Host parasite interactions between *Ichthyobodo necator* (Henneguy, 1883) and farmed salmonids

by

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DEDICATION

This work is dedicated to my wife Heather, without whose kindness, constant encouragement and many sacrifices this work could neither have been started nor completed.

CONTENTS

		Page
List of Tables		(i)
List of Figures		(ii)
Acknowledgements		(v)
Abstract		(vii)
CHAPTER 1	General Introduction and Literature Review	
1.1	General	1
1.2	Taxonomy of *Ichthyobodo necator*	1
1.3	Biology and life history of *Ichtyhobodo necator*	5
1.3.1	Life forms	5
1.3.1.1	Free swimming form	5
1.3.1.2	The attached form	6
1.3.1.3	The saprophagous form	6
1.3.1.4	Cyst form	6
1.3.2	Morphology and size	7
1.3.3	Reproduction	9
1.3.4	Feeding methods	10
1.4	Host parasite interactions	11
1.4.1	Clinical signs of costiasis	11
1.4.2	Hosts and geographic range	12
1.5	The effect of environmental variables	15
1.5.1	Temperature	15
1.5.2	pH	15
1.5.3	Salinity	16
1.6	Chemical treatment of *Ichthyobodo* infestations	17
1.7	Summary	20
CHAPTER 2	Seasonal infestations of farmed salmonids with *Ichthyobodo necator*	
2.1	Introduction	21
2.2. 2.2.1	Almondbank and Unilever sites	22
2.2.2	Materials and methods	23
2.2.3	Results	23
2.2.4	Discussion	23

2.3/

Page

CHAPTER 2 (cont.)

2.3 2.3.1 Howietoun Trials 24
2.3.2 Materials and Methods 25
2.3.2.1 Salmon 25
2.3.2.2 Rainbow trout 26

2.4 Results 27

2.4.1 Salmon fry 27
2.4.2 Rainbow trout fry 29
2.4.3 Rainbow trout 1+ 33
2.4.4 Appearance of Ichthyobodo 33
2.4.5 Correlations 35
2.4.6 Environmental variables 35
2.4.7 Other parasites 37
2.4.8 1979-1980 Data 38

2.5 Discussion 38

2.6 Summary of trials at Almondbank, Lochailort and Howietoun 42

CHAPTER 3 Pathogenesis and autoradiographic studies of the epidermis of salmonids infested with Ichthyobodo necator

3.1 Introduction 43

3.2 Materials and methods 44

3.2.1 Fish 44
3.2.1.1 Sequential pathology of costiasis in salmon alevins 44
3.2.1.2 Pathology of costiasis in salmon smolts 44

3.3 3.3.1 Autoradiography 45
3.3.1.1 In vivo study 45
3.3.1.2 In vitro study 45

3.4 Results 46

3.4.1 Salmon alevin pathology 46
3.4.2 Salmon smolt pathology 47
3.4.3 Rainbow trout alevin autoradiography 48
3.4.4 Salmon smolt autoradiograpy 49

3.5 Discussion 50

CHAPTER 4 Endocrinological aspects of Ichthyobodo necator infestations

4.1 Introduction 54

4.1.1 Oestrogens 54
4.1.2 Androgens 55
4.1.3 Corticosteroids 56
4.1.4 The effect of androgens and oestrogens on fish 57
4.1.5 The effect of corticosteroids on fish 59

4.2/

CHAPTER 4 (cont.) Page

4.2 Proprietory corticosteroid trial 60

4.2.1 Materials and Methods 60
4.2.2 Results 63
4.2.3 Discussion 65

4.3 Hydrocortisone and testosterone trial 65

4.3.1 Materials and Methods 65
4.3.1.1 Hydrocortisone 66
4.3.1.2 Testosterone 66
4.3.1.3 Fish 67
4.3.1.4 Inoculation 67
4.3.1.5 Sampling 69
4.3.1.6 Steroid assays 69
4.3.1.6.1 Testosterone 70
4.3.1.6.2 Cortisol 73
4.3.1.7 Histopathology 75

4.4 Results 76

4.4.1 Ectoparasites 76
4.4.2 Growth 84
4.4.3 Testosterone Assays 84
4.4.4 Cortisol Assay 86
4.4.5 Histopathology 90

4.5 Discussion 93

CHAPTER 5 Overall Discussion and Summary 104

References 110

APPENDICES 1 to 7

LIST OF TABLES

Table		Page
1	The taxonomic status of Ichthyobodo necator	2
2	Hosts of Ichthyobodo	13
3	Geographical distribution of Ichthyobodo infestations	14
4	Chemicals used in treatment of Ichthyobodo infestations	18-19
5	Intensity of Ichthyobodo infestations on the gills of Atlantic salmon fry	28
6	Intensity of Ichthyobodo infestations on skin and gills of 0+ rainbow trout	30
7	Weekly percentage mortality of the 0+ rainbow trout	32
8	Cumulative percentage mortality of 0+ rainbow trout infested with Ichthyobodo	32
9	Intensity of Ichthyobodo infestations on skin and gills of 0+ rainbow trout	34
10	Correlation of transformed Ichthyobodo levels on 0+ fish with other variables	36
11	Dilutions of proprietory corticosteroids inoculated into rainbow trout	62
12	Ectoparasite infestation attributable to proprietory corticosteroid injections	64
13	Hydrocortisone/testosterone trial. Brands allotted to each treatment	68
14	Coding used in Table 15	77
15	Results from implantation experiment	78-82
16	Mean testosterone levels of implanted and control fish	85
17	Cortisol levels in implanted and control fish	87
18	Cortisol levels and presence of Ichthyobodo on 0+ and 1+ rainbow trout at 5°C	89
19	Mean epidermal thickness of control and implanted fish	91
20	Mean goblet cell count/unit area of control and implanted fish	91

LIST OF FIGURES

Figure

1 Interference phase contrast micrograph of free living form of Ichthyobodo

2 Interference phase contrast micrograph of free living form of Ichthyobodo. Side view

3 Interference phase contrast micrograph of attached form of Ichthyobodo

4 Scanning electron micrograph of Ichthyobodo attached to epidermal cells of a salmonid host

5 Scanning electron micrograph of Ichthyobodo showing ventral view of the parasite

6 Scanning electron micrograph of Ichthyobodo shown the dorsal surface of the parasite

7 Prevalence of Ichthyobodo on 0+ salmon at Almondbank salmon farm

8 Prevalence of Ichthyobodo on 0+ salmon at Lochailort salmon farm

9 Intensity of Ichthyobodo infestations on 0+ salmon gills

10 Isometric diagram of weekly percentage prevalence of Ichthyobodo infestation on salmonids

11 Intensity of Ichthyobodo infestations on 0+ rainbow trout gills

12 Intensity of Ichthyobodo infestations on 0+ rainbow trout skin

13 Weekly percentage mortalities of 0+ rainbow trout caused by Ichthyobodo

14 Cumulative mortalities of 0+ rainbow trout caused by Ichthyobodo

15 Intensity of Ichthyobodo infestations on 1+ rainbow trout skin

16 Mean weekly water temperatures over the time period of the study

17 Prevalence of other parasites on 0+ rainbow trout

18 Prevalence of other parasites on 1+ rainbow trout

19 Ichthyobodo attached to the cuff of skin sheltered by the operculum

20 Ichthyobodo attached to the underside of a pectoral fin

Figure

21 Ichthyobodo attached to the peripheral cells of the pseudobranch

22 Hyperplastic epidermis with Ichthyobodo attached to the peripheral cells

23 Low power light micrograph to show progressive thickening of epidermis infested by Ichthyobodo

24 Normal uninfested skin

25 Strongly basophilic nuclei of peripheral cells beneath Ichthyobodo infestation

26 Effete ghost cells showing characteristic sucked out appearance

27 Spongiosis of epidermis beneath Ichthyobodo infestation

28 Extensive epidermal erosion leaving an incomplete layer of cells above the basal membrane

29 Normal salmon smolt epidermis

30 Heavy labelling of [^{3}H] thymidine of the outermost squamus cells

31 Heavy labelling with [^{3}H] thymidine of remaining cells in the eroded epidermis

32 Ichthyobodo displaying uptake of [^{3}H] thymidine throughout the cytoplasm

33 Very heavy labelling of Ichthyobodo 4 days post infection

34 Hyperplasia of the salmon smolt epidermis

35 Salmon smolt epidermis showing discrete clusters of heavily labelled cells

36 Standard curve of percentage binding of testosterone antiserum to testosterone versus quantity of testosterone in solution

37 Standard curve of percentage binding of cortisol antiserum to cortisol versus amount of cortisol in solution

38 Testosterone levels in implanted and control fish

39 Cortisol levels in implanted and control fish

40 Normal epidermis of immature rainbow trout

41 Acidophilic cell in epidermis of rainbow trout skin

Figure

42 Skin of normal rainbow trout showing scales in stratum spongiosum

43 Flask shaped goblet cells in epidermis of cortisol-treated fish

44 Ichthyobodo attached to epidermis of cortisol-treated fish

45 Extensive spongiosis and sloughing of epidermal cells in Ichthyobodo-infested cortisol-treated fish

46 Virtual complete breakdown of epidermis of cortisol-treated, Ichthyobodo-infested fish

47 Hyperplasia of epidermis of testosterone-treated rainbow trout

48 Epidermal ridges in testosterone-treated rainbow trout

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ABSTRACT

The literature on Ichthyobodo necator is reviewed.

The prevalence and intensity of Ichthyobodo infestations on farmed salmonids was investigated on three farms over a period of two years. The infestations were found to be markedly age dependent. Peak infestations and related mortalities occurred in the first eight weeks after first feeding. Both mortalities and infestations declined to zero shortly after this period with no chemotherapy. Ichthyobodo reappeared on 0+ and appeared for the first time on 1+ fish after a drop of water temperatures to less than 10°C. Many of the 1+ fish had started to mature.

It is suggested that some form of host defence mechanism operates which limits the Ichthyobodo infestations in farmed salmonids.

The sequential pathology of Ichthyobodo infestations of the skin of 0+ and 1+ salmon and rainbow trout was studied. Areas of greatest shelter from water currents were found to be most commonly infested and no parasites were found attached to the epidermis on the head of the fish. The parasite caused hyperplasia of the malphigian cells and exhaustion of the goblet cells below infestations, followed by spongiosis of the underlying epidermis. The epidermal plaque then sloughed off leaving a single layer of cells attached to the basement membrane. Cell kinetic studies showed that Ichthyobodo caused the cells immediately below infestations to divide, a markedly different pattern from that of normal teleost epidermal cell proliferation. The possibility that the parasite secretes some form of digestive enzyme is postulated. In areas where sloughing had occurred, the remaining malphigian cells were seen to be in the process of division.

Various endocrinological aspects of Ichthyobodo infestations were investigated. Three corticosteroids and one androgen were injected or implanted into 1 year old rainbow trout. Implantations of hydrocortisone led to very heavy Ichthyobodo infestations. Radio immune assays showed that the level of cortisol and testosterone in the serum of implanted fish was similar to that which would occur when salmonids mature. There appears to be a clear link between cortisol levels in the serum and Ichthyobodo infestation.

The host response to Ichthyobodo is discussed and it is concluded that cortisol may suppress the host's defence mechanism to Ichthyobodo.

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

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1.1 GENERAL

Ichthyobodo necator or Costia necatrix as it is more commonly known is a very important protozoan parasite of cultured fish throughout the world. Its importance is often underestimated in the scientific literature probably because it is so often missed by scientists and fish farmers alike because of its small size and transparency when observed in the normal light microscope. However, it is probably the major cause of death of cultured salmonid fry in Scottish fish farms; even the viral disease IPN seldom causes such acute mortality as does Ichthyobodo. Because of its predilection for first feeding fry salmonid farmers generally assume that an Ichthyobodo infection is extant before investigating other possible causes and thus excessive treatment with formalin, often unnecessarily, is the norm in most farms. For such an economically important parasite there has been relatively little research carried out on aspects of its biology. Much of the literature on Ichthyobodo was published in the first half of the twentieth century and concentrated on trying to establish the taxonomic status of the parasite. Other reports of Ichthyobodo in the literature briefly mention the parasite in species lists for infestations of groups of fish or in parasite lists for specific countries.

1.2 TAXONOMY OF ICHTHYOBODO NECATOR

Ichthyobodo necator is a protozoan flagellate of the order kinetoplastida; its currently accepted taxonomic status is shown in Table 1. The exact taxonomic status has caused many problems over the

TABLE 1

The Taxonomic Status of Ichthyobodo Necator

Phylum	:	Protozoa
Class	:	Mastigophora
Order	:	Kinetoplastida
Suborder	:	Bodonina
Family	:	Bodonidae
Genus	:	Ichthyobodo
Species	:	necator

last century and was not fully clarified until 1969 by the electron microscopy studies of Joyon and Lom. Ichthyobodo necator was first described by Henneguy in 1883 and more fully in 1884 and he named it Bodo necator. In 1890 the parasite was relegated to the genus Costia by Leclerq, who called it Costia necatrix, as the genus Bodo contained flagellates with only two flagellae, whereas Henneguy reported the parasite to have three flagellae, two short and one long. Weltner (in Nitsche and Weltner, 1894) described an ectoparasitic flagellate on goldfish Carassius auratus L and on the basis of four flagellae placed it in the Tetramiditae and called it Tetramitus nitschei; Moroff in 1904 showed this parasite to be identical to Costia necatrix. The name Costia, however, was already preoccupied by a genus of Hymenoptera created by Kirchner in 1867 and was therefore invalid under the rules of zoological nomenclature. According to Joyon and Lom (1969), Pinto in 1928 proposed replacement of the name Costia by the name Ichthyobodo. Several other inappropriate generic designations have been used for Ichthyobodo but Costia necatrix became the accepted synonym and is still used today by most fish farmers and aquarists. Ichthyobodo necator is however the correct name according to the strict code of zoological nomenclature.

Recent electron microscope studies by Schubert (1966) and Joyon and Lom (1966) and (1969) confirmed that Ichthyobodo necator is a kinetoplastid of the family Bodonidae, and not in the Proteromonadidae (Grell, 1956; Reichenow, 1928) or the Tetramitididae (Lemmermann, 1914; Hall, 1953; Kudo, 1945, and Minchin, 1922).

However Joyon and Lom (1969) called the parasite Ichtyobodo necator and most subsequent authors have dropped the "h". However it seems that Ichtyobodo was a lapse of spelling on Joyon's part as

the French pronounce "th" as "t". However as Pinto (1928) had spelt it Ichthyobodo and subsequently Grassé in 1952, who pointed out the preoccupation of the name Costia in his 'Traite de Zoologie', also spelled Ichthyobodo with an "h", this will be the spelling throughout this thesis. Personal communications with J. Lom (co-author of the Joyon and Lom paper) and K. Vickerman support this spelling of Ichthyobodo necator.

Kinetoplastids are well demarcated from the rest of the class mastigophora by possession of a conspicious extra nuclear deoxyribunucleic acid (DNA) organelle, the kinetoplast. This organelle is still the largest repository of extra nuclear genetic material known in any cell (Vickerman, 1976) and consequently has attracted much attention from molecular biologists. The kinetoplast is found within the single mitochondrion usually close to the basal bodies of the flagellae.

Bodonine kinetoplastids differ from Trypanosomatina kinetoplastids by possessing two flagellæ as opposed to the single locomotory flagellum of the latter.

The family Bodonidae are bodonine flagellates in which the recurrent flagellum is free from the body surface, which are phagotrophic and ingest food through a cytostome which opens close to the flagellar pocket.

The number of flagellae which Ichthyobodo possesses and which caused so many problems for earlier workers has been shown to be two, one long and one short flagellum. The quadriflagellar form of Ichthyobodo has been shown to be the predividing form (Joyon and Lom, 1969) and thus the biflagellate normal form establishes Ichthyobodo as a true bodonid.

The genus Ichthyobodo is unusual in the kinetoplastida in that its members possess several kinetoplasts dispersed throughout the reticular mitochondrion, and not just in the region of the flagellar basal bodies. The only other kinetoplastid reported to have similar multiple kinetoplasts is Crytobia vaginalis another bodonine flagellate found in leeches Hirudo medicinalis (Vickerman, 1974).

Only two species of Ichthyobodo have been described, Ichthyobodo necator and Ichthyobodo pyriformis, the latter species was described by Davis in 1943 on rainbow trout Salmo gairdneri Richardson and brook trout, Salvelinus fontinalis (Mitchill) and by Heckman (1974) on golden trout, Salmo aquabonita. This species is supposedly smaller than Ichthyobodo necator and pyriform in shape. However, most authors (Tavolga and Nigrelli, 1947; Vickerman, 1976 and Becker, 1977) doubt the existence of Ichthyobodo pyriformis and consider that it is a small form of Ichthyobodo necator as the size range falls within the highly variable size range of Ichthyobodo necator. For brevity Ichthyobodo necator will be shortened to Ichthyobodo from now on.

1.3 BIOLOGY AND LIFE HISTORY OF ICHTHYOBODO

1.3.1 Life forms

Very little work has been carried out on the life cycle and biology of Ichthyobodo, however several forms of the parasite have frequently been described.

1.3.1.1 The free swimming form (Figs. 1 and 2)

This form of the parasite is ovoid or ellipsoid in shape and bears two flagellae in most cases but in some cases four. The parasite swims with hesitant spiralling movements by beating the flagellae. Most authors think that this is only a transitory stage for swimming from host to host or for moving about on the host.

FIGURE 1

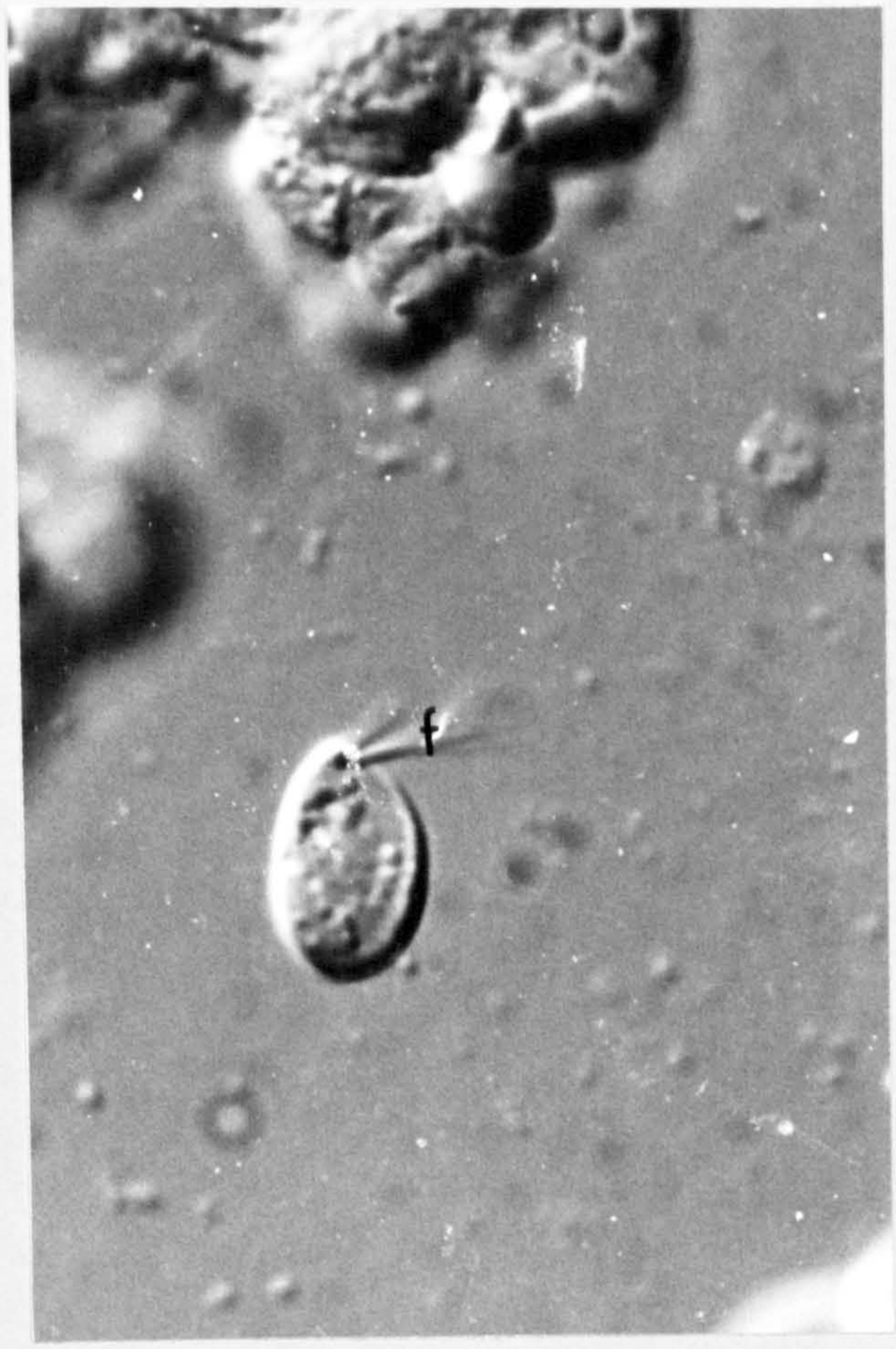
Interference phase contrast micrograph of free living form of Ichthyobodo × 1000

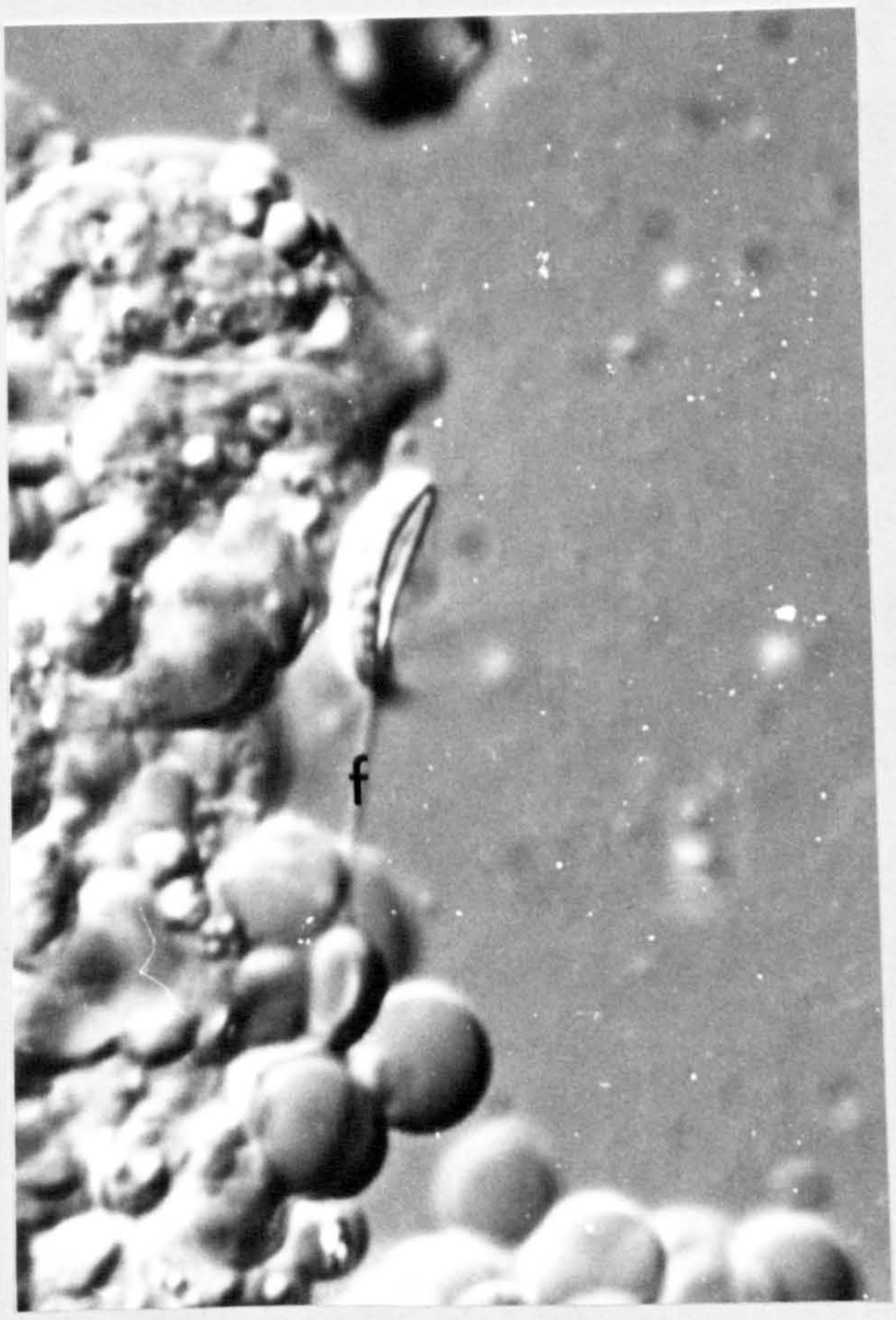
f = flagellae

FIGURE 2

Interference phase contrast micrograph of free living form of Ichthyobodo. Side view × 1000

f = flagellae


f


f

1.3.1.2 The attached form (Figs. 3, 4, 5, 6)

The fixed parasitic form is more pyriform in shape and the flagellae are less noticeable than in the free living form. The parasite attaches to the epidermal cells of the gills and skin of the host and feeds on the cell contents. The area of skin around the dorsal fin (Tavolga and Nigrelli, 1947) and the tips of the secondary lamellae (Fish, 1940) have been reported to be the most favoured sites of attachment. As many as fifteen parasites have been reported attached to a single cell (Fish, 1940); however one to three are more normal. Becker (1977) has suggested that the parasite can detach or attach to cells readily and that it swims away by twirling the tips of the flagellae against the body groove.

1.3.1.3 Saprophagous form

A third, saprophagous, form has been suggested by Tavolga and Nigrelli (1947) which they claim feeds on detached and decaying cells and scales on the bottom of the tank or pondin which the host lives. They reported that 10 to 100 such individuals were attached to each scale. No other authors have reported this phenomenom, however, and Bauer (1959) doubted the existence of a saprophagous phase as he thought that the parasites died fairly rapidly after the death of the host.

1.3.1.4 Cyst form

The existence of a resistant cyst has been frequently postulated. In 1904 Moroff described a cyst and most authors appear to have repeated his description and drawing without actually describing cysts themselves. Most descriptions of encystment in the Russian literature indicate that the flagellae disappear and the parasite rounds up and a cyst wall is produced (Bauer, personal communication).

FIGURE 3

Interference phase contrast micrograph of attached form of Ichthyobodo × 1000

N = nucleus

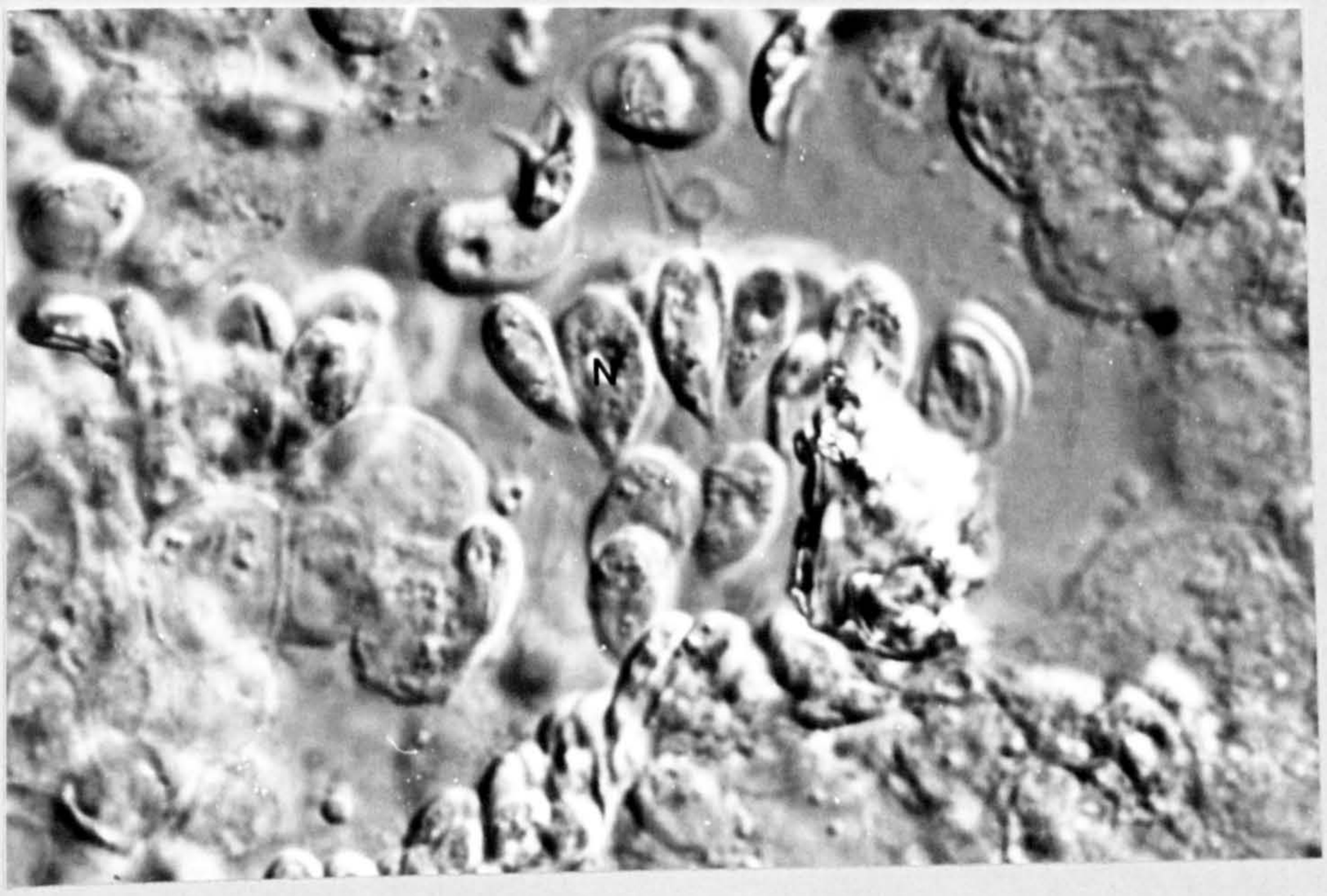


FIGURE 4

Scanning electron micrograph of Ichthyobodo attached to epidermal cells of a salmonid host

× 700

FIGURE 5

Scanning electron micrograph of Ichthyobodo showing ventral view of the parasite.

Note flagellae (F) and attachment organelle (A)

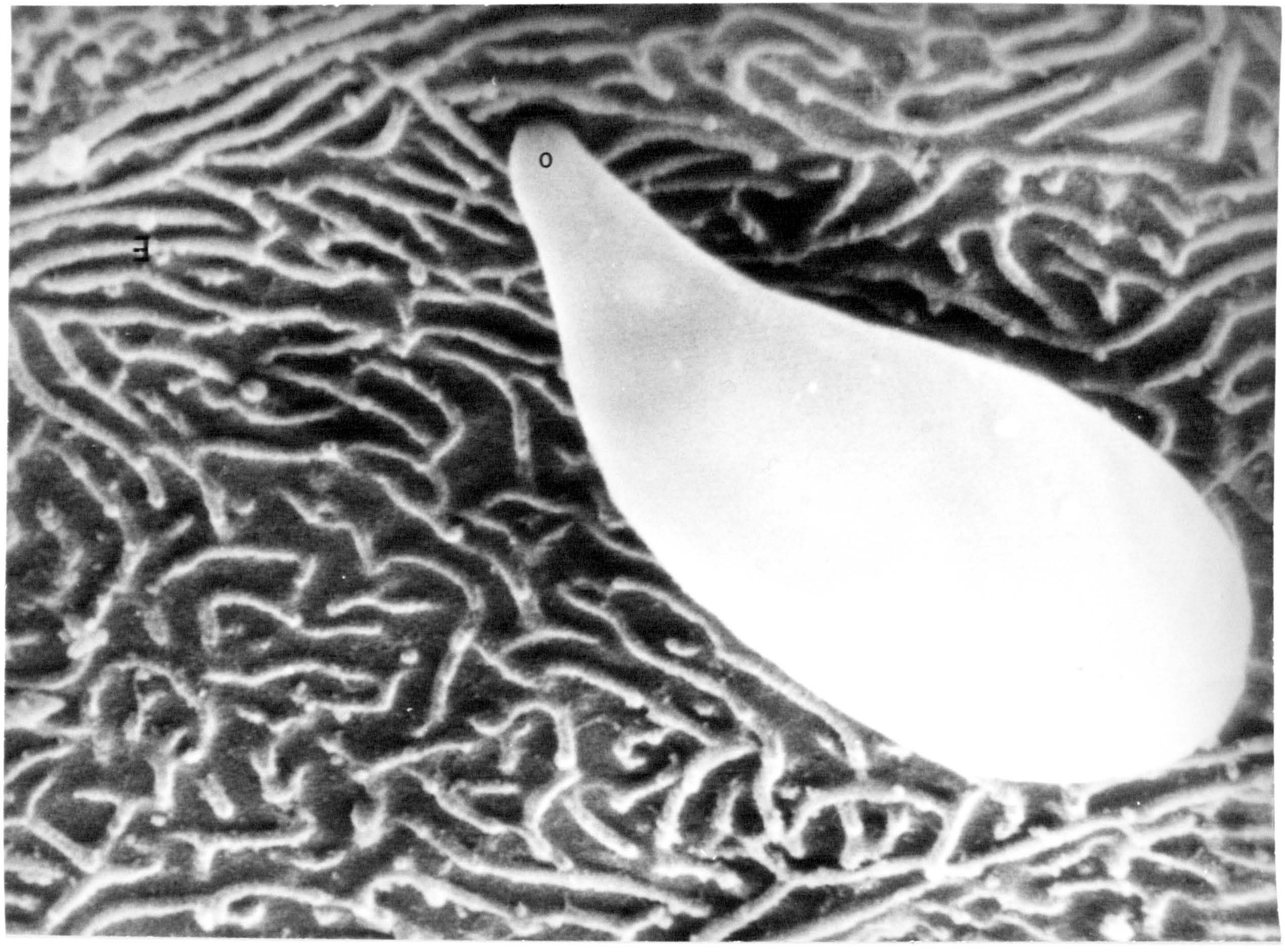
× 5250


A
T

FIGURE 6

Scanning electron micrograph of Ichthyobodo showing the dorsal surface of the parasite. Note attachment organelle (O) and epidermal ridges on the surface of the host cell (E)

× 10,500


O
E

This is similar to the rounding up of the parasite that occurs when the parasite encounters unfavourable conditions, for example in a wet preparation on a microscope slide. However a cyst wall is not produced in these circumstances; the parasite apparently dies. Neither Benisch (1936) nor Tavolga and Nigrelli (1947) found any evidence for a resistant cyst, although the latter thought that certain changes in the environment such as temperature, osmotic and light conditions etc. might bring about encystment. Bauer (1959) quoted Tack (1949) as recording the appearance of Ichthyobodo in isolated trout ponds supplied with spring water and in which fish were absent prior to stocking with these trout. However it is conceivable that Ichthyobodo was introduced with the stocked fish as Bauer (1959) and other Russian authors have indicated that Ichthyobodo can survive on larvae long before the yolk sac has been absorbed and indeed Hlond (1963) has described the presence of Ichthyobodo on eggs of carp.

Therefore, until the presence of a cyst wall can be convincingly defined, preferably by electron microscopy, the question of whether an Ichthyobodo cyst exists or not will remain open. However the circumstantial evidence existing and the occurrence of cysts in related protozoans such as Bodo sp. would indicate that a cyst stage is likely.

1.3.2 Morphology and size

Ichthyobodo is a small parasite with a reported size range of 5-18 µm long (mean length of 7.85 µm, Fish (1940), 9.57 µm, Tavolga and Nigrelli (1947) and 9.63 µm, Andai (1933)) and 2.5-14.54 µm wide, (mean width 4.35 µm, Tavolga and Nigrelli, 5.12 µm, Fish and 7.49 µm Andai). The quadriflagellate form is reported to be the largest

form with a more uniform size and rounded shape. The mean size for the quadriflagellate form reported by Andai was 14.59 µm long and 14.33 µm wide.

The shape of the parasite varies between the free swimming form and the attached form. The free swimming form is reniform in shape and has a convex dorsal surface and concaveventral surface, whereas the fixed form is more pyriform with a slight twist which gives the parasite a comma-like appearance. Both forms have a prominent groove transversing the posterior two thirds of the ventral surface, in which lie the flagellae. The flagellae arise from the kinetoplast at the anterior end of the groove. The biflagellate form has two flagellae of unequal length, with the longer being on average 25 µm and the shorter being 20 µm (Andai, 1933). In the quadriflagellate form there are two short and two long flagellae, with the short being approximately half the length of the long (12.1 µm versus 24.9 µm).

At the anterior end of the free swimming form of the parasite is a cytostome and this is highly modified in the fixed form to form a flat plate which attaches the parasite to the outer surface of the host cell.

Few structural details can be seen inside the living parasite apart from the contractile vacuole filling and emptying, and a number of dark short bacillary or spherical granules. However when properly fixed and stained with a suitable stain such as Heidenheim's haematoxylin (Andai, 1933) several internal structures can be seen. The nucleus which measures 1.36 µm - 4.36 µm diameter (Andai, 1933) with a mean of 1.9 µm (Fish, 1940) is found just below the middle of

the body, and is composed of a dark spherical block of chromatin surrounded by a clear halo. When stained with Romanowsky's stain a large number of basophilic bodies are seen distributed throughout the cytoplasm. There is also a large basophilic body at the base of the flagellum (Vickerman, 1976). This is the kinetoplast as described in the section on taxonomy. Food vacuoles can also be seen in the cytoplasm.

Studies with the electron microscope by Schubert (1966) and Joyon and Lom (1966 and 1969) have provided ultrastructural details that were not possible using the resolving power of the conventional microscope.

1.3.3 Reproduction

Very little work has been carried out on the reproduction of *Ichthyobodo*. However Bauer (1959) has suggested that *Ichthyobodo* reproduces asexually by longitudinal division, the four flagellae form recorded by several authors presumably being the predividing form. Andai (1933) reported that 6.8% of the population were quadriflagellate whereas Benisch (1936) reported 12.5% to be quadriflagellate. Tavolga and Nigrelli (1947) reported that division occurs whilst the parasite is attached to the cell and that dividing individuals are rare, being on average only one per five hundred non-dividing individuals. However it is likely that this ratio increases when conditions are ideal for reproduction. In searching for a site where reproduction might take place off the host Tavolga and Nigrelli found that the percentage of dividing individuals was as high as three to five percent of the population that were attached to scales lying on the bottom of the fish tank.

There have been no reports on the life span of Ichthyobodo and the reports of survival off the host vary enormously. Fish (1940) reported that Ichthyobodo would survive for only five minutes under a cover slip, whereas Benisch (1936) suggested that the rounding up of the parasite under a cover slip takes approximately half an hour and may be caused by anaerobic conditions, as he found that this did not happen when the parasites were placed in a watch glass. Becker (1977) has suggested that the parasite can only survive free in the water for about an hour. Most of the reports of length of survival have referred to survival under a cover slip, a somewhat unnatural model! However Benisch (1936) reported that carp which had been dead for thirty hours were still infested with living Ichthyobodo.

1.3.4 Feeding methods of Ichthyobodo

The method by which Ichthyobodo feed has caused much confusion. Bauer (1959) cited Alekseev (1914) who thought that Ichthyobodo fed on bacteria in dying epithelial tissue, whereas Tavolga and Nigrelli (1947) suggested that the parasites fed on dying epithelial cells, extruded macrophages and mucus. They thought it unlikely that Ichthyobodo fed on bacteria since bacterial populations are comparatively low in well conditioned water and because Ichthyobodo were thought not to survive in areas where putrefaction was taking place such as on dead fish. However it was not until the electron microscopy work of Joyon and Lom (1966 and 1969) and Schubert (1966) that some clues to the method of feeding were found. Joyon and Lom (1966) suggested that the anterior end of the parasite attaches to the host epidermal cells by forming a plate to which it attaches to the membrane of the epidermal cell. Schubert (1966) suggested that the cellular membrane of Ichthyobodo consists of two membranes

enclosing a fibrillar layer. The fibrils in this layer run lengthwise and converge into a stalk at the anterior end of the parasite. When the parasite is preparing to attach to a cell it moves backwards with the long flagellae extended. Schubert suggested that the flagellae aid in site selection but that the attachment organ is a flat plate which sticks to the epidermal cell surface although it can be withdrawn by the microtubules whilst free swimming. The disc is apparently withdrawn by the contractile microtubules. In 1968 Schubert suggested that the limiting membrane of the parasite and host fish fitted closely together and that the adhesion power was sufficient for a secure fastening; formerly it had been supposed that an excreted glue helped to fix the parasite to the host. The actual method of feeding is still controversial. Joyon and Lom (1969) suggested that a sucking organelle containing the cytostome tube forms from the plate and penetrates the cell and sucks up the cell contents, whereas Schubert (1968) described finger like processes which penetrated deeply into the host cell. The parasite then appeared to suck up small parts of the epidermal cell. The appearance of the ingested material changed during this process which has been interpreted as due to the beginning of digestion.

1.4 HOST PARASITE INTERACTIONS

1.4.1 Clinical Signs of Costiasis

The symptoms of Ichthyobodo infestation generally known as costiasis, which have been reported by most authors have been:

(i) a reduction in appetite of the fish and general listlessness;

(ii) flashing or scraping of the body surface against the substrate, presumably in an attempt to dislodge the irritating parasite:

(iii) a progressive increase in fish mortalities and in the more advanced stages of the infestation the appearance of a greyish white

film over the body and fins of the fish (Fish, 1940). Other signs that have been described are destruction of the fins and the fish struggling to the surface of the water (Bauer, 1959). Savage (1935) described severely infested fry struggling to maintain their normal position; as soon as they relaxed they turned on their backs and floated to the surface, weakly bending from side to side. Savage also described the frayed and notched fins of infested fish. However, in Scottish fish farms there are generally no obvious signs except progressive daily mortalities usually associated with floating dead fish.

1.4.2 Hosts and geographic range of Ichthyobodo

The hosts of Ichthyobodo are many and varied. The hosts reported in the literature are shown in Table 2, but it would appear that Ichthyobodo can infest virtually any fresh-water fish and the high stocking densities used in most forms of aquaculture appear to exacerbate the condition as there have been few reports of Ichthyobodo infestations in wild fish populations. However, most parasite lists of wild fish do not include protozoan parasite and therefore Ichthyobodo may be more prevalent in wild fish than is currently supposed, as a recent study by Bullock and Robertson (1982) has shown that Ichthyobodo were present in large numbers on wild plaice, when routinely sampled for experimental purposes.

Ichthyobodo infestations are not merely confined to fish, as there have been reports of infestations on frogs and tadpoles (Bauer, 1959) and axolotols (Vickerman, 1976).

The geographical distribution of Ichthyobodo is virtually world-wide (see Table 3) and is particularly prevalent where the high

TABLE 2

Hosts of *Ichthyobodo*

Host	Species	Reference
Sockeye Salmon	*Oncorhyncus nerka* (Walbaum)	Johnston (1972)
Atlantic Salmon	*Salmo salar* L.	Ellis & Wootten (1978)
Pacific Salmon	All species	Wood (1974)
Brown Trout	*Salmo trutta* L.	Franke (1908)
Rainbow Trout	*Salmo gairdneri* Richardson	Davis (1953)
Brook Trout	*Salvelinus fontinalis* (Mitchill)	Davis (1953)
Common Carp	*Cyprinus carpio* L.	Bauer (1959)
Common Carp	*Cyprinus carpio* L.	Migala (1971)
Goldfish	*Carrasius arratus* (L)	Osborne (1966)
Goldfish	*Carrasius arratus* (L)	Benisch (1936)
Tench	*Tinca tinca* L.	Roth (1910)
Channel Catfish	*Ictalurus punctatus* (Rafinesque)	Allison (1963)
Pond fishes	Various	Hora & Pillay (1962)
Crucian carp	*Carrassius vulgaris* L.	Schaperclaus (1954)
Swordtail	*Xiphophorus helleri* Meckel	Tavolga & Nigrelli (1947)
Guppy	*Lebistes reticulatus* L.	" "
Platyfish	*Platypoecilus maculatus* Günther	" "
Tilapia	*Tilapia macrocephala* L.	" "
Gilthead	*Chrysophris auratus* L.	Penso (1953)
Snakehead	*Ophiocephalus striata* L.	Personal observation
Tilapia	*Sarotherodon spilurus* L.	" "
Pike	*Esox lucius* L.	Franke (1908)
Sturgeon	*Acipenser sturio* L.	Astakhova (1975)
Plaice	*Pleuronectes platessa* L.	Bullock & Robertson (1982)

TABLE 3

Geographical distribution of Ichthyobodo infestations

Country	Author
Europe:	
Trout culture in Britain	Roberts & Shepherd (1974)
Trout culture in France	Leger (1909)
Trout culture in Germany	Schubert (1966)
Carp culture in Poland	Migala (1971)
Carp culture in Germany	Schaperclaus (1954)
N. America:	
Trout culture in Canada	Savage (1935)
Pacific Salmon culture in USA	Wood (1974)
Warm water pond fish culture Southern USA	Meyer (1966)
" "	Rogers (1969)
Trout culture USA	Davis (1953)
Channel Catfish culture USA	Allison (1963)
Goldfish culture USA	Osborne (1966)
Asia:	
Carp culture in Korea	Chun (1976)
Trout and Carp culture in Russia	Bauer (1959)
Atlantic Salmon culture in Russia	Bauer and Strelkov (1959)
Trout culture Japan	Sano, Personal communication
Pond fish culture Japan	Susuki (1938)
Trout culture in Kashmir	Gopalkrishan (1966)
Pond fish culture in Indopacific region	Hora and Pillay (1962)
Africa:	
Pond fish culture in Israel	Sarig (1966)

stocking densities used in fish culture encourage the transmission of the parasite from host to host. To date there have been no known reports of Ichthyobodo in the Southern hemisphere.

1.5 THE EFFECT OF ENVIRONMENTAL VARIABLES ON ICHTHYOBODO INFESTATIONS

1.5.1 Temperature

There is very little information on the effect of various environmental parameters on Ichthyobodo infestations. However the fact that Ichthyobodo infestations are found at temperatures ranging from 2°C - 38°C (2°C - Schaperclaus, 1929; 20-22°C - Hlond, 1953; 2-30°C - Benisch, 1936; up to 38°C Tavolga and Nigrelli, 1947) indicates that the parasite is not temperature specific. Bauer (1959)and Becker (1977) have suggested that the parasite multiplies more rapidly at temperatures above 20°C; however the fact that Ichthyobodo infestations are so prevalant at Scottish fish farms, where the temperature seldom exceeds 20°C, suggests that there are probably local strains of Ichthyobodo which are adapted to the local water temperatures prevalent at each location. There has been no work reported indicating that Ichthyobodo from warm water fish can survive in cold water conditions on temperate fish.

1.5.2 pH

There have been several reports in the literature which have shown that Ichthyobodo can survive in water at pH levels of between 4.5 and 7.5 (Schaperclaus, 1929, 4.5-5.8; Bauer, 1959, 5-5.5; Benisch, 1936, 6.8-7.2; Hlond, 1963, 7.2-7.5; and Migala, 1971 at pH less than 7.5). It can therefore be concluded that Ichthyobodo survives best in waters of low pH, a factor which Bauer (1959) considers distinguishes Ichthyobodo from other fresh-water fish

parasites. Benisch (1936) suggested that at low pH destruction of the skin and gills occurs and thus probably facilitates invasion by the parasite although this now seems unlikely.

The range of pH in which Ichthyobodo can survive is generally the same as the range reported for the survival of fish (Munro, 1978) and this is generally true of temperature also, and therefore it would seem that the parasite is adapted to the environmental conditions in which its host is living. It is likely that any major change from the normal will stress or debilitate the fish and thus facilitate Ichthyobodo infestations.

1.5.3 Salinity

Although Ichthyobodo is generally regarded as a freshwater parasite, there have been several cases of infestations in sea water reported (Penso, 1953; Bullock and Robertson, 1982; Wood, 1974; and Ellis and Wootten, 1978). However, the authors of the last two papers considered that the fish were harbouring Ichthyobodo when they were transferred to sea water, although the sea water did not adversely affect the Ichthyobodo as Wood found that the infestations increased after transfer. Bullock and Robertson (1982) suggested that Ichthyobodo infestations of plaice may have been cross-infestations from local aquaculture facilities, which would indicate that the parasite can transfer from sea water acclimated fresh water fish to fully marine fish.

No other reports of the effects of environmental variables on Ichthyobodo have been reported in the literature; however it is interesting to note that Franke (1908) suggested vigorous aeration of the aquarium water as a method of preventing Ichthyobodo infestation,

thus suggesting that Ichthyobodo do not thrive in well-aerated water. However, it seems more likely that the condition of the fish improved in the more suitable conditions, and they were thus more able to resist the parasite, although turbulent water may be less conducive to parasite attachment.

1.6 CHEMICAL TREATMENT OF ICHTHYOBODO INFESTATIONS

When Ichthyobodo infestations are heavy and the resultant mortalities unacceptable most aquaculturists resort to chemotherapy to eliminate the parasite. There have been many reports in the literature on treatment methods for protozoan parasites including Ichthyobodo and these are summarised in Table 4.

As can be seen from the table, the most frequently recommended treatment is formalin addition to the water at concentrations of 1:4000 - 1:6000. Care must be taken with formalin treatment in warm water, however, as formalin is a reducing agent which readily combines with free oxygen to form formic acid. At the recommended concentration formalin is capable of binding the total dissolved oxygen in a tank, (Helms, 1967); therefore aeration is always recommended during formalin treatment. There have been several reports in the literature on the pathological effects of formalin on young fish and these have been reviewed by Wedemeyer (1971). There are reports in Scandinavia (Gunnes, pers. comm.) that a formalin resistant strain of Ichthyobodo has been produced as a result of regular prophylactic treatment. Therefore formalin is not the ideal solution for treatment of Ichthyobodo and new methods for its control should be sought.

Acknowledging the drawbacks of formalin, Wood (1974) has recommended that the parasite should be discouraged by high water

TABLE 4 Chemicals used in treatment of Ichthyobodo Infestations (Modified from Hoffman and Meyer (1974))

Host	Treatment	Dosage	Method	No. of Applications	Frequency	Author's Report of Success	Remarks	References
Pond fishes	Acetic acid, glacial	2,000 ppm	D	1	...	Effective	...	Hora & Pillay, 1962
Salmonids	Acetic acid, glacial	2,000 ppm	D 1 min or less	1	...	Effective	....	Davis, 1953
Trout	Acetic acid, glacial	2,000 ppm	"	1	...	Effective	...	Savage, 1935
Ictalurus punctatus	Formalin	10-15 min	|	1	...	Effective	...	Allison, 1963
Pond fishes	Formalin	400 ppm	F 10 min	1	...	Effective	...	Hora & Pillay, 1962
Salmonids	Acetic acid, glacial	2,000 ppm	D 1 min	...	As needed	Effective	...	Davis, 1953
Unidentified	Aquarol	80 ppm	|	3	Daily	Effective	...	Amlacher, 1961 Reichenbach-Klinke, 1966
Unidentified	Aureomycin	0.13 ppm	|	1	...	Effective	...	Amlacher, 1961b
Unidentified	Chloramin	10 ppm	|	1	...	Effective	Better than 66 ppm	Schäperclaus, 1954
Unidentified	Chloramin	66 ppm	F 2-4 hrs	1	...	Effective	...	Schäperclaus, 1954
Cyprinus carpio	Chloramine-B	20 ppm	|	1	...	Effective	...	Goncharov, 1966
Pond fishes	Copper sulfate	100 ppm	F 10 min	?	As needed	Effective	....	Amlacher, 1961
Carassius auratus	Copper sulfate	500 ppm	D 1-2 min	?	As needed	Effective	...	Osborn, 1966
Carassius auratus	Copper sulfate	0.5-1.0 ppm	|	?	As needed	Effective	Use with caution in soft waters	Osborn, 1966
Trout	Boric acid	2,000 ppm	F 1 hr		Weekly	Not effective		Fish, 1946
Trout	Formalin	400 ppm	F 15 min	1	...	...	Killed fish	Leger, 1909
Tinca tinca	Formalin	Less than 400 ppm	F 15 min	1	...	Effective	...	Roth, 1910
.....	Formalin	.....	....	..	...	.....	....	Zschiesche, 1910
Trout	Formalin	350 ppm	F 15 min	1	...	Effective	...	Plehn, 1924
Trout	Formalin	166 ppm	F 1 hr	..	As needed	Effective	Alternate weeks	Fish, 1940
Unidentified	Formalin	200-500 ppm	D 30-45 min.	1	...	Effective	...	Schäperclaus, 1954
Unidentified	Formalin	1,000 ppm	D 15 min	1	...	Effective	...	Schäperclaus, 1954
Oncorhynchus sp.	Furanace	1 ppm	|	?	?..	Not effective	...	Amend, 1969
Ictalurus punctatus	Gentian violet	0.3 ppm	|	1	...	Inhibitory	Toxic to many species of fish	F. Meyer, Unpubl.

Table 4 (continued)

Host	Treatment	Dosage	Method	No. of Applications	Frequency	Author's Report of Success	Remarks	References
Unidentified	Globucid	2,000 ppm	F 24 hrs	1	...	Effective	...	Schäperclaus, 1954
Cyprinus carpio	Lysol	200 ppm	D 30 sec	1	...	Effective	Not as good as formalin	Schäperclaus, 1954
Trout, Cyprinus carpio	Malachite green oxalate	0.1-0.15 ppm	|	2-3	Alternate days	Effective	...	Amlacher, 1961b
Unidentified	Methylene blue	3 ppm	|	1	...	Effective	...	Amlacher, 1961b
Carassius vulgaris	Micropur	10 ppm	F 24 hrs	1	...	Effective	...	Schäperclaus, 1954
Salmonids	PMA	2 ppm	F 1 hr	1	...	Effective	Toxic to Salmo gairdneri	Burrows & Palmer, 1949
Ictalurus punctatus	PMA	2 ppm	F 1 hr	1	...	Effective	...	Clemens & Sneed, 1959
Unidentified	Potassium permanganate	10 ppm	F 1 hr	?	?	Effective	Toxic to Stizostedion sp.	Fish, 1933
Unidentified	Potassium permanganate	10 ppm	F 90 min	?	As needed	Effective	...	Amlacher, 1961b
Cyprinus carpio	Potassium permanganate	1,000 ppm	D 30-45 sec	?	As needed	Effective	...	Schäperclaus, 1954; Amlacher, 1961b; Reichenbach-Klinke, 1966
Pond fishes	Potassium permanganate	1,000 ppm	F 10 min	As needed	Daily	Effective	...	Amlacher, 1961b
Unidentified	Potassium permanganate	10 ppm	F 30 min	?	As needed	Effective	...	Reichenbach-Klinke,
Cyprinus carpio	Quicklime	2,000 ppm	D 5 sec	1	...	Effective	...	Schäperclaus, 1954
Cyprinus carpio	Quinine hydrochloride	20 ppm	F 24 hrs	1	...	Effective	Toxic to fish	Schäperclaus, 1954
Cyprinus carpio	Sodium chloride	10,000 ppm	D 15-30 min	As needed	Daily	Effective	May recur	Schäperclaus, 1954
Cyprinus carpio	Sodium chloride	175,500 ppm	D 3 min	As needed	Daily	Effective	May recur	Schäperclaus, 1954
Cyprinus carpio	Sodium chloride	25,000 ppm	D 30 sec	As needed	Daily	Effective	May recur	Schäperclaus, 1954
Unidentified large fish	Sodium chloride	25,000 ppm	F 10-15 mins	As needed	Daily	Effective	...	Amlacher, 1961
Cyprinus carpio	Temperature	Raise to 32°C	P 5 days	1	...	Effective	Parasite cannot live above 30°C	Schäperclaus, 1954
In vitro	Ultraviolet light	212,400 MWS/cm^2	P	1	...	Effective	...	Vlesanko, 1969

flows and good husbandry and prophylactic treatment with formalin given just prior to the time that Ichthyobodo has been a problem in the past.

1.7 SUMMARY

From the literature review it can be seen that although there have been numerous papers published in which Ichthyobodo is mentioned most have been related to the taxonomy and morphology of the parasite. Very little work has been carried out on the interaction between the parasite and its host and the work which has been carried out in this field has generally been on tropical fish or carp. The effect of the parasite on its host has largely been ignored and its importance to the fish farming industry is just becoming recognised.

The aim of the present study therefore has been to evaluate the importance of Ichthyobodo infestations in cultured Salmonids in Scotland and to assess which parts of the life cycle are most prone to infestation or conversely at what time of the year the parasite is most prevalent. The pathology of the condition has been studied including the parasite's effect at the cellular level. Experimental infestations were induced using corticosteroids to try to define some aspects of the host defence mechanism to the parasite.

The work is subdivided into chapters which attempt to provide the most logical description of the work although they do not necessarily relate to the chronological order in which the study was carried out.

CHAPTER 2

SEASONAL INFESTATIONS OF FARMED SALMONIDS WITH ICHTHYOBODO NECATOR

CHAPTER 2

SEASONAL INFESTATIONS OF FARMED SALMONIDS WITH *ICHTHYOBODO NECATOR*

2.1 INTRODUCTION

Although there have been many studies on the seasonal fluctuations in abundance of fish parasites, most have been on wild fish or have ignored propozoan parasites, possibly because of their small size and the difficulty of observing them in fixed tissue.

In order to assess seasonal fluctuations of *Ichthyobodo* on farmed salmonids it was decided to study the changes in level of *Ichthyobodo* infestations with time on different ages of cultivated salmonids under typical farming conditions on three commercial salmon farms in Scotland.

A similar study of parasitic protozoa on carp by Migala (1971) had shown that *Ichthyobodo* appeared on the gills and skin of carp fry during the first two weeks of his study only, but he gives no explanation of this phenomenon.

As salmon and rainbow trout are the most important commercially farmed species of fish in Scotland the importance of *Ichthyobodo* infestation to these species was studied over a one year period. In addition most of the material presented for routine diagnosis by the Institute of Aquaculture from Scotland and the north of England over this period was examined for *Ichthyobodo*.

The three farms sampled for this part of the study were

1. Almondbank experimental salmon farm, Almondbank, Perthshire. (0+ salmon)

2. Unilever Research Ltd. Salmon Farm, Lochailort, Inverness-shire. (0+ and 1+ salmon)

3. Howietoun Fish Farm, Sauchieburn, Stirling. (0+ salmon, 0+ and 1+ rainbow trout)

Howietoun was used as a general base to give more regular samples at peak periods and also to provide experimental facilities because of its close proximity to the University. The Almondbank and Unilever salmon farms were sampled less frequently to provide supporting information and to see if traits seen at Howietoun were typical.

2.2.1 Almondbank and Unilever Salmon Sites Sampling

Description of sites

Almondbank:

This farm is run by the Department of Agriculture and Fisheries for Scotland as an experimental salmon smolt producing farm whose remit is to research salmon physiology, ecology and behaviour. The hatchery is sited at Almondbank in Perthshire on the river Almond which is a tributary of the River Tay. The salmon are reared from first feeding to the smolt stage in one or two years using 1 and 2m^2 circular tanks. The tanks are supplied with unfiltered river water of average pH 6.9 and total hardness of 47 ppm.

The fish sampled were from a two metre square tank containing 4,000 salmon whose parents were both grilse of 70.5 cms and 63.6 cms.

Unilver Research Ltd. Lochailort

This farm is a smolt rearing unit run by Unilever Research Ltd., and is situated at Lochailort, Inverness-shire; most of the work carried out here is commercial research into growth improvement and physiology of smolting. The salmon are reared in two metre square and four metre square tanks drawing unfiltered water from the River Ailort which has an average pH of 6.8 and total hardness of 10 ppm.

The fish sampled were taken from a two metre square tank containing 6,000 salmon which had hatched from a pool of eggs from selected domesticated broodstock.

2.2.2 Materials and Methods

Twenty fish were sampled from each site at monthly intervals. The fish were measured, weighed, sexed and the prevalence of parasites, in particular Ichthyobodo, recorded. In addition water temperatures and pH and hardness were taken at the time of sampling.

Fish were killed by a blow on the head and mucus scrapings and gill arches were examined for the presence of Ichthyobodo at × 100 and × 400 magnification.

2.2.3 Results

The results are shown in Figs. 7 and 8. Ichthyobodo levels were low over the period sampled and the parasite generally did not cause high mortalities as the fish were treated with formalin as soon as the parasite manifested itself. However the only time the parasite was recorded was in the first seven weeks after the commencement of first feeding at Lochailort, when 100% incidence was recorded at six weeks post first feeding and in the winter period at both sites when the water temperature had dropped to 1°C. The most common parasites encountered at both sites were Trichodina sp. and members of the Schyhphidia complex.

2.2.4 Discussion

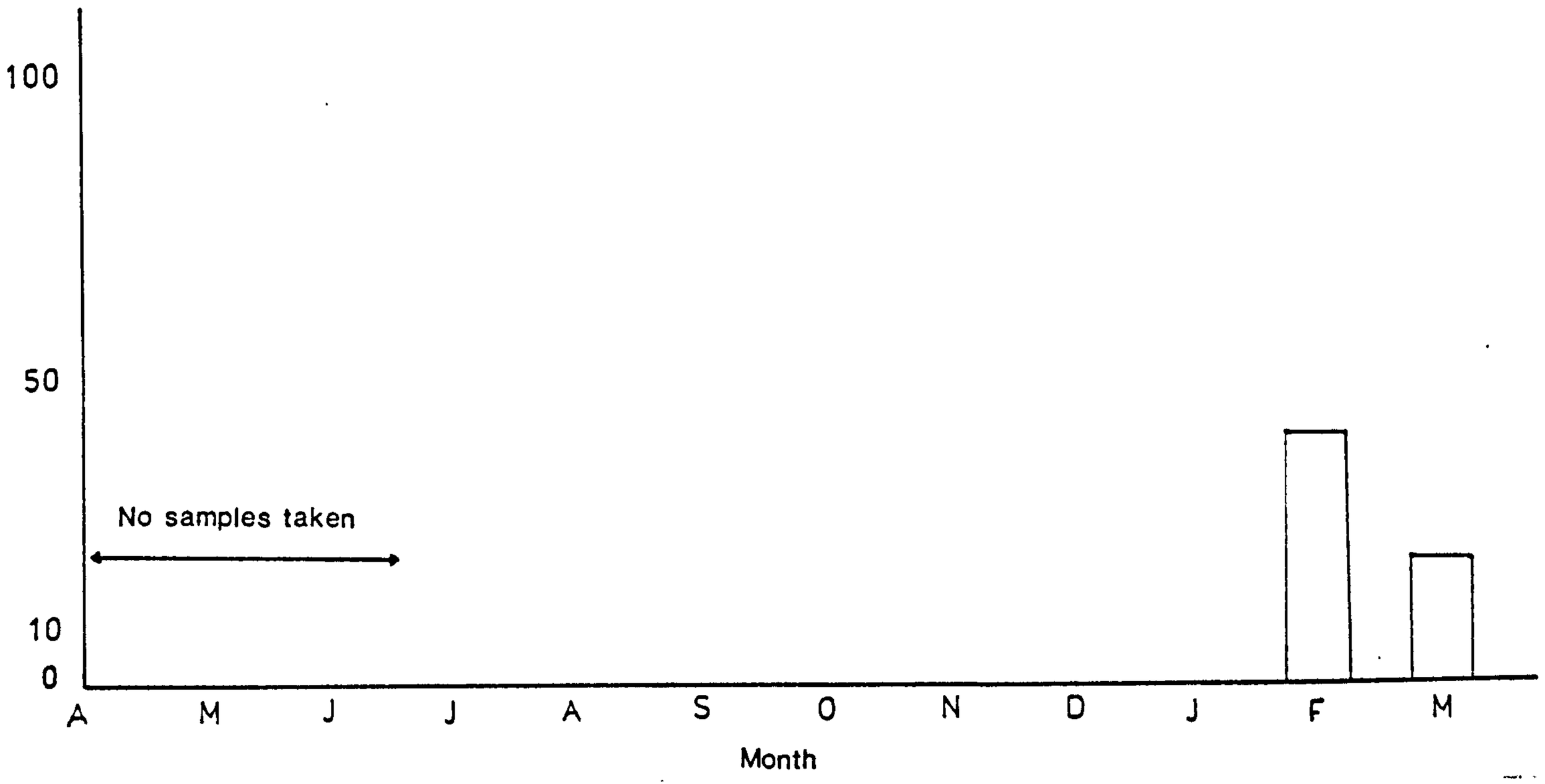
Because of limitations imposed on the sampling programme it was felt that these sites were not sampled frequently enough to highlight real trends in the parasite fluctuation. However, the general trend appeared to be a rise in the incidence of Ichthyobodo shortly after

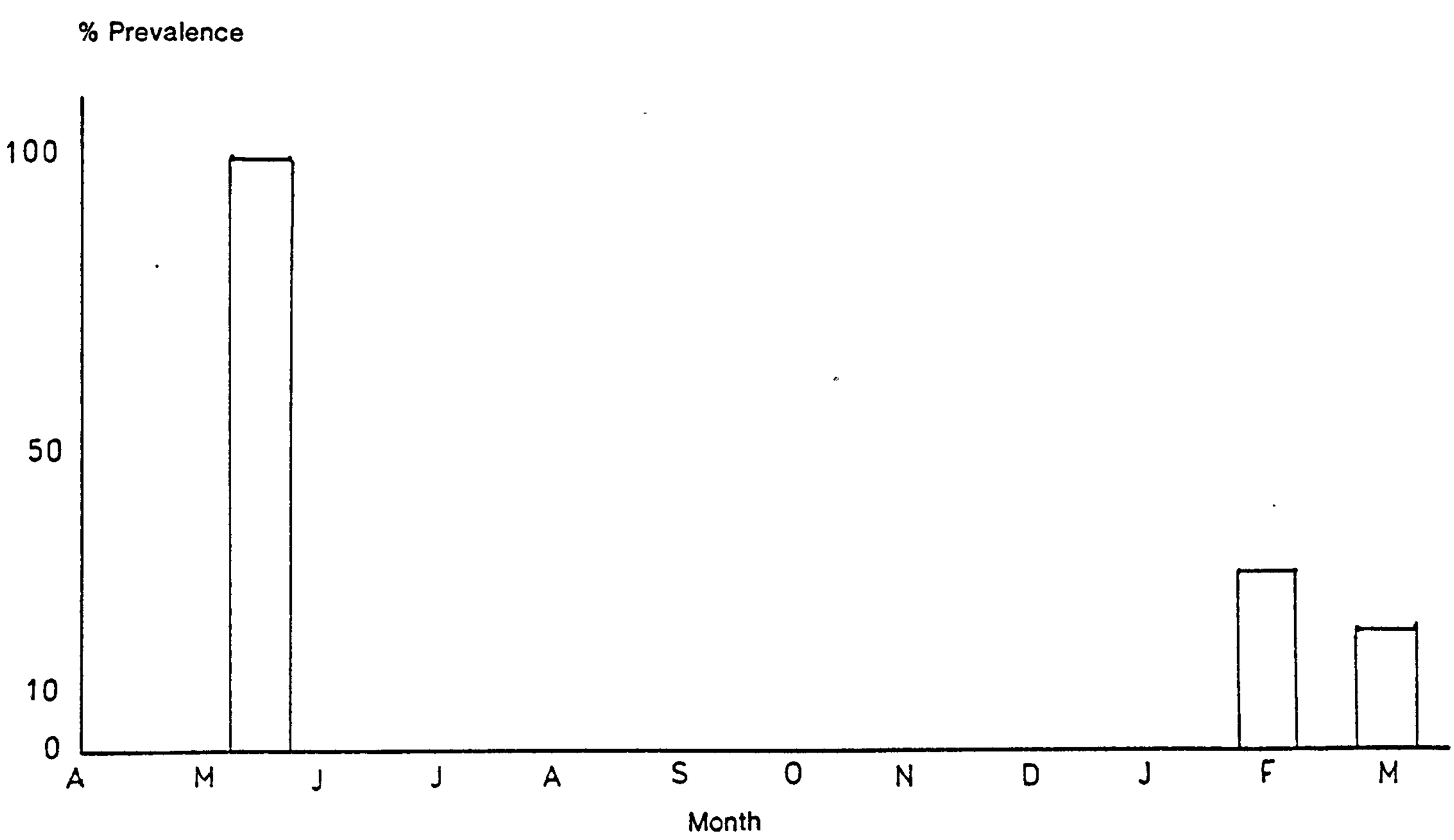
FIGURE 7

Prevalence of Ichthyobodo on 0+ Salmon at Almondbank salmon farm.

FIGURE 8

Prevalence of Ichthyobodo on 0+ Salmon at Lochailort salmon farm.


% Prevalence
100
50
10
0
No samples taken
A
M
J
J
A
S
O
N
D
J
F
M
Month


% Prevalence
100
50
10
0
A
M
J
J
A
S
O
N
D
J
F
M
Month

first feeding, followed by disappearance of the parasite and then reinfestation of the fish after a period of cold water temperature.

It is surprising that no Ichthyobodo were recorded at Almondbank after first feeding. However the first sample was not obtained until six weeks after first feeding because of notifiable disease restrictions and it is conceivable that Ichthyobodo had been present earlier but had disappeared by the time the fish were sampled. In an unpublished study of parasites on Almondbank salmon, Wootten and Smith of DAFS found that Ichthyobodo prevalence increased from 4% two weeks after first feeding to 45% incidence six weeks after first feeding and subsequently declined to zero ten weeks after first feeding. The appearance of Ichthyobodo at both sites during cold water temperature also correlated with the findings of Wootten and Smith who noted a reappearance of Ichthyobodo after a period of cold water temperatures.

It was of interest to note that the incidence of Ichthyobodo on the fish at both sites during the winter was always on the smaller fish of the sample, those destined to be two-winter smolts.

2.3.1 HOWIETOUN TRIALS

The sampling programme from the other two fish farms used in the study had shown that the parasite was present on first feeding salmon, but it was felt that the sampling programme was not intensive enough to assess short-term trends.

Therefore, a more intensive sampling programme was devised at Howietoun fish farm, which is situated five miles west of the University of Stirling, to try to assess how Ichthyobodo infestation fluctuated over an eight month period on two different species of

salmonid and using two different age classes of fish and to try to evaluate the reasons for any fluctuation.

2.3.2 Materials and Methods

0 group salmon, 0 group rainbow trout and one-year old rainbow trout were used in this study which lasted from May to December 1978. All experiments were carried out under standard cultivation conditions at Howietoun fish farm situated at Sauchieburn, Stirling. The fish were fed a commercially prepared diet at 5% body weight per day, using automatic feeders which delivered food at two minute intervals during daylight hours during first feeding and at ten minute intervals thereafter.

The salmon were held under artificial lighting with a photo-period simulating the natural light cycle, the rainbow trout in natural light. No chemotherapy was administered to the fish during the period of study. Fish mortalities and temperatures were recorded daily and pH and hardness weekly.

2.3.2.1 Salmon

Six thousand Atlantic salmon first feeders, which had previously been incubated in spring water, were reared from May 4th, 1978 onwards in concrete raceway type tanks of 0.1 m^3 volume, with a water flow of 9 litres/min. The fish were held at an initial stocking density of 1,000 fish per tank. The stocking density was halved at the end of first feeding when the fish averaged one gram in weight. Twenty to thirty fish were sampled weekly for the first ten weeks, and then every other week thereafter, for the presence of Ichthyobodo on the gills. The fish were decapitated, measured and weighed and four gill arches were removed and mounted in water under a cover slip. The slides were left for ten minutes to standardise

counts and then the number of Ichthyobodo on the four gill arches were counted.

2.3.2.2 Rainbow Trout

This trial was started on the 14th June 1978, six weeks after the salmon. First feeding rainbow trout and one year old rainbow trout were compared. Twenty thousand rainbow trout first feeders which had previously been reared in spring water and 2,000 one-year old rainbow trout (12 cm long) previously held in burn water on the same fish farm, and which had presumably experienced Ichthyobodo before, were used. The one-year old fish were treated with 1:5000 formalin before the trial commenced to eliminate any parasites.

The 0+ fish were held in fibreglass 2 meter^2 tanks at an initial stocking density of 20,000 fish per tank with a water level of 20 cms and flow of 36 litres/min. The population was split into two tanks with 7,500 in each when the fish averaged 1 gm in weight (16th August = 65 days). The fish were further split into populations of 3,000 per tank on the 18th September (98 days).

The 1+ fish were held in identical tanks at the same flow rates at an initial stocking density of 1,000 fish per tank. The stocking density was halved on the 18th September, 1978 (98 days). Ten to twenty 0+ fish were sampled weekly until week 13 and then sampled every other week. Ten 1+ fish were sampled weekly for the first month and then bi-weekly or monthly for the rest of the study.

The method of sampling was as for the salmon but in this case the skin was sampled also. Tavolga and Nigrelli (1947) suggested that the highest concentration of Ichthyobodo occurred around the dorsal fin. Therefore, a scrape of one centimetre was made at the

base of the dorsal fin with a scalpel. This was then smeared thinly on to a slide mounted in water and a 22 × 22 mms cover slip placed above the smear. The slide was examined after ten minutes.

Twenty different fields of 2 mm^2 were examined and the number of Ichthyobodo in each counted. Counts of 20 and above were recorded as 20. The mean for each fish was recorded and after examination of all the fish a mean for the whole sample could be obtained.

2.4 RESULTS

A logarithmic transformation of log (n + 1) for all intensity data was used as this reduced the dependence of the variance on the mean. The resulting data was t-tested for significance.

Prevalence data was tested using a Fisher exact test on a two by two contingency table.

2.4.1 Salmon Fry

From Table 5 and Figure 9 it can be seen that Ichthyobodo levels increased weekly to a peak four weeks after commencement of first feeding. The numbers of Ichthyobodo declined very sharply between weeks 4 and 5 and from week 6 onwards declined until by week 12 no Ichthyobodo were seen on the gills. No Ichthyobodo were seen on the gills of these fish for the remainder of the study.

The prevalence of Ichthyobodo infestations is shown in Figure 10a. The prevalence peaked at week 3 when 93% of the fish examined were infested with Ichthyobodo. Between weeks 4 and 5 the prevalence declined significantly ($p < 0.001$, 1 d.f.) to 24% infection. By week 12 the prevalence had declined to zero.

No accurate quantitative data of weekly mortality is available

TABLE 5

Intensity of Ichthyobodo infestations on the gills of Atlantic salmon fry

Week	Mean (n)	Mean log (n + 1)	SE
1	0	0	0
2	1.633	0.288	0.057
3	16.667	0.751	0.107
4	35.419	0.870	0.144
5	1.133	0.187	0.058
6	1.067	0.221	0.049
7	0.071	0.022	0.015
8	0	0	0
9	0	0	0
10	0.2	0.060	0.060
11	0.2	0.060	0.060
12	0	0	0
↓			
30	0	0	0

n = number of Ichthyobodo on four gills

FIGURE 9

Intensity of Ichthyobodo infestations on 0+ salmon gills.

(vertical bars = $\bar{X}$ ± S.E.)

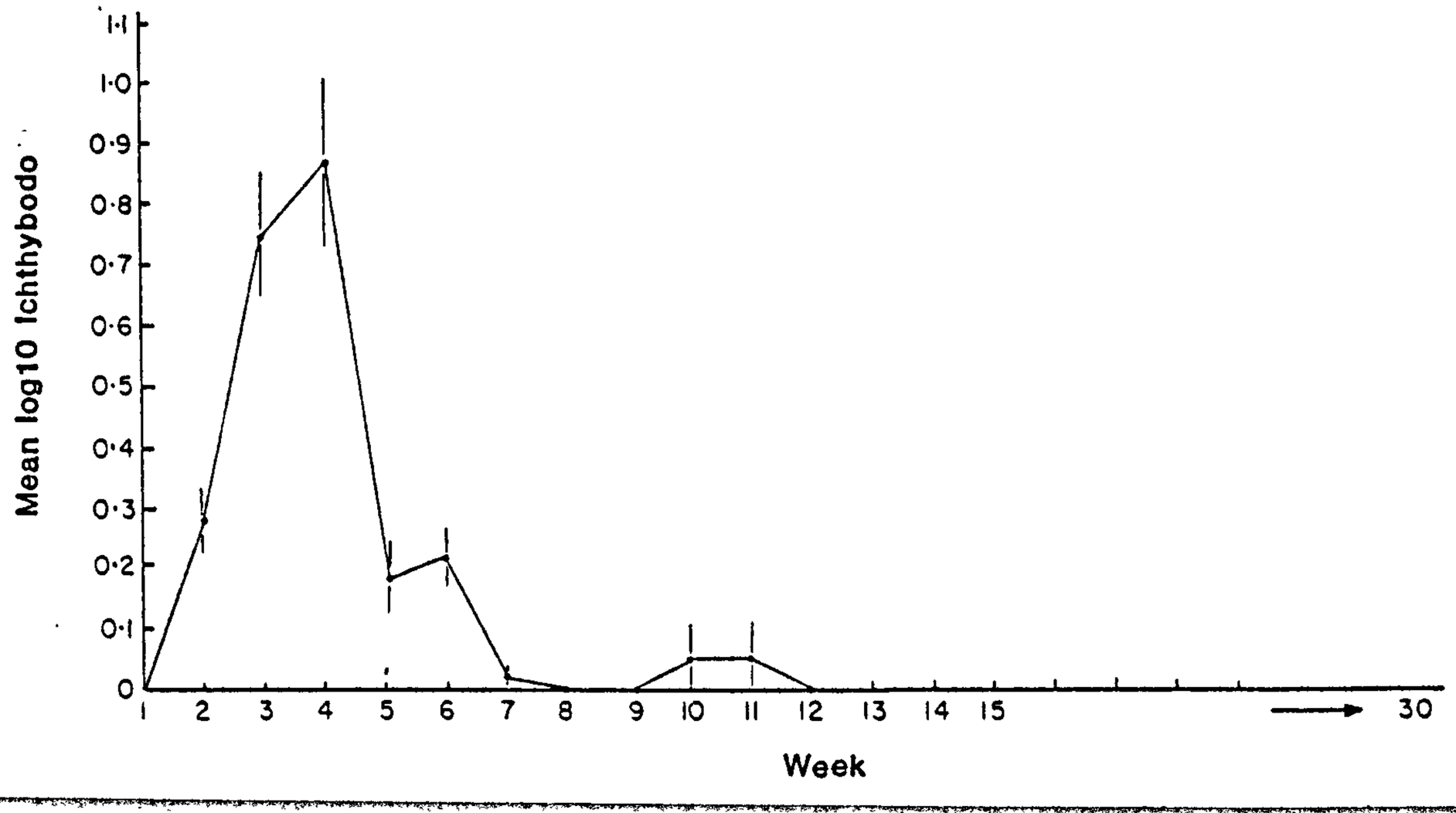

Mean log10 Ichthybodo
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 30
Week

FIGURE 10

Isometric diagram of weekly percentage prevalence of

Ichthyobodo infestation on:

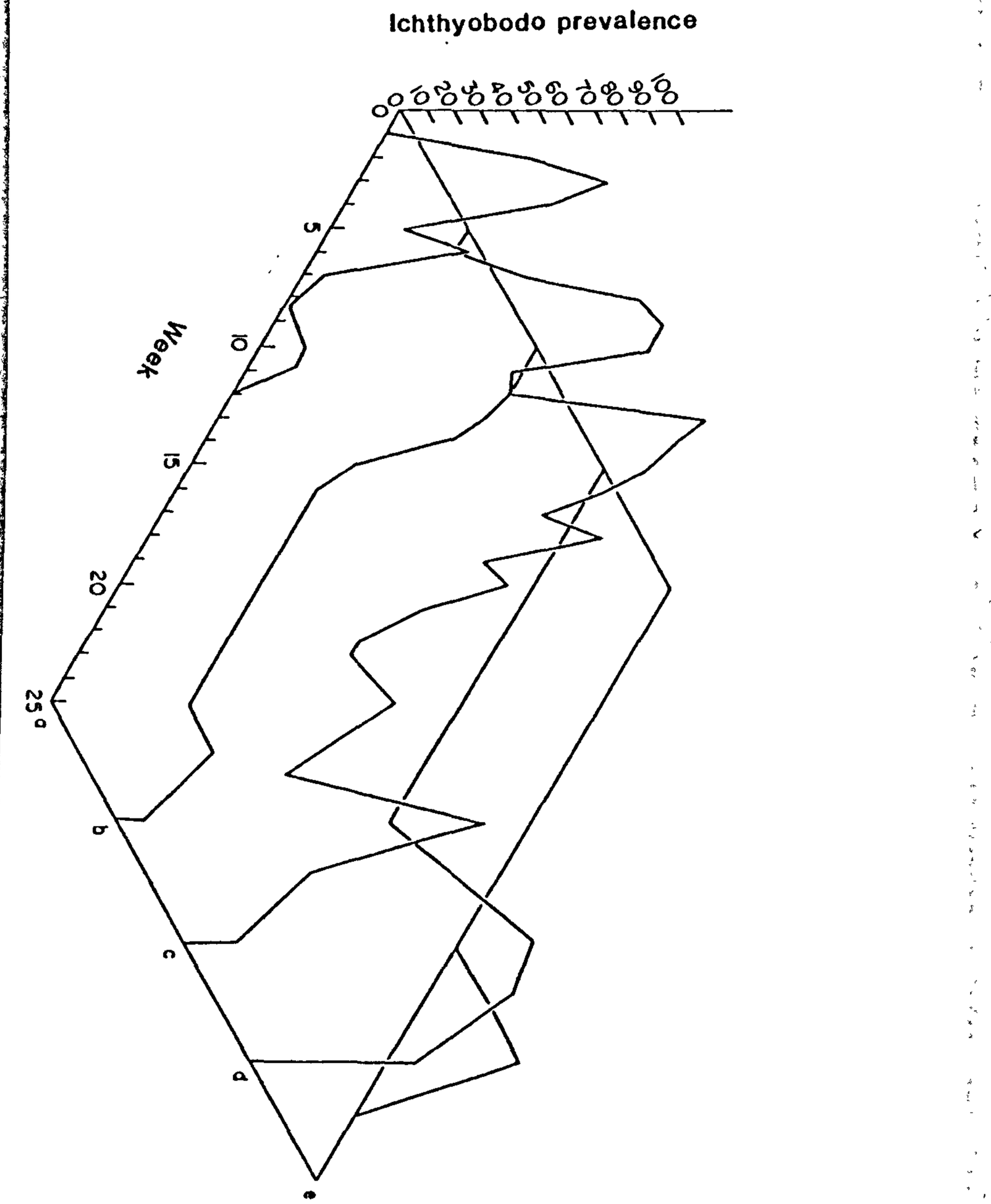
(a) 0+ salmon gills

(b) 0+ rainbow trout gills

(c) 0+ rainbow trout skin

(d) 1+ rainbow trout skin

(e) 1+ rainbow trout gills


Ichthyobodo prevalence
Week
5
10
15
20
25
a
b
c
d
e

for the salmon fry; however, the mortalities peaked at an estimated 7.6% per week by week 7 and by week 13 this had dropped to 0.2% per week.

2.4.2 Rainbow Trout Fry

From Table 6 and Figure 11 it can be seen that there were significant weekly increases in the number of Ichthyobodo on the gills to week 4. The intensity of infestation then declined sharply between weeks 5 and 7. Between weeks 7 and 8, there was an anomalous insignificant increase ($t = 0.46$, $p = 0.65$, d.f. = 29). The intensity of Ichthyobodo infestation then declined to zero by week 11. No Ichthyobodo were seen on the gills for the next eleven weeks, then on weeks 22 and 25 small numbers were encountered again.

The pattern of infestation on the skin was similar to that on the gills (see Table 6 and Figure 12); however, no Ichthyobodo were recorded until week 3, one week later than on the gills. The intensity of infestation reached a peak at week 4 and then numbers declined significantly between weeks 4 and 7. As in the gills, there was an insignificant increase in the intensity of infestation between weeks 7 and 8 ($t = 1.21$, $p = 0.236$, d.f. = 29). The intensity of infestation declined thereafter until by week 13 no Ichthyobodo were recorded on the skin. Between weeks 13 and 20 there were minor fluctuations in Ichthyobodo numbers on the skin. On week 20 there was a relatively large increase in numbers of Ichthyobodo present on the skin; the intensity of infestation did not change significantly for the next five weeks.

TABLE 6

Intensity of Ichthyobodo infestations on skin and gills of 0+ rainbow trout

	Gills			Skin		
Week	Mean 'n'	Mean log (n + 1)	SE	Mean 'n'	Mean log (n + 1)	SE
1	0	0	0	0	0	0
2	2.5	0.238	0.144	0	0	0
3	10.55	0.677	0.203	1.77	0.3615	0.093
4	23.2	1.040	0.194	7.4	0.5837	0.189
5	17.95	1.018	0.113	4.16	0.4167	0.108
6	2.615	0.310	0.114	1.58	0.2365	0.096
7	1.938	0.282	0.090	1.756	0.2019	0.093
8	3.33	0.351	0.120	4.06	0.3930	0.129
9	0.467	0.132	0.045	1.536	0.1518	0.091
10	0.300	0.060	0.060	0.30	0.0829	0.036
11	0	0	0	0.14	0.0252	0.025
13	0	0	0	0	0	0
15	0	0	0	0.13	0.04	0.020
18	0	0	0	0	0	0
20	0	0	0	0.42	0.1467	0.029
22	0.33	0.140	0.093	0.15	0.0528	0.027
25	0.20	0.048	0.048	0.15	0.0477	0.033

n = number of Ichthyobodo

FIGURE 11

Intensity of Ichthyobodo infestations on

0+ rainbow trout gills

(vertical bars = $\overline{X}$ ± S.E.)

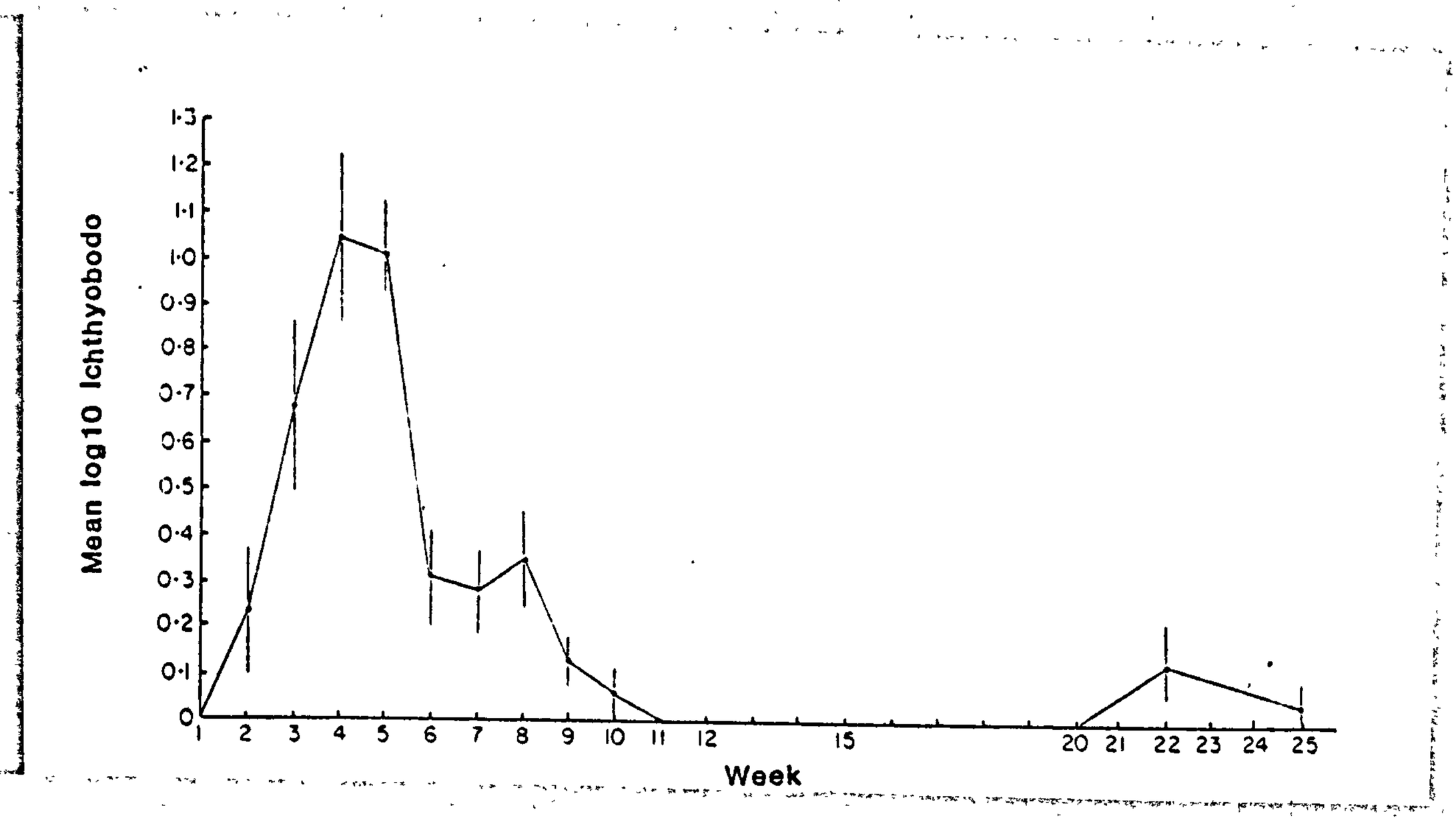
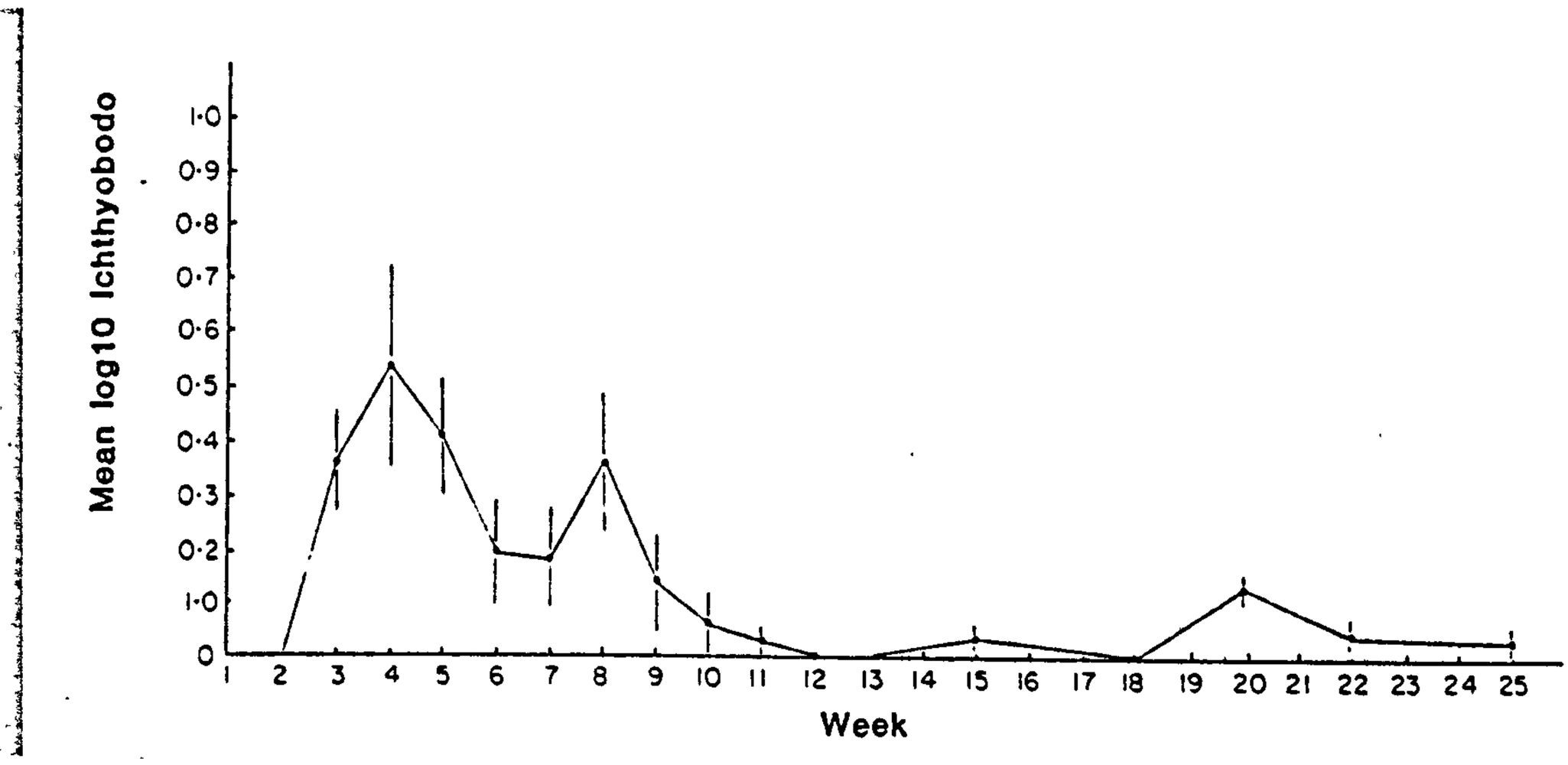

Mean log10 Ichthyobodo
1·3
1·2
1·1
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
1 2 3 4 5 6 7 8 9 10 11 12 15 20 21 22 23 24 25
Week

FIGURE 12

Intensity of Ichthyobodo infestations on

0+ rainbow trout skin

(vertical bars = $\bar{X}$ ± S.E.)


Mean log10 Ichthyobodo
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
1·0
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Week

The prevalence data for skin and gill is shown in Figures 10b and 10c. The prevalence of Ichthyobodo infestation on the gills rose rapidly from the start of the trial to weeks 4 and 5 when 90% of the fish examined were infested with Ichthyobodo. Between weeks 5 and 6 there was a highly significant decrease in prevalence of Ichthyobodo on the gills ($p < 0.01$, 1 d.f.). The prevalence then declined smoothly to zero by week 11. There was a similar pattern of infestation on the skin, peaking at week 3 with 78% of the fish infected. The decline was not as smooth as on the gills, with insignificant increases between weeks 7 and 8 and 9 and 10. The infestation had dropped to zero by week 13 and the prevalence until week 20 was low. There was a marked increase to 80% infestation at week 20; this had dropped to 30% by week 22 and remained at this level until the end of the study.

The weekly percentage mortality of the rainbow trout fry is shown in Table 7 and Figure 13 and the cumulative mortality in Table 8 and Figure 14. Mortalities had risen to nearly 1% per week by week 4. By week 5 there was a very sharp increase in mortalities to 3.44% per week. By week 8 the mortalities had peaked at 4.58% of the surviving population dying in a week (this represented 784 fish). After week 8 the mortalities declined rapidly to 1.5% by week 12. By week 15 the mortalities had returned to normal fish farm levels at one or two fish per tank per day. Between weeks 1 and 15, 24.67% of the original stock died. In the next thirteen weeks only 1.75% of the remaining stock died. The small peak at week 16 probably represents losses due to handling when reducing stocking density.

TABLE 7

Weekly percentage mortality of the 0+ rainbow trout

Week	% Mortality	Week	% Mortality	Week	% Mortality
1	0.44	10	2.28	19	0.08
2	0.51	11	1.59	20	0.08
3	0.57	12	1.62	21	0.07
4	0.82	13	1.14	22	0.14
5	3.44	14	0.37	23	0.1
6	3.43	15	0.27	24	0.14
7	3.05	16	0.63	25	0.03
8	4.58	17	0.30	26	0.03
9	3.82	18	0.13	27	0.02
				28	0.02

TABLE 8

Cumulative percentage mortality of 0+ rainbow trout infested with Ichthyobodo

Week	% Mortality	Week	% Mortality	Week	% Mortality
1	0.44	10	25.21	19	31.34
2	0.95	11	26.80	20	31.42
3	1.52	12	28.42	21	31.49
4	2.34	13	29.56	22	31.63
5	8.05	14	29.93	23	31.73
6	11.48	15	30.20	24	31.87
7	14.53	16	30.83	25	31.90
8	19.11	17	31.13	26	31.93
9	22.93	18	31.26	27	31.95
				28	31.97

FIGURE 13

Weekly percentage mortalities of 0+ rainbow trout caused by Ichthyobodo

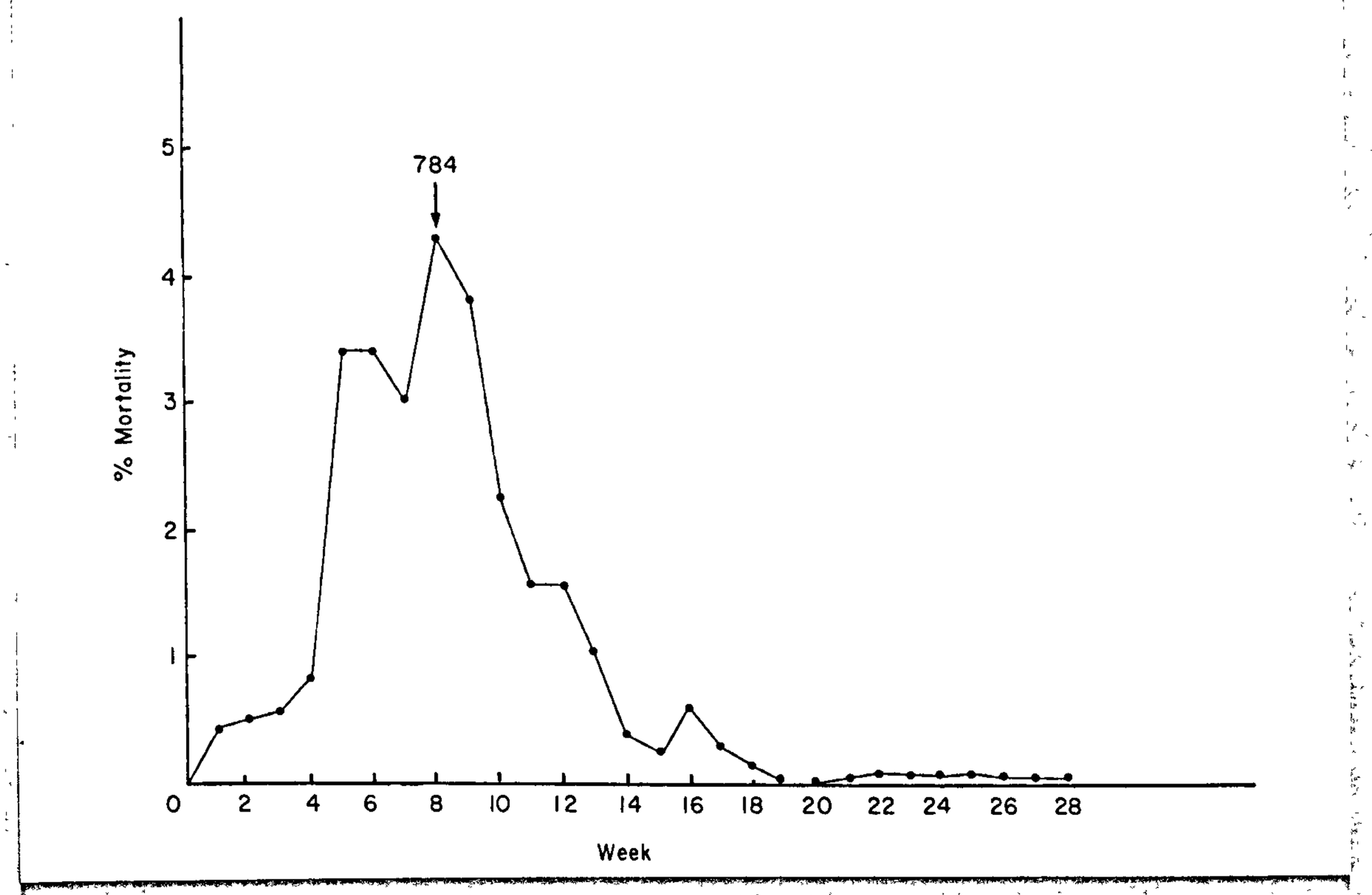
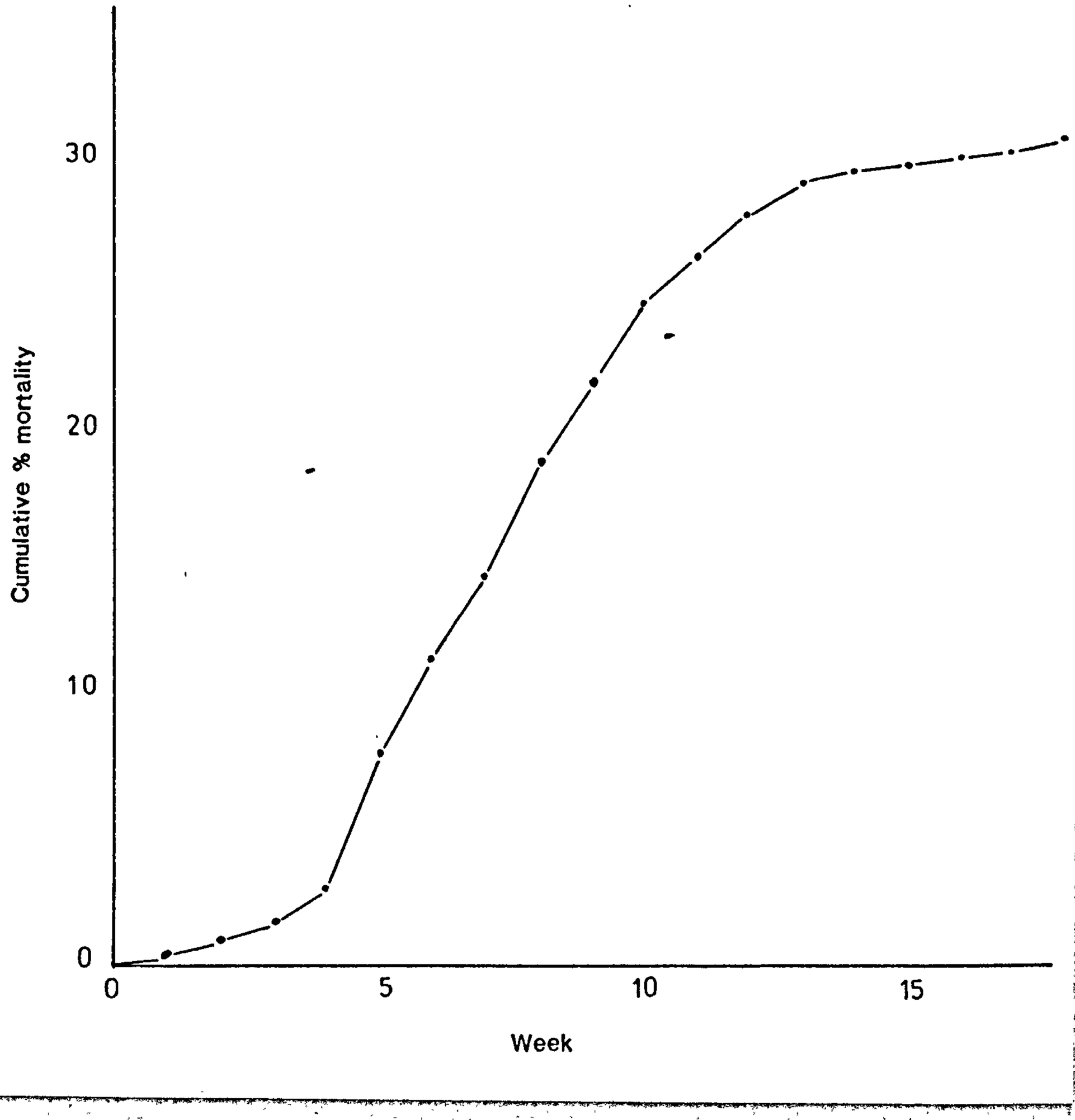

784
% Mortality
0
1
2
3
4
5
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
Week

FIGURE 14

Cumulative mortalities of rainbow trout caused by Ichthyobodo


Cumulative % mortality
30
20
10
0
0
5
10
15
Week

The only apparent cause of death in both the salmon fry and rainbow trout fry was the Ichthyobodo infestations. The only clinical signs of cause of death were fish floating belly upwards, erratic swimming, some erosion around the dorsal fin area and frayed fins. Heavily infested fish usually sought out the areas of the tank with least flow and the gills were seen to be beating very rapidly. Dying fish would often suddenly spiral to the surface rapidly and then sink to the bottom of the tank, apparently dead.

The fish which died were generally in good condition and did not show the characteristic pinhead appearance of starved fish which had not come on to feed properly. Histopathological studies confirmed the absence of any other aetiological cause of death.

2.4.3 Rainbow Trout 1+

No Ichthyobodo were encountered on the gills or skin of these fish until week 20 when moderately heavy infestations were seen on the skin of 80% of the fish examined; intensity and prevalence remained high until the end of the study (see Table 9 and Figures 15 and 10d). Likewise, in week 20, 44% of the fish had small numbers of Ichthyobodo on the gills (Fig. 10e); no Ichthyobodo were seen on the gills after this.

The mortality pattern of these fish was very different to that of the 0+ fish as on average only one fish per week died in each tank, even during the later stages of the trial when relatively large numbers of Ichthyobodo were seen on the skin of these fish.

2.4.4 Appearance of Ichthyobodo

The Ichthyobodo were observed under phase and interference phase contrast microscopy and samples measured by means of an eyepiece

TABLE 9

Intensity of Ichthyobodo infestations on skin and gills of 1+ rainbow trout

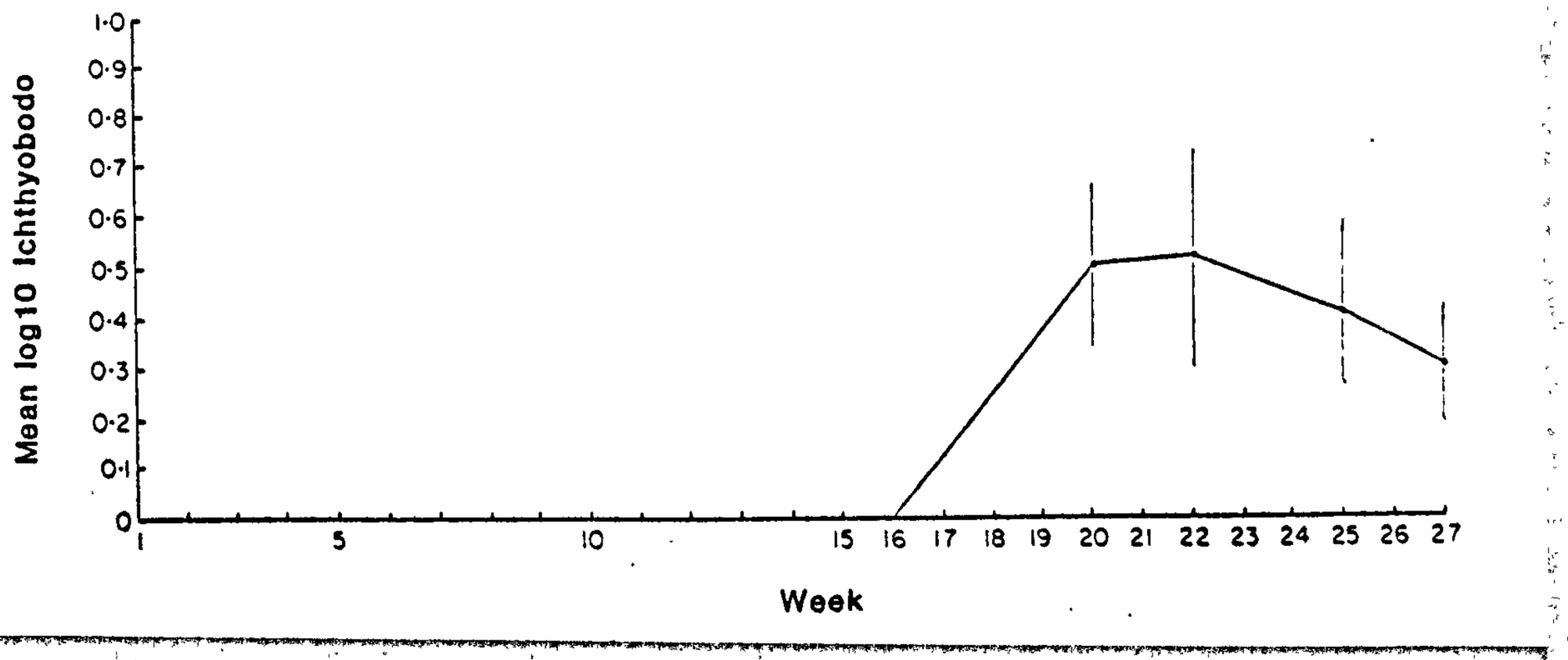
Week	Gills			Skin		
	Mean 'n'	Mean log (n + 1)	SE	Mean 'n'	Mean log (n + 1)	SE
No Ichthyobodo until Week 20						
20	2.66	0.367	0.148	4.11	0.507	0.140
22	0	0	0	4.20	0.511	0.208
25	0	0	0	3.90	0.412	0.151
27	0	0	0	2.00	0.30	0.12

n = number of Ichthyobodo

FIGURE 15

Intensity of Ichthyobodo infestations on 1+ rainbow trout skin.

(vertical bars = $\overline{X}$ ± S.E.)


Mean log10 Ichthyobodo
1·0
0·9
0·8
0·7
0·6
0·5
0·4
0·3
0·2
0·1
0
1
5
10
15
16
17
18
19
20
21
22
23
24
25
26
27
Week

graticule. The size of the parasite varied from individual to individual but was always in the range 5-15 µm long and 2-7.5 µm wide. No trends in size were obvious over the period of the study. It was found that ten minutes under a cover slip was the optimum time for counting the parasite as most had detached from the host cell by this time, which greatly facilitated counting. It was very unusual to observe the "free form" of the parasite immediately after the scrape was taken, most being attached.

2.4.5 Correlations

A matrix of correlation coefficients for all pairs of variables was computed for the 0+ fish. The most relevant correlations are shown in Table 10. There were highly significant correlations in both salmon and rainbow trout fry between Ichthyobodo levels on gills and skin and mortalities, and a highly significant -ve correlation between Ichthyobodo levels and passage of time when taken over the whole study.

The presence or lack of a relationship between the condition of 0+ fish (as expressed by condition factor $w/L^3 \times 100$) and the intensity of Ichthyobodo infestation was tested. No correlation was found between the condition of the fish and the intensity of infestation on the gills or skin of the salmon and rainbow trout.

When comparing the skin and gill data for the 0+ rainbow trout, although the skin infestation seemed more prolonged there was a good overall correlation between the two.

2.4.6 Environmental Variables

Mean weekly temperatures are represented graphically in Figure 16. During weeks 0-12, the critical times for Ichthyobodo infesta-

TABLE 10

Correlation of transformed Ichthyobodo levels on 0+ fish with other variables

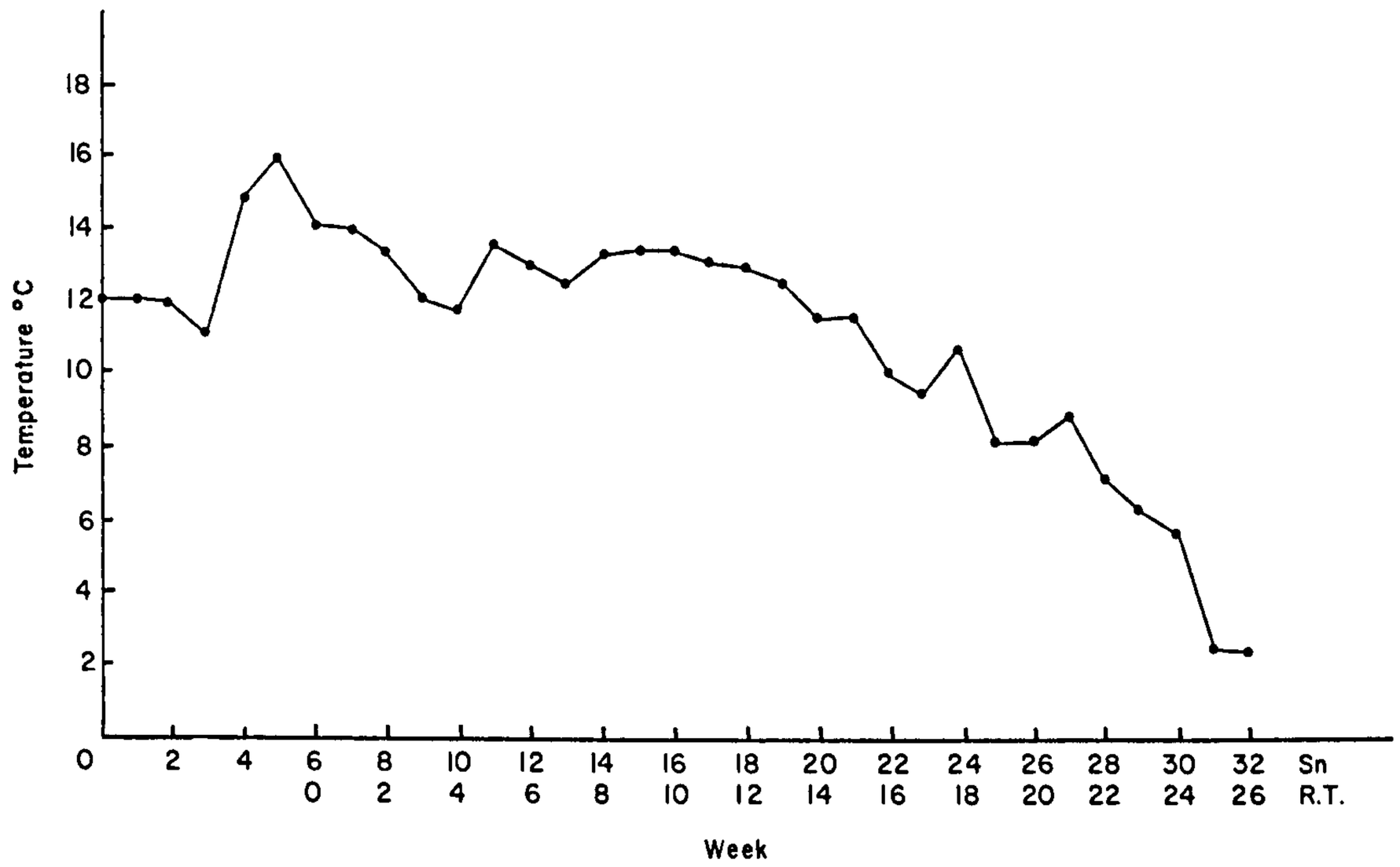
Variables	r	p	
Colog 1 (RT)/Mortality	0.21	0.002	Colog 1 = Ichthyobodo on gills
Colog 2 (RT)/Mortality	0.24	0.0003	Colog 2 = Ichthyobodo on skin
Colog 1 (Sn)/Mortality	0.394	0.00001	Confact = Condition factor
Colog 1 (RT)/week	-0.446	0.00001	
Colog 2 (RT)/week	-0.312	0.00001	
Colog 1 (Sn)/week	-0.333	0.00001	
Confact/Colog 1 (RT)	0.01	0.385	
Confact/Colg 2 (RT)	0.05	0.248	
Confact/Colog 1 (Sn)	0.016	0.41	
Colog 1/Colog 2	0.580	0.00001	

FIGURE 16

Mean weekly water temperatures over the time period of the study

Sn = salmon

RT = rainbow trout


Temperature °C
18
16
14
12
10
8
6
4
2
0
2
4
6
0
8
2
10
4
12
6
14
8
16
10
18
12
20
14
22
16
24
18
26
20
28
22
30
24
32
26
Sn
R.T.
Week

tions, the mean weekly temperature varied only 2°C (12-14°C) for the rainbow trout fry, and 5°C (11-16°C) for the salmon. Temperatures started declining from the end of August (week 11 RT and 17 Sn) and the mean weekly temperatures dropped below 10°C from the end of October onwards (week 19 RT and 25 Sn). pH varied between 6.5 - 7.5, the lowest pH being recorded after heavy rain but generally the variation was between 7.4 - 7.5. The pH did not vary outside this range between weeks 0-12. Total hardness at 24.5 ppm $CaCO_3$ did not change significantly over the time period studied.

2.4.7 Other Parasites on 0+ and 1+ Rainbow Trout

Apart from Ichthyobodo, the following ectoparasites were observed in low numbers:- One Gyrodactylus sp., and the following protozoa, Bodo sp., Chilodonella sp., Ichthyophthirius multifiliis, Trichophrya piscium, Trichodinids and the following members of the Scyphidia complex: Scyphidia, Glossatella, Epistylis. Their seasonal fluctuations are shown in Figures 17 and 18.

In general there was a higher incidence of other parasites on the 1+ rainbow trout than on the 0+ rainbow trout, the reverse of the case with Ichthyobodo. Scyphidians were the only parasites which reached anything like the incidence and intensity of Ichthyobodo infestations. The incidence of other parasites was generally low and appeared to fluctuate throughout the trial.

Ichthyophthirius multifiliis and Ichthyobodo necator were the only parasites recorded on the 0+ fish and not on the 1+ fish before week 20.

None of the parasites showed the same rapid rise of infestation followed by decline to extinction as Ichthyobodo.

FIGURE 17

Prevalence of other parasites on 0+ rainbow trout.

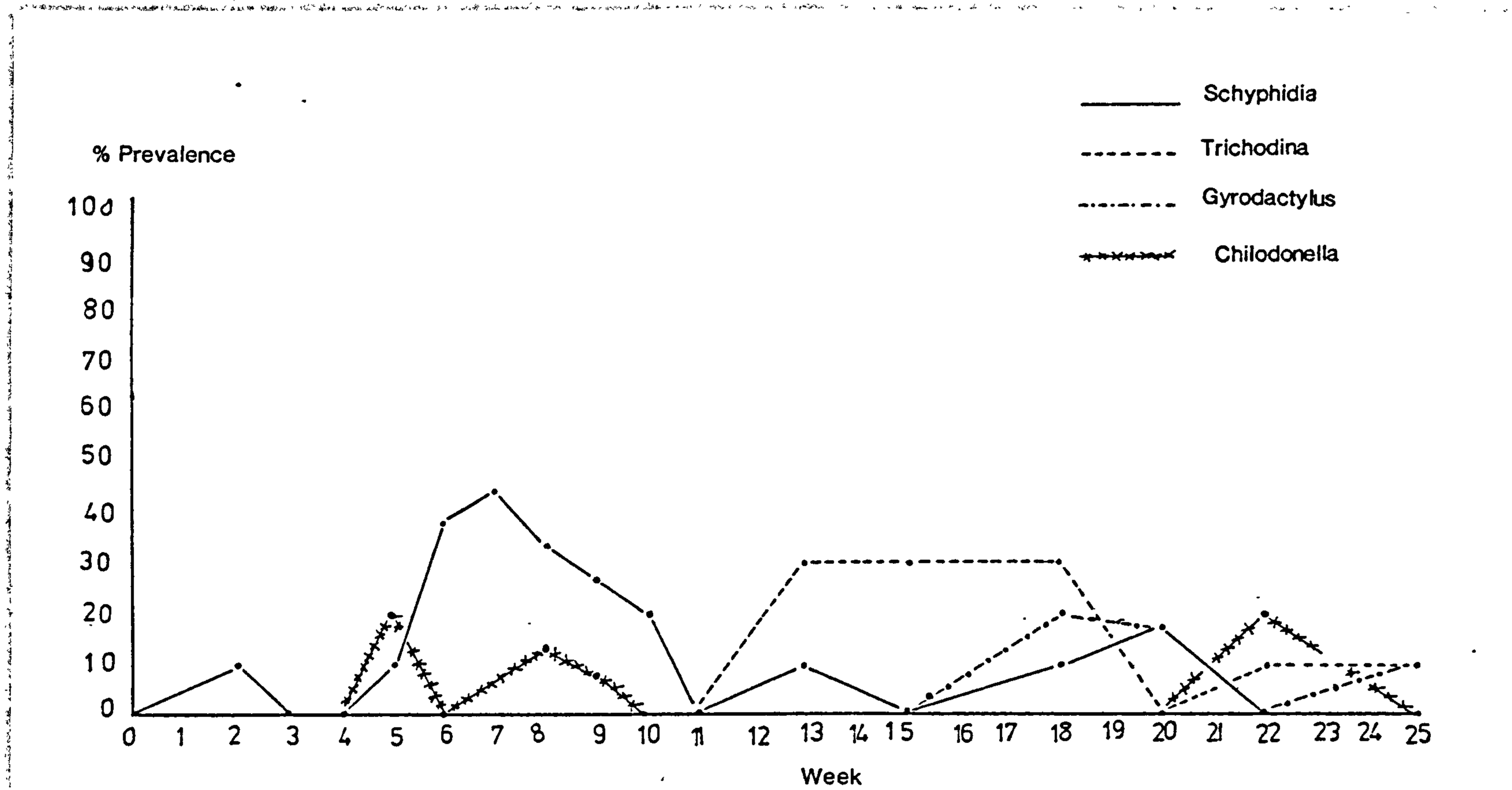
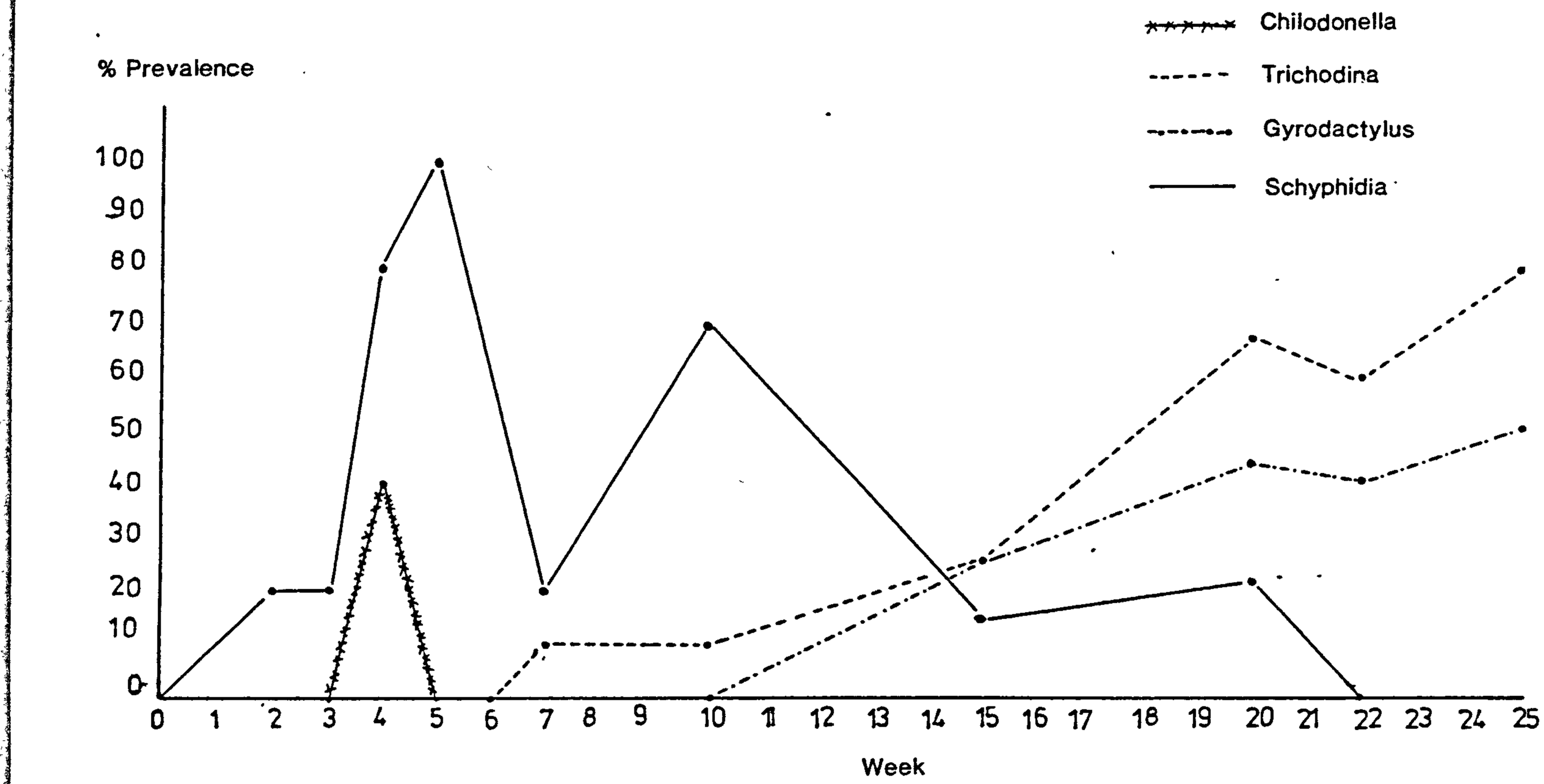

% Prevalence
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Week
Schyphidia
Trichodina
Gyrodactylus
Chilodonella

FIGURE 18

Prevalence of other parasites on

1+ rainbow trout


% Prevalence
100
90
80
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Week
Chilodonella
Trichodina
Gyrodactylus
Schyphidia

These other parasites also did not appear to have the same deleterious effect on the host as Ichthyobodo as there were very low mortalities in the 1+ rainbow trout and the mortalities on the 0+ fish had dropped to low levels by week 15 when these other parasites were extant.

2.4.8 1979-1980 Data

A similar study was carried out on 0+ rainbow trout from May 1979-80 to assess if the trends shown in this study were normal. These are summarised in APPENDIX 1. There was a very good overall correlation between mortalities and Ichthyobodo number fluctuations in both years.

2.5 DISCUSSION

The present investigation has clearly shown that costiasis, the disease caused by Ichthyobodo necator, when left untreated, can be a serious pathogen of first feeding salmonids, causing up to 25% mortality. Much higher mortalities caused by Ichthyobodo have been described by Bauer (1958), who quotes a case of 100% mortality caused by costiasis of carp in spawning ponds in the Ukraine, and Pickering and Richards (1980) have described mortalities of 50% in untreated brown trout infested with Ichthyobodo.

The Ichthyobodo infestation showed a marked dependency on the age of the host as the one-year old rainbow trout examined did not show the same pattern of infestation or mortality as the first feeding fry. This phenomenon has been described before in carp by Lyamain (in Bauer 1959) who noted the consistently lighter infections of Ichthyobodo on older carp. Wood (1974) also noted the parasite's preference for attacking fry and young fingerlings.

The results obtained show a clearly defined periodicity displayed by Ichthyobodo infestations on first feeding salmonid fry, typified by a rapid increase in numbers of Ichthyobodo on skin and gill to a peak at four weeks, followed by a decline to zero on the gills in eleven weeks, and insignificant numbers on the skin by thirteen weeks, and virtual absence for the next twelve weeks in which other parasites were present.

The reasons for the decline in numbers of Ichthyobodo are at present unknown but it would appear that there is either some skin or gill change which prevents the parasite colonising or some form of immunity developing. As far back as 1908, Franke observed the 'mysterious disappearance' of Ichthyobodo on brook trout, whilst Tavolga and Nigrelli (1947) found that aquarium fish developed relative immunity to superinfestation of Ichthyobodo and suggested that immunity developed through premunition. Lyamain (in Bauer, 1959) suggested a form of age immunity to Ichthyobodo in older carp. The concept of immunity to parasitic protozoa in fish is not new and there have been an increasing number of reports in the literature recently. Hines and Spira (1974) described immunity to Ichthyophthirius multifiliis on carp, Bower and Woo (1977) immunity of rainbow trout to Cryptobia catostomi, Nagel and Summerfelt (1977) apparent immunity of goldfish to Pleistophora ovariae. All of these protozoans either live intracellularly or, like Ichthyobodo, feed from the host. The appearance of Ichthyobodo on the 1+ rainbow trout and reappearance on the 0+ rainbow trout after a decrease in temperature would appear to be a particularly significant finding related to a temperature effect on the host defence mechanisms. One such relationship, which is already well

defined is the effect of low temperature in depressing the immune response. Avtalion, Wojdani, Malik, Shahrabani and Duczyminer (1973) have reviewed the effect of environmental temperature on the immune response of fish and found that in general the immune response is reduced at low temperatures. The high prevalence and intensity of Ichthyobodo on the 1+ rainbow trout at this time in the study may also be explained by the fact that many of these fish had started to mature by week 20. It is known that as the fish mature there is an increase in circulating corticosteroids in both sexes (Whitehead, Bromage and Forster, 1978; Whitehead, Bromage, Breton and Billard, 1978; Whitehead Bromage, Forster and Matty, 1978; Breton, Jalabert, Fostier and Billard, 1975; Jalabert and Breton 1980; Gross, 1976; Fostier, Weil, Tezin, Breton and Jalabert, 1978; Hane and Robertson, 1959; Lambert, Bosman, van der Kurk and van Oordt, 1978; Scott, Bye and Baynes, 1980). These compounds are known to be immuno-suppressants in mammals (Monjan and Collector, 1977)

The pattern of infestation on the skin and gills of the rainbow trout fry appeared to be slightly different. Although there was a good overall correlation, the infestation on the skin started later and declined more slowly. There seemedto be three phases to the Ichthyobodo infestations on the fry, (1) infestation of gills; (2) infestation of gills and skin; (3) infestation of skin only. When Ichthyobodo was seen in very large numbers on the skin of the 1+ rainbow trout, very few were seen on the gills.

The mortality pattern was certainly correlated to the infestation of Ichthyobodo although there was some hysteresis between peak of parasite infestation and peak mortality. The infestation of the skin seems to be of equal importance as the gill infestations

and thus it cannot be assumed, as Savage (1935) did, that the cause of death is by respiratory failure due to the parasite on the gills only.

Previous authors, i.e. Amlacher (1970) and Bauer (1958) have suggested that Ichthyobodo is a parasite of debilitated fish in poor condition; whilst this is probably true of older fish, broodstock and kelts, the results presented here suggest that this is not the case in first feeding salmonids. There was found to be no correlation between condition of fish and levels of Ichthyobodo on skin and gills of the 600 fish examined; fish in good condition were just as likely to be severely infested as fish in poor condition. If there is an immune response to Ichthyobodo it is possible that debilitated fish are immunosuppressed and therefore cannot resist the Ichthyobodo.

The data obtained on environmental variables showed that Ichthyobodo infestation occurred at temperatures between 3.5°C-16°C. This confirms with other findings that Ichthyobodo is not temperature specific. Hlond (1963) described massive infestations of carp eggs and larvae at 20-22°C, Benisch (1936) found the parasites in temperatures ranging from 2-29°C. Tavolga and Nigrelli (1947) claimed that the parasite was active at 38°C, whereas Amlacher (1970) considered the upper lethal temperature to be 30°C. As the pH did not change significantly throughout the period of study it is unlikely that this was responsible for any effect on the level of Ichthyobodo infestation.

2.6 SUMMARY OF TRIALS AT ALMONDBANK, LOCHAILORT AND HOWIETOUN

It would appear from the studies of the three sites (one of which was duplicated over a two year period) that salmonid fry are particularly susceptible to Ichthyobodo infestations for the first few weeks of first feeding when, if untreated, they can lead to very severe mortalities. However, even when left untreated a form of immunity seems to set in after 4-6 weeks and the parasite numbers and fish mortalities drop off to zero.

The parasite appears to be capable of recolonising the fish after a period of cold water temperatures and/or in conjunction with sexual maturation. Although the parasite infestation would appear to be seaonal this probably only reflects the time of year when the fish are susceptible. It is not clear whether this is caused by diminution of an acquired immunity by environmental, hormonal or other stressors or lack of immunocompetence in the case of the fry. (Ellis (1977) has suggested that the immune system is "primed" by food acting on antigens in the gut just after first feeding.)

It would appear likely therefore that if young first feeding fish were available throughout the year, which can now occur by importation of southern hemisphere eggs, or by photoperiod manipulation of broodstock, Ichthyobodo infestations would occur not only in Spring, the traditional time for first feeding in Britain, but throughout the year.

CHAPTER 3

PATHOGENESIS AND AUTORADIOGRAPHIC STUDIES OF THE EPIDERMIS OF SALMONIDS INFESTED WITH ICHTHYOBODO NECATOR

CHAPTER 3

PATHOGENESIS AND AUTORADIOGRAPHIC STUDIES OF THE EPIDERMIS OF SALMONIDS INFESTED WITH *ICHTHYOBODO NECATOR*

3.1 INTRODUCTION

It is very surprising that for such a common and damaging parasite, the pathology of the condition caused by infestation of *Ichthyobodo* and its pathological effects on the host's skin have remained poorly documented. Apart from a brief mention in early papers by Fish (1940) and Tavolga and Nigrelli (1947), virtually all of the published information on *Ichthyobodo* has been motivated towards clarifying its taxonomic status. Ellis and Wootten (1978) briefly described the pathology of *Ichthyobodo* infestations on the gills of Atlantic salmon, *Salmo salar* L., smolts in sea water, but did not describe the effect of the parasite on the skin.

The work described in Chapter 2 has indicated that the infestation of the skin by *Ichthyobodo* is as important in causing mortality in juvenile farmed salmonids, as gill infestation. Thus, it was decided to attempt to assess the effect of the parasite on the skin of salmonids of varying ages under controlled conditions, and to study the cell kinetics of the infested epidermis, and to compare them to those observed in normal and wounded epidermes of other teleost species (Bullock, Marks and Roberts, 1978a and b; Bullock and Roberts, 1980). For a review of normal salmonid skin see Roberts, Shearer, Elson and Munro (1970), Roberts and Bullock (1980) and Pickering and Richards (1980).

3.2 MATERIALS AND METHODS

3.2.1 Fish

3.2.1.1 Sequential pathology of costiasis in salmon alevins

One hundred salmon alevins previously held in spring water at Howietoun fish farm were held in recirculating fresh water at 12°C. These alevins had hatched from eggs which had been disinfected with 50 ppm Buffodine; an iodophor compound, which although previously used as an antiviral agent also kills Ichthyobodo. Earlier work had shown that Ichthyobodo could be transmitted from broodstock to eggs in the stripping process. Fifty 0+ rainbow trout heavily infested with Ichthyobodo were placed in the same tank. Five fish were sampled daily for a period of fourteen days and fixed in phosphate buffered formalin and processed for paraffin wax sections at 5 microns. The sections were then stained with haematoxylin and eosin, and alcian blue (pH 2.5), see Appendix 2.

3.2.1.2 Pathology of costiasis in salmon smolts

Thirty salmon smolts were treated with 200 ppm formalin two weeks prior to commencement of the trial to eliminate any ectoparasites. The fish were checked to confirm the absence of any parasites and then freeze branded and placed in an aquarium containing recirculating water at 12°C. Ten severely stressed smolts, heavily infested with Ichthyobodo were placed in the water. Two of the branded smolts were sacrificed and sampled daily for the next fourteen days and blocks of skin and gills processed as above.

3.3 AUTORADIOGRAPHY

3.3.1 *In vivo* study

Because of the unavailability of salmon alevins at the time of year that this part of the study commenced, rainbow trout alevins heavily infested with *Ichthyobodo* were used. Fifty heavily infested alevins were anaesthetised with iced water (Mittal and Whitear, 1978) and then injected intraperitoneally with tritiated thymidine (H^3) to give a final dosage of 20 μm Ci per gram body weight. The fish were then allowed to recover and placed in an aquarium containing recirculating fresh water at 12°C.

Five fish were sampled at intervals of 16 hours, 40 hours, 3, 4, 5, 6, 7 and 8 days respectively. The fish were fixed in phosphate buffered formalin and processed for paraffin wax sectioning at 5 μm. Sections were dipped in Ilford K2 emulsion and exposed in the dark for sixty days at 4°C. The sections were then processed in Kodak D76 developer and subsequently stained in haematoxylin and eosin. Estimates of labelling were obtained from examination of the sections under oil immersion at × 100 magnification. Nuclear labelling of more than six grains per nucleus over background was required before a positive result was recorded.

3.3.1.2 *In vitro* study

Because of the lack of suitability of the *in vivo* technique for large fish an *in vitro* method was used to study the epidermal changes in the smolts which had been severely stressed.

Two of the smolts maintained at 10°C and heavily infested with *Ichthyobodo* were killed and blocks of skin and muscle were dissected from the fish and placed in sterile petri dishes containing 10 mls

of Eagle's (Glasgow modification) tissue culture medium (Paul, 1975) see Appendix 3, and 0.2 mls of tritiated thymidine (specific activity of 15-30 Ci/mmol) was added to each dish and incubated at 10°C. Blocks of skin and muscle were removed from the media at 12, 24 and 48 hours and fixed for histology and autoradiography as described above.

3.4 RESULTS

3.4.1 Salmon Alevin Pathology

Within three days of exposure to the infested rainbow trout, Ichthyobodo were seen attached to the epidermis of the alevins; large numbers were not seen until day 7, but by day 12 all fish sampled showed moderately heavy Ichthyobodo infestations.

The areas of skin infestations showed a focal non-random distribution, several discrete areas being invariably infested. These foci were the cuff of the skin sheltered by the operculum (Fig. 19), the pectoral and pelvic fins (Fig. 20) and the area of skin subjacent to the dorsal fin. Because of the method of sectioning, infestations of the gills were difficult to ascertain; however, when the alignment was correct moderate numbers of Ichthyobodo were seen attached to the secondary lamellae of the gills and also to the pseudobranch (Fig. 21). The skin on the head of the alevins anterior to the operculum was never seen to be infested by Ichthyobodo over the duration of this study.

The presence of Ichthyobodo led to marked hyperplasia of the malphigian cells of the epidermis underneath the infested area, and by way of contrast the almost complete disappearance of goblet cells in the same area (Fig. 22). Uninfested skin in the immediate

FIGURE 19

Ichthyobodo attached to the "cuff" of skin sheltered by the operculum

HαE × 320

I = Ichthyobodo

O = operculum.

FIGURE 20

Ichthyobodo attached to the underside of a pectoral fin

HαE × 320

I = Ichthyobodo


O

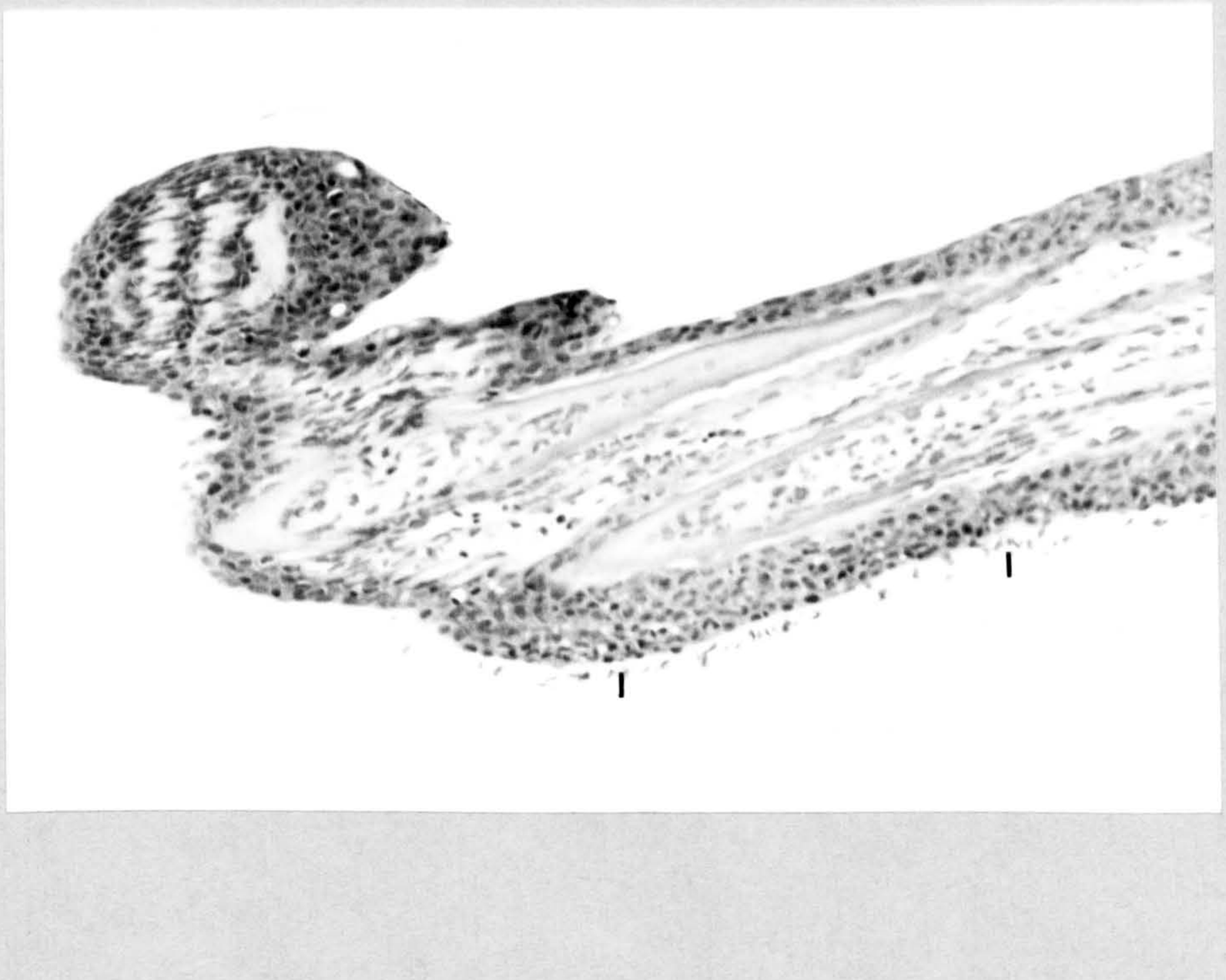


FIGURE 21

<u>Ichthyobodo</u> attached to the peripheral cells

of the pseudobranch

HαE × 410

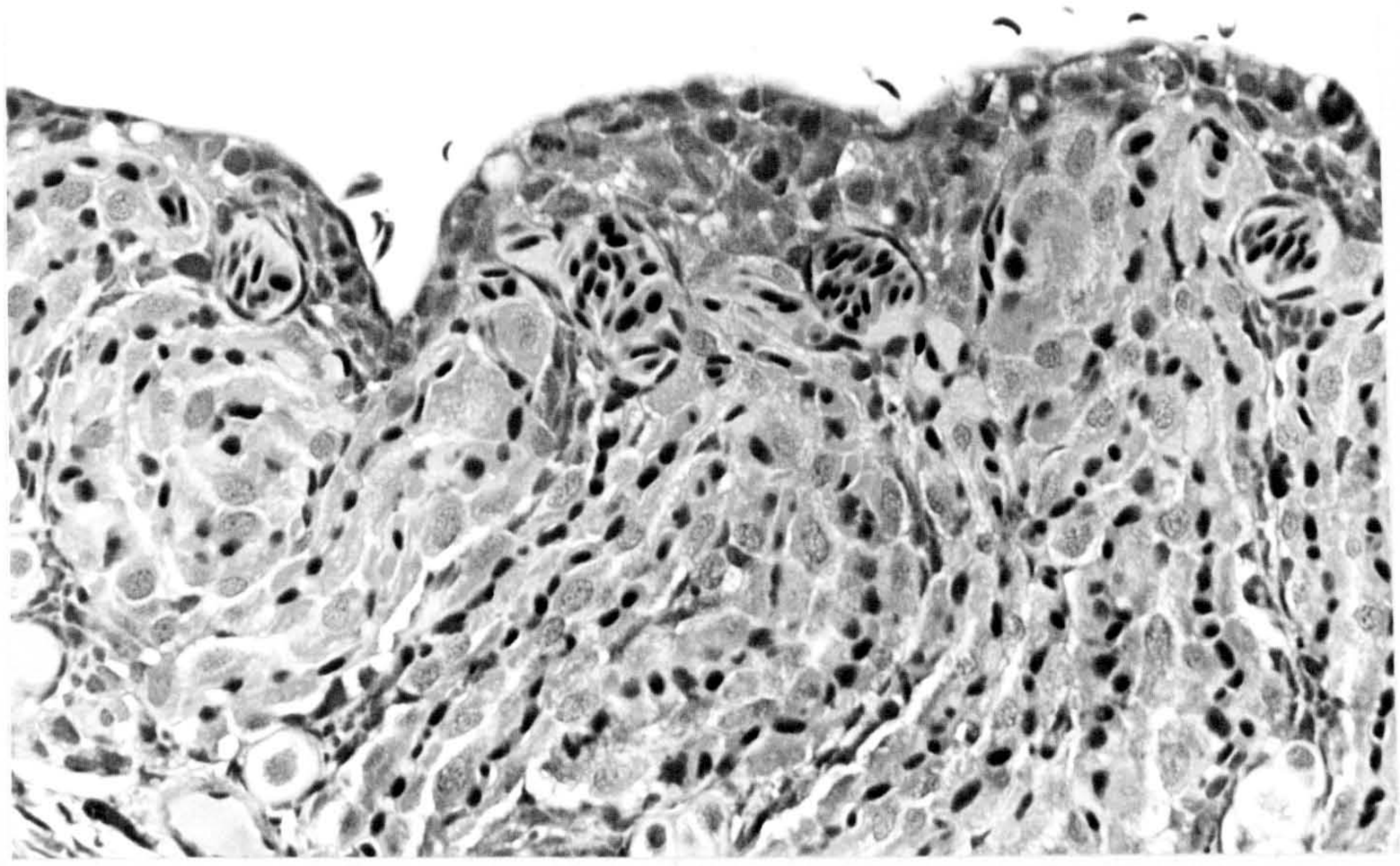
FIGURE 22

Hyperplastic epidermis with <u>Ichthyobodo</u>

attached to peripheral cells.

Note the lack of goblet cells.

HαE × 410



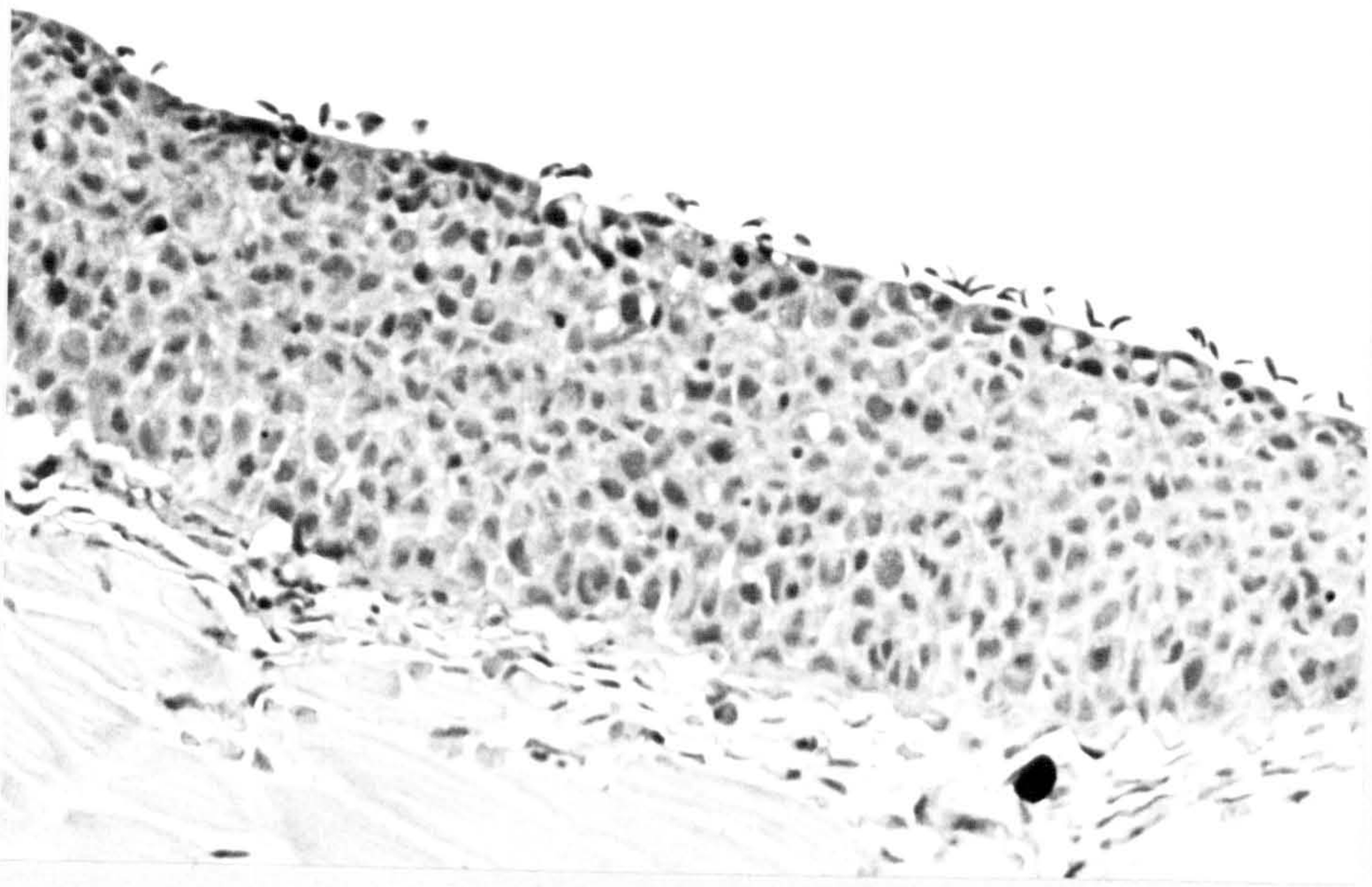
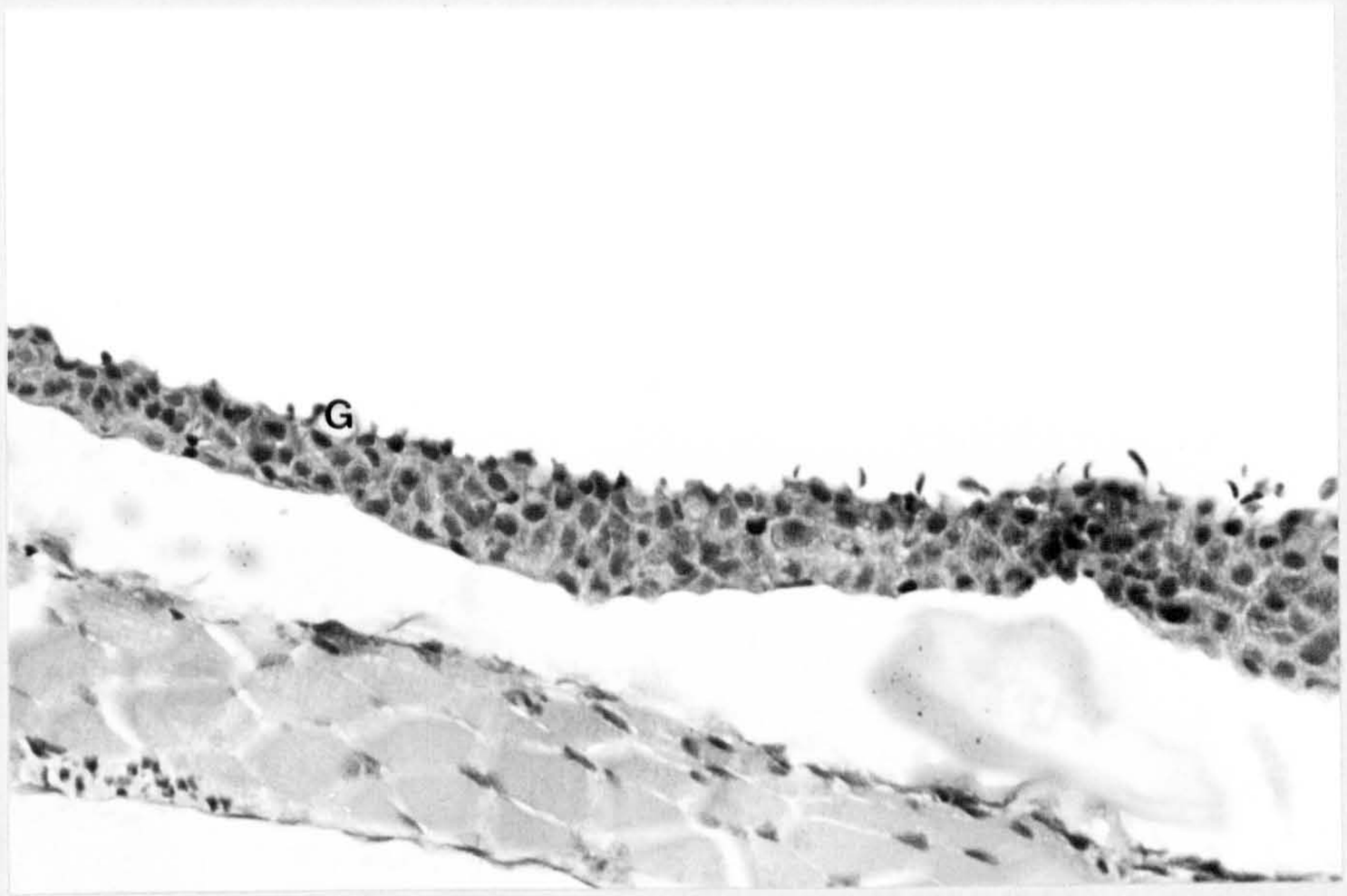


FIGURE 23

Low power light micrograph to show progressive thickening of epidermis infested by Ichthyobodo. Note decline of goblet cells (G) in infested area.
HE × 320.

FIGURE 24

Normal uninfested skin, note goblet cells (G)
(H&E × 410)


G

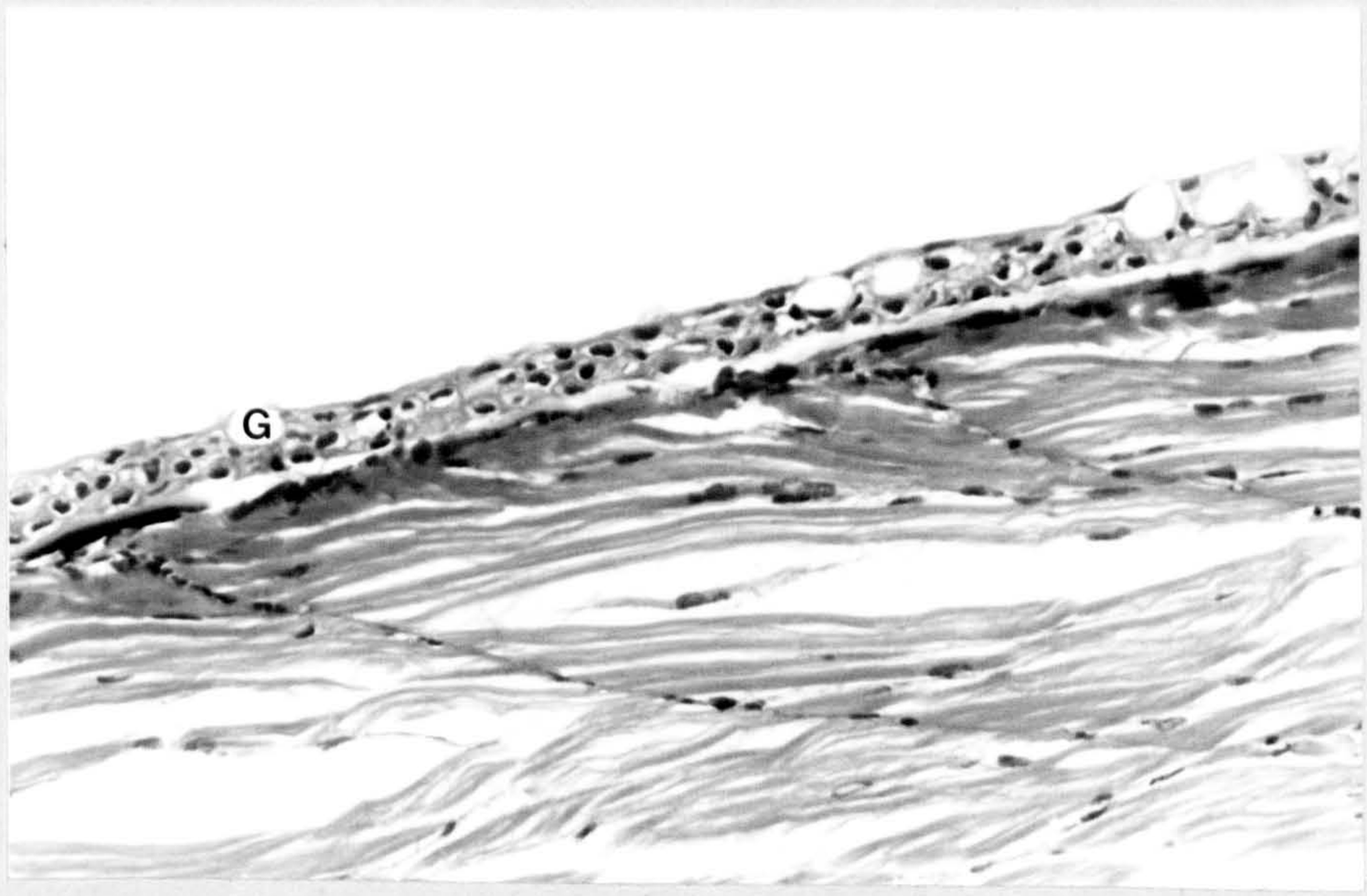

G

FIGURE 25

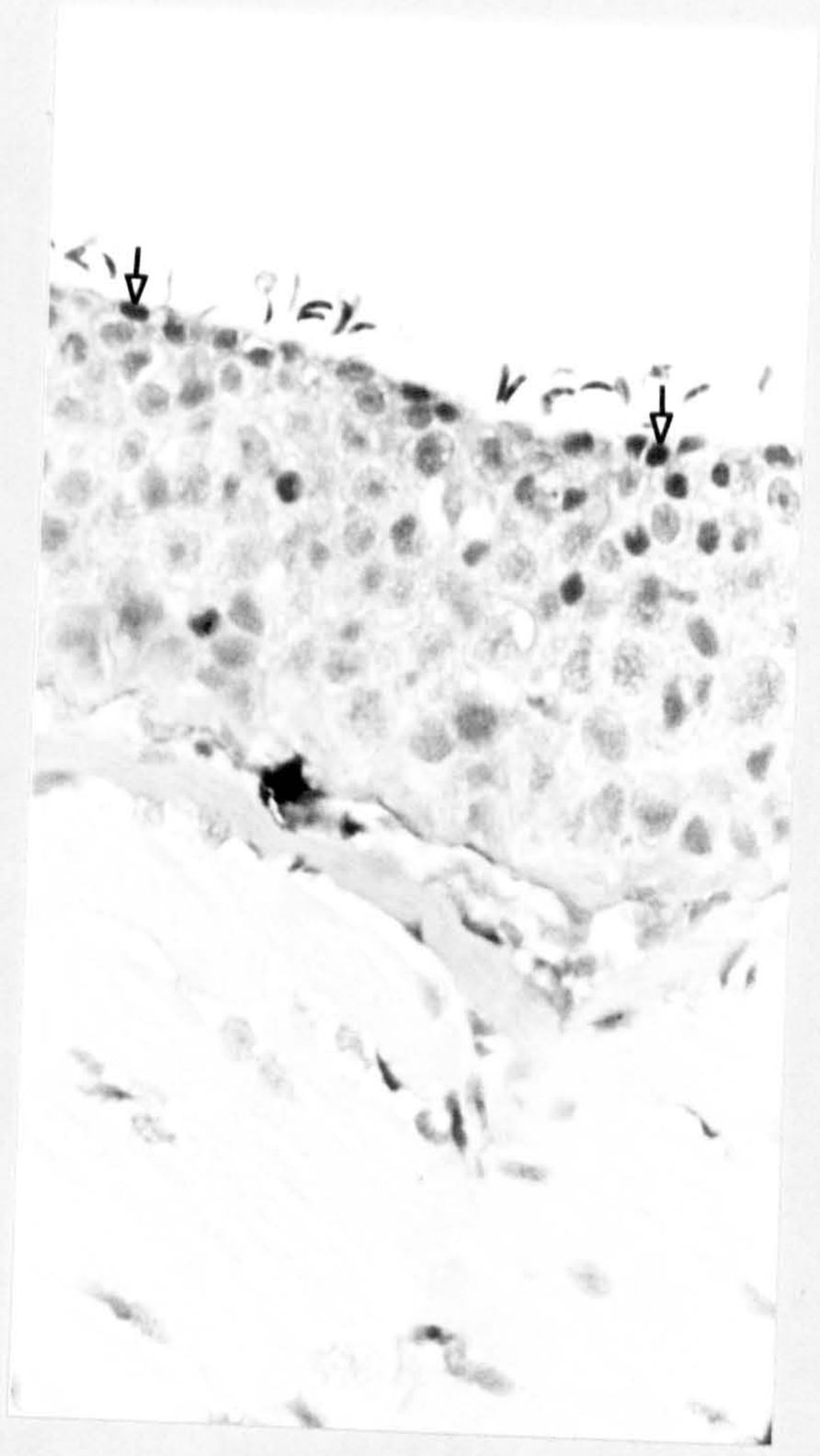
Strongly basophilic nuclei (arrowed) of periphal cells beneath Ichthyobodo infestation.

(HαE × 500)

FIGURE 26

Effete ghost cells (arrowed) showing characteristic 'Sucked out appearance'. Note the leucocytes (L) migrating through the hyperplastic epidermis.

(HαE × 500)



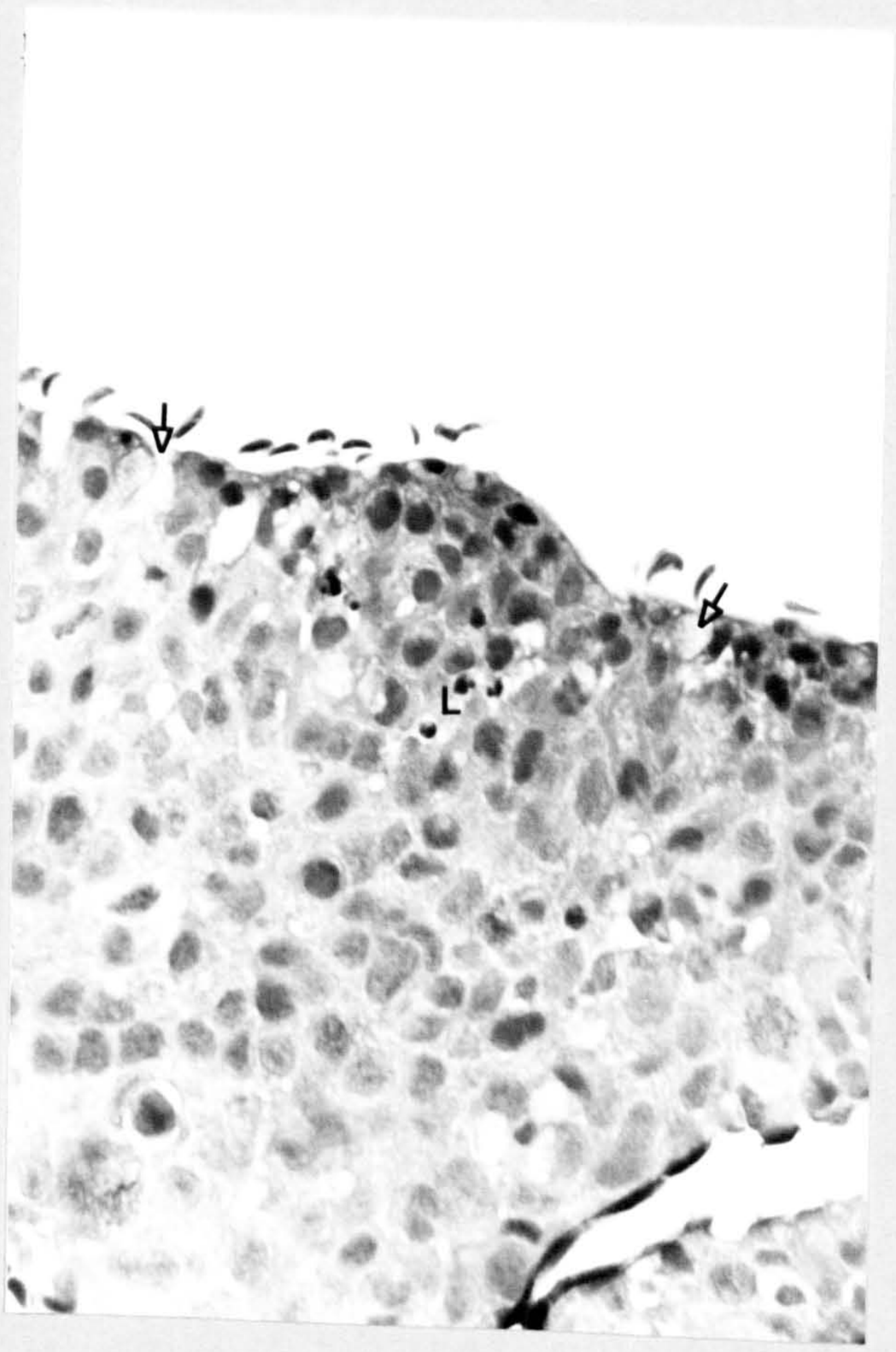


FIGURE 27

Spongiosis (S) of epidermis beneath

Ichthyobodo infestation.

(HαE, × 410)

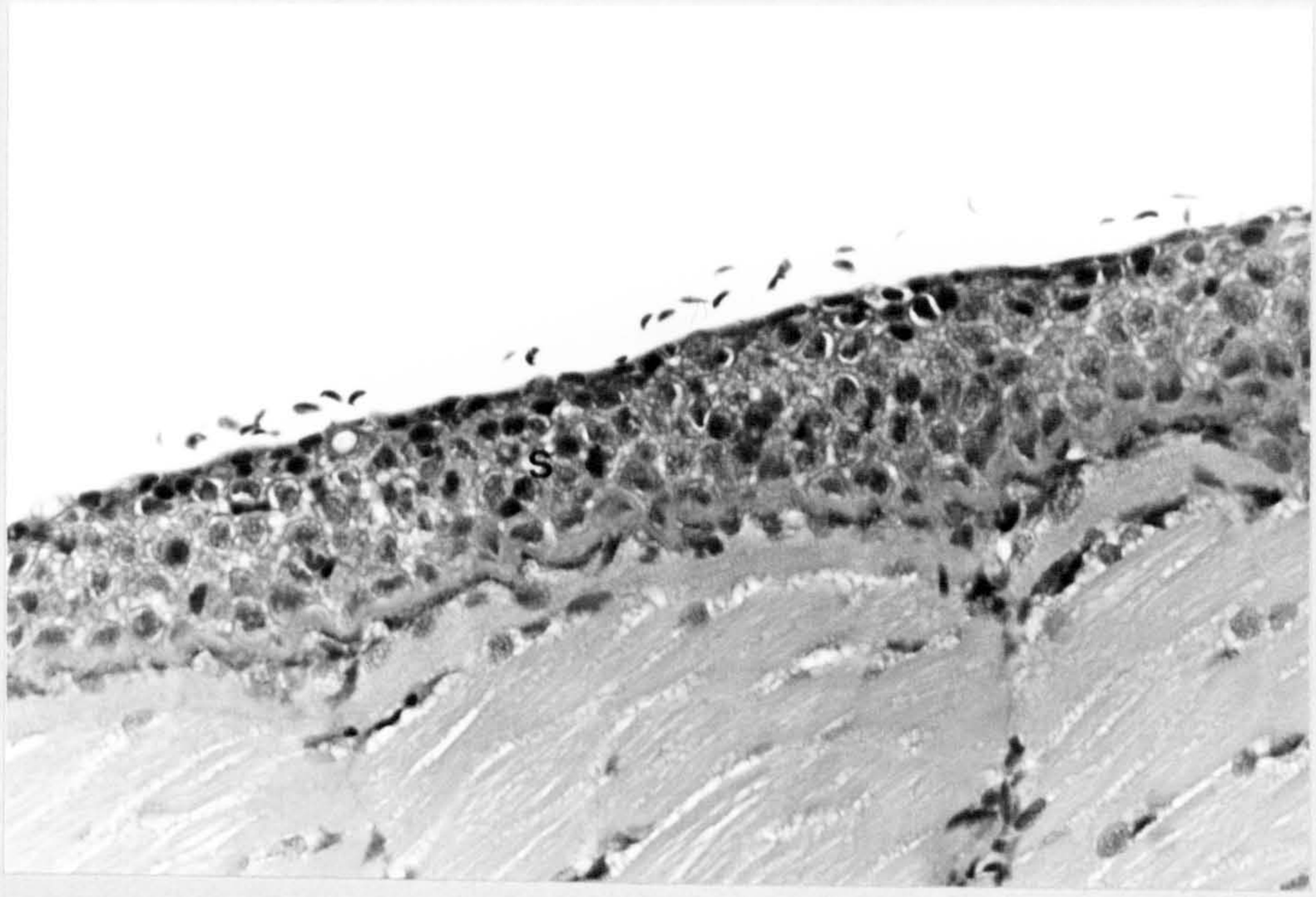
FIGURE 28

Extensive epidermal erosion leaving an

incomplete layer of cells above the basal

membrane.

(HcE, × 410)


S

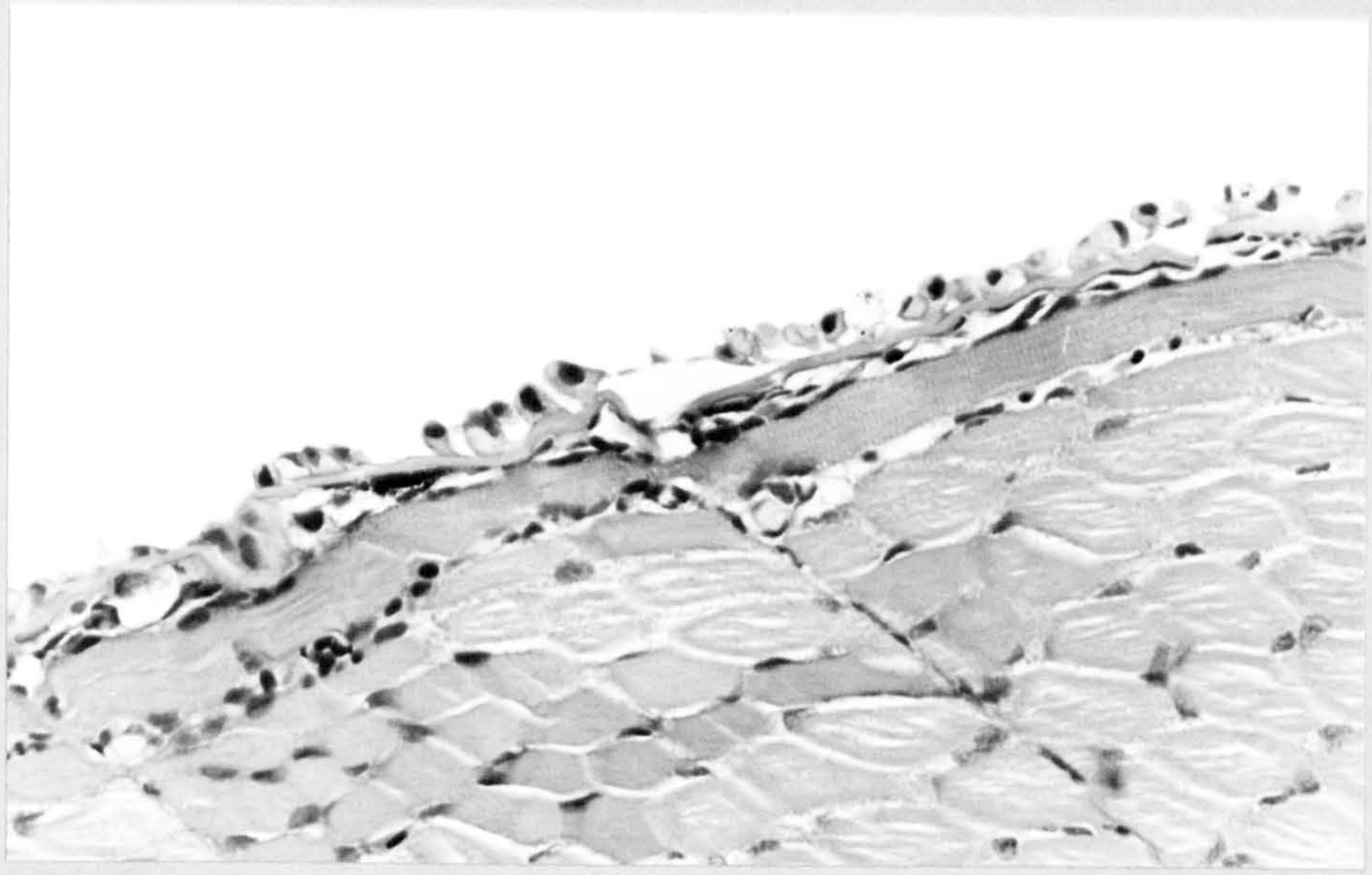


FIGURE 29

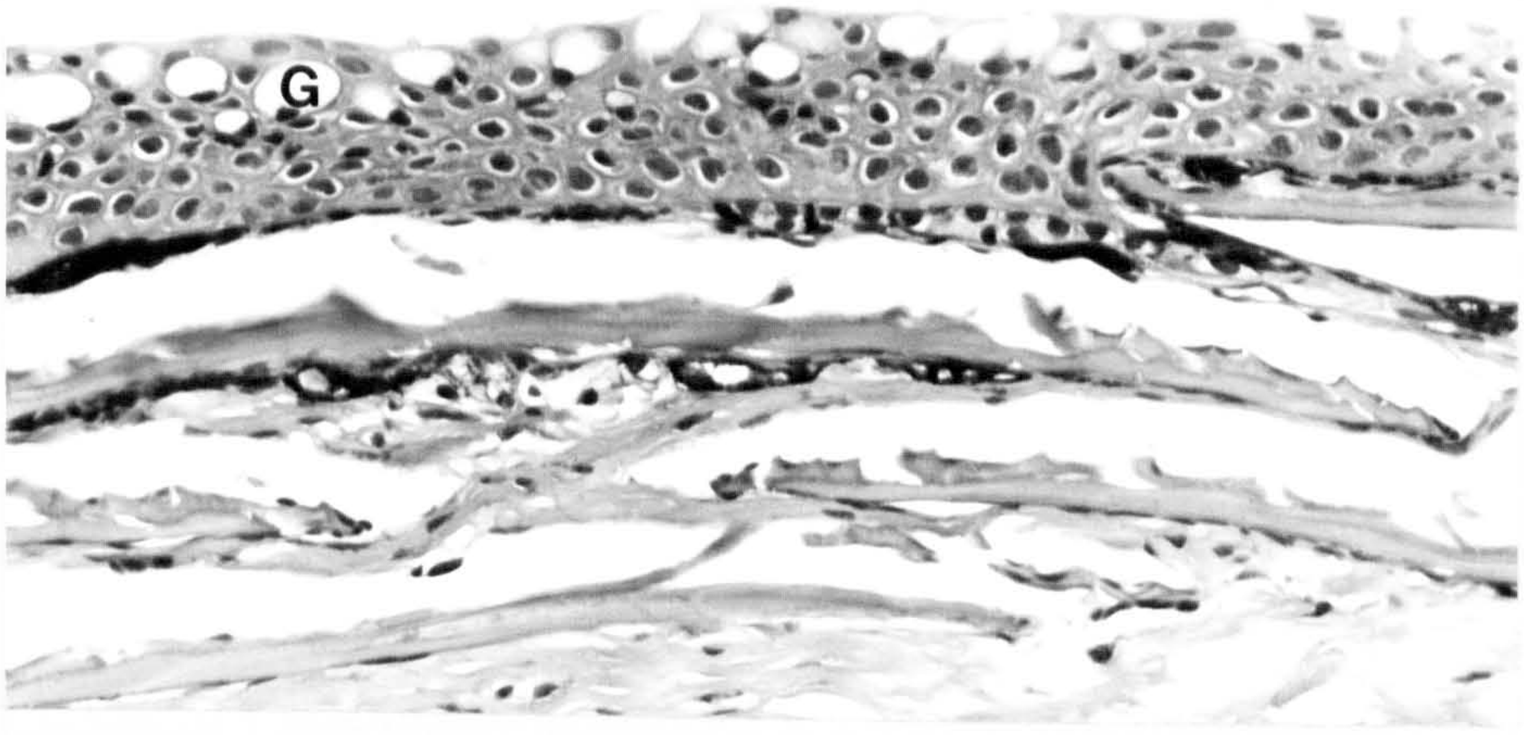
Normal salmon smolt epidermis.
Note numerous goblet cells (G).

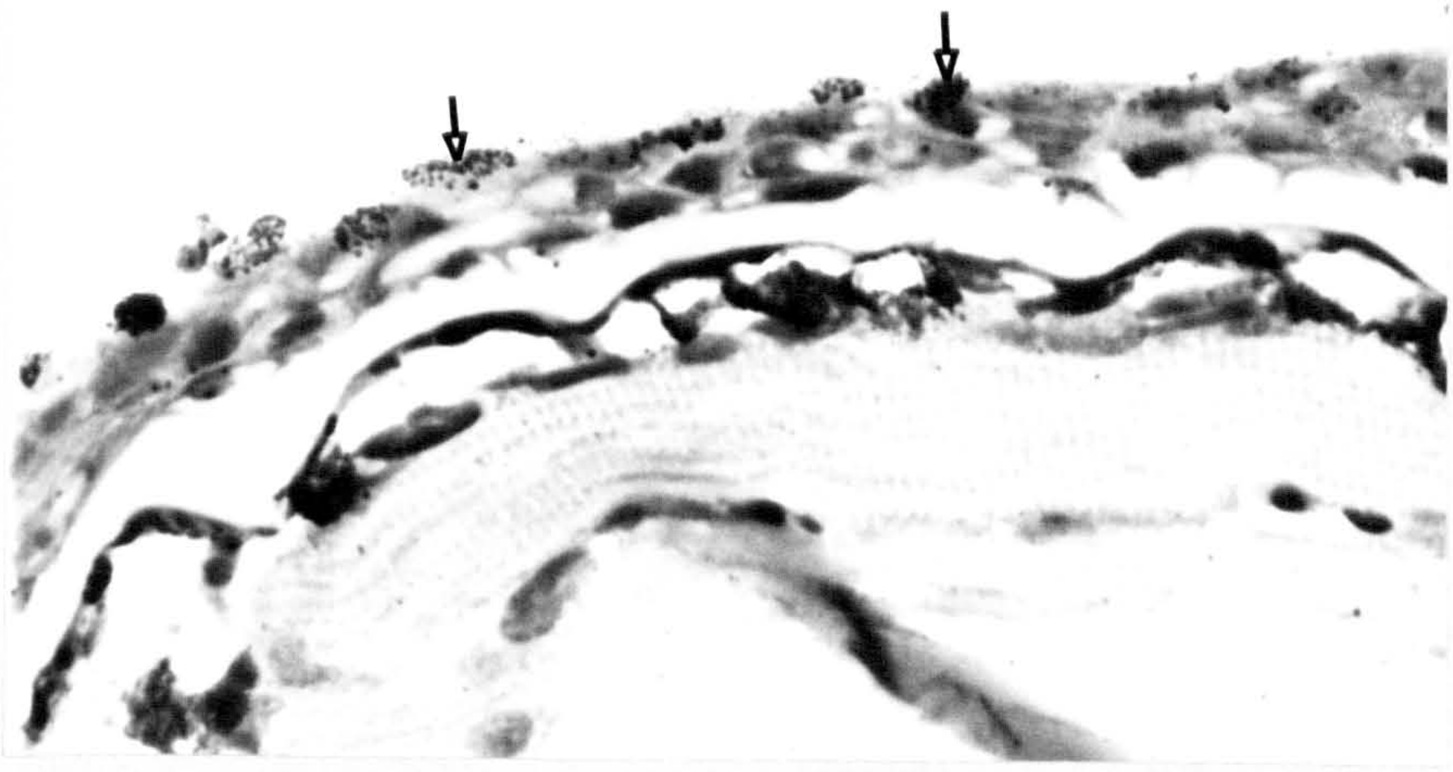
(HαE, × 650)

FIGURE 30

Heavy labelling with [^{3}H] thymidine of the
outermost squamous cells (arrowed)

(HαE, × 650)


G



proximity of the hyperplastic areas was of normal thickness and had normal complements of goblet cells (Fig. 23). Hyperplastic areas of epidermis were usually ten to fifteen cells thick whereas uninfested areas were on average three cells thick (Fig. 24).

The epidermal cells immediately below the Ichthyobodo organisms showed strongly basophilic pyknotic degenerative nuclei (Fig. 25) and peripheral cells to which Ichthyobodo were attached were frequently effete 'ghost' cells with no nuclear chromatin and were eosinophilic (Fig. 26). Sections stained with alcian blue showed that these cells did not possess sulphated mucins characteristic of goblet cells.

By days 10, 11 and 12 the hyperplastic areas of epidermis below heavy Ichthyobodo infestations frequently showed slight spongiosis, vacuolation and loss of cytoplasmic and nuclear detail in the supra-basal layers (Fig. 27). In several cases marked epidermal erosion down to the dermis was apparent with only the skeletal remnants of the malphigian cells left (Fig. 28). From the appearance of the epidermis it would seem that large areas had sloughed off just above the basal layer.

3.4.2 Salmon Smolt Pathology

None of the smolts became infested with Ichthyobodo during the period of this study and the skin appeared to be normal in all of them and was characterised by the presence of large numbers of goblet cells (Fig. 29).

3.4.3 Rainbow Trout Alevin Autoradiography

The most obvious feature of this study was the very marked degree of epidermal erosion associated with the parasite, and the virtual disappearance of goblet cells in nearly all of the fish examined. However as in the salmon alevin study the epidermis of the head of the fish was normal and uninfested irrespective of the degree of epidermal erosion of the skin behind the head.

Large numbers of Ichthyobodo were present on all of the fish, and again the most heavily infested area was invariably just ventral to the dorsal fin. Parasites were not seen on areas where sloughing had occurred, being confined to areas of relatively thick epidermis.

The uptake of 3H thymidine by predividing cells was very obvious. Several features were apparent in their distribution:- labelled cells were distributed throughout the epidermis but were most frequently found in the middle to outer layers beneath heavy infestations of Ichthyobodo, rather than in the supra basal layer. In many cases the cells of the outermost squamous rather than epidermal layer were virtually all heavily labelled (Fig. 30). However the areas with the most frequently labelled cells were those areas where epidermal erosion was greatest. Indeed in those areas where the epidermis had been eroded to the thickness of a single cell, virtually every cell was labelled (Fig. 31).

The Ichthyobodo attached to the epidermal cells also showed high frequency of labelling (Fig. 32). The label was generally scattered throughout the cytoplasm. Density of labelling of the parasites increased, until by four days post injection of 3H

FIGURE 31

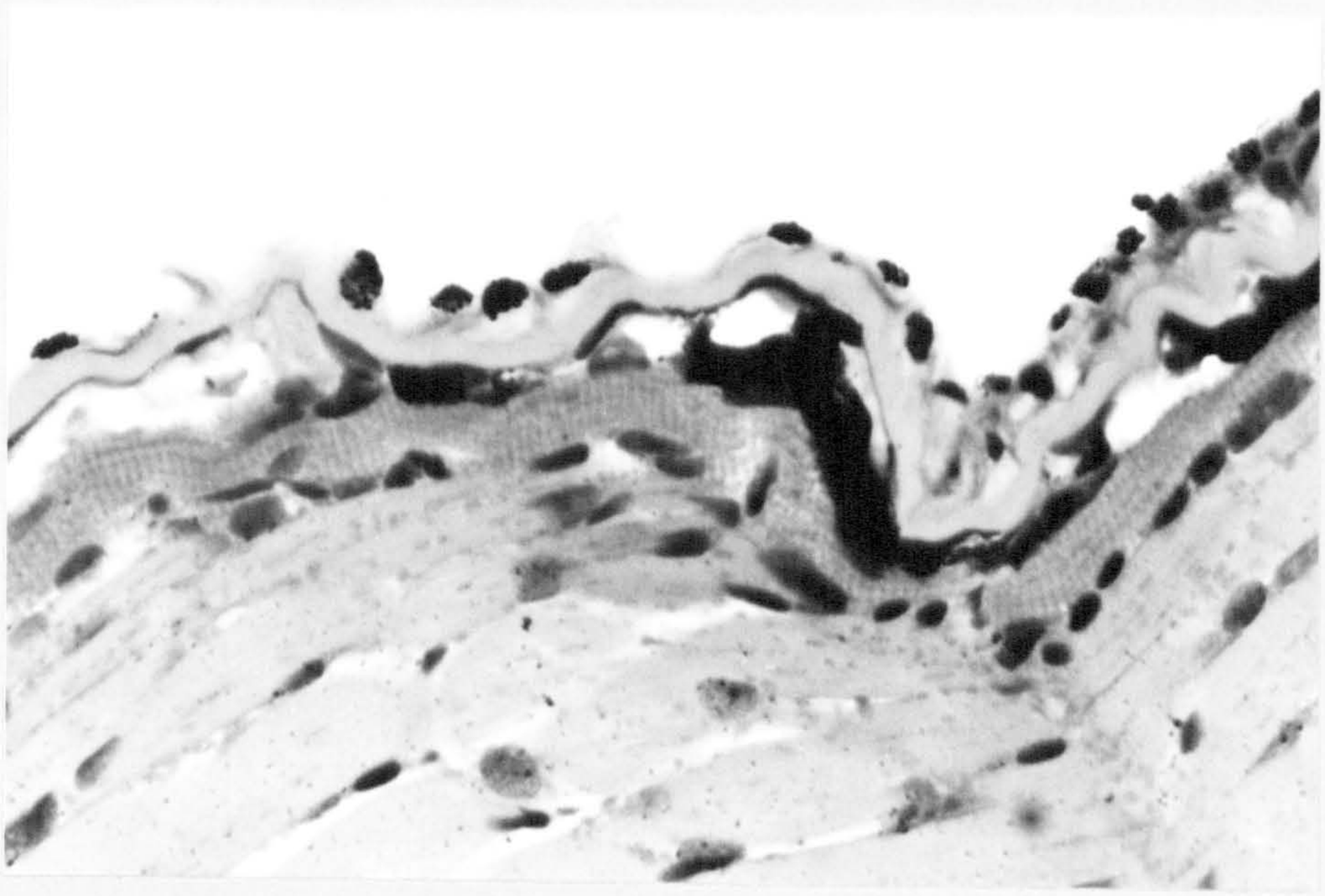
Heavy labelling with [^{3}H] thymidine of remaining cells in the eroded epidermis.

(H&E, × 650)

FIGURE 32

Ichthyobodo displaying uptake of [^{3}H] thymidine throughout the cytoplasm.

(H&E, × 650)



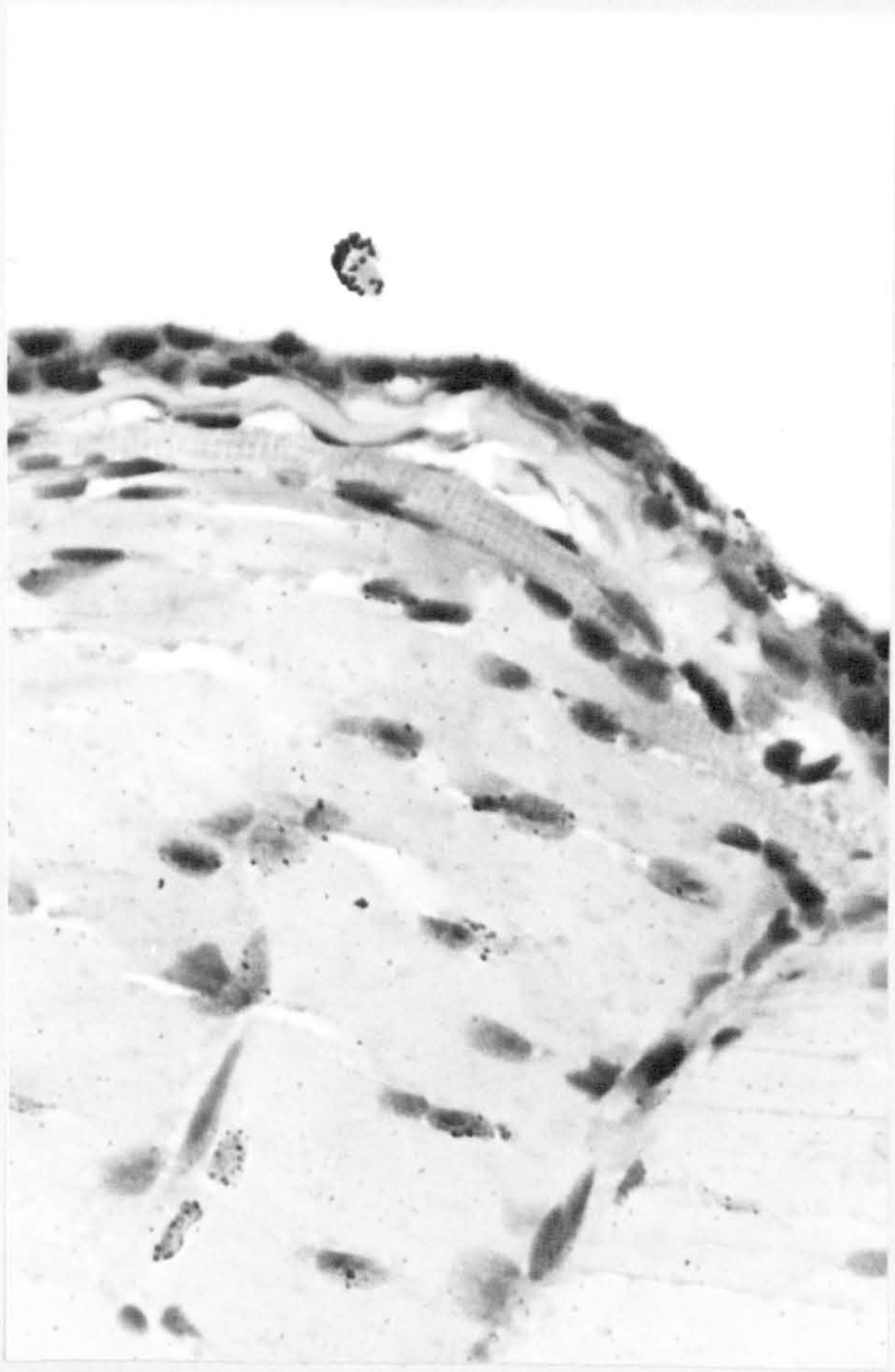


FIGURE 33

Very heavy labelling of Ichthyobodo 4 days post infection. The label is scattered throughout the parasite.

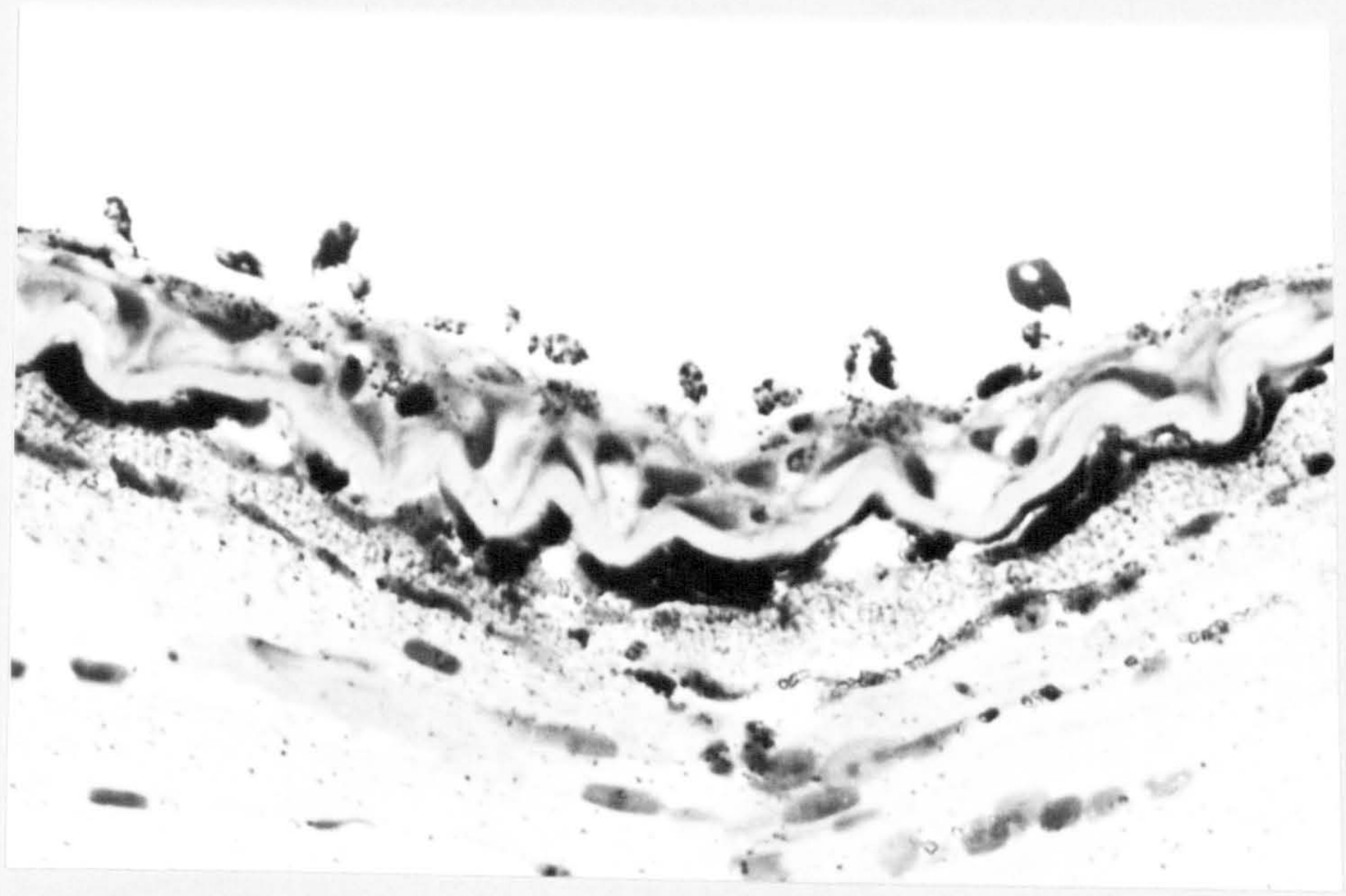
(H&E, × 600)

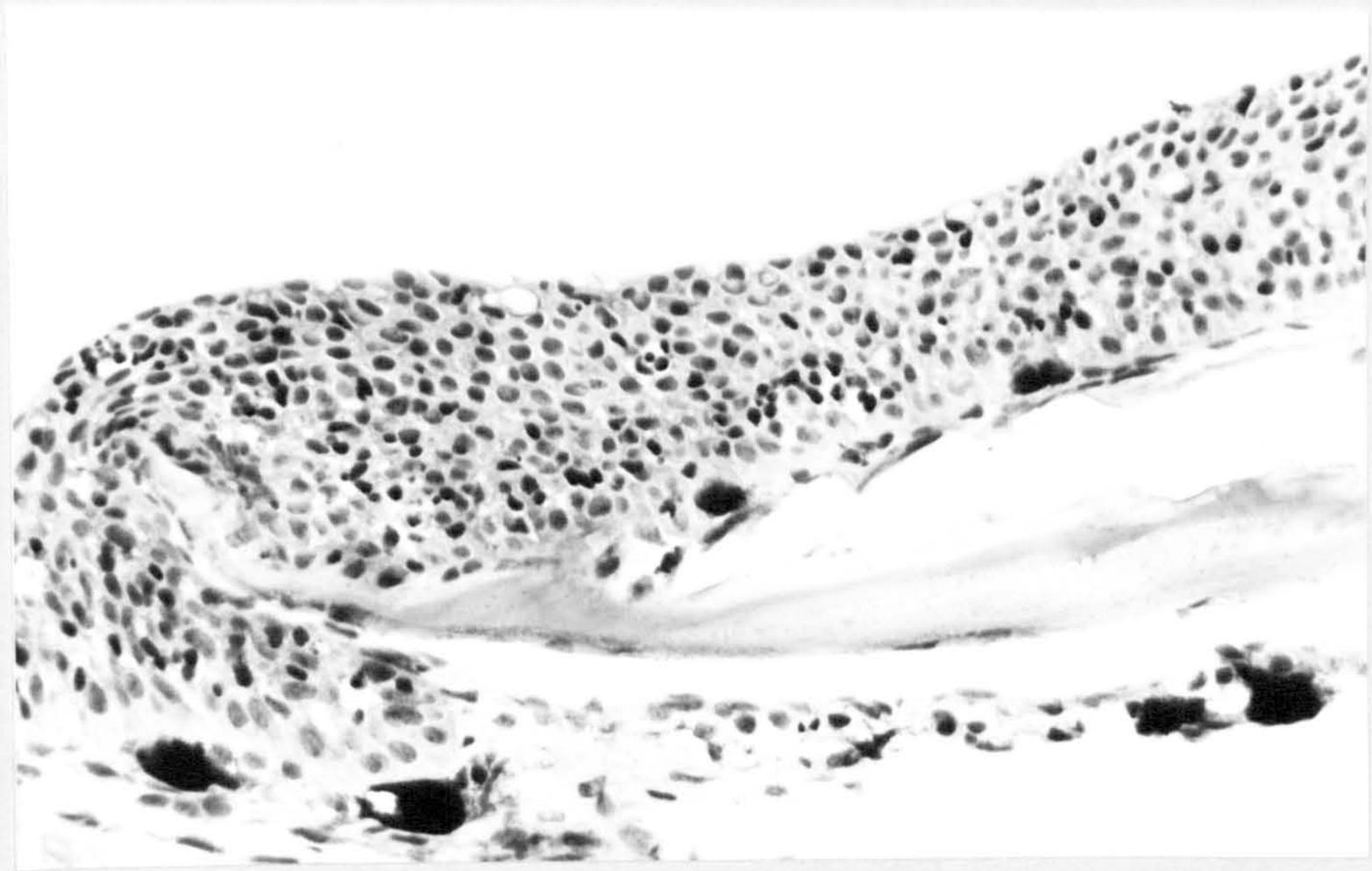
FIGURE 34

Hyperplasia of the salmon smolt epidermis.

Note lack of goblet cells.

(H&E, × 350)





thymidine many of the Ichthyobodo were virtually replete with label (Fig. 33). The labelling appeared to be scattered throughout the parasite and did not seem to be confined to the nucleus or to the kinetoplasts.

3.4.4 Salmon Smolt Autoradiography

These fish had heavy infestations of Ichthyobodo on the skin before sacrificing. Previous studies had shown that Ichthyobodo would not survive in the tissue culture medium, as they almost invariably detached from the skin, rounded up and contractile motion ceased, and thus it was not surprising that at sampling very few Ichthyobodo were found to be still attached. However in a few cases there appeared to be the enucleated remnants of Ichthyobodo above the epidermis.

The explants of the skin of these smolts showed extensive hyperplasia (Fig. 34) and very few goblet cells were present (cf. Fig. 29). However there were also numerous discrete areas of thin epidermis (two to three cells deep) as opposed to the ten to fifteen cell deep areas of the hyperplastic epidermis.

Labelling of the epidermal cells was similar to that seen in the 0+ rainbow trout, with heaviest labelling occurring in the middle layers as opposed to the supra-basal layers. However, there were also discrete areas of very heavy labelling (Fig. 35). These were almost invariably associated with the thinner areas of epidermis mentioned above. Statistical analysis showed that areas of very heavy labelling were significantly thinner than areas with no labelling (unrelated $t = 19.26$, $p < 0.001$, $df = 230$) (Meddis, 1975). Unlike the situation seen in the 0+ rainbow trout, there was no labelling of surface epithelial cells in these explants.

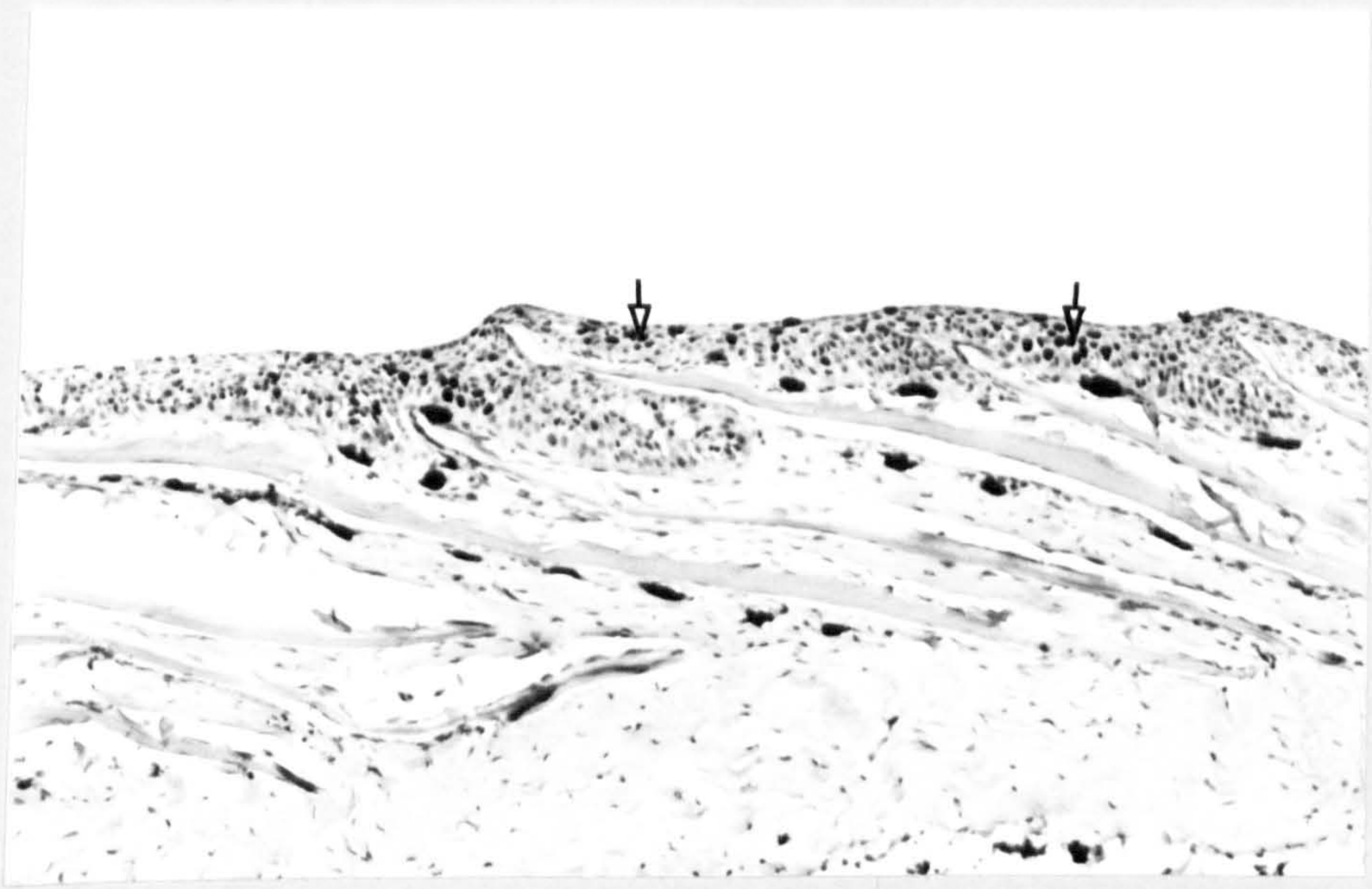
FIGURE 35

Salmon smolt epidermis showing discrete clusters of heavily labelled cells (arrowed). Note how thin these areas are compared to Figure 34. (H&E, × 125)

FIGURE 35

Salmon smolt epidermis showing discrete clusters of heavily labelled cells (arrowed). Note how thin these areas are compared to Figure 34.

(HαE, × 125)



3.5 DISCUSSION

The distribution of Ichthyobodo on the surface of all of the infested fish was distinctive with a pattern of heavy infestation correlated with sites of protection such as the trailing edges of the pectoral fins and the operculum. The pattern of development of such infestations is not known with certainty but supports the hypothesis suggested in Chapter 2 that infestation arises via the respiratory inflow of water across the gills, with initial infestation of gills and branchial chamber, extending to the body surface. In the later stages of Ichthyobodo infestation the fish seek out areas of minimal water flow and such conditions would readily provide the opportunity for the more generalised extension of infestation with this minute parasite, which would presumably be impossible in any fast water stream. With such a mechanism of per-branchial infestation the dorsum of the head would naturally be virtually inaccessible.

The relative time scale for the depletion of goblet cells and localised hyperplasia of the epidermis at sites of maximal infestation was not resolved in this study. It is however possible to speculate that the first response to the Ichthyobodo is the exhaustion of the mucus from the goblet cells and their subsequent sloughing, a feature which has already been recorded in brown trout, Blackstock and Pickering (personal communication), followed by reactive hyperplasia after the mucoid response has failed to resolve the condition. Similar goblet cell depletion as a result of parasitic infestation has been reported in heavy Argulus infestations of the skin and Ergasilus infestation of the gills of carp (Neuhaus, 1929). Likewise Hines and Spira (1974) reported goblet cell hyperplasia followed by depletion in Ichthyophthirius

multifiliis infestation of mirror carp. The efficacy of parasite removal with sloughed mucus has been reported by several workers (Lester, 1972; Willougby and Pickering, 1977), whilst Roberts and Bullock (1976) have reviewed the literature on hyperplastic response to various skin irritants.

The assumption by some authors that the greyish sheen which appears on the flanks of Ichthyobodo infested fish is caused by excess mucus production is not supported by this study, as the mucus producing cells were very rapidly depleted. It would appear that the grey sheen is most likely to be necrotic cells and parasites in the process of being sloughed off.

The activity of the parasite on the surface was marked principally by the generalised degenerative changes in the uppermost cells of the epidermis. The nuclei of the cells under the Ichthyobodo layer were densely basophilic and often surrounded by clear haloes. This did not appear to be directly related to individual parasites and indeed in some cases was two cells beneath the Ichthyobodo infestation, this therefore lends credence to the possibility that the parasite secretes some form of digestive enzyme or toxic substance which leads to necrosis of the outermost cells.

The destruction of the integrity of the outermost level of the epidermis, with its tight junctions and its osmotic barrier,led to a distinctive intra and extra-cellular oedema,presumably associated with inflow of hypoosmotic water from the surrounding aqueous habitat. This resulted, within twelve days of infestation, in degeneration and sloughing of virtually the entire hyperplastic epidermal plaque, leaving at most a single layer of basal cells over the area. Where the infestation is severe and the areas of

desquamation are therefore extensive the likeliest cause of death is osmoregulatory breakdown and resultant haemodilution, as described by Richards and Pickering (1979).

The complete absence of pathological change or even low level infestation in the smolts exposed to high levels of Ichthyobodo confirms the findings of Chapter 2 that post-fry are normally refractory to the parasite although the precise reason for this is at present not known. The infested smolts used to attempt to induce the infestation, which were also used for autoradiography, came from a severely stressed group presented by a salmonid farmer for diagnosis. Stressed fish invariably show increased cortisol levels (Fagerlund, 1967; and Wedemeyer, 1969).

The epidermis of teleost fish is markedly different from that of higher vertebrates in that it is unkeratinised and is vital to its outer layer. Hendrikson (1967), using tritiated thymidine, demonstrated that cells at all levels of the epidermis of goldfish are capable of mitosis and thus duplication. Bullock et al, (1978a, b) and Bullock and Roberts (1980) confirmed Hendrickson's finding but also found that in plaice the main focus of epidermal proliferations was in the suprabasal layer and labelling at the surface was very limited. Roberts (personal communication) has indicated that this is true of salmonids also. The presence of Ichthyobodo induced a major change in this pattern with extensive labelling of the surface cells taking place. This increased activity may have been in response to an irritant in the form of a digestive enzyme elaborated by the organism, a more likely possibility than simple replacement of damaged cells which normally develops from the suprabasal layer. Erosion, leaving only the

basal cells intact, leads to extensive labelling in this area, but this is more easily explained, even in the complete absence of parasites, by a requirement to increase epidermal thickness.

There was an anomaly in the labelling of the infested smolt skins, where the extreme outer cells were not labelled as in the alevins. The entire epidermis of an alevin is likely to be more active than that of a smolt but by itself that is unlikely to be a complete explanation.

The uptake of label by the parasite was a very distinctive feature of this study. It was not generally associated with the parasite nucleus and the label appeared to be concentrated by the parasite, suggesting the possibility of preferential nucleic acid ingestion by feeding organisms. This limited light microscopy evidence supports the elegant work of Schubert (1966 and 1968) who suggested that the parasite feeds on the host cell by protruding 'finger-like processes' into the cell and sucking out parts of the cell contents. If there is preferential nucleic acid ingestion this would explain the presence of the effete ghost cells with no nuclear chromatin. Because of the density of labelling seen in the latter part of this study it seems likely that the parasite browses from cell to cell, presumably dividing from time to time.

CHAPTER 4

ENDOCRINOLOGICAL ASPECTS OF

ICHTHYOBODO NECATOR INFESTATIONS

CHAPTER 4

ENDOCRINOLOGICAL ASPECTS OF *ICHTHYOBODO NECATOR* INFESTATIONS

4.1 INTRODUCTION

The results described in Chapter 2 have indicated that *Ichthyobodo* infestations appear to increase after a period of low water temperature and on sexually mature and maturing rainbow trout. The possible reasons for this phenomenon will be discussed in this chapter.

There have been several reports of severe ectoparasitic epizootics on sexually maturing or mature fish in the literature (Cope, 1958; Becker and Katz, 1965; White, 1975 and Pickering and Christie, 1980). In addition Richards and Pickering (1978), have shown that the incidence of infestation of brown trout by the parasitic fungus *Saprolegnia diclina* Type 1 is greater in sexually mature fish compared with immature fish sampled from the same water.

The onset of sexual maturation and the following spawning cycle is a particularly stressful time for the fish. As they become mature they undergo complex hormonal changes and associated changes in the levels of sex steroids and corticosteroids; this may influence the host's resistance to infestations.

4.1.1 Oestrogens

In 1961 Cedard, Fontaine and Nomura recorded marked changes in oestrogen levels in Atlantic salmon during gonad maturation and Eleftherieu, Boehlke and Tiemeier (1966) described similar changes in the channel catfish. Wingfield and Grimm (1977) showed that

oestradiol levels increased to a peak in mature female plaice and fell just prior to spawning.

In a review of the seasonal variation in sex steroids of female rainbow trout Scott, Bye and Baynes (1980) found a similar seasonal cycle of 17β oestradiol levels to that described by Whitehead, Bromage and Forster (1978), with levels of oestradiol peaking in November and falling away sharply at ovulation in January.

4.1.2 Androgens

Likewise, androgen levels have been shown to alter during the maturation of fish. Eleven keto-testosterone and 11-β-hydroxy testosterone levels have been shown to increase in both male and female sockeye salmon during maturation, (Grajcer and Idler, 1961; Schmidtt and Idler, 1962; and Idler, Horne and Sangalang, 1971). Idler et al (1971) showed that 11-keto-testosterone and 11-β-hydroxy testosterone increased in level as maturation proceeded but levels of testosterone remained relatively constant. Schreck, Flickinger and Hopwood (1972), reported an increase in androgen levels in fully mature male rainbow trout. More recently Campbell, Walsh and Idler (1976) found that testosterone levels in the winter flounder Pseudopleuronectes americanus Walbaum changed only slightly in males during the annual cycle but that 11-keto-testosterone levels rose dramatically near the time of spawning, and also testosterone levels were highest just prior to spawning in the females. Wingfield and Grimm (1977) obtained similar results with plaice but found that testosterone levels reached a peak just before spawning and then dropped rapidly before spawning took place.

Scott, Bye, Baynes and Springate (1980) found that levels of both 11-keto-testosterone and testosterone increased in male rainbow

trout over their second summer and testosterone levels peaked in November, subsequently declining, whereas 11-keto testosterone levels peaked in February and then declined rapidly.

Scott, Bye and Baynes (1980) found similar seasonal increases in testosterone levels in mature female rainbow trout to Scott, Bye, Baynes and Springate (1980) in mature male rainbow trout, with levels peaking in November. However, 11-keto testosterone levels only increased very slightly in mature female rainbow trout. A recent study by Hunt, Simpson and Wright (1982) has shown that, as in rainbow trout, mature Atlantic salmon held in captivity show peak testosterone levels at full maturation during October and November, followed by a rapid decline.

4.1.3 Corticosteroids

Many authors have suggested that sexual maturation in salmonids (particularly the anadromous salmonids) is associated with hyperplasia of the interrenal gland and increased production of corticosteroids in particular hydrocortisone and cortisone, and 17-hydroxy-corticosteroids (Robertson and Wexler, 1959; Hane and Robertson, 1959; Idler, Ronald and Schmidt, 1959; Phillips, Holmes and Bondy, 1959; Schmidt and Idler, 1962; Donaldson and Fagerlund, 1970; and Heyl and Carpenter, 1972). However, Idler and Truscott (1972) in a review of the subject have suggested that interpretation of these data is difficult as several factors could lead to the high level of corticosteroids recorded, for example the stress of capture, motor activity of migration and moribund condition of post spawned fish. Whatever the cause of this change in level of circulating corticosteroids the majority of reports suggest that in general corticosteroid levels do increase in mature fish. There are reported exceptions however, (Leloup-Hatey, 1964, and Fagerlund, 1967).

Recent work by Wingfield and Grimm (1977) on plaice has indicated that cortisol levels reach their maximum during the peak spawning period not only in mature males and females but also in non-breeding immature fish. They suggested that plasma cortisol levels would rise in any species of fish which because of inadequate food intake in the winter are required to mobilise stored energy whether for locomotion, gonad maturation, spawning or, in immature fish, merely basal maintenance. Similar winter increases in cortisol levels in immature fish have been found by Fontaine and Leloup-Hatey (1954); T. Murphy (personal communication); P. Langhorne (personal communication); and Olivereau (1975) in juvenile Atlantic salmon.

However, Pickering and Christie (1981) found that although there were marked increases in cortisol levels in immature 2+ brown trout, they found no increase in level of cortisol over the period October to December in immature 1+ brown trout. A report by Terkalin-Shimony, Ilan, Yaron and Johnston (1980) was unable to correlate the state of ovarian development in female Tilapia aurea L. with circulating cortisol, whereas Cook, Stacey and Peter (1980) demonstrated a curve in serum cortisol prior to the onset of ovulation in goldfish. As a result of these two apparently conflicting reports, Pickering and Christie (1981) concluded that the control of maturation and ovulation in teleost fish differs markedly between species. There may also be differences in cortisol levels of immature fish between species.

4.1.4 The Effect of Androgens and Oestrogens on the Skin

The effects of androgens and oestrogens on the skin of fish is well documented. There is a generalised hyperplasia of the skin

of both male and female fish during the spawning season (Pickering, 1977) with a decrease in goblet cells in the epidermis of male fish. This has been attributed to increases in the levels of androgens and oestrogens of maturing fish. Testosterone is produced in small quantities in maturing female fish as well as males and though oestrogens can cause a hyperplastic response these low levels of testosterone may have an added effect (Richards, 1979). This hyperplastic response has been reproduced artificially by the injection of androgens and oestrogens (Idler, Bitners and Schmidt, 1961; McBride and Overbeeke, 1971; and Yamazaki, 1972). Most of the work carried out on fish has utilised testosterone or 17-α-methyl testosterone (Donaldson and Fagerlund, 1969; Overbeeke and McBride, 1971; and McBride and Overbeeke, 1971). Richards (1979) has shown that injection with 17-α-methyl testosterone leads to skin thickening and some reduction in mucus cell numbers; this is similar to the condition reported in wild male brown trout by Pickering (1977), although no reduction in goblet cell numbers in females was reported.

It is conceivable that the decrease in goblet cell numbers seen in male fish may affect the resistance of the fish to parasitaemias as the protective properties of fish mucus have been well documented. It has been shown to contain immunoglobulins and C reactive proteins (Fletcher and White, 1973a; Baldo and Fletcher, 1973; and Bradshaw, Richards and Sigel, 1970) and lysozyme which has bacteriocidal properties (Fletcher and Grant, 1968; Fletcher and White, 1973b; and Murray and Fletcher, 1976). In addition Willoughby and Pickering (1977) have shown that the continual sloughing off of mucus carries away fungal spores germinating on the cuticle.

4.1.5 The Effect of Corticosteroids on Fish

The effect of high levels of corticosteroids on the skin of salmonids is not well documented but most authors who have described high levels of cortisol in the anadromous salmonids describe general atrophy of the epidermis of the skin. The use of corticosteroids in veterinary medicine as anti-inflammatory agents is well accepted and their effect in reducing natural resistance to parasitic infections in mammals is well documented (Robinson, 1961; Mathies, 1962; Campbell and Collette, 1962; and Esch, 1967). In addition corticosteroids have been shown to increase the survival times and favourably alter morphologic development of various parasites (Oliver, 1962; Michel and Sinclair, 1969; and Sinclair, 1970).

There is evidence that inoculation of corticosteroids may facilitate skin infestations of fish, as Robertson, Hane, Wexler and Rinfret (1963) showed that intraperitoneal implants of hydrocortisone and cholesterol in the form of pellets in immature trout resulted in heavy infestations of Ichthyophthirius multifiliis and eventual death of heavily infested fish. Roth (1972) performed similar experiments with Catostomus commersonii commersonii Lacépede and found that injecting fish with 1 mg of cortisol or cortisone facilitated fungal growth on the fish. It has been reported that disease and fungal infections common in post-spawned Pacific salmon may produce high plasma cortisol levels (Fagerlund, 1967). However, Idler and Truscott (1972), suggested that elevated levels of cortisol in the post-spawned salmon may contribute to the development of a diseased condition by a diminution of the immunological defence mechanism rather than be a result of the effects of the disease.

The foregoing evidence suggests that as salmonids mature they experience increased levels of androgens, oestrogens and corticosteroids. As these compounds may alter the fish's resistance to skin infestations by reducing the immune and inflammatory response, and altering the structure of the epidermis of the skin and its goblet cell component (the contents of which may have antibiotic properties), it was decided to investigate whether inoculation with these compounds would lead to the same decrease in resistance of immature rainbow trout to Ichthyobodo as seen in the maturing fish in the previous trials described in Chapter 2.

There is little documented evidence of the effect of oestrogens on the skin structure, whereas testosterone has been reported to produce skin changes and in addition is found in maturing male and female rainbow trout. Therefore, one androgen and a variety of corticosteroids were tested.

The androgen used was testosterone because of its general availability and because of its effectiveness in simulating maturation changes (T.H. Simpson, personal communication). Three corticosteroids were tested - these were hydrocortisone, prednisolone and flumethasone.

4.2 PROPRIETORY CORTICOSTEROIDS TRIAL

4.2.1 Materials and Methods

Two proprietary anti-inflammatory corticosteroids were obtained for inoculating the fish in this trial.

Prednivet is a short-lasting anti-inflammatory compound containing 10 mg of prednisolone BP per ml of solution - the manufacturers (Willow Francis) claim that it is five times as active as hydro-

cortisone. Prednisolone acetate was used by McCarthy (1976) for detecting carriers of furunculosis (Aeromonas salmonicida) infection.

Fluvet is a long-lasting anti-inflammatory corticosteroid claimed by the manufacturers (Syntex Agribusiness) to be effective for up to three weeks in mammals. Fluvet contains 2 mg of flumethasone per ml of solution.

Both compounds are in the form of injectable, aqueous suspensions. McCarthy used prednisolone acetate at a concentration of 20 mg per kg of fish, and the recommended dosage of flumethasone is 1 mg per 5 kg body weight. The fish which were used in this trial weighed between 35-40 grams, and therefore the quantity of steroid in its undiluted form that could be injected into each fish would have been too small to administer accurately. Therefore, both solutions were diluted with sterile distilled water to give an inoculation of 0.1 ml to each fish. The quantities of active ingredient and the dilutions used are shown in Table 11. Two controls were used; these were fish injected with 0.1 ml of sterile distilled water and fish which were given no inoculation. Two hundred fish, which had previously been selected from a large pool of fish for uniformity of size, were used, fifty for each trial. All fish were anaethestised in 25 p.p.m. benzocaine prior to being allotted to their respective treatments. Two days prior to commencement of the trial all the fish were treated with 1:5000 formalin to eradicate any existing ectoparasites.

The fish which were inoculated were inoculated intra - peritoneally with 0.1 mls of their respective solutions just anterior to the pelvic fins, and each treatment of fifty fish was

TABLE 11 - Dilutions of proprietary corticosteroids inoculated into rainbow trout

Treatment	No. of Fish	Total Weight kg	Mean Weight gms	Dose mg/kg	Quantity of Diluted Solutions Injected mls	Strength of Diluted Solution mg/ml	Amount of Active Ingredient per Fish mg
Prednivet	50	2.03	40.6	20	0.1	8.12	0.81
Fluvet Depot	50	2.03	40.6	0.2	0.1	0.08	0.008
Distilled Water	50	1.58	31.6	-	0.1	-	-
Controls	50	1.72	34.30	0	0	-	0

then placed in a concrete raceway tank of 0.1 metre^3 volume with a through flow of 30 litres of water per minute. All of the fish were fed a commercially prepared diet using automatic feeders. Five fish from each trial were sampled for ectoparasites on days 1, 5, 8 and 20 after inoculation, by the methods described in Chapter 2. The trial started on May 13th, 1979, and was terminated on June 2nd, 1979.

4.2.2 Results

The results are shown in Table 12. It was clear on day 1 that the formalin treatment had not killed off *Bodo* sp. or *Gyrodactylus* sp.; however, there were no *Ichthyobodo* present on any of the fish samples on day 1. On day 5, one of the five flumethasone treated fish had *Ichthyobodo* present on the skin. However, by day 8, one of the five fish injected with prednisolone and one of the five fish injected with flumethasone had *Ichthyobodo* infestations, that of the fish injected with prednisolone being a heavy infestation. By day 20 all of the fish injected with flumethasone had light infestations of *Ichthyobodo* but none of the prednisolone injected fish had *Ichthyobodo* infestations. None of the control fish showed any *Ichthyobodo* infestations throughout the trial. However, most of the treatment had *Gyrodactylus* sp. and *Bodo* sp. infestations throughout the trial. Previous investigation had shown that the strainsof *Gyrodactylus* sp. and *Bodo* sp. present at Howietoun were very difficult to remove with conventional formalin treatment.

TABLE 12

Ectoparasite infestation attributable to proprietary corticosteroid injections

Day	Ichthyobodo %	Grade	Other Parasites %
PREDNIVET			
1	0	0	100
5	0	0	100
8	20	10	100
20	0	0	100
FLUVET			
1	0	0	100
5	20	1	40
8	20	2	80
20	100	2	20
INJECTED CONTROL			
1	0	0	100
5	0	0	40
8	0	0	20
20	0	0	20
CONTROL			
1	0	0	100
5	0	0	20
8	0	0	80
20	0	0	25

4.2.3 Discussion

It was clear from this trial that the corticosteroid preparations were having an effect in promoting Ichthyobodo infestations with the degree of infestations of Ichthyobodo increasing over the period of the trial in the fish injected with fluvet, whereas the prednisolone appeared to have very little long-term effect on the fish. This is not surprising as the prednisolone is a short-term anti-inflammatory compound which does not remain in circulation for long periods, whereas the fluvet is designed to give a slow steady release of the corticosteroid over a three-week period.

It was clear from the results of the trial that corticosteroids could affect the host response to Ichthyobodo and that a longer-term more detailed study was necessary using a method of corticosteroid presentation that allowed steady release of the corticosteroid over a period of up to five weeks without the additional stress of reinjecting the fish after three weeks. It was also felt that it was desirable to monitor the corticosteroid levels in the inoculated and control fish.

4.3 HYDROCORTISONE AND TESTOSTERONE TRIAL

4.3.1 Materials and Methods

Robertson et al (1963) had developed a method of slow release of hydrocortisone into fish by incorporating hydrocortisone into a cholesterol pellet which was insoluble in body fluids. The hydrocortisone levels had remained high in the host for up to five weeks and therefore this method was adopted to present hydrocortisone and testosterone to the fish.

4.3.1.1 Hydrocortisone

Hydrocortisone and cholesterol were mixed in a ratio of 1:2 in ethyl ether to ensure complete mixing. The solution was stirred continuously with a glass rod in a fume cupboard whilst blowing air onto the solution until all the ether had evaporated to leave a well-mixed white powder.

Robertson et al (1963) had found that doses of 40 mg of hydrocortisone to rainbow trout of 80-110 gms had produced skin infections and thus two doses of 40 mg and 20 mg were decided on as the fish used in this trial averaged 40 gms. Therefore, 0.24 gms of the mixture of hydrocortisone and cholesterol were weighed out and compressed into a disc using seven tons pressure in a hand press. A hard disc of 0.24 gms was produced containing cholesterol and hydrocortisone in the ratio of 2:1. The disc was then quartered to give pellets of composition of approximately 20 mg hydrocortisone and 40 mg cholesterol. Thus one-quarter of a disc was used for a single dose of 20 mg and one-half disc for a 40 mg dose of hydrocortisone.

4.3.1.2 Testosterone

Richards (1979) had produced skin changes similar to those seen in maturing fish, by injecting 0.5 mg testosterone in arachis oil into fish every three days. As this trial was to last six weeks the equivalent dose would be 7 mg of testosterone. Thus two doses were decided on : 7 mg and 3.5 mg. To produce a pellet that was easily handled and would not break up, a ratio of 14 mg testosterone to 86 mg of cholesterol was used to produce a disc of 100 mg, using the same method as the hydrocortisone/cholesterol disc. One-quarter of a disc was used for a single dose of 3.5 mg testosterone and

one-half a disc used for a double dose of 7 mg testosterone.

A control pellet comprising cholesterol only was made by the same method; a 240 mg disc was quartered to produce a 60 mg pellet of cholesterol only.

4.3.1.3 Fish

One-year old rainbow trout which had had prior exposure to Ichthyobodo and were presumably resistant to the parasite as examination had shown no Ichthyobodo present, were used in this trial. One thousand fish were placed in a 2 meter^2 tank at a flow rate of 32 litres/minute and held under normal cultivation conditions for one month prior to treatment with 1:5000 formalin to eliminate any ectoparasites two days before commencement of the trial.

4.3.1.4 Inoculation

The trial commenced on the 19th September 1979.

Fish were netted out of the tank into individual plastic bins containing a solution of 25 ppm benzocaine. When the fish had ceased moving they were removed from the anaesthetic and a small incision was made into the peritoneal cavity with a number 11 scalpel blade just posterior to the pectoral fin. Either one or two of the required pellets were placed in the peritoneal cavity, which was not sutured. The fish were then freeze-branded on the dorsal surface just behind the head in order to identify the treatment when sampling. The fish were then returned to the tank. The whole procedure took not more than five minutes per fish and no mortalities were seen the next day and there was no evidence of rejected pellets. The treatment and brand allotted to each treatment is shown in Table 13. Three controls were used:-

TABLE 13

Hydrocortisone/testosterone trial -
Brands alloted to each treatment

Treatment	No. of Fish	Brand
Hydrocortisone		
40 mg	28	0
20 mg	28	1
Testosterone		
7 mg	26	2
3.5 mg	25	3
Cholesterol Control	28	6
Brand Only Control	27	8
Control	1800	No brand

1. Fish - cholesterol pellet only.

2. Fish which had been anaesthetised and branded but no operation.

3. The remainder of the fish in the tank which had received no treatment whatsoever.

4.3.1.5 Sampling

Five fish from each of the seven groups were sampled weekly over a five-week period. Fish were netted individually out of the tank with as little disturbance to the rest of the tank as possible. The fish were not anaesthetised prior to capture as this increases serum cortisol levels (T.H. Simpson, personal communication; Wedemeyer, 1970a). Each fish was killed by a blow on the head and blood taken from the caudal vein within two minutes of capture. A skin scrape was then taken from around the dorsal fin area and the fish was then weighed. The sex and state of maturity, general appearance of healing of incision and brand were noted. A skin sample of 2 cm square was taken from the opposite side to the skin scrape and one whole gill arch removed and fixed in 10% buffered formalin. Finally the amount of internal damage caused by the pellet was noted. The skin scrape was examined after 10 minutes for the presence of Ichthyobodo and other ectoparasites as described in Chapter 2. The blood was stored at 4°C overnight and the serum removed the next day by centrifugation at 1500g for 10 minutes. The serum was then stored at -70°C until steroid assay could be performed.

4.3.1.6 Steroid Assays

The serum samples were analysed for the presence of cortisol and testosterone using the radioimmune assay (RIA) technique in the

endocrinology laboratory of Dr T. H. Simpson of the Marine Laboratory, Aberdeen.

4.3.1.6.1 Testosterone

A rapid assay technique worked out by Dr Simpson and his staff was used for the testosterone assay. This technique gives slightly higher results than the conventional techniques as the serum containing the testosterone is not purified using thin layer chromotography, and therefore there is some cross-reaction between 11-keto testosterone in particular and the testosterone anti-serum used. However, as the fish used were immature, it was thought that 11-keto testosterone levels would be low and therefore that the technique would be accurate enough for the purposes of this trial.

The technique compares the binding of testosterone antisera to testosterone in solutions of known amounts of testosterone with the binding to solutions containing unknown amounts of testosterone.

Standards

A standard curve of picogrammes of testosterone per assay versus percentage binding was calculated using seven standards containing known levels of testosterone in phosphate buffer. The level of testosterone in 200 microlitre samples of each of the seven standards was as follows - 0, 10.2, 25.5, 50, 102, 255 and 510 picogrammes.

200 µl replicates of each standard were added to 1.5 ml polypropylene tubes (Sarstedt U.K. Ltd) using Eppendorf pipettes. To each tube was added 100 µl of tritiated testosterone (3H) (approx. 20,000 DPM) (Radiochemicals Centre, Amersham), and 100 µl of

testosterone anti-serum (Steranti Research, Ltd.). The tubes were capped and the solutions were mixed in a rotamixer and incubated in a water bath at 37°C for 30 minutes. After the incubation period the solutions were allowed to cool for 30 minutes at 4°C. Once the solutions had equilibrated, 500 µl of charcoal/dextran solution was added to the tubes; they were then placed in a rotator and rotated for 40 minutes at 4°C; the tubes were then centrifuged at 2000g for ten minutes in a cold room at 4°C. 400 µl of the resulting supernatant was removed from each tube and added to 800 µl of distilled water in labelled counting vials. Ten mls of Unisolve was added to each vial and the contents shaken well. The vials were washed with meths before placing in a Nuclear Chicago Mk III scintillation counter where the disintegrations per minute (DPM) were counted for each vial.

To estimate the quantity of label in the 3H testosterone 100 microlitres of 3H testosterone was added to 1100 µl of distilled water in a counting vial; to this was added 10 mls of Unisolve and the solution counted as above.

Background radiation was counted as above, after adding 1200 µl of distilled water to 10 mls of Unisolve.

The percentage binding was calculated, using the formula shown in Appendix 4, and a standard curve was drawn (see Fig. 36).

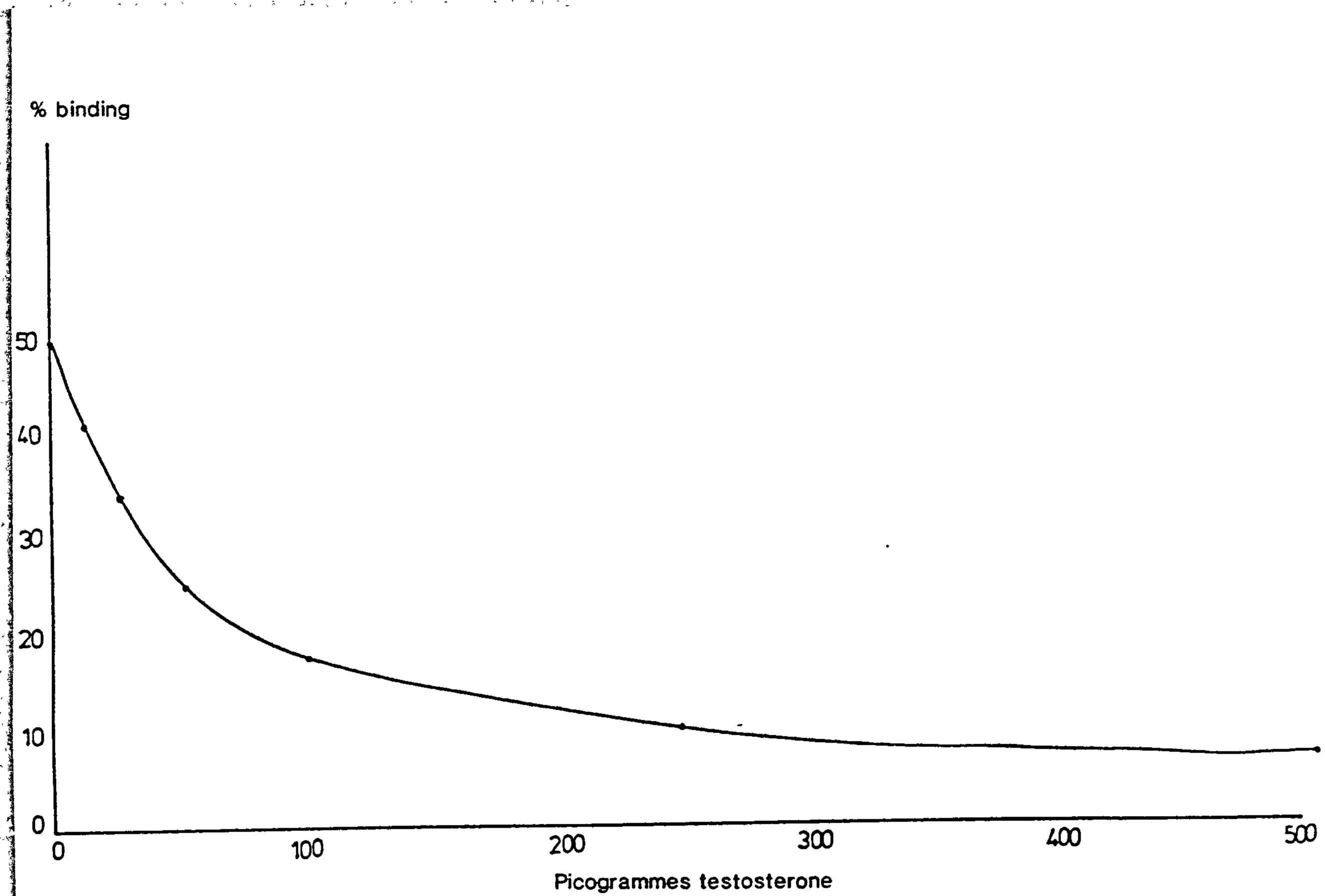
Unknowns

The serum from the testosterone-implanted fish and the branded controls were assayed for testosterone levels.

The serum was thawed and 20 µl replicates were transferred into glass 1 ml tubes, 180 µl of buffer was added to the serum and

FIGURE 36

Standard curve of percentage binding of testosterone antiserum to testosterone versus quantity of testosterone in solution.


% binding
50
40
30
20
10
0
0
100
200
300
400
500
Picogrammes testosterone

then 1.5 mls of ethyl acetate was added to the tubes which were then rotated for 30 minutes. The tubes were then briefly centrifugated to bring down the precipitate and the supernatant in which the steroids were dissolved was taken off, using an Eppendorf pippette. It was very important at this stage to take as much of the supernatant as possible, as all of the steroid is contained in the supernatant. The supernatant was put into labelled glass tubes supported on a heating plate and the liquid evaporated off with a stream of nitrogen. 500 µl of buffer was added to the steroid left in the tube after the ethyl acetate had been evaporated off. The solution was then rotated for 5 minutes to ensure thorough mixing of the steroid with the buffer.

Assay

Trials had shown that the implanted fish had testosterone levels which were greater than the maximum point on the standard curve when using conventional dilutions. Therefore, for the implanted fish 20 µl of buffer extract plus 180 µl of buffer were pipetted into polypropylene assay tubes; for the control fish 200 µl of buffer extract was added to the polypropylene assay tubes. 100 µl of testosterone anti-serum and 100 µl of tritiated testosterone was added to each assay tube and the assay continued as for the standards.

The percentage binding was calculated, using the formula shown in Appendix 4, and the picogrammes assay read off the standard curve.

Thus the quantity of testosterone in 20 µl of serum was determined using the formulas in Appendix 5, and by multiplying up was expressed as microgrammes/100 mls of serum.

4.3.1.6.2 Cortisol

This assay is again based on the radioimmune assay technique but differs in several ways from the testosterone rapid assay. The technique depends on comparing the binding by cortisol antisera to cortisol in solutions of known amounts of cortisol with binding on solutions containing unknown amounts of cortisol.

Standards

Seven standard solutions were made up, containing the following amounts of cortisol per 100 µl:- 1.05, 0.788, 0.525, 0.389, 0.263, 0.105, 0.0525, 0.0105 and 0 ng. One hundred µl of the standard was added to 500 µl of tritiated cortisol (Radiochemicals Centre, Amersham); this gave approximately 20,000 DPM per assay in 1 ml glass tubes and was allowed to equilibrate overnight at 4°C.

The following morning 400 µl of 25% aqueous trichloro acetic acid was added to each tube. The tubes were then capped and the contents mixed in a rotamix and then centrifuged at 200 RPM. Two hundred µl of the supernatant weretaken off and placed into 5 ml polypropylene tubes and 3.8 mls of buffer added to each tube. The tubes were capped and the contents well mixed on a rotamix, 600 µl of the above solution were then pipetted into polypropylene assay tubes and 600 µl into counting vials. To the latter were added 600 µl of distilled water and 10 mls of Unisolve and the resultant solution was then counted in the scintillation counter to calculate the recovery. To the 600 µl of TCA extract was added 100 µl of cortisol anti-serum (Steranti, Ltd.). The tubes were then mixed well on a rotamix and incubated for 45 minutes in a water bath at 37°C. The tubes were then allowed to equilibrate at 4°C for 30 minutes before 200 µl of dextran/charcoal suspension was added to each tube; they were then

mixed on a rotator for 30 minutes at 4°C. The tubes were centrifuged for 10 minutes at 2000g in a 4°C constant temperature room. Five hundred µl of supernatant was then carefully taken off and pipetted into counting vials containing 700 µl of distilled water; 10 mls of Unisolve was then added to each vial which was shaken well and placed in a scintillation counter for counting. The DPM for the recovery vials were taken from the printout, and using the formula in Appendix 6, the percentage recovery calculated. The DPM for the assay were taken off the printout and the percentage binding was calculated for each standard. A standard curve of ng/assay versus percentage binding was then drawn (see Fig. 37).

Unknowns

Three groups of sera were assayed. They were serum from the two levels of cortisol-implanted fish, serum from the cholesterol control fish (to show the stress caused by the implant) and serum from the normal control.

Tests had shown that the level of cortisol in the implanted fish was very high and therefore different quantities of serum and chemicals were used for the different groups of sera.

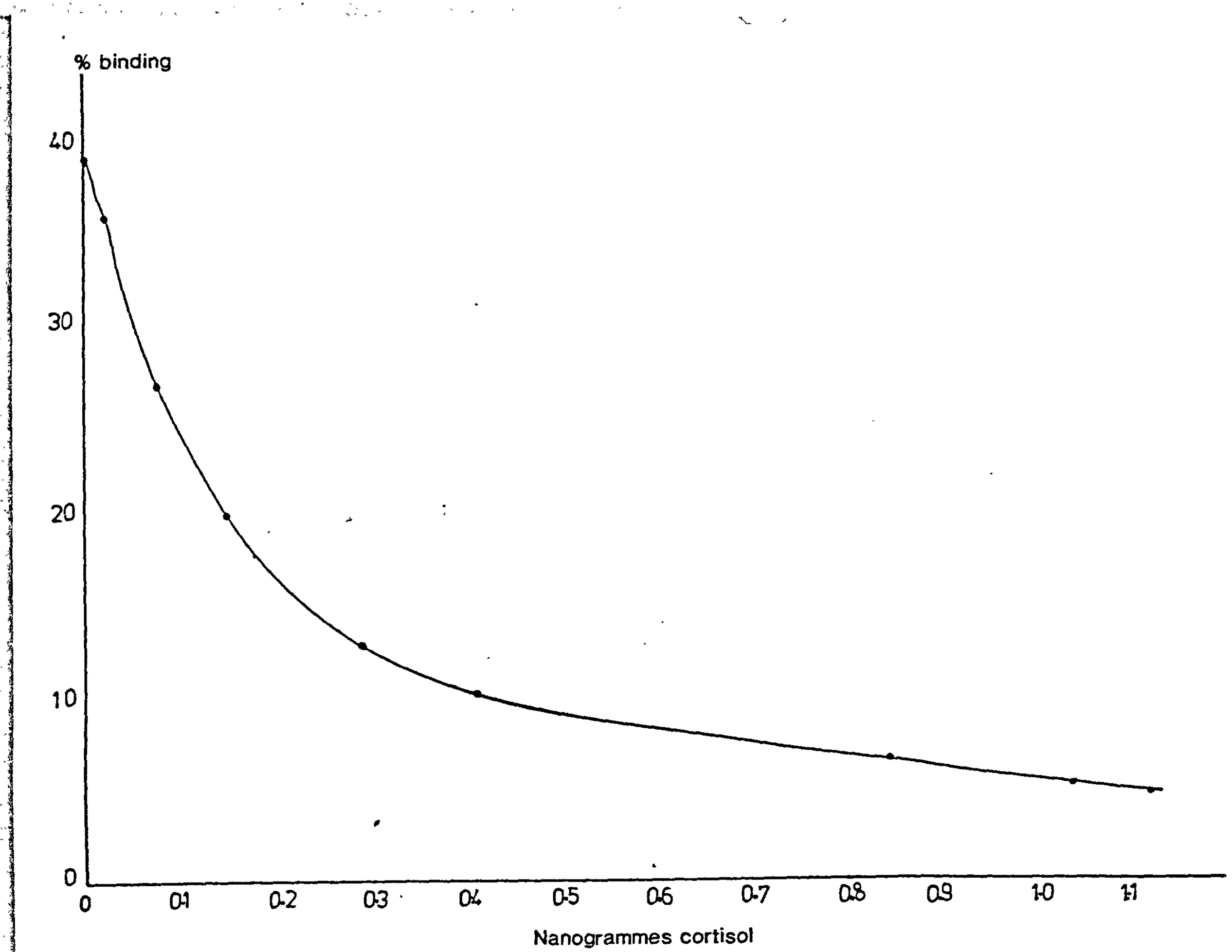
Assay

Cortisol implants

10 µl of serum was added to 90 µl of buffer and 500 µl of tritiated cortisol in glass tubes. The samples were allowed to equilibrate overnight and the next day 400 µl TCA was added to each tube. After centrifugation 200 µl of supernatant was taken off and added to 3.8 mls of buffer. The assay was then continued as for the standards.

FIGURE 37

Standard curve of percentage binding of cortisol antiserum to cortisol versus amount of cortisol in solution.


% binding
40
30
20
10
0
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
1·1
Nanogrammes cortisol

Cholesterol controls and normal controls

Ten µl of serum was added to 100 µl of tritiated cortisol in glass tubes and equilibrated overnight. The next morning 80 µl of TCA was added to each tube. After centrifugation 100 µl of the supernatant was taken off and mixed with 1.9 mls of buffer. The assay was then continued as for the standards.

The percentage binding was worked out using the formulas in Appendix 6, and the nanogrammes per assay worked out from the standard curve. The quantity of cortisol in the serum was then calculated, using the formulas in Appendix 7 and expressed as ug/ 100 mls.

All the samples were done in replicate and the mean of the two taken. A separate standard curve was drawn for each day's assay.

4.3.1.7 Histopathology

The skin and gill samples from each treatment were processed as described in Appendix 1, and 5 µm thick sections were cut and stained with Haemotoxylin and Eosin and Alcian Blue.

Mean epidermal thickness was calculated for each fish by measuring the epidermis at 20 random points on the section. Mean goblet cell number per unit area was calculated by counting the number of goblet cells in a 355 µm length of epidermis at ten random points on the section.

4.4 RESULTS

4.4.1 Ectoparasites

The results of the examination of the skin smears is shown in Tables 15a - e. The coding used in Table 15 is explained in Table 14.

One week after commencement of the trial small numbers of protozoan parasites, namely Scyphidia sp., Trichodina sp. and Bodo sp. were observed on three out of five of the double-dose hydrocortisone-treated fish. No parasites were seen on any of the other fish examined.

By week 2, eight out of ten of the hydrocortisone-treated fish examined showed light infestations of ectoparasites; two out of these eight fish had light Ichthyobodo infestations on the skin. Two of the double-dose testosterone-treated fish, one cholesterol and one normal control showed light ectoparasite infestations (Scyphidia sp. and Trichodina sp.) but not Ichthyobodo.

By week 3, nine out of ten of the hydrocortisone-treated fish examined had Ichthyobodo infestations which varied in intensity from light to massive; in general the intensity of the Ichthyobodo infestation of the double-dose hydrocortisone-treated fish was greater than that of the single-dose. Six out of ten of these fish also had other light ectoparasite infestations. None of the other fish sampled in week 3 showed any Ichthyobodo on the skin when examined, although one of the single-dose testosterone-treated fish had small numbers of Scyphidia sp.

By week 4 all of the fish treated with hydrocortisone had massive Ichthyobodo infestations; some 2 mm^2 fields had as many as

TABLE 14

Coding used in Table 15

*	=	Occasional Ichthyobodo	1 per 10 fields
**	=	Light Ichthyobodo	1 - 3 per field
***	=	Moderate Ichthyobodo	10 per field
****	=	Heavy Ichthyobodo	20 - 100 per field
*****	=	Massive Ichthyobodo	100+ per field

This code applies for other parasites also.

TABLE 15 Result from implantation experiment.

TABLE 15 a Week 1 26.9.79

Treatment		Weight	Sex	Heal-ing	Les-ion	Ichthy-obodo	%	Others	%
Brand 0	1	38.0	♂	✓	x	0		*	
40 mg	2	42.6	♂	✓	x	0		*	
Hydrocortisone	3	22.61	♂	x	x	0	0	0	60
	4	43.3	♂	✓	x	0		0	
	5	37.0	♀	✓	x	0		*	
Brand 1	1	37.0	♂	x	x	0		0	
20 mg	2	34.8	♂	✓	x	0		0	
Hydrocortisone	3	33.8	♀	✓	x	0	0	0	0
	4	33.5	♂	x	x	0		0	
	5	24.7	♂	✓	x	0		0	
Brand 2	1	37.0	♂	✓	x	0		0	
7 mg	2	29.3	♀	✓	x	0		0	
Testosterone	3	40.2	♂	x	x	0	0	0	0
	4	23.0	♀	x	x	0		0	
	5	27.0	♂	✓	x	0		0	
Brand 3	1	29.7	♀	✓	x	0		0	
3.5 mg	2	43.0	♂	✓	x	0		0	
Testosterone	3	31.8	♀	✓	x	0	0	0	0
	4	32.5	♂	✓	x	0		0	
	5	27.3	♂	✓	x	0		0	
Brand 6	1	42.0	♀	x	x	0		0	
Cholesterol	2	43.1	♂	x	x	0		0	
Control	3	29.4	♂	✓	x	0	0	0	0
	4	35.0	♂	x	x	0		0	
	5	31.9	♀		x	0		0	
Brand 8	1	15.0	♀	-	x	0		0	
Brand	2	31.8	♀	-	x	0		0	
Only	3	17.3	♀	-	x	0	0	0	0
	4	36.8	♂	-	x	0		0	
	5	28.9	♂	-	x	0		0	
Control	1	34.4	♂	-	-	0		0	
Fish	2	24.0	♀	-	-	0		0	
	3	26.1	♂	-	-	0	0	0	0
	4	37.0	♂	-	-	0		0	
	5	51.3	♀	-	-	0		0	

TABLE 15b

Week 2 3.10.79

Treatment		Weight	Sex	Healing	Lesion	Ichthyobodo	%	Others	%
Brand 0	1	35.0	♀	✓	✓✓	0		*	
40 mg	2	29.0	♂	✓	✓	0		*	
Hydrocotrisone	3	37.5	♀	✓	✓✓	0	0	0	80
	4	31.2	♂	x	✓✓	0		*	
	5	34.9	♀	x	✓✓	0		*	
Brand 1	1	32.2	♀	✓	✓✓	0		**	
20 mg	2	21.5	♂	✓	✓✓	0		**	
Hydrocortisone	3	53.9	♂	✓	✓✓	*	40	**	80
	4	22.2	♂	x	x	*		*	
	5	26.0	♂	✓	✓	0		0	
Brand 2	1	62.5	♂	✓	x	0		*	
	2	71.0	♂	✓	x	0		0	
7.0 mg	3	29.4	♂	x	x	0	0	0	40
Testosterone	4	34	♀	✓	x	0		*	
	5	35	♂	✓	x	0		0	
Brand 3	1	41.0	♂	✓	x	0		0	
	2	65	♂	✓	x	0		0	
	3	18.1	♀	✓	x	0	0	0	0
	4	35	♀	✓	x	0		0	
	5	34	♂	✓	x	0		0	
Brand 6	1	23	♀	✓	✓✓	0		0	
	2	45.6	♀	✓	x	0		*	
Cholesterol	3	42.5	♂	x	✓✓	0	0	0	20
Control	4	29.3	♀	x	✓	0		0	
	5	66	♀	✓	✓✓	0		0	
Brand 8	1	42.7	♂	-	x	0		0	
	2	56.8	♀	-	x	0		0	
	3	45.2	♂	-	x	0	0	0	0
	4	35.1	♂	-	x	0		0	
	5	36.2	♀	-	x	0		0	
Control	1	32.8	♀	-	x	0		0	
	2	55.1	♀	-	x	0		*	
	3	35.9	♀	-	-	0	0	0	20
	4	72.0	♂	-	-	0		0	
	5	33.6	♀	-	-	0		0	

TABLE 15c

Week 3 11.10.79

Treatment		Weight	Sex	Heal-ing	Les-ion	Ichthy-obodo	%	Others	%
Brand 0	1	20	♂	x	✓	****		**	
40 mg	2	27.5	♀	x	✓	****		**	
Hydrocortisone	3	26.4	♂	x	✓✓	*****	100	0	60
	4	30	♀	x	✓✓	****		****	
	5	27.5	♂	x	✓✓	****		0	
Brand 1	1	24.0	♀	x	✓✓	**		**	
20 mg	2	21.6	♀	x	✓	***		0	
Hydrocortisone	3	27.0	♂	x	✓	0	80	**	60
	4	35.7	♀	✓	✓	***		**	
	5	30.0	♂	x	✓✓	****		0	
Brand 2	1	58.2	♂	✓	x	0		*	
7 mg	2	43.0	♂	✓	x	0		0	
Testosterone	3	32.8	♂	✓	x	0	0	0	20
	4	35.5	♂	✓	x	0		0	
	5	40.6	♂	✓	x	0		0	
Brand 3	1	58.2	♂	✓	x	0		0	
3.5 mg	2	35.2	♂	✓	x	0		0	
Testosterone	3	34.7	♂	✓	x	0	0	0	0
	4	31.3	♀	✓	x	0		0	
	5	32	♂	x	✓	0		0	
Brand 6	1	37.0	♀	x	✓	0		0	
	2	32.3	♀	x	✓	0		0	
Cholesterol	3	31.6	♂	✓	✓	0	0	0	0
Control	4	30.6	♀	x	x	0		0	
	5	30	♀	x	✓	0		0	
Brand 8	1	24.5	♂	-	x	0		0	
Brand	2	19.1	♀	-	✓	0		0	
Only	3	31.0	♀	0	x	0	0	0	0
	4	30	♀	-	x	0		0	
	5	29.5	♂	0	✓	0		0	
Control	1	38	♂	-	x	0		0	
	2	70	♂	-	x	0		0	
	3	51	♂	-	x	0	0	0	0
	4	40.5	♂	-	x	0		0	
	5	55	♂	-	x	0		0	

TABLE 15d

Week 4 18.10.79

Treatment		Weight	Sex	Heal-ing	Les-ion	Ichthy-obodo	%	Others	%
Brand 0	1	32.0	♂	x	√√	*****		****	
40 mg	2	23.3	♂	√	√	*****		****	
Hydrocortisone	3	38.0	♀	x	√	****	100	**	100
	4	44.5	♀	x	√√	*****		**	
	5	30	♂	√	√	*****		**	
Brand 1	1	22	♀	√	√√	****		**	
20 mg	2	28	♀	x	√	*****		**	
Hydrocortisone	3	30	♂	√	√√	*****	100	**	100
	4	38.4	♂	x	√	***		*	
	5	24.0	♂	√	√√	***		**	
Brand 2	1	44	♀	√	√	*		*	
	2	51.7	♂	√	√	0		0	
7 µg	3	47.6	♂	√	x	0	20	0	20
Testosterone	4	44	♀	√	x	0		0	
	5	45	♂	√	x	0		0	
Brand 3	1	56.5	♀	x	x	0		0	
3.5 mg	2	25.5	♂	x	x	0		*	
Testosterone	3	25.0	♂	x	x	0	0	0	20
	4	30	♂	x	x	0		0	
	5	36.5	♂	x	x	0		0	
Brand 6	1	27	♀	x	x	0		0	
	2	20	♂	√	x	0		0	
Cholesterol	3	43.4	♀	√	x	0	0	0	0
	4	36.9	♂	x	√	0		0	
	5	40.0	♀	√	x	0		0	
Brand 8	1	60.2	♀	-	x	0		0	
Brand	2	27	♀	-	x	0		0	
Only	3	24.5	♂	0	x	0	0	0	0
	4	40	♀	-	x	0		0	
	5	44	♂	-	x	0		0	
Control	1	43	♂	-	-	0		0	
	2	58.4	♂	-	-	*	20	*	80
	3	57.0	♂	-	-	0		*	
	4	40	♂	-	-	0		*	
	5	44.2	♂	-	-	0		*	

TABLE 15e

Week 5 25.10.79

Treatment		Weight	Sex	Heal-ing	Les-ion	Ichthy-obodo	%	Others	%
Brand 0	1	33.55	♀	✓	✓	****		0	
40 mg	2	54	♂	x	✓	***	100	0	0
Hydrocortisone	3								
	4								
	5								
Brand 1	1	30.28	♀	✓	✓✓	*****		*	
20 mg	2	32.22	♀	x	✓✓	****	100	0	25
Hydrocortisone	3	29.13	♂	x	✓	**		0	
	4	61.7	♂	x	✓	**		0	
	5								
Brand 2	1	25	♂	✓	x	0		0	
	2	63.5	♀	✓	x	0		*	
75 mg	3	27.4	♀	x	x	0	0	0	20
Testosterone	4	30.0	♀	x	x	0		0	
	5	33.2	♂	✓	x	0		0	
Brand 3	1	35	♂	✓	x	0		0	
3.5 µg	2	40.6	♀	✓	x	0		*	
Cholesterol	3	38.7	♂	✓	x	0	0	0	20
	4	39.4	♀	✓	x	0		0	
	5	33.2	♂	✓	x	0		0	
Brand 6	1	43.5	♀	x	✓	0		*	
Cholesterol	2	50.2	♂	✓	x	0		0	
	3	55.1	♀	✓	x	0	0	*	40
	4	25.5	♂	✓	x	0		0	
	5	18.34	♀	x	x	0		0	
Brand 8	1	43.5	♀	-	x	0		0	
Brand	2	37.8	♂	-	x	0	0	0	0
only	3	38.8	♂	-	x	0		0	
	4	39.4	♀	-	x	0		0	
	5	38.56	♂	-	x	0		0	
Control	1	38.8	♂	-	-	0		0	
	2	38.0	♂	-	-	0		0	
	3	53.0	♂	-	-	0	0	0	0
	4	47.9	♂	-	-	0		0	
	5	28.8	♀	-	-	0		0	

500 - 1000 Ichthyobodo visible

With such heavy infestations it was impossible to ascertain any difference in intensity of infestation between the single and double-dose hydrocortisone-treated fish. All of these hydrocortisone-treated fish also had infestations of other ectoparasites (Scyphidia sp.; Trichodina sp., Gyrodactylus sp. and Chilodonella sp.) varying from light to heavy. One of the double-dose testosterone and one of the normal controls had very light infestations of Ichthyobodo. One of these fish, the normal control, was a precociously mature male. Several of the other fish also showed light ectoparasite infestations but none of them showed any Ichthyobodo.

By week 5, the last week of the trial, several of the hydrocortisone-treated fish had died, and thus the sample was incomplete; however, all of the six fish examined showed Ichthyobodo infestations ranging from light to massive. The intensity of infestation, however, was generally slightly less than was seen in week 4. One of these fish also showed light ectoparasitic infestations other than Ichthyobodo. None of the other treatments or controls showed any Ichthyobodo on the skin although several showed small numbers of Scyphidia sp. and Trichodina sp..

Therefore, in summary, with the exception of two fish in week 4, one of which was mature, the only fish which showed Ichthyobodo infestations over the five week period were the hydrocortisone-treated fish. In addition the prevalence and intensity of infestation of parasites other than Ichthyobodo was almost invariably greater in the hydrocortisone-treated fish than in any of the other treatments.

The method of presentation of the steroids appeared to be very successful as all of the fish examined throughout the trial had retained their pellets. The method of inserting the pellet did not appear to have any detrimental effect on the fish, and healing of the incision varied with the treatment. In general the testosterone-treated fish showed the best healing and the hydrocortisone-treated the worst. In addition from week 2 onwards most of the hydrocortisone-treated fish had developed lesions around the brand mark, on some occasions the lesion was very extensive. Lesions were only seen occasionally in the other treatments and again the testosterone-treated fish were the least affected.

4.4.2 Growth

The best overall growth and appearance of the fish was seen in the testosterone-treated fish and the poorest growth was seen in the cortisol-treated fish; these fish had a very ragged and darkly pigmented appearance.

4.4.3 Testosterone Assays

The mean weekly levels of testosterone recorded in the implanted fish and the branded controls are shown in Table 16 and Figure 38. It can be seen that the testosterone levels of the implanted fish were very high when compared to the controls throughout the period of the trial, and ranged from a mean of 5.17 µg/100 mls of serum to 13.65 µg/100 mls of serum. The highest level of testosterone recorded in the 7 mg implant was 13.65 µg/100 mls in week 2 and for the 3.5 mg/implant 11.42 µg/100 mls in week 3. The levels declined after this to 5.17 µg/100 ml and 6.11 µg/100 mls respectively by week 4. The levels of testosterone

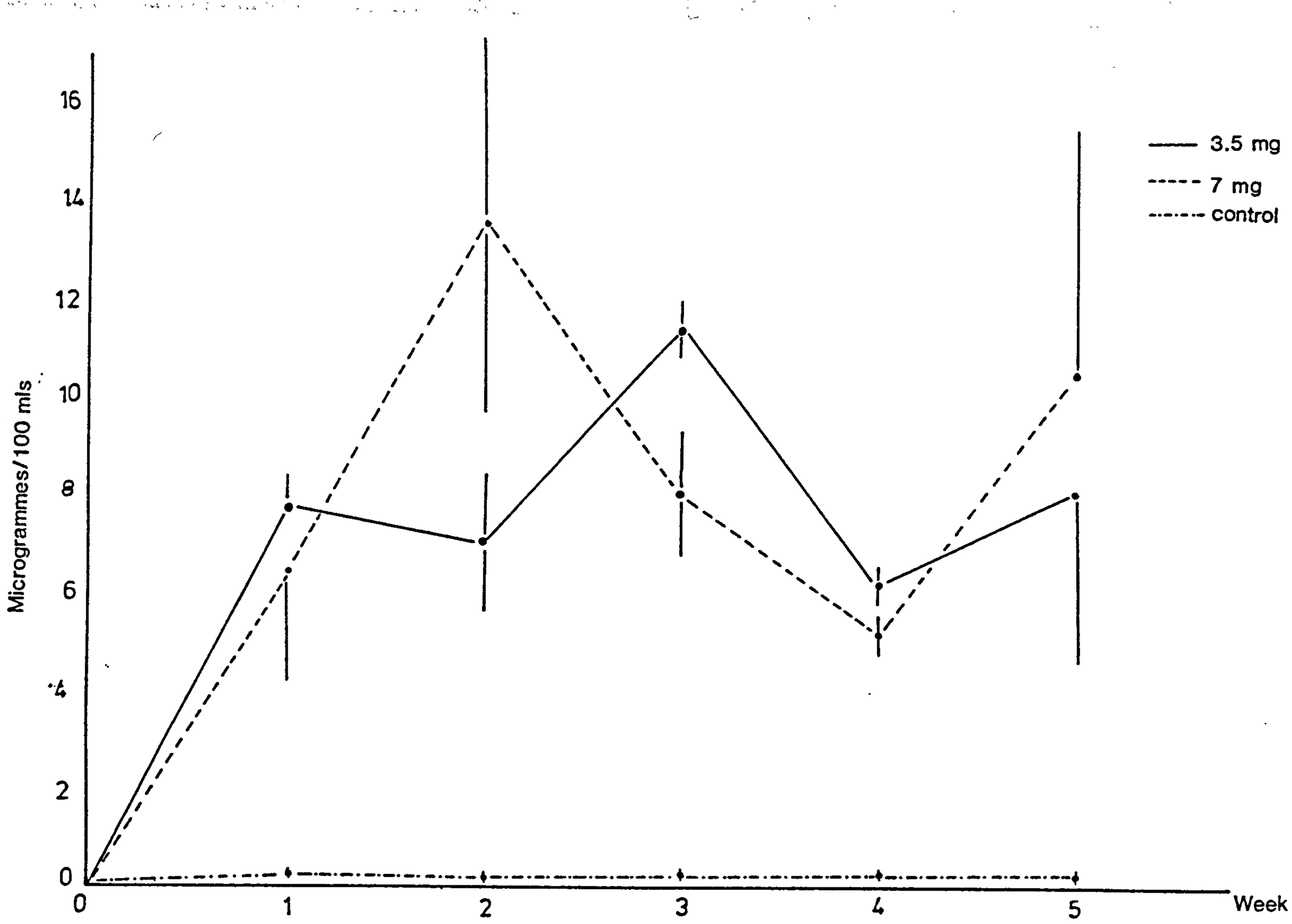
TABLE 16

Mean Testosterone Levels of Implanted and Control Fish

Week	7 mg testosterone	3.5 testosterone	Control	Temperature
1	6.48 ± 2.47 µg/100 ml	7.83 ± 0.92 µg/100 ml	0.23 ± 0.05 µg/100 ml	11°C
2	13.65 ± 3.95 "	7.05 ± 1.45 "	0.19 ± 0.06 "	11°C
3	8.05 ± 1.27 "	11.42 ± 0.50 "	0.23 ± 0.04 "	11°C
4	5.17 ± 0.22 "	6.11 ± 0.50 "	0.21 ± 0.02 "	9°C
5	10.68 ± 4.92 "	8.09 ± 3.59 "	0.19 ± 0.187 "	9°C

FIGURE 38

Testosterone levels in implanted and control fish.


16
14
12
10
8
6
4
2
0
Micrommes/100 mls
0
1
2
3
4
5
Week
3.5 mg
7 mg
control

increased slightly in both levels of implant by week 5. Variability was large within a sample which could probably be explained by the small size of the sample and individual variation in uptake between fish.

The level of testosterone in the serum of the control fish varied very little over the duration of the trial, and mean values fluctuated between 0.19 - 0.23 µg/100 mls of serum over the five week period. There was very little variation in testosterone levels between the fish in a group, and no overall increase in level over the duration of the study.

4.4.4 Cortisol Assay

The mean weekly levels of cortisol recorded in the cortisol-implanted fish, the cholesterol controls and the normal controls are shown in Table 17 and Figure 39. As can be seen,very high levels of cortisol were reached one week after implantation (374 µg/100 ml in the 40 mg implanted fish and 102 µg/100 ml in the 20 mg implanted fish). The levels subsequently declined but remained very high at around the 100 µg/100 ml level in the double-dose implant, and 70 µg/100 mls in the single dose fish, but declined to 45.4 µg/100 ml by week 5. As in the testosterone implants, variation within a weekly sample was high,probably because of the small size of sample. From week 3 the cortisol-implanted fish serum samples were pooled, as great difficulty was experienced in taking blood samples from these fish. The quantity of serum obtained was very small and was pooled at the time of assay.

The cortisol levels in the cholesterol implants and the controls were low throughout the trial; significantly however the

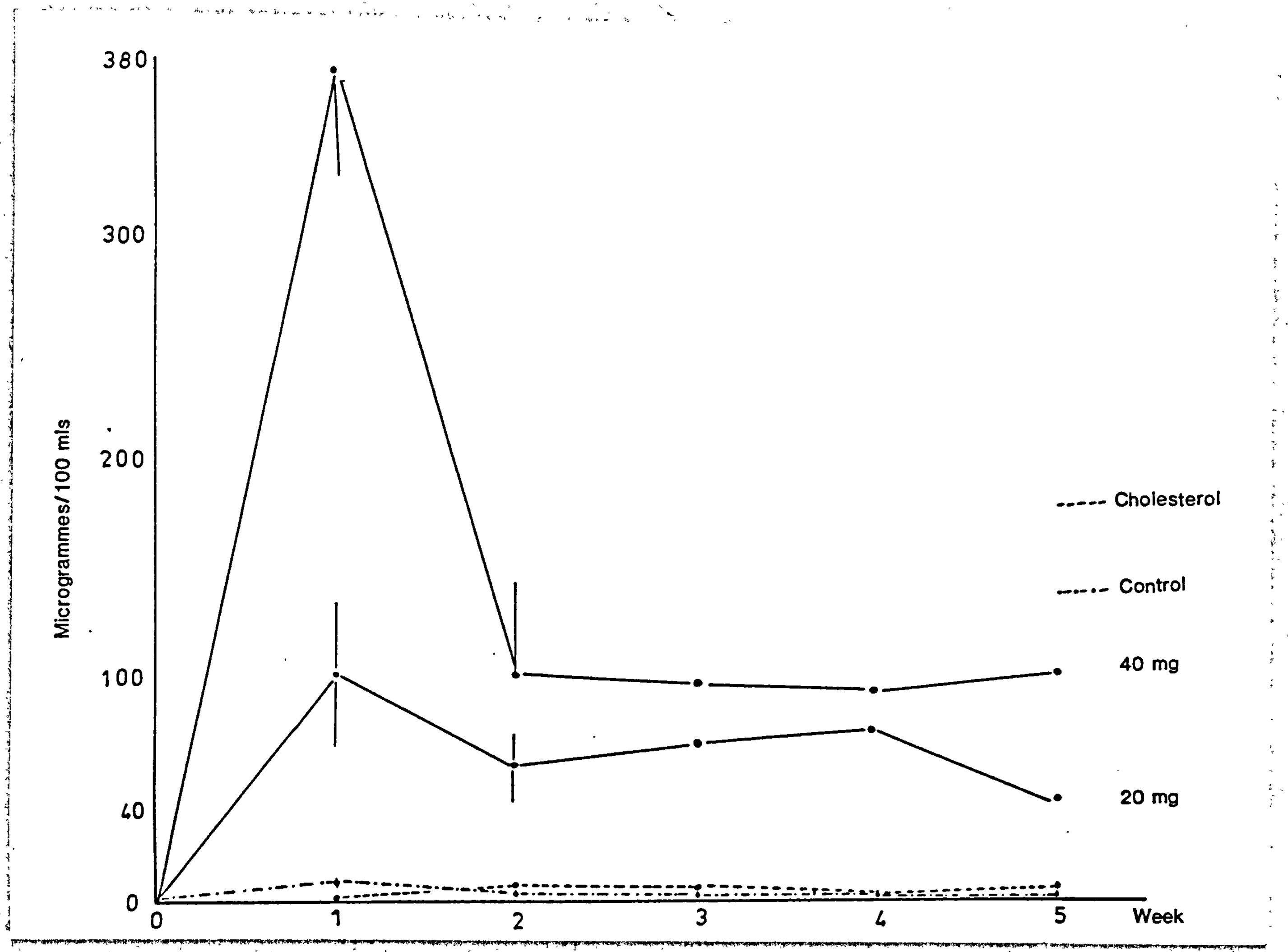
TABLE 17

Cortisol levels in implanted and control fish

Week	40 mg Cortisol	20 mg Cortisol	Cholesterol	Control	
0				9.01 ± 1.64 µg/100 ml	12°c
1	374 ± 58 µg/100 ml	103 ± 35 µg/100 ml	0.84 ± 0.59 µg/100 ml	3.2 ± 0.50 µg/100 ml	11°C
2	104 ± 41.5 "	62.5 ± 13.81 "	6.83 ± 2.99 "	2.24 ± 0.85 "	11°C
3	98 µg/100 ml (Pooled)	72 µg/100 ml (Pooled)	3.2 ± 1.2 "	1.82 ± 0.75 "	11°C
4	94.4 µg/100 ml (Pooled)	77 µg/100 ml (Pooled)	1.00 ± 0.5 "	1.75 ± 0.8 "	9°C
5	102 µg/100 ml (Pooled)	45.4 µg/100 ml (Pooled)	3.44 + 1.61 "	1.6 ± 0.7 "	9°C
23	-	-	-	0 + 6.00 µg/100 ml 1 + 5.5 µg/100 ml	5°C

FIGURE 39

Cortisol levels in implanted and control fish.


380
300
200
100
40
0
Microgrammes/100 mls
0
1
2
3
4
5
Week
Cholesterol
Control
40 mg
20 mg

cortisol levels of the control fish were at their highest on the day of commencement of the trial. This was probably as a result of the stress of disturbing the fish during the setting up of the trial when the fish were netted out over the period of a day. By week 1 the levels had markedly declined and they dropped from then until the end of the trial (3.2 µg/100 ml on week 1 - 1.6 µg/100 ml on week 5). On week 5 a comparison was carried out between the control fish and similar fish in an adjacent tank which had not been sampled weekly. The cortisol levels in both were similar (1.6 µg/100 ml in the sample tank and 1.22 µg/100 ml in the unsampled tank).

The cholesterol-implanted fish showed higher levels of cortisol than the controls but they were still relatively low throughout the trial. It is likely that the operation technique and the presence of the implant led to the higher levels of cortisol than the controls.

Serum samples taken in March, 1980, eighteen weeks after the end of the trial, after a period of prolonged low water temperatures, showed higher cortisol levels than recorded in the controls at the end of the trial (see Table 18). Both 0+ and 1+ fish had elevated cortisol levels, and in both ages of fish the fish with the highest cortisol levels also had moderate Ichthyobodo infestations. The 1+ fish with the highest cortisol level (31.12 µg/100 mls) was considered to be moribund and therefore cannot be considered to be representative of the group.

TABLE 18

Cortisol levels and presence of Ichthyobodo on 0+ and 1+ Rainbow Trout at 5°C

Fish		Cortisol µg/ml (100)	Ichthyobodo
0+	1	0	0
	2	3.7	***
	3	1.011	0
	4	18.32	**
	5	6.95	*
1+	1	31.12	***
	2	1.26	0
	3	5.9	0
	4	11.37	***
	5	3.412	0

4.4.5 Histopathology Results

Control Fish

The sections of these fish appeared to be normal and showed the typical teleost skin picture (Fig. 40) with normal complement of malphigian and goblet cells and the occasional so-called acidophilic cell (Fig. 41). The scales were obvious in scale pockets in the stratum spongiosum (Fig. 42) and there were some deposits of melanin in the basement membrane of the epidermis. Various unidentified cell types were seen in the epidermis. The occasional lymphocyte was also seen but these cells were not a feature of this study. In general the skin thickness and goblet cell count per unit area increased over the trial period (see Tables 19 and 20).

Cortisol-treated Fish

These fish showed an increase in the number of goblet cells per unit area in comparison to the control fish over the first two weeks, with numbers starting to decline by week 3 (see Table 20).

The shape of the goblet cells was slightly different to that of the controls, being larger and more flask-shaped (see Fig. 43). By week 3 the first Ichthyobodo were seen in the sections and in such sections breakdown of the integrity of the epidermal surface and decline of the goblet cells were becoming apparent (Fig. 44). By week 4 spongiosis of the mid zone of the epidermis was apparent (Fig. 45) and in most of the fish the epidermal integrity had broken down completely (Fig. 46) and the scales had eroded and detached from the stratum spongiosum followed, by subsequent waterlogging of the underlying muscle. Breakdown of the epidermis and loss of goblet cells appeared to be correlated to the Ichthyobodo infestation rather than the effect of the cortisol as

TABLE 19

Mean epidermal thickness (μm) of control and implanted fish

Epidermal Thickness	Control	Cortisol 20 mg	Cortisol 40 mg	Testosterone 3.5 mg	Testosterone 7 mg
0	95.6	-	-	-	-
1	95.19	96.3	95.5	126.80	102.50
2	90.16	97.1	96.8	123.60	145.30
3	126.15	120.11	121.23	121.80	154.90
4	125.51	-	-	120.07	142.00
5	125.27	-	-	143.60	172.40

TABLE 20

Mean goblet cell count/unit area
(350 μm × 350 μm length of epidermis)
of control and implanted fish

Count	Control	Cortisol 20 mg	Cortisol 40 mg	Testosterone 3.5 mg	Testosterone 7 mg
0	5.79	-	-	-	-
1	6.52	7.1	7.51	9.9	10.71
2	8.53	9.0	9.10	9.4	16.6
3	9.77	3.51	2.51	10.17	15.90
4	11.40	-	-	12.32	15.40
5	11.36	-	-	13.375	21.6

FIGURE 40

Normal epidermis of immature rainbow trout.

G = goblet cells

B = basement membrane

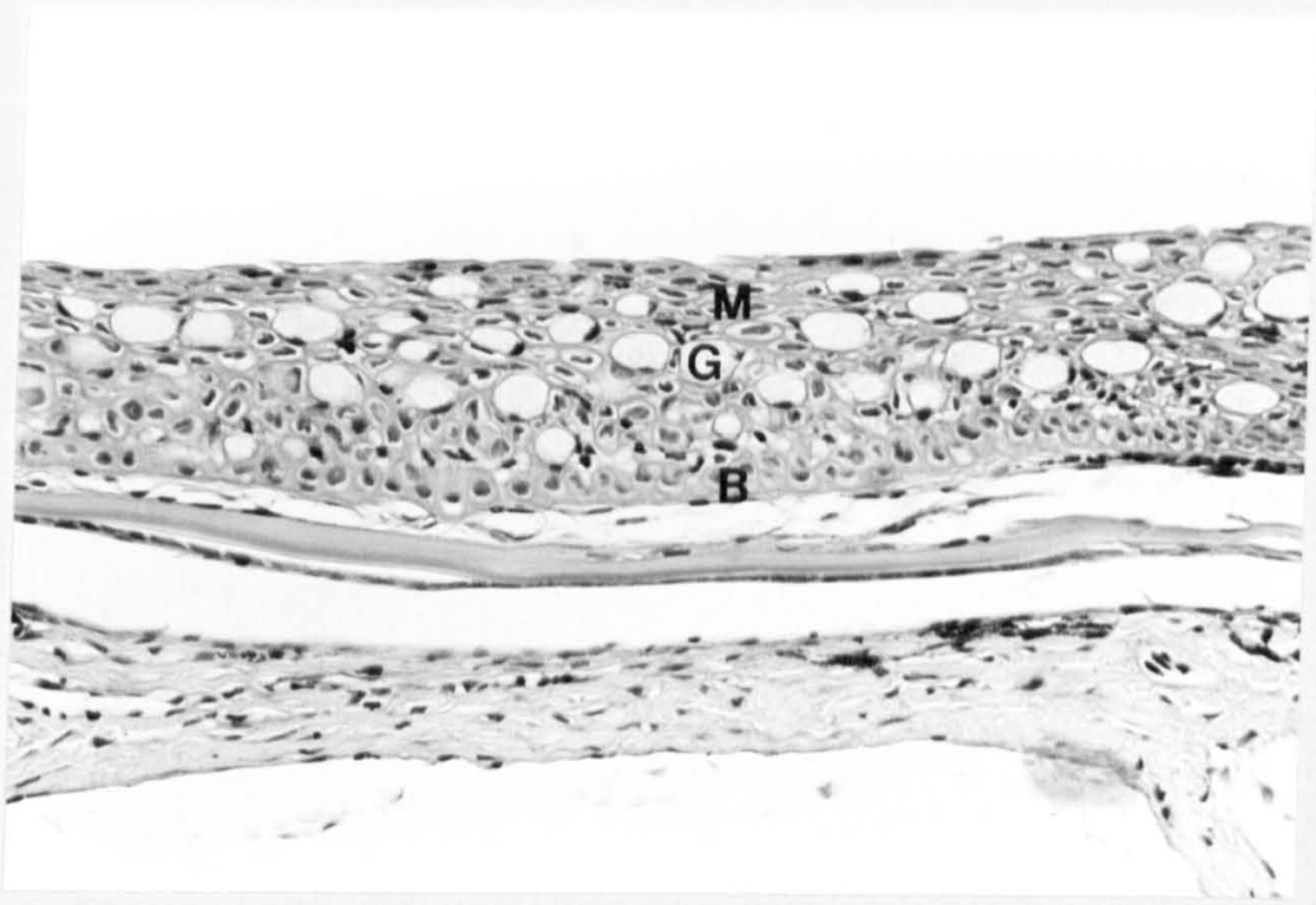
M = malphigian cells

HαE × 320

FIGURE 41

Acidophilic cell (A) in epidermis of rainbow trout skin.

HαE × 550


M
G
B

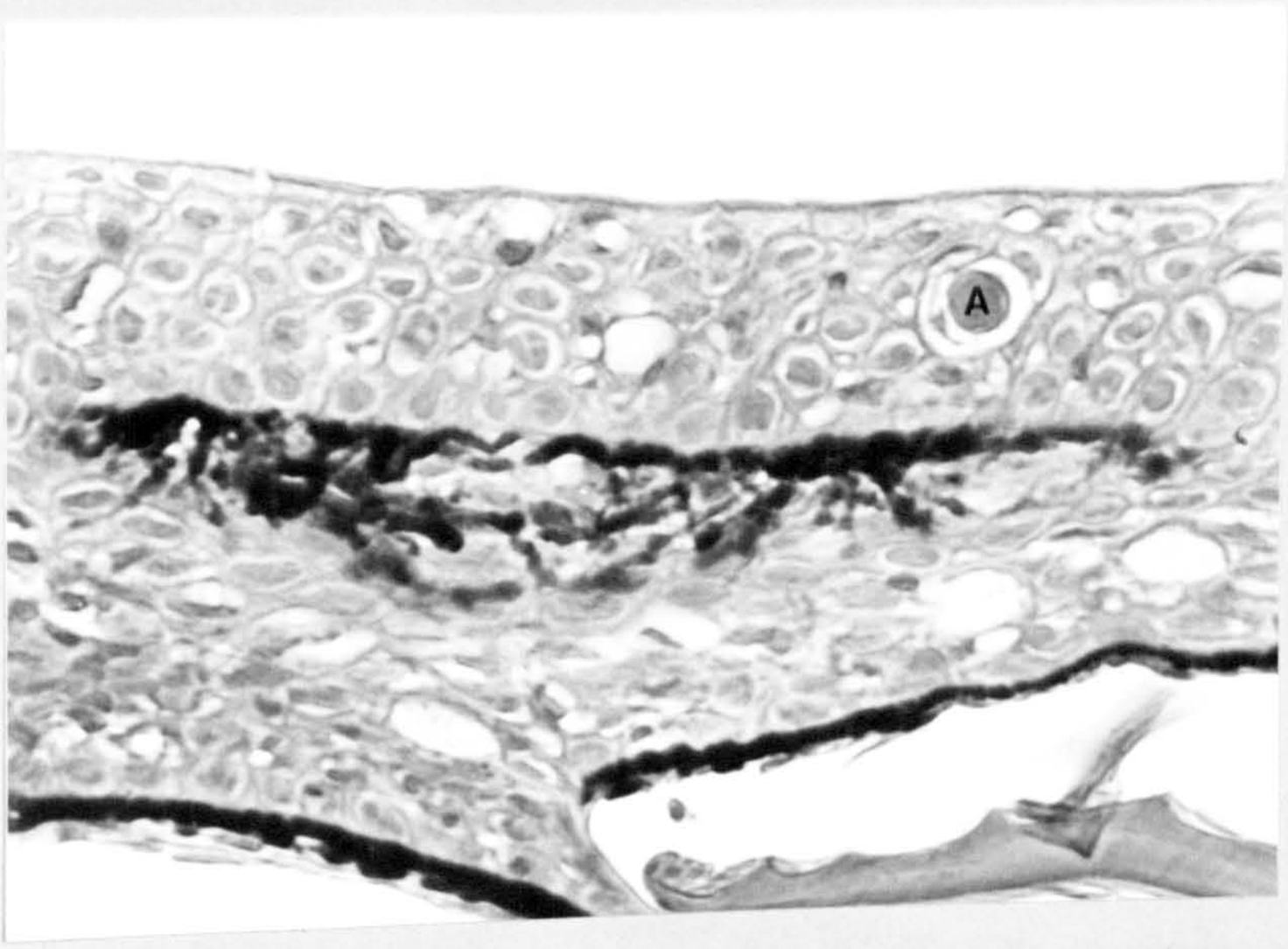

A

FIGURE 42

Skin of normal rainbow trout showing scales (S)

in stratum spongiosum.

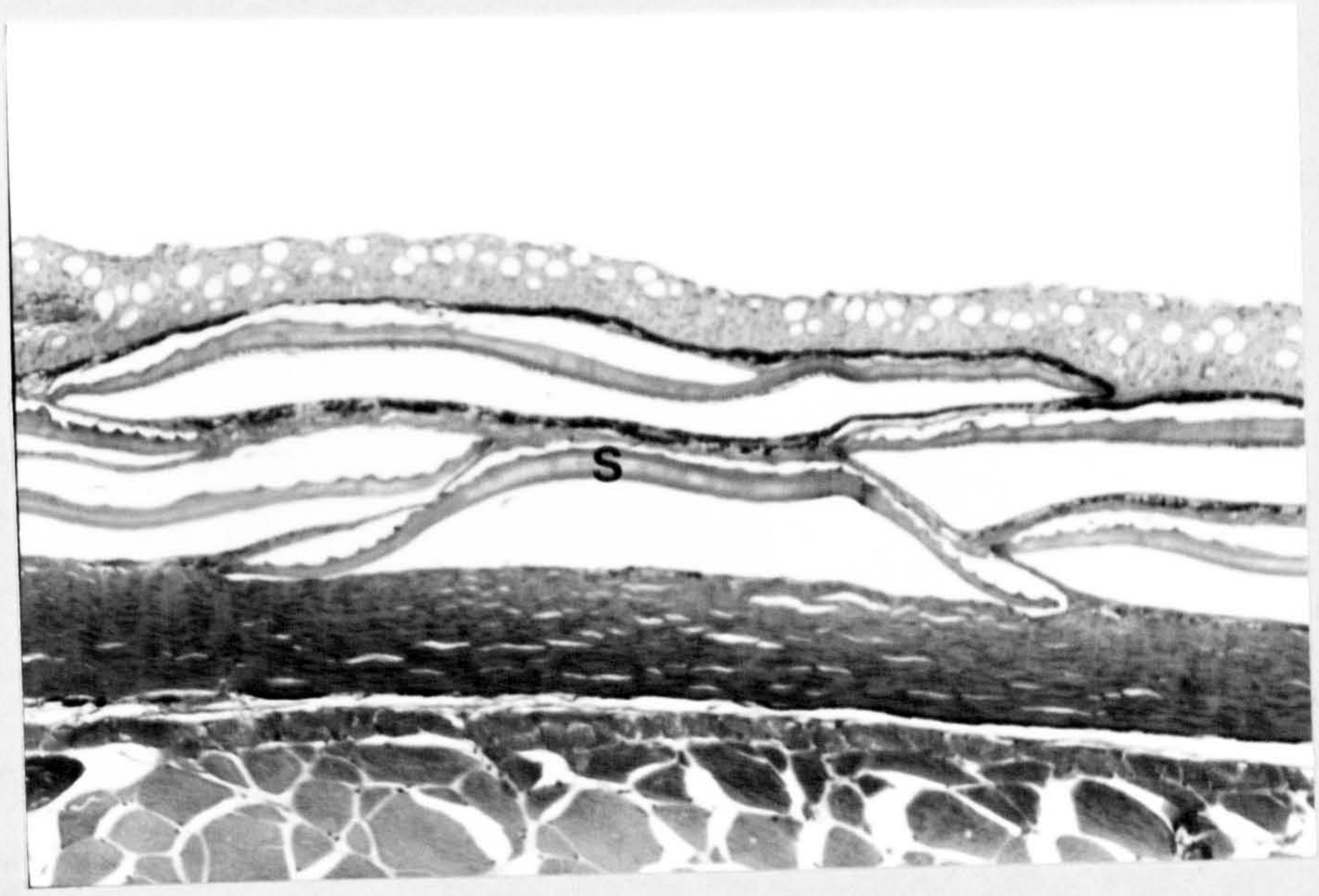
HαE × 125

FIGURE 43

Flask-shaped goblets cells (G) in epidermis

of cortisol-treated fish.

HαE × 500


S


G
G

FIGURE 44

Ichthyobodo (I) attached to epidermis of cortisol - treated fish. Note slight spongiosis (S) of epidermis beneath Ichthyobodo.

HαE × 320

FIGURE 45

Extensive spongiosis and sloughing of epidermal cells in Ichthyobodo-infested,cortisol-treated fish.

HαE × 320


I
S

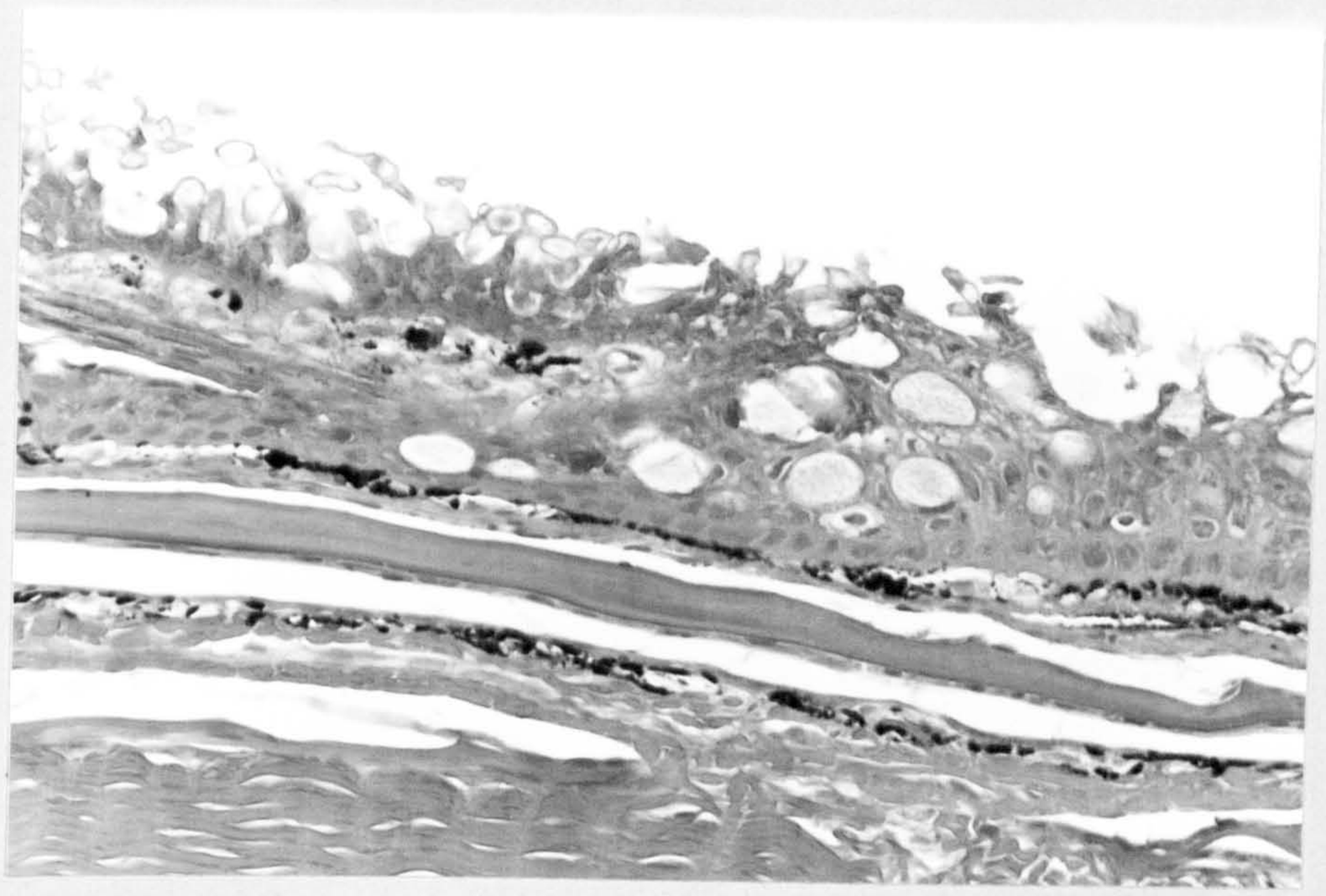
FIGURE 46

Virtual complete breakdown of epidermis

of cortisol-treated, Ichthyobodo -

infested fish.

HαE × 500



it was not seen until week 3, when the Ichthyobodo were present on the fish, although cortisol levels had been very high from the end of week 1.

The pathological changes seen in the Ichthyobodo-infested cortisol-treated fish were very similar to those described in Chapter 3, with marked degenerative changes of the malphigian cells characterised by markedly basophilic nuclei.

Testosterone-treated Fish

There was a progressive thickening of the epidermis and increase in goblet cell count for both the single-dose and double-dose testosterone-treated fish over the time course of the trial. In addition both epidermal thickness and goblet cell count were markedly greater in the testosterone-treated fish than the control fish and this was seen to be most extreme in the double-dose testosterone fish (see Tables 19 and 20 and Fig. 47). As would be expected there was a good correlation between increased epidermal thickness and increased goblet cell count. The stratum compactum appeared to be much thicker but less compact in the testosterone-treated fish and in some cases the scales abutted into the epidermis to form pronounced ridges (Fig. 48). There was however no breakdown of the epidermis as seen in the cortisol - treated fish.

FIGURE 47

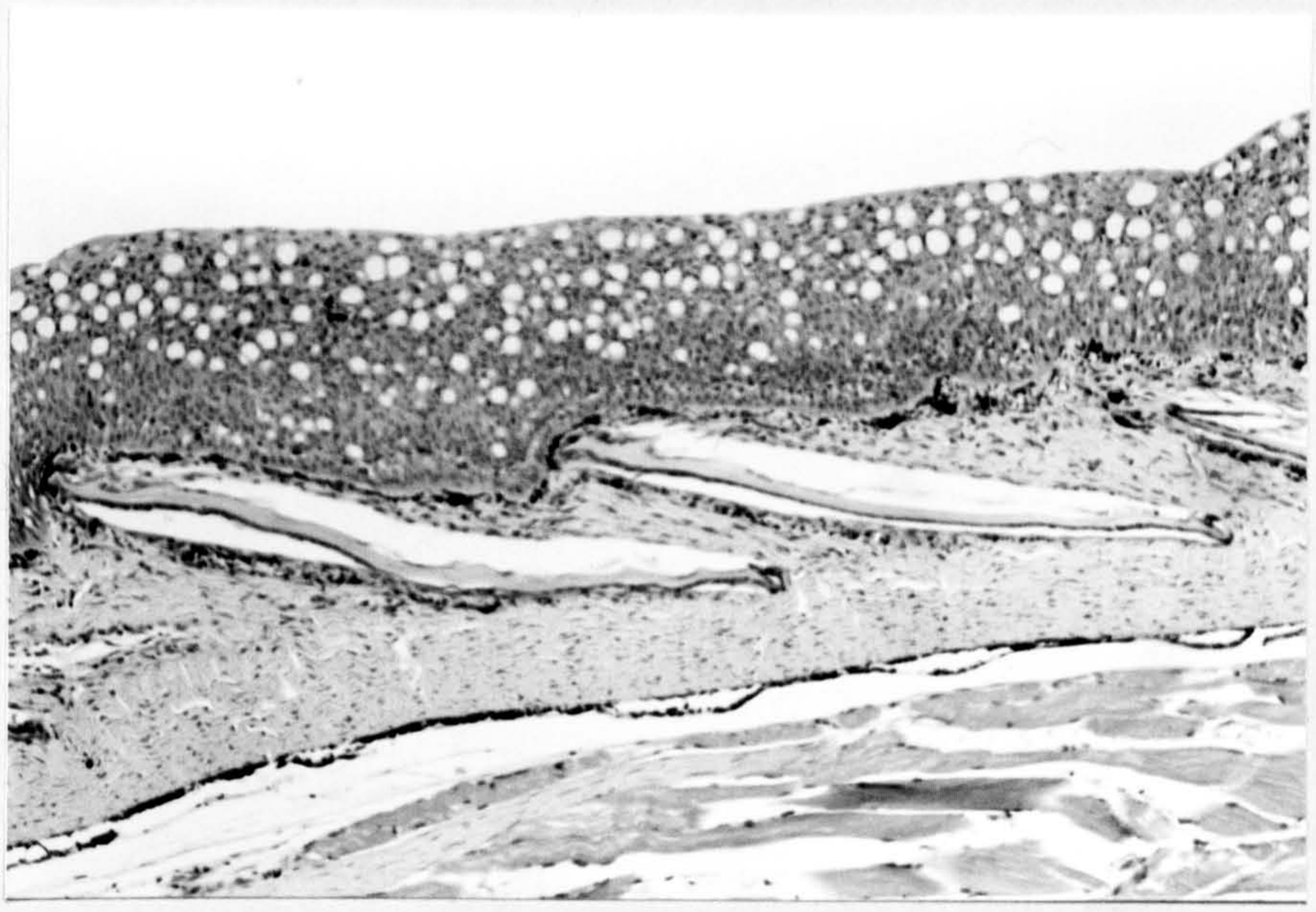
Hyperplasia of epidermis of testosterone-treated rainbow trout. Note also hypertrophy of goblet cells.

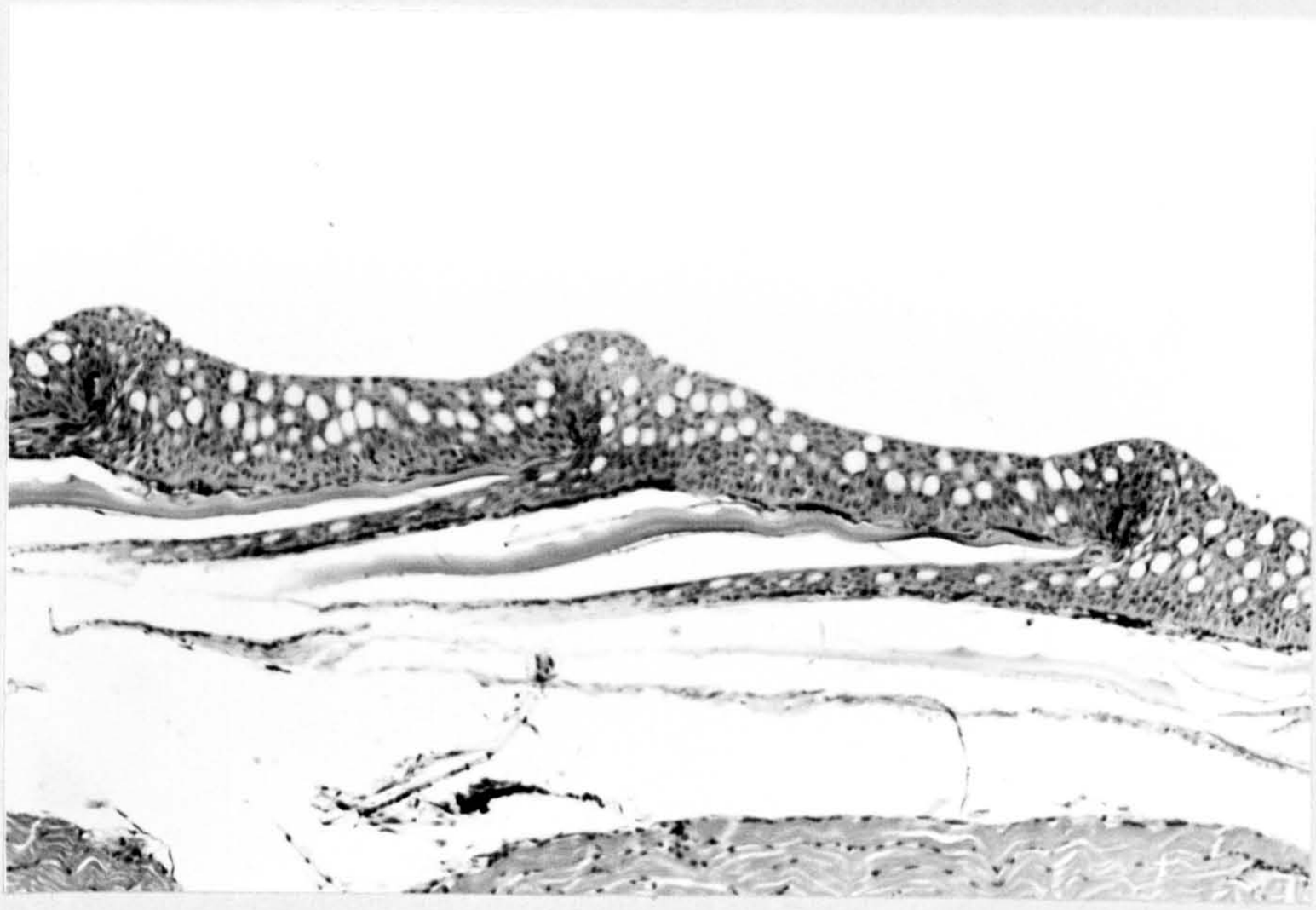
HαE × 125

FIGURE 48

Epidermal ridges in testosterone-treated rainbow trout.

HαE × 125





4.5 DISCUSSION

The trial was clearly successful in promoting heavy Ichthyobodo infestations in the cortisol-treated fish only and not in the testosterone-treated or control fish. The level of Ichthyobodo seen by week 4 of the trial was heavier than any seen throughout this study. It is significant that the Ichthyobodo levels did not increase immediately but took up to three weeks before heavy infestations were seen. This time scale is very similar to that seen in the natural infections in the rainbow trout first feeders as described in Chapter 2. The level of infestation appeared to peak at week 4 and showed a slight decline by week 5. It is unfortunate that the trial had not been planned to go on for longer as it would then have been possible to determine whether Ichthyobodo levels declined completely when, as Robertson et al (1963) have shown, the cortisol levels, using the same type of implant as used in this study, declined after five weeks.

No Ichthyophthirius multifiliis or Saprolegnia infestations were seen on the cortisol-implanted fish as reported by Robertson et al (1963) and Roth (1972) in similar trials. However, Ichthyophthirius multifiliis is not particularly prevalent at Howietoun, particularly at the water temperatures extant during the trial, and fungal infestations have never been a feature of any of the trials carried out during this study. The most prevalent ectoparasites seen in the cortisol-treated fish other than Ichthyobodo were Trichodina sp. and Scyphidia sp.. There have been no suggestions in the literature of any form of protection by salmonids to these parasites and it is therefore surprising that their presence was a feature of the cortisol-treated fish and not

the untreated fish. However, these protozoans are an ubiquitous feature of the parasite fauna at Howietoun and it is likely that they are opportunist parasites which take advantage of stressed fish.

The increase in length and weight of the testosterone-treated fish was generally greatest of all the implanted fish. This is likely to have been caused by the anabolic effect of testosterone which has frequently been reported by other authors (Hirose and Hibiya, 1968; McBride and Fagerlund, 1973; Simpson, Wright and Fraser, 1974; and Yu, Sinnhuber and Hendricks, 1979).

Likewise, the poorest growth was shown by the cortisol-treated fish, and in the later stages the stress effect of the large numbers of Ichthyobodo probably caused anorexia and cachexia of these fish.

The method of presentation of cortisol to the fish was clearly successful, as indicated by the very high levels of cortisol found in the serum after one week. The initial very sharp rise in cortisol levels in the first week is not surprising as much of the cortisol would be on the outside of the pellet. The levels had dropped significantly by week 2, presumably as the implant slowly released the cortisol from inside. The initial levels of the 40 mg implants were higher than those reported by Robertson et al (1963). However, the fish used in this trial were approximately half the size of the fish used by them. Singley and Chavin (1975) considered that a meaningful expression of circulating cortisol levels in teleosts must consider the mass of the animal as they found significantly different levels of cortisol in goldfish of different

weight groups - they were, however, looking at very low levels in unstressed fish.

The range of cortisol levels in both implants remained between 40-100 µg/100 mls for the next three weeks. These levels are slightly higher than some of the levels reported for 17-hydroxy corticosteroids in mature/spawning salmonids, for example sockeye salmon 39.4 µg/100 ml (Schmidt and Idler, 1962), coho salmon, Oncorhyncus Kisutch Walbaum 31.5 µg/100 mls (Robertson and Wexler, 1959) and Atlantic salmon 24.3-58.5 µg/100 mls (Heyl and Carpenter, 1972), but are very similar to the range of 33.8-104 µg/100 ml reported by Robertson, Krupp, Thomas, Favour, Hane and Wexler, (1961), in spawning non-migratory rainbow trout. Even the highest level of cortisol recorded in this study was similar to that reported in spawning Pacific salmon by Hane and Robertson (1959) and Idler, Ronald and Schmidt (1959). Therefore it would appear that the cortisol levels reached in the cortisol-implanted fish could be considered to be physiological rather than pharmacological and the effect that these high levels of cortisol had on the fish would be similar to those that might be expected to occur naturally when salmonids mature.

The cortisol level of the control fish at 1.6-3.3 µg/100 mls was quite low and there was a gradual decrease over the study this is similar to the values of 2.7 µg/100 mls reported by Hanes and Robertson (1959) in immature hatchery-reared rainbow trout and 0.5-5.5 µg/100 mls, also in immature rainbow trout (Rance, Baker and Webley, 1982), and also that of 3.31 µg/100 mls reported in hatchery-reared Atlantic salmon (T. Murphy - personal communication), but lower than that reported in immature European eels, Anguilla

anguilla (10 µg/100 ml) by Lidman, Dave, Johansson-Sjobeck, Larsson and Lewander (1979) and Fundulus heteroclitus L (4.24 + 0.48 µg/100 mls) by Leach and Taylor (1977).

However, Pickering and Christie (1981) reported much lower levels of 0.06 µg/100 mls in one year old hatchery reared brown trout over the period October-December, although they found levels of 0.7 µg/100 mls in two year old brown trout. It would, however, appear to be of little value to compare the resting levels of different species reported by other workers, as the method of sampling (Fagerlund, 1967; Strange, Schreck and Golden, 1977), the size of the fish (Singley and Chavin, 1975) and the time of day when sampling (Singley and Chavin, 1971; Rance, Baker and Webley, 1982) can alter cortisol levels. Indeed, even the act of removing fish from an experimental population is enough to cause elevation of cortisol levels in the remaining fish (Redgate, 1974; Spieler and Noeske, 1979).

The high levels of cortisol reported in mature salmonids is thought to be caused by hyperplasia and hypertrophy of the cells of the interrenal gland (adrenocortical tissue) in the head kidney. However, as well as increased production, Idler and Truscott (1972) have suggested that cortisol levels may be elevated by impaired clearance of the steroid, particularly in the moribund post-spawned Pacific salmon.

The serum samples taken in March eighteen weeks after the end of the trial from 0+ and 1+ rainbow trout, were revealing. They were taken after a period of prolonged low water temperatures (0-3°C) and both sets of fish had elevated levels in comparison with

the control fish at the end of the trial (0+ mean 6.00 µg/100 ml and 1+ mean 5.5 µg/100 mls); in addition the fish with the highest levels of cortisol were also infested with Ichthyobodo and generally the higher the cortisol level the greater the Ichthyobodo infestation. However, it has not been resolved in this study whether Ichthyobodo appeared as a consequence of the period of low temperature and their presence led to an increase in cortisol levels as seen in Saprolegnia-infested fish (Pickering and Christie, 1981; Fagerlund, 1967) and also in Cryptobia and B.K.D.-affected fish (Donaldson, 1981) or whether the low temperatures caused an elevation of cortisol which suppressed the host response to Ichthyobodo and allowed colonisation. This latter version would agree with the limited evidence available which suggests that cortisol levels increase over the winter period as a response to low temperature and poor feeding (Wingfield and Grimm, 1977; Olivereau, 1975; T. Murphy - personal communication; and Mcleay, 1975).

It would appear that the cortisol implantations were abolishing the host's response to Ichthyobodo but which aspect of the host defence system is not immediately clear. The effect of cortisol implants on rainbow trout organs has been described by Robertson et al (1963), who found degenerate changes in most of the organs, including the skin. Most pronounced were degeneration of the pituitary gland, atrophy of adrenocorticoid cells, a marked decrease in the number of lymphocytes in the thymus with complete involution in fish receiving large doses and pronounced reduction of lymphocytes in the spleen. Significantly they also found a significant decrease in lymphocytes in the thymus and spleen of

starved fish. They first noted the loss of lymphocytes from the thymus and spleen two weeks after cortisol implantation (Ichthyobodo appeared after three weeks in this trial). In 1958 Weinreb had shown that there was a decrease in the number of circulating lymphocytes in rainbow trout following hydrocortisone injections. Mcleay (1973) reported that cortisol and dexamethesone injections to juvenile coho salmon caused a marked decrease in the numbers of circulating small lymphocytes but an increase in the number of large lymphocytes. However, he found no change in the number of circulating erythrocytes. In addition Johansson-Sjobeck, Dave, Larsson, Lewander and Lidman (1978) found similar results with cortisol-treated eels and suggested that there was a negative correlation between the number of lymphocytes and the level of circulating free cortisol.

Mammalian studies have shown that adrenocorticosteroid secretions regulate the synthesis and secretion of pituitary ACTH (Yates, 1967; Motta, Fraschini, Piva and Martini, 1968), and affect the number of circulating white blood cell types (Dougherty and White, 1943 and 1945). The lymphocytolytic action of corticosteroids is well recognised in mammalian studies (Dougherty and White, 1945; Dougherty, 1960; and Burton, Storr and Dunn, 1967), and therefore the lymphophenia in fish caused by corticos-teroids was presumed by Mcleay (1973) to be a direct consequence of the lymphocytolytic action of corticosteroids. Lymphocyte dissolution characterised by pyknosis, karyorrhexis or a shedding of the cytoplasm occurs in the lymphoid tissue of mammals following a single injection of adrenocorticotrophic or adrenocortical hormones (Dougherty and White, 1945). The lymphophenia is thought to be caused by interference with protein synthesis in lymphoid

tissue by the cortisol which ultimately results in the dissolution of the lymphocytes. The effect of the cortisol appears to be very specific to lymphocytes as other leucocytes such as neutrophilo granulocytes have been shown to increase with cortisol injections (Johansson-Sjobeck et al, 1978).

The role of the lymphocyte as a central feature in the fish defence mechanism is now well accepted although its precise mode of action is still poorly understood. However, it is known that after the injection of an antigen into rainbow trout at the time of appearance of antibodies in the serum large numbers of cells with the dimension of small lymphocytetes, possessing considerable amounts of cytoplasmic antibody, have been observed in the propnephros of the kidney (Ellis, 1978).

Therefore, by deduction it would seem likely that the cortisol is inhibiting potential antibody formation by the fish host and although this area is still very unclear recent work on mammals by Monjan and Collector (1977) demonstrated that when mice were exposed to a daily circulatory stressor an increase in plasma cortisol was correlated with a decrease in the ability of lymphocytes to lyse target cells.

The possible type of host response to Ichthyobodo is discussed more fully in Chapter 5 but it is interesting to speculate that if antibodies are involved in an immune response to Ichthyobodo their synthesis by lymphocytes would not appear to be local as the presence of lymphocytes in the skin has not been a feature of this study in either this chapter or in Chapter 3. However, Ellis and Wooten (1978) described a massive infiltration of

lymphocytes at the base of the primary lamellae of Ichthyobodo - infested Atlantic salmon in sea water, although they did not describe any changes in the skin.

The histopathological results were interesting in that the high levels of cortisol had very little effect on the skin structure apart from a slight initial thickening with an increase in goblet cell numbers. This is similar to the results obtained by McBride and Van Overbeeke (1971), but different from those of Robertson et al (1963) who described general atrophy of the skin. However, these latter authors did not indicate at what stage the atrophy occurred as breakdown of the skin occurred in this study after the Ichthyobodo infestation. This may have been the case with their study as Ichthyopthirius multifuliis was extant in the later stages of their trial.

Much of the work on the effect of high corticosteroid levels on fish has concentrated on changes in the interrenal gland,gut and pituitary gland rather than skin changes; also much of the work is complicated by the presence of other hormones associated with sexual maturation.

The change in goblet cell shape of cortisol-treated fish seen in this study has not been previously described and may have been caused by a change of the composition of the mucus; although no histochemical studies were carried out it is likely that this would have proved enlightening in order to ascertain if there was any change in the protein content of the mucus.

The skin changes in the cortisol-treated fish after week 3, when Ichthyobodo infestations were present on the fish, were very similar

to those described in Chapter 3 and it seems likely that these were caused by the presence of Ichthyobodo rather than cortisol. Indeed the damage was so great it is likely that the affected fish would have died had the trial been planned to continue for a longer period.

Clearly the cortisol implantation technique is a very successful method for producing Ichthyobodo infestations in fish normally refractive to this parasite, and it is likely that this method would also promote infestations of other parasites such as monogeneans which elicit a host response from fish (Lester, 1972).

The technique will also be invaluable for many immunological techniques where the effect of the host's immune mechanism must be suppressed.

The role of cortisol is becoming increasingly recognised in its involvement in stressed fish and their susceptibility to disease (Wedemeyer, 1970b) and its involvement in many physiological processes, for example; Langhorne and Simpson (1981) noted increased cortisol levels in pre-smolts prior to sea water transfer but found significantly lower cortisol levels in non-smolting parr at the same time of year. The author of this study has noted increased susceptibility of salmon smolts to Ichthyobodo just prior to sea water transfer, whereas parr in the same population are refractory to the parasite.

In summary, there is a clear link between cortisol blood levels and Ichthyobodo infestation on farmed salmonids but much more work is required to elucidate the processes involved.

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Host–parasite interactions between *Ichtyobodo necator* (Henneguy, 1883) and farmed salmonids

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Abstract. The prevalence and intensity of *Ichtyobodo necator* infestations on farmed salmonids was investigated over a 7-month period. The infestations were found to be markedly age dependent. *Ichtyobodo* infestations peaked at 4 weeks after commencement of first feeding and mortalities peaked at 4·6% per week at 8 weeks in the 0+ fish. Both infestations and mortalities showed a marked decline to zero shortly after these periods with no chemotherapy. No *Ichtyobodo* were seen on the 1+ fish until late in the study after a drop in temperature to below 10°C, when they reappeared on the 0+ fish also. *Ichtyobodo* were found at temperatures between 3·5°C and 16°C and in the 0+ fish appeared irrespective of the condition of the fish.

It is suggested that some form of host defence mechanism is operating which limits the *Ichtyobodo* infestations in farmed salmonids.

Introduction

The bodonid flagellate *Ichtyobodo necator* = *Costia necatrix* (Henneguy, 1883) Pinto, 1928, is probably the most damaging protozoan parasite of Scottish salmonid farming. It has been reported to be an important pathogen of fish cultured in the Northern Hemisphere (Bauer 1959; Meyer 1966; Sarig 1966; Gopalkrishnan 1966).

Bauer (1959) claimed that *Ichtyobodo* can be found on practically all freshwater fish and under experimental conditions will establish on salamanders and frog tadpoles. *Ichtyobodo* has also been reported infesting fish in sea water (Penso 1953; Wood 1974). Dale Becker (1977) has reviewed much of the literature on *Ichtyobodo*.

Although there have been many studies of the seasonal fluctuations in abundance of fish parasites, most have been on wild fish or have ignored protozoan parasites, possibly because of their small size and difficulties in observing them in fixed tissue. Migala (1971) described a seasonal study of parasitic protozoa on carp *Cyprinus carpio* L. and found that *Ichtyobodo* appeared on the gills and skin of carp fry during the first two weeks of the study only; however, he gave no explanation for this phenomenon.

The aim of the present study was to observe the changes in levels of *Ichtyobodo* infestations with time on different ages of cultivated salmonids and to try to evaluate the reasons for any fluctuations.

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The method of presentation of testosterone was also successful and did not involve the stress of repeatedly handling the fish to inject testosterone which would probably have elevated cortisol levels and thus defeated the point of the trial. The method, however, did not promote infestation of Ichthyobodo and it is not known if high testosterone levels affected the cortisol levels in the fish as the serum samples of the testosterone-treated fish were only assayed for testosterone.

The levels of testosterone in the serum of the control fish at 0.19-0.23 µg/100 mls are very similar to those reported by Whitehead, Bromage, Breton and Matty (1979) of 0.41 ± 0.04 µg/100 mls and Scott, Bye, Baynes and Springate (1980) 0.2-0.3 µg/100 mls for immature rainbow trout.

Likewise the maximum levels of testosterone in the testosterone-implanted fish (13.65 µg/100 mls) was very similar to that recorded by Scott et al (1980) 10-15 µg/100 mls in maturing male rainbow trout, and even higher levels (21.1 µg/100 mls) have been reported in pre-ovulating female rainbow trout (Scott, Bye and Baynes, 1980). They are also similar to maximum levels reported in plaice 12.1 µg/100 mls, Wingfield and Grimm, 1977) and winter flounder 14 µg/100 mls (Campbell, Walsh and Idler, 1976). Therefore, it can be assumed that the levels of testosterone in the implanted fish were similar to those experienced by naturally maturing rainbow trout. The effect of testosterone on the skin was similar to that described by Yamasaki (1972), McBride and Van Overbeeke (1971) and Richards (1979), with an increase in epidermal thickness and increase in goblet cell numbers and size. The increased thickness of the stratum compactum in this study was interesting

as it was similar to that described in mature male brown trout from Loch Leven (Richards, 1979). However, both Yamasaki (1972) and Richards were unable to reproduce this thickened stratum compactum by injecting fish with androgens, primarily methyl testosterone.

The testosterone implantations did not lead to reduced goblet cell numbers and therefore reduced mucus secretion as described in mature male brown trout (Pickering, 1977) and therefore it is not possible to speculate whether the sexually mature males' susceptibility to ectoparasites infestation (see Chapter 2 and Pickering and Christie, 1980) is caused by the goblet cell depletion. However, it would appear that 11-keto testosterons is the androgen responsible for the reduction in goblet cell numbers seen in mature male brown trout (Richards, 1979) and therefore further work is required with inoculations of this hormone, using the cholesterol implantation technique to ascertain if androgens play a role in promoting Ichthyobodo infestations in mature male fish.

CHAPTER 5

OVERALL DISCUSSION AND CONCLUSIONS

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The previous chapters have considered the importance and effect of Ichthyobodo necator on farmed salmonids in Scotland. The parasite is clearly a very significant pathogen of salmonid fry in the relatively high stocking densities used in fish farming and causes very extensive damage to the epidermis of the host fish. This damage is very severe because the epidermis is the fish's barrier to inflow of the surrounding water and outflow of vital ions and protein. Any breakdown of the epidermis is very serious and it is postulated that fish die due to fatal haemodilution. Unfortunately, the haemodilution effect could not be assessed by testing the osmolality on an osmometer as carried out by Richards (1979) as the fish which were killed by Ichthyobodo were generally less than one gram in weight and serum samples were so small that analysis by conventional techniques was impossible. More work is required in this field, possibly using microosmometry techniques which might be derived from those currently use in invertebrate physiology. It was clear from the study that many of the signs of Ichthyobodo infestation are similar to those seen in fungal infestations of salmonids, i.e. floating mortalities, affected fish seeking out areas of slow water with dorsal and caudal fins protruding from the water. The overall impression was that of heavily infested fish being more buoyant than the surrounding water.

Ichthyobodo necator is one of the few protozoan ectoparasites that cause significant mortalities of farmed fish, the other major pathogen being Ichthyophthirius multifiliis which is primarily

a warmer water pathogen. Although ectoparasites are a normal feature of cultured fish, it is unusual for them to kill the fish per se. The parasites normally appear due to a change in the fish's environment; for example, silt and organic detritus in the water supply often lead to an accumulation of mucus on the gills and skin of cultured salmonids. This forms a substrate for myxobacterial and other infestations on which in turn various opportunist parasites such as Chilodonella sp. and Trichodinids can feed. Members of the Schypidia complex may be very prevalent on fish in suboptimum conditions but are scarcely seen on fish in good condition in fast-flowing water. However, Ichthyobodo necator differs from these parasites in that it can be considered to be a primary pathogen which feeds directly on the epidermal cells of its host, causing the degenerative changes described in Chapter 3, and appears on salmonid fry irrespective of the conditions of the host, water flow or environmental conditions.

Also, unlike most ectoparasitic protozoa the Ichthyobodo infestations showed a marked seasonal periodicity in the late spring and winter period. Several authors have suggested spring and summer blooms of parasites (Bauer, 1959; Rogers, 1969; Meyer, 1970) but in many cases increasing water temperatures and decreasing oxygen levels provide ideal conditions for sudden epidemics of parasitic, bacterial or viral nature. This does not seem to be the case with Ichthyobodo infestations as it seems likely that the parasite's ability to colonise its host is dependent on the host's ability to suppress the infestation, which in this study was greatest in the summer after initial infestation and poorest in the winter following a period of low temperature and sexual maturity. It is interesting to note that Lom (1969) described similar winter

infestations of "layers of protozoa" on fish in ponds after the long months of hibernation in which the fish suffered lack of food and oxygen and decreased quality of water.

The area of host/parasite response in fish is poorly understood and appears to vary with host and parasite (Bauer, 1958; Sniesko, 1969). Whilst several authors have detected antibodies specific to naturally occurring parasites (McVicar and Fletcher, 1970; Molnar and Bercksi, 1965; McArthur, 1978; Harris, 1972; and Cottrell, 1977) the protective properties of these antibodies are unknown. The presence of detectable antibodies specific to parasites in a host does not necessarily imply immunity of the host to that parasite, and conversely Kennedy and Walker (1969) reported a protective immune response by dace, Leuciscus leuciscus, to larvae of the intestinal cestode Carophyllaeus laticeps, but could not demonstrate circulating antibodies. However, all of the previous mentioned work has been carried out on metazoan parasites which are relatively easy to observe, collect and prepare antigenic extract from. Because of their greater bulk and intrahost location it is much more likely that the host will regard their presence as foreign than as that of a single-celled ectoparasite.

Immune responses to parasitic protozoa of poikilothermic vertebrates are even less well understood even if immunity is taken in its broadest sense (Lom, 1969), and research on the immune reaction of poikilotherms to parasitic protozoa lags behind research on zoonotic parasites and those of the economically more important warm-blooded animals. The disappearance of a parasite from its host is not always caused by an immune response by the host to that parasite as various ecological factors may interplay and cause

parasitic infestations to fluctuate. Factors such as temperature, dissolved oxygen levels, pH, feed availability, etc. can lead to an increased degree of infection by changing the rate of the parasites' development, as noted by Wagner (1960) in Ichthyophthirius multifiliis infestations. Climatic conditions may even cause sudden declines in ectoparasite infestations as Dubinin, quoted by Lom (1969), described increased salinity caused by evaporation in summer pools eliminating Ichthyobodo necator and Trichodina infestations from their hosts. Also a degree of immunity may be exhibited in concurrent invasions when one parasite eliminates another. Lom (1969) has quoted Russian literature which claims to have found inverse relationships between the myxosporidians Phyllodostimum and Myxidum lieberkuhni in pike and also a similar relationship between Dactylogyrus and Trichodina, although Noble (1963) disputes this finding, claiming that he found a greater incidence of Trichodina in fish infested with monogeneans, resulting perhaps from tissue damage. This increase in Trichodina infections with monogenean infestations is supported by the findings in Chapter 2. However, none of these factors would seem to explain the disappearance of Ichthyobodo from its host seen in this study.

Allowing for all the non-specific mechanisms which have been suggested there is an increasing amount of evidence of specific immune responses to parasitic protozoa in fish and much of this work has centred on determining the method of resistance to subsequent reinfestation of fish previously infested with Ichthyophthirius multifiliis (Bauer, 1953; Beckert and Allison, 1964; Putz, 1964; and Hines and Spira, 1974), as it is such an important pathogen of cultured fish throughout the world.

There is a great deal of circumstantial evidence available that protective molecules are found in the mucus and serum of the host. Hines and Spira (1974) described immobilisation of Ichthyophthirius multifiliis by mucus washings from carp immune to the parasite and Nigrelli (1935) showed that Epibdella melleni immersed in mucus from fish normally refractory to Epibdella died within five hours whereas parasites immersed in mucus from susceptible hosts survived for 18-24 hours.

Mucus has been shown to contain lysozome (Fletcher and Grant, 1968) a heat-labile component presumed to be complement (Harrell, Etlinger and Hodgins, 1976) as well as immunoglobulins (Fletcher and Grant, 1969; Di Conza and Halliday, 1971; Bradshaw, Richard and Sigel, 1971; and Di Conza, 1971). However, the method of transfer of these molecules to the mucus is still unclear, although Kearn (1976) has suggested the possibility of local synthesis of antibody at the mucus surfaces.

With the accumulating evidence of specific immune responses to parasites and the circumstantial evidence presented in this study, a specific immune response to Ichthyobodo necator by farmed salmonids is the most likely form of host-response, as the parasite infestations have been shown to decline rapidly after initial infestation and to reappear after periods of cold water temperatures on sexually mature and immature fish, both of which have elevated corticosteroid levels (which are known to be immunosuppressive). The parasite also establishes on previously immune fish (but not control fish) which have artificially high levels of cortisol after hydrocortisone implantation. Whether the winter susceptibility to Ichthyobodo is caused by increased corticosteroid levels causing

decreased lymphocyte production, as has been suggested by Mcleay (1975), or purely a diminution of the immune response at low temperatures (Avtalion, 1969; Avtalion et al 1969; Paterson and Fryer, 1974; Fryer, Pitcher, Sanders, Rohovec, Zinn, Groberg and McCoy, 1976; and Avtalion, Wishkovsky and Katz, 1980) is speculative but it is likely to be caused by a combination of those two factors.

Assuming that there is an immune response to Ichthyobodo the antigenic component can only be guessed at; however the autoradiographic work described in Chapter 3 suggested that Ichthyobodo was concentrating the DNA component of the host cells, suggesting enzymic breakdown of the epidermal cells as cells several layers beneath Ichthyobodo infestations were necrotic. It is likely that these enzymes would be antigenic to the host.

Clearly much more work is required to elucidate the type of, or even presence of, an immune response by fish to Ichthyobodo as if this is established it will provide the much needed incentive to produce a commercial vaccine, which would appear to be the safest form of eradication of this parasite from fish farming.

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APPENDICES

APPENDIX 1

1979 - 1980 Ichthyobodo samples on 0+ rainbow trout

Week	Gills		Skin	
	Mean (log n+1)	S.E.	Mean (log n+1)	S.E.
1	0	0	0	0
2	0.177	0.02	0.02	0.01
3	0.3	0.18	0.23	0.13
4	1.6	0.2	0.89	0.15
5	1.26	0.22	0.81	0.12
6	0.98	0.17	0.37	0.13
7	1.54	0.2	0.93	0.12
8	1.55	0.12	0.84	0.12
9	0.65	0.19	0.43	0.14
10	0.76	0.10	0.48	0.26
11	0	0	0.13	0.11
12	0	0	0.02	0.10
13	0	0	0	0
14	0	0	0.06	0.05
15	0	0	0.08	0.08
16	0	0	0	0
17	0	0	0	0
18	0	0	0	0
19	0	0	0	0
20	0	0	0	0

Appendix 1 (cont.)

Mortalities associated with Ichthyobodo 1979 - 1980

Start 10,000 fish

Week	Mortality	%	Cumulative	%
1	81	0.81	81	0.81
2	61	0.614	142	1.42
3	261	2.71	303	3.03
4	936	9.75	1239	12.39
5	374	4.31	1611	16.11
6	190	2.29	1801	18.01
7	79	0.97	1880	18.80
8	99	1.23	1997	19.97
9	279	2.52	2258	22.58
10	234	3.06	2492	24.92
11	80	1.08	2572	25.72
12	49	0.66	2621	26.21
13	7	0.09	2628	26.28
14	1	0.03	2629	26.29
15	3	0.04	2632	26.32
16	2	0.03	2636	26.36
17	0	0.03	2636	26.36
18	1	0.01	2637	26.37
19	3	0.04	2640	26.40
20	2	0.03	2642	26.47

APPENDIX 2

Processing and Staining Procedures

Histological Methods

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
"	1 hour

Appendix 2 (cont.)

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.

Appendix 2 (cont.)

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

APPENDIX 3

Glasgow Modification of Eagle's Medium

AMINO ACIDS	mg/litre
L-Arginine HCl	42.0
L-Cystine	24.0
L-Glutamine	292.0
L-Histidine HCl	19.2
L-Isoleucine	52.4
L-Leucine	52.4
L-Lysine HCl	73.1
L-Methionine	15.0
L-Phenylalanine	33.0
L-Threonine	47.6
L-Tryptophan	8.0
L-Tyrosine	36.2
L-Valine	46.8

VITAMINS	
D-Calcium panthothenate	2.0
Choline Chloride	2.0
Folic Acid	2.0
i-Inositol	3.5
Nicotinamide	2.0
Pyridoxal HCl	2.0
Riboflavin	0.2
Thiamin HCl	2.0

APPENDIX 4

Calculations used in the RIA of testosterone

Percentage binding of standards

Read off DPM in 100 µl of label = X

Read off DPM in assay, this is the DPM in 400 µl

Multiply by 2.25 to give DPM in total volume of dextran/charcoal extract = Y

Therefore, percentage binding = $\frac{Y \times 100}{X}$

APPENDIX 5

Estimation of testosterone in unknowns

Calculate binding as for standards

Read pg/assay off standard curve

Correct for recovery (if known)

Subtract blank

Then pg/assay = pg/20 µl of buffer eluate

Therefore, pg/500 µl of buffer eluate = pg/assay × 25

This represents the amount of testosterone in 20 µl of serum

Therefore, pg/100 µl serum = pg/assay × 125

Therefore, ug/100 ml = $\frac{\text{pg/100 µl}}{1000}$

APPENDIX 6

Calculations used in cortisol radioimmune assay

Standards

To work out percentage recovery.

Read off DPM for 500 µl label from print-out = X

Therefore, DPM in assay = $\frac{X \times 0.2 \times 600}{4000}$ = Y

Percentage recovery = $\frac{\text{DPM in 600 µl}}{Y} \times 100$

To work out the quantity of cortisol put in in the label.

Specific activity of 3H cortisol 232 mc/mg or 232 uc/ug.

1 uc = 2.22×10^6 DPM

So Y DPM = $\frac{1 \times Y}{2.22 \times 10^6}$ uc = Z

If 232 uc = 1ug

Therefore, Z uc = $\frac{1 \times Z}{232}$ = A ng

Standards must be corrected for total amount of cortisol.

Therefore ng put in = ng put in standard + A.

The recovery must be taken into account also.

Therefore, ng put in = $\frac{\text{ng in standard + A} \times \text{Recovery}}{100}$

Percentage binding = $\frac{\text{DPM in total charcoal supernatant} \times 100}{\text{DPM in 600 µl aliquots}}$

Where DPM in total charcoal supernatant = $\frac{\text{DPM in 500 µl} \times 900}{500}$

The standard curve is plotted with corrected ng put in versus percentage binding.

APPENDIX 7

Estimation of cortisol in unknowns

Cortisol implants

The percentage binding is worked out using the formulas described on the previous page.

Using the percentage binding the ng/assay are read off the standard curve.

This figure is then corrected for recovery.

The ng in 500 µl label (A) are then subtracted.

The corrected ng/assay X = the ng in 600 µl.

Therefore in 4 mls of buffer extract there are $X \times \frac{4000}{600}$ ng = Y ng

This corresponds to the ng in 200 µl TCA supernatant.

Therefore in 1 ml TCA extract = Y × 5 ng

This is ng in 10 µls of serum.

Multiply by 10 to give ug/100 mls.

Cholesterol controls and normal controls

Work out percentage binding, then read off ng/assay off curve, correct for recovery and subtract ng in label.

Therefore ng/assay (corrected) in 600 µl = X.

In 2 mls therefore = $\frac{X \times 2000}{600}$ = Y

In 190 µl total therefore = $\frac{Y \times 190}{100}$ ng

This is ng/10 µl of serum.

Multiply by 10 to give ug/100 mls/

Journal of Fish Diseases (1979) **2**, 481–491

Host–parasite interactions between *Ichtyobodo necator* (Henneguy, 1883) and farmed salmonids

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Abstract. The prevalence and intensity of *Ichtyobodo necator* infestations on farmed salmonids was investigated over a 7-month period. The infestations were found to be markedly age dependent. *Ichtyobodo* infestations peaked at 4 weeks after commencement of first feeding and mortalities peaked at 4·6% per week at 8 weeks in the 0+ fish. Both infestations and mortalities showed a marked decline to zero shortly after these periods with no chemotherapy. No *Ichtyobodo* were seen on the 1+ fish until late in the study after a drop in temperature to below 10°C, when they reappeared on the 0+ fish also. *Ichtyobodo* were found at temperatures between 3·5°C and 16°C and in the 0+ fish appeared irrespective of the condition of the fish.

It is suggested that some form of host defence mechanism is operating which limits the *Ichtyobodo* infestations in farmed salmonids.

Introduction

The bodonid flagellate *Ichtyobodo necator* = *Costia necatrix* (Henneguy, 1883) Pinto, 1928, is probably the most damaging protozoan parasite of Scottish salmonid farming. It has been reported to be an important pathogen of fish cultured in the Northern Hemisphere (Bauer 1959; Meyer 1966; Sarig 1966; Gopalkrishnan 1966).

Bauer (1959) claimed that *Ichtyobodo* can be found on practically all freshwater fish and under experimental conditions will establish on salamanders and frog tadpoles. *Ichtyobodo* has also been reported infesting fish in sea water (Penso 1953; Wood 1974). Dale Becker (1977) has reviewed much of the literature on *Ichtyobodo*.

Although there have been many studies of the seasonal fluctuations in abundance of fish parasites, most have been on wild fish or have ignored protozoan parasites, possibly because of their small size and difficulties in observing them in fixed tissue. Migala (1971) described a seasonal study of parasitic protozoa on carp *Cyprinus carpio* L. and found that *Ichtyobodo* appeared on the gills and skin of carp fry during the first two weeks of the study only; however, he gave no explanation for this phenomenon.

The aim of the present study was to observe the changes in levels of *Ichtyobodo* infestations with time on different ages of cultivated salmonids and to try to evaluate the reasons for any fluctuations.

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0140-7775/79/1100-0481 $02.00

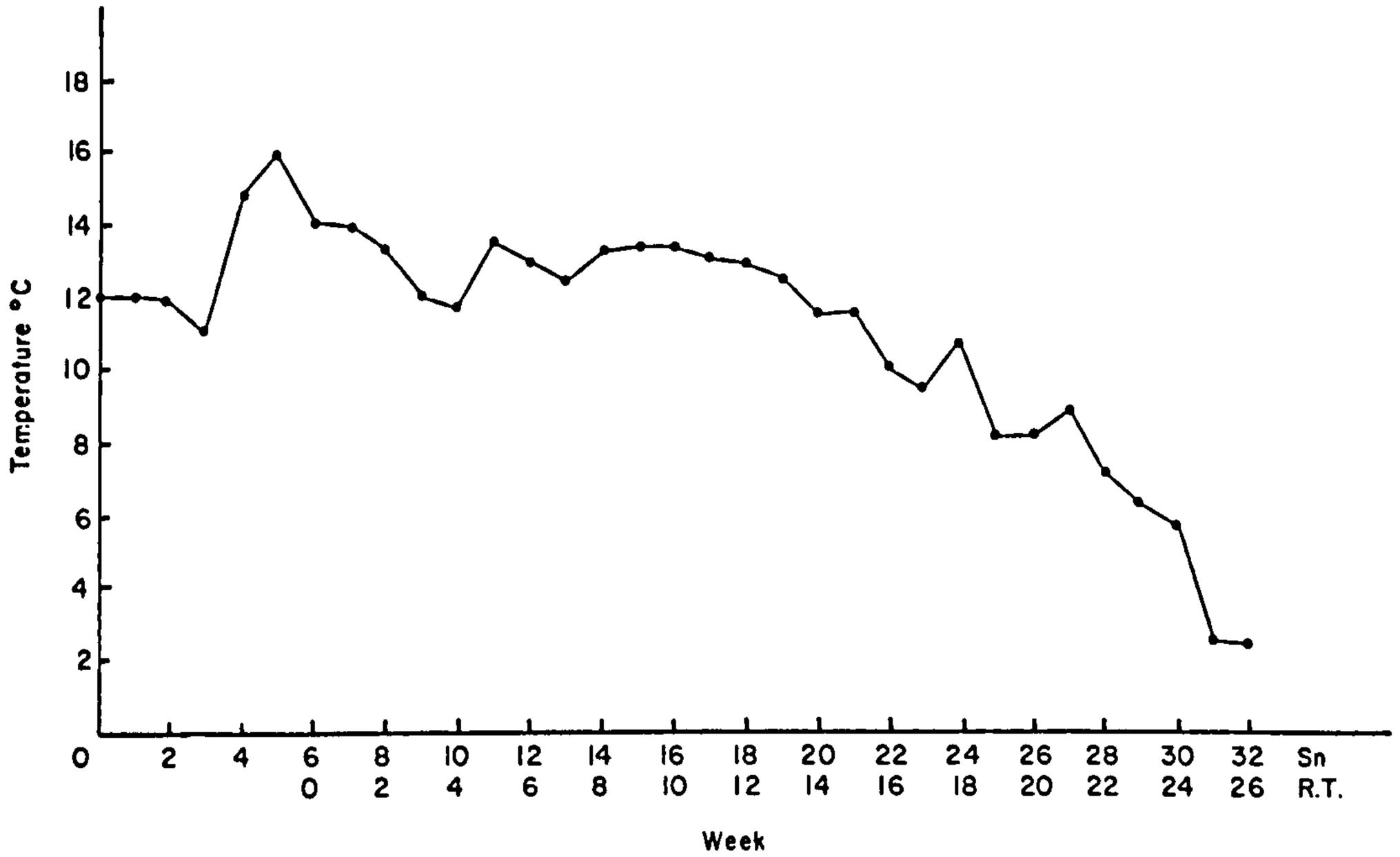

Figure 1. Mean weekly water temperatures over the time period studied. Sn = salmon, RT = rainbow trout.

Materials and methods

0 group salmon *Salmo salar* L., 0 group rainbow trout *Salmo gairdneri* Richardson and 1-year-old rainbow trout were used in this study which lasted from May to December 1978. All experiments were carried out under standard cultivation conditions on a commercial fish farm in central Scotland. The fish were fed on a commercially prepared diet at 5% body weight per day using automatic feeders.

The salmon were held under artificial lighting with a photoperiod simulating the natural light cycle the rainbow trout in natural light. No chemotherapy was administered to the fish during the period of study. Fish mortalities and temperatures were recorded daily and pH and hardness weekly.

Mean weekly temperatures are represented graphically in Fig. 1. During weeks 0–12, the critical times for *Ichtyobodo* infestations in this study, the mean weekly temperature varied only 2°C (12–14°C) for the rainbow trout fry, and 5°C (11–16°C) for the salmon. Temperatures started declining from the end of August (week 11 rainbow trout and 17 salmon) and the mean weekly temperatures dropped below 10°C from the end of October onwards (week 19 rainbow trout and 25 salmon); pH varied between 6·5 and 7·5, the lowest pH being recorded after heavy rain, but generally the variation was between pH 7·4 and 7·5. The pH did not vary outside this range between weeks 0 and 12. Total hardness at 24·5 ppm $CaCO_3$ did not change significantly over the time period studied.

Salmon

Six thousand Atlantic salmon first feeders which had previously been incubated in spring water, were reared from 4 May 1978 onwards in concrete raceway tanks of 0·1 m^3 volume, with a water flow of 9 l/min. The fish were held at an initial stocking density of 1,000 fish per tank. The stocking density was halved at the end of first feeding when the fish averaged 1 g in weight. Twenty to thirty fish were sampled weekly for the first 10 weeks and then every other week thereafter, for the presence of *Ichtyobodo* on the gills. The fish were decapitated, measured and weighed and four gill arches were removed and mounted in water under a cover slip. The slides were left for 10 min to standardize counts and then the number of *Ichtyobodo* on the four gill arches was counted.

Rainbow trout

This trial was started on 14 June 1978, 6 weeks after the salmon. First feeding rainbow trout and 1-year-old rainbow trout were compared. Twenty thousand rainbow trout first feeders which had previously been reared in spring water and 2,000 1-year-old rainbow trout, measuring 12 cm, previously held in burn water on the same fish farm, and which had presumably experienced *Ichtyobodo* before, were used. The 1-year-old fish were treated with 1:5000 formalin before the trial commenced to eliminate any parasites.

The 0+ fish were held in fibreglass 2 m^2 tanks at an initial stocking density of 20,000 fish per tank with a water level of 20 cm and flow of 36 l/min. The population was split into two tanks with 7,500 in each when the fish averaged 1 g in weight (16 August = 65 days). The fish were further split into populations of 3,000 per tank on 18 September (98 days).

The 1+ fish were held in identical tanks at the same flow rates at an initial stocking density of 1,000 fish per tank. The stocking density was halved on 18 September 1978 (98 days). Ten to twenty 0+ fish were sampled weekly until week 13 and then sampled every other week. Ten 1+ fish were sampled weekly for the first month and then bi-weekly or monthly for the rest of the study.

The method of sampling was as for the salmon but in this case the skin was sampled also. Tavolga & Nigrelli (1947) suggested that the highest concentration of *Ichtyobodo* occurs around the dorsal fin. Therefore, a scrape of 1 cm was made at the base of the dorsal fin with a scalpel. This was then smeared thinly on to a slide mounted in water and a 22 × 22 mm cover slip placed above the smear. The slide was examined after 10 min.

Twenty different fields of 2 mm^2 were examined and the number of *Ichtyobodo* in each counted. Counts of 20 and above were recorded as 20. The mean for each fish was recorded and after examination of all the fish a mean for the whole sample could be obtained.

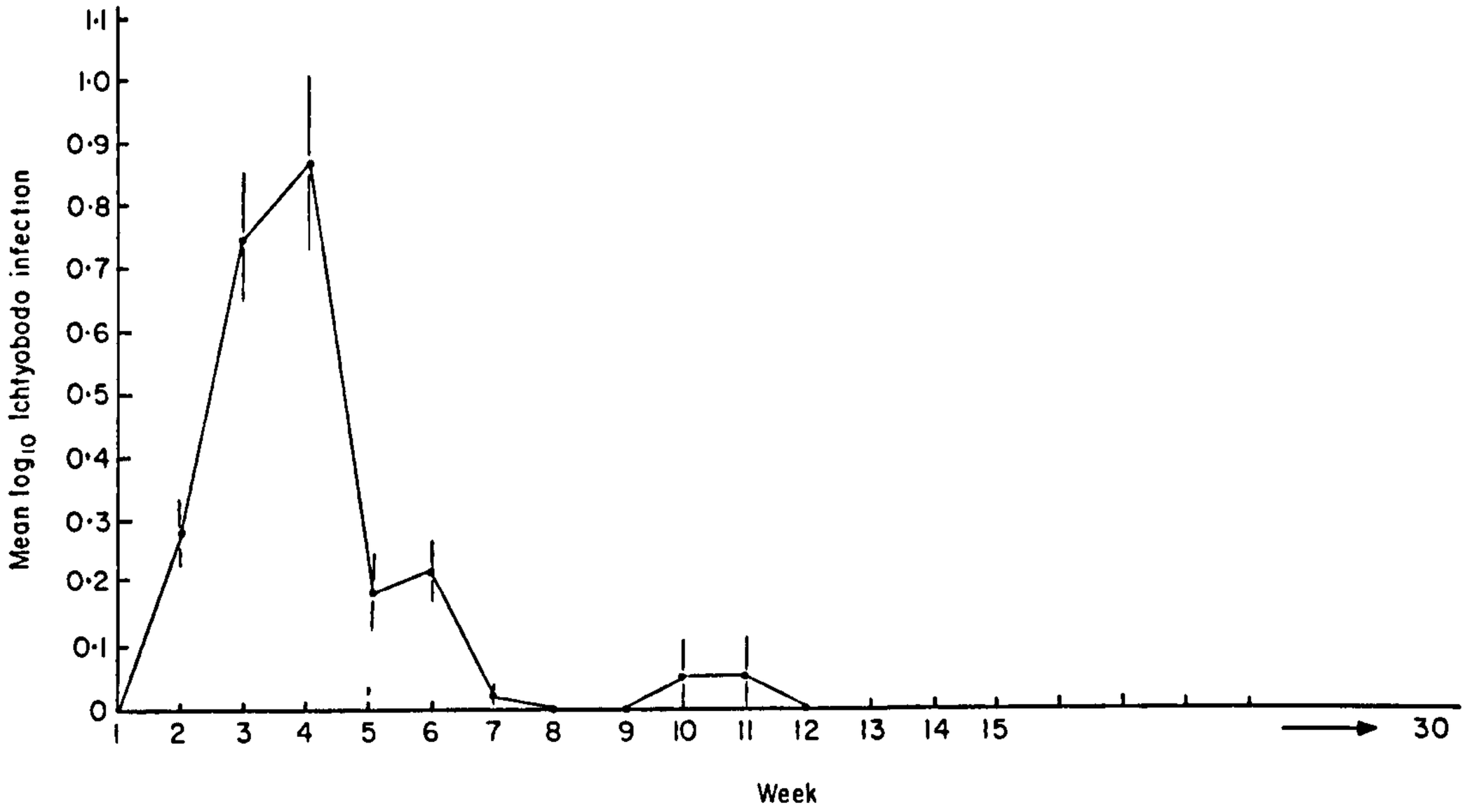

Figure 2. Intensity of *Ichtyobodo* infestations on 0+ salmon gills. (Vertical bars = $\bar{x} \pm$ s.e.)

Results

A logarithmic transformation of log $(n+1)$ for all intensity data was used as this reduced the dependence of the variance on the mean. The resulting data was *t*-tested for significance.

Prevalence data was tested using a Fisher exact test on a two by two contingency table.

Salmon first feeders

From Fig. 2 it can be seen that *Ichtyobodo* levels increased weekly to a peak 4 weeks after commencement of first feeding. The numbers of *Ichtyobodo* declined very sharply between weeks 4 and 5 and from week 6 onwards declined until by week 12 no *Ichtyobodo* were seen on the gills. No *Ichtyobodo* were seen on the gills of these fish for the remainder of the study.

The prevalence of *Ichtyobodo* infestations is shown in Fig. 6a. The prevalence peaked at week 3 when 93% of the fish examined were infested with *Ichtyobodo*. Between weeks 4 and 5 the prevalence declined significantly ($P<0{\cdot}001$, 1 d.f.) to 24% infection. By week 12 the prevalence had declined to zero.

No accurate quantitative data of weekly mortality is available for the salmon fry; however, the mortalities peaked at an estimated 7·6% per week by week 7 and by week 13 this had dropped to 0·2% per week.

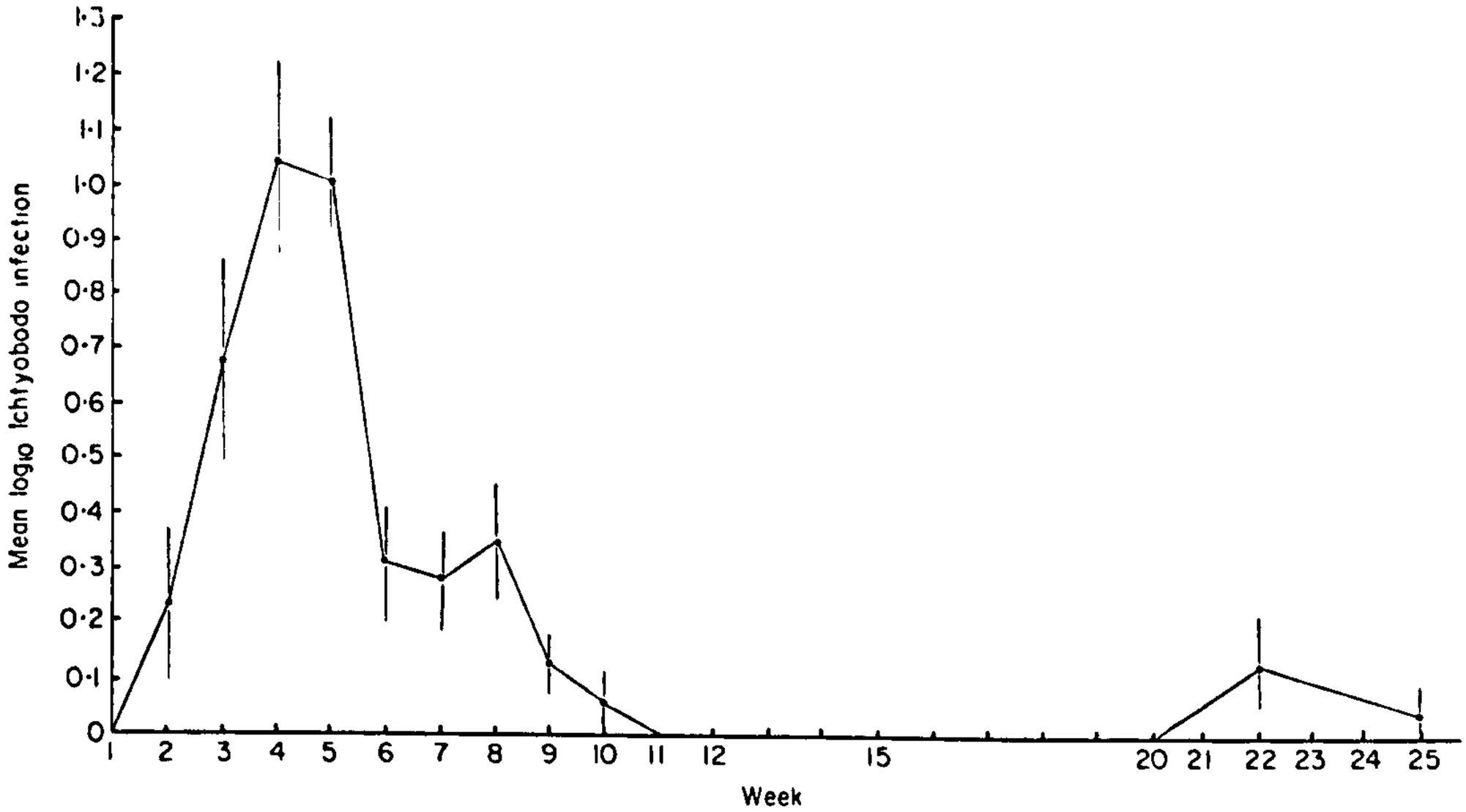

Figure 3. Intensity of *Ichtyobodo* infestations on 0+ rainbow trout gills.

Rainbow trout fry

From Fig. 3 it can be seen that there were significant weekly increases in the number of *Ichtyobodo* on the gills to week 4. The intensity of infestation then declined sharply between weeks 5 and 7. Between weeks 7 and 8 there was an anomalous insignificant increase ($t = 0{\cdot}46$, $P = 0{\cdot}65$, d.f. $= 29$). The intensity of *Ichtyobodo* infestation then declined to zero by week 11. No *Ichtyobodo* were seen on the gills for the next 11 weeks, then in weeks 22 and 25 small numbers were encountered again.

The pattern of infestation on the skin was similar to that on the gills (see Fig. 4); however, no *Ichtyobodo* were recorded until week 3, 1 week later than on the gills.

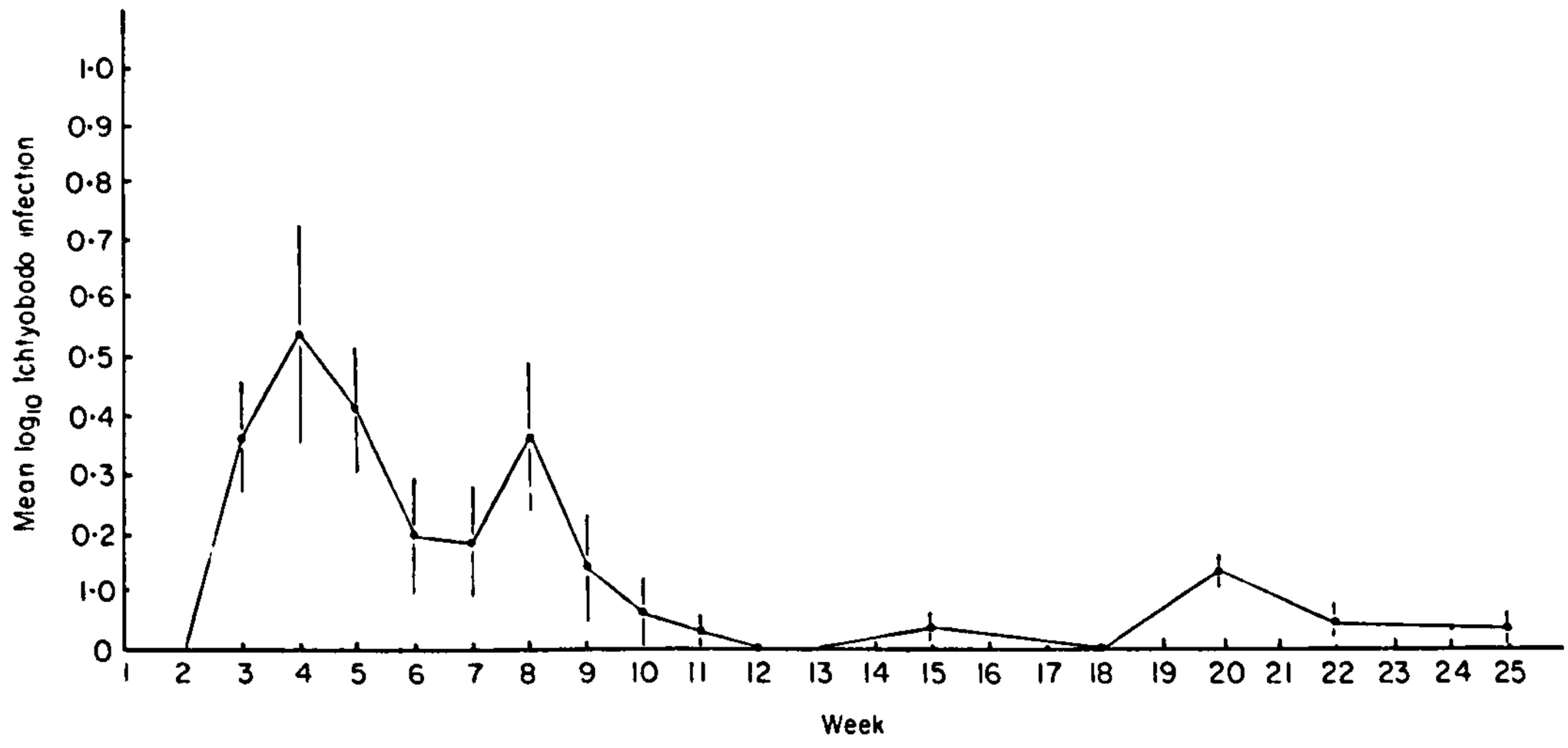

Figure 4. Intensity of *Ichtyobodo* infestations on 0+ rainbow trout skin.

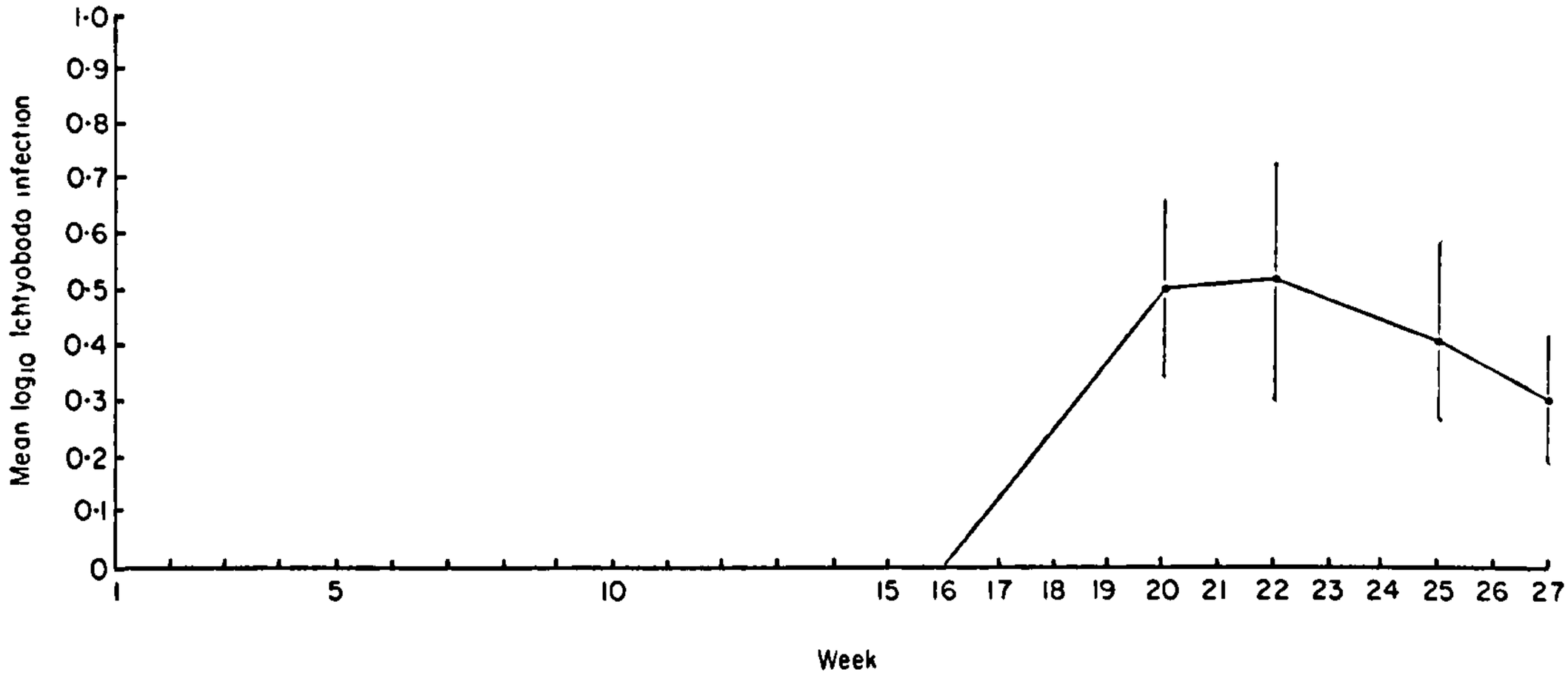

Figure 5. Intensity of *Ichtyobodo* infestations on 1+ rainbow trout skin.

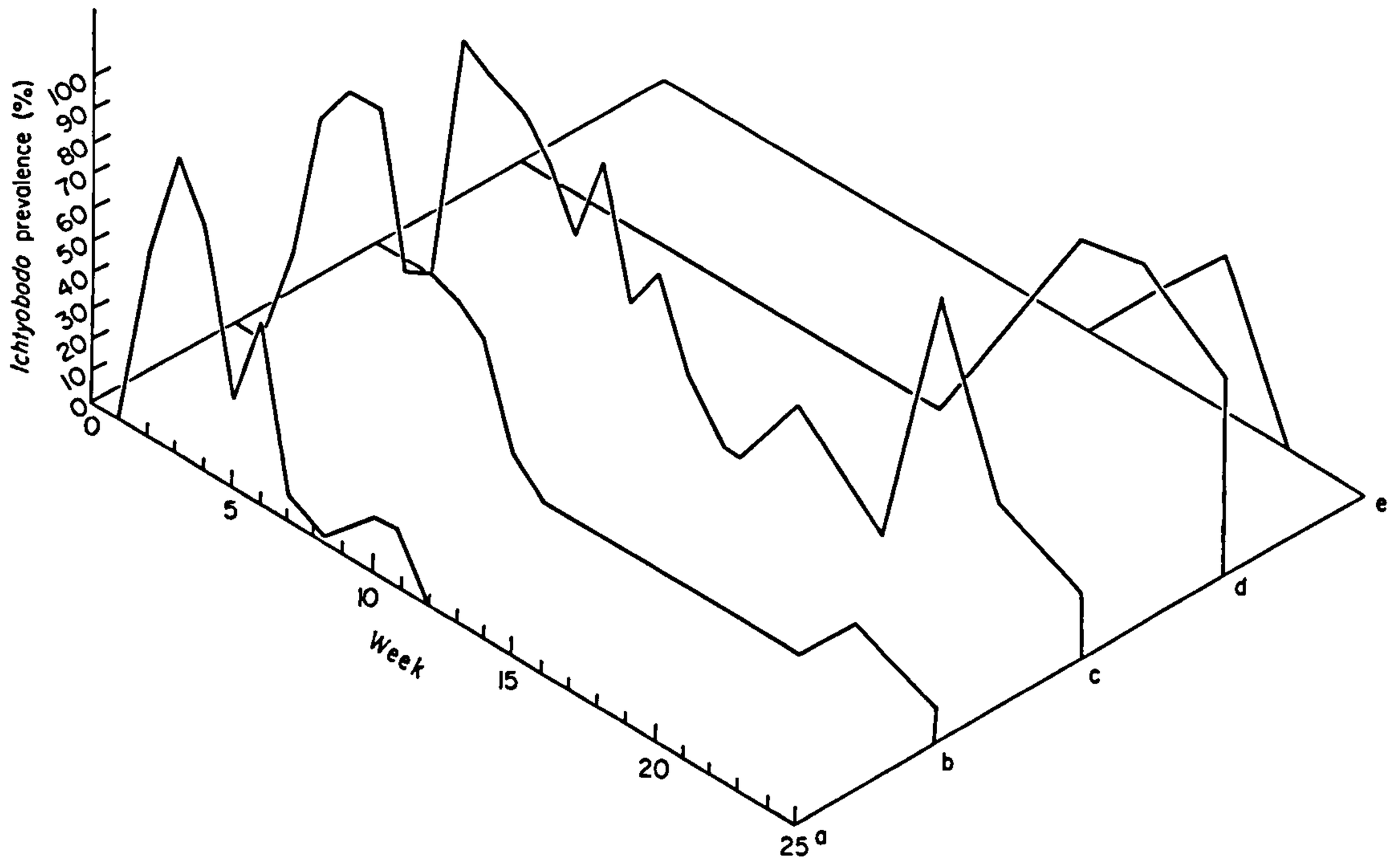

Figure 6. Isometric diagram of weekly percentage prevalence of *Ichtyobodo* infestation on: (a) 0+ salmon gills; (b) 0+ rainbow trout gills; (c) 0+ rainbow trout skin; (d) 1+ rainbow trout skin; (e) 1+ rainbow trout gills.

The intensity of infestation reached a peak at week 4 and then numbers declined significantly between weeks 4 and 7. As in the gills, there was an insignificant increase in the intensity of infestation between weeks 7 and 8 ($t = 1{\cdot}21$, $P = 0{\cdot}236$, d.f. = 29). The intensity of infestation declined thereafter until, by week 13, no *Ichtyobodo* were recorded on the skin. Between weeks 13 and 20 there were minor fluctuations in *Ichtyobodo* numbers on the skin. On week 20 there was a relatively large increase in numbers of *Ichtyobodo* present on the skin; the intensity of infestation did not change significantly for the next 5 weeks.

Although the skin infestation seemed more prolonged there was a good overall correlation between skin and gill infestations ($r = 0{\cdot}580$, $P<0{\cdot}001$).

The prevalence data for skin and gill is shown in Fig. 6b and c. The prevalence of *Ichtyobodo* infestation on the gills rose rapidly from the start of the trial to weeks 4 and 5 when 90% of the fish examined were infested with *Ichtyobodo*. Between weeks 5 and 6 there was a highly significant decrease in prevalence of *Ichtyobodo* on the gills ($P<0{\cdot}01$, 1 d.f.). The prevalence then declined smoothly to zero by week 11. There was a similar pattern of infestation on the skin peaking at week 3 with 78% of the fish infested. The decline was not as smooth as on the gills with insignificant increases between weeks 7 and 8 and 9 and 10. The infestation had dropped to zero by week 13 and the prevalence until week 20 was low. There was a marked increase to 80% infection at week 20; this had dropped to 30% by week 22 and remained at this level until the end of the study.

The weekly mortality of the rainbow trout fry is shown in Fig. 7. Mortalities had risen to nearly 1% per week by week 4. By week 5 there was a very sharp increase in mortalities to 3·4% per week. By week 8 the mortalities had peaked at 4·6% of the surviving population dying in a week (this represented 784 fish). After week 8 the mortalities declined rapidly to 1·5% by week 12. By week 15 the mortalities had returned to normal fish farm levels at one or two fish per tank per day. Between weeks 1 and 15, 24·7% of the original stock died. In the next 13 weeks only 1·8% of the remaining stock died. The small peak at week 16 probably represents losses due to handling when reducing stocking density.

The only apparent cause of death in both the salmon fry and rainbow trout fry was the *Ichtyobodo* infestations. Clinical signs observed included fish floating belly upwards, erratic swimming, some erosion around the dorsal fin area and frayed fins. The fish which died were generally in good condition and did not show the characteristic pinhead appearance of starved fish which had not come on to feed properly. Histopathological studies confirmed the absence of any other aetiological cause of death.

Rainbow trout 1+

No *Ichtyobodo* were encountered on the gills or skin of these fish until week 20 when moderately heavy infestations were seen on the skin of 80% of the fish examined; intensity and prevalence remained high until the end of the study (see Figs 5 & 6d).

c

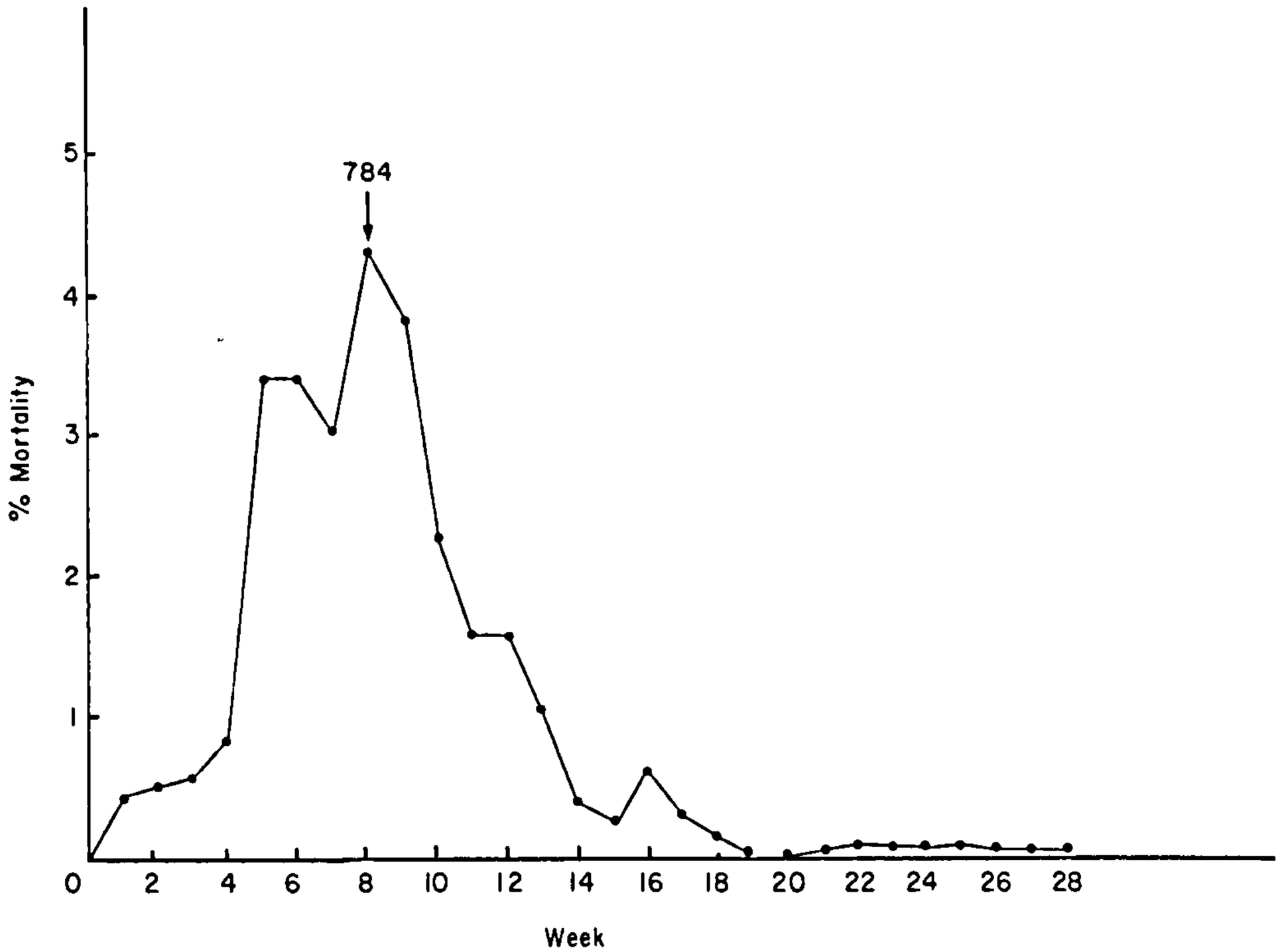

Figure 7. Weekly percentage mortalities of 0+ rainbow trout caused by *Ichtyobodo*.

Likewise, in week 20 44% of the fish had small numbers of *Ichtyobodo* on the gills (Fig. 6e); no *Ichtyobodo* were seen on the gills after this.

The mortality pattern of these fish was very different from that of the 0+ fish as on average only one fish per week died in each tank, even during the later stages of the trial when relatively large numbers of *Ichtyobodo* were seen on the skin of these fish.

Condition of fish and Ichtyobodo *infections*

The presence or lack of a relationship between the condition of the 0+ fish (as expressed by condition factor W/L3 × 100) and the intensity of *Ichtyobodo* infestation was tested. No correlation was found between condition of the fish and intensity of infestation on the gills of 0+ salmon ($r = 0{\cdot}016$, $P = 0{\cdot}41$), 0+ rainbow trout ($r = 0{\cdot}01$, $P = 0{\cdot}383$) and skin of 0+ rainbow trout ($r = 0{\cdot}05$, $P = 0{\cdot}248$).

Other parasites

Apart from *Ichtyobodo* the following ectoparasites were observed in low numbers throughout the trial on the 0+ fish. One *Gyrodactylus* sp, and the following protozoa, *Bodo* sp., *Chilodonella* sp., *Ichthyophthirius multifiliis*, *Trichophrya piscium*, *Trichodina* sp. and the following members of the *Scyphidia* complex: *Scyphidia*, *Glossatella*, *and Epistylis*.

It is of interest to note that the 1+ fish had similar light infestations of the above parasites but no *Ichtyobodo* until the late stages of the trial.

Discussion

The present investigation has clearly shown that costiasis, the disease caused by *Ichtyobodo necator*, when left untreated, can be a serious pathogen of first feeding salmonids causing up to 25% mortality.

The *Ichtyobodo* infestations showed a marked dependency on the age of the host as the 1-year-old rainbow trout examined did not show the same pattern of infestation or mortality as the first feeding fry. This phenomenon has been described before in carp by Lyamain (in Bauer 1959) who noted the consistently lighter infestations of *Ichtyobodo* on older carp. Wood (1974) also noted the parasites' preference for attacking fry and young fingerlings. The author has observed that this phenomenon occurs in other salmonid farms in Scotland and the north of England also.

The results obtained show a clearly defined periodicity displayed by *Ichtyobodo* infestations on first feeding salmonid fry, with a rapid increase in parasite numbers to a peak at 4 weeks, followed by a decline to insignificant numbers after 11 and 13 weeks on the gills and skin respectively, and virtual absence thereafter.

The reasons for the decline in numbers of *Ichtyobodo* is at present unknown but it would appear that there is either some skin or gill change which prevents the parasite colonizing or some form of immunity develops. Franke (1908) observed the 'mysterious disappearance' of *Ichtyobodo* on *Salvelinus fontinalis* (Mitchill), whilst Tavolga & Nigrelli (1947) found that aquarium fish developed relative immunity to superinfestation of *Ichtyobodo* and suggested that immunity developed through premunition. Lyamain (in Bauer 1959) suggested a form of age immunity to *Ichtyobodo* in older carp. The concept of immunity to parasitic protozoa in fish is not new and there have been an increasing number of reports in the literature recently (Hines & Spira 1974; Bower & Woo 1977; Nagel & Summerfelt 1977). The appearance of *Ichtyobodo* on the 1+ rainbow trout and re-appearance on the 0+ rainbow trout after a decrease in temperature may be related to the effect of low temperature in depressing the immune response (Avtalion, Wojdani, Malik, Shahrabini & Duczyminer 1973). The high prevalence and intensity of *Ichtyobodo* on the 1+ rainbow trout from week 20 onwards may also be explained by the fact that many of these fish had started to mature. It is known that as fish mature there is an increase in circulating corticosteroids (Hane & Robertson 1959); these compounds are known to be immunosuppressants in mammals (Monjan & Collector 1977).

Previous authors, i.e. Amlacher (1970) and Bauer (1958), have suggested that *Ichtyobodo* is a parasite of debilitated fish in poor condition; whilst this is probably true of older fish, broodstock and kelts, the results presented here suggest that this is not the case in first feeding salmonids. There was found to be no correlation between condition of fish and levels of *Ichtyobodo* on skin and gills of the 600 fish examined; fish in good condition were just as likely to be severely infested as fish in poor con-

dition. If there is an immune response to *Ichtyobodo* it is possible that debilitated fish are immunosuppressed and therefore cannot resist the *Ichtyobodo*.

The pattern of infestation on the skin and gills of the rainbow trout fry appeared to be slightly different. Although there was a good overall correlation, the infestation on the skin started later and declined more slowly. There seemed to be three phases to the *Ichtyobodo* infestations on the fry: (1) infestations of gills, (2) infestation of gills and skin, and (3) infestation of skin only. When *Ichtyobodo* was seen in very large numbers on the skin of the 1+ rainbow trout very few were seen on the gills.

The mortality pattern was certainly correlated to the infestation of *Ichtyobodo* although there was some hysteresis between peak of parasite infestation and peak mortality. The infestation on the skin seems to be of equal importance as the gill infestations and thus it cannot be assumed, as Savage (1935) did, that the cause of death is by respiratory failure due to the parasite on the gills only. *Ichtyobodo* causes severe necrosis of the epidermal cells and the cytoplasmic content of those that survive appears to be greatly reduced (R. J. Roberts, personal communication). This epidermal damage is sufficiently severe to postulate that the fish dies because of osmoregulatory breakdown, followed by haemodilution and circulatory failure, as in fungal infections (Richards & Pickering 1979).

The data obtained on environmental variables showed that *Ichtyobodo* infestations occurred at temperatures between 3·5°C and 16°C. This conforms with other findings that *Ichtyobodo* is not temperature specific (Benisch 1936; Tavolga & Nigrelli 1947; Hlond 1963). As the pH did not change significantly throughout the period of study it is unlikely that this was responsible for any effect on the level of *Ichtyobodo* infestation.

It would appear that the effect of environmental variables and starvation are of secondary importance to the host's state of susceptiblility and immunocompetence in limiting *Ichtyobodo* infestations.

Further work is now being carried out to elucidate the mechanisms involved in the form of host resistance that appears to take place in *Ichtyobodo* infestations of farmed salmonids.

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Pathogenesis and autoradiographic studies of the epidermis of salmonids infested with *Ichtyobodo necator* (Henneguy, 1883)

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Abstract. The sequential pathology of *Ichtyobodo necator* infestations of the skin of 0+ and 1+ salmon and rainbow trout was studied. Areas of greatest shelter from water currents were found to be most commonly infested and no parasites were found attached to the epidermis on the head of the fish. The parasite caused hyperplasia of the malphigian cells and exhaustion of the goblet cells below infestations, followed by spongiosis of the underlying epidermis. The epidermal plaque then sloughed off leaving a single layer of cells attached to the basement membrane. Cell kinetic studies showed that *I. necator* caused the cells immediately below infestations to divide, a markedly different pattern from that of normal teleost epidermal cell proliferation. The possibility that the parasite secretes some form of digestive enzyme is postulated. In areas where sloughing had occurred, the remaining malphigian cells were seen to be in the process of division.

Introduction

The bodonid flagellate *Ichtyobodo necator* (=*Costia necatrix*) (Henneguy, 1883) is an ubiquitous and significant protozoan ectoparasite of farmed freshwater fish. Robertson (1979) has described its seasonal population cycle and significance in salmonid culture on a Scottish fish farm while Becker (1977) has reviewed much of the earlier literature on the taxonomy and host range of *I. necator*. It is, however, surprising that for such a common and damaging parasite, the pathology of the condition caused by infestation, which is known as costiasis, and its pathological effects on the host's skin have remained poorly documented. Apart from a brief mention in early papers by Fish (1944) and Tavolga & Nigrelli (1947), virtually all of the published information on *I. necator* has been motivated towards clarifying its taxonomic status, which was not finally determined until the electron microscope studies of Joyon & Lom (1969). Ellis & Wootten (1978) briefly described the pathology of *I. necator* infestations on the gills of Atlantic salmon, *Salmo salar* L., smolts in sea water, but did not describe the effect of the parasite on the skin.

The work of Robertson (1979) has indicated that the infestation of the skin by *I. necator* is as important as gill infestation in causing mortality in juvenile farmed

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salmonids. Thus, it was decided to attempt to assess the effect of the parasite on the skin of salmonids of varying ages under controlled conditions, to study the cell kinetics of the infested epidermis and to compare them to those observed in normal and wounded epidermis of other teleost species (Bullock, Marks & Roberts 1978a,b; Bullock & Roberts 1980).

Materials and methods

Fish

Sequential pathology of costiasis in salmon alevins. One hundred Atlantic salmon alevins, previously held in spring water with no prior exposure to *I. necator*, were held in recirculating fresh water at 12°C; 50 0+ rainbow trout, *Salmo gairdneri* Richardson, heavily infested with *I. necator*, were placed in the same tank. Five salmon were sampled daily for 14 days and fixed in phosphate buffered formalin and processed for paraffin wax sections at 5 μm. The sections were then stained with haematoxylin and eosin (H&E) and alcian blue (pH 2·5).

Pathology of costiasis in salmon smolts. Thirty salmon smolts were treated with 200 ppm formalin 2 weeks prior to commencement of the trial to eliminate any ectoparasites. The fish were checked to confirm the absence of any parasites and then freeze branded and placed in an aquarium containing recirculating water at 12°C. Ten severely stressed smolts, heavily infested with *I. necator* were placed in the water. Two of the branded smolts were killed and sampled daily for the next 14 days and blocks of skin and gills processed as above.

Autoradiography

In vivo *study.* Because of the unavailability of salmon alevins at the time of year that this part of the study commenced, rainbow trout alevins heavily infested with *I. necator* were used. Fifty heavily infested alevins were anaesthetized with iced water (Mittal & Whitear 1978) and then injected intraperitoneally with tritiated thymidine to give a final dosage of 20 μm Ci/g body weight. The fish were then allowed to recover and placed in an aquarium containing recirculating fresh water at 12°C.

Five fish were sampled at intervals of 16 h, 40 h, 3, 4, 5, 6, 7 and 8 days, respectively. The fish were fixed in phosphate buffered formalin and processed for paraffin wax sectioning at 5 μm. Sections were dipped in Ilford K2 emulsion and exposed in the dark for 60 days at 4°C. The sections were then processed in Kodak D76 developer and subsequently stained in haematoxylin and eosin. Estimates of labelling were obtained from examination of the sections under oil immersion at × 100 magnification. Nuclear labelling of more than 6 grains per nucleus over background was required before a positive result was recorded.

In vitro *study.* Because of the unsuitability of the *in vivo* technique for large fish,

an *in vitro* method was used to study the epidermal changes in the smolts which had been severely stressed.

Two of the smolts maintained at 12°C and heavily infested with *I. necator* were killed and blocks of skin and muscle were dissected from the fish and placed in sterile petri dishes containing 10 ml of Eagle's (Glasgow modification) tissue culture medium (Paul 1975) and 0·2 ml of tritiated thymidine (specific activity of 15–30 Ci/mmol) was added to each dish and incubated at 12°C. Blocks of skin and muscle were removed from the media at 12, 24 and 48 h and fixed for histology and autoradiography as described above.

Results

Salmon alevin pathology

Within 3 days of exposure to the infested rainbow trout, *I. necator* were seen attached to the epidermis of the alevins; large numbers were not seen until day 7, but by day 12 all fish sampled showed moderately heavy *I. necator* infestations.

The areas of skin infestations showed a focal non-random distribution, several discrete areas being invariably infested. These foci were the cuff of skin sheltered by the operculum, the pectoral and pelvic fins and the area of skin subjacent to the dorsal fin. Because of the method of sectioning, infestations of the gills were difficult to ascertain; however, when the alignment was correct, moderate numbers of *I. necator* were seen attached to the secondary lamellae of the gills and also to the

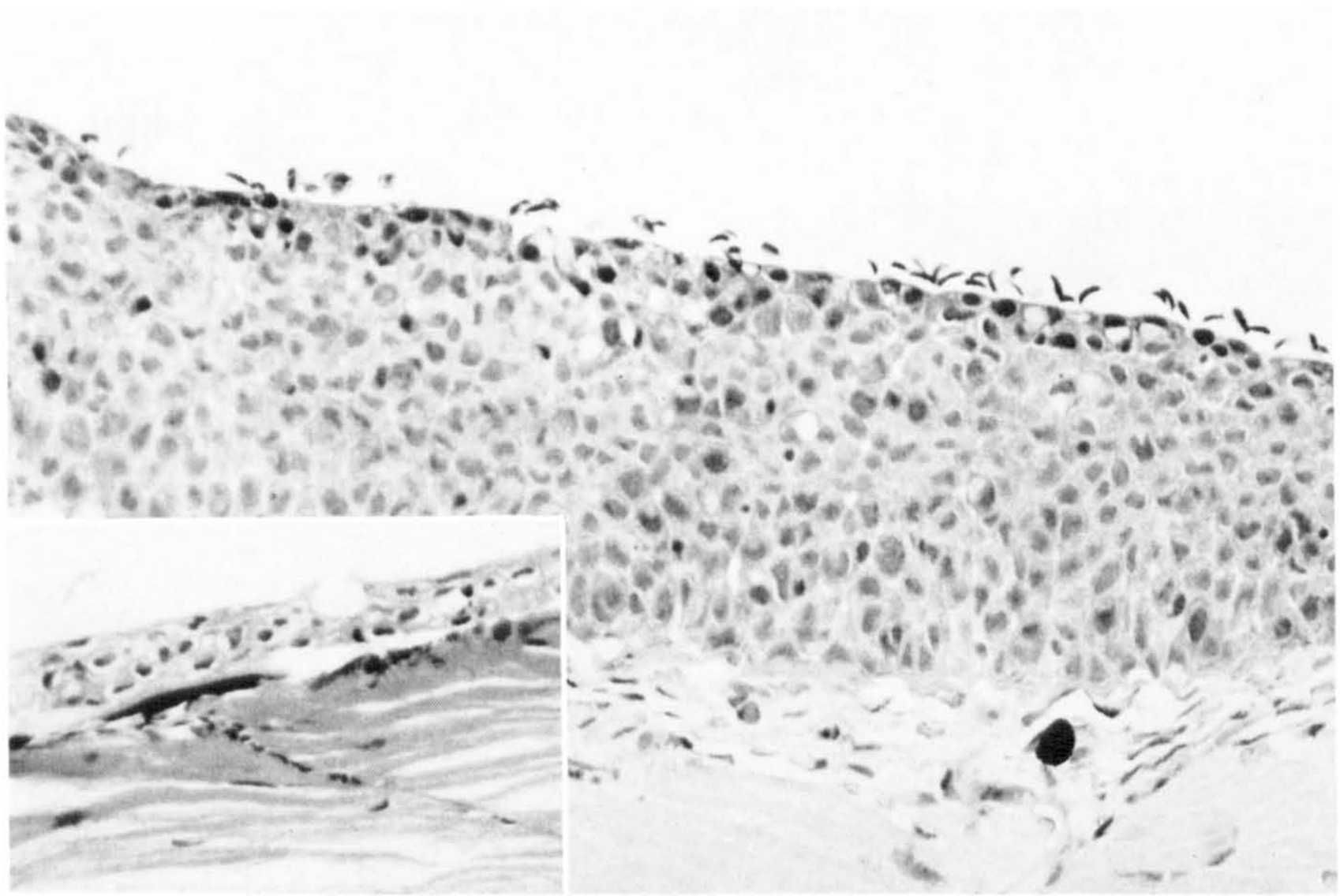

Figure 1. Hyperplastic epidermis with *I. necator* attached to peripheral cells. Note the lack of goblet cells. Inset shows normal uninfested epidermis from the same fish (H&E, × 410).

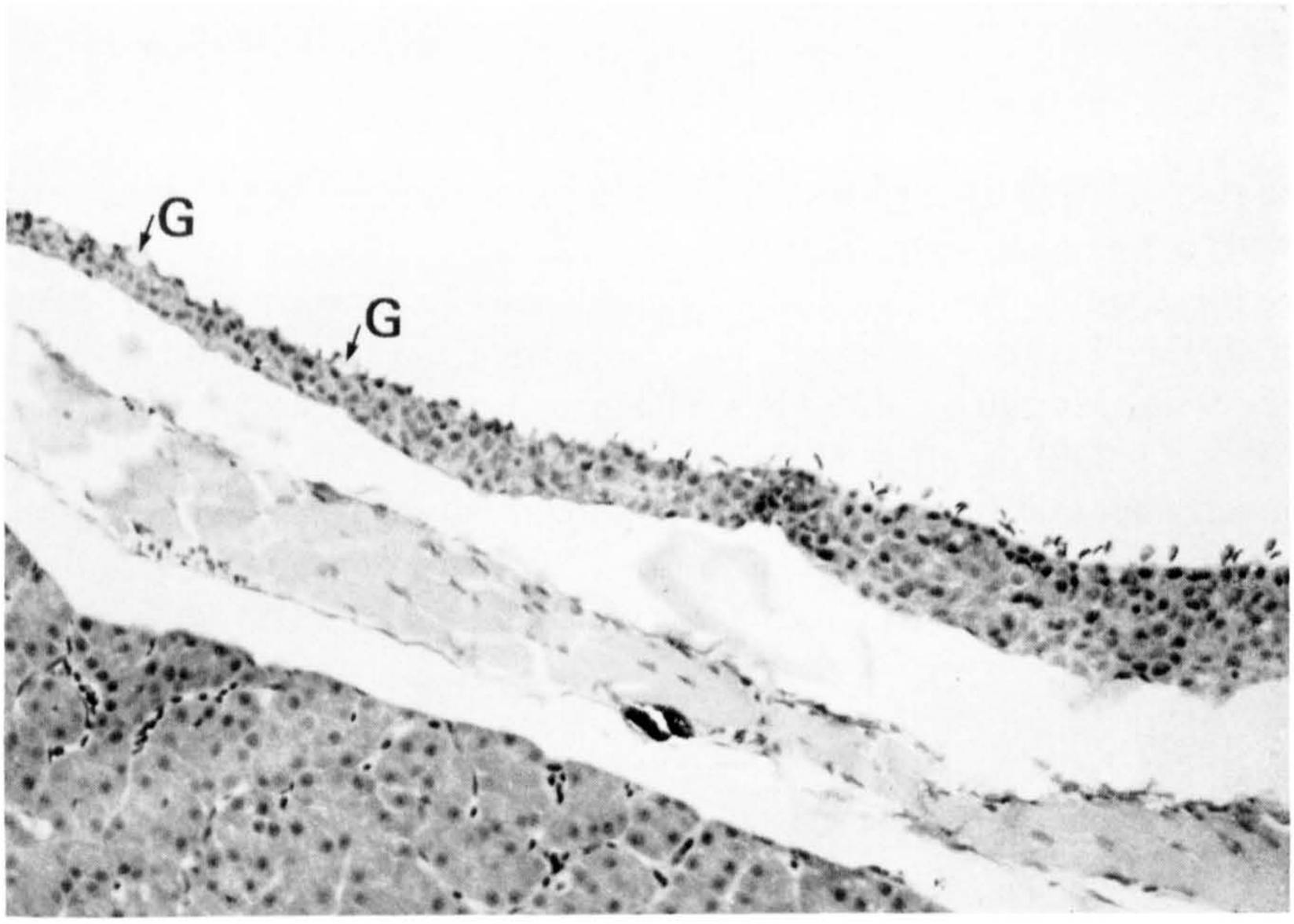

Figure 2. Low power light micrograph to show the progressive thickening and decline of goblet cells (G) infested by *I. necator* (H&E, × 320).

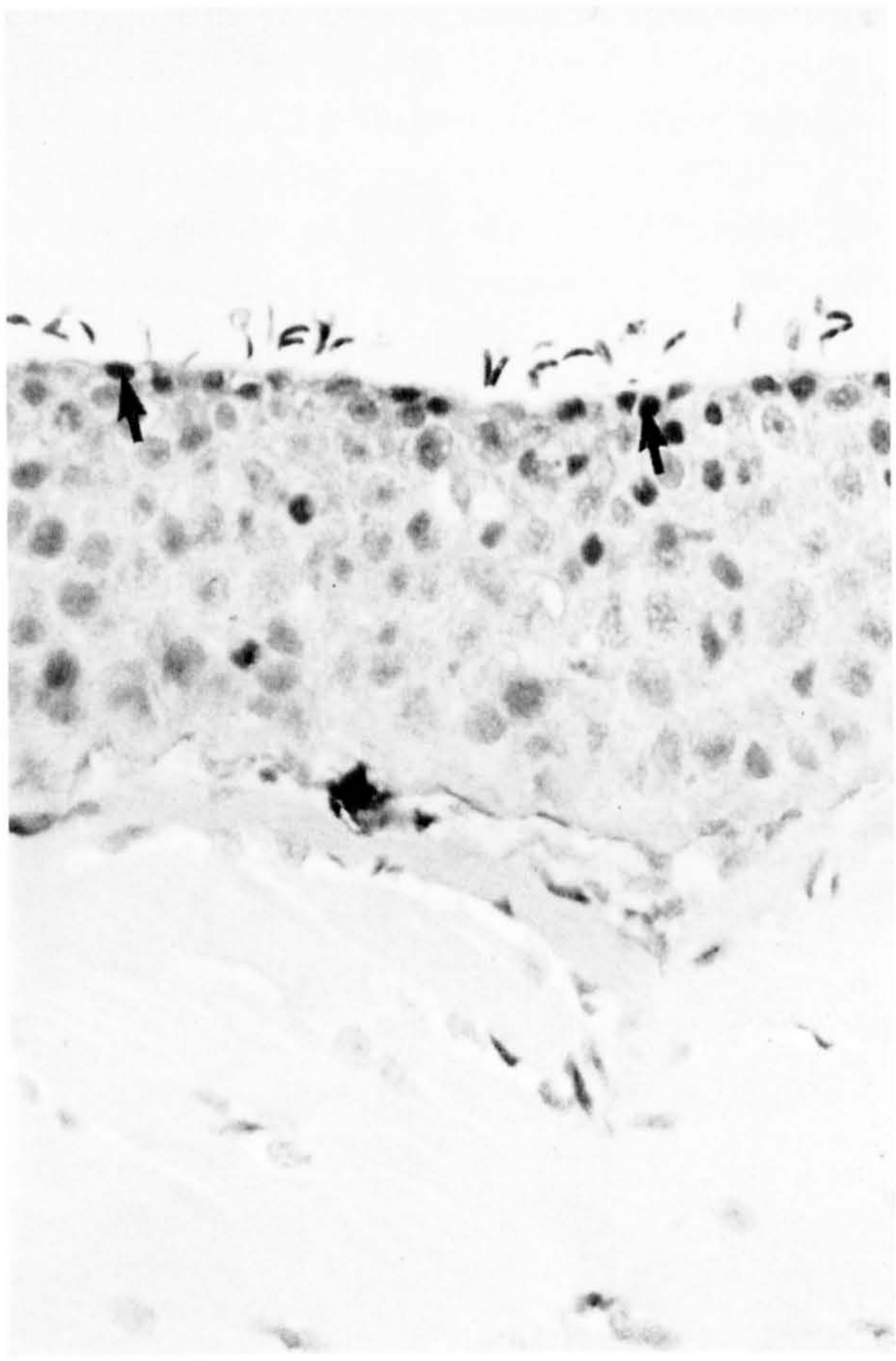

Figure 3. Strongly basophilic nuclei (arrowed) of peripheral cells beneath *I. necator* infestations (H&E, × 500).

Figure 4. Effete ghost cells (arrowed) showing characteristic 'sucked out appearance'. Note also the leucocytes (L) migrating through the hyperplastic epidermis (H&E, ×500).

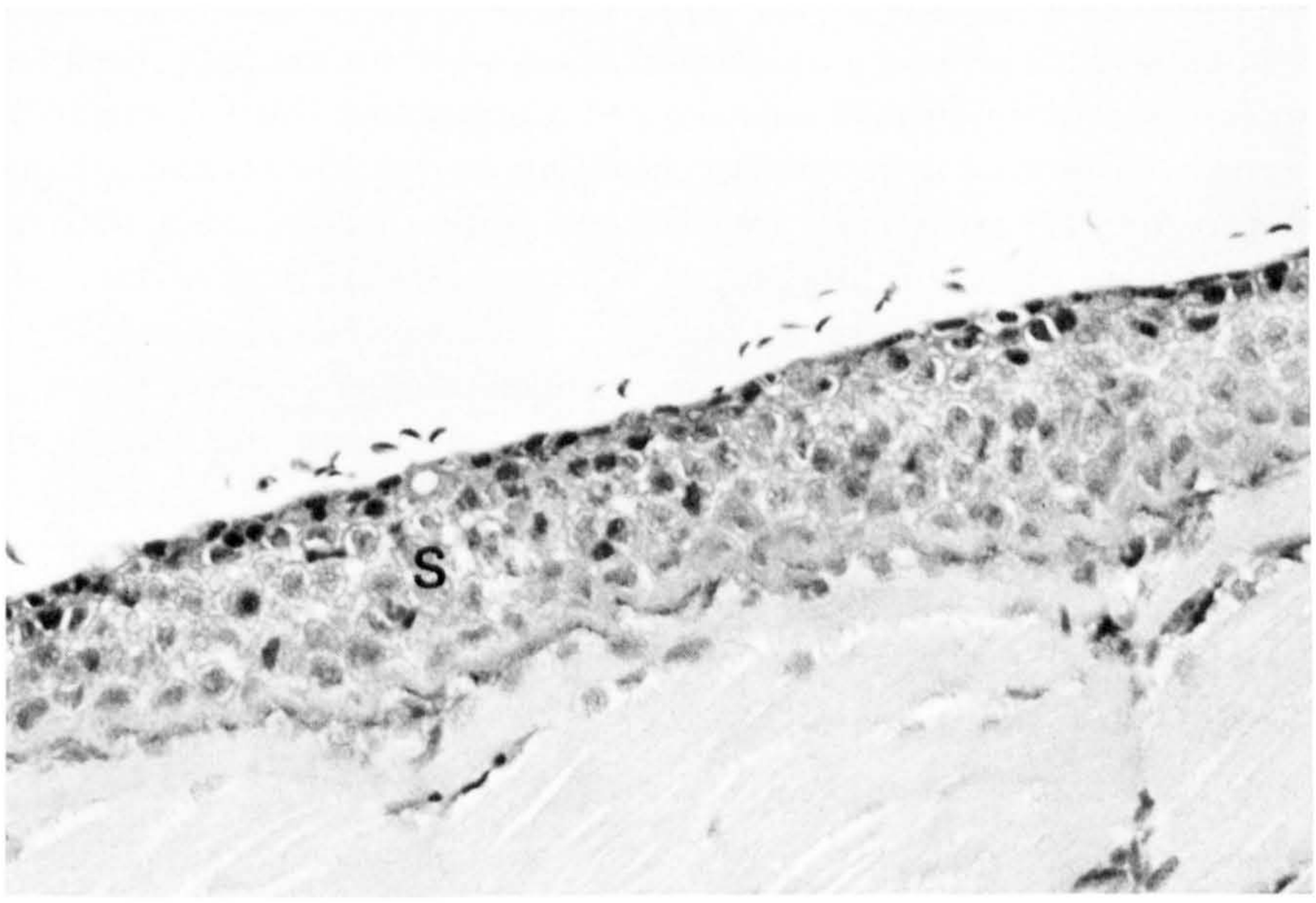

Figure 5. Spongiosis (S) of hyperplastic epidermis beneath *I. necator* infestation (H&E, ×410).

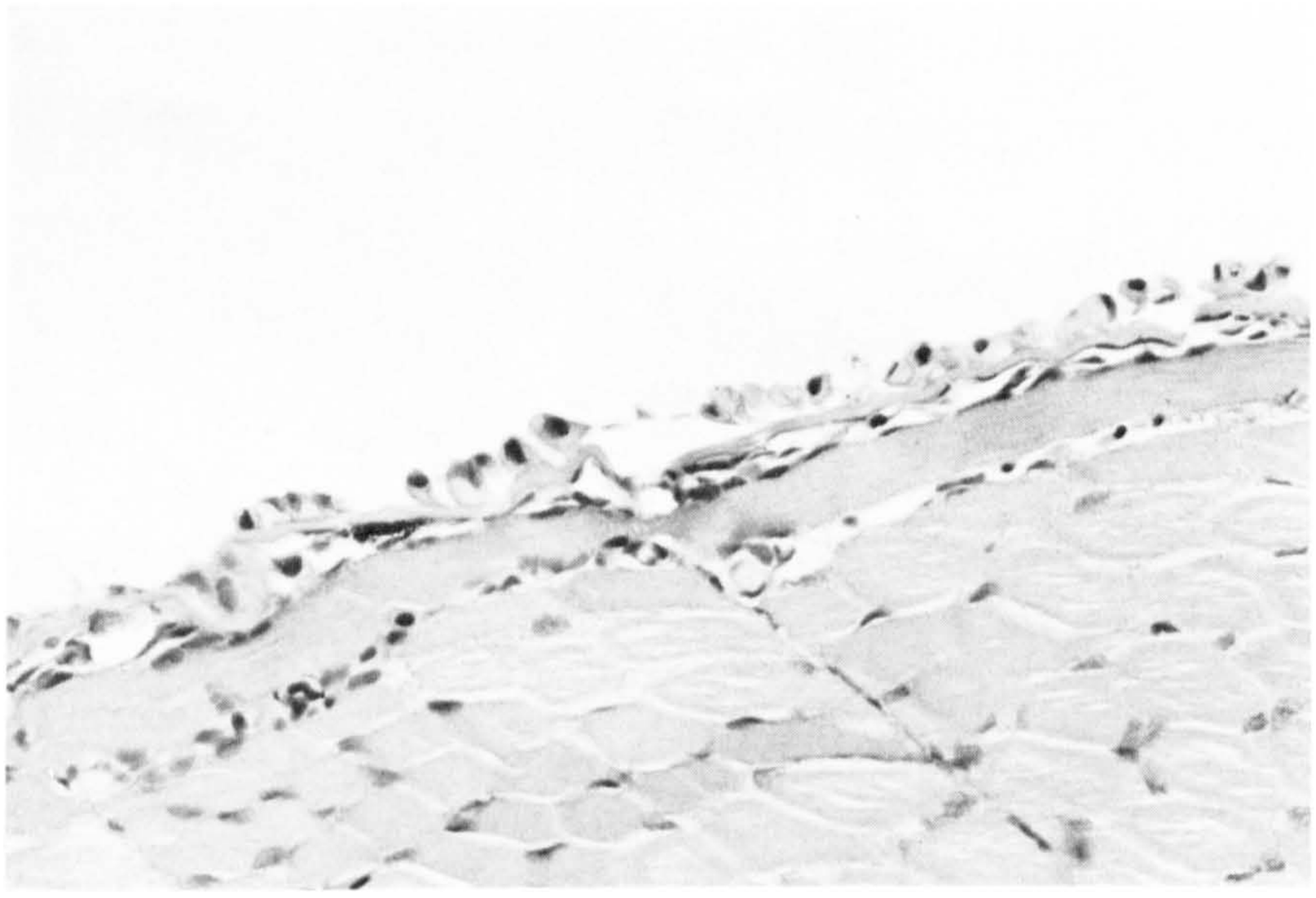

Figure 6. Extensive epidermal erosion leaving an incomplete layer of cells above the basal membrane (H&E, ×410).

pseudobranch. The skin on the head of the alevins anterior to the operculum was never seen to be infested by *I. necator*.

The presence of *I. necator* led to marked hyperplasia of the malphigian cells of the epidermis underneath the infested area and, by way of contrast, the almost complete disappearance of goblet cells in the same area (Fig. 1). Uninfested skin in the immediate proximity of the hyperplastic areas was of normal thickness and had the normal complements of goblet cells (Fig. 2). Hyperplastic areas of epidermis were usually ten to fifteen cells thick whereas uninfested areas were on average three cells thick.

The upper layer of epidermal cells immediately below the *I. necator* organisms showed strongly basophilic pyknotic degenerative nuclei (Fig. 3) and peripheral cells to which *I. necator* were attached were frequently effete 'ghost' cells, with no nuclear chromatin, and were eosinophilic (Fig. 4). Sections stained with alcian blue showed that these cells did not possess sulphated mucins characteristic of goblet cells.

By days 10, 11 and 12 the hyperplastic areas of epidermis below heavy *I. necator* infestations frequently showed slight spongiosis, vacuolation and loss of cytoplasmic and nuclear detail in the suprabasal layers (Fig. 5). In several cases marked epidermal erosion down to the dermis was apparent with only the skeletal remnants of the malpighian cells left (Fig. 6). From the appearance of the epidermis it would seem that large areas had sloughed off just above the basal layer.

Salmon smolt pathology

None of the smolts became infested with *I. necator* during the period of this study and the skin appeared to be normal in all of them and was characterized by the presence of large numbers of goblet cells.

Rainbow trout alevin autoradiography

The most obvious feature of this study was the very marked degree of epidermal erosion associated with the parasite and the virtual disappearance of goblet cells in nearly all of the fish examined. However, as in the salmon alevin study the epidermis

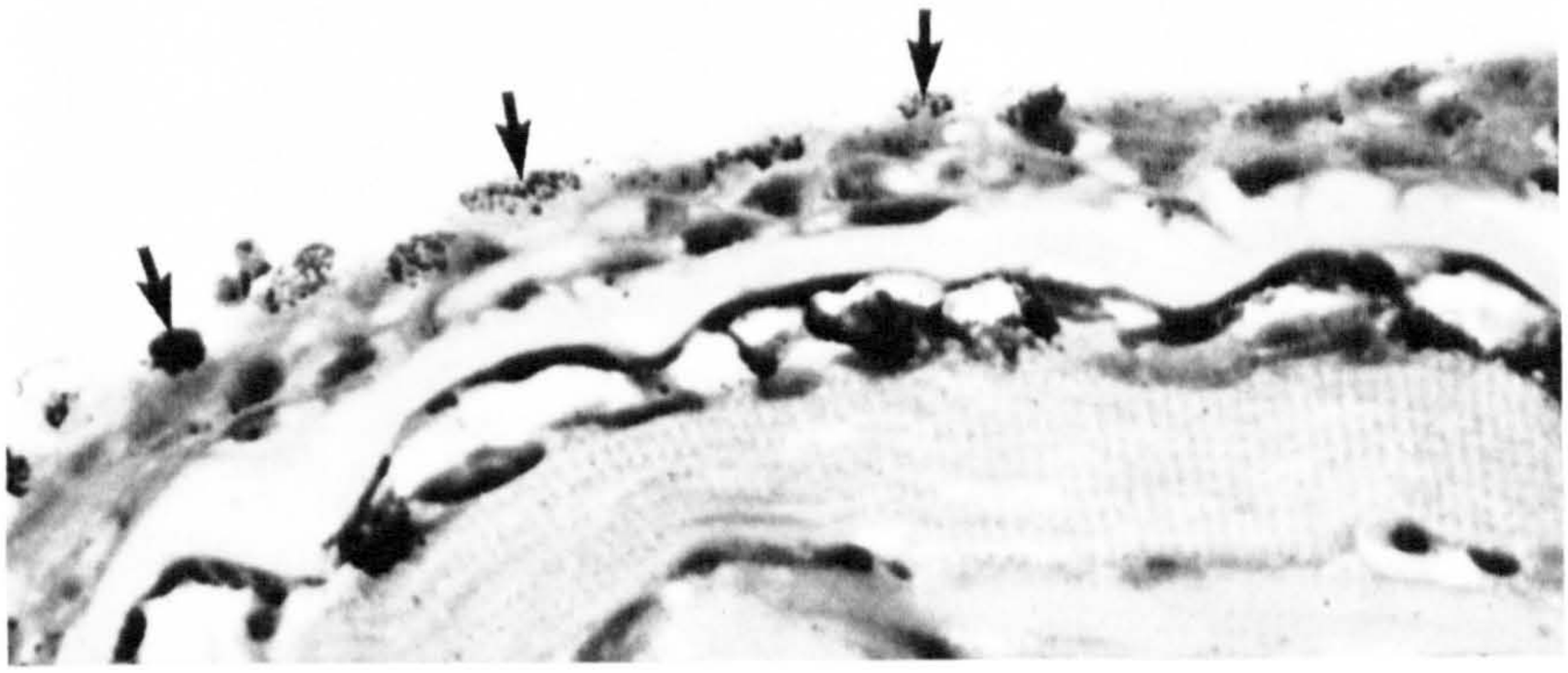

Figure 7. Heavy labelling with [^{3}H] thymidine of the outermost squamous cells (arrowed) (H&E, $\times 650$).

Figure 8. Heavy labelling of all remaining cells in the eroded epidermis (H&E, $\times 650$).

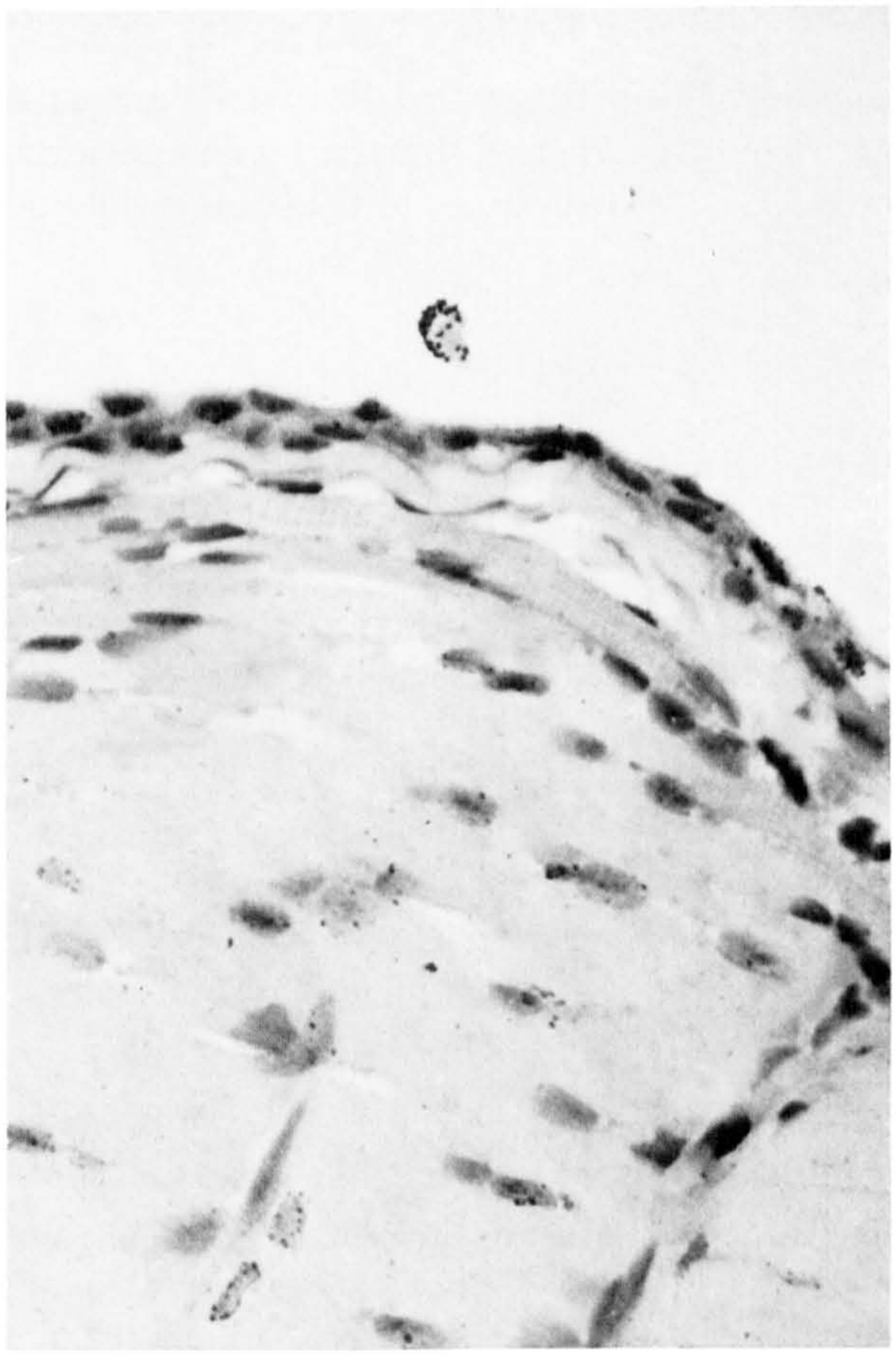

Figure 9. *I. necator* displaying uptake of [^{3}H] thymidine throughout the cytoplasm (H&E, ×650).

of the head of the fish was normal and uninfested irrespective of the degree of epidermal erosion of the skin behind the head.

Large numbers of *I. necator* were present on all of the fish and again the most heavily infested area was invariably just ventral to the dorsal fin. Parasites were not seen on areas where sloughing had occurred, being confined to areas of relatively thick epidermis.

The uptake of [^{3}H]thymidine by pre-dividing cells was very obvious. Several features were apparent in their distribution; labelled cells were distributed throughout the epidermis but were most frequently found in the middle to outer layers beneath heavy infestations of *I. necator* rather than in the suprabasal layer. In many cases the cells of the outermost squamous rather than the epidermal layer were virtually all heavily labelled (Fig. 7). However, the areas with the most frequently labelled cells were those areas where epidermal erosion was greatest. Indeed, in those areas where the epidermis had been eroded to the thickness of a single cell, virtually every cell was labelled (Fig. 8).

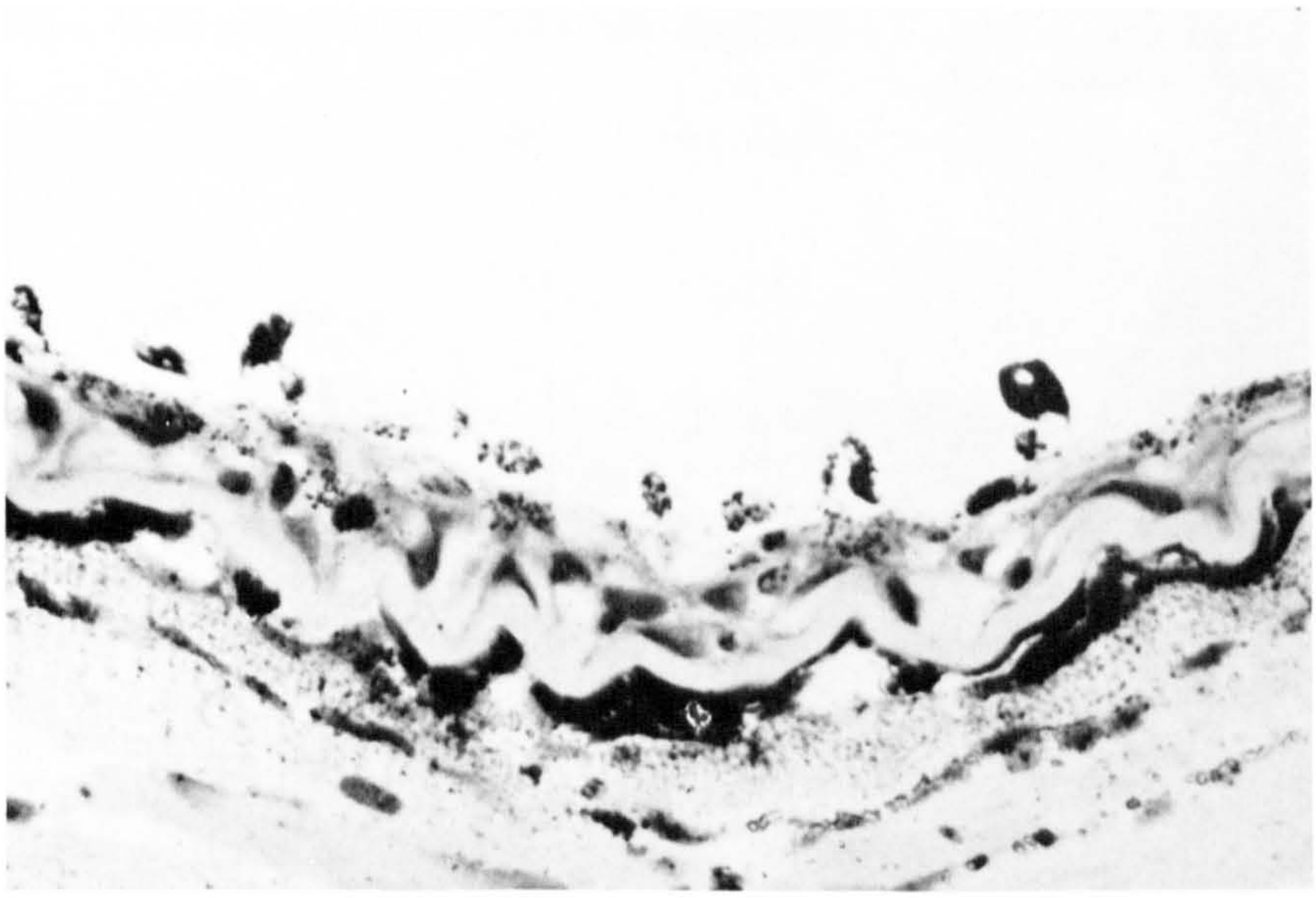

Figure 10. Very heavy labelling of *I. necator* 4 days post-infection. The label is scattered throughout the parasite (H&E, ×600).

The *I. necator* attached to the epidermal cells also showed a high frequency of labelling (Fig. 9). The label was generally scattered throughout the cytoplasm. Density of labelling of the parasites increased, until by 4 days post-injection of [^{3}H]thymidine many of the *I. necator* were virtually replete with label (Fig. 10). The labelling appeared to be scattered throughout the parasite and did not seem to be confined to the nucleus or to the kinetoplasts.

Salmon smolt autoradiography

These fish had heavy infestations of *I. necator* on the skin before they were killed. Previous studies had shown that *I. necator* would not survive in the tissue culture medium as they almost invariably detached from the skin, rounded up and contractile motion ceased; thus it was not surprising that at sampling very few *I. necator* were found to be still attached. However, in a few cases there appeared to be the enucleated remnants of *I. necator* above the epidermis.

The explants of the skin of these smolts showed extensive hyperplasia (Fig. 11) and very few goblet cells were present (*c.f.* inset). However, there were also numerous discrete areas of thin epidermis (two to three cells deep) as opposed to the ten to fifteen cell deep areas of the hyperplastic epidermis.

Labelling of the epidermal cells was similar to that seen in the 0+ rainbow trout, with heaviest labelling occurring in the middle layers as opposed to the suprabasal layers. However, there were also discrete areas of very heavy labelling (Fig. 12). These were almost invariably associated with the thinner areas of epidermis mentioned above. Statistical analysis showed that areas of very heavy labelling were signifi-

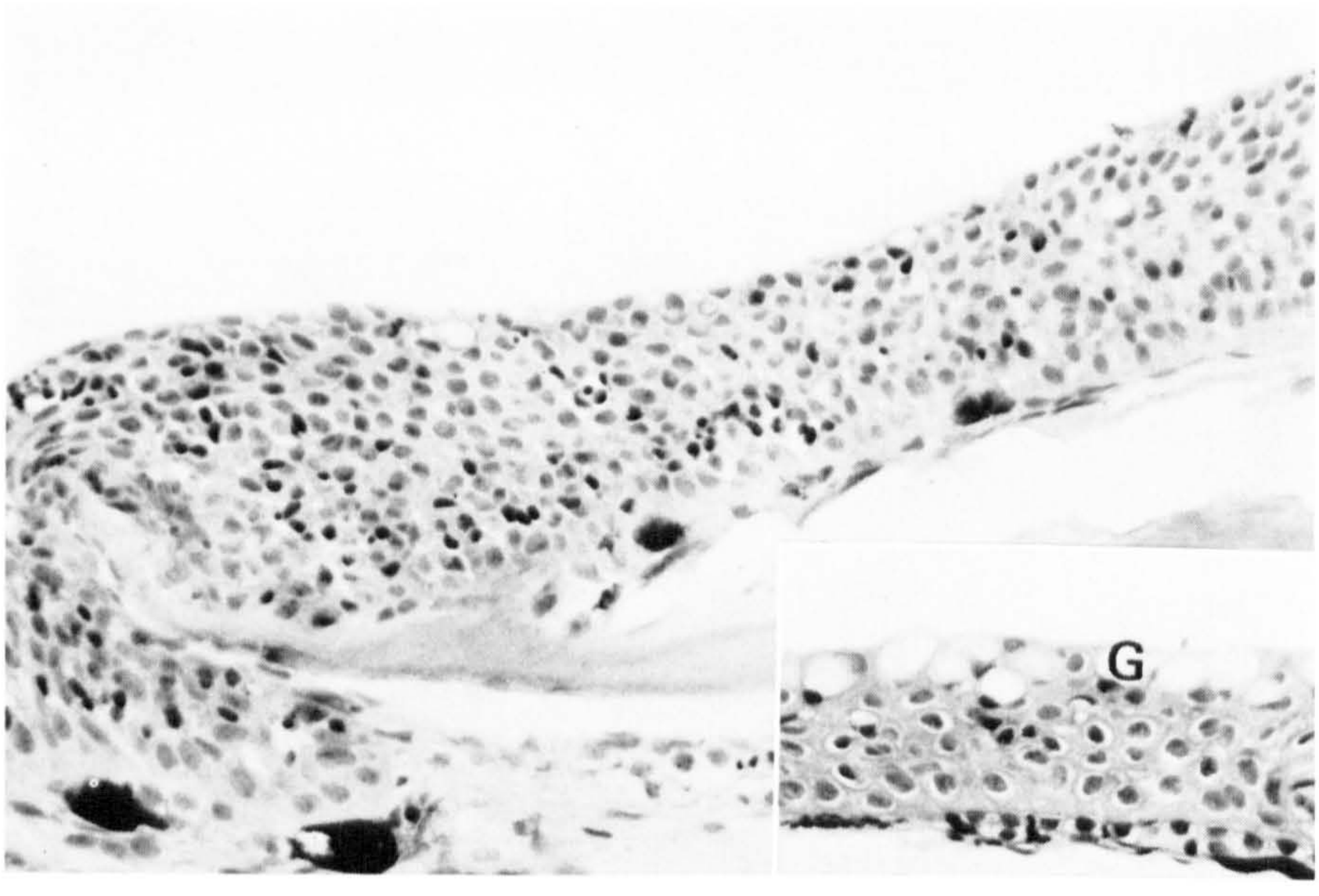

Figure 11. Hyperplasia of the salmon smolt epidermis. Note the lack of goblet cells. Inset shows normal smolt epidermis (G = goblet cells) (H&E, ×350).

Figure 12. Salmon smolt epidermis showing discrete clusters of heavily labelled cells (arrowed). Note how thin these areas are compared to Fig. 11 (H&E, ×320).

cantly thinner than areas with no labelling (unrelated $t = 19{\cdot}26$, $P < 0{\cdot}001$, df = 230; Meddis 1975). Unlike the situation seen in the 0+ rainbow trout, there was no labelling of surface epithelial cells in these explants.

Discussion

The distribution of *I. necator* on the surface of all of the infested fish was distinctive with a pattern of heavy infestation correlated with sites of protection such as the trailing edges of the pectoral fins and the operculum. The pattern of development of such infestations is not known with certainty but supports the hypothesis first suggested by Robertson (1979) that infestation arises via the respiratory inflow of water across the gills, with initial infestation of gills and branchial chamber, extending to the body surface. In the later stages of *I. necator* infestation the fishes seek out areas of minimal water flow and such conditions would readily provide the opportunity for the more generalized extension of infestation. With such a mechanism of per-branchial infestation the dorsum of the head would naturally be virtually inaccessible.

The relative time scale for the depletion of mucous cells and localized hyperplasia of the epidermis at sites of maximal infestation was not resolved in this study; it is, however, possible to speculate that the first response to the *I. necator* is the exhaustion of the mucus from the goblet cells and their subsequent sloughing, a feature which has already been observed in brown trout, *Salmo trutta* L., by N. Blackstock & A. D. Pickering (personal communication), followed by reactive hyperplasia after the mucoid response has failed to resolve the condition. The efficacy of parasite removal with sloughed mucus has been reported by several workers (Lester 1972; Willoughby & Pickering 1977), whilst Roberts & Bullock (1976) have reviewed the literature on hyperplastic response to various skin irritants.

The activity of the parasite on the surface was marked principally by the generalized degenerative changes in the uppermost cells of the epidermis. This did not appear to be directly related to individual parasites and therefore lends credence to the possibility that the parasite secretes some form of digestive enzyme or toxic substance which leads to necrosis of the outermost cells.

The destruction of the integrity of the outermost level of the epidermis, with its tight junctions and its osmotic barrier led to a distinctive intra- and extra-cellular oedema presumably associated with inflow of hypo-osmotic water from the surrounding aqueous habitat. This resulted, within 12 days of infestation, in degeneration and sloughing of virtually the entire hyperplastic epidermal plaque, leaving at most a single layer of basal cells over the area. Where the infestation is severe and the areas of desquamation are therefore extensive the likeliest cause of death is osmoregulatory breakdown and resultant haemodilution, as described by Richards & Pickering (1979).

The complete absence of pathological change or even low level infestation in the smolts exposed to high levels of *I. necator* confirms the findings of Robertson (1979)

that fish of this age are normally refractory to the parasite although the precise reason for this is not known. The infested smolts used to attempt to induce the infestation, which were also used for autoradiography, came from a severely stressed group presented by a salmonid farmer for diagnosis. Stressed fish invariably show increased cortisol levels (Fagerlund 1967; Wedemeyer 1969). Robertson (unpublished observations) has found that similar levels of cortisol to those reported from stressed fish leads to heavy *I. necator* infestations.

In normal fish skin the main focus of epidermal proliferations is the suprabasal layer (Bullock *et al.* 1978a,b; Bullock & Roberts 1980) and labelling at the surface is very limited. The presence of *I. necator* induced a major change in this pattern with extensive labelling of the surface cells taking place. This increased activity may have been in response to an irritant elaborated by the organism, a more likely possibility than simple replacement of damaged cells which normally develop from the suprabasal layer. Erosion, leaving only the basal cells intact, leads to extensive labelling in this area, but this is more easily explained, even in the complete absence of parasites, by a requirement to increase epidermal thickness.

There was an anomaly in the labelling of the infested smolt skins, where the extreme outer cells were not labelled as in the alevins. The entire epidermis of an alevin is likely to be more active than that of a smolt but this is unlikely to be a complete explanation.

The uptake of label by the parasite was a very distinctive feature of this study. It was not generally associated with the parasite nucleus and the label appeared to be concentrated by the parasite suggesting the possibility of preferential nucleic acid ingestion by feeding organisms. Our limited light microscopy evidence supports the elegant work of Schubert (1966) who suggested that the parasite feeds on the cell by protruding 'finger-like processes' into the cell and sucking out parts of the cell contents. Because of the density of labelling seen in the latter part of this study it seems likely that the parasite browses from cell to cell.

Further work is now in progress to ascertain the actual cause of death of the host and the nature of the defence mechanism which prevents the parasite infesting 1+ fish.

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