THE APPLICATION OF NEW BIOSYSTEMATIC TECHNIQUES IN THE DISCRIMINATION OF THE GENUS *GYRODACTYLUS* (MONOGENEA) ON SALMONOID FISH

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To My Family

With Love

DECLARATION

I hereby declare that this thesis has been composed by myself and is the result of my own investigations. It is neither been accepted, nor has been submitted for any other degrees. All the sources of information have been duly acknowledged.

ABSTRACT

Prior to 1989 the total number of *Gyrodactylus* species recorded for all British freshwater fish numbered 20. The fauna present on the British Salmonidae was poorly documented and frequently not identified to species level. The European free market, created in 1992, resulted in legislative changes allowing the movement of live fish stocks, albeit under strict disease monitoring conditions, into the UK. One stipulation maintains that the fish stock be free of the ectoparasitic monogenean *Gyrodactylus salaris* Malmberg, 1957, a parasite made notifiable in the UK in 1987 (Diseases of Fish Act, 1937) owing to its pathogenicity and damage to Norwegian salmon populations in 38 rivers. Although this parasite has been reported since 1957 throughout mainland Europe, its occurrence in the UK was unknown.

This project set out to make a national survey of British salmonids and investigated 250 sample sites, examined four salmonid hosts, Atlantic salmon *Salmo salar*, brown trout *Salmo trutta*, rainbow trout *Oncorhynchus mykiss* and Arctic charr *Salvelinus alpinus*. Seventy of the sites were found to be positive for *Gyrodactylus*. Distinctions were made between wild and farmed fish and prevalence, abundance and intensity data collected for comparison. Species determination within the genus *Gyrodactylus* is based upon subtle differences in hook morphology and has long posed a taxonomic problem. The discrimination of collected specimens was based on two platforms. The initial approach used classical morphometrics from the light microscope, the results being processed using multivariate analyses to separate species. The second approach analysed morphometric data collected from scanning electron micrographs. This was made possible by the development of a sclerite release technique utilising a source of ultrasound to liberate hooks from surrounding tissue and a subsequent flotation stage which permitted flat preparations. Sonication of fresh and frozen material retained the structures that would be lost by enzymatic digestion. The description of new morphometric parameters using digital image analysis allowed the subtle differences in hamuli and marginal hook shape to be discriminated when analysed using principal components analysis (PCA).

Four species were identified following multivariate and morphological analyses of opisthaptoral sclerites. *G. truttae* Gläser, 1975 was found to occur on *S. trutta* and

G. derjavini Mikailov, 1975 was found to occur on *S. trutta*, *S. salar* and *O. mykiss*. Two hitherto undescribed forms, one on *S. salar* and one on *S. alpinus* which may be a new species are described. In addition, two forms of *G. derjavini* from *S. salar* and *S. alpinus* and one form of *G. truttae* from *S. trutta* are described. Sub-populations of *Gyrodactylus* sp. were found to be determined by the pattern of distribution of the host; *S. salar*. The two sub-populations were divided into a southern celtic population (Morph 1) and a northern boreal population (Morph 2). Water temperature, was found to be an important environmental parameter influencing sclerite size. The principal component analyses identified key characters which could discriminate *G. salaris* from the native British species using novel parameters based upon both single elements and the full complement of sclerites. Of these new parameters, the hamulus angle and the size of the marginal hook sickle aperture were the most discriminating. Electronmicrographs of hamuli were traced using a digitising tablet and prepared for image processing. The hamulus angle was measured on original hook images and on enhanced (skeletonised) images using an image analyser. Skeletonisation investigated the reliability of the hook angle as a taxonomic criterion by the removal of possible age-related sclerotisation of the hamulus. Statistical analysis of the data revealed that there are significant differences in hook angle between some species.

The isolation of sclerites by sonication enabled their elemental composition to be investigated. The hamuli and marginal hooks were found to have a high sulphur content, indicative of a keratin-like substance. The ventral bar composed of sulphur and calcium is weakly keratinised. The hamulus and the ventral bar were also found to contain vanadium, the significance of which is unknown. The detailed morphology and composition of the individual sclerites is discussed in relation to the functional mechanics of the entire haptoral complex.

The protein profiles of *G. salaris*, *G. truttae* and *G. derjavini* were investigated using SDS-PAGE gel electrophoresis, with four proteins common to the three species. Two antibodies raised against *G. salaris* were found using Western blots.

The chaetotaxy of argentophilic structures on three species of the genus *Gyrodactylus* was investigated to ascertain the usefulness of this technique in distinguishing species of this genus. Chaetotaxy maps were prepared for *G. salaris*

from Scandinavia and compared to native species of *Gyrodactylus* parasitising salmonids in Britain. A formula for the arrangement of the sensilla analogues and the evolutionary position of the genus *Gyrodactylus* is commented upon.

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Lastly, to gyrodactylids everywhere, long may you thrive!.

CONTENTS

List of tables

List of figures

CHAPTER 1: INTRODUCTION

Historical perspective

Gyrodactylus species are ectoparasitic monogeneans parasitising the skin and gills of fish as shown in Figure 1.1. It is usually distinguished taxonomically by examination of the sclerotised portions of the haptoral attachment apparatus. The first major attempt to study the species of *Gyrodactylus* Nordmann, 1832 in the UK was by Harris (1983) who made a comprehensive survey of the fauna of the three-spined stickleback, *Gasterosteus aculeatus* L. in Britain. He recorded ten species from this host, three of which represented new British host records. This raised the total number of *Gyrodactylus* species recorded for all British freshwater fish to 20; these are listed in Table 1.1. Prior to the present study the species of *Gyrodactylus* occurring on British salmonids, was unknown despite this being an economically important group of fish. The European free market, created in 1992, has resulted in legislative changes concerning the movement of fish and allowed the importation of live fish stocks, albeit under strict disease monitoring conditions, into the UK. The disease assessment of a particular fish stock requires that it be free of the ectoparasitic monogenean *Gyrodactylus salaris* Malmberg, 1957; a species made notifiable in the UK in 1987 under the Diseases of Fish Act, 1937. Although the potential for this parasite to spread throughout mainland Europe was recognised, it was unknown as to whether it was already resident within the UK. Since salmonids form the basis of the aquaculture industry in the UK and are vital to the sport fishing activities, a survey was necessitated. Johnsen & Jensen (1988) believed that *Gyrodactylus salaris* was introduced into the River Vefsna in northern Norway via infected Baltic stocks of Atlantic salmon smolts *Salmo salar* L. from infected hatcheries in Sweden during the period 1975-1977. It was first recorded in the River Lakselva, Misvær, Norway in 1975 and, dispersed rapidly throughout its new host population, the Atlantic salmon, as a result of its rapid rate of reproduction. A heavily infested salmon parr is shown in Figure 1.2. Since being reported in Norway, *G. salaris* has been found on salmonids in other parts of Europe and, in one case, on *Pleuronectes flesus* L. in Norway. Table 1.2 lists the reports of this parasite so far in Northern Europe. It is said to be responsible for an estimated loss of 300 tons of salmon parr in 1985 (Mo, 1987), and as a result of the impact of this parasite on juvenile salmon in Norway, a major

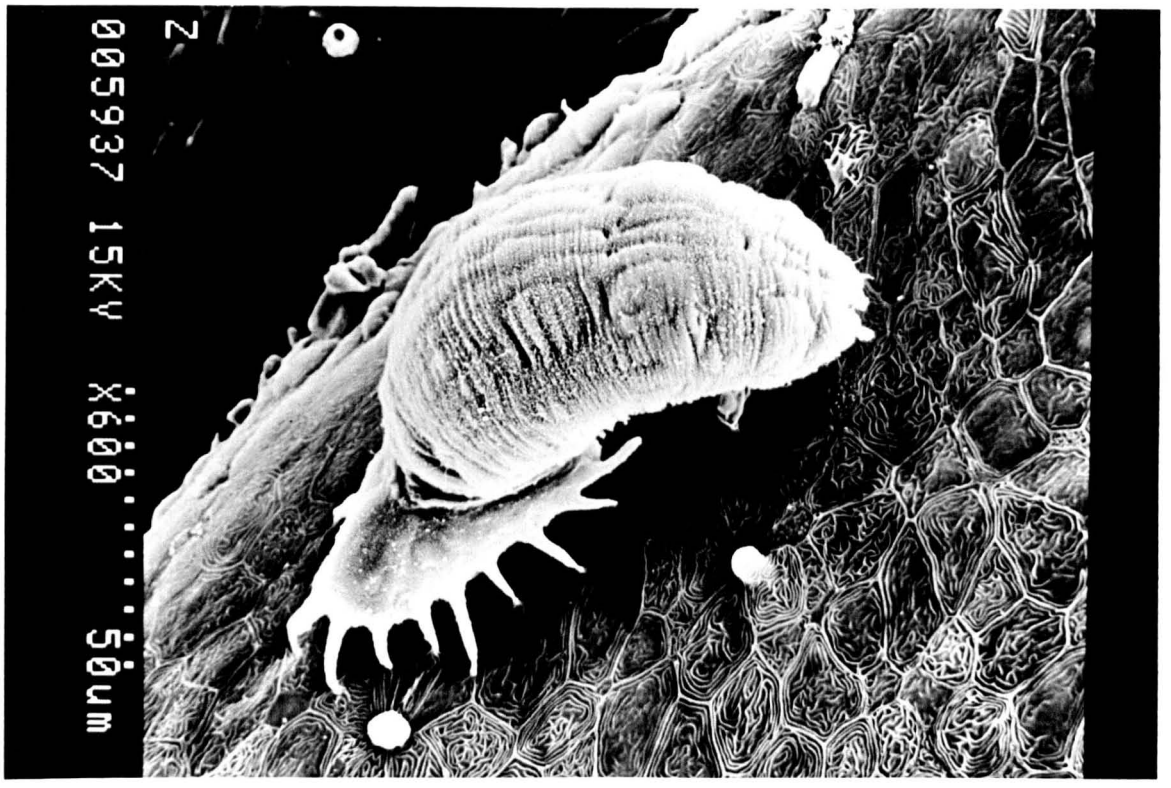

Figure 1.1: *Gyrodactylus* Nordmann, 1832 on the skin of a salmonid (x600).

Figure 1.2: Heavy infestation of *Gyrodactylus salaris* Malmberg, 1957 on the pectoral fin of an Atlantic salmon parr (x8).

Table 1.1: The British *Gyrodactylus* fauna

Species	Host	Reference
G. alexanderi Mizelle & Kritsky, 1967	*Gasterosteus aculeatus*	Harris, 1983
G. aphyae Malmberg, 1956	*Phoxinus phoxinus*	Chubb, 1964; Powell, 1966; Harris, 1983
G. arcuatus Bykhowsky, 1933	*Gasterosteus aculeatus*	Chubb, 1964, 1970; Powell, 1966; Harris, 1983
G. branchicus Malmberg, 1964[1]	*Gasterosteus aculeatus*	Harris, 1983
G. elegans Nordmann, 1832	*Abramis brama*	†Nicoll, 1924; Sproston, 1946; Anderson, 1971
	Carassius carassius	Sproston, 1946
	Cyprinus carpio	Nicoll, 1924
	Esox lucius	Nicoll, 1924
	Gasterosteus aculeatus	Bradley, 1861[2]; Houghton, 1862[2]; Cobbold, 1862[2]; Sproston, 1946[2]; Dawes, 1947[2]; Treasurer, 1974[3]
	†*Leuciscus leuciscus*	Nicoll, 1924
	†*Noemacheilus barbatulus*	Nicoll, 1924
	†*Phoxinus phoxinus*	Nicoll, 1924
	Pungitius pungitius	Dawes, 1947
	Rutilus rutilus	Nicoll, 1924; Anderson, 1971
G. gasterostei Gläser, 1974	*Gasterosteus aculeatus*	Harris, 1983
G. gracilis Kathariner, 1894	*Cyprinus carpio*	Nicoll, 1924
	Gobio gobio	Nicoll, 1924
	Scardinius erythrophthalmus	Nicoll, 1924
G. laevis Malmberg, 1956	*Phoxinus phoxinus*	Powell, 1966; Harris, 1983
G. limneus Malmberg, 1956	*Phoxinus phoxinus*	Powell, 1966; Harris, 1983
G. lucii Kulakovskaya, 1952	*Esox lucius*	Campbell, 1974
G. macronychus Malmberg, 1956	*Phoxinus phoxinus*	Powell, 1966; Harris, 1983
G. medius Kathariner, 1894	*Carassius carassius*	Anderson, 1971
	Cyprinus carpio	Nicoll, 1924
	Phoxinus phoxinus	Baylis, 1928; Sproston, 1946; Rawson, 1952
	Rutilus rutilus	Chubb, 1965
G. minimus Malmberg, 1956	*Phoxinus phoxinus*	Harris, 1983
G. pavlovskyi Ergens & Bychowsky, 1967	*Noemacheilus barbatulus*	Harris, 1983
G. pungitii Malmberg, 1964	*Gasterosteus aculeatus*	Powell, 1966[4]; Chubb, 1970; Harris, 1980a
	Pungitius pungitius	Harris, 1983
G. rarus Wegener, 1909	*Gasterosteus aculeatus*	Chappell, 1969[2]; Wootten, 1973; Harris, 1983

G. rogatensis Harris, 1985	*Pungitius pungitius*	Nicoll,1924; Wootten, 1973[2]; Harris, 1983a
G. salaris Malmberg, 1957	*Cottus gobio*	Harris, 1983a, 1985
G. sedelnikowi Gvosdev, 1950	*Salmo trutta*	Campbell, 1974[5]
G. truttae Gläser, 1974	*Noemacheilus barbatulus*	Harris, 1983a
G. turnbulli Harris, 1983	*Salmo trutta*	Malmberg, 1987b
Gyrodactylus sp.	*Poecilia reticulata*	(Harris, p.c.)
	Alburnus alburnus	Nicoll, 1924
	Carassius carassius	Nicoll, 1924
	Gasterosteus aculeatus	Vickers, 1951[2]; Hopkins, 1959[2]; Chubb, 1964[2]; Madan, 1965[2]; Arme & Owen, 1967[2]; Lyons, 1969[2]; Dartnall *et al.*, 1972[2]; Shillcock, 1972[2]
	Gobio gobio	Shillcock, 1972
	Leuciscus leuciscus	Shillcock, 1972
	Lota lota	Nicoll, 1924
	Oncorhynchus mykiss	Sommerville, 1983; Naich & Bennett, 1989
	Pungitius pungitius	Vickers, 1951; Dartnall, 1973[6]
	Rutilus rutilus	Shillcock, 1972
	Salmo salar	Wootten & Smith, 1980; Wootten & Sommerville, 1989
	Salmo trutta	Nicoll, 1924; Aderounmu, 1966; Wootten & Sommerville, 1989
	Tinca tinca	Nicoll, 1924

[1] Synonym of *G. bychowskyi* Sproston, 1946 (see Harris, 1983).

[2] Species most probably *G. gasterostei* Gläser, 1974 and/or *G. arcuatus* Bykhowsky, 1933 (see Harris, 1983).

[3] Specimens identified as being *G. arcuatus* (see Harris, 1983).

[4] Specimens identified as being *G. gasterostei* (see Harris, 1983).

[5] Mis-identification, more likely *G. truttae* Gläser, 1974 or *G. derjavini* Mikailov, 1975.

[6] Species more likely to be *G. pungitii* Malmberg, 1964 and/or *G. rarus* Wegener, 1909.

† Identification of the *Gyrodactylus* species in question, is tentative.

Table 1.2: Reports of *Gyrodactylus salaris* in Northern Europe.

Host	Country	References
Salmo trutta	R. Topla & R. Osoblaha, Czech.	Ergens, 1961[1]
Oncorhynchus mykiss	R. Topla & R. Osoblaha, Czech.	Ergens, 1961[1]
Salvelinus fontinalis	R. Topla & R. Osablaha, Czech.	Ergens, 1961[1]
Salmo trutta m. fario	R. Seret, Yugoslavia	Kukakovskaja, 1967
Salmo gairdneri irideus	R. Pliva, Black Sea coast	Zitnan & Cankovic, 1970[1,3]
Salmo trutta m. *fario*	R. Buna, Adriatic coast	Zitnan & Cankovic, 1970[1,3]
S. thymus obusirestris oxyrhynchus	R. Buna, Adriatic coast	Zitnan & Cankovic, 1970[1,3]
Salmo trutta	Norway	Malmberg, 1973
S. trutta; S. fontinalis & O. mykiss	Czechoslovakia	Rehulka, 1973[2]
Salmo salar	R. Moravice, Ukraine	Tesarcik & Ivasik, 1974
Salmo trutta m. *fario*	R. Moravice, Ukraine	Tesarcik & Ivasik, 1974
Oncorhynchus mykiss	Slovakia	Zitnan, 1974
Salmo salar	L. Lagoda, Russia	Ergens, 1983[4]
O. mykiss & S. alpinus	Norway	Tanum, 1983
Salmo salar	R. Morrum, Finland	Rimaila-Parnaneri & Wiklund, 1987
Salmo salar	Bosnia-Hercegovina	Imamovic, 1987
Salmo trutta	Bosnia-Hercegovina	Imamovic, 1987
Oncorhynchus mykiss	Norway	Malmberg, 1987a,b,c; Mo, 1987
Oncorhynchus mykiss	Sweden & Finland	Malmberg, 1987; Malmberg & Malmberg, 1987
Platichthys flesus	Norway	Mo, 1987
Salmo salar	Germany	Schmahl, 1988
Salmo salar	R. Kemijoki, Finland	Rintamaki, 1988
Oncorhynchus mykiss	Norway	Mo, 1988; Malmberg, 1988
Salmo salar	Norway	Bakke et al., 1989
Salmo salar	Finland	Malmberg, 1989; Rintamaki, 1989
Salmo salar	N. Europe	Malmberg, 1989; Bylund, 1989
Oncorhynchus mykiss	Norway	Malmberg, 1989; Eken & Garnås, 1989
Salmonoidea sp.	Karelia & Kola, Russia	Rumyantsev, 1989
Oncorhynchus mykiss	Germany	Lux, 1990
Salmo salar	Norway	Mo, 1991
Oncorhynchus mykiss	Spain	Santamarina *et al.*, 1991

investigative effort into its pathogenic potential and host-specificity was instigated by The Norwegian Directorate for Nature Management, in 1986. Furthermore, the relatively long life-span of *G. salaris*, 31 days at 6°C, 53 days at 2.5°C (Jansen, 1989), in comparison to *G. bullatarudis* Turnbull, 1956, 5.5 days at 21°C (Scott & Nokes, 1984), coupled with its ability to survive without a host (8 days at 4°C) (Bakke *et al.*, 1991), posed the question, "To what extent could other fish species possibly aid its transmission?". The susceptibility of several fish species, which co-habit with Atlantic salmon in the water bodies of Scandinavia, to *G. salaris* has been investigated, i.e. *Lampetra planeri* (Bloch, 1784), *Rutilus rutilus* (L., 1758) and *Perca fluviatilis* L., 1758 (Bakke *et al.*, 1990b), *Phoxinus phoxinus* (L., 1758) (Bakke & Sharp, 1990), and *Anguilla anguilla* (L., 1758) (Bakke *et al.*, 1991).

The rapid spread and devastating impact of *G. salaris* on stocks of wild Norwegian salmon has led to its being regarded as a particularly serious pathogen. The introduction of monogeneans with the importation of live fish stocks (transfaunation) is not limited to *G. salaris* and has occurred on numerous occasions (McVicar, 1975; Bauer & Hoffman, 1976; Buchmann *et al.*, 1987). However, the only known introductions of monogeneans have been with cultured fishes, such as the movement of grass carp *Ctenopharyngodon idella* (Valenciennes, 1884) into the former Soviet Union which introduced *G. ctenopharyngodonis* Lin Mo-en, 1962 (Musselius, 1988). Similarly, the introduction of European eels *Anguilla anguilla* (L., 1758) and Japanese eels *Anguilla japonica* Temminck & Schlegel also into the Soviet Union, brought a further three or four species of *Gyrodactylus* (*G. katharineri* Malmberg, 1964, *G. sprostonae* Lin Mo-en, 1962 and *G. anguillae* Ergens, 1960) (Musselius, 1988). Of these, *G. sprostonae* was responsible for carp mortalities during summer, whilst *G. katharineri* was found to be responsible for mortalities in carp in the winter months (Golovin, 1977).

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[1] Ergens, 1961; Lucky, 1963; Rehulka, 1973; Cankovic & Kiskarolj, 1967 and Zitnan & Cankovic, 1970 believed to be another species, not *G. salaris* (Tanum, 1983; Halvorsen & Hartvigsen, 1989).

[2] Mo (1983) believed this to be *G. truttae*, however, Malmberg (1987b) believes that *G. truttae* is also erroneous but Rehulka's specimens resemble another, *Gyrodactylus* sp. closely related to *G. salaris*.

[3] Ergens (1983) believed the species represented here, was *G. truttae*.

[4] Malmberg (1987b) indicated that *G. thymalli* Zitnan, 1960 closely resembled *G. salaris* and the specimen identified as *G.* sp. by Ergens (1983) was most probably *G. salaris*.

Several species of *Gyrodactylus* have been shown to be responsible for the death of a wide variety of captive fish species (Atkins, 1901; Embody, 1924; Guberlet *et al.*, 1927; Gowanloch, 1927; Yin & Sproston, 1948; Turnbull, 1956; Lewis & Lewis, 1963; MacKenzie, 1970; Amatyakul, 1972; Lester & Adams, 1974; Ogawa & Egusa, 1980; Hoffman, 1981; Cone & Odense, 1984; Cusack & Cone, 1986a). The presence of *Gyrodactylus* has also been linked to outbreaks of viral, bacterial and fungal diseases (Snieszko & Bullock, 1968; Brown & Gratzek, 1980; Heggberget & Johnsen, 1982; Cone & Odense, 1984; Cusack & Cone, 1985, 1986b).

The manner in which *Gyrodactylus* is believed to kill fish fry is through disruption of the skin leading to osmotic imbalance (Cusack & Cone, 1986a). In situations where population growth goes unchecked, parasite movement across the host surface, detaching and re-attaching its armed opisthaptor, results in mechanical damage to the epidermis; there is, in addition, damage due to feeding. The extent and rate at which this damage is inflicted may exceed the host's natural ability for repair. The Norwegians believed that the rapid spread of gyrodactyliasis through Norway's waterways, infecting over 38 rivers and 11 hatcheries, called for drastic measures. The extent to which the Norwegian Government was prepared to go in curbing the further spread of *G. salaris* included the use of rotenone. Treatment of the River Vikja, W. Norway, removed the entire fish stock and the parasite with it. Restocking proved successful, such that the salmon population subsequently increased (Dolman, 1987). On the basis of the above information, it is essential to have a precise diagnosis of the causative agent if such drastic measures are to be implemented on a large scale. The action of rotenone is non-specific, removing all organisms possessing gills. If it is to be used as the final means to eradicate *G. salaris* within a river system, then there must be no mistake in identifying it accurately. Furthermore, *G. salaris* has a relatively low host specificity compared to some other species in the genus, and it is not restricted to Atlantic/Baltic salmon alone, having been recorded from at least five salmonid hosts plus some non-salmonids.

The techniques in current use for discriminating the species of *Gyrodactylus*, of which there are an estimated 400 species, are based on specimens prepared by the ammonium-picrate glycerine method (Malmberg, 1970). However, in certain cases, it is not possible to use slides prepared in this way and examined under high power light microscopy to resolve subtle differences between closely related species, e.g. between *G. salaris* Malmberg, 1957 and *G. thymalli* Zitnan, 1960. This study was conducted

to ascertain how many species of *Gyrodactylus* parasitise native salmonids, and to identify them. In particular, it was important to specify whether *G. salaris* was resident in the UK and, if present, the extent of its distribution. The information would be gathered from a nationwide survey of British salmonids and the species found, described and presented for use in taxonomic keys. The determination of species identity from hook morphology has traditionally been accomplished by the use of whole mounted specimens viewed under the light microscope. This approach is complicated by two major problems: the lack of resolution of the light microscope and the scattering of light by tissue above and below the intended plane of focus. Improved methods of preparing and viewing the attachment sclerites might be expected to overcome these problems such that subsequent morphological analyses could discriminate one species from another with greater accuracy. One approach to the improvement of sclerite visualisation is to clarify the sclerite structure by methods such as the use of improved staining techniques and the digital processing of images. This would improve the accuracy of the morphometric data but would not overcome the problems of resolution. A further approach is to try and remove the tissue surrounding the sclerites and possibly view the specimens by means of an instrument with better resolving power e.g. SEM.

The morphometric data collected could then be subjected to multivariate analysis in order to attempt to separate the specimens of *Gyrodactylus*. Mathematical separation of the gyrodactylids would then be cross-referenced to the data on hook morphology, to allow identification of species. Non-statistical methods were sought to explore the differentiation of *G. salaris* from other species of *Gyrodactylus*, i.e. the elemental composition of the sclerites using X-ray analysis or the protein profile of the parasite, as determined by the use of electrophoresis.

The importance of *G. salaris* as a notifiable disease requires that the techniques used should not only be able to discriminate *G. salaris* from other species of *Gyrodactylus* infecting salmonids, but should at the same time be readily usable by non-specialists working in the field of disease diagnostics. The final technique chosen should therefore be simple in its application e.g. a staining technique that would indicate species-specific characteristics. Chaetotaxy appeared to be a good candidate for this purpose providing that chaetotaxy maps could be prepared for reference. However, these would need to be simplified for use by non-specialists in gyrodactylid taxonomy. A study was therefore proposed which would explore these possibilities.

CHAPTER 2: DISTRIBUTION AND CHARACTERISATION OF SPECIES OF *GYRODACTYLUS* PARASITISING SALMONIDS IN THE UK.

Introduction

The parasitic fauna of British freshwater fishes has been compiled from literature surveys by several authors (Nicoll, 1924; Rawson, 1952; Chappell & Owen, 1969; Chubb, 1970; Kennedy, 1974); in addition, detailed profiles of the parasite fauna of various lakes, lochs, etc. also exist (Copland, 1957; Chubb, 1963; Campbell, 1974). Whilst the gyrodactylid fauna of the Gasterosteidae (Harris, 1983), Cyprinidae, Cobitidae and the Percidae is documented to some extent (see Chapter 1, Table 1.1), those collected from the Salmonidae and other salmonoids in Britain have received little attention. Malmberg (1987) reported the presence of *G. truttae* Gläser, 1974 on British brown trout and also found a species of *Gyrodactylus* closely resembling, but not identical with *G. derjavini* Mikailov, 1975, present on Scottish Atlantic salmon. The reports of *Gyrodactylus* that have been recorded for salmonids in the UK include the following: wild brown trout (Aderounmu, 1966; Wootten & Sommerville, 1989); wild and farmed Atlantic salmon (Wootten & Sommerville, 1989); and farmed rainbow trout (Sommerville, 1983; Naich & Bennett, 1989). Campbell (1974) reported finding *G. salaris* Malmberg, 1957 on brown trout in Loch Lomond: although this was a mis-identification, it was unknown for certain whether or not *G. salaris* was already resident within the UK. The pullulation of records of *G. salaris* on the Continent (see Chapter 1, Table 1.2) indicated that this species could intrude into the UK where expertise to discriminate *G. salaris* from native species of *Gyrodactylus* was lacking. Nineteen species of *Gyrodactylus* have been described on salmonoids worldwide (see Table 2.1), five of these are from Northern Europe (*G. salaris* Malmberg, 1957; *G. derjavini* Mikailov, 1975; *G. truttae* Gläser, 1974; *G. thymalli* Zitnan, 1960; *G. lavareti* Malmberg, 1978). The following investigation presents the results of a national survey of native British salmonoids and the use of a variety of techniques for the determination of the specimens collected. The relationships of the resident species of *Gyrodactylus* are discussed.

Table 2.1: Species of *Gyrodactylus* recorded from salmonoids worldwide.

Species	Host
G. asiaticus Ergens, 1978	*Brachymystax lenok* (Pallas)
G. avalonia Hanek & Threlfall, 1969 (syn. *G. lairdi*; *G. memoralis*; *G. terranovae*)	*Oncorhynchus mykiss* (Walbaum); *Salvelinus fontinalis* (Mitchill)
G. birmani Konovalov, 1967	*S. alpinus* (Linnaeus)
G. bohemicus Ergens, 1992	*O. mykiss*, *S. fontinalis*
G. brachymystacis Ergens, 1978	*B. lenok*
G. brevis Crane & Mizelle, 1967	*O. mykiss* (accidental infection)
G. bychowski Sproston, 1946	*S. salar* Linnaeus (returning)
G. colemanensis Mizelle & Kritsky, 1967	*S. fontinalis*; *O. mykiss*
G. derjavini Mikailov, 1974	*Salmo trutta caspius* (Kessler); *S. trutta* Linnaeus; *S. salar*; *S.t.* m. *lacustris* Linnaeus; *O. mykiss*; *S. trutta* m. *fario* L.; *S. fontinalis*; *S. t. caspius* m. *fario* L.; *S. trutta oxianus* Kessler
G. lavareti Malmberg, 1978	*Coregonus lavaretus* (Linnaeus); *C. nasus* (Pallas)
G. lenoki Gussev, 1953	*B. lenok*
G. magnus Konovalov, 1967	*Thymallus arcticus grubei* natio *mertensi* Valenciennes; *T. arcticus* (Pallas)
G. masu Ogawa, 1986	*O. masou* (Brevoort); *O. rhodurus* Günther; *O. mykiss*
G. nerkae Cone, Beverley-Burton, Wiles & MacDonald, 1983	*O. nerka* (Walbaum)
G. salaris Malmberg, 1957	*Salmo salar*; *O. mykiss*; *S. trutta* (see table 1.2)
G. salmonis Yin & Sproston, 1948	*O. kisutch* (Walbaum); *S. clarki* Richardson; *O. mykiss*; *S. salar*; *S. fontinalis*
G. taimeni Ergens, 1971	*Hucho taimen* (Pallas)
G. thymalli Zitnan, 1960	*Thymallus thymallus* (Linnaeus); *T. arcticus grubei* natio *mertensi*
G. truttae Gläser, 1974	*Salmo trutta* m. *fario*; *O. mykiss*; *S. fontinalis*; *S. salar*

Materials and methods

A national survey of 227 sample sites throughout the British Isles (shown in Figures 2.1-2.6) was undertaken between May 1990 and April 1992, including both wild and farmed populations of salmonoids. This investigation attempted to cover all the species of salmonoids resident in the UK, but concentrated especially on *Salmo salar*, *S. trutta*, *Oncorhynchus mykiss* and *Salvelinus alpinus*. Fish, which ranged in age from 0-1+ and length 5-15 cm, were collected by a variety of means, including electro-fishing, gill-netting, line fishing and the sampling of farm cages. A minimum sample of 20 fish was collected at each site. The skin, fins and gills, plus the oral and nasal cavities, were examined from each fish. Fish were killed by insertion of a pointed needle into the brain via the upper eye in the case of small fish, or by a blow to the cranium, and then examined in local water under an Olympus binocular microscope. Live gyrodactylids were removed by means of mounted needles, placed onto a clean glass slide in a drop of water and a coverslip was placed on top. Excess water was then removed using filter paper in order to leave a flat preparation, with the marginal hooks lying flat. A single drop of ammonium picrate-glycerine (Malmbergs fixative) was added to the edge of the coverslip. Drawn by capillary action under the coverslip this fluid fixes and clears the parasite. The coverslip was then sealed using a slide mountant such as Glyceel (Gurr) or Pertex (Cellpath), and the slides given a reference number. Fish samples received from the National River Authority were collected and fixed in 80% alcohol by the river biologists; in this instance, collected worms required washing in distilled water to remove the alcohol prior to slide preparation.

The measurements of the sclerites or hard parts of the *Gyrodactylus* opisthaptor required for diagnostic purposes were performed on 388 specimens (178 from Atlantic salmon; 127 from brown trout; 83 from rainbow trout and 10 from Arctic charr) prepared using the ammonium picrate-glycerine technique (Malmberg, 1970). A BH2 Olympus binocular microscope with phase contrast was used to make drawings and measurements for light microscope studies (x 100, oil immersion) (see Figure 2.7). Further samples were obtained from those sites found to be *Gyrodactylus* positive and this subsequent material was processed for scanning electron microscopy.

Techniques for the preparation of live and alcohol-fixed material for the scanning electron microscope are given in Chapter 6.

The results from both the light and scanning electron microscope investigations are considered together. The British material was used to compare with other species of *Gyrodactylus* collected from salmonoids in other parts of the Holarctic region. These were obtained either as slide or alcohol-preserved collections deposited in The Natural History Museum, London (BMNH) or as part of private collections. The non-British material was as follows:-

i) *G. salmonis* Yin & Sproston, 1946 (1 specimen) on *Salvelinus fontinalis* (Nova Scotia) (BMNH reg no. 1990.6.19.20-22).

ii) *G. colemanensis* Mizelle & Kritsky, 1967 (2 specimens) on *Oncorhynchus mykiss* (Nova Scotia) (BMNH reg no. 1990.6.19.20-22). Alcohol (80% ethanol) fixed material from *Salvelinus fontinalis* in Nova Scotia (Dr D. Cone).

iii) *G. truttae* Gläser, 1974 (7 specimens) on *Salmo trutta* (from Czechoslovakia) (BMNH reg no. 1990.7.23.2-3) and *G. truttae* Gläser, 1974 (5 specimens) on *Salmo trutta* from the River Chess, England (Dr P. Harris, private collection).

iv) *G. derjavini* Mikailov, 1975, alcohol (80% ethanol) fixed material from *Salmo trutta*, River Dalälven, Sweden (Dr G. Malmberg).

v) *G. salaris* Malmberg, 1957, alcohol (80% ethanol) fixed material from *Salmo salar* from the Rivers Ätran and Beukaforsen, Sweden (Dr G. Malmberg).

Figures 2.7-2.9 outline the relative portions and morphometric measurements made on both the hamuli and marginal hooks that are used to discriminate the salmonid gyrodactylids at the two levels of microscopical examination.

Results

Distribution

Of the 227 salmonoid sites sampled, 69 were found to be positive for

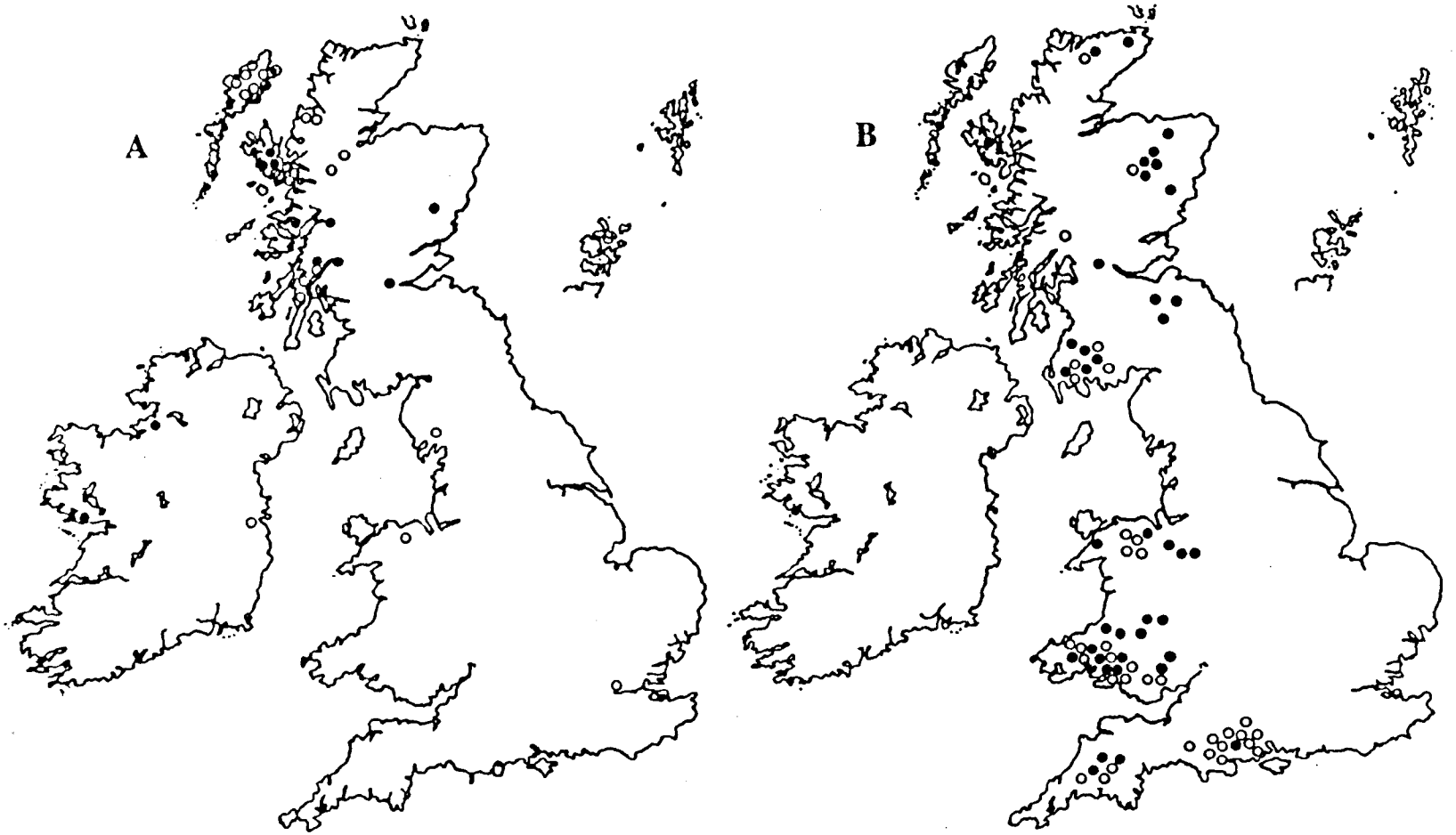


Figure 2.1: Geographical distribution of Atlantic salmon sites sampled for *Gyrodactylus*. A = farmed sites and B = wild sites sampled. (Solid black circles indicate positive sites and the white circles negative for *Gyrodactylus*).

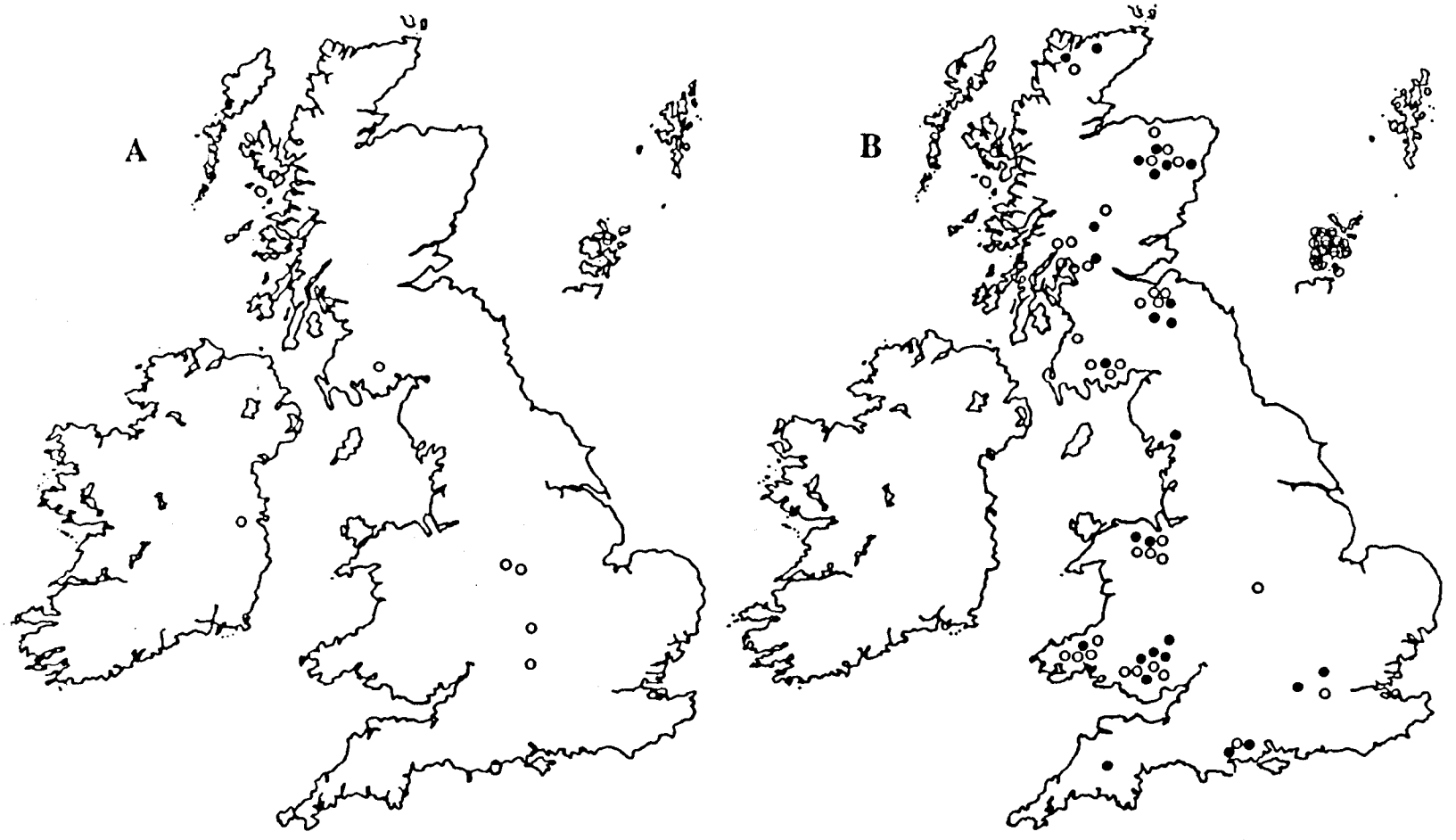


Figure 2.2: Geographical distribution of brown trout sites sampled for *Gyrodactylus*. A = farmed sites and B = wild sites sampled. (Solid black circles indicate positive sites and the white circles negative for *Gyrodactylus*).

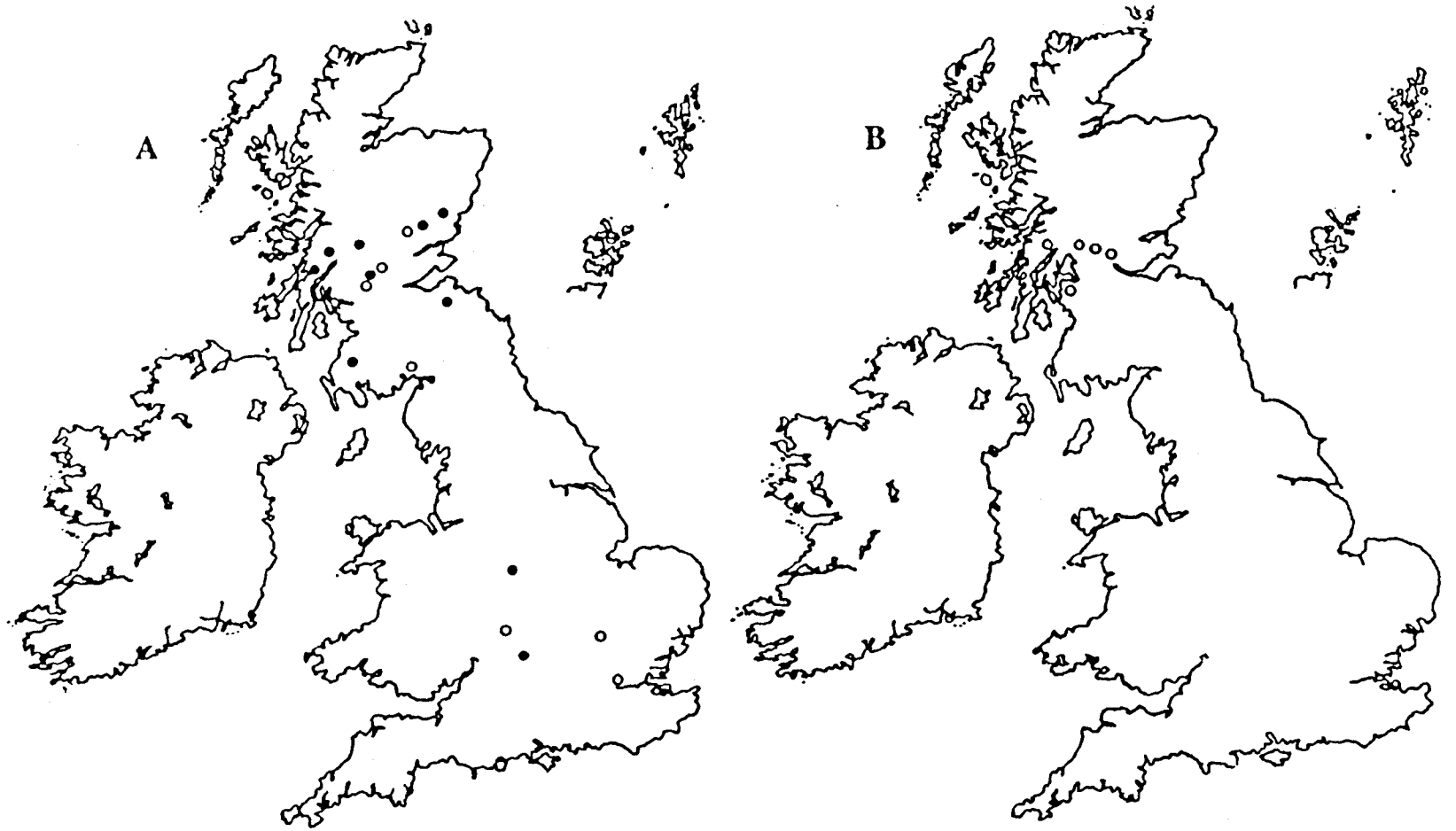


Figure 2.3: Geographical distribution of rainbow trout sites sampled for *Gyrodactylus*. A = farmed sites and B = wild sites sampled. (Solid black circles indicate positive sites and the white circles negative for *Gyrodactylus*).

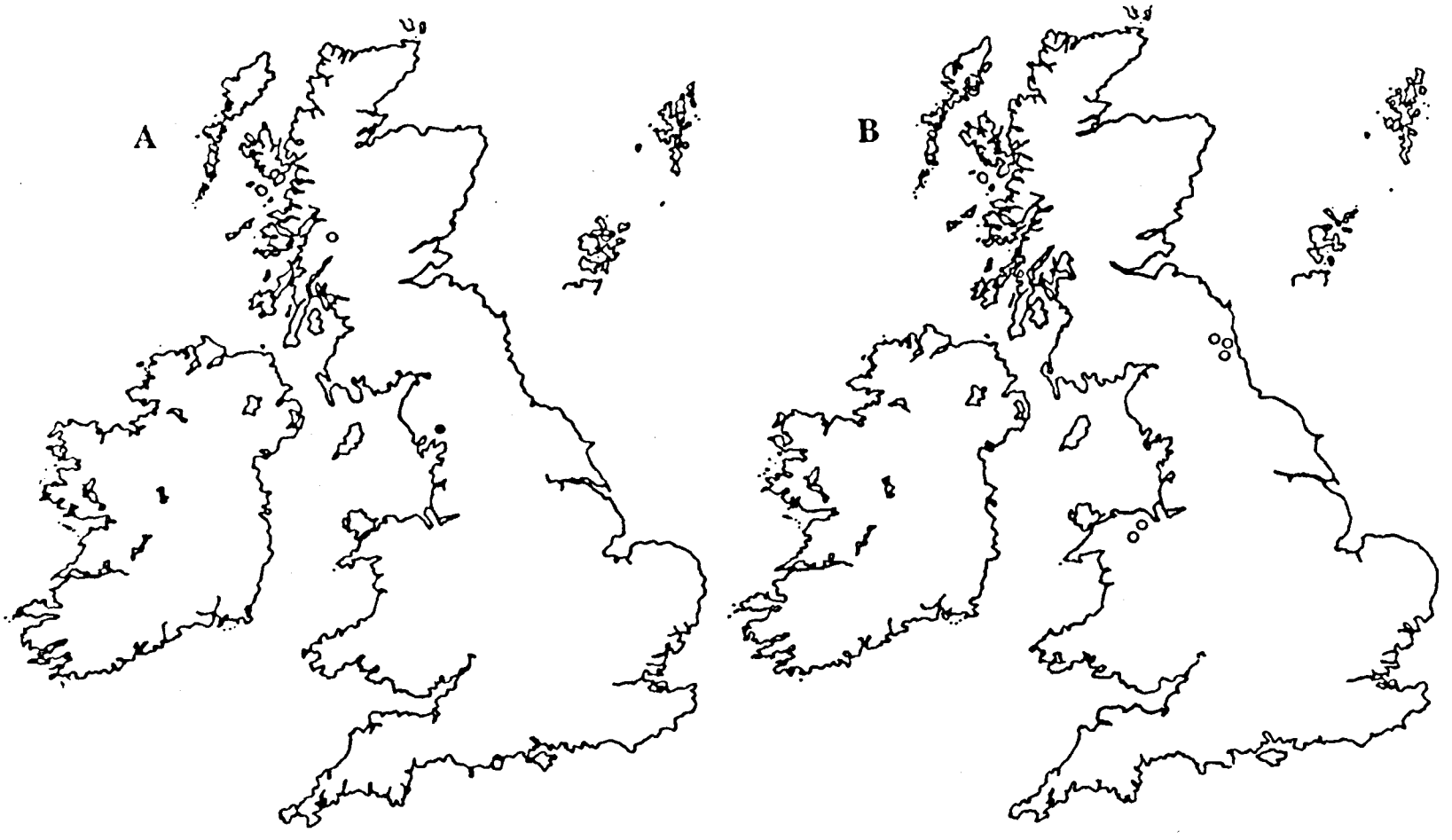


Figure 2.4: Geographical distribution of charr (A) and sea trout (B) sites sampled for *Gyrodactylus* (Solid black circles indicate positive sites and the white circles negative for *Gyrodactylus*).

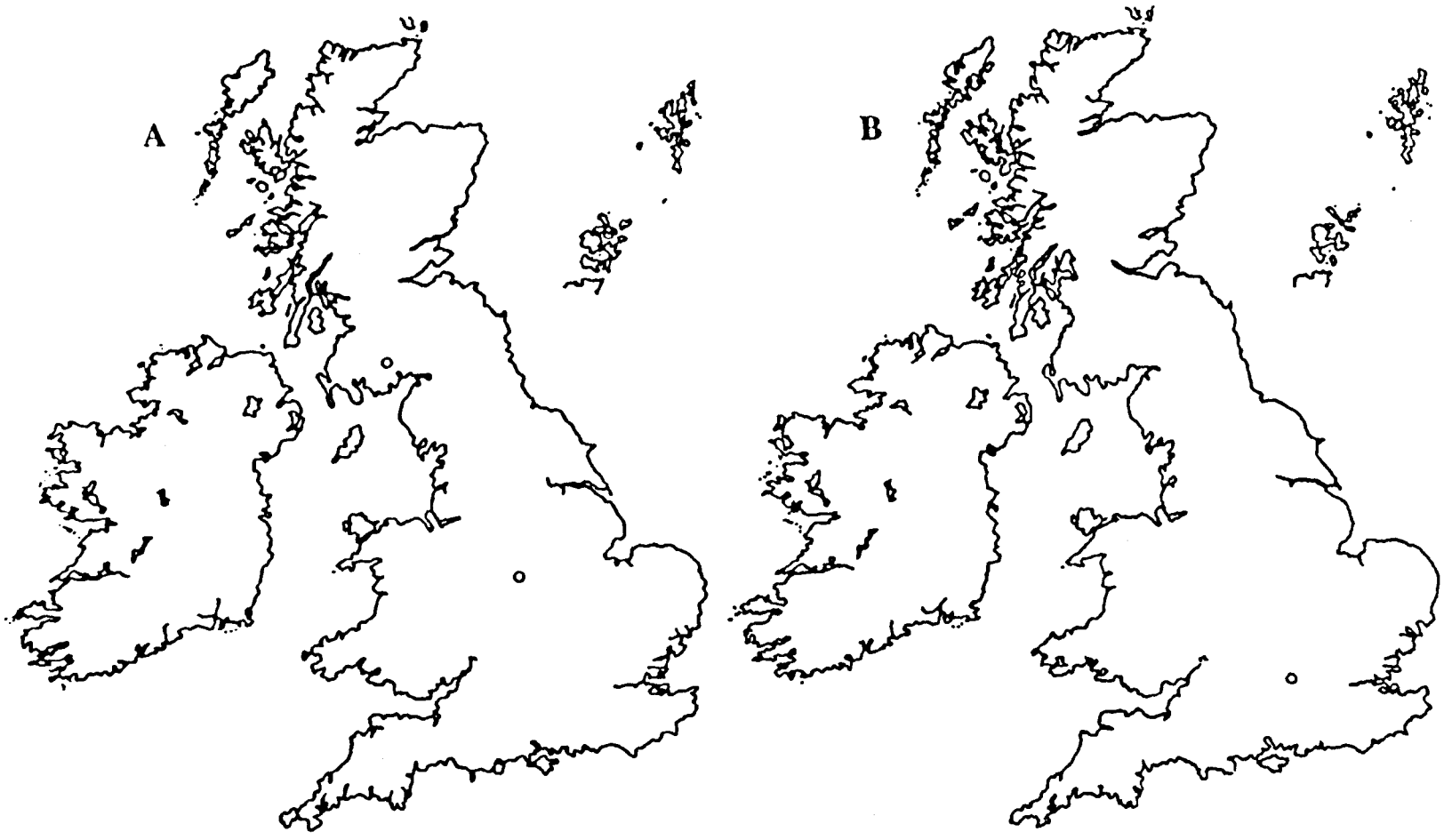


Figure 2.5: Geographical distribution of brook trout (A) and grayling (B) sites sampled for *Gyrodactylus* (Solid black circles indicate positive sites and the white circles negative for *Gyrodactylus*).

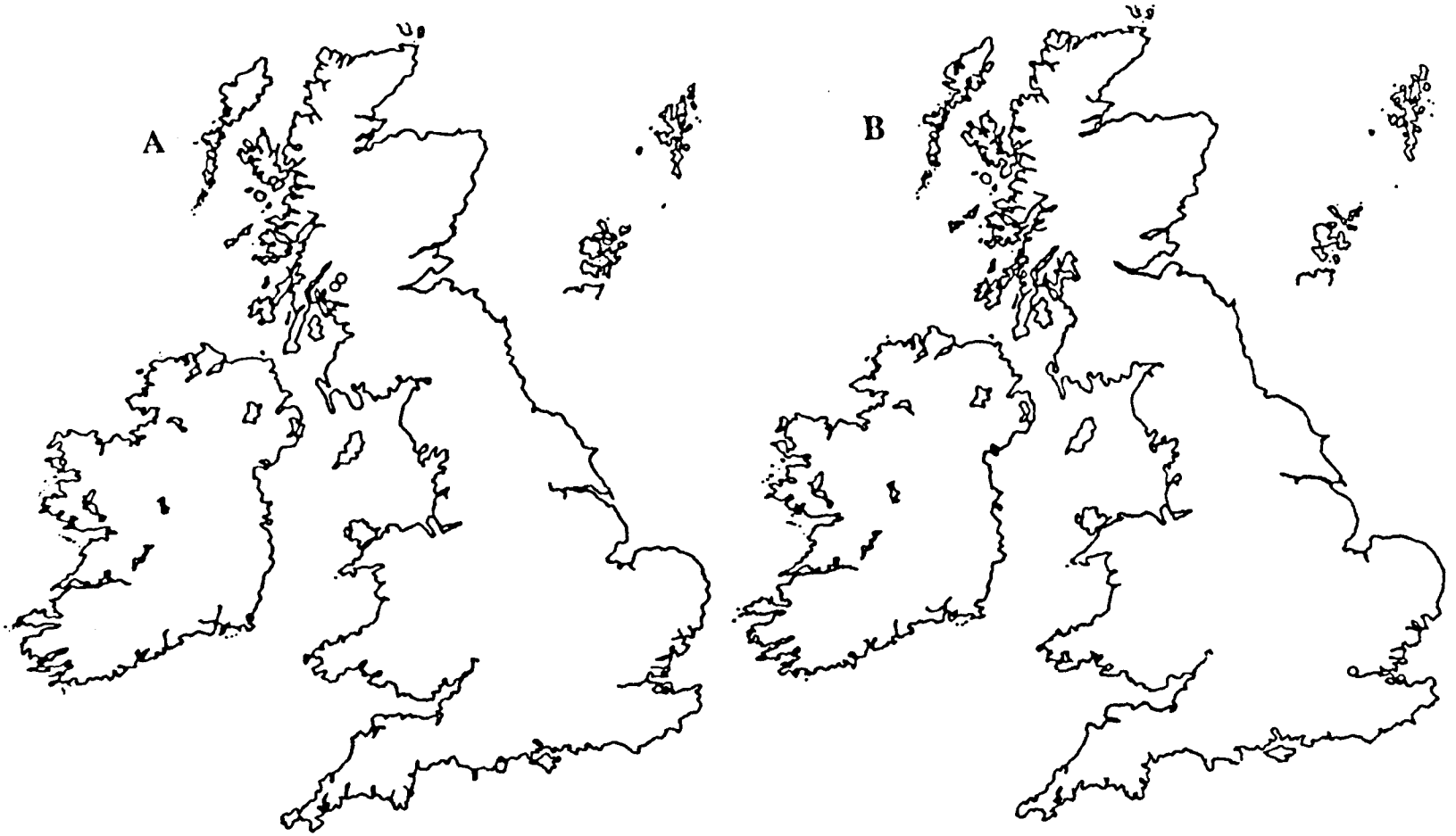


Figure 2.6: Geographical distribution of powan (A) and smelt (B) sites sampled for *Gyrodactylus* (Solid black circles indicate positive sites and the white circles negative for *Gyrodactylus*).

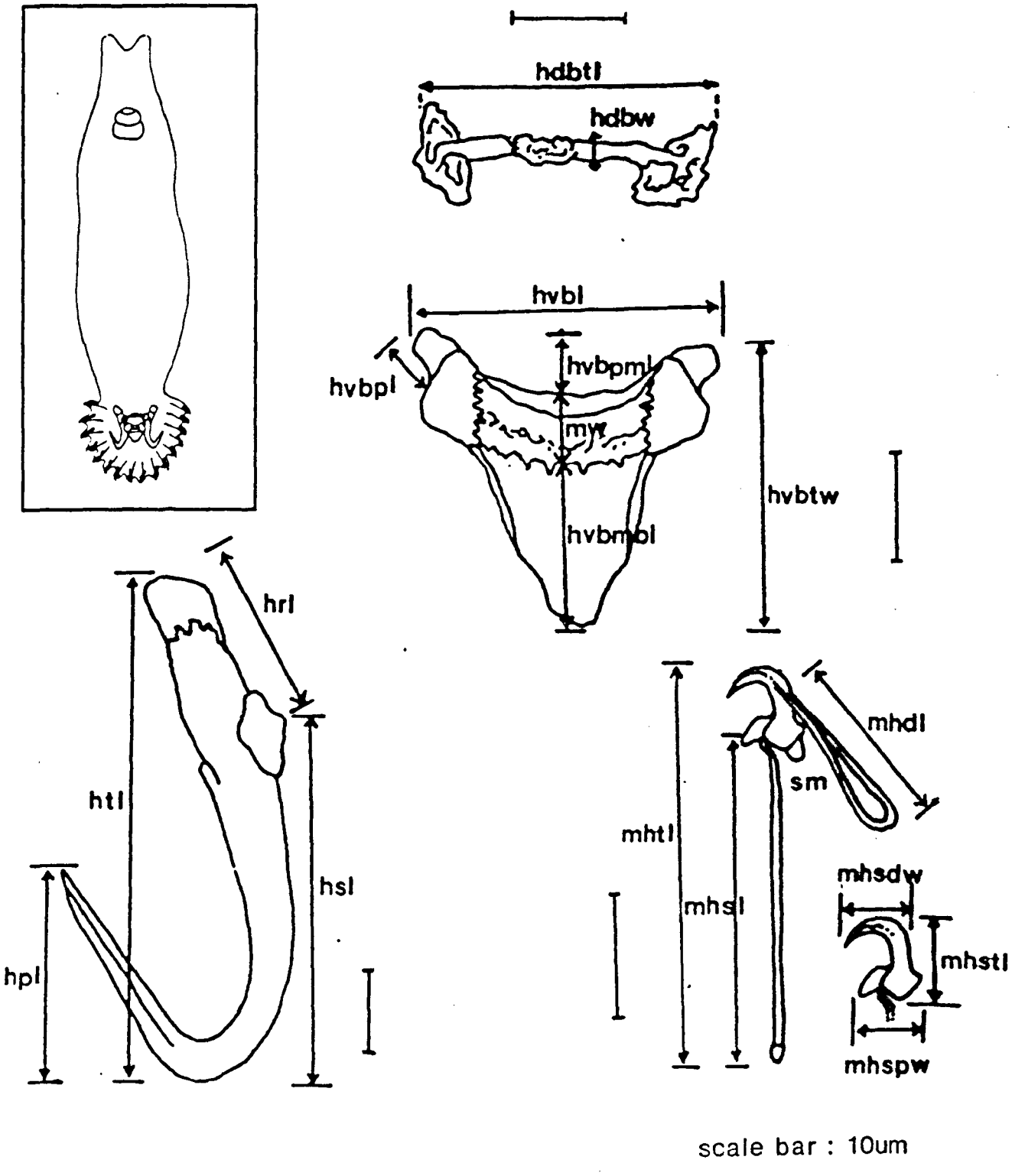
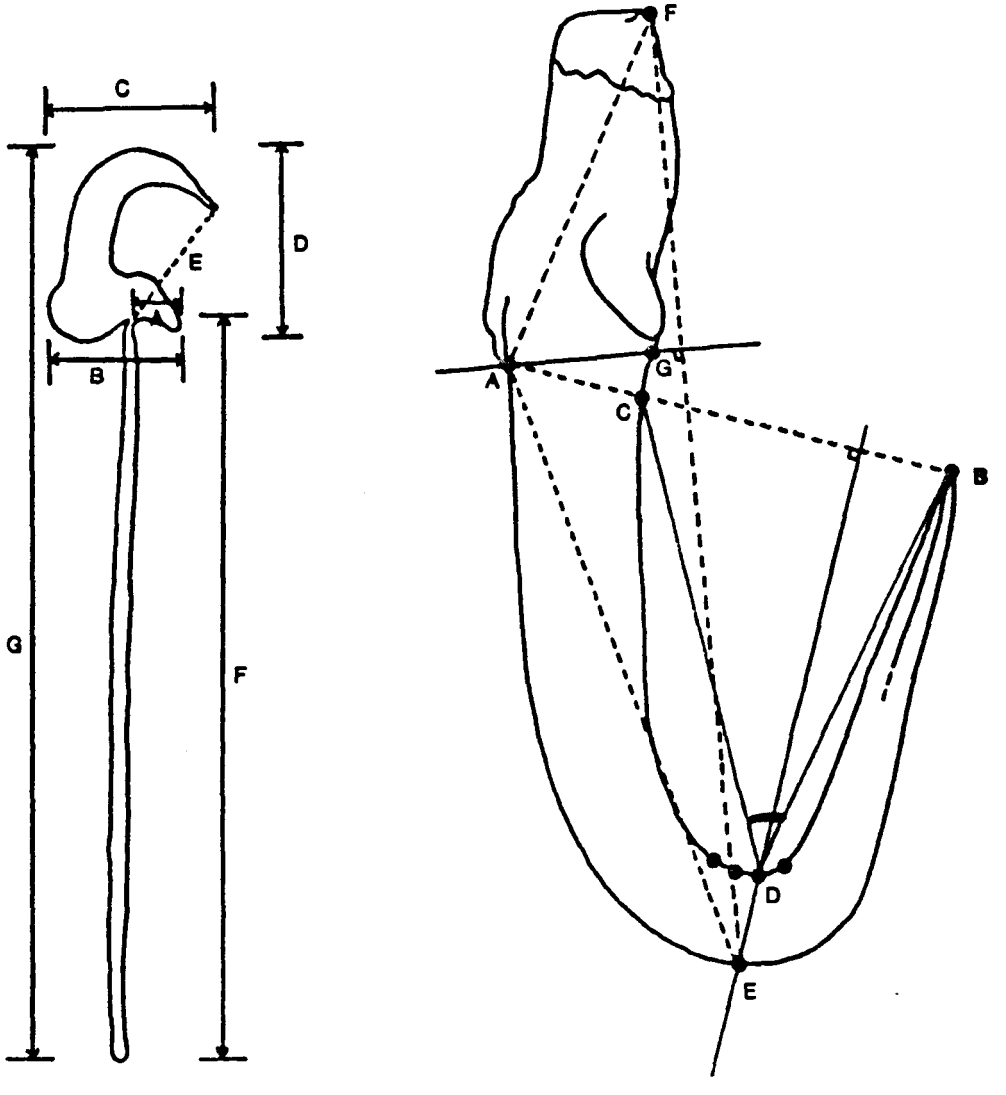


Figure 2.7: Schematic illustration of the *Gyrodactylus* attachment complex showing the morphometric features used as variables measured using the light microscope. See the abbreviations list for the full nomenclature given in Appendix 2 on page 318 (pullout).



Marginal hook measurements

Hamulus measurements

Figure 2.8: Schematic illustration of the morphometric features measured on extracted hamuli and marginal hooks using scanning electron microscopy. See the abbreviations list for the full nomenclature given in Appendix 2.

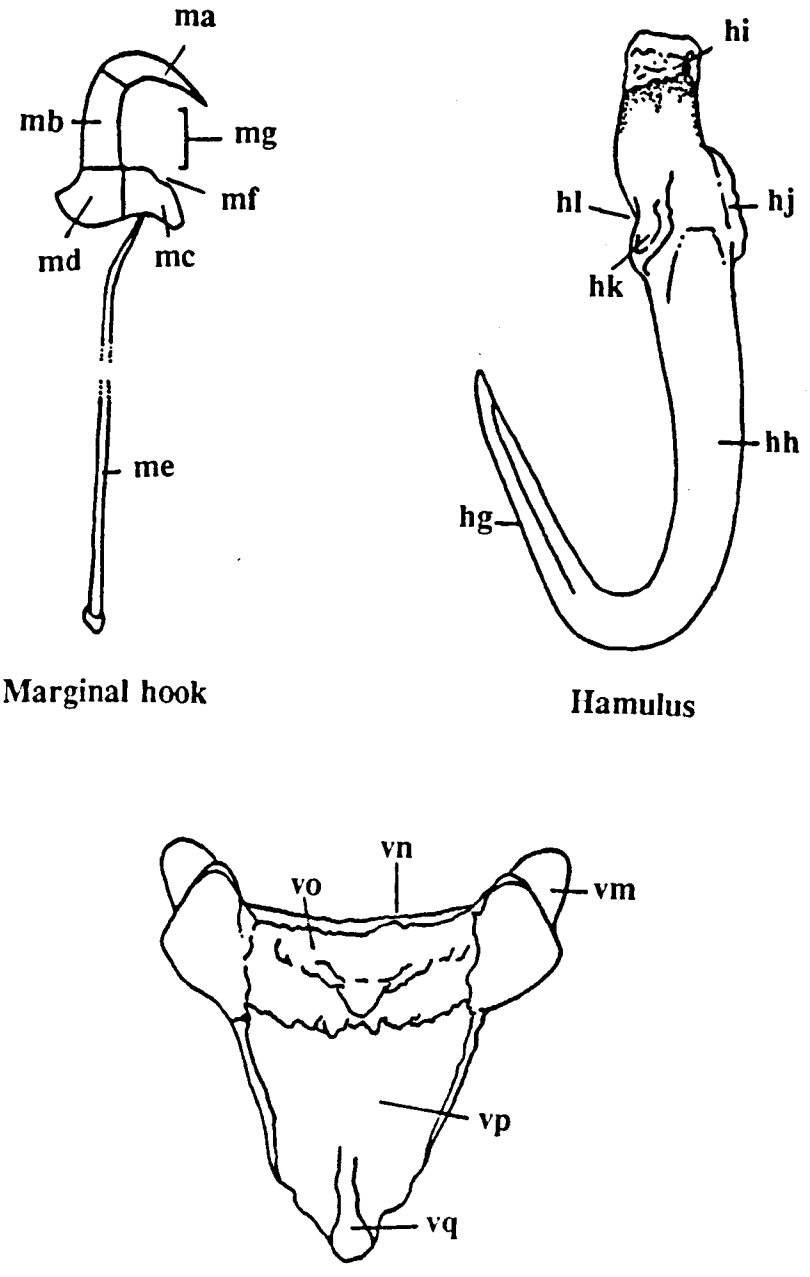


Figure 2.9: Schematic illustration of the sclerite divisions used as a basis of discriminating species of *Gyrodactylus*. See the abbreviations list for the full nomenclature given in Appendix 2.

Gyrodactylus. These are shown in Figures 2.1-2.6. Of the nine species of salmonoid sampled, however, only *Salmo salar*, *S. trutta*, *Oncorhynchus mykiss* and *Salvelinus alpinus* were found to harbour *Gyrodactylus* infections (see Table 2.2). Although attempts were made to sample larger numbers of the other species, unfortunately too few were obtained to make conclusions regarding their role as hosts for *Gyrodactylus*.

In the case of Atlantic salmon 47.2% of the sites were infected: of the 35 farmed sites 31.4% were positive, whereas, of the 73 wild salmon sites, 54.8% were positive. Only wild populations of brown trout were found to be infected (35.7%), whilst 58.8% of the farmed populations of rainbow trout were positive. Of the two sites where Arctic charr were sampled only one was found to have *Gyrodactylus*. The abundance of parasites (total number of parasites divided by the total number of hosts examined) is shown in Table 2.3. The mean intensity (number of parasites divided by the total number of infected hosts) is shown in Table 2.4 and the prevalence (number of infected fish divided by the total number of fish sampled x100) is given in Table 2.5. The maximum intensity of parasite infection observed on a wild fish was 70 individuals on an Atlantic salmon (Table 2.5); however, on farmed fish, a single charr specimen was recorded as having 219 parasites (Table 2.5). The farmed populations represent an artificial system and numbers may have been affected by preventative or therapeutic treatments for parasites or other infections, such that they are not directly comparable. However, when the parasite numbers from wild salmon and wild brown trout are compared (see Table 2.6), there appears to be very little difference. The data for salmon and trout co-existing within the same river system are given in Table 2.7 and suggests that where one salmonid population is infected with *Gyrodactylus*, it is likely that a second salmonid population is also infected.

Characterisation

Initial studies using the light microscope indicated that there were five morphotypes parasitising the salmonids in the samples. However, closer examination of the *Gyrodactylus* specimens suggested that there were seven morphotypes native to UK salmonids.

The seven morphotypes found were as follows:

Table 2.2: UK site survey data.

Species	Number of sites examined		Sites positive for *Gyrodactylus*			
	Farmed	Wild	Farmed		Wild	
			No.	%	No.	%
Salmo salar	35	73	11	31.4	40	54.8
Salmo trutta	6	78	-	-	28	35.9
Oncorhynchus mykiss	17	5	10	58.8	-	-
Salvelinus alpinus	1	1	1	100	-	-
Salmo trutta trutta	2	4	-	-	-	-
Coregonus lavaretus	-	1	-	-	-	-
Salvelinus fontinalis	2	-	-	-	-	-
Osmerus eperlanus	-	1	-	-	-	-
Thymallus thymallus	-	1	-	-	-	-

Table 2.3: Parasite abundance.

Species	No. of fish examined	No. of parasites	Abundance
Salmo salar	413 (Farmed)	160	0.39
	330 (Wild)	585	1.77
Salmo trutta	70 (Farmed)	0	0.00
	402 (Wild)	765	1.90

Table 2.4: Parasite intensity.

Species	No. of infected fish		No. of parasites		Mean intensity	
	wild	farmed	wild	farmed	wild	farmed
Salmo salar	72	24	588	160	6.3	6.7
Salmo trutta	98	-	765	0	7.8	0.0
Oncorhynchus mykiss	-	93	-	292	0.0	3.1
Salvelinus alpinus	-	11	-	404	0.0	36.7

Table 2.5: Parasite prevalence and abundance.

Species	No. of fish examined	Prevalence	Abundance	Range	Mean intensity
Salmo salar	330 (W)	28.2	1.77	1 - 70	6.29
	413 (F)	5.8	0.39	1 - 23	6.75
	743 (T)	15.1	1.00		6.39
Salmo trutta	402 (W)	24.4	1.90	1 - 48	7.81
	70 (F)	0.0	0.00	-	0.00
	472 (T)	20.8	1.62		7.81
Oncorhynchus mykiss	17 (W)	0.0	0.00	-	0.00
	271 (F)	34.3	1.11	1 - 75	3.14
	288 (T)	32.4	1.01		3.14
Salvelinus alpinus	3 (W)	0.0	0.00	-	0.00
	13 (F)	92.3	31.08	1 - 219	33.67
	16 (T)	75.0	25.25		33.67

Abbreviations: (W) = wild fish; (F) = farmed fish; (T) = wild + farmed fish.

Table 2.6: A comparison of the abundance of *Gyrodactylus* on wild salmon and brown trout.

Species	No. of fish examined	No. of parasites	Abundance
Salmo salar	330	585	1.77
Salmo trutta	402	765	1.90

Table 2.7: Table of wild sample sites with mixed salmonid populations illustrating that where one salmonid species within a river is infected with *Gyrodactylus*, a second species present is also infected and vice versa.

Host and *Gyrodactylus* presence	No of sites
Salmo salar (+ve) & *Salmo trutta* (+ve)	13
Salmo salar (-ve) & *Salmo trutta* (+ve)	4
Salmo salar (+ve) & *Salmo trutta* (-ve)	3
Salmo salar (-ve) & *Salmo trutta* (-ve)	9

Morphotype	Host
1	Atlantic salmon, brown trout, rainbow trout, and Arctic charr
2	Atlantic salmon
3	Atlantic salmon
4	Brown trout
5	Brown trout
6	Arctic charr
7	Arctic charr

Data from some of the species of *Gyrodactylus* obtained from other collections for example, *G. salaris*, *G. derjavini* and *G. colemanensis* were introduced for comparison with the species found in the U.K and were used as reference points.

The findings of the light microscope (LM) and scanning electron microscope studies (SEM) will be considered together.

Morphotypes

Morph 1:

Host: wild *Salmo salar*; wild *S. trutta*; hatchery reared *O. mykiss*; *Salvelinus alpinus* (hatchery reared from original wild broodstock, Lake Ennerdale).
Number of specimens measured: 241 (L); 69 marginal hooks; 45 hamuli (SEM)

Specimens collected from the River Beukaforsen, Sweden, identified by Dr Malmberg as *G. derjavini* were used as standards for comparisons with the collections. Figures 2.10 and 2.11 show the marginal hooks and the hamuli liberated from Swedish *G. derjavini*, whilst Figures 2.12 and 2.13 show the sclerites released from Morph 1 on Welsh salmon and Figures 2.14-2.16 from farmed rainbow trout.

The marginal hooks for each of the British gyrodactylids were compared with all of the salmonoid species of *Gyrodactylus* and are illustrated in Figure 2.17, thus the subtle differences in marginal hook form are clearly shown. In *G. derjavini* Mikailov, 1975 and Morph 1 the overall morphology of the marginal sickle proper constitutes a very robust structure, more so than that of either *G. salaris* Malmberg,

1957 (shown in Figure 2.26-2.28) or *G. truttae* Gläser, 1974 (Morph 4, shown in Figure 2.21e-j). The heel of the sickle proper (see Figure 2.10b or 2.21d), as in *G. truttae* and Morph 4 (Figure 2.21h), is pronounced; however, the base of the marginal sickle forming the toe and heel appears deeper in *G. derjavini*, resulting in a more rounded heel, whilst that of *G. truttae* and Morph 4 have a longer heel in relation to that of *G. derjavini*.

The shaft and point of the marginal hook sickle proper is broad, the point stopping abruptly in line or just beyond the toe of the sickle proper to give a stout, deep blade. The toe of the sickle proper is very triangular in shape, its base being in line with the attachment point between the sickle proper and the marginal hook shaft, and in some cases the toe is marked by an indentation on the upper surface (this is arrowed in Figure 2.10d for example). A sickle membrane is visible in preparations for the light microscope as seen in Figure 2.25h; however, in the specimens extracted by the enzyme digestion technique (Figure 2.10) this structure has been lost. This morph clearly fits the characters of *G. derjavini* and represents the major form found to parasitise Atlantic salmon in England, S. Wales and S.W. Ireland.

The morphometrics for the light based studies are given in Tables 2.8-2.9, whilst data obtained for the hamuli and marginal hooks from electron microscope studies which is much more accurate are given in Tables 2.10 and 2.11, respectively. The marginal hooks of Morph 1 were also recovered from *Gyrodactylus* parasitic on brown trout, these are shown in Figure 2.21a-d and on Arctic charr as shown in Figure 2.25g-h. Figure 2.12k represents an unusual marginal hook recovered from an unidentified species of *Gyrodactylus* from an otherwise wholly Morph 1 population.

The ventral bars of Morph 1 parasitising rainbow trout are shown in Figure 2.16. The ventral bar shows a high degree of variability in overall morphology; however, the medial ridge in the membrane of Morph 1 is more noticeable than in Morph 4 (*G. truttae*) (Figure 2.23) and the membrane appears more pointed and elongate compared to the rounded and shorter membrane in Morph 4 (*G. truttae*).

It is believed that Morph 1 represents *G. derjavini* Mikailov, 1975, being identical to the standards in the form of the sclerites.

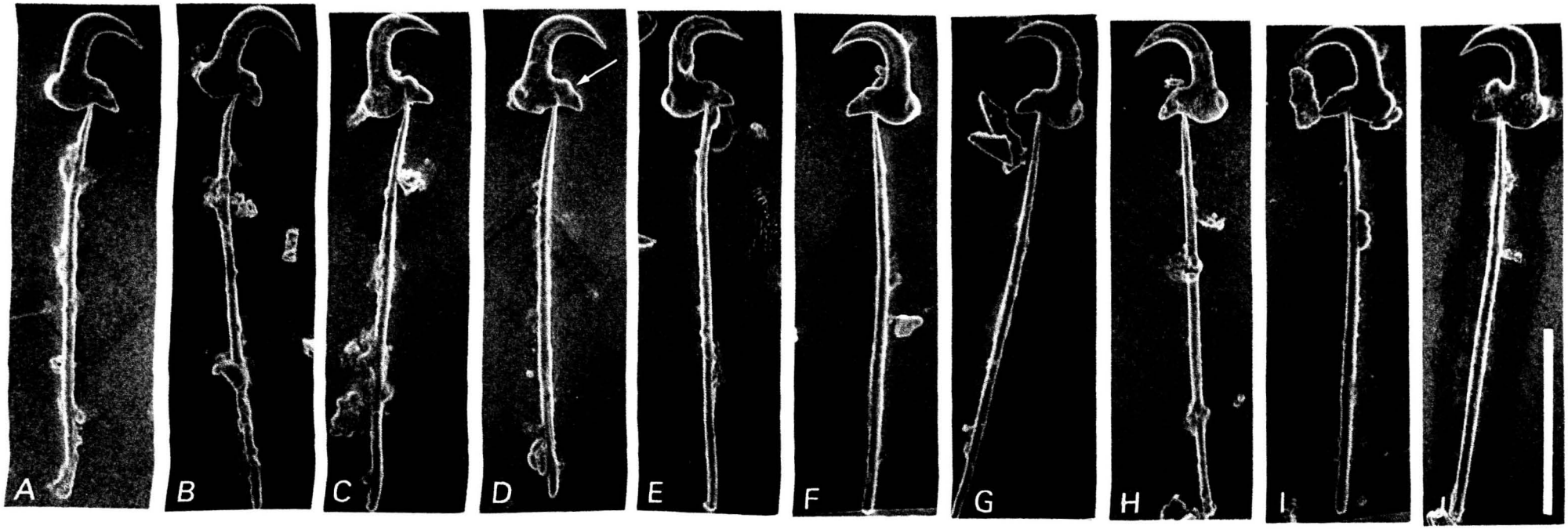


Figure 2.10: *G. derjavini* Mikailov, 1975 marginal hooks from the River Beukaforsen, Sweden brown trout extracted by digestion. Scale bar: 12.0 µm. The arrow indicates the indentation on the upper surface of the marginal sickle toe.

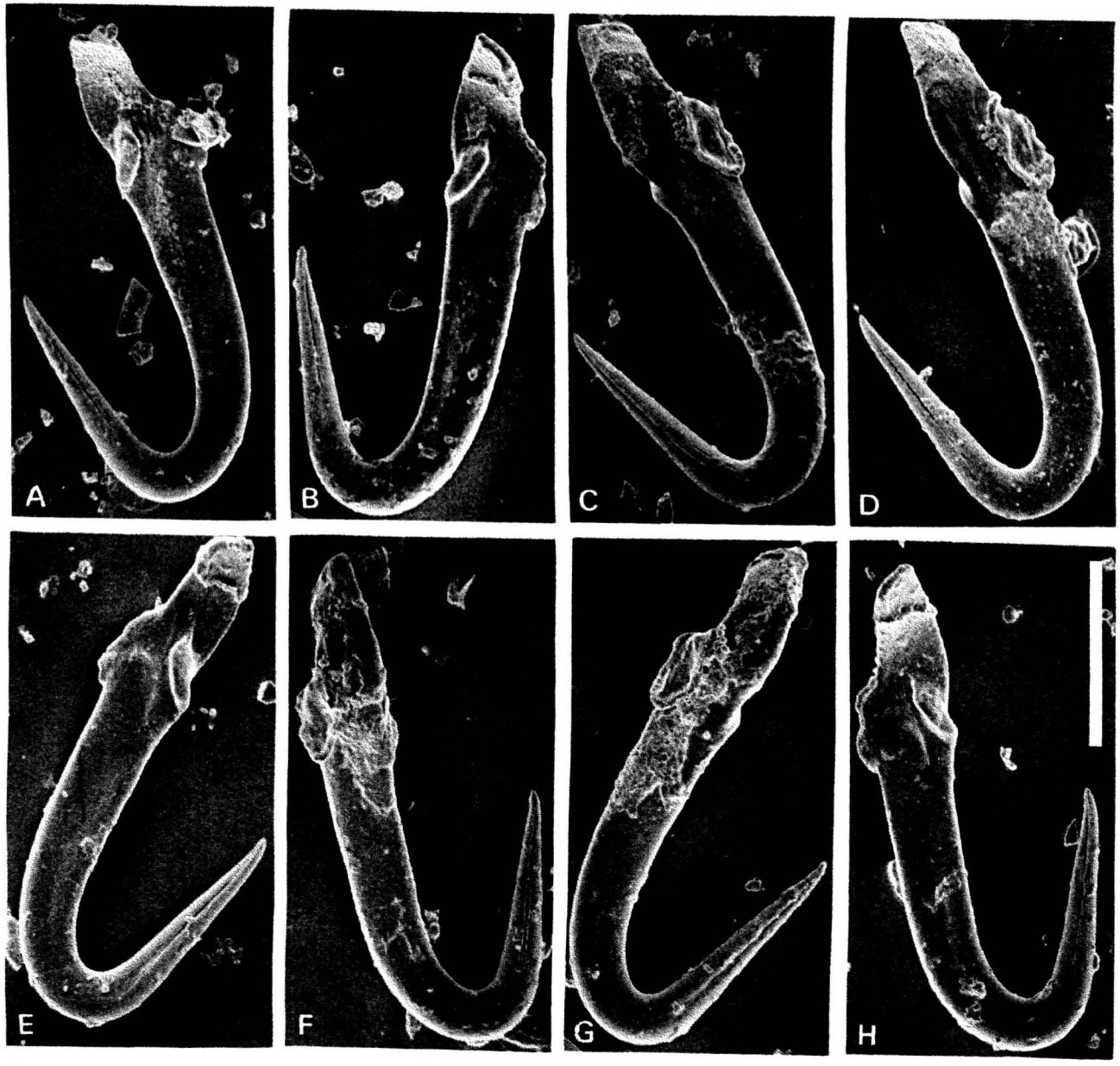


Figure 2.11: *G. derjavini* Mikailov, 1975 hamuli from the River Beukaforsen, Sweden brown trout extracted by digestion. Scale bar: 20.0 μm.

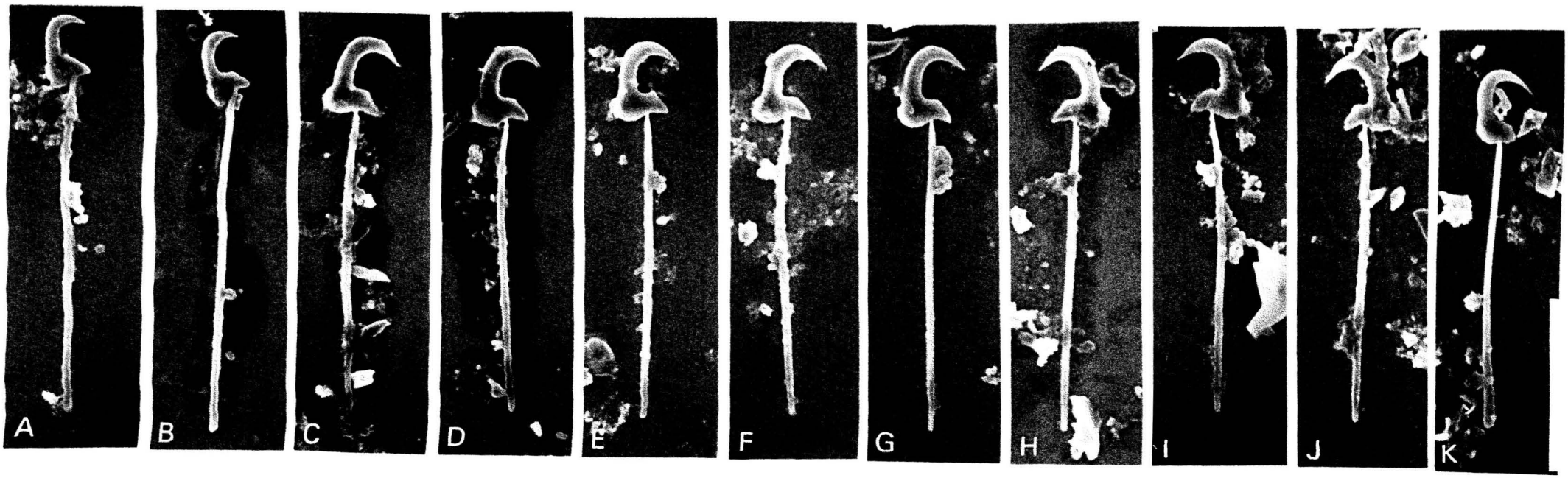


Figure 2.12: Morph 1 marginal hooks from Mags Yr Afon, Wales salmon extracted by digestion. Scale bar: 13.6 μm.

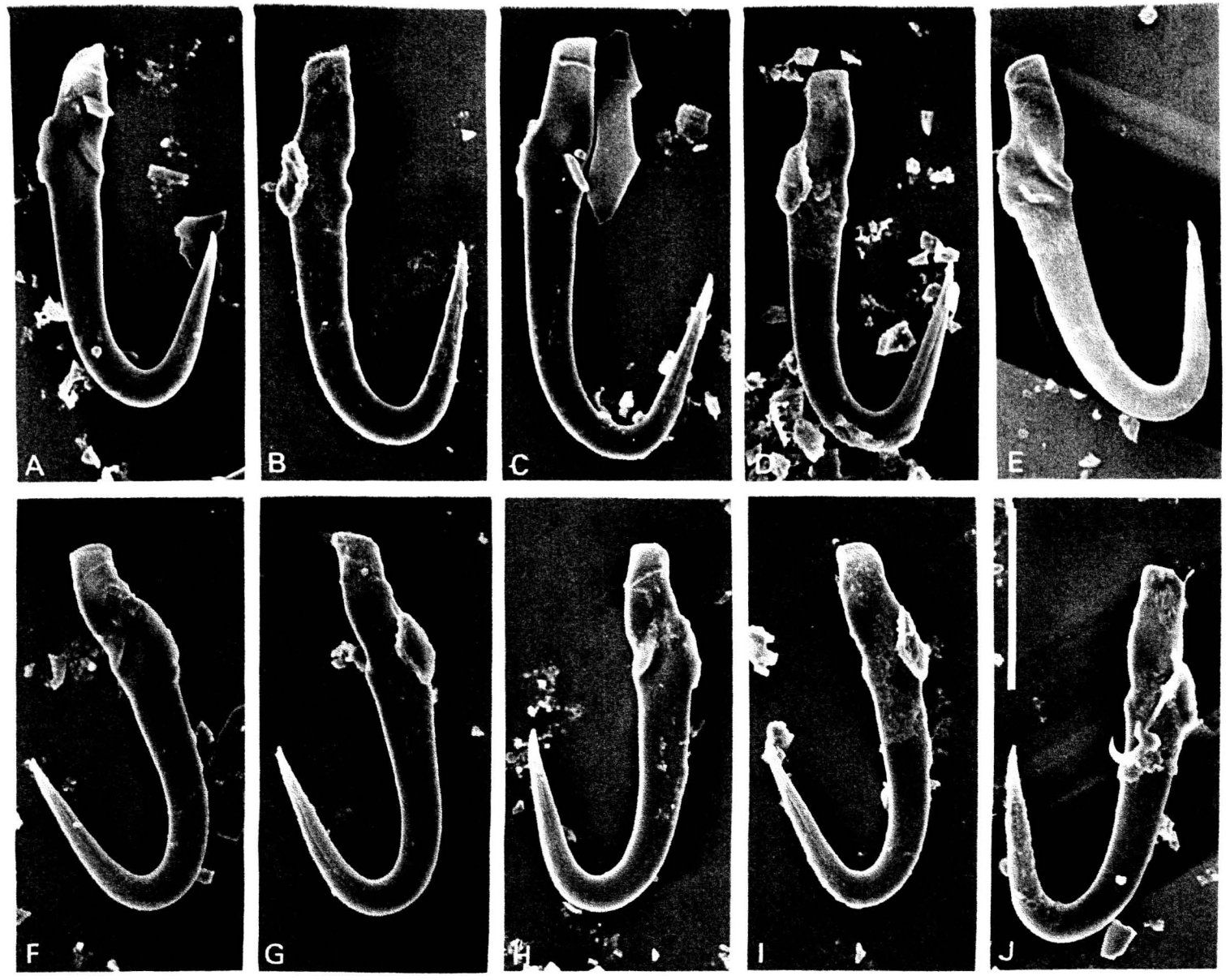


Figure 2.13: Morph 1 hamuli from Mags Yr Afon, Wales salmon extracted by digestion. Scale bar: 23.1 μm.

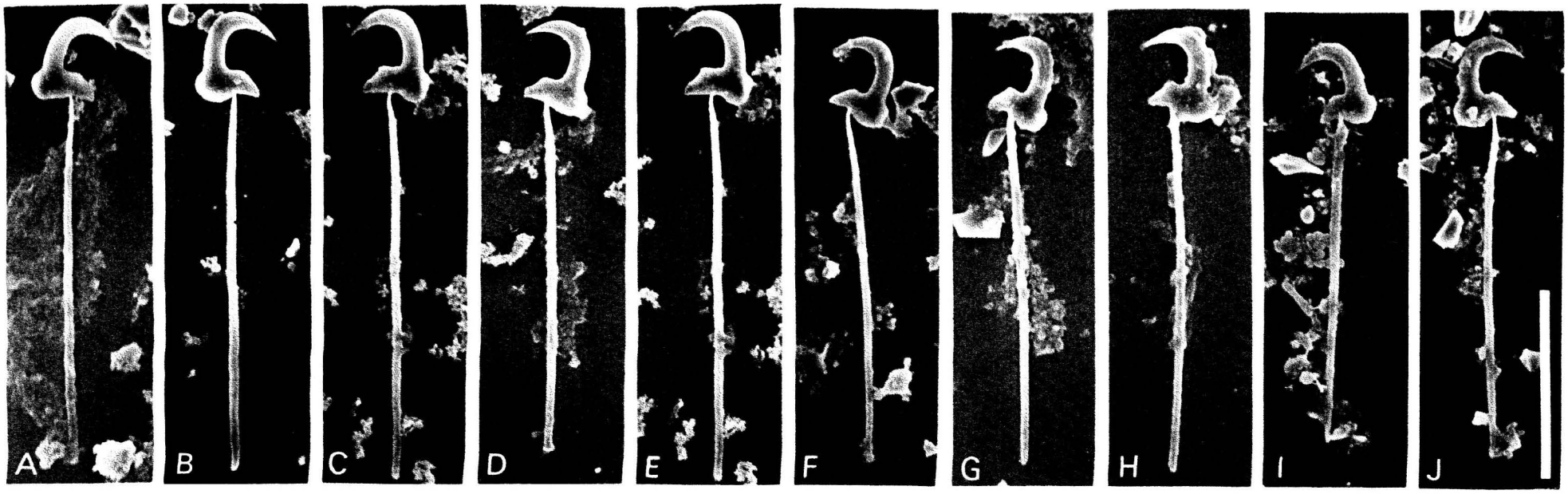


Figure 2.14: Morph 1 marginal hooks extracted by digestion from two Scottish rainbow trout farms (Loch Awe & R. South Esk). Scale bar: 13.6 μm.

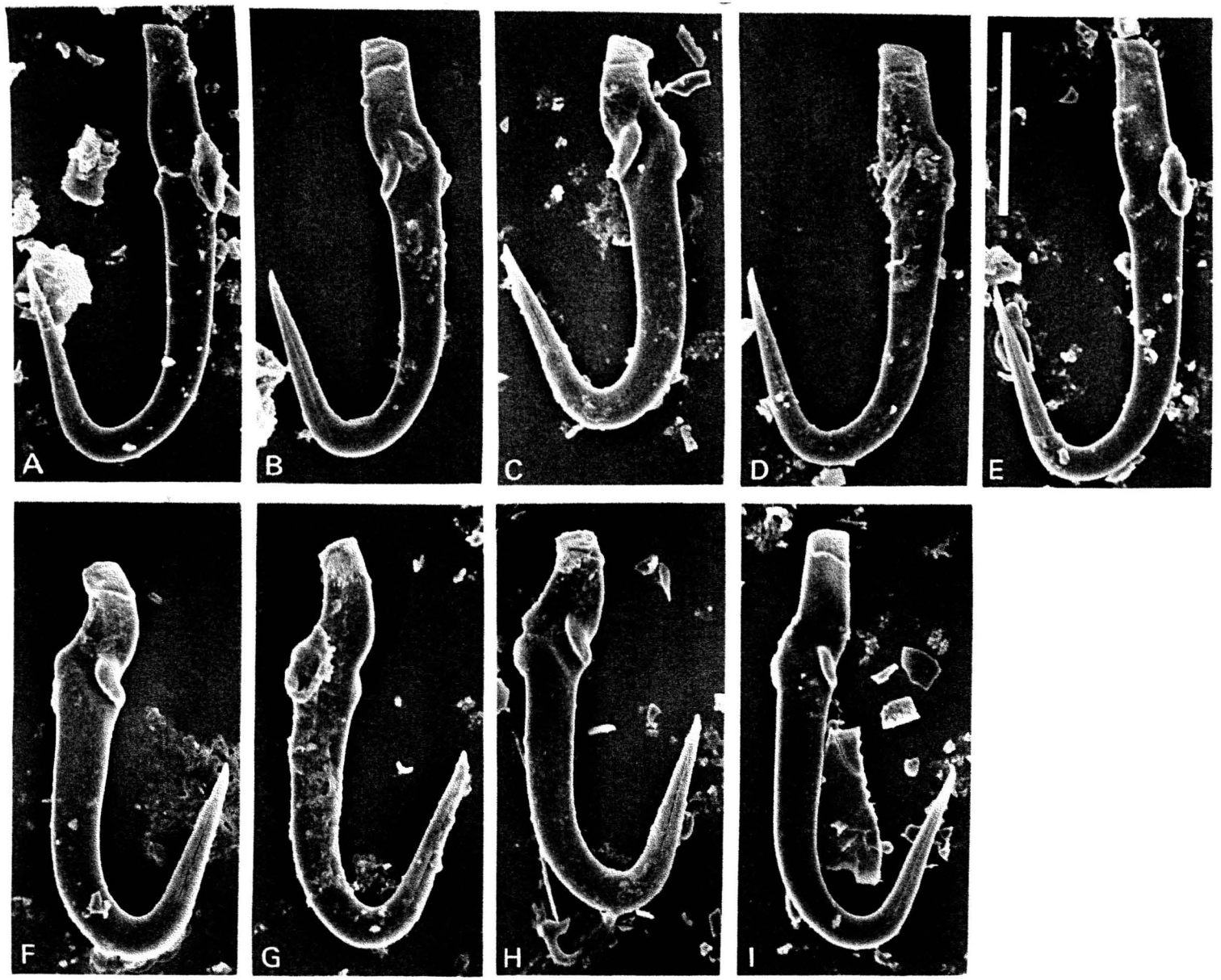


Figure 2.15: Morph 1 hamuli extracted by digestion from two Scottish rainbow trout farms (Loch Awe & R. South Esk). Scale bar: 23.1 μm.

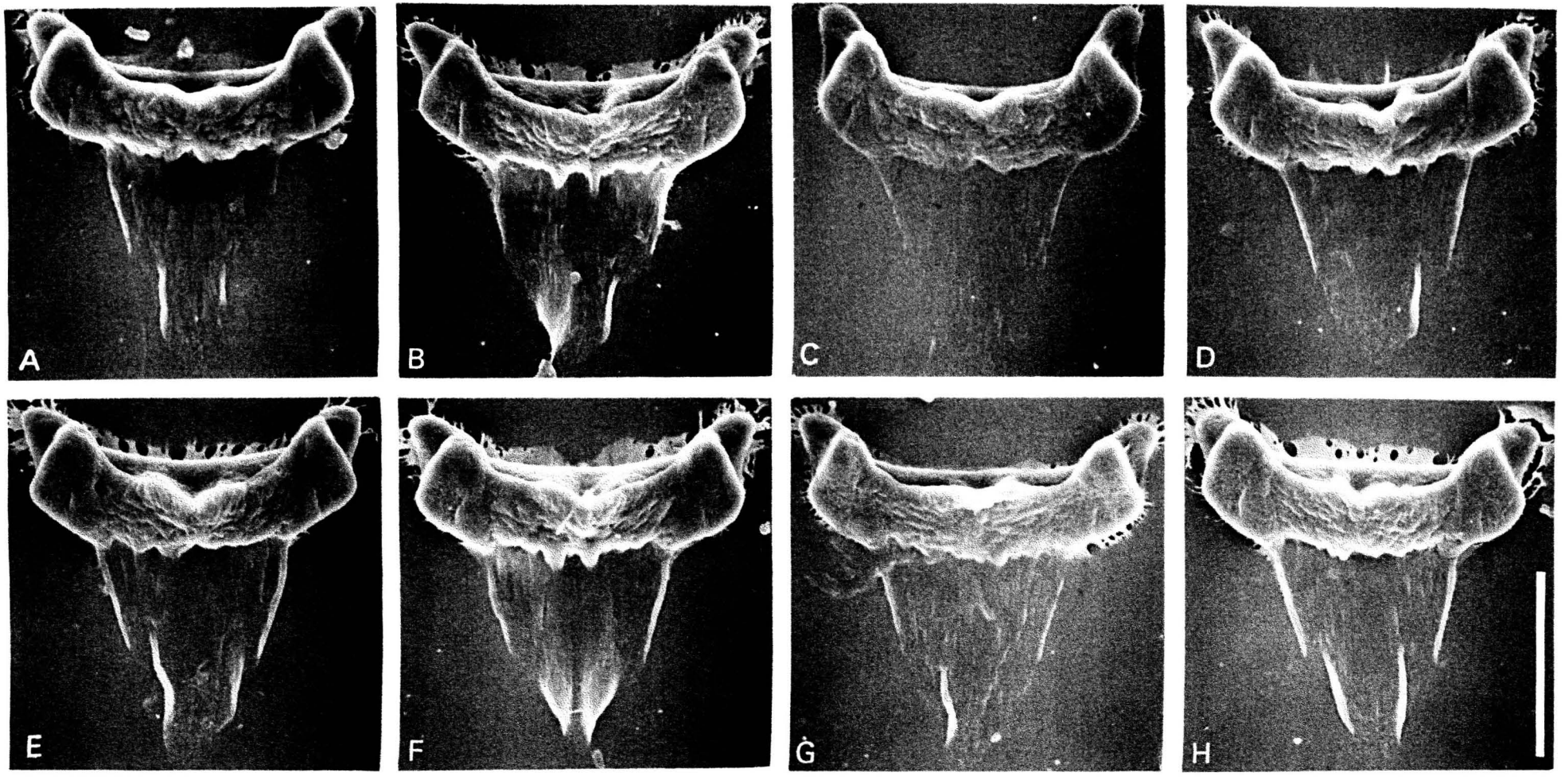


Figure 2.16: Morph 1 ventral bars extracted by sonication from Loch Awe, Scotland farmed rainbow trout. Scale bar: 13.6 μm.

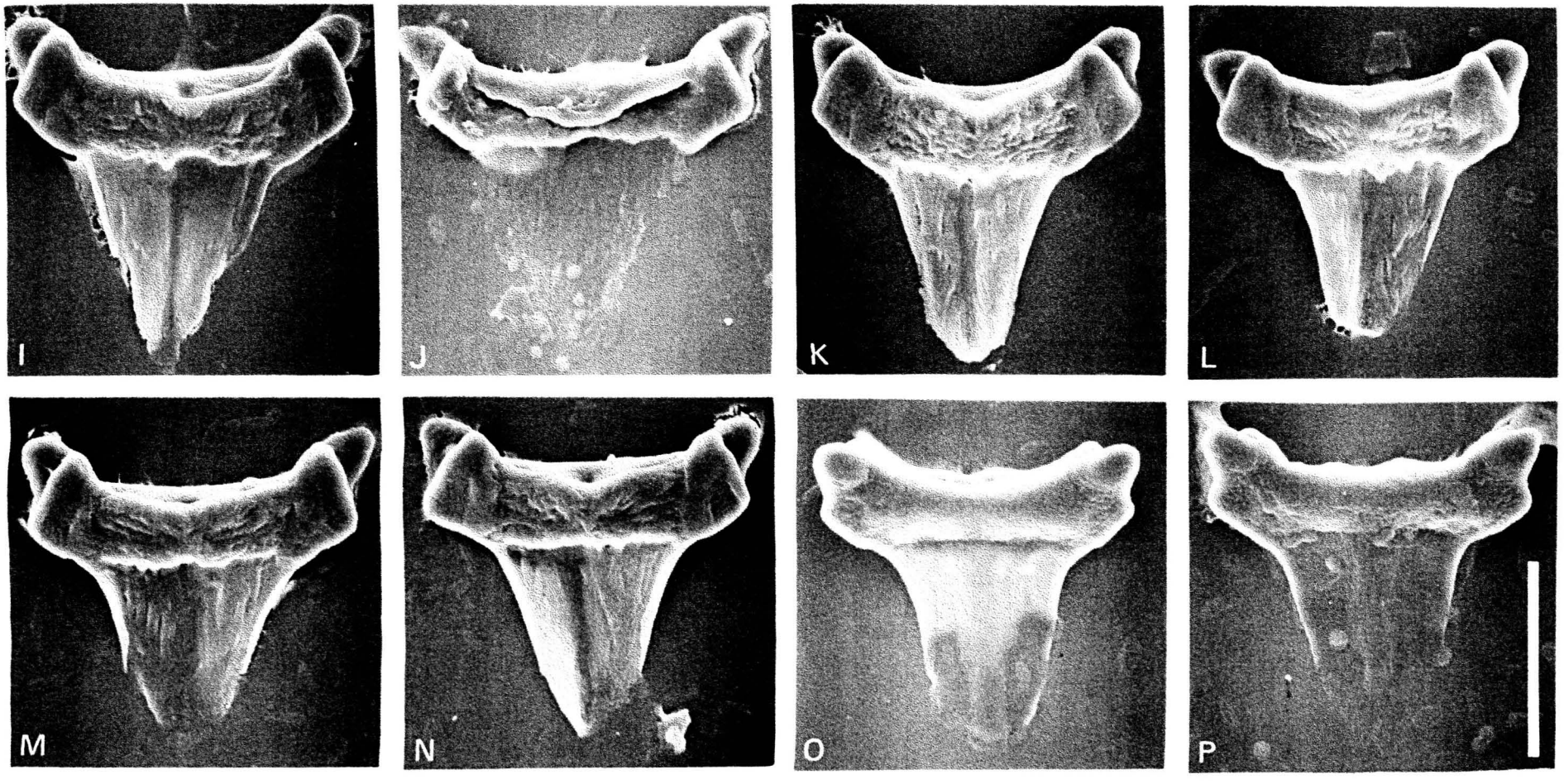


Figure 2.16: Morph 1 ventral bars extracted by sonication from Loch Awe, Scotland farmed rainbow trout. Scale bar: 13.6 μm.

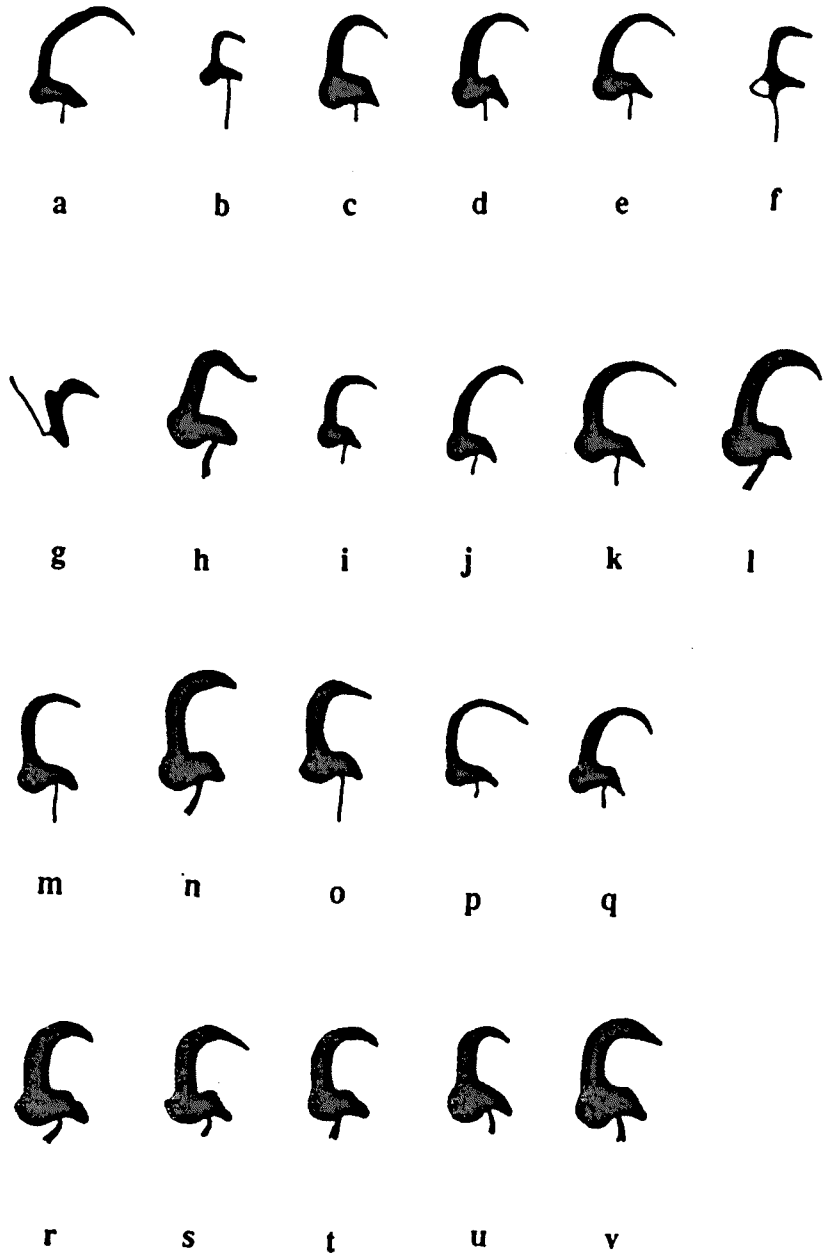


Figure 2.17: Marginal hooks of the salmonoid species of *Gyrodactylus* with particular attention to those found in the UK.

Key:

a = *G. asiaticus* Ergens, 1978
b = *G. avalonia* Hanek & Threlfall, 1969
c = *G. birmani* Konovalov, 1967
d = *G. bohemicus* Ergens, 1992
e = *G. brachymystacis* Ergens, 1978
f = *G. brevis* Crane & Mizelle, 1967
g = *G. bychowski* Sproston, 1946
h = *G. colemanensis* Mizelle & Kritsky, 1967
i = *G. lavareti* Malmberg, 1978
j = *G. lenoki* Gussev, 1953
k = *G. magnus* Konovalov, 1967
l = *G. masu* Ogawa, 1986
m = *G. nerkae* Cone *et al.*, 1983
n = *G. salaris* Malmberg, 1957
o = *G. salmonis* Yin & Sproston, 1948
p = *G. taimeni* Ergens, 1971
q = *G. thymalli* Zitnan, 1960
r = *G. derjavini* Mikailov, 1975 and Morph 1 & 3
s= *G. truttae* Gläser, 1974 and Morphs 4 & 5
t = *Gyrodactylus* sp. (Morph 2)
u = *Gyrodactylus* sp. (Morph 6)
v = *Gyrodactylus* sp. (Morph 7)

Table 2.8: Sclerite dimensions of *Gyrodactylus* Morph 1 and Morph 3 collected from Atlantic salmon (*Salmo salar*) using LM based studies.

Variable	Morph 1 from other British salmon			Morph 3 from L. Coulin & Tralaig salmon		
	Mean	St.dev.	Range	Mean	St.dev.	Range
Hamulus:						
Total length	56.1	4.0	46.9 - 65.6	74.6	0.8	73.5 - 76.2
Shaft length	40.1	2.7	32.5 - 48.1	53.1	1.9	50.6 - 56.1
Point length	28.1	2.2	23.8 - 33.4	32.8	1.6	30.0 - 35.6
Root length	16.6	2.1	12.5 - 23.1	21.8	1.4	20.5 - 23.7
Dorsal bar:						
Total length	24.5	3.2	16.3 - 33.8	36.1	3.3	30.8 - 40.3
Width	2.4	0.3	1.3 - 3.3	3.4	0.5	2.4 - 4.0
Ventral bar:						
Total length	27.7	2.0	21.9 - 36.9	34.6	0.8	34.0 - 35.6
Total width	24.0	1.9	18.8 - 28.1	26.2	2.5	23.7 - 30.0
Middle width	6.8	1.0	4.4 - 10.0	8.9	0.5	7.9 - 9.5
Process-Middle length	3.0	0.4	1.3 - 3.8	3.7	0.5	3.2 - 4.7
Process length	3.3	0.9	0.6 - 5.0	5.0	0.5	4.0 - 5.5
Membrane length	13.9	1.5	9.4 - 18.2	17.5	2.4	14.2 - 21.3
Marginal hook:						
Total length	31.3	1.9	25.0 - 35.6	42.3	0.9	40.3 - 43.5
Shaft length	25.4	1.7	20.0 - 30.0	34.4	1.0	32.4 - 35.6
Sickle length	6.7	0.4	5.6 - 7.7	8.7	0.1	8.7 - 9.1
Sickle dorsal width	4.8	0.3	3.8 - 5.6	5.9	0.4	5.5 - 6.3
Sickle proximal width	5.1	0.3	4.1 - 5.6	6.4	0.3	5.9 - 6.7
Filament loop	12.1	1.1	6.3 - 17.5	15.6	0.5	15.0 - 16.6
Number measured	168			10		

Table 2.9: Sclerite dimensions of *Gyrodactylus* Morph 1 from two populations of Atlantic salmon (*Salmo salar*) in the U.K. using LM based studies.

Variable	*Gyrodactylus* sp. from Scottish salmon			*Gyrodactylus* sp. from Welsh salmon		
	Mean	St.dev.	Range	Mean	St.dev.	Range
Hamulus:						
Total length	58.1	3.7	49.7 - 65.6	52.7	2.7	46.9 - 59.4
Shaft length	41.4	2.5	36.3 - 48.1	37.7	1.7	32.5 - 41.3
Point length	29.2	1.9	24.4 - 33.4	26.2	1.4	23.8 - 29.4
Root length	17.2	2.1	12.5 - 23.1	15.7	1.8	12.5 - 19.4
Dorsal bar:						
Total length	26.1	2.9	19.4 - 33.8	22.0	2.4	16.3 - 27.8
Width	2.4	0.3	1.3 - 3.3	2.4	0.3	1.3 - 2.8
Ventral bar:						
Total length	28.4	1.7	23.8 - 36.9	26.6	1.7	21.9 - 30.0
Total width	24.6	2.0	20.6 - 28.1	22.9	1.4	18.8 - 26.3
Middle width	7.3	0.9	5.0 - 10.0	6.3	0.7	4.4 - 8.8
Process-Middle length	3.0	0.4	1.3 - 3.8	2.9	0.4	2.2 - 3.8
Process length	3.4	1.1	0.6 - 5.0	3.0	0.7	1.9 - 5.0
Membrane length	13.9	1.5	10.6 - 18.2	13.7	1.4	9.4 - 16.3
Marginal hook:						
Total length	32.3	1.7	28.1 - 35.6	29.7	1.2	25.0 - 31.9
Shaft length	26.3	1.6	22.5 - 30.0	24.1	1.1	20.0 - 26.3
Sickle length	6.8	0.4	5.6 - 7.6	6.4	0.4	5.6 - 7.5
Sickle dorsal width	4.8	0.3	4.1 - 5.6	4.7	0.3	3.8 - 5.0
Sickle proximal width	5.1	0.3	4.4 - 5.6	5.0	0.3	4.1 - 5.6
Filament loop	12.1	1.0	8.8 - 17.5	12.1	1.1	6.3 - 13.8
Number measured	86			58		

Table 2.10: Marginal hook dimensions for several species of *Gyrodactylus* (Morph 1) parasitising salmonids measured from electronmicrographs.

Variable	Morph 1 from *Salmo salar* Mean	St.dev.	Range	Morph 1 from *Oncorhynchus mykiss* Mean	St.dev.	Range
Proximal width	4.850	0.467	3.46 - 5.58	4.369	0.668	3.23 - 5.58
Toe length	1.926	0.178	1.62 - 2.23	1.756	0.338	1.21 - 2.34
Distal width	4.947	0.537	3.39 - 5.78	4.553	0.682	3.32 - 5.41
Sickle length	6.818	0.636	5.37 - 7.84	6.416	0.605	5.43 - 7.14
Sickle aperture distance	5.292	0.343	4.83 - 6.07	4.895	0.497	4.10 - 5.84
Total length	32.485	2.667	28.80 - 37.22	31.834	1.806	26.22 - 35.35
Shaft length	26.338	2.332	23.13 - 30.70	26.143	1.657	20.81 - 28.19
Number of specimens measured	21			30		

Variable	Morph 1 from *Salmo trutta* (British) Mean	St.dev.	Range	*G. derjavini* from *Salmo trutta* (Swedish) Mean	St.dev.	Range
Proximal width	4.878	0.246	4.49 - 5.27	4.792	0.154	4.52 - 5.08
Toe length	1.854	0.124	1.70 - 2.02	1.928	0.127	1.77 - 2.18
Distal width	5.173	0.380	4.68 - 5.86	5.144	0.180	4.80 - 5.41
Sickle length	6.924	0.110	6.80 - 7.12	6.821	0.199	6.57 - 7.28
Sickle aperture distance	5.253	0.187	4.99 - 5.58	5.293	0.166	5.10 - 5.57
Total length	32.506	0.984	30.91 - 34.05	32.562	0.553	31.48 - 33.47
Shaft length	26.356	0.791	25.19 - 27.45	26.501	0.550	25.46 - 27.45
Number of specimens measured	8			10		

Table 2.11: Hamuli dimensions for several species of *Gyrodactylus* (Morph 1) parasitising salmonids measured from electronmicrographs.

Variable	Morph 1 from *Salmo salar* (Scottish) Mean	St.dev.	Range	Morph 1 from *Salmo salar* (Welsh) Mean	St.dev.	Range
Shaft-point length	18.889	1.419	16.71 - 22.44	14.789	1.435	13.31 - 18.45
Hamulus angle	36.131	2.083	31.24 - 38.59	38.335	2.079	35.56 - 42.69
Point length	32.174	1.201	29.86 - 34.72	25.898	0.843	24.47 - 27.34
Shaft length	39.553	2.412	35.16 - 43.59	31.170	1.999	29.02 - 35.87
Total length	61.562	4.861	53.29 - 70.42	48.860	2.356	46.12 - 53.96
Root length	25.034	2.603	22.01 - 30.08	21.206	1.293	19.47 - 23.07
Hamulus width	8.635	1.235	7.28 - 11.82	6.986	0.307	6.45 - 7.37
Number of specimens measured	15			30		

Variable	Morph 1 from Swedish *Salmo trutta* Mean	St.dev.	Range	Morph 1 from *O. mykiss* (Scottish) Mean	St.dev.	Range
Shaft-point length	15.477	0.623	14.23 - 16.16	17.194	1.201	14.06 - 18.56
Hamulus angle	36.910	1.477	35.54 - 38.29	37.835	2.270	32.33 - 40.92
Point length	28.630	0.649	27.13 - 29.15	29.602	1.617	26.62 - 32.42
Shaft length	33.723	0.603	32.59 - 34.60	36.070	2.253	32.30 - 40.45
Total length	53.049	1.530	49.80 - 55.12	56.106	3.163	49.56 - 61.58
Root length	22.381	1.355	19.38 - 23.95	23.265	2.130	19.87 - 26.42
Hamulus width	7.796	0.374	7.22 - 8.52	7.521	0.726	6.47 - 8.75
Number of specimens measured	10			30		

Morph 2:

Host: wild and farmed *Salmo salar* (Scotland, N. England, N. Ireland)

Number of specimens measured: No light microscope measurements taken; 20marginal hooks; 9 hamuli (SEM).

The morphology of the marginal hook of Morph 2 shown in Figure 2.18 differs slightly from Morph 1 (*G. derjavini*). The heel of Morph 2 is less pronounced, less rounded, giving the impression that the base of the sickle proper is not as deep as in Morph 1. The marginal sickle proper differs from Morph 1 (*G. derjavini*), Morph 2 has a flat base whilst that of *G. derjavini* undulates, arching in the centre. Although the shaft and point of the sickle proper follow the same curve as in *G. derjavini* (Morph 1), these parts are more slender, such that the shaft appears longer and the aperture of the sickle more open. The point of the sickle proper is in line with the toe, such that the curve describing the inside shape of the sickle proper is quite square, whereas it is more rounded in Morph 1 (*G. derjavini*) (Figure 2.10). The measurements for the marginal hook are given in Table 2.12. The morphology of these two marginal hooks are compared in Figure 2.17.

The hamuli (Figure 2.19) are indistinguishable from those of Morph 1 (*G. derjavini*) (Figure 2.11), the ranges of measurements coinciding with those of *G. derjavini* and *G. truttae* (Table 2.13). The ventral bars released from Morph 2 parasitising Atlantic salmon in the River Allan, Stirling, are shown in Figure 2.20. The ventral bar processes are more pointed and more elongate than those of Morph 4 (*G. truttae*). The membrane also appears thinner, although to what extent this is an artefact of the sonication technique is unknown.

Morph 2 is most closely related to *G. derjavini* Mikailov, 1975 in the shape of the anchors and ventral bar and is close to *G. salaris* Malmberg, 1957, but it can be readily distinguished from both these species by the shape of the marginal hook.

Morph 3:

Host: *Salmo salar* (L. Coulin & L. Tralaig, Scotland)

Number of specimens measured: 10 (LM); no specimens examined with the SEM

Collections of this morphotype were made in 1987 from farmed Atlantic salmon. The morphology of the sclerites closely resembles that of Morph 1 (*G. derjavini*) but can be isolated on the basis of size alone. This represents the largest British *Gyrodactylus* specimens found, the hamuli exceeding 75µm in length and the marginal hooks 40µm in length. Although, these sites were resampled on several occasions, no further *Gyrodactylus* specimens were found with which to make an SEM study. This morph is most closely related to Morph 1 (*G. derjavini*), differing only in its large size. Table 2.8 compares the sclerite dimensions of Morph 3 with those of *Gyrodactylus* from all other UK salmon.

Morph 4:

Hosts: Wild *Salmo trutta* (England, Scotland and Wales).

Number of specimens measured: 117 (LM); 16 marginal hooks, 10 hamuli (SEM).

The marginal hook is notable for its prominent, long heel (Figures 2.21e-j, 2.24). The point and shaft of the sickle proper are slender throughout their lengths, the point tapering well beyond the toe. The morphology of the marginal hooks of this morph closely resembles that of *G. truttae* Gläser, 1974. The aperture or face of the sickle proper, as defined by the distance between the sickle point and the sickle toe, is more open in *G. truttae* and Morph 4 as a result of a longer shaft portion to the sickle shaft, although the extent to which the point drops on the curve is apparently the same in both Morph 1 (*G. derjavini*) and Morph 4 (see Figure 2.21e-j). The toe is very slender, angular in shape and drops below the point of attachment of the sickle proper with the marginal hook shaft: this is also seen in Figure 2.23 d-j for Morph 5. A sickle membrane is present.

The hamuli are shown in Figure 2.22 and are virtually indistinguishable from Morph 1 (*G. derjavini*) (Figure 2.11). The ventral bars of Morph 4 (Figure 2.23) are seen to possess a broader median portion to the ventral bar proper than occurs in Morph 1. Furthermore, the presence of a transverse depression between the ventral bar processes, giving the ventral bar a "stepped" appearance (Figure 2.23g, k) is seen only occasionally in Morph 4 but is more obvious in Morph 1. The dimensions of the opisthaptoral sclerites for LM observations are given in Table 2.14 and for SEM

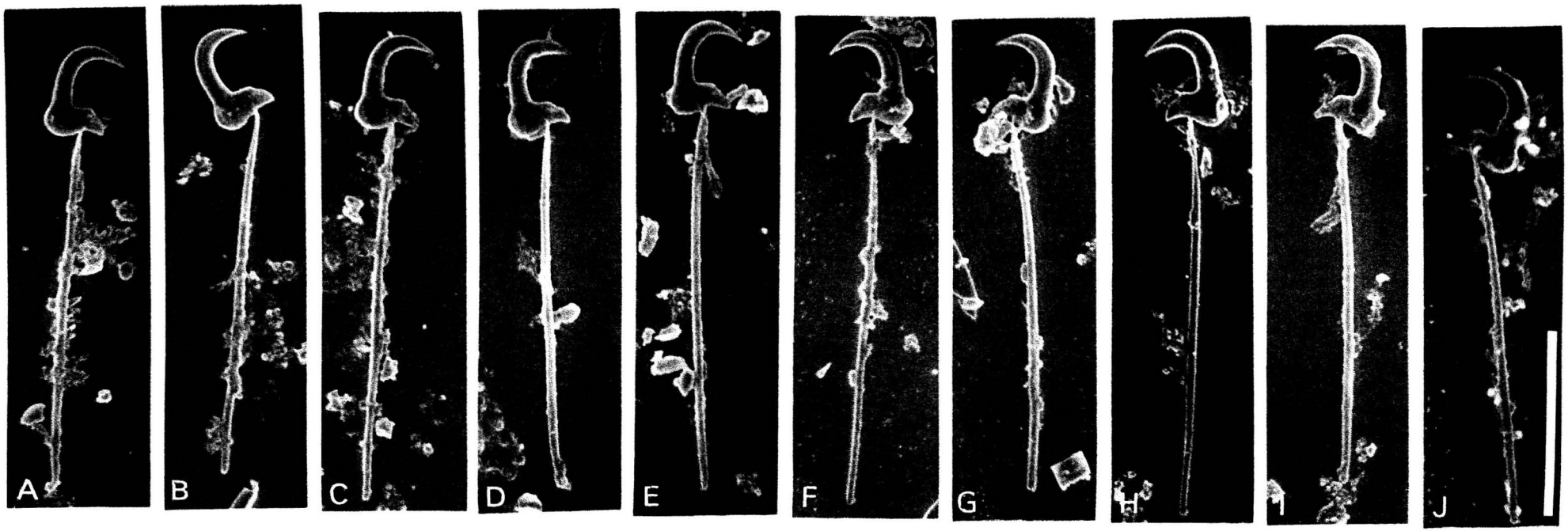


Figure 2.18: *Gyrodactylus* sp. (Morph 2) marginal hooks extracted by digestion from the River Snizort, Isle of Skye, Scotland salmon. Scale bar: 13.6 μm.

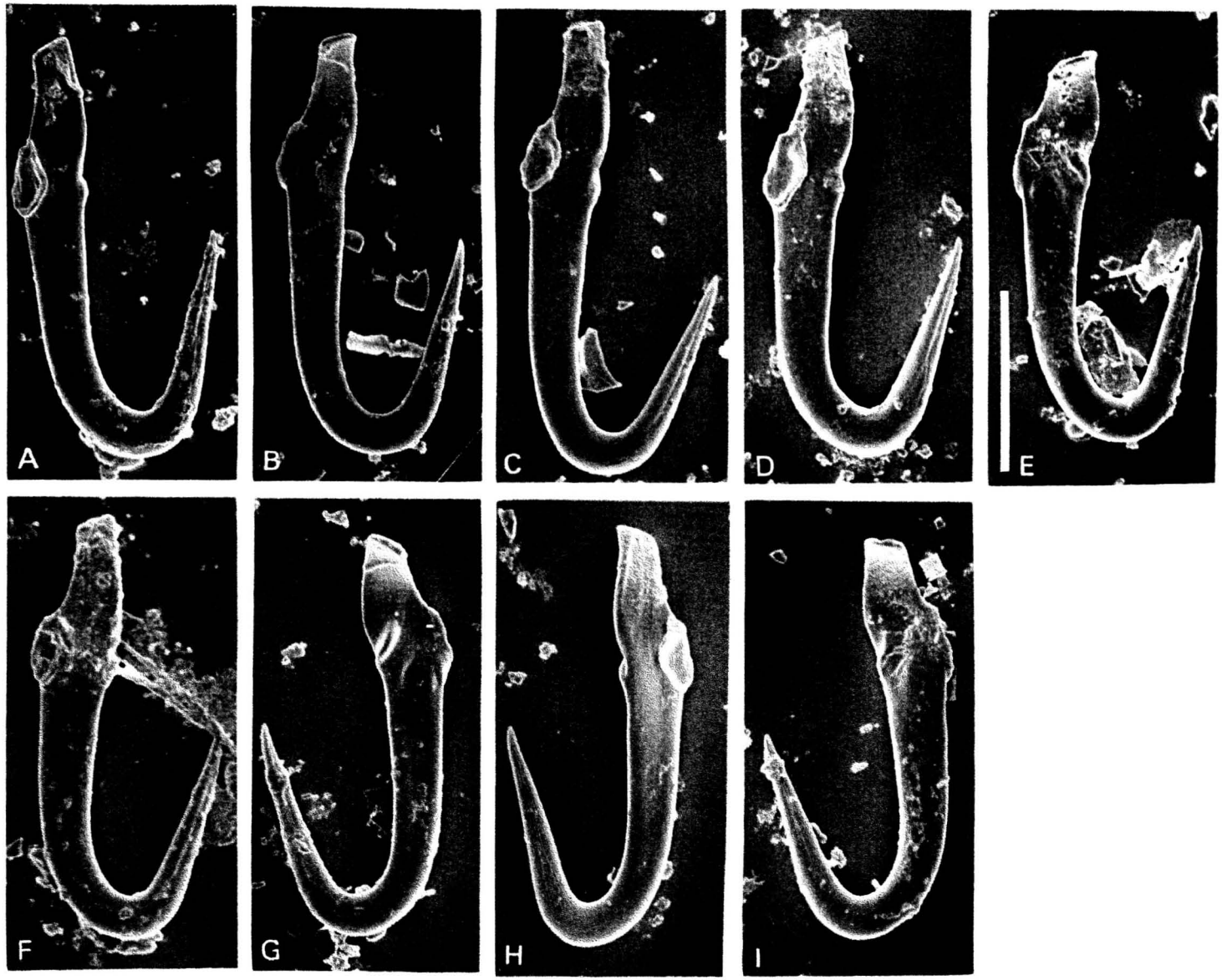


Figure 2.19: *Gyrodactylus* sp. (Morph 2) **hamuli extracted by digestion from the River Snizort, Isle of Skye,** Scotland salmon. Scale bar: 23.1 µm.

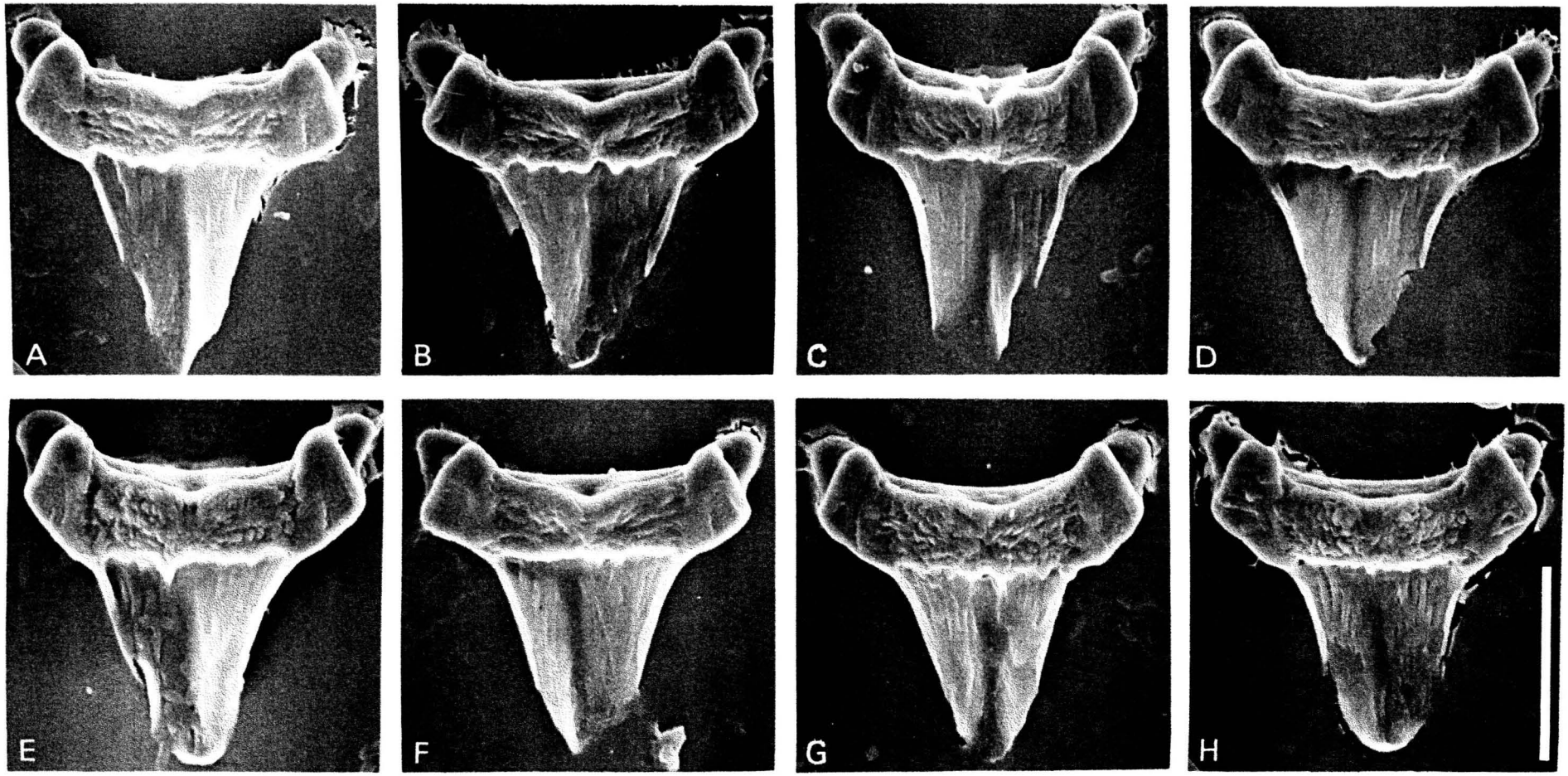


Figure 2.20: Ventral bars released by sonication from *Gyrodactylus* sp. (Morph 2) on salmon in the River Allan, Scotland. Scale bar: 13.6 μm.

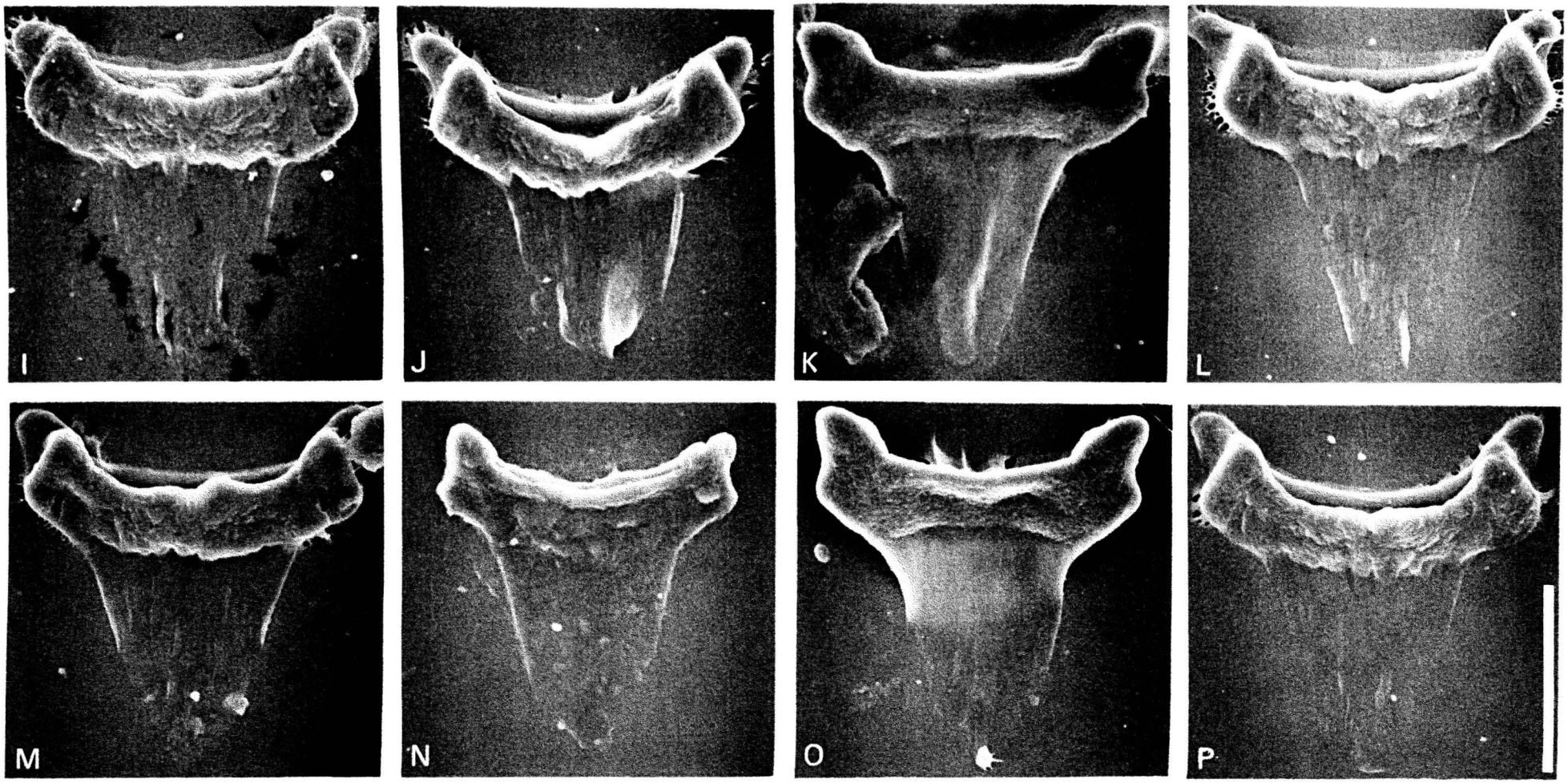


Figure 2.20: Ventral bars released by sonication from *Gyrodactylus* sp. (Morph 2) on salmon in the River Allan, Scotland. Scale bar: 13.6 μm.

Table 2.12: Marginal hook dimensions from SEM micrographs for several species of *Gyrodactylus* parasitising salmonoids.

Variable	*Gyrodactylus sp.* (Morph 2) from *Salmo salar* Mean	St.dev.	Range	*G. salaris* from Swedish *Salmo salar* Mean	St.dev.	Range
Proximal width	4.535	0.477	3.34 - 5.13	5.594	1.143	4.02 - 8.21
Toe length	1.793	0.287	1.16 - 2.29	2.210	0.590	1.58 - 3.72
Distal width	4.725	0.550	3.36 - 5.47	6.713	1.329	5.19 - 9.79
Sickle length	6.839	0.469	5.77 - 7.67	8.817	1.084	7.53 - 11.15
Sickle aperture distance	5.358	0.542	4.50 - 6.24	6.849	0.693	5.70 - 8.23
Total length	32.891	1.486	28.38 - 35.51	40.393	2.578	36.80 - 46.50
Shaft length	26.777	1.274	22.65 - 28.95	32.748	1.913	29.99 - 37.59
Number of specimens measured	20			24		

Variable	*G. colemanensis* from Canadian *Salvelinus fontinalis* Mean	St.dev.	Range
Proximal width	4.154	0.137	4.02 - 4.42
Toe length	1.546	0.132	1.34 - 1.72
Distal width	4.125	0.267	3.55 - 4.48
Sickle length	5.954	0.225	5.65 - 6.30
Sickle aperture distance	4.570	0.189	4.19 - 4.83
Total length	31.103	0.538	30.26 - 31.87
Shaft length	25.835	0.578	24.88 - 26.83
Number of specimens measured	10		

Table 2.13: Hamulus dimensions measured from SEM micrographs for several species of *Gyrodactylus* parasitising salmonoids.

Variable	Morph 4 from *Salmo trutta* Mean	St.dev.	Range	*G. salaris* from Swedish *Salmo salar* Mean	St.dev.	Range
Shaft-point length	16.090	0.429	15.17 - 16.75	24.383	2.467	20.49 - 30.95
Hamulus angle	38.609	0.868	37.72 - 40.05	42.418	1.654	38.92 - 45.09
Point length	28.568	0.526	27.61 - 29.25	32.988	1.811	29.17 - 36.49
Shaft length	35.737	0.703	34.48 - 36.69	46.962	3.060	40.71 - 52.55
Total length	56.461	1.374	54.11 - 58.63	68.171	4.642	60.28 - 77.50
Root length	23.377	1.239	21.39 - 25.16	28.492	2.382	24.93 - 34.12
Hamulus width	7.471	0.327	7.01 - 7.99	9.655	1.169	8.87 - 14.11
Number of specimens measured	10			20		

Variable	*G. colemanensis* from Canadian *Salvelinus fontinalis* Mean	St.dev.	Range	Morph 2 from *Salmo salar* Mean	St.dev.	Range
Shaft-point length	17.525	0.736	16.48 - 18.52	16.164	1.207	15.00 - 19.05
Hamulus angle	47.799	2.047	44.60 - 50.87	36.589	1.675	34.51 - 39.66
Point length	22.879	0.674	21.51 - 23.77	31.080	0.798	30.13 - 32.64
Shaft length	32.262	0.846	30.53 - 33.01	34.388	2.139	32.77 - 39.53
Total length	47.196	1.024	45.27 - 48.20	54.267	3.549	50.24 - 62.69
Root length	17.774	0.820	16.60 - 19.02	23.051	1.794	21.11 - 26.20
Hamulus width	7.008	0.188	6.79 - 7.32	7.742	0.358	7.27 - 8.53
Number of specimens measured	10			9		

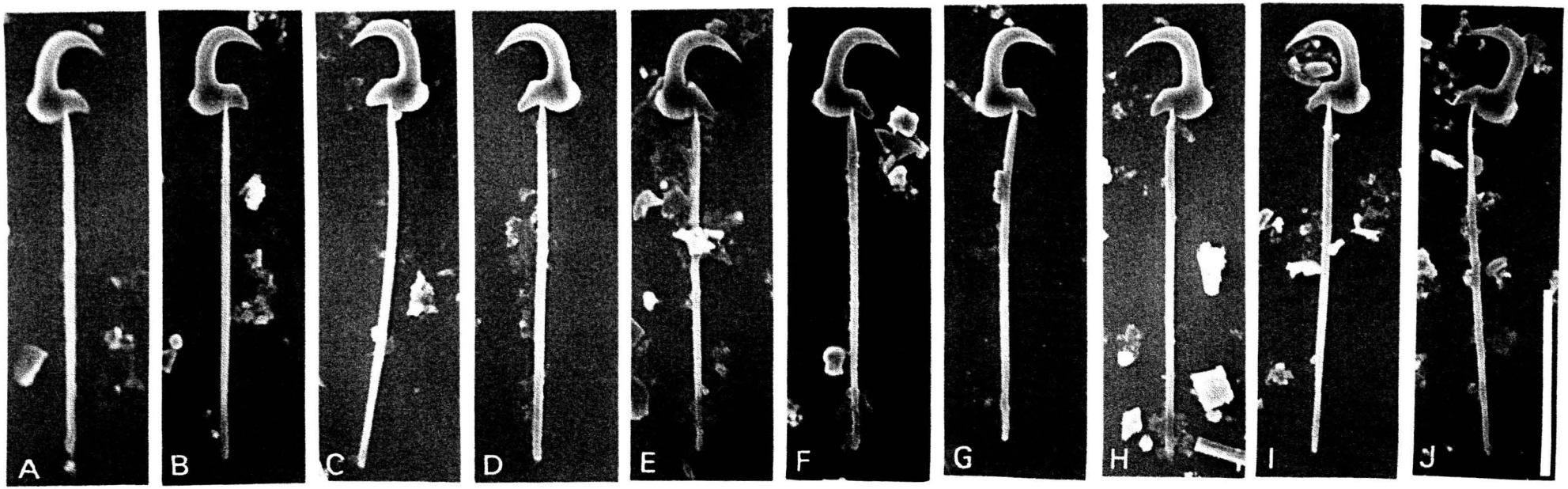


Figure 2.21: Morph 1 (a-d) and Morph 4 (e-j) marginal hooks extracted by digestion from the River Manor, Scotland brown trout. Scale bar: 13.6 μm.

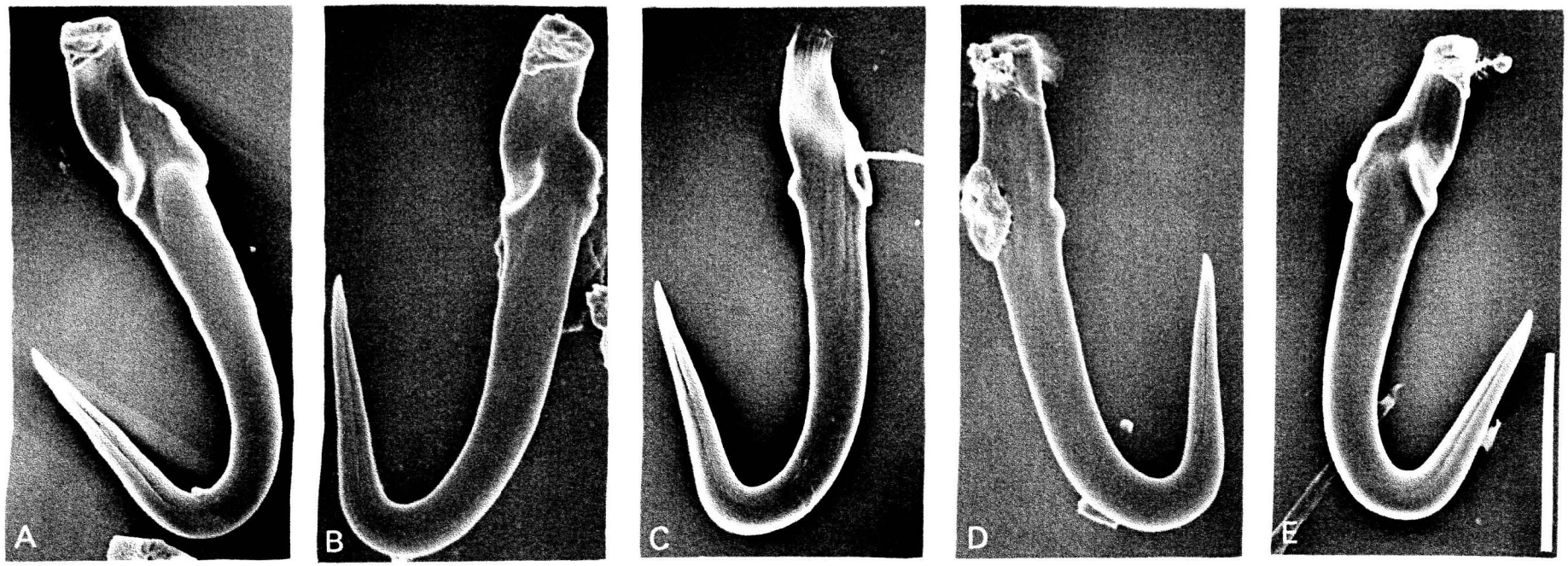


Figure 2.22: Morph 5 hamuli extracted by sonication from Loch Airthrey, Scotland brown trout. Scale bar: 23.1 μm.

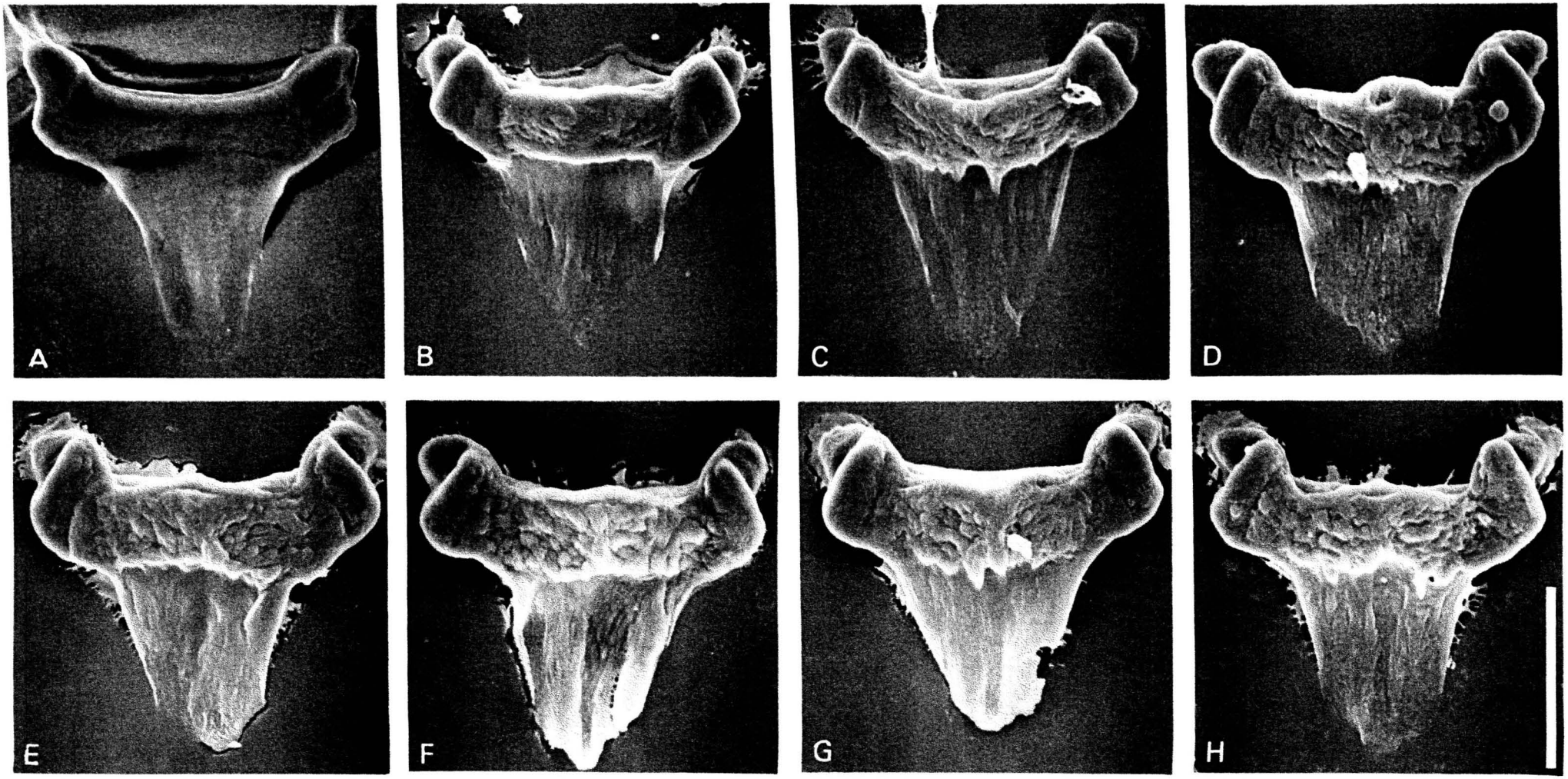


Figure 2.23: Morph 5 ventral bars extracted by sonication from Loch Airthrey, Scotland brown trout. Scale bar: 13.6 μm.

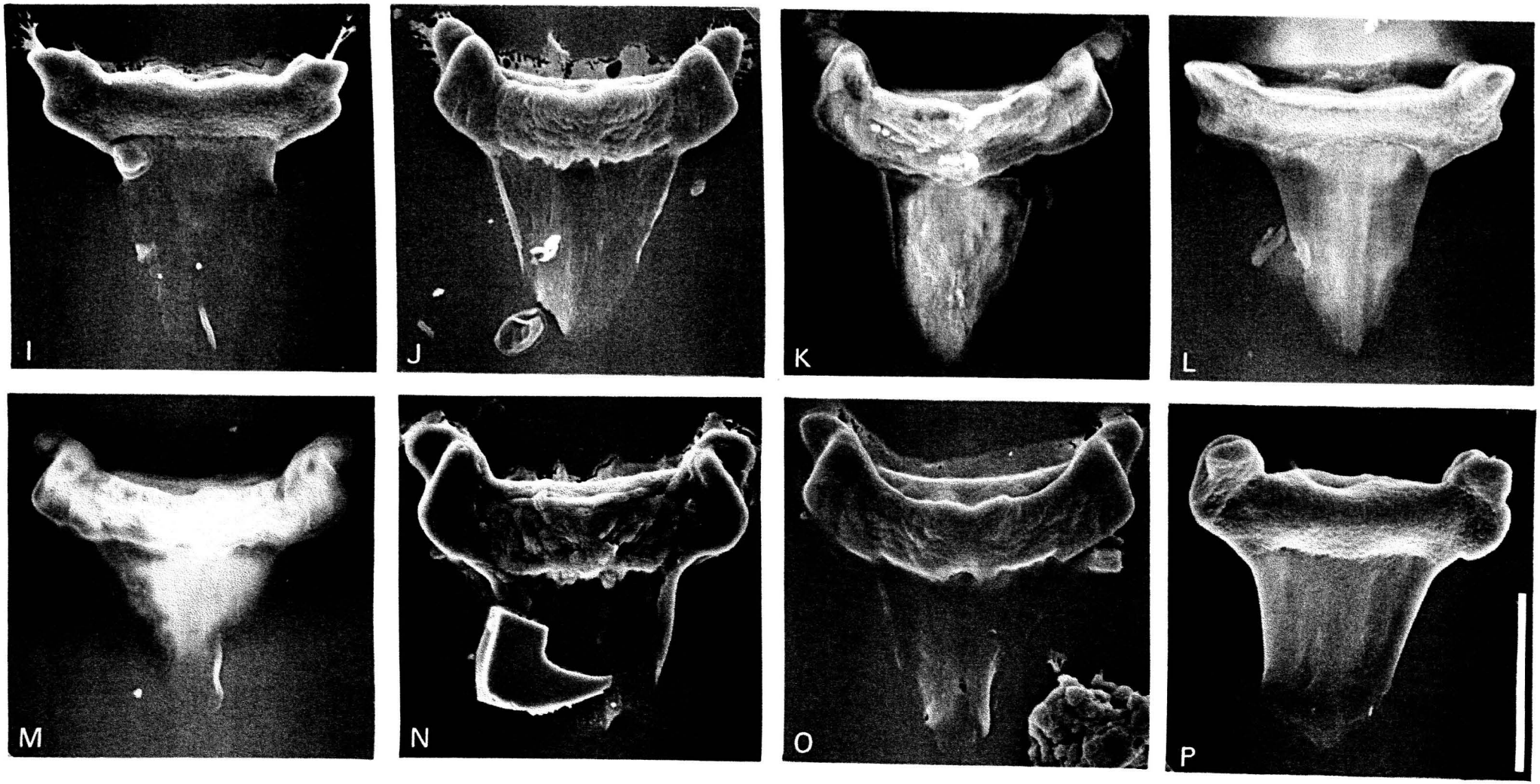


Figure 2.23: Morph 5 ventral bars extracted by sonication from Loch Airthrey, Scotland brown trout. Scale bar: 13.6 μm.

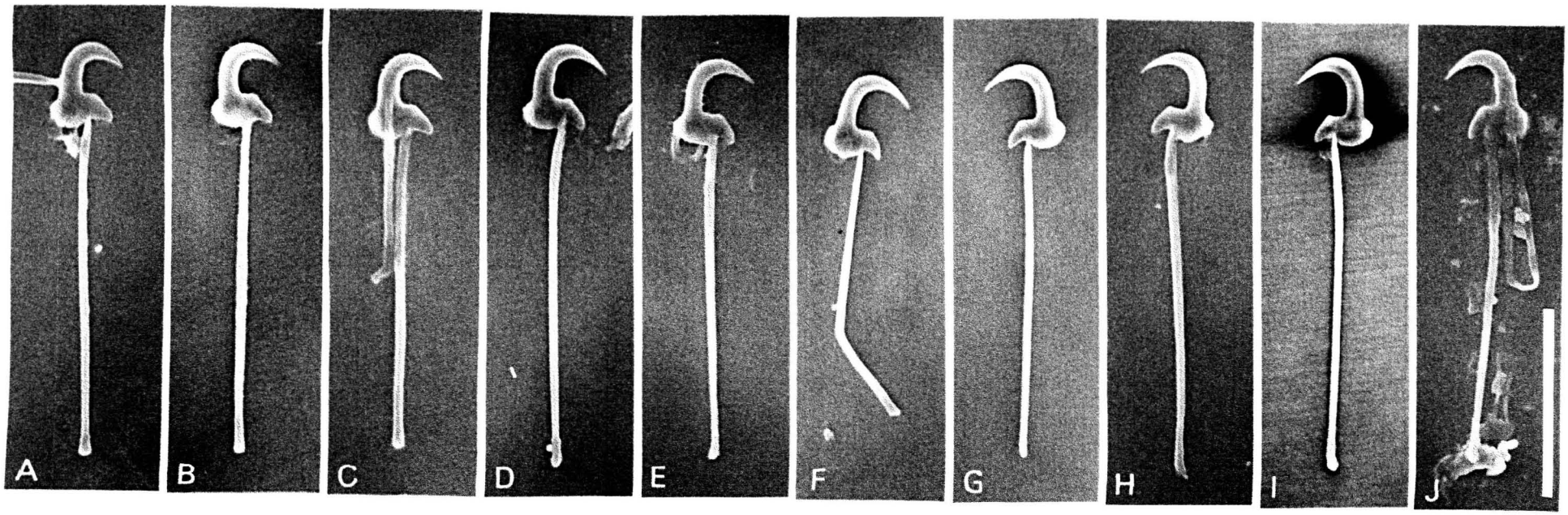


Figure 2.24: Morph 5 (d-j) marginal hooks extracted by sonication from Loch Airthrey, Scotland brown trout. The marginal hooks a-c represent Morph 1. Scale bar: 15.0 μm.

observations of the marginal hook in Table 2.15. It was concluded that Morph 4 is identical with *G. truttae* Gläser, 1974 on the basis of the marginal hook morphology and sclerite dimensions.

Morph 5:

Host: Wild *Salmo trutta* from Loch Airthrey, Stirlingshire, Scotland.
Number of specimens measured: 10 (LM); 7 marginal hooks (SEM); no hamuli were measured (SEM).

Samples of brown trout were taken from Loch Airthrey, Stirling, at the same time as other collections of British brown trout. Although there is no doubt that this morph is *G. truttae*, the specimens from this one particular site differed slightly from other populations of Morph 4 (*G. truttae*) in the morphology of its marginal hooks. This variant has proportionately small marginal hook sickle proper in relation to the length (Figure 2.24d-j). The total length is 58.13 ± 2.048µm, sickle length 5.76 ± 0.412µm, and the sickle distal and proximal widths are 3.42 ± 0.505µm and 4.07 ± 0.479µm, respectively. These results are consistent for the 20 specimens measured by both LM and SEM (Figure 2.24d-j). This morphotype was part of a mixed infection from *S. trutta* in Loch Airthrey, which occurred alongside specimens of Morph 1 (*G. derjavini*) (Figure 2.24a-c).

Morph 6:

Host: *Salvelinus alpinus* (hatchery reared from original wild broodstock, Lake Ennerdale, N. England).
Number of specimens measured: No LM observations; 3 marginal hooks (SEM).

The discrimination and description of this form is based on the morphology of just three marginal hooks collected for SEM studies from a mixed infection on Arctic charr. This morphotype (Figure 2.25a-c) was found alongside two other morphotypes, Morph 7 (Figure 2.25d-f) and Morph 1 (*G. derjavini*) (Figure 2.25g-h). The heel of the marginal hook sickle proper is not as pronounced as in both Morph 1 (Figure 2.10) and Morph 4 (Figure 2.24). The base of the sickle, although deep, has a short toe

Table 2.14: Sclerite dimensions (LM) of *Gyrodactylus* collected from British brown trout (*Salmo trutta*).

Variable	Morph 4 from British brown trout Mean	St.dev.	Range	Morph 5 from L. Airthrey brown trout Mean	St.dev.	Range
Hamulus:						
Total length	60.9	3.1	50.0 - 66.3	58.1	2.1	54.7 - 60.6
Shaft length	44.1	2.2	35.9 - 48.8	39.6	1.2	38.4 - 42.5
Point length	31.0	1.7	26.3 - 38.1	28.8	1.4	25.9 - 30.6
Root length	17.5	1.8	8.8 - 21.3	18.9	1.7	16.3 - 20.9
Dorsal bar:						
Total length	25.3	2.3	21.3 - 30.6	27.3	1.8	24.4 - 30.0
Width	2.2	0.3	1.3 - 3.1	2.1	0.3	1.9 - 2.5
Ventral bar:						
Total length	29.2	1.7	23.8 - 34.4	27.3	1.5	23.4 - 28.8
Total width	25.1	2.2	20.0 - 29.4	24.0	1.5	21.3 - 25.6
Middle width	7.2	0.9	5.3 - 9.4	7.3	0.9	5.6 - 8.8
Process-Middle length	2.9	0.5	1.3 - 4.4	3.1	0.2	2.8 - 3.4
Process length	3.6	0.6	2.2 - 5.0	4.5	0.6	3.4 - 5.0
Membrane length	14.4	1.8	9.4 - 18.8	12.2	1.1	10.0 - 13.8
Marginal hook:						
Total length	30.9	1.3	26.3 - 34.4	32.5	0.8	30.9 - 33.8
Shaft length	25.0	1.2	20.6 - 27.8	27.4	1.0	25.3 - 29.1
Sickle length	6.7	0.4	5.6 - 7.5	5.8	0.4	5.6 - 6.9
Sickle dorsal width	5.0	0.3	4.1 - 5.6	3.4	0.5	3.1 - 4.7
Sickle proximal width	5.1	0.2	4.4 - 5.6	4.1	0.5	3.8 - 5.3
Filament loop	12.4	1.0	8.8 - 14.4	10.8	1.0	8.8 - 12.2
Number measured	117			10		

Table 2.15: Marginal hook dimensions from SEM micrographs for several species of *Gyrodactylus* parasitising salmonoids.

Variable	Morph 4 from *Salmo trutta* Mean	St.dev.	Range	Morph 5 from *Salmo trutta* (L. Airthrey) Mean	St.dev.	Range
Proximal width	4.627	0.190	4.43 - 5.21	4.947	0.158	4.71 - 5.16
Toe length	1.676	0.156	1.39 - 1.89	1.687	0.068	1.57 - 1.74
Distal width	5.275	0.450	3.90 - 5.80	5.595	0.125	5.37 - 5.76
Sickle length	6.938	0.158	6.60 - 7.25	7.157	0.181	6.94 - 7.43
Sickle aperture distance	5.386	0.258	4.93 - 5.80	5.418	0.217	5.23 - 5.79
Total length	31.157	0.630	30.31 - 32.59	32.848	0.929	31.61 - 34.19
Shaft length	24.983	0.634	24.04 - 26.30	25.873	1.669	23.09 - 27.77
Number of specimens measured	16			7		

Variable	Morph 6 from *Salvelinus alpinus* Mean	St.dev.	Range	Morph 7 from *Salvelinus alpinus* Mean	St.dev.	Range
Proximal width	3.503	0.306	3.27 - 3.85	4.260	0.014	4.25 - 4.27
Toe length	1.337	0.093	1.23 - 1.40	1.720	0.198	1.58 - 1.86
Distal width	3.050	0.269	2.81 - 3.34	4.950	0.580	4.54 - 5.36
Sickle length	5.560	0.190	5.35 - 5.72	6.650	0.127	6.56 - 6.74
Sickle aperture distance	4.187	0.095	4.09 - 4.28	5.315	0.177	5.19 - 5.44
Total length	30.307	0.361	29.90 - 30.59	32.265	0.148	32.16 - 32.37
Shaft length	25.573	0.471	25.04 - 25.93	26.400	0.184	26.27 - 26.53
Number of specimens measured	3			2		

Table 2.16: Sclerite dimensions based on LM observations of *Gyrodactylus* spp. collected from British arctic charr (*Salvelinus alpinus*) and farmed rainbow trout (*O. mykiss*).

Variable	*Gyrodactylus* spp. from British rainbow trout Mean	St.dev.	Range	*Gyrodactylus* sp. from L. Ennerdale charr Mean	St.dev.	Range
Hamulus:						
Total length	56.8	2.8	53.1 - 61.3	58.8	1.8	56.3 - 61.3
Shaft length	40.3	2.0	36.3 - 43.8	42.8	1.0	40.6 - 44.4
Point length	29.1	1.6	25.6 - 31.3	29.6	1.4	26.9 - 31.3
Root length	17.2	2.0	13.1 - 25.3	16.6	2.3	13.1 - 19.7
Dorsal bar:						
Total length	26.0	3.0	19.4 - 30.6	25.8	2.9	18.8 - 28.8
Width	2.7	0.4	1.6 - 3.5	2.5	0.0	2.5 - 2.5
Ventral bar:						
Total length	26.6	1.4	21.9 - 30.6	28.6	1.4	25.6 - 30.6
Total width	24.4	1.5	21.3 - 27.8	24.1	0.9	22.8 - 26.3
Middle width	6.7	0.9	5.3 - 9.7	6.9	0.4	6.3 - 7.5
Process-Middle length	2.9	0.9	1.7 - 3.8	3.2	0.2	3.1 - 3.4
Process length	3.7	0.6	2.5 - 5.1	3.4	0.6	2.5 - 4.4
Membrane length	14.1	1.3	9.4 - 16.9	13.8	1.2	12.5 - 16.3
Marginal hook:						
Total length	31.6	1.5	28.2 - 35.6	31.4	1.1	28.8 - 32.5
Shaft length	25.7	1.4	22.1 - 30.3	25.2	1.1	22.8 - 26.3
Sickle length	6.6	0.5	5.6 - 7.8	6.9	0.3	6.3 - 7.5
Sickle dorsal width	4.8	0.4	3.4 - 5.6	5.0	0.2	4.7 - 5.3
Sickle proximal width	5.2	0.3	4.4 - 5.9	5.1	0.2	5.0 - 5.6
Filament loop	11.6	1.2	8.1 - 13.1	12.5	0.7	11.6 - 13.8
Number measured	73			10		

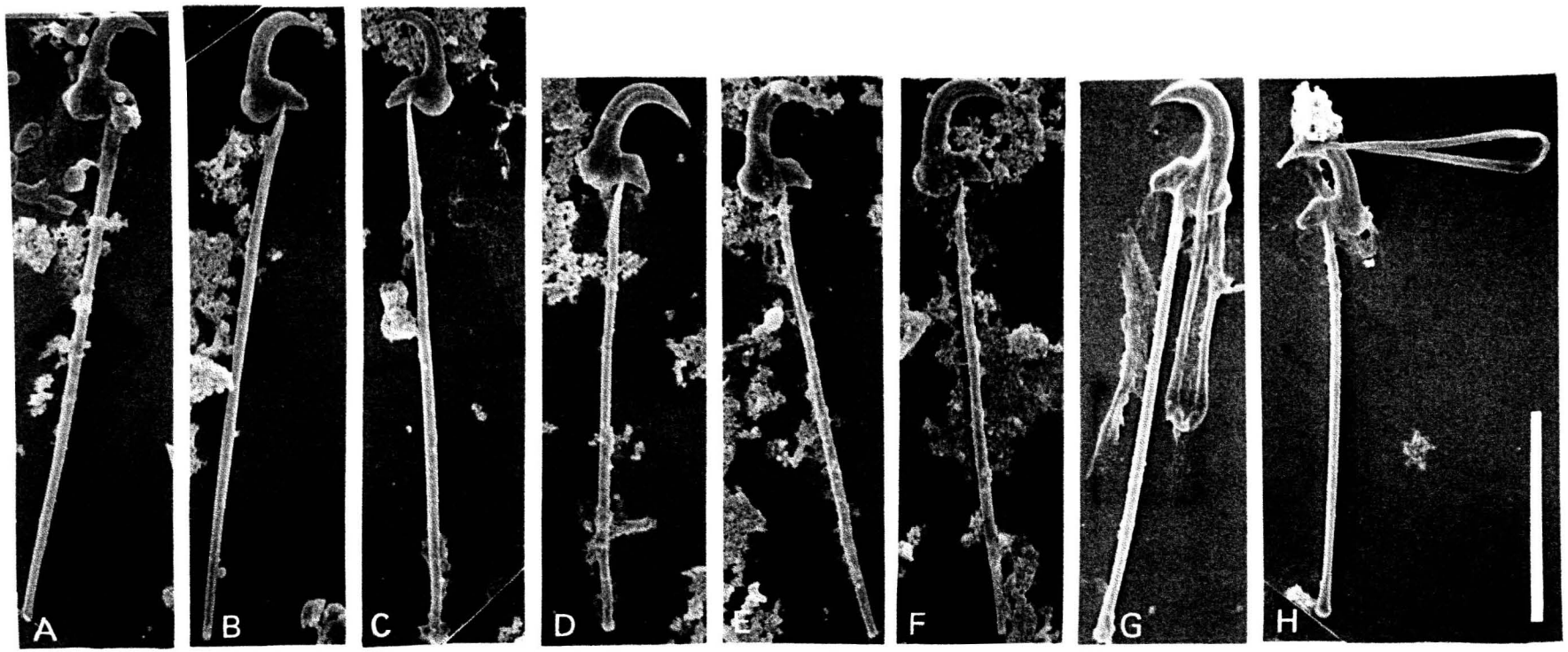


Figure 2.25: The marginal hooks of Morphs 1 (g-h), Morph 6 (a-c) & Morph 7 (d-f) extracted by sonication from Lake Ennerdale, England arctic charr. Scale-bar: a-c = 10 µm; d-g = 12.0 µm and h = 13.6 µm.

portion which lacks the distinctive upper flattened portion before turning into the shaft of the sickle proper. The shaft and point of the sickle proper is slender, the point short and the curve not as marked as for Morph 1 or Morph 4. The sickle is small in proportion to the total length of the marginal hook, and the lack of a full curve in the sickle proper gives the sickle a wider aperture or face than Morph 1 or Morph 4 (Figure 25a-c). The marginal hook also resembles Morph 2 and *G. salaris* (see below) in having a narrow sickle blade and unpronounced heel; however, the characteristic triangular base of the sickle proper is unique to this morph. It is believed that these marginal hooks represent an as yet undescribed species of *Gyrodactylus.*

Morph 7:

Host: *Salvelinus alpinus* (hatchery reared from original wild broodstock, Lake Ennerdale, N. England).

Number of specimens measured: 3 marginal hooks (SEM).

This description is based on the morphology of three marginal hooks, analysed using the SEM, which closely resemble Morph 1. These marginal hooks have a deep sickle base and a triangular toe analogous to that of Morph 1; however, the heel is not as pronounced, the shaft slightly less robust and the sickle point more tapering, resulting in a slightly more open aperture to the marginal sickle (Figure 2.25d-f). It is believed these may represent Morph 2 (Figure 2.18) normally parasitic on Atlantic salmon, which appears to have a distribution through N. England, N. Ireland and Scotland, however more specimens are required.

***G. salaris* Malmberg, 1957**

Host: *Salmo salar* (River Ätran system, Sweden) supplied by Dr Malmberg.

Number of specimens measured: No LM measurements; 24 marginal hooks, 20 hamuli (SEM).

The shape of the marginal hook sickle is quite characteristic and is shown in Figures 2.26 and 2.27. The base of the sickle proper is shallow, the heel weakly pronounced, and the toe narrow and level with the heel base. The shaft of the sickle proper is long

and slender, and the point tapers to a point beyond the level of the toe, but not to the extent of that of Morph 4. The shape of the inner curve of the sickle proper encompasses an oval shape described by the long, narrow form of the sickle. Summer (Figure 2.26) and winter (Figures 2.27) collections differed, the winter form appearing to exhibit a more elongate sickle proper (Table 2.12).

The hamuli of *G. salaris* (Figure 2.28) can be differentiated from other species by its size, being the largest of the "salmonoid" *Gyrodactylus* forms studied. Table 2.13 gives the dimensions measured under the SEM. One new feature discriminating the hamulus of this species from the other "salmonoid" gyrodactylids investigated here is the relative position of the ventral and dorsal bar attachment points (Figure 2.9). The ventral bar attachment point appears as a flattened region close to the junction between the root and shaft portions of the hamulus (Figure 2.28d). This junction manifests itself as an indentation on the inner curve of the hamulus. Its relative position, opposite the lower third of the adjacent dorsal bar attachment point (or cap), differs from the other species of *Gyrodactylus* studied, where the position of the indentation marked by the ventral bar attachment point is opposite the mid-point of the dorsal bar attachment point (Figure 2.28).

***G. colemanensis* Mizelle & Kritsky, 1967**

Host: *Salvelinus fontinalis* (Nova Scotia) supplied by Dr D. Cone.
Number of specimens measured: No LM measurements; 10 marginal hooks, 10 hamuli (SEM).

G. colemanensis, a North American species, was studied here alongside the European "salmonoid" *Gyrodactylus* species because of its marked differences. *G. colemanensis* can be clearly differentiated from the European *Gyrodactylus* spp. in several ways, including hamulus and marginal hook form and overall size.

The marginal hooks (Table 2.14; Figure 2.29) can be clearly differentiated from the European salmonoid gyrodactylid species. The heel of the sickle proper is pronounced and the sickle shaft is narrow, with the point turning sharply to produce a sickle form with a square internal line.

This sharply contrasting species serves to illustrate how subtle are the

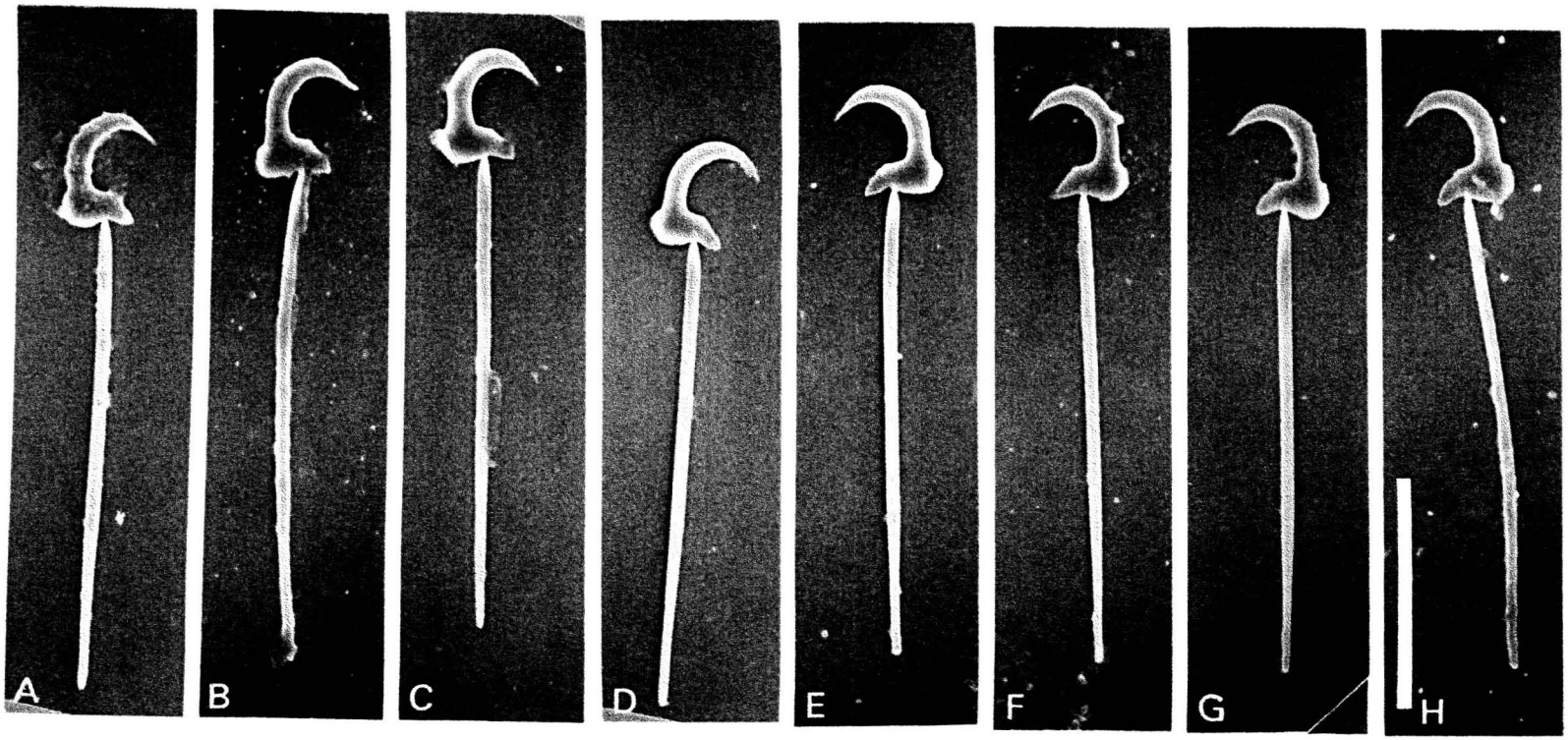


Figure 2.26: *G. salaris* Malmberg, 1957 marginal hooks (summer) extracted by digestion from River Högvadsån, Sweden salmon. Scale bar: 15.0 μm.

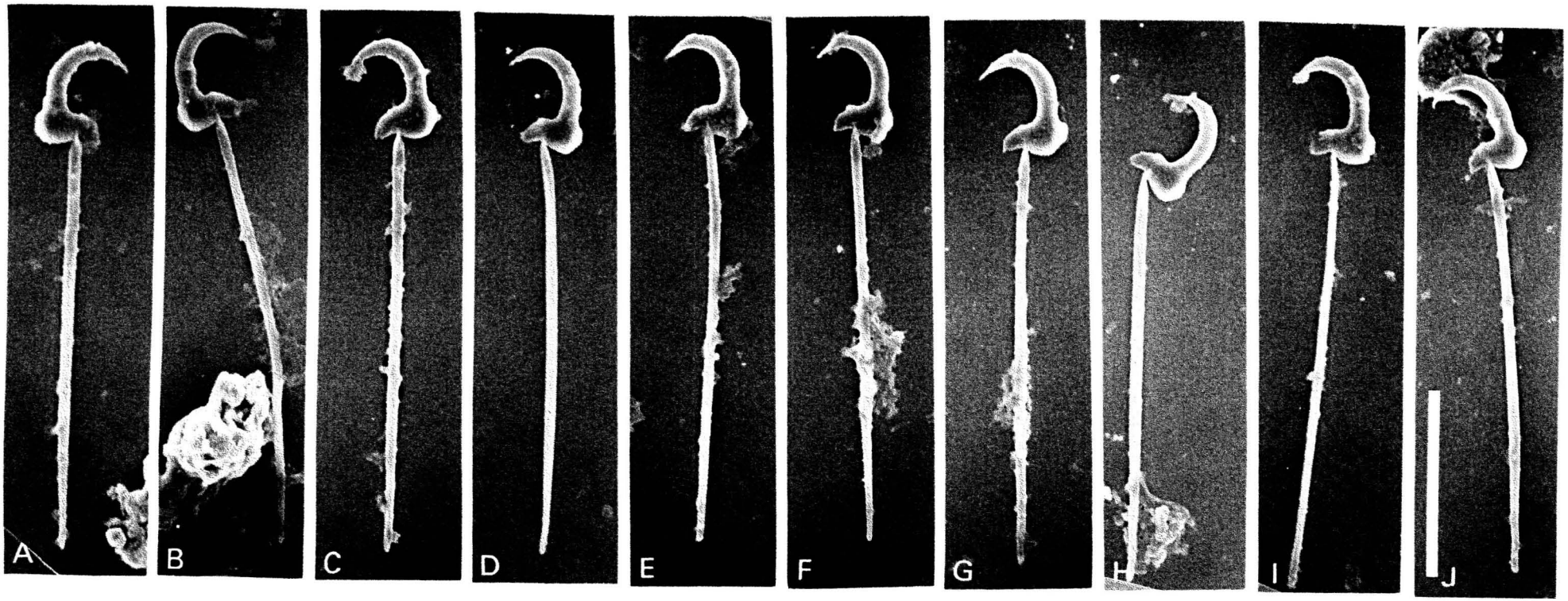


Figure 2.27: *G. salaris* Malmberg, 1957 marginal hooks (winter) extracted by digestion from the River Högvadsån, Sweden salmon. Scale bar: 15.0 μm.

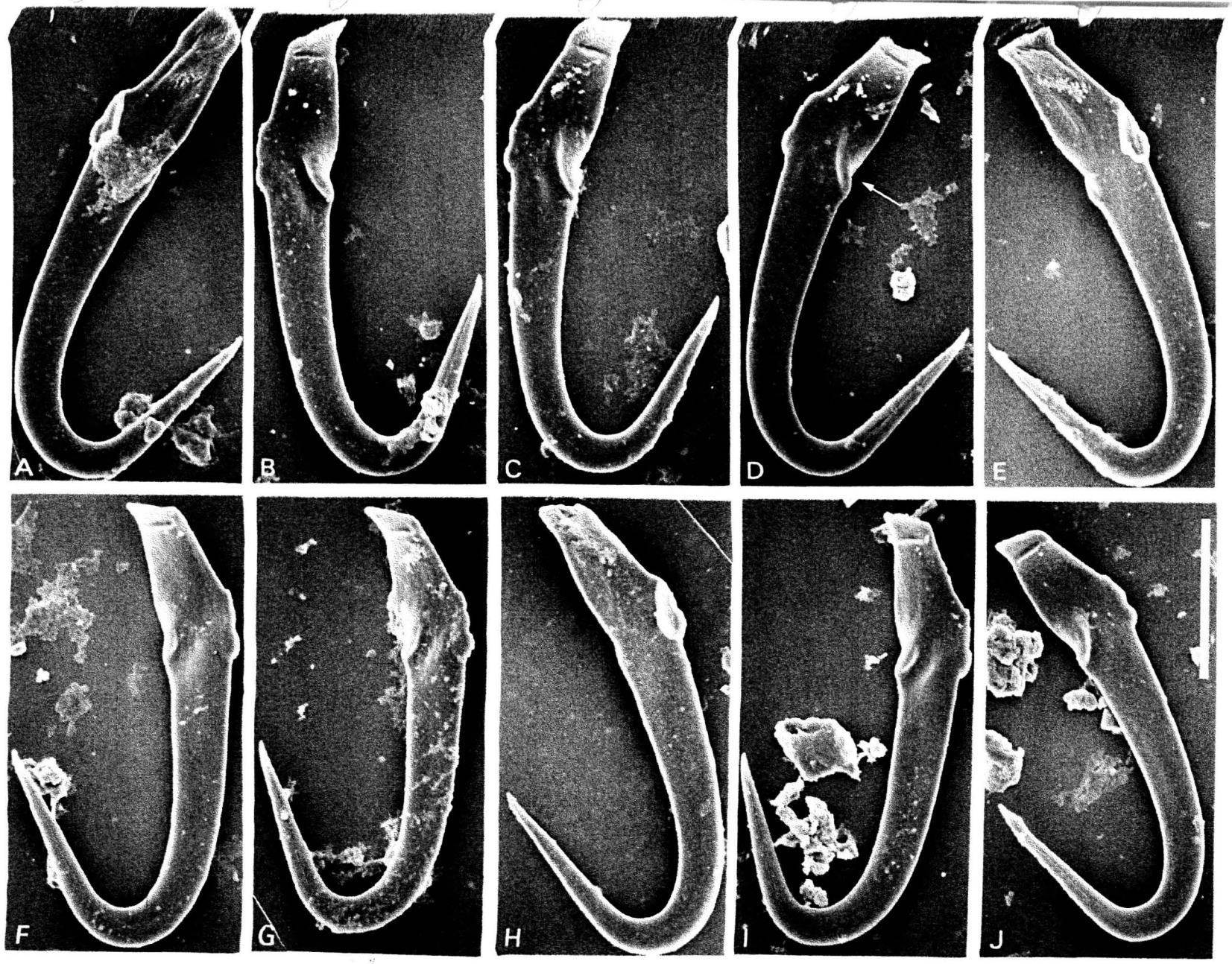


Figure 2.28: *G. salaris* Mallmberg, 1957 hamuli (winter) extracted by digestion from the River Säveån, Sweden salmon. Scale bar: 25.0 µm. The arrow indicates the ventral bar attachment point, a region close to the junction between the root and shaft portions of the hamulus.

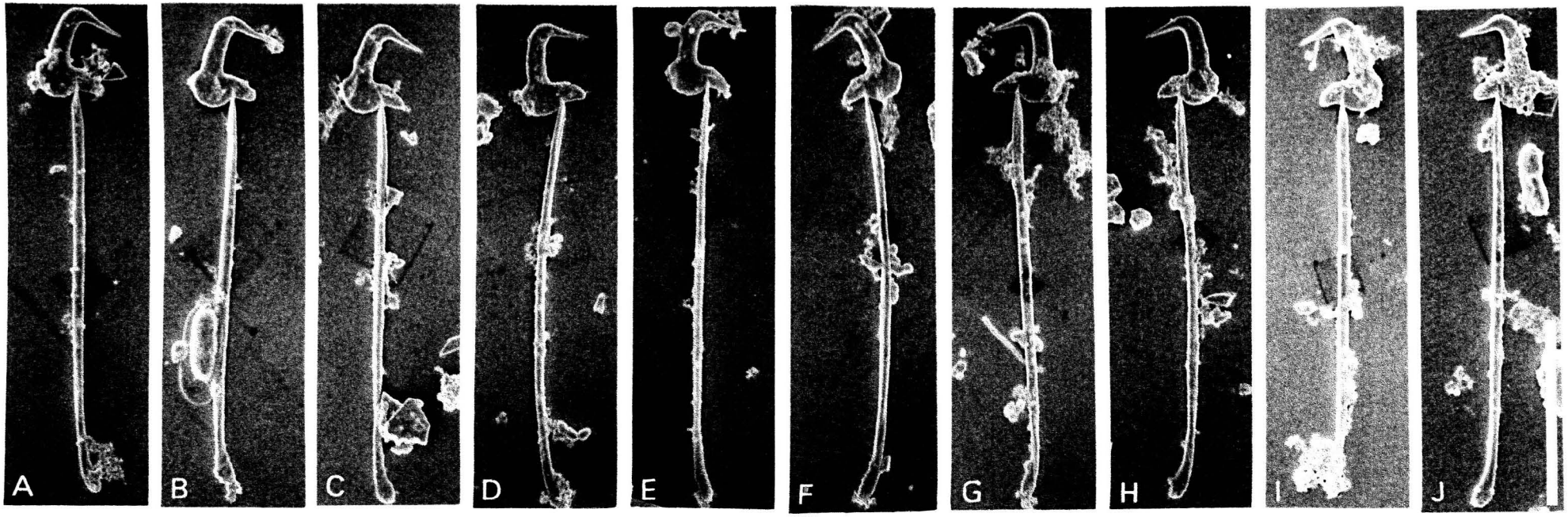


Figure 2.29: *G. colemanensis* Mizelle & Kritsky, 1967 marginal hooks extracted by digestion from Nova Scotian brook trout. Scale bar: 12.0 µm.

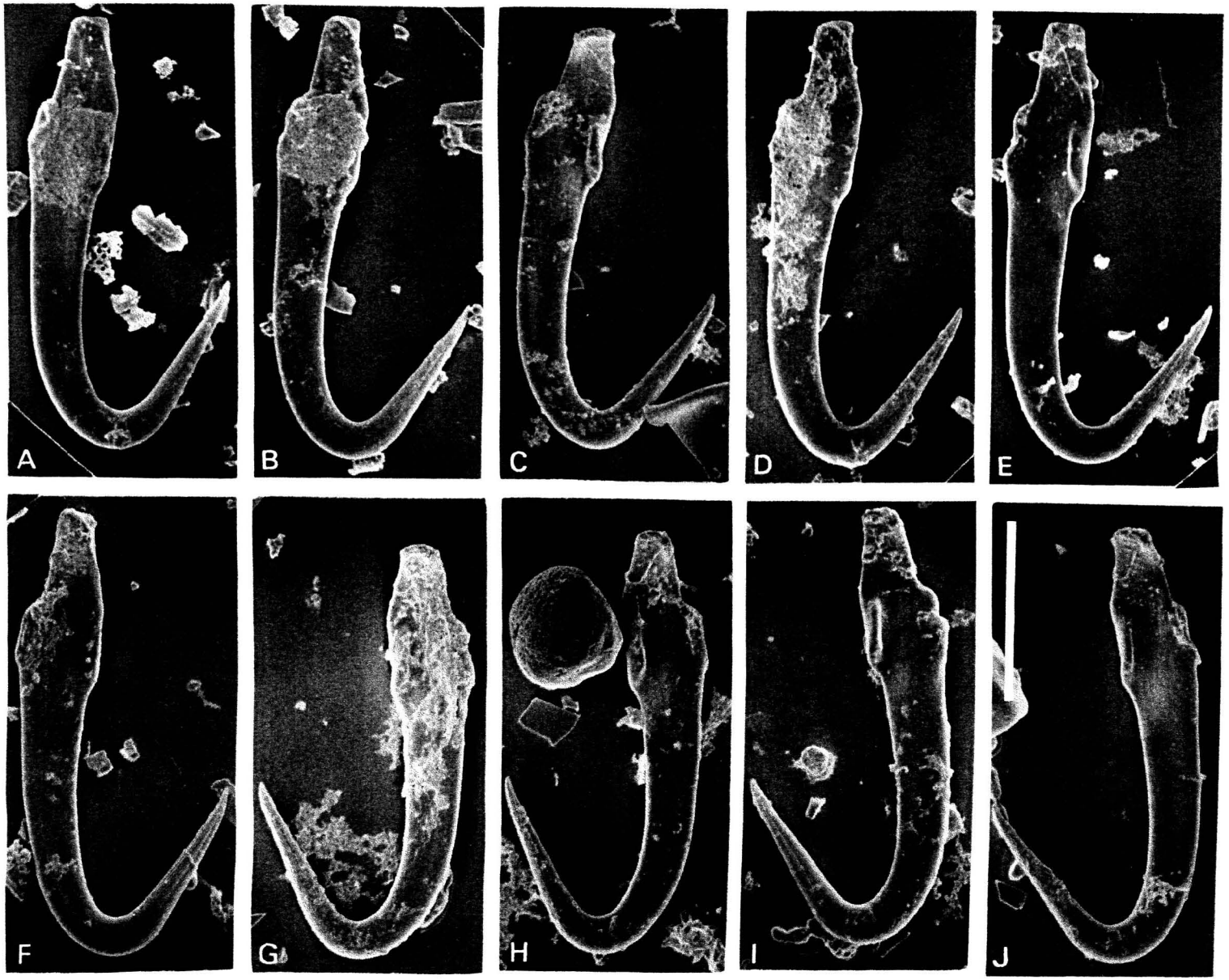


Figure 2.30: *G. colemanensis* Mizelle & Kritsky, 1967 hamuli extracted by digestion from Nova Scotian brook trout. Scale bar: 20.0 μm.

differences in hook morphology which exist between the native species of *Gyrodactylus*. The hamuli represent the smallest studied (Table 2.13) amongst the salmonoid gyrodactylids. In addition to its short, open point, the hamulus can be discriminated on two further morphological features. The ventral bar attachment point on the hamulus is elongate, giving this region a square appearance. Secondly, the root portion of the hamulus is relatively small and tapers (Figure 2.30).

Discussion

The systematics of the genus *Gyrodactylus* Nordmann, 1832 is based principally on morphological variation in the shape of the marginal hook (Malmberg, 1970). However, species of *Gyrodactylus* parasitising the salmonoids exhibit marginal hooks with low variability in their form, such that the discrimination of these species from literary accounts is confusing. The issue is further confused by the influence of environmental parameters, temperature and salinity, which contribute some variability in the size and form of the marginal hook (Mo, 1991a,b,c). Secondly, there is variation within any species of *Gyrodactylus* collected from different host species. The re-examination of *G. derjavini*, *G. truttae*, *G. salaris* and the new morphotypes identified using electron microscopy has permitted the elucidation of the subtle differences between these species at much higher levels of accuracy than was possible previously using light microscopy.

Malmberg (1987a,b) recorded the presence of *G. truttae* on *Salmo trutta* in the UK, whilst *G. derjavini* parasitises *S. trutta* and *O. mykiss* in Sweden, Norway, Denmark and Italy. In the survey these two species were found to co-exist on the same host, *S. trutta*, an observation which was consistent throughout the British Isles, although *G. truttae* always represented the dominant species, accounting for 80-90% of all specimens collected from brown trout. Studies with the light microscope and the SEM revealed 7 morphotypes parasitising the British salmonids. *G. derjavini* was found on hatchery-reared populations of *O. mykiss*, but was the only species of *Gyrodactylus* found to infect this host. *G. derjavini* represents the major species on *S. trutta* in Sweden, whereas in the UK it represented approximately 10% of all

Gyrodactylus specimens collected from *S. trutta*. *G. derjavini* was also found to infect a third host, *Salmo salar*, and, although this was not the only species found on this host, it was the dominant species found on *S. salar* in England and Wales. Malmberg (1987b) observed that Scottish Atlantic salmon carried a species similar to, but not identical with, *G. derjavini*. It would appear that this unidentified species is identical with Morph 2. This morphotype was found to parasitise only Scottish Atlantic salmon, and judging from evidence based on the marginal sickle shape this may represent a new species.

Morph 3 from *S. salar* is most probably also *G. derjavini*, differing from Morph 1 only on the basis of size. This morph was found at two sites, but only on a single occasion. Morph 4 is identical to *G. truttae* Gläser, 1974 and is found on wild *S. trutta* throughout the UK. Morph 5 from Loch Airthrey *S. trutta* is identical to Morph 4 but was differentiated on the basis of having very long marginal hooks.

It is clear that *Salvelinus alpinus* (the Lake Ennerdale population) in the UK is parasitised by at least two species, namely Morph 1 (*G. derjavini*) (Figure 2.25g-h) and Morph 6 (*Gyrodactylus* sp) (Figure 2.25 a-c), an as yet undescribed species; it is not clear whether the third morph from this host (Morph 7) (Figure 2.25 d-e), which closely resembles Morph 2, represents a third species. In summary, the species of *Gyrodactylus* found on British salmonoids are:

Morph 1 = *G. derjavini* Mikailov, 1975

Morph 2 = undescribed form closely related to *G. derjavini*

Morph 3 = giant variant of *G. derjavini*

Morph 4 = *G. truttae* Gläser, 1974

Morph 5 = *G. truttae* variant

Morph 6 = undescribed form from *S. alpinus*

Morph 7 = variant of *G. derjavini* on *S. alpinus*

The discrimination of the three morphs on *S. alpinus* proved impossible at the level of the light microscope, and the measurements are given as a single table (Table 2.16). It is only when the marginal hooks were examined under the SEM that the differences in the three morphs became evident.

The hamuli collected from Morph 1 on Atlantic salmon (Table 2.11) were the

largest from the three hosts studied. They also exhibited a large variability in their overall measurements, but, as the specimens of Morph 1 were collected over a period of two years, the differences in water temperature throughout the year might explain this variability, since Mo (1991a,b,c) showed that temperature made a significant contribution to variability in *G. salaris* in Norway. The large range of measurements for each of the sclerites, therefore, makes the differentiation of *G. truttae* from *G. derjavini* problematical when the measurements alone are considered. The detail that can be obtained from electron-micrographs has allowed the Scottish and Welsh populations of *G. derjavini* to be distinguished (Table 2.11). However, there is one drawback of using the enzyme digestion and sonication techniques (see Chapter 6) to liberate the sclerites. Each technique requires pooled samples of gyrodactylid worms (approx. 30), thus, following their release from the host tissue, it is impossible to determine which marginal hooks belong to which hamuli. Samples processed for electron microscopy where possible were collected from a single host and pooling specimens from several fish was generally kept to a minimum. Although mixed infections might occur, the sample was however considered as a single population. Malmberg (1970) used the form of the marginal hooks as a basis for the systematics of the genus *Gyrodactylus* and it can be seen here that individual marginal hooks can be discriminated and identified to the species level when using electronmicroscopy. However, this clear distinction does not exist for the hamuli and so the measurements given in the tables may represent mixed populations and hamulus data should therefore be considered with this in mind. This point is important, especially for Morph 1 and Morph 4 in the UK, each of which appears to have two populations characterised on slight differences in the form of their marginal hooks. The Scottish salmon form of Morph 1 appears to be the largest and the Welsh salmon form the smallest, when the hamuli are considered alone, but their discrimination from Morph 4 (*G. truttae*), which is very close morphologically, can prove to be problematical. A clearer discrimination is given for the marginal hooks, where the morphology is distinctive and the morphometric data are accurate and reliable.

Gyrodactylids collected from hatchery reared Arctic charr stock (developed from eggs stripped from wild fish) were maintained on water from a borehole; Atlantic salmon on the same water supply at the time of sampling were devoid of

Gyrodactylus. The morphotypes identified from Arctic charr were Morph 1 (*G. derjavini*), Morph 6 and Morph 7, and represent the sampling of only a single population of charr (Lake Ennerdale). A more complete survey should aim to sample other charr populations, e.g. Loch Lomond, Loch Awe and Lakes Windermere, Ennerdale, Coniston, etc. Grayling *Thymallus thymallus*, powan *Coregonus lavaretus* (Loch Eck) and the isolated highland loch populations of relict brown trout and wild brown trout fry from the Shetland and Orkney Islands should also be studied.

According to Bychowsky (1957), the majority of monogeneans (74%) occur on one host only, i.e. they are monoxenic, and most of the others are found in fish belonging to the same genera, i.e. these are oioxenic. The findings of Bakke *et al.* (1992) with regard to *Gyrodactylus* spp. were in close agreement, since of the 319 species recorded, 235 (73.6%) were found to infect a single host, 48 (15.05%) two hosts, 19 (5.96%) three hosts, 4 (1.25%) four hosts and 13 (4.08%) more than four hosts. However, when the 18 true "salmonoid" species of *Gyrodactylus* are considered (Table 2.1), 7 (38.89%) were found to infect a single host, 6 (33.33%) two hosts, 1 (5.56%) three hosts, 1 (5.56%) four hosts, and 3 (16.67%) more than four hosts. Table 2.1 shows that *G. truttae* parasitises four salmonoid hosts (*Salmo trutta*, *S. salar*, *Oncorhynchus mykiss* and *Salvelinus fontinalis*), *G. salaris* parasitises at least five hosts (see Table 1.2), and *G. derjavini* parasitises nine salmonoid hosts (Table 2.1). This, however, does not include accidental infections recorded in the wild, for which *G. derjavini* infection is attributed to a further two occasional hosts, *Chondrostoma cyri* Kessler, 1877 and *Cyprinus carpio* L. (see Ergens, 1983b). Records of accidental infections on salmonoids include: *G. aphyae* Malmberg, 1957 (see Mo, 1983) and *G. phoxini* Malmberg, 1957 (see Mo, 1988) on *S. salar*; *G. arcuatus* Bychowsky, 1933 on *S. salar* (see Tanum, 1983; and the present survey); *G. gobii* Schulmann, 1953 on *O. mykiss* (see Lux, 1990), and *G. macronychus* Malmberg, 1957 on *S. trutta* (see Mo, 1983). The evidence presented here would suggest that the "salmonoid" gyrodactylids appear to show a lower level of host specificity than occurs generally in the genus, and it is notable that *G. derjavini*, *G. salaris* and *G. salmonis*, all with four or more hosts, are known to cause losses to captive salmonoids (Johnsen, 1978; Cone & Odense, 1984).

If one considers all the salmonoid gyrodactylids (Figure 2.17), the following

can be distinguished from the other species of *Gyrodactylus* by their characteristic marginal hooks. *G. asiaticus* Ergens, 1978 and *G. lenoki* Gussev, 1953, both parasites of the genus *Brachymystax*, are noted as having angular points and a large open face to their marginal sickle proper. The North American species *G. avalonia* Hanek & Threllfall, 1969, *G. colemanensis* and *G. nerkae* Cone *et al.*, 1983 (*G. brevis* Crane & Mizelle, 1967 was an accidental infection), which are considered true parasites of the genus *Oncorhynchus*, have distinctive marginal hook sickles. *G. avalonia* has a large, rectangular heel to the marginal hook; *G. colemanensis* is typified by the presence of a kink in the point of the marginal hook proper (Figures. 2.17 & 2.29); and *G. nerkae* has a very slight heel and narrow shaft and point of the marginal hook proper. Although *O. mykiss* was introduced into Europe during the late 1800s, no true "oncorhynchid" parasites became established in Europe. The record of *G. bychowskyi* Sproston, 1946, on returning Atlantic salmon (Mo, 1988), represents a marine gyrodactylid and, therefore, the form of the marginal hook differs from the other species, all of which are freshwater forms. *G. taimeni* Ergens, 1971, a parasite of *Hucho* spp., is characterised by a narrow, elongate marginal hook point. Similarly, the marginal hooks of *G. brachymystacis* Ergens, 1978, *G. magnus* Konovalov, 1967 and *G. thymalli* Zitnan, 1960 are similar morphologically, all having marginal hook points that extend beyond the toe of the marginal hook sickle.

The hooks of Morph 2 resembled those of *Gyrodactylus birmani* Konovalov, 1967, *G. salaris* and *G. thymalli* in approximate morphological form but differ in size and by subtle differences in the shape of the marginal hook. The discrimination of Morph 2 from *G. derjavini* Mikailov, 1975 and *G. truttae* Gläser, 1974 was discussed earlier. The range of measurements for these four species are given in Table 2.17. Morph 2 represents the smallest form of the four in both the size of the total marginal hook length and the sickle size. By comparison, the marginal hook sickle of *G. birmani* like Morph 2 has a slender heel but the base of the marginal hook is deeper and more robust (see Figure 2.17c c.f. Figure 2.17t). The differentiation of *G. salaris* from *G. thymalli* presents a taxonomic problem; however, the marginal hooks of *G. thymalli* (Figure 2.17r) have a pronounced heel and angular toe which permit its discrimination from those of Morph 2. The marginal hooks of *G. salaris* differ from Morph 2 in the size and open face of the sickle.

In conclusion, the survey indicates that upto seven morphotypes of *Gyrodactylus* may be present on the British salmonoids. The precise identity of these will be determined by use of computational analysis on both morphological form of the sclerites and point to point morphometrics in the following chapters.

Table 2.17: Comparison of Morph 2 with *Gyrodactylus birmani*, *G. salaris* and *G. thymalli*.

Structure	Morph 2	*G. birmani*	*G. salaris*	*G. thymalli*
Total marginal length	28 - 36	41 - 45	34 - 41	37 - 44
Marginal sickle length	5 - 8	7 - 8	8 - 9	7 - 9
Total hamulus length	50 - 62	68 - 76	61 - 69	75 - 84
Hamulus shaft length	32 - 40	52 - 59	48 - 49	57 - 65
Hamulus point length	30 - 33	33 - 40	30 - 37	33 - 39
Hamulus root length	21 - 26	20 - 23	20 - 21	23 - 30

CHAPTER 3: MULTIVARIATE ANALYSIS OF SOME MORPHOMETRICAL FEATURES OF SPECIES OF *GYRODACTYLUS* PARASITISING BRITISH SALMONOID FISHES.

Light microscope based studies

Introduction

Within the Gyrodactylidae, most species identifications are based on the interpretation of sclerite form and the comparison of single variable ranges using, in particular, the marginal hooks. In the case of the genus *Gyrodactylus*, however, the range of measurements for a commonly used variable often overlaps with the measurement range of another species. The interpretation of these may be further complicated by factors, such as seasonal changes, or variations in local environmental parameters which may influence hook morphology (Kulemina, 1977; Mo, 1991a,b,c). In view of this, when comparing two species, ideally one must have available, specimens that cover the full range of temperatures, hosts and other environmental parameters. Not only would this contribute to an understanding of which portions of the sclerites exhibit the most variation, but would also allow us to ascertain those variables which enable true discrimination of the two species under comparison. Within the genus *Gyrodactylus* there exists a number of taxonomically "close" groups. These "close" groups are defined as comprising species which differ from each other only by subtle variations in hook morphology, for example, the *G. elegans*-complex, the *G. phoxini*-complex (see Malmberg, 1970) and the north European "salmonoid" *Gyrodactylus* species. For example, within the *G. wageneri*-group, the differentiation between *G. salaris* Malmberg, 1957 and *G. thymalli* Zitnan, 1960 is a particular problem and has recently been the subject of discussion by Malmberg (1987) and Bakke & Jansen (1991).

The application of multivariate analysis is an attempt to isolate species or to reveal information contributing to an understanding of the relationships between the species of *Gyrodactylus* by considering all the measured morphometric variables simultaneously. The introduction of mathematical modelling applied to morphometric

data has, in certain cases, proven to be a powerful means of discriminating taxonomically close species. Bray & des Clers (1991) used principal components analysis (PCA) in series with a stepwise linear discriminant analysis to prove the existence of five oioxenic species of lepocreadiid digeneans. Silan & Maillard (1989) differentiated two species of monogeneans infecting the sea bass, *Dicentrarchus labrax* L., *Diplectanum aequans* (Wagener, 1857) Diesing, 1858 and *D. laubieri* Lambert & Maillard, 1974, both of which have similar growth rate dynamics, by the application of statistical techniques to the morphometric data. The use of PCA has also been used effectively to discriminate species of the digenean genera *Rhipidocotyle* (Gibson *et al.*, 1992) and *Diplostomum* (Brady, 1989; Höglund & Thulin, 1992). In this study, the species of *Gyrodactylus* present on native salmonoids could not be resolved using traditional taxonomic techniques, thus the application of PCA to the morphometric data was used in an attempt to ascertain the number of species present and to make an assessment of the morphological variation within the identified species. Three fundamental questions were addressed: (1) is it possible to differentiate groups in the genus *Gyrodactylus* parasitising salmonoids by modelling morphometric data, taking into account host-species and local environmental parameters, such as temperature or salinity; (2) if it is possible to isolate species using PCA, then is it feasible to use this method to separate *G. salaris* from other species present on salmonoids in the UK; and (3) is *G. salaris* indeed present in the UK?

Materials and Methods

During the period 1989-1991, a survey of some 250 locations throughout the UK (see Chapter 2, Figures. 2.1-2.6) were investigated for *Gyrodactylus* infection in four salmonoid hosts, *Salmo salar*, *S. trutta*, *Oncorhynchus mykiss* and *Salvelinus alpinus*. The survey included both wild and farmed populations. Specimens were collected as outlined in Chapter 2. Table 3.1 provides a list of sample sites and the number of specimens examined from each site. These specimens were prepared for light microscope studies by mounting in ammonium picrate glycerine according to Malmberg (1970). Wherever possible, 10 specimens from each locality were prepared for the analysis. In the case of some sites, where more material was available, i.e. the

Table 3.1: A list of British sample sites positive for *Gyrodactylus* and the number of specimens examined from each site.

Locality	Sample site	Host species	Worms measured
(a) Scotland			
Borders	North Esk	*Salmo salar*	5
	River Manor	*Salmo salar*	10
	River Tweed	*Salmo salar*	10
	River Manor	*Salmo trutta*	10
	River Tweed	*Salmo trutta*	10
	S. Esk, Brechin	*O. mykiss*	10
	Gala Selkirk	*O. mykiss*	1
Central	Howietoun	*Salmo salar*	5
	Loch Airthrey	*Salmo trutta*	10
	Lake Menteith	*O. mykiss*	10
	Almondbank	*O. mykiss*	2
Dumfries	River Nith	*Salmo salar*	10
Grampian	River Avon	*Salmo salar*	6
	River Fiddich	*Salmo salar*	6
	River Avon	*Salmo trutta*	4
	River Fiddich	*Salmo trutta*	7
Highlands	Halkirk burn	*Salmo salar*	5
	River Shin	*Salmo salar*	1
	Storr loch	*Salmo salar*	2
	Halkirk burn	*Salmo trutta*	10
	River Loth	*Salmo trutta*	7
	Loch Ericht	*O. mykiss*	4
Strathclyde	Loch Arienas	*Salmo salar*	7
	Loch Avich	*Salmo salar*	10
	Loch Coulin	*Salmo salar*	8
	Kilmartin	*Salmo salar*	9
	River Noodle	*Salmo trutta*	8
	Loch Awe	*O. mykiss*	20
	Loch Kendoon	*O. mykiss*	10
Tayside	Lavers burn	*Salmo salar*	1
	Loch Tralaig	*Salmo salar*	2
	Lavers burn	*Salmo trutta*	9
	Loch Butterstone	*O. mykiss*	10

(b) Wales			
N. Dyfed	Afon Bargod	*Salmo salar*	10
	Afon Brefi	*Salmo salar*	10
	Afon Brenig	*Salmo salar*	10
	Afon Cledlyn	*Salmo salar*	3
	River Grannell	*Salmo salar*	4
S. Dyfed	Floodvale	*Salmo salar*	2
	Rofwar Tywi	*Salmo salar*	1
	Blotweth	*Salmo trutta*	2
	Llanwrda Dulais	*Salmo trutta*	2
	Melindwr	*Salmo trutta*	8
	River Twrch	*Salmo trutta*	5
Glamorgan	River Neath	*Salmo trutta*	10
Pembroke	Angof Glanrhyd	*Salmo salar*	1
	Angof Wern	*Salmo salar*	2
	Deepford Brook	*Salmo salar*	1
	Llangolman	*Salmo salar*	4
	Mags Yr Afon	*Salmo salar*	10
	Pont Hywel	*Salmo salar*	2
	Spittal Brook	*Salmo trutta*	6
(c) England			
North	Lake Ennerdale	*Salvelinus alpinus*	10
Severn-Trent	Brailsford	*O. mykiss*	3
	Donnington	*O. mykiss*	3
South-West	Coryton Bridge	*Salmo salar*	10
	River Ohement	*Salmo salar*	8
	River Tiddy	*Salmo trutta*	4
Thames	River Chess	*Salmo trutta*	3
	River Wye	*Salmo trutta*	10
Wessex	Bere Regis	*Salmo trutta*	1
	River Piddle	*Salmo trutta*	1
(d) Ireland			
	Loch Atlan	*O. mykiss*	3
	West coast	*O. mykiss*	3
(e) Standards			
	G. colemanensis	*O. mykiss*	2
	G. salmonis	*O. mykiss*	1
	G. truttae	*Salmo trutta*	7

natural level of infection exceeded 10 specimens per fish, all specimens were removed into a watch glass and 10 parasites were selected randomly and prepared for the analysis. This sampling strategy, and a small number of specimens, was adhered to in order to make the initial analysis manageable.

Specimens were measured under oil immersion (x100) with an eye-piece graticule (100x0.01mm divisions) using an Olympus BH2 binocular microscope. A series of 18 measurements were made on the attachment organ of 399 specimens; these are shown in Chapter 2, Figure 2.7 in accordance with the suggestions of Malmberg (1970) and Harris (1983). Some specimens of *Gyrodactylus* infecting salmonoids outside the UK were utilised in order to act as references in PCA standards. These included: (i) *G. salmonis* Yin & Sproston, 1948 (1 specimen) on *Salvelinus fontinalis* from Nova Scotia (BMNH cat. no. 1990.6.19.20-22), provided by Dr D. Cone; (ii) *G. colemanensis* Mizelle & Kritsky, 1967 (2 specimens) on *Oncorhynchus mykiss* from Canada (BMNH cat. no. 1990.6.19.20-22), provided by Dr D. Cone; and (iii) *G. truttae* Gläser, 1974 (7 specimens) on *Salmo trutta* from Czechoslovakia (BMNH cat. no. 1990.7.23.2-3), provided by Dr R. Ergens.

Principal components analysis (PCA)

There were 399 original specimens, each having 18 measured parameters, Figure 2.7 illustrates these parameters.

All the data were included in the first PCA test. Principal components analysis expresses the relationship between the 18 measured parameters or variables. Each axis of the PCA plot is ordered by the amount of variation they explain, the x-axis shows the variable that explains the most variation within the sample; the next axis plotted at right angles to this, the y-axis shows the second best variable with respect to the amount of variation it explains. The third best variable would be placed at right angles to the first two axes, the fourth at right angles to the resultant of the first three, and so on. This form of analysis summarises as much of the information, i.e. the variability between specimens in the data set, as accurately as possible using only a few components, usually the first three or four. The first component has the longest axis, the second is the next largest and is perpendicular to the first, and the third is the

next largest in the size hierarchy and arises perpendicular to the first two components. To calculate the first principal component, all the variables (coefficients) are made equal, so once the components with these coefficients have been calculated then the total variance on the components is the same as the total variance on the original variables. The component loadings are the covariances of the original variables. If each of the loadings is squared and added up for each component then this will give the variance accounted for by each component. The eigenvector values calculated by the analysis explain how much each character contributes to each axis in the PCA and the eigenvalues explain how much each axis contributes to the overall variation.

Histograms were produced for each variable separately from the raw data to determine whether the data have a normal distribution and are shown in Figure 3.1. Some of these histograms do not display a normal distribution (bell-shaped curve) but are irregular; these may be due to two factors: (1) the size of the structure: if small the number of size class allocations becomes reduced due to the limitations of resolution of the measuring device - in this case 0.5μm is the limit of the resolution of the light microscope under oil immersion; and (2) size hierarchy (natural variation) due to different species in the data set: since the latter was found to be the case, a logarithmic transformation of the data was used to correct the situation, making the variance independent of the mean; this also made the frequency distributions skewed to the right more symmetrical as shown in Figure 3.2.

Each specimen run in a PCA is given a score, the position of each specimen being dictated by all other specimens in the analysis. If the scores are plotted as a two dimensional plot, specimens that are similar are located at the centre of the plot, i.e. close to origin (0,0) whilst these that exhibit the greatest variability are located furthest from the origin.

Cluster Analysis

A cluster analysis was performed on the first PCA plot. This detects natural groupings within the data set. The manner in which these groups are produced is by calculating some measure of dissimilarity between the specimens. In order to calculate this dissimilarity, the data are standardised across the individual structures measured for

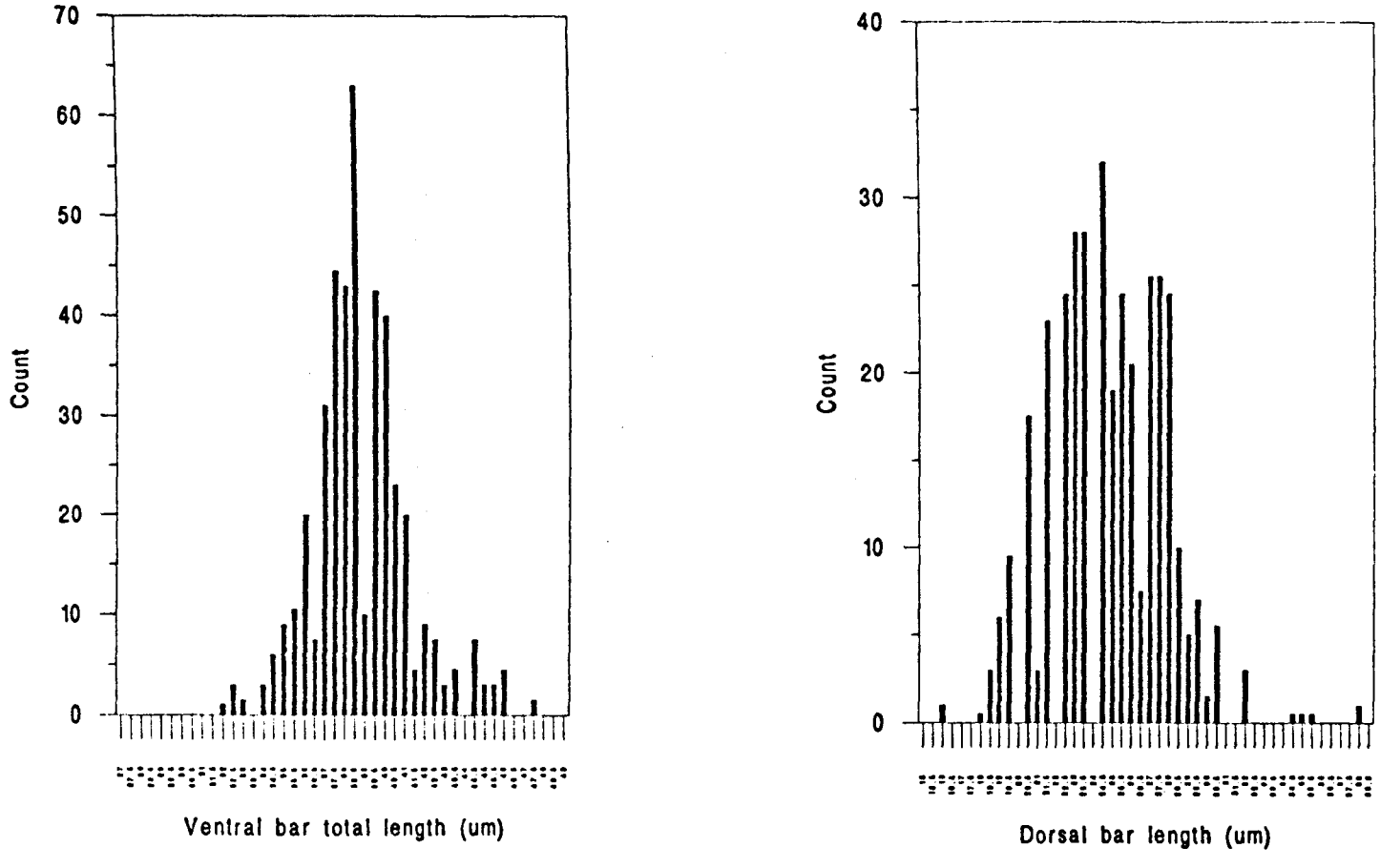


Figure 3.1: Histograms of several measured sclerite structures (non-logged data) showing irregular distributions. The ventral bar total length and the dorsal bar length. The x-axis represents the size of the measured structure (μm) and the y-axis represents the number of specimens for each measurement class.

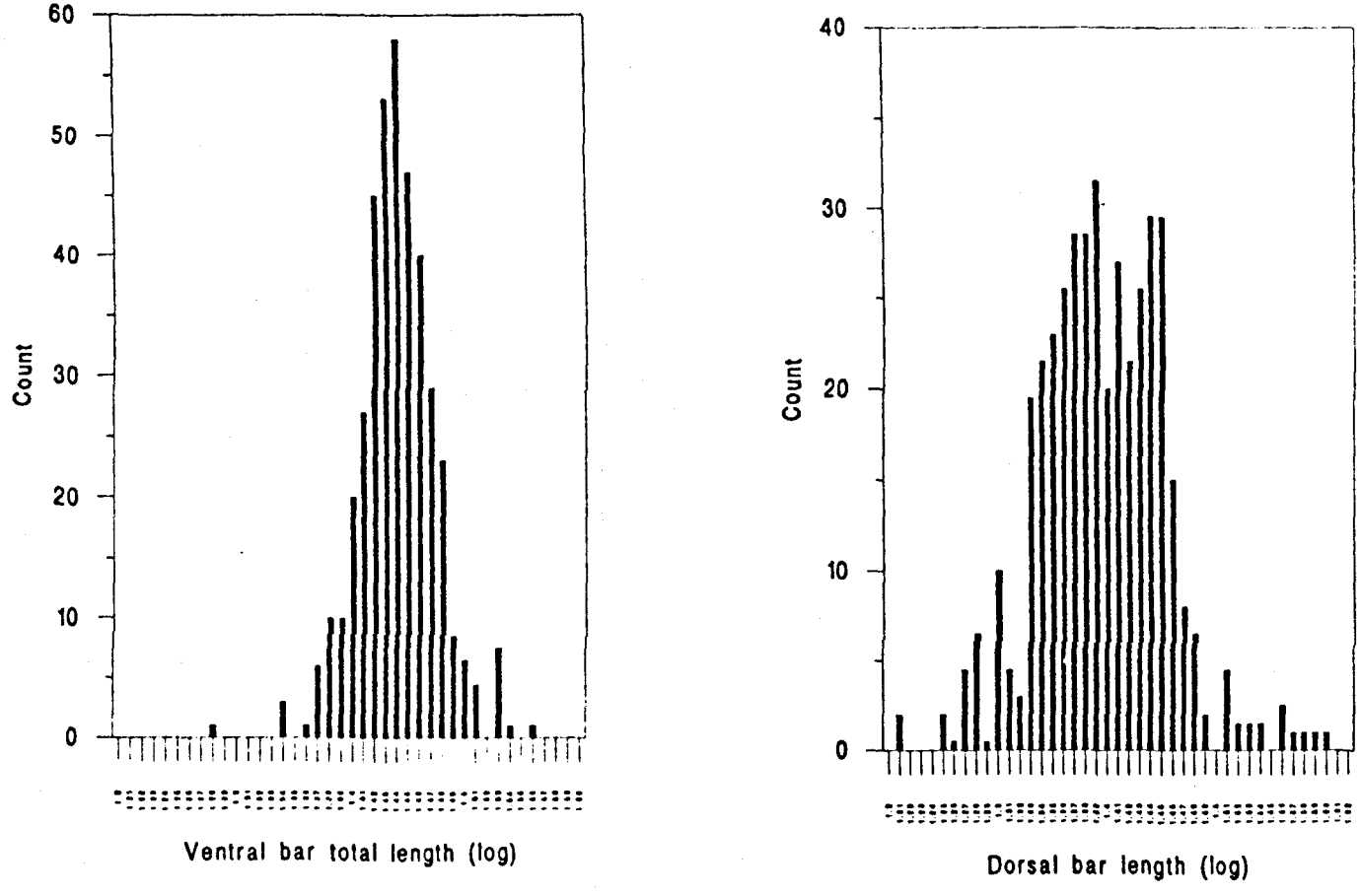
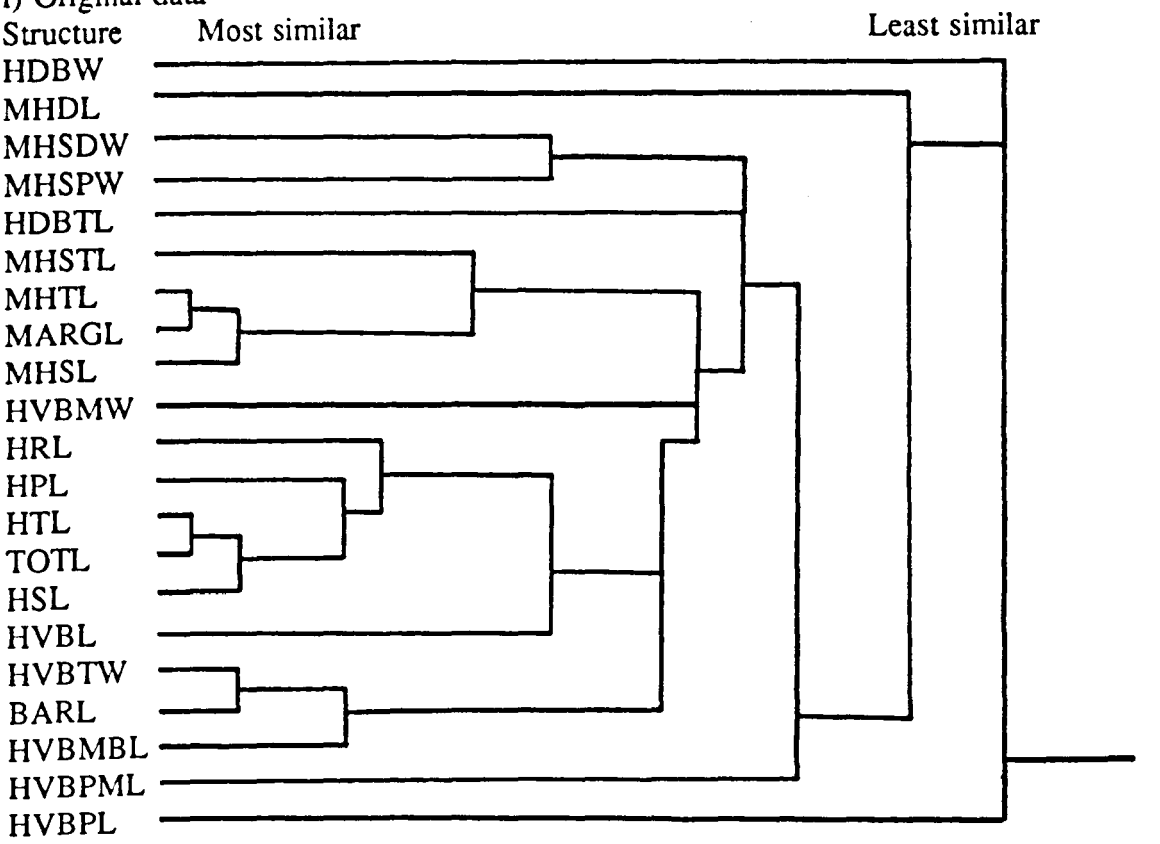


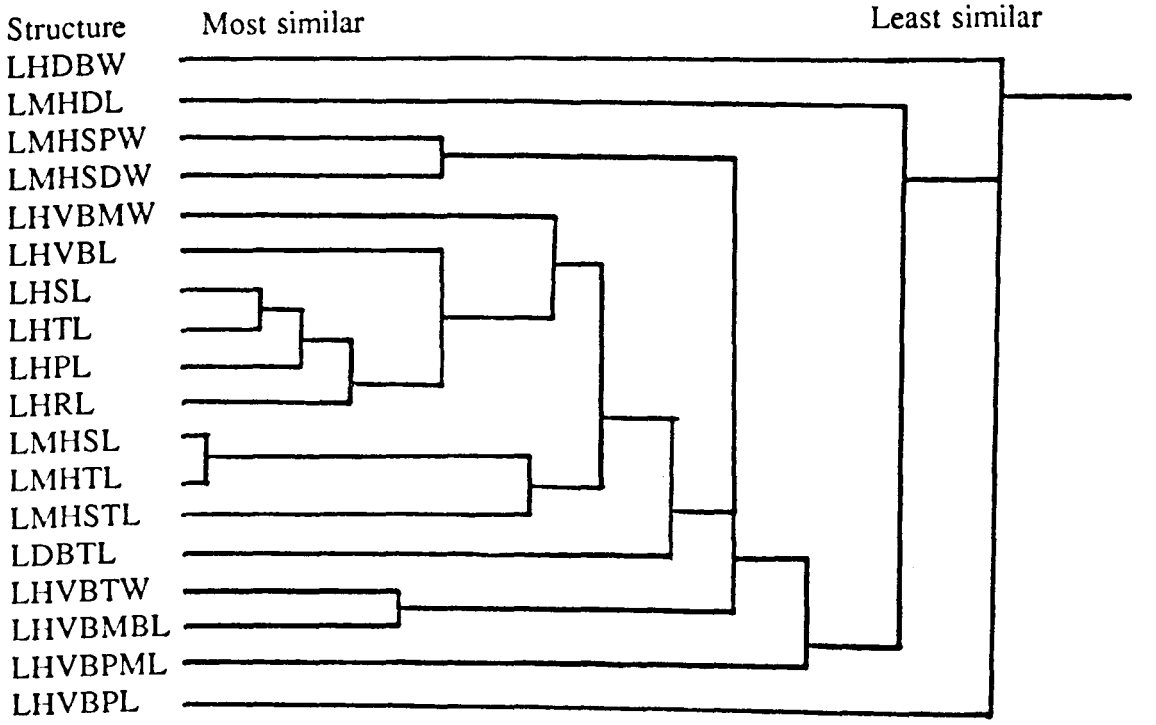
Figure 3.2: Histograms of two measured sclerite structures showing a better distribution following logarithmic transformation of the data. The ventral bar total length and the dorsal bar length. The x-axis represents the log size of the measured structure (μm) and the y-axis represents the number of specimens in each measurement class.

Figure 3.3: A dendrogram showing the relationship of dissimilarities between structures of the haptoral compliment. (Abbreviations see list in Appendix 2).

i) Original data



ii) Log transformed data



the analysis; this gives all the measurements on a common scale. Pearson's correlation can be used as the basis for dissimilarity, the single linkage method of nearest neighbour being given as a dendrogram of dissimilarities between structures of the haptoral complement is shown in Figure 3.3. The cluster analysis is interactive, and is instructed to look for 2 clusters, then 3 clusters, 4, 5 etc. up to 15 clusters (in this case). The "optimal" number of groupings within the PCA is given by the F-ratio and to a lesser extent the value of probability in the summary statistics for the number of clusters pulled out by the analysis. Here the analysis looks at the first three factors operating in the PCA (this information is obtained from the variance explained by the components and the percent of total variance explained); here it is anticipated that the first few factors explain a high percentage of the total variance explained by the components. A plot of the PCA coefficients displays this information (Figure 3.6). The lower the probability value, the greater the chance of any one specimen belonging to that group. An optimal number of clusters, or groupings, is given by a high F-ratio in all three factors before decreasing again (the number of clusters is determined conservatively, retaining those specimens which may contribute to the separation of specimens in a subsequent PCA test). For this reason multiple analyses are performed to ensure that the highest F-ratio is observed.

It should be stated that PCA and cluster analysis are not definitive, but are a compromise acting as an exploratory guide through the data. The clusters indicated by the analysis, were cross-referenced back to the original specimens and re-examined. The clusters or morphs generated by the analyses were only confirmed following direct morphological comparison of the specimens using the scanning electron microscope. These morphs are given in Chapter 2.

Results

PCA 1

The results of the first PCA (n = 399; 69 sites) are shown in Figure 3.4. Table 3.2 gives the values of covariance for PCA 1 where the coefficient of variation (CV) is given by the standard deviation divided by the mean x 100. The component loadings

for PCA 1 are given in Table 3.3. Table 3.4 gives the variance explained by the components and the percentage of the total variance explained. The first three factors here accounting for 62.13% of the total variance explained. The cluster analysis suggested three natural groups within the specimens run in the first PCA 1 (Figure 3.4) (factor 1 plotted against factor 2) and are as follows:

(i) Loch Shin (1 specimen on *S. salar*); this was removed from the data set as an outlier, having a hamulus length of only 38μm (Figure 3.5).

(ii) Loch Coulin (7 specimens on *S. salar*) and Loch Tralaig (2 specimens on *S. salar*) represent Morph 3; these were removed from the data set, having hamulus lengths that exceeded 75μm.

(iii) The third grouping of 388 specimens from 66 localities was used for a second PCA analysis.

It was necessary to remove all the outliers in each instance as their presence within a data set influences the relative position of all other specimens in the correlation matrix.

PCA 2

The 388 remaining specimens from the first PCA were then run in a second analysis, PCA 2 (logged data). In this analysis six major groupings appeared and the results were also analysed in the form of a cluster analysis. From PCA 2, six clusters were identified using cluster analysis, the three factors here account for 50.82% of the total variance explained. Several groupings were removed as outliers, these were:

(i) River Grannell (specimen 46; 1 of 4 specimens collected from this site on *S. salar*). This specimen was removed as having a large ventral bar process-middle length (LHVBPML) and the marginal hooks which are narrow for their length.

(ii) a) Donnington (specimen 207; 1 of 3 on *O. mykiss*).

b) Loch Airthrey (specimens 269-278; on *S. trutta*).

c) Loch Butterstone (specimen 353; 1 of 10 on *O. mykiss*).

All the above specimens were removed having a large ventral bar process-middle length (LHVBPML) and long, thin marginals. Specimen 277 did not fit into this category but was removed with the other specimens for that site (Figure 3.7).

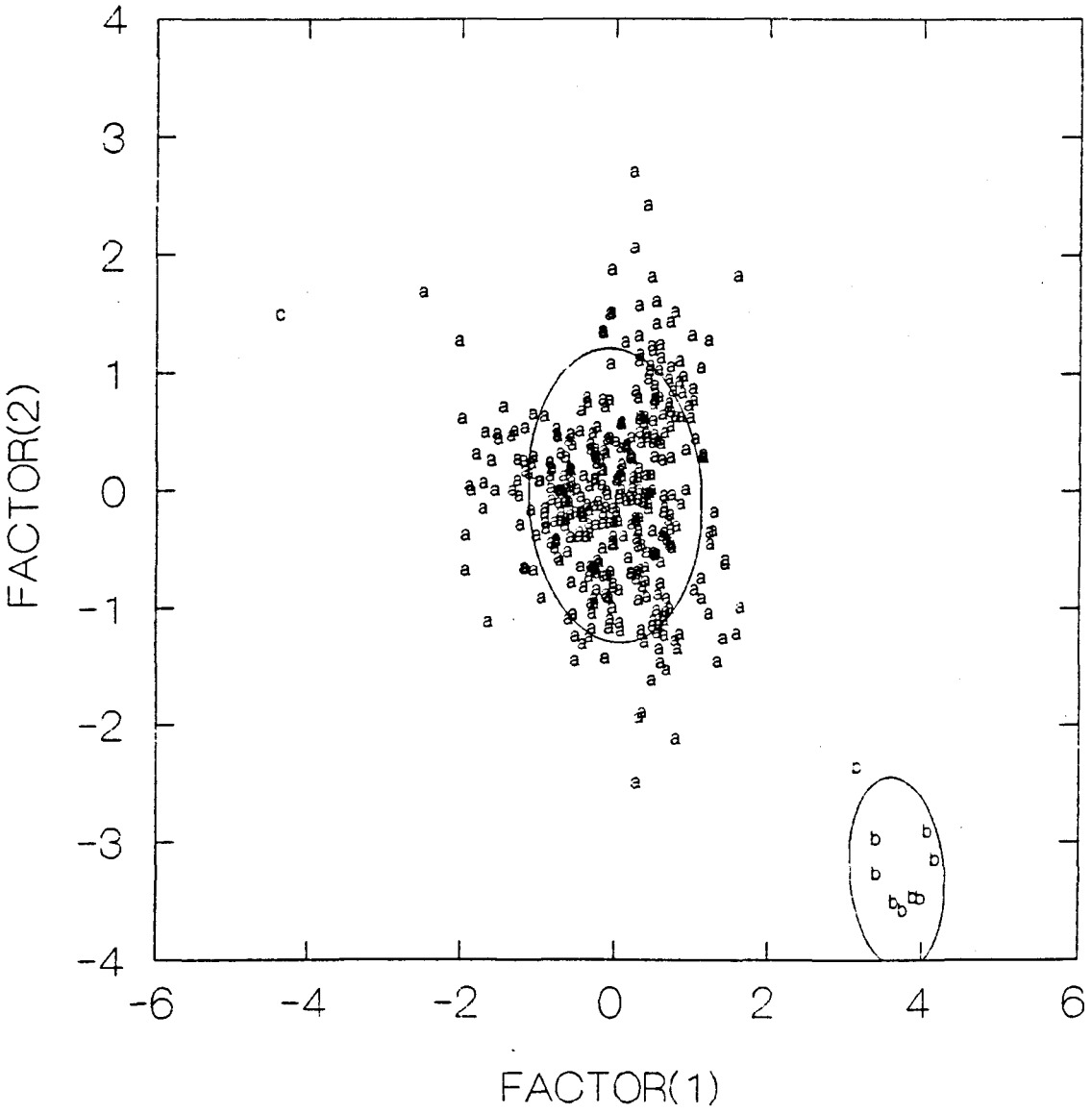


Figure 3.4: Map of the 399 specimens of *Gyrodactylus* collected from British salmonoids as shown in the first plane of the PCA 1. Each specimen is identified by its cluster number as calculated using Cluster Analysis: a = *Gyrodactylus* sp. from all UK; b = Morph 3 from Lochs Coulin and Tralaig; and c = *Gyrodactylus* sp. from Loch Shin.

Table 3.2: The values of covariance for the first PCA (n=399 specimens)

STRUCTURE	MEAN	S.D	C.V
LHTL	4.062	0.084	0.021
LHSL	3.727	0.083	0.022
LHPL	3.377	0.091	0.027
LHRL	2.836	0.133	0.047
LHDBTL	3.228	0.133	0.041
LHDBW	0.848	0.184	0.216
LHVBL	3.334	0.080	0.024
LHVBTW	3.194	0.081	0.025
LHVBMW	1.937	0.143	0.074
LHVBPML	1.225	0.294	0.240
LHVBPL	1.074	0.200	0.186
LHVBMBL	2.643	0.123	0.047
LMHTL	3.449	0.074	0.021
LMHSL	3.240	0.080	0.025
LMHSTL	1.901	0.082	0.043
LMHSDW	1.574	0.104	0.066
LMHSPW	1.629	0.075	0.046
LMHDL	2.486	0.115	0.046

Table 3.3: Component loadings for the first PCA (n=399 specimens)

STRUCTURE	1	2	3
HTL	0.905	0.215	-0.251
HSL	0.833	0.206	-0.183
HPL	0.732	0.305	-0.337
HRL	0.665	0.185	-0.285
HDBTL	0.646	-0.076	0.185
HDBW	0.341	-0.469	0.254
HVBL	0.709	0.079	-0.228
HVBTW	0.578	0.593	0.444
HVBMW	0.631	0.205	-0.278
HVBPML	0.333	0.163	0.522
HVBPL	0.171	-0.047	0.345
HVBMBL	0.432	0.287	0.475
MHTL	0.804	-0.446	0.166
MHSL	0.747	-0.464	0.179
MHSTL	0.729	-0.306	0.016
MHSDW	0.532	-0.110	-0.345
MHSPW	0.504	-0.404	-0.039
MHDL	0.406	-0.335	-0.155

Table 3.4: Eigenvalues and proportion of the variance explained by the first four principal components for the first PCA (n=399 specimens).

Eigenvalue	% of total variance	cumulative %
9.04	43.06	43.06
2.16	10.30	53.36
1.84	8.77	62.13
1.72	8.18	70.31

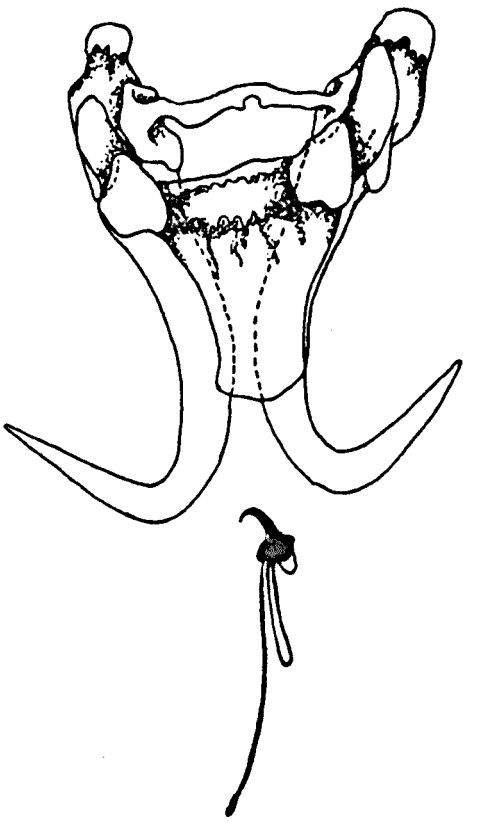


Figure 3.5: *Gyrodactylus arcuatus* from Loch Shin identified and isolated by the first PCA. Scale bar: 20µm

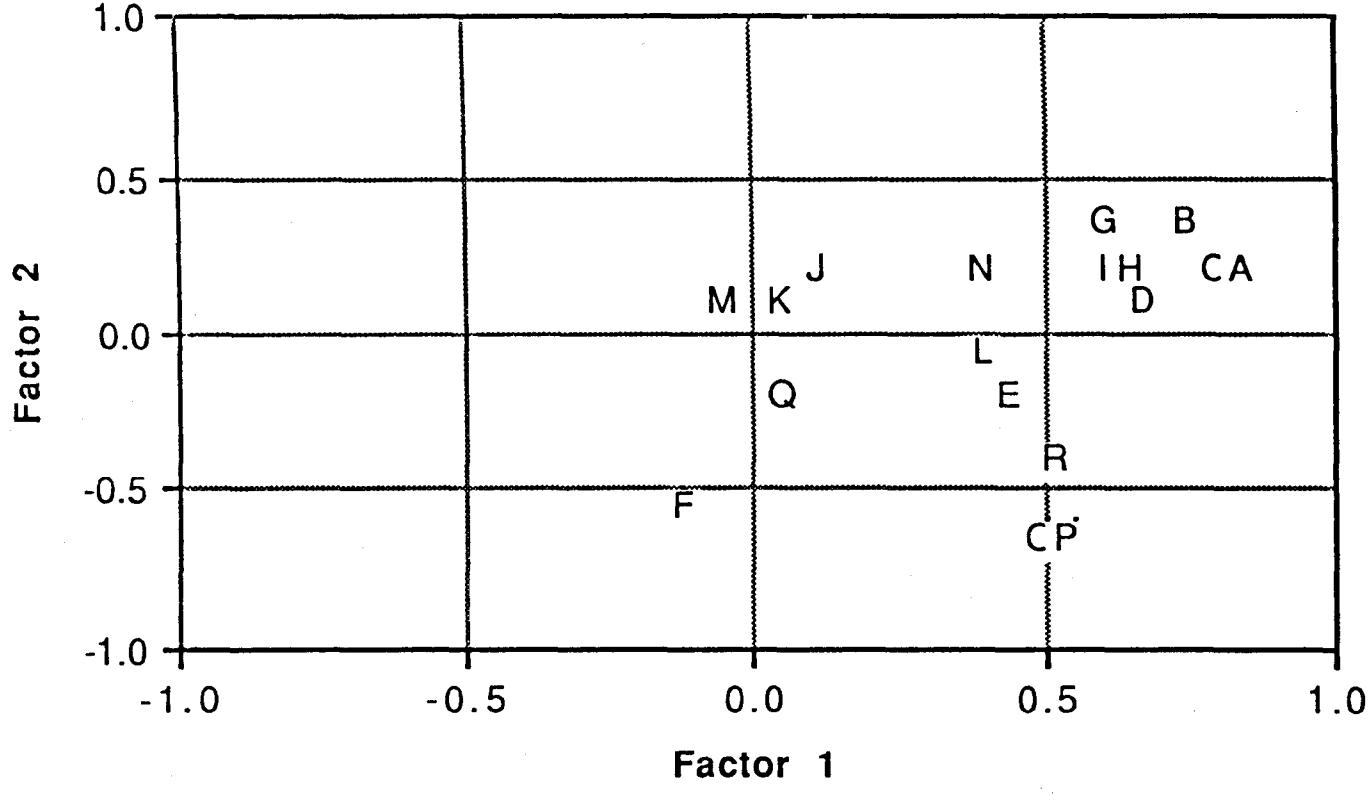


Figure 3.6: A plot of the PCA coefficients (factor scree plot). Abbreviations: A = Total hamulus length; B = Hamulus shaft length; C = Hamulus point length; D = Hamulus root length; E = Dorsal bar total length; F = Dorsal bar width; G = Ventral bar length; H = Ventral bar total width; I = Ventral bar middle width; J = Ventral bar process to middle length; K = Ventral bar process length; L = Ventral bar membrane length; M = Marginal hook total length; N = Marginal hook shaft length; O = Marginal hook sickle length; P = Marginal hook sickle distal width; Q = Marginal hook sickle proximal width; R = Marginal hook domus length.

[These first two groupings appear to represent morphological variants, anomalous worms that were unassigned to any of the clusters pulled out so far, but which have been removed because of their influence on the correlation matrix.]

(iii) *G. colemanensis* (internal standard); specimens 176-177 were isolated as a separate group by the PCA and removed from the data set. These specimens were discriminated from other specimens in the analysis on the basis of these two specimens having very large ventral bar to process-middle lengths (Figure 3.8).

PCA 3

The 15 specimens identified in PCA 2 were removed and the remaining 373 individuals (64 sites) subjected to a third analysis, PCA 3 (logged data), and corresponding cluster analysis. From the F-ratio values seven groupings were identified. No outliers were removed at this stage as the clusters radiated out from a central point with an apparent high degree of overlap. The first three factors here accounted for 49.453% of the total variance explained.

The data for 10 clusters were then examined, analysing factor 1 and factors 2 and 3 separately in order to ascertain the extent to which each of these factors was responsible for the clusters generated and, more importantly, which point to point measurements of the haptoral complement yield the largest eigenvalues and explain the most variance. This explains the amount of variation exhibited between clusters on PCA 3, i.e whether or not incremental steps in terms of "measurement sizes" can be detected between the clusters.

The factors operating in F1, F2 and F3 were calculated from the component loadings and factor score coefficients for the three factors on PCA 3. From the factor loading plots of the component loadings for PCA 3, it can be calculated that Factor 1 is a function of length (a continuum) of the separate structures. Factor 2 is principally explained by the marginal hook total length and shaft length and to some extent by the dorsal bar width. Factor 3, is explained by the ventral bar membrane length and total width. Having identified these structures as being influential on the clusters generated, it was then necessary to return to the raw data for each of these structures within each cluster and determine whether the clusters had discrete ranges of measurements. The raw data indicated that there were some loose associations in

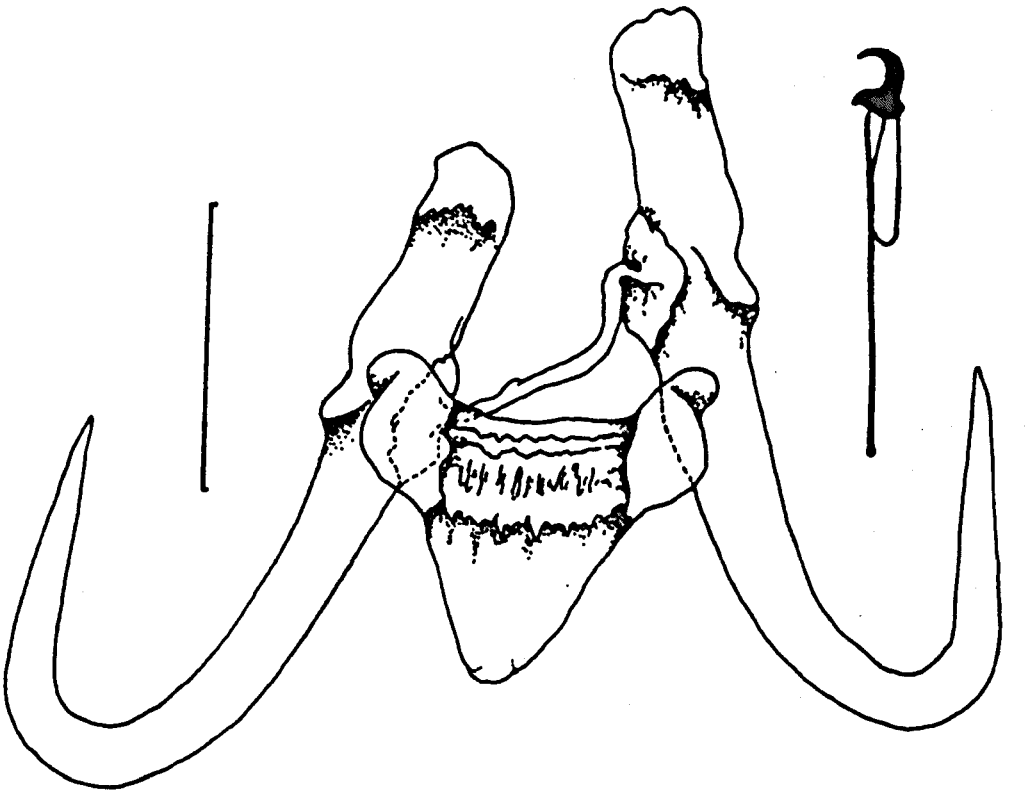


Figure 3.7: *Gyrodactylus truttae* (Morph 5) from Loch Airthrey brown trout identified by the PCA analysis as having a large bar process-middle length and long, thin marginal hooks. Scale bar: 20µm.

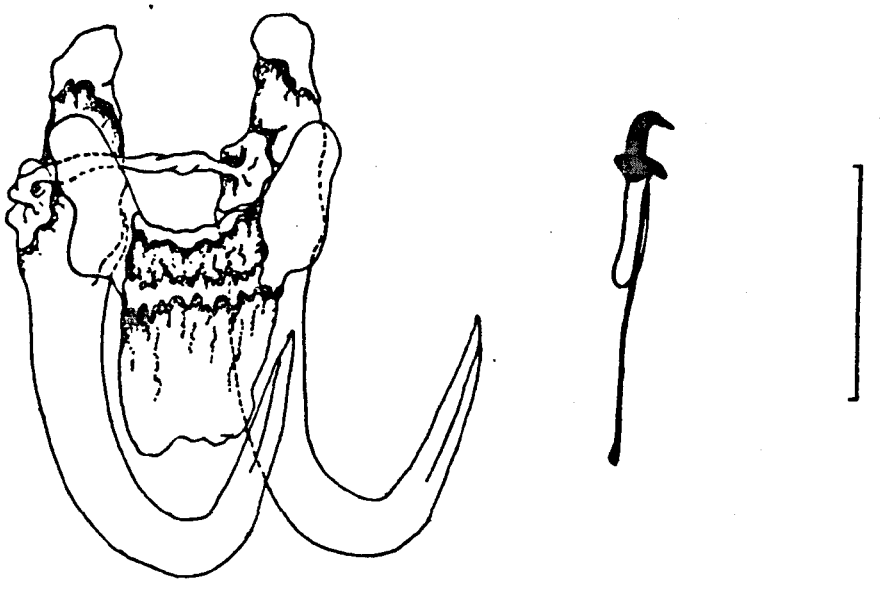
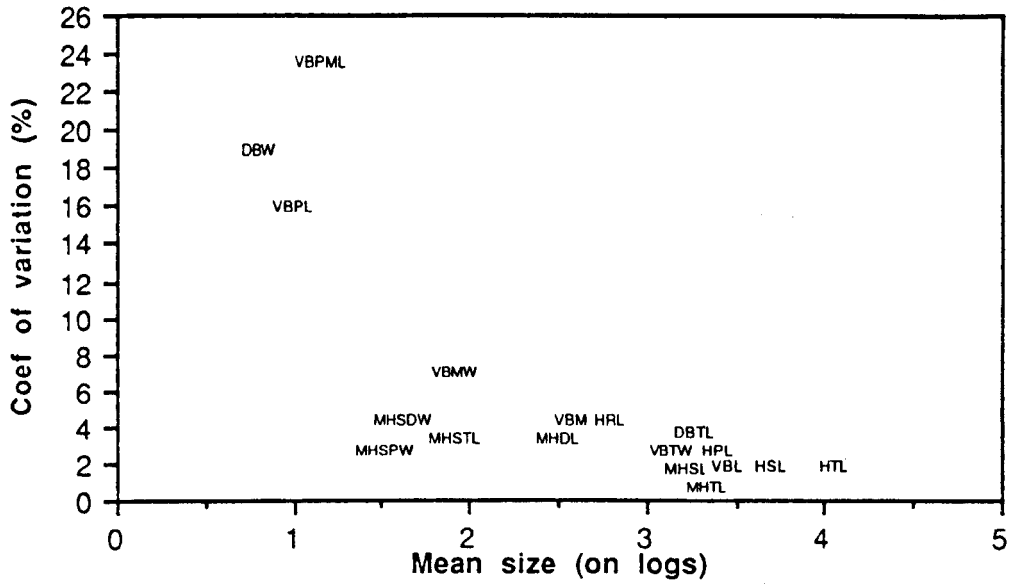


Figure 3.8: *G. colemanensis* discriminated from the other specimens of *Gyrodactylus* within the PCA analysis on the basis of having a very large ventral bar to process-middle length. Scale bar: 20µm.

Factors 1 and 2 for the clusters generated. Although it was not discrete, it was found that there was a gradual increase in the mean value for each of the measured variables within each identified cluster. However, since the clusters identified displayed a large degree of overlap, the range of measurements for each variable within each cluster would also be expected to show large degrees of overlap.

In order to calculate the main concentration of points within a cluster, the mean(± 1 std. dev.) for each factor variable was plotted on PCA plots for seven clusters. It was found that the bulk of the observations were situated close to the central point of each factor, so anything close to the middle of these axes has a variance close to zero on each factor axis examined by the graph. As a consequence, a separate species or morphological variant would be obscured by all the surrounding data. A different form could only be recognised, therefore, at the periphery of the data set, as in the cases of those already highlighted by, and subsequently omitted from, the PCA which were different enough to place these specimens at the extremes of the log axis when plotted against one another. Further analysis was required to clarify the present observations, since there was no justification for the removal of any further outliers at this stage. The relationship of the coefficient of variation (CV) with mean size (on logged data) was examined in order to determine which structural components exhibited the least variability. Figure 3.9 shows the CV plotted against the mean size. The correlation of each morphometric variable against one another was also investigated on the logged data.

The original data set comprised specimens from four hosts; however, specimens of *Gyrodactylus* from *Salvelinus alpinus* were not equally represented (only 10 specimens) in relation to the number of gyrodactylids collected from the other salmonoids. PCA 3 identified the single specimen of *G. salmonis*, operating as an internal standard (Figure 3.10), as an outlier due to the possession of very long marginal hooks (43µm long *vs* an average of 33µm in other specimens), and this single specimen was removed together with the specimens from *S. alpinus* (Figure 3.11). This left the data from three host species, *Salmo salar*, *S. trutta* and *O. mykiss* for further analysis.



Gyrodactylus on salmonids

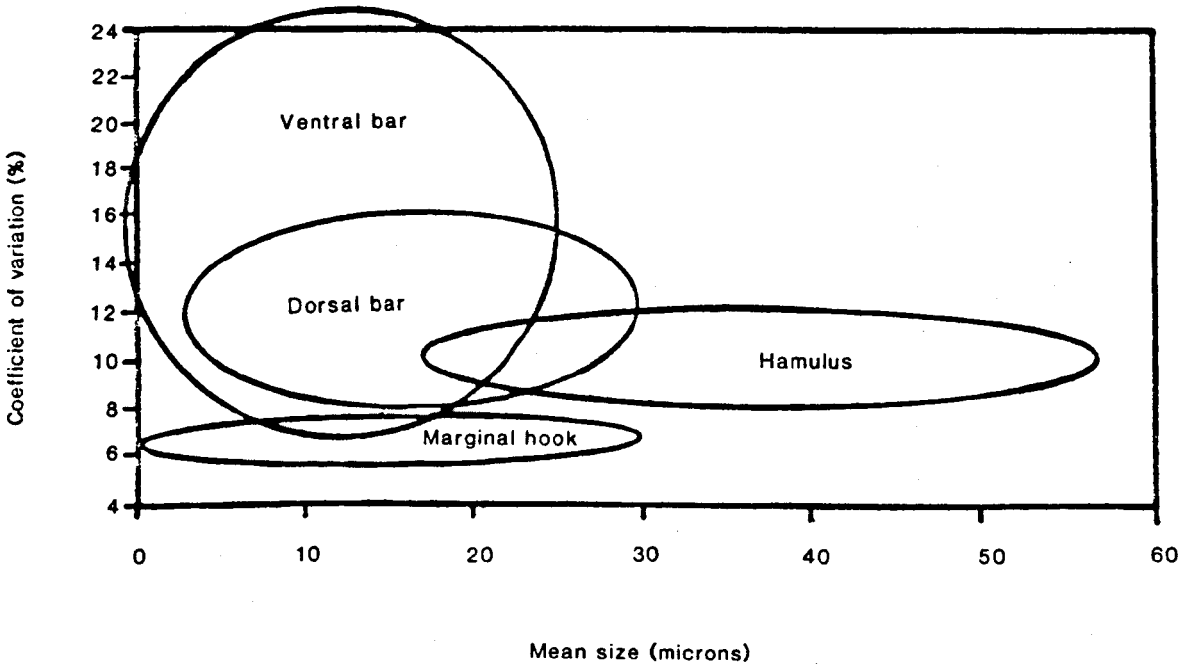


Figure 3.9: The coefficient of variation plotted against the mean size for each structural component.

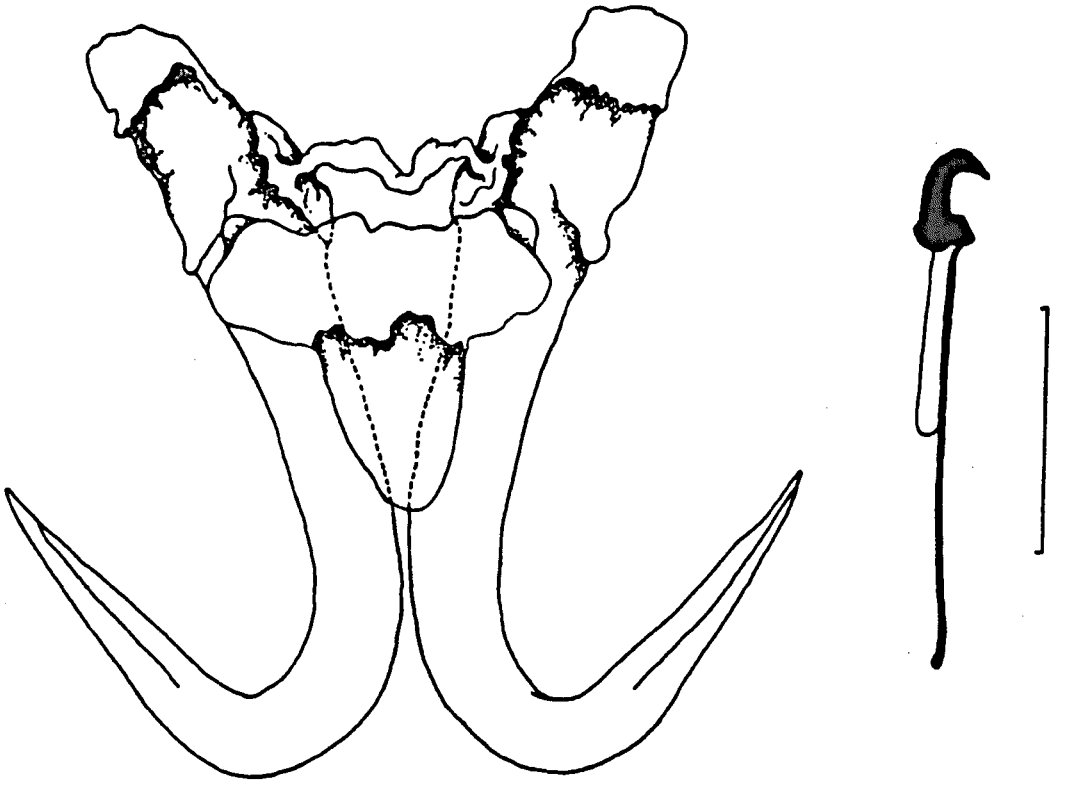


Figure 3.10: *G. salmonis* on *Salvelinus fontinalis* from Nova Scotia, a form with very long marginal hooks identified as an outlier of the third PCA analysis. Scale bar: 20µm.

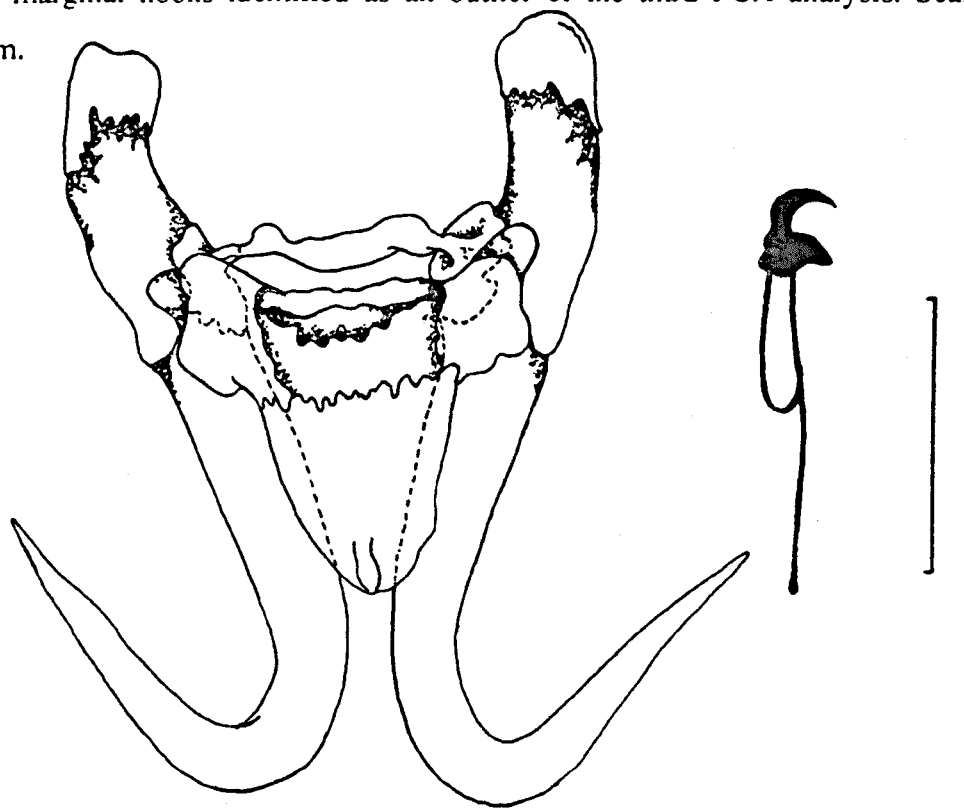


Figure 3.11: *Gyrodactylus* Morph 6 collected from *Salvelinus alpinus* in Lake Ennerdale excluded from the PCA analysis. Scale bar: 20µm.

PCA 4

The remaining 362 specimens from PCA 3 were then given a weighting so that the number of specimens parasitising each host species were equally represented in the analysis.

The weighting was calculated thus:

Total no. of specimens / no. of host species = 362 / 3 = 120.66

(i) 167 *S. salar* specimens	120.66 / 167 = 0.720
(ii) 124 *S. trutta* specimens	120.66 / 124 = 0.973
(iii) 71 *O. mykiss* specimens	120.66 / 71 = 1.700

These weighted data were run in PCA 4, the cluster analysis (F-ratio values) indicating four groups. When the specimens within these groups were analysed, one group was composed of *S. trutta* specimens, a second composed of *S. salar* specimens and a further two groups that were composed of a mixture of gyrodactylids collected from all three salmonid hosts. The first three factors (principal components) accounted for 50.16% of the total variance explained.

In order to study the separate morphometric variables in relation to each host species, a series of histograms (coefficient of variation against the mean) were produced for each variable. Figure 3.12 shows the ventral bar process length, an example of a structure showing bimodality, and Figure 3.13 shows the hamulus total length, an example of a structure showing low variability. It was hoped that this would explain some of the variability and determine any bimodality within the results. The results of these histograms (ranges of logged raw data used on a standardised variable scale, i.e the mean is equal to 0 and the standard deviation to 1) are briefly discussed below.

Ventral bar

Ventral bar length (HVBL): The worms from all three host species appeared to show the same degree of variability in this structure.

Ventral bar total width (HVBTW): This parameter showed greater variability and more so in salmon and brown trout. The reason for this may be due to the fact that the total

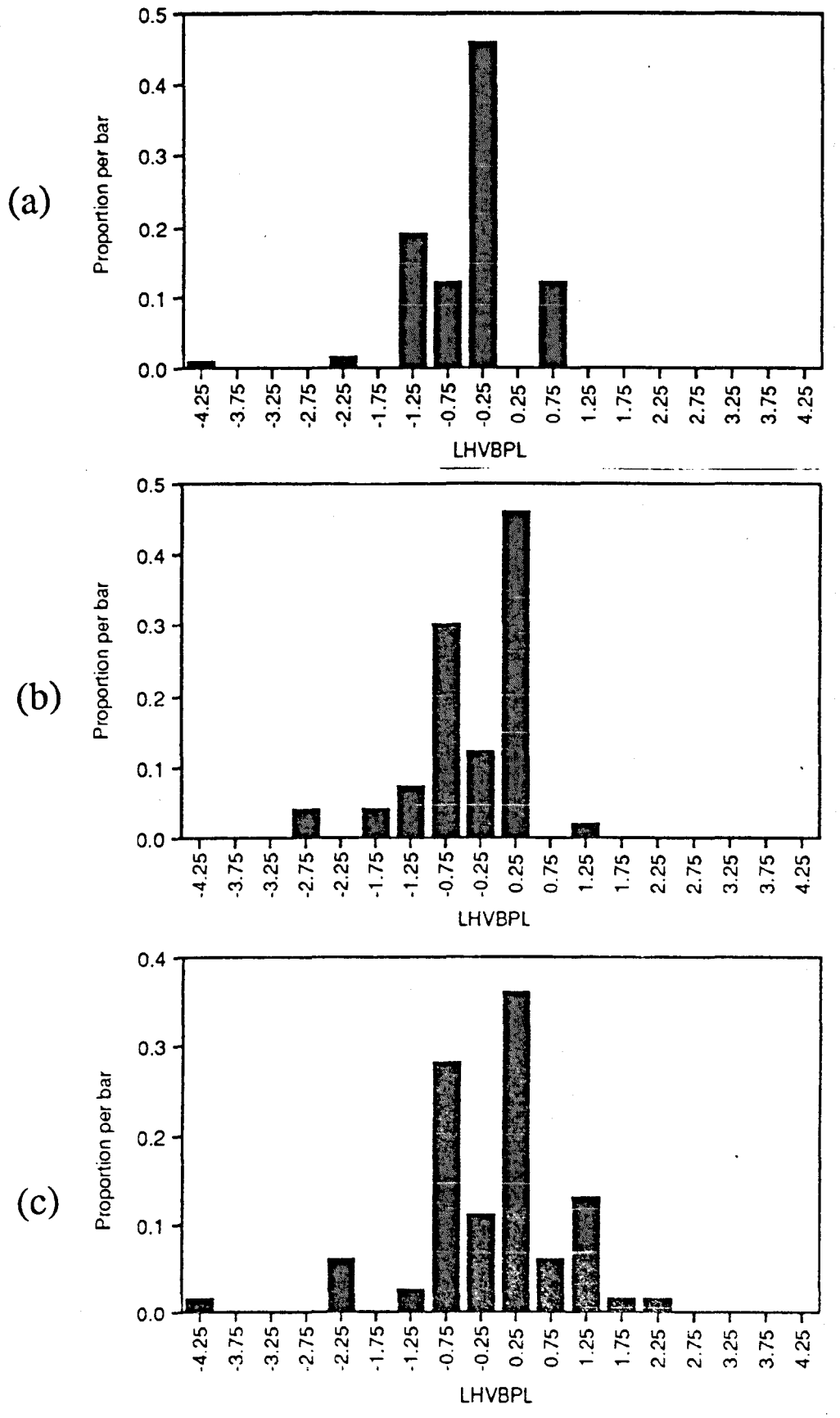


Figure 3.12: The ventral bar process length, a structure showing some bimodality for the three salmonids sampled: (a) salmon, (b) brown trout and (c) rainbow trout.

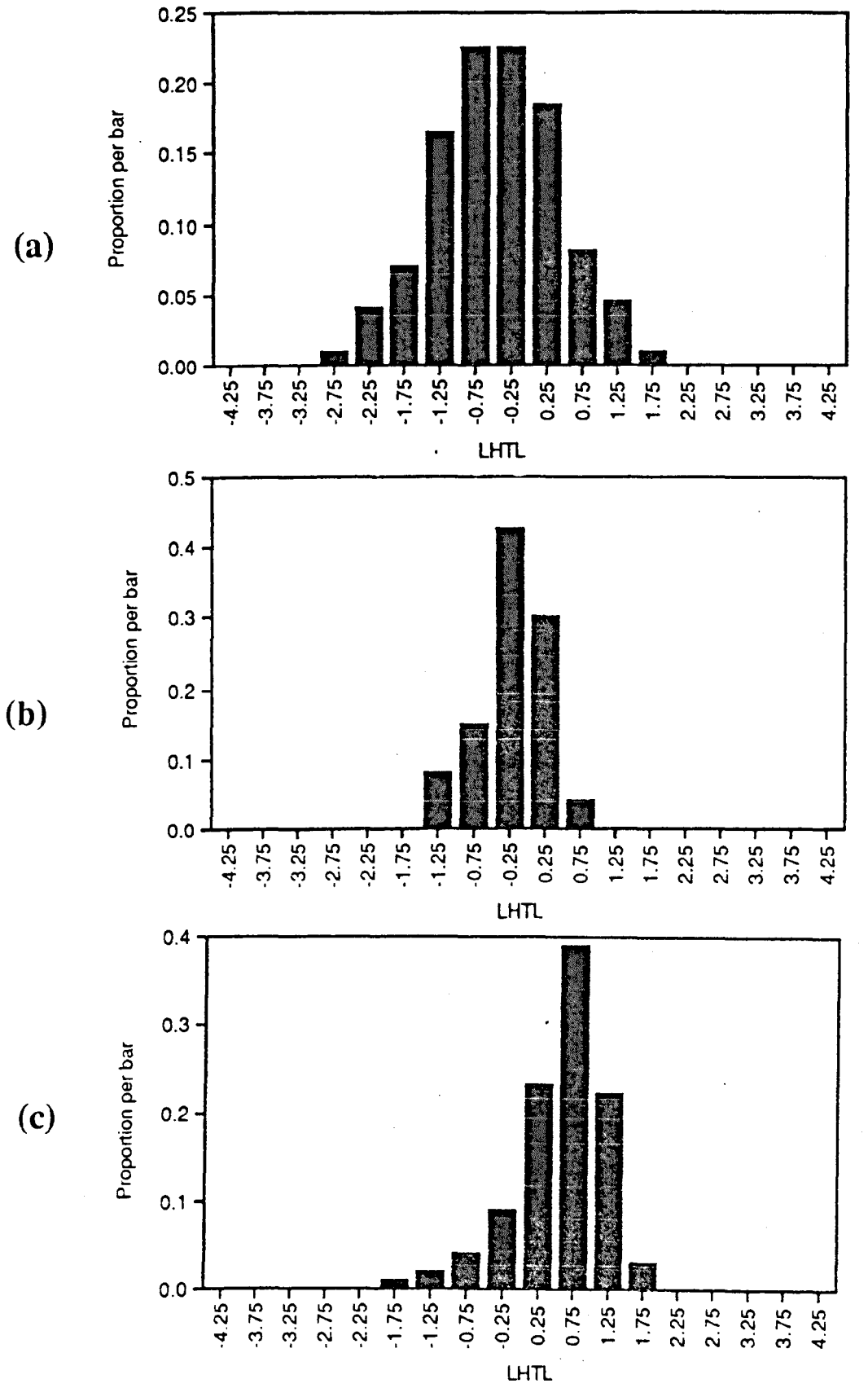


Figure 3.13: The hamulus total length, a structure showing low variability for the three salmonids sampled: (a) salmon, (b) brown trout and (c) rainbow trout.

width includes the ventral bar membrane and the membrane's distal point was often difficult to discern.

Ventral bar process to mid-length (HVBPML): This structure was more consistent in rainbow trout than in the other two hosts in which it had a greater variability.

Ventral bar median width (HVBMW): As in the case of HVBTW, this is a function of the total ventral bar width and displayed a high degree of variability. Both the salmon and brown trout specimens exhibited some bimodality. Due to the problems encountered in measuring this structure, it was deemed to be unreliable for discriminating species. For this reason, it was removed from the analysis in order to see a clearer picture of the structures which were responsible for the "forms" of *Gyrodactylus* exposed by the PCA.

Ventral bar membrane length (HVBMBL): A function of the ventral bar width, this membrane is a larger structure and, therefore, has a smaller variability, although there were some indications of bimodality in the material from brown trout and salmon.

Ventral bar process length (HVBPL): This represents one of the smallest structures measured in the haptoral complement, with a small range in the size of measurements. The process length exhibited a high degree of variability within the histogram (Figure 3.12), and did not, therefore, have a normal distribution. There are two possible reasons for this variability. Firstly, it is a small structure (2-3μm) such that, when measured with the light microscope whose accurate resolution is approximately 0.5μm, the number of measurement "score" classes is small and any error becomes more significant. Secondly, the ventral bar process arises from the median portion of the ventral bar at a tangent to it, such that defining its point of origin becomes difficult and consequently a source of measurement error. It must be concluded that this parameter does not represent a reliable taxonomic criterion.

Dorsal bar

Dorsal bar width (HDBW): This represents the smallest structure measured in the haptoral complement. It exhibited a high variability which was also possibly due to the limitations of the light microscope. The point of measurement for this is taken at the mid-point along the dorsal bar. The mid-point of the dorsal bar has previously been found to exhibit several intraspecific morphological variations (Malmberg, 1970;

Harris, 1983) and is, therefore, probably too unreliable to warrant inclusion in the analysis.

Dorsal bar total length (HDBTL): There appeared to be some degree of bimodality in the specimens from all three salmonid hosts. Some of the variation may be attributable to the fact that this bar is flexible, adjusting to maintain the hamuli in position for effective attachment to the host.

Marginal hook

Marginal hook total length (MHTL): The variability exhibited in this structure was lower than in all other structures.

Marginal hook shaft length (MHSL): This is a function of the marginal hook total length and, similarly, had a low variability. However, the variability in the gyrodactylids from salmon was much greater than in those from the other hosts.

Marginal hook filament loop length (MHDL): The filament loop, which functions as a sclerotised guard at the rear of the sickle, is flexible, being attached to the marginal hook sickle by an as yet undetermined mechanism. This system allows the sickle to travel through several planes during the process of attachment to the host. It is for this reason that there is a degree of variability.

Marginal hook sickle length (MHSTL) and marginal hook sickle widths (MHSPW & MHSDW): The sickle length and both sickle widths are less than 10μm in size and are, therefore, unreliable criteria. Malmberg (1970) demonstrated that it is its shape rather than differences in overall size alone that makes the marginal hook a useful feature for differentiating species.

Hamulus

All portions of the hamulus (Figure 3.13), total, point, root and shaft lengths were well behaved and exhibited a low variability.

The major result of this analysis was that the variables in the haptoral complement below 10μm in size were likely to produce a confusing picture when used for species determination; this was due to the limitations of the light microscope as a measuring device at this level. The structures which fell within this category were:

dorsal bar width (HDBW), ventral bar process length (HVBPL), ventral bar process mid-length (HVBPML), ventral bar median width (HVBMW), marginal hook sickle length (MHSTL), and marginal hook sickle distal (MHSDW) and proximal (MHSPW) widths. Several additional structures exhibited a degree of bimodality which was again possibly a function of size and microscope limitations. The structures within this category were all less than 20μm in size and the viability of such as taxonomic criteria is questionable, especially when they may not be completely flat, thus introducing further error in point to point measurements. These structures were the ventral bar membrane length (HVBMBL), ventral bar process length (HVBPL), marginal hook filament loop length (MHDL) and hamulus root length (HRL). Some of this variation may be explained as follows: the hamulus root, which represents a region of unconsolidated hook material, changes in shape as a result of fixation with ammonium picrate glycerine (P.D. Harris pers. comm.); the filament loop of the marginal hook occurs at various angles from the hook proper and this may cause small deviations in measurement; and the ventral bar membrane is a delicate structure located within the body of the haptor with its distal point is often difficult to discern. The conclusion of this part of the analysis was, therefore, that all variables with measurements of less than 20μm taken on the light microscope were unreliable and should be excluded from subsequent analyses.

PCA 5

A fifth PCA was then run, having removed those variables whose point to point measurements were less than 20μm. The eight variables used in this analysis were: hamulus total length (HTL), hamulus shaft length (HSL), hamulus point length HPL), dorsal bar total length (HDBTL), ventral bar total length (HVBL), ventral bar total width (HVBTW), marginal hook total length (MHTL) and marginal hook shaft length (MHSL). From the cluster analysis summary statistics (F-ratio) three clusters were indicated as optimal. Here the first two factors accounted for 69.330% and the first three accounted for 80.476% of the variance. A breakdown of the clusters identified according to host species (*S. salar* 167 specimens, *S. trutta* 124 and the *O. mykiss* 71) is given in Table 3.5.

PCA 5 was then repeated for three clusters on just factors 1 and 2, which

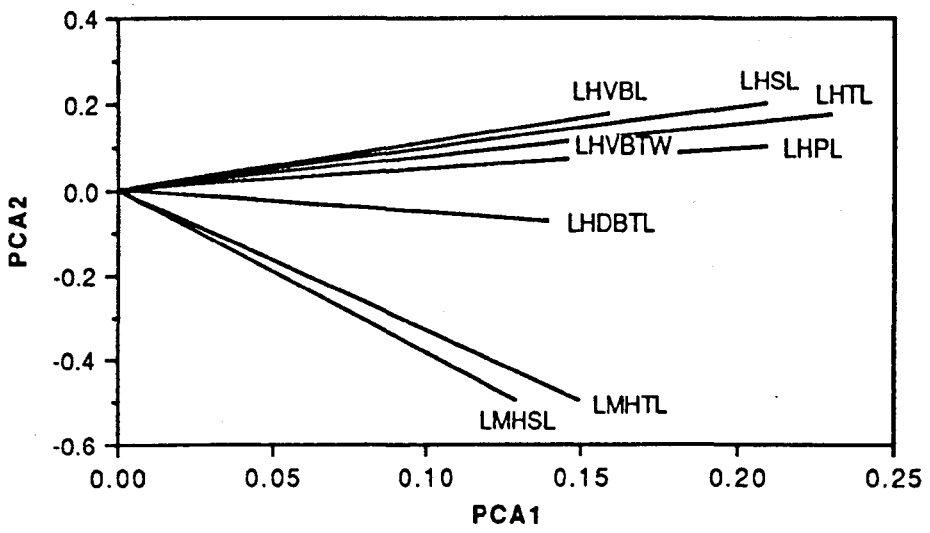
Table 3.5: A breakdown of the clusters identified according to host species for the fifth PCA (n=362 specimens).

	Host species	specimens in cluster	% of total specimens in cluster	% of all specimens on host species
CLUSTER 1:	*S. salar*	83	71.6	49.7
	S. trutta	8	6.9	6.5
	O. mykiss	25	21.6	35.2
CLUSTER 2:	*S. salar*	70	49.0	41.9
	S. trutta	34	23.8	27.4
	O. mykiss	39	27.3	54.9
CLUSTER 3:	*S. salar*	14	13.6	8.4
	S. trutta	82	79.6	66.1
	O. mykiss	7	6.8	9.9

explain 69.33% of the total variance. There were no significant differences between PCA 4 and PCA 5, and the removal of Factor 3 resulted only in the transposition of eight specimens. A plot of the PCA coefficients shown in Figure 3.14, shows the eight lines intersecting at "0, 0" representing the variables retained for PCA 5. The length of each vector on the plot is proportional to its contribution to the PCA. The angle between any two of these vectors is inversely proportional to the correlation between them. Figure 3.14 shows the longest axes to be the hamulus total length, hamulus shaft length and hamulus point length (LHTL, LHSL & LHPL). This corresponds to the amount of variation these structures explain between specimens, of which the hamulus total length describes the most variation. The largest angle is between the hamuli structures and the marginal hook total and shaft lengths, which would suggest that these structures vary independently of one another. This confirms that the marginal hook acts principally along the y-axis (through Factor 2) whilst the hamulus acts principally along the x-axis (through Factor 1).

PCA 6

The final discrimination was achieved in the sixth PCA, where data on 11 variables (hamulus total length, hamulus shaft length, hamulus point length, ventral bar length,



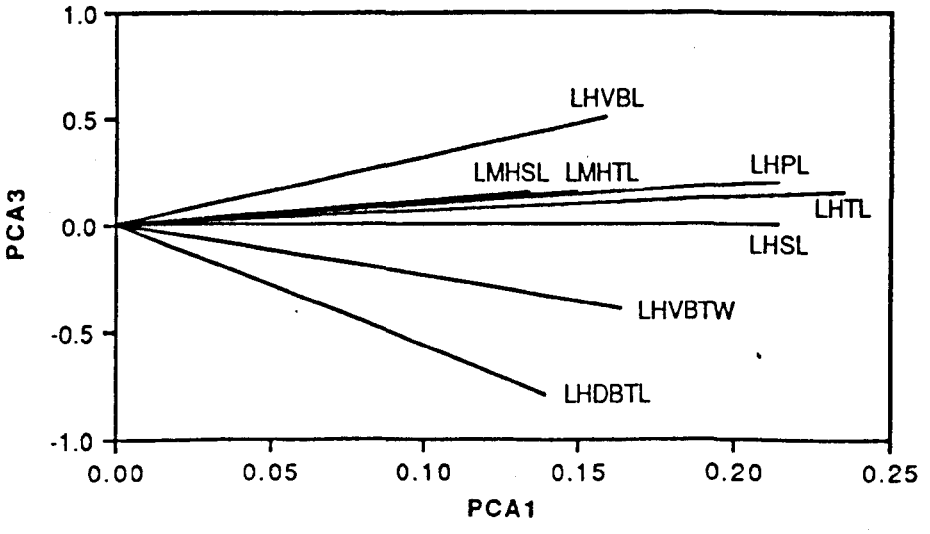


Figure 3.14: A plot of the PCA coefficients for the fifth PCA.

ventral bar width, marginal hook total length, marginal hook shaft length, marginal hook sickle length, marginal hook sickle proximal and distal widths and the marginal hook domus length) were utilised. The importance of the marginal hook in the discrimination of the salmonoid gyrodactylids called for three additional parameters to be added for the following analysis (PCA 6), these were the marginal hook sickle length and the marginal hook sickle proximal and distal widths. This used those features of the hamulus and ventral bar found to be reliable taxonomic criteria coupled with all of the measurements for the marginal hook used by Malmberg as basic taxonomic criteria for discriminating species of *Gyrodactylus*. The correlation matrix on the 11 variables used in PCA 6 is given in Table 3.6. The component loadings are given in Table 3.7, the variance explained by the components is given in Table 3.8 and the summary statistics for the cluster analysis performed on PCA 6 is given in Table 3.9.

The plot of points obtained from this PCA were then distinguished by host and an ellipse incorporated to encompass 50% of the points for each host as shown in Figure 3.15.

Discussion

The final PCA identified two clusters (Figure 3.15 & Table 3.9), one of worms present on wild *S. trutta* and a second cluster of worms parasitic on both wild and farmed *S. salar* and *O. mykiss*. These specimens were cross referenced to original numbered slides so that they could be re-examined. The species parasitic on *S. trutta* were considered to be *G. truttae* Gläser, 1974 Morph 4 (Figure 3.16), whereas those parasitic on the other two hosts, *S. salar* and *O. mykiss*, closely resemble *G. derjavini* Mikailov, 1975 (Figure 3.17). Malmberg (1987) noted in a study of a *Gyrodactylus* found on salmonids that specimens from *S. salar* from Scotland were of a *G. derjavini* form which differed from the form found in Scandinavia. When the distribution of specimens within the salmon cluster was analysed more closely, there appeared to be some further internal separation which may explain the oval shape of the ellipse. There appeared to be a bipolar effect within the ellipse, which contained 50% of the points, with Scottish localities being pulled towards one end of the ellipse and Welsh

Table 3.6: Correlation matrix for the 11 variables in PCA 6 (n = 362 specimens).

	HTL	HSL	HPL	HVBL	VBTW
Hamulus total length	1.000				
Hamulus shaft length	1.000	1.000			
Hamulus point length	0.814	0.815	1.000		
Ventral bar length	0.567	0.567	0.485	1.000	
Ventral bar total width	0.513	0.513	0.446	0.296	1.000
Marginal hook total length	0.337	0.340	0.341	0.187	0.217
Marginal hook shaft length	0.276	0.279	0.298	0.140	0.152
Marginal hook sickle length	0.369	0.369	0.384	0.210	0.220
Marginal hook distal width	0.350	0.349	0.341	0.230	0.159
Marginal hook proximal width	0.013	0.014	0.046	-0.003	0.003
Marginal hook filament loop	0.005	0.004	-0.055	0.140	-0.103

	MHTL	MHSL	MHSTL	MHSDW	MHSPW
Marginal hook total length	1.000				
Marginal hook shaft length	0.963	1.000			
Marginal hook sickle length	0.542	0.434	1.000		
Marginal hook distal width	0.082	0.087	0.093	1.000	
Marginal hook proximal width	0.106	0.126	0.054	0.403	1.000
Marginal hook filament loop	0.098	0.101	-0.064	-0.021	0.070

	MHDL
Marginal hook filament loop	1.000

Table 3.7: Component loadings on the first three principal components for PCA 6 (n=362 specimens).

STRUCTURE	1	2	3
Hamulus total length	0.888	0.292	0.093
Hamulus shaft length	0.794	0.382	0.095
Hamulus point length	0.835	0.210	0.039
Ventral bar length	0.620	0.286	0.049
Ventral bar total width	0.601	0.226	0.165
Marg. hook total length	0.607	-0.764	0.066
Marg. hook shaft length	0.538	-0.787	0.032
Marg. hook sickle length	0.579	-0.393	0.073
M. hk. sic. distal width	0.424	0.207	-0.698
M. hk. sic. prox. width	0.135	-0.129	-0.865
M. hk. filament loop len.	0.053	-0.107	-0.138

Table 3.8: Eigenvalues and proportion of the variance explained by the first three principal components for PCA 6 (n=362 specimens).

Eigenvalue	% of total variance	Cumulative percentage
4.06	36.87	36.87
1.84	16.70	53.57
1.32	11.95	65.52
1.11	10.07	75.59

Table 3.9: Summary statistics for the cluster analysis on PCA 6 (n=362 specimens).

VARIABLE	BETWEEN SS	DF	WITHIN SS	DF	F-RATIO	PROB
FACTOR 1	221.248	2	139.752	359	284.174	0.0
FACTOR 2	226.930	2	134.070	359	303.825	0.0

	VARIABLE	Min	Mean	Max	No. of specimens
CLUSTER 1	FACTOR 1	-0.83	0.51	2.17	117
	FACTOR 2	-3.12	-0.86	0.05	
CLUSTER 2	FACTOR 1	-0.52	0.59	2.02	143
	FACTOR 2	0.13	1.08	3.95	
CLUSTER 3	FACTOR 1	-3.01	-1.12	-0.12	102
	FACTOR 2	-1.30	0.03	1.88	

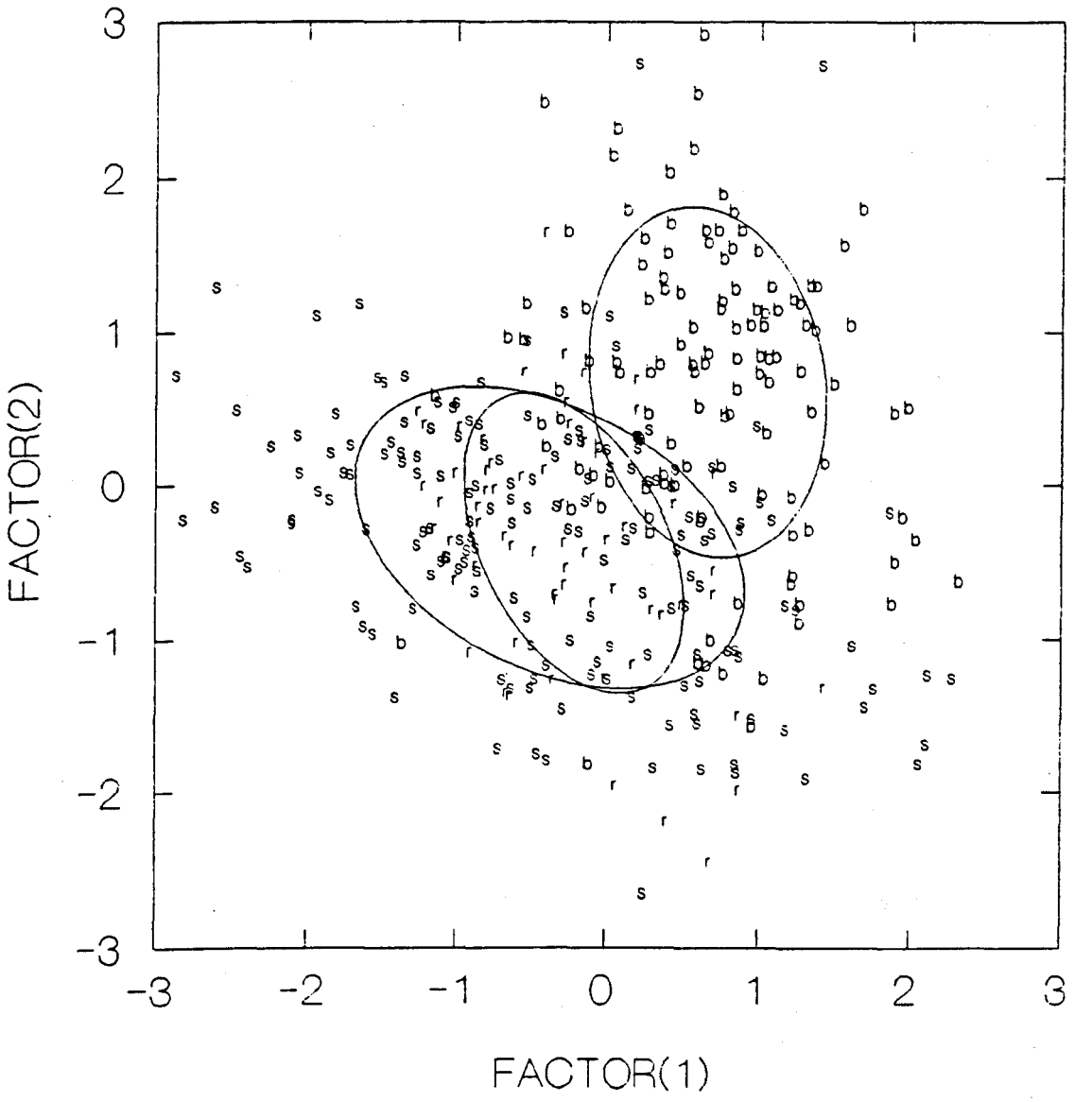


Figure 3.15: Map of the 362 specimens in the first plane of the Principal Component Analysis (PCA 6). Each specimen is identified by its host letter: s = *Gyrodactylus* sp. from *Salmo salar*; b = *Gyrodactylus* sp. from *Salmo trutta*; and r = *Gyrodactylus* sp. from *Oncorhynchus mykiss*. Ellipses incorporate 50% of the specimens for each host.

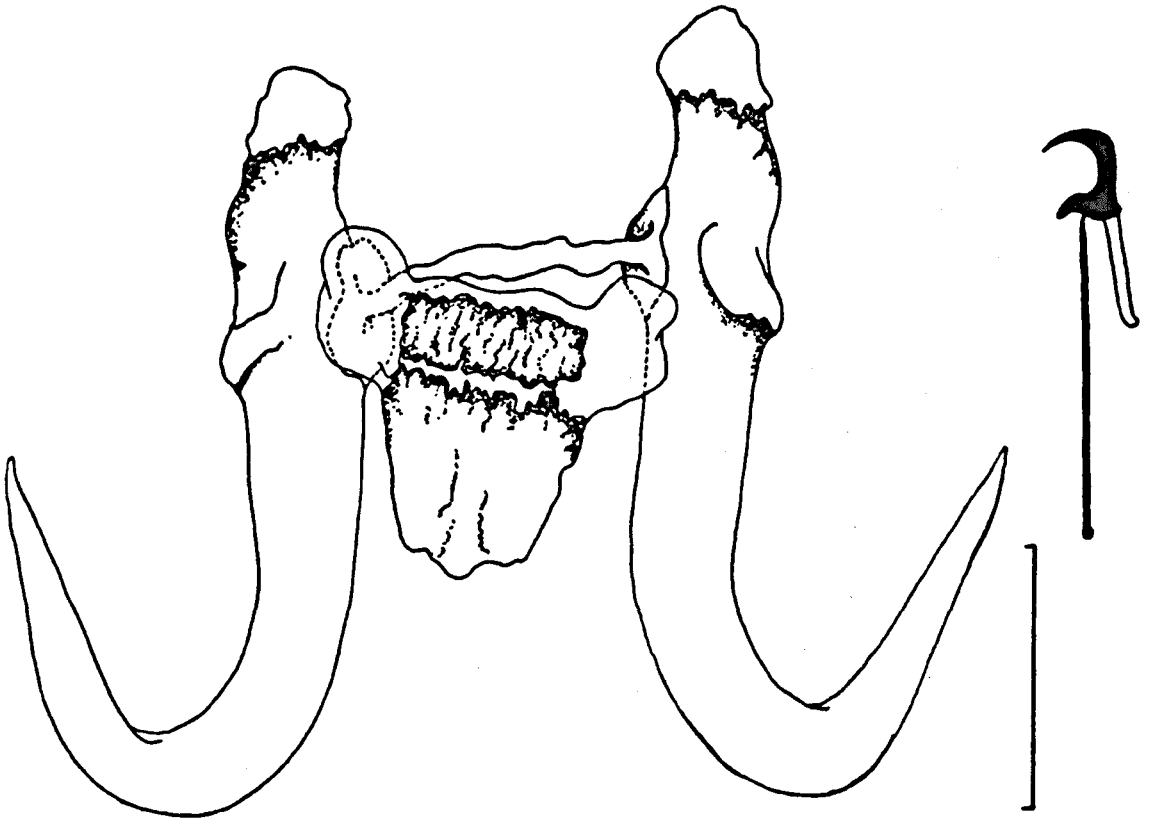


Figure 3.16: *Gyrodactylus truttae* Morph 4 parasitic on British brown trout. Scale bar: 20µm.

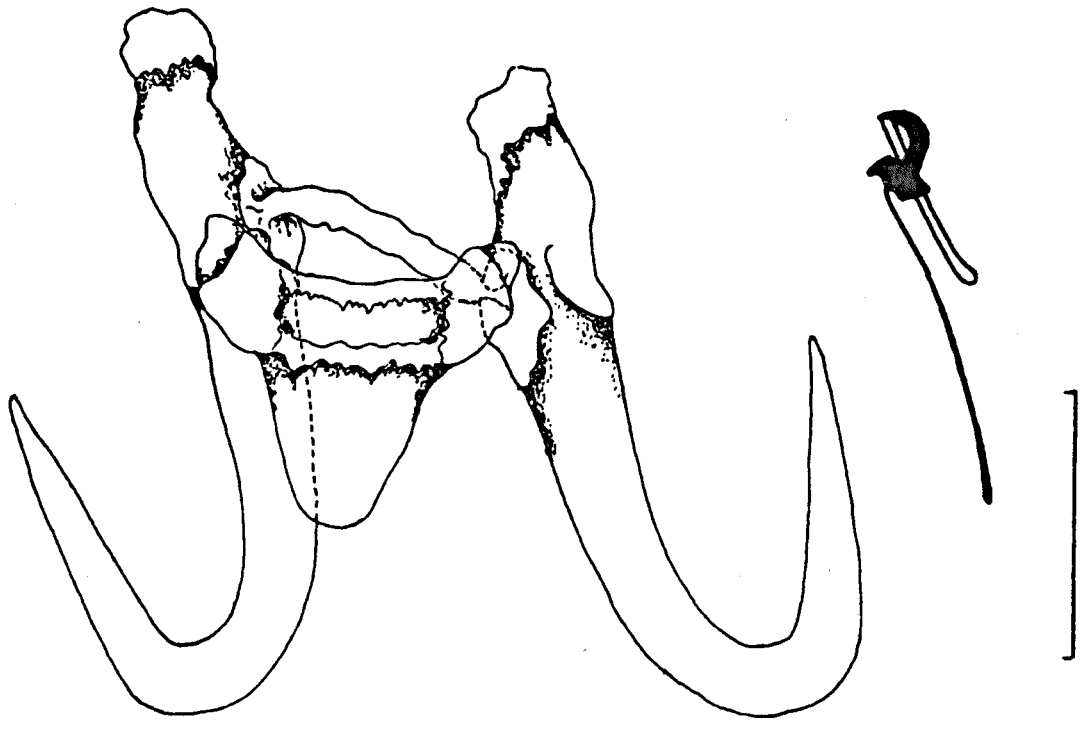


Figure 3.17: *Gyrodactylus derjavini* Morph 1 parasitic on British Atlantic salmon and rainbow trout. Scale bar: 20µm.

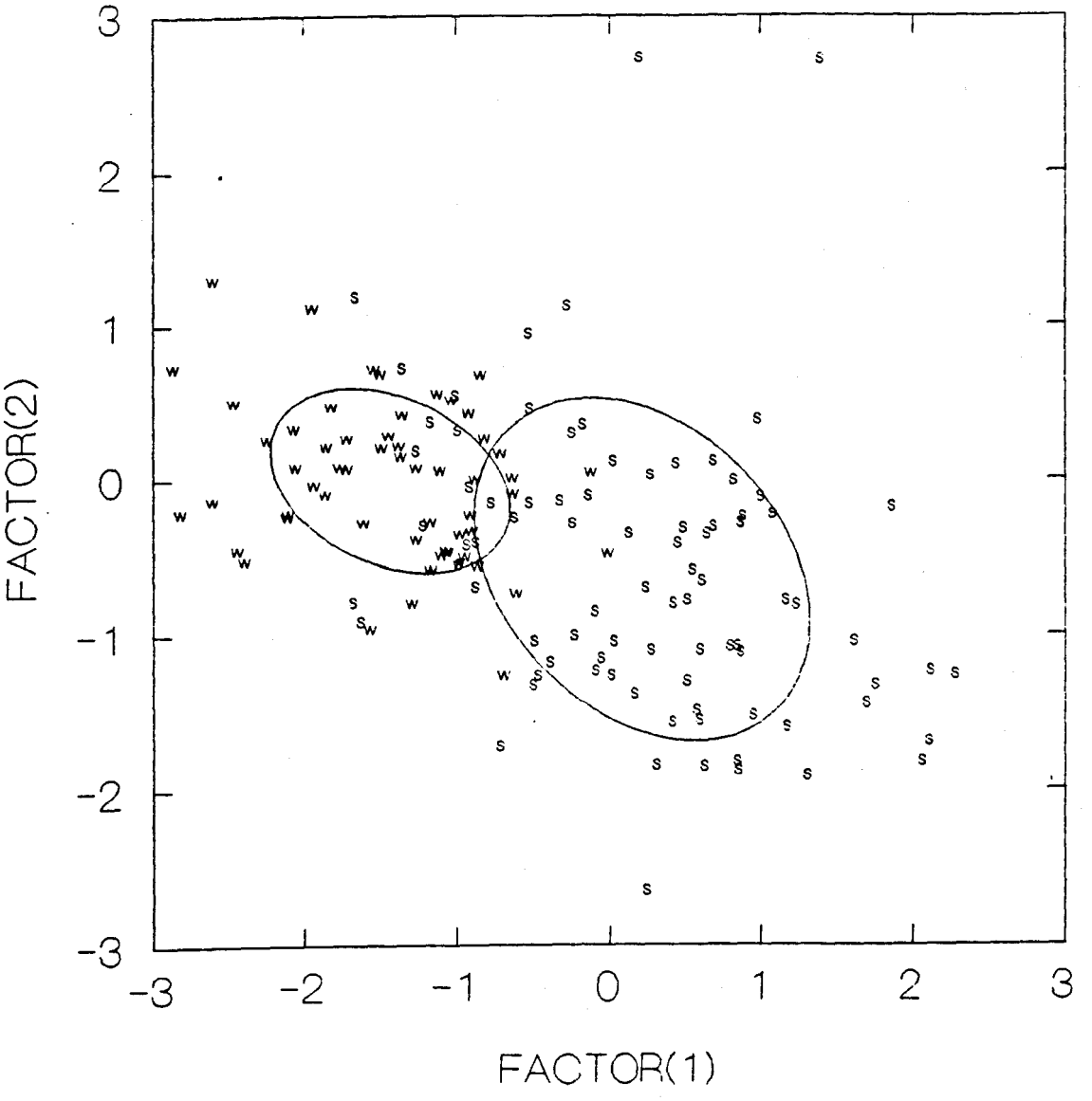


Figure 3.18: Map of the specimens of *Gyrodactylus* collected from wild Atlantic salmon in the first plane of the Principal Components Analysis. Each specimen is identified by its host's locality: s = *Gyrodactylus* sp. from Scottish wild salmon; and w = *Gyrodactylus* sp. from Welsh wild salmon. Ellipses incorporate 70% of the host's specimens.

forms towards the other (Figure 3.18). The cluster analysis on PCA5 recognized three clusters. The first cluster was made up of 50% salmon specimens, and the ratio of these in relation to Scotland: Ireland: England: Wales was in the percentage ratio of 82.6: 7.2: 5.8: 4.3. The third cluster, made of approximately 70% salmon specimens was similarly split in the ratio 26.5: 1.2: 8.4: 63.9, respectively. The second cluster was predominantly *S. trutta* specimens. It was evident, even at the fifth PCA, that the analysis was able to discriminate between Scottish and Welsh forms of the parasite resembling *G. derjavini* Morph 1. Members of the genus *Gyrodactylus* are known to be host specific and, therefore, the possibility of there being two populations of *G. derjavini* posed the question, "are there any host differences which could influence the parasite populations ?".

Payne, Child & Forrest (1971) proposed the existence of two races of Atlantic salmon in the British Isles, a northern 'boreal' race and a south western 'celtic' race identified on the basis of differences in gene loci. It would appear that, at this level of examination, the strong polar effect within the specimens resembling *G. derjavini* Morph 1 is mirroring the division they saw in the Atlantic salmon in the UK. The Irish forms appeared to be placed closer to the Scottish forms of "*G. derjavini*" than to the Welsh and Southern English forms. The relationship of these apparent subpopulations of "*G. derjavini*" on Atlantic salmon will be analysed further in Chapter 4.

Consequently, it appears that the population "*G. derjavini*" parasitic on *Salmo salar* may follow the geographical distribution of its host. Host specificity enabled Gibson (1972), MacKenzie (1985, 1990) and Hemmingsen *et al.* (1991) to use parasites as biological tags, with hosts being traced back to their place of origin. Although the two populations of "*G. derjavini*" appear to be the only species on Atlantic salmon in the UK, it is not possible to identify whether a salmon host belongs to celtic or boreal stock from its species of *Gyrodactylus*, since it is not possible to discriminate the two populations of *G. derjavini* Morph 1 effectively using PCA based on measurements made with the light microscope.

However, since one specimen within a PCA is ultimately influenced by all other specimens entered into the analysis, in order to reveal the real distribution patterns within the *Gyrodactylus* specimens parasitic on salmon, it is necessary to

remove those found on the brown trout and rainbow trout.

The possibility that *G. derjavini* Morph 1 is composed of more than one species, or that it occurs as different morphotypes of the same species which are both present on salmon, might explain some of the observed variability for each morphological character when the histograms of the coefficient of variation versus the mean are considered. For example, the marginal hook, the principal feature in current use as a taxonomic criterion (Malmberg, 1970), is conservative, although some variables, such as the marginal hook shaft, do exhibit some variation and bimodality: this may be a result of the presence of two populations.

Within the brown trout specimens an interesting situation was seen, as shown in Figure 3.19. It appears, in this instance, that geographical location and host are also factors exposed by the PCA model. The inclusion of internal standards for *G. truttae* from *Salmo trutta* in the River Chess, UK and the River Malse, Czechoslovakia (denoted by the letter c on Figure 3.19) served their function in enabling the recognition of specimens parasitic on native British brown trout. The specimens from the River Malse were displaced to the periphery of the brown trout plot, with two of the original seven specimens having been rejected as outliers. This might suggest that the population of *Gyrodactylus* on brown trout and indeed of brown trout itself in the United Kingdom differs from the mainland European populations of *Gyrodactylus* on brown trout and European populations of brown trout. The specimens from brown trout in Czechoslovakia might represent the sampling of trout from a different genetic origin or pool. However, the separation of the English specimens represents a different problem. Two of the English sites positive for *G. truttae* Morph 4 were located towards the periphery of the PCA plot. The input of the English *G. truttae* Morph 4 specimens, which were few in number, into the analysis was masked by those from Scotland and Wales. It would require further sampling of English brown trout to give the English specimens an equal representation in the PCA model before making statements as to the precise nature of *G. truttae*'s Morph 4 distribution.

More research is required to decide whether the two clusters of Morph 1 represent distinct *Gyrodactylus* forms or whether some of the variability observed within and between clusters is due to the natural variability of a small sample size, resulting from differences in environmental parameters, such as water temperature.

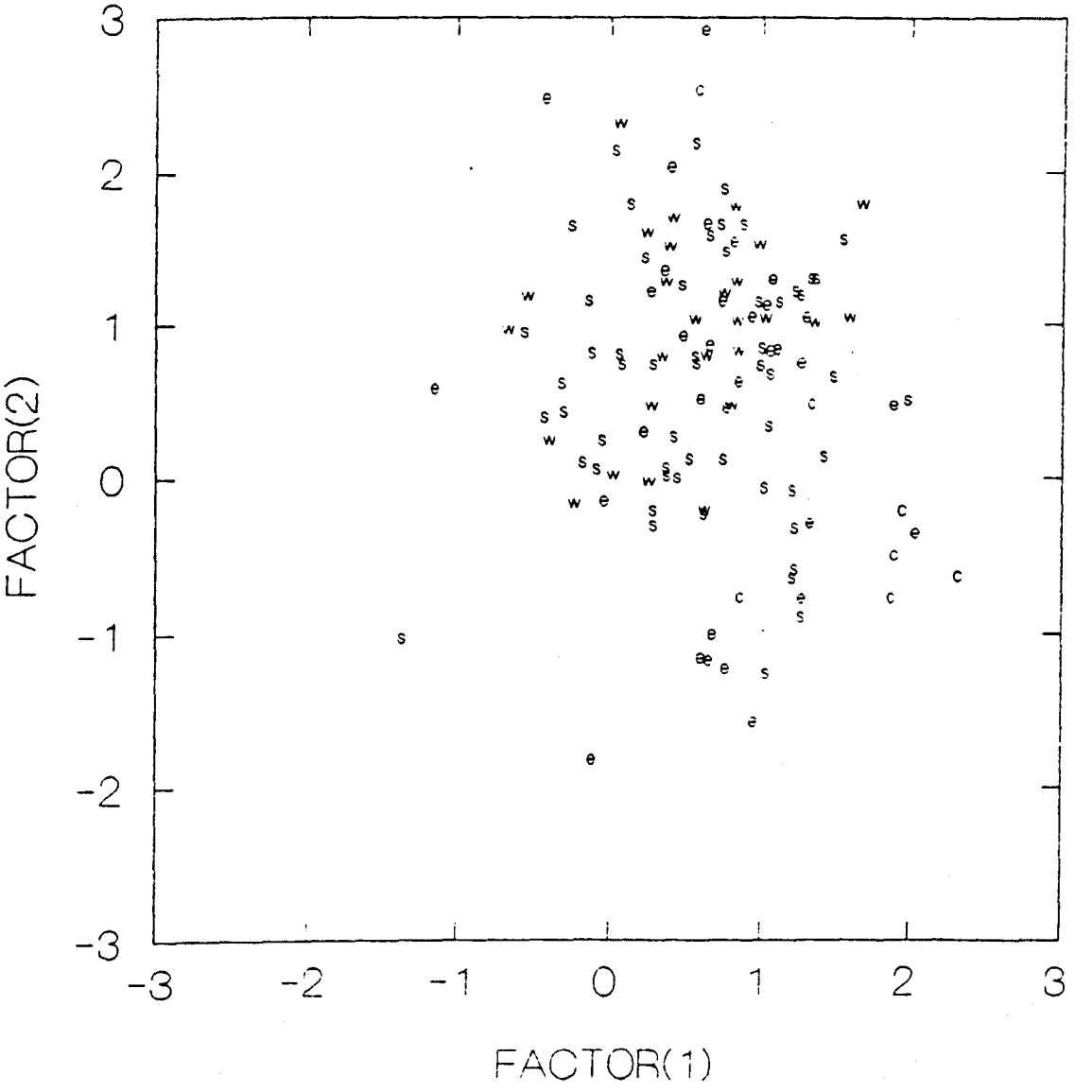


Figure 3.19: Plot of the 124 specimens of *Gyrodactylus* collected from *Salmo trutta*, as shown in the first plane of the PCA. Each specimen is identified by the host's locality: c = *G. truttae* from the River Malse, Czechoslovakia; e = *Gyrodactylus* sp. from England; s = *Gyrodactylus* sp. from Scotland; and w = *Gyrodactylus* sp. from Wales.

Summary and conclusions

From the analysis performed on measurements of the sclerotised hard parts of the attachment organ of the monogenean ectoparasite *Gyrodactylus* using light microscopy the following conclusions were possible:

The combination of using PCA and cluster analysis on the most significant PCA coordinates is an effective way of identifying groups of specimens on the basis of multivariate morphometric features.

From PCA 1, three clusters were pulled out on the basis of the size of the hamulus. These were Loch Shin (1 specimen from *Salmo salar*) having a hamulus length of 38μm, verified as a specimen of *G. arcuatus* Bychowsky, 1933, which is normally parasitic on three-spined sticklebacks *Gasterosteus aculeatus* and almost certainly present as a result of an accidental infection; and Loch Tralaig and Loch Coulin Morph 3 (2 and 7 specimens, respectively from *Salmo salar*) having hamulus lengths that exceeded 75μm.

In PCA 2 six clusters were indicated. Four specimens were removed from the data-set on the basis of having a large ventral bar process to mid-length (HVBPML) and long, thin marginal hooks. In addition to these, Loch Airthrey Morph 5 (10 specimens from *Salmo trutta*), *Gyrodactylus colemanensis* Mizelle & Kritsky, 1967 (2 specimens from Canadian *S. fontinalis*) and three outliers from three different sites were also removed.

PCA 3 suggested seven clusters but no outliers were removed directly from this analysis. The specimens collected from *S. alpinus* (10 specimens) and *Gyrodactylus salmonis* Yin & Sproston, 1948 (1 specimen) were removed prior to PCA 4.

PCA 4 was run on just the three hosts that were well represented within the data-set, namely *Salmo salar*, *Oncorhynchus mykiss* and *Salmo trutta* and weighted equally. In PCA 4, four clusters were recognised, one represented almost entirely by *Salmo salar* specimens, one predominantly composed of *Salmo trutta* specimens, and the remaining 2 clusters were a mixture of specimens from all three hosts. Correlations and histograms of the separate structures revealed that sclerites below 20μm in size, when measured with the light microscope, were unreliable in the analysis due to the small number of measurement classes in which they fell. Furthermore, there was

evidence that small structures have a high variability and cannot, therefore, be used reliably.

PCA 5 was run after removing all those structures smaller than 20μm in size, leaving eight reliable variables, with the result that three clusters were identified (the first 3 factors accounting for over 80% of the total variance explained). Of these clusters, one appears to be material from a mixture of all three host species but 50% of the specimens came from *Salmo salar*. Cluster 2, is pre-dominantly *Salmo trutta*, whilst cluster 3 is predominantly *Salmo salar*.

PCA 6 included eleven variables with a coefficient of variation <10%, the other seven variables being excluded. Specimens were identified by incorporating 50% of the points by ellipse and superimposing the results for each of the three hosts. The analysis revealed that two forms were apparent parasitic on British salmonids, one which appeared to be *G. derjavini* Morph 1 on *S. salar* and *O. mykiss* and one believed to be *G. truttae* Morph 4 on *S. trutta*.

Examination of the distribution of specimens within each cluster with respect to geographical location suggested that different populations of *G. derjavini* Morph 1 might exist in Scottish and Welsh salmon. In the case of *S. trutta* specimens, the Czechoslovakian forms of *G. truttae* were displaced from the main body of *G. truttae* Morph 4 specimens, and there also appeared to be some drift of the English *G. truttae* Morph 4 specimens away from those from the other localities. To what extent this distribution reflects morphological variants requires further investigation.

CHAPTER 4: MULTIVARIATE ANALYSIS OF SOME MORPHOMETRIC FEATURES OF SPECIES OF *GYRODACTYLUS* PARASITISING BRITISH *SALMO SALAR*.

Introduction

The findings of the first analysis reported in Chapter 3 indicated that two species of *Gyrodactylus* von Nordmann, 1832 were found to parasitise the three salmonid hosts sampled in the 69 British localities investigated. *G. truttae* Gläser, 1974 Morph 4 was found to parasitise native wild brown trout *Salmo trutta* and a second species tentatively identified as *G. derjavini* Mikailov, 1975 Morph 1 was found to parasitise both wild Atlantic salmon *Salmo salar* and farmed rainbow trout *Oncorhynchus mykiss*. Furthermore, within the PCA distribution pattern of *G. derjavini* Morph 1 on the wild salmon, there was a component of bimodality which reflected geographical location. The result of the model, based on the eight most reliable taxonomic criteria proposed for *Gyrodactylus* (Chapter 3), suggested the existence of two populations which mirror the celtic and boreal races of *Salmo salar* resident in the United Kingdom proposed by Payne, Child & Forrest (1971). Since this pattern of distribution was apparent when the salmon data were considered simultaneously with specimens of *Gyrodactylus* parasitic on brown trout and rainbow trout (Chapter 3), the following study considers the distribution of the species parasitic on Atlantic salmon in isolation, in an attempt to reveal any underlying information concerning their PCA distribution such as geographical location or temperature.

Materials and Methods

The previous survey (Chapter 3) used material from Atlantic salmon from 31 sites positive for *Gyrodactylus*. The present survey, carried out in response to the initial results obtained, was expanded to include a further 14 sites positive for *Gyrodactylus*, which added a further 86 specimens to the analysis to give a total of 253 specimens.

These sites are listed below:

(a) Scotland	
Grampian	River Don
Dumfries	Crawick Water, Shinnel Water, Carron Water, Cross Water Luce, River Cree (at Penkiln & Palnure)
Isle of Skye	River Snizort, Kilmarie Hatchery, Storr Loch
(b) Wales	
Caernarfon	Afon cwm Mynacl
(c) England	
Severn-Trent	Rhyd-Ros-Lan, Mochdre Brook, River Carno

Fry and parr of Atlantic salmon (6-12 cm) were collected by electro-fishing from August, 1990 - December, 1991, with 20 fish being collected from each site where possible. The water temperature was measured with a precision of 0.1°C at each site. Fish were killed and parasites prepared according to Chapter 2. A total of eight measurements, found to be the reliable characteristics in Chapter 3, were measured using an Olympus BH2 binocular microscope with phase-contrast and a drawing tube. The measurements taken in accordance with Malmberg (1970) (see Chapter 2; Figure 2.1) were: hamulus total length, hamulus shaft length, hamulus point length, dorsal bar length, ventral bar length, ventral bar total width, marginal hook total length and marginal hook shaft length.

The measurements taken were fed into a principal components analysis (PCA)(Systat version 5.3, 1991). Because all the data were not normally distributed, it was necessary to log-transform them. Specimens separated by principal components analysis were then analysed using cluster analysis to detect groupings within the data-set, either by direct manipulation of the model or by determining natural grouping of the specimens. The separation of the specimens was investigated to ascertain whether differences that existed between specimens were related to the geographical location of the host and whether the effect of temperature had any influence on this result.

Results

The morphometric data obtained from the light microscope study were analysed using PCA and cluster analysis. The correlation matrix on the 8 measurements taken on the opisthaptoral hard parts of *Gyrodactylus* are shown in Table 4.1. The component loadings for the first three principal components are given in Table 4.2. As can be seen in Table 4.2, component 1 exhibits all the characters, increasing in the same direction except the hamulus shaft length. The hamulus shaft and the ventral bar length are the key variables acting through component 2, whilst there is a relative decrease in total length and shaft length of the marginal hook. Component 3 indicates that for an increase in the length of certain characters, there is a relative decrease in the width. The variance explained by the components (eigenvalues) and the percentage of the total variance explained, are given in Table 4.3. The results given in Tables 4.1 and 4.2, therefore, explain the general behaviour of the variables in this analysis. The extension of this study was principally aimed at determining whether the species of *Gyrodactylus* parasitic on British Atlantic salmon mirrors the distribution suggested for different races of this host by Payne, Child & Forrest (1971). The first assumption was based on the existence of a Scottish and a Welsh population of Atlantic salmon. Eire and England (to some extent) were for the time being not considered separately in this study because of the few positive *Gyrodactylus* sites obtained and the paucity of salmon habitats in England compared with those in Scotland and Wales.

The distribution of the Scottish and Welsh specimens were examined in this analysis by assigning a weight factor to material from the two regions and comparing the separation. The results of this are shown in Figure 4.1, where the two ellipses show a large degree of overlap of some 25-30%. When the specimens collected from English salmon were run against those for Scotland and Wales, they grouped, such that they were positioned at the interface of both the two clusters also shown in Figure 4.1.

Although Payne, Child & Forrest were able to sample 11 sites, which formed the basis of their proposal for the existence of two salmon races, the nature of their study did not allow them to predict a boundary (if one exists) separating the two populations of salmon. However, assuming that two races of salmon do exist, some

Table 4.1: Correlation matrix on the eight reliable structures made on the *Gyrodactylus* spp parasitising British salmon.

Variable	HTL	HSL	HPL	DBTL	VBL
HTL	1.000				
HSL	-0.075	1.000			
HPL	0.767	0.028	1.000		
DBTL	0.468	0.129	0.398	1.000	
VBL	0.466	0.377	0.414	0.341	1.000
VBTW	0.462	0.036	0.391	0.428	0.219
MHTL	0.662	-0.208	0.536	0.429	0.179
MHSL	0.631	-0.253	0.520	0.410	0.156

Variable	VBTW	MHTL	MHSL
VBTW	1.000		
MHTL	0.414	1.000	
MHSL	0.369	0.977	1.000

Table 4.2: Component loadings for the first three principal components.

Variable	1	2	3
Hamulus total length	0.879	0.044	0.217
Hamulus shaft length	-0.052	0.845	-0.074
Hamulus point length	0.791	0.132	0.288
Dorsal bar total length	0.649	0.248	-0.421
Ventral bar length	0.475	0.675	0.319
Ventral bar total width	0.615	0.105	-0.602
Marginal hook total length	0.857	-0.360	0.028
Marginal hook shaft length	0.834	-0.404	0.055

Table 4.3: Eigenvalues and the percentage of the variance explained for the Principal Components Analysis.

	Variance explained by components	% of total variance	cumulative percentage
Factor 1	3.86	48.23	48.23
Factor 2	1.55	19.42	67.65
Factor 3	0.78	9.77	77.41

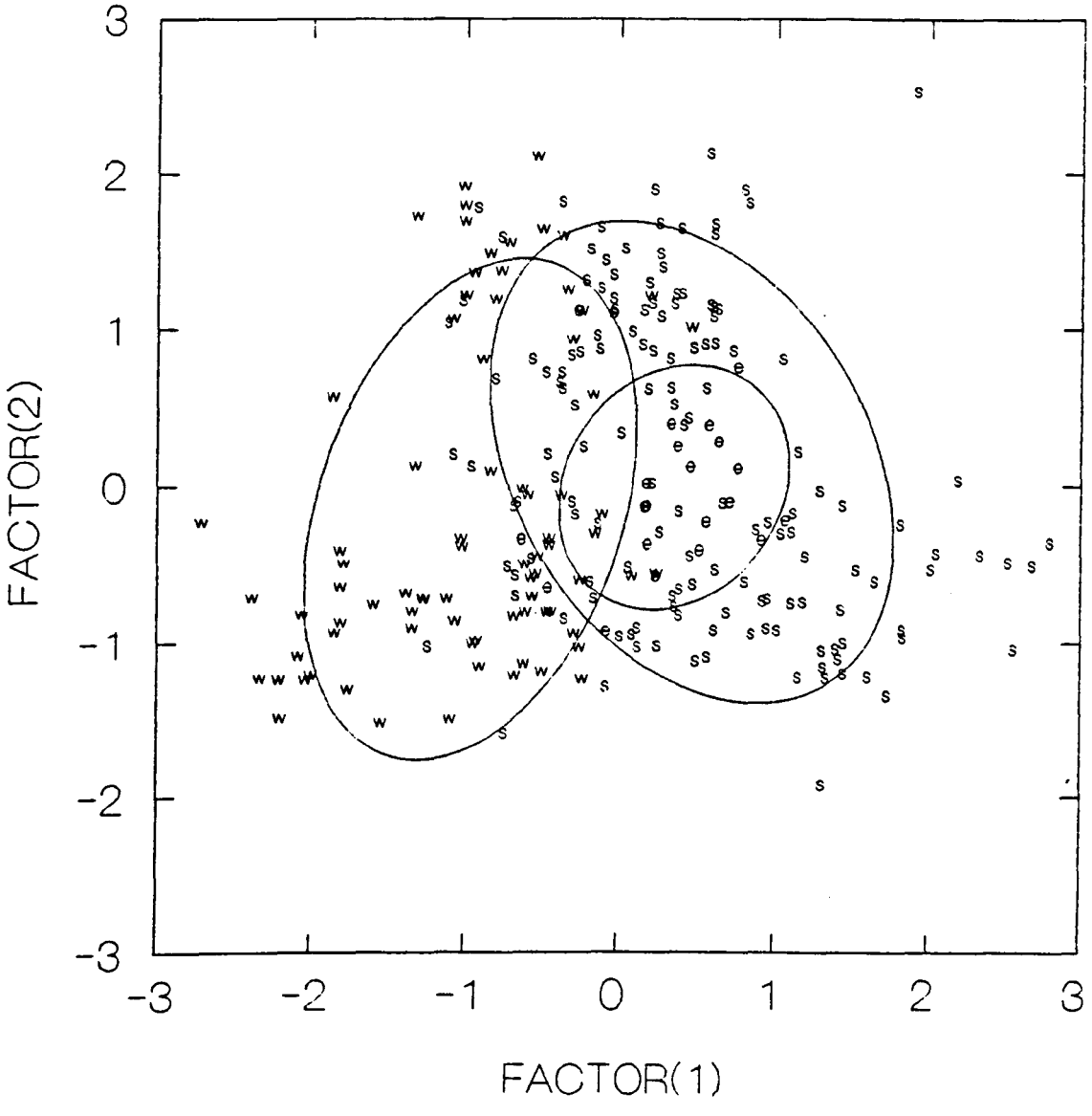


Figure 4.1: Separation of *Gyrodactylus* specimens with respect to host distribution. Ellipse incorporates 70% of the points. Abb: e = English specimens; s = Scottish specimens and w = Welsh specimens.

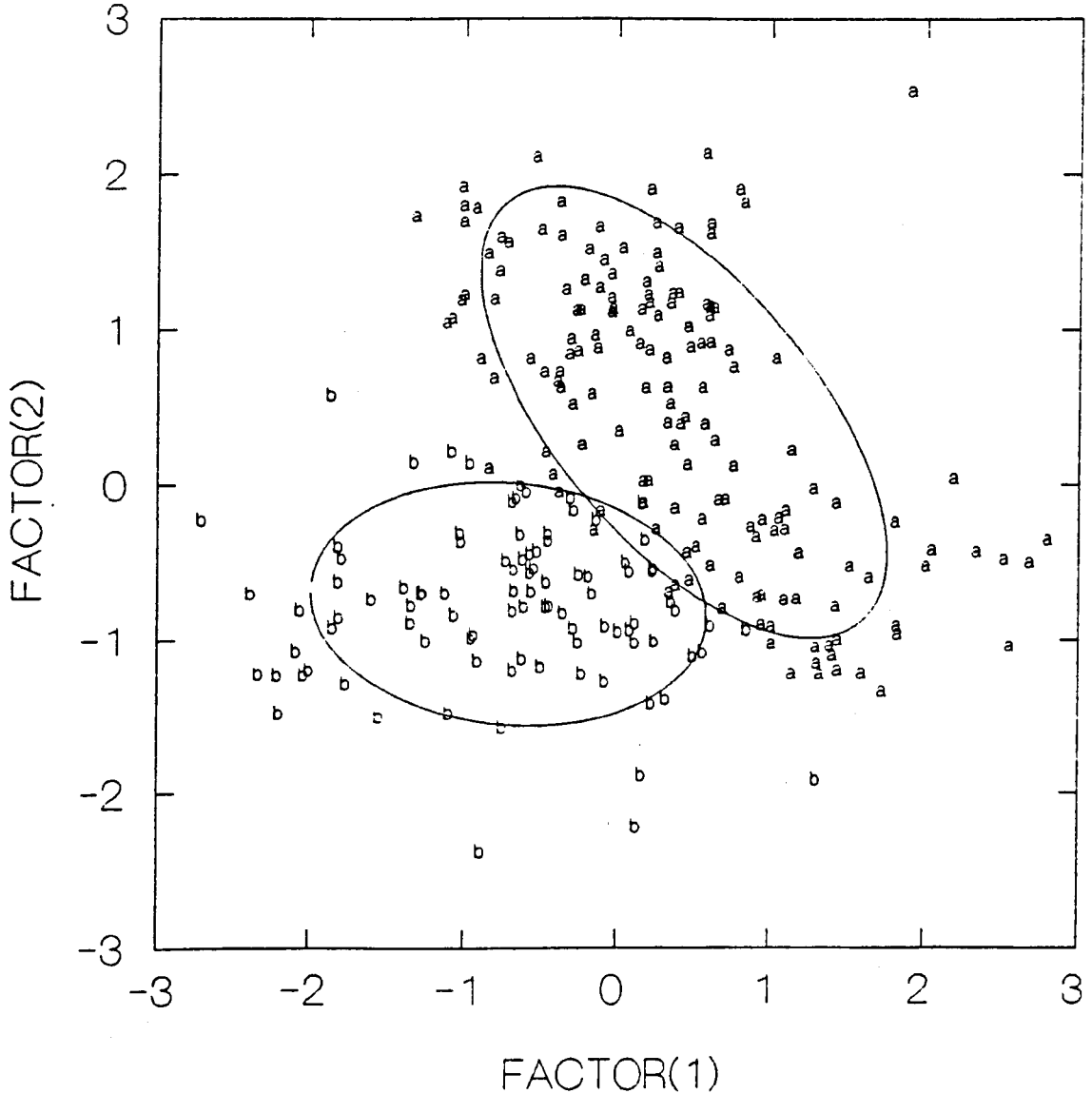


Figure 4.2: Cluster analysis detecting two natural clusters within the salmon specimens. Ellipse incorporates 70% of the specimens. Abb: a = predominantly Scottish specimens and b = predominantly Welsh specimens.

indication of geographical distribution between the two races might be given by the cluster analysis. The two populations of *Gyrodactylus* were sought by looking for two clusters from the factor scores of the PCA and then the geographical locality of each specimen within each cluster analysed; these are shown in Figure 4.2. The clusters in this situation only had about a 5% overlap, the ellipses having a directional component arising at an angle to one another. The breakdown of each of these two clusters revealed that the first cluster contained 117 Scottish, 28 Welsh, 15 English and 2 Irish specimens which represents 79.59%, 35.0%, 75.0% and 33.33% of the total number of specimens entered for each geographical region, respectively. Cluster 2 had 30 Scottish, 52 Welsh, 5 English and 4 Irish specimens, representing 20.41%, 65.0%, 25.0% and 66.67%, respectively. If the distribution of cluster 1 (denoted by the letter a) alone is considered (Figure 4.2), there appears to be a bipolar effect within this cluster, with the distribution of the specimens orientated towards the poles of the ellipse. This suggested that there may be three sub-populations of *Gyrodactylus* on British salmon, a Welsh and possibly two Scottish populations, or some other factor influencing this distribution. The cluster analysis was then repeated, looking for three natural clusters within the factor score data, the results are shown in Figure 4.3. Three clusters were identified and showed little overlap. The relative composition of each of these clusters in terms of geographical region were:

Cluster 1: 64 Scottish, 22 Welsh, 5 English, 0 Irish given as 43.54%, 27.50%, 25.0% and 0.0% respectively (denoted by the letter a).
Cluster 2: 20 Scottish, 56 Welsh, 3 English, 1 Irish given as 13.61%, 70.0%, 15.0% and 16.67% respectively (denoted by the letter b).
Cluster 3: 63 Scottish, 2 Welsh, 12 English, 5 Irish given as 42.86%, 2.50%, 60.0% and 83.33% respectively (denoted by the letter c).

These results indicate the presence of a Welsh population, a Scottish population and a zone where these two populations meet. However, Mo (1991a, 1991b, 1991c) and earlier authors (Malmberg, 1962; Kulemina, 1974, 1977; Ergens, 1975, 1976; Ergens & Gelnar, 1985) have shown that the influence of temperature can produce artifacts. Temperature affects the rate of growth of the sclerites during the worm's development,

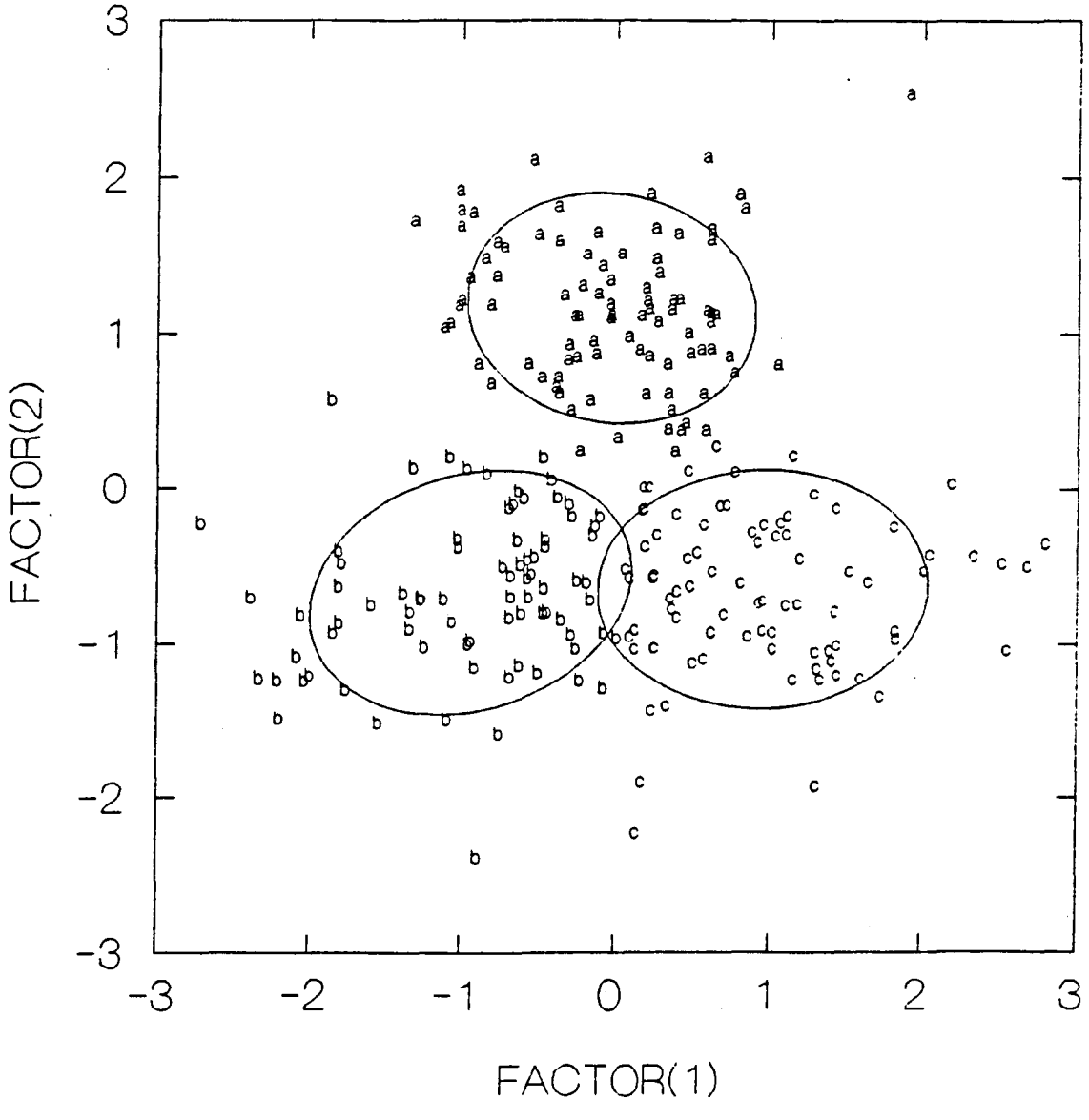


Figure 4.3: Cluster analysis showing three natural clusters within the *Gyrodactylus* specimens on salmon. Ellipses incorporate 70% of the specimens. a = cluster 1, b = cluster 2 and c = cluster 3.

such that the sclerites are smaller during the warm summer months and larger in winter when the development of the embryo is slower. Specimens of a single species of *Gyrodactylus*, when collected over the period of a year and subsequently analysed by such a model as used here, might appear to be two species, when in actual fact it represents a summer sample and a winter sample.

The effect of temperature was therefore examined. The Welsh samples were collected through a long summer (1991), which was peaked by a period of high water temperatures in excess of 18°C. They were, therefore, split into summer low temperatures (late August - September, 16.1-14.0°C) and summer high temperatures (late July - early August, 17.5-18.8°C). The results of this analysis are shown in Figure 4.4. In this instance, the effect of temperature can be seen to produce a clear separation of the two groups of Welsh specimens, such that it appears that there are two species, rather than one, parasitising Welsh Atlantic salmon. Similarly, the Scottish specimens were separated into winter collections (October 1990 - May 1991, 2.0-9.0°C) and summer collections (July - early September 1991, 13.0-18.5°C). The separation of the Scottish specimens also shown in Figure 4.4 follows a pattern similar to the Welsh situation, both collection periods are separated, with the ellipses moving in the same direction. The fact that the ellipses for both winter and summer collections are in line, the Scottish to the right of the PCA plot, the Welsh to the left, suggests that a single species is most probably represented on Scottish salmon samples and a single species on Welsh salmon. This is contrast to the populations found on sticklebacks where, for example, the population of three-spined sticklebacks in Loch Airthrey, Stirlingshire was found to be parasitised by *G. gasterostei* Gläser, 1974 throughout the summer, to then disappear and be replaced by *G. arcuatus* Bychowsky, 1933 in autumn. In the latter case, it would be expected that the ellipses for the two species would arise at right angles to one another.

Discussion

The genetic variation within the Salmonidae has been investigated by *inter alia* Payne *et al.* (1971), Payne (1974), Child *et al.* (1976), Ryman (1981), Ståhl (1981, 1983, 1987), MacCrimmon & Claytor (1985, 1986), Ferguson (1989), Verspoor & Jordan

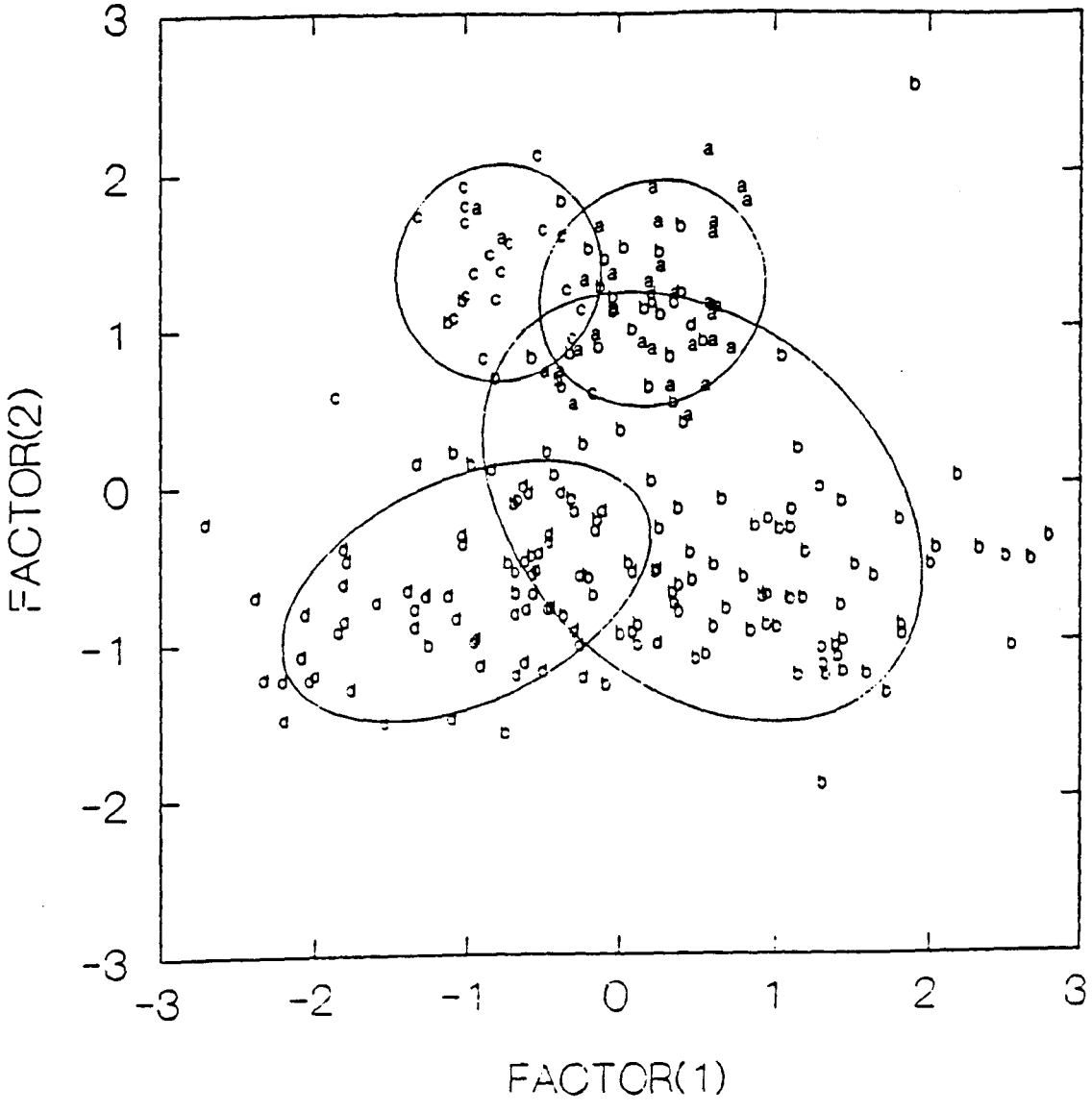


Figure 4.4: Discrimination of *Gyrodactylus* specimens with respect to location and temperature. The total hamulus length is the key variable acting along the x-axis and the hamulus shaft length acting along the y-axis. Ellipses incorporate 70% of the specimens. Abb: a = Scottish summer collection; b = Scottish winter collection; c = Welsh summer high temperature collection and d = Welsh summer low temperature.

(1989), Davidson *et al.* (1989) and Stephen & McAndrew (1990) on both sides of the Atlantic. Payne *et al.* (1971) using transferrin phenotypes, and in particular the Tf2 gene, found salmon populations in the British Isles to have different frequencies of this gene correlated with geographical distribution. A high frequency of Tf2 was found in the south and a low frequency in the north, this boundary bisected south-west Ireland from south-east Ireland and passed through the northern tip of Wales and the North of England (Figure 4.5). The difference between samples from north Wales and the River Lune in Lancashire were, however, only just significant ($0.05 > P > 0.01$). This suggested a northern boreal and a southern celtic population, plus possibly a third population in Devon. This was confirmed by Child *et al.* (1976), who suggested that the latter population is possibly related to a present day European stock and a remnant of the boreal race isolated following the Würm III glaciation.

The division between the two major races of Atlantic salmon correlates with the extent of glaciation by the Würm I and II ice caps (Figure 4.5), which would have pushed the salmon stocks southwards. Then, as the ice receded, recolonisation would have come from the relict celtic stock, then in the south, moving along the west coast of Ireland northwards and into the Skagerrak. With the advent of the Würm III glaciation, ice caps were produced over the north of Britain and Scandinavia, again pushing resident salmon populations southwards. The boreal Rhine Sea population of salmon, however, having been isolated by this event, recolonised northern Britain as the ice caps receded.

Investigations by Thorpe & Mitchell (1981) proposed three major salmon stocks in Europe, Baltic, Northern and Western. The British/Irish population, as part of the Western stock, together with the French and Icelandic sub-divisions of this stock, was considered by these authors to be comprised of at least 74 independent stocks in the UK. These findings were confirmed by Ståhl (1983, 1987), who reported differences in Baltic and Atlantic salmon populations, which, it was suggested, could possibly be due to genetic drift in pre-glacial refugial populations. Verspoor & Jordan (1989) state that variation in gene loci is due to natural selection and a genetic adaptation to local environment, such that a divergence of British Atlantic salmon from those of Iceland and southern mainland Europe (France and Spain) occurred.

Ryman (1983) found that the varibility in salmonids overall was low, with 60-

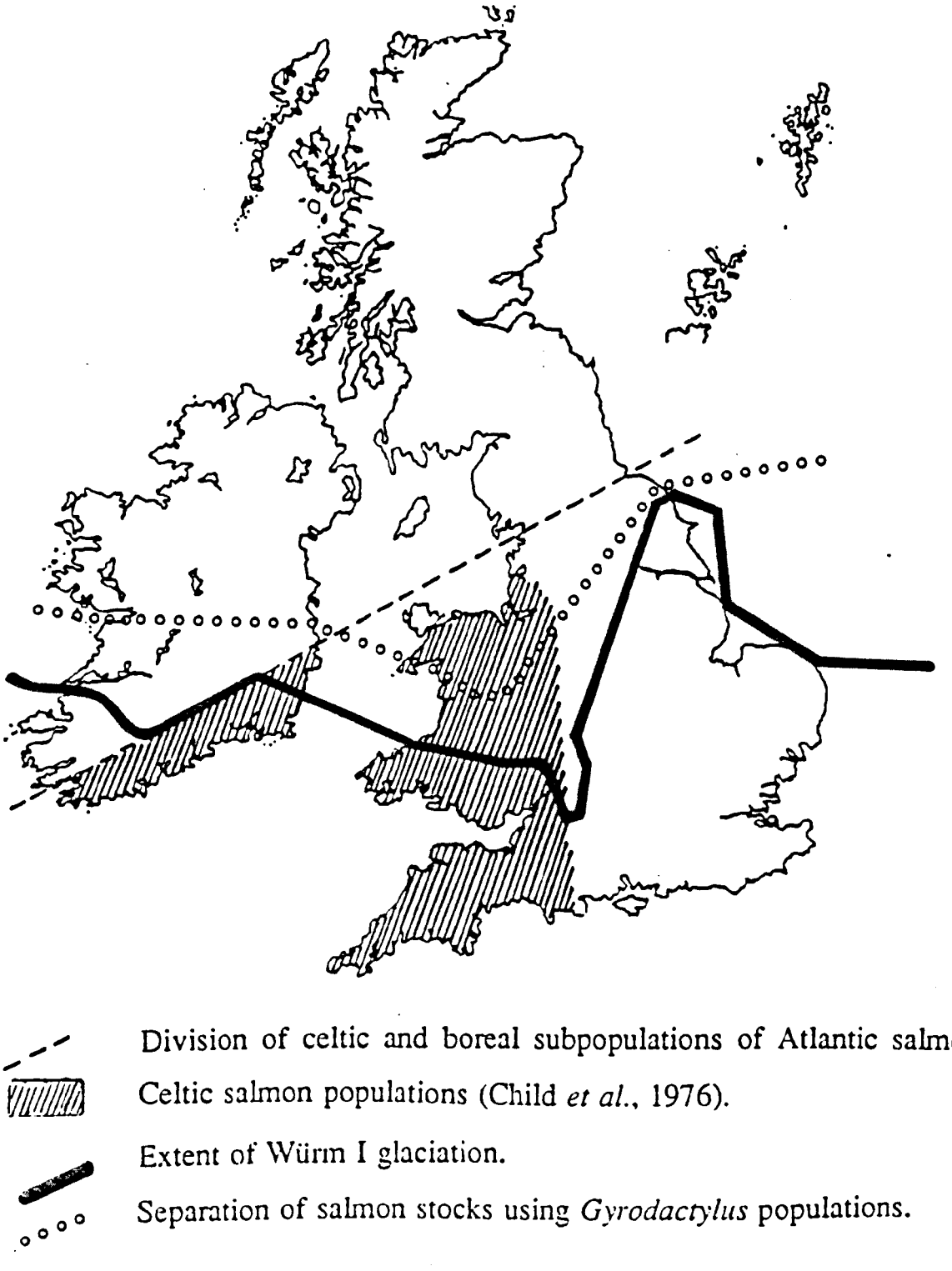


Figure 4.5: Distribution of races of Atlantic salmon and *Gyrodactylus* spp. (adapted from Payne *et al.*, 1971 and Child *et al.*, 1976).

80% of that genetic variability attributable within local populations of Atlantic salmon. This implied that 20-40% of genetic variation is shared between all populations of salmon. Davidson *et al.* (1989) suggested this was due either to a poorer than expected ability of salmon to return to their native stream or to the present population of Atlantic salmon being recently derived from a common population. Furthermore, it was suggested that, if the last ice age occurred only 6,000-13,000 years ago, there has not been sufficient time for new mutations to spread through specific populations (Cross & Ward, 1980; Davidson *et al.*, 1989).

However, further evidence for the recognition of salmon stocks has come from the use of diploid chromosome number (2n) and the total number of chromosome arms (NF). Although salmon with 2n = 58 and NF = 74 have been found in Wales (usually 2n = 56/57) (Rees, 1967) and Scandinavia (Nygren *et al.*, 1972), this represents the predominant situation found in Scottish salmon (Hartley & Horne, 1984).

The documented evidence for apparent discrete races of salmon existing in the UK raises the question as to whether species of *Gyrodactylus* have a similar pattern of distribution, representing examples of co-evolution with their host. Several investigations (Kabata, 1963; Margolis, 1965; Pippy, 1969; Scott, 1969; Gibson, 1972; Hemmingsen *et al.*, 1991; and Siddall *et al.*, 1991) have found that parasites might be used as biological tags or indicators. In a similar fashion, the identity of *Gyrodactylus* species parasitic on Atlantic salmon might allow us to predict whether its host is of boreal or celtic stock. Although, the number of brown trout and Atlantic salmon hybrids is low in natural river-systems (they may account for 1% of the salmon population in rivers of north-eastern Britain) (Payne *et al.*, 1972), the sampling of hybrids in this study was believed to be negligible. Hybrids found at the time of sample collection were returned directly to the river to prevent confusion within the analyses. According to the national survey (Chapter 2) where morphometric data and scanning electron microscopy were used to confirm the identity of the *Gyrodactylus* species, it appeared that there were two forms parasitic on Atlantic salmon in the UK. The two forms, although very close to *G. derjavini* Mikailov, 1975 (Chapter 2, Figures 2.10-2.11) collected from brown trout in the River Dalälven, Sweden, differed slightly, depending upon whether they were from Scottish (Chapter 2, Figure 2.18-2.19) or

Welsh salmon (Chapter 2, Figure 2.12-2.13). The Welsh form, Morph 1 from Mags Yr Afon appeared to be closest in morphological appearance to the Swedish *G. derjavini* species. The marginal hook sickle proper for Morph 1 was slightly smaller 6.4 µm long *vs* 6.8 µm for *G. derjavini*, however, as previously discussed, temperature may affect relative size. The sickle base (heel and toe) appeared less robust in the Welsh specimens and the origins of the blade were also slightly narrower and the point more tapered. This results in a hook which has a longer shaft portion to the blade and a more open face of the sickle, but the sickle point still remains in line with the toe. The overall appearance, although still robust, was slightly smaller than *G. derjavini*. The Scottish form, Morph 2 (Chapter 2, Figure 2.18) from the River Snizort, Isle of Skye, was clearly different. The heel was less pronounced and the sickle shape was similar to that of the Welsh form; however, the sickle shaft was narrower and longer, resulting again in a more open face to the marginal sickle. This species clearly differed from *G. truttae* Gläser, 1974 Morph 4, which has a defined heel and a very narrow tapering sickle point extending beyond the level of the toe, such that the point drops to produce a more closed sickle face (compare with specimens collected from Loch Airthrey, Scotland, Chapter 2, Figure 2.24).

Discrimination of the two morphotypes by manipulation of the cluster analysis to group specimens by geographical location, i.e. Scotland, Wales and England (Figure 4.1) showed that there was a large degree of overlap (25-30%). However, the use of cluster analysis to detect two natural groupings within the data (Figure 4.2) gave a clearer separation of the two clusters; subsequent analysis of these showed one to be predominantly composed of Scottish specimens and the other predominantly Welsh specimens. Furthermore, there appeared to be a directional component within the analysis, with the clusters arising at an angle to one another being discriminated on the basis of the Welsh specimens having a shorter total hamulus and hamulus shaft length and shorter marginal hooks. When this was analysed further, looking for three clusters (Figure 4.3), the clusters obtained were discriminated not only on a geographical basis but by temperature as well. Cluster 1 (indicated by a in Figure 4.4) was principally Scottish specimens collected from the warm months (June - early September), cluster 2 (indicated by c in Figure 4.4) was Welsh specimens collected from the same period, whilst cluster 3 (indicated by b in Figure 4.4) was Scottish

specimens collected from the cold months (October - May). However, when this effect of temperature was manipulated within the model (Figure 4.4), the discrimination by temperature was clear for the Welsh specimens. Nevertheless, it is interesting to note that the Welsh specimens collected during the summer period of high temperatures are marked by having larger shaft lengths than those collected during the summer low temperatures, since higher temperatures were shown to result in smaller sclerites by Mo (1991a). It is calculated that a collection of Welsh winter worms would be placed towards the -3,-3 coordinates on Figure 4.4.

From the PCA plot (Figure 4.4) it is interesting to note that the Welsh specimens have lower factor 1 scores than the Scottish specimens, while they have similar ranges for factor 2. The hamulus total length contributes the most variation to factor 1 whilst marginal hook total length is the major contributor to factor 2. The measurements of the hamulus total length from scanning electronmicrographs (Chapter 2, Table 2.11) shows that the Morph 2 form from Scottish salmon has a mean hamulus length of 61.56 μm, whilst the *G. derjavini* Morph 1 form from Welsh salmon has a mean hamulus length of only 48.86 μm.

The situation of the English forms at the interface of the Scottish and Welsh specimens (Figure 4.1) may possibly indicate that the parasites from southern Britain, namely those parasitic on salmon in the River Tamar (south-west catchment) and the River Piddle (Dorset), are of both celtic and boreal stock, the latter form being from an isolated boreal population of Atlantic salmon. The results of the PCA and cluster analysis are in close agreement with the findings of Payne *et al.* (1971) and Child *et al.* (1976). The boundary running just above north Wales, separating the Scottish sub-population of Morph 2 from the Welsh sub-population Morph 1, is however, tentative. This region may not only mark the boundary between the two sub-populations but may also be a zone where there is a possibility of finding both morphotypes on salmon. This boundary may be resolved by resampling the major salmon rivers down the west coast of Britain, and the determination of the relative prevalence of each morph.

Studies of genetic variation in brown trout by Stephen & McAndrew (1990) revealed that trout populations were found to be positively correlated with height above sea level for the Ldh-5(100) allele, which is common in Scottish ancestoral

brown trout. Ferguson & Fleming (1983) suggested a similar scenario for brown trout to the salmon situation, whereby, following the post-glacial period, rivers and lakes in Britain and Ireland were colonised by brown trout with this ancestral allele. However, this was later replaced by another migratory brown trout, the ancestral form surviving in isolated waterbodies or protected by impassable falls. Although attempts were made to make the present survey a study of all waterbodies and all possible salmonid populations isolated by hydrographic features, such as the River Loth population (Sutherland, Scotland), the samples collected may have been migratory brown trout which have only recently become isolated. An extension of the survey should ideally incorporate a larger number of isolated mountain lochs, sampling true ancestral brown trout forms, and only then could possible differences in the *Gyrodactylus* faunas on brown trout be determined.

In summary, it appears that Atlantic salmon resident in the UK are parasitised by a species of *Gyrodactylus* which closely resembles *G. derjavini*. This species exhibits two morphotypes, a Welsh or "celtic" form, Morph 1, and a Scottish or "boreal" form, Morph 2, which closely mirrors the pattern of distribution for the races of salmon in the British Isles.

The study has also indicated the influence of temperature/season on specimens of *Gyrodactylus*,since those collected from Scottish salmon in summer separated from those collected in the winter months. The discrimination of the Welsh specimens is clearer, with gyrodactylids collected from water differing by only 2°C being separated by the PCA. Chapter 3 indicated that *G. derjavini* parasitised both Atlantic salmon and rainbow trout. Although the species of *Gyrodactylus* parasitic on salmon in the UK has been found to be composed of two morphotypes, a further study should investigate whether a similar situation exists on rainbow trout.

CHAPTER 5: MULTIVARIATE ANALYSIS OF SOME MORPHOMETRICAL FEATURES OF PUTATIVE SPECIES OF *GYRODACTYLUS* PARASITISING BRITISH *SALMO SALR* AND *ONCORHYNCHUS MYKISS*.

Introduction

The influence of host was considered to be a key factor in the discrimination of gyrodactylids on British salmonids as shown in PCA 1 (Chapter 3). Separation of specimens of the genus *Gyrodactylus* Nordmann, 1832 represents a taxonomic conundrum; the "salmonid" gyrodactylids were found to be morphologically very similar, and the national survey of the gyrodactylid fauna of the three salmonid hosts will inevitably include a range of variants for each species. The initial discrimination (Chapter 3) was achieved by the incorporation of a host factor, assuming strict host-specificity. Nevertheless, if some of the variation was reduced by restricting the data to two samples, could the model discriminate a population of *G. derjavini* on Atlantic salmon *Salmo salar* Linnaeus, 1758 from a population on rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792) based on the parasite's morphology?

This model was set up to test: (1) whether the species of *Gyrodactylus* parasitic on salmon could be differentiated from those parasitic on rainbow trout; (2) whether populations of *Gyrodactylus* sp. on individual rainbow trout could be discriminated within a single sample; and (3) whether populations of *Gyrodactylus* exist at different loci on heavily parasitised fish and whether these could be distinguished, i.e. a head population from a tail population.

At the start of this investigation, studies with the light microscope, together with the results of the first principal components analysis (PCA)(Chapter 3) experiment, suggested that *G. derjavini* Mikailov, 1975 Morph 1 was parasitic on both Atlantic salmon and rainbow trout. However, with the development of the sclerite extraction techniques (Chapter 6), new evidence enforced a reassessment of the situation. Examination of the sclerites by scanning electron microscopy (SEM) (Chapter 2) clearly showed differences in marginal hook morphology between the species of *Gyrodactylus* parasitic on salmon and rainbow trout. Malmberg (1987) and

Wootten & Sommerville (1989) believed that Atlantic salmon from Scotland possibly harboured an undescribed form (Morph 2 in Chapter 2) closely resembling *G. derjavini* (Morph 1 in Chapter 2). The species on Scottish salmon differed from *G. derjavini* Morph 1 in the shape of the heel of the marginal hook sickle, as discussed in Chapter 2. Such subtle differences between these two species prevented their discrimination when data collected using the light microscope within the PCA model were utilised. The findings from the initial survey resulted in the discovery of populations of Atlantic salmon which carried only the morph 2 variant. In the following study, a population of *G. derjavini* Morph 1 is compared with a population of Morph 2 to see whether these two morphs can be discriminated.

Materials and Methods

Two populations of salmonid fish were sampled, one of Atlantic salmon *Salmo salar* from a fish farm on a tributary of the River Allan, Stirlingshire, and the second, rainbow trout *Oncorhynchus mykiss* from a fish farm on Loch Awe, Scotland. Both farm sites were sampled on consecutive days in December 1991, when the water temperature was 4.5°C. Fish (10.4 - 12.6 cm) were killed by a blow to the cranium and examined under an Olympus binocular microscope for the presence of gyrodactylids. Individual specimens of *Gyrodactylus* were removed and mounted on glass slides using the ammonium picrate-glycerine method (Malmberg, 1970). Each glass slide was given a code number cross-referenced to its exact position on the host. The distribution of *G. derjavini* Morph 1 on rainbow trout is illustrated in Figure 5.1 and the divisions of the bands used are indicated. A total of eight morphometric measurements were taken on the sclerites of *Gyrodactylus* as follows: hamulus total length, hamulus shaft length, hamulus point length, dorsal bar total width, ventral bar total length, ventral bar total width, marginal hook total length and marginal hook shaft length. The measurements were taken using an eye-piece graticule on an Olympus BH2 binocular microscope using oil immersion. A total of 477 specimens of *Gyrodactylus* were measured (from one rainbow trout with 322 specimens, and three Atlantic salmon with 120, 24 and 11 individuals, respectively).

The data were fed into a multivariate analysis package Systat (version 5.0,

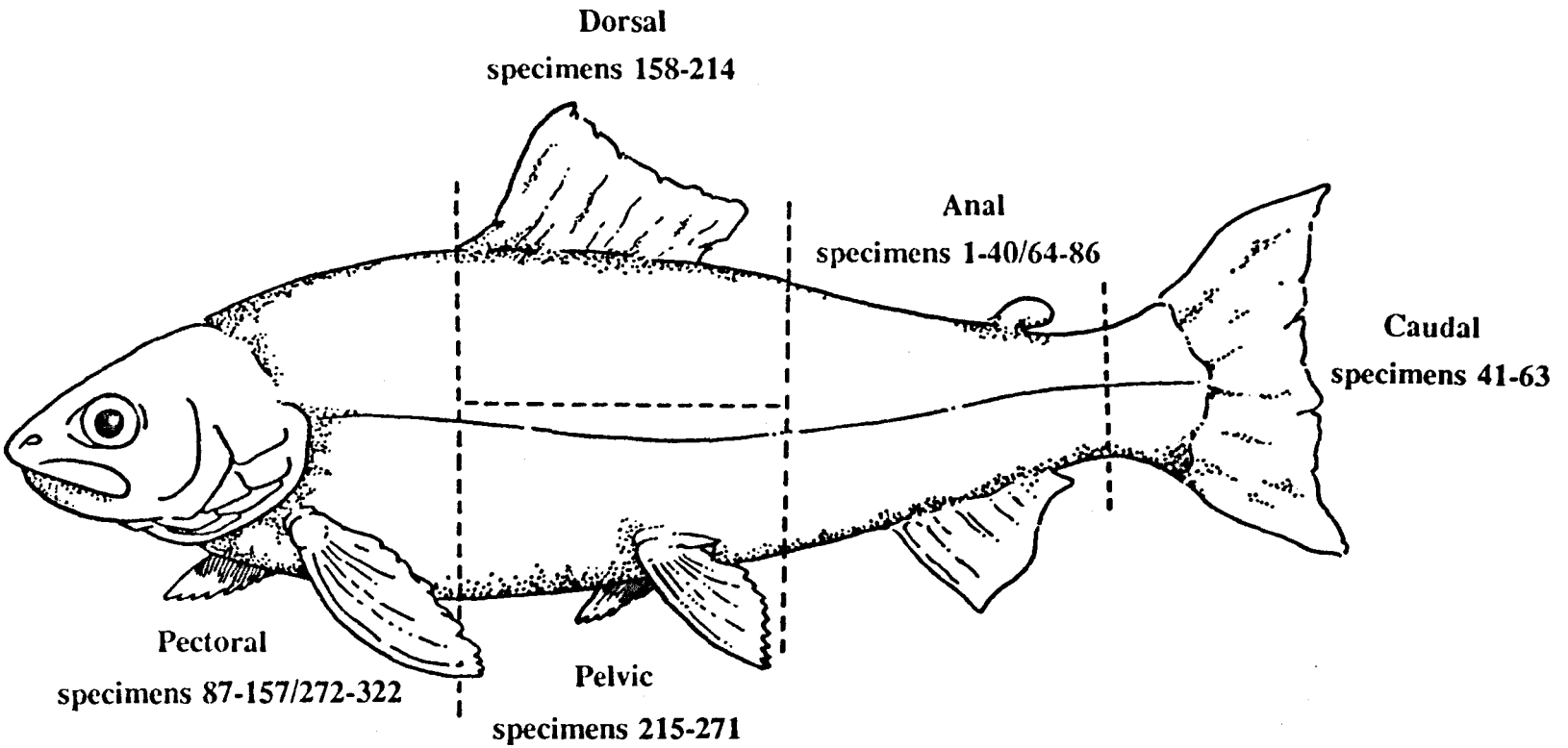


Figure 5.1: Distribution of *Gyrodactylus* on a heavily parasitised rainbow trout. The specimens were numbered 1-322 for the analysis and their position noted.

Table 5.1: Pearsons correlation matrix on the measured sclerites of *Gyrodactylus* spp.

Structure	HTL	HSL	HPL	DBTW	VBTW
HTL	1.000				
HSL	0.795	1.000			
HPL	0.402	0.222	1.000		
DBTW	0.229	0.155	0.124	1.000	
VBTW	0.405	0.406	0.065	0.087	1.000
VBBTL	0.130	0.099	0.164	0.058	0.014
MHTL	0.523	0.476	0.399	0.183	0.194
MHSL	0.471	0.427	0.374	0.150	0.199

Structure	VBBTL	MHTL	MHSL
VBBTL	1.000		
MHTL	0.131	1.000	
MHSL	0.151	0.944	1.000

Table 5.2: The component loadings for the first four principal components.

Structure	1	2	3	4
HTL	0.836	-0.288	0.098	0.060
HSL	0.771	-0.400	0.032	0.121
HPL	0.541	0.384	0.084	0.016
DBTW	0.305	-0.025	0.558	-0.761
VBBTL	0.229	0.380	0.674	0.536
VBTW	0.450	-0.652	0.050	0.176
MHTL	0.847	0.320	-0.293	-0.089
MHSL	0.817	0.347	-0.308	-0.058

Table 5.3: Percentage of variation explained by the components.

	Variance explained by components	% of total variance	Cumulative percentage
FACTOR 1	3.320	41.96	41.96
FACTOR 2	1.183	14.79	55.75
FACTOR 3	0.967	12.09	67.84
FACTOR 4	0.926	11.58	79.42

Table 5.4: Cluster analysis on PCA1.

VARIABLE	BETWEEN SS	DF	WITHIN SS	DF	F-RATIO	PROB
FACTOR (1)	323.857	6	152.143	470	166.742	0.00
FACTOR (2)	321.632	6	154.368	470	163.210	0.00
FACTOR (3)	340.536	6	135.464	470	196.918	0.00

CLUSTER 1

Variable	Min	Mean	Max	St.Dev	No.
FACTOR (1)	-2.26	-0.48	0.83	0.73	55
FACTOR (2)	-2.56	-1.29	-0.46	0.52	
FACTOR (3)	-0.24	0.76	1.70	0.49	

CLUSTER 2

Variable	Min	Mean	Max	St.Dev	No.
FACTOR (1)	-2.12	-2.12	-2.12	0.00	1
FACTOR (2)	-7.55	-2.12	-7.55	0.00	
FACTOR (3)	-13.56	-13.56	-13.56	0.00	

CLUSTER 3

Variable	Min	Mean	Max	St.Dev	No.
FACTOR (1)	-0.29	0.62	1.98	0.54	103
FACTOR (2)	-2.12	-0.33	0.68	0.58	
FACTOR (3)	-1.95	-0.61	0.18	0.45	

CLUSTER 4

Variable	Min	Mean	Max	St.Dev	No.
FACTOR (1)	-1.45	-0.20	0.82	0.49	115
FACTOR (2)	-0.56	0.25	1.49	0.49	
FACTOR (3)	-0.39	0.64	2.12	0.49	

CLUSTER 5

Variable	Min	Mean	Max	St.Dev	No.
FACTOR (1)	-2.10	0.24	2.58	0.80	45
FACTOR (2)	0.85	1.81	4.03	0.72	
FACTOR (3)	-2.39	-0.74	1.19	0.76	

CLUSTER 6

Variable	Min	Mean	Max	St.Dev	No.
FACTOR (1)	0.68	1.60	3.60	0.48	55
FACTOR (2)	-1.59	-0.26	1.65	0.64	
FACTOR (3)	-0.58	0.39	1.66	0.55	

CLUSTER 7

Variable	Min	Mean	Max	St.Dev	No.
FACTOR (1)	-3.08	-1.07	-0.16	0.49	103
FACTOR (2)	-1.16	0.16	1.77	0.55	
FACTOR (3)	-1.86	-0.26	0.88	0.54	

Table 5.5: Composition of clusters.

Cluster	Rainbow trout		Atlantic salmon	
	no.	%	no.	%
Cluster 1	40	12.40	15	9.68
Cluster 2	1	0.31	0	0.00
Cluster 3	57	17.70	46	29.68
Cluster 4	104	32.30	11	7.10
Cluster 5	18	5.59	27	17.42
Cluster 6	1	0.31	54	34.84
Cluster 7	101	31.37	2	1.29

1990), and principal components analysis (PCA) and cluster analysis were used to predict some of the associations between the two host species studied.

Results

Pearsons correlation was performed on the measured morphometric data (Table 5.1) and indicated that the sclerite lengths were all correlated together, i.e. the total hamulus length, hamulus shaft length, marginal hook total length and the marginal

hook shaft length.

A PCA analysis of the data (Tables 5.2-5.3) indicated that the first three factors explain 67.84% of the variation. Cluster analysis was then used to look for natural groupings, of which 7 clusters were indicated by the summary statistics (Table 5.4 and the composition of each cluster in Table 5.5).

From the cluster analysis there appeared to be a slight indication of discrimination between the species of *Gyrodactylus* parasitic on rainbow trout, i.e. *G. derjavini* Mikailov, 1975 Morph 1 and that on Atlantic salmon, i.e. *Gyrodactylus* sp. Morph 2. The evidence for this is seen in clusters 4 and 7 (Table 5.5), which are predominantly rainbow trout specimens and represent 63.67% of all specimens measured, whilst cluster 6 is predominantly Atlantic salmon specimens (34.84% of all measured). Since the use of cluster analysis to detect natural groupings within the specimens has indicated some separation of the two forms and host, the cluster analysis was then manipulated to look for two clusters weighted for host (Figure 5.2). The resulting pattern was similar. In Figure 5.2 the analysis separated the specimens on the basis of host-specificity, each ellipse incorporating 70% of the specimens. This distribution matches the findings of the earlier cluster analysis test (Chapter 3) with the ellipses overlapping by approximately 30% (the first cluster analysis found 63.67% of rainbow trout specimens to be separated from the salmon specimens). No separation of the salmon specimens from different individual salmon hosts was apparent (Figure 5.3).

The ability to recognise different populations based on their position on a host was investigated by using the rainbow trout population. Firstly the salmon specimens were removed from the analysis, as the presence of these specimens ultimately affect the relative position of all other specimens. Secondly, a single specimen of *Gyrodactylus* parasitic on a rainbow trout, which was identified as an outlier (cluster 2) by the cluster analysis was removed on the basis that the overall size of this specimen was considerably smaller than all other rainbow trout specimens. The remaining 321 specimens were then analysed using PCA and cluster analysis. The results from Pearsons correlation performed on the morphometric data on the specimens entered in the second PCA are shown in Table 5.6.

The component loadings for the first four principal components are given in

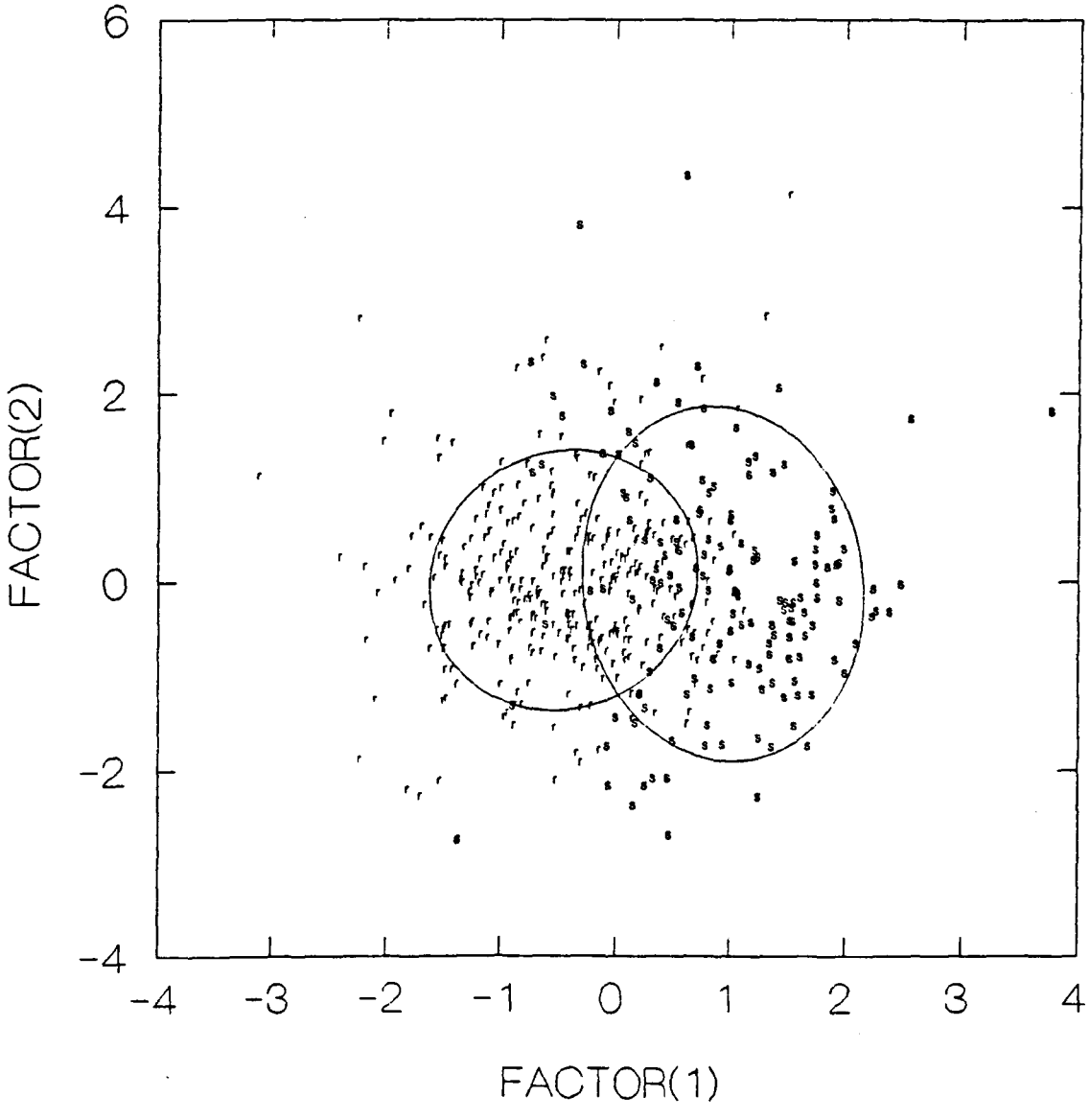


Figure 5.2: Discrimination of *Gyrodactylus* specimens with respect to salmonid host. Abbreviation: r = rainbow trout; s= Atlantic salmon. Ellipses incorporate 70% of the specimens.

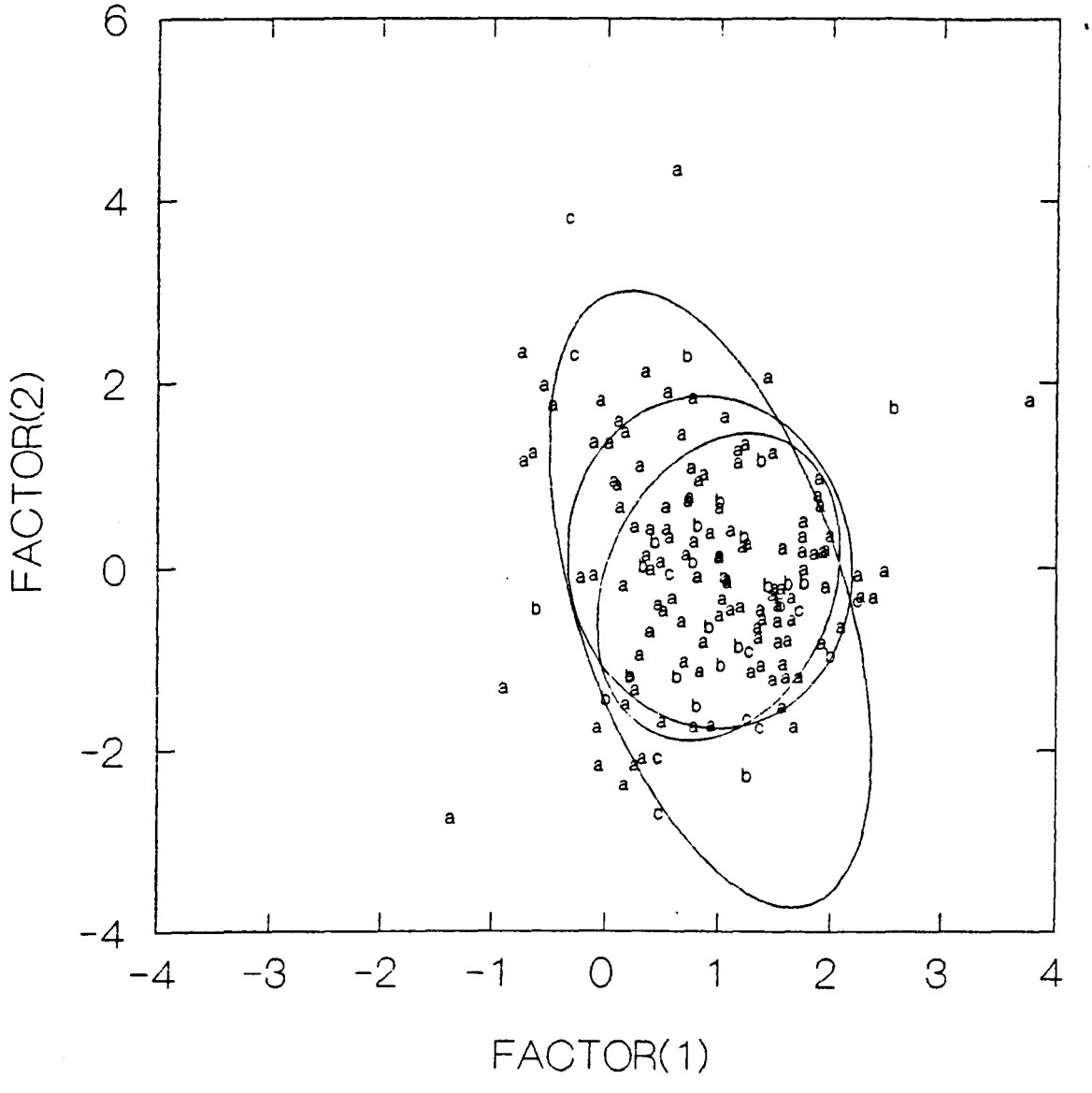


Figure 5.3: Distribution of *Gyrodactylus* Morph 2 specimens with respect to individual salmon hosts. The hosts are labelled a,b,c. The ellipses incorporate 70% of the specimens.

Table 5.6: Pearsons correlation matrix on n = 321 specimens.

	HTL	HSL	HPL	DBTW	VBTW
HTL	1.000				
HSL	0.693	1.000			
HPL	0.166	-0.063	1.000		
DBTW	0.147	0.028	0.028	1.000	
VBTW	0.311	0.344	-0.083	0.009	1.000
VBBTL	0.352	0.217	0.206	0.148	0.133
MHTL	0.396	0.361	0.098	0.132	0.135
MHSL	0.322	0.289	0.092	0.090	0.148

	VBBTL	MHTL	MHSL
VBBTL	1.000		
MHTL	0.303	1.000	
MHSL	0.260	0.908	1.000

Table 5.7: Component loadings of the first four principal components (PCA2, n = 321).

Structure	1	2	3	4
HTL	0.769	-0.293	0.302	0.059
HSL	0.700	-0.504	0.048	0.061
HPL	0.170	0.499	0.593	0.437
DBTW	0.214	0.206	0.317	-0.877
VBBTL	0.533	0.182	0.442	0.017
VBTW	0.410	-0.589	0.012	0.001
MHTL	0.815	0.363	-0.397	-0.004
MHSL	0.766	0.389	-0.458	0.024

Table 5.8: Percentage of variation explained by the components.

	Variance explained by components	% of total variance	Cumulative percentage
FACTOR (1)	2.858	35.72	35.72
FACTOR (2)	1.294	16.18	51.90
FACTOR (3)	1.109	13.86	65.76
FACTOR (4)	0.969	12.12	77.88

Table 5.7. Table 5.8 gives the variance explained by the components and the cumulative percentage explained. The data were analysed using cluster analysis looking for 5 clusters (ie. anal, caudal, pelvic, pectoral and dorsal fins). The summary statistics are shown in Table 5.9. Table 5.10 summarises the composition of the 5 clusters with respect to the fin region of the host.

The results (Table 5.10) suggest that there is some slight grouping of specimens from a particular fin region. In the case of cluster 1, over 55% are from the anal fin, 47% of the specimens in cluster 2 are from the dorsal fin, and 35% of those in cluster 3 are from the pectorals. The caudal fin is not equally represented, having only 23 specimens collected from this region of the body and, therefore, if the specimens from the caudal fin represent one population on the host, the sample size is not large enough to make any clear statements. The gyrodactylids belonging to each particular fin were identified and the ellipses of the cluster analysis were set to encompass 70% of the specimens for each site; the results are shown in Figure 5.4. The ellipses produced overlap one another to quite a large degree; however, the caudal and anal fin specimens appear to be displaced slightly when compared in relation to one another. This observation is in agreement with the results for the anal fin shown in Table 5.9.

Discussion

Several authors have reported individual fish have differing susceptibility to *Gyrodactylus* (Parker, 1965; Shulman, 1982; Scott, 1985; Madhavi & Anderson, 1985; Cusack, 1986; Bakke *et al.*, 1990, 1991); the situation in fish farms is that a few fish harbour relatively high numbers of *Gyrodactylus*, whereas most fish carry relatively few parasites (Scott, 1982, 1985; Cone & Odense, 1984; Cusack, 1986). This study analysed the specimens of *Gyrodactylus* collected from only four fish, the single rainbow trout (see Chapter 2) having a very high parasite burden of 322 individuals. Parasite populations on cultured fish are over dispersed, since with many fish concentrated within a confined area, the parasite spreads rapidly such that all fish become parasitised. As the probability of any one fish being infected by more than one source/fish is high, can the populations resulting from each of these infections be

Table 5.9: Summary statistics for *G. derjavini* Morph 1 specimens from rainbow trout.

Variable	BETWEEN SS	DF	WITHIN SS	DF	F-RATIO	PROB
FACTOR(1)	193.293	4	126.707	316	120.515	0.000
FACTOR(2)	130.569	4	189.431	316	54.453	0.000
FACTOR(3)	148.117	4	171.883	316	68.077	0.000
FACTOR(4)	138.304	4	181.696	316	60.134	0.000

CLUSTER 1

Variable	Min	Mean	Max	St.Dev	No.
FACTOR(1)	-0.08	0.98	2.23	0.58	75
FACTOR(2)	-2.19	-0.50	0.67	0.59	
FACTOR(3)	-2.75	-0.82	0.46	0.67	
FACTOR(4)	-1.29	0.08	2.43	0.68	

CLUSTER 2

Variable	Min	Mean	Max	St.Dev	No.
FACTOR(1)	-1.28	0.06	1.19	0.51	79
FACTOR(2)	-2.28	-0.28	1.15	0.76	
FACTOR(3)	-0.31	0.90	3.14	0.65	
FACTOR(4)	-1.18	0.67	2.35	0.77	

CLUSTER 3

Variable	Min	Mean	Max	St.Dev	No.
FACTOR(1)	-2.30	-0.88	0.46	0.57	76
FACTOR(2)	-2.40	-0.42	1.08	0.76	
FACTOR(3)	-1.63	-0.06	1.58	0.72	
FACTOR(4)	-3.20	-0.77	0.85	0.77	

CLUSTER 4

Variable	Min	Mean	Max	St.Dev	No.
FACTOR(1)	-0.79	0.65	2.56	0.72	48
FACTOR(2)	-0.23	1.15	4.25	0.88	
FACTOR(3)	-1.38	0.51	2.06	0.83	
FACTOR(4)	-2.66	-0.74	0.97	0.74	

CLUSTER 5

Variable	Min	Mean	Max	St.Dev	No.
FACTOR(1)	-3.65	-0.99	0.54	0.85	43
FACTOR(2)	-0.75	0.80	3.49	0.91	
FACTOR(3)	-3.34	-0.71	1.30	0.86	
FACTOR(4)	-0.40	0.84	3.27	0.83	

Table 5.10: Composition of identified clusters.

	Percentage found on fin (no.)				
	Anal	Caudal	Dorsal	Pectoral	Pelvic
Cluster (1)	55.56 (35)	26.09 (6)	1.75 (01)	16.39 (20)	23.21 (13)
Cluster (2)	17.46 (11)	8.70 (2)	47.37 (27)	17.21 (21)	32.14 (18)
Cluster (3)	14.29 (09)	34.78 (8)	15.79 (09)	35.25 (43)	12.50 (07)
Cluster (4)	3.17 (02)	0.00 (0)	14.04 (08)	20.49 (25)	23.21 (13)
Cluster (5)	9.52 (06)	30.43 (7)	21.05 (12)	9.02 (11)	12.50 (07)

detected? The principal factor determining whether we have the ability to recognise these sub-populations of *Gyrodactylus* is the number of cross infections that have occurred for any one particular fish and the age of the epizootic in the cage under investigation. The recognition of sub-populations on farmed fish using this model is, therefore, difficult. Although the occurrence of self-fertilisation is known in the Monogenea (Bychowsky, 1957; Jackson, 1982), *Gyrodactylus* spp. are known to reproduce by a mixture of reproductive strategies. *Gyrodactylus* has been observed in single worm infections to reproduce parthenogenetically for up to 20 consecutive generations without sexual reproduction being recorded (Lester & Adams, 1974); this observation is not unique and has been observed for other monogeneans (Wagener, 1860; Katheriner, 1904; Minouchi, 1936; Malmberg, 1956; Braun, 1966; Nollen, 1983). However, with population growth, the availability of mates increases such that sexual reproduction can occur (Bychowsky, 1957; Kearn, 1970; Llewellyn, 1983; Harris, 1983, 1989). Harris (1988, 1989) calculated that, for *G. turnbulli* Harris, 1986 copulation was related to the mean nearest neighbour distance of the parasite population, and that mating was observed when this distance was less than 0.3 mm (this observation was made in 70% of populations when the total parasite number exceeded 40 individuals). Mating between gyrodactylids is "promiscuous" in that coupling takes place with several partners. This adds a large component of variation and complexity and decreases the clarity with which we can delineate populations, for, although single worm infections give rise to clones, once a threshold number of parasites is reached, the probability of a mating encounter increases; furthermore, the

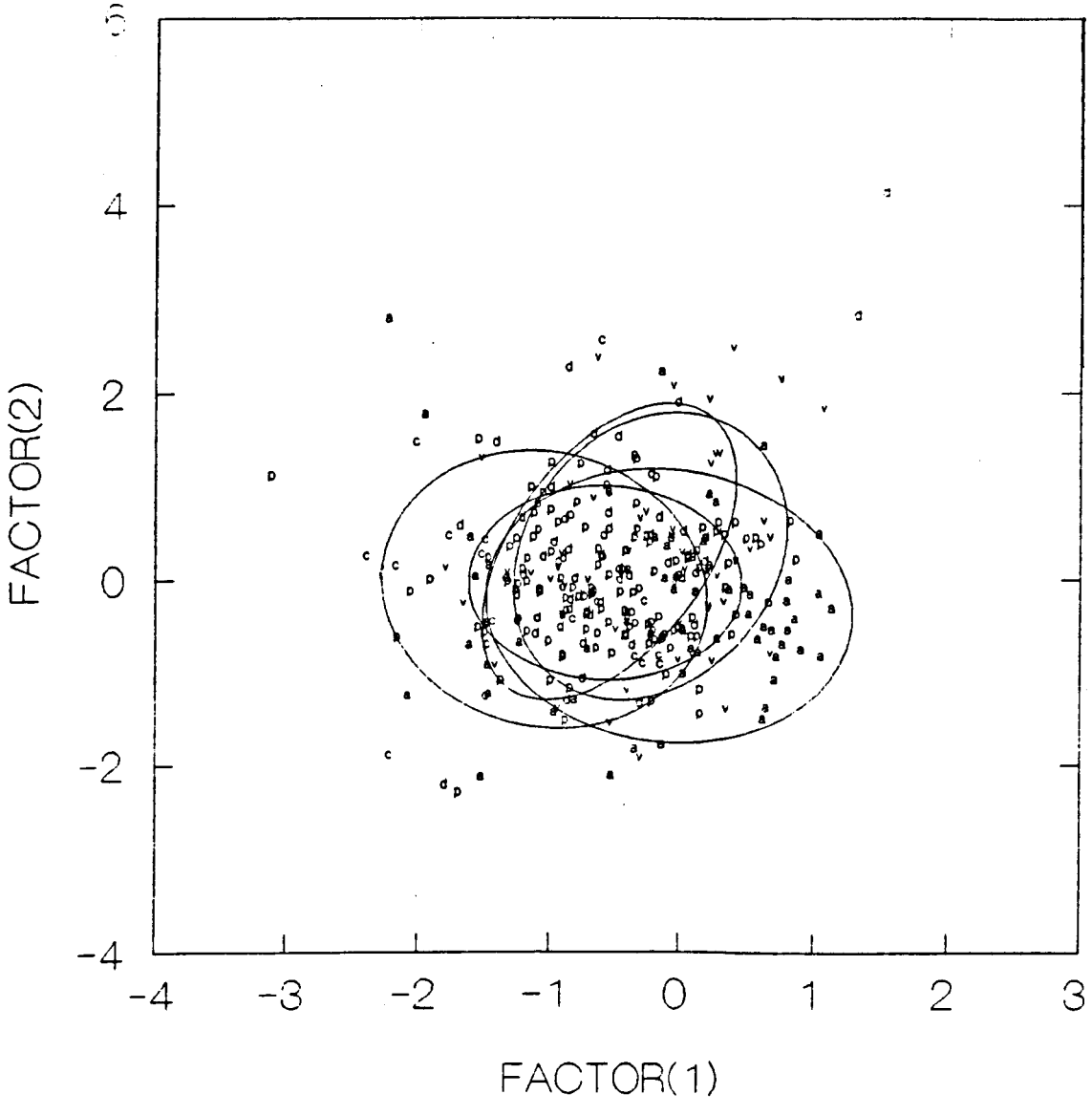


Figure 5.4: Distribution of *Gyrodactylus* with respect to microhabitat on a heavily parasitised rainbow trout host. Abbreviation: a = anal fin; c = caudal fin; d = dorsal fin; p = pectoral fins; v = ventral pair.

range of genetic variation also increases and with it the range of variation in hook morphology. However, Scott & Robinson (1984) stated that *G. turnbulli* follows a cyclical pattern of growth and decline, as a result of which Harris (1989) believed that an infection arising from a single parasite was unlikely to reach the threshold number where mating is then possible. This means that the intensity of *G. turnbulli* infections on wild fish would tend to be low, and the occurrence of cross-insemination would be rare (Scott & Anderson, 1984). Therefore, it should be feasible to use the present model to discriminate two populations of *Gyrodactylus* on a single fish which arise from single worm infections where the extent of genetic variation is low due to the low prevalence of sexual reproduction. Such a situation may explain Figure 5.4, where the gyrodactylids present on the caudal fin are discriminated to some extent from those on the anal fin, although the ellipses do overlap by some 30%. Certain species of *Gyrodactylus* have preferred microhabitats; for example *G. bullatarudis* Turnbull, 1956 and *G. turnbulli*, both parasites of the guppy *Poecilia reticulata*, become localised on the anterior (head and opercula) and the caudal peduncle, respectively.

In this study farmed fish were used, and the probability of gyrodactylids having originated from several fish is high, with the additional possibility of further specimens transferring during the course of the infection. The introduction of a large number of gyrodactylids from various origins onto a naïve fish suggests that the population growth should soon reach a level where mating would occur. The ability to discriminate founder populations is then rapidly lost as mating continues and increases the amount of genetic variation within the population. The results in Figure 5.4 could be interpreted in this way where the gyrodactylids collected from the dorsal, pelvic and pectoral fins overlap to a large degree. According to Harris (1988) and Cone & Cusack (1989), *Gyrodactylus* specimens generally migrate to the fins, such that the parasite population establishes and becomes concentrated within these sites. Response to a directional cue, possibly water currents (Cone & Cusack, 1988), not only brings the parasite to a locality where the probability of finding a mate is higher but where the worms are in a position for effective transmission to new hosts as the fins touch. To clarify whether it is possible to identify two or more founder populations of *Gyrodactylus* on a single host, the experiment should be repeated but using wild fish. The number of possible encounters with an infected fish being

considerably less than in cultured salmonids.

The fish samples in this study were collected during December 1991, at a water temperature of 4.5°C; low water temperatures are known to affect both the birth and death rates of *Gyrodactylus*: *G. salaris* has a mean lifespan of 33.7 days at 2.5°C (Jansen & Bakke, 1991); *G. alexanderi* Mizelle & Kritsky, 1967, 71 days at 7°C (Lester & Adams, 1974); and *Gyrodactylus* sp. 30+ days at 4-6°C (Ikezaki & Hoffman, 1957). If *G. salaris* is representative of other "salmonid" gyrodactylids, then at 6-13°C the average number of offspring per parasite (2.4 offspring/parasite, maximum of 4 offspring/parasite, Jansen & Bakke, 1991) is higher than that reported for other species of *Gyrodactylus* (Lester & Adams, 1974; Scott, 1982). Therefore, at low temperatures parasite longevity is increased, the number of offspring is higher and a reduction in the rate of transmission has been observed (Bakke *et al.*, 1990), such that under these conditions the population gradually increases and the genetic variation within a population also increases.

In conclusion, farmed fish do not represent an ideal basis on which to discriminate morphologically closely related species, since the close interaction between many individuals permits the transmission of parasites from several sources, whereas wild fish may only encounter a low number of infected hosts. Cage culture allows the infection to spread rapidly, to such an extent that on fishes with heavy parasite burdens sexual reproduction is possible and the identification of the founder populations is no longer possible. Although the sample size in this study was small, there is an indication that the analysis does permit some discrimination between the two salmonid hosts and their species of *Gyrodactylus*. In this case, however, it was not possible to study populations present on individual Atlantic salmon. Nevertheless, on the heavily parasitised rainbow trout, there was some indication that a proportion of the parasites found on the anal and caudal fins may have originated from the population(s) on the dorsal, pelvic and pectoral fins.

Chapters 3-5 have been based on the ability of the multivariate analyses to discriminate species of *Gyrodactylus* parasitic on salmonoids by considering all the measured morphometric variables simultaneously. The morphometric data used includes only gross dimensional data for each of the sclerites; however, when the species to be separated differ by subtle variations in their hook morphology, then a

precise means of analysing the hook shape is required. A means of releasing the sclerites from the surrounding tissue such that they could be subsequently analysed on a higher platform of accuracy would permit a clearer discrimination of the salmonoid gyrodactylids.

CHAPTER 6: AN SEM STUDY OF THE HAPTORAL SCLERITES OF THE GENUS *GYRODACTYLUS* NORDMANN, 1832 (MONOGENEA) FOLLOWING EXTRACTION BY DIGESTION AND SONICATION TECHNIQUES.

Introduction

Species determinations of the genus *Gyrodactylus* are presently based upon subtle variations in hook morphology (Malmberg, 1970), but for certain species these have posed taxonomic problems when carried out using light microscopy (Bakke & Jansen, 1991). The ambiguity caused by the inability to make complete and meaningful measurements of the most subtle key characters used for this group has emphasised the need for the development of novel techniques for accurate species identification. In Chapter 3 it was found that the lowest limit of resolution with the light microscope was 0.5μm. It was subsequently found that structures below 20μm in size proved too unreliable to include within the multivariate analysis because of the too few measurement classes on which to base discrimination.

Mo & Appleby (1990) found that the hard parts of the opisthaptor could be collected using enzymatic digestion of the freshly collected worms which are then examined by scanning electron microscopy. In this way the precise structure and shape of the hooks could be determined more accurately. However, the use of proteolytic enzymes is destructive in that some of the structures associated with the hooks are lost, thus permitting close examination of only the main parts of the hamuli and marginal hooks. Since the lost sclerites, notably the bars, may be important taxonomic features, this technique has a major disadvantage. Furthermore, the fact that only fresh material may be processed in this way limits the value of the technique.

In this chapter techniques were examined which permit the use of both fresh and preserved material. One technique is a modification of the enzymatic digestion method and the other one uses the application of a low amplitude sonicating source to remove soft surrounding tissue, thus making available taxonomically important structures that would otherwise be destroyed by proteolytic enzyme digests.

Materials and methods

The following material was collected: fresh specimens of *Gyrodactylus gasterostei* Gläser, 1974 and *Gyrodactylus arcuatus* Bychowsky, 1933 from three-spined sticklebacks *Gasterosteus aculeatus* (L.) from Airthrey Loch, Stirling; fresh specimens of *G. derjavini* Morph 1 from rainbow trout *Oncorhynchus mykiss* (Walbaum) from Loch Awe, Scotland; and frozen specimens of *Gyrodactylus* sp. Morphs 6 and 7 from charr *Salvelinus alpinus* (L.) from Lake Ennerdale, in Cumbria, England.

Fish infected with gyrodactylids were killed by insertion of a needle into the brain in the upper part of the eye (Malmberg, 1970), and infected fins removed and transferred to a watch-glass with distilled water. A 1 in 1500 solution of 2-phenoxyethanol was used as a parasite anaesthetic to aid dislodgement of the parasites from the fins. Live parasites were collected in batches of a minimum of 30-40, washed free of any adhering fish mucus in 0.2 M phosphate buffer, and transferred to mild acid cleaned, MSE pointed 10ml glass centrifuge tubes. They were then processed by one of a variety of enzymatic digestion and sonication techniques.

Digestion technique

The method described by Mo & Appleby (1990) uses a 1mg per ml pepsin (BDH pepsin A, E.C. 3.4.23.1) in 0.7mM hydrochloric acid solution on fresh or frozen specimens. However, it was found necessary to keep the amount of digestive fluid added to the gyrodactylids to a minimum (3-5 ml), because undissolved particulate matter from the digestive fluid preparation concentrates in the subsequent centrifugation stages and interfered with the preparation. When this occurred it was remedied by filtration of the peptic fluid.

In addition, alcohol (80%) and formalin (2%, 5% and 10%) fixed *Gyrodactylus* material was pre-washed in several changes of distilled water in order to remove the fixative before being subjected to prolonged incubation times, 24, 48, 72 and 144 hours, at 37°C in the digestive fluid. Solutions of 10% potassium hydroxide for 24, 72 and 144 hours at room temperature and 40% (w/v) solution of sodium carbonate (Maillard *et al.*, 1982) for 24 and 48 hours, were also tested for their efficacy in releasing sclerites.

Following a single exposure to the pre-selected digestion chemical and conditions, the resultant material was then processed as for the post-sclerite release procedures.

An additional digestion step for formalin fixed material was tested as follows: material was pre-washed for 2 hours in distilled water to remove any fixative, and the parasites were then digested for 24 hours at 37°C in pepsin fluid. Following the pepsin digestion, formalin-fixed parasites were centrifuged at 6,000 rpm for 5 minutes and the supernatant decanted. The pellet was resuspended with distilled water and spun again and the supernatant siphoned off. The pellet was again resuspended this time with 0.25mg/ml trypsin (BDH trypsin, pancreatic, E.C. 3.4.21.4) in 0.0016M tris pH 7.8 containing 0.049% BSA, and allowed to digest for 24 hours at 37°C. After this the material was again washed and replaced with a second pepsin digest for 24 hours and then processed as for the post-sclerite release procedures in preparation for scanning electron microscopy.

Sonication techniques

Fresh and frozen gyrodactylids were collected in pointed glass centrifuge tubes in 3ml distilled water and sonicated by one of two means. (1) The sonicating probe was inserted into the centrifuge tube, which contained the gyrodactylids, and sonicated on ice. Three bursts of 9 seconds at a low peak to peak amplitude of 9.5 μm were used in a MSE 150 W ultrasonic disintegrator (point sonicator) at a nominal frequency of 20 KHz fitted with an exponential titanium probe. The resultant ultrasonic energy was concentrated by increasing the amplitude of vibration during the process by use of a conical probe (end diameter 1/8") through which the ultrasound was emitted. (2) Centrifuge tubes were placed in a wire rack in a sonic water bath (Kerry Pulsatron 125) connected to a 240 V power supply with a continuous power output of minimum 100 W. This was operated at a mean frequency of 40 KHz. Both fresh and frozen specimens of *Gyrodactylus* from *Salvelinus alpinus* were used to test the viability of using frozen material for the sonication method. Specimens were frozen in distilled water, kept at -70°C until required, thawed, rinsed with distilled water and sonicated.

Post-sclerite release procedures

Following sclerite release, the samples from both fresh and frozen material either still in the digestive fluid or the resultant sonicate, were then centrifuged, still in their original pointed glass centrifuge tubes, at 6,000 rpm for 5 minutes and the supernatant decanted. The pellet was resuspended with distilled water and spun again; this was repeated several times. The final pellet was agitated in a minimum amount of water, pipetted onto 11 mm round coverslips and allowed to air dry. The coverslips were then screened for sclerites under the light microscope (Olympus BH2 stereo microscope) and the positions marked by means of adhesive paper pointers, prior to being sputtercoated with 15 nm of gold (using a Polaron E5000 sputtercoater). They were then examined under a (Hitachi S800) field emission scanning electron microscope (SEM) at an accelerating voltage of 8 kV.

Results

Digestion technique

Sclerites free of any adhering material were recovered from fresh and frozen material after 24 hours digestion and from alcohol-fixed specimens after 72 hours pepsin treatment at 37°C. However, all concentrations of formalin-fixed material exposed to a single pepsin treatment failed to release their sclerites by this method up to and beyond the exposure times recommended for alcohol-fixed specimens. The use of potassium hydroxide and sodium carbonate also yielded unsatisfactory results. However, the pepsin and trypsin combined digestion did release a large proportion of formalin-fixed marginal hooks, although the hamuli required further treatment to completely remove some remaining tissue remnants.

Sonication technique

The sonic water bath was preferred in practice over the point disintegrator for two reasons: (i) the percentage return of individual hooks was considerably higher, i.e. (70-80% for the sonic water bath as compared to only around 10% for the point disintegrator; and (ii) the resultant sonic energy was more controllable.

Fresh and frozen specimens prepared for the sonic water bath method were

found to require differential periods of sonication in order to release the sclerites from their surrounding tissue. The fresh specimens of the gyrodactylids infecting charr required only 20 minutes sonication, whilst the frozen material collected from charr required up to 25 minutes to release all of the sclerites. This was only in part dependent upon whether the specimens were fresh or frozen, since fresh gyrodactylids from sticklebacks required only 1 minute sonication. However, sonication of alcohol (80%) and formalin (2%, 5% and 10%) fixed material failed to release the sclerites.

The following observations and comments, are mainly based upon sonicated material. For the first time, the complete ultrastructure of the ventral bar membrane and also the dorsal bar could be examined and accurate morphometric data obtained from SEM micrographs. Figure 6.1 gives a diagrammatic representation of the sclerites and illustrates the nomenclature used below.

Ventral bar

The major accessory bar, or ventral bar as shown in Figures 6.6(E)-6.9(H), is situated ventrally to the paired median hooks or hamuli, and the ventral bar processes (Figure 6.1 vbp) arising from the ventral bar proper correspond to the smooth flattened area on the hamulus (Figure 6.1 vbap).

The terminal point and structural shape of the ventral bar membrane is often difficult to discern in preparations for the light microscope, depending on gyrodactylid age. The terminal point of the ventral bar membrane is arrowed in Figure 6.7(F). Observations on the sonicated material suggest that, *in situ*, the dorsal and ventral surfaces of the ventral bar are not bilaterally symmetrical. Dorso-ventrally it consists of three levels in agreement with the observations made by Malmberg (1970) (Figure 6.1B1 and B2). In the three species studied here, the ventral surface of the bar proper has, at its anterior end, a depression which runs to 1-1.5 μm deep at the mid-point between the origins of the ventral bar processes. This depression is arrowed in Figure 6.6(E). In addition, the ventral bar processes originate dorso-laterally to the plane of the ventral bar proper and at a tangent to it laterally. Micrographs of partly sonicated material (with some remnants of tissue still in association with the sclerites) (Figure 6.4(C)) suggest that these ventral bar processes may have some connection with the

folds (Figure 6.1A vbap) seen on the hamulus, even in species with large processes such as *G. arcuatus* (Figure 6.4(C)). However, the precise nature of the connection made at these two connection points (Figure 6.1A vbap) requires further study.

Apart from these two points of attachment, there may also be a second system of attachment between these two points of contact, as suggested by Malmberg, 1970, since the dorsal surface of the bar appears to have the ventral bar processes (vbp) and the mid-portion of the ventral bar proper (vbpr) within the same plane (Figure 6.1B1). A double system of attachment points securing the ventral bar in two planes would in effect produce a securing plate maintaining the position of the hamuli within the gyrodactylid opisthaptor.

The mid-portion of the ventral bar proper typically has an irregular surface in contrast to the smooth surfaces of the ventral bar processes and the extremities of the ventral bar proper. From this irregular mid-portion a number of thickened ramifications extend posteriorly across the posterior membrane for almost its entire length. This is best demonstrated in bars obtained from specimens of *G. arcuatus* (Figures 6.7(F)-6.9(H)).

The lateral margins of the ventral bar membrane are thickened for almost two thirds of its length (Figure 6.6(E)-6.7(F)). The structure of this membrane is thickened at its origin (anterior end) and tapers off, yielding a delicate structure at its distal point. In *G. arcuatus* the central region of the membrane is irregularly perforated between the thickened ridges.

Dorsal bar

Each hamulus is secured in position by a second point of attachment provided by the dorsal bar (Figure 6.10(I)), which is situated dorsally in relation to the hamuli *in situ*. Although the dorsal bar as a whole is flexible, the central portion of this sclerite appears to exhibit one or two morphological variants or morphs within a certain species, as indicated by Malmberg (1970). In the specimens of *G. gasterostei* (Figures 6.2(A)-6.3(B)) taken from Airthrey Loch, this middle portion is 1.7-1.95 μm thick and appears as a heavy deposition of keratin-like protein (*) having no precise structure. In *Gyrodactylus* Morph 1 on *O. mykiss* from Loch Awe, the middle portion of the dorsal bar seems to have two processes arising 11 μm apart. These processes are

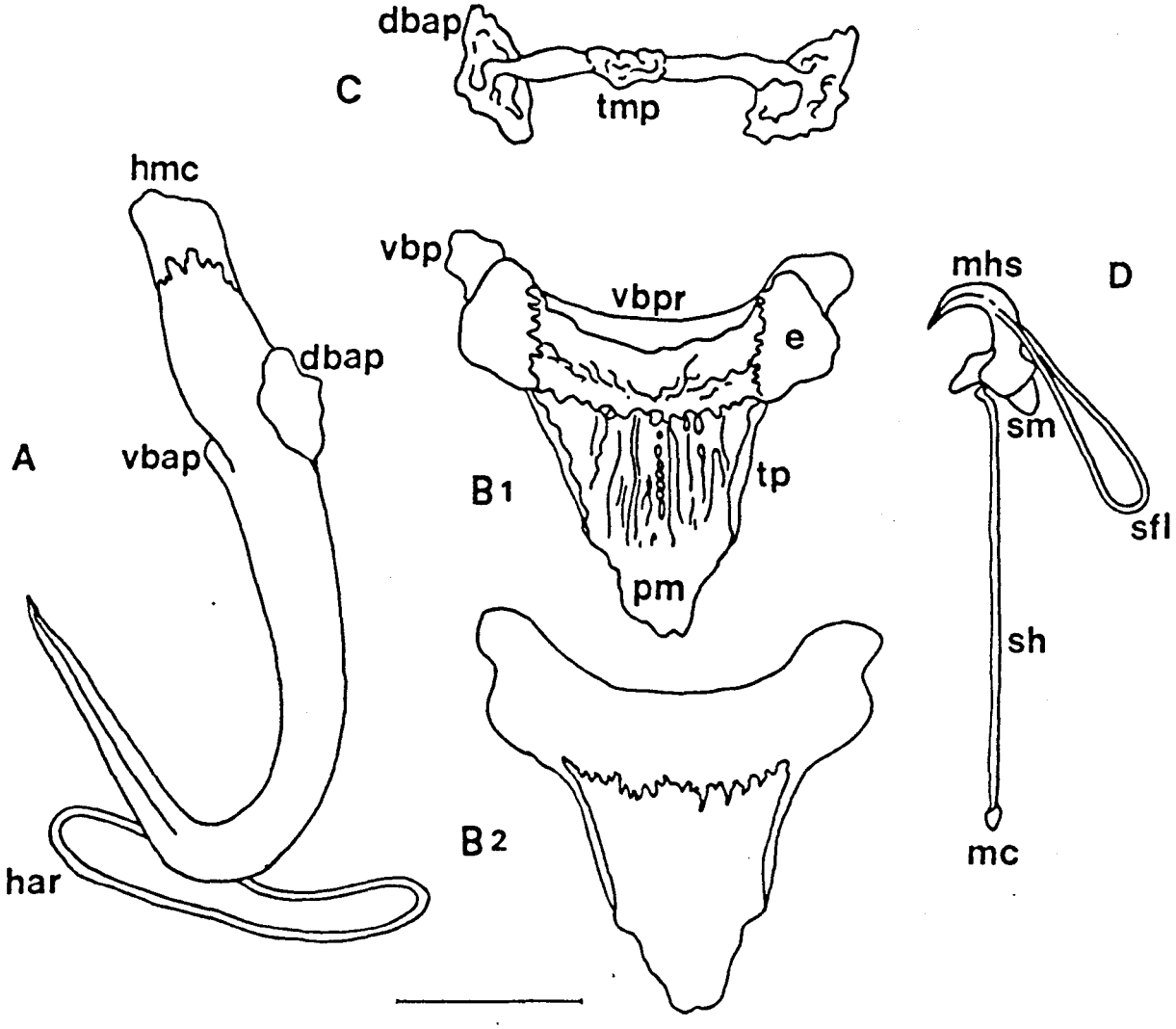


Figure 6.1: Nomenclature of the haptoral sclerites extracted by the sonication technique; (A) hamulus and hamulus ring (B) tentative diagrammatic representation of the respective (1) ventral and (2) dorsal surfaces of the ventral bar (C) dorsal bar, and (D) marginal hook. Hamulus: **hmc**, hamulus muscle cap; **dbap**, dorsal bar attachment point; **vbap**, ventral bar attachment point; **har**, hamulus aperture ring. Ventral bar; **vbp**, ventral bar processes; **vbpr**, ventral bar proper; **e**, extremities of ventral bar proper; **tp**, thickened lateral border; **pm**, posterior membrane. Dorsal bar; **dbap**, dorsal bar attachment point; **tmp**, thickened mid-point. Marginal hook; **mhs**, marginal hook sickle; **sfl**, sickle-filament loop; **sm**, sickle-membrane; **sh**, shaft; **mc**, muscle cap. **Scale bar:** 5µm.

labelled in Figure 6.10(I) by an "x".

While the mid-portion of the dorsal bar appears to vary in structure, its two attachment points made with the hamuli are more characteristic. The dorsal bar attachment point (Figure 6.11(J)) appears as a cap, one on the inner surface of each hamulus, the cap surface being irregular. Each cap forms the dorsal border between the posterior part of the hamulus root and the anterior part of the hamulus shaft. The dorsal bar originates directly from the apical portion of the attachment cap. It then extends diagonally forward following the plane of the hamulus shaft *in situ*, before turning ventrally (Figure 6.11(J)) and then transversally, where it links with the corresponding attachment point on the other hamulus. The two caps or attachment points of the dorsal bar, together with the attachment points of the ventral bar, maintain the hamuli in an effective position for the process of attachment to its host.

Hamuli

In hamuli obtained from digested *Gyrodactylus* specimens, the attachment area for the cap, or dorsal bar attachment point, is visible but the caps and the dorsal bar proper and its origins are lost. This observation is consistent with the findings of Mo & Appleby (1990). In specimens obtained from partly sonicated material, some of the associated musculature remains intact and still attached to the muscle cap of each hamulus root. In some specimens the ligaments between these muscle caps which keep the hamuli in the correct orientation, were present. The ligaments are marked in Figure 6.5(D) by an "l".

Marginal hooks

The marginal hooks have been widely used as important criteria in taxonomy of the gyrodactylids (Malmberg, 1970). The development of the digestion technique has

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* Although the precise chemical composition of monogenean hooks is still unclear, the investigations of Lyons (1966) and Kayton (1983) demonstrated that the hamuli contained a sulphur component. The term "keratin-like proteins" is applied here, in the absence of a definitive determination and without direct reference to this point.

further enhanced the likelihood of these sclerites being used as diagnostic features. Close examination of the sonicated marginal hook sickle reveals a sickle heel; this is a thickened region clearly marking a transition to a thinner region, the toe (see Chapter 2 Figure 2.9). At this transition point the marginal hook shaft joins the hook sickle. The shape of the ventral (anterior) portion of the heel may influence the movement and joint of the sickle.

The sonication technique used here retains three additional portions of the marginal hook shown in Figure 6.12(K), the sickle filament loop, the sickle membrane and the muscle cap of the shaft which are lost (Figure 6.13(L)) using the digestion techniques. The filament loop of the marginal hook of *Gyrodactylus* sp. collected from *Salvelinus alpinus* measures 0.6 μm in thickness and is uniform along its length. The filament loop appears to have some structural adaptation at its connection with the sickle proper (Figure 6.1D), presumably for the guidance of the marginal hook in the correct line through its aperture in the opisthaptor when the sickle is in the process of attachment to the host epidermis. This, however, requires closer investigation. Furthermore, the marginal hook sickle membrane was retained, the peripheral margin of the membrane being thickened (Figure 6.12(K)). Analogous to the hamulus root, the end of the marginal hook shaft possesses a cap for the attachment of muscle fibres. In the sonicated preparations, however, no specimens with muscle fibres attached to the shaft cap were retrieved.

Hamulus aperture rings

Ellipsoid rings which were recognised as the hamulus aperture rings (Figure 6.5(D)) were recovered in the sonicated preparations. These rings form and strengthen the openings through which the hamuli protrude ventrally from the opisthaptor. Although in the literature the rings are occasionally included in drawings of the whole hook complement, they are not considered as part of the hook set. These structures were retained in the sonicated material, although they were not resistant to digestion and their composition remains to be determined. It is unknown whether this structure arises from a local thickening in the body wall about the hamulus opening, or is similar in composition to the bars of *Gyrodactylus*. No such analogous structures were found for the marginal hooks, but this function may be served by the sickle filament loop.

Discussion

One of the major problems when making slides of monogeneans for light microscopy is the achievement of a correctly orientated specimen with all the hardparts lying flat in order to permit accurate measurements and drawings. The employment of enzymatic and sonication techniques to liberate the sclerites from the surrounding tissue and their subsequent flotation in water permits these structures to lie flat as the sonicate dries. This achieves a considerable improvement of the existing sclerite preparation method. Furthermore, scanning electron micrographs of these sclerites allows precise measurements to be made. The limitations of the resolution of the light microscope is approximately 0.5 μm, which means that some of the smaller measurements taken from the haptoral complement fall into only a few measurement classes. Thus, for a structure such as the mid-point width of the dorsal bar, which in *G. gasterostei* is only about 1.5-2.0 μm wide, an error in using the eyepiece graticule on the light microscope would effectively be a 25% measurement error. Morphometric measurements taken from electron micrographs with the aid of a digital image analyser allowed extremely accurate measurements to be made. Electron micrographs can easily be enlarged and, in addition to the classical point to point system of collecting morphometric data in most species descriptions, novel parameters, such as area, can be investigated by the application of new digital instrumentation, e.g. to marginal hooks.

The techniques for the preparation of haptoral sclerites of *Gyrodactylus* species for scanning electron microscopy were improved by the application of sonication. For the first time it was possible to study the precise structure of various portions of the haptoral complement, and these are discussed in Chapter 11.

The connection of the dorsal bar to the hamuli allows for flexibility as the two hamuli move together or apart during movement. Whilst the dorsal bar cap on the hamulus maintains a characteristic form, the dorsal bar mid-point appears as an irregular deposition of scleroprotein. Although the manner in which the dorsal bar forms is unknown, the structure of the mid-point might suggest that it arises as an outgrowth from the hamulus, the two halves then fusing. Malmberg (1970) noted that a single species may exhibit several morphological forms with regard to this centre

piece, these forms possibly arising as a result of the way in which the two halves of the dorsal bar fuse. Figure 6.14 presents a diagrammatic representation of how this might occur. The developing dorsal bar extends, when contact with the other half is made, the whole structure thickens as the embryo matures. If the two halves fail to align correctly then it is feasible to predict the morphological forms that are observed.

A suspensory ligament between the hamuli was seen for the first time. The hamuli are secured together by the dorsal bar; however, in some preparations the dorsalbar appears damaged or even absent. The hamuli require a second system to maintain them in a position effective for attachment to prevent the hamuli rotating.

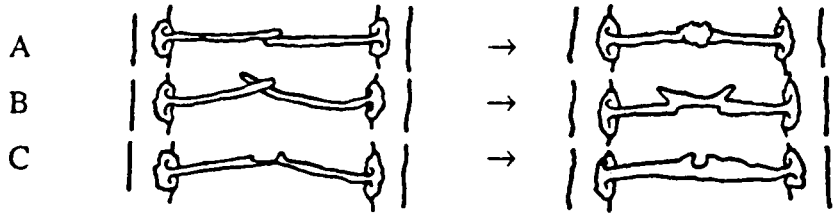


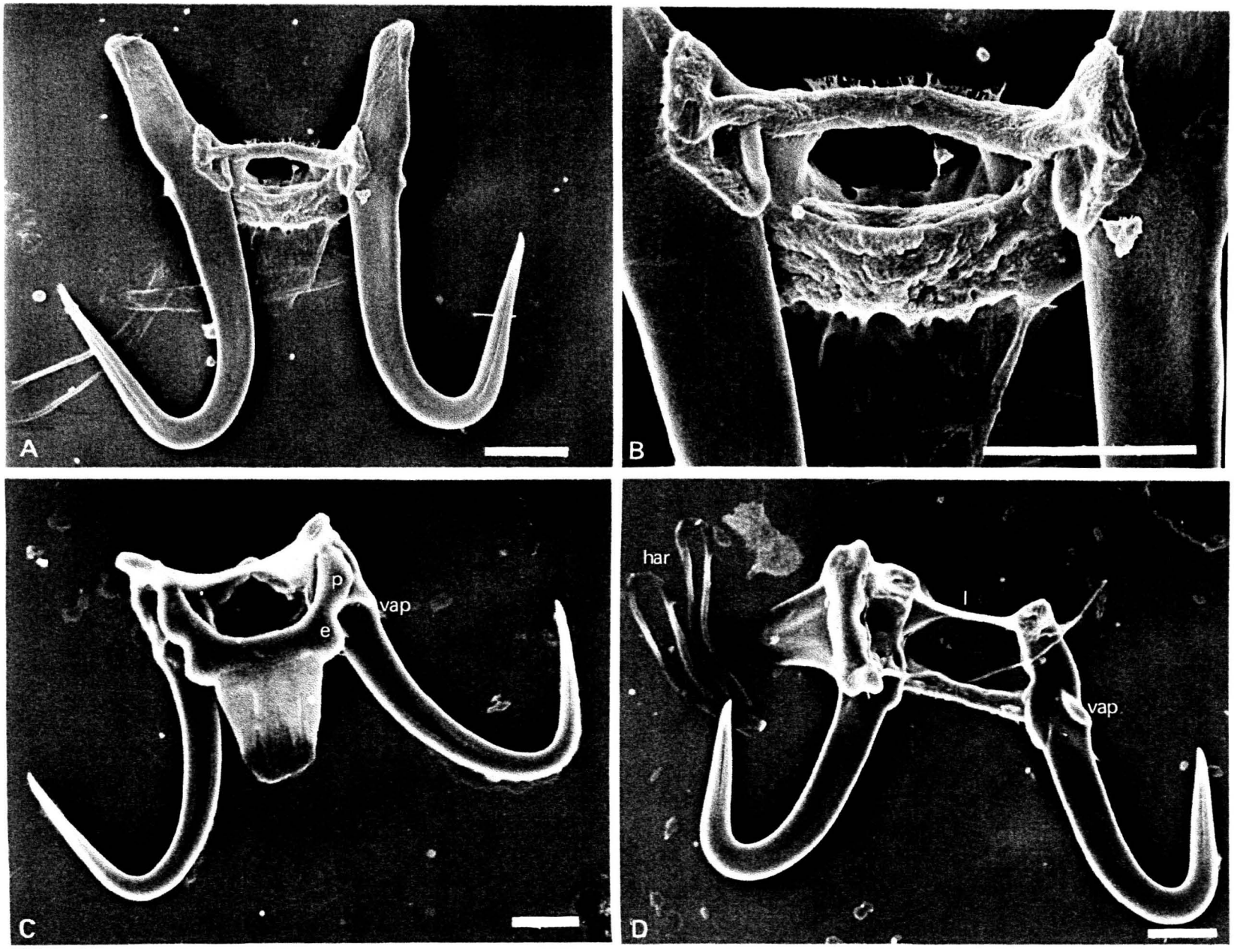
Figure 6.14: Formation of the dorsal bar mid-point. The primordia of the two halves of the dorsal bar extend and make contact with the other half. The two halves fuse at their contact point and then undergo secondary thickening.

The ligament fulfils this requirement, preventing the hamuli from splaying open, but retains the elastic component allowing the hamuli to move together as they are retracted into the opisthaptor.

Although the marginal hooks of *Gyrodactylus* are known to be hinged at the point where the shaft joins the sickle proper just anterior to the heel of the sickle (Malmberg, 1990), the mechanism is still unclarified. Using electronmicrographs the marginal hook sickle proper is noted as having a thick rounded heel and a thinner toe. It is believed that the end at which the marginal hook shaft joins the marginal sickle terminates in a bi-pronged-like structure, such that the sickle locates itself between the two prongs, and the anterior edge of the thicker heel follows closely the curve of the prong (Figure 6.13(L)). This form of articulation allows for the rotation of the marginal sickle within a single plane. The extent of rotation is dictated by the sickle filament loop attached to the sickle which in turn is joined to muscles within the opisthaptor. Although the precise connection of the sickle filament loop with the sickle

is unknown, it is not a rigid joint but has been observed at various points along the length of the sickle blade. Two grooves are observed running down each side of the sickle blade (Figure 6.13(L)) and it may be that a peg on the inner surface of the filament loop locates with the groove such that it acts as a runner. The shaft of the marginal hook anchored in the body of the opisthaptor has its sickle free to be guided by the action of the muscles pulling on the sickle filament loop through its aperture in the opisthaptor. It is postulated that, in addition to the pegs, the anterior portion of the sickle filament loop terminates in a sheath through which the sickle blade passes. Examination of the filament loop under high magnification with the electron microscope (x30,000+) is inconclusive, although it does suggest that some specialised structure exists. Silver nitrate staining of fresh worms (Chapter 12) shows that the ring through which the marginal sickle point emerges from the opisthaptor is argentophilic. It is not known if this ring represents the anterior portion of the sickle filament loop and is joined to the tegument. Extrusion of the marginal hook sickle point would then be effected by a combination of the muscles pulling on the sickle filament loop and the end of the marginal shaft. The aperture rings through which the points of the hamulus emerge are only recovered using the sonication technique (Figure 6.2(A) and 6.5(D)). The sickle filament loop is also only retained in sonicated preparations; therefore, it may be reasonable to suggest that the anterior portion of the filament loop is analogous to the aperture rings of the hamulus.

The enzymatic digestions tested allowed both fresh, frozen and alcohol-fixed material to be examined, but some sclerites, such as the dorsal and ventral bars, were lost by this technique. The sonication of fresh and frozen material retained these accessory bars, including the ventral bar membrane, the sickle filament loop and the membrane of the marginal hooks and even ligaments associated with the sclerites. This technique allowed for the distinction of subtle differences in hook shape, which will be studied further. Such differences might contribute to the search for distinguishing characters between gyrodactylid species.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Figures 6.2-6.5.

Figure 6.2(A): *Gyrodactylus gasterostei* Gläser, 1974. Arrangement of the central sclerites following sonication, the hamuli joined by the two accessory bars. One of the rings through which the hamulus protrudes from the haptor is also visible.

Figure 6.3(B): *G. gasterostei.* Higher magnification to show the alignment of the two accessory bars to their respective points of attachment on the hamulus.

Figure 6.4(C): *G. arcuatus* Bychowsky, 1933. Dorsal view demonstrating the alignment of the ventral bar processes (p) and positioning of the extremities of the ventral bar proper (e) to coincide with the ventral bar attachment points seen as flattened surfaces (vap) on the hamuli.

Figure 6.5(D): *Gyrodactylus* Morph 1. Sonicated hook set with the ventral bar displaced at 90° revealing the ventral bar attachment points (vap). The muscle caps of the hamuli can be seen, joined by a ligament (l) attached to the muscle cap surfaces of the hamuli. The hamulus aperture rings (har) are clearly visible. Scale bars: 5µm.

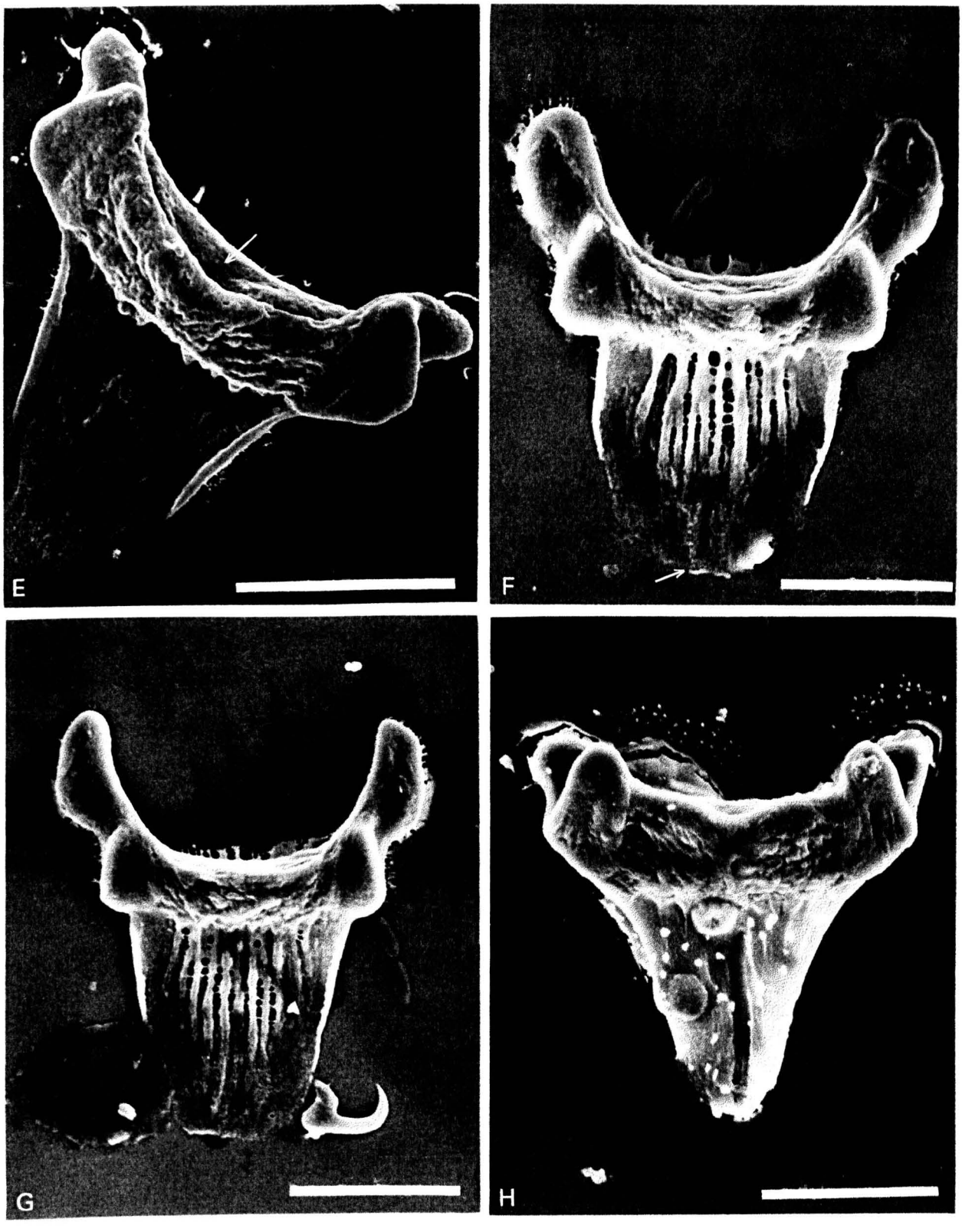
Figures 6.6-6.9.

Figure 6.6(E): Sonicated ventral bar of *G. gasterostei* Gläser, 1974. The thickened lateral borders of the ventral bar membrane can be seen. The depression running between the ventral bar processes is arrowed.

Figure 6.7(F): Sonicated ventral bar from *G. arcuatus* Bychowsky, 1933 from *Gasterosteus aculeatus.* The mid-portion of the bar proper has an irregular surface compared to the ventral bar processes and the ends of the ventral bar proper. Thickened ramifications extend posteriorly over the membrane. Between the ridges perforations of the membrane are seen. The terminal edge of the membrane is arrowed.

Figure 6.8(G): Sonicated ventral bar of *G. arcuatus* Bychowsky, 1933 showing variation in shape, notably of the membrane compared to Figure 6.7. At the right posterior end of the ventral bar membrane a marginal hook sickle is seen.

Figure 6.9(H): Sonicated ventral bar of *Gyrodactylus* Morph 1 from *O. mykiss*, the ventral bar membrane has a medial ridge. Scale bars: 5μm.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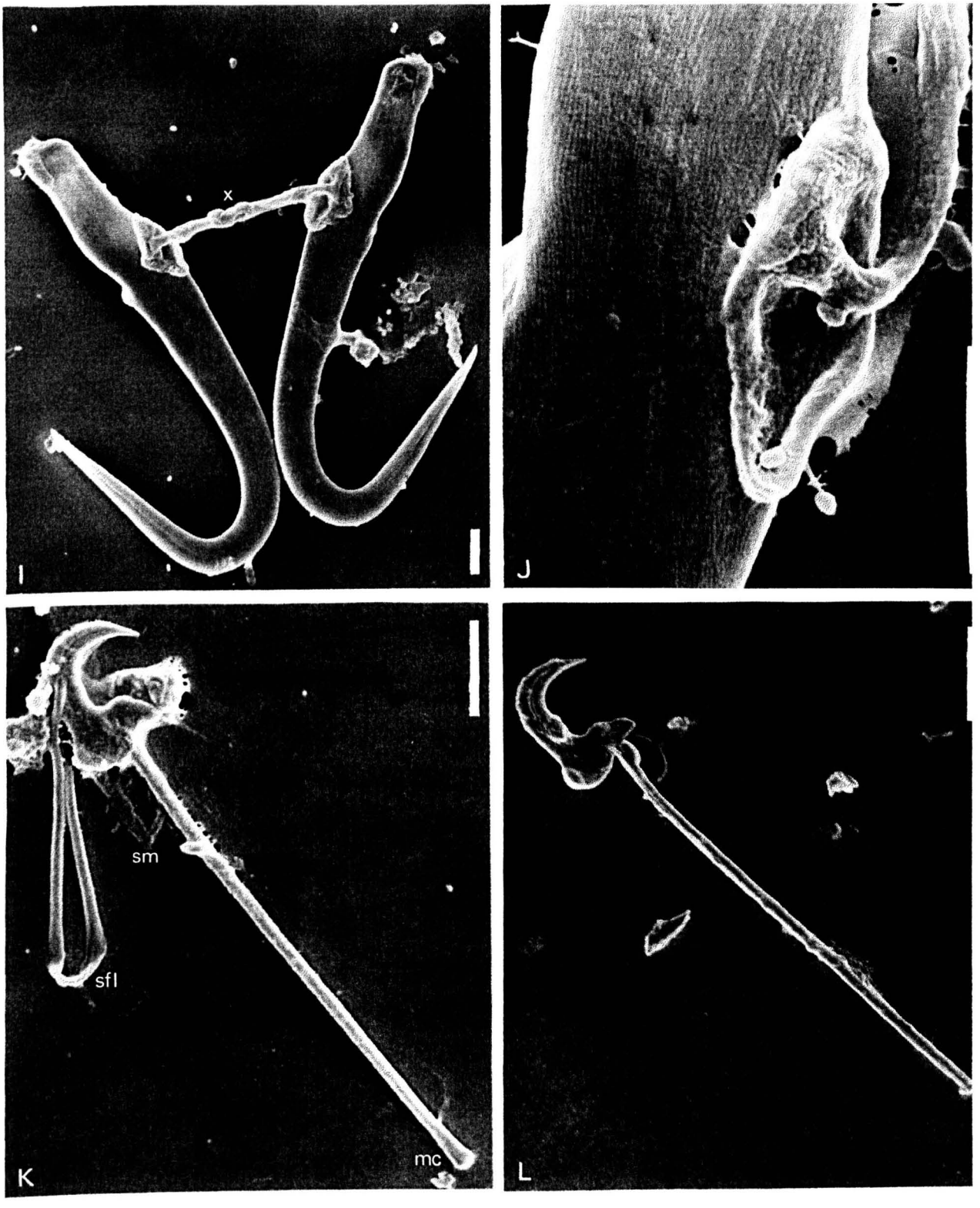
Figures 6.10-6.13.

Figure 6.10(I): *Gyrodactylus gasterostei* Gläser, 1974. Sonicated specimen with the dorsal bar (db) retained and its central portion appearing to be an irregular deposition of keratin-like protein. The processes arising on the dorsal bar are marked by an "x".

Figure 6.11(J): *G. gasterostei.* Dorsal bar attachment cap on the hamulus retained by the sonication technique. The origin and structure of the dorsal bar proper is clearly seen.

Figure 6.12(K): *Gyrodactylus* sp. from *Salvelinus alpinus.* A sonicated marginal hook with the three additional structures otherwise lost in conventional digestion techniques: the sickle filament loop (sfl), the sickle membrane (sm) and the muscle cap on the hook shaft (mc).

Figure 6.13(L): *Gyrodactylus* sp. from *Salvelinus alpinus.* Marginal hook recovered following digestion showing the absence of its associated structures. Scale bars: 5μm.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CHAPTER 7: THE DISCRIMINATION OF *GYRODACTYLUS SALARIS* FROM SPECIES OF *GYRODACTYLUS* PARASITISING BRITISH SALMONIDAE USING NOVEL MORPHOMETRIC PARAMETERS: A SCANNING ELECTRON MICROSCOPY STUDY.

Introduction

The results of morphometric studies using light microscopy and multivariate analyses to discriminate species of the genus *Gyrodactylus* suggested the presence of seven morphotypes parasitising UK salmonids (Chapters 2 & 3). Two of these were found on brown trout *Salmo trutta*. One form, Morph 4, was believed to be *G. truttae* Gläser, 1974, and was found throughout the United Kingdom. The second brown trout form, Morph 5, was believed to be a *G. truttae* variant, and was found in just a single locality. Although the latter form closely resembled Morph 4, it had long, thin marginal hooks. Similarly, three morphotypes were found on Atlantic salmon *Salmo salar*. *Gyrodactylus* Morph 1, believed to be *G. derjavini* Mikailov, 1975, occurred on *Salmo salar* (English and Welsh), *Salmo trutta* and on *Oncorhynchus mykiss*. The second salmon morphotype, Morph 3, a *G. derjavini* variant, was found at just two localities and was the largest of all the forms found, having hamuli exceeding 75µm in size compared to 60-65µm for all other salmon forms. The third salmon morphotype, Morph 2, found on Scottish and Northern Irish salmon, appears to represent a hitherto undescribed species which, although resembling *G. derjavini*, differs in the shape of the marginal hook. The last two morphotypes, both occurred on *Salvelinus alpinus*: Morph 6 was an as yet undetermined form and Morph 7 was close to *G. derjavini*.

The existing system of biosystematics for the genus *Gyrodactylus* relies upon the determination of subtle differences in the marginal hook morphology and the comparison of measurement ranges for each morphometric variable. However, an examination of the literature shows that for closely related species, there are no discrete ranges on which species discrimination might easily be based (Malmberg, 1987; Bakke *et al.*, 1991). A new system based on new variables is, therefore, clearly required. The liberation of the opisthaptoral sclerites by digestion (Mo & Appleby,

1990) provided the opportunity to study some of these structures at very high magnification under the scanning electron microscope (SEM). This technique was refined and improved by using sonication to the extent that even some of the more delicate structures such as the ventral bar were also available for study (Chapter 6).

However, whereas previously (Chapter 3-5) the basis of discrimination within the PCA model used morphological variables from all four forms of sclerite (hamulus, marginal hook, ventral bar and dorsal bar) analysed simultaneously, an attempt was made to discriminate species using information gathered from just one of these sclerites. For example, the use of the hamulus or marginal hook only could considerably simplify and improve the analysis. Although the sonication and/or digestion technique allows closer examination of these structures, they do have the limitation that only single structures can be analysed, since the technique uses pooled samples and there is no means of cross-referencing marginal hooks to hamuli from the same specimen.

A simplified method able to discriminate *G. salaris* from the other species would considerably assist the legislation in the UK, which requires that the occurrence of this parasite is notified as a result of the problems associated with *G. salaris* Malmberg, 1957 in Scandinavia in relation to both farmed and wild populations of salmonids since the early 1970s.

The aim of this study, therefore, was to define morphometric parameters which would discriminate this particular species of *Gyrodactylus* from all other salmonid gyrodactylids studied in Northern Europe.

Materials and methods

Sites known to harbour large numbers of *Gyrodactylus* on the four salmonid hosts, *Salmo salar*, *Salmo trutta*, *Oncorhynchus mykiss* and *Salvelinus alpinus*, together with those sites identified in Chapter 3 as harbouring unique morphotypes, were sampled (Table 7.1). Juvenile fish (0+ and 1+ year classes) were sampled since these were found to be more predisposed to *Gyrodactylus* infection. In addition, representative material of *G. derjavini* from Swedish brown trout, *G. salaris* from Swedish Atlantic salmon and *G. colemanensis* from *Salvelinus fontinalis* from Canada was collected in

80% alcohol for use in the comparative study. The fresh material was then sonicated and the alcohol-fixed material was digested using proteolytic enzymes. The techniques are described in Chapter 6.

Released sclerites were photographed using an Hitachi S800 field emission scanning electron microscope. Photomicrographs were obtained for 10 hamuli and 10 marginal hooks for each locality randomly selected from a pool of 30-40 gyrodactylids. Fourteen characters were measured, seven for the hamulus and seven for the marginal hook (see Chapter 2, Figure 2.2). These measurements include those which were shown to be reliable criteria (Chapter 3), i.e. hamulus point length, hamulus shaft length, hamulus total length, marginal hook shaft length and marginal hook total length. The use of the SEM allowed parameters, such as the marginal hook sickle length and the distal and proximal widths of the marginal hook sickle, to be measured to a great degree of accuracy, i.e. 0.01 μm compared to 0.5 μm on material prepared for the light microscope. In addition, the enlargement and clarity of these electronmicrographs permitted the use of new parameters in the analysis: these include the hamulus shaft to point distance, the hamulus angle, the hamulus shaft width, the marginal hook sickle toe length and the point to marginal hook shaft distance. These morphometric variables were measured using an image analysis system (IMCO 10 Intel 80386 / 80387 math coprocessor) linked to a digitizing tablet and dual EGA monitors (Kontron Electronics Ltd) and using the Videoplan morphometrics software package (Kontron). The origin of each measurement is defined as follows:

Hamulus characters

(A) Hamulus shaft to point distance (CB) (see Chapter 2, Figure 2.2 for abbreviations or Appendix 2). The interface between the shaft and root portions of the hamulus is indicated by the base of the dorsal bar attachment cap (A). If this point is connected to the hamulus point (B), then the shaft-point distance is given as the distance between B and the point (C) where the line AB touches the inner curve of the hamulus.

(B) Hamulus angle (CDB). A series of lines are dropped to the inner curve of the hamulus from the two points of origin (C) and (B). The hamulus angle (CDB) is given as the smallest recorded angle from a number (c. 7) of replicate measurements (CDB).

(C) Hamulus point (BE). The classical measurement of this character requires a point

Table 7.1: *Gyrodactylus* populations sampled, the hosts and location they were collected from.

Host	Locality	Population code number	
		Hamulus	Marginal
Oncorhynchus mykiss	Loch Butterstone, Scotland	1	1
Salmo salar	River Nith, Scotland	2	2
Salmo trutta	River Neath, Wales	3	3
Salvelinus alpinus	Lake Ennerdale, England	4	4
S. fontinalis (G. colemanensis)	Nova Scotia, Canada	5	5
S. salar (G. salaris)	River Beukaforsen, Sweden	6	6
S. trutta (G. derjavini)	River Dalälven, Sweden	7	7
Salmo trutta	River Manor, Scotland	8	8
Oncorhynchus mykiss	River South Esk, Scotland	9	9
Salmo salar	Mags Yr Afon, Wales	10	10
Salmo salar	River Snizort, Skye, Scotland	11	11
Oncorhynchus mykiss	Loch Awe, Scotland	12	12
S. salar (G. salaris)	River Ätran, Sweden	13	13
S. salar (G. salaris)	River Lier, Norway	14	15
Salmo salar	River Allan, Scotland	15	16
Salmo trutta	Loch Airthrey, Scotland	16	14

to be chosen on the hamulus curve, marking the interface between the point and shaft portions of the hamulus. In this account, it is given as a perpendicular line dropped from the line (CB) through the apex of the hamulus angle (D) to a point on the outer edge of the hamulus curve, given as point (E). The hamulus point is, therefore, given as the distance (BE).

(D) Hamulus shaft (AE). This is given by the distance (AE) connecting the base of the dorsal bar attachment point to the hamulus point-shaft interface at point (E).

(E) Total hamulus length (FE). This is given as the most proximal point on the hamulus root (F) to the hamulus shaft-point interface point (E).

(F) Hamulus root (AF). The measurement of this character is less precise, as it requires an objective decision as to the most proximal point on the hamulus (F). This region shows a high degree of variability, being a zone of elongation it strongly influences the total hamulus length, which is a key discriminative character.

(G) Hamulus width (AG). A perpendicular line dropped from the line FE through the hamulus shaft-root interface point (A), such that the hamulus width is the distance along this axis from point (A) to the inside of the hamulus curve.

Marginal hook characters

The following characters are in accordance with the definitions of Malmberg (1970) and Harris (1983) and are shown in Chapter 2 (Figure 2.2): marginal hook total length (G); marginal hook shaft length (F); marginal hook sickle length (D); and marginal hook sickle proximal (B) and distal (C) widths. Two characters are defined below.

Marginal hook toe (A). This is given as the proximal portion of the marginal hook sickle that extends from the junction with the marginal hook shaft at a right angle from the shaft parallel to the direction of the marginal hook sickle point.

Marginal hook sickle aperture distance (E). This is the distance from the sickle point to the junction between the sickle and the marginal hook shaft.

Results

Since the data did not conform to a pattern of normal distribution, it was log transformed; the cosine of the hamulus angle was taken in order to convert this to a

linear function. The data for these characters were firstly compared using the mean with 95% confidence limits in order to ascertain whether any of these new measurements were able to discriminate *G. salaris*. The results for the hamulus are given in Figures 7.1a-i and for the marginal hook in Figures 7.4a-h. The measurements were then imported into a multivariate analysis package (Systat®, version 5.3, 1991) and a principal components analysis (PCA) performed in an attempt to discriminate *G. salaris* from native British forms using the hamulus and marginal hook variables separately. The area of the hamulus shaft and point, hamulus root and the marginal hook sickle, although not included within the multivariate analysis were likewise investigated. The results of each structure will now be considered separately.

The hamulus

A total of seven characters, shaft-point distance, hamulus angle, point length, shaft length, total length, root length and hamulus width on 140 specimens were subjected to the analysis. The correlation matrix (Table 7.2) for the hamulus characters shows that the angle varies independently of the shaft with some indication of it having an inverse relationship with the shaft-point length. Table 7.3 gives the component loadings for the hamuli variables and Table 7.4 indicates the variance explained by the components and the percentage of the total variance explained for the first three components. The first three factors in Table 7.4 account for 93.76% of the total variance explained and the first two factors for 89% of the variance. Figure 7.2 shows the relationship of each of the measured variables (PCA coefficients) as factor plots for the first three factors. A factor plot explains how each of the measured variables behave for the first three principal components. A variable with a high component loading (i.e. near to 1) or low component loading (i.e. near to -1) indicates that variable is having a strong influence on the separation of the specimens within the analysis. Variables with component loadings that are close to zero would indicate that the variable is contributing less or little to the separation of the specimens within that principal component. Table 7.3 shows that the total hamulus length is the key variable along the first principal component, the hamulus angle along the second and the hamulus width along the third principal component.

The scores from the principal components analysis were then imported into a

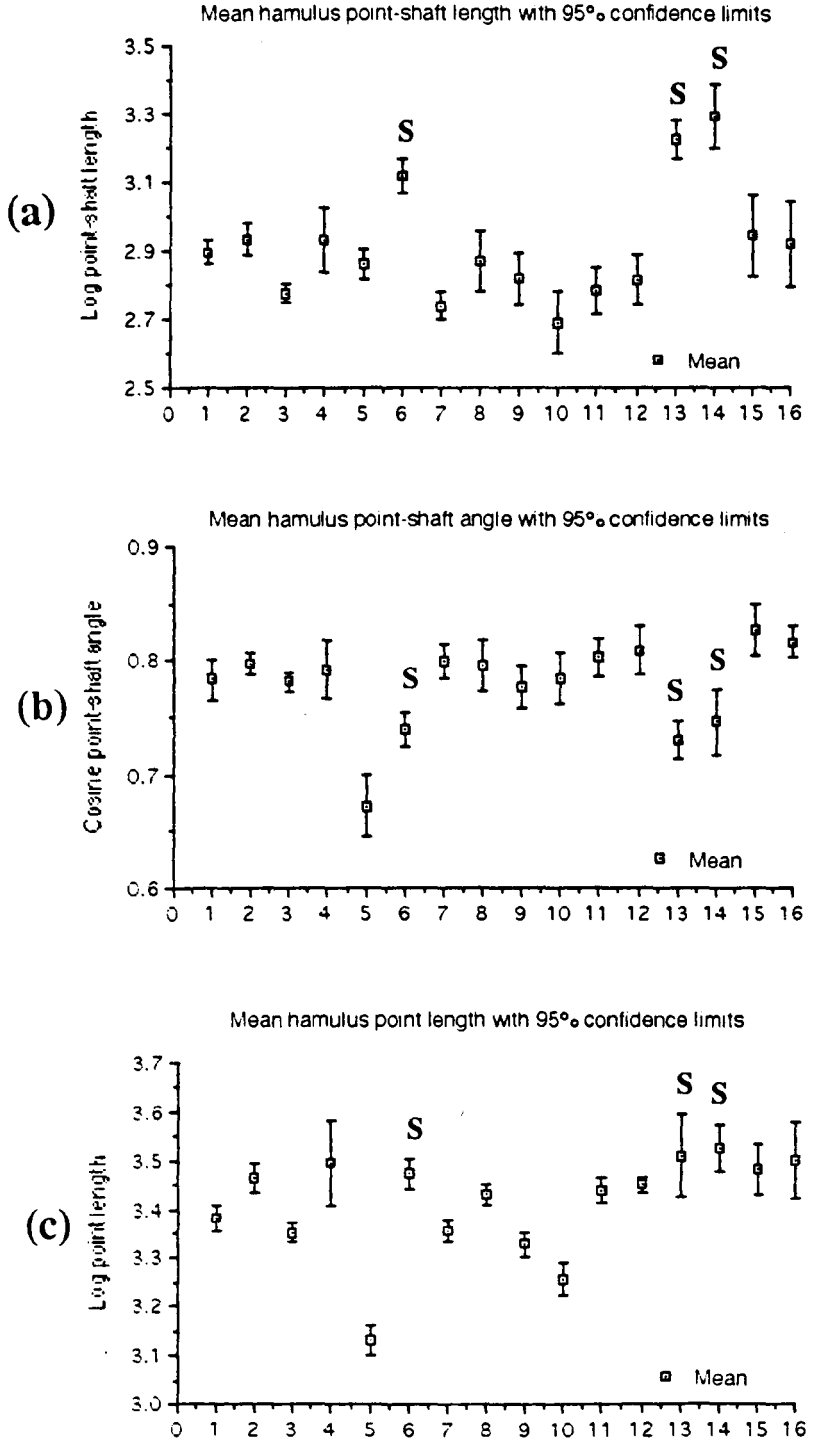
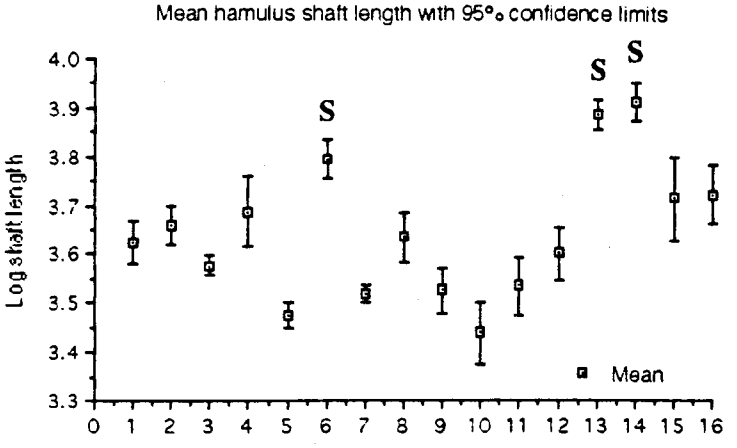
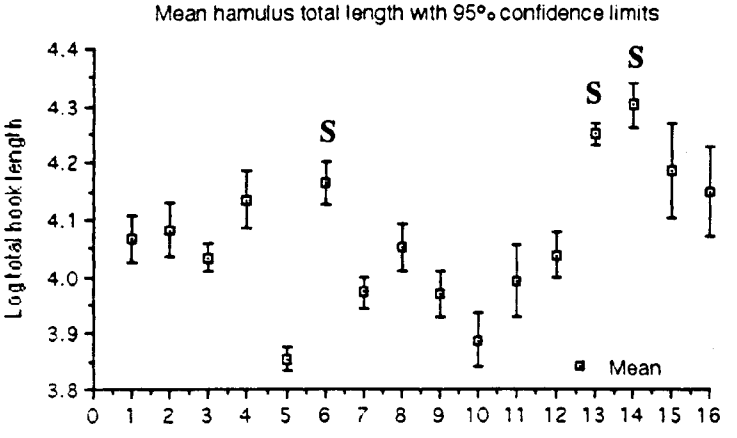


Figure 7.1a-i: Histograms of the hamulus character variables for several species of *Gyrodactylus*. The mean with 95% confidence limits are given along the y-axis, whilst the x-axis relates to the *Gyrodactylus* population sampled as given in Table 7.1. The *G. salaris* populations are highlighted by (S).

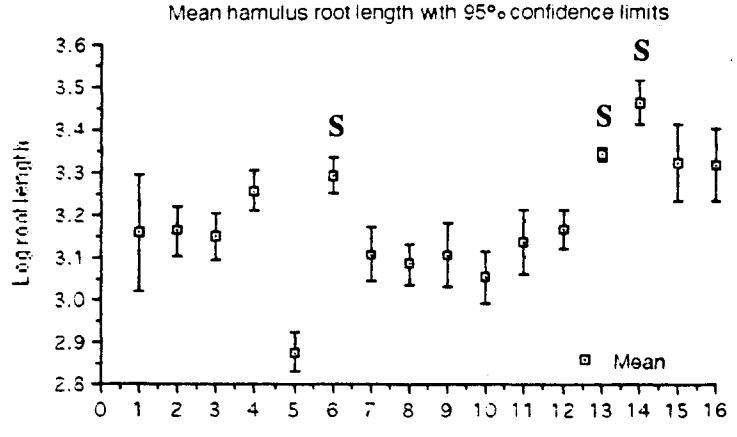
(d)


Mean hamulus shaft length with 95% confidence limits
Log shaft length
4.0
3.9
3.8
3.7
3.6
3.5
3.4
3.3
S
S
S
Mean
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

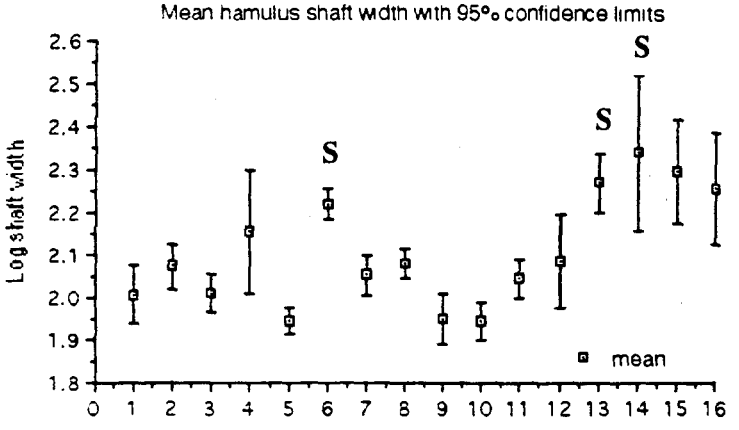
(e)


Mean hamulus total length with 95% confidence limits
Log total hook length
4.4
4.3
4.2
4.1
4.0
3.9
3.8
S
S
S
Mean
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

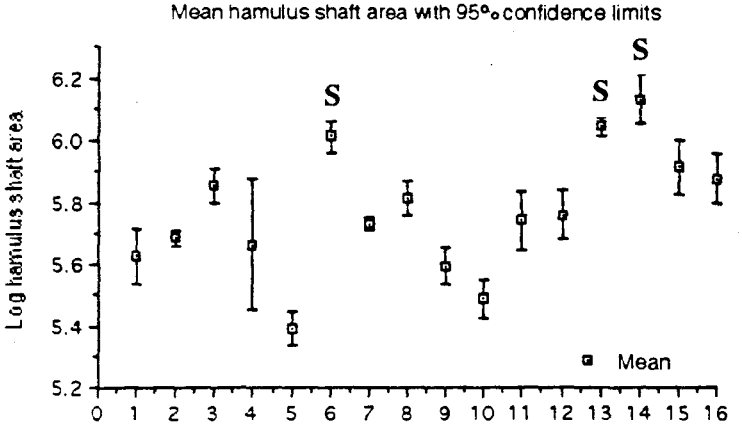
(f)


Mean hamulus root length with 95% confidence limits
Log root length
3.6
3.5
3.4
3.3
3.2
3.1
3.0
2.9
2.8
S
S
S
Mean
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

(g)


Mean hamulus shaft width with 95% confidence limits
Log shaft width
2.6
2.5
2.4
2.3
2.2
2.1
2.0
1.9
1.8
S
S
S
mean
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

(h)


Mean hamulus shaft area with 95% confidence limits
Log hamulus shaft area
6.2
6.0
5.8
5.6
5.4
5.2
S
S
S
Mean
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

(i)

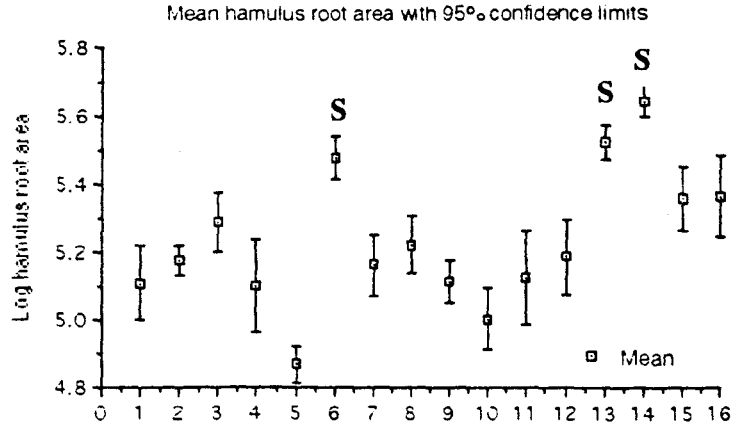

Mean hamulus root area with 95% confidence limits
Log hamulus root area
5.8
5.6
5.4
5.2
5.0
4.8
S
S
S
Mean
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Table 7.2: Correlation matrix for the hamulus variables.

Variable	shft-pt	angle	point	shaft	total	root	width
Shaft-point length	1.000						
Hamulus angle	-0.399	1.000					
Point length	0.492	0.477	1.000				
Shaft length	0.886	-0.033	0.745	1.000			
Total length	0.786	0.130	0.834	0.934	1.000		
Root length	0.605	0.239	0.751	0.747	0.879	1.000	
Hamulus width	0.593	0.154	0.685	0.769	0.769	0.699	1.000

Table 7.3: The component loadings for the hamuli variables for the first three principal components.

Variable	1	2	3
Shaft-point distance	0.802	-0.556	0.099
Hamulus angle	0.140	0.972	0.025
Point length	0.859	0.387	0.114
Shaft length	0.950	-0.199	0.024
Total length	0.979	-0.009	0.113
Root length	0.884	0.145	0.148
Hamulus width	0.848	0.055	-0.525

Table 7.4: Variance explained by the components and the percentage of total variance explained for the first three principal components for the *Gyrodactylus* hamuli specimens.

	variance explained by components	% of total variance	cumulative percentage
Factor 1	4.76	68.01	68.01
Factor 2	1.47	20.97	88.98
Factor 3	0.33	4.78	93.76

Table 7.5: Summary statistics for the cluster analysis performed on the PCA for the *Gyrodactylus* hamuli specimens.

Variable	Between SS	DF	Within SS	DF	F-Ratio	Prob
Factor (1)	102.076	4	36.924	135	93.302	0.00
Factor (2)	109.062	4	29.938	135	122.948	0.00
Factor (3)	91.077	4	47.923	135	64.142	0.00

	Variable	Min	Mean	Max	No. of Specimens
Cluster 1	Factor (1)	-1.86	-1.66	-1.48	10
	Factor (2)	-3.03	-2.33	-1.64	
	Factor (3)	-1.58	-1.01	-0.25	
Cluster 2	Factor (1)	-1.11	-0.00	1.29	47
	Factor (2)	-1.08	0.14	1.79	
	Factor (3)	0.22	0.94	2.69	
Cluster 3	Factor (1)	0.76	1.48	2.21	19
	Factor (2)	-2.21	-1.22	-0.44	
	Factor (3)	-1.59	0.14	1.59	
Cluster 4	Factor (1)	-1.76	-0.53	0.40	51
	Factor (2)	-0.36	0.57	1.43	
	Factor (3)	-2.20	-0.31	0.39	
Cluster 5	Factor (1)	0.31	1.21	2.74	13
	Factor (2)	-0.58	0.84	1.59	
	Factor (3)	-3.16	-1.61	-0.34	

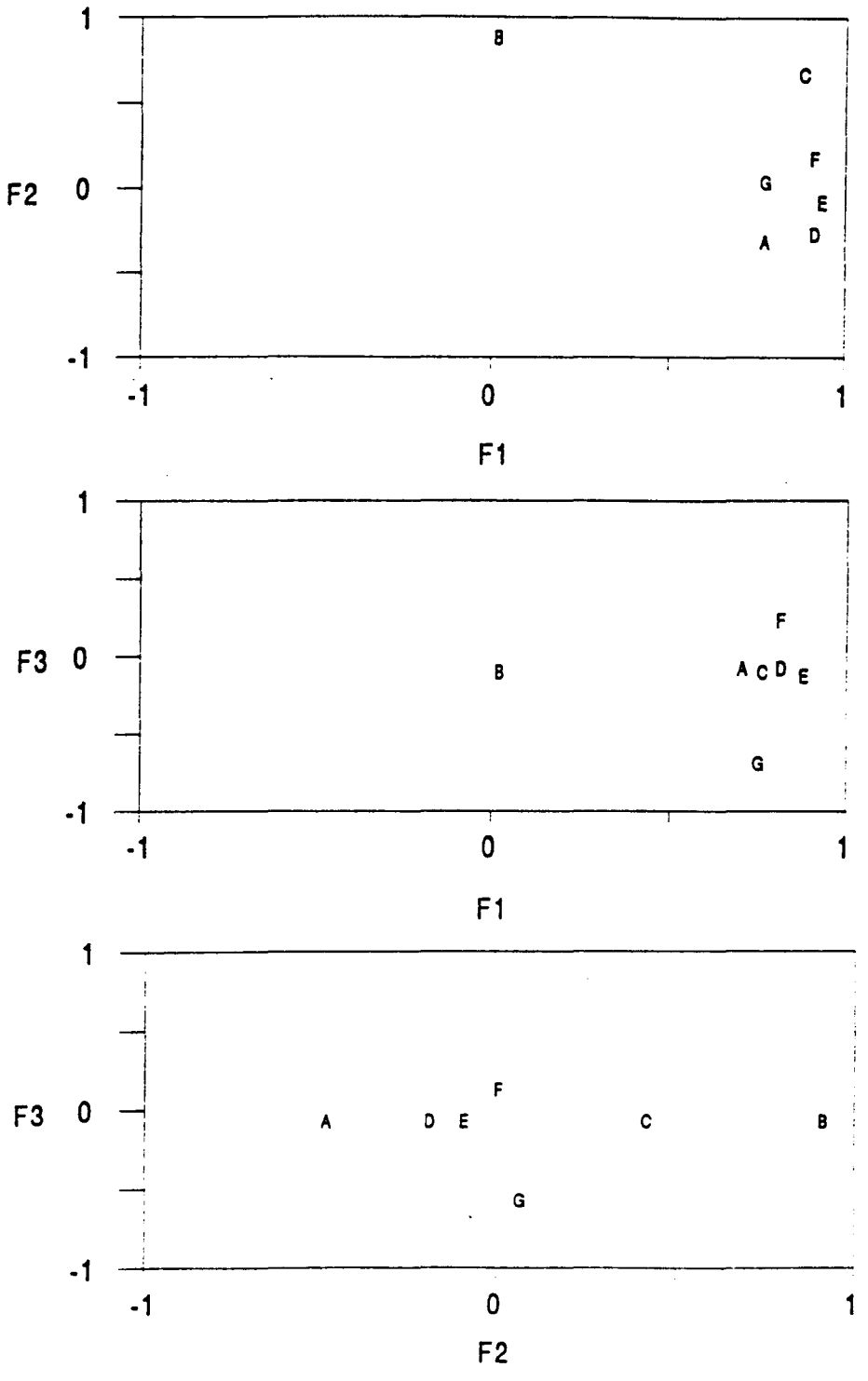


Figure 7.2: Factor plot of the hamulus variables for the first three principal components. Abbreviations: A = shaft-point length, B = hamulus angle, C = point length, D = shaft length, E = total length, F = root length and G = hamulus width.

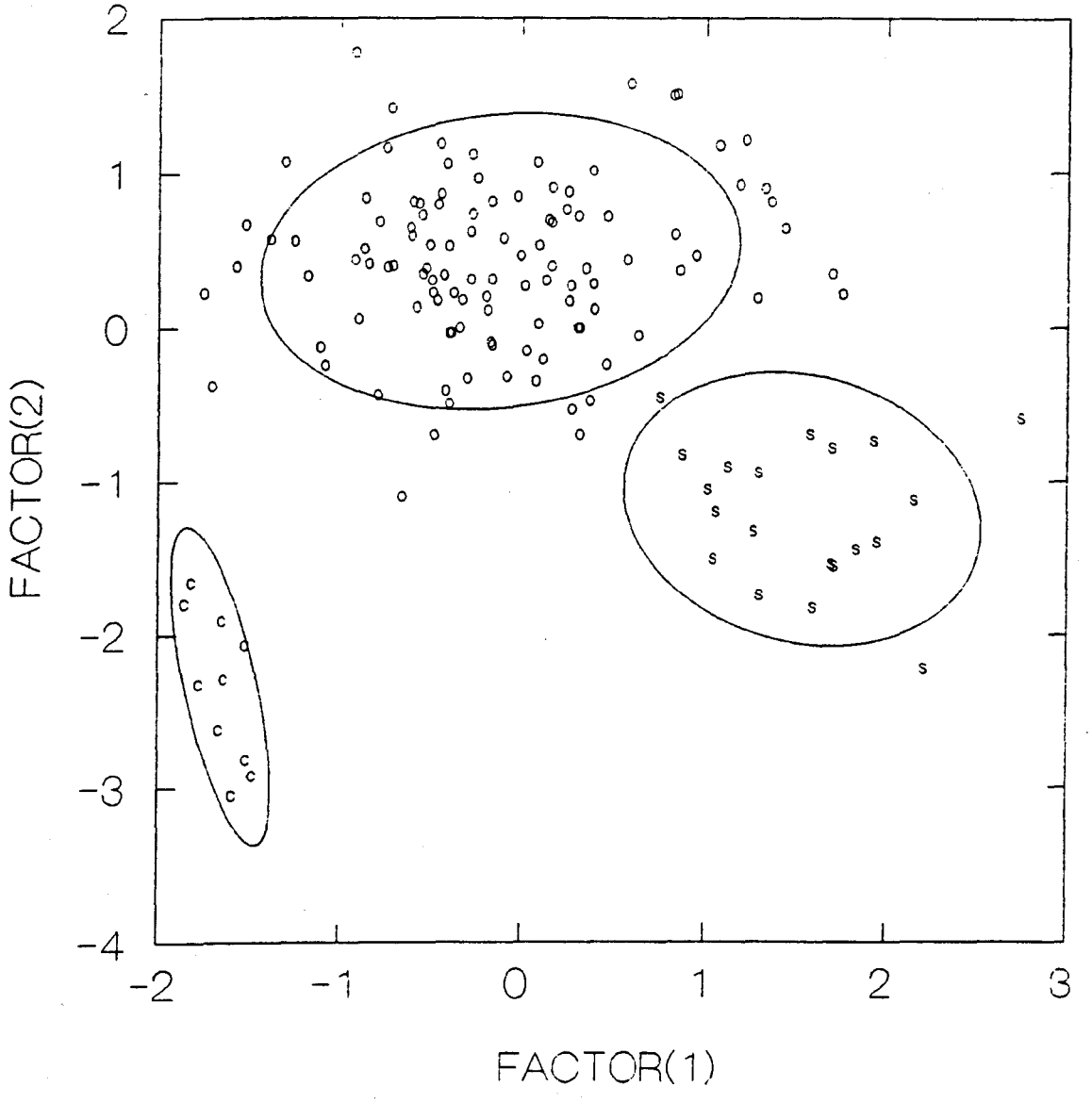


Figure 7.3: PCA plot of the *Gyrodactylus* hamuli specimens. Abbreviations: S = *G. salaris*, C = *G. colemanensis* and O = *Gyrodactylus* species native to British salmonids. Ellipses incorporate 80% of the specimens.

cluster analysis in an attempt to detect natural groupings within the data. The summary statistics (Table 7.5) indicated five clusters within the data. The PCA plot (Figure 7.3) indicates the presence of three groups, *G. salaris*, *G. colemanensis* and the remaining *Gyrodactylus* specimens as compared to the six clusters obtained from the cluster analysis.

The marginal hook

Malmberg (1970) used differences in the morphology and morphometrics of the marginal hooks as a basis for the systematics of this genus. Although this analysis still retains the key morphometric variables used for the light microscope, the examination of these structures at a higher plane might permit the incorporation of additional variables and also reduce some of the noise surrounding the "salmonid" species of *Gyrodactylus* which, in part, results from the relatively crude measurements generated by the light microscope. Five of the original measurements were retained, namely the proximal and distal widths of the sickle, the length of the sickle, the shaft length and the total length, and two new measurements, the toe length and the sickle aperture distance, were incorporated. The histograms of the marginal hook character variables for the studied species of *Gyrodactylus* are shown in Figure 7.4a-h.

The correlation matrix for the variables of the marginal hook are shown in Table 7.6 and suggests that they vary in the same direction. Table 7.7 shows that the separation of the marginal hook specimens is based on the length of the sickle and its aperture size within the first principal component. The shaft length and the total length of the marginal hook were the variables that separated the specimens in the second principal component, whilst the length of the sickle toe was mainly responsible in the third principal component. Figure 7.5 shows the factor plot for the marginal hook variables.

Table 7.8 gives the variance explained by the components and the percentage of the total variance explained.

The relationship of the character variables are shown in the factor plots (Figure 7.5), and the PCA plot is shown in Figure 7.6. The scores from the principal components analysis were then analysed by cluster analysis with eight clusters indicated by the analysis. The summary statistics are shown in Table 7.9.

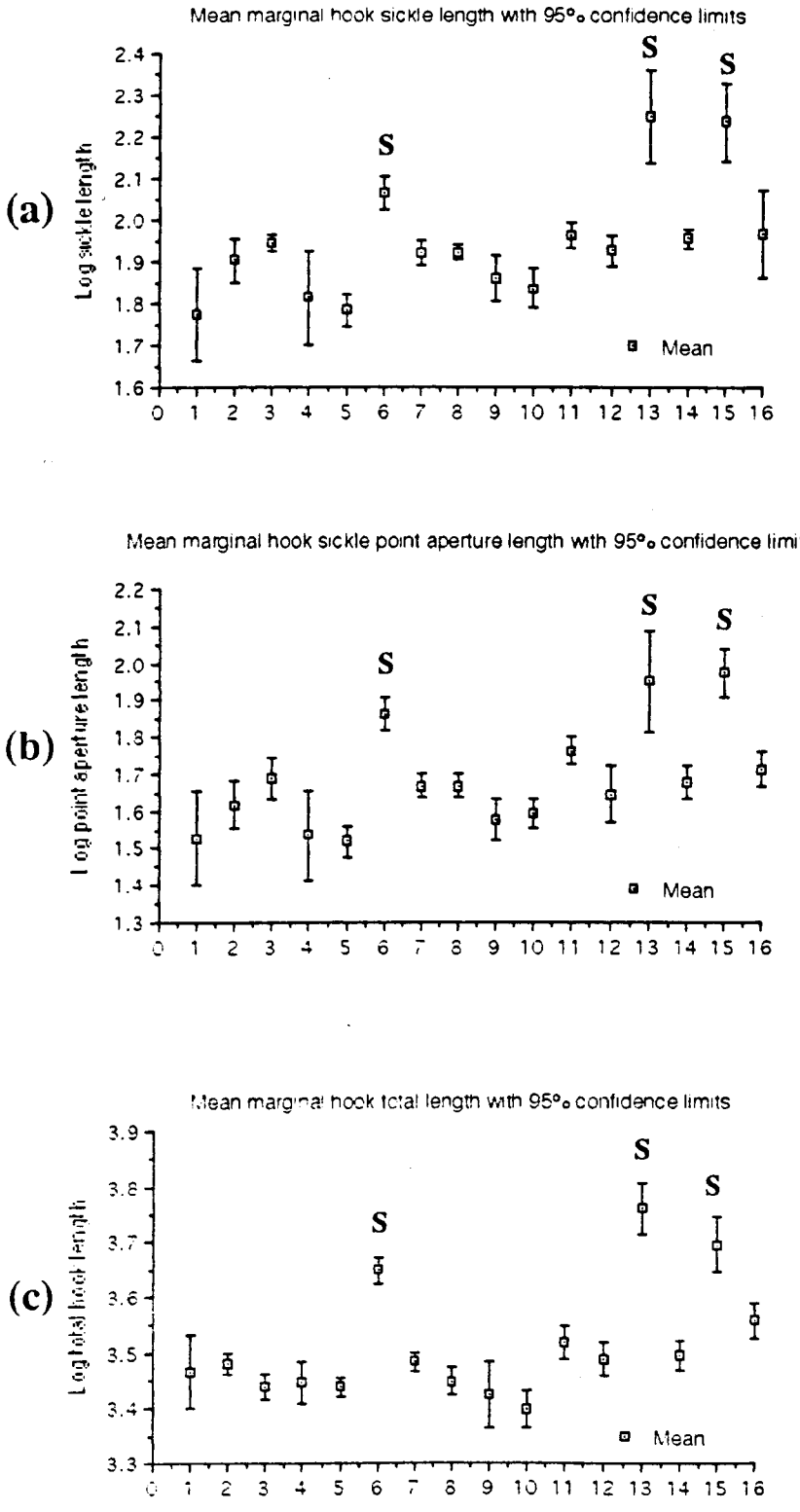
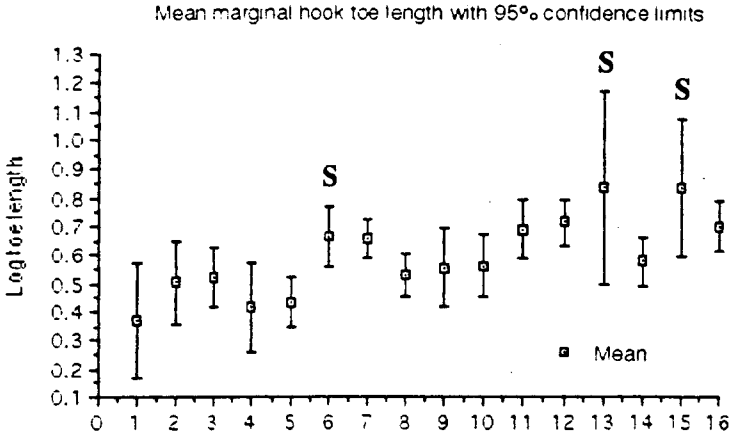
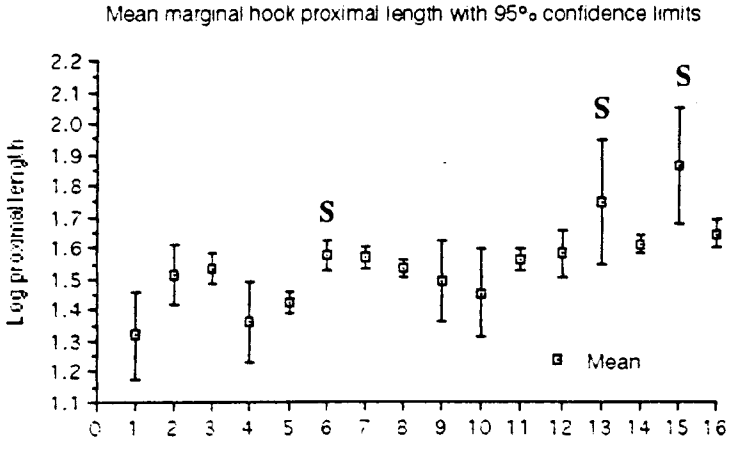


Figure 7.4a-h: Histograms of the marginal hook character variables for several species of *Gyrodactylus*. The mean with 95% confidence limits are given along the y-axis, whilst the x-axis relates to the *Gyrodactylus* population sampled as given in Table 7.1. The *G. salaris* populations are highlighted by (S).

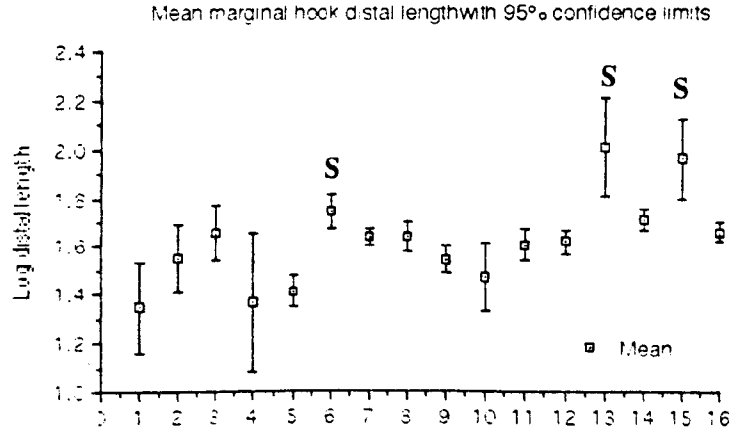
(d)


Mean marginal hook toe length with 95% confidence limits
Log toe length
S
S
S
Mean
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

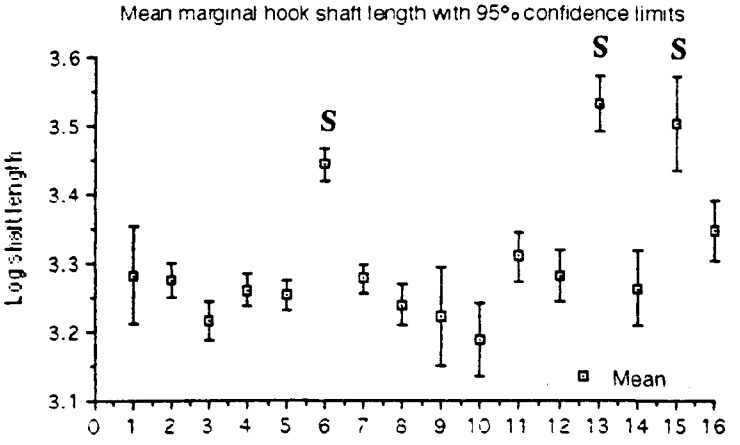
(e)


Mean marginal hook proximal length with 95% confidence limits
Log proximal length
S
S
S
Mean
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

(f)


Mean marginal hook distal lengthwith 95% confidence limits
Log distal length
S
S
S
Mean

(g)


Mean marginal hook shaft length with 95% confidence limits
Log shaft length
3.6
3.5
3.4
3.3
3.2
3.1
S
S
S
Mean
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

(h)

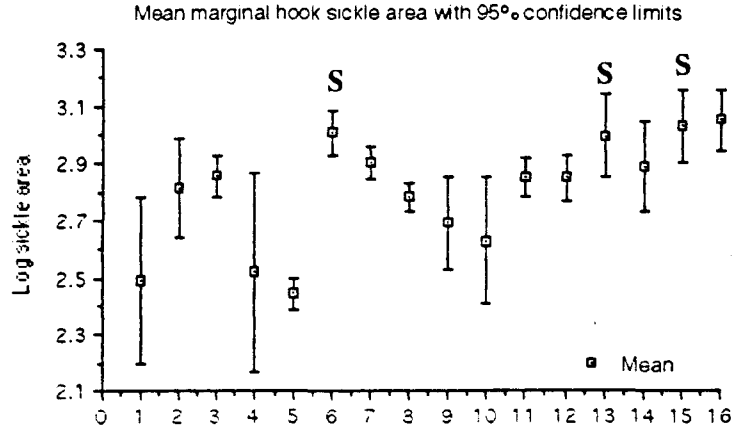

Mean marginal hook sickle area with 95% confidence limits
Log sickle area
3.3
3.1
2.9
2.7
2.5
2.3
2.1
S
S
S
Mean
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Table 7.6: Correlation matrix for the marginal hook variables.

	prox	toe	distal	sickle	apert.	total	shaft
1) proximal width	1.000						
2) toe length	0.823	1.000					
3) distal width	0.787	0.668	1.000				
4) sickle length	0.823	0.720	0.827	1.000			
5) aperture distance	0.705	0.676	0.804	0.905	1.000		
6) total length	0.629	0.556	0.663	0.816	0.768	1.000	
7) shaft length	0.539	0.488	0.578	0.715	0.675	0.962	1.000

Table 7.7: The component loadings for the marginal hook variables for the first three principal components.

Variable	1	2	3
1) Proximal width	0.869	0.356	0.146
2) Toe length	0.804	0.409	0.359
3) Distal width	0.875	0.193	-0.308
4) Sickle length	0.954	0.028	-0.133
5) Sickle aperture	0.910	-0.003	-0.268
6) Total length	0.883	-0.444	0.094
7) Shaft length	0.810	-0.544	0.174

Table 7.8: Variance explained by the components and the percentage of the total variance explained for the first three principal components for the *Gyrodactylus* marginal hook specimens.

	variance explained by components	% of total variance	cumulative percentage
FACTOR 1	5.34	76.27	76.27
FACTOR 2	0.83	11.79	88.06
FACTOR 3	0.37	5.34	93.40

Table 7.9: Summary statistics for the cluster analysis performed on the PCA for the *Gyrodactylus* marginal hook specimens.

Variable	Between SS	DF	Within SS	DF	D-Ratio	Prob
Factor (1)	125.443	7	24.557	143	104.356	0.00
Factor (2)	100.388	7	49.612	143	41.337	0.00
Factor (3)	123.025	7	26.975	143	93.171	0.00

	Variable	Min	Mean	Max	No. of Specimens
Cluster 1	Factor (1)	-0.96	0.03	0.65	43
	Factor (2)	-0.96	0.40	1.96	
	Factor (3)	0.32	0.86	1.97	
Cluster 2	Factor (1)	0.70	1.28	2.78	16
	Factor (2)	-2.69	-1.46	-0.45	
	Factor (3)	-1.70	-0.56	0.47	
Cluster 3	Factor (1)	2.63	2.83	3.17	3
	Factor (2)	-0.21	0.40	1.24	
	Factor (3)	0.38	0.78	1.30	
Cluster 4	Factor (1)	-0.78	-0.15	0.50	47
	Factor (2)	-0.48	0.43	1.76	
	Factor (3)	-0.97	-0.30	0.24	
Cluster 5	Factor (1)	-2.02	-1.39	-0.86	18
	Factor (2)	-2.60	-1.49	-0.23	
	Factor (3)	-0.06	0.67	1.31	
Cluster 6	Factor (1)	-1.92	-0.58	0.06	19
	Factor (2)	-0.46	0.66	1.40	
	Factor (3)	-2.67	-1.47	-0.72	
Cluster 7	Factor (1)	2.06	2.24	2.42	3
	Factor (2)	-1.32	-0.69	0.44	
	Factor (3)	-3.02	-2.10	-1.58	
Cluster 8	Factor (1)	2.87	3.00	3.13	2
	Factor (2)	0.07	0.42	0.77	
	Factor (3)	2.50	3.02	3.54	

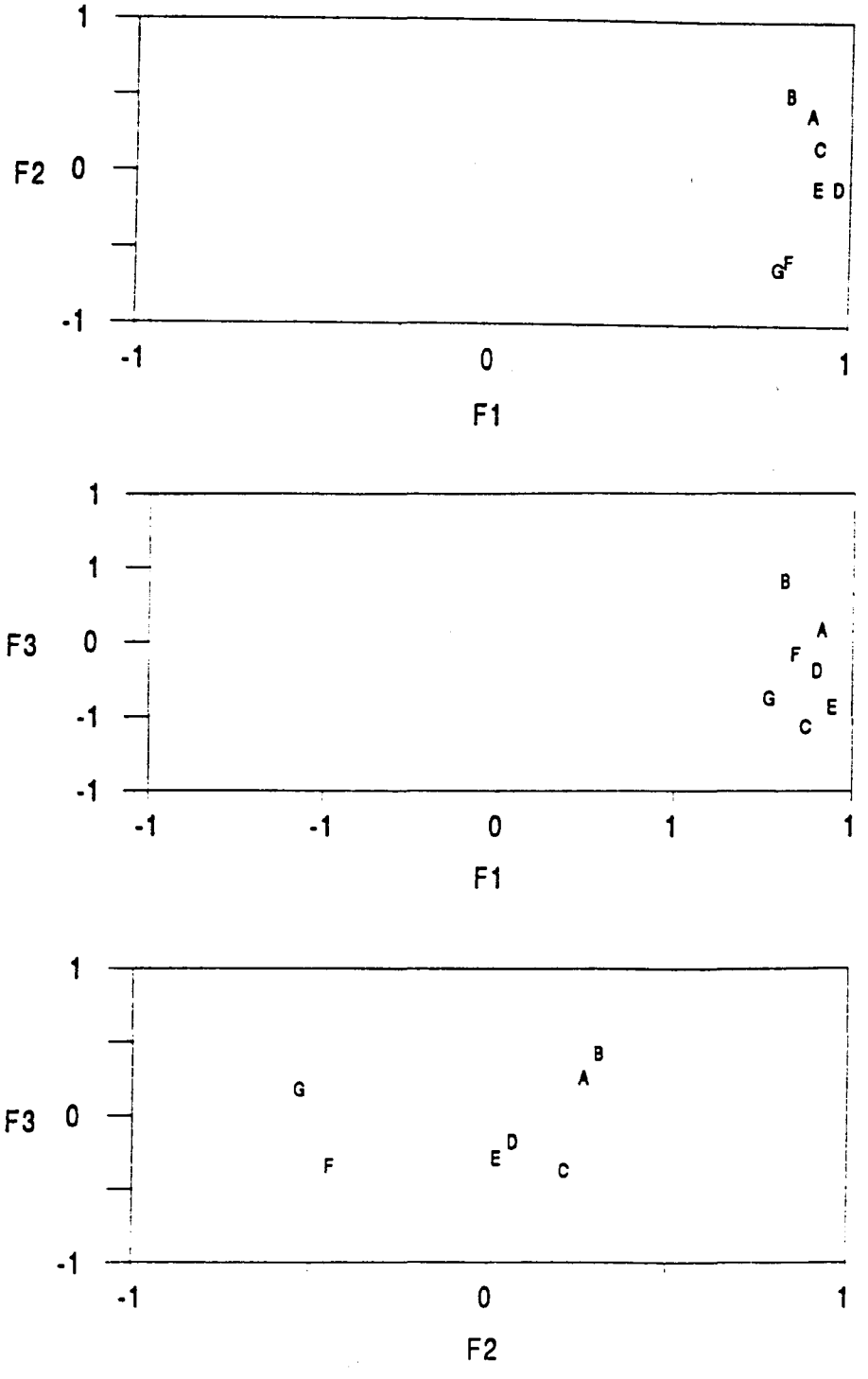


Figure 7.5: Factor plot of the marginal hook variables for the first three principal components. Abbreviations: A = proximal width, B = toe length, C = distal width, D = sickle length, E = sickle aperture, F = total length and G = shaft length.

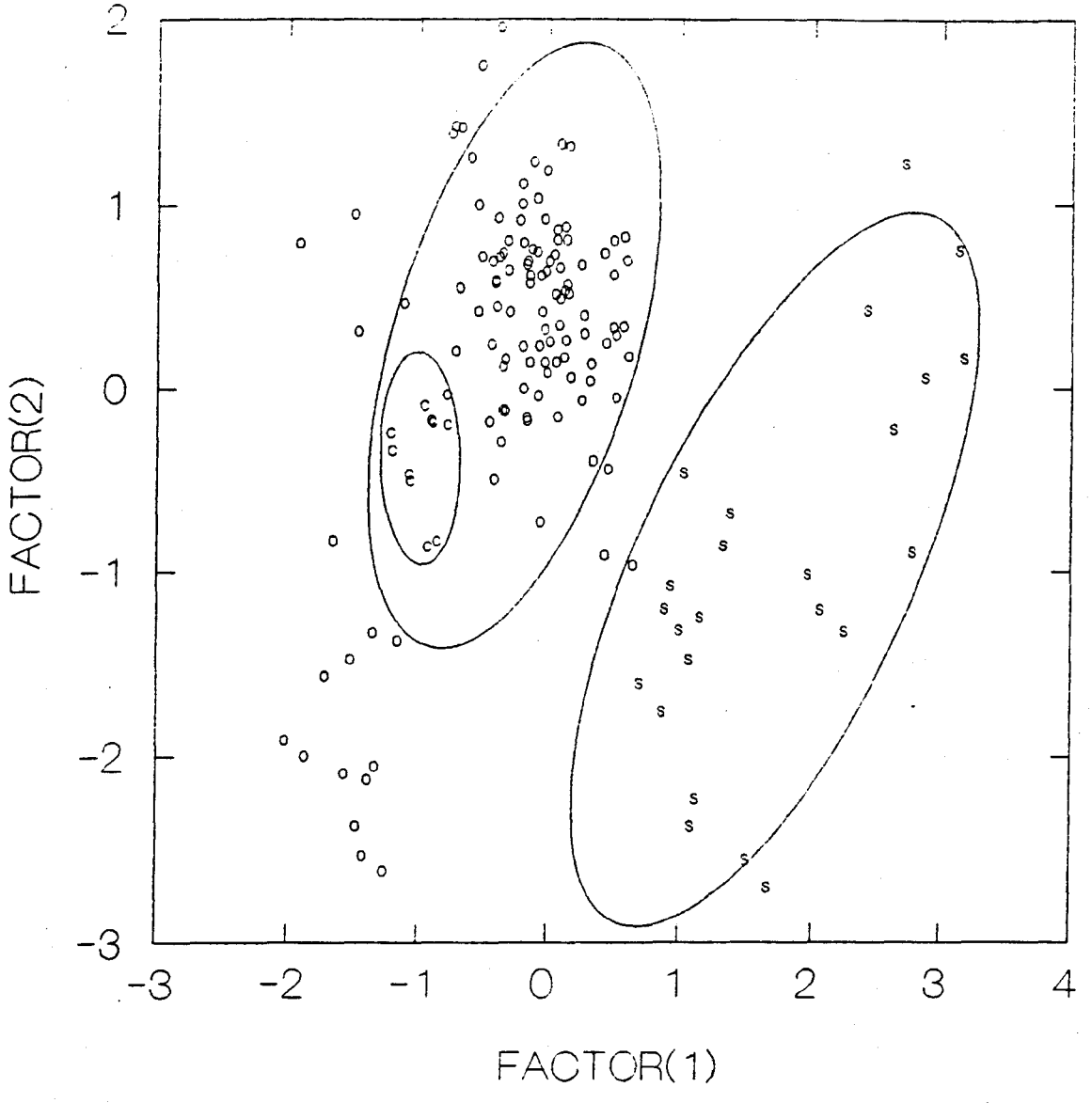


Figure 7.6: PCA plot of the *Gyrodactylus* marginal hook specimens. Abbreviations: S = *G. salaris*, C = *G. colemanensis* and O = *Gyrodactylus* species native to British salmonids. Ellipses incorporate 80% of the specimens.

Discussion

The hamulus

Comparison of the histograms of plots of the mean and 95% confidence limits for each of the characters reveals that certain species of "salmonid" *Gyrodactylus* can be discriminated. *G. salaris* is differentiated from other species studied here on the basis of the hamulus point-shaft distance (BC) and hamulus angle (CDB). Both of these parameters follow the description of the hamulus shape, providing two pieces of information describing a triangle encompassed within the area of the shaft and point (CDB) (Chapter 2, Figure 2.8). *G. colemanensis* from Nova Scotia is also readily distinguished on the basis of its hamulus angle. The value of the hamulus angle as a key discriminative variable is discussed elsewhere (Chapter 8). The overall size of *G. salaris* contributes to its determination, as indicated by the histograms for the shaft length and total length. The area of the shaft and root portions of the hamulus were also calculated separately due to the large range of variability exhibited by the hamulus root. Nevertheless, *G. salaris* can be discriminated on the basis of both portions for both the Swedish and Norwegian populations of this parasite. Conversely, *G. colemanensis* represents a species of smaller overall size, as reflected by the hamulus point length, hamulus total length and root length, and the size effect is similarly manifested in small areas being calculated from digitised hooks for both the shaft and root portions of the hamulus. The native species of *Gyrodactylus* cannot, however, be differentiated from each other by single morphometric variables, but it should be noted that Morph 1 (*G. derjavini*) parasitising Welsh salmon (Mags Yr Afon) appears to be consistently smaller for most of the variables in this study (Figure 7.1 site 10).

When the factor plots (Figure 7.2) and the component loadings are analysed, the first principal component suggests that all the variables increase together, except for the hamulus angle which, although increasing, does not do so to the same degree. In the second principal component, an increase in the angle is observed at the cost of a decrease in the shaft-point distance, whereas the third principal component indicates that, for slight increases in the length variables, there is a decrease in the hamulus width.

The cluster analysis identified five clusters within the PCA data. Of these, one cluster included all *G. colemanensis*, a second all *G. salaris* and the last three clusters a combination of the remaining species. One specimen of *G. salaris* (from the River Lierelva) was not separated to the same extent as the others and remained close to the native British forms of *Gyrodactylus* (Figure 7.3). This specimen, however, was well displaced from the remaining three clusters and lies closer to the bulk of *G. salaris* specimens than to the British specimens. The discrimination of *G. salaris* is, therefore, by the total hamulus length operating along the first principal component and the hamulus angle along the second principal component.

The marginal hook

The relatively large marginal hooks of *G. salaris* permit clear discrimination of this species from the other *Gyrodactylus* species studied. The classical characters, such as the sickle length, total length and the shaft length proved to be the most useful distinguishing features. The range of measurements for the proximal width of the marginal sickle had a large degree of overlap, whilst the distal width behaved more conservatively, discriminating *G. salaris* from the River Ätran (Sweden) and the River Lierelva (Norway) from the other species. Of the two new variables used, the sickle point aperture proved most valuable, again discriminating *G. salaris* with its open sickle point. The toe length proved, however, to be too variable and, therefore, in this instance of little use. On the basis of these isolated measurements no other species or localities were distinguishable.

The factor plots (Figure 7.5) and component loadings indicate that all the measured characters vary together; however, the second principal component placed width-related variables (toe length, proximal and distal widths) on one side and characters describing length (total length and shaft length) on the other.

Eight groupings were suggested by the cluster analysis, four of these clusters isolating all of the specimens of *G. salaris*, as shown by the PCA plot (Figure 7.6). No pattern of species discrimination was discernible within the remaining clusters. In this instance *G. salaris* was discriminated on its relatively large size, principally the length of the sickle, operating along the first principal component and the shaft length operating along the second.

Conclusions

The release of sclerites from the body tissue of *Gyrodactylus* and their examination under SEM has allowed some redescription of various point to point morphometrics. The ability of some of these new measurements, notably the hamulus angle, has in this study, proved to be a useful character in the discrimination of *G. salaris* from native species of *Gyrodactylus* found on British salmonids. The hamulus angle was also able to discriminate *G. colemanensis* from Canadian brook trout. The higher magnification of images has enabled precise measurements of the marginal hook sickle aperture to be taken, the key marginal hook parameter used to distinguish *G. salaris* from other *Gyrodactylus* species parasitising British salmonids.

CHAPTER 8: THE HAMULUS ANGLE OF SPECIES OF *GYRODACTYLUS* AS A RELIABLE TAXONOMIC CRITERION: WITH COMMENTS UPON ITS SIGNIFICANCE TO THE MECHANISM OF ATTACHMENT AND TO PATHOGENICITY, A STUDY USING DIGITAL IMAGE ANALYSIS.

Introduction

Although the structural elements, and notably the marginal hooks embedded within the opisthaptor of gyrodactylids have been shown to have species specific characteristics (Malmberg, 1970), the extent to which other morphological features as the pharynx and cirrus contribute to variation between species is in question. Malmberg (1970) stated that environmental factors, such as temperature, chemical composition and water salinity, may contribute to subtle variations in the marginal hooks and anchors of species of *Gyrodactylus* von Nordmann, 1832. Several authors (Ergens, 1975, 1976; Ergens & Gelnar, 1985; Kulemina, 1974, 1977; Malmberg, 1962) have reported the influence of temperature on hook structure, as have studies into seasonal variation of *G. salaris* (Mo, 1991a,b,c). Such studies have shown that at higher temperatures there is a decrease in the overall sclerite size which is dictated, in part, by an increase in the rate of embryogenesis (Kulemina, 1988). Consequently, colder temperatures are associated with an increase in sclerite size, coupled with an increased parasite longevity. However, the relationship between sclerite structural development and increased parasite life-span still requires investigation. Baron (1968) proposed the theory that developing hooks become hollow and undergo secondary thickening; this thickening was believed by Crusz (1948) to be deposited externally. The external contribution has been postulated as coming from two sources, from the tegument surrounding the hamuli (Crusz, 1948) or from the water medium (Malmberg, 1970). According to the first theory, thickening could arise either from the hook core material or from externally deposited material. It seems unlikely that differences in local water parameters would be responsible directly for significant differences in the chemical composition of portions of the sclerite, such as the hook points of the hamuli and marginal hooks which are exposed to the external environment. This is due to the fact that the relatively short life-span of the gyrodactylids would not permit any significant

bioaccumulation to occur. However, changes in the water parameters might account for seasonal differences within and between populations, as might the mucus of its host. Sclerite composition might possibly be influenced by both the microhabitat and parasite feeding.

Initial studies using multivariate analysis on morphometric data to discriminate species of *Gyrodactylus* spp. from salmonids (Chapter 7) suggested that the hamulus angle alone appeared to be a useful criterion for distinguishing *G. salaris* from native British forms. However, the reliability of this variable to discriminate *G. salaris* from other species of *Gyrodactylus* is questionable if the hamulus angle is affected by the external deposition of material to the hooks as the worm ages. An attempt was therefore made to reduce the possible influence of increased external deposition to the hamulus with age or microclimate by the use of image analysis techniques on scanning electron micrographs of hamuli isolated by sonication.

A second feature for discriminating the "salmonid" gyrodactylids relates to the size of the opisthaptor. Since the preparation techniques for light and scanning electron microscopy use worms removed from the surface of the fish, the accuracy of any measurements taken will be affected by variations in compression during slide preparation and in contraction caused by dehydration of the soft parts. Once removed from the host epidermis, the haptors of *Gyrodactylus* contract and curl up. Therefore, one means of obtaining an accurate representation of the opisthaptoral size would be to look at attachment wounds or haptors still attached to the host epidermis. The attachment wounds are clearly marked by the penetration into the epidermis of the 16 marginal hooks as the gyrodactylid maintains its position on the host. If the parasite is fixed *in situ* and then removed at a late stage during the electron microscopy fixation process, then it might be anticipated that the extent of tissue contraction might be reduced to give a truer representation of the size of the opisthaptor. It would then be possible to determine whether species can be distinguished on the size of their footprint, i.e. opisthaptoral size.

The aims of this part of the study, therefore, were: (a) to compare the hamulus angle of *Gyrodactylus* hamuli extracted by sonication (Chapter 6) to hamuli that were "skeletonised", digital image analysis techniques were used to remove the effect of age related growth to the hamuli, eg. the material deposited on to the external surface of

the hook, by reducing the hamulus image to a linear form i.e. skeletonised; and (b) to measure and compare the attachment wounds and/or detached haptors fixed *in situ* on the host epithelium for several species of *Gyrodactylus*, using digital image analysis.

Materials and methods

Hamulus angle

Electronmicrographs of hamuli obtained from sonicated specimens of *Gyrodactylus* were obtained from a range of salmonids. These are listed in Table 8.1.

Table 8.1: A list of the salmonoid populations sampled from which samples of *Gyrodactylus* hamuli were collected and analysed using digital image analysis.

***Gyrodactylus* species**	**Host**	**Location**
G. derjavini Morph 1	wild *S. salar*	R. Nith, Dumfries, Scotland
	farmed *S. salar*	R. Allan, Stirlingshire, Scotland
Gyrodactylus Morph 2	wild *S. salar*	Mags Yr Afon, Pembroke, Wales
	farmed *S. salar*	R. Snizort, Isle of Skye, Scotland
G. truttae Morph 4	wild *S. trutta*	R. Manor, Borders, Scotland
	wild *S. trutta*	R. Neath, W. Glamorgan, Wales
G. truttae Morph 5	wild *S. trutta*	L. Airthrey, Stirlingshire, Scotland
G. derjavini Morph 1	farmed *O. mykiss*	L. Awe, Strathclyde, Scotland
	farmed *O. mykiss*	L. Butterstone, Tayside, Scotland
	farmed *O. mykiss*	R. South Esk, Borders, Scotland
Gyrodactylus Morphs 6 & 7	farmed *S. alpinus*	L. Ennerdale, L. District, England
G. colemanesis	farmed *S. fontinalis*	Nova Scotia
G. derjavini	wild *S. trutta*	R. Dalälven, Sweden
G. salaris	wild *S. salar*	R. Beukaforsen, Sweden
	wild *S. salar*	R. Ätran, Sweden
	wild *S. salar*	R. Lierelva, Norway

Hamuli were released by sonication and prepared for scanning electron microscopy according to the protocol of Chapter 6.

For each population of *Gyrodactylus* studied, ten hamuli were selected from a pool of 60-80 hamuli. Photomicrographs were obtained using a Hitachi S800 field emission scanning electron microscope operating at an accelerating voltage of 8kV. The micrographs of the hamuli were analysed and processed by means of an IMCO

10 Intel 80386DX computer fitted with a maths coprocessor linked to a digitising tablet and dual multisync 3D VGA monitors (Kontron Electronics Ltd). The imaging program VIDAS 2.1 (Kontron) allows interactive modification of the recorded images; in this case photo-enlarged copies of the hamuli were reproduced using the digitising tablet. This image was then filled to produce a solid structure, a form analysable by the program. This image was then subjected to serial skeletonisation in order to reduce each hamulus to a linear form. The process is such that single pixels are stripped away until the shape of the object is preserved only as a line of a single pixel width, resulting in a skeletal image. The process does not influence overall image size, i.e. length, as the program allows interaction to maintain the endpoints. The final product, as a resultant vector of the original binary image, is exported to produce a hard copy. These images were then processed to calculate the internal angle of the hamuli. A second imaging package, Videoplan (Kontron), in addition to being a graphics program, permits the subsequent calculation of linear or complex, convoluted morphometrics. In this instance, an angle between two lines defined by a single point of origin on the inside of the hamulus curve and two registration points, one being the hamulus point and the second being the point on the inner curve of the hamulus intersected by a line drawn between the base of the dorsal bar attachment point and the hamulus point (Figure 8.1). This point can be marked on hooks to be skeletonised as an intersecting line (see Figure 8.1, line marked X^1-X^2; X^3 = mid-point); the subsequent conversion of the image to a skeleton will retain this line without having an effect on the hamulus angle. A total of seven measurements were recorded for each specimen, the smallest recorded angle being taken as the apex or hamulus angle.

Angles were recorded from both natural unprocessed hamuli and on linear images resulting from the process of skeletonisation for comparison. Statistical tests were carried out on the results using the Kruskal-Wallis and Dunn's non-parametric tests.

Opisthaptor size

During routine collection of gyrodactylid material, fin samples with attached *Gyrodactylus derjavini* on rainbow trout from Loch Awe, *G. truttae* on brown trout from a stream leading into Loch Airthrey, and *G. gasterostei* Gläser, 1974, included

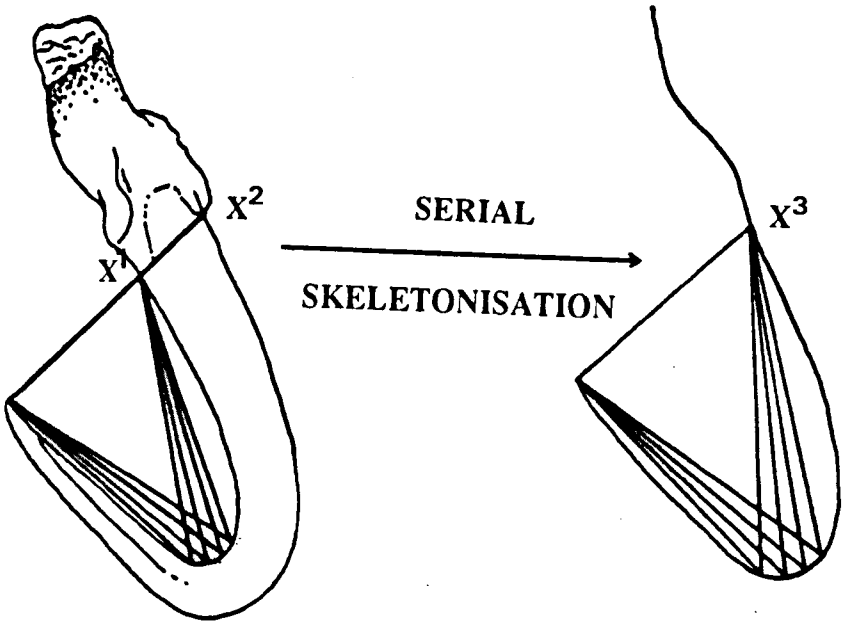


Figure 8.1: Measurement of the hamulus angle in natural and skeletonised hooks. The region marked X-X represents the line connecting the hamulus point to the base of the dorsal bar attachment point.

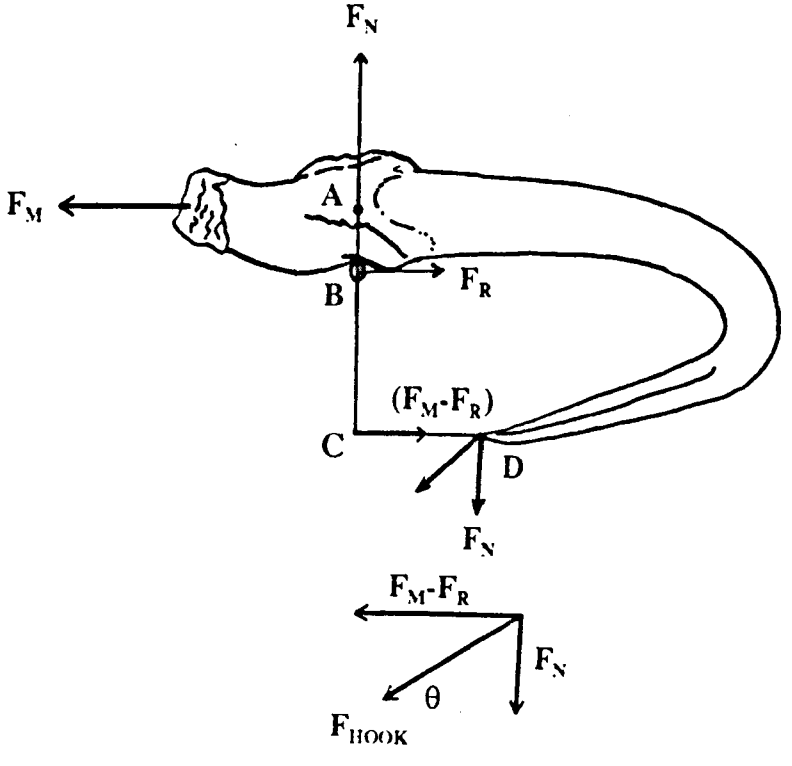


Figure 8.2: The lines of force acting through the hamulus.

for comparison, from three-spined sticklebacks *Gasterosteus aculeatus* also from Loch Airthrey were taken and placed directly into 3% glutaraldehyde in 0.2M cacodylate buffer. Fin samples with parasites attached were allowed to fix for several days before their transfer to 0.2M cacodylate buffer for an equal time period. Samples were then passed through a graded acetone series (70%, 90%, 95%, 100%, 100%) to 100% acetone. Fin specimens were screened for gyrodactylids and the majority of worms removed using a pointed needle to expose their attachment sites or the worm was broken leaving only the haptor attached. They were then critical point dried and sputter coated with 15nm of gold (using a Polaron E5000 sputtercoater). Fin specimens were examined under a Philips field emission scanning electron microscope (SEM) at an accelerating voltage of 15 kV and the attachment sites photographed.

In addition to the fin samples, SEM micrographs of *G. salaris* footprints published in the literature by Malmberg & Malmberg (1986) and *G. alexanderi* Mizelle & Kritsky, 1967 by Lester (1972) were measured for their opisthaptoral size. These attachment sites were defined by the use of a digital image analysis system using the morphometrics package Videoplan.

Results

Hamulus angle

The results of the determination of hamulus angle given in Table 8.2 did not constitute a normal distribution and so the Kruskal-Wallis and Dunn's tests were used to analyse the data.

The test value H for Kruskal-Wallis is 94.195. This was greater than χ^2 at 0.001 (level of significance (χ^2 = 37.697) and, therefore, the null hypothesis that the 16 lots of material do not differ significantly was rejected. The differences are shown in Table 8.3, as calculated using Dunn's non-parametric test. Similarly, there were significant differences between species for the skeletonised hamuli (Kruskal-Wallis test value H = 79.097, χ^2 at 0.001 = 30.578); these differences are shown in Table 8.4.

Opisthaptor size

The area covered by the opisthaptor permitted discrimination of certain species of

Table 8.2: Comparison of the hamulus angle for entire and skeletonised hamuli for several populations of *Gyrodactylus*.

Species	Number measured	Entire hooks mean°	SD	Skeletonised mean°	SD
G. derj Morph 1 (salmon, Mags Yr Afon)	10	38.37	2.09	41.81	2.17
G. derj Morph 1 (salmon, River Allan)	5	34.21	2.39	37.68	3.28
Gyrodactylus Morph 2 (salmon, River Nith)	10	37.16	0.96	39.50	1.36
Gyrodactylus Morph 2 (salmon, River Snizort)	9	36.58	1.67	40.57	1.92
G. truttae Morph 4 (brown trout, River Manor)	10	37.34	2.20	40.25	1.89
G. truttae Morph 4 (brown trout, River Neath)	10	38.65	0.85	41.13	1.31
G. truttae Morph 5 (brown trout, Loch Airthrey)	10	35.31	1.38	39.10	1.68
G. derj Morph 1 (rainbow trout, L. Butterstone)	10	38.46	1.56	41.14	1.63
G. derj Morph 1 (rainbow trout, Loch Awe)	10	36.00	2.19	40.03	2.17
G. derj Morph 1 (rainbow trout, River South Esk)	10	39.10	1.77	41.76	1.84
Gyrodactylus sp. (arctic charr, Lake Ennerdale)	6	37.63	2.31	39.11	2.24
G. colemanensis (brook trout, Nova Scotia)	10	47.78	2.05	49.75	1.81
G. derjavini (brown trout, River Dalälven)	10	36.97	1.47	40.69	1.82
G. salaris (salmon, River Beukaforsen)	10	42.44	1.25	44.61	1.31
G. salaris (salmon, River Ätran)	5	43.20	1.42	44.44	1.52
G. salaris (salmon, River Lier)	5	41.75	2.46	43.97	2.23

Table 8.3: Significant differences in hamulus angle (unskeletonised) for several populations of *Gyrodactylus*.

Species	Kruskal-Wallis test values for significance														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *G. derj* (Butterstone)	-														
2. *G. derj* (River Nith)	1.50	-													
3. *G. trut* (River Neath)	0.03	1.53	-												
4. *G.* sp. (L. Ennerdale)	0.95	0.35	0.97	-											
5. *G. cole* (Nova Scotia)	3.07	**4.57**	3.05	**3.61**	-										
6. *G. sal* (Beukaforsen)	2.18	**3.68**	2.15	2.84	0.90	-									
7. *G. derj* (R. Dalälven)	1.59	0.09	1.62	0.43	**4.67**	**3.77**	-								
8. *G. trut* (River Manor)	1.34	0.16	1.37	0.21	**4.42**	3.52	0.25	-							
9. *G. derj* (South Esk)	0.22	1.72	0.19	1.14	2.86	1.96	1.81	1.56	-						
10. *G. derj* (Mags Yr Afon)	0.49	1.01	0.52	0.52	**3.57**	2.67	1.10	0.85	0.71	-					
11. *G. derj* (River Snizort)	1.92	0.46	1.95	0.75	**4.92**	**4.04**	0.38	0.62	2.14	1.45	-				
12. *G. derj* (Loch Awe)	2.38	0.87	2.40	1.11	**5.45**	**4.56**	0.79	1.04	2.59	1.89	0.39	-			
13. *G. sal* (River Ätran)	1.99	3.21	1.97	2.61	0.52	0.21	3.29	3.08	1.81	2.39	**3.54**	**3.93**	-		
14. *G. sal* (River Lier)	1.41	2.64	1.39	2.09	1.10	0.37	2.71	2.51	1.23	1.81	2.97	3.35	0.50	-	
15. *G. derj* (River Allan)	2.78	1.55	2.80	1.70	**5.29**	**4.55**	1.48	1.68	2.95	2.38	1.14	0.84	**4.13**	**3.63**	-
16. *G. trut* (L. Airthrey)	3.06	1.56	3.08	1.70	**6.13**	**5.23**	1.46	1.72	3.27	2.56	1.05	0.68	**4.48**	**3.91**	028

Significant figures are shown in bold

Levels of significance: 0.05 = 3.529 0.01 = 3.935 0.005 = 4.098 0.001 = 4.456

Table 8.4: Significant differences in the hamulus angle of skeletonised hooks for several populations of *Gyrodactylus*.

Species	Kruskal-Wallis test values for significance														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. *G. derj* (Loch Awe)	-														
2. *G. derj* (River Nith)	0.71	-													
3. *G. trut* (River Neath)	0.95	1.66	-												
4. *G. cole* (Nova Scotia)	**4.61**	**5.32**	**3.66**	-											
5. *G. derj* (Mags Yr Afon)	1.44	2.15	0.49	3.17	-										
6. *G. derj* (Butterstone)	0.98	1.70	0.03	**3.63**	0.46	-									
7. *G. trut* (River Manor)	0.01	0.73	0.94	**4.60**	1.43	0.97	-								
8. *G. derj* (River Snizort)	0.45	1.14	0.47	**4.03**	0.95	0.51	0.44	-							
9. *G. derj* (R. Dalälven)	0.55	1.26	0.40	**4.06**	0.89	0.43	0.54	0.08	-						
10. *G. derj* (South Esk)	1.55	2.26	0.60	3.06	0.11	0.56	1.54	1.06	1.00	-					
11. *G. sal* (Beukaforsen)	**3.56**	**4.27**	2.61	1.05	2.12	2.58	**3.55**	3.02	3.01	2.01	-				
12. *G.* sp. (L. Ennerdale)	0.79	0.17	1.61	**4.77**	2.03	1.63	0.79	1.16	1.26	2.12	**3.87**	-			
13. *G. sal* (River Lier)	2.47	3.05	1.69	1.30	1.29	1.66	2.46	2.05	2.02	1.20	0.44	2.90	-		
14. *G. derj* (River Allan)	1.20	0.62	1.97	**4.96**	2.37	2.00	1.21	1.55	1.65	2.46	**4.11**	0.42	3.17	-	
15. *G. sal* (River Ätran)	2.87	3.45	2.09	0.90	1.69	1.69	2.86	2.44	2.42	1.60	0.04	3.26	0.35	3.52	-
16. *G. trut* (L. Airthrey)	0.93	0.22	1.88	**5.54**	2.37	2.37	0.94	1.35	1.48	2.48	**4.49**	0.02	3.22	0.44	**3.62**

Significant figures are shown in bold

Levels of significance: 0.05 = 3.529 0.01 = 3.935 0.005 = 4.098 0.001 = 4.456

Table 8.5: Discrimination of species of *Gyrodactylus* by opisthaptor size.

Species	no.	G. sal	G. alex	G. gast	G. trut
G. salaris[1]	5	-			
G. alexanderi[2]	5	0.22	-		
G. gasterostei[3]	4	**3.61**	**3.83**	-	
G. truttae[3]	6	1.29	1.52	2.67	-
G. derjavini[3]	10	2.71	**2.97**	1.60	1.50

Significant figures are shown in bold.

Levels of significance: 0.05 = 2.807 0.01 = 3.291 0.005 = 3.481 0.001 = 3.891

Annotation: [1]SEM of footprints from Dr G. Malmberg, Zoology Department, University of Stockholm; and Malmberg & Malmberg (1986); [2]SEM footprints measured from Lester (1972); [3]original.

Table 8.6: Summary of hamulus angles for unskeletonised hamuli from several species of *Gyrodactylus*.

Species	no.	mean°	SD	range
G. salaris	20	42.42	1.65	38.92 - 45.09
G. colemanensis	10	47.79	2.05	44.60 - 50.88
G. derjavini (rainbow trout)	30	37.89	2.28	32.33 - 40.89
G. derjavini (Atlantic salmon)	44	36.90	2.00	31.24 - 42.69
G. truttae	30	37.08	2.05	33.38 - 40.89
G. sp. (arctic charr)	6	37.61	2.31	35.21 - 40.45

Gyrodactylus, namely the separation of members of the *G. wageneri*-group from species parasitising three-spined sticklebacks. Again, the data collected did not conform to a natural distribution and were analysed using Kruskal-Wallis and Dunn's tests. Species did differ significantly, the Kruskal-Wallis test value being H = 22.534 (χ^2 at 0.001 = 18.476). The species discriminated on the basis of opisthaptor size are indicated in Table 8.5.

Discussion

Tables 8.3-8.4 show that the hamulus angle of *G. salaris* and *G. colemanensis* varied significantly from nearly all the populations of *Gyrodactylus* parasitising British salmonoids. The ability of the hamulus angle to discriminate *G. salaris* from other closely related species warrants its inclusion within the list of variables presently used to describe the morphometric dimensions of the sclerites of the genus *Gyrodactylus*. Furthermore, Tables 8.3-8.4 show that there were few differences between unprocessed and skeletonised hooks. This would suggest that, if deposition of material continues to be added as the worm matures, then it appears to have little effect on the hamulus angle in this case.

At its position close to the junction in monogenean evolution between the monopisthocotyleans and polyopisthocotyleans, *Gyrodactylus* represents an unusual group. It is viviparous, a feature which, according to Llewellyn (1981) represents, in evolutionary terms, an advanced reproductive strategy. However, it fails to exhibit any post-larval development, retaining its larval morphological features to live as a neotenous adult. Post-oncomiracidial development in polyopisthocotyleans is marked by the development of a genito-intestinal canal and blood feeding; however, *Gyrodactylus*, an epidermal grazer, lacks this canal. During the development of the oncomiracidium of most monogeneans there is a switch from the marginal hooks as the key attachment mechanism to the hamuli or to the post-larval development of sclerites to form clamps. *Gyrodactylus*, in contrast, retains the use of marginal hook attachment throughout its life. When the morphology of these sclerites is examined in relation to their function, questions arise as to whether (a) the hamulus angle plays a role in parasite attachment, (b) the angle is linked to the pathogenicity associated

with some species, i.e. *G. salmonis* Yin & Sproston, 1948 (Cone & Odense, 1984; Cusack & Cone, 1986) and (c) the differences in hamulus angle are a feature of inter-specific variation. Kearn (1964) showed in a capsalid monogenean that, although the hamuli do penetrate the epidermis, they also act as props, allowing the opisthaptor to attach mainly by suction: in the capsalid, the hamuli contribute more to attachment than the marginal hooks. The marginal hooks merely prevent the flanges of the opisthaptor migrating inwards thus reducing the efficiency of the suctorial action. *Gyrodactylus*, however, uses the marginal hooks as the major component of its attachment mechanisms, since, by their action, the protruding points of the hamuli compress the underlying epidermis to an extent that they may penetrate this tissue (Lester, 1972). In this manner the hamuli of *Gyrodactylus* act as a subsidiary attachment mechanism. Furthermore, the body design of *Gyrodactylus* does not, according to Lester (1972), permit the parasite to initiate direct penetration of the anchors, since the hamuli are large in relation to the body and consequently there is insufficient muscle force generated to actively induce hamulus penetration. The inability of the hamuli to actively penetrate the host's tissue by their own action would imply that any penetration is a result of the force exerted by the marginal hooks or perhaps less likely external forces, i.e. water movement or fish behaviour acting on the worm and causing the anchors to embed. The hamuli of the species investigated in this study do not actively embed and any penetration is passive; yet some of the more primitive gyrodactylid groups do attach using the hamuli, creating a deep wound, while the marginals act as stabilizers (Harris, 1982). Further evidence comes from the gill species *G. rarus* Wegener, 1909, whose hamuli are actively embedded into the host tissue and whose marginal hooks provide further support by gripping the gills firmly, maintaining the parasite's position (Harris, 1982).

Pugachev (pers. comm.) has analysed the hook mechanics of several lower monogeneans and found that the force generated by the anchor on host epidermis was dependent on the angle between the point and root portions of the hamuli. The present results show that the relatively large hamulus angles of *G. salaris* and *G. colemanensis* can be discriminated from the species of *Gyrodactylus* resident on British salmonids. Since *G. salaris* (see Johnsen, 1978) and *G. colemanensis* (see Cone & Cusack, 1988) are both known to cause losses in salmonid populations, the size of the hamulus angle

and the possible force of attachment was investigated. If the forces acting on the hamulus of several *Gyrodactylus* species are briefly examined (Figure 8.2), the only force for attachment (omitting the possible downward pressure contributed by the opisthaptoral muscles) would have to be generated by the extrinsic muscles attached via tendons to the hamulus roots; this "force of penetration" (F_{HOOK}) is discussed below.

The force (Figure 8.2) acting through the hamulus point (F_{HOOK}) can be calculated as:

$$F_M.AC - F_R.BC - F_N.CD = 0$$

where:

$$F_R = \mu_D F_N$$

[μ_D = the coefficient of dynamic friction; F_N = forces acting about "rotation" point; F_M = force of muscle action pulling on the hook; F_R = resistance to sclerite movement; A = centre of the hamulus about its rotation point B; BC = downward force perpendicular through B to a point C (horizontal with the hamulus point, D); CD = distance from the perpendicular at point C to the hamulus point (F_M - F_R)]

thus:

$$F_{HOOK} = \sqrt{(F_M - F_R)^2 + F_N^2}$$

and the angle through which the force acts is therefore given by:

$$\text{Tan } \theta = \frac{F_N}{F_M - F_R}$$

This briefly summarises the main forces acting upon the hamulus. The actual operation of the hook mechanics is far more complex with hook rotation, elasticity of the extrinsic muscles, resistance imposed by the host epidermis to penetration, and, most importantly, the contribution of the marginal hooks transmitted through the opisthaptor to the hamuli. It is interesting to note, however, that if we consider four

species, namely *G. colemanensis*, *G. derjavini*, *G. truttae* and *G. salaris*, and use hook morphometric data, the following can be noted. The hamulus angle for these species is in the order *G. derjavini* < *G. truttae* < *G. salaris* < *G. colemanensis* (36.58° < 37.10° < 42.46° < 47.78°). If we assume that the force pulling on the extrinsic muscles is equal for all species, i.e. F_M = 10 Newtons (N) and the resistinal friction of the two sclerites, the hamulus and the ventral bar, moving over one another is also constant, i.e. μ_D = 0.5 (resistinal friction for bone against bone), then the values of F_{HOOK} are as follows: *G. colemanensis* < *G. salaris* < *G. truttae* < *G. derjavini* (11.81 N < 11.90 N < 12.30 N < 15.31 N). These are effective through the following angles 67.33° < 67.84° < 69.90° < 80.82°, respectively.

The values, however, assume a constant force operating on the hamulus and only summarise the principal forces; they are, therefore, unlikely to be a true representation. If these were responsible for hamulus penetration independent of the influence of the marginal hook, then it would imply that the smaller the hamulus angle the greater the penetration power. However, as shown by Lester (1972), when the angle through which the penetrating force acts is considered, the hamuli are influenced by marginal hook attachment and thus the contribution of these marginal hooks must be considered. This raises the question whether a relationship between hamulus and marginal hook morphology exists, i.e. the structure of one influencing the structure and performance of the other. The hamuli in these species do not actively penetrate host epidermis, but serve as props pushing up the roof of the opisthaptor. The height through which the opisthaptor roof is raised would therefore be dictated by the size of the hamulus angle and the force transferred through to the marginal hooks embedded in the opisthaptor. Therefore, the greater the height, the greater the force of marginal hook attachment. Braun (1966) and Kollmann (1967) have given literary accounts of the movement of marginal hooks. Braun's model showed the sickle of the marginal hook of *G. wageneri* Malmberg, 1956 to be rigid and the component of movement to be derived from the flexibility of the marginal hook shaft, the degree of flexing being responsible for the movement of the marginal hook point through its aperture for attachment. Kollmann's model, using *G. cyprini* Kollmann, 1967, showed that the marginal hook sickle point was moveable about its attachment point with the marginal hook shaft by means of a hinge. Attachment by the marginal hook is then

achieved by rotation of the sickle head within a single plane. The arrangement of the marginal hooks buried deep in the opisthaptor forms a circle such that any force exerted by the marginal hooks will be transferred in part to the hamuli.

If the theory relating hamulus angle to penetration force is true, then the real power of attachment must be the marginal hooks. The force of this attachment can be seen in the case of *G. salmonis* on rainbow trout, which lodges its marginal hooks so deep into the host epidermis that extensive fin damage results (Cone & Odense, 1984). The real relationships would, therefore, be between the force of marginal hook muscle contraction and hamulus angle and, to a lesser extent, opisthaptoral size. However, the analysis showed that species of *Gyrodactylus* parasitising salmonids cannot be discriminated on the basis of area covered by the opisthaptoral size, but the hamulus angle does readily discriminate *G. salaris* from other salmonid species of *Gyrodactylus*. A large angle does not increase the likelihood of pathological damage to the epidermis or imply a stronger force of attachment directly. However, the power of attachment comes through the marginal hooks, the efficiency of which may be maximised by several contributory factors, of which the hamulus angle is one possibility.

It appears that most age-related growth in the sclerites of *Gyrodactylus* is reflected in the form of elongation of the root portion of the hamuli, as the hook angle appears to be determined before birth, with growth in the hooks of adults occurring at the hook base, i.e. the root. Furthermore, chemical treatments which cleave disulphide bridges are shown to have an effect initially in the root portion, the region most prone to dissolution (Lyons, 1966). For this reason, the root represents an area where the production of prekeratin has yet to undergo consolidation and hardening, and consequently it exhibits a high variability when a cross-section of a population is studied. This study has shown that, even if sclerotisation of the hamuli is by an external process, *G. salaris* can be discriminated from native species of *Gyrodactylus* parasitising salmonids and from the North American species *G. colemanensis* on the basis of hamulus angle (see Table 8.6).

CHAPTER 9: MULTIVARIATE ANALYSIS OF SOME MORPHOMETRICAL FEATURES OF *GYRODACTYLUS SALARIS* MALMBERG, 1957.

Introduction

Gyrodactylus Morph 1 closely resembling *G. derjavini* Mikailov, 1975 was found to be parasitic on Atlantic salmon *Salmo salar* L. in British waterways (Chapter 4). The population was shown to be divided into two sub-populations, a northern boreal form and a southern English/Welsh, celtic form. The separation of the hosts dates back 6,000 - 13,000 years to the last glaciation and it is believed that this has resulted in the two morphotypes of *Gyrodactylus* (Morph 1 and Morph 2) evident today. A multivariate analysis performed on the British salmon *Gyrodactylus* species suggested that the clusters incorporating each morphotype could be further sub-divided (Chapter 4). However, it was found that seasonal water temperature was responsible for apparent sub-divisions. In the case of *G. salaris*, the size variation in the sclerites is well documented (Tanum, 1983; Mo 1991a,b,c). Mo (1991a,b,c) discussed the problem of temperature, which affected the size of hooks of *G. salaris* to such an extent that samples collected during the summer and winter appeared as two closely related but separate species. The precise origin of *G. salaris* is still unknown, whether it arose on salmonids in the Baltic or whether it is derived from an ancestral, or a now geographically isolated, species. If *G. salaris* is to be clearly discriminated from species of *Gyrodactylus* parasitising British salmonids, then it is important that we are aware of, or investigate, all the possible factors that may contribute to variability in the morphology of *G. salaris* sclerites. To begin investigating the variability of *G. salaris*, it is important to know how much variation exists between populations in closely situated rivers, and in addition, whether the hooks of *G. salaris* are influenced by the host factors such as its hormonal state. The multivariate analysis has shown to be sufficiently sensitive to discriminate two closely related species of *Gyrodactylus* (Morph 1 and Morph 2) (Chapter 4), and even different founder populations of the same species on a single host. This suggests that it might be sensitive enough to discriminate different populations of *G. salaris* in different river systems. Halvorsen

& Hartvigsen (1989) thought that *G. salaris* probably originated from the Baltic and had only recently moved into Norway, spreading along the coastline. This study attempts to determine whether different populations of *G. salaris* can be distinguished from one another in one and the same river and from different rivers using multivariate techniques. Two river systems were sampled for *G. salaris*, the River Ätran close to the Norwegian border and to Atlantic salmon, and the River Säveån which is closer to the Baltic to ascertain if *G. salaris* on Baltic salmon could be differentiated from *G. salaris* on Atlantic salmon. From this it might be possible to ascertain whether the spread of *G. salaris* has indeed occurred recently or whether it has been long established and we have only recently become aware of its presence.

Materials and Methods

Specimens of *G. salaris* were collected by Dr G. Malmberg from Atlantic salmon *Salmo salar* throughout the period May, 1991 to April 1992 from three river systems in Sweden: the River Högvadsån (May, 1991; Sept., 1991; Nov., 1991 precocious i.e. males which are mature pre-smolt fish and male fish showing normal development; March, 1992); the River Ätran at Fageredsån (Nov., 1991) and Nydala (April 1992) and the River Säveån (May, 1991), all in the county of Halland. Unfortunately, Baltic salmon were unattainable from the River Säveån at this time. The fish, caught by electro-fishing, were killed by a blow to the cranium, placed directly into 80% ethanol and sent to the UK. Individual specimens of *Gyrodactylus* were then removed, collected in batches of 30-40, washed in distilled water to remove the ethanol before treatment with proteolytic enzymes, and the sclerites prepared for SEM according to the procedure of Chapter 6.

Photomicrographs were obtained of ten hamuli and ten marginal hooks for each locality and collection period using an Hitachi S800 field emission scanning electron microscope (accelerating voltage 8kV). A total of 14 measurements (seven for the hamulus and seven for the marginal hook) were taken using an image analysis system linked to a digitising tablet, according to the procedure outlined in chapter 8.

The morphometric data for the hamuli and the marginal hooks were analysed separately using principal components analysis (PCA) and cluster analysis to ascertain

whether the *G. salaris* samples could be separated with respect to locality or season.

Results

Hamulus

The morphometric data for the hamuli were analysed using PCA. Since these data and those for the marginal hook did not give a normal distribution, they were log transformed (the cosine of the angle was used in order to give it a linear function). The relationships of the seven measured variables for the hamulus are given in the correlation matrix in Table 9.1. It can be seen that the hamulus shaft, point and total lengths vary in the same direction, whilst the hamulus angle varies inversely with the hamulus shaft-point length and that the hamulus angle also varies independently of the shaft length. The component loadings of the PCA on the first four principal components are given in Table 9.2.

For component 1 it is apparent that all the measured variables increase together except for the angle which decreases (denoted by the negative sign). The variables explaining the length features of the hamulus, however, account for a greater proportion of the variability between specimens than the variables explaining the width. In component 2 the reverse is observed with the width dimensions, i.e. the angle and the hamulus width, being the key variables responsible for the separation of specimens. The root, which increases inversely in relation to the hamulus width, is the key variable acting through component 3, whilst component 4 suggests that changes in the growth rate of the root and hamulus width are the principal variables.

The Table 9.3 gives the variance and percentage of variation explained by the components.

Marginal hook

The marginal hook morphometric data were also subjected to analysis by PCA. The correlation matrix for the seven measured variables is given in Table 9.4. This indicates that the marginal hook shaft varies in the same direction as the total length of the marginal hook and the sickle length, but to a lesser degree. The toe length is observed to vary independently of the sickle distal width, total length and the shaft length (denoted by values close to zero). The component loadings on the first three

Table 9.1: Pearson's correlation matrix on the hamulus variables.

Variable	Shft-Pt	Angle	Point	Shaft	Total
Shaft-Point	1.000				
Hamulus angle	-0.440	1.000			
Point length	0.232	-0.113	1.000		
Shaft length	0.346	-0.006	0.352	1.000	
Total length	0.289	-0.193	0.489	0.553	1.000
Root length	0.099	-0.127	0.117	-0.014	0.224
Hamulus width	-0.072	-0.040	0.063	0.185	0.079

Variable	Root	Width
Root length	1.000	
Hamulus width	-0.049	1.000

Table 9.2: Component loadings for the hamulus variables

Variable	1	2	3	4
Shaft-point	0.641	-0.436	-0.320	0.228
Hamulus angle	-0.427	0.638	0.439	0.236
Point length	0.675	0.197	0.233	0.082
Shaft length	0.712	0.431	-0.076	0.208
Total length	0.811	0.170	0.220	0.004
Root length	0.275	-0.373	0.645	-0.538
Hamulus width	0.149	0.513	-0.447	-0.699

Table 9.3: Percentage and variance explained by the components

	Variance explained by components	% of total variance	Cumulative percentage
Factor 1	2.31	33.03	33.03
Factor 2	1.25	17.90	50.93
Factor 3	1.02	14.56	65.49
Factor 4	0.94	13.37	78.86

principal components (Table 9.5) indicate that the pattern is one where all the marginal hook dimensions increase together, but where the length dimensions increase at a faster rate than the widths (all have positive values). The toe length, appears however, to act independently. Component 2 shows that for an increase in the growthrate of the point-shaft length, there is a decrease in the rate of growth for the width dimensions of the marginal hook. The sickle-shaft distance increases as a result of slight length increase in the sickle, rather than the width. Component 3 shows that, while there is a marked decrease in the growth rate in the toe length of the marginal hook, there is a slight increase in the growth rate of the sickle length and distal width.

The Table 9.6 gives the variance and percentage of variation explained by the components. The scores from the PCA were then analysed using cluster analysis in order to detect whether the populations of *G. salaris* could be separated on the basis of locality and seasonality. Firstly, the influence of locality was investigated in relation to the Rivers Fageredsån, Nydala and Högvadsån, which all belong to the Ätran River system which empties into the Kattegatt at Falkenberg, and the River Säveån, which is located some 90 km north, entering the Kattegatt at Gothenburg. The model was manipulated to ascertain whether a distance of 90km was great enough to allow the discrimination of the two populations of *G. salaris*. The results for the hamulus (Figure 9.1) and the marginal hook (Figure 9.2) indicate that there is a large degree of overlap for the hamuli, and the marginal hooks for the Säveån region are almost totally encompassed by all other marginal hook specimens.

A similar pattern of distribution for both the hamuli and the marginal hooks was found when the state of host maturity was investigated, i.e. specimens of *Gyrodactylus* collected from a precocious male and compared with gyrodactylids collected from normal males (Figures 9.3-9.4). Thirdly, the effect of temperature/seasonality was investigated. The samples of *G. salaris* were collected for comparative studies with British *Gyrodactylus* species, and, therefore, a full sampling programme in order to study the effects of seasonality was not initially intended, resulting in gaps in the data. The collections were split into summer (June - September) and winter (October - May) collections and compared. The results, shown for the hamuli (Figure 9.5) and the marginal hook (Figure 9.6), indicate that both are generally smaller in size during the summer months and that the two groups clustered

Table 9.4: Pearson's correlation matrix for the marginal hook variables.

Variable	Proximal	Toe	Distal	Sickle	Pt-Sht
Proximal width	1.000				
Toe length	0.146	1.000			
Distal width	0.348	0.027	1.000		
Sickle length	0.152	-0.078	0.148	1.000	
Point-shaft length	-0.025	0.123	-0.070	0.330	1.000
Total length	0.322	0.032	0.252	0.524	0.391
Shaft length	0.255	0.021	0.248	0.470	0.476

Variable	Total	Shaft
Total length	1.000	
Shaft length	0.843	1.000

Table 9.5: Component loadings for the marginal hook variables.

Variable	1	2	3
Proximal width	0.433	-0.677	-0.099
Toe length	0.079	-0.197	-0.932
Distal width	0.382	-0.684	0.190
Sickle length	0.686	0.196	0.234
Point-shaft length	0.562	0.545	-0.312
Total length	0.903	0.035	0.045
Shaft length	0.895	0.109	0.020

Table 9.6: Percentage and Variance explained by the components.

	Variance explained by components	% of total variance	Cumulative percentage
Factor 1	2.74	39.15	39.15
Factor 2	1.31	18.76	57.91
Factor 3	1.07	15.27	73.18

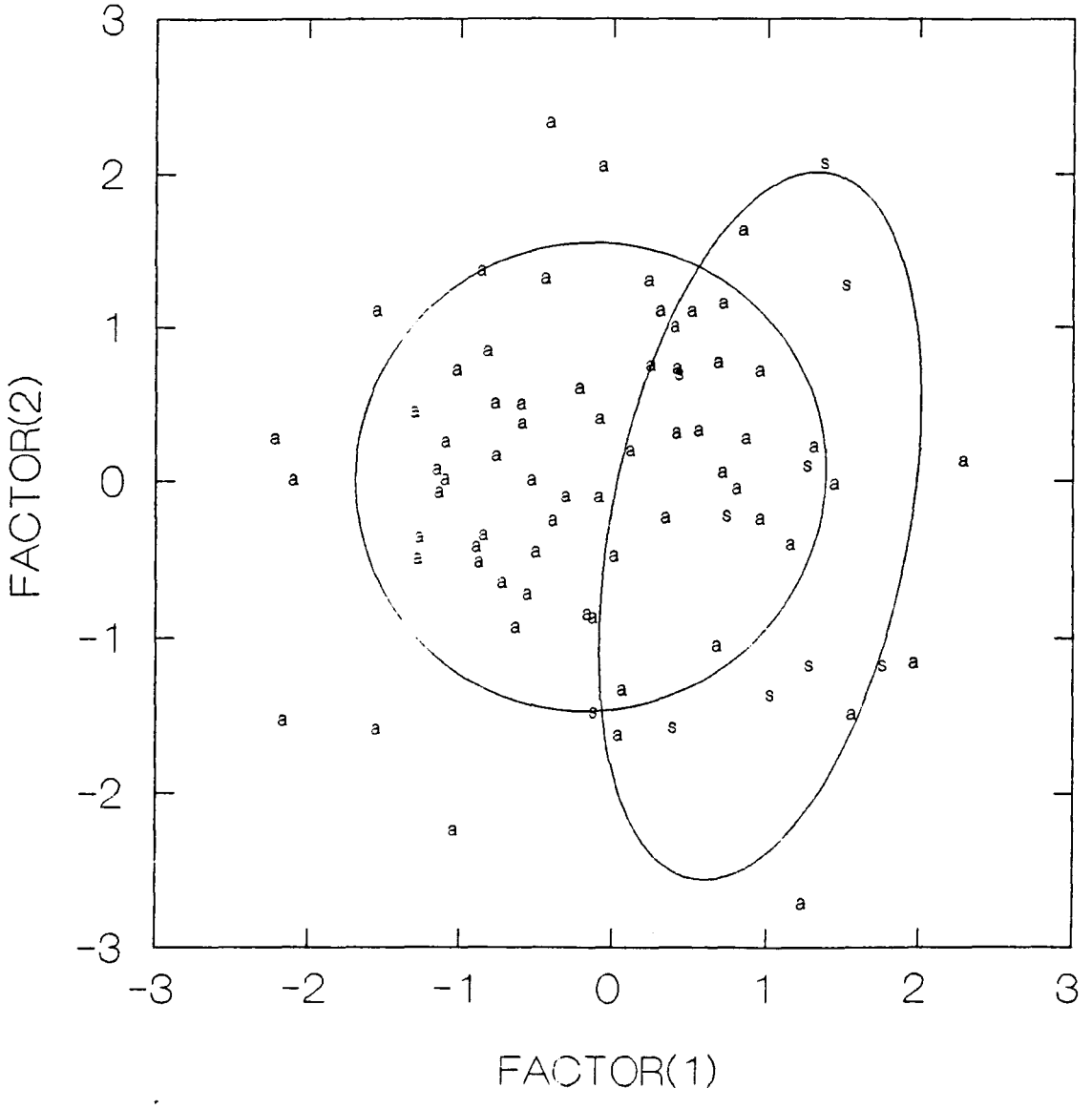


Figure 9.1: Comparison of hamuli from two river populations of *G. salaris*. The River Säveån (05/1991) (s) against the River Ätran (05/1991-04/1992) (a) in Sweden. Ellipses incorporate 70% of the specimens.

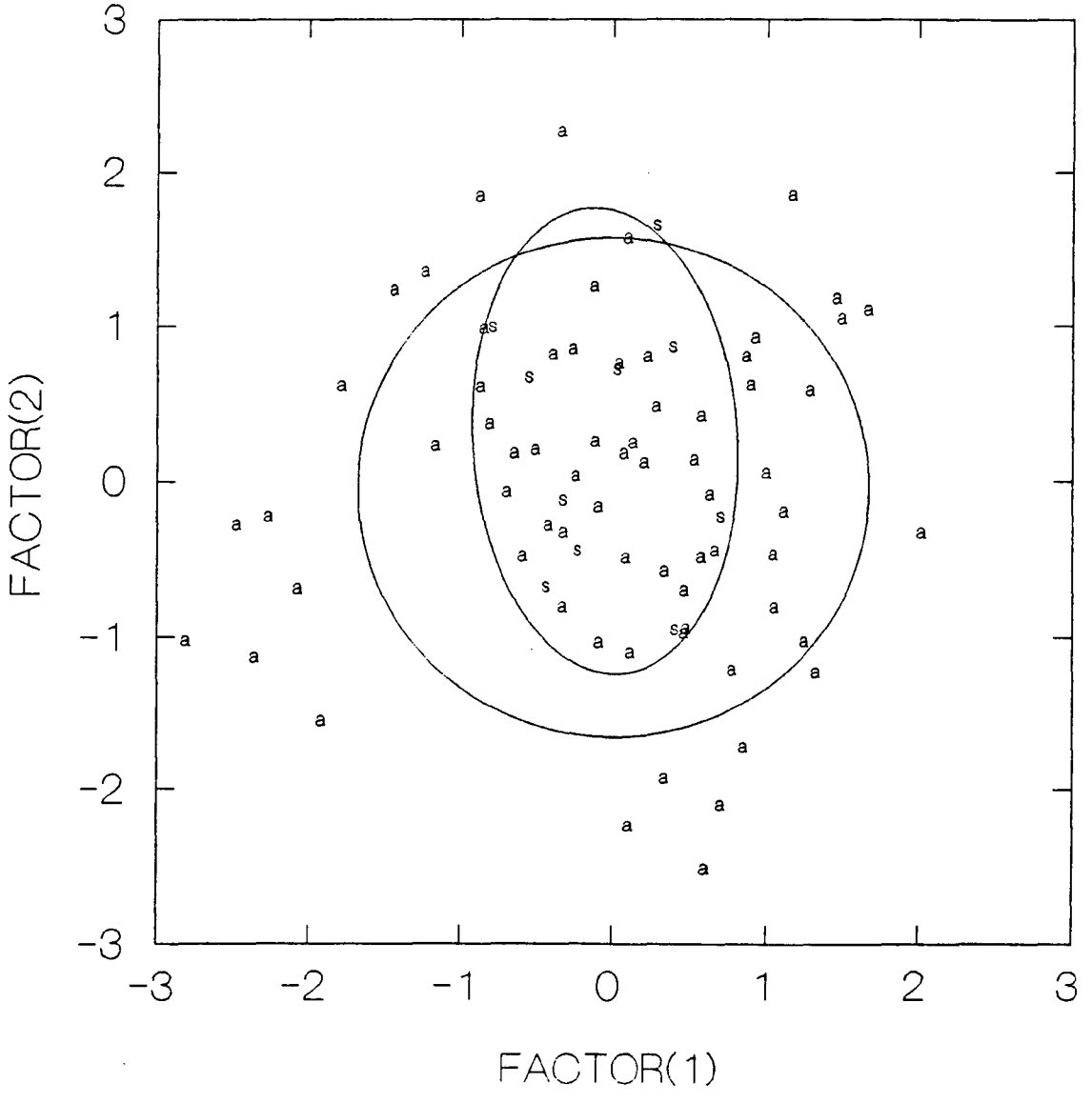


Figure 9.2: Comparison of marginal hooks from two river populations of *G. salaris.* The River Säveån (05/1991) (s) against the River Ätran (05/1991-04/1992) (a) in Sweden. Ellipses incorporate 70% of the specimens.

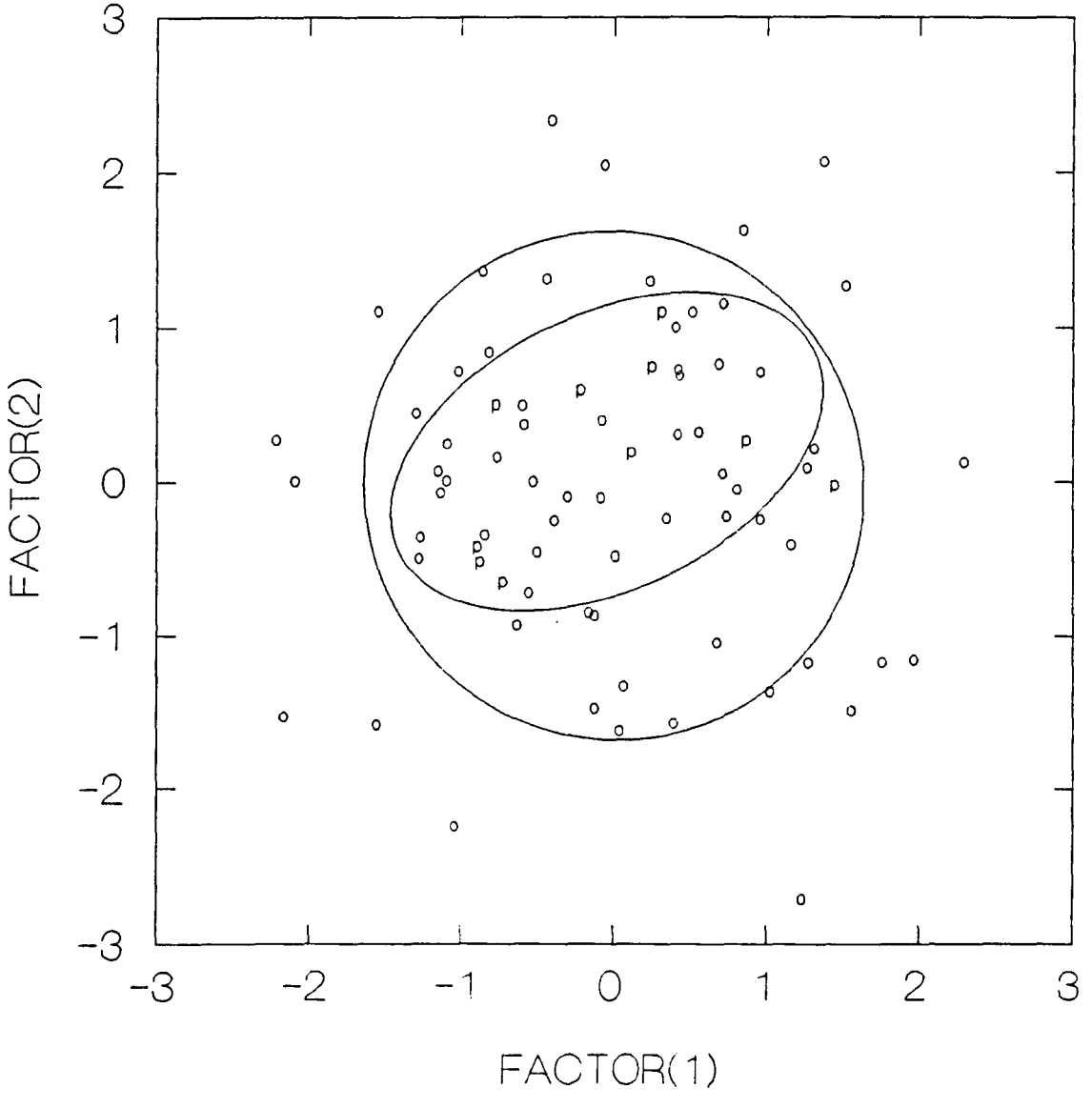


Figure 9.3: Comparison of *G. salaris* hamuli collected from precocious Atlantic salmon host (p) against a normal developed host (o). Ellipses incorporate 70% of the specimens.

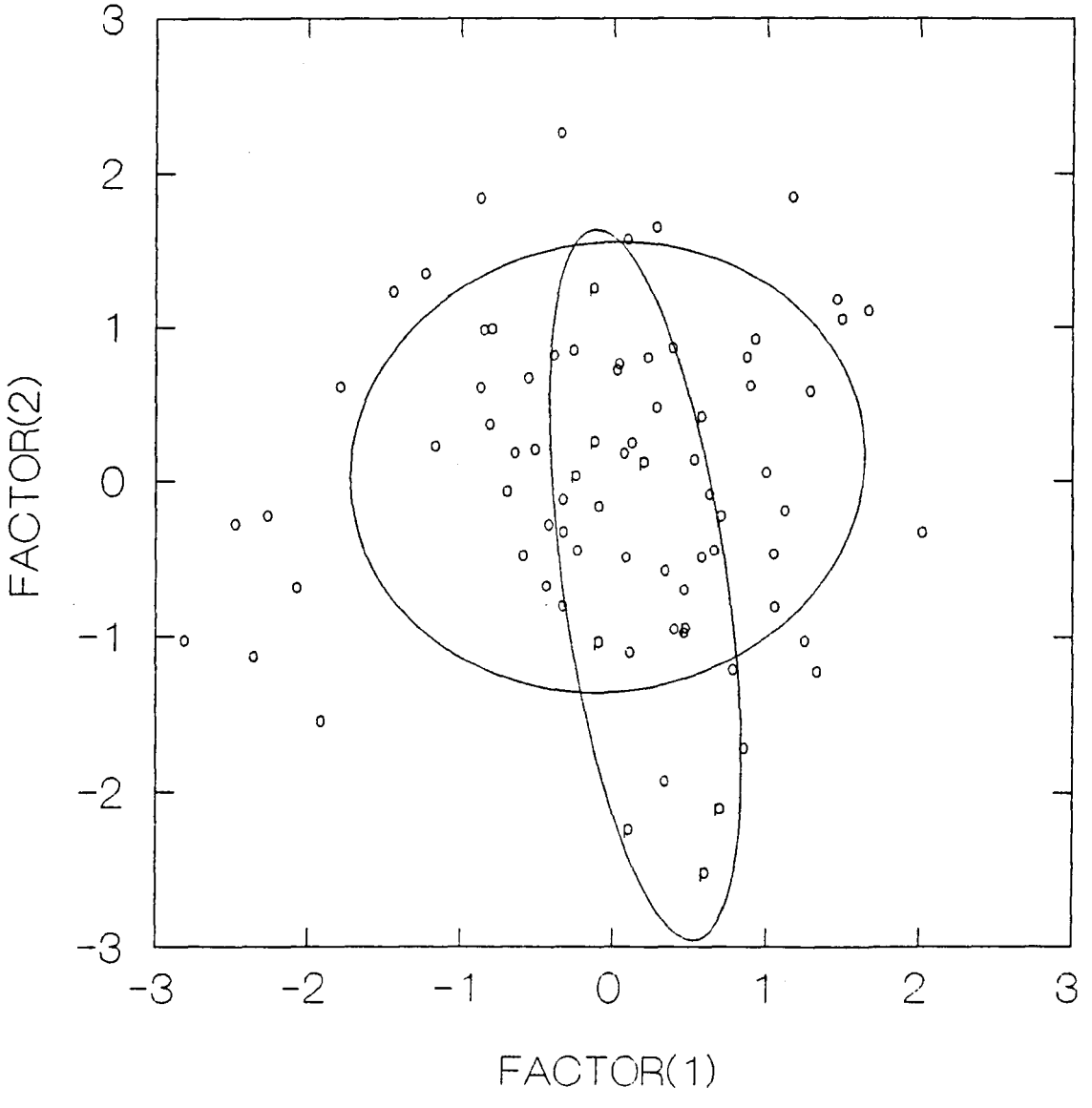


Figure 9.4: Comparison of *G. salaris* marginal hooks collected from precocious Atlantic salmon host (p) against a normal developed host (o). Ellipses incorporate 70% of the specimens.

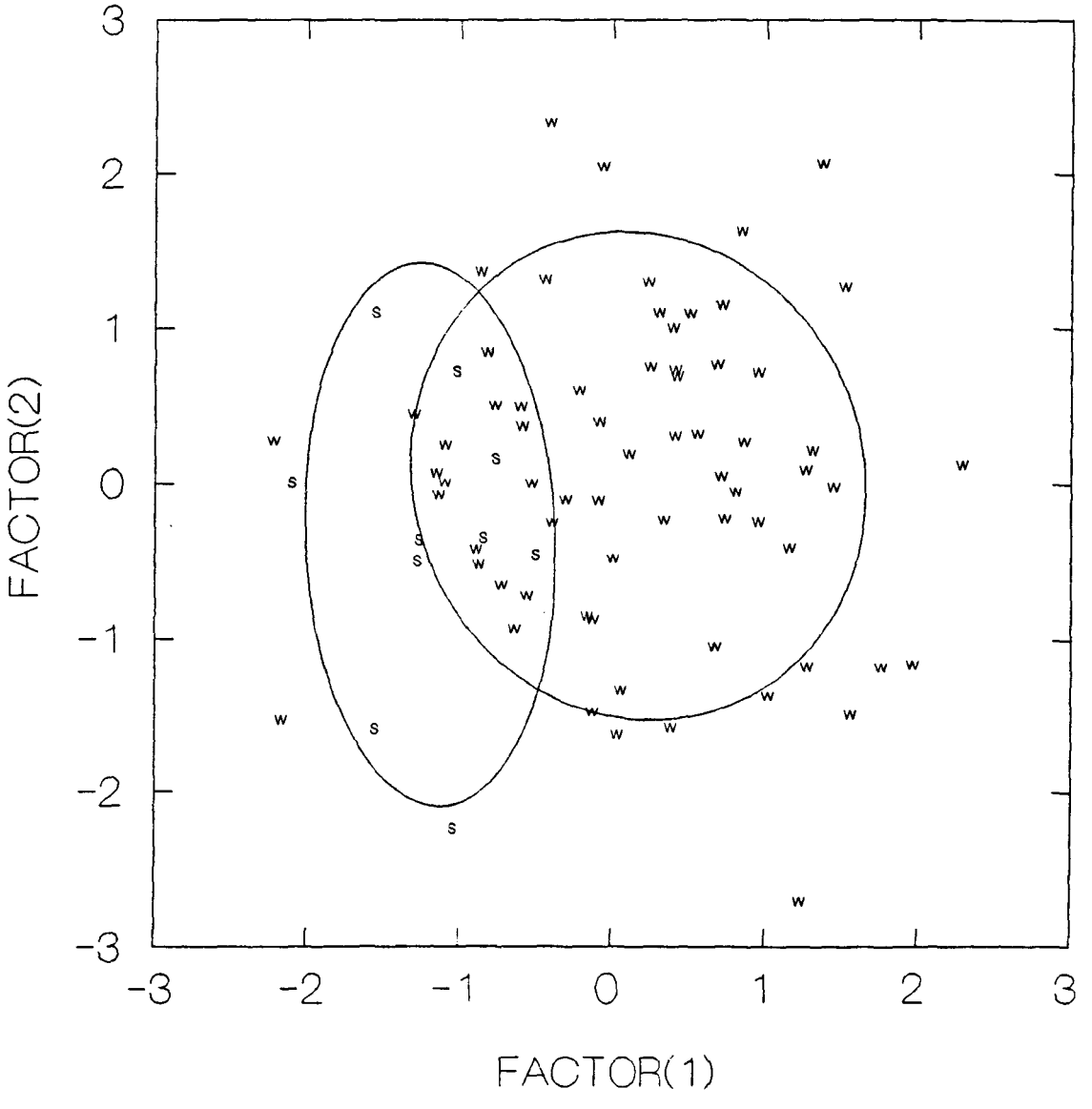


Figure 9.5: Distribution of hamuli from summer collections (06/1991-09/1991) (s) of *G. salaris* against winter collections (05/1991; 10/1991-04/1992) (w). Ellipses incorporate 70% of the specimens.

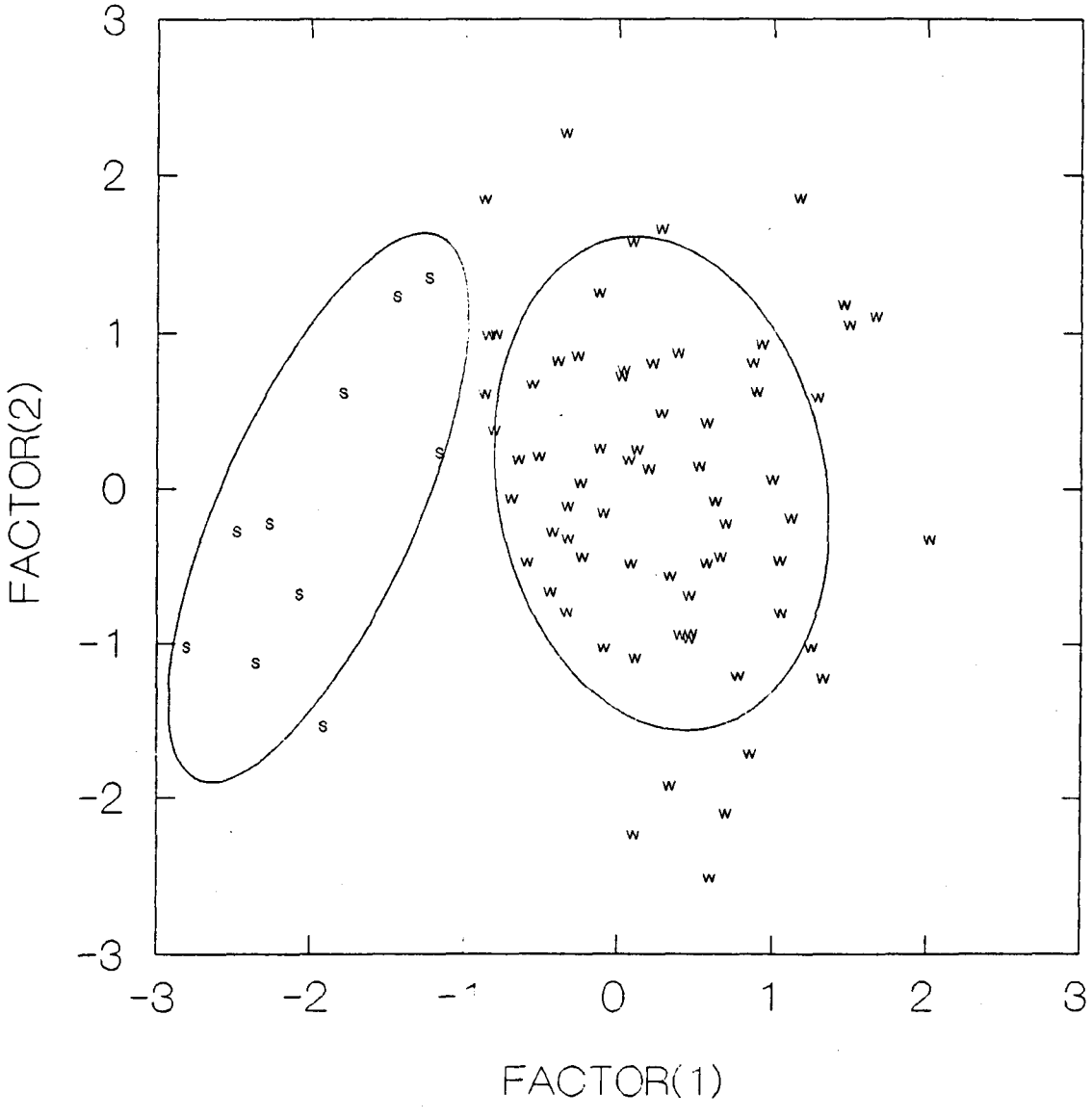


Figure 9.6: Distribution of marginal hooks from summer collections (06/1991-09/1991) (s) of *G. salaris* against winter collections (05/1991; 10/1991-04/1992) (w). Ellipses incorporate 70% of the specimens.

separately in the case of the marginal hooks.

Discussion

The results of the study of seasonality on the separation of *G. salaris* populations are in good agreement with the observations of Mo (1991a), who stated that a single species sampled at the extremes of a temperature range can yield what appears to be two separate species. This is particularly evident when the marginal hook is considered, as shown by the PCA plot in Figure 9.6. The hamuli and marginal hooks separated on the basis of the overall small size of the summer collections and thus confirm the observations of several authors for different species (Malmberg, 1962, 1970; Kulemina, 1974, 1977; Ergens, 1975, 1976; Solomatova & Luzin, 1977; Tanum, 1983; Ergens & Gelnar, 1985; Mo, 1991a).

The influence of the hormonal state of the host over the population of *G. salaris* was less clear cut. The precocious male had a comparatively high infestation, and it is possible to speculate that parasite numbers might increase while the fish is under a state of stress. Nevertheless, it appeared that this population was indistinguishable from those parasitising fish developing normally.

The lack of separation of the populations of *G. salaris* with respect to locality may be explained by several factors. Firstly, the distance between the two river systems might be too short (90 km) for any significant differences in the two populations to show. These sample sites from the Kattegatt are effectively on the boundary between the Atlantic and the Baltic; the Baltic region possibly being the origin of *G. salaris* (Halvorsen & Hartvigsen, 1989). The Baltic and Atlantic stock of salmon found today are believed to have been separated after the last glaciation which occurred some 6,000 - 13,000 years ago. If *G. salaris* was present on the relict population of salmon prior to the glaciation period, then two forms of *G. salaris* or morphotypes might be expected, one on Baltic salmon and the other on Atlantic salmon. However, no evidence to confirm this has been found. The lack of separation of the *G. salaris* in the sample sites situated 90km apart might suggest that either they originate from the same source and or that *G. salaris* has spread along the Kattegatt coast. Although, the Kattegatt represents a zone where the two stocks of salmon once

overlapped, it is problematical whether enough time has passed for the *Gyrodactylus* from the two populations in these rivers to have developed distinct variations which allow their separation by a model such as that used here. Halvorsen & Hartvigsen (1989) suggested that *G. salaris*, or its ancestor, may have been present in the refuge population which, following the glaciation, later repopulated both the East Atlantic and the Baltic.

Further evidence to support the hypothesis that all Scandinavian *G. salaris* originated from a source entering the Baltic, the spread of which has occurred only recently, is sought using chaetotaxy in Chapter 13. The low variability in sclerite form between the two sample sites, as shown by the PCA analysis, may possibly be explained by the movement of fish from the Baltic across Scandinavia.

In summary, the findings of this investigation, in agreement with those of several authors, are that the sclerites of *G. salaris* and the marginal hooks in particular, can be discriminated on the basis of seasonality (water temperature). In addition, isolated populations of *G. salaris*, sampled and analysed using PCA, could not be discriminated. It is considered that these results support the hypothesis that *G. salaris* has recently parasitised the salmonids in these regions, from an origin within the Baltic area.

Chapters 2-9 have sought to discriminate *G. salaris* on the basis of morphometric and multivariate analyses. Therefore, Chapters 10-13 will investigate non-computational techniques.

CHAPTER 10: A STUDY OF THE COMPOSITION OF THE SCLERITES OF *GYRODACTYLUS* NORDMANN, 1832 USING X-RAY ELEMENTAL ANALYSIS

Introduction

The precise chemical composition of monogenean sclerites still remains to be confirmed, despite tentative suggestions by Lyons (1966) and Kayton (1983). Lyons (1966) investigated the biochemical composition of sclerites of several monogeneans and proposed that the sclerites were of a distinct scleroprotein similar to that found in vertebrate wool (alpha keratin). The presence of keratin in invertebrate structures has been suggested in numerous papers (Brown, 1950; Krishanan, 1969; Hackman, 1971). Keratin was suggested to be present in the hooks of the cestode *Cysticercus longicollis* Rudolphi, 1810 (see Baron, 1968), *Echinococcus granulosus* Batsch, 1786 (see Gallagher, 1964), *Hymenolepis citelli* McLeod, 1933 (see Collin, 1968) and *Taenia crassiceps* Zeder, 1800 (see Mount, 1970). The presence of keratin in cestodes was confirmed in the studies of Dvorak (1969a,b) on *Hymenolepis microstoma* (Dujardin, 1845) and Swiderski (1973) on *Catenotaenia pusilla* Goeze, 1782. Crusz (1948) also showed evidence that species of the larval "genus" *Cysticercus* had a chitinaecous substance in their hooks. Kayton (1983) argued the case against its presence in *Gyrodactylus* or for its misidentification.

In this study, all the sclerites of three species of *Gyrodactylus* were freed from surrounding tissue using the methods devised in Chapter 6, allowing the composition of the rigid sclerites, such as the hamuli and the marginal hooks, to be compared with the flexible sclerites, such as the dorsal and ventral bars. In addition, the sclerites were further analysed and compared to ascertain whether the composition of each sclerite varies between species.

Materials and methods

The following species were examined: *Gyrodactylus salaris* Malmberg, 1957 on *Salmo salar* taken from the river Högvadsån, Sweden (Nov., 1991); *G. derjavini* Mikailov,

1975 Morph 1 from *Salmo salar* taken from a Stirlingshire fish farm (Jan., 1992); and *G. colemanensis* Mizelle & Kritsky, 1967 from *Salvelinus fontinalis* from Nova Scotia, Canada (June, 1991). The sclerotised hard parts of the three species of *Gyrodactylus* were recovered from whole parasites in batches of 20-30 for each species. If worms were alcohol-fixed, hooks were released by the digestion technique, or, if the worms were live, sonication was used as the preparative technique (these procedures are discussed in full in Chapter 6). Hooks were released and washed with distilled water before being dropped, in suspension, onto graphite stubs. These were cut from a graphite rod 10mm in diameter and made into discs 4-5mm in thickness; discs were then polished using emery paper and velin tissue. Prepared discs were allowed to air dry and were then mounted in groups of four onto a glass slide. Glass slides were then coated with amorphous carbon using a Nanotech Carbon coater (140mA, 120 MilliTorr, $2x10^{-4}$ Torr) depositing approx. 12 nm of carbon.

A number of standards were also produced to test alongside the sclerites of *Gyrodactylus*. These included samples of scleroproteins and chitins taken from known sources such as human nail, human hair, crab shell and purified chitin. Hooks were then analysed using an Hitachi S2500 / LINK AN10 / 55S system in which an energy dispersive X-ray analysis system is combined with a scanning electron microscope to allow the composition of samples to be determined. The system is such that elements below the atomic number 11 cannot be detected as they are absorbed by the X-ray detector window.

The process by which a quantitative analysis is executed involves the use of X-rays which are emitted in a beam approx 1.5 μm in diameter at an accelerating voltage of 15kV and used to bombard the specimen with electrons at an angle of incidence of 45°. The X-ray count was maximised by ensuring that a high count rate (over 600 counts per second) was observed, thus ensuring that the beam was analysing the specimen only, rather than background. Data for each portion of the sclerite were collected over a period of 50 seconds real time and the results analysed and processed by a 386-computer and printer in series.

During this analysis the relative proportions of elements in different portions of the sclerotised hard parts were determined, the relative proportions of each element being calculated for 100g of sample. It should be noted that the relevant

concentrations of each element were compared, but the values produced by the analysis are in part dictated by the thickness of the structure being analysed. This constituted a problem when taking measurements for the ventral bar membrane and making a comparison with the hamulus shaft, for example, because the X-rays burned through the membrane with the result that a certain percentage of the count was derived from the background. Secondly, certain parts of the sclerite cannot be analysed due to their small size, since the minimum sampling area is 2 μm in diameter; unfortunately, this prevented the study of the hamulus point, the dorsal bar and the shaft of the marginal hook. Due to some of the limitations imposed by the size of these sclerites, background counts were regularly taken from which "actual" hook compositions could be calculated.

The regions analysed by X-ray elemental analysis are shown in Figure 10.2. The relevant concentrations of the major elements were analysed using a statistical package, STPROG3 (J. Bron, University of Stirling, 1992). The data did not conform to a normal distribution and was, therefore, analysed using a nonparametric Dunn's test for any significant differences in elemental composition between the species of *G. colemanensis*, *G. salaris* and *G. derjavini* Morph 1.

Results

The X-ray analysis resulted in various peaks of X-ray intensity above the background noise such is shown in Figure 10.1. The composition of each sclerite is given separately.

For the marginal hooks, peaks were located at 1.0, 1.74, 2.02, 2.30, 2.61 and 3.68 keV (X-ray energies for each respective element). These peaks correspond to the elements sodium, silicon, phosphorus, sulphur, chlorine and calcium, with major peaks appearing for sulphur and calcium. The results for the three species of *Gyrodactylus* studied are given in Table 10.1.

Four regions of the hamulus were examined, the dorsal bar attachment point, the ventral bar attachment point, the hamulus root and the hamulus shaft (Figure 10.2). The results for these regions are given in Tables 10.2-10.5. X-ray analysis yielded additional peaks, to those detected in the marginal hooks, at 3.30 and 4.95 keV, which

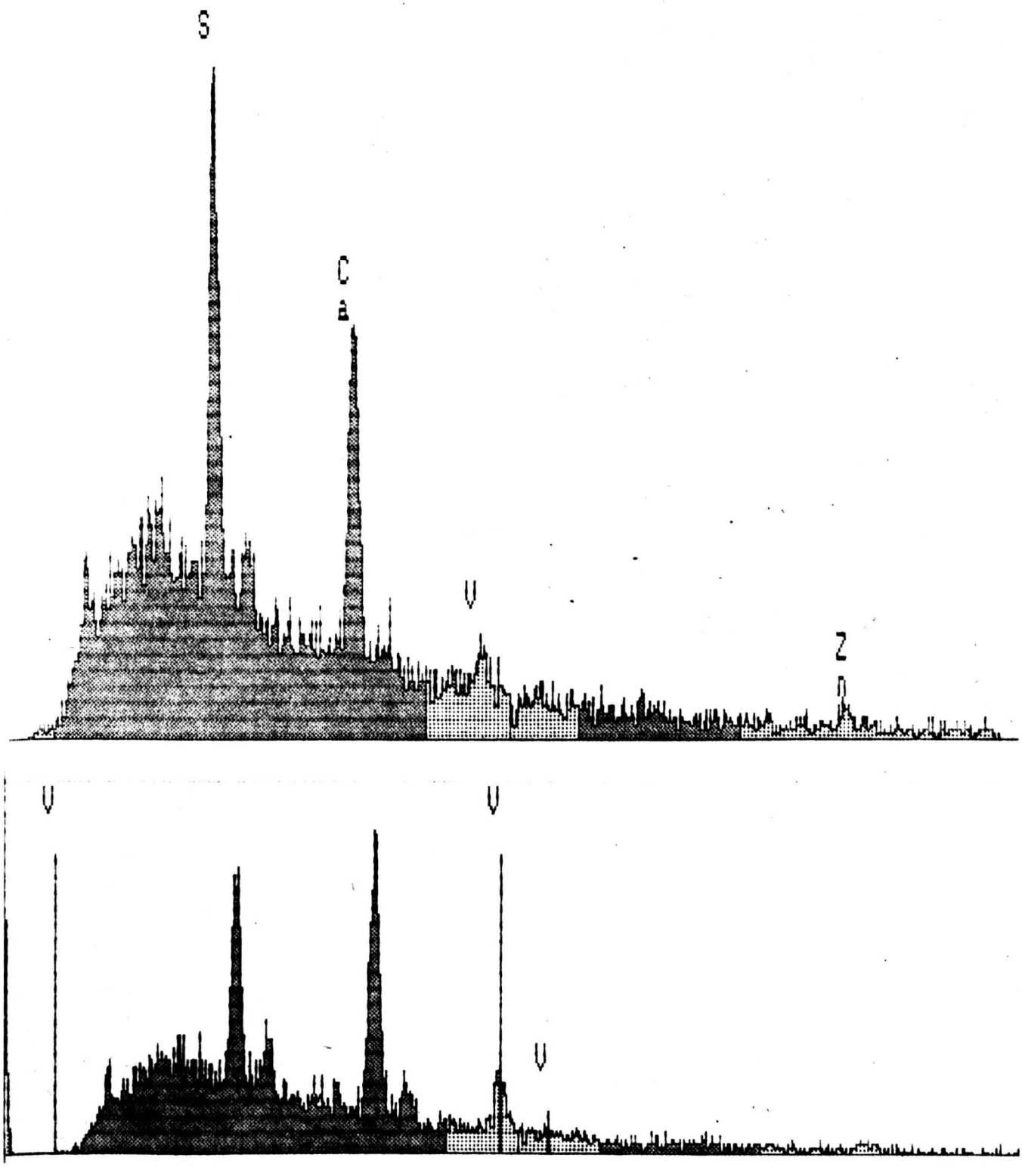


Figure 10.1: Elemental composition from *G. derjavini* ventral bar proper (middle). The appearance of the peaks vanadium (V) and zinc (Zn) are compared to sulphur (S) and calcium (Ca); the result for a second specimen (below) indicating the presence of vanadium.

Table 10.1: Elemental composition (g per 100g of tissue analysed) for the marginal hook sickle for three species of *Gyrodactylus*.

ELEMENT	*G. salaris*			*G. colemanensis*			*G. derjavini*		
	Mean (g) n = 5	SD	%	Mean (g) n = 4	SD	%	Mean (g) n = 5	SD	%
Sulphur	1.0728	0.3005	57.67	1.2768	0.1276	59.00	1.5956	0.5827	66.05
Phosphorus	0.0858	0.0197	4.61	0.0813	0.0118	3.76	<0.075	0.0041	3.10
Calcium	0.3062	0.1549	16.46	0.3420	0.0611	15.80	0.2560	0.1180	10.60
Chlorine	0.1282	0.0531	6.89	0.1828	0.0809	8.45	0.1896	0.0872	7.85
Silicon	0.0510	0.0091	2.74	0.0550	0.0034	2.45	<0.058	0.0034	2.40
Potassium	<0.062	0.0000	3.33	<0.062	0.0000	2.87	<0.065	0.0038	2.69
Sodium	0.0732	0.0155	3.94	<0.083	0.0006	3.84	0.0954	0.0178	3.95
Vanadium	<0.081	0.0000	4.35	<0.081	0.0000	3.74	<0.081	0.0000	3.35

% = Percentage of analysable portion.

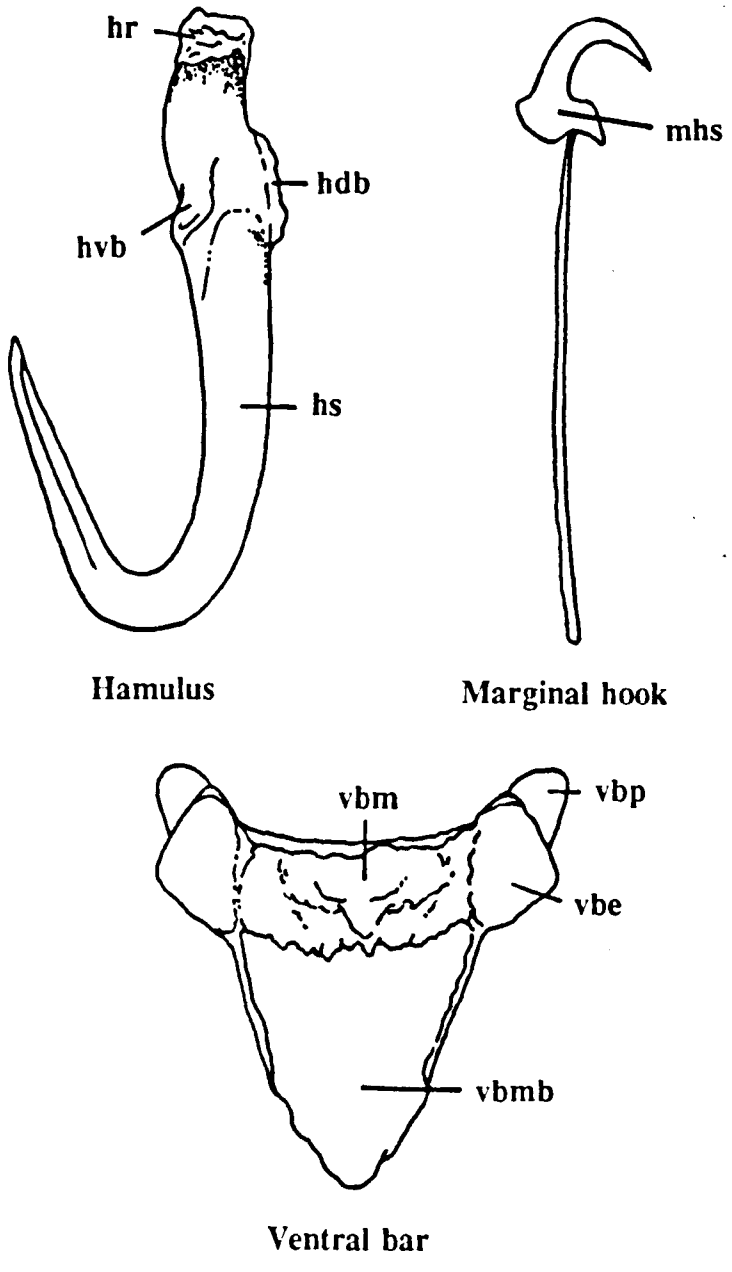


Figure 10.2: Schematic diagram illustrating the points of analysis on the sclerites of *Gyrodactylus*.

Abbreviations: hdb = hamulus dorsal bar attachment point; hr = hamulus root; hs = hamulus shaft; hvb = hamulus ventral bar attachment point; mhs = marginal hook sickle; vbe = ventral bar extremities; vbm = ventral bar middle; vbmb = ventral bar membrane; vbp = ventral bar processes.

Table 10.2: Elemental composition (g per 100g of tissue analysed) for the dorsal bar attachment point on the hamulus for three species of *Gyrodactylus*.

ELEMENT	*G. salaris*			*G. colemanensis*			*G. derjavini*		
	Mean (g) n = 5	SD	%	Mean (g) n = 5	SD	%	Mean (g) n = 5	SD	%
Sulphur	5.5772	0.8782	88.51	4.4814	2.0476	80.55	5.4874	1.7047	90.48
Phosphorus	0.1970	0.0320	3.13	0.1814	0.1808	3.26	0.0934	0.0067	1.54
Calcium	0.1564	0.0215	2.48	0.4486	0.3942	8.06	0.1302	0.0923	2.15
Chlorine	0.0684	0.0071	1.09	0.1956	0.1997	3.52	0.0834	0.0228	1.38
Silicon	0.0616	0.0067	0.98	0.0612	0.0059	1.10	0.0640	0.0024	1.06
Potassium	<0.062	0.0000	0.98	<0.068	0.0085	1.22	0.0757	0.0115	1.25
Sodium	0.0844	0.0112	1.34	0.1248	0.0746	2.24	0.1306	0.0499	2.15
Vanadium	0.0943	0.0231	1.50	0.0027	0.0027	0.05	0.0000	0.0000	0.00

% = Percentage of analysable portion.

Table 10.3: Elemental composition (g per 100 g of tissue analysed) for the ventral bar attachment point on the hamulus for three species of *Gyrodactylus*.

ELEMENT	*G. salaris*			*G. colemanensis*			*G. derjavini*		
	Mean (g) n = 5	SD	%	Mean (g) n = 5	SD	%	Mean (g) n = 5	SD	%
Sulphur	6.2908	1.0941	91.04	5.1496	1.0144	86.79	6.6700	1.1225	92.94
Phosphorus	0.2176	0.0971	1.61	0.0994	0.0252	5.17	<0.097	0.0033	1.25
Calcium	0.1110	0.0369	3.15	0.3070	0.0853	1.68	0.0895	0.0156	1.36
Chlorine	0.0698	0.0077	1.01	0.1232	0.0840	2.08	0.0948	0.0449	1.32
Silicon	0.0646	0.0051	1.36	<0.060	0.0033	1.77	<0.064	0.0013	1.28
Potassium	<0.062	0.0000	0.90	0.0896	0.0378	1.51	<0.069	0.0000	0.96
Sodium	0.0942	0.0127	0.93	0.1050	0.0317	1.00	<0.092	0.0073	0.90
Vanadium	0.0000	0.0000	0.00	0.0000	0.0000	0.00	0.0000	0.0000	0.00

% = Percentage of analysable portion.

Table 10.4: Elemental composition (g per 100g of tissue analysed) for the hamulus root for three species of *Gyrodactylus*.

ELEMENT	*G. salaris*			*G. colemanensis*			*G. derjavini*		
	Mean (g) n = 5	SD	%	Mean (g) n = 5	SD	%	Mean (g) n = 5	SD	%
Sulphur	5.2160	1.2451	80.00	2.5550	1.0120	69.87	3.1848	0.8936	82.88
Phosphorus	0.4230	0.1143	6.49	0.0808	0.0093	2.21	0.0834	0.0067	2.17
Calcium	0.5078	0.2195	7.79	0.4962	0.3434	13.57	0.1694	0.1014	4.41
Chlorine	<0.068	0.0080	1.04	0.2380	0.2112	6.51	0.1330	0.2112	3.46
Silicon	0.0656	0.0081	1.01	0.0610	0.0109	1.67	0.0938	0.0757	2.44
Potassium	<0.062	0.0000	0.95	0.0634	0.0031	1.73	0.0690	0.0000	1.80
Sodium	0.0874	0.0146	1.34	0.0814	0.0100	2.23	0.1092	0.0516	2.84
Vanadium	0.0900	0.0155	1.38	<0.081	0.0000	2.22	0.0000	0.0000	0.00

% = Percentage of analysable portion.

Table 10.5: Elemental composition (g per 100g of tissue analysed) for the hamulus shaft for three species of *Gyrodactylus*.

ELEMENT	*G. salaris*			*G. colemanensis*			*G. derjavini*		
	Mean (g) n = 5	SD	%	Mean (g) n = 5	SD	%	Mean (g) n = 5	SD	%
Sulphur	5.2522	0.2910	88.82	4.8670	1.5570	85.62	7.0012	0.8135	93.32
Phosphorus	0.1776	0.0492	3.00	0.1392	0.1098	2.45	0.0984	0.0032	1.31
Calcium	0.1180	0.0337	2.00	0.2578	0.1231	4.54	0.0970	0.0229	1.29
Chlorine	0.0686	0.0063	1.16	0.1116	0.0522	1.96	0.0724	0.0030	0.97
Silicon	0.0664	0.0119	1.12	0.0682	0.0179	1.20	0.0710	0.0129	0.95
Potassium	<0.062	0.0000	1.05	0.0712	0.0206	1.25	<0.069	0.0000	0.92
Sodium	0.0872	0.0063	1.47	0.0878	0.0264	1.54	0.0934	0.0057	1.24
Vanadium	<0.081	0.0000	1.37	0.0818	0.0018	1.44	0.0000	0.0000	0.00

% = Percentage of analysable portion.

Table 10.6: The elemental composition of the ventral bar processes of *G. derjavini* Morph 1.

ELEMENT	Ventral bar processes		
	Mean (g)(n = 2)	SD	Percentage
Sulphur	0.3085	0.1860	36.64
Phosphorus	0.0715	0.0007	8.49
Calcium	0.2240	0.0255	26.60
Silicon	0.0750	0.0212	8.91
Sodium	<0.075	0.0000	8.91
Potassium	0.0000	0.0000	0.00
Chlorine	0.0880	0.0000	10.45

% = Percentage of analysable portion.

Table 10.7: The elemental composition of the ventral bar proper (end) of *G. derjavini* Morph 1.

ELEMENT	Ventral bar proper		
	Mean (g) (n = 4)	SD	Percentage
Sulphur	0.6820	0.3607	31.24
Phosphorus	0.0758	0.0043	3.47
Calcium	0.7283	0.7721	33.36
Silicon	0.1130	0.0884	5.18
Sodium	0.2435	0.1280	11.15
Potassium	0.0930	0.0000	4.26
Chlorine	0.2475	0.0983	11.34

% = Percentage of analysable portion.

Table 10.8: The elemental composition of the ventral bar proper (middle) of *G. derjavini* Morph 1.

ELEMENT	Ventral bar proper (middle)		
	Mean (g) (n = 4)	SD	Percentage
Sulphur	1.0428	0.1579	35.37
Phosphorus	0.0795	0.0024	2.70
Calcium	1.0795	0.5985	36.62
Silicon	0.0635	0.0058	2.15
Sodium	0.3640	0.0472	12.35
Potassium	0.0695	0.0007	2.36
Chlorine	0.2493	0.0924	8.46

% = Percentage of analysable portion.

Table 10.9: The elemental composition of the ventral bar membrane of *G. derjavini* Morph 1.

ELEMENT	Ventral bar membrane		
	Mean (g) (n = 2)	SD	Percentage
Sulphur	0.1870	0.0368	31.04
Phosphorus	0.0705	0.0007	11.70
Calcium	0.1325	0.0092	21.99
Silicon	0.0565	0.0021	9.38
Sodium	0.0980	0.0184	16.27
Potassium	0.0000	0.0000	0.00
Chlorine	0.0580	0.0028	9.63

% = Percentage of analysable portion.

indicated the presence of low levels of potassium and vanadium.

The ventral bar is only liberated by sonication, the sonication technique which requires live material (Chapter 6) and, since *G. derjavini* Morph 1 was the only species available live at the time of the analysis, it was studied only in this species, as the digestion technique destroys this sclerite. An elemental analysis was performed on four regions, the ventral bar processes, the ends of the ventral bar proper, the middle of the ventral bar proper and the ventral bar membrane (Figure 10.2). Major peaks of X-ray intensity were located at 2.30 and 3.68 keV, representing sulphur and calcium respectively, in addition to minor peaks at 1.0, 1.74, 2.02, 2.61 and 3.30 for sodium, silicon, phosphorus, chlorine and potassium, respectively for all parts of the ventral bar. Additional peaks were recorded for zinc, 8.58 keV, for the middle of the ventral bar proper on a single specimen, and for vanadium, 4.95 keV, for the same region and also for the ventral bar extremities on several specimens (Figure 10.1). The precise composition of each portion of the ventral bar from *G. derjavini* Morph 1 is given in Tables 10.6-10.9.

The relative concentration of the two major elements revealed by the X-ray analysis study, sulphur and calcium, were analysed using Dunn's nonparametric test for differences in elemental loadings between species and within a species for each relevant portion of the hamulus and marginal hook.

G. colemanensis parasitising *Salvelinus fontinalis* in Nova Scotia was discriminated from *G. salaris* and *G. derjavini* Morph 1 on *Salmo salar* on both the calcium and sulphur content of various hamulus regions (Table 10.10). The elements listed in Table 10.11 were not significant, but had Q values close to the tabled values for rejecting Ho (Q 0.05 = 2.394).

Intraspecific differences identified the sulphur content of the ventral bar and the marginal hook in all three species as differing significantly. However, most of the observed differences for *G. salaris* were attributable to the calcium content (Table 10.12). For regions within a species, it appears that the marginal hook was differentiated from the other sclerites in most cases by the sulphur and calcium levels. The discrimination of the marginal hook sickle from other sclerite portions is believed to be a result of the relative thickness of each of the measured portions. The marginal hook is thinner; therefore, it is possible that part of the analysable portion by the X-

Table 10.10: Interspecific differences in the sulphur and calcium composition of three species of *Gyrodactylus*.

Species *vs* Species	Sclerite zone	Element	Sig.
G. colemanensis vs G. salaris	Hamulus root	Sulphur	P<0.05
G. colemanensis vs G. derjavini	Hamulus DB att. pt.	Calcium	P<0.05
G. colemanensis vs G. derjavini	Hamulus VB att. pt.	Calcium	P<0.05
G. colemanensis vs G. derjavini	Hamulus shaft	Sulphur	P<0.05
G. colemanensis vs G. derjavini	Hamulus shaft	Calcium	P<0.05

Table 10.11: Interspecific differences in the sulphur and calcium composition of three species of *Gyrodactylus* found not to be significant, but with Q values close to the value for rejecting Ho.

Species *vs* Species	Sclerite zone	Element
G. colemanensis vs G. salaris	Hamulus VB att. pt.	Calcium
G. salaris vs G. derjavini	Hamulus shaft	Sulphur
G. salaris vs G. derjavini	Hamulus root	Calcium

Table 10.12: Intraspecific differences in the sulphur and calcium compositions of different sclerites in three species of *Gyrodactylus*.

Species	Sclerite zone *vs* zone	Element	Sig.
G. colemanensis	Ham. VB att. pt. *vs* Marginal	Sulphur	P<0.05
G. salaris	Ham. VB att. pt. *vs* Marginal	Sulphur	P<0.005
G. derjavini	Ham. VB att. pt. *vs* Marginal	Sulphur	P<0.05
G. derjavini	Hamulus shaft *vs* Marginal	Sulphur	P<0.005
G. salaris	Ham. VB att. pt. *vs* Ham. root	Calcium	P<0.01
G. salaris	Ham. VB att. pt. *vs* Marginal	Calcium	P<0.05
G. salaris	Hamulus shaft *vs* Hamulus root	Calcium	P<0.05

Table 10.13: Intraspecific differences in the sulphur and calcium compositions of different sclerites in three species of *Gyrodactylus* found to be not significant, but with Q values close to the value for rejecting Ho.

Species	Sclerite zone *vs* zone	Element
G. colemanensis	Hamulus shaft *vs* Marginal hook	Sulphur
G. salaris	Marginal *vs* Hamulus DB att. pt.	Sulphur
G. salaris	Hamulus shaft *vs* Marginal hook	Calcium

ray beam is lost to the background. The elements given in Table 10.13 were not significant, but had Q values close to the tabled values for rejecting Ho (Q 0.05 = 2.807).

Discussion

The X-ray analysis beam measures an area 1.5 μm in diameter; however, the elemental composition data tend to be influenced by specimen thickness and this, therefore, can pose a problem when comparing the relative proportions of the elements in the various regions of the sclerite. This was particularly relevant to the ventral bar, for in cases such as the ventral bar proper, the beam would record a count based on sclerite material alone, whereas in others, such as the membrane which is very thin, background interference contributed to the count. The proportion of the ventral bar proper analysable by the beam (elements higher than the atomic number 11) was 2.948% (sum of the means), whereas the membrane contained only 0.603% of the analysable elements. This does not necessarily infer that the membrane has a higher organic (C:N:O:H) component, which in comparison represents only 20.4% of the elements detected for the ventral bar proper, since it is an artefact caused by structural thickness. Direct comparison between these zones was, therefore, not possible. On the other hand, structures of comparable thickness or deeper than the area analysed by the beam can be compared, i.e. the marginal hooks and the hamuli.

The nature of the *Gyrodactylus* sclerite structure has also to be considered. Monogenean sclerites have developed from a number of origins. The concentric rows of spines on the pseudohaptor of *Acanthocotyle lobianchi* Monticelli, 1888 and *Diplectanum aequans* (Wagener, 1857) Diesing, 1858 are hollow and believed to be of epidermal origin (Shaw, 1981). Some hooks become modified, adapting in shape with the different stages of the life-cycle; for example, the post-oncomiracidial development of the posterior hooks on the terminal lappet of *Gastrocotyle trachuri* Beneden & Hesse, 1863, which was suggested by Llewellyn (1963) to be an adaptation to the host surface. Similarly, clamp structures in higher monogeneans arise as a post-larval feature; these were found by Ramalingam (1973) to contain dityrosine. Previous work by Shinn (unpublished) found monogenean sclerites to have a

secondary organisation; using specimens of *Entobdella*, *Calicotyle*, *Kuhnia*, *Gastrocotyle*, *Gyrodactylus* and *Amphibdella*, it was revealed that the hamuli were composed of two or more layers. Birefringence studies using the polarising microscope showed that there is a sheath of retardation of 500 nm protecting the hamulus point, whilst the region underlying this was in the order of 450 nm. These two regions under light microscopy are estimated to yield a combined lamina 6 μm thick in *Entobdella soleae*. A study of the marginal hooks of *Gastrocotyle* revealed, however, only a single layer of retardation of 500 nm. This was confirmed by serial sectioning in the case of the hamuli, showing a thickened lamina and central core. Similarly, Collin (1968) showed the hooks of *Hymenolepis citelli* McLeod, 1933 to be composed of three layers: an outer granular layer extending from the base; a fibrous central portion of electron dense material; and a central core of laminated crystalline material.

The presence of sulphur indicates a keratin-like substance which is resistant to the action of proteolytic enzymes, a property which is utilised in the extraction of the hamuli and marginal hooks (Mo & Appleby, 1990; Chapter 6). The presence of a keratin-like protein in the sclerites was shown by Shinn (unpublished), using a mercaptoethanol buffer (5% 2-mercaptoethanol, 15% glycerine, 1% SDS and 0.0625M Tris buffer, pH 6.8) which is used to disrupt keratin. Mercaptide-forming compounds, even in low concentrations and mild conditions, are highly selective and show a great affinity for SH groups. The effect of temperature on mercaptoethanol was shown by Dvorak (1969a) to influence the rate of reaction. At 70°C there is a gradual dissolution of the root portion of the sclerite with the shaft gradually turning brown.

An investigation by Kayton (1983) on a species of *Gyrodactylus* parasitising Utah chub *Gila atraria* Girard (Cyprinidae) utilised X-ray elemental analysis and revealed that the hamuli had significant amounts of sulphur, potassium and calcium. In Kaytons account, it appeared that part of the apparent elemental composition of the hamuli had been derived from the tegument surrounding the hamuli, through which only the points emerge, as a result of the preparative technique used. In the present study, the principal analysable elements composing the marginal hooks and hamuli appear to be sulphur and calcium. Potassium was rather poorly represented in relation to the other elements in these structures (less than 3.0% of the analysable portion). Although it is possible that a part of the marginal hook count may have suffered from

interference from the background, 1.86-2.42% of the total composition was still analysable by the X-ray system. When this fraction is broken down into the contributions made by each element, it is found that sulphur constitutes 57.67-66.05% and calcium 10.6-16.46% of the measurable portion.

The hamuli present a clearer picture, since the X-ray analysis was able to determine 5.68-7.50% of the total hamulus composition. Although the contribution of sulphur was proportionally higher in the hamuli than in the marginal hooks, the concentration varied when different regions of the hamulus were compared. It was found that the root portion of the hamulus had the lowest sulphur content of 69.87-82.88% (Table 10.4) compared to the shaft, ventral and dorsal bar points which had higher sulphur contents of 80.55-93.32%.

The differential sulphur content may be correlated to the different function of each portion of the hamulus. The hamuli are slightly bilaterally flattened, with sclerite fibres being laid down parallel to the longitudinal axis of the hook. The deposition of sulphur-based proteins within this plane constitutes a resilient system able to endure the forces stressed upon it. The distribution of these proteins, therefore, reflects the orientation and strength of these forces. Furthermore, histological transverse sections through the hamuli of *E. soleae* (Shinn, unpublished) have revealed the flanks to be thickened, thus reinforcing the sclerite along its length. The forces stressed upon the hamuli are the action of the opisthaptoral muscles pulling on the root portion of the hamulus and the resistance of the host epidermis acting at the point of the hamulus. The presence of reinforced flanks on the hamuli would, therefore, transfer the forces to which it is subjected towards its extremities down its entire length, thus spreading the force. It is not surprising, therefore, that there appears to be a link between mechanical strength and sulphur concentration.

The ventral bar attachment point arises from the shaft as a flattened region. The precise nature of this point of articulation between the shaft and the ventral bar is still in question, but the ventral bar processes possibly have some connection with the folds that have been observed on the hamuli at this point (Malmberg, 1970). The dorsal bar attachment point, however, represents a true connection. Chapter 6 has shown that the dorsal bar is linked to a cap located on the inner surface of the hamulus, a structure lost during proteolytic enzyme digests. This point represents the

transition from the hamulus shaft to the root; the root of the hamulus is shown to have a slightly lower sulphur component. The lower sulphur content is, however, replaced by a higher calcium fraction, which may add to strengthening the sclerite in the absence of sulphur. Since the point and shaft of the hamulus have already achieved their final configuration at birth, growth and age-related changes occur only within the root, a zone shown to have high morphological variability (Mo, 1991a,b,c). Differences in the sulphur concentration of the root may, therefore, be attributable to the deposition of pre-keratin which would subsequently undergo consolidation, hardening and transformation into keratin.

The minimum analysable area covered by the X-ray beam unfortunately prevented the study of the hamulus point, but it is believed that this region would yield a zone with an even higher sulphur content. Dennell (1960) stated that sclerotised protein is found most abundantly in areas of cuticle that are subject to abrasion. This is likely to be true for the hamulus point, since this is the only portion that emerges from the body of the worm and must, therefore, constitute a resistant system in a hostile environment, i.e embedded in host tissue as an attachment mechanism and able to withstand the shearing forces stressed upon it.

G. colemanensis and *G. derjavini* Morph 1 appear to have the greatest differences in terms of the calcium composition of the dorsal bar attachment point, ventral bar attachment point and shaft regions of the hamulus, and of the sulphur composition of the shaft region. *G. salaris* could be differentiated from *G. colemanensis* on the basis of differences in the sulphur loadings of the hamulus root. However, although not statistically significant, *G. salaris* did appear to differ from *G. derjavini* Morph 1 (Q values Dunn's test) in its shaft sulphur and root calcium levels, and from *G. colemanensis* in the calcium content of the ventral bar attachment point of the hamulus.

The observed statistical differences between species may be explained by a combination of several factors. Firstly, elements incorporated into the sclerite depend upon the composition of the body fluids of *Gyrodactylus*, which in turn depend upon the parasite's feeding or absorption from the environment. Secondly, the deposition of elements depends upon the environment, in this case water and/or fish mucus, and, therefore, differences between species may be due to differences in the ionic

composition of the parasite's microhabitat. Thirdly, the rate of reproduction is related to temperature, and different water temperatures may be related to differential growth (Mo, 1991a) and thus differential rates for the incorporation of elements into the sclerites. A low water temperature would permit a longer period for sclerite development and all precursors may be drawn from the body or, alternatively, a longer period may allow for the accumulation of elements to measurable and significant levels. In contrast, at high water temperatures, there is an increased rate of reproduction and smaller sclerites; these sclerites may accumulate elements from the environment into the protein complex prior to the hardening of the pre-keratin. Furthermore, a low water temperature influences parasite longevity, the longer survival allowing a greater period of time for elements to be accumulated from the environment.

Ratios of certain elements (Mg, Ca, Sr, Ba) have been used to estimate both ionic ratios of the external medium and temperature in the determination of evolutionary relationships between animals, palaeoclimatology and oceanic history (Dodd, 1967). Although information relating to water temperature is lacking for the samples of *G. salaris* from Sweden and *G. colemanensis* from Nova Scotia, it may be assumed that water temperature may be involved in the observed statistical differences. The collection of *G. colemanensis* in summer could be discriminated from *G. salaris*, and especially from *G. derjavini* Morph 1 collected from the colder months on the basis of differences in the sulphur and calcium content of the hooks. There were no significant differences between the two species, *G. salaris* and *G. derjavini* Morph 1, collected from populations of Atlantic salmon in similar water temperatures. However, the sulphur content of the hamulus shaft and the calcium content of the hamulus root for *G. salaris* and *G. derjavini* Morph 1 gave Q values close to the tabled significant levels when analysed using Dunn's test. If there is a real difference between the two species, it may also be accounted for by the age of the parasite at the time of examination or differences in the local water composition. Baron (1968), working with the cestode *Taenia crassiceps* Zeder, 1800, proposed the hypothesis that, during development, cestode hooks elongate, become hollow and then undergo secondary thickening to assume their final shape. Crusz (1948) believed that this secondary thickening was developed as an external deposition via a hypertrophied tegumentary

layer surrounding the hook.

The significance of sulphur has been discussed above. The presence of -S-S- and -SH bonds produced during the process of exoskeletal structural hardening results in the formation of rigid structures resistant to attack from proteolytic enzymes (Brown, 1950). Lyons (1966) concluded that the haptoral sclerites, principally the hamuli of monogeneans, are comparable to vertebrate α-keratin, which, according to Stryer (1981), contains significant amounts of tryptophan. However, this contradicts the findings of Ward & Lundgren (1954), whose examination of human epidermis and hair, as examples of vertebrate α-keratins, indicated low levels of tryptophan in comparison to other amino acids. Kayton (1983) tenuously argued for the presence of a collagenous protein rather than a keratin on the evidence of Lyons' (1966) finding that tryptophan was lacking in the hamuli of *Entobdella soleae*. This was confirmed by *E. soleae* having a 10-20% glycine and a 3-6% proline content, which, according to Stryer (1981), is more characteristic of a collagen, in the form of β-keratin; but, although this has comparable amounts of glycine, it lacks proline. However, certain α-keratins do contain proline; for example, the epicuticle of wool and hair - glycine 0.7, proline 2.5 (figures given as g per 100g protein) (Golden, 1954); wool and hair cuticle - glycine 4.0, sulphur 5.9, proline 8.5 (Ward, 1952); wool cortical membrane - glycine 3.2, proline 5.6 (Golden, 1954); and spindle cells - proline 9.1, sulphur 7.5 (Lindley, 1947, 1948). Furthermore, the values given by Lyons (1966) were estimations derived from amino acid chromatograms from samples which would be composed of hamulus cortex as well as the hamulus sheath, the area of real interest.

The sclerites of *Gyrodactylus* are also notably rich in calcium. Kayton (1983) believed the ventral and dorsal bars to be different in composition to the hamuli and marginal hooks, and Mo & Appleby's (1990) use of proteolytic enzymes to release the sclerites of *Gyrodactylus* failed to recover these bars. Although they still contain a large sulphur component, their calcium content is considerably greater than that of the hamuli and marginal hooks, ranging from 22% in the ventral bar membrane to 37% in the ventral bar proper. The high sulphur content of the marginal hook and hamulus which constitutes its rigid structure is replaced by a combination of calcium and sulphur. Neville (1975) stated, in relation to the pro-cuticle of crustaceans, "the more calcium, the less protein."

The function of the ventral bar differs somewhat from that of the hamuli. The ventral bars maintain the hamuli in a position which aids the worm's attachment to the host. The primary function of the hamuli and marginal hooks is attachment and these have been adapted to achieve mechanical strength. In the sclerites of *Entobdella soleae* the molecules are laid down parallel to the long axis of the hook, the fibres constituting the point having rotated through 12°; this produces a resilient system (Shinn, unpublished). The ventral and dorsal bars are able to flex, and have been developed to maintain the position and effectiveness of the hamuli. It is suggested, that the use of a strong calcium chelating agent, such as EGTA, may be used to remove some of the calcium portion and examine the structural architecture beneath. Calcium as a structural component of the ventral bar might possibly suggest that the ventral bar is a form of chitin.

The ventral bars also appear to contain significant quantities of the following ions: chloride, 8.5-11.3%; sodium, 8.9-16.3%; phosphorus, 2.7-11.7%; silicon, 2.2-9.4%; and potassium, 0.0-4.3%. Chloride, sodium and potassium ions are ubiquitous in the body, serving a role in the ionic and osmotic balance of tissues. Roche *et al.* (1963) considered that chloride has an effect on the rate of some of the enzymatic processes, membrane phenomena and the swelling of colloids.

It is interesting to note that portions of the ventral bar of *G. derjavini* Morph 1 and the hamuli of *G. salaris* and *G. colemanensis* contains the element vanadium. The presence of this element is unusual, especially when the amount incorporated into the sclerites is considered and with no background levels of vanadium detectable. The significance of its presence is unknown; however, the blood of a tunicate, *Phallusia mammillata* (Cüvier) (Ascidiacea), has been found to yield large quantities of vanadium in the pigment (approx. 18.5% as V_2O_5) as do many other ascidians (Vinogradov, 1953). Henze (1932) suggested that vanadium present in the blood pigment is a divalent compound acting as a hydrogen donator. George (1930) found the blood of some ascidian cells, called vanadocytes, to contain vanadium. He believed that vanadium is bioaccumulated from silt during the process of freshwater filtration. In addition, the blood of the genus *Amphioxus* contains a vanadium complex rather than a porphyrin, as in haemoglobin of other vertebrates.

Gyrodactylus is not unique within the phylum Platyhelminthes in possessing

different types of sclerites that vary in their chemical nature. Llewellyn's (1970) account of monogenean evolution stated that the early polyopisthocotylean retained its 16 peripheral marginal hooks, but marginal hook pair 1 underwent modification to lose its domus and at the same time four lappets became modified to form gripping lamellae. According to Lyons (1966), the latter attachment organs possessed a skeletal framework of two types, a keratinised proteinaceous component stabilised by disulphide bridges and a pair of transversely placed non-keratinised peripheral sclerites. The monopisthocotylean genus *Chimaericola*, which possesses 12 marginals, having modified marginal pair 1 and a pair of hamuli during its evolutionary development, appears to have given rise to two families: the protodicylobothriids which retained the keratinised median sclerite but lost the peripherals, and the protomazocraeids which lost the keratinised sclerite but maintained the non-keratin peripherals (Llewellyn, 1970). However, Lyons (1966) stated that the clamp structures of various monogeneans were devoid of sulphur, as are the accessory bars (ventral and dorsal bars) of the dactylogyrid *Amphibdelloides maccallumi* (Johnston & Tiegs, 1922) Price, 1937 and the gyrodactylid *G. elegans*, but this study has shown that the accessory bars do indeed contain a sulphurous component.

Although this study has shown that there is a sulphurous component of the ventral bar in *Gyrodactylus*, it raises the question of whether its presence in this structure represents new evidence relevant to the theory of Llewellyn. That is, does *Gyrodactylus* represent a point in evolution where the sclerites are at an intermediary stage between the heavy sulphur-bearing hamuli and the sulphur-free clamps (according to Lyons, 1966)? However, although the accessory bars have a sulphur component, they are weakly keratinised, having calcium as the major compositional element. Although the nature of scleroproteins and their chemical composition is variable in nature, the bars still appear to represent a problem in terms of identifying their composition. *Gyrodactylus* may be an intermediate link in evolution, in that it represents an adaptation developed to enhance function, exchanging the loss of sulphur for an increase in calcium to maintain a rigid structure. It is possible that the hooks of the monopisthocotyleans have possibly shifted away from the sulphur bearing and keratin based sclerites to develop hooks of a different composition, such as the sulphur-free clamps and the chitin-like accessory bars of *Gyrodactylus*. If this is true,

then *Gyrodactylus* may well represent a genus at a junction in phylogenetic terms close to the base of the polyopisthocotylean line.

CHAPTER 11: A NOTE ON THE MECHANISM OF ATTACHMENT IN SPECIES OF THE GENUS *GYRODACTYLUS* NORDMANN, 1832.

Introduction

The mode of attachment has been discussed for several monogeneans (Llewellyn, 1956a,b, 1957, 1958; Kearn, 1964, 1966; Paling, 1966), including species of *Gyrodactylus* Nordmann, 1832 (Lester, 1972; Cone & Odense, 1984; Cone & Wiles, 1989). In the latter papers, however, the emphasis has been on the placement of the hooks with respect to its host rather than on the mechanics of attachment and the contribution of each of the sclerites to this process. *Gyrodactylus* represents a monogenean genus which, although it has possessed two accessory bars, the ventral and dorsal bars, throughout its evolutionary history, it retains the larval characteristic of using the 16 relatively large marginal hooks as the principal mode of attachment, whilst the two larger hamuli rarely penetrate the host epidermis. The role of the hamuli and the accessory bars are herein considered in relation to the marginal hooks and the relationship between sclerite function and their elemental composition, as outlined in Chapter 10, is commented upon.

Materials and Methods

Scanning electron micrographs of the opisthaptoral sclerites from several species of *Gyrodactylus* Nordmann, 1832 released by the sonication technique, outlined in Chapter 6, were examined.

Observations

The development of techniques to liberate the sclerites from the body tissue of *Gyrodactylus* permitted a closer look at their structure (Mo & Appleby, 1990; Chapter 6). Subsequent examination of these sclerites by X-ray elemental analysis in order to determine their chemical composition has enabled some comment on their possible origin (Chapter 10). The major elements in the ventral bar analysable by X-ray

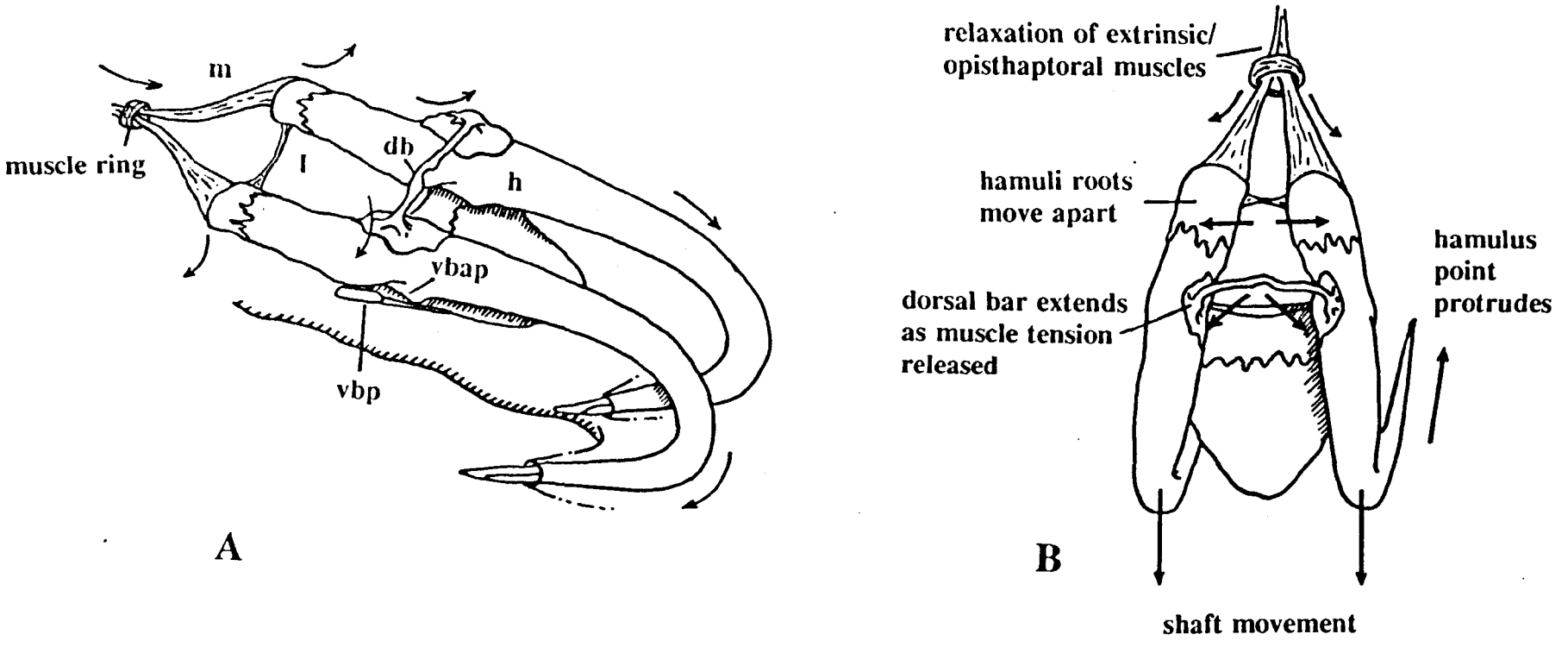
analysis were found to be calcium and sulphur, which accounted for a total of 80% of the analysable elements. The relationship between the large calcium content and its function was discussed in Chapter 10. The information so far indicates that the ventral bar controls the alignment of the hamuli and maintains them in a position such that they can contribute to the attachment of the worm. Secondly, the position of the ventral bar beneath the hamuli acts as a plate protecting the tegument from damage by spreading the downward force of the hamuli and the overlying opisthaptoral muscles (Cone & Odense, 1988). Finally, the large calcium component of the ventral bar may form part of the skeletal structure, having replaced some of the sulphur. The dissolution of this structure by proteolytic enzymes confirmed its differing chemical composition from heavily sclerotised hamuli and marginal hooks, i.e. it is not keratinaceous. The calcification of the ventral bar by the internal deposition of calcium in order to produce a rigid structure anchored firmly in the opisthaptor, raises the question of how it functions in relation to the hamuli. The ventral bar extends posteriorly under the hamuli, and the lateral margins of the membrane are thickened, possibly to prevent the membrane from being damaged during the process of hook movement. The extent of sclerotisation in the membrane differs from one species to the next; however, this membrane is further strengthened by irregular ridges as seen in the ventral bars of *G. arcuatus* (see Chapter 6, Figure 6.7(F)) or as a medial ridge in *Gyrodactylus* sp. Morph 1 from rainbow trout (see Chapter 6, Figure 6.9(H)).

Studies with the SEM (Chapter 6) have highlighted regions of morphological adaptation on the hamuli. Two of these, the dorsal bar attachment point and the ventral bar attachment point, were seen as oval, flattened regions located *in situ* ventrally on the hamuli. The ventral bar attachment point rises obliquely to the longitudinal axis of the hamulus such that the lower edge of this region occurs at the junction between the root and shaft and extends in the direction of the hamulus point. Chapter 6 indicated that, although the ventral bar obviously locates with this region and the ventral bar processes may possibly have some muscle association, either with this region or with the neighbouring folds occasionally observed on the hamuli, the precise function of the association between these structures is not fully understood. The ventral bar attachment point represents the point of rotation, since the position of the hamuli is affected by the relaxation and contraction of the opisthaptoral peduncle

muscles pulling the hamuli at their attachment points (the hamulus muscle caps) over the fixed ventral bar, i.e. over its processes. The manner in which the ventral bar processes act as runners, and possibly correspond closely to observed folds in the hamuli, is illustrated in Figure 11.1-11.2.

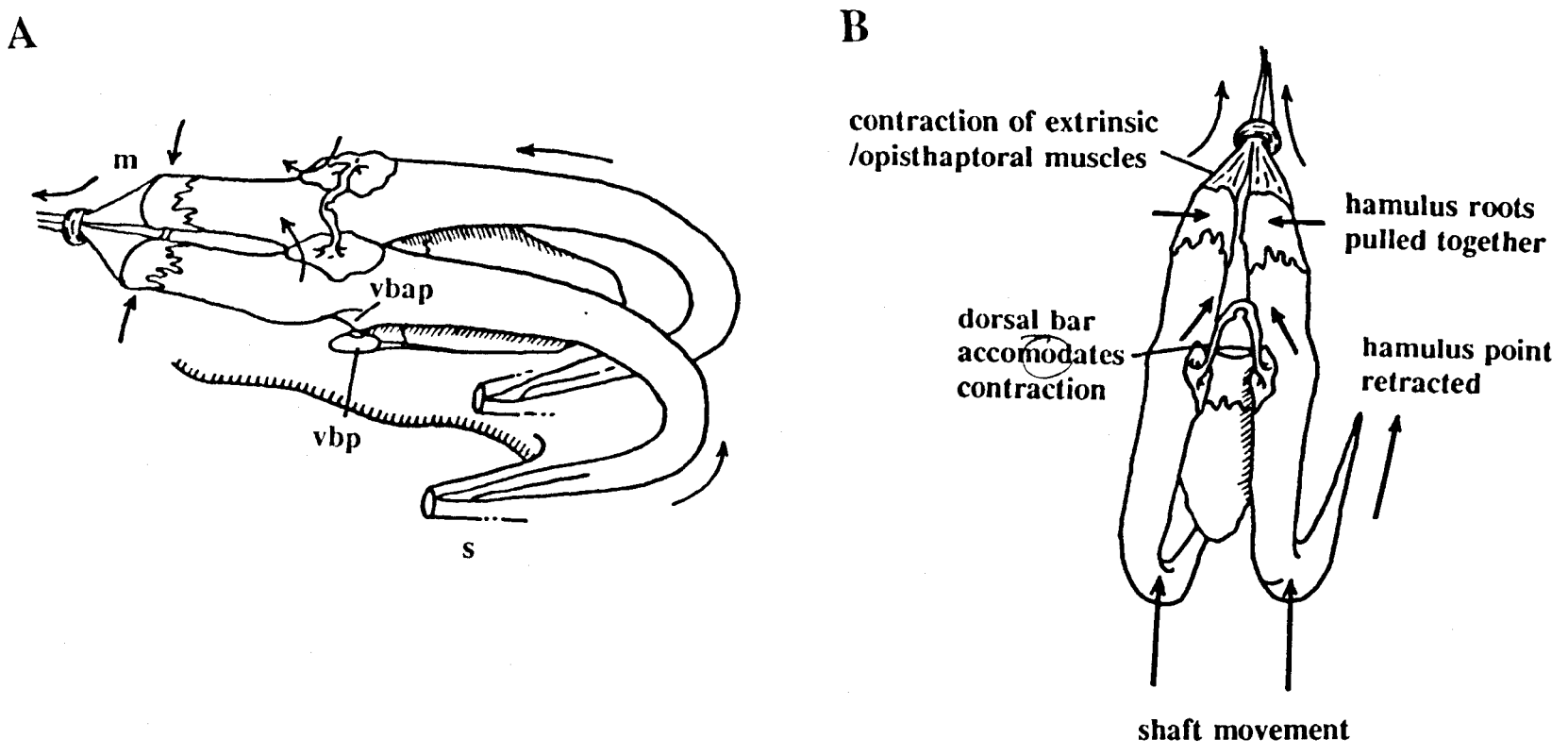
In species, such as those in the subgenus *G. (Gyrodactylus)*, where the ventral bar processes are lacking or are considerably reduced, their function may be served by the extremities of the ventral bar proper. Relaxation of the opisthaptoral peduncle muscles allows the hamulus point to move anteriorly and ventrally from its position in the tegument, such that the ventral bar processes are situated at the apex of the ventral bar attachment point. The apex is defined as the edge of the ventral bar attachment point closest to the root portion of the hamulus and is *in situ* dorsal to the base of the ventral bar attachment point and the origin of the hamulus shaft.

The dorsal bar, which is flexible, compensates for the action of the opisthaptoral muscles on the hamuli. The actual chemical nature of the dorsal bar is unknown, its analysis being prohibited by its structural size and the limitations imposed by the X-ray analysis beam (Chapter 10). Kayton (1983) tested the accessory bars of *Gyrodactylus* using 2,2'dihydroxy-6,6'-dinaphthyl disulphide, specific for sulphuryl groups, and performic acid alcian blue, specific for sulphur-bearing amino acids, with negative results; but he concluded that they were of similar composition. Lyons (1966) also found the accessory bars of certain monogenean genera to be devoid of cystine sulphur. It is believed, however, that the sulphur component of this structure will, like the ventral bar, prove to be low, resulting in a loss of rigidity in favour of an increase in flexibility. The dorsal bar appears to be able to relax or extend, adapting as the hamuli extend and protrude through the tegument (Figure 11.1a-b), and also possibly act as a guide. When the peduncle muscles are contracted, the dorsal bar flexes (Figure 11.2a-b), compensating for the hamulus roots being drawn together, and the hamuli are retracted. As they are drawn back across the ventral bar processes the hamuli are pulled dorsally and the oblique surface of the ventral bar attachment points causes them to move upwards. Although, this flattened region is small, its association with the ventral bar processes acts as a pivot; its position at the base of the hamulus shaft causes a rotation of several degrees which is transmitted through to the hamulus point. This movement is sufficient to extend and



EXTRUSION OF HAMULI

Figure 11.1: The mechanism of hamuli extrusion. A (side view) and B (overhead view) illustrating the ventral bar processes (vbp) situated at the apex of the ventral bar attachment point (vbap), such that the hamuli are exposed.



RETRACTION OF HAMULI

Figure 11.2: The mechanism of hamuli retraction. A (side view) and B (overhead view) illustrate the contracted opisthaptoral muscles (m) which pull the hamuli (h) back within the sheath (s), such that the ventral bar processes are at the base of the ventral bar attachment point.

retract the hamulus point from its protective sheath.

Discussion

The muscle system associated with the hamuli of *Gyrodactylus* operates, not as a mechanism to aid the penetration of the hamuli, but as a means of disengaging the sclerites from the host's surface (Kearn, 1966). In most monogeneans the marginal hooks represent the main attachment mechanism in the larval stages; however, as the adult develops, the hamuli develop to become the principal attachment mechanism (Llewellyn, 1963). The comparatively large marginal hooks of *Gyrodactylus*, however, retain this essentially larval function throughout life. The marginal hooks have been observed in *G. colemanensis* Mizelle & Kritsky, 1967 to pierce a host epidermal cell and anchor to an aggregation of microfilaments, becoming firmly attached (Cone & Wiles, 1989). The force of the attachment of these 16 marginal hooks is such that the combined force generated is able to push the hamuli either into the epidermis or compress the underlying epidermis (Lester, 1972). Although, the principal attachment mechanism is the penetration of the marginal hooks, it is feasible that a small component may be contributed by the hamuli. The hamuli of *Entobdella soleae* van Beneden & Hesse, 1863 are raised vertically by the contraction of extrinsic muscles pulling on the accessory sclerites, such that the association of the accessory sclerites with the hamuli embedded in the ventral surface of the opisthaptor are lifted to produce a suction pressure. The efficiency of suctorial attachment is maintained by the marginal hooks which prevent the inward migration of the opisthaptoral margins (Kearn, 1964). The hamuli of *Gyrodactylus*, fixed firmly in a single plane by the combination of the dorsal and ventral bars, constitute a box-like configuration, giving a rigid structure. The combined effect of the 16 marginal hooks exert such a force of attachment that the edges of the opisthaptor are compressed tightly against the epidermis of the host, to the extent that the hamuli may penetrate. Extrusion of the hamuli or relaxation of the opisthaptoral muscles may, therefore, following marginal hook attachment, lift the ventral surface of the opisthaptor, further adding to the efficiency of attachment by inducing a suction pressure. The hamuli are able to adapt to attachment by rotation about the ventral bar attachment point on the ventral bar

processes, as discussed above. Contraction of the opisthaptoral muscles would, therefore, retract the hamuli, lowering the opisthaptor and reducing the force of attachment.

CHAPTER 12: THE DETERMINATION OF PROTEIN PROFILE IN THREE SPECIES OF *GYRODACTYLUS* (MONOGENEA) PARASITISING SALMONIDS.

Introduction

The elucidation of protein profiles has made a vital contribution to the discrimination of helminth species, especially in instances when there is intraspecific variation in morphological characters or when the generic allocation of species is dubious, e.g. schistosomes (Southgate & Knowles, 1975), amphistomes (Southgate *et al.*, 1985, 1989), the Diplostomidae (Brady, 1989), ascaridoid worms (Bullini *et al.*, 1986) and gyrocotylids (Bristow & Berland, 1988). In addition, gas chromatography has successfully been employed when attempting to acheive species specific fatty acid profiles for the nematode genus *Philometra* (Juhasz & Molnar, 1987, 1988) and a gyrocotylidean species (Berland *et al.*, 1990). There appear to be no records of such work having previously been carried out on monogeneans of the genus *Gyrodactylus* von Nordmann, 1832.

Both electrophoretic and chromatographic techniques depend on relatively large amounts of tissue which are devoid of host contamination. The abundance of gyrodactylid parasites alleviates the problem of obtaining sufficient material; however, there are inherent problems of host contamination. *Gyrodactylus* inhabits the mucus layer of its host's epidermis, its body being coated with a biofilm of mucus. Prior to analysis it is necessary to remove as much mucus as possible. Secondly, parasites removed directly from the host often take with them a plug of host epidermis held within the haptor which may affect the results of the analysis. The third source of contamination is from the parasite's gut contents, which contain host epidermis, and this source is the most difficult to eliminate.

The use of SDS-PAGE gel electrophoresis was investigated to ascertain whether it was possible to obtain an overall protein profile, indicating the size of the major proteins present. Secondly, Western blots were used to determine whether it was possible to raise *G. salaris* species specific antibodies in rabbits and to identify the proteins raised. Thirdly, Isoelectric focusing (IEF), superceeding gel electrophoresis

Thirdly, Isoelectric focusing (IEF), superceeding gel electrophoresis because of its improved sensitivity, resolution and reproducibility (Arbuthnott & Beeley, 1975), was also tested. IEF has the advantage of requiring smaller aliquots to run gels, with isoenzymes being separated through a pH gradient, moving to their respective isoelectric points. Subsequent histochemical staining shows high specificity for the isoenzyme under test, reducing the possibility and ambiguity of non-specific staining.

Materials and methods

Sample collection

A variety of gyrodactylid species were obtained as follows: specimens of *Gyrodactylus* spp. were collected from koi carp *Carassius auratus* L. (tanks held at the Institute of Aquaculture, University of Stirling) and from Arctic charr *Salvelinus alpinus* (*Gyrodactylus* Morph 6) from Lake Ennerdale, the Lake District, England, and samples of *G. salaris* were obtained from Atlantic salmon *Salmo salar* from the River Drammen, Tromso, Norway. Live specimens were collected, from fish which had been anaethetised with Chlorbutanol (stock solution: 200g β,β,β-trichloro-tert-butyl alcohol dissolved in 1l 96% ethanol; working solution: 4 ml stock solution dissolved in 2l dechlorinated distilled water). They were then washed in distilled water, and batches of 20 individuals were kept in a minimal volume of double-distilled water (approx. 20 µl) and frozen and stored at -70°C in liquid nitrogen. Samples of *G. salaris* were collected in Norway, placed into a reducing buffer containing 10% v/v 2-mercaptoethanol (see Appendix 1) and boiled for 5 minutes prior to transporting to the UK (sample provided by Dr. K. MacKenzie, SOAFD). Contamination of the specimens with fish mucus was reduced by one of two means, either by immersing fins in liquid nitrogen and snapping parasites off leaving the haptor attached *in situ* or by narcotising the parasites such that they readily detached. The protein profiles of these three species of *Gyrodactylus* were studied using gel electrophoresis and iso-electric focusing because of their relatively high abundance, on individual fish. Specimens of *G. derjavini* Morph 1 were also collected from *S. salar*

blots only.

Sample preparation

Live samples collected in 20µl of distilled water and stored in liquid nitrogen were thawed and the tissue macerated using an eppendorf pestle. To the homogenate, 20µl of double strength reduction buffer was added and the sample boiled. The samples of *Gyrodactylus*, which were held in reduction buffer, were boiled together with molecular weight standards to give a calibration curve for molecular weight on SDS-PAGE gels. The molecular weights of the markers (supplied by Pharmacia LKB, Uppsala, Sweden) were solubilised by the addition of 0.5 ml distilled water and 0.5 ml sample reduction buffer (the marker proteins used are given in Appendix 1).

Sodium dodecyl sulphate gel electrophoresis (SDS-PAGE)

Gels were run on the Phast System (PharmaciaLKB, Uppsala, Sweden) using pre-made homogenous 12.5 gradient media SDS-PAGE gels bonded to a transparent polyester backing (Pharmacia). Phast gel buffer strips for SDS-PAGE (0.20M Tricine, 0.20M Tris, 0.55% SDS and pH 7.5) were also acquired (Pharmacia). Samples were prepared as noted above and 1 µl of sample applied to the loading comb and applicator. Gels were focused at 250V, 10.0mA, 3.0W for 60Vh and the electrophoresis bed cooled and stabilized at 15°C prior to loading samples. Samples were run at this setting for 30 minutes when bed conditions maintained protein separation at 60V, 0.1mA, 0.5W.

Gels were immediately developed in the development unit of the Phast system and stained using silver nitrate optimised for SDS-PAGE. The following sequence was then followed: (1) they were then passed through a wash solution (10% ethanol, 5% acetic acid) for 2 mins, then again for 4 mins at 50°C; (2) into 5% glutaraldehyde (protein sensitisation) for 6 minutes at 50°C; (3) each gel was then again washed in the 10% ethanol, 5% acetic acid solution at 50°C for 3 and 5 minutes followed by two 2 minute washes in distilled water; (4) stained with 0.4% silver nitrate at 40°C for 6.5 minutes followed by two washes in distilled water; (5) transferred to developer at 30°C (1 ml 2% formalehyde in 250 ml 2.5% sodium carbonate) until protein banding appeared to be of a sufficiently visible intensity; (6) the gel background was then reduced to enhance the protein profile (2.5g sodium thiosulphate pentahydrate, 3.7g

Tris.HCl added to 100ml distilled water at 30°C); (7) the reaction was stopped by transfer to 5% glycerine for 5 minutes at 50°C; and (8) stored in distilled water, photographed when wet and then stored after drying. The limits of sensitivity for this SDS-PAGE separation for standard proteins is 0.3-0.5 ng protein per band.

Western blotting

A rabbit (New Zealand White) was given an initial injection of 50 *G. salaris* parasites which had been sonicated and emulsified in Freund's complete adjuvent. The injection was given to the rabbit subcutaneously over several sites (18 Dec., 1990). A booster (50 parasites in Freund's incomplete adjuvent) was given two weeks after the initial innoculation (15 Jan., 1991), a primary test bleed was taken two weeks after the booster, and then a total bleed of 70 ml was collected four weeks after the initial injection.

Western blots were performed using Pharmacia polyacrylamide gels (PAA4/30, 4.9 x 82 x 82 mm, separating proteins in the range 50,000-2,000,000 MW) run in a Pharmacia electrophoresis tank linked to an LKB Biochem 2103 1000V power supply and an LKB ultrorac 7000 cooling system. Gels were allowed to equilibrate in a non-reducing SDS buffer (see Appendix 1) for 1 hour, 30W, 70V, before sample loading. Samples in sample buffer were boiled for 5 minutes, following which 10 µl of sample was loaded.

Gels were pre-run at 300V for 10 minutes, driving the sample through the loading gel into the stack gel, before running for 3.5 hours in a field strength of 150V. The bromophenol blue marking the extent of electrophoretic migration leaves the gel after about 2 hours.

Biotinylated alkaline phosphatase (Pierce, Crewe) used as a calibration marker, has a strong affinity for Avidin (see Appendix 1). The calibration marker is supplied lyophilised as a 1mg mixture and is reconstituted in 0.5ml distilled water to give a 2mg/ml concentration in 10mM phosphate, 150mM sodium chloride, and 0.02% sodium nitrate to give a solution of pH 7.2.

Run gels were then marked and equilibrated with transfer buffer (see Appendix 1) for 15 minutes. The gel was blotted with nitrocellulose and filter paper, and 30V (approx 0.1 A) applied overnight supplied by a Biorad 250V power supply, followed

by 60V (approx 0.2 A) for 1-2 hours the following day before terminating electroblotting.

Staining of nitrocellulose

(i) Immunostaining

Nitrocellulose blots were immersed in blocking solution for an hour, followed by two 5 minute washes in TTBS before immersion in the first antibody solution, rabbit anti-*Gyrodactylus* serum (see Appendix 1), for either 1, 2 or 4 hours. Following this the blots were washed in two 5-minute TTBS washes before adding the second antibody solution, goat anti-rabbit IgG alkaline phosphatase conjugate (see Appendix 1), for an hour. Two further 5-minute washes in TTBS and a 5 minute wash in TBS were carried out before colour development (30 minutes to 4 hours). The blots were stopped by transference to distilled water, and then photographed whilst still wet. Preliminary tests following a one hour immersion in the first antibody solution showed faint banding which might indicate non-specific staining and this was tested by omitting the rabbit anti-*Gyrodactylus* serum from the procedure.

(ii) Protein Staining

The nitrocellulose membrane was transferred into PBS at 37°C for 30 minutes, followed by three 15 minute changes before incubation in Aurodye for 2-4 hours. Developed membranes were stopped by the addition of distilled water, and then photographed whilst still wet.

Dot Blots

Dot blots were performed on three samples of *Gyrodactylus* parasitising salmonids to ascertain whether: (i) antibodies to *G. salaris* were raised in rabbits; (ii) a titre could be obtained for any antibodies raised; and (iii) any antibodies raised were unique to *G. salaris* or common to all three species of *Gyrodactylus* studied.

Specimens of *G. salaris* were collected from laboratory raised stocks of *G. salaris* on salmon (water temp. 12-13°C) from the River Lier in Norway; these were removed from anaethetised fish. Specimens of *G. derjavini* Morph 1 from rainbow

trout and Atlantic salmon were also prepared for comparison by dot blot.

Discs of nitrocellulose were cut, marked, soaked in distilled water and allowed to dry before loading 1-2µl of whole raw parasite homogenate (50 parasites in 150-200µl distilled water). Concentrations of neat, 1 in 2, 1 in 5, 1 in 10, 1 in 25, 1 in 50, 1 in 100, 1 in 500 and 1 in 1,000 parasite homogenate were loaded onto the nitrocellulose and allowed to dry for 10-15 minutes. A series of total blotting solutions were tried in an attempt to obtain clear results, solutions of 2.5% blotto (2.5g commercial dried skimmed milk powder in 100ml distilled water), 2% bovine serum albumin (2g BSA in 100ml phosphate buffered saline (PBS)) or 2% casesin (2g casesin in 100ml PBS) were tried. The nitrocellulose was blotted for 1.5 hours before rinsing with 0.5% blotto (0.5g skimmed milk powder in 100ml distilled water) for 10 minutes. The nitrocellulose was allowed to dry slightly before loading 1-2µl of antibody solution (serum obtained from the final rabbit bleed diluted to the relevant concentration with 1 in 10 foetal calf serum: PBS solution) and then left for 1 hour. Following this the nitrocellulose was rinsed with 0.5% blotto for 10-15 minutes before loading the goat anti-rabbit conjugate (this was diluted 1 in 100 with the 1 in 10 foetal calf serum:PBS solution) and left for 1 hour.

A series of controls were set up alongside the test reactions; these controls were as follows: (i) Ag and conjugate only, (ii) Ab and conjugate only, and (iii) conjugate only. These were added at the relevant times. After exposure to the conjugate the nitrocellulose was again rinsed with 0.5% blotto for 15 minutes prior to developing the dot blot. The developer used was 6mg of 3-3 diaminobenzidine (DAB) added to 10mls of PBS or TBS supplemented with 30µl of hydrogen peroxide, and the dot blot was allowed to develop for 10 minutes. The reaction was then stopped by the addition of 0.5% blotto solution for 10 minutes and, after the blot was allowed to dry, it was photographed.

Iso-electric focusing

Batches of 50 *G. salaris* were picked off Norwegian Atlantic salmon fry and placed into 10µl of distilled water, frozen and stored at -70°C. Prior to use, samples were thawed and the worms were macerated and centrifuged at 10,000g, 0°-4°C for 5 minutes. The supernatant was used for analysis and could be refrozen at -20°C until

required. An LKB Multiphor System was used to run polyacrylamide gels (2mm thickness, pH range 4.5-9.5) (see Appendix 1), loading 2µl of sample. Electrofocusing was carried out at 1,200V and 30W, with 50mA current; gels were run for 2 hours, the plate being maintained at 2°-4°C by circulating a cooling solution of 75% ethylene glycol in the base of the apparatus. Gels were tested for acid phosphatase, adenylate kinase, α-glycerophosphate dehydrogenase, hexokinase, lactate dehydrogenase, malate dehydrogenase, phosphoglucose isomerase and phosphoglucomutase; each isoenzyme was developed using a stain specific for its unique pathway sequences (see Appendix 1).

Results

SDS-PAGE

Initial runs on SDS-PAGE gels suggested that a concentration of about 60 parasites in 20 µl of distilled water was required, before adding an equal volume of sample buffer, in order obtain bands of a suitable intensity for calculating protein molecular weights.

The molecular weights of the proteins identified by the SDS-PAGE method are presented in Table 12.1. For comparison Table 12.2 and Figures 12.1-12.2 illustrate the results obtained from Western blots stained for protein using Aurodye. Table 12.3 gives the molecular weights for the proteins isolated by the immunostaining with the anti-*Gyrodactylus* rabbit serum calculated from the gels shown diagrammatically in Figures 12.3-12.4. The bands stained for antibodies raised against *Gyrodactylus* (Figure 12.5) were faint for *G. salaris* and several bands were visible in the samples of *Gyrodactylus* sp. collected from charr and koi carp. Since the presence of non-specific staining was in question, and the goat blocking solution possibly being responsible, the test was repeated four times as follows: (i) Protein stain with Aurodye; (ii) first antibody solution (anti-*Gyrodactylus* rabbit sera) omitted to determine whether non-specific staining was occurring; (iii) incubation period with the first antibody solution increased to two hours; and (iv) incubation period with the first antibody solution increased to four hours.

Table 12.1: Molecular weights of proteins identified for three species of *Gyrodactylus* using SDS-PAGE.

Charr fin	Charr *G.* sp.	*G. salaris*	Koi *G.* sp.	Koi fin
117,500	162,200	105,900	164,100	162,200
112,200	147,900	102,300	162,200	133,400
110,900	121,600	93,300	149,600	123,000
107,200	117,500	75,900	112,200	116,100
102,300	112,200	74,100	105,900	112,200
93,300	101,200	70,800	102,300	93,300
84,600	93,300	63,100	93,300	91,200
67,600	91,200	58,500	91,200	77,600
64,600	78,500	53,100	84,600	70,800
61,700	72,100	33,900	83,200	65,300
58,600	67,600	29,900	77,600	64,600
56,900	64,600	28,200	75,900	58,600
56,200	58,500	27,200	70,800	48,400
48,400	56,900	25,700	68,400	44,200
47,900	48,400	24,800	56,200	30,900
44,400	47,900	23,400	53,100	
28,200	33,900	21,600	48,400	
25,700	32,900	16,400	47,900	
	30,900	15,700	44,400	
	28,200	14,900	35,500	
	25,700	13,800	33,900	
	22,400		30,900	
	21,400		14,900	
	14,900		14,400	
	13,200		13,800	

Table 12.2: Molecular weights of proteins for three species of *Gyrodactylus* calculated from Western blots stained for protein using Aurodye.

Charr fin	Charr *G.* sp.	*G. salaris*	Koi *G.* sp.	Koi fin
121.600	113.000	49.500	131.800	126.600
116.800	112.200	44.200	126.600	120.200
113.000	100.000	41.700	121.600	113.500
112.200	70.800	37.000	112.200	112.200
93.300	65.300	35.900	102.300	108.400
79.400	62.400	32.500	95.000	104.700
69.200	61.000	30.000	93.300	98.900
64.600	60.300	29.200	81.300	87.100
62.400	58.200	26.000	79.400	86.100
61.700	55.700	18.400	75.900	81.300
61.000	49.000	17.600	71.600	79.400
58.200	47.900	16.200	66.800	75.900
56.200	46.200	15.100	62.400	71.600
57.500	45.700	12.700	61.700	67.600
53.100	44.200		58.900	65.300
50.700	43.700		57.500	62.400
47.900	41.700		56.900	61.000
45.700	36.300		53.700	58.200
42.400	34.700		51.600	53.700
35.500	32.500		50.700	52.500
34.700	31.600		49.000	50.700
33.100	30.900		48.100	49.000
32.500	26.000		46.200	48.100
31.600	24.800		44.200	45.700
27.500	23.000		43.700	44.200
26.000	18.800		40.700	40.700
24.000	17.200		36.900	38.000
18.900	16.200		36.300	37.200
17.400			35.100	34.700
16.200			33.500	33.500
			31.300	33.100
			29.200	32.500
			28.800	31.200
			27.500	30.000
			26.500	27.200
			26.000	21.400
			24.800	18.400
			22.600	18.200
			21.100	17.200
			17.600	16.700
			16.700	16.400
			15.300	13.200
			13.700	
			13.300	
			12.600	

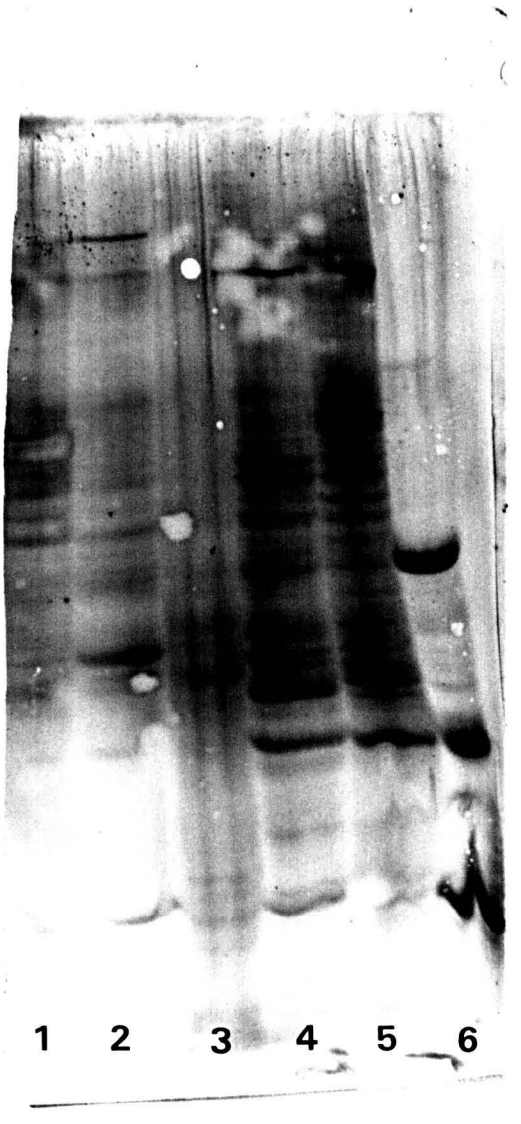


Figure 12.1: Nitrocellulose blot stained for protein with Aurodye.
Lane: 1 = *Salvelinus alpinus* fin sample. 2 = *Gyrodactylus* sp. (Morph 6) from *S. fontinalis*. 3 = *Gyrodactylus salaris*. 4 = *Gyrodactylus* sp. from *Carassius auratus*. 5 = *Carassius auratus* fin sample. 6 = alkaline phosphatase calibration marker.

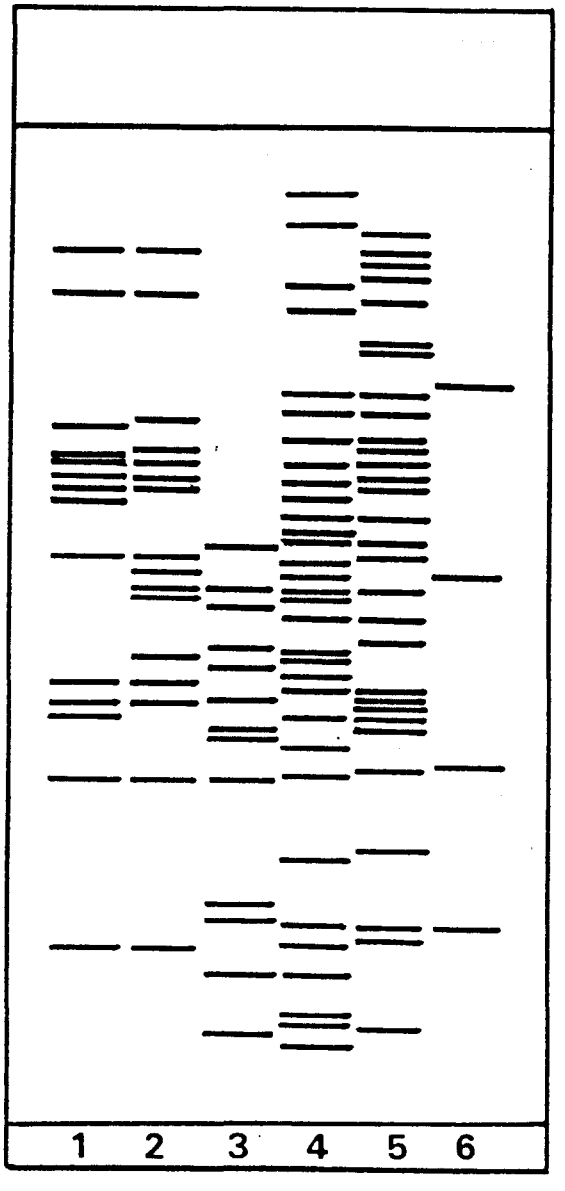


Figure 12.2: Diagram taken from the nitrocellulose blot stained for protein with Aurodye. Lanes (left to right): 1 = *Salvelinus alpinus* fin sample. 2 = *Gyrodactylus* sp. (Morph 6) from *S. alpinus*. 3 = *Gyrodactylus salaris*. 4 = *Gyrodactylus* sp. from *Carassius auratus*. 5 = *C. auratus* fin sample. 6 = alkaline phoshatase calibration marker.

Table 12.3: Molecular weights of proteins identified following immunostaining with the anti-*Gyrodactylus* rabbit serum.

Charr fin	Charr *G.* sp.	*G. salaris*	Koi *G.* sp.	Koi fin
75,900	81,300	31,600	102,300	104,700
18,600	75,900	24,700	91,200	78,500
	47,900		74,100	74,100
	42,400		62,400	65,300
	38,000		61,000	61,000
	36,900		36,300	53,700
	32,500		27,500	40,700
	31,600		25,700	32,500
	28,800		24,500	31,300
	27,500		16,000	30,000
	24,000			16,000
	18,400			
	16,700			

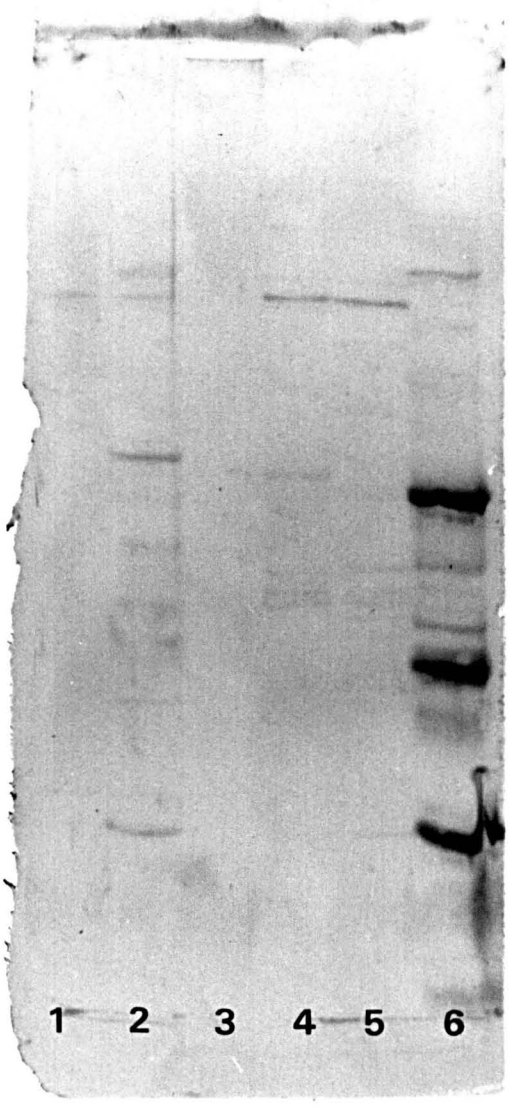


Figure 12.3: Nitrocellulose blot stained with anti-*Gyrodactylus* rabbit sera (1 hour). Lanes (left to right): 1 = *Salvelinus alpinus* fin sample. 2 = *Gyrodactylus* sp. (Morph 6) from *S. alpinus*. 3 = *Gyrodactylus salaris*. 4 = *Gyrodactylus* sp. from *Carassius auratus*. 5 = *C. auratus* fin sample. 6 = alkaline phosphatase calibration marker.

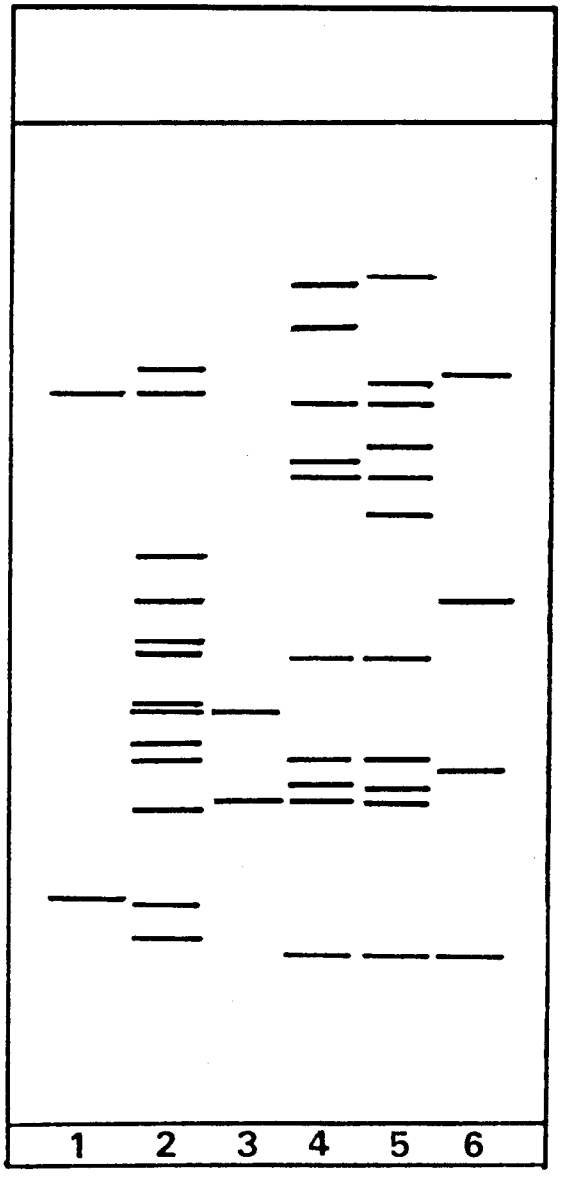


Figure 12.4: Diagram taken from the nitrocellulose blot stained with anti-*Gyrodactylus* rabbit sera (1 hour). Lanes (left to right): 1 = *Salvelinus alpinus* fin sample. 2 = *Gyrodactylus* sp. (Morph 6) from *S. alpinus*. 3 = *Gyrodactylus salaris*. 4 = *Gyrodactylus* sp.from *Carassius auratus*. 5 = *C. auratus* fin sample. 6 = alkaline phosphatase calibration marker.

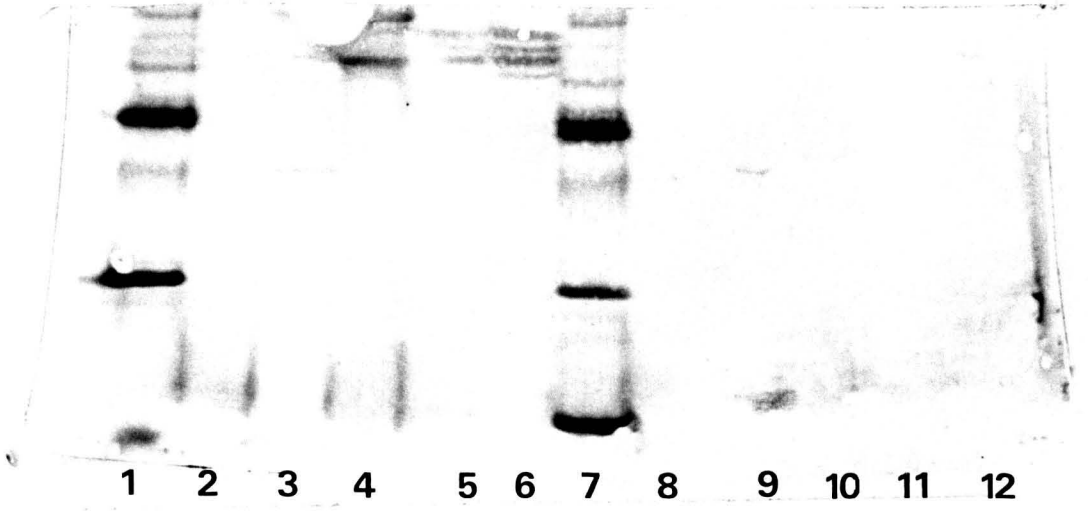


Figure 12.5: Nitrocellulose blot stained with anti-*Gyrodactylus* rabbit sera (2 hours). Unfortunately sliced the wrong way, but has been included here to demonstrate the increased staining intensity with increased incubation. Lanes (left to right): 1 + 7 = alkaline phosphatase calibration marker. 2 + 8 = *Salvelinus alpinus* fin sample. 3 + 9 = *Gyrodactylus* sp. (Morph 6) from *S. alpinus*. 4 + 10 = *Gyrodactylus salaris*. 5 + 11 = *Gyrodactylus* sp. from *Carassius auratus*. 6 + 12 = *C. auratus* fin sample.

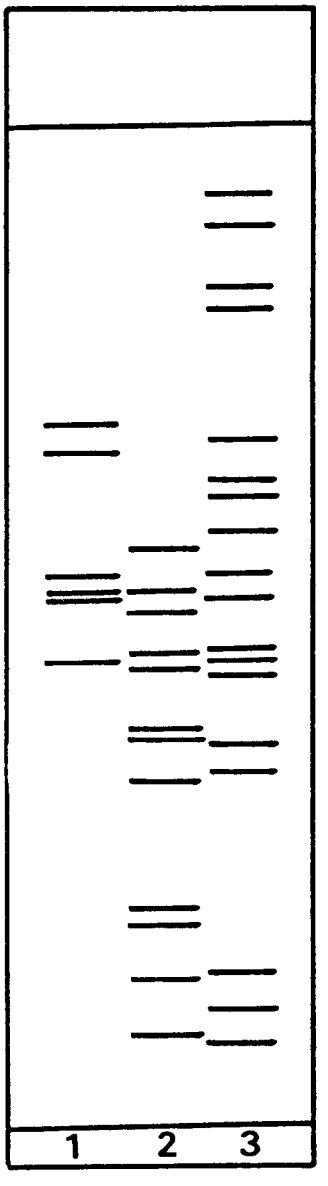


Figure 12.6: Diagram taken from the nitrocellulose blot stained for protein. This shows the bands unique to the *Gyrodactylus* sample being run (i.e. *Gyrodactylus* sample bands minus homologous bands present in the host fin sample). 1 = *Gyrodactylus* sp. (Morph 6) from *Salvelinus alpinus*. 2 = *G. salaris*. 3 = *Gyrodactylus* sp. from *Carassius auratus*.

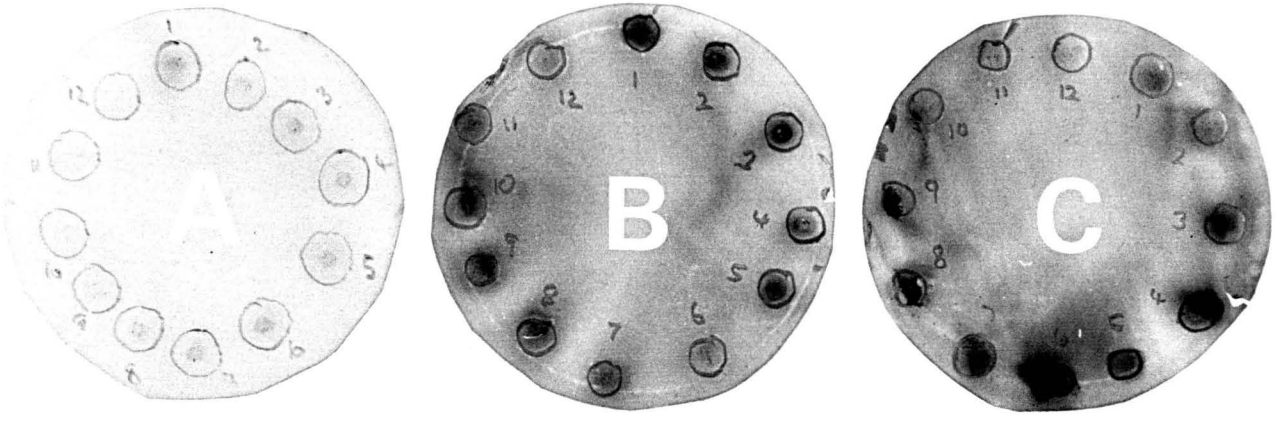


Figure 12.7: Dot blots for *Gyrodactylus salaris* (A) and *G. derjavini* (Morph 1) (B). The concentrations tested for are neat, 1 in 2, 1 in 5, 1 in 10, 1 in 25, 1 in 50, 1 in 100, 1 in 500 and 1 in 1000.

The results of the repeat protein stain, are however, in close agreement with those given in Table 12.2. The removal of the first antibody solution still resulted in some faint bands being present on the nitrocellulose blot when developed. However, when the incubation period was increased to two hours plus, the bands for *G. salaris* did become more intense, see Figure 12.5. The protein bands unique for *Gyrodactylus* are summarised in Figure 12.6.

Dot Blots

Blots obtained from *G. salaris*, using 2.5% blotto solution as the total blotting solution, yielded a good Ab response, with the Ab response giving a titre of between 1 in 500 and 1 in 1000 dilution of rabbit Ab sera.

A second series of dot blots was performed in order to determine whether the antibodies raised in the rabbit were unique to *G. salaris* or a general reaction to the proteins of *Gyrodactylus* when a rabbit is innoculated with whole parasite homogenate, as might be expected. All reactions proved positive for both of the other species of *Gyrodactylus* tested and for all three initial blotting solutions tried. The reaction obtained from *G. derjavini* Morph 1 from salmon, gave a titre of around 1 in 25 antibody dilution compared to *G. derjavini* Morph 1 from rainbow trout, which yielded a stronger reaction with a positive titre of 1 in 1000 Ab dilution observed. These are shown in Figure 12.7.

Discussion

A number of problems arose initially with the collection of *Gyrodactylus* material for electrophoretic studies, due in part to the small size and the nature of gyrodactylid attachment. In order to obtain sufficient material for an electrophoretic run a pooled sample of up to 60 individuals per run had to be collected; this automatically prevented natural variation being studied. Furthermore, because of the low intensity levels on wild fish, collections from several fish hosts have to be pooled in order to achieve sufficient numbers.

The following discussion will be directed primarily towards those results obtained for *G. salaris* and how it compares to other gyrodactylid species, this being

the species that needs a definitive test owing to its pathogenicity and the legislative requirements.

The results extrapolated from the SDS-PAGE gels varied slightly but agreed for four proteins of molecular weight 102,300, 33,900, 28,200, and 25,700 Daltons (D). However, the length of the separation bed on the Phastsystem is only 37mm, and, therefore, bands separated on this system provided only a preliminary indication of what constituted the samples. The nitrocellulose blots stained for protein yielded better results due to the sensitivity of the gold stain, the increased bed separation length and the more stable, slower running conditions. The Western blot confirmed the presence of proteins of molecular weight 30,000, 26,000, 16,000, and 15,000 D in the SDS-PAGE gels. The major bands in the Western blot were found to be molecular weight proteins of 36,900, 32,500, 17,600, and 16,200 D.

It appeared that the immunostaining showed a response to two *G. salaris* proteins, one of molecular weight 31,600 and a second of 24,200 D. When the results from this blot were compared with those from the SDS-PAGE gels, they corresponded to proteins of molecular weight 30,900 and 24,800 respectively. They also corresponded to proteins of molecular weights 32,500 (major band) and 26,000 D when compared to the protein bands derived from the Aurodye stained Western blots. It is suggested that the antibody proteins raised against *G. salaris* are of molecular weights 31,700 (SD = 650; n=3) and 25,200 (SD = 590; n=3).

The protein profiles given in Figure 12.6, are unique to the *Gyrodactylus* sample being run, i.e bands also present in fish host tissue were removed at this stage on the assumption that they were caused by contamination. There were homologous bands present between *G. salaris* and the two other species, but these were few.

Bands obtained from the immunostaining were faint, and, when the blot was repeated without anti-*Gyrodactylus* rabbit serum, some faint bands were also seen. However, increasing the incubation time with the first antibody increased the band intensity, but the results appeared to be inconsistent, with only half of the gel responding. It is possible that the antibodies raised to *Gyrodactylus* were low and a longer immunisation period or larger inoculum is required before the titre of antibody present can be measured. However, when this was repeated in order to confirm the molecular weights of the proteins to which antibodies had been raised, a non-specific

binding reaction was suggested. A repeat trial of this failed to confirm the presence of the two faint bands originally identified. It is, therefore, with some degree of caution that these results are presented; it is possible that problems with the technique may be solved by increasing the number of individual gyrodactylids in the intial and booster inoculations.

With reference to the results obtained for the Dot blots of *G. salaris* against the two species of *Gyrodactylus* infecting salmonids in the UK, some comment on the precise composition of the rabbit inoculum should be made. The specimens of *G. salaris* were collected from wild fish in the River Drammen, Norway rather than from laboratory-established stocks. The reason why such strong responses, i.e good titres of antibody, were obtained for *G. derjavini* Morph 1 from rainbow trout and *G. derjavini* Morph 1 from salmon may be due to the fact that the initial population was not solely *G. salaris* but a mixed population of *G. salaris* and *G. derjavini*. A mixed population taken, homogenised and then injected into the rabbit would therefore give rise to antibodies to all three species. Ideally, fish taken in the wild should have been held in laboratory conditions where *G. salaris* has been observed to outcompete other species, leaving a pure population of *G. salaris* on the fish. However, the Norwegian salmon used as the source of *G. salaris*, was taken directly from the River Drammen and it is feasible that these fish harboured mixed populations of *G. salaris* and *G. derjavini*.

This study was instigated as a pilot study to assess the potential of these techniques for identifying *G. salaris*, since it is now a notifiable disease. Iso-electric focusing investigated the presence of eight enzymes namely acid phosphatase, adenylate kinase, α- glycerophosphate dehydrogenase, hexokinase, lactate dehydrogenase, malate dehydrogenase, phosphoglucose isomerase and phosphoglucomutase. Although up to 50 specimens per run were tried, the system failed to yield useable results. Improvements in the sample collection and preparation maximising the amount of tissue free from contamination by host tissue may alleviate some of the problems which arose in these investigations. However, the major problem was collecting enough tissue to obtain protein bands. It is likely that approaching the *G. salaris* problem using various molecular biological techniques will ultimately provide further evidence to confirm the results of the morphological studies

as the recent development of PCR (polymerase chain reaction) means that single specimens can be investigated. The technique used in the present study yielded little useful data in relation to the amount of time invested.

All that can be concluded is that there appears to be two antibody proteins raised against *G. salaris*, one of 31,700 and another of 25,200 D. The use of dot blots confirmed the presence of good antibody titres, 1 in 1000 for *G. salaris*; 1 in 25 for *G. derjavini* from salmon and 1 in 1000 for *G. derjavini* from rainbow trout. The explanation for such strong titres is not known, i.e. whether they were a response to common proteins of *Gyrodactylus* or whether the initial collection of the *G. salaris* sample was a mixed infection of both *G. salaris* and *G. derjavini.*

CHAPTER 13: ARGENTOPHILIC STRUCTURES AS A DIAGNOSTIC CRITERION FOR THE IDENTIFICATION OF *GYRODACTYLUS* (MONOGENEA) PARASITISING SALMONIDS WITH COMMENTS ON THE LIGHT WHICH THIS TECHNIQUE SHEDS UPON THE SYSTEMATIC RELATIONSHIPS OF THE GENUS.

Introduction

Gyrodactylus salaris Malmberg, 1957 though highlighted in Norway as a result of the problems it has caused on wild salmon parr, is not restricted to salmonids there, but has been recorded in other parts of Europe, with reports of its occurrence on salmon *Salmo salar* in Russia (Ergens, 1983) and Bosnia-Hercegovinia (Imamovic, 1987). It is also known from brown trout *Salmo trutta* in Bosnia-Hercegovinia (Imamovic, 1987) and N. Europe (Malmberg, 1973), charr *Salvelinus alpinus* in Norway (Tanum, 1983), on rainbow trout *Oncorhynchus mykiss* in Germany (Lux, 1990) and possibly Spain (Santamarina *et al.*, 1991). It is possible that it has been spread from Scandinavia/Sweden where it was first reported to other parts of Europe with cultured salmonids. On the other hand, the recent interest in this species generated by the problems in Norway may have stimulated research in this area in other parts of the world. There is also potential for its introduction to the UK, especially since there are frequent importations resulting from salmonoid aquaculture. It poses a potential threat not only to the salmon industry in the UK in terms of lost revenue but also to wild populations. MacKenzie & Bakke (1992) exposed Atlantic salmon of Scottish origin to *G. salaris* and found them to be as susceptible to *G. salaris* as the Norwegian salmon, indicating that the worm is potentially a danger to salmon in the UK. This parasite's pathogenicity (Johnsen, 1978) coupled with its speed of reproduction warranted its inclusion on the UK list of notifiable diseases in 1987 (brought within the control provisions of the Diseases of Fish Act, 1937) (Anon, 1987). Although *Gyrodactylus* spp. are frequently identified on farmed salmonid stocks in the UK, sporadic outbreaks of gyrodactyliasis on farms have been controlled by formalin treatments. Because of the ease of this treatment procedure, there has been no incentive to identify the parasites to species level. However, prior to the survey work

carried out as part of this study, it was not known whether *G. salaris* was already resident in the UK. In view of the threat posed by this pathogen, it is essential, in order for the law to be practicable, that there are methods which permit its easy recognition by non-specialists responsible for translocation of fish and assessment of fish health.

Current techniques used in the identification of *Gyrodactylus* species involve the discrimination of minute differences in the attachment hooks. The elucidation of these differences required the development of specialised techniques to liberate the hooks from the body tissue in order to permit subsequent examination under the scanning electron microscope. These were refined and used satisfactorily in Chapter 6. Subsequent analysis of the specimens to discriminate between species and/or indicate a species identity required the input of a large number of specimens, was very laborious and involved the use of sophisticated, expensive equipment. The time and facilities required to make accurate species identifications is not always available to diagnosticians, and consequently a potentially simpler method of recognising these species was sought.

The importance of chaetotaxy as a means of discriminating species and providing information contributing to the understanding of systematics and phylogenetics has been demonstrated by a number of authors in relation to the Digenea. For example, Wagner (1961) found considerable differences in the distribution patterns of papillae of three species of schistosome cercariae, Mohandas (1971) discussed the taxonomic importance of integumentary papillae for species of echinostome and xiphidio-cercariae, and Richard (1971) established a system of nomenclature for the digenean cercariae on the basis of the argentophilic structures. Since that time, there has been much interest in using this technique to resolve some of the problematical trematode genera. Niewiadomska & Moczon (1982), for example, were able to relate the sensillary patterns for *Diplostomum* cercariae with the topography of their nervous system and its commissures.

Combes & Lambert (1975) used this technique on monogeneans and established a system of nomenclature for the expression of monogenean sensillary patterns. The body of the larval worm was divided into bands corresponding to the transverse nerve commissures. In this manner it was found possible to discriminate

closely related species of *Polystoma* by minor variations in the basic chaetotaxy of this genus. Maeder (1973) used chaetotaxy in part to differentiate between three "polystomatid larvae" from the Ivory Coast, *Polystoma grassei* Combes & Knoepffler, 1966, *P. dorsalis* Maeder, Euzet, & Combes, 1970 and *P. ebriensis* Maeder, 1973. *P. grassei* differed from the other two species in lacking a single 2 x 2 group of postero-dorsal sensilla on the dorsal surface and a 2 x 2 group postero-laterally on the ventral surface.

The utilisation of maps of argentophilic structures has also made a useful contribution to the discrimination and systematics of taxa at higher taxonomic levels, from the subordinal level down to the generic level (Richard, 1971). Since the work of Richard (1971) many papers have followed: for example Eklu-Natey *et al.* (1985) on the digeneans *Apatemon*, *Trichobilharzia* and several species of the Diplostomidae; Khotenovski (1975) on *Diplozoon*; Tinsley & Owen (1975) on *Protopolystoma*; Tinsley (1978) on *Eupolystoma*; Combes & Lambert (1972) on the Polyopisthocotylea; Lambert (1977b) on *Ergenstrema mugilis*; Lambert (1977c) on *Ancyrocephalus paradoxus*; Lambert (1978a) on *Tetraonchus monenteron*; and Lambert (1978b) on the Capsalidae. Nevertheless, chaetotaxy has not always been totally discriminating. For example, Lie (1966) found the distribution and numerical pattern of sensilla in two closely related echinostome cercariae to be similar, and Lambert & Bourgat (1978) concluded that chaetotaxy did not provide enough information to permit the differentiation of genera of polystomatids, although they were able to conclude that *Polystoma*, *Metapolystoma* and *Eupolystoma* should be in the same family.

The success of chaetotaxy in discriminating certain problematic trematode species warranted an exploration of its application to the *Gyrodactylus* situation. An assessment of the technique was made and the chaetotaxy maps of *G. salaris* from Scandinavia were determined. These were compared to those species of *Gyrodactylus* parasitising native British salmonids to ascertain whether species specific maps exist.

The study was extended to look at possible variation between gyrodactylid populations in relation to geographical location, their host species and local environmental parameters.

Table 13.1: Populations of *Gyrodactylus* sampled and sensillary patterns compared by use of chaetotaxy.

Site	Species	Host	Location
1	*G. salaris*[1]	*S. salar*	River Ätran, Högvadsån/Fageredsån, Halland, Sweden
2	*G. salaris*[2]	*S. salar*	River Lierelva, Buskerud, SE Norway
3	*G. derjavini* Morph 1	*S. salar* (c)	River Allan, Stirlingshire, Scotland
4	*G. derjavini* Morph 1	*O. mykiss* (c)	Loch Awe, Perthshire, Scotland
5	*G. truttae* Morph 4	*S. trutta*	River South Wey, Berkshire, England
6	*G. truttae* Morph 4	*S. trutta*	River Allan, Stirlingshire, Scotland
7	*G. truttae* Morph 5	*S. trutta*	Loch Airthrey, R. Allan, Stirlingshire, Scotland
8	*G. turnbulli*	*P. reticulata*	Lab. cultures, Natural History Museum, England
9	*G. gasterostei*	*G. aculeatus*	Loch Airthrey, R. Allan, Stirlingshire, Scotland
10	*G. gasterostei*	*G. aculeatus*	R. Wey, Reading, Berkshire, England
11	*Gyrodactylus* sp.[3]	*C. auratus* (c)	Fish pond, Bedfordshire, England

(c) = cultured

1 = courtesy of Dr. G. Malmberg

2 = courtesy of Dr. T.A. Bakke

3 = possibly *G. elegans indicus* complex Tripathi, 1957

Materials and methods

Parasite origin

Table 13.1 shows the species of *Gyrodactylus* collected for examination and comparison using chaetotaxy. The species of *Gyrodactylus* from each host and locality was verified by examination of slide preparations, electronmicrographs and from simultaneous multivariate analyses (Chapters 3,4 and 7). Non-salmonid gyrodactylids were used for comparison. Since only a few of the non-salmonid gyrodactylids were successfully stained, the chaetotaxy map for these forms are presented only tentatively and are included only as part of the comparative study.

Collection of parasites

Fish were killed by insertion of a mounted needle into the brain via the upper part of the eye, and fins parasitised with gyrodactylids were removed. The body surface and gills were was also examined in local water for the presence of parasites. Infected fins were briefly washed in 0.2M phosphate buffer in order to remove excess mucus prior to staining.

Staining procedure

Parasites were processed *in situ* on the fin and placed directly into 0.5% silver nitrate at 65-70°C in the dark for 5 minutes. The fins were then washed in 5-10 changes of distilled water. Each side of the fin was subsequently submerged in distilled water and exposed to UV light (325nm) for 5 minutes each side. They were then again washed in several changes of distilled water before being placed into a solution of 90% alcohol and 10% glycerine. The alcohol was allowed to evaporate leaving the fins in glycerine. The parasites were then picked off the fins and mounted in glycerine. An excess of glycerine was used when mounting the parasites for two reasons: (i) to prevent the parasites from being flattened too much under the weight of the coverslip; and (ii) to allow enough depth to gently roll the parasite in order to achieve the correct orientation, i.e. the parasite was laid dorso-ventrally in a straight line, so as to maximise the number of sensilla visible in any one focal plane. Prepared slides were kept in the dark in a fridge (4°C) until drawings were made, usually within 3 days, by

means of a drawing tube and they were then photographed. Chaetotaxy patterns were still discernible on slide preparations maintained in the dark and at low temperature after 6 months. A minimum of 50 specimens were prepared for each sample site where possible and 10-15 drawings were made from randomly selected preparations. When a species specific map was derived for these it was checked against the remaining 35-40 specimens. Any points not conforming were noted. When all maps were complete the slides were further compressed and examined under phase contrast so that drawings of the marginal hooks could be made in order to confirm the species determinations.

Results

Although the total number of sensilla varied slightly from species to species, the sensilla conformed to consistent patterns, being symmetrical about the sagittal axis of the worm. Figure 13.1 shows a generalised specimen of *Gyrodactylus* showing the zonation used and the nomenclature of the groups of sensilla. When the ventral sensillary maps of *G. salaris* were compared with those for the two species native to the United Kingdom, i.e. *G. derjavini* Morph 1, *G. truttae* Morph 4 and *G. truttae* variant Morph 5, it could be seen that there was a high degree of homology between the three species but more so between the two UK species.

Within each species of gyrodactylid there appeared to be a component of intraspecific variation, most of which was confined to the anterior end of the worm. There were some subtle differences between *G. derjavini* Morph 1 collected from sites 3 and 4, *G. truttae* Morph 4 from sites 5 and 6, and *G. truttae* variant Morph 5 from site 7. The latter population of gyrodactylids, recovered from Loch Airthrey trout, were shown by light microscope studies and multivariate analyses (PCA and cluster analysis) in Chapter 3 to deviate from other brown trout forms, this population typically had long, thin marginal hooks.

To begin to find some form of order in the sensilla pattern, structural body landmarks were identified as a guide to locate key clusters of sensilla. The number of sensilla on the opisthaptor (Figure 13.1) were few in number and were relatively consistent amongst the species studied here. Furthermore, when the specimen was

rolled to achieve the correct orientation, the opisthaptor invariably twisted. It did not therefore represent a satisfactory locus for discriminating sensilla patterns. The opening of the pharynx and the excretory pores at the anterior were heavily stained by silver nitrate and acted as good reference points. The pharyngeal opening is present on the ventral surface; by focusing through the specimen the excretory pores could be located just posterior to the pharynx on the dorsal surface. The arrangement of sensilla at the anterior end of *Gyrodactylus* was complex, with many sensilla located around the spike sensillum and pharynx (Figure 13.1). Slight differences in the chaetotaxy pattern for a species may occur as a result of the loss of a single sensillum through damage, duplication or because it is not visible due to being situated at the interface between the dorsal and ventral surfaces and thus compressed during the flattening process. Most differences in the anterior region occurred on the ventral surface which bears the most sensilla, whilst few differences were found on the dorsal surface. The dorsal surface sensilla were mapped first, starting with the sensilla around the excretory pores and then working along the length of the worm. Although in some cases both ventral and dorsal surface sensilla were visible simultaneously, establishing the dorsal surface pattern first enabled the ventral map to be elucidated.

The system of nomenclature used in Figure 13.1 was applied as a result of examination of some 300 stained specimens of *Gyrodactylus*. It attempts to use clusters that appear to form naturally, rather than attempting to relate sensillary patterns to the underlying nervous system as done previously by Richard (1971) and Combes & Lambert (1975).

The ventral and dorsal maps for each gyrodactylid species at each locality are discussed below.

Intraspecific variation between G. salaris populations

(i) The ventral surface

The chaetotaxy maps of the two populations of *G. salaris* studied showed some similarities, as shown in Figures 13.2-13.3. In the antero-lateral zone (al) as indicated on Figure 13.1, reading anteriorly from the mouth and moving anteriorly, both the Swedish (site 1) in Figure 13.2 and Norwegian (site 2) in Figure 13.3 have 4+2

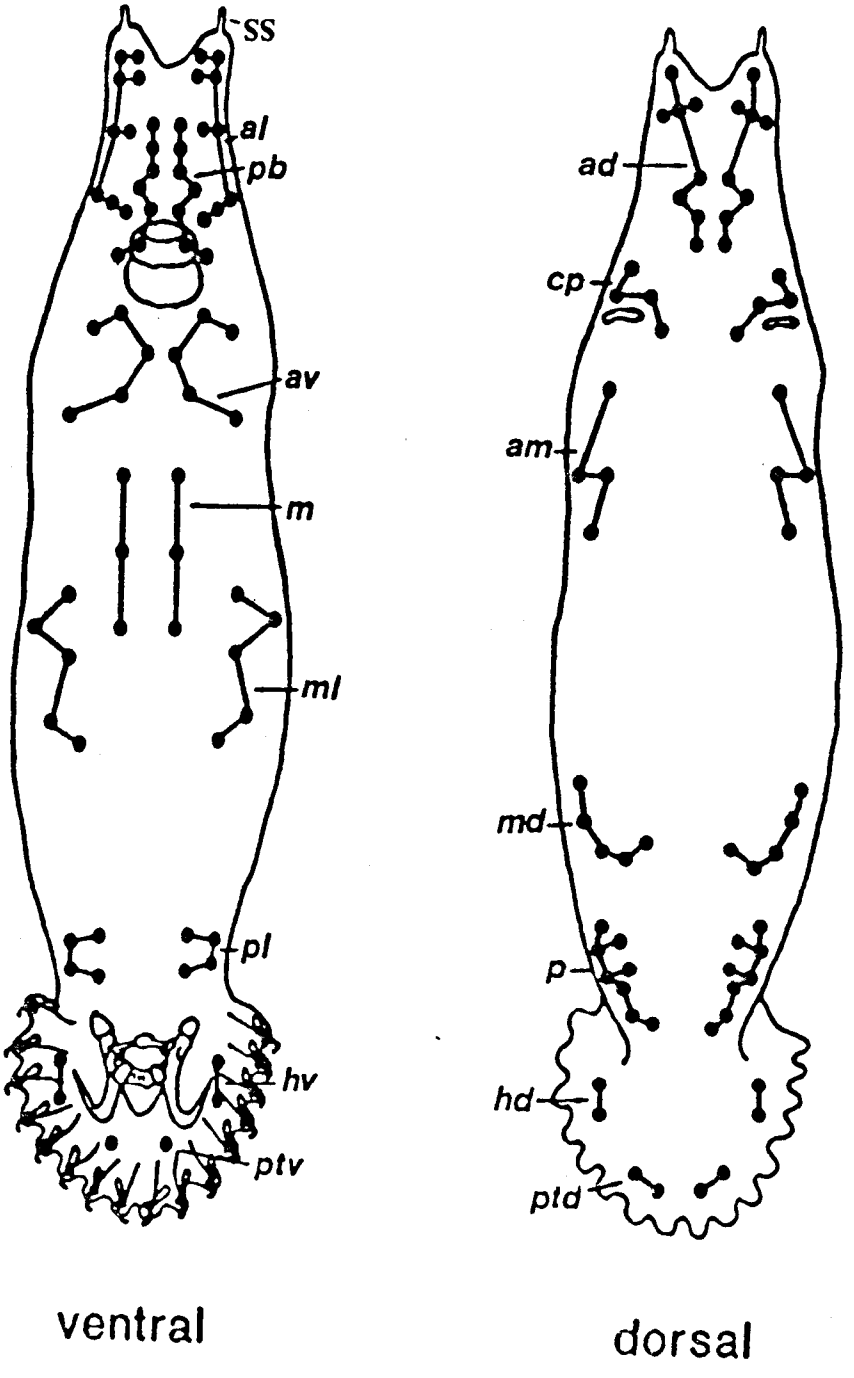


Figure 13.1: Diagram of generalised *Gyrodactylus* showing zones and nomenclature of sensilla. Abbreviations are given in Appendix 2.

arrangements. The third cluster in this zone differs slightly, for, while those from site 2 (11 observations) had a consistent cluster of 4 sensilla, the prevalence of a 4th sensillum was less than 70% for the Swedish population (7 observations).

The most characteristic feature of the *G. salaris* maps was seen in the zone posterior to the pharyngeal complex, the anterior zone on the ventral surface (av, Figure 13.1) with a cluster of 5 sensilla. However, in the Swedish material, the post-pharyngeal sensillum no. 2 (anterior sensillum no. 2 in Figure 13.4) was often more marginally displaced, such that its distinction from the dorsal surface sensillum circumporal no. 3 was difficult; these two apparent sensilla are possibly one and the same, their position on the lateral margin of the body making their designation difficult. Nevertheless, it can be concluded that, for *G. salaris*, there is a cluster of 5 sensilla in the anterior zone on the ventral surface and 5 sensilla in the circumporal zone on the dorsal surface. The 5th sensillum in this anterior zone normally lies anterior to the median cluster (m, Figure 13.1).

Both populations were similar in terms of the medio-lateral (ml) and postero-lateral (pl, Figure 13.1) zones, having 6 and 5 sensilla respectively; however, in the latter zone the prevalence of these points was not as consistent in the Norwegian population (70%) as in the Swedish population (100%).

With regard to the haptor, the Swedish population shown in Figure 13.2, could be differentiated from the Norwegian population shown in Figure 13.3 by the presence of an extra haptoral (h, Figure 13.1) sensillum.

(ii) The dorsal surface

On the dorsal surface both populations exhibit 5 circumporal (cp, Figure 13.1) sensilla but differ in the median zone (md). This zone in the Swedish population is composed of 2 clusters, a laterally placed cluster of 3 sensilla and a centrally positioned cluster of 4, whilst the median zone in the Norwegian specimens is comprised of a single cluster of 6 medio-laterally placed sensilla. A second important difference occurs in the postero-dorsal (pd) group of sensilla, which in *G. salaris* is unique among the salmonid gyrodactylids studied in having a cluster of 9 sensilla rather than the 8 present in *G. derjavini* Morph 1, *G. truttae* Morph 4 and *G. truttae* variant Morph 5 (as shown in Figures 13.5-13.9). Further variation exists in the dorsal haptoral zone

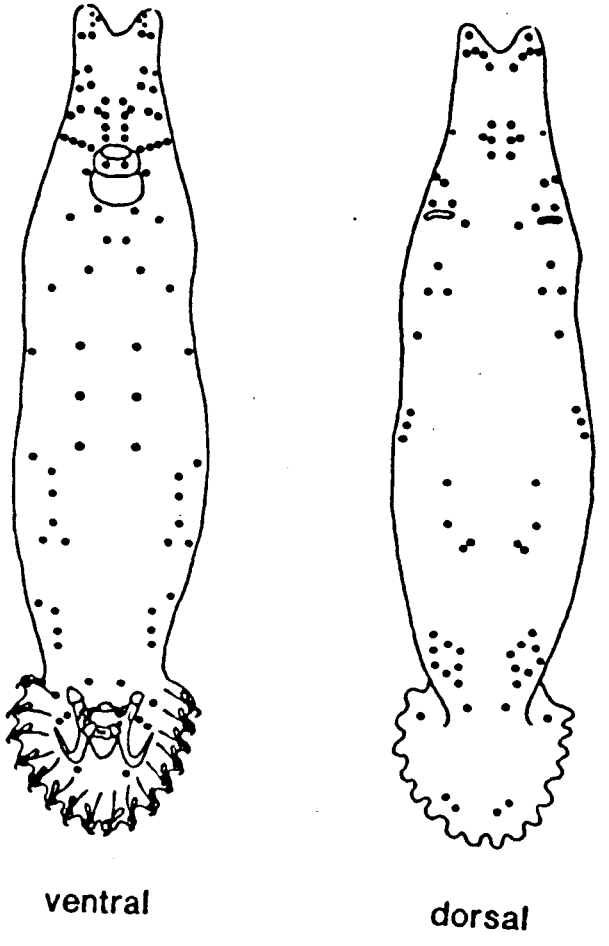


Figure 13.2: Chaetotaxy map of *G. salaris* on *Salmo salar* from the River Ätran, Sweden (Site 1).

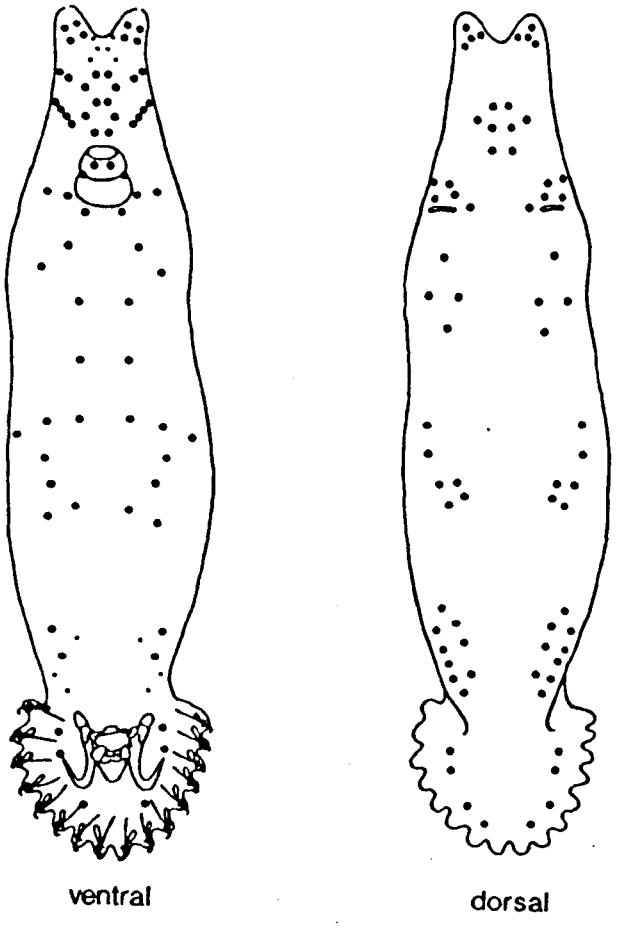


Figure 13.3: Chaetotaxy map of *G. salaris* on *Salmo salar* from the River Lierelva, Norway (Site 2).

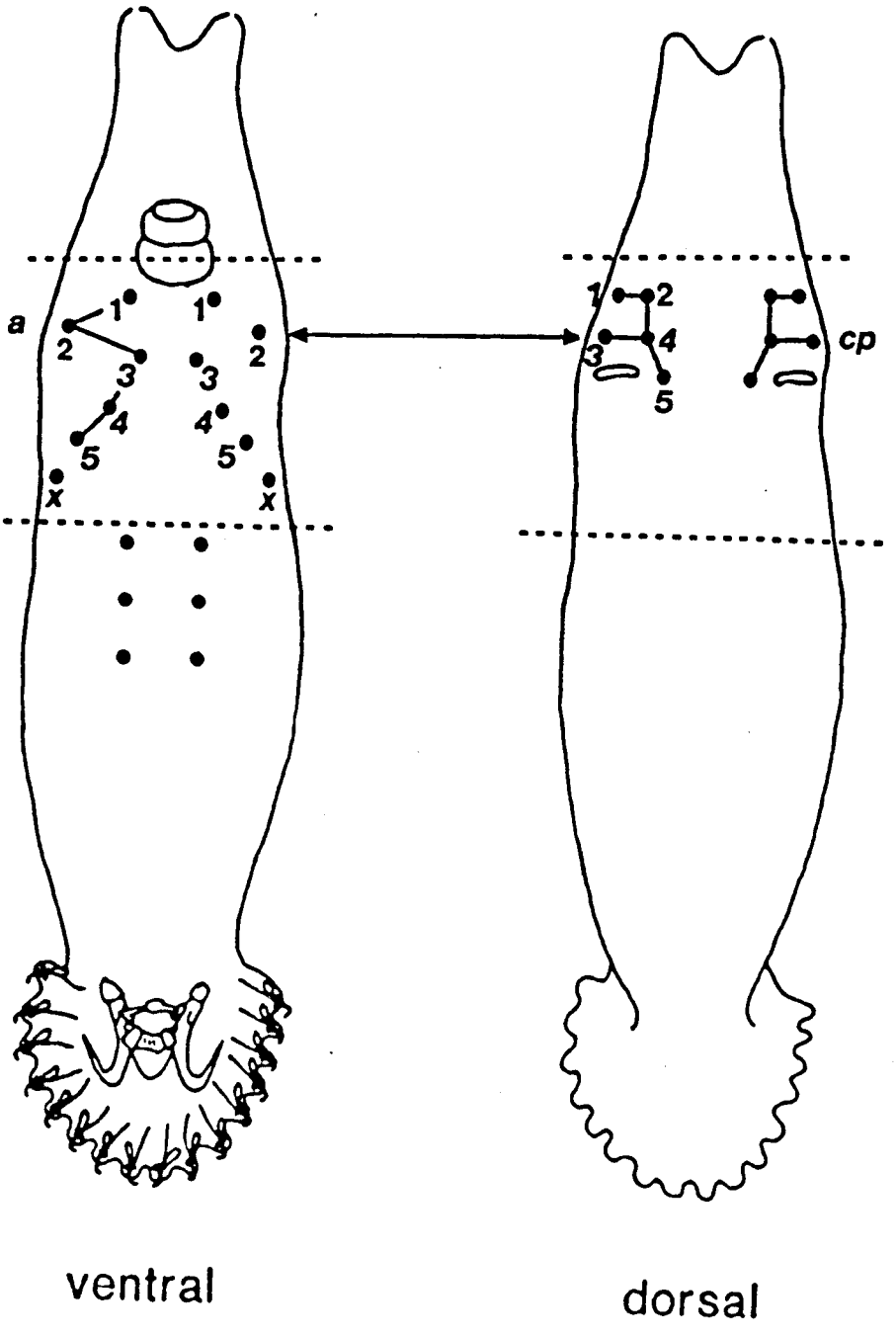


Figure 13.4: *G. salaris* from the River Ätran, Sweden illustrating the problem in the distinction of the anterior (a) 2 sensillum from the circumporal (cp) 3 sensillum in this region.

(hd, Figure 13.1), with the Swedish population of *G. salaris* having a single sensillum.

Intraspecific variation between G. derjavini Morph 1 populations

The results of the multivariate analyses (Chapter 3-5) showed that, within the UK, *G. derjavini* Morph 1 is parasitic on two salmonid hosts, *Salmo salar* and *Oncorhynchus mykiss*. There was therefore, an opportunity to investigate the influence of host on the sensillary patterns of the parasite. In this study, fish stocks sampled were restricted to Scotland, since the multivariate analyses indicated the possible existence of two *G. derjavini* Morph 1 subpopulations related to the geographical distribution of the host when English and Welsh material was included. The exclusion of all but the Scottish specimens not only served to reduce variation in environmental parameters but also permitted *G. derjavini* Morph 1 from the two hosts it parasitises to be examined concurrently.

(i) The ventral surface

The antero-lateral (al) configuration is variable in both populations, each having an equal likelihood of possessing 2, 3 or 4 sensilla in the posterolateral set (pl), as shown in Figures 13.5-13.6. The anterior region (av) apparently varies in relation to the host, the population on salmon having a cluster of 6 (n = 15) (Figure 13.5) and that on rainbow trout has a cluster of 5 or 6 (n = 17) (Figure 13.6), although the 6th point was found to have a prevalence of only 50%. The medio-lateral (ml) zones are similar in both populations, although in the salmon population the entire medio-lateral zone has a prevalence of less than 70%, as shown in Figure 13.5 by the smaller sensilla. The only real differences are in the postero-lateral (pl) zone, where there are only 2 sensilla in the salmon population against 4 in the rainbow trout, and the haptoral zone (hv), where there are only 3 sensilla on the haptor of specimens from Atlantic salmon from the River Allan fish farm (site 3) (Figure 13.5) and 6 sensilla in total on the specimens from Loch Awe rainbow trout (site 4) (Figure 13.6).

(ii) The dorsal surface

Dorsally, both populations are similar except for the presence of one extra median

(md) sensillum and one fewer in the postero-dorsal (pd) cluster on specimens from salmon in the River Allan fish farm (site 3) (Figure 13.5).

As in the case of *G. salaris*, it appears that the dorsal chaetotaxy pattern is relatively consistent for the species irrespective of the host.

Intraspecific variation between G. truttae Morph 4 populations

In this study 2 rivers supporting only brown trout as the resident salmonid were investigated, the River Wey in Berkshire (site 5) (n = 10) and a second river forming a tributary of the River Allan (site 6) (n = 10). The chaetotxy maps of *G. truttae* are shown in Figures 13.7 and 13.8. The population of *Gyrodactylus* parasitic on brown trout in Loch Airthrey (site 7) (n = 10), as shown in Figure 13.9, was found to include a morphological variant of *G. truttae*, Morph 5, with long, thin marginal hooks, when analysed by multivariate analysis. For this reason, this locality was included in the chaetotaxy study in order to determine whether the deviation was reflected in its sensillary pattern.

(i) The ventral surface

Figures 13.7-13.9 show that the distribution of the sensilla in the antero-lateral (al) zones of this species appear to be quite variable, although the total number is relatively constant. Due to the fact that the compression of these worms varied during the preparation of the slides, it was impossible to determine to what extent the spatial distribution of the sensilla was influenced by this and, hence, the precise configuration. As in the case of the other gyrodactylids studied, this zone poses some problems, not only because of the large number of sensilla in the zone and their concentration, but also due to their position close to the lateral margin of the body.

The anterior zone (av) was consistent for all three localities with 6 sensilla in each zone; in both the River Wey (Figure 13.7) and in the Loch Airthrey populations (Figure 13.9) there was a 7th sensillum arising parallel to the median (m) sensillum no. 1. The medio-lateral (ml) and posterolateral (plv) zones were similar in all three populations and the haptoral complex (hv+ptv) appeared highly variable.

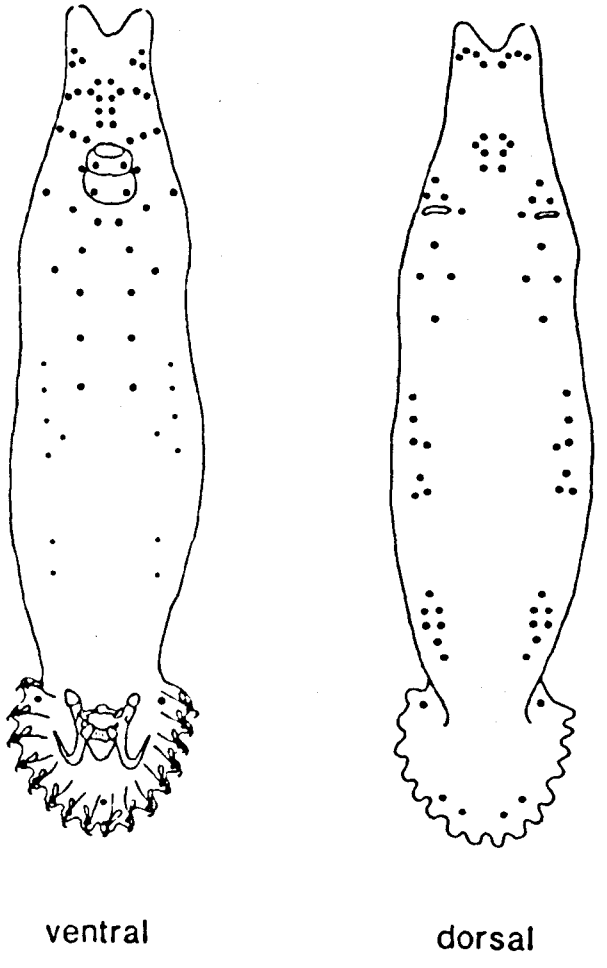


Figure 13.5: Chaetotaxy map of *G. derjavini* Morph 1 on *S. salar* from the River Allan, Scotland (Site 3).

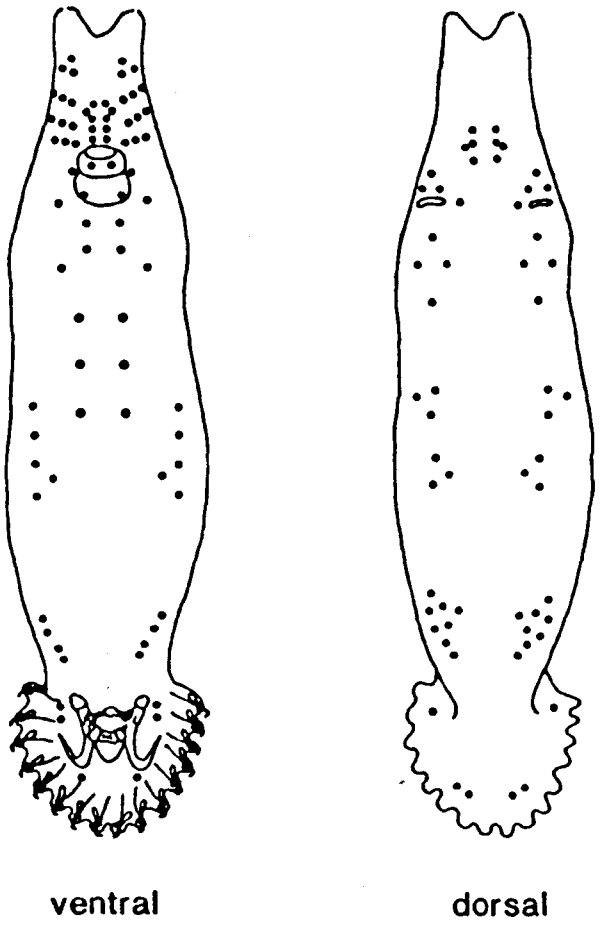
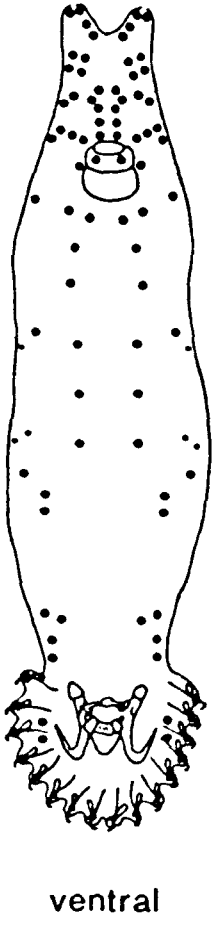
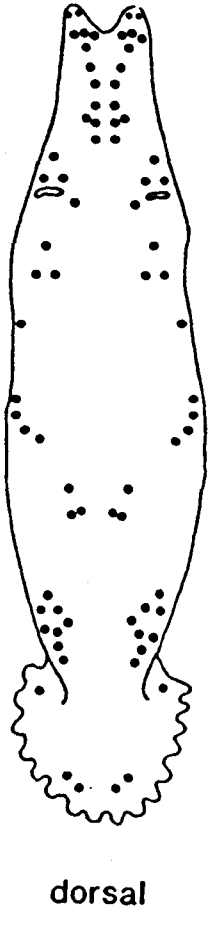


Figure 13.6: Chaetotaxy map of *G. derjavini* Morph 1 on *O. mykiss* from Loch Awe, Scotland (Site 4).

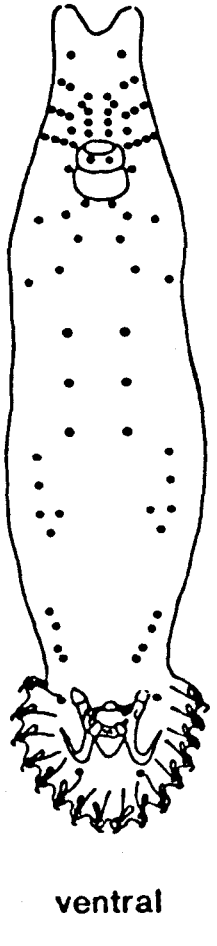


ventral

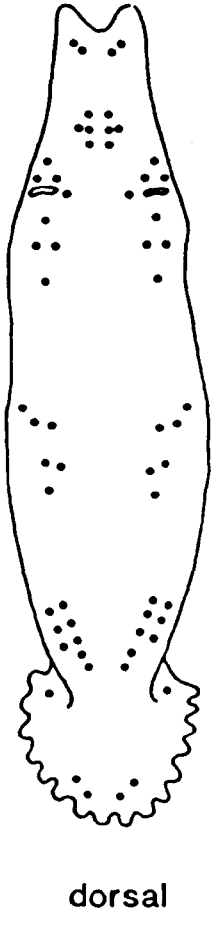


dorsal

Figure 13.7: *G. truttae* Morph 4 from *S. trutta* from the River Wey, England (Site 5).

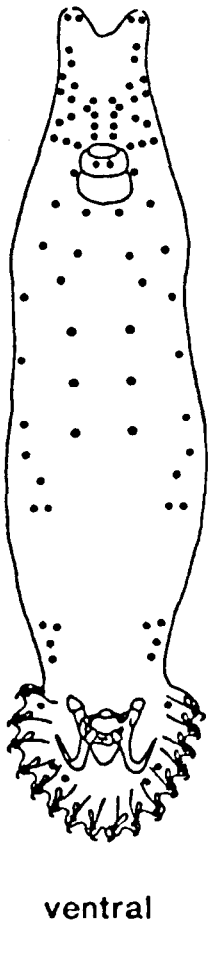


ventral

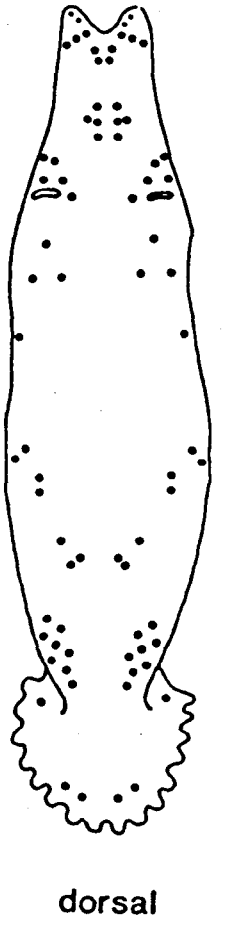


dorsal

Figure 13.8: *G. truttae* Morph 4 from *S. trutta* from the River Allan, Scotland (Site 6).



ventral



dorsal

Figure 13.9: *G. truttae* Morph 5 from variant from *S. trutta* from Loch Airthrey, Scotland (Site 7).

CHAETOTAXY MAPS OF *G. truttae*

(ii) The dorsal surface

The population resident on *Salmo trutta* in Loch Airthrey (site 7) (Figure 13.9) had 5 sensilla in its circumporal (cp) zone, a cluster of 4 anterior to the opening of the excretory pore and a single sensillum lying laterally to the oral aperture. Of the cluster of 4, the most anterior and laterally positioned sensillum (no. 1) lay close to the lateral margin of the body. Gyrodactylids from this locality could be differentiated from those from the other sites in that the most posterior antero-lateral (al) set on the ventral surface had only 3 sensilla: it is possible that the extra circumporal sensilla belongs to this set and, if so, suggests a 4-2-4 antero-lateral (al) configuration. The only other slight difference lay in the anterior-most median (md) cluster of sensilla, where *G. truttae* Morph 4 from the River Allan had only 3 sensilla, as shown in Figure 13.8, in comparison to 4 in the other two cases as shown in Figures 13.7 and 13.9. The postero-dorsal (pd) set and the haptoral complex (hd+ptd) did not vary in the specimens from the three sites.

Interspecific variation between gyrodactylids from salmonids

In the species of *Gyrodactylus* from salmonids studied, there existed homologous zones of sensilla where the configuration of sensilla appeared to have a low degree of variation. The peribuccal (pb, Figure 13.1) zone on the ventral surface always had a cluster of 5 sensilla anterior to the opening of the mouth and 2 sensilla posterior to this. Perhaps the most obvious cluster was in the ventro-median zone (m, Figure 13.1), seen as a line of 3 sensilla, which is also present in other families of the Monogenea. Dorsally, the region directly anterior to the pharyngeal region typically possessed a cluster of 4 sensilla which formed the posterior portion of the antero-dorsal zone (ad). Other homologous zones include the antero-median (am) set of 4 sensilla and in the dorso-haptoral complex (hd+ptd, Figure 13.1), the haptoral (hd) and postero-terminal (ptd) portions having one and 2 sensilla, respectively.

The anterolateral (al) cluster of sensilla on the ventral surface exhibited the greatest variability. Table 13.2 shows the arrangement of sensilla for this region in the 10 populations of *Gyrodactylus* investigated. Such variability might be explained by the concentration of sensilla around the spike sensillum and the effect of pressure on

the slide preparation with respect to the number of sensilla visible or undetected on the lateral margin of the body.

In consideration of the interspecific differences, Tables 13.3 and 13.4 present the chaetotaxy formulae for the ventral and dorsal surface for each of the 10 populations of *Gyrodactylus* investigated, Table 13.5 summarises those zones which are most discriminating and the diagrams in Figures 13.10-13.11 highlight these differences.

G. salaris could be differentiated from the salmonid gyrodactylids occurring in Britain by means of combinations of sensillary groupings. The most useful zones to examine for distinguishing features were the anterior zone (av, Figure 13.1) of 5 sensilla and the medio-lateral (ml) zone of 6 sensilla on the ventral surface plus the postero-dorsal (pd) zone of 9 sensilla (Table 13.5). Other differences are more subtle. The region posterior to the median (md, Figure 13.1) zone in *G. salaris* differed from that in the other two species in arrangement, but to what extent this was due to specimen preparation is not known. There exists, therefore, some degree of variability between species and, although intraspecific variation was greater in zones such as the haptoral complex (hv+ptv) and the ventral antero-lateral (al) region, sensillary patterns were otherwise relatively consistent.

Although the arrangements of sensilla occurring at the anterior end of the body have been described as labelled in Figure 13.1, the precise arrangements are still questionable. The region between the mouth and the cephalic lobes has, the greatest concentration of sensillary structures and, therefore, the allocation and identification of a single sensillum becomes somewhat problematical. The results suggest that the zone of sensilla posterior to the mouth will be most useful for identification keys, since the sensilla are less concentrated and the patterns are clearly exhibited. Ventrally, all three species studied showed a very high degree of homology (Figure 13.10), *G. salaris* differing only in that the zone immediately posterior to the mouth had only 5 sensilla versus 6 in the corresponding zones of *G. derjavini* Morph 1 and *G. truttae* Morph 4.

A similar situation existed for the dorsal maps (Figure 13.11), although the differences were not so clearly exhibited, since they appeared to involve arrangement rather than presence or absence. When the chaetotaxy patterns of the non-salmonid

Table 13.2: The arrangement of the ventral surface sensilla in the region anterior to the mouth.

Species	Site No.	Arrangement	Total
(a) Salmonid gyrodactylids			
G. salaris (salmon, R. Ätran, Sweden)	1	4-2-3-2	11
G. salaris (salmon, R. Lierelva, Norway)	2	4-2-4	10
G. derjavini (salmon, R. Allan)	3	3-2-3	8
G. derjavini (rainbow trout, L. Awe)	4	3-3-3-3	12
G. truttae (brown trout, South Wey)	5	1-3-2-4-2-2	14
G. truttae (brown trout, R. Allan)	6	4-1-3-2-1	11
G. truttae (brown trout, L. Airthrey)	7	3-2-4-2-2	13
(b) Non-salmonid gyrodactylids			
G. turnbulli (guppy, BM(NH) culture)	8	1-3-2-3-1-2-1-3-2	18
G. gasterostei (three-spined stickleback)	9	3-2-2-1-2	10
Gyrodactylus sp. (shubunkin goldfish)	10	3-3-1-3-2	12

gyrodactylids are compared, more obvious differences became apparent. The "salmonid" gyrodactylids investigated here, *G. salaris*, *G. derjavini* Morph 1 and *G. truttae* Morph 4, all belong to the *G. wageneri*-complex. Members of this group are distinguished by the morphological characteristics of their marginal hooks which bear a distal portion wider than the proximal portion, hamuli with folds, ventral bars with short processes, small cirrus spines in a single arched row and long pharyngeal processes (Malmberg, 1970). *G. salaris* is included within the *G. wageneri*-complex for the purposes of this paper, following its initial allocation to this group by Malmberg (1956). However, this species has subsequently been moved into the *G. salaris*-complex by Malmberg (1962), along with *G. thymalli* Zitnan, 1960, *G. brachymystacis* Ergens, 1978, *G. lenoki* Gusev, 1953, *G. asiaticus* Ergens, 1978 and *G. magnus* Konovalov, 1967, on the basis of the ventral bar being wider in relation to that of members of the *G. wageneri*-complex.

G. gasterostei Gläser, 1974 is a member of the *G. wageneri*-complex, but, when compared to the "salmonid" gyrodactylids, its sensillary pattern differed in a number of respects (Figure 13.12). Ventrally, the peribuccal (pb) sensillary arrangement was slightly more spaced in the region posterior to the mouth, but more obviously, the anterior part of this peribuccal zone in the "salmonid" species typically bore 5 of the 7 sensilla, whereas *G. gasterostei* possessed 6. The anterior (av) zone of 5 sensilla of *G. gasterostei* also differed in its configuration, and the median (m) zone was bordered by 2 sensilla. The medio-lateral (ml) zone was displaced posteriorly and comprised 2 sets of 3 sensilla.

Dorsally, the circumporal (cp) zone comprised only 3 sensilla, lacking the typical no. 4 sensillum of the salmonid forms positioned medially between the excretory pores. The median (md) zone was marked by a single sensillum, but the postero-dorsal (pd) zone was similar to that of the "salmonid" forms bearing 8 sensilla. The haptor (hd) zone bore only 3 sensilla, their prevalence being less than 70%.

G. turnbulli Harris, 1986 (Figure 13.12) (Site 8) belongs to the *G. eucaliae*-complex and again the chaetotaxy pattern differed from the "salmonid" gyrodactylids, but it was analogous to *G. gasterostei* (Figure 13.13) (Site 9+10) in that the anterior portion of the peribuccal (pb) zone (Figure 13.12) had 6 sensilla and 2 posterior to the opening of the mouth. There appeared to be a 9th sensillum lying laterally to the oral

Table 13.3: Comparison of the chaetotaxy formulae of *Gyrodactylus* spp. for various zones on the ventral surface. The figures refer to the number of those sensilla having a prevalence greater than 70% on constructed chaetotaxy maps.

Species	Site No.	pb	al	av	mv	ml	pl	hv	ptv
G. salaris (salmon, R. Ätran, Sweden)	1	7	11	5	3	7	4	4	1
G. salaris (salmon, R. Lierelva, Norway)	2	7	10	5	3	6	3	2	1
G. derjavini (salmon, River Allan)	3	7	8	6	3	0	0	1	1.5
G. derjavini (rainbow trout, Loch Awe)	4	7	12	5	3	5	4	2	1
G. truttae (brown trout, South Wey)	5	7	13	6	3	5	4	2	0
G. truttae (brown trout, River Allan)	6	7	11	6	3	5	4	1	1
G. truttae (brown trout, Loch Airthrey)	7	7	13	6	3	5	4	1	2
G. turnbulli (guppy, Natural History Museum culture)	8	8	18	8	3	5	4	3	1
G. gasterostei (three-spined stickleback, Reading)	9	7	10	6	3	8	4	2	0
Gyrodactylus sp. (shubunkin goldfish, private aquarium)	10	6	12	4	3	0	5	2	0

Abbreviations: pb = peribuccal; al = antero-lateral; av = anterior (ventral); m = median (ventral); ml = medio-lateral; pl = postero-lateral; hv = haptoral (ventral); ptv = postero-terminal (ventral).

Table 13.4: Comparison of chaetotaxy maps of *Gyrodactylus* spp. for various zones on the dorsal surface.

Species	Site No.	ad	cp	am	md	pd	hd	ptd
G. salaris (salmon, R. Ätran, Sweden)	1	9	5	4	7	9	1	2
G. salaris (salmon, R. Lierelva, Norway)	2	8	5	4	6	9	2	2
G. derjavini (salmon, River Allan)	3	8	4	4	7	8	1	2
G. derjavini (rainbow trout, Loch Awe)	4	4	4	4	6	8	1	2
G. truttae (brown trout, South Wey)	5	12	4	4	7	8	1	2
G. truttae (brown trout, River Allan)	6	6	4	4	6	8	1	2
G. truttae (brown trout, Loch Airthrey)	7	11	5	4	7	8	1	2
G. turnbulli (guppy, Natural History Museum culture)	8	8	4	4	3	9	4	0
G. gasterostei (three-spined stickleback, Reading)	9	4	3	3	1	8	0	0
Gyrodactylus sp. (shubunkin goldfish, private aquarium)	10	14	3	1	3	6	2	1

Abbreviations: ad = anterior (dorsal); am = antero-median; cp = circumporal; md = median (dorsal); pd = posterior (dorsal); hd = haptoral (dorsal); ptd = posteroterminal (dorsal).

Table 13.5: A key to differences between species of *Gyrodactylus* parasitising salmonids as indicated by chaetotaxy.

Ventral		
anterior	5 sensilla	*G. salaris*
	6+ sensilla	*G. derjavini* Morph 1 or *G. truttae* Morph 4
medio-lateral	5 sensilla	*G. derjavini* Morph 1 or *G. truttae* Morph 4
	6 sensilla	*G. salaris*
postero-lateral	4 sensilla	*G. salaris*, *G. derjavini* Morph 1 or *G. truttae* Morph 4
	5 sensilla	possibly *G. salaris*
Dorsal		
circumporal	4 sensilla	*G. derjavini* Morph 1 or *G. truttae* Morph 4
	5 sensilla	*G. salaris* or possibly *G. truttae* Morph 4
posterior	8 sensilla	*G. derjavini* Morph 1 or *G. truttae* Morph 4
	9 sensilla	*G. salaris*

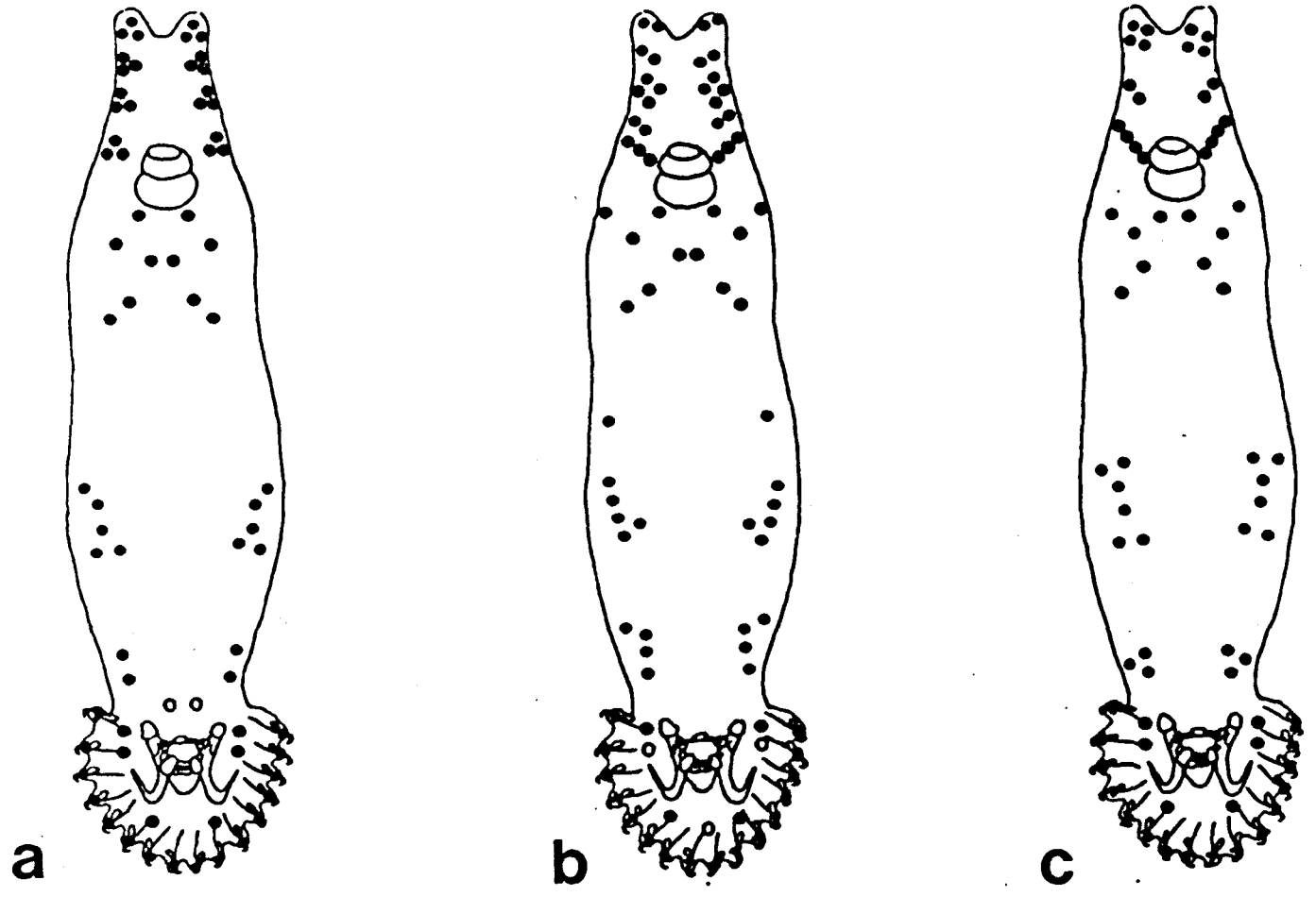


Figure 13.10: Differences in the ventral surface chaetotaxy maps for three studied populations of salmonid *Gyrodactylus*. Key: A = *G. derjavini* Morph 1; B = *G. truttae* Morph 4 and C = *G. salaris*.

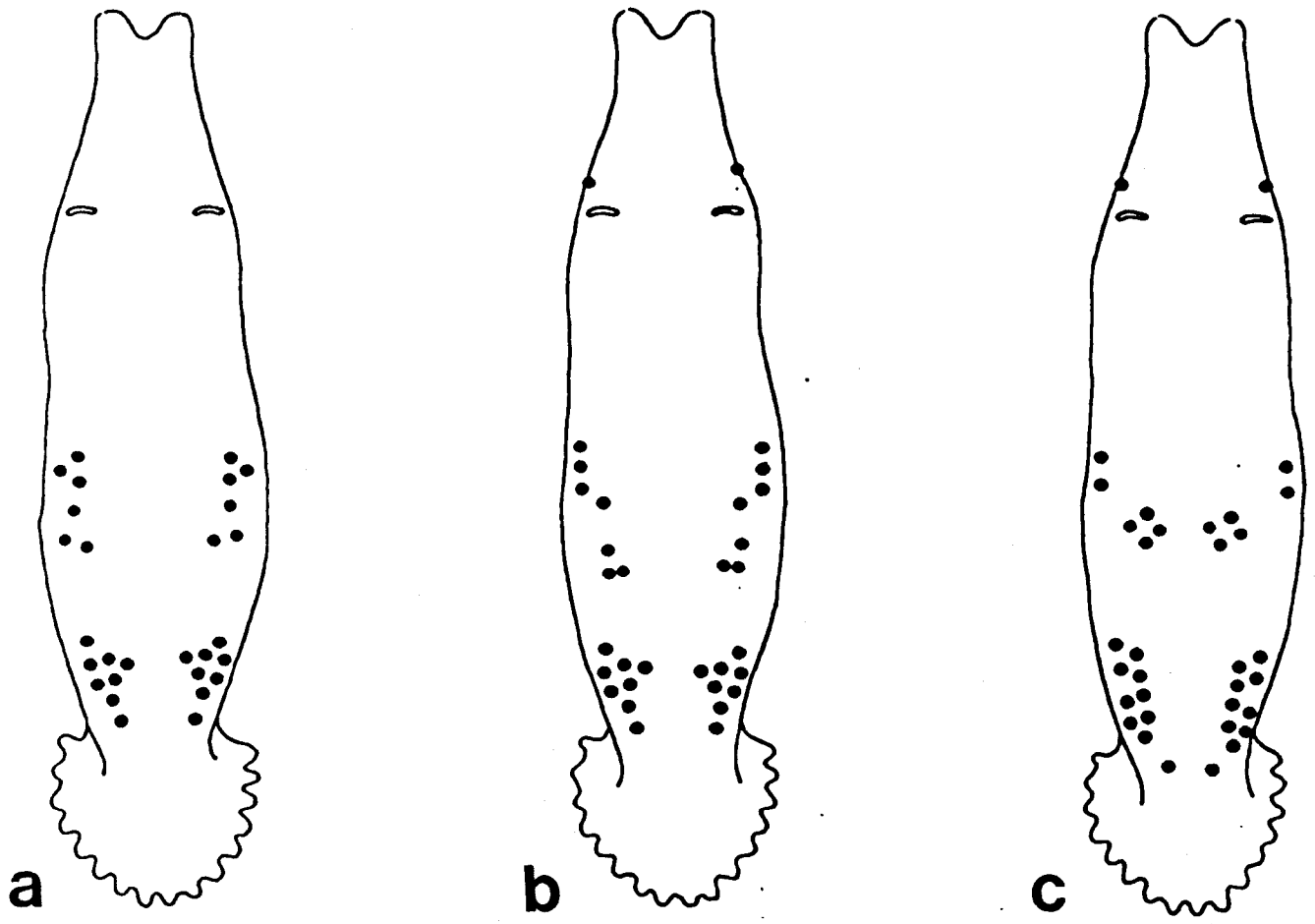


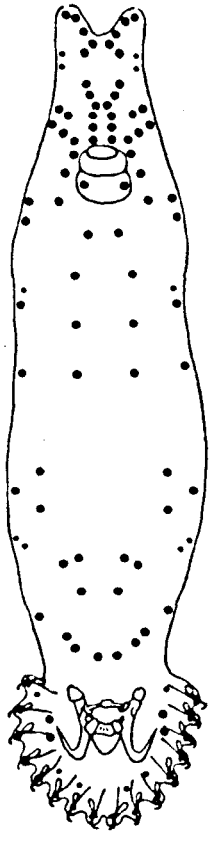
Figure 13.11: Differences in the dorsal surface chaetotaxy maps for three studied populations of salmonid *Gyrodactylus*. Key: A = *G. derjavini* Morph 1; B = *G. truttae* Morph 4 and C = *G. salaris*.

aperture but it may be that this belonged to the posterior-most part of the antero-lateral zone. The anterior (av) zone differed in having 9 sensilla in a unique configuration. Dorsally, the differences tended to be in the posterior portion of the worm. The median (md) and postero-dorsal (pd) zones were close together, bearing 3 and 7 sensilla respectively, and the postero-dorsal set was more anteriorly positioned. The haptoral complex (hd+ptd) arrangement was unique and appeared to be variable in configuration.

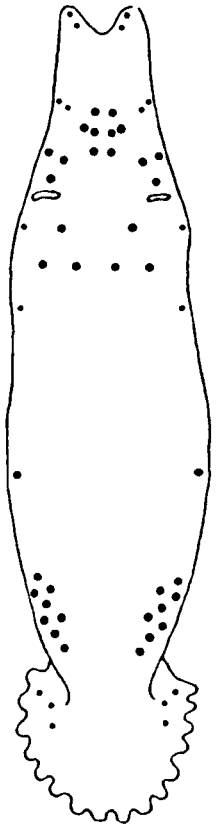
The chaetotaxy maps for *Gyrodactylus* sp., possibly belonging to the *G. elegans*-complex (*G. elegans* and *G. phoxini* group), recovered from a specimen of *Carassius auratus* (Figure 13.14) (Site 11) will be commented on only briefly due to the small number of adequately stained specimens available. The apparent differences were as follows: ventrally, the peribuccal (pb) zone was analogous to that of the "salmonid" gyrodactylids with a 5 anterior and 2 posterior arrangement, the anterior (av) zone was deficient, bearing only 2 sensilla, the medio-lateral (ml) zone had 3, and postero-lateral (pl) zone had 6 sensilla, and dorsally, the circumporal (cp) zone bore 4, the antero-median (am) zone had 2, the median (md) zone had 3 and the postero-dorsal (pd) zone bore 6 sensilla.

Discussion

A comparison of chaetotaxy maps of *Gyrodactylus*, an apparently neotenous monogenean, and larval monogeneans cannot be undertaken because, although some larval sensillary aggregations are retained, most form unique patterns. Furthermore, Lambert (1981) found that the larval chaetotaxy pattern of some monogeneans is modified on attachment to its host, by the disappearance of a group of dorsal sensilla. In larvae of monogeneans, two kinds of sense organ are known to open through the tegument (Fournier, 1981), single uniciliate and compound multiciliate receptors. Although argentophilic staining does not differentiate between these two kinds, two types of sensilla on silver nitrate stained specimens were observed. The first type was utilised to construct the chaetotaxy maps; these large, dark, toroid sensilla conform to a pattern and are symmetrical about the longitudinal axis of the worm. The second type of sensilla appear as small, randomly distributed clusters and were not included

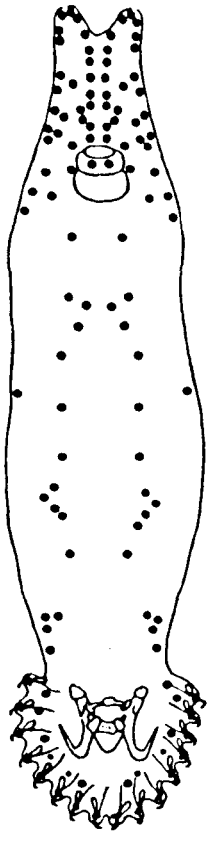


ventral

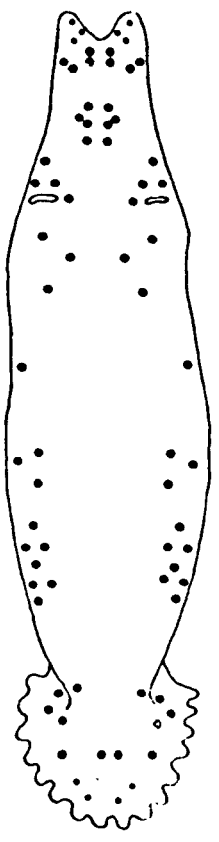


dorsal

Figure 13.12: Chaetotaxy map of *G. turnbulli* Harris, 1986 from *Poecilia reticulata* held in aquaria at The Natural History Museum (Site 8).

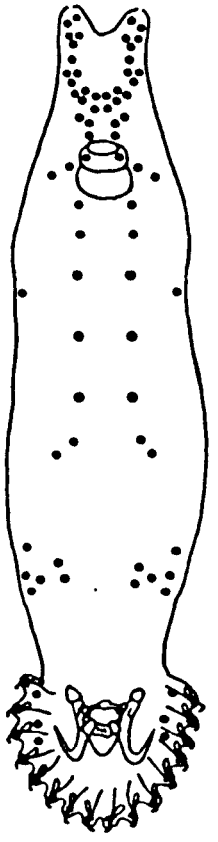


ventral

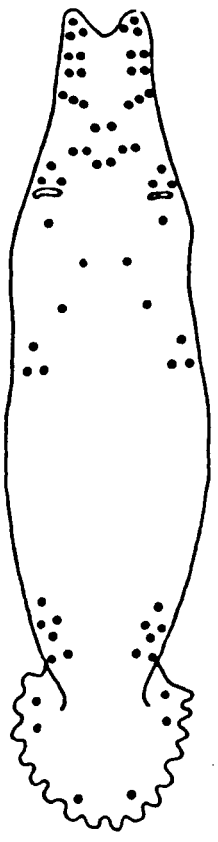


dorsal

Figure 13.13: Chaetotaxy map of *G. gasterostei* Gläser, 1974 from *Gasterosteus aculeatus* in the River Wey, Berkshire (Site 9+10).



ventral



dorsal

Figure 13.14: Chaetotaxy map of *Gyrodactylus* sp. Nordmann, 1832 from *Carassius auratus* from a Bedfordshire fish pond (Site 11).

in the maps or chaetotaxy formula. Although some 50 specimens of *Gyrodactylus* were used in the construction of each map from each geographical site, it should be noted that the average number of specimens found on individual salmonids from the UK was low (0.493 specimens per fish, based on data from 250 sites), and therefore new born specimens were not available for staining and examination. However, considering the viviparous mode of reproduction and disregarding the possibility of the existence a "larval stage", the chaetotaxy formula for *Gyrodactylus* would appear to be consistent throughout life.

Some points are worth noting regarding the construction of chaetotaxy maps for *Gyrodactylus* spp. One problem associated with the elucidation of these patterns was caused by the viviparity of these monogeneans. The presence of an embryo within the uterus makes the sensillary pattern difficult to discern. This is not because the embryo's sensilla are evident, since this is not the case, but because the embryo adds bulk to the specimen, preventing a wholly flattened preparation. It should also be noted that the haptoral sensilla are probably unreliable criteria on which to make species comparisons for two reasons. Firstly, the fibrous sheaths through which the points of the hamuli and thickenings through which the marginal hooks emerge from the body of the opisthaptor were also argentophilic, or at least appeared to be. All the openings on the body of *Gyrodactylus*, the pharyngeal aperture, the excretory pores and the apertures through which the hamuli and marginal hooks emerge, stain with silver nitrate. It is unlikely that these structures are argentophilic, but that infiltration of silver nitrate occurs which is not removed by the washing steps of the staining procedure. A tactile receptor would be useful in such a position, but investigations using the SEM have failed to show any affiliated structures, although one specimen examined under the SEM did indicate the presence of two structures believed to be sensilla on the anterior-most edge of the opisthaptor of *G. gasterostei*, but no other analogous structures were found. Secondly, if all mucus is not removed from the parasite by adequate washing in phosphate buffer prior to staining, an otherwise clear sensillary pattern may be marred, since mucus also takes up the silver stain. As both Combes & Lambert (1975) and Tinsley (1978) noted, individual sensilla may be duplicated, missing or displaced. It is important, therefore, that chaetotaxy maps be constructed from a minimum of 10 individuals to ensure the elucidation of complete

sensillary arrangements.

Both the *G. salaris* populations were from wild Atlantic salmon, and, although the Norwegian River Lierelva stock had been maintained under laboratory conditions, the characteristics unique to *G. salaris* were retained. These are a postero-dorsal (pd) cluster of 9 and, ventrally, an anterior (av) cluster of 5 sensilla, plus a medio-lateral (ml) group of 6 sensilla (Figure 13.2-13.3). Although, possible host-mediated differences, i.e. Baltic versus Atlantic strains of salmon, have not as yet been investigated for *G. salaris*, the results from these two populations indicated that there is a good homology in the chaetotaxy patterns irrespective of geographical location.

Chaetotaxy and the evolutionary relationships of Gyrodactylus

In order to apply the chaetotaxy patterns for *Gyrodactylus* spp. it is necessary to firstly consider the origin of each species within the family Gyrodactylidae. In this work, six species of *Gyrodactylus* were investigated from three major branches of the *Gyrodactylus* evolutionary tree proposed by Malmberg (1970). The species *Gyrodactylus* sp. (possibly *G. elegans* from *Carassius auratus*) and *Gyrodactylus* sp. (from *Phoxinus phoxinus*) belong to the *G. elegans*-complex (*G. elegans*-group and *G. phoxini*-group), which contains apparently primitive species belonging to the nominal subgenus *G. (Gyrodactylus)* and parasitising primitive fish host species, i.e. Ostariophysi (mainly Cyprinidae). *G. turnbulli* belongs to the *G. eucaliae*-complex, a primitive group arising early on in the development of the subgenus *G. (Metanephrotus)*, which, together with *G. (Paranephrotus)* and *G. (Limnonephrotus)*, are the most advanced subgenera. The remaining species *G. salaris*, *G. derjavini* Morph 1, *G. truttae* Morph 4 and *G. gasterostei* are members of the *G. wageneri*-complex and belong to the subgenus *G. (Limnonephrotus)*. Within this group it is possible to use host inference, since *G. gasterostei* is parasitic on gasterosteids, a group which is phylogenetically more advanced than the salmonoids. From the information gathered from this study, no trends in the sensillary pattern within the genus *Gyrodactylus* in relation to their evolution could be elucidated.

It appears from the analysis, that the "salmonid" gyrodactylids within the *G. wageneri*-complex show a slight intra-specific variability in the configuration of the sensillary pattern, but the inter-specific differences are more clear. Most notable are

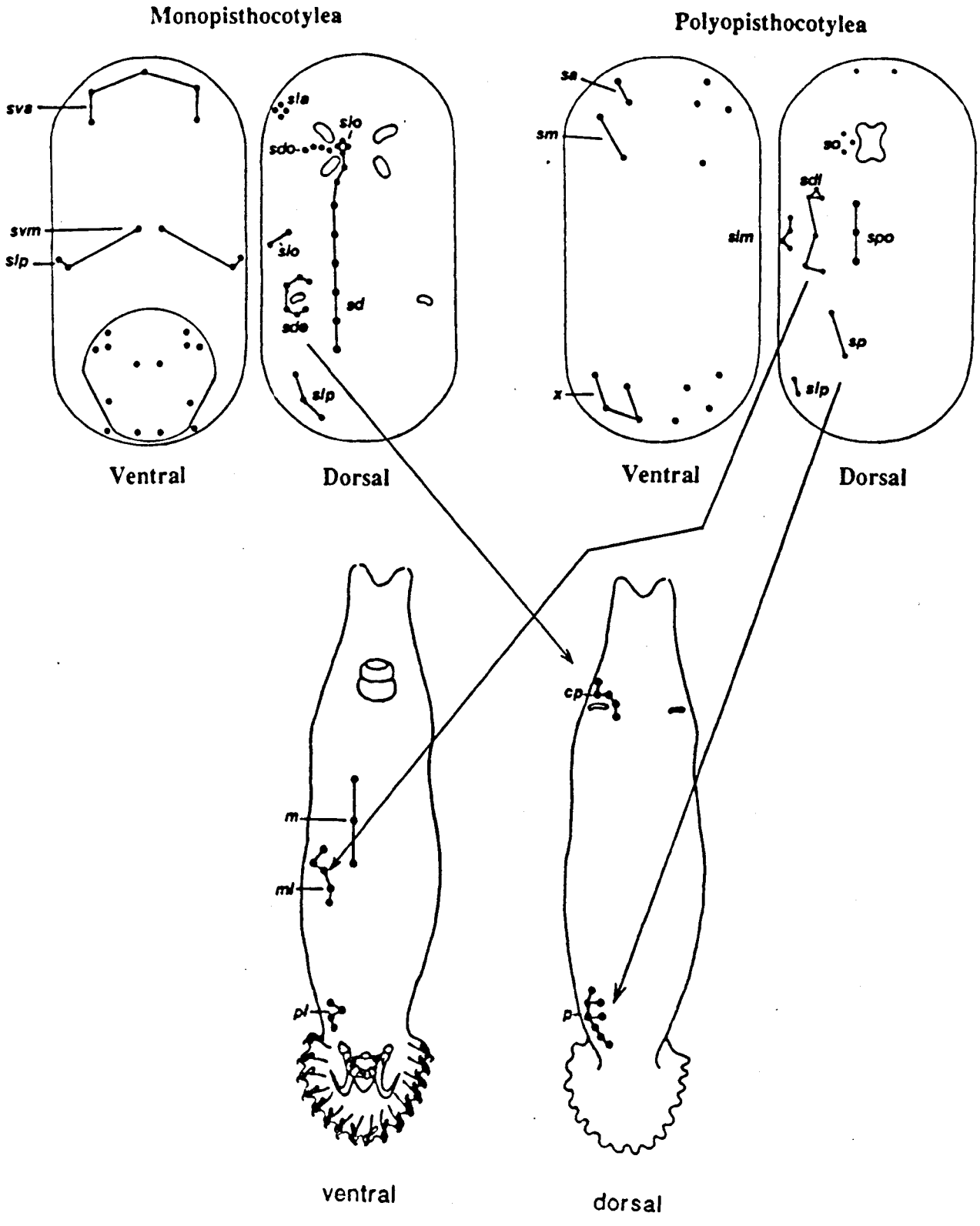


Figure 13.15: Comparison of the sensillary patterns of a monopisthocotylean *Diplectanum aequans*, a polyopisthocotylean *Microcotyle mormyri* (adapted from Lambert, 1980) and *Gyrodactylus*.

those between *G. salaris* and other members outside the *G. wageneri*-complex. The agreement in sensillary patterns for the Norwegian and Swedish populations of *G. salaris* suggests species-specific patterns and a genetic basis. Short & Kuntz (1976) were able to show differences in *Schistosoma rodhaini* and *S. mansoni* cercarial chaetotaxy patterns; although they have similar total numbers of papillae, they were differentiated on the dorsal configurations. Their study also looked at isolated populations in Puerto Rica and Kenya and, in the case of both species, found the chaetotaxy maps to be in close agreement.

Although the present study also investigated species-specific sensillary arrangements, the influence of the host was also considered. The differences between *G. derjavini* Morph 1 and *G. truttae* Morph 4 within the UK appear to be subtle and require a more detailed study. The results suggest that these two *G. wageneri*-complex members evolved recently relative to many other members of the genus (Malmberg, 1970) and differences between them in chaetotaxy patterns have not developed. Subtle differences in spatial configuration may make discrimination possible between these two species. Cabaret *et al.* (1990) analysed this aspect, measuring intersensillary distances and, with subsequent multivariate analysis, were able to discriminate between four species of schistosome cercariae. Similarly, Euzet & Lambert (1974) were able to differentiate between *Diplozoon paradoxum* von Nordmann, 1832 and *D. gracile* Reichenbach-Klinke, 1961 by differences in the spatial configuration of two sensilla posterior to the mouth.

Lyons (1969), using the thiocholine method, was able to stain and map the nervous system of *Gyrodactylus* sp., but did not examine its relationship to the distribution of sensilla. Combes & Lambert (1975) attempted to produce a system of sensillary nomenclature for the larval monogeneans analogous to that which Richard (1971) had developed for digenean cercariae. They also used transverse commissures of the nervous system to divide the body into equal bands. However, this system proved difficult to apply to the genus *Gyrodactylus* for a number of reasons. Using a system dividing the body into equal portions broke up normal aggregations of sensilla and introduced an element of confusion in the description of the precise location of single sensilla. Furthermore, Combes & Lambert's system was designed for larval monogeneans, but *Gyrodactylus*, an unusual monogenean, may be a neotenic adult

(Lambert, 1980); therefore, although it does possess analogous clusters, the chaetotaxy pattern is quite unique. Lambert (1979, 1980) applied this system to two species of *Gyrodactylus*, but the regions which he considered analogous to those in the larval Polyopisthocotylea are debatable. Tinsley & Owen (1975), unaware of the paper of Combes & Lambert (1975), described the constellar arrangements of the oncomiracidium of *Protopolystoma* sensilla according to their natural grouping.

Lambert (1977a) highlighted the sensillary characteristics unique to the Polyopisthocotylea and described the analogous regions in the genus *Gyrodactylus*. Three dorsal post-ocular sensilla (spo) [also known as the "sensilles post-oculaires", see Lambert, (1978c), and "dorsal interocular sensilla", see Tinsley & Owen, (1975)] (Figure 13.15) were linked to the ventro-median (mid-set of six) zone (m), although the reason why this analogous cluster of sensilla is found on the ventral surface in *Gyrodactylus* is questioned. On the attachment of the oncomiracidium to its host, the dorsal sensilla (sd) in the monopisthocotyleans and the post-ocular sensilla (spo), distinguishable on the basis of size (Lambert, 1980) in the polyopisthocotyleans, disappear; similarly these sensilla in the genus *Gyrodactylus* are noticeable by the fact that they are slightly larger than those comprising the rest of the sensillary arrangement. In addition, the six dorso-lateral sensilla (sdl) on polyopisthocotyleans may equally correspond closely to the ventral medio-lateral (ml) cluster or the dorsal circumporal (cp) cluster described for the "salmonid" gyrodactylids, and the postero-lateral (slp) and posterior (sp) zones on the dorsal surface of the polyopisthocotyleans correspond with the postero-dorsal region (pd) of the "salmonid" gyrodactylids. The postero-ventral cluster of sensilla on the polyopisthocotylean (marked "x", unnamed by Lambert (1977a) in Figure 13.15) may be analogous to the postero-lateral region (pl) in *Gyrodactylus*. The dorsal region (sde) indicated on the map of the monopisthocotylean (Figure 13.15) may be analogous to the circumporal zone (cp) on *Gyrodactylus*. The ventral surface of *Microcotyle mormyri* (Lorenz, 1878), proposed by Lambert (1977a) as a typical polyopisthocotylean, bears no zones comparable with those on either surface of *Gyrodactylus*. However, on the monopisthocotylean *Diplectanum aequans* Wagner, 1857, the sensillary clusters, interocular (sio), dorsal (sd) and circumporal (sde) on the dorsal surface, are apparently comparable to the polyopisthocotylean clusters, periocular (so), postocular (spo) and dorso-lateral (sdl),

respectively (see Figure 13.15) (Lambert, 1977a).

Llewellyn (1963) stated that members of the genus *Gyrodactylus* were monopisthocotyleans on the basis that they lacked a genito-intestinal canal and are epidermal grazers, whereas the Polystomatidae (now placed in the Polyopisthocotylea), which together with Gyrodactylinea formed the order Gyrodactylidea (Bychowsky, 1957) on the basis of similarities in their hook configurations, have a genito-intestinal tract and are blood-feeders, and, therefore, belong to the Polyopisthocotylea. Baer & Euzet's (1961) classification of the Monogenea separated these two groups into the Gyrodactylidae (Gyrodactyloidea: Monopisthocotylea) and Polystomatidae (Polystomatoidea: Polyopisthocotylea). Subsequently, Lambert (1979; 1980) suggested that the gyrodactylids arose by neoteny from a polyopisthocotylean, so explaining both the loss of the genito-intestinal canal and its epidermal feeding (features of the Monopisthocotylea). Lambert (1980) stated that, *Gyrodactylus* has a chaetotaxy pattern which excludes them from the Monopisthocotylea, as they must have descended from ancestral stock close to the origin of the Polyopisthocotylea. However, other morphological characters suggest a placement of *Gyrodactylus* close to the origin of the Polyopisthocotylea. Lyons (1972, 1977) suggested a division between the suborders of the Monogenea based on the presence of a dense fibrous zone in the external plasma-membrane of the polyopisthocotylean tegument. Malmberg (1970) considered that the excretory system of *Gyrodactylus,* although having monopisthocotylean characteristics, was similar to that of the polypisthocotyleans. Considering the chaetotaxy alone, it appears that both *Gyrodactylus* spp. and *Polystoma* spp. have zones of sensilla that place them within the Polyopisthocotylea; namely, the presence of postocular sensilla (spo) in *Polystoma* (Maeder, 1973; Combes & Lambert, 1975) and the comparable group in *Gyrodactylus* (Lambert, 1979) plus, the ventral median zone (m) outlined in the present study.

Lambert (1979, 1980) recommended the inclusion of *Gyrodactylus* within the Polyopisthocotylea based on the presence of a group of sensilla, i.e. the post-ocular (spo) zone as shown in Figure 13.15. However, this group is in fact further displaced in the case of *Gyrodactylus* and is actually on the opposite surface, i.e. the ventral surface, recognised by their size and consistent pattern of a 3x2 group of sensilla. Lambert's identification of the spo group in *Gyrodactylus* is questionable, however,

since the spatial distribution and configuration of this group and size of the sensilla in this region are not consistent with the present findings, as indicated in Figure 13.16. The findings here suggest that the ventral median (m) cluster is analogous to the spo sensilla. In his 1980 paper, Lambert discussed the chaetotaxy map of *Gyrodactylus* sp. from *Phoxinus phoxinus* in relation to the earlier work (1979) as shown in Figure 13.16. The 1980 paper is in close agreement with the current findings on the sensillary arrangement, whereas the first account seems confused in relation to the antero-dorsal (ad) zone, which appears to include the circumporal region (cp). Unfortunately, the basis for this group's homology to the sdl and spo regions in the Polyopisthocotylea is based on evidence from the 1979 paper. Fournier (1981) discussed two kinds of sense organ from *Polystoma integerrimum* Fröhlich, 1791, single uniciliate and compound multiciliate receptors, and suggested that the multiciliate receptors differ from those occurring on monogeneans with fish hosts, since the latter have modified short cilia which are more representative of chemosensory structures.

The oviparous *Ooegyrodactylus farlowellae* Harris, 1983 is closely related to the genus *Gyrodactylus* and their chaetotaxy (Harris, 1983) is similar. This agrees with Llewellyn's theory (1981) that the Gyrodactylidae are an isolated group which arose early on during the evolution of the Monogenea.

Comparison of Gyrodactylus with Ooegyrodactylus.

Comparison of the "salmonid" species of *Gyrodactylus* with the observations of Harris (1983) on *Ooegyrodactylus farlowellae* from *Farlowella amazonum* (Loricaridae), as seen in Figures 13.17-13.18, indicates certain homologies in the sensillary patterns common to both genera. Harris' observations suggested that it was the dorsal surface which bears the greatest number of sensilla, although in the "salmonid" *Gyrodactylus* spp. the reverse is found. It seems unusual that the latter is not also the case in *Ooegyrodactylus*, which also maintains a position in close proximity to its host where touch receptors must provide the major portion of the sensory input in the absence of photo-sensitive eye-spots present in other monogeneans. There is a concentration of sensilla about the cephalic lobes and the zone impinging on the mouth and region of the cirrus on the ventral surface (Figure 13.17). The pattern anterior to the mouth (pb) in both genera is similar, except that in the "salmonid" *Gyrodactylus* spp. there is an

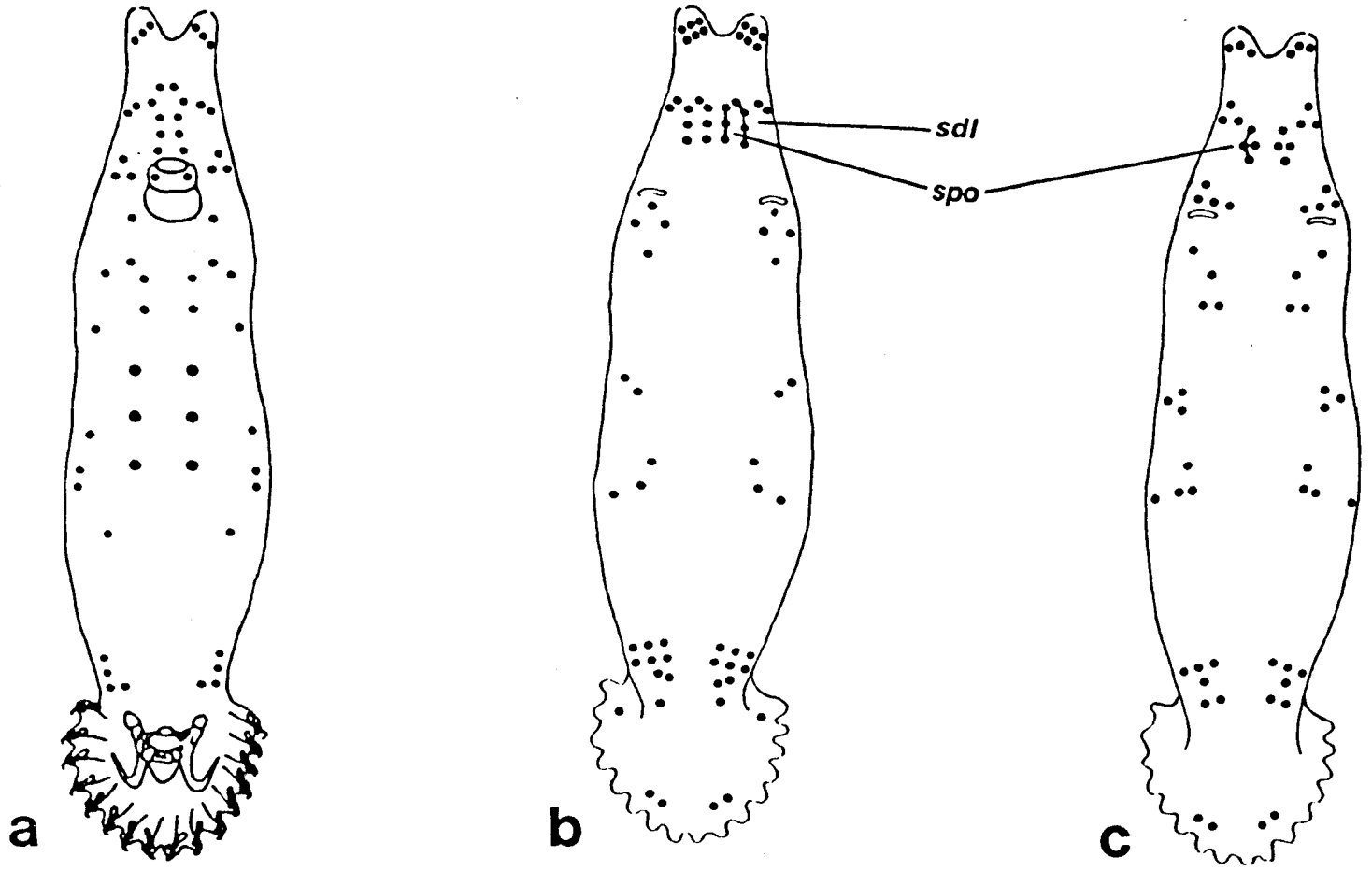


Figure 13.16: The location of the post-ocular sensilla (SPO). A = Ventral surface of *Gyrodactylus* sp. from *Carassius auratus*, the structure of the polyopisthocotylean SPO sensilla are analogous to the median (m) cluster with respect to their size and arrangement. B = Dorsal surface of *Gyrodactylus* sp. from *Carassius auratus* (from Lambert, 1979) and C = Dorsal surface of *Gyrodactylus* sp. from *Phoxinus phoxinus* (from Lambert, 1980), the spo sensilla in these accounts are believed to be analogous to the anterior (ad) cluster.

extra pair. The arrangement of sensilla on the ventral surface of the "salmonid" *Gyrodactylus* species corresponds more closely to those on the dorsal surface of *Ooegyrodactylus* in the region extending between the cephalic lobes and the pharynx. This concentration about the cephalic lobes is also seen on the anterior extremity of the dorsal surface of the worm, the large number of sensilla within this region making it difficult to determine a precise formula for this zone. Gyrodactylids are epidermal grazers moving across the surface of their host using a looping movement and attaching by the opisthaptor and the cephalic lobe region (acting as an adhesive prohaptor). The functions of attachment and feeding explain the requirement for a large number of sensilla in the latter zone.

Harris (1983), in his description of *Ooegyrodactylus*, identified sensilla arranged into four transverse bands, with additional clusters around the cephalic lobes, mouth, genital aperture and opisthaptor. Harris did not specify the manner in which these bands were classified; however, using the system of sensillary nomenclature for the dorsal surface of *Gyrodactylus* as given in Figure 13.1, each band will incorporate the following clusters: 1 = the cephalic lobes to, and including, the circumporal (cp) region; 2 = the antero-median (am) zone; 3 = the median (md) zone; 4 = the postero-dorsal (pd) zone; and 5 = the haptoral (hd+ptd) complex. These appear to have unique patterns for each genus, but in the cases of the third and fourth bands there exists a large degree of homology. The number of sensilla in each band is the same for each species, but it is not possible to determine to what extent the spatial distribution of each sensilla within a cluster accounts for differences between genera (Figure 13.18). The fourth band in the "salmonid" *Gyrodactylus* spp. is observed as an elongate cluster of eight sensilla, whereas in *Ooegyrodactylus* there exists a cluster of five in a similar arrangement to the anterior five in *Gyrodactylus* and a slightly posteriorly displaced cluster of three (Figure 13.18). An examination of the ventral surface shows that there really exists only one homologous band, the mid-set (m) of three pairs of sensilla in band 3. The fourth band in *Ooegyrodactylus* is, however, devoid of any sensilla, whereas the "salmonid" *Gyrodactylus* spp. have two by two pairs of sensilla. The opisthaptor is essentially similar, with both genera possessing at least four sensilla on both surfaces, one pair in the anterior region of the opisthaptor, level with the roots of the hamuli (hv), and a second pair near the posterior margin in close proximity to

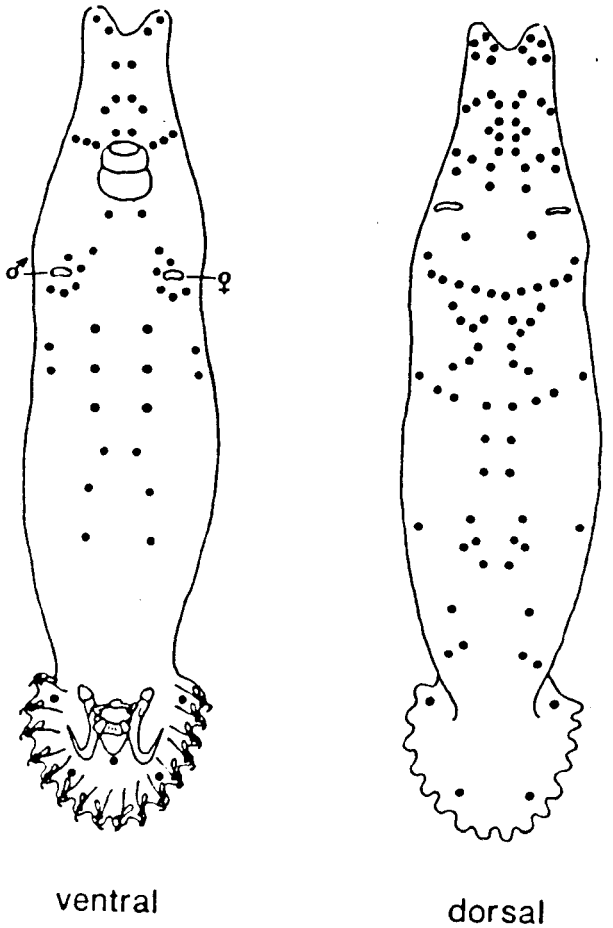


Figure 13.17: Chaetotaxy map for *Ooegyrodactylus farlowellae* (adapted from Harris, 1983).

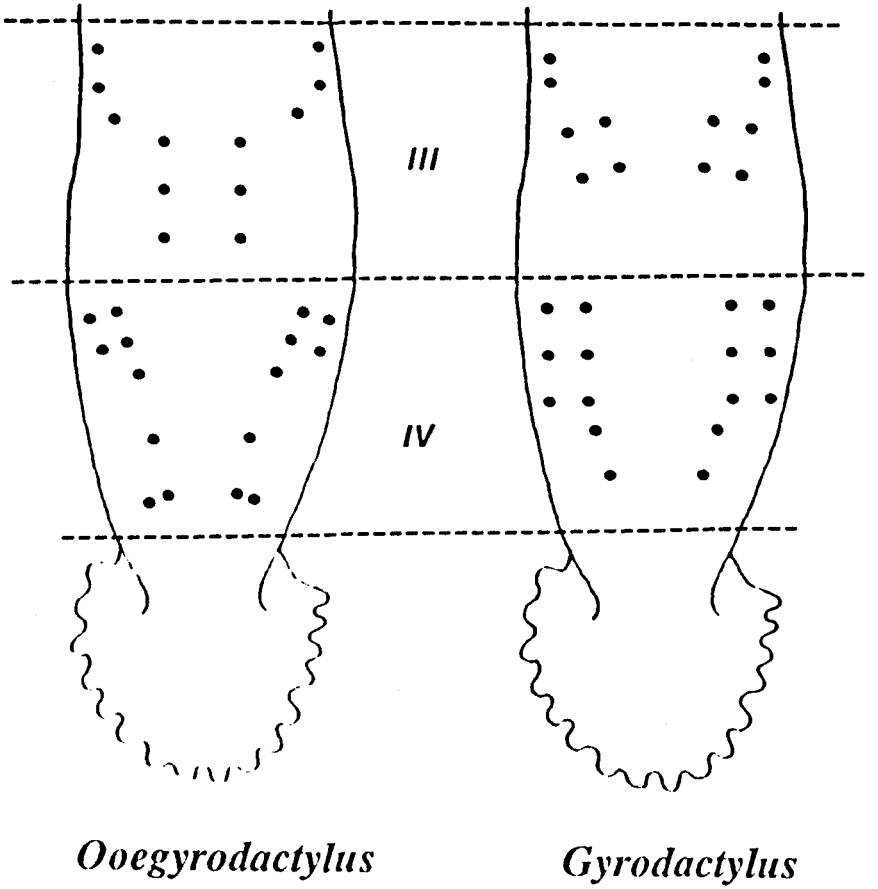


Figure 13.18: Comparison of the third and fourth dorsal bands *Ooegyrodactylus* (modified from Harris, 1983) and *Gyrodactylus* (generalised).

the point where the hamulus point emerges from the body of the opisthaptor (ptv).

Summary and conclusions

The production of species-specific maps for *G. salaris*, *G. derjavini* and *G. truttae* will provide an aid to diagnosticians using silver staining as a simple technique to identify *G. salaris*. Although, the construction of each map required the input of many specimens, it has resulted in defining the key clusters of sensilla on which to base the discrimination of *G. salaris*. *G. salaris* can be differentiated from *G. derjavini* Morph 1 and *G. truttae* Morph 4 using chaetotaxy, although, the latter two species cannot be readily distinguished (Figure 13.10-13.11). The ventro-median (m) mid-set is the most readily usable homologous region in the "salmonid" species of *Gyrodactylus*, and it is this set of three pairs of sensilla which appear to form one of the major distinctions in the systematics and phylogenetics of the Monogenea in terms of chaetotaxy. These three pairs are present in the polyopisthocotyleans, but are absent in the monopisthocotyleans so far studied which have a large set of dorsal sensilla not present in the former group. Comparisons of sensillary patterns, especially the ventro-medial set, therefore, support the hypothesis that *Gyrodactylus* is a polyopisthocotylean genus.

CHAPTER 14: SUMMARY AND CONCLUSIONS.

The sampling programme looked at the four principal species of salmonid fishes, salmon *Salmo salar*, brown trout *Salmo trutta*, rainbow trout *Oncorhynchus mykiss* and Arctic charr *Salvelinus alpinus* at 250 separate locations throughout the UK. Of the sites sampled, 47.23% of the salmon sites were found to be positive for *Gyrodactylus*, 35.7% of the brown trout sites, 58.2% of the rainbow trout sites and 1 site for Arctic charr. Only wild brown trout were found to be infected with *Gyrodactylus*, while in contrast, only farmed populations of rainbow trout and Arctic charr were infected. It was interesting to note that wild caught salmon were more likely (54.8%) to be infected with *Gyrodactylus* than salmon reared in culture (31.4%). The abundance of *Gyrodactylus* on wild salmon was also found to be considerably higher (1.77 parasites/fish) than on farmed salmon (0.39 parasites/fish). This may reflect the application of control methods in fish farms.

Specimens were prepared for light microscopy and the sclerites of the opisthaptor were measured using traditional morphometrical parameters. The data collected was subjected to multivariate analysis (Principal Components Analysis, PCA) in order to differentiate the species found. The application of multivariate analysis was an attempt to isolate species or reveal information contributing to understanding the relationships between these species of *Gyrodactylus* by considering all the measured morphometric variables simultaneously. Four hundred specimens of *Gyrodactylus* were collected from three salmonid hosts, salmon, brown trout and rainbow trout. Eighteen morphometric parameters were included in the first PCA. The number of gyrodactylids collected from the Arctic charr were few in number and were not entered in the analysis but dealt with separately. The addition of type specimens of *Gyrodactylus* species from specialist collections tested the ability of the PCA to isolate different species by acting as known standards. Further separation of the remaining specimens was achieved by removing unreliable morphometric characters, those below 15µm in size or of high variability. After deliberation, eight measurements were used for subsequent analysis. Following the removal of the unreliable structures, separation of the remaining specimens was not possible. At this juncture, it was necessary to assume that the *Gyrodactylus* specimens displayed strict host specificity, enabling them to be

separated by host. A plot of the PCA scores indicated that two species were present, one on rainbow trout and salmon and a second species on brown trout. The separation of these specimens was by the length of the marginal hook as the key factor acting along the x-axis and the total hamulus length acting along the y-axis. According to the key characters, *G. derjavini* was identified as the species on salmon and rainbow trout and *G. truttae* on brown trout. Of the 1800+ fish sampled none were found to harbour *G. salaris*.

Closer examination of the distribution of points on the PCA plot, indicated a polar effect with the *Gyrodactylus* from Welsh salmon pulled towards one end of an ellipse and the *Gyrodactylus* from Scottish salmon towards the other end. In addition, three other morphotypes were revealed in the earlier PCA tests: *Gyrodactylus* morph 3 from Loch Tralaig and Loch Coulin was isolated on the basis of having very large hamuli; morph 5 on *S. trutta* was isolated on the basis of characteristic morphological features; and a fifth form recognised by the analysis was a single specimen of *Gyrodactylus arcuatus* probably arising via an accidental infection from a gasterosteid. Further investigation of the distribution of the salmon specimens revealed that the polar effect noted above was due to the geographical distribution of the host species, and this interesting observation required substantiating. The survey was therefore, extended by including an additional 14 sites positive for *Gyrodactylus* on *S. salar*. A cluster analysis performed on the morphometric data for 31 sites indicated three clusters, one group of specimens, principally Welsh and a second group of specimens, mainly Scottish. A third group comprised a mixture of the two. The third cluster separated as a result of the influence of water temperature, mainly on the Welsh specimens. Water temperature influenced the size of the sclerites which tended to be larger in winter and smaller in the summer. It was also found that the hamuli of the Welsh salmon specimens tended to be smaller overall.

The distribution of the two subpopulations of the *Gyrodactylus* from salmon, appeared to be mimicking the distribution of its host. The celtic (southern) population of salmon was parasitised by *G. derjavini* Morph 1 and the boreal (northern) population of salmon was parasitised by a species closely resembling, but not identical to, *G. derjavini* Morph 1. Thus supporting evidence for the separation of *S. salar* during the Würm glaciations.

Having ascertained that two morphs of *Gyrodactylus* were present on salmon according to geographical location, data was analysed to see if the *G. derjavini* on salmon could be discriminated from those on rainbow trout. A total of 322 specimens of *Gyrodactylus* collected from rainbow trout were compared with 155 specimens collected from salmon using multivariate analyses. It was found that the specimens were indeed discriminated with respect to host. Morph 2 parasitised Scottish salmon, whilst Morph 1 parasitised English and Welsh salmon and rainbow trout. Morph 2 showed consistently different marginal hooks which were unique and thus this may constitute a previously undescribed species.

The ability to recognise founder populations based on their position on a host was investigated using a heavily parasitised rainbow trout. The ellipse for each fin on the PCA plot overlapped one another to a large degree, although the caudal and anal fin specimens were slightly displaced. It would appear that such a technique might be able to discriminate separate populations on wild fish, where the picture may be clearer since the interchange of parasites between wild fish is lower than would be expected for intensively cultured rainbow trout.

The identification of morphs measured using the light microscope derived data were not separable on the strength of the morphometric measurements alone, but were separated by the host fish from which they were collected. It was necessary therefore, to develop a technique which enabled accurate measurements at the EM level to be made. Thus, a technique was devised which liberated the sclerites from the body tissue thereby ensuring flattened specimens which could be analysed using the SEM. This was a major improvement over earlier techniques which were based on the action of proteolytic enzymes to digest away the tissue and free the sclerites. This, however, had a major disadvantage in that not all the sclerites were resistent to enzymatic attack. Structures such as the ventral and dorsal bars were lost, as were structures associated with the marginal hooks and hamuli, such as membranes, filament loops and muscle caps. It was found that by the use of ultrasound in the process of sonication all of the sclerites were shaken free from adhering tissue undamaged thus revealing details of structures previously unobserved. The incorporation of centrifugation and flotation techniques enabled the sclerites to be cleaned and prepared for SEM. Subsequent examination by SEM revealed the structural architecture and formation of each of the

sclerites in detail, allowing for the precise measurement and the description of potentially new taxonomic characters.

It was then possible to perform multivariate analyses on data collected from digitised images of hamuli and marginal hook electronmicrographs with greater accuracy. The new morphometric parameters described included the internal angle of the hamulus, the hamulus width and the aperture size of the marginal hook. Marginal hooks and hamuli were analysed separately; this was because, following sonication, it was impossible to state which hamulus belonged to which marginal hook. The resultant PCA analysis and plots showed that it was possible to discriminate every specimen of *G. salaris* from the British forms of *Gyrodactylus*, using both marginal hooks and hamuli. It was also found that single morphometric parameters could themselves be discriminating in some cases. *G. derjavini* and *G. truttae*, however, proved difficult to separate from one another on the basis of the measured characters, but were differentiated on the morphology of their marginal hooks.

The hamulus angle alone, described as the angle through which the hamulus point turns away from the hamulus shaft, was found to discriminate *G. salaris* from the other gyrodactylids studied. *G. salaris* was found to have a hamulus angle in excess of 42°, while the forms of *Gyrodactylus* found in the UK survey had a hamulus angle of less than 39°. The value of this character was tested further by comparison of both winter and summer collections of *G. salaris* with the British material, and by skeletonising the hamulus using image enhancement techniques to remove the effect of age-related thickening. It was found again that all specimens of *G. salaris*, regardless of season or locality, could be discriminated from the British gyrodactylids, but the British species of *Gyrodactylus* could not be differentiated from each other using the hamulus angle.

The ability to make accurate measurements when using digital analysis and electronmicrographs, raised the question of how discriminating are the new morphometric parameters; could they, for example, separate isolated populations of the same species of *Gyrodactylus*. The origin of *G. salaris* is believed to be within the Baltic and one hypothesis is that it has spread along natural water systems to the North Sea and hence onto Atlantic salmon. Two river systems were sampled for *G. salaris*, the River Ätran close to the border with Norway which is closer to the North

Sea where the Atlantic salmon is found and the River Säveån, S. Sweden which is closer to the Baltic to see if *G. salaris* from the two stocks of salmon could be separated. This would help elucidate the spread of *G. salaris* from Baltic to Atlantic salmon. Unfortunately, Baltic salmon were unattainable from the River Säveån at the time of sampling but summer and winter collections of *G. salaris* on Atlantic salmon were analysed from these two localities which are 90km apart. The results showed that it was possible to separate specimens from the two collection periods, the hooks being larger in winter and smaller in summer, but it was not possible to separate *G. salaris* from the two localities. In this instance the distance between the two sites may have been too small; it is possible that sites separated by a greater distance may yield a different result. Further studies are required to compare parasites from both Baltic and Atlantic salmon.

The isolation of sclerites using the sonication technique also allowed the material to be useable for X-ray elemental analysis. The elemental composition of hamuli, marginal hooks and ventral bars of several species of "salmonid" *Gyrodactylus* were examined for the identification of species specific differences. The principal analysable elements composing the marginal hook were found to be sulphur (57.7-66.1%) and calcium (10.6-16.5% of the measurable portion). In the hamulus the sulphur content was proportionally higher at 69.9% for the hamulus root to 93.3% for the shaft and ventral bar attachment regions of the hamulus. The ventral bar, previously believed to be devoid of sulphur, was found to have sulphur but a lower content (31.0-36.6%), than the hamulus. However, the calcium fraction was found to be more substantial (22.0-36.6%) than in the hamulus. Surprisingly, the ventral bar and the hamulus contained vanadium (0.05-2.22%), whose significance is unknown, but it may have been bioaccumulated from the environment. The high sulphur content of the marginal hook and the hamulus indicated the presence of a keratin-like substance, which is resistant to the action of proteolytic enzymes and results in a rigid structure which produces mechanical strength. The ventral and dorsal bars, able to flex in order to maintain the position and effectiveness of the hamuli, are weakly keratinised. The ventral bars appear to represent an intermediary stage between the heavy sulphur bearing sclerites of the monopisthocotyleans and the sulphur-free clamps of the polyopisthocotyleans. It is suggested that the accessary bars of

Gyrodactylus have exchanged a portion of the sulphur component for calcium, while maintaining a rigid structural strength. The relative proportions of the elements in different species of *Gyrodactylus* were not significantly different from each other within the salmonid forms.

The role of the individual sclerites and their function in relation to the whole haptoral complex was then considered, drawing on the information collated from studies on elemental composition and morphological structure. The heavy sulphur bearing hamuli and marginal hooks show adaptation to the directional stresses placed upon them since these molecules orientate along the principal stress axis which runs between the opisthaptoral muscles pulling at one end and the resistance of the tissue at the other. This arrangement minimises the shearing stress experienced by the hamulus. The ventral bar, is not subjected to directional forces such as the pull of muscles, but appears to act as a plate guiding the hamuli as they move back and forth during the process of attachment and detachment, in addition to preventing the hamuli from tearing through the opisthaptor. Although it was not possible to analyse the dorsal bar with the X-ray system, it is believed that the molecular composition differs from that of the other sclerites because it is able to flex to accommodate the movement of the hamuli, preventing the hamuli from rotating and maximising their efficiency in the process of attachment.

A pilot study attempted to discriminate three species of *Gyrodactylus* on the basis of differences in their protein profiles. Specimens of *G. salaris*, *G. derjavini* and *G. truttae* were subjected to SDS-PAGE gel electrophoresis. Four proteins of molecular weight 102,300, 33,900, 28,200 and 25,700 Daltons were found to be common to the three species of *Gyrodactylus*. The possibility of finding species specific proteins to discriminate *G. salaris* from the other salmonid gyrodactylids was tested using Western blotting. The presence of antibodies raised in rabbits specifically against *G. salaris* were investigated against the protein profiles of *G. truttae* and *G. derjavini*, by using a Western blot. Immunostaining showed a response to two *G. salaris* proteins, one of 31,700 Daltons and the other of 25,200 Daltons. A preliminary investigation into the use of iso-electric focusing to discriminate species of the genus *Gyrodactylus* was made, but no positive results were obtained as this requires further exploration.

Current techniques used to identify *Gyrodactylus* species are based on the ability to discriminate minute differences in the shape of the marginal hook. The development of techniques, such as sonication, and the revision of the morphometrics used on the liberated sclerites using an image analysis system, enabled the differentiation of the "salmonid" gyrodactylids. The extraction and processing of these sclerites, although yielding accurate data, were very laborious procedures. Neither time nor sophisticated facilities for such analyses are available to diagnosticians requiring quick species determinations. The technique of silver staining surface sensilla structures (chaetotaxy) was therefore explored. Species specific maps were produced and compared. Following this analysis, a revised system of nomenclature for the sensillar patterns for the genus *Gyrodactylus* was proposed, using clusters of sensilla that aggregate normally, rather than attempting to relate sensillar patterns to the underlying nervous system.

The aim of this part of the study was to produce reference maps for comparison of specimens by non-specialists in order to identify *G. salaris* with certainty. By drawing attention to the key areas or groups of sensilla used in the discrimination, *G. salaris* can now be differentiated from *G. derjavini* Morph 1 and *G. truttae* Morph 4 in Britain. On the ventral surface *G. salaris* differed by having 5 sensilla in its anterior set and 6 sensilla in the medio-lateral set compared to 6+ and 5 sensilla respectively in these zones in *G. truttae* Morph 4 and *G. derjavini* Morph 1. On the dorsal surface, *G. salaris* differed from *G. truttae* Morph 4 and *G. derjavini* Morph 1 by having 9 sensilla in its posterior set compared to 8.

Analogous sensillar aggregations unique to the Polyopisthocotylea and the Monopisthocotylea were found within the generalised chaetotaxy maps for the genera *Gyrodactylus* and *Ooegyrodactylus*. In particular, the large post-ocular sensilla (SPO) in the polyopisthocotyleans, which disappear on attachment to its host, are analogous to the ventro-median mid-set of 6 (3x2) sensilla on these two genera. This mid-set is absent in monopisthocotyleans and appears to form one of the major distinctions in the systematics and phylogenetics of the Monogenea, supporting the hypothesis that *Gyrodactylus* has a greater affinity to the polyopisthocotyleans.

In conclusion, this study has provided a range of techniques whereby the seven morphs of *Gyrodactylus* identified from British salmonids can be discriminated. Morph

1 *G. derjavini* was found on salmon, rainbow trout and brown trout. Morph 2 a hitherto undescribed morph, which may constitute a new species of *Gyrodactylus* was found on Scottish salmon. Morph 3, found on Scottish salmon, was identical with *G. derjavini* but was identified on the basis of having large hamuli. Morph 4 and Morph 5 *G. truttae* were found to parasitise brown trout. Morph 6 from Arctic charr also represents a previously undescribed species of *Gyrodactylus* and Morph 7, closely resembling *G. derjavini*, was also was found on Arctic charr. Amongst the new techniques found to be most effective in discriminating these species were morphological studies of the sclerites following sonication, multivariate analysis and chaetotaxy. Furthermore, these techniques have elucidated the key factors, i.e. the hamulus angle, the aperture size of the marginal hook and the distribution of sensilla to discriminate *G. salaris* from the British *Gyrodactylus* species. These results have important implications, not only to facilitate the recognition of *G. salaris* and to curb its translocation to other salmonid stocks, but for related taxonomic problems associated with other problematic genera of monogeneans.

APPENDIX 1

Specimen reduction buffer (double strength)

Tris	0.242g
EDTA	0.058g
SDS	2.000g
0.5% bromophenol blue	1 ml
Glycerine	40 ml
2-mercaptoethanol	10 ml

Distilled water to 100 ml (pH adjusted with NaOH (1M) to pH 8.0.

SDS-PAGE calibration curve molecular markers

phosphorylase b	94,000
albumin	67,000
ovalbumin	43,000
carbonic anhydrase	30,000
trypsin inhibitor	20,100
alpha lactalbumin	14,400

Electrophoresis Buffer (x10 strength, pH 7.4)

Tris	96.8g
EDTA	11.8g
Sodium acetate	32.8g
SDS	40.0g

Distilled water to 1900 ml
pH adjusted to 7.4 with approx 35-40ml acetic acid
Distilled water to 2000 ml

Working Electrophoresis Buffer

Stock buffer	310 ml

Distilled water to 3100 ml

Biotinylated alkaline phosphatase molecular weight markers

	Mol.Wt.
Transketolase (yeast)	81,000
Creatine phosphokinase (rabbit muscle)	40,500
Phoshoglycerate mutase (rabbit muscle)	29,000
Myoglobin (horse)	17,500

Stock Transfer Buffer (x10 strength)

Tris	48.4g
Glycine	225.2g
Distilled water to 2000 ml	

Working Transfer Buffer

Stock transfer buffer	400 ml
Methanol	800 ml
Distilled water	2800 ml

Immunostaining reagents

i) Tris Buffered Saline (TSB)

Tris	4.84g
Sodium chloride	58.48g
Distilled water	1900 ml
pH adjusted to 7.5 with conc. HCl (approx 3 ml)	
Distilled water to 2000 ml	

ii) Tween Tris Buffered Saline (TTSB)

Tween 20	0.5 ml
TBS	1000 ml

iii) Nitrocellulose blocking solution

10 ml Goat serum (Scottish Antibody Production Unit, Edinburgh)
90 ml TTBS

iv) First Antibody Solution

25 ml	Nitrocellulose blocking solution
0.25 ml	Rabbit anti *Gyrodactylus* serum
0.025 ml	Avidin, alkaline phosphatase conjugated to stain molecular markers (Pierce)

v) Second Antibody Solution (1 in 3000 dilution)

25 ml	TTBS
0.0083 ml	Goat anti rabbit IgG (whole molecule) alkaline phosphatase conjugate

vi) Colour Development Solution

Solution A

0.7 ml	N,N-dimethyl-foramide (DMF)
0.3 ml	Distilled water
30 mg	Nitro blue Tetrazolium (NBT) (Sigma)

Solution B

15 mg	5,Bromo-4 Chloro 3 Indolyl phosphate (p-Toludine salt) (BCIP) (Sigma)
1 ml	N,N-dimethylforamide

Add solutions A and B to 100ml carbonate buffer (1 capsule in 100ml distilled water to yield a 0.05M buffer, pH 9.6) use immediately.

Protein staining reagents

i) Phosphate Buffered Saline (PBS) (0.5ml per cm sq. membrane)

One phosphate buffered saline tablet (Sigma) (sodium chloride 137mmol; potassium chloride 2.7 mmol, and phosphate buffer 10mmol) to yield a solution of pH 7.4.

200 ml	distilled water
0.3%	Tween 20

ii) Aurodye (0.2ml per cm sq. membrane)

This solution comes pre-prepared from Amersham (Hull).

Iso-electric focusing

Gel preparation

72 ml distilled water
15g sucrose added to
20 ml 29.1% acrylamide
20 ml 0.9% NN methylenebisacrylamide
8.2 ml Ampholine mixture

Place under vacuum for 5 minutes, add 0.8ml 5mg% riboflavin prior to pipetting solution into gel moulds. Moulds are placed under a white fluorescent light for 2 hours to ensure complete photopolymerisation.

Isoenzyme stains

Acid phosphatase

0.33g	sodium acetate
0.15g	α-naphthyl acid phosphate
0.05g	black K salt
50 ml	distilled water

Stain for 1 hour, decant stain, then spray gel surface with concentrated ammonium hydroxide.

Adenylate kinase

50 ml	0.2M Tris-HCl, pH 8.0
6 ml	0.1M $MgCl_2.6H_2O$
0.03g	adenosine 5'-diphosphate
0.1g	D(+)-glucose
20 units	hexokinase
40 NAD units	G6PDH
2 ml	10mg/ml NAD
1 ml	5mg/ml NBT
1 ml	5mg/ml PMS

Stain is prepared as a gel overlay

Glucose-6-phosphate Isomerase (GPI)

50 ml	0.2M Tris-HCl, pH 7.0
5 ml	0.1M $MgCl_2.6H_2O$
0.04g	D-fructose-6-phosphate
40 NAD units	G6PDH
2 ml	10mg/ml NAD
1 ml	5mg/ml NBT
1 ml	5mg/ml PMS

Stain is prepared as a gel overlay

Glycerol-3-phosphate Dehydrogenase (G3PDH)

50 ml	0.2M Tris-HCl, pH 8.0
1g	DL-α-glycerophosphate, pH 8.0
1 ml	0.1M $MgCl_2$
1 ml	10mg/ml NAD
1 ml	5mg/ml NBT
1 ml	5mg/ml PMS

Hexokinase

50 ml	0.2M Tris-HCl, pH 8.0
1 ml	1M $MgCl_2$
0.25g	adenosine 5'-triphosphate
5g	D(+)-glucose
80 NAD units	G6PDH
2 ml	10mg/ml NAD
1 ml	5mg/ml NBT
1 ml	5mg/ml PMS

Incubate gel in the dark at 37°C.

L-Lactate Dehyrogenase (LDH)

50 ml	0.2M Tris-HCl, pH 8.0
8 ml	1M lithium lactate, pH 8.0
1 ml	10mg/ml NAD
1 ml	5mg/ml NBT
1 ml	5mg/ml PMS

Malate Dehydrogenase (MDH)

50 ml	0.2M Tris-HCl, pH 8.0
5 ml	2M DL-malic acid
1 ml	10mg/ml NAD
1 ml	5mg/ml NBT
1 ml	5mg/ml PMS

Phosphoglucomutase (PGM)

50 ml	0.2M Tris-HCl, pH 8.0
5 ml	0.1M $MgCl_2$
0.1g	α-D-glucose-1-phosphate
40 NAD units	G6PDH
2 ml	10mg/ml NAD
1 ml	5mg/ml NBT
1 ml	5mg/ml PMS

Stain may be prepared as a gel overlay

APPENDIX 2: Abbreviations

a	= marginal hook toe length
ad	= anterior dorsal
ae	= hamulus shaft length
ag	= hamulus width
al	= antero-lateral (ventral)
am	= antero-median
av	= anterior ventral
b	= marginal hook proximal width
be	= hamulus point length
c	= marginal hook distal width
cb	= hamulus shaft-point length
cdb	= hamulus angle
cp	= circumporal (dorsal)
d	= marginal hook sickle length
e	= marginal hook sickle aperture distance
f	= marginal hook shaft length
fa	= hamulus root length
fe	= hamulus total length
g	= marginal hook total length
hd	= haptoral dorsal
hdbtl	= dorsal bar total length
hdbw	= dorsal bar width
hg	= hamulus point
hh	= hamulus shaft
hi	= hamulus root
hj	= hamulus dorsal bar attachment point
hk	= hamulus ventral bar attachment point
hl	= hamulus indentation marking the lower edge of the ventral bar attachment point
hpl	= hamulus point length
hrl	= hamulus root length
hsl	= hamulus shaft length
htl	= hamulus total length
hv	= haptoral ventral
hvbl	= ventral bar length
hvbmbl	= ventral bar membrane length
hvbmw	= ventral bar middle width
hvbpl	= ventral bar process length
hvbpml	= ventral bar process to mid length
hvbtw	= ventral bar total width
lhdbtl	= dorsal bar total length (log value)
lhdbw	= dorsal bar width (log value)
lhpl	= hamulus point length (log value)
lhrl	= hamulus root length (log value)

lhsl	= hamulus shaft length (log value)
lhtl	= total hamulus length (log value)
lhvbl	= ventral bar length (log value)
lhvbmbl	= ventral bar membrane length (log value)
lhvbmw	= ventral bar middle width (log value)
lhvbpl	= ventral bar process length (log value)
lhvbpml	= ventral bar process to mid length (log value)
lhvbtw	= ventral bar total width (log value)
lmhdl	= marginal hook filament loop length (log value)
lmhsdw	= marginal hook sickle distal width (log value)
lmhsl	= marginal hook shaft length (log value)
lmhspw	= marginal hook sickle proximal width (log value)
lmhstl	= marginal hook sickle length (log value)
lmhtl	= marginal hook total length (log value)
ma	= marginal hook point
mb	= marginal hook shaft of the sickle proper
mc	= marginal hook toe
md	= median dorsal (chaetotaxy)
md	= marginal hook heel
mc+md	= marginal hook foot/base of the sickle proper
me	= marginal hook shaft
mf	= marginal hook indentation noted in the toe of certain species
mg	= marginal hook aperture
mhdl	= marginal hook filament loop length
mhsdw	= marginal hook sickle distal width
mhsl	= marginal hook shaft length
mhspw	= marginal hook sickle proximal width
mhstl	= marginal hook sickle length
mhtl	= marginal hook total length
ml	= medio-lateral (ventral)
mw	= ventral bar middle width
p	= posterior (dorsal)
pb	= peri-buccal (ventral)
pl	= postero-lateral (ventral)
ptd	= postero-terminal dorsal
ptv	= postero-terminal ventral
sm	= sickle membrane
ss	= spike sensillum
vm	= ventral bar processes
vn	= ventral bar transverse depression
vo	= ventral bar median portion
vp	= ventral bar membrane
vq	= ventral bar medial ridge

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