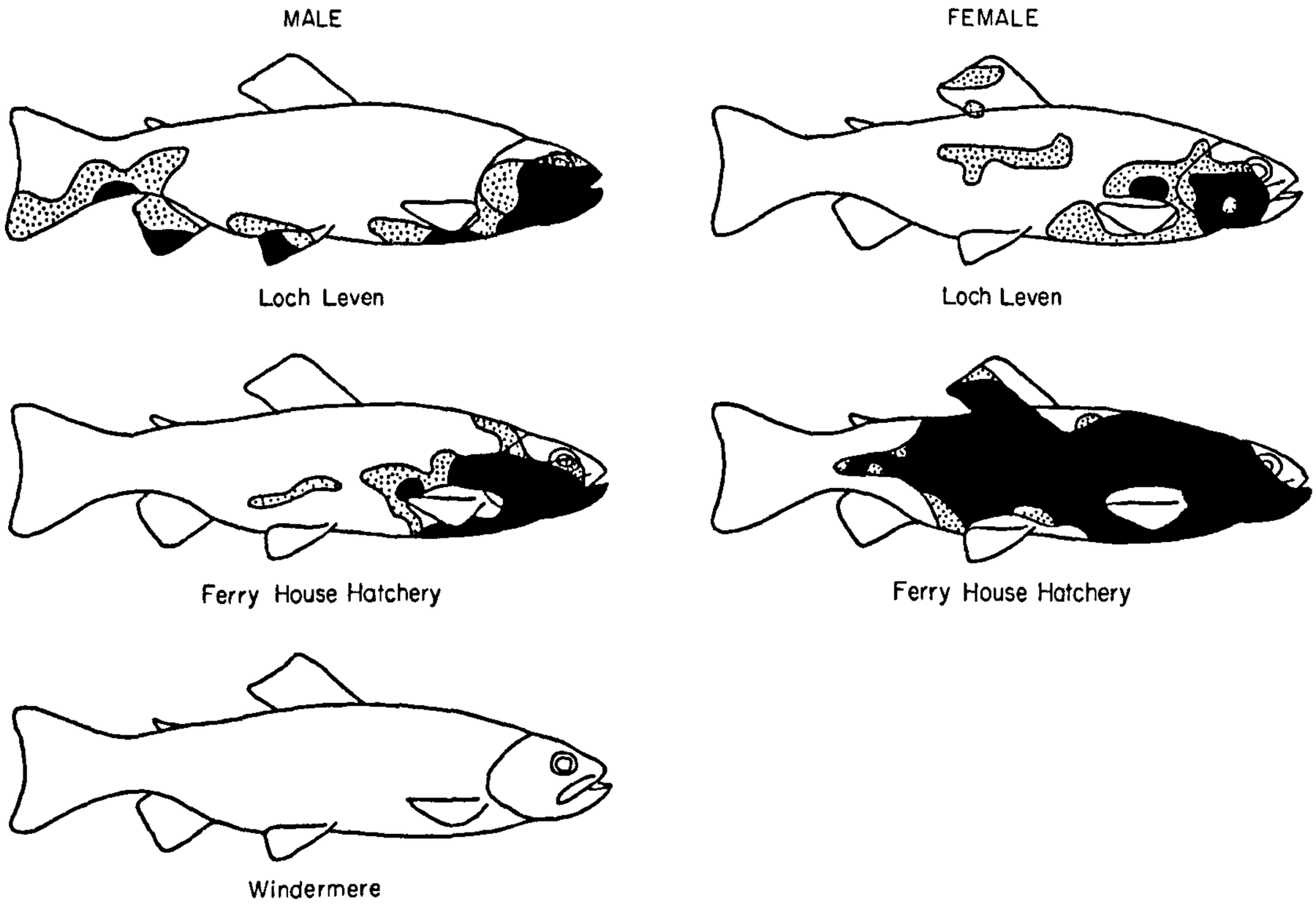

Figure 2. Uninfected areas on the body and fins of sexually mature brown trout from three different populations. Shaded areas are significantly less frequently infected than the rest of the body. Black: $P<0{\cdot}001$; stippled: $P<0{\cdot}05$.

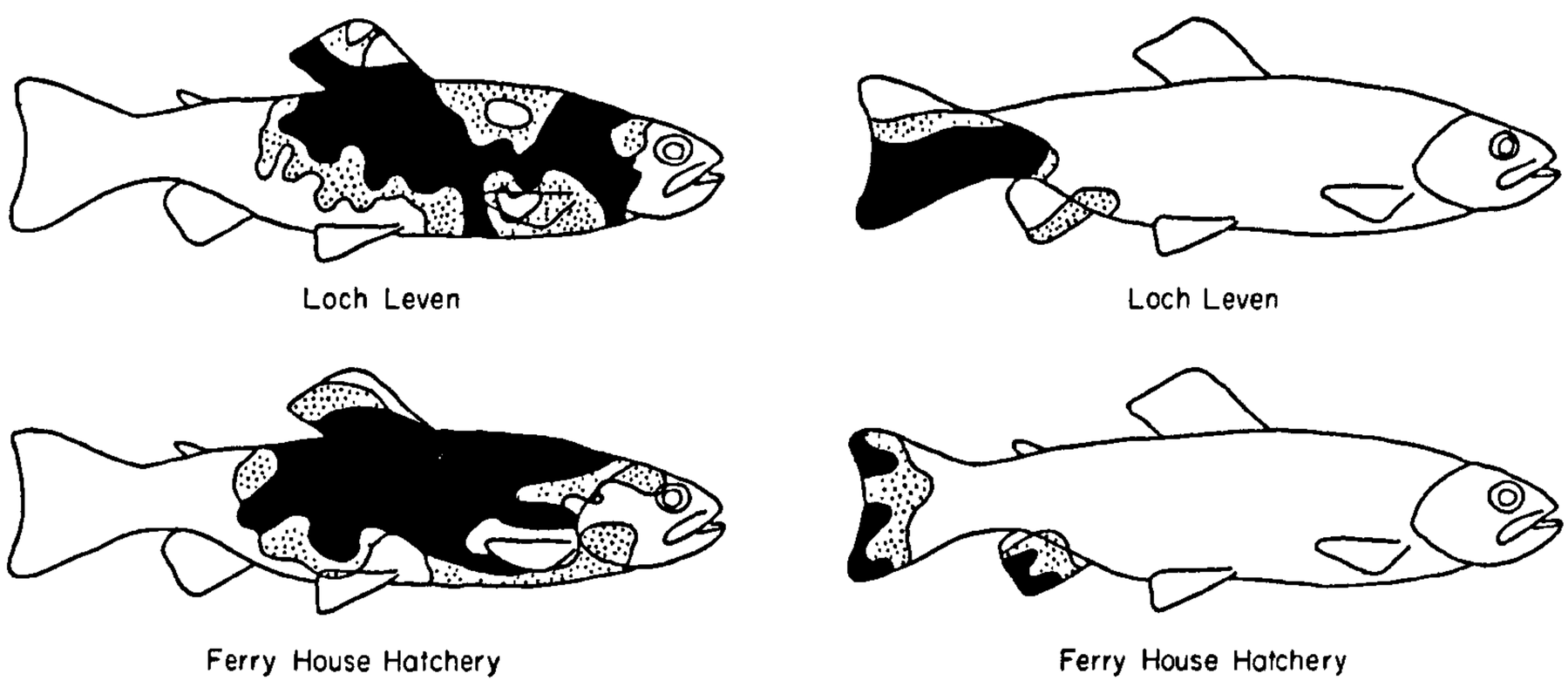

Figure 3. A statistical comparison of the patterns of infection between male and female brown trout from Loch Leven and Ferry House Hatchery. Black: $P<0{\cdot}001$; stippled: $P<0{\cdot}05$.

Table 4. Frequency of fungal infection on immature, ripe and spent brown trout taken by gill nets from Windermere during October–January, 1967–76

	Male			Female		
	Immature	Ripe	Spent	Immature	Ripe	Spent
Clean	124	22	7	47	24	22
Infected	13	34	6	1	6	18

of the males which are significantly more frequently infected than the females. Furthermore, the tail and ventral fin of the females from both groups of fish are significantly more infected than the males. This similarity is all the more striking when one recalls that the hatchery-reared fish are significantly more frequently infected on the fins than are the wild fish from Loch Leven (Fig. 1).

Discussion

Isolates from a sample of fungal-infected fish all proved to be *Saprolegnia* spp. The majority of these were further identified as *Saprolegnia diclina* Type 1 (Willoughby 1978) but the remaining isolates were sexually sterile at both 7°C and 20°C. *Saprolegnia diclina* Type 1 has been previously referred to as *Saprolegnia* Type 1 (Willoughby 1969) and *Saprolegnia parasitica* (Coker 1923; Kanouse 1932). Thus, fungal infection of the salmonid fish used in the present investigation appeared to be associated

with a particular *Saprolegnia* strain (or group of closely related strains) with a low degree of homothallic sexuality, thus supporting the observations of Neish (1977) and Willoughby & Pickering (1977). This is in contrast to the situation with the perch in which a variety of aquatic fungi (representing several genera) may colonize lesions on the body surface (Pickering & Willoughby 1977).

An aeromonad epidemic of brown trout in Loch Leven was described by Thorpe & Roberts (1972) in which at least 7700 adult fish were thought to have died. This mortality occurred during the spawning season of 1970–71 and was confined to sexually mature adults. Many of the fish which were affected were also suffering from severe fungal infections. During the spawning runs of 1973–74 and 1974–75 a smaller number of fungal-infected fish were obtained from the traps and there was no recurrence of the massive mortalities of 1970–71. Bacteriological investigations revealed that only a small number (seven out of sixty fish samples) were suffering from bacterial septicaemia in contrast to the findings of Thorpe & Roberts in 1970–71. Thus, the majority of the fish examined from Loch Leven during the present investigation did not have primary bacterial infections but we cannot discount the possibility that the *Saprolegnia* was secondary to viral or parasitic infections.

Small numbers of bacteria may be isolated from apparently healthy fish, particularly during the colder months of the year. The bacteria present were of a variety of types with *Aeromonas hydrophila* predominating, possibly a reflection of the normal intestinal or skin flora at the time of sampling (Bisset 1948). Clearance of bacteria from tissues of rainbow trout *Salmo gairdneri* Richardson, has been shown to be temperature-dependent, and the time taken for clearance is much slower at low temperatures (Finn & Neilsen 1971). The prevalence of bacteria in Loch Leven brown trout at lower temperatures may possibly be explained in the same manner.

Saprolegnia-infected brown trout from Loch Leven and Windermere had 20–25% of the body surface covered with fungus at the time of capture. By comparison, up to 50% of the body surface of hatchery-reared brown trout was infected. This difference in the severity of the infection is probably the result of differences in the numbers of separate fungal colonies found on the fish. Hatchery-reared fish had a significantly greater number of individual colonies growing on the body than did wild fish. It seems likely that this is at least partly a reflection of the background level of fungal spores within the water. Under hatchery conditions the background spore count may rise to over 20,000 spores/l (Willoughby & Pickering 1977) whereas the normal spore count does not exceed 5000 spores/l in Windermere (Willoughby 1962) and 4000/l in Loch Leven (R. H. Richards, unpublished).

A variety of sampling techniques were used to assess the frequency of infection of salmonid populations in the wild and under hatchery conditions. The effectiveness of a gill net (used to sample trout and char from Windermere) is dependent upon the activity of the fish and we have little information upon the selectivity of this technique particularly with respect to diseased fish. Thus, caution must be used when interpreting data from gill nets in terms of the total population from which the sample was taken. There can be little doubt that the traps set to catch brown trout in the spawning streams of Loch Leven were highly selective for diseased fish. Direct

observation confirmed that the more active, uninfected fish could avoid or escape from the traps. Thus, the data provided by this technique give an erroneously high impression of the incidence of infection within the spawning population of Loch Leven. Hatchery fish were sampled by total capture of all the fish within an infected pond and therefore these data are an accurate record of the total population within that particular pond but do not reflect the situation in the rest of the hatchery.

With these considerations in mind, it still seems clear from the results that sexual maturity of the brown trout favours the occurrence of *Saprolegnia* infection in both sexes. This is in agreement with the opinion of Roberts & Shepherd (1974) and confirms the observations of White (1975). Furthermore, it is also evident that under certain conditions sexually mature male brown trout and char are more frequently infected than sexually mature females. This is particularly true of ripe fish prior to spawning but this sexual difference may not be apparent in spent fish. In salmonid fish, it is common for the survival rate of spawning females to be greater than that of males despite the apparently greater metabolic demand for gonadal maturation in the female. This sexual difference in mortality has been noted for the char (Le Cren & Kipling 1963), the Atlantic salmon *Salmo salar* L. (Hutton 1925; Netboy 1968) and the sea-trout (Lamond 1916).

In addition to the difference in frequency of infection of sexually mature male and female brown trout a marked difference in the pattern of infection occurs. This is not simply a reflection of the severity of the infection because it occurs when the percentage area of the body infected by the fungus is similar in the two sexes (Loch Leven fish—Table 2, Fig. 1). A statistical comparison of the patterns of infection reveals that there are large areas on the flanks of the sexually mature, male brown trout which are significantly more frequently infected than corresponding areas on the female. Equally, there are areas on the tail and ventral fin of the female which are more frequently infected than the corresponding areas on the male. White (1975) also found that spawning female brown trout had significantly more fungal infection in the caudal area and that the males were more frequently infected anteriorly. Thus, the present investigation confirms and extends this observation.

It has been suggested that the infection on the tail of the female results from damage inflicted during redd digging (White 1975). This conclusion is supported by our own observation that the incidence of infection in mature female brown trout from Windermere appears to increase after spawning. However, hatchery-reared, mature, female brown trout were also infected on the caudal and ventral fins but in this case the fish did not have a suitable substrate for spawning. It is possible that mature females make abortive attempts at redd building and spawning in the hatchery tanks as eggs are freely shed into the water and very few fish become egg-bound.

White (1975) suggests that the fungal infection on the body of the male brown trout results from abrasion during spawning behaviour and wounds received during territorial defence. This is certainly a possibility when the fish are on the spawning grounds but it seems unlikely to be a complete explanation. The data from Windermere indicate that many fish become infected in the lake before they begin their migration to the spawning grounds. In this connection it is interesting to note that a

sexual dimorphism exists in the structure of the skin of mature sea-trout (Stoklosowa 1966, 1970) and hatchery-reared, brown trout (Pickering 1977). One characteristic of this dimorphism is a reduction in the number of mucus-producing goblet cells in the epidermis of the male fish during the spawning period. Since mucus may help to protect the fish from fungal infection by removing viable spores from the body surface (Willoughby & Pickering 1977) we suggest that changes in the structure of the epidermis of sexually mature, male brown trout may increase its vulnerability to *Saprolegnia* infection.

The present investigation has shown that hatchery-reared brown trout of both sexes are particularly prone to fungal infection on the fins. Damage to the fins frequently occurs when fish are kept under crowded conditions and in these circumstances bacterial fin-rot may be a common occurrence. Furthermore, the concentration of mucous cells of both hatchery-reared and wild salmonid fish is significantly lower on the fins than on the rest of the body (Pickering 1974). Any or all of these factors could contribute to the vulnerability of the fins of hatchery-reared fish to fungal infection.

In all groups of fish the anterior regions, particularly around the head, operculum and pectoral areas, were significantly less infected than the rest of the body. The bony areas of the undamaged head may provide a less suitable substrate for the establishment of fungal colonies than the softer tissues elsewhere. It is also interesting that a concentration gradient of mucous cells from anterior (high concentration) to posterior (low concentration) has been reported for the brown trout (Pickering 1974).

Further work is now being directed towards epidermal/fungal spore interactions on salmonid fish in an attempt to define more clearly the factors responsible for colonization of fish skin by *Saprolegnia*, to factors influencing the structure of the salmonid epidermis and to the mechanisms by which *Saprolegnia* ultimately kills its teleost host.

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Changes in serum parameters of *Saprolegnia*-infected brown trout, *Salmo trutta* L.

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Abstract. Fungal infection of both wild and hatchery-reared sexually mature brown trout, *Salmo trutta* L., was caused by *Saprolegnia diclina* Humphrey Type 1. The major ions in the serum of infected fish were all significantly reduced in concentration and it is suggested that the primary cause of death was osmoregulatory breakdown resulting in a lethal haemodilution. Severe hypoproteinaemia and a significant reduction in the albumin/globulin ratio were reflected in the electrophoretogram of the serum proteins from infected fish.

Introduction

Fungal infections of teleost fish are generally considered to be secondary conditions associated with other diseases or with physical damage. Nevertheless, a small body of evidence indicates that in certain situations aquatic fungi may act as primary pathogens (Tiffney 1939; Vishniac & Nigrelli 1957). Pathogenic fungi have been isolated from a wide variety of teleost fish (Scott & O'Bier 1962; Wilson 1976) with as many as four different species of fungi occurring together in a mixed colony on a single fish (Pickering & Willoughby 1977). In salmonid fish, however, fungal infections often involve a single strain (or group of closely related strains) of *Saprolegnia* (Neish 1977; Willoughby 1968, 1969, 1971). Willoughby (1978), in a recent analysis of diseased salmonids from Windermere, refers to this particular fungus as *Saprolegnia diclina* Humphrey Type 1. Sexually mature brown trout, *Salmo trutta* L., are particularly vulnerable to fungal infection and a significant difference exists between the pattern of infection on male and female fish (White 1975; Richards & Pickering 1978). In our experience, fungal infection frequently results in the death of the fish but the immediate cause of this mortality has not been clearly demonstrated. There can be little doubt that infection of small fish may result in a lethal invasion of internal organs by fungal hyphae (Bootsma 1973; Nolard-Tintigner 1973) but in the case of large salmonid fish the infection would appear to be restricted to the body surface or superficial musculature (Stuart & Fuller 1968). Impaired osmoregulatory capabilities

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have been demonstrated in fungal-infected Atlantic salmon *Salmo salar* L. with ulcerative dermal necrosis (Gardner 1974).

The present investigation considers the changes in serum osmotic pressure, ionic content and protein patterns of the brown trout in relation to the degree of *Saprolegnia* infection during the spawning season.

Materials and methods

Fish

Fifty-three, sexually mature, brown trout (mean weight 1074·0 g) were taken by traps from the streams supplying Loch Leven and from the outlet to the River Leven during the periods October–December 1973–1975 (for details of techniques and trapping sites see Thorpe & Roberts 1972). Eighteen out of 29 males and 15 out of 24 females had obvious signs of fungal infection. Twenty uninfected brown trout were also obtained from Loch Leven by beach seine during the periods March–June 1973–1975.

Thirty, sexually mature, 2 and 3 year-old, brown trout (mean weight 128·5 g) from the Freshwater Biological Association's hatchery at the Ferry House were used during the period October–December 1976. Eighteen out of 21 males and six out of nine females showed obvious signs of fungal infection. The fish were reared in large (1500 l), outdoor, fibreglass tanks supplied with a constant flow of Windermere lakewater (normal temperature range 3–14°C), and fed with a commercial food in the form of dry pellets.

Each fish was anaesthetized in MS222 (Sandoz), weighed and measured. The distribution of any fungal infection was carefully mapped on a standard chart outlining the body and fins and the percentage area of the fish infected by the fungus was calculated from this chart (for details of technique see Richards & Pickering 1978). After taking a blood sample from the caudal peduncle the fish was killed by spinal section and the sex and state of maturity determined.

Serum analyses

Blood samples (1–5 ml) were allowed to clot in centrifuge tubes at ambient temperature for 30 min and then spun down at 2000 rpm and the serum removed. When a delay of more than 30 min between sampling and centrifugation was inevitable, the blood samples were stored on ice.

Sodium and potassium were measured with a Corning Eel Flame Photometer (Model 430) and calcium and magnesium with a Unicam SP 90A Atomic Absorption Spectrophotometer. Total protein concentration was determined using the Biuret method and serum albumin levels were measured with an autoanalyser using the bromocresol green technique (Northam & Widdowson 1967). Osmotic pressure was measured with an Advanced Instruments Osmometer.

Polyacrylamide gel electrophoresis was carried out according to the method

described as System 1 by Norris & Ribbons (1971), but it was found necessary to add 30 λ temed to each 32 ml gel mixture to aid gelling.

Serum samples were diluted 1:10 with distilled water and 5 μl samples together with 5 μl of tracking dye (0·05% bromophenol blue) were layered in a sucrose solution (80 g/100 ml) on to the gel and a current of 4 milliamps/tube passed for 1½ h. Gels were stained with Coomassie Brilliant Blue R250 and destained in 10% methanol/10% acetic acid.

A semi-quantitative estimate of the protein composition of the serum was achieved with a Unicam SP 1800 Densitometer at 544 nm. From the traces obtained, the relative movement and relative area of each peak were calculated.

Microbiology

During the course of this investigation, five randomly selected fungal isolates from infected brown trout from each locality were kindly identified by Dr L.G. Willoughby (FBA). The isolates were initially grown in glucose-yeast extract–penicillin-streptomycin (GY–PS) agar, transferred to sterile hemp seeds and then incubated with sterile lake water at 7°C to encourage the formation of sexual structures (for details of techniques see Willoughby & Pickering 1977).

Bacterial samples were taken from the spleen, liver and kidney of both fungal-infected and non-infected brown trout from Loch Leven and plated out on to nutrient agar (Difco) and incubated at 20°C. Isolates were subsequently purified by subculture and identified biochemically according to Bergey's *Manual of Determinative Bacteriology* (8th edn, 1974).

Results

The five fungal isolates taken at random from different, infected fish were all identified as *Saprolegnia diclina* Type 1 (see Willoughby 1978). Since there was no evidence of severe bacterial infection in any of the fish used during the present investigation, it would seem that the fungi were not secondary pathogens associated with a primary bacterial infection.

Saprolegnia infection of brown trout from the spawning streams of Loch Leven resulted in significant reductions in the serum concentrations of sodium, potassium, calcium, magnesium and protein (Table 1). Moreover, there was a direct, and highly significant, correlation between the serum sodium concentration and the degree of infection of the fish when expressed as the percentage of the body surface area colonized by *Saprolegnia* (Fig. 1). A similar, significant, correlation was found between the serum osmotic pressure and the degree of infection of hatchery-reared brown trout (Fig. 2). When these two correlations were standardized by expressing the serum osmotic pressure and sodium concentration as a function of the values for uninfected fish, a comparison of the two regression lines by analysis of variance showed that there was no significant difference between either the slopes of the two lines or their

Table 1. Composition of the blood serum of *Saprolegnia*-infected and non-infected brown trout from Loch Leven. Results expressed as mean ± S.E.M. (n). A 't' test comparison was made between infected and non-infected male and female fish sampled in the period October–December. Significant differences are denoted: * $p<0{\cdot}05$, † $p<0{\cdot}01$, ‡ $p<0{\cdot}001$

Sample	Sex	Na mM/l	K mM/l	Ca mM/l	Mg mM/l	Protein g/100 ml	Albumin: globulin ratio
Non-infected	♂	164·4	1·8	2·5	0·8	5·8	0·48
brown trout		±1·7 (10)	±0·06 (10)	±0·03 (10)	±0·01 (10)	±0·2 (10)	±0·01 (10)
March–June	♀	165·3	1·8	2·5	0·8	5·4	0·50
		±1·4 (10)	±0·05 (10)	±0·03 (10)	±0·01 (10)	±0·3 (10)	±0·01 (10)
Non-infected	♂	162·4	2·1	2·2	0·8	7·4	0·49
brown trout		±1·5 (11)	±0·09 (11)	±0·10 (11)	±0·01 (11)	±0·5 (11)	±0·01 (11)
October–December	♀	166·0	2·1	2·3	0·8	6·0	0·47
		±1·1 (9)	±0·07 (9)	±0·06 (9)	±0·01 (11)	±0·2 (11)	±0·01 (9)
Infected	♂	130·2‡	1·3*	1·5‡	0·5‡	3·3‡	0·25‡
brown trout		±4·7 (18)	±0·2 (18)	±0·1 (12)	±0·07 (14)	±0·4 (18)	±0·04 (18)
October–December	♀	131·1‡	1·2*	1·6‡	0·5†	3·4†	0·28†
		±4·7 (15)	±0·3 (15)	±0·14 (12)	±0·07 (12)	±0·6 (15)	±0·05 (15)

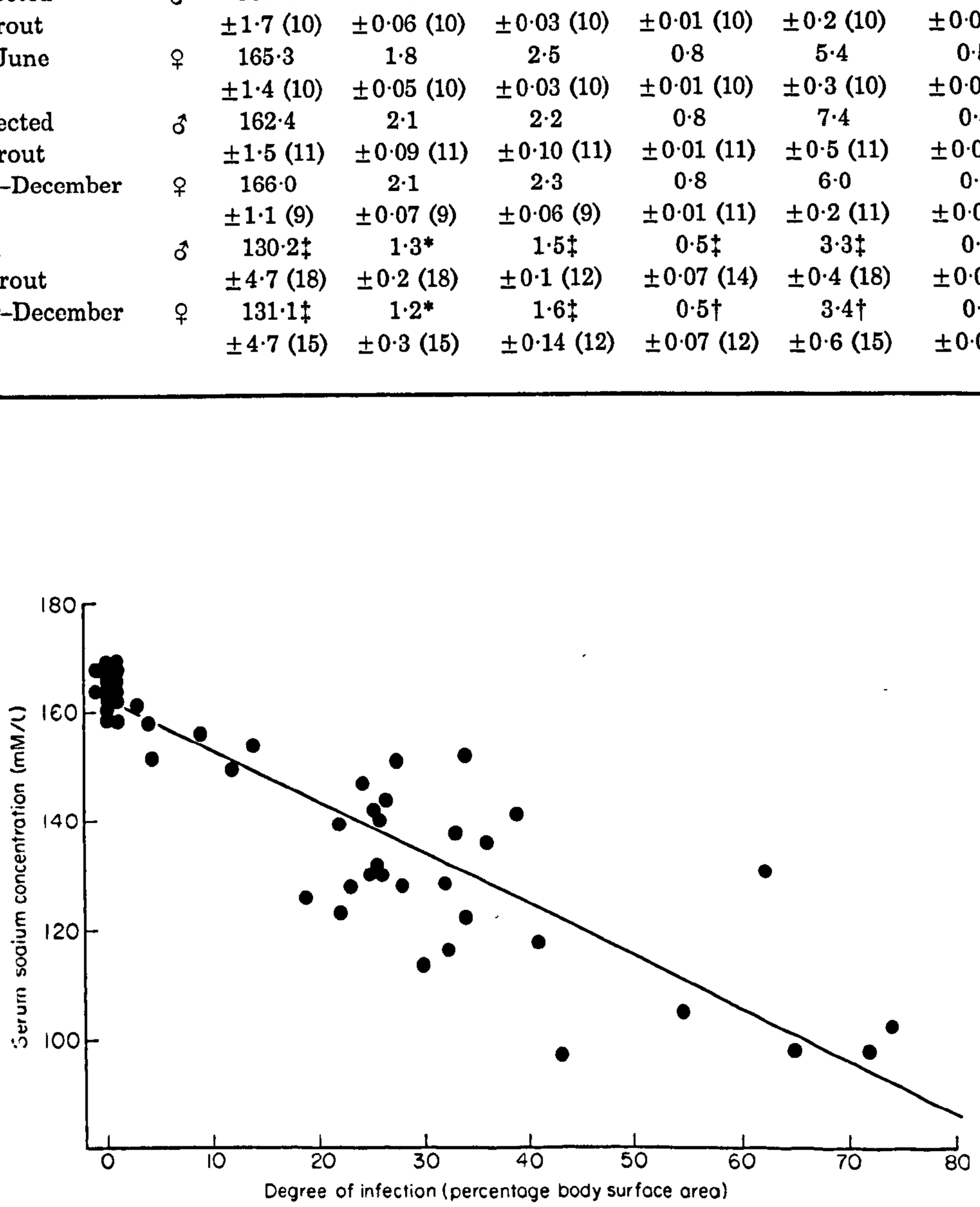

Figure 1. Correlation between the concentration of sodium in the blood serum of *Saprolegnia*-infected brown trout from the spawning streams of Loch Leven and the degree of infection expressed as the percentage body surface area of the fish covered by fungus. Regression analysis $y = -0{\cdot}94x + 161{\cdot}7$. Correlation coefficient $r = 0{\cdot}884$ ($p<0{\cdot}001$). Significance of the regression $p<0{\cdot}001$.

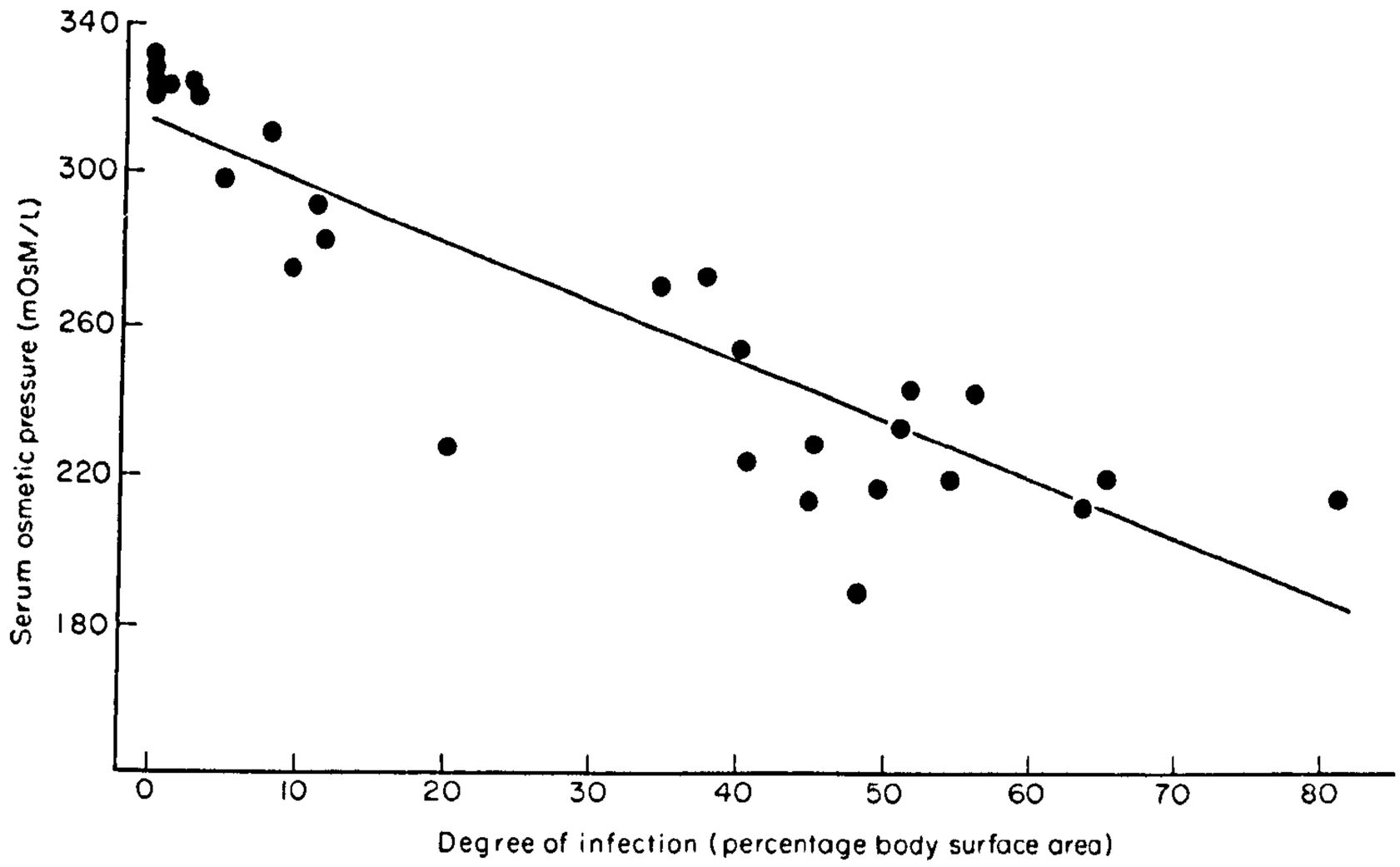

Figure 2. Correlation between the serum osmotic pressure of *Saprolegnia*-infected, hatchery-reared brown trout and the degree of infection expressed as the percentage body surface area of the fish covered by fungus. Regression analysis $y = -1{\cdot}64x + 313{\cdot}8$. Correlation coefficient $r = 0{\cdot}906$ ($p < 0{\cdot}001$). Significance of the regression $p < 0{\cdot}001$.

intercepts on the y axis. No apparent differences in response to *Saprolegnia* infection were noted between male and female fish but the mean proportional reduction in concentration of each ion as a result of infection varied between 20% for sodium and 40% for potassium.

A variation is also indicated in the proportional reduction in concentration of serum proteins by the significant decrease in albumin/globulin (A/G) ratio associated with fungal infection (Table 1). No consistent differences were noted between the electrophoretic patterns of male and female fish at either time of year (March–June and October–December). Thus, for the purpose of further analysis, the data obtained from both sexes were pooled. Electrophoresis revealed a total of 18 separate protein peaks from the serum of both infected and non-infected fish (Fig. 3) when identified by their mobilities relative to bromophenol blue. However, in view of the wide variation in serum electrophoretogram between individual brown trout, no attempt was made to identify individual peaks as particular serum proteins. Peak 1 was the only peak present in all the fish examined. The mean number of protein peaks from the serum of non-infected fish ($8{\cdot}95 \pm 0{\cdot}30$ (21), range 7–11) was not significantly different to that of infected fish ($9{\cdot}05 \pm 0{\cdot}28$ (21), range 7–11). In addition, χ^2 analysis failed to show any significant difference in the frequency of occurrence of any single protein peak between infected and non-infected fish. When estimates of the areas of individual peaks were made it was found that 10 out of the 18 peaks were significantly reduced in the infected fish. Moreover, if the peaks are pooled into four groups (group 1 = peaks 1, 2, 3, 4, 5; group 2 = peaks 6, 7, 8, 9; group 3 = peaks 10, 11, 12, 13; group 4 = peaks 14, 15, 16, 17, 18) in order of decreasing electrophoretic mobility,

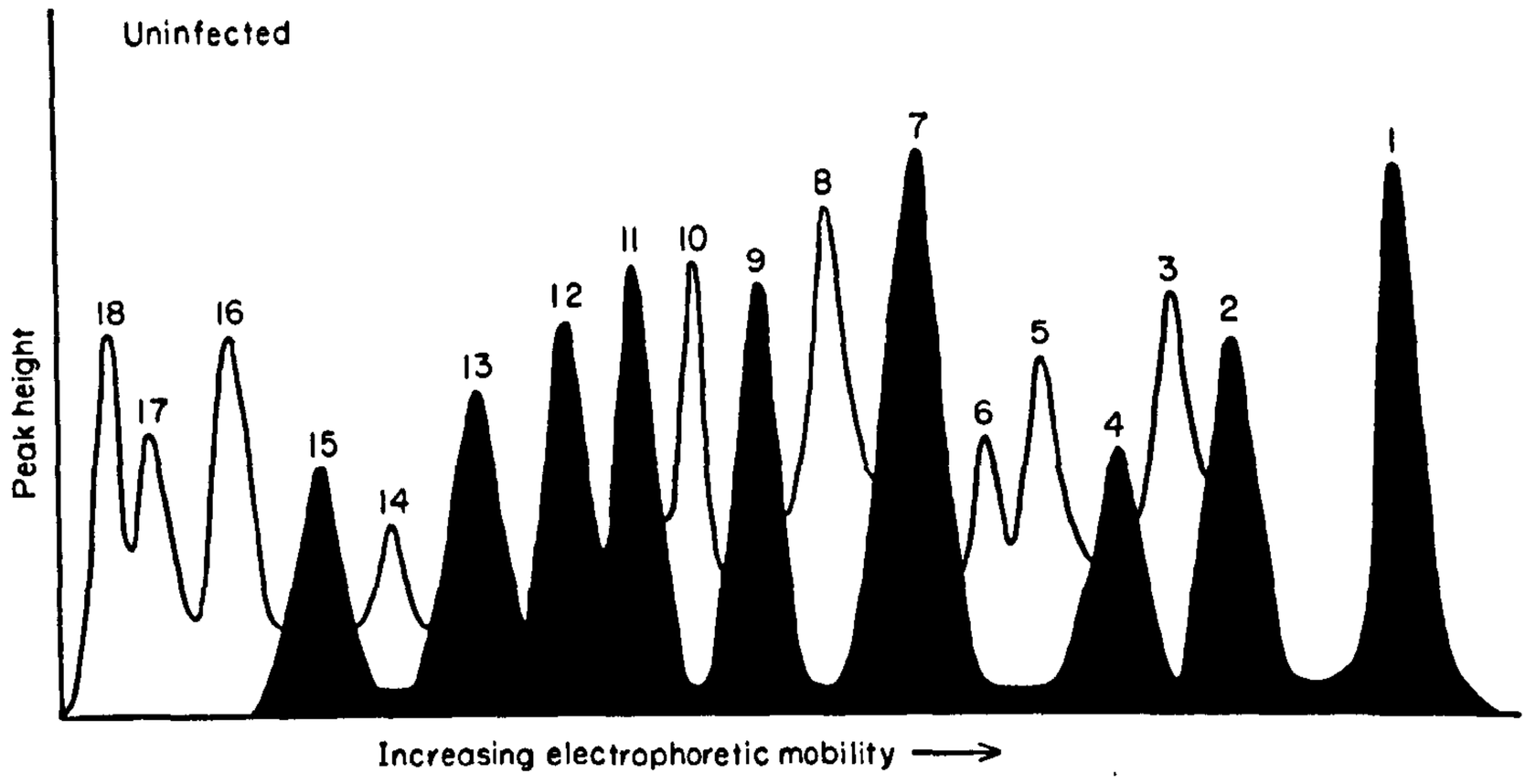

Figure 3. Diagrammatic representation of the serum electrophoretograms for uninfected and *Saprolegnia*-infected brown trout from the spawning streams of Loch Leven. The diagrams are based upon the mean dimensions of each peak for all the fish examined. Peaks were identified by their mobilities relative to bromophenol blue. Major peaks (occurring in over 50% of the fish) are represented by solid shading and minor peaks (occurring in less than 50% of the fish) are represented in white.

it can be shown that there are significant decreases in the mean area of the first three groups ($p < 0{\cdot}001$, Student's 't' test) but not in group 4, the group of slowest moving proteins.

Discussion

In the present investigation, all five fungal isolates taken at random from infected brown trout were identified as *S. diclina* Type 1 (Willoughby 1978). This is consistent

with previous observations on salmonid fish (Willoughby 1968, 1969, 1971; Neish 1977; Richards & Pickering 1978). In these earlier studies, however, the taxonomic status of the isolates was deferred pending more information and several of the *Saprolegnia* isolates failed to produce sexual organs and could not, therefore, be identified further. More recent ultrastructural evidence indicates that there may be a close relationship between the sexually sterile *Saprolegnia* isolates and *S. diclina* Type 1 (Pickering, Willoughby & McGrory 1979). This apparently specific association between salmonid fish and a particular fungus (or group of closely related fungi) contrasts markedly with the findings of Pickering & Willoughby (1977) on fungal-infected perch, *Perca fluviatilis* L. In this case a variety of different aquatic fungi with representatives from several genera were isolated.

We failed to find any evidence that the fungal infection of mature brown trout from Loch Leven was associated with a primary bacterial infection. However, from the work of Richards & Pickering (1978) it would seem likely that physical damage to the integument, incurred during the migration and subsequent spawning of brown trout in the streams supplying Loch Leven, could promote saprolegniasis.

The mean serum osmotic pressure (325·8 mOsm/l) of uninfected fish used in present work was identical to that reported by Gordon (1959) for the brown trout in fresh water. The normal sodium concentration (164 mM/l) was slightly higher than some reported values (Phillips & Brockway 1958; Gordon 1959), but Oduleye (1976) has shown that the plasma sodium concentration may vary between 110 and 187 mM/l depending upon the water temperature, calcium concentration and upon the time of year. Our serum potassium values would appear to be somewhat lower than previously reported values (Phillips & Brockway 1958; Oduleye 1976), but are within the range determined by Gordon (1959). Serum calcium and magnesium values for uninfected brown trout in fresh water were similar to those of Phillips & Brockway (1958).

Saprolegnia infection produced highly significant decreases in the serum concentration of all the ions measured. Indeed, significant correlations were demonstrated between the degree of infection (expressed as the percentage body surface area covered by the fungus) and the serum osmotic pressure and sodium concentration. The similarity of these two correlations supports the conclusion that the reduction in serum osmotic pressure is predominantly caused by reductions in the serum sodium and chloride concentrations. The loss of serum electrolytes in severe cases of *Saprolegnia* infection was so great as to decrease the sodium concentration from 164 mM/l to less than 100 mM/l. It has been shown that a similar drop in serum sodium concentration associated with acid water-induced osmoregulatory failure is fatal to brown trout (Leivestad, Hendrey, Muniz & Snekvik 1976). Clearly, this loss of electrolytes and protein from the damaged integument will depend not only upon the extent of the fungal colonization over the body surface of the fish but also upon the length of time the fish has been infected. We have no information from the present investigation concerning the length of time for which individual fish have been infected, but from our experience of hatchery-reared brown trout it would seem that fungal infection can cause mortality within 3 days. We suggest that under these conditions the pri-

mary cause of death is haemodilution. This conclusion supports the work of Gardner (1974) who related changes in blood serum of Atlantic salmon to UDN and *Saprolegnia* infection.

However, the changes in composition of the blood serum cannot be totally accounted for by a simple haemodilution due to an increased osmotic influx of water, because all the ion-species were not reduced by equivalent proportions. For example, the mean potassium concentration was reduced by 40% whereas the mean reduction of sodium was only 20%. Serum potassium reduction is a characteristic stress response in certain teleost fish (Oikari & Soivio 1975) and there can be little doubt that fungal infection in salmonid fish is a severe stress factor (Mazeaud, Mazeaud & Donaldson 1977). Calcium and magnesium were also reduced by a greater proportion than sodium in the infected fish.

The severe hypoproteinemia associated with *Saprolegnia* infection in the brown trout is similar to that noted by Mulcahy (1969) and Gardner (1974) for UDN infected salmon and by Carbery (1970) and Mulcahy (1971) for UDN infected brown trout. In the present work, this serum protein depletion was proportionally greater than any of the ionic changes so far discussed. Gardner (1974) found a dissociation between ionic and protein (measured as total nitrogen) regulation in UDN infected salmon as indicated by their ability to maintain a normal serum ionic composition, but not a normal protein level, when held in brackish water.

Protein concentration in the extravascular fluid has been shown to be similar to the plasma protein concentration in the marine teleosts *Gadus morhua* L. and *Pleuronectes platessa* L. and is thought to be due to high capillary permeability (Hargens, Millard & Johansen 1974). If such is the case in the brown trout, any loss of protein from the extravascular fluid at sites of fungal damage may be expected to be reflected by a similar rapid decrease in plasma proteins and may explain the rapidity with which the fungal-infected fish can die.

A major factor contributing to the hypoproteinemia is the relative reduction in the serum albumin levels as shown by the decrease in A/G ratio. However, the technique used to measure the albumin levels (bromocresol green) has been criticized on the grounds of specificity (Webster 1974; Webster, Bignell & Attwood 1974) and consequently the A/G ratios obtained during the present investigation must be treated with some reserve. Nevertheless, a decrease in the serum A/G ratio is also characteristic of UDN infected brown trout (Carbery 1970) and of Sacramento River chinook disease virus (SRCDV ≡ IHNV) infection in rainbow trout, *Salmo gairdneri* Richardson (Klontz, Yasutake & Parisot 1965). Thus it seems likely that a reduction in the A/G ratio may be a characteristic response of fish to many different diseases.

Electrophoretic analysis of the serum proteins of uninfected and *Saprolegnia*-infected brown trout failed to reveal any consistent qualitative changes associated with infection. This agrees with the observations of Mulcahy (1971) for UDN infected brown trout and contrasts sharply with her work on UDN infected salmon (Mulcahy 1969) in which the disease was associated with a specific electrophoretic pattern. We have provided some electrophoretic evidence that the drop in serum total

protein concentration is associated with the faster migrating proteins and that the slower moving bands are not reduced. Although we have made no attempt to characterize and identify individual protein peaks, by analogy with other work both on fish and mammals it would seem likely that the fastest moving proteins include the albumins and that the slower moving proteins are predominantly globulins. Thus this general change in electrophoretic pattern supports our observation that the A/G ratio is decreased in *Saprolegnia*-infected fish.

It is interesting to hypothesize that the relative increase in slower moving globulins is partly related to immunoglobulin production by the fish in response to the fungus. However, further work is needed on the characterization of serum proteins in healthy and *Saprolegnia*-infected fish before any hypothesis may be seriously tested.

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