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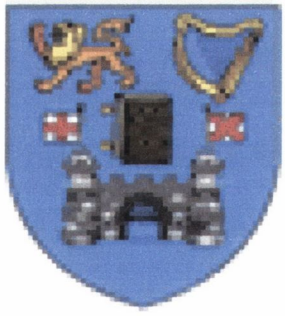
AN INVESTIGATION OF STRAIN DIFFERENCES IN *POMPHORHYNCHUS LAEVIS* (ACANTHOCEPHALA): AN ECOLOGICAL, MORPHOLOGICAL AND MOLECULAR APPROACH.

ELLEN MARIA O'MAHONY



A thesis submitted in fulfilment of the degree of Doctor of Philosophy to Trinity College, University of Dublin.

October 2002.

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DECLARATION

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Ellen Maria O'Mahony.

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SUMMARY

Kennedy, Bates and Brown (1989) stated that in the British Isles the acanthocephalan, *Pomphorhynchus laevis* could be separated into three strains, an English, Irish and marine strain. Much ecological evidence exists in support of this hypothesis. The three strains use different hosts to complete their life cycles (Hine and Kennedy, 1974a; Kennedy, 1984; Molloy, Holland and O'Regan, 1995), they are located in different sections of the definitive host's gut (Broughton, Hine and Kennedy, 1976; Fitzgerald and Mulcahy, 1983; Kennedy, 1984) and geographically they are largely confined to different areas, though the marine and English freshwater strains do overlap in certain river estuaries (Hine and Kennedy, 1974a; Kennedy, 1984; Guillen-Hernandez and Whitfield, 2001). In the thesis presented here, ecological and other evidence will be presented in support of the strain hypothesis.

Four samples of brown trout were collected per year over a two-year period from L. Feeagh, Co. Mayo. This enabled a detailed study of the ecology of the Irish strain. Differences between the Irish and other strains, which were highlighted in previous studies, were confirmed (Fitzgerald and Mulcahy, 1983; Molloy et al, 1995). One of the most striking differences between the Irish strain and others is its seasonal pattern of growth and maturation. This may be due to the limited breeding potential of the intermediate host, compared to similar animals in Britain, which affects the transmission dynamics of the parasite (Fitzgerald, 1987). It may also be due to low temperatures preventing growth of the parasite over the winter period (Brown, 1989).

Morphological analysis of the Irish strain from L. Feeagh and the English strain from the river Avon, Hampshire, England was conducted using new techniques. Instead of the traditional method of using an eyepiece graticule attached to a microscope to make measurements, a digital camera was used to photograph worms prepared on slides. Measurements were then taken from these photographs using an image analysis programme, NIH Image v6.01. The English strain possessed significantly longer trunks, necks, bulbs and proboscides and also wider trunks and bulbs than the Irish strain. When the English strain was compared to Irish worms from the summer season only, there were no longer any significant differences in trunk length and neck width between the two. In addition, the Irish strain now had significantly longer and wider proboscides than the English strain. Therefore season was found to be a confounding variable. Two characters were found to be more

consistent in identifying the two populations. In the English strain, the stoutest hooks are mostly found at the sixth or fifth position in a hook row and in the Irish strain they are found at the fourth or fifth position. If one examines more than one row on each individual proboscis, one should find at least one of the stoutest hooks at the fourth position on Irish *P. laevis* and at the sixth position in English *P. laevis*. The English strain has more or equal numbers of anterior than posterior hooks, while the opposite is true of the Irish. These two characters should distinguish between the two populations.

A mitochondrial gene, subunit I of cytochrome c oxidase was partially sequenced from three Irish populations of *P. laevis*, one Scottish, three English and one Italian population. Populations were grouped by host. *P. laevis* from brown trout from Ireland, England and Scotland were very closely related, showing only a mean sequence divergence of 0.7%. The two populations of *P. laevis* from English chub and bullhead were similar to each other (0.35% divergence). These two groups, the brown trout group and the chub/bullhead group were 2.2% different. That these groups were distributed by host rather than by geography contravened the strain hypothesis, which stated that *P. laevis* in Ireland might differ from *P. laevis* in England due to different selection pressures in Ireland. Despite this, an AMOVA analysis favoured a division of genetic variation by host rather than geography. High genetic heterogeneity was found in the Italian population, which was composed of two groups, which were 18.5% different. This striking difference may be due to the impact of ice ages on genetic diversity or the existence of cryptic species. Further work needs to be done to corroborate this.

The hypothesis that *P. laevis* exists as two freshwater strains in Britain and Ireland was confirmed by morphological and molecular analysis. How these strains were formed requires further investigation.

Whatever you can do or dream you can, begin it.
Boldness has genius, power and magic in it.

Begin it now

- Goethe

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CHAPTER 1

INTRODUCTION

&

LITERATURE REVIEW

Introduction

Kennedy, Bates and Brown (1989) stated that in the British Isles the acanthocephalan, *Pomphorhynchus laevis* could be separated into three strains; two freshwater strains (one in England and one in Ireland) and a marine strain. The three strains use different hosts. Their preferred sites in the alimentary canals of the vertebrate host differ and they show differences in their patterns of distribution (Hine and Kennedy, 1974; Kennedy, Broughton and Hine, 1978; Kennedy 1984). Attempts at separating the marine strain from the freshwater strains at a morphological level have proven unsatisfactory due to conservation of characters and a large degree of individual variability (Gibson, 1972; Brown, 1987). The inability to separate the strains morphologically has led to confusion about the exact taxonomic status of the three strains (Kennedy *et al*, 1989). A recent study of the ecology of the marine strain in flounders in the tidal Thames discovered the sympatric coexistence of both the marine and freshwater strains (Guillen-Hernandez and Whitfield, 2001). Despite morphological difficulties, the two strains were identified based on colour, size and niche in the alimentary canal. The first study to investigate differences at the molecular level between the marine and English freshwater strains was successful (Munro, Reid and Whitfield, 1990). A large degree of homogeneity was found between the two samples when their genomes were exposed to restriction endonucleases. Subsequent hybridisation of these digests, with a probe consisting of various ribosomal DNA repeat sequences from *Caenorhabditis elegans*, did show some differences in the pattern of hybridisation between the two strains. The first study to compare the morphology of Irish and English strains has also resulted in the discovery of some distinguishing characteristics (Edwards, unpublished observations).

In light of these studies, it was decided to compare the English and Irish freshwater strains of *P. laevis* using a holistic approach. Results of the ecological, morphological and molecular comparisons of these two strains will be reported in this thesis. These results will be used to gain insight into the status of strain differentiation within the species *P. laevis*.

AIMS

The aims of this project are

1. to document the growth and maturation cycle of an Irish population of *P. laevis*. This will enable a direct comparison with a) previous studies of other *P. laevis* strains and b) the first ecological study of the same Irish population.
2. to record morphological characters including trunk, neck, bulb, proboscis and hooks of an Irish and English population of *P. laevis* using a digital camera and image analysis. This will enable a) the development of a new technique to conduct morphological studies, b) the documentation of morphological variability of a wild population of Irish *P. laevis* from brown trout, which has never before been conducted and c) a direct morphological comparison between the Irish and English freshwater strains of *P. laevis.*
3. to sequence a mitochondrial gene from various populations of *P. laevis* in Ireland, the U.K. and Italy. This will be the first time an acanthocephalan mitochondrial gene is sequenced. This will enable a) the development of a protocol to successfully sequence subunit I of the cytochrome c oxidase gene of *P. laevis*, b) the typing of this gene in many populations of *P. laevis,* c) a direct comparison between all populations, which will reveal levels of heterogeneity within and between populations and d) the identification of strains of *P. laevis*.

Ecology

Many ecological differences between the three proposed strains have been documented. These differences include geographical distribution, host utilization, seasonal pattern of growth and female maturation and the position in the intestine of the definitive host.

Distribution

When considered as a complete unit, the species *P. laevis* is widespread throughout the palaearctic (Hine and Kennedy, 1974a). *P. laevis* has been found in many regions of the former USSR and in most countries throughout the rest of Europe, including Britain and Ireland (Petrochenko, 1956; Kennedy, 1974; Holland and

Kennedy, 1997; Moravec *et al*, 1997). Britain and Ireland represent the Western most extreme of the range of *P. laevis*. Within this broad range, the three strains have relatively narrow and exclusive distributions. The marine strain is located in the Baltic Sea, the Black, Caspian and Aral Seas, the English Channel and the North Sea estuaries in Eastern Scotland (Kennedy, 1984). The marine strain has also been recorded in the Thames (Munro, Whitfield and Diffley, 1989). The Irish strain is found in rivers and lakes throughout Ireland and recently it has been recorded in Northern Ireland (Holland and Kennedy, 1997; Evans, Matthews and McClintock, 2001). The distribution of the British strain is much more localised than that of the Irish strain and *P. laevis* on the continent. *P. laevis* is limited to rivers in the South of England and is virtually absent from Wales and Scotland, though recently it has been recorded in the West of Scotland (Hine and Kennedy, 1974a; Mackenzie, pers. comm., Lyndon, pers. comm.) In England, stable infections are restricted to the rivers Severn, Thames and the Avon Stour (Kennedy *et al*, 1989) though it has spread to other rivers there, *e.g.* the rivers Otter and Culm (Kennedy, 1996; Kennedy, in press). Hine and Kennedy (1974a) could not explain this localised distribution in terms of the distribution of hosts, as both the preferred definitive hosts, barbel (*Barbus barbus*) and chub (*Leuciscus cephalus*) and the intermediate host *Gammarus pulex* are widespread throughout Britain. Kennedy *et al* (1989) explained the peculiar distribution of *P. laevis* in Britain, using strain differentiation as the cornerstone of their hypothesis. By thinking of *P. laevis* in Britain and Ireland as three strains rather than as one homogeneous species, the differences in *P. laevis* distribtution can be attributed to differences in dispersal capabilities exhibited by the strains.

Host Utilization

Acanthocephalans are relatively generalist parasites. On the continent, *P. laevis* was found in 43 different fish hosts by Petrochenko (1956). *P. laevis* from Britain and Ireland was found in 21 hosts (Kennedy, 1974) and Irish *P. laevis* was described from 15 hosts (Holland and Kennedy, 1997). The lesser number of hosts of *P. laevis* in Britain and Ireland reflects the long term effect of past glaciation events on the British and Irish fauna. The incursion of glaciers into northern Europe resulted in a diminished distribution of most flora and fauna, including that of freshwater fish, limited to southern Europe. As the glaciers retreated, new habitats were opened for

recolonisation. In Britain and Ireland only some freshwater fish species were able to recolonise before the creation of the English Channel and the Irish Sea, which became barriers to further recolonisation.

This perceived level of generalism in host use by *P. laevis* is misguiding. When growth and maturity of the parasite are taken as indicators of host suitability, very few of the fish from which *P. laevis* has been described, actually play a role in the perpetuation of this parasite (Hine and Kennedy, 1974a). Hine and Kennedy (1974a) describe three categories of host for British *P. laevis*. The first group comprises fish, *e.g.* bullhead (*Cottus gobio*), in which only low numbers of *P. laevis* are found and the parasite neither grows nor matures. The second group comprises fish, *e.g.* brown trout (*Salmo trutta*) and dace (*Leuciscus leuciscus*), in which *P. laevis* is more common and grows and matures, though gravid females were rarely found. The final group represents the preferred definitive hosts, barbel and chub (*Leuciscus cephalus*). In these hosts, infections reach high densities, *P. laevis* regularly attains its maximum size and females are often gravid. The terminology relating to the ability of fish to host *P. laevis* has been updated by Lyndon and Kennedy (2001). Hosts in which acanthocephalans grow, but do not reach their maximum size, and in which they also mature regularly, are known as suitable hosts. Additional hosts are fish in which acanthocephans grow a little and few females become gravid. Acanthocepahlans in accidental hosts neither grow beyond their size as a cystacanth nor mature. *P. laevis* is found in many fish which feed on its intermediate host due to the fact that the cystacanth everts in the presence of a common component of bile (Kennedy *et al*, 1978). Some of these unsuitable hosts, if small, instead of being dead ends for the parasite, may act as paratenic hosts and pass the parasite onto a larger preferred host, if eaten. For example, sheatfish (*Siluris glanis*) of less than 20cm in length could transmit *P. laevis* to bigger fish of the same and different species (Dezfuli, 1992). These fish possessed small immature worms in their intestines and cystacanths and less developed worms in intraperitoneal sites. *P. laevis* is therefore more specific than once thought and in accordance with this, the three strains use different definitive and intermediate hosts.

Barbel and chub are the preferred definitive host of the English strain and *G. pulex* acts as the intermediate host. The Irish strain uses brown trout and *G. duebeni* to complete its life cycle. Flounder acts as the preferred definitive host for the marine strain and possibly *G. locusta* or *G. zaddachi* or both act as the intermediate host (Kennedy, 1984). The English strain is more similar to the continental population of *P.*

laevis as barbel and chub are the preferred definitive hosts there (Moravec and Scholtz, 1991; Moravec *et al*, 1997; Galli *et al*, 1998). On the continent, *P. laevis* is able to use whatever gammarid species is locally abundant, *i.e. G. roeselli*, *G. fossarum*, *Echinogammarus stammeri* (Dezfuli *et al*, 1991; Moravec *et al*, 1997; Lyndon and Kennedy, 2001). Only the salmonids, brown trout and salmon, and the eel are thought to be native to Ireland (Wheeler, 1977; Holland and Kennedy, 1997). Neither chub nor barbel exist there and in their absence *P. laevis* uses brown trout as a preferred definitive host and does so more successfully than documented for the English strain (Hine and Kennedy, 1974a). The ability of the English strain to use brown trout as a preferred definitive host has been observed at one location, the river Otter in Devon (Kennedy, 1996). This also took place in the absence of chub and barbel.

Flounder, which can and do regularly invade freshwater, were found in the river Avon containing the English strain as opposed to the marine strain (Kennedy, 1984). The identification of the strain was based upon the fact that the parasites were very small (females averaged 2.18mg in weight) and females were not gravid, which is in stark contrast to the higher weights and gravidity of *P. laevis* found in flounders in the Baltic Sea (Kennedy, 1984). To investigate some ecological aspects of the English strain in flounders, Kennedy (1984) conducted laboratory infections of flounders with the English strain. Again parasites grew very little, the largest female weighing 4.65mg and none of the females became gravid. These observations support the division of *P. laevis* from freshwater in Britain and from various marine habitats in Europe, into separate strains.

Seasonal Patterns in Growth and Maturation

In barbel and chub in the parts of Europe where *P. laevis* is the dominant parasitic species, both the prevalence and intensity of the infections remained high and stable throughout the year (table 1.1). In infections of barbel and chub in the R. Danube and the R. Rokytna, any fluctuations in the prevalence and intensity during the year were random and not related to season. In the river Danube, in Hungary, eggs were produced by female *P. laevis* throughout the year, apart from the winter months (Moravec *et al*, 1997). In the river Rokytna, gravid females made up between 20 and 30% of the entire *P. laevis* population throughout the year (Moravec and Scholtz, 1991).

Table 1.1: A comparison of important parameters concerning *P. laevis* infections of a number of hosts.

HOST	LOCATION	PREVALENCE	INTENSITY	% FEMALES GRAVID	REFERENCE
Barbel	R. Danube	99%	2 – 491	*	Moravec et al, 1997
Chub	R. Rokytna	68%	1 – 97	40 – 60%	Moravec & Scholtz, 1991
Chub	R. Severn & R. Worfe	100%	1 – 207	60%	Brown, 1989
Dace	R. Avon	65 – 89%	*	10 – 85%	Hine & Kennedy, 1974
Flounder	R. Thames	100%	1 – 218	50%	Munro et al, 1989
Flounder	R. Thames	20 – 100%	1 – 22	17.4%	Guillen-Hernandez & Whitfield, 2001
Brown Trout	L. Feeagh	*	*	5 – 73%	Molloy et al, 1995

* indicate missing data.

The population dynamics of the English strain mirrors that seen in European populations. In chub collected from the rivers Severn and Worfe, prevalence and infection intensity remained high throughout the year (Brown, 1989). The intermediate host, *G. pulex,* was also infected throughout the year. Dace, in the river Avon, were found to be infected throughout the year and the prevalence of the infection remained high and stable throughout the year also (Hine and Kennedy, 1974b). The intermediate host in this river was found always to be infected and a high proportion of the larval parasites found were at the cystacanth stage. Gravid female *P. laevis* accounted for at least 60% of the female population in the river Severn at all times of the year (Brown, 1989). In chub, in the river Avon, most of the females were fully mature with eggs at all times (Hine and Kennedy, 1974b). The situation in dace was different, as a high proportion of females were only mature in the summer from April to August. Dace is not considered to be a preferred definitive host of *P. laevis* and the parasite did not grow very well in this host (Hine and Kennedy, 1974a).

Samples of flounders, collected from the river Thames in two separate studies revealed a high prevalence and intensity of the marine strain of *P. laevis* (table 1.1).

The population dynamics of the Irish strain differs from that seen in the European populations of *P. laevis* and the English strain. The prevalence and intensity of *P. laevis* in brown trout in L. Feeagh, was higher in summer than winter (Molloy, Holland and O'Regan, 1995). In the river Shournagh, the prevalence of *P.laevis* in

brown trout peaked in May at 85%, from a baseline prevalence of approximately 20 to 50% during the rest of the year (Fitzgerald and Mulcahy, 1983). The seasonal periodicity apparent in the growth and maturation of the Irish strain is more striking. Worms are significantly longer and heavier in the summer than the rest of the year (Molloy *et al*, 1995). Overall, Irish *P. laevis* averaged 7mm in length and 7mg in weight, which contrasts with an average length and weight of 8.2mm and 13.0mg in the summer. The seasonal fluctuation in the size of Irish *P. laevis* is in contrast to the occurrence of heavy worms (females averaging > 36mg in weight, males averaging > 20mg) in the population of the English strain in the river Severn all year round (Brown, 1989). Another striking difference between the English and Irish strains is that the number of gravid females in L. Feeagh also fluctuates with season. The percentage of females at stage 3 was found to increase through the year from a low of < 5% in March to a high of 73% in September, followed by a drop to almost 10% in October (Molloy *et al*, 1995). In both the rivers Avon and Severn, gravid females made up the largest portion of females all year round (Hine and Kennedy, 1974; Brown, 1989). In the river Severn, the percentage of females at stage 3 never dropped below 60% (Brown, 1989).

In a longterm study of the *P. laevis* / dace system in the river Avon, the prevalence and intensity of infection were found to be stable and any fluctuations remained within the boundaries of annual change (Kennedy and Rumpus, 1977). This constancy was unexpected, as a density dependent control mechanism of *P. laevis* populations in goldfish, *Carassius auratus*, could not be identified in the laboratory (Kennedy, 1974). Kennedy (1972) found a relationship between the establishment of *P. laevis* and temperature, as a lower percentage of worms established at higher temperatures. Neither establishment nor growth were affected by the density of the population in goldfish (Kennedy, 1972, 1974). The effect of temperature on the establishment of *P. laevis* could not alone explain the stability of the infection of *P. laevis* in the wild. Temperature, in association with changes in host diet were thought to control the population (Kennedy, 1972). In the summer lower rates of establishment at higher temperatures is compensated for by a higher rate of feeding and therefore a higher intake of parasites. In winter, higher rates of establishment at lower temperatures is offset by decreased feeding. In this way, *P. laevis* populations are thought to exist in a state of dynamic equilibrium (Kennedy, 1972). In contrast, Brown (1986) found that establishment reached a ceiling level at densities higher than those tested by Kennedy (1972, 1974), which was also dependent on the size of the fish host. Bates and Kennedy

(1991b) found that threshold levels of establishment were overcome by the growth of the host, which led to more niches being made available over time. Therefore temperature, fish feeding behaviour and the infection pattern in the intermediate host are the most important factors influencing the lack of a seasonal cycle in the population dynamics of the English strain of *P. laevis*.

The seasonal pattern of the prevalence, growth and maturation of the Irish strain is unexpected. Growth and maturation of *P. laevis* were found to follow a seasonal cycle in dace in the river Avon, but this is considered an unsuitable host and gravid females are very rare in this host (Hine and Kennedy, 1974b). In summer, the Irish strain grows to a size comparable to that of the English strain and a large proportion of females are at stage 3, which supports the suitability of brown trout as a host for *P. laevis*. The population dynamics of the intermediate host differs to that of *G. duebeni* in Britain. In the river Shournagh, *G. duebeni* bred once a year (Fitzgerald and Mulcahy, 1983). In certain locations in Britain this gammarid was found to breed twice a year (Fitzgerald, 1987). The difference in the availability of young, susceptible gammarids to infection by *P. laevis* acanthors between Britain and Ireland may explain the difference in seasonal patterns (Fitzgerald, 1987).

Position in the Gut of the Definitive Host

The infection of cyprinids by the English strain is centred in an anterior part of the gut (Kennedy, Broughton and Hine, 1976). If one considers the oesophagus as 0% and the anus as 100%, most worms in cyprinids are found in an area of the gut between 20 and 33%. In an experimental infection of brown trout and rainbow trout with the English strain, worms were found at 14% in the brown trout gut and at 46% in the gut of rainbow trout. The Irish strain is found in a much more posterior part of the gut. Most of the Irish strain sampled from brown trout in the river Shournagh were found between 50 and 60% of the gut (Fitzgerald and Mulcahy, 1983). Irish *P. laevis* in brown trout in L. Feeagh were found at an average position of 77% (Molloy *et al*, 1995). The marine strain is located in the lowest reaches of the gut *i.e.* the rectum. The rectum is the most commonly used site by the marine strain in flounders from both the Baltic Sea and the river Thames (Kennedy, 1984; Munro *et al,* 1989).

Despite the distinctly different sites used by the three strains, *P. laevis* is not known to actively select a site within the gut. Rather, site selection and the distribution

of *P. laevis* is a result of liberation and activation processes (Kennedy *et al*, 1976). In cyprinids, the cystacanth is liberated from the intermediate host through physical action carried out by pharyngeal teeth. Salmonids do not possess these teeth and liberation results purely from enzymatic digestion (Kennedy *et al*, 1976).

Once liberated, the cystacanth is activated in the presence of bile (Broughton, 1974). The opening of the bile duct in cyprinids is positioned just posterior to the oesophagus in the intestinal swelling, while in salmonids this opening is situated even further down the intestine (Kennedy *et al*, 1976). Therefore host physiology plays a large part in determining the position of *P. laevis*.

Morphology

The Acanthocephala comprises a group of pseudocoelomate invertebrates, all of which are obligate parasites. These parasites are so adapted to their way of life that they possess very few of the organs considered critical to the survival of free living animals (Van Cleave, 1941; Petrochenko, 1956). Externally they do not possess any recognisable sense organs but rely on tiny papillae for information about their environment. Their main external feature, for which they are named, is a proboscis covered in a multitude of recurved hooks. The proboscis is important as it anchors the adult to the wall of the alimentary canal of its vertebrate definitive host as well as allowing the acanthor to move around within the intermediate host. Internally the Acanthocephala do not have locomotory organs or a digestive system. Their bodies have become little more than a housing for the male and female reproductive systems (Van Cleave, 1941). In addition to their limited anatomical features, all acanthocephalans follow the same basic two host life cycle (though some also use paratenic hosts) (Schmidt, 1985). The intermediate host is always an arthropod and the definitive host is a vertebrate. Despite their homogeneity, the Acanthocephala have succeeded in colonising most vertebrate groups in all major habitats (Kennedy, 1993).

Acanthocephalan taxonomy

The loss of so many organs, as a result of their adaptation to parasitism, has left a limited number of features upon which to base their taxonomy. The phylum is divided into three classes, the Archiacanthocephala, the Eoacanthocephala and the

Palaeacanthocephala. The higher taxa are separated mainly by the organisation and composition of certain tissues such as the proboscis receptacle, cement glands and the lacunar system (the nearest thing to a circulatory system which the Acanthocephala possess) (Meyer, 1931, 1932, 1933; Van Cleave, 1936; Bullock, 1969). Species identification has relied heavily on external morphology. Acanthocephala therefore have been and continue to be a challenge to taxonomists, as the proboscis is the most prominent external feature. The size and shape of the proboscis, the number of hooks and their size and shape, as well as the arrangement of the hooks are considered to be very useful characters, when identifying acanthocephalans (Van Cleave, 1941; Petrochenko, 1956).

Despite the difficulties inherent in acanthocephalan taxonomy, the number of species described has increased greatly over the last century. Acanthocephalans were first described from eels by Redi in 1684. At the turn of the twentieth century, less than 100 species were known, today this number stands at over 1000 (Petrochenko, 1956; Kennedy, 1993). Unfortunately, a problem with the identification of new species has slowly become apparent over this period. The problem is associated with the plasticity exhibited by the external features upon which acanthocephalan taxonomy is based. Van Cleave (1941) states that Lühe in 1912 was one of the first to observe variability in the number of longitudinal rows of hooks. Specimens of *Acanthocephalus ranae* ranged from having twelve to twenty longitudinal rows. Descriptions of new species, based on small numbers of specimens, have contributed to the difficulty of classification. By sampling small numbers of specimens from only particular areas of a parasite's range, researchers have confounded the problem of phenotypic variability. This has led to descriptions of new species, which later became invalidated when the full extent of the variation of an existing species was known (Bullock, 1962; Amin, 1975a; Amin, 1985). Semanas and Trejo (1997) redescribed *Acanthocephalus tumescens* due to insufficient original descriptions. These researchers complained about earlier conflicting reports stating different numbers of longitudinal rows and hooks per row for this species. *Fessisentis vancleavi* required further redescription due to the original report being based on only 15 specimens (Buckner and Nickol, 1972).

Phenotypic variability

Acanthocephalans have also been shown to differ in the degree of phenotypic plasticity they display. Members of the Eoacanthocephalans such as *Neoechinorhynchus* sp, *Octospinfer* sp and *Gracilisentis* sp display three circles of six, eight and twelve hooks respectively and almost never deviate from these formulae (Van Cleave, 1941). Conscious of variability in other genera, Buckner and Nickol (1975) questioned the status of *Moniliformis moniliformis* and *M. clarki* as separate, distinguishable species. They compared specimens of the two species from their respective natural hosts and also from experimental hosts. Though the characters of both species differed slightly between experimental and natural hosts, the distinctiveness of the two species was confirmed. Due to the large variability exhibited by species of *Fessisentis*, species were also compared across a range of hosts and geographic areas (Buckner and Nickol, 1979). Despite slight changes in character dimensions between host species, four species of *Fessisentis* in the USA remained distinctive.

Conversely, some acanthocephalan genera exhibit large degrees of variability, in particular the genus *Acanthocephalus* (Amin, 1975b). This genus comprises 44 species (Amin, 1985). In-depth studies on three members of this group found in the USA, based on large numbers of parasites, revealed significant overlap in certain characters (Bullock, 1962; Amin, 1975a, b). For the earlier half of the twentieth century, *A. dirus* was the only member of this genus in North America. In the latter half of the century two more species were described (Bullock, 1962; Amin, 1975a). *A. jacksoni* and *A. parksidei* overlapped in all characters such as body length, proboscis size, size of proboscis receptacle, size of leminisci *etc.* Females of *A. jacksoni* possessed hooks arranged in 12 to 18 longitudinal rows, with 7 to 10 hooks per row. Females of *A. parksidei* possessed 10 to 16 longitudinal rows with 8 to 11 hooks per row. Due to the extent of the variation and overlap exhibited by these two putative species, they are now considered synonyms of the original *A. dirus* (Amin, 1985).

Morphological variability within the Acanthocephala can be attributed to the following factors:

Host: Acanthocephalans are considered generalist parasites and are capable of surviving in a wide range of hosts (Bullock, 1962; Amin, 1974; Hine and Kennedy, 1974, Kennedy, 1993; Amin and Dailey, 1998). Despite this ability to survive in many

hosts, they will generally only grow to their maximum size and reproduce in relatively few hosts (Hine and Kennedy, 1974; Samuel, Nickol and Meyes, 1976). If the host, which an acanthocephalan has infected, restricts the ability of the parasite to mature then it is not surprising to find that morphological features differ between hosts (Bullock, 1962; Amin, 1975b). The exact mechanism through which growth of acanthocephalans is influenced by different hosts is largely unknown.

Geography: Geographical variation is closely linked to the nature of the host. Fish species are known to range over quite vast areas and for this reason so do their parasites. Bullock (1962) found more hooks on *A. jacksoni* in New Hampshire than Massachusetts. Amin and Redlin (1998) found Taiwanese populations of *Mediorhynchus papillosus* to have more hooks and more rows of spines than American populations. *M. papillosus* from Taiwan also had smaller probosces, hooks, spines and receptacles than conspecifics from the USA. Genetic drift, combined with a reduced gene flow between geographically distant populations may be a source of morphological variation. Adaptation of the host to a different environment, may also result in consequent adaptations of the parasite.

Sexual dimorphism: Females tend to be larger than males in most acanthocephalan species. They are not just bigger but their proboscides are generally larger; they possess more and longer hooks (Bullock, 1962). *A. jacksoni* females are twice the size of the males and also have a much larger proboscis (Bullock, 1962). Females stay in the host longer than males (who generally die after copulation) in order to incubate the growing embryos. A larger holdfast and more hooks may help considerably in surviving the rigours of the vertebrate intestinal environment (Petrochenko, 1956).

Age: Acanthocephalans are known to grow considerably once they have established themselves in the intestinal environment of a suitable host (Brown, 1987). Amin (1975b) found body size of *A. parksidei* to be directly related to developmental stage, which in turn is an indicator of age. Amin (1975b) also observed an increase in the length of the proboscis with age and slight increases in hook length with age. These observations contravene assumptions made by previous researchers on the constancy of proboscis and hook size (Van Cleave, 1941; Petrochenko, 1956). Both Van Cleave (1941) and Petrochenko (1956) believed the proboscis remained constant and unchanged by the growth of the acanthocephalan throughout the period from cystacanth to adult. Possibly because the proboscis is so essential to acanthocephalan systematics

that it seemed more critical that it did not grow, which increased its reliability as a character. It is easy to accept the assumption that hooks do not grow, because of the hard nature their composition. This hard surface material is now known to be composed of polysaccharide and phenolic acid joined by o-glycoside links (Anantaraman and Ravindranath, 1973). It is also known that the inside of the hook is composed of living material containing mitochondria, vacuoles and endoplasmic reticulum (Hutton and Oetinger, 1980). It seems plausible, in light of this information, that hooks may continue to grow, to some degree, after they are formed in the acanthella.

Genetic Variation: Acanthocephalan species contain separate sexes and only sexually reproduce. As a result, one would expect to find individuals of the same species exhibiting slight differences in shape and size.

The Genus *Pomphorhynchus*

The taxonomic difficulties associated with the Acanthocephala are also true of the genus *Pomphorhynchus* and particularly the species *P. laevis*. *P. laevis* has a wide distribution, being found throughout Europe, from the former USSR in the east, to Ireland in the west (Petrochenko, 1956; Holland and Kennedy, 1997). It is found in a variety of fish hosts and aquatic environments. Across its host and geographic range, its description has varied. Petrochenko (1956) described it as having 18 to 20 longitudinal rows of hooks, with 12 to 13 hooks per row. In a key to the freshwater fish acanthocephalans of Britain, *P. laevis* was described as having 13 to 20 longitudinal rows with 8 to 13 hooks per row (Brown, Chubb and Veltkamp, 1986). Though the ranges overlap, they differ considerably. *P. laevis* varies so much that Brown (1987), from specimens from only two fish in England, found longitudinal rows ranging in number from 14 to 18, with 8 to 13 hooks per row.

These wide ranging characteristics have caused so many problems with the identification of *Pomphorhynchus* species in Europe that another species was erected, *P. tereticollis*. This acanthocephalan is found in brackish and marine locations and its morphology overlaps considerably with *P. laevis* (Petrochenko, 1956; Brown, 1987). They are thought to differ in how the proboscis hooks vary in shape along a longitudinal row. Some researchers think the two species synonymous while others think them separate (Brown, 1987). Engelbrecht (1957) considered *P. laevis* and *P. tereticollis* as subspecies and even described a third, *P. laevis forma intermedius*. The genus

Pomphorhynchus is so variable, that out of the 23 species in the group, at least nine of them overlap with the description of *P. laevis* (Brown, 1986; Cordonnier and Ward, 1967; Schmidt and Hugghins, 1973). As mentioned in an earlier section, *P. laevis* is currently thought to exist as three strains, though this has not been confirmed morphologically (Kennedy, 1984).

A similar situation occurred in the USA. *P. rocci* was described as a new species, distinct from *P. bulbocolli* (Cordonnier and Ward, 1967). This was based on the fact that the former is found in estuarine habitats and the latter in freshwater. It was also thought that morphologically they were quite distinct. When specimens of *Pomphorhychus* from freshwater were found exhibiting the estuarine morphology and vice versa, the status of the new species was cast into doubt (Samuel *et al*, 1976). The species description of *P. rocci* was based on only 12 specimens and its erection as a new species was based on a comparison with an incomplete description of *P. bulbocolli* (Samuel *et al*, 1976). Despite these setbacks, further investigations into the morphology of *P. bulbocolli* and *P. rocci* revealed them to be distinct species (Huffman and Nickol, 1978; Gleason and Huffman, 1981).

To those researchers who have looked beyond their homogeneity, the Acanthocephala have revealed themselves to be quite diverse in many ways. The limited nature of their external morphological features and the variability exhibited by them, has provided a great challenge for taxonomists. It is clear that new species cannot now be described based on few specimens. Extensive studies covering as much of a proposed species range as possible must be conducted (Amin, 1975b). It is also clear that slight morphological differences alone cannot be used to separate species. Morphological analyses must be conducted in conjunction with ecological, molecular and other studies (Petrochenko, 1956).

Genetics

Strain Hypothesis

The current hypothesis regarding strain formation in *P. laevis* is described in full by Kennedy *et al* (1989). The hypothesis consists of three tenets, which combined explain the distributional peculiarities exhibited by *P. laevis* in the British Isles. The first of these tenets regards postglacial events in Europe. During the last ice age,

freshwater fish, along with many other animals, are thought not to have survived in Britain and Ireland (Varley, 1967). At this time, species mostly survived in refugia in southern Europe (Hewitt, 1996). With the retreat of the glaciers, eastern-flowing rivers such as the river Thames were connected to the river Rhine on the continent (Wheeler, 1977). During this time, *P. laevis* is thought to have been distributed throughout European freshwaters, where cyprinids and *Gammarus* species acted as its definitive and intermediate hosts, respectively. It is suggested that *P. laevis* split into three stocks during the formation of the North Sea, the disappearance of Doggerland and the severing of the Thames-Rhine link. On the continent, *P. laevis* remained virtually unchanged as one stock. The population in the river Thames, in its isolation, became the English strain. The marine strain was also formed through isolation and its unique life cycle.

The second tenet involves the stocking of other rivers in England with cyprinids infected with *P. laevis* from the river Thames (Kennedy *et al*, 1989). It is thought that *P. laevis* colonised Ireland in this way also, though only within the last 500 years. Due to the lack of suitable hosts there, *P. laevis* adapted to the native salmonids and *G. duebeni*. The more widespread distribution of *P. laevis* in Ireland, when compared to England, is thought to be as a result of its use of euryhaline hosts, which would allow it to colonise new rivers, via the sea.

The final tenet, which is mostly applicable to the English strain, is possible competition between *P. laevis* and other acanthocephalans (Kennedy *et al*, 1989; Bates and Kennedy, 1991a). There is a tendancy for one acanthocephalan species to dominate a river or a part of it and often the distributions of acanthocephalans in Britain do not overlap. This is taken as evidence of resource partitioning, in response to interspecific competition in the past and may explain why *P. laevis* does not occur in some areas where its hosts are found in abundance.

The Biological Species Concept and Strains

A definition of the term 'species', that is equally applicable to all organisms, does not exist. The arrival of the concept of evolution is largely responsible for this (Barton, 2001). In pre-darwinian times, the term 'species' was much easier to define. Organisms that seemed very similar to each other and yet different from others could be grouped naturally and called a species (Hey, 2001). That species are not static but

dynamic in both space and time is a commonly known fact and evolutionary theory has provided an explanation and a process for this continuous change. Because species are continuously evolving, whether due to random genetic drift or selection, a definition of species covering every stage of divergence does not seem possible (Barton, 2001).

The most widely applied species definition today is the Biological Species Concept (BSC). Species, as defined by Dobzhansky and Mayr, are "groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" (Kunz, 2002). It is obvious that this definition can only apply to sexually reproducing organisms, which excludes all asexually reproducing ones, including many parasitic species (Macko, 1978; Thompson and Lymbery, 1990; de Meeus, Michalakis and Renaud, 1998; Kunz, 2002). Because *P. laevis* is a sexually reproducing species it seems that the BSC can still be applied to it. But the BSC has limitations even when considering sexually reproducing species (Kunz, 2002).

The cornerstone of the BSC is the requirement for species to be reproductively isolated from each other. In reality, many species are not (de Meeus *et al*, 1998; Kunz, 2002). However, this does not mean that species in reproductive contact lose their distinctiveness. In fact, it is possible to maintain divergent genomes while exchanging genetic information (de Meeus *et al*, 1998; Kunz, 2002). Genomes, which are adapted to different ecological niches, are capable of this (de Meeus *et al*, 1998; Kunz, 2002). In some cases, hybridisation between species can lead to the creation of a new species (de Meeus *et al*, 1998). Another problem with the BSC is that even within a species, gene flow is not homogeneous over the whole range of the species (Kunz, 2002). This is especially true if a species is spread over a wide geographical range, as individuals in close contact with each other will tend to breed together (Kunz, 2002). Under these circumstances, the morphological characteristics of the species can be maintained by the force of similar selection pressures across the species' range (Kunz, 2002). The final failure of the BSC to be discussed here is that reproductive isolation is not a prerequisite for speciation (de Meeus *et al*, 1998). Speciation can occur in sympatry, which is speciation taking place in the same geographic area. This can occur through differential habitat preferences and other processes.

Since the advent of modern molecular techniques, large degrees of genetic variation have been found between species and even between individuals (Thompson and Lymbery, 1990; Kunz, 2002). This is true also for parasites. This raises a problem about defining species because the degree of genetic variability of parasites is not

always reflected in morphological variability (Thompson and Lymbery, 1990). The question arises, "what degree of difference is sufficient to put two objects into different species taxa?" (Kunz, 2002). Because it is such a difficult question to answer, parasitologists have begun to use the term 'strain'. Strain does not have a strict definition but is used in instances where different populations of a species display different characteristics and the significance of these differences, from a taxonomic point of view, is unclear. Strains in parasitic species have been defined by genetic and biological characteristics (Thompson and Lymbery, 1990). The term strain is often used when describing populations with different pathogenic potential or with different epidemiological characteristics. The term is used most appropriately when genetic variation lies more between populations from different hosts/areas, rather than within populations (Thompson and Lymbery, 1990). *P. laevis* strains differ ecologically. How they differ morphologically and genetically will be established in this thesis.

P. laevis, as a species, displays epidemiological characteristics which might favour speciation. *P. laevis* is a trophically transmitted parasite, which uses a gammarid as an intermediate host. These are a common food source for a variety of fish species. This point, coupled with *P. laevis'* ability to survive in a variety of definitive hosts, might favour sympatric speciation. In general, Acanthocephala are not particularly speciose, relative to other groups, such as the cestodes (Kennedy, 1993). In the British Isles very few acanthocephalan species are found in the same habitat, therefore sympatric speciation does not seem to operate. Acanthocephala must therefore evolve through some other means.

The Effects of Ice Ages on Genetic Structure

Postglacial events in Europe and their effect on the distribution of fish hosts in the British Isles, is central to the current theory of strain formation in *P. laevis*. Large scale phylogeographic studies have been carried out for many species in Europe (reviewed in Hewitt, 1996; Taberlet et al, 1998) including two important definitive hosts of *P. laevis*; chub and brown trout (Durand, Persat and Bouvet, 1999; Bernatchez, Guyomard and Bonhomme, 1992). These phylogeographic studies have provided evidence for predictions on genetic structure by the contraction/expansion model (Taberlet *et al*, 1998).

Large scale fluctuations in the Earth's climate since the Tertiary period (65 million years ago) led to a series of major ice ages in the Quarternary period (2.4 mya to the present) (Hewitt, 1996). Throughout the last 700,000 years, ice ages have been occurring roughly every 100,000 years. Orbital eccentricity of the earth around the sun seems to be the cause of this periodicity in the occurrence of ice ages, according to the Milankovitch theory (Hays, Imbrie and Shackleton, 1976). The last ice age lasted around 100,000 years with the present interglacial period having begun 18,000 BP (Hewitt, 1996). During the last ice age, the Scandinavian ice sheet extended to a latitude of 52°N, covering most of Ireland, Britain and Northern Europe. Large mountain ranges, such as the Pyrenees and the Alps were also covered by large ice sheets.

Obviously movements of ice on such a large scale had major effects on the distributions of organisms throughout Europe. Areas covered in ice were denuded of their flora and fauna. Species which did survive suffered severely contracted ranges and were forced into southerly located refugia (Hewitt, 1996). Therefore, during cold periods, species tended to move South and during warm interglacials, species moved North. These fluctuating ranges affected the genetic structure of species. Such effects can be predicted by the contraction/expansion model (Hewitt, 1996).

As the ice retreated, large areas became available which allowed colonisation northward from the Southern refugia. This rapid colonisation is known as leading edge expansion and was probably carried out by long distance dispersers (Hewitt, 2000). Once a colony was established, it expanded very quickly to fill the area. This colony would then allow dispersers to move even further North and repeat the cycle until a barrier was met. These repeated founding events led to a loss of alleles and an increase in homozygosity with the distance covered (Hewitt, 1996, 2000). In the refugia themselves, genomes tend to be extremely diverse, due to the varied topography provided by mountain ranges, which encourages speciation (Hewitt, 1996, 2000). Therefore, genomes are predicted to be divided geographically across Europe due to colonisation from different refugia and sorting of past mutations (Hewitt, 1996, 2000).

The effect of ice ages on chub and brown trout can be seen from their present day genetic structure. From a study of a 600bp section of cytochrome b in chub, four lineages could be ascertained (Durand *et al*, 1989). Two lineages were seen in the South of Europe, an Adriatic and an Aegean lineage and two in the North, East and West. Chub in the West of Europe are thought to have been colonised from the Danube

in two steps, the second of which led to the colonisation of Britain (Durand *et al,* 1998). Northern chub populations exhibit very low genetic diversity. This is in contrast to the high genetic diversity in Italy where haplotypes differ between rivers (Durand *et al*, 1998)

The genetic stucture of brown trout can be interpreted in a similar way. The study of 640bp of the mitochondrial control region revealed five phylogenetic assemblages (Bernatchez *et al*, 1992). Again populations across the Atlantic basin were characterised by monomorphism, while high levels of diversity characterised Southerly located populations such as those along the Mediterranean and Adriatic Seas and in Corsica. Brown trout vary morphologically and ecologically across Europe. Populations are specialised ecologically into anadromous, fluviatile and lacustrine groups. Interestingly, no congruence between genetic structure and ecology was found, though the opposite was true for geographic morphs (Bernatchez *et al,* 1992). Though Irish and British populations were not included in this study, these would be expected to be genetically identical to the other populations in Northern Europe with very low levels of diversity.

The genetic structure of the host would have implications for *P. laevis*. Being an obligate parasite, *P. laevis* would have depended on its hosts for survival through ice ages and would have suffered similar range expansions and contractions.

Molecular Studies within the Acanthocephala

From the point of view of population genetics, only four such studies have been published for the Acanthocephala. The most relevant to this work was that carried out on English freshwater and marine strains of *P. laevis* (Munro *et al*, 1990). The main results were summarised in the introduction to this chapter. When the entire genome of both strains were subjected to RFLP (restriction fragment length polymorphism) analysis, with various restriction enzymes, the genomes were revealed to be relatively homogeneous. This method was extended by then hybridising a ribosomal DNA probe to the digest, which revealed some differences in the pattern of hybridisation. This led to the conclusion that there may be a certain degree of diversity within the species *P. laevis* at the level of the DNA.

The first electrophoretic study of its kind on the Acanthocephala was used in an attempt to distinguish a new species *Acanthocephaloides geneticus* from an exisiting

one, *A. propinquus* (de Buron *et al*, 1986). The two species use different hosts, but slight morphological differences were not considered enough to name a new species. A study of 18 isozymes uncovered discernable differences between the two proposed species. The two proposed species were found to be homozygous for different alleles in 5 of the 18 loci. It was concluded that a complete mating isolation existed between the two species, since no heterozygotes were found, and *A. propinquus* may represent an as yet undiscovered species complex.

RAPD (random amplified polymorphic DNA) analysis and isoenzyme analysis enabled the recognition of congeneric species of *Neoechinorhynchus* (Aho *et al*, 1992; Dezfuli and Tinti, 1998). Three primers gave rise to species specific patterns which allowed *N. pseudemydis* to be distinguished from both *N. emydis* and *N. emyditoides* (Dezfuli and Tinti, 1998). Prior to this method, female morphology was used to identify these acanthocephalans, though not very successfully and males were unidentifiable.

Currently, interest in the population genetics of the Acanthocephala is growing. Tina Hassu (2001) at the University of Jyvaskyla, Finland, is using a variety of DNA techniques to compare locally adapted populations of *Acanthocephalus lucii*. In the United States, Michelle Steinhauer (2001), in collaboration with Professor Brent Nickol, is investigating the phylogeography of *Leptorhynchoides thecatus* in the Southern states and is attempting to sequence the entire mitochondrial genome of this species. France *et al* (2002) working at the University of Charleston, South Carolina, U.S. are studying the diversity of mitochondrial cytochrome genes among representatives of two classes of acanthocephalans, the Palaeacanthocephala and the Eoacanthocephala.

The remainder of the studies on the Acanthocephala are those carried out by phylogeneticists. Phylogeneticists are interested in how higher organismal taxa are related to each other and how they have evolved over the course of time. Relationships between the Acanthocephala and other pseudocoelomates have long puzzled researchers. Studies on the 18S RNA gene have shown the Aschelminthes to be a polyphyletic group (Winnepenninckx *et al*, 1995). This taxon includes the Acanthocephala and other pseudocoelomate animals. This study also showed a close relationship between the Acanthocephala and the Rotifera. The relationship was confirmed by Garey *et al* (1996) who suggested the bdelloid rotifers and the Acanthocephala be united under one superclass, the Leminiscea. This is apparently the first study to reveal a potential extant free-living relative of a parasitic group.

Mitochondrial DNA Studies in other Helminths

In this study, subunit I of the cytochrome c oxidase gene (COI) was sequenced in order to determine diversity between the English and Irish freshwater strains of *P. laevis*. Mitochondrial DNA (mtDNA) is often used for within species studies, due to its fast evolutionary rate, compared to that of DNA, and the usefulness of its haploid state and lack of recombination (Bowles, Blair and McManus, 1992). Some DNA studies in other helminth groups will be presented to demonstrate the flexibility and usefulness of this approach.

Cestoda

The genus *Echinococcus* was once thought to consist of 16 species, but now only 4 are regarded as being taxonomically valid (Bowles *et al*, 1992). Intraspecific variation, due to differences in epidemiology, pathology and control of cystic hydatid disease, was observed in one species *E. granulosus* (Zhang *et al*, 1998; Bowles *et al*, 1992). Due to the unreliability of morphological characters, strain differentiation was examined using direct sequencing of the COI. Bowles *et al* (1992) defined an *Echinococcus* strain as being an "intraspecific group of genetically distinguishable isolates which also share biological features of actual or potential significance in the control of hydatid disease". Nine strains of *E. granulosus* are now recognised (Bowles *et al*, 1992; Zhang *et al*, 1998). Despite the very low number of nucleotide substitutions found between some strains, the status of these strains was upheld, due to the corresponding important epidemiological differences. Two distinct strains of *E. granulosus* in Iran were also identified, using mitchondrial DNA (Zhang *et al*, 1998).

A taeniid unlike the other known species, *Taenia saginata* and *T. solium,* was found in Asia (Bowles and McManus, 1994). RFLP analysis of COI and ITS1 (internal transcribed spacer 1, ribosomal DNA) distinguished Asian taenia from the other two species, while direct sequencing of these genes revealed that Asian taenia closely resembled *T. saginata*. It was felt, based on the evidence, that Asian taenia was not a separate species, but either a subspecies or strain of *T. saginata*. The first case of *T. solium* infection in black bears was confirmed using direct sequencnig of COI and RFLP analysis (Theis *et al*, 1996)

Digenea

Molecular analysis, at the DNA sequence level, has determined relationships within and among a plethora of digenean species. Chinese and Philippine strains of *Schistosoma japonicum* were found to be very similar at the nuclear and mitochondrial DNA level (Bowles *et al*, 1993). Their COI sequences differed at only 2 out of 372 positions, while interspecific COI sequences were different by 13 to 21%. The level of homogeneity discovered was in contrast to clear morphological and epidemiological differences and to the results of an isoenzyme study. From the results of that study, the divergence of the two strains was estimated at between one and two million years. The inconsistency of the molecular and non-molecular studies was possibly due to rapid phenotypic change, which itself was due to differences in host availability and geographical location without concomitant genotypic change.

COI sequence analysis, in conjunction with sequence analysis of ITS1 and ITS2, revealed the close relationship between a new species *Schistosoma mayalensis* and *S. mekongi* (Blair *et al*, 1997). A study of the triploid and diploid forms of Fasciola in Japan revealed homogeneity within both NDI (NADH dehydrogenase, subunit I) and COI sequences (Itagaki *et al*, 1998). Even distinct species such as *F. gigantica* and *F. hepatica* possessed relatively similar NDI and COI sequences. The NDI sequences of these two parasites differed by 1.4% and their COI sequences differed by between 0.6 and 1.1%. The Japanese forms were found to be closer to *F. gigantica*. In a further study of the Japanese *Fasciola* strains, some were found to identify with *F. hepatica* and others with *F. gigantica* from an RFLP study of their mtDNA (Itagaki *et al,* 2001). This led to the hypothesis that Japanese triploids were composed of two genetic lineages, corresponding to *F. hepatica* and *F. gigantica*. An attempt to distinguish between triploid and diploid forms of *Paragonimus westermani* was conducted through RFLP analysis of mtDNA (Agatsuma *et al*, 1994). Two enzymes did reveal a distinct pattern for the two forms. It was hypothesised that triploids arose from the hybridisation between Japanese diploids and non-Japanese diploids. Low levels of heterogeneity imply a recent origin.

A study of strains of *Echinostoma* species is more reminiscent of the *Echinococcus* study. Few morphological characters exist to differentiate such species, apart from the number of spines around the oral sucker (Morgan and Blair, 1998). This method is unreliable as it is not always possible to make a count. Sequences of ITS1

and ITS2 as well as two mitochondrial sequences, those of NDI and COI uncovered heterogeneity among new isolates. Isolates that were between 0 and 3.6% different from a recognised species, were considered to be strains of that species. NDI is known to differ by between 12.3 and 30.5% between species, within the genus *Echinostoma*.

Molecular data has also revealed cryptic diversity among morphologically similar parasites. ITS1 sequencing revealed the existence of two cryptic species within each of two recognised digenean species, spread amongst three definitive host species in the Mediterranean (Jousson, Bartoli and Pawlowski, 2000). One of the newly discovered cryptic species from both original species occurred sympatrically and shared two host species, while the other cryptic species occurred in one host. With further sequencing of the host COI gene, speciation within the digenean parasites was found not to be influenced by coevolutionary processess, but rather host dietary preferences.

Sequencing has also helped resolve taxonomic difficulties within two families Campulidae and Nasitrematidae (Fernandez *et al*, 1998). These trematodes parasitise marine mammals, cetaceans in particular. A phylogeny, based on both tRNAlys (a transfer RNA encoding gene) and a mitochondrial gene, revealed the monophyly of these families. Suggestions were made to lose the familial status of the Nasitrematidae, as its type species was found to be a member of the Campulidae, and to correct the current subfamilial division of the Campulidae.

Nematoda

Researchers working with nematodes suffer the same problems as those working with acanthocephalans and many other parasitic groups, stemming from the lack of diagnostic characters. The uncertain species status of *Haemonchus placei* and *Haemonchus contortus*, parasites of domestic ruminants, was overcome by sequence analysis of a mitochondrial gene, ND4 (Blouin *et al*, 1997). At this locus, intraspecific variation was within 2% and average interspecific variation was 15.6%. The extent to which these species hybridise in the wild was another setback to taxonomists. No evidence of introgression was found in the ND4 gene. Despite the ability of *H. placei* and *H. contortus* to hybridise under laboratory conditions, hybridisation does not seem to occur in the wild.

Another study, analysing ND4 sequences in 5 species of nematodes, found high levels of genetic variability within species (Zarlenga *et al*, 1998). The lack of

population genetic structure found in parasites of sheep and cattle contrasted with that found in a parasite of deer. Host movement was thought to be a major influence of genetic structure. High intraspecific diversity was attributed to the unnatural level of movement of domestic sheep and cattle due to human activity. This was substantiated by the geographically related genetic structure observed for a deer nematode. 98% of total genetic diversity was found to be within populations from a previous study on *Ostertagia ostertagi*, a parasite of cattle (Blouin *et al*, 1992).

The first study of the ND4 sequence of heterorhabditid nematodes was able to clearly distinguish four species (Liu, Berry and Blouin, 1999). Generally these species are very similar morphologically and are also highly variable phenotypically. Three of the species were found to be polymorphic and two initially separate species were found to be conspecifics.

The usefulness of mitochondrial sequence data seems most appropriate when examining closely related species or intraspecific strains. Attempts at employing COI sequences for phylogenetic research of the Ostertagiinae, the Strongylinae and the Cyathostominae were not very successful (Zarlenga *et al*, 1998; McDonnell *et al*, 2000). In these instances sequencing ITS1 was more informative. Mitochondrial sequence between distantly related animals tends to be very variable. Another problem is the A, T bias exhibited by the sequence. This tends to lead to saturation of sites within the sequences, as there are fewer possible character states (McDonnell *et al*, 2000).

This study therefore represents a comprehensive investigation of three aspects of the biology of the freshwater strains of *P. laevis*. Previous studies on species and strain identification have advocated such an approach due to the unreliability or lack of morphological markers (Bowles *et al*, 1992; Nascetti *et al*, 1993; Jacquiet *et al,* 1995; Ponce Gordo and Cuesta Bandera, 1997). Due to the novelty of sequencing an acanthocephalan mitochondrial gene, it was not possible to do the same using a nuclear gene. Anderson (2001) warned of the pitfalls of drawing conclusions on speciation from just one marker. It is hoped that the combination of genetic and morphological markers might offset that danger. Also the lack of information regarding the genetics of the Acanthocephala, make interpretation of the results a little difficult.

CHAPTER 2

THE ECOLOGY OF *POMPHORHYNCHUS LAEVIS* IN BROWN TROUT, L. FEEAGH

2.1 INTRODUCTION

Most of the evidence in support of the strain hypothesis has come from studies of the ecology of *P. laevis* in various locations throughout its range. In Ireland *P. laevis* cycles through *G. duebeni* and brown trout (Fitzgerald and Mulcahy, 1983; Brown, Chubb and Veltkampf, 1986). This combination of hosts differs to that used by *P. laevis* elsewhere. In Britain and on the continent, chub and barbel are the definitive hosts and the locally abundant gammarid acts as an intermediate host (Hine and Kennedy, 1974a; Petrochenko, 1956). The marine strain completes its life cycle through flounder and *G. zaddachi* (Kennedy, 1984; Munro *et al*, 1989). One of the first peculiarities noticed about the English strain was its very restricted distribution there (Hine and Kennedy, 1974a). The latest study concerning the ecology of the freshwater and marine strains in England has revealed that the two strains do live in sympatry in flounder and that niche adjustments in mixed infections may result in reproductive isolation (Guillen-Hernandez and Whitfield, 2001). This is the strongest evidence yet for the existence of separate marine and freshwater strains in England.

In Ireland, *P. laevis* has been studied very little compared with English and continental populations. Only two in-depth studies have concentrated on the population biology of the parasite and only one has studied the parasite at the infracommunity level (Fitzgerald and Mulcahy, 1983; Molloy *et al*, 1995). Findings from these studies have shown further differences between the Irish strain and *P. laevis* elsewhere. The most important difference revealed is the seasonal cycle of growth and maturation shown by Irish *P. laevis*, which contrasts with the seasonal stability of the parasite in Britain (Hine and Kennedy, 1974b; Brown, 1989) and on the continent (Moravec and Scholtz, 1991).

This study is only the second to investigate a variety of aspects of the population biology of *P. laevis* in Ireland. The main aim of this study is to outline in detail the yearly cycle of growth and maturation of the adult phase of *P. laevis* in Ireland and to compare this with previous studies of other populations. This study is more comprehensive than that conducted by Molloy *et al* (1995) as all individual parasites were examined and two years of sampling were conducted. Because it is the second investigation of the L. Feeagh population it will enable a direct comparison with the results of Molloy *et al* (1995) and a chance to document any change in the population over time.

2.2 MATERIALS AND METHODS

Sampling Procedures

The population biology of *P. laevis* from wild brown trout (*Salmo trutta*) in L. Feeagh, Co. Mayo (west of Ireland) was studied over the years 1997 and 1998. Sampling was carried out by Dr. Ciaran Byrne (2000), as part of his PhD study into the parasite community structure of wild and stocked brown trout. The community structure of *P. laevis* in both types of trout was examined as this was an important member of the parasitic fauna. A sample of brown trout was recovered from the lake in April, May, August and November of 1997 and May, August and November of 1998.

L. Feeagh is a low-lying, oligotrophic freshwater lake of glacial origin (Byrne, 2000). Figure 2.2.1 shows where it is situated within the Burrishoole catchment. The lake has an area of 410ha. Its maximum depth is 43m and it is located at an altitude of 14m above sea level. The water of L. Feeagh is neutral to acidic (pH = 6.3 – 7.0) and distinctly coloured, due to peat siltation. The temperature of the lake usually falls in the range of 3 to 20°C.

Fish were captured from L. Feeagh by beach seine netting and trapping (Byrne, 2000). Beach seine netting was begun by first fixing one end of the net (60m long) onshore with a towline. The net was then allowed to unfurl from a boat in a semi-circle. Both ends of the net were hauled simultaneously, drawing the net into a circle. The net was then hauled in slowly. When near the shore, bottom lines were also pulled so as to trap fish in the holding bag attached to the centre of the net. Full upstream and downstream traps are present at L. Feeagh and fish caught in them were also included in the study.

Fish were deep frozen until a full parasitological examination could be carried out. A description of the fish dissection can be found in Byrne (2000) and Byrne *et al* (2002).

Treatment of Parasites

Individuals of *P. laevis*, once removed intact from the gut of the host, were relaxed in tap water for a 24 hour period in order to evert the proboscis. Parasites were

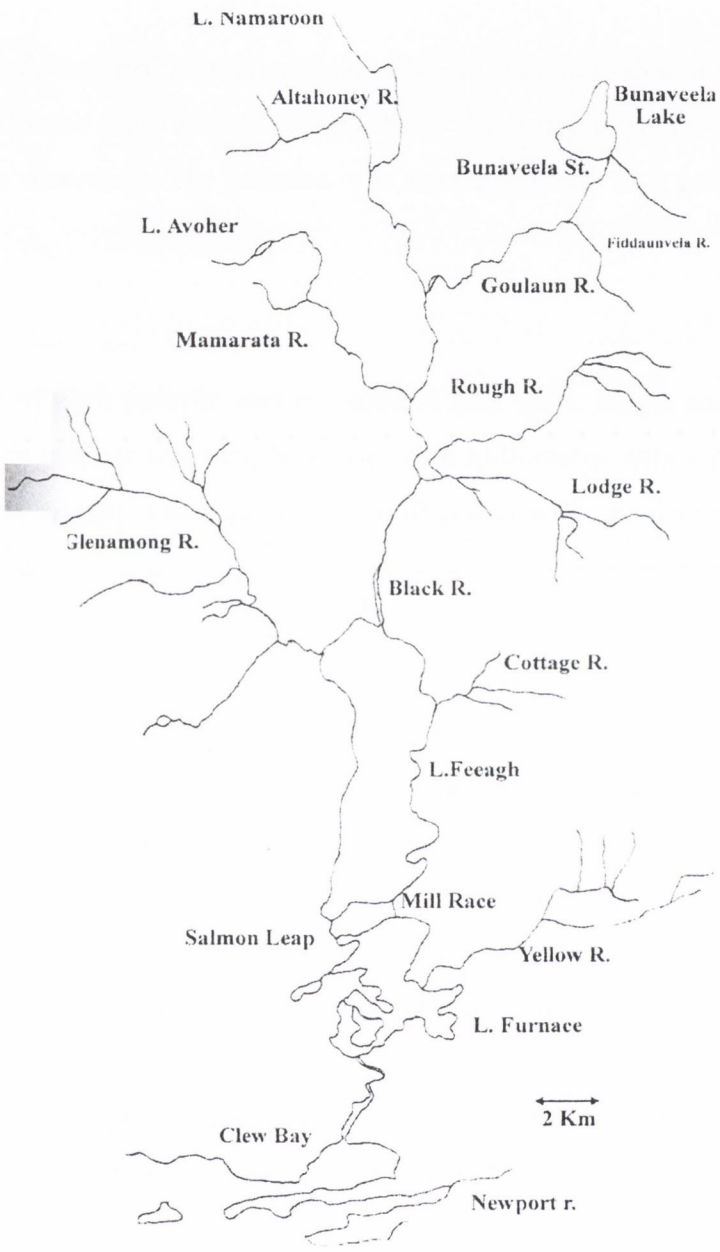


Fig. 2.2.1 Map of L. Feeagh and the Burrishoole catchment area. After Byrne, 2000.

fixed in 10% formalin and left in the fixative for 24 to 48 hours. They were then washed in 70% ethanol and stored in fresh 70% ethanol and 4% glycerol.

Four biological parameters were recorded from each worm. These parameters were position in the host's gut, size, sex and maturity status.

Position

This was determined at post mortem. The gut was measured in centimetres from the oesophagus to the anus and the length from the oesophagus to the position of each parasite was recorded. The position was then expressed as a percentage of total gut length.

Size

The size of each parasite was recorded in two ways, length and weight. The total length of each parasite was simply measured in millimetres with a ruler. The weight as the fixed wet weight was measured in milligrams with an electronic Mettler-Toledo AG245 balance.

Sex

The sex of each parasite was noted at the same time as the maturity status was being assessed. After cutting the worm in half with a fine scissors in saline (0.9%), the posterior half was roughly flattened under a coverslip on a microscopic slide and viewed at a magnification of 100 times. Males were easily distinguished by the presence of two ovoid testes lying in series in the middle of the body followed by three pairs of cement glands, also in series at the end of the body. Females were distinguished by the absence of these structures and the presence of ligament sacs containing reproductive products. The ligament sacs may be ruptured allowing the eggs and ovarian balls to float freely within the body cavity.

Maturity Status

Female acanthocephala progress through three easily recognisable stages of maturation during their lifetime. Germ cells are incorporated into a tissue known as an ovarian ball, which is in fact a true ovary. During the period when the ovarian balls are fragmenting and prior to fertilisation, the females are in the first stage of maturity or stage I. The second stage (stage II) is reached when a female has mated with one or

more males and some of her oocytes have been fertilised. This stage is easily recognisable by the appearance of developing eggs in the ligament sacs. These eggs can be first seen developing within the ovarian ball and consist of an embryo enclosed by an oval shaped membrane. When at least one of the eggs has become fully developed, the female has reached the third stage or stage III. At the final stage of development, the egg contains an acanthor surrounded by three membranes some of which develop polar elongations (Crompton, 1985).

Females were assigned to one of the three stages by cutting open each worm and viewing the reproductive products under a light microscope (Bates and Kennedy, 1991b).

Statistical Analysis

All analyses were carried out on Data Desk version 6.0. Three different levels of probability are referred to in the results. * is equivalent to $p < 0.05$, ** is equivalent to $p < 0.01$ and *** is equivalent to $p < 0.001$. Position and weight were not normally distributed and therefore were log transformed prior to statistical analyses such as t-test or analysis of variance. Length, weight and position were compared between males and females using a student t-test. Length, weight and position were compared between females at the three stages of maturity with a one-way analysis of variance. Scheffe Post Hoc tests were also conducted so as to determine exactly at which stage of maturity females differed in length *etc.* The effect of year and season on the proportion of females at the three stages of maturity was examined using a chi-squared analysis. The effect of extrinsic factors such as year, season, parasite and host sex on the length, weight and position of worms was examined using a four-way interaction analysis of variance. When conducting an analysis of variance on a data-set using four factors, sample sizes become very reduced. For example there are only 14 male worms in 16 male fish from April in 1997 (see table 2.3.1, section 2.3). This reduction in the data occurs asymmetrically so there are large discrepancies in sample sizes between samples. For this reason, results from the four way analyses of variance must be treated with caution. Tests were therefore made more stringent and significance was only accepted at the $p = 0.01$ levels.

2.3 RESULTS

General Observations

739 worms were examined from 113 wild brown trout in 1997 and 416 worms were examined from 64 brown trout in 1998. The community parameters, such as prevalence, mean abundance and mean intensity for *P. laevis* are described in detail in Byrne (2000). When both years are considered together, prevalence was 48.33% in April. Prevalence in May and August was high at 90.63% and 73.86% respectively. In November *P. laevis* infected 46.67% of fish. The mean intensity (± SD) of *P. laevis* in brown trout in L. Feeagh, Co. Mayo over the years 1997 and 1998 was 6.4 ± 7.03 worms per fish. The sex ratio of *P. laevis* was 1.16:1 in favour of females.

Size and Position

Female worms had a mean length (± SD) of 9.6 ± 3.12mm and ranged in length from 2 to 21mm. Male worms were on average (± SD) 8.2 ± 2.71mm long and ranged in length from 2 to 18mm. Length was normally distributed among both sexes with overall skewness and kurtosis values of 0.55 and 0.38 respectively (figure 2.3.1). Female worms were significantly longer than males ($t = 7.742$, $df = 1110$, $p \leq 0.0001$).

Female worms had a mean weight (± SD) of 6.6 ± 6.71mg and ranged in weight from 0.5 to 51mg. Male worms weighed an average (± SD) of 4.7 ± 4.35mg and ranged in weight from 0.5 to 40mg. Weight was not distributed normally and a positive skew can be seen from figure 2.3.2. Weight had an overall skewness value of 3.04 and a kurtosis value of 12.63. Female worms were significantly heavier than males ($t = 6.395$, $df = 1108$, $p \leq 0.0001$).

Females were found at a mean position (±SD) of 77.9 ± 12.67% in the gut and ranged over the entire length of the gut. Males had a mean position (±SD) of 79.9 ± 12.09% and ranged from 10 to 100%. A negative skew can be seen from the distribution of position (figure 2.3.3). Position had a skewness value of –2.6 and a kurtosis value of 13.66. Females and males occurred at similar positions in the gut ($t = 0.17$, $df = 892$, $p = 0.87$).

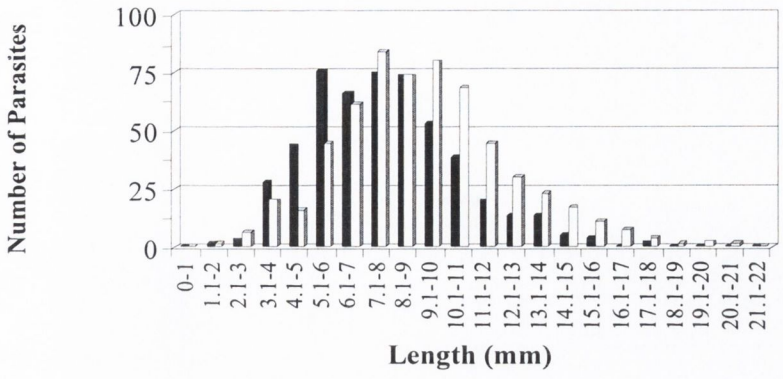


Fig. 2.3.1: Frequency distribution of the length of male and female Irish *P. laevis*.

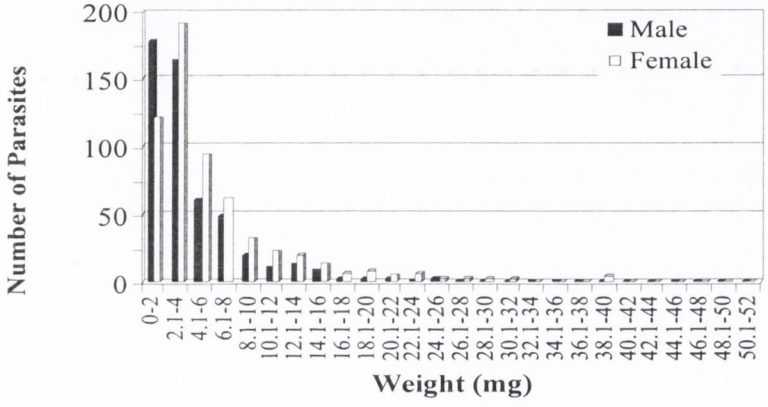


Fig. 2.3.2: Frequency distribution of the weight of male and female Irish *P. laevis*.

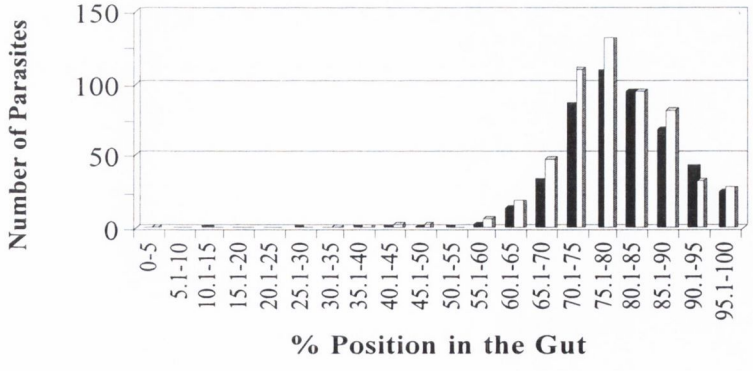


Fig. 2.3.3: Frequency distribution of the position of male and female Irish *P. laevis*.

Maturity Status

The majority of females were at the first stage of maturity (49.9%). At this stage females have yet to be fertilised. 20.6% of females were at stage 2 which is when fertilisation has taken place and embryos are beginning to develop. Less than a third of the females overall (29.5%) were fully mature (stage 3). At stage 3, embryos are fully developed and ready to be shed into the environment.

Females at stage 1 had a mean length (±SD) of 8.3 ± 2.55mm (figure 2.3.4). At stage 2 females had a mean length (±SD) of 8.9 ± 2.73mm and at stage 3 they had a mean length (±SD) of 12.1 ± 2.82mm. Significant differences in length were found between females at different stages of maturity ($F = 112.3$, $df = 566$, $p \leq 0.0001$). Females at stage 3 were significantly longer that females at stage 1 (Scheffe Post Hoc test, $p \leq 0.0001$) and stage 2 (SPH test, $p \leq 0.0001$). Length did not differ between females at the first and second stages (SPH test, $p = 0.1$). Females at stage 1 weighed 4.3 ± 4.26mg on average and females at stage 2 had a mean weight (± SD) of 5.3 ± 5.12mg (figure 2.3.5). Again stage 3 females were the largest with a mean weight (± SD) of 11.5 ± 8.54mg. Weight also differed significantly between females at different stages of maturity ($F = 109.1$, $df = 565$, $p \leq 0.0001$). Females at stage 2 were significantly heavier than females at stage 1 (SPH test, $p = 0.0019$) and stage 3 females were significantly heavier than both stage 2 (SPH test, $p \leq 0.0001$) and stage 1 females (SPH test, $p \leq 0.0001$). Stage 1 females were found at a mean position (± SD) of 76.1 ± 16.9% (figure 2.3.6). Females at stage 2 were found lowest in the gut on average at 81.1 ± 7.78% and stage 3 females were found at a mean position (± SD) of 77.9 ± 7.27%. Position differed significantly among females ($F = 6.1$, $df = 543$, $p = 0.0025$). Females at stage 2 were found to be in a significantly different position to females at stage 1 (SPH test, $p = 0.0027$).

Extrinsic Factors

Year, Season and Host Sex

Factors examined for their effects on *P. laevis* were year, season and host sex (table 2.3.1). Mean intensity fluctuated with season (figure 2.3.7). In 1997 the mean

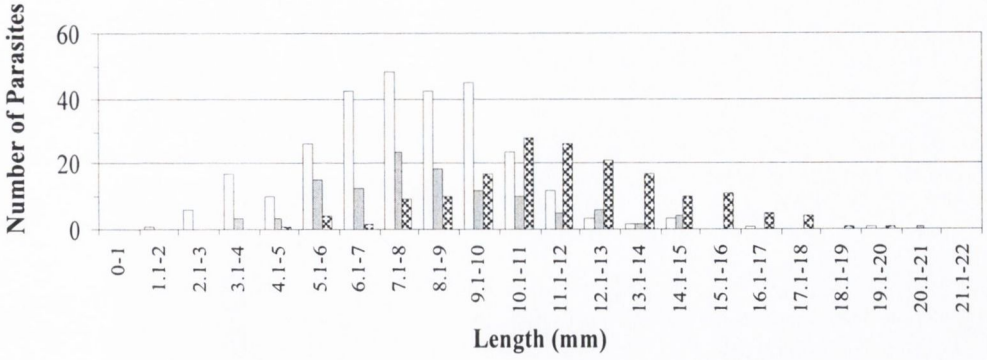


Fig. 2.3.4: Frequency distribution of the length of Irish female *P. laevis* at three stages of maturity.

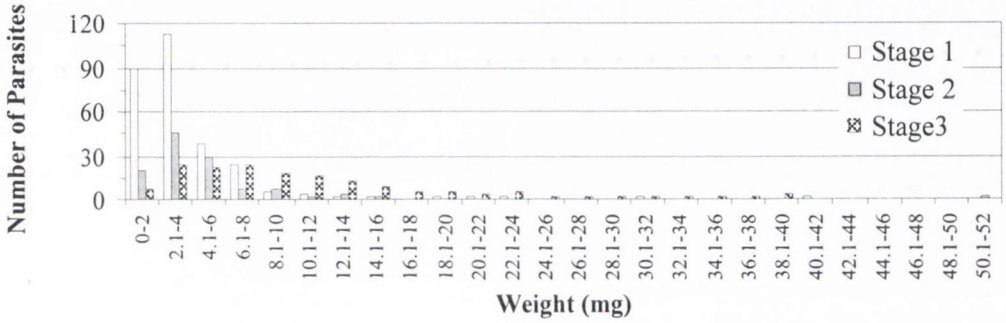


Fig. 2.3.5: Frequency distribution of the weight of Irish female *P. laevis* at three stages of maturity.

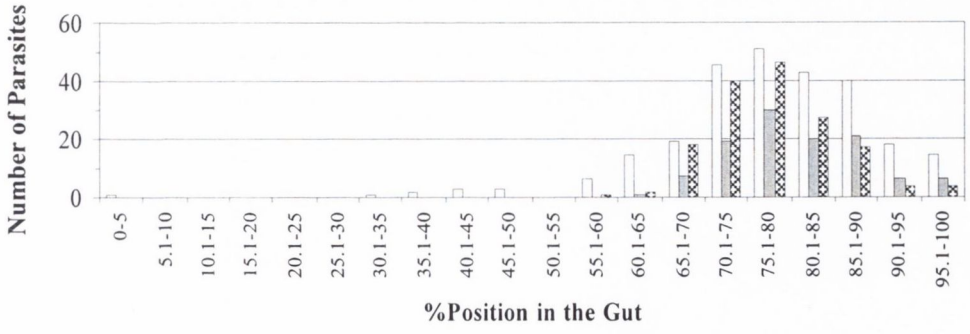


Fig. 2.3.6: Frequency distribution of the weight of Irish female *P. laevis* at three stages of maturity.

intensity increased from April to May and decreased from May to August. This decrease was followed by another increase in November. This seasonal trend was consistent in 1998 though the mean intensity was generally lower in all months (April excluded) than in 1997. A three way ANOVA revealed that of the factors year, season and host sex, only season had any significant effect on intensity ($F = 3.6$, $df = 175$, $p = 0.0147$). Mean intensity in August was found to be significantly lower than in May (SPH test, $p = 0.033$).

Sex ratio (females/males) also fluctuated with season though not consistently (figure 2.3.8). In 1997 the number of females increased from April to November. The opposite trend is seen in 1998 with females decreasing in number from May to November. Overall there were generally more females than males. When considering sex ratio together with the seasonal changes in mean intensity, the stability in sex ratio between May and August in 1997 can be interpreted as demonstrating an equal loss of males and females as intensity decreased at this time of year. For the same period in 1998, more females seem to have been lost than males.

Table 2.3.1: The number of male and female hosts (N_H), number of parasites (N_P), mean intensity ± standard deviation (MI) and parasite sex ratio (f/m) for each season in 1997 and 1998. Stars refer to missing data.

		1997				1998			
		N_H	N_P	MI	f/m	N_H	N_P	MI	f/m
April	**Female**	23	121	5.3±5.94	1.06	*	*	*	*
	Male	16	39	2.4±1.93	1.77	*	*	*	*
May	**Female**	16	161	10.1±8.15	1.19	10	97	9.7±11.38	1
	Male	14	123	8.8±5.16	1.06	15	99	6.6±6.47	1.44
August	**Female**	19	125	6.6±7.62	1.23	16	75	4.7±3.79	1
	Male	18	117	6.5±7.41	1.19	9	33	3.7±3.46	1.31
November	**Female**	3	33	11.0±7.81	1.2	9	35	3.9±5.44	1.27
	Male	4	20	5.0±3.56	1	5	77	15.4±17.21	1.08

- *Worm Length*

The length of males and females also fluctuated seasonally (figure 2.3.9). Worms were similar in length in April and May and increased to a mean length (± SD) of 10.3 ± 2.47mm (females) and 12.2 ± 2.81mm (males) in August. Worms subsequently decreased in length in November. A four way ANOVA revealed a statistical significance in the seasonal change in length (table 2.3.2). The largest statistical difference was found between length in August and all of the other months

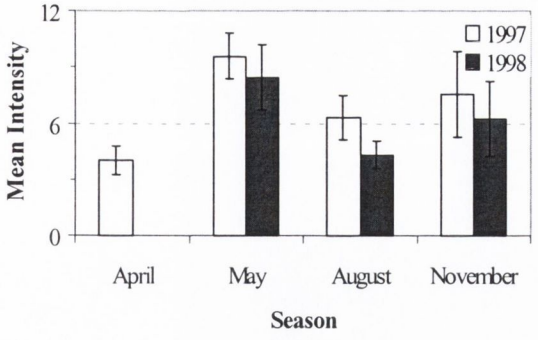


Fig. 2.3.7: Change in mean intensity (± standard error) of *P. laevis* with season for 1997 and 1998.

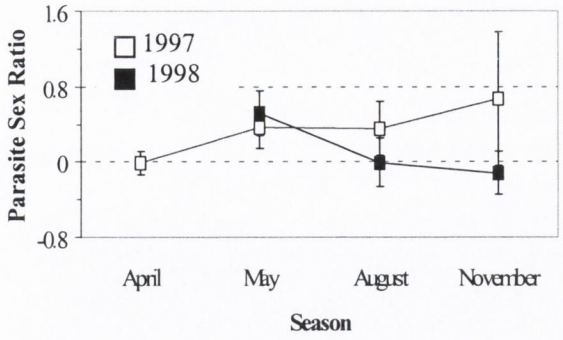


Fig. 2.3.8: Change in mean sex ratio (± standard deviation) with season for 1997 and 1998.

(SPH test, $p \leq 0.0001$). Worms were also significantly longer in November than in May and April (SPH test, Nov > May - $p = 0.049$, Nov > Apr - $p = 0.016$). Worm length did not differ significantly between years alone though there was an interaction between year and season. Worms in 1998 were longer than those in 1997 for May (Sheffe Post Hoc test, $p \leq 0.0001$) and August (SPH test, $p = 0.006$). Host sex did not affect worm length. As was mentioned earlier in the chapter, female worms are significantly longer than males. Worm sex did not interact significantly with any other factor.

Table 2.3.2: Results of a 4-way ANOVA of worm length, weight and position (males and females) using year, season, host sex and parasite sex as factors.

	LENGTH			WEIGHT			POSITION		
	F-ratio	df	p	F-ratio	df	p	F-ratio	df	p
YEAR	3.1	1, 1055*	NS	15.0	1, 1053	***	6.2	1, 1033	NS
SEASON	144.9	3, 1055	***	107.7	3, 1053	***	14.8	3, 1033	***
YEAR*SEASON	14.8	2, 1055	***	0.8	2, 1053	NS	1.5	2, 1033	NS
HOST SEX	3.4	1, 1055	NS	0.7	1, 1053	NS	7.4	1, 1033	**
YEAR*HOST SEX	5.4	1, 1055	NS	4.1	1, 1053	NS	10.0	1, 1033	**
SEASON*HOST SEX	3.2	3, 1055	NS	4.4	3, 1053	**	5.5	3, 1033	***
PARASITE SEX	77.9	1, 1055	***	44.4	1, 1053	***	5.2	1, 1033	NS
YEAR*PARASITE SEX	1.1	1, 1055	NS	0.0	1, 1053	NS	6.6	1, 1033	NS
SEASON*PARASITE SEX	2.0	3, 1055	NS	1.2	3, 1053	NS	4.5	3, 1033	**
HOSTSEX*PARASITE SEX	4.5	1, 1055	NS	2.9	1, 1053	NS	1.6	1, 1033	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

- *Worm Weight*

As with length and intensity, season had the greatest effect on worm weight (figure 2.3.10, table 2.3.2). Worms in August were significantly heavier than those in all other months (SPH test, Aug > Apr – $p = 0.04$, Aug > May and Nov – $p \leq 0.0001$). No significant differences were found in worm weight between any of the other months.

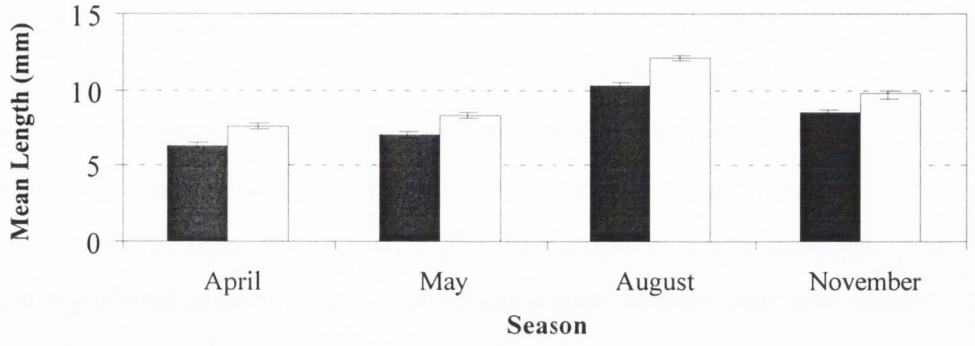


Fig. 2.3.9 The change in the mean length (± standard error) of male and female Irish *P. laevis* with season.

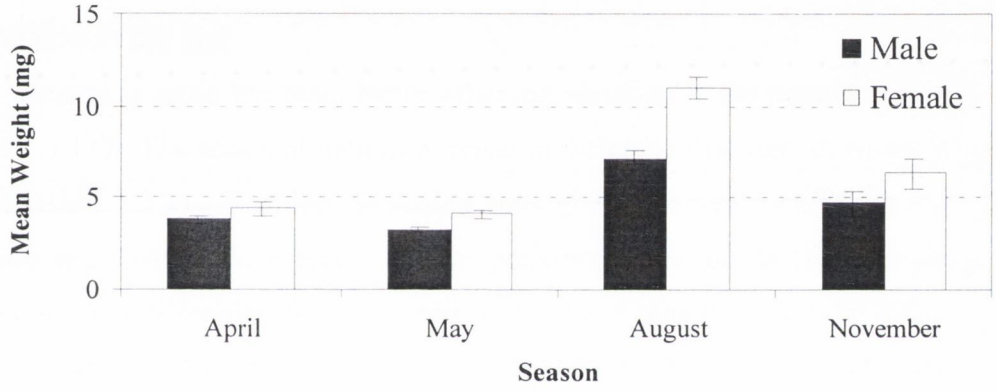


Fig. 2.3.10 The change in the mean weight (± standard error) of male and female Irish *P. laevis* with season.

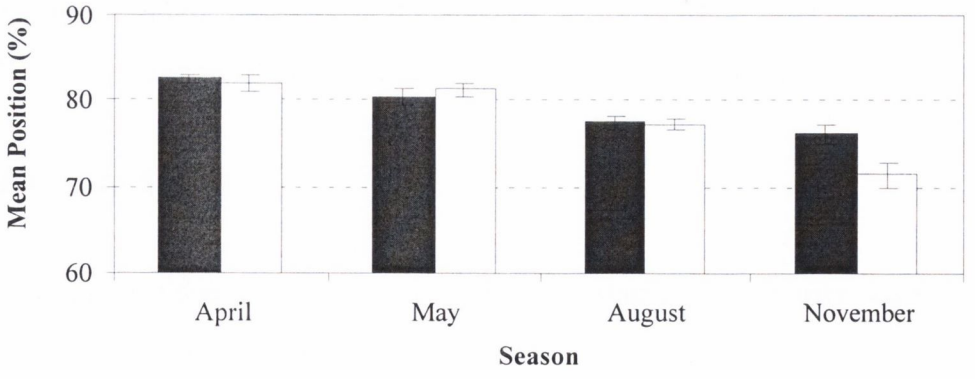


Fig. 2.3.11 The change in the mean position (± standard error) of male and female Irish *P. laevis* with season.

Unlike length, weight differed between years. Overall, worms were significantly heavier in 1998 than in 1997. Weight did not differ with host sex. A significant interaction was found between host sex and season. The seasonal pattern was again prominent with no significant differences between the sexes within each month except for a weak interaction between host sex in May and November. Overall, worms were only significantly heavier in August. The interaction between host sex and season revealed a significant difference between worm weight in both male and female hosts in May and male hosts in November (SPH test Female May > Male Nov – $p = 0.04$, Male May > Male Nov – $p = 0.003$). Worms in female hosts in November did not differ from worms in May. Female worms were significantly heavier than males. There were no interactions between parasite sex and either season, year or host sex.

- *Position in the gut*

Season is again the main factor affecting variation in the population of *P. laevis* (figure 2.3.11). The seasonal pattern in position differs to that seen in worm length and weight (table 2.3.2). Position decreased throughout the year, which means that the infection was centred in a more anterior position in the gut as the year progressed. Position did not differ significantly between April and May though it did differ between all other months (SPH test, Apr > Aug – $p = 0.02$, Apr > Nov – $p = 0.001$, May > Aug – $p = 0.007$, May > Nov – $p \leq 0.0001$, Aug > Nov – $p = 0.02$). Position differed overall with host sex as worms were found nearer the anus in female hosts. Position did not differ between male and female worms though an interaction was found between parasite sex and season. The seasonal pattern was again clear though males in April were significantly more posterior in the gut than males in May (SPH test, $p = 0.034$). Males did not differ in position between May, August and November.

Maturity Status

The proportions of females at the three stages of maturity also differed significantly with season (figure 2.3.12). In April, May and November there were less females at stages 2 and 3 and more at stage 1 than expected by the null hypothesis ($\chi^2 = 267$, $df = 6$, $p \leq 0.0001$). The null hypothesis states that the female population should be comprised mostly (>60%) of females at the third stage of maturity during all months with the remainder of the population roughly divided between stages 1 and 2.

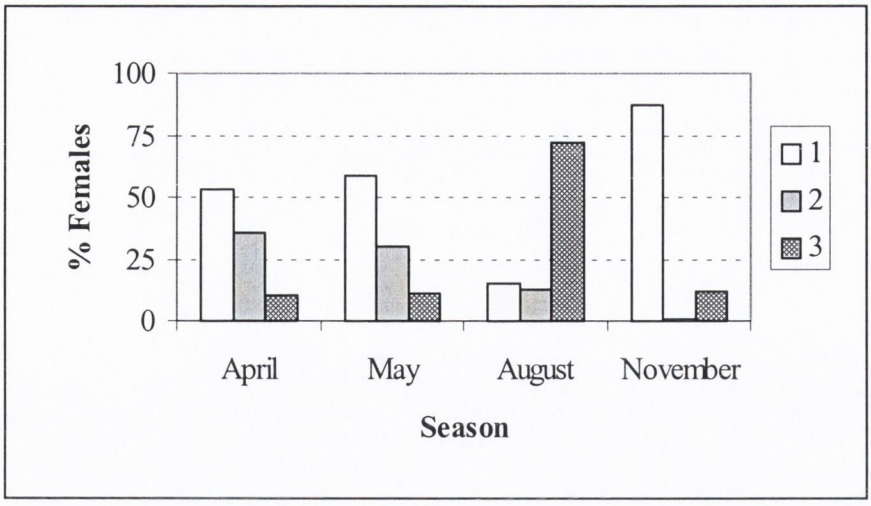


Fig. 2.3.12: Barchart showing the change in the percentage of females at each stage of maturity with season. Data from 1997 and 1998 are combined.

This is based on observations of *P. laevis* in its definitive host, chub in England (Brown, 1989; Hine and Kennedy, 1974). This situation was most pronounced in November. In August the opposite was true as the composition of the female population corresponded to that predicted by the null hypothesis.

Female maturity also differed between years (χ^2 = 8.002, df = 2, p = 0.018). The null hypothesis states that the composition of the female population should be similar between years. There were more females at stages 2 and 3 in 1997 and less stage 1 females than expected. The opposite was true for 1998. Female maturity did not differ between male and female hosts.

Table 2.3.3: Results of a 4-way ANOVA of worm length, weight and position (females only) using year, season, host sex and stage of maturity as factors.

	LENGTH			WEIGHT			POSITION		
	F-ratio	df	p	F-ratio	df	p	F-ratio	df	p
YEAR	4.3	1, 537*	NS	3.6	1, 536	NS	1.3	1, 520	NS
SEASON	25.8	3, 537	***	17.6	3, 536	***	4.9	3, 520	**
YEAR*SEASON	12.1	2, 537	***	4.3	2, 536	NS	1.4	2, 520	NS
HOST SEX	1.7	1, 537	NS	0.8	1, 536	NS	15.2	1, 520	***
YEAR*HOST SEX	2.6	1, 537	NS	0.7	1, 536	NS	10.7	1, 520	**
SEASON*HOST SEX	7.0	3, 537	***	5.5	3, 536	***	5.9	3, 520	***
MATURITY STATUS	23.1	2, 537	***	25.9	2, 536	***	0.2	2, 520	NS
YEAR*MATURITY STATUS	4.7	2, 537	**	6.8	2, 536	**	0.6	2, 520	NS
SEASON*MATURITY STATUS	0.9	6, 537	NS	1.1	6, 536	NS	1.4	6, 520	NS
HOST SEX*MATURITY STATUS	5.4	2, 537	**	4.4	2, 536	NS	0.5	2, 520	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

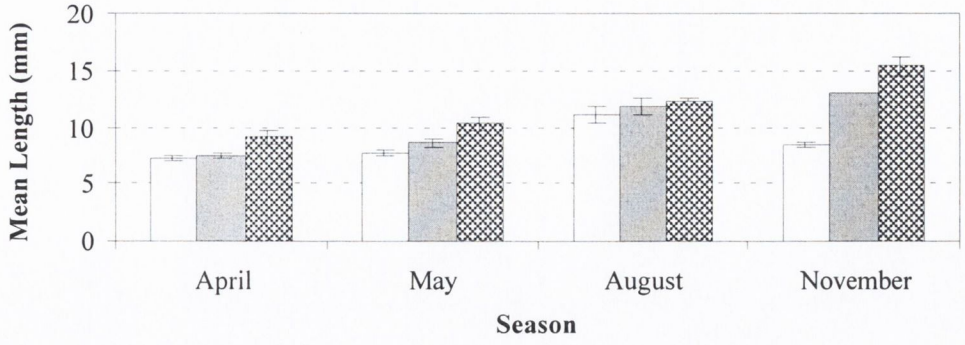


Fig. 2.3.13: The change in the mean length (± standard error) of female Irish *P. laevis* at three stages of maturity with season.

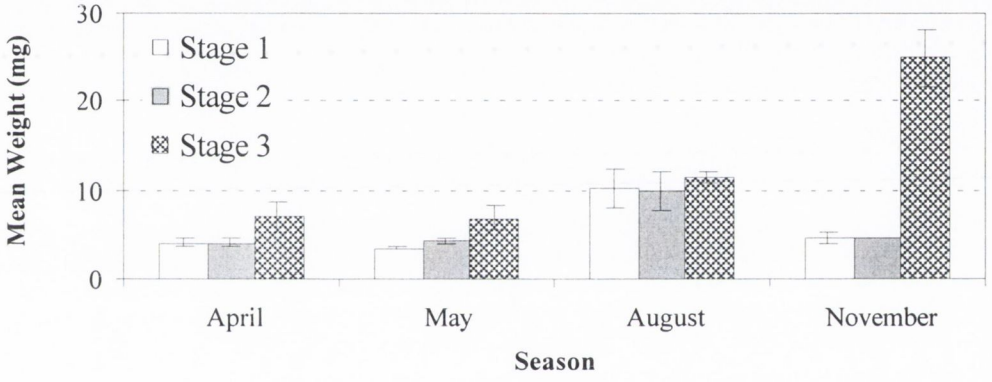


Fig. 2.3.14: The change in the mean weight (± standard error) of female Irish *P. laevis* at three stages of maturity with season.

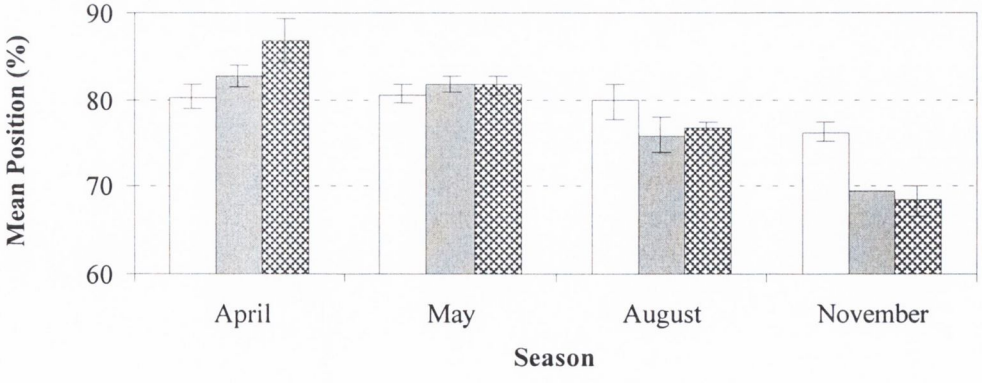


Fig. 2.3.15: The change in the mean position (± standard error) of female Irish *P. laevis* at three stages of maturity with season.

- *Length*

The effects of factors such as year, season and host sex on the length, weight and position of females were examined for differences between stages of maturity (table 2.3.3). The seasonal change in the length of females (figure 2.3.13) followed a similar pattern as that followed by the length of both males and females (figure 2.3.9). Significant differences were only found between August and April (SPH test, $p = 0.014$) and August and May (SPH test, $p \leq 0.0001$). An interaction emerged between year and season. Again this followed the same trend as length overall (males and females). Length in May and April 1997 was significantly lower than all other groups. Length did not differ between male and female hosts though an interaction was found between host sex and season. Male fish in August had significantly longer worms than females (SPH test, $p \leq 0.0001$). Female fish in November harboured longer worms than fish from both sexes in May (SPH test, Female Nov > Female May – $p = 0.017$, Female Nov > Male May – $p = 0.033$).

Female length differed significantly with stage. Stage 3 females and stage 2 females were significantly longer than stage 1 females (SPH test, 3>1 - $p \leq 0.0001$, 2 > 1 – $p = 0.028$). Females did not differ in length between the second and third stages. Significant interactions were found between year and maturity status and host sex and maturity status. Females at stage 3 in 1998 were significantly longer than those in 1997 (SPH test, 3 1998 > 3 1997 – $p = 0.012$). Stage 3 females in 1998 were also longer than stage 2 females in 1998 (SPH test, $p = 0.03$) and 1997 (SPH test, $p = 0.041$). Stage 1 females were longer in male fish than female fish (SPH test, $p = 0.01$). Only stage 2 females in male fish were longer than stage 1 females from fish of both sex (SPH test, 2 Male > 1 Male – $p = 0.044$, 2 Male > 1 Female – $p = 0.0007$). Stage 3 females were longer than stage 1 females from fish of both sex (SPH, $p \leq 0.0001$) and stage 2 females from female hosts (SPH, $p = 0.039$). Stage 3 females from male and female hosts did not differ in length from stage 2 females from male fish.

- *Weight*

Female weight (figure 2.3.14) followed a similar pattern as for males and females together (figure 2.3.10). In August females were significantly heavier than those in May (SPH test, $p \leq 0.0001$). No other differences were statistically significant. Female weight did not differ with year. Host sex alone did not affect the weight of females though an interaction was found between host sex and season. Females in

August were significantly heavier in male fish than female fish (SPH test, $p = 0.0003$). Female worms in male fish in August were significantly heavier than those from either sex in May (SPH test, $p \leq 0.001$) and those from male hosts in November (SPH test, $p = 0.03$). The maturity of the worm affected its weight as females at stage 3 were significantly heavier than those at stage 1 (SPH test, $p \leq 0.0001$) and stage 2 (SPH test, $p = 0.017$). Maturity status interacted with year in a similar manner as for female length. This interaction followed the trend of stage 3 females being heavier than those at the other stages of maturity though stage 3 females in 1998 were significantly heavier than those in 1997 (SPH test, $p = 0.007$).

- *Position*

The position of female worms (figure 2.3.15) changed with season in a similar manner to the population as a whole (figure 2.3.11). The mean position decreased significantly throughout the year (table 2.3.3). The significant decrease in position through the year is solely due to females in May being located nearer the anus than in August (SPH test, $p = 0.02$). Host sex also affected the position of females worms. Females worms were located nearer the anus in female hosts than male hosts. Host sex interacted with both year and season. Female worms were found significantly lower in the gut in female hosts in 1998 than male hosts in 1998 (SPH test, $p \leq 0.0001$), male hosts in 1997 (SPH test, $p = 0.018$) and female hosts in 1997 (SPH test, $p = 0.035$). The interaction between host sex and season revealed a significant difference in the position of females between hosts in November. Females were found in a significantly more posterior position in female hosts in November (SPH test, $p = 0.005$) and in May (SPH test, $p = 0.01$). Position did not vary with the maturity status of the female.

Host Size and Condition

No relationship was found between worm weight and host size (Pearson Product Moment correlation, male hosts = -0.06, female hosts = -0.008) and worm length and host size (PPM correlation, male hosts = 0.003, female hosts = 0.033). Gut length was used as the measure of host size. A weak positive correlation was found between worm weight and host condition (PPM correlation, male hosts = 0.272, female hosts = 0.229) and also between worm length and host condition (PPM correlation, male hosts = 0.297, female hosts = 0.38).

Intrinsic Factors

Intensity

The length and weight of males and females at the three stages of maturity, plotted against intensity are shown in figure 2.3.16 a and b. There does not seem to be any relationship between intensity and worm length nor between intensity and worm weight for either male or female *P. laevis*.

The effect of intensity on the proportion of maturing females in the population was examined in two seasons, May and August. These months were chosen as they represent the two extremes of female maturity status. In May very few females are mature, though all three maturity stages are still represented and in August, most females are mature. In figure 2.3.17 a and b the mean percentage of males, stage 1 females *etc.* are plotted against each level of intensity (standard error not shown). Again there does not seem to be any relationship between the percentage of maturing or mature females and intensity. The composition of the population differed between May and August as there was in general a higher percentage of males in the population in May. Despite this difference, the percentage of maturing or mature females only differed seasonally and fluctuated randomly with intensity.

Position

The change in composition of the population along the gut was examined in each of the four seasons (figure 2.3.18 a – d). From April to August, the niche of *P. laevis* is quite specific as the majority of worms are within the 70 to 90 % section of the gut. In August females matured throughout this preferred section. In November the niche is slightly less specific with worms having a wider distribution. Maturing and mature females therefore do not seem to have a more specific niche than the population as a whole.

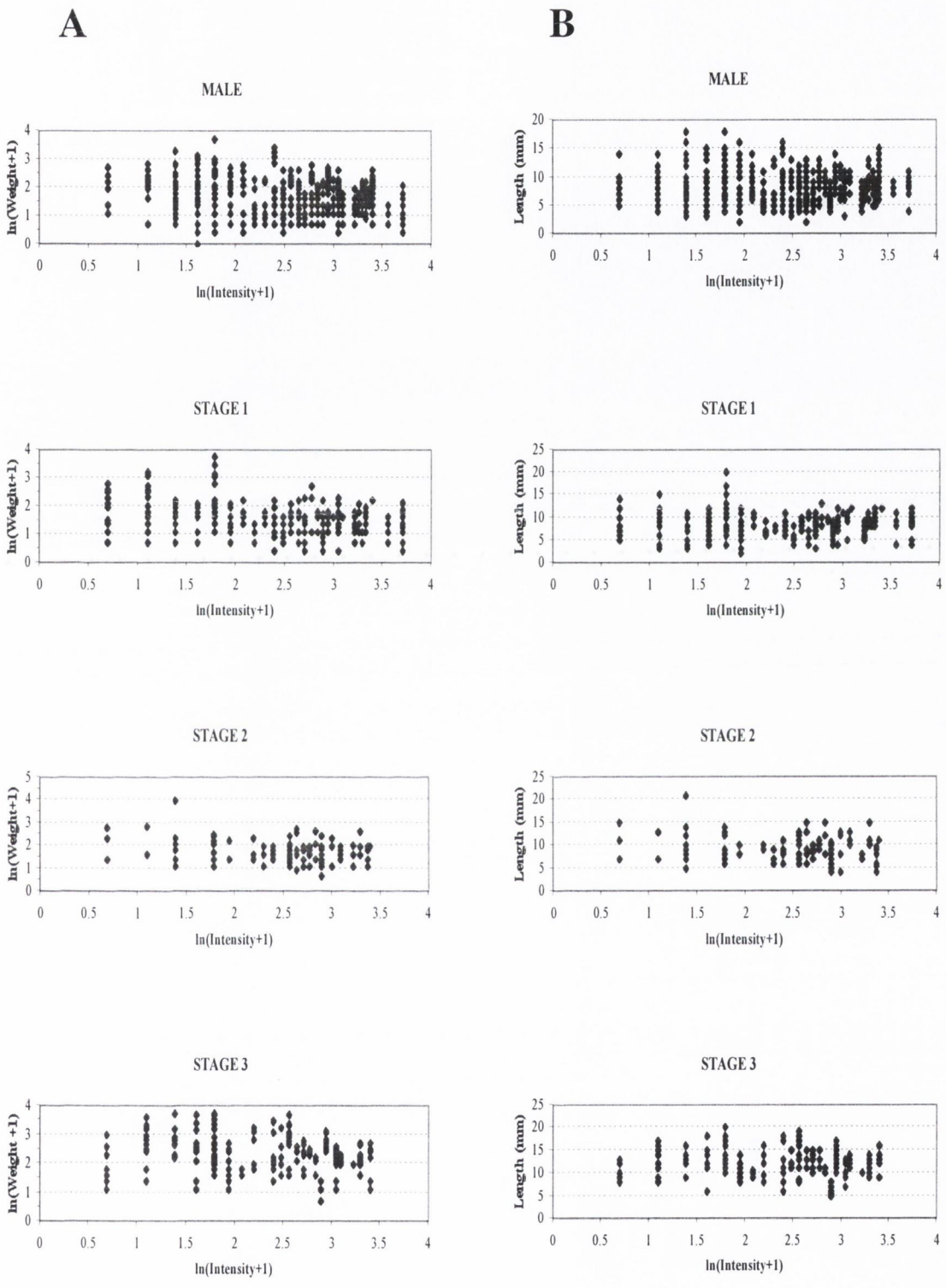


Fig. 2.3.16: Scatterplot of **A)** the weight and **B)** the length of male and female Irish *P. laevis* against intensity.

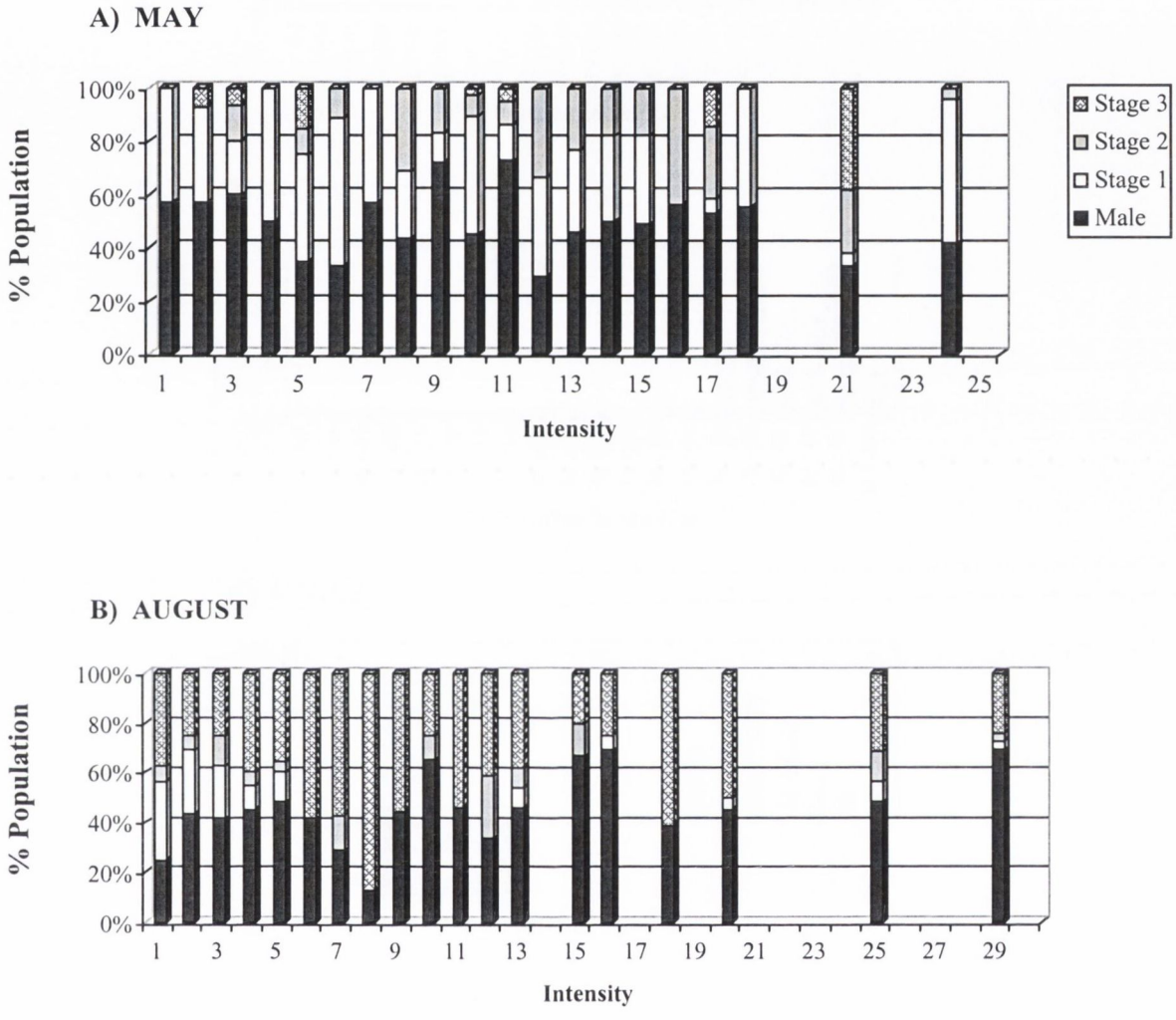


Fig. 2.3.17 Barchart showing the mean percentage composition of the *P. laevis* population at different levels of intensity in **A)** May and **B)** August (error bars not included).

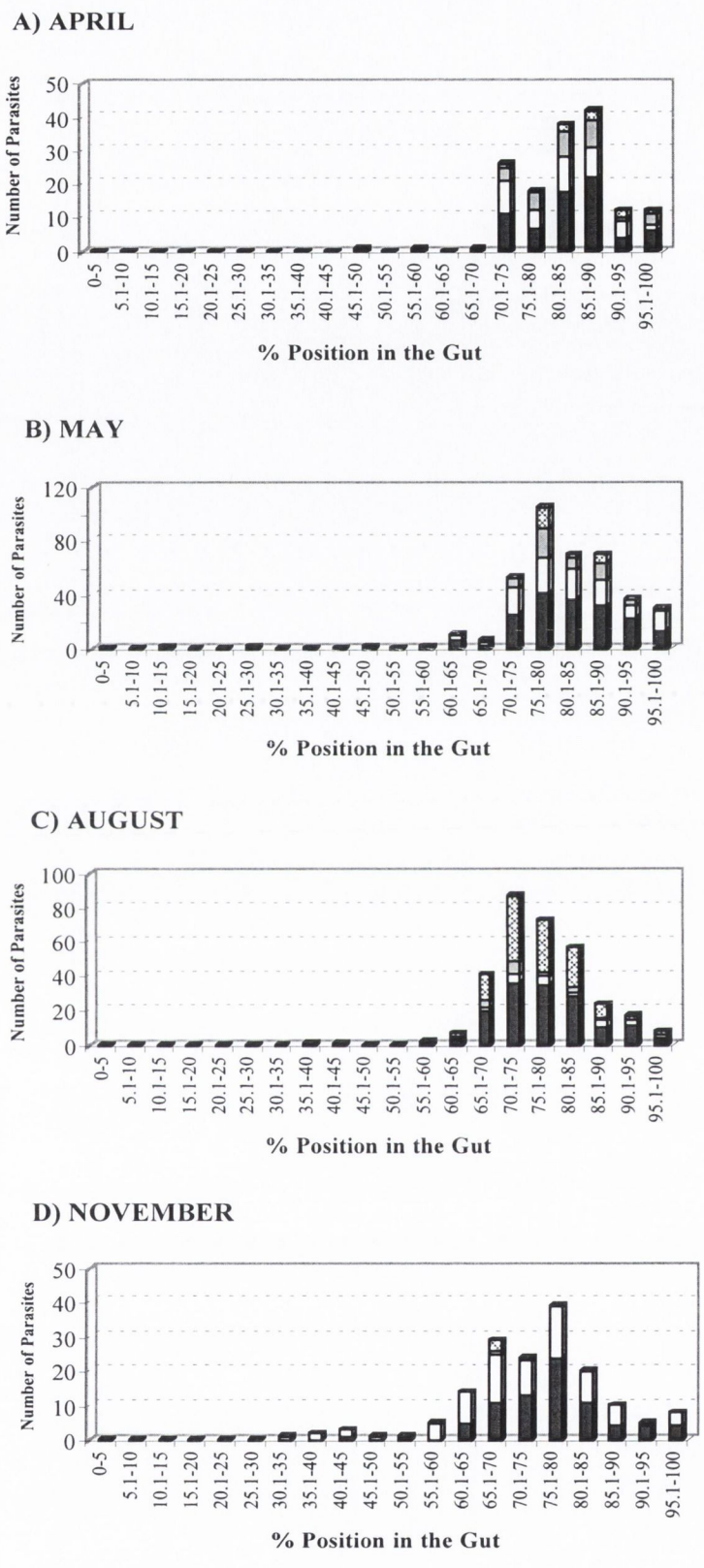


Fig. 2.3.18: Barchart showing the composition of the *P. laevis* population at different positions along the gut in **A)** April, **B)** May, **C)** August and **D)** November (error bars not included).

■ MALE □ STAGE 1 ▒ STAGE 2 ⊠ STAGE 3

2.4 DISCUSSION

Only two studies concerning the biology of *P. laevis* in Ireland have been published. Prior to these studies only distributional records were available (Kane, 1966; Kennedy, 1974). The first study revealed information on the dynamics of a community of *P. laevis* over a period of one year (Fitzgerald and Mulcahy, 1983). Changes in prevalence and intensity of *P. laevis* in trout and salmon (*Salmo salar* L.) were recorded on a monthly basis. The most recent study on the biology of *P. laevis* was carried out in L. Feeagh (Molloy *et al*, 1995). This study also included changes in prevalence and intensity of the infection of brown trout on a seasonal scale. It also contributed new information about *P. laevis* at the infracommunity level. Information on length, weight and the position of individual worms was recorded. The maturity status of females was also recorded. The present study is even more thorough as data has been recorded from every worm sampled in 1997 and 1998, rather than from subsamples. The aims of this ecological study of *P. laevis* in Ireland were two fold. The first aim of this study is to compare these results with those concerning populations of *P. laevis* from Britain and Europe. The second aim of the present study was to confirm the results reported by Molloy *et al* (1995).

Size and Position

The average length, weight and position recorded for *P. laevis* from L. Feeagh compare very well with the findings of Molloy *et al* (1995) (table 2.4.1). Females were both longer and heavier on average than males. This level of sexual dimorphism is common among acanthocephalans (Crompton, 1985). Females remain in the host longer to incubate the growing embryos, which also contributes to their larger size (Petrochenko, 1956).

Molloy *et al* (1995) recorded *P. laevis* at a mean position of 77% and within a range of 60 to 100%. This finding is fully comparable with this study. Fitzgerald and Mulcahy (1983) recorded the highest infection intensities of *P. laevis* from the 60 to 70% region. It can be concluded therefore that *P. laevis* in Ireland is found in the posterior end of the gut of brown trout.

Table 2.4.1: A comparison of the length and weight of males and females and a measure of female maturity of *P. laevis* infections of a number of hosts.

HOST	LOCATION	LENGTH	WEIGHT	% FEMALES GRAVID	REFERENCE
Brown Trout	L. Feeagh	2 – 21mm	0.5 – 51mg	10 – 75%	This thesis
Brown Trout	L. Feeagh	1 – 20mm	1 – 10mg	5 – 73%	Molloy et al, 1995
Chub	R. Severn & R. Worfe	*	20 – 46mg	60 - 72%	Brown, 1989
Chub	R. Avon	4 – 24mm	*	58 – 80%	Hine & Kennedy, 1974

* indicate missing data.

The size of *P. laevis* in L. Feeagh falls within the range recorded from chub by Hine and Kennedy (1974a) (table 2.4.1). The weight of the Irish strain is comparable to the weight of *P. laevis* from chub in the river Severn (table 2.4.1). Brown (1989) took measurements from parasites, which were not fixed as in this study and this may account in some way for the consistently higher weights recorded by him. Kennedy (1996) recorded a mean weight of 17.2 ± 15.9mg for gravid females found in brown trout in the river Otter. This value is higher than the 11.5 ± 8.4mg recorded for Irish females at the third stage of maturation, though certainly comparable.

The English strain is found in much more anterior position in the gut of its definitive hosts (Kennedy *et al*, 1976). Position in the gut can be explained in terms of how the cystacanth is liberated and activated (Kennedy *et al*, 1978). In cyprinids, these processes can occur more anteriorly in the intestine due to the presence of pharyngeal teeth, which facilitate the earlier liberation of the parasite than in salmonids. Differences in the availability of nutrients along the gut may affect the ability of *P. laevis* to grow to its maximum size. The posterior position of the Irish strain may reduce the nutrients available to it, as its salmonid host will have more time to absorb nutrients. Though why newly infected parasites are lost anteriorly over winter is then difficult to explain (see discussion of seasonal effects on position).

Maturity Status

Overall the percentage of females at different stages of maturity was again similar to that reported by Molloy *et al* (1995) (table 2.4.1). From 510 females

examined, 60% of them were at stage1, 15.7% were at stage 2 and the remaining 24.3% were at the third stage of maturation. The number of Irish females at stage 3 is very low when compared with the English strain. In chub in the river Avon gravid females were found at all times of year (Hine and Kennedy, 1974b). In chub in the river Severn a similarly high percentage of females were fully mature (Brown, 1989).

In this study stage 3 females were significantly heavier and longer than females at the other stages of maturity. Molloy *et al* (1995) suggested that the size of the parasite influenced its maturity status. Brown (1989) found that size was only related to age and he stated that size had no bearing on the maturation of *P. laevis*. He found immature females, grown experimentally in rainbow trout, of similar age and size to fully mature females. Findings made in the present study support Brown's observation. Stage 3 females continued to increase in size in November (figure 2.3.14). Most mature females are lost between August and November but those which were not, grew to a larger size. Also in August, the weight of all females at each of the three stages increased to the same degree. Therefore age determines size independently of maturity status.

Statistically significant differences in the positions of females at different stages of maturity were found. Only a 5% difference was found between the mean positions of stage 1 and stage 2 females. Therefore it is doubtful if this level of difference has any significant biological meaning. Stage 3 females might be found slightly more anteriorly in the gut than the other females due to loss of old females, which generally happens from the posterior end. Likewise stage 1 females may be higher in the gut if they were recently established.

Extrinsic Factors

Three factors year, season and host sex were examined for influences on the intensity, size, position and maturity status of Irish *P. laevis*. Season was undoubtedly the most important factor and will be discussed first.

Season

Season was found to be the main cause of fluctuations in intensity, size, position and maturity status observed in Irish *P. laevis*. The size of all worms and the gravidity

of females peaked dramatically in August. Intensity fluctuated throughout the year but a peak was found in May. The mean position of *P. laevis* became more anterior throughout the year. The observations reported here are the same as those reported by Molloy *et al* (1995). Prevalence was low in spring and high throughout summer. Intensity also peaked in spring. Molloy *et al* (1995) also reported significant increases in the size of worms and the proportion of gravid females during the summer. These summer peaks contrast with the stability of the English strain and European populations. In definitive hosts in Britain and Europe, prevalence and intensity *P. laevis* remain high and constant throughout the year (Hine and Kennedy, 1974a; Rumpus and Kennedy, 1977; Brown, 1989; Moravec and Scholtz, 1991; Moravec *et al*, 1997). Heavy worms are found throughout the year in the rivers Severn and Avon and most females are gravid (Hine and Kennedy, 1974b; Brown, 1989).

Studies have revealed the stability of populations of the English strain over time to be a dynamic equilibrium between recruitment of new individuals and the mortality of the old ones (Kennedy, 1972; Brown, 1989). In Britain and Europe, different generations of *P. laevis* overlap. Brown (1989) commented on observing very small and very big individuals all through the year. This is not the case in Ireland as there clearly seems to be just one generation per year. New individuals are probably recruited in November as this month corresponds to an increase in intensity, a huge reduction in the average size of individuals, a peak in the number of immature females and a more anterior position in the gut. When worms are recruited, they are positioned throughout the length of the gut and this causes a decrease in the average position of the population. In April intensity is lower and position has become more posterior. This may indicate the loss of young parasites unable to establish successfully. Parasites have not grown but there is a small proportion of stage 3 females. There is little change seen between April and May apart from a slight increase in size. Everything changes between May and August. Worms increase in size. Most of the females are gravid at this time. The decrease in intensity and mean position suggest a loss of old parasites from the posterior end of the gut. Acanthors are probably shed throughout the summer months and infect the intermediate host at this time.

Earlier, in the section entitled maturity status it was stated that when compared directly to the English strain, a relatively small proportion of Irish females are fully mature. If season is now taken into account, in the summer almost 75% of Irish females are gravid. This figure compares well with those quoted earlier for *P. laevis* from the

rivers Avon and Severn. It also compares well with the proportion of females found to be gravid in brown trout in the river Otter which was 71% (Kennedy, 1996).

How does season influence the population dynamics of Irish *P. laevis*? Temperature changes with season. Temperature affects both the parasite and its hosts (Chubb, 1982). Brown (1989) showed experimentally that *P. laevis* does not grow very much at low temperatures and can survive for long periods of time in this state. The Irish strain does not seem to grow at all between November and April. This lack of growth is most likely mediated by changes in the definitive host. Brown trout must feed very little in winter. The intermediate host is probably also affected by temperature. Fitzgerald (1987) observed only one generation of *G. duebeni* per year in the river Shournagh. This contrasted with the ability of *G. duebeni* in parts of Britain to have two generations per year. Fitzgerald (1987) found that *G. duebeni* reproduced in the winter months and produced the next generation throughout the following spring. From spring onwards therefore, young susceptible gammarids were available for infection by *P. laevis*. The same yearly cycle has also been determined for *G. duebeni* in a stream in Dublin (McLoughlin, 2001). The effect of temperature on the population dynamics of the intermediate host could easily have an effect on *P. laevis* as the parasite is fully reliant on this host for transmission to the definitive host.

Year and Host Sex

Year and host sex were not expected to affect the population biology of Irish *P. laevis*. The host sampled were very young, mostly 1+ years old and sexually immature. The weight of male and female *P. laevis* was greater in 1998. This may be explicable by the dates on which fish were sampled between years. Obviously it would be preferable to carry out sampling at the same date each year in order to facilitate a comparison between years. It was not possible logistically to do this and samples were taken in 1998 as close to the corresponding date in 1997 as possible. Fish were sampled from the 7th to the 13th of August in 1997 and from the 21st to the 28th in 1998. Fish eat more and metabolise faster in the summer and *P. laevis* also grows and matures faster at higher temperatures (Kennedy, 1972; Brown, 1989). An extra two weeks of growth in the summer of 1998 may have been enough to result in heavier worms in 1998. The effect of any interaction between year and another factor may also be explained in this manner.

More females were found at stage 3 of maturation in 1997. This probably reflects random fluctuations in the exact composition of female *P. laevis*. Position was unaffected by year alone, but an interaction between year and host sex did have an effect. Worms were found more posteriorly attached in female fish in 1998. The biological significance of this is unclear. This difference might be based on purely stochastic changes.

Host sex alone seemed to affect the position of *P. laevis*. Worms seem to be located in a more posterior position in female hosts. The more anterior position in males might mean that *P. laevis* finds it easier to establish in male brown trout or that worms are lost first from males. The adaptive significance of this is unclear.

Host size and Condition

Neither the length nor weight of Irish *P. laevis* was found to be influenced by the size of the host. The smallest gut may provide sufficient space for *P. laevis* to fulfill its growth potential or the hosts sampled did not differ in size enough to elicit a difference in *P. laevis*. Host condition was weakly correlated with an increase in the size of *P. laevis*. This relationship may be affected by season. Fish eat more in the summer and presumably put on weight, which benefits their overall condition. Since *P. laevis* also grows in the summer, the relationship may be dependent on both the host and the season.

Intrinsic Factors

No relationship was found between the size and maturation of *P. laevis* and the intensity of infection. Kennedy (1974) showed density independent growth in an experimental infection of goldfish by *P. laevis*. Bates and Kennedy (1991b) stated that though establishment may be affected at high densities, growth and maturation of established worms remains unaffected. They theorised that the continuing availability of new niches due to the growth of the host was responsible.

Mature females were found in a section of the gut from 70 to 90%. This is obviously the preferred site of Irish *P. laevis* in brown trout. Maybe females do not mature outside of this site due to low population densities there.

Conclusions

P. laevis cycles through different definitive and intermediate hosts in Ireland than in Britain and continental Europe. It exhibits a distinct seasonal cycle in its growth and maturation. *P. laevis* is found in the posterior end of the gut of brown trout in Ireland. Though on average lengths and weights of Irish *P. laevis* are lower than those of their English counterparts, when season is adjusted for, length and weight become comparable between the two strains. In summer the ability of Irish *P. laevis* to grow and mature provides clear evidence of the suitability of brown trout as a host. The weight of gravid Irish females in summer was lower compared to that of gravid females from brown trout from the river Otter (Kennedy, 1996). The shorter growing season in Ireland must be responsible for this discrepancy as the host species is the same. With further research on host diet and the relationship between *P. laevis* and its intermediate host in L. Feeagh, it is possible that the seasonal dynamics can be explained in ecological terms. Seasonality in the growth and reproduction of *P. laevis* in dace in Rriver Avon was seen as evidence for the lack of suitability of this host (Hine and Kennedy, 1974b). This is not the case for brown trout in Ireland. The ecological evidence certainly supports the existence of a different strain of *P. laevis* in Ireland. This may answer the question of why *P. laevis* is not found more regularly in brown trout elsewhere.

CHAPTER 3

A STUDY OF THE MORPHOLOGICAL VARIATION WITHIN AN IRISH AND ENGLISH POPULATION OF *POMPHORHYNCHUS LAEVIS*

3.1 INTRODUCTION

No previous work has succeeded in identifying the three proposed strains of *P. laevis* on a morphological basis. Such is the phenotypic plasticity exhibited by populations of *P. laevis* that some researchers have proposed the splitting of the species into various subspecies (Engelbrecht, 1957) and even into different species. Some parasitologists thought the marine strain to be so different that a new species, *P. tereticollis* was erected (Petrochenko, 1956). This morphological classification was based on size and shape differences between the anterior and posterior hooks. In *P. tereticollis* the anterior and posterior hooks are much more alike than in *P. laevis*. Such a slight difference did not prove useful when identifying new populations of the marine strain and so *P. tereticollis* is not recognised as a distinct species by all acanthocephalan taxonomists (Petrochenko, 1956; Gibson, 1972).

It has been suggested that there may be some genetic exchange between marine and freshwater strains due to the frequent observations of flounder, the preferred definitive host of the marine strain, in rivers infected with freshwater *P. laevis* (Kennedy *et al*, 1989). Gene flow therefore may be preventing the morphological divergence of the marine and freshwater strains. A large degree of variation in the morphology of *P. laevis* even within a single population has been uncovered (Brown, 1987). Such variability has hindered the classification of *P. laevis* and its strains.

The morphology of a wild Irish and a wild English population of *P. laevis* have never been compared. The aim of this chapter is to study in detail the morphological variability of both populations. Factors already identified as causing morphological variation such as the sex of the parasite, the size of the parasite and the season in which parasites are sampled will be examined. Characters measured will be traditional ones such as hook number, proboscis size *etc.* so as to aid comparison with previous studies. Two studies conducted by A.F. Brown (1987) and P.A. Edwards (Unpublished) will be used for direct comparisons as these studies represent the only carried out on *P. laevis* to date. The study conducted by Edwards remains unpublished. It was carried out at Exeter University in the late 1980's under the supervision of Professor Clive Kennedy. The study was a detailed one and involved a comparison of the morphology of experimentally grown English and Irish *P. laevis*. Rainbow trout (*Oncorhynchus mykiss*) were infected in the laboratory with *P. laevis* cystacanths and the subsequent infections were followed over a 200 day period.

3.2 MATERIALS AND METHODS

Collection

Irish *Pomphorhynchus laevis* included in the morphological study were collected as part of the sampling effort for the ecological study (please refer to section 2.2 in chapter 2). 76 females and 64 males from brown trout (*Salmo trutta*) were chosen from three of the sampling months (plate 3.2.1). 31 worms were sampled in November 1997, 65 in May 1998 and 44 in August 1998. These were all collected from the same catchment, L. Feeagh in Co. Mayo. English *P. laevis* were sampled from chub (*Leuciscus cephalus*), a preferred definitive host, which were caught in the river Avon, Hampshire, England (figure 3.2.1). Chub were sampled in August 2000. 44 female worms and 39 male worms were included in the study.

Preparation

Both fish and worms from both locations were treated in the same manner as those included in the ecological study (please refer to section 2.2 in chapter 2). Irish worms were fixed in 10% formalin for 24 to 48 hours after their removal from the gut. Worms were then removed from the fixative, washed in 75% ethanol and stored in 75% ethanol and 4% glycerol. The English worms were also fixed in the aforementioned manner but while they were still attached to the gut. It was difficult to remove fixed worms intact from the gut due to the brittleness induced by the fixative and this is reflected in the smaller sample size for English *P. laevis*.

The specimens included in the study were chosen based on criteria outlined by Huffman and Bullock (1975). Only worms with a fully everted proboscis and displaying one longitudinal row in profile with all hooks intact were used. Worms were prepared by clearing in lactophenol for a duration of between one and two hours depending on the size and opacity of the worms. They were then mounted temporarily on a slide and held in place under a coverslip with some clear nail varnish.

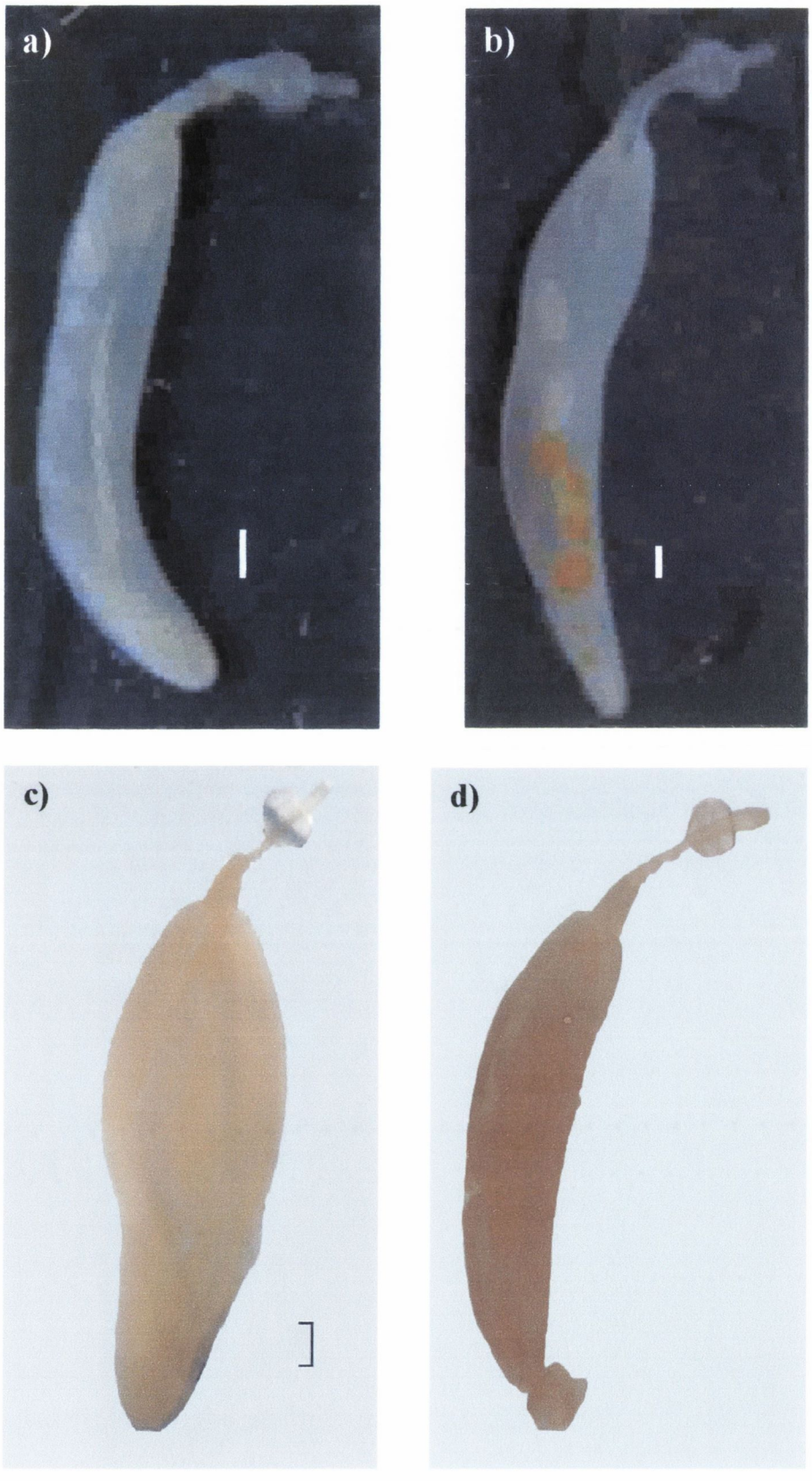


Plate 3.2.1 : a) Female and **b)** male Irish *P. laevis*. **c)** Female and **d)** male English *P. laevis*. Scale bars equal to 1mm.

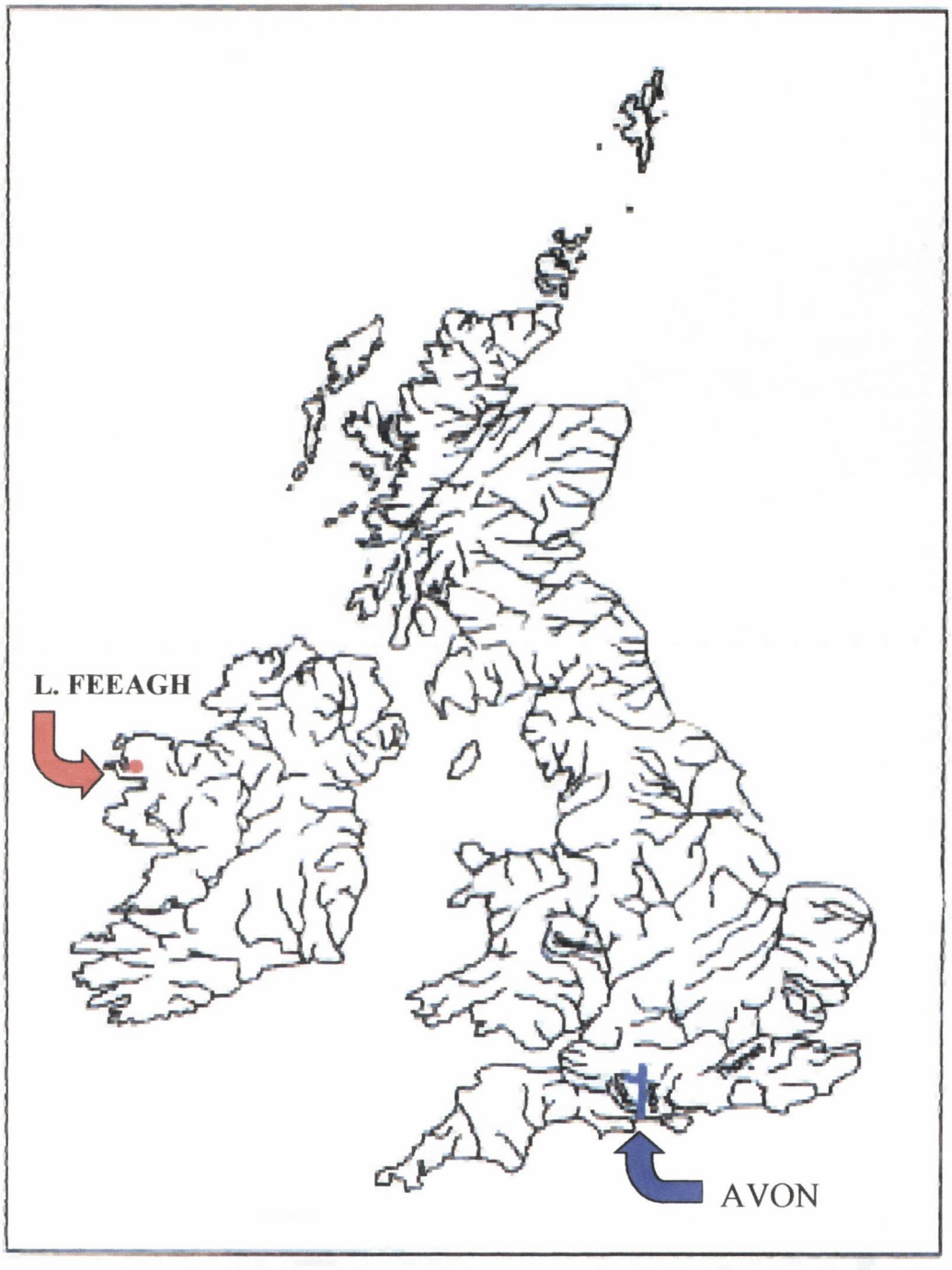


Fig. 3.2.1 : Location of the Irish and English populations of *P. laevis* sampled for the morphological study. Adapted from Kennedy *et al*, 1989.

Characters and how they were measured

The characters measured and recorded are listed in table 3.2.1. The number of hooks per row can alternate by one between neighbouring longitudinal rows. In order to simplify matters the number of hooks per row recorded for each worm is the number counted from the longitudinal row under scrutiny. Some derived characters were also used. Shape of the non-hook characters is simply the width of the character divided by the length. Hook area and hook shape were derived from the length and width of the hook (Huffman and Bullock, 1975). Hook area is (length x width) / 2, which is based on the area of a triangle. Hook shape, which better represents the stoutness of a hook, is simply width/length. Another derived character described in chapter 4 is anterior /posterior ratio. Figure 3.2.2 shows the location of non-hook characters on the worm.

Table 3.2.1: Characters measured and recorded on each individual *P. laevis*.

NON-HOOK CHARACTERS	DERIVED CHARACTERS
Total Length (mm)	Body Shape
Trunk Length and Width (mm)	Neck Shape
Neck Length (mm) and Width (μm)	Bulb Shape
Bulb Length and Width (μm)	Proboscis Shape
Proboscis Length and Width (μm)	Anterior /Posterior Ratio
HOOK CHARACTERS	Hook Area and Shape (for all n hooks)
Number of Hooks per Longitudinal Row, n	
Number of Anterior and Posterior Hooks	
Number of Longitudinal Rows	
Hook Position in the row (for all n hooks)	
Length and Width (μm) (for all n hooks)	

Hook Width: The starting point of the line along which hook width was measured is the point of inflexion of the inner surface (edge nearest the proboscis) of the hook (figure 3.2.3 a). To find the direction of the line along which the measurement was made, a line was drawn through the root of that hook, along its longer axis. The hook width was then measured along a line, starting at the point of inflexion of the inner surface, running parallel to the longer axis of the hook root and terminating at the point on the outer surface of the hook, through which the line runs.

Hook Length: The starting point of the line along which hook length was measured is the tip of the hook (figure 3.2.3 b). The line follows the direction of the inner edge of the hook and continues towards the outer surface of the hook to the point where it meets the root, which is the termination point of the line. If the line, as it runs parallel to the inner edge of the hook, does not bisect the termination point directly, then to find the point, a further instruction was followed. A line was drawn perpendicularly from the desired termination point toward the line of measurement. The intersection point of these two lines is the new termination point.

Measurements were made with the aid of a digital camera and computer software. A Nikon Coolpix 900 attached to a Nikon phase contrast microscope was used to photograph the characters in question for each worm. The images were then uploaded onto an IMAC computer. A public domain image analysis package, NIH

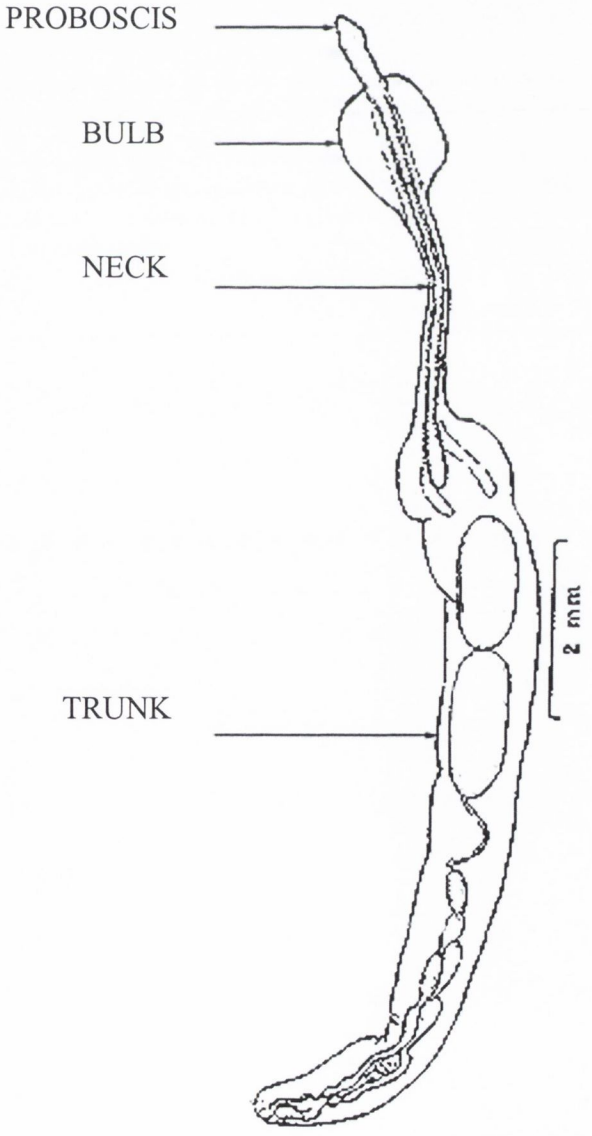


Fig. 3.2.2: Diagram of male *P. laevis* showing the probosics, bulb, neck and trunk. Adapted from Cordonnier and Ward, 1967.

Image v6.2 was used to digitally measure the characters from the images digitally. The software was calibrated to make 'real' measurements by using images of a slide micrometer taken at magnifications of 10 times, 20 times and 40 times and translating the number of pixels into micrometers (μm).

Hooks chosen for comparison

It was initially intended to compare all the characters measured between the samples. This proved to be difficult to achieve with the hooks. As described by Petrochenko (1956) the hooks on the proboscis of *P. laevis* change in appearance as one moves from the top to the bottom of the proboscis nearest the bulb. The first hooks are known as the anterior hooks and these are generally the longest hooks on the proboscis (plates 3.2.2 and 3.2.3). The number of anterior hooks varies between individuals. The last anterior hook is the stoutest hook, short in length but quite wide, and this hook is always found near the middle of the row. The posterior hooks are very different from the anterior hooks and therefore it is very easy to distinguish between the two sets of

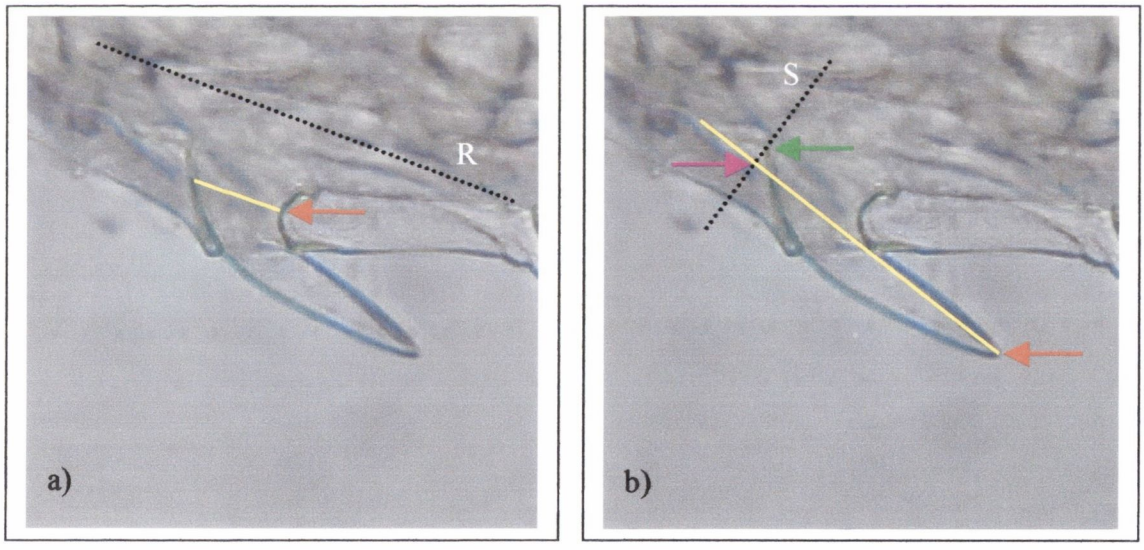


Fig 3.2.3: a) The orange arrow shows the starting point for the measurement of hook width. The yellow line is hook width, which runs parallel to the hook root axis shown by the line labelled R. **b)** The orange arrows shows the starting point for the measurement of hook length. The yellow line is hook length, which runs parallel to the inner edge of the hook. The green arrow shows the point where the hook meets the root and the pink arrow shows the end of the line for measurement, where it intersects with the line labelled S.

hooks. The posterior hooks are much smaller in length and very narrow and they may also have a less well developed root than the anterior hooks. The last hook is known as the basal hook. This is longer than the posterior hooks, almost on the scale of an anterior hook, though not quite as wide or long. This hook protrudes through the top of the bulb, which makes this hook the most difficult to measure accurately.

More importantly though, the number of hooks per row differs substantially between worms and this makes it impossible to compare the dimensions of each hook between all individuals. For example, how does one compare the tenth hook between all worms, if for a worm with 9 hooks there is no such hook or for a worm with 10 hooks it is the basal hook, whereas in a worm with 12 hooks it is an ordinary posterior hook. Various methods could have been used to overcome the problem. It was finally decided to include only those hooks which were common to all worms regardless of their number of hooks per row. These hooks are the first three anterior hooks, the stoutest hook, three of the posterior hooks, 7, 8 and 9 (excluded for worms with only 9 hooks) and the basal hook. This course of action was chosen for its simplicity and because the maximum amount of variation was retained.

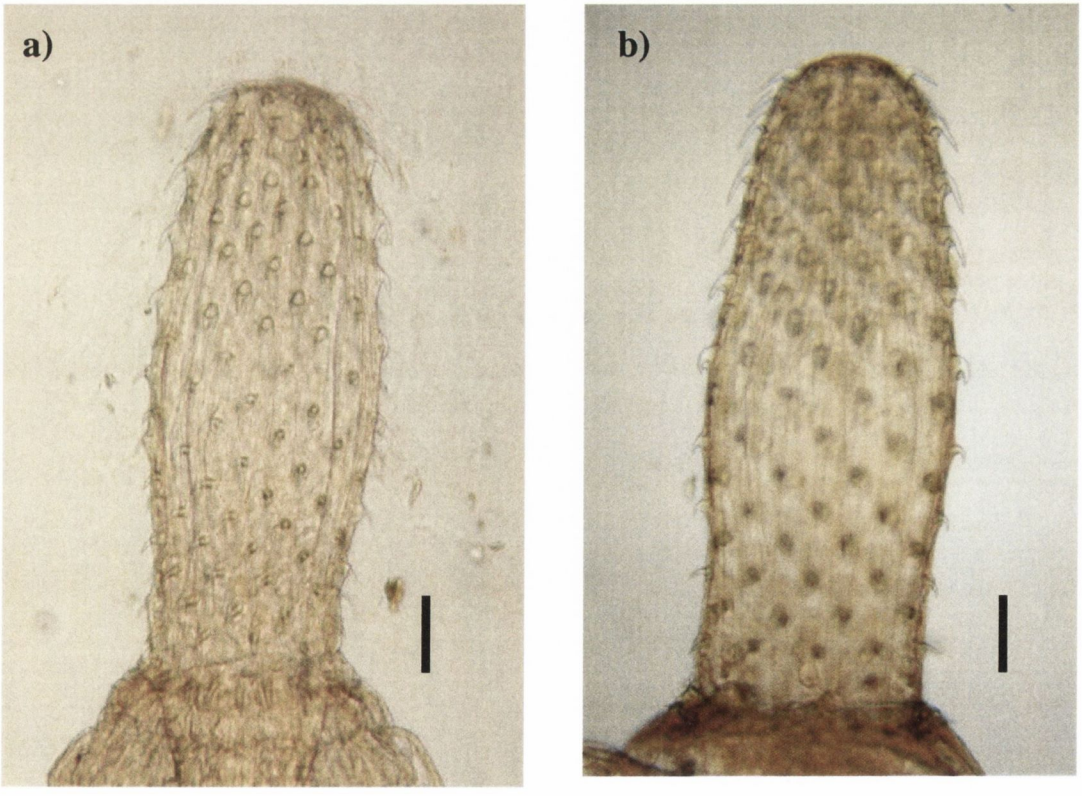


Plate 3.2.2: Typical probosces of *P. laevis* with 11 hooks per row from a) Co. Mayo, Ireland and b) Hampshire, England. Scale bar equals 100 μm.

Scale bars equal 100 μm.

Statistical Analysis

All analyses were carried out on Data Desk version 6.0. Three different levels of probability are referred to in the results. * is equivalent to $p < 0.05$, ** is equivalent to $p < 0.01$ and *** is equivalent to $p < 0.001$. Some of the characters in the Irish population were non-normally distributed *i.e.* total length, trunk length, trunk width, body shape, neck shape and bulb shape. These variables were all $\log_{10}$-transformed prior to statistical analysis. The coefficient of variation was used to examine the variation within the non-hook characters. This measure is the standard deviation divided by the mean, multiplied by 100. The coefficient increases with an increase in variability.

Continuous variables such as the measurements of the non-hook characters, hook length, hook width, hook area and hook shape, were compared between males and females using a student t-test. Two-way analysis of variance (general linear model) was used to compare characters between season and parasite sex, hook number and parasite sex and country and parasite sex. In order to ascertain the relationship between two continuous variables such as total worm length and proboscis length, linear regression was carried out. Chi-squared analysis was used when comparing categorical data *i.e.* hook number, number of rows of hooks, positions of longest and stoutest hooks. In some situations, certain categories were omitted from the analysis due to small cell size *e.g.* worms with 13, 18 and 19 rows of hooks were not included when comparing row number between Irish males and females because the number of observations was less than 2.

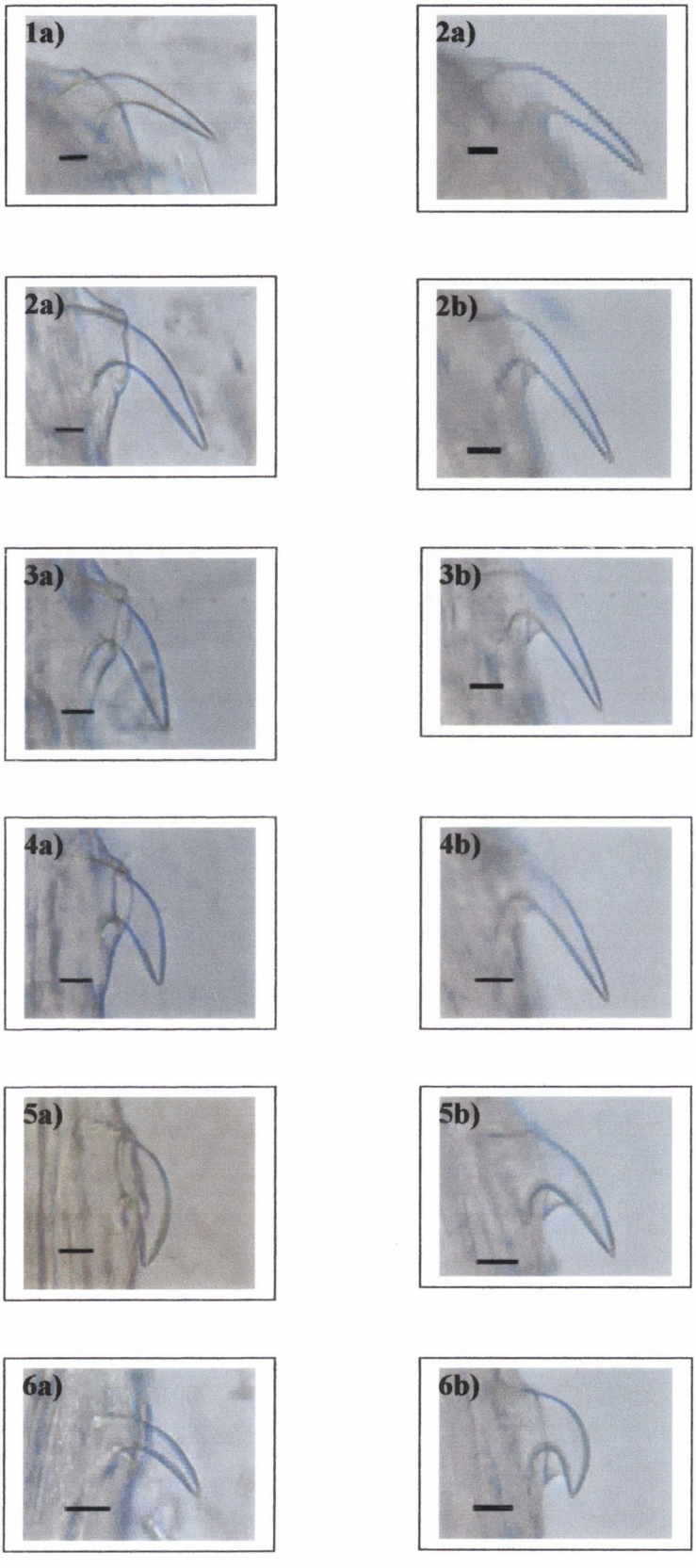


Plate 3.2.3 (1 – 6): The first six hooks on the proboscis (shown in plate 2) of *P. laevis* from a) Ireland and b) England. Scale bar equals 10 μm.

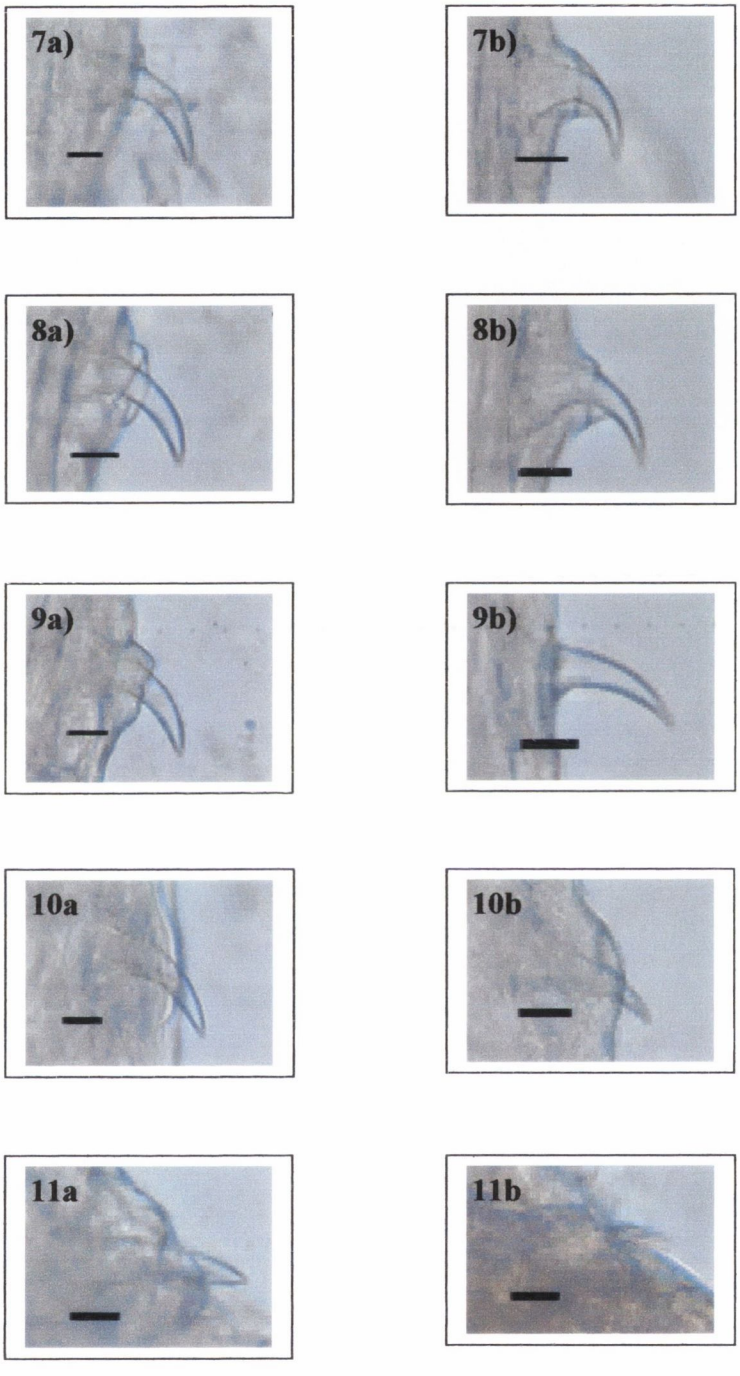


Plate 3.2.3 (7 – 11): The last five hooks on the proboscis (shown in plate 2) of *P. laevis* from a) Ireland and b) England. Scale bar equals 10 μm.

3.3 RESULTS

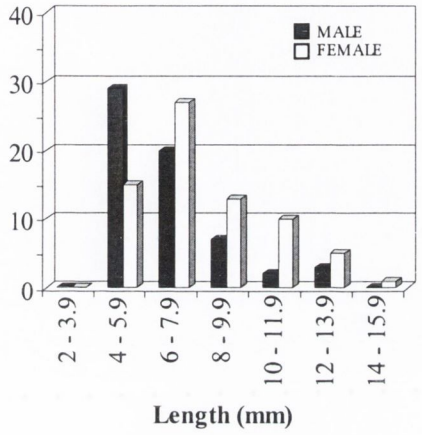
Non-Hook Characters

Distribution and Variability

The length and weight of the Irish sample are represented in figure 3.3.1. Irish *P. laevis* has a mean length of 10.9 ± 2.84 mm and a mean weight of 7.8 ± 7.73 mg. Both variables are skewed to the right, though weight shows a more extreme distribution (skewness = 2.6, kurtosis = 9.3). Figure 3.3.2 shows the corresponding length and weight distributions of the English sample of *P. laevis*. English *P. laevis* has a mean length of 12.56 ± 2.91 mm and a mean weight of 15.7 ± 11.97 mg. Weight is skewed (skewness = 1.56, kurtosis = 1.96) though in contrast to the Irish sample length is normally distributed. The remaining non-hook characters of Irish *P. laevis* are shown in figures 3.3.3 and 3.3.4. Skewness, kurtosis and other statistics pertaining to both populations are presented in tables 3.3.1 and 3.3.2. Trunk length and trunk width are non-normally distributed showing a slight skew to the right as did length. The length and width of the neck, bulb and proboscis are normally distributed. The shape of the body, neck and bulb also show a slight skew to the right with proboscis shape displaying a very peaked normal distribution. Figures 3.3.5 and 3.3.6 depict the remaining non-hook characters of English *P. laevis*. The lengths and widths of the English characters mostly exhibit a normal distribution with the exception of bulb shape.

The coefficients of variation for non-hook characters are generally greater for Irish *P. laevis* (tables 3.3.1 and 3.3.2). In both populations, trunk length and width show the highest levels of variability exhibited by the non-hook characters. Bulb width shows the second greatest amount of variation in the Irish sample, though not in the English sample. The coefficients of variation of total length, neck length and width and bulb length fall between 20% and 29% for both samples. The lowest levels of variation are shown by proboscis length and width. The variability of these English characters seems to be lower than that of the Irish characters.

a)



b)

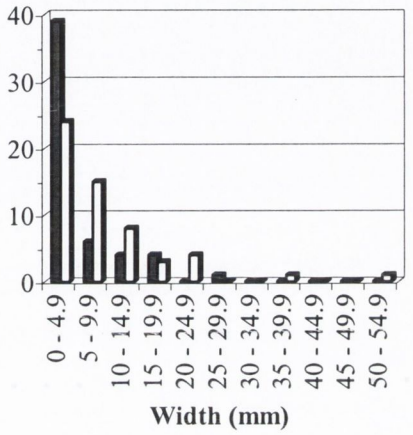
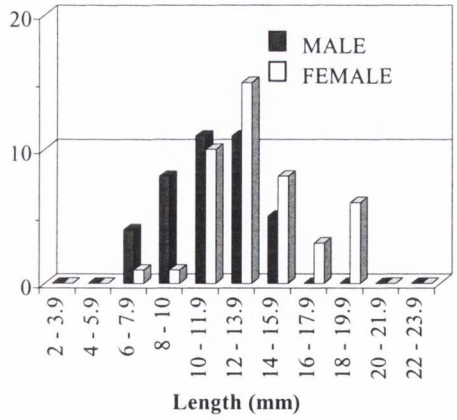


Fig. 3.3.1: Frequency distribution of **a)** total length and **b)** weight for male and female *P. laevis* of Irish origin.

a)



b)

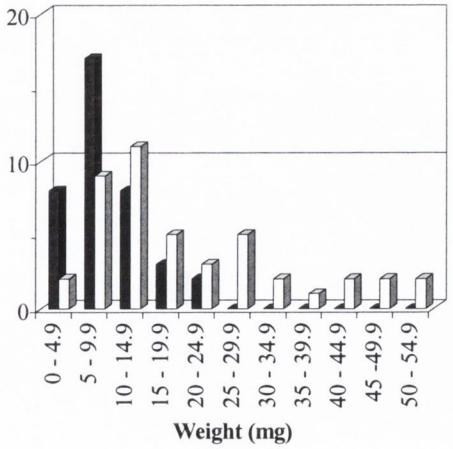


Fig. 3.3.2: Frequency distribution of **a)** total length and **b)** weight for male and female *P. laevis* of English origin.

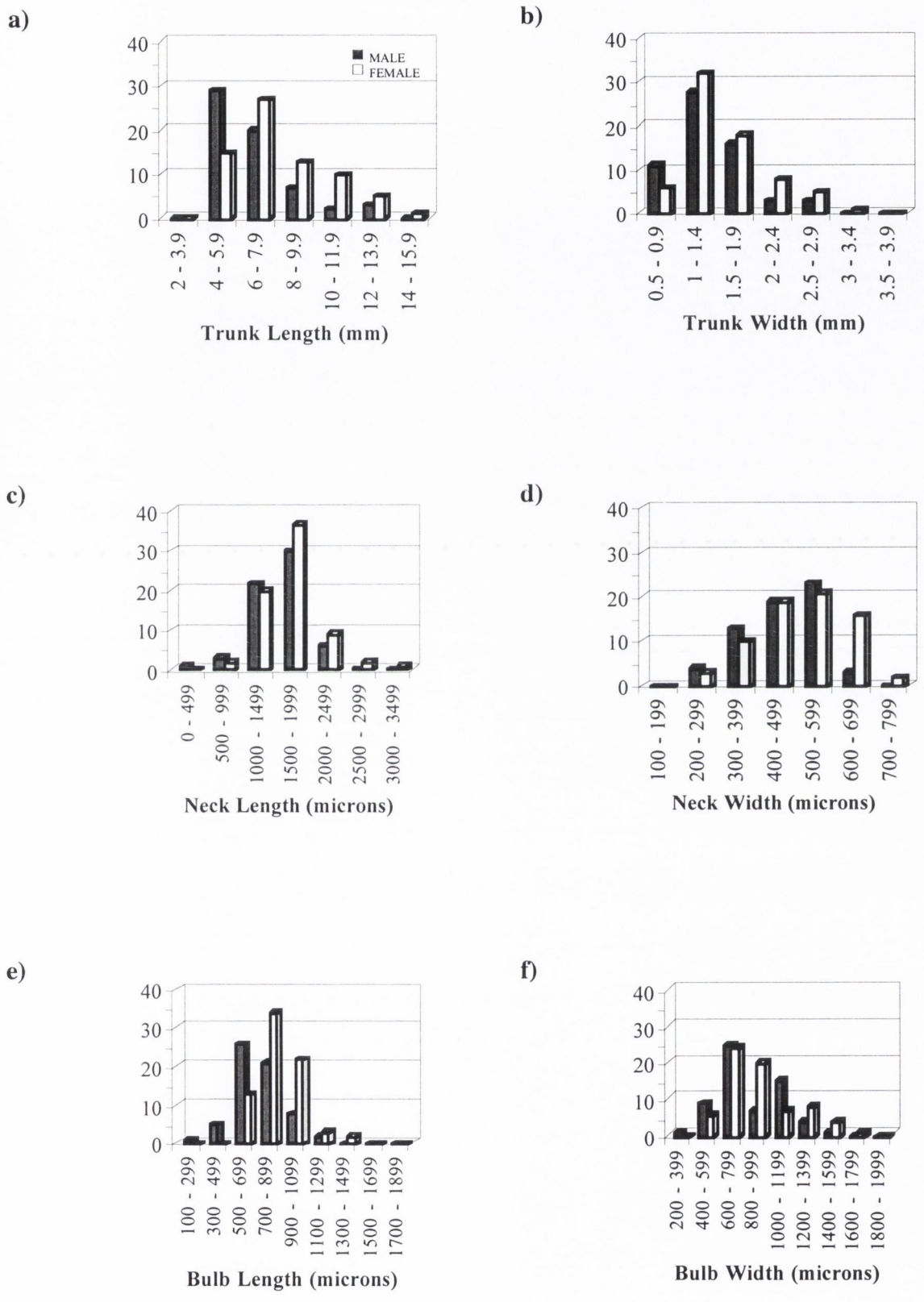


Fig. 3.3.3(a -f) Frequency distribution of trunk length, trunk width, neck length, neck width, bulb length and bulb width for male and female *P. laevis* of Irish origin.

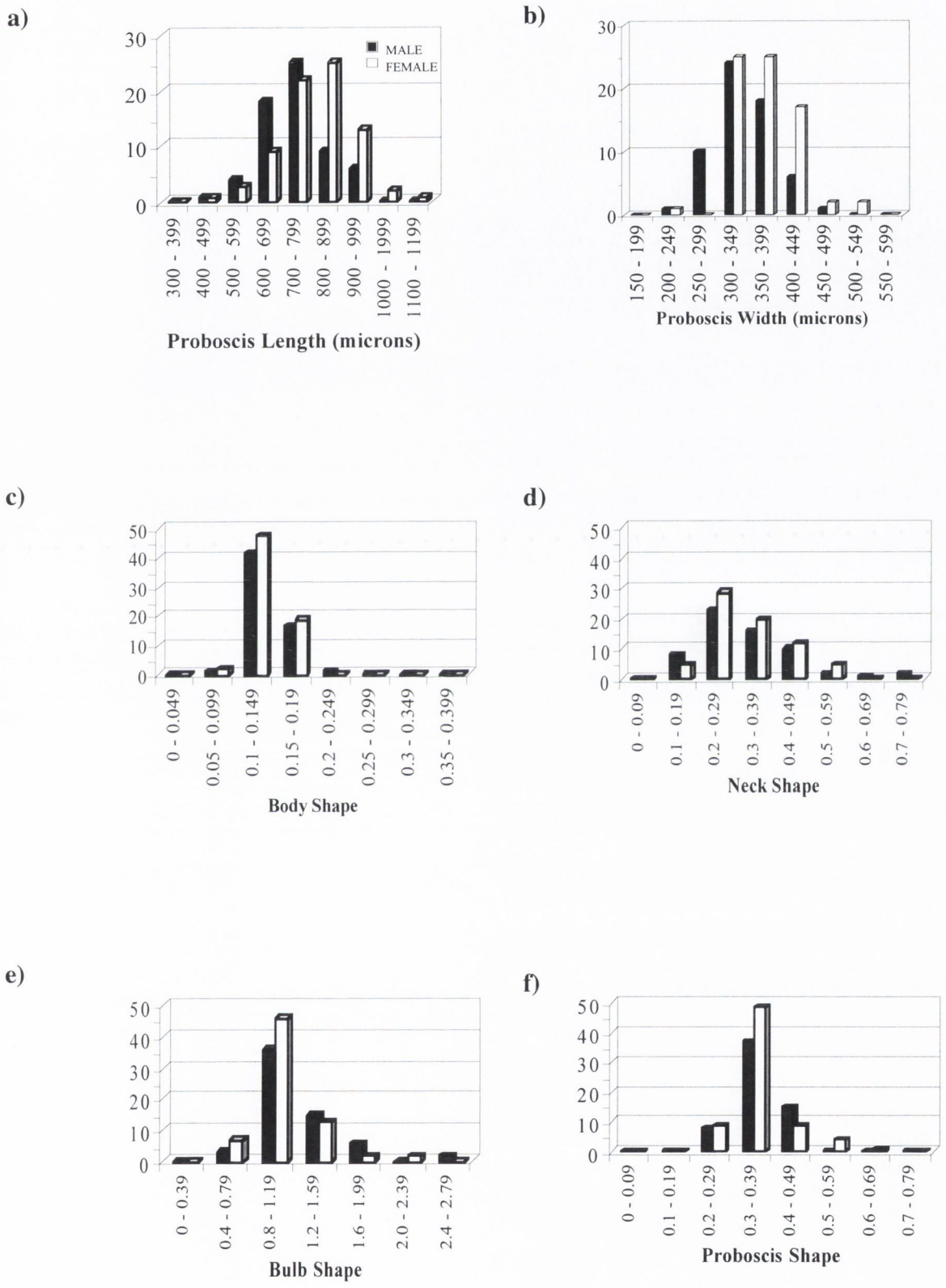


Fig. 3.3.4 (a – f) Frequency distribution of proboscis length, proboscis width, body shape, neck shape, bulb shape and proboscis shape for male and female *P. laevis* of Irish origin.

Table 3.3.1: Summary statistics of non-hook characters for male and female *P. laevis* of Irish origin.

(N = sample size, t = student t test statistic, p = probability, CV = coefficient of variation)

	FEMALE		>	MALE	T-test		ALL		
	N	Mean ± SD.	N	Mean ± SD.	t	p	Skewness	Kurtosis	CV
TOTAL LENGTH†	70	12.1 ± 2.7	61	9.7 ± 2.5	3.6	***	0.85	0.48	26.0
TRUNK LENGTH†	71	8.1 ± 2.5	61	6.9 ± 2.2	3.3	**	1.13	0.63	32.1
TRUNK WIDTH†	70	1.6 ± 0.6	61	1.4 ± 0.5	2.1	*	1.04	0.38	34.6
NECK LENGTH†	71	1.7 ± 0.4	62	1.5 ± 0.4	2.5	*	0.36	1.09	24.7
NECK WIDTH	71	514.0 ± 111.4	62	461.1 ± 100.3	2.9	**	-0.18	-0.63	22.2
BULB LENGTH	74	861.2 ± 182.9	63	719.8 ± 177.8	4.6	***	0.48	0.87	24.2
BULB WIDTH	70	914.6 ± 288.5	62	835.7 ± 249.3	1.7	NS	0.50	0.12	32.8
PROBOSCIS LENGTH	76	809.9 ± 127.0	63	736.3 ± 102.8	3.7	***	0.41	0.88	15.7
PROBOSCIS WIDTH	72	373.4 ± 50.2	60	338.9 ± 45.1	4.1	***	0.44	0.94	14.1
BODY SHAPE	69	0.14 ± 0.02	61	0.14 ± 0.02	1.2	NS	0.57	-0.03	-
NECK SHAPE	71	0.3 ± 0.1	62	0.3 ± 0.1	0.1	NS	0.89	0.76	-
BULB SHAPE	70	1.1 ± 0.3	62	1.2 ± 0.4	1.9	NS	1.71	4.29	-
PROBOSCIS SHAPE	72	0.5 ± 0.1	60	0.5 ± 0.1	0.6	NS	1.20	3.30	-

† units = millimeters, remaining characters measured in micrometers

- a coefficient of variation was not calculated for derived characters.

Table 3.3.2: Summary statistics of non-hook characters for male and female *P. laevis* of English origin.

(N = sample size, t = student t test statistic, p = probability, CV = coefficient of variation)

	FEMALE		>	MALE	T-test		ALL		
	N	Mean ± SD	N	Mean ± SD	t	p	Skewness	Kurtosis	CV
TOTAL LENGTH†	44	13.7 ± 3.0	39	11.3 ± 2.3	4.321	***	0.34	0.10	23.2
TRUNK LENGTH†	44	10.0 ± 2.7	39	7.7 ± 2.0	4.129	***	0.55	0.08	29.9
TRUNK WIDTH†	39	2.9 ± 0.8	34	2.1 ± 0.4	5.596	***	0.96	0.69	29.4
NECK LENGTH†	42	2.0 ± 0.4	38	1.9 ± 0.4	0.582	NS	-0.14	-0.22	20.5
NECK WIDTH	42	486.3 ± 146.1	38	426.6 ± 101.7	2.136	*	0.04	0.36	28.3
BULB LENGTH	44	1046.9 ± 242.0	39	916.4 ± 216.3	2.595	*	0.74	1.19	24.2
BULB WIDTH	44	1038.9 ± 178.9	37	924.8 ± 230.9	2.449	*	0.34	1.55	21.4
PROBOSCIS LENGTH	44	827.5 ± 110.8	39	769.3 ± 50.8	3.133	**	-0.42	4.66	11.5
PROBOSCIS WIDTH	43	331.5 ± 32.2	39	314.2 ± 32.7	2.419	*	0.37	0.24	10.3
BODY SHAPE	39	0.21 ± 0.04	34	0.19 ± 0.03	2.858	**	0.63	0.65	-
NECK SHAPE	41	0.3 ± 0.1	38	0.2 ± 0.1	0.655	NS	0.75	0.96	-
BULB SHAPE	44	1.0 ± 0.3	37	1.0 ± 0.2	0.066	NS	0.95	0.72	-
PROBOSCIS SHAPE	44	0.4 ± 0.1	39	0.4 ± 0.0	0.916	NS	0.65	0.74	-

† units = millimeters, remaining characters measured in micrometers.

- a coefficient of variation was not calculated for derived character

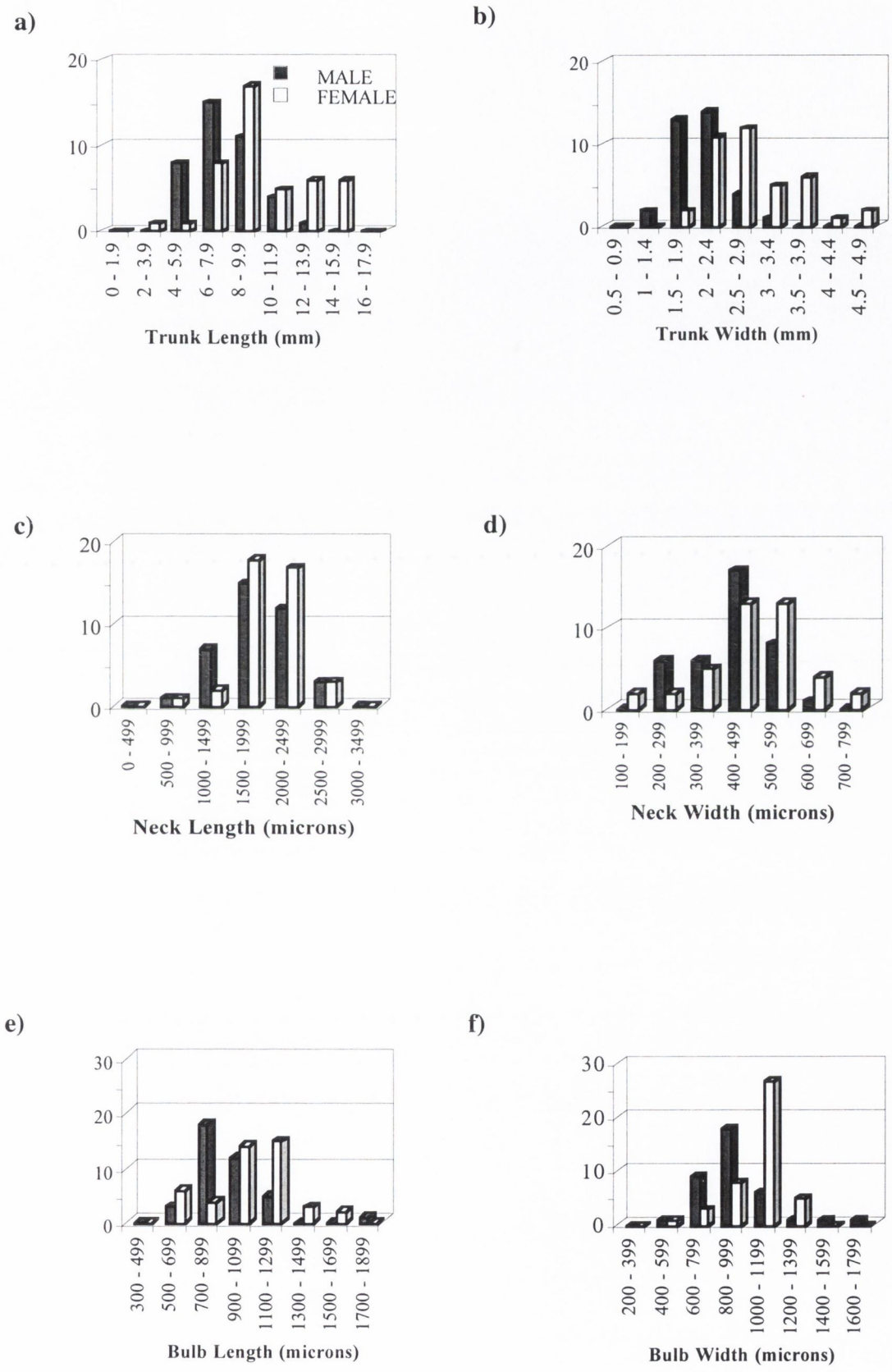


Fig. 3.3.5 (a -f) Frequency distribution of trunk length, trunk width, neck length, neck width, bulb length and bulb width for male and female *P. laevis* of English origin.

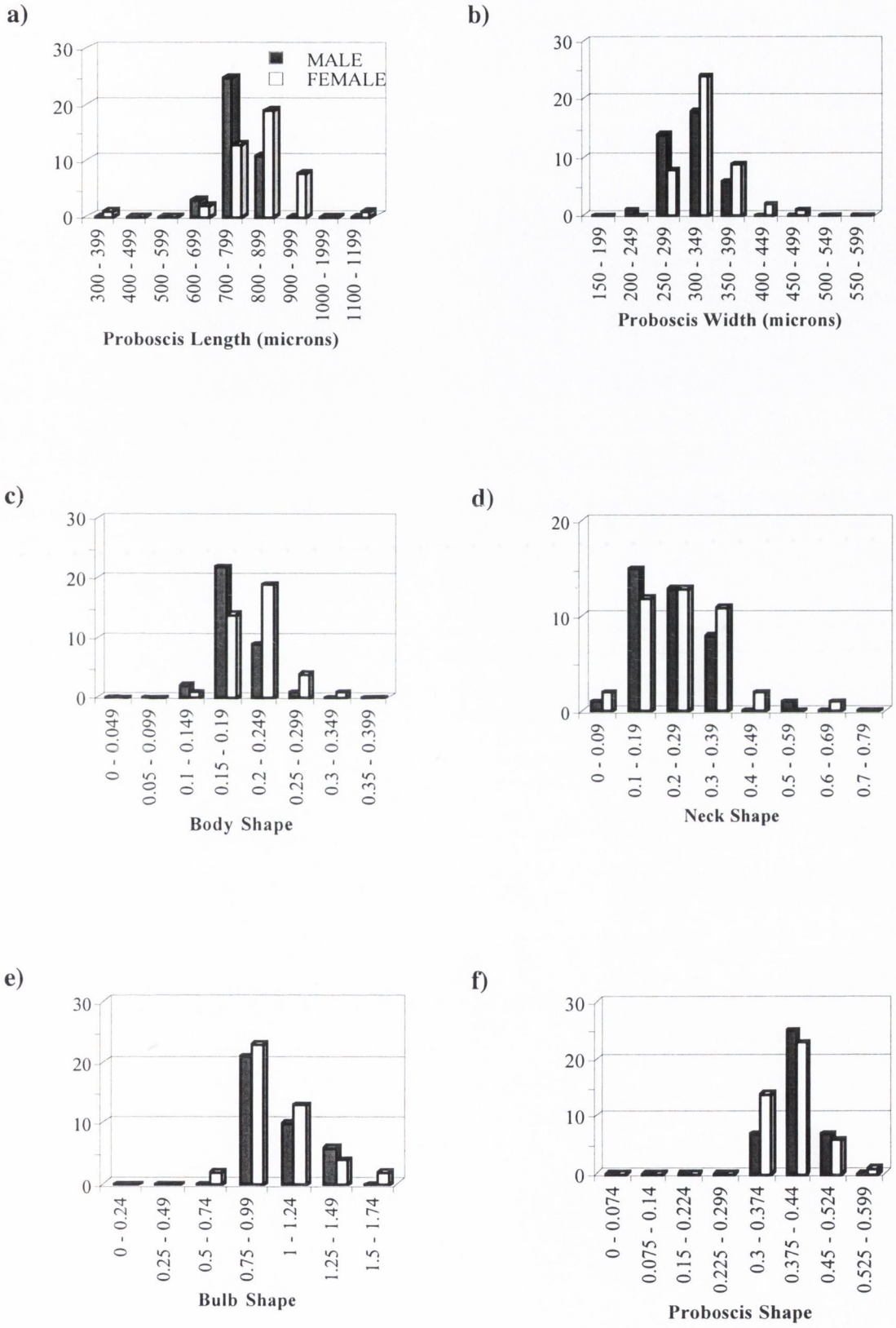


Fig. 3.3.6(a – f) Frequency distribution of proboscis length, proboscis width, body shape, neck shape, bulb shape and proboscis shape for male and female *P. laevis* of English origin.

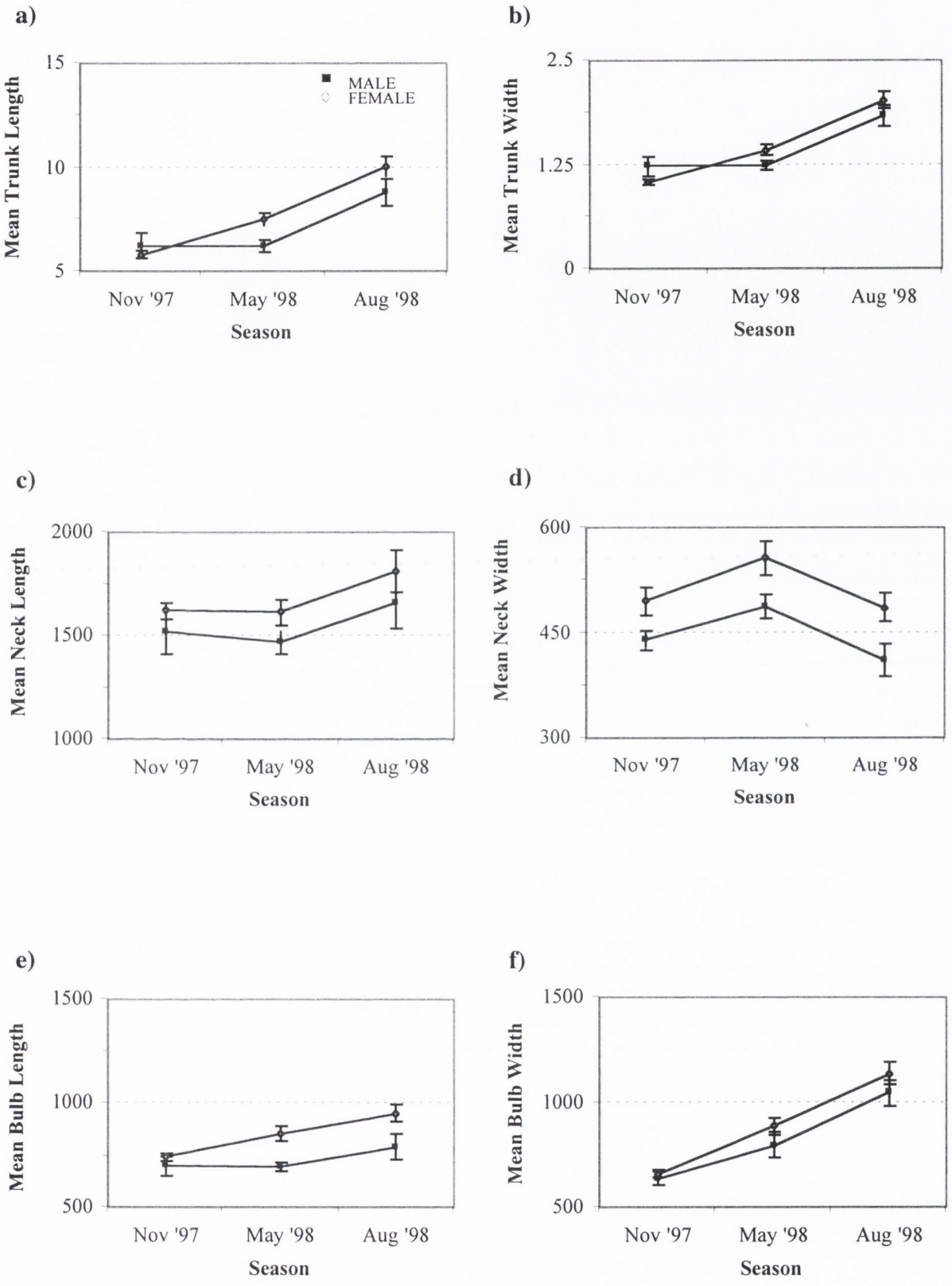


Fig. 3.3.7(a - f) Change in mean trunk length, mean trunk width, mean neck length, mean neck width, mean bulb length and mean bulb width with season for male and female *P. laevis* of Irish origin. Error bars represent standard error.

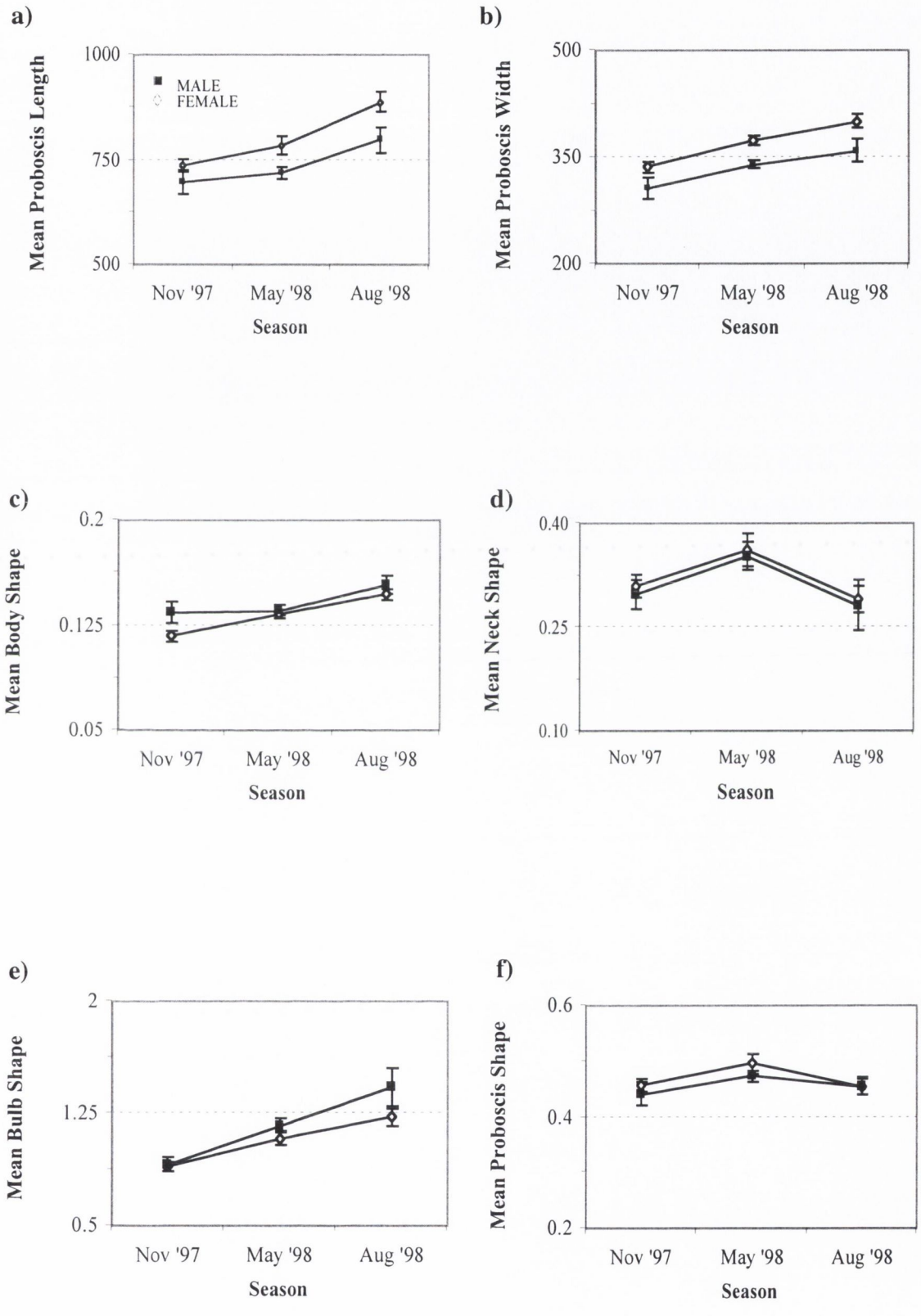


Fig. 3.3.8(a - f) Change in mean proboscis length, mean proboscis width, mean body shape, mean neck shape, mean bulb shape and mean proboscis shape with season for male and female *P. laevis* of Irish origin. Error bars represent standard error.

Sex

A clear sexual dimorphism was exhibited by most of the non-hook characters of both Irish and English *P. laevis* (tables 3.3.1 and 3.3.2). Females tend to be significantly larger than males in most characters, with some exceptions. In the Irish sample bulb width does not differ between the sexes. Nor was any degree of sexual dimorphism found in the neck length of English *P. laevis*. In both cases, organ shape does not differ between males and females except for the body shape of English *P. laevis,* which is another sexually dimorphic character.

Seasonal effects within the Irish sample

As stated in materials and methods Irish *P. laevis* used for the morphological study was sampled during three different months, November 1997, May 1998 and August 1998. Statistically significant differences (Two-way ANOVA) in all non-hook characters were found between the three months (table 3.3.3).

Table 3.3.3: Results showing the effects of season and parasite sex on the non-hook characters of Irish *P. laevis* (Two-way ANOVA).

	SEX			SEASON			SEX*SEASON		
	F – Stat	df	p	F – Stat	df	p	F – Stat	df	p
TOTAL LENGTH	6.3	1, 130*	*	30.1	2, 130	***	2.1	2, 130	NS
TRUNK LENGTH	4.4	1, 131	*	34.6	2, 131	***	2.5	2, 131	NS
TRUNK WIDTH	0.5	1, 130	NS	42.6	2, 130	***	3.0	2, 130	NS
NECK LENGTH	3.1	1, 132	NS	3.2	2, 132	*	0.1	2, 132	NS
NECK WIDTH	11.0	1, 132	**	6.8	2, 132	**	0.1	2, 132	NS
BULB LENGTH	15.1	1, 136	***	7.0	2, 136	**	1.2	2, 136	NS
BULB WIDTH	2.8	1, 131	NS	36.0	2, 131	***	0.3	2, 131	NS
PROBOSCIS LENGTH	11.0	1, 138	**	13.9	2, 138	***	0.5	2, 138	NS
PROBOSCIS WIDTH	18.5	1, 131	***	14.5	2, 131	***	0.2	2, 131	NS
BODY SHAPE	4.2	1, 129	*	11.6	2, 129	***	1.1	2, 129	NS
NECK SHAPE	0.3	1, 132	NS	6.1	2, 132	**	0.0	2, 132	NS
BULB SHAPE	2.9	1, 131	NS	20.7	2, 131	***	0.6	2, 131	NS
PROBOSCIS SHAPE	1.2	1, 131	NS	3.4	2, 131	*	0.3	2, 131	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

Table 3.3.4 presents Scheffe Post Hoc tests, which were based on the results of the ANOVA tests presented in table 3.3.3. Each column of table 3.3.4 shows whether a character in one month is significantly bigger than another month. As can be seen from figures 3.3.7 and 3.3.8 and table 3.3.4, characters increased in size from November to August, in most cases. Most of these size increases took place between May and August. Growth patterns were similar for males and females. Total length, trunk length, trunk width, bulb length, and proboscis length were significantly greater in August than May and November. Neck length was also significantly greater in August than May. Bulb width and proboscis width were significantly greater in August than May and also significantly greater in May than November. Body shape and bulb shape also followed the established growth trend. Neck width and neck shape followed a different pattern. These characters were significantly larger in May than August. No change in proboscis shape was found over the course of the sampling period. No interactions were found between sex and season.

Table 3.3.4: Results of comparisons of the non-hook characters of Irish *P. laevis* between the three sampling periods (Scheffe Post-Hoc test). Dashes represent outcomes that did not occur, *e.g.* if length was greater in August than May, then a dash is present in the MAY > AUG column.

	AUG > MAY	AUG > NOV	MAY > NOV	MAY > AUG
	p	p	p	p
TOTAL LENGTH	***	***	NS	-
TRUNK LENGTH	***	***	NS	-
TRUNK WIDTH	***	***	NS	-
NECK LENGTH	*	NS	NS	-
NECK WIDTH	-	-	NS	**
BULB LENGTH	*	**	NS	-
BULB WIDTH	***	***	***	-
PROBOSCIS LENGTH	***	***	NS	-
PROBOSCIS WIDTH	*	***	**	-
BODY SHAPE	**	***	NS	-
NECK SHAPE	**	NS	NS	-
BULB SHAPE	**	***	***	-
PROBOSCIS SHAPE	NS	NS	NS	-

Table 3.3.5: Regression of non-hook characters with total length for male and female *P. laevis* of Irish origin.

	FEMALE			MALE		
	R^2 (%)	t-ratio	p	R^2 (%)	t-ratio	p
TRUNK LENGTH	**96.5**	43.0	***	**94.2**	31.0	***
TRUNK WIDTH	**79.9**	16.3	***	**70.5**	11.9	***
NECK LENGTH	33.2	5.82	***	29.3	4.95	***
NECK WIDTH	6.4	-2.17	*	0.9	-0.75	NS
BULB LENGTH	**44.9**	7.39	***	**59.7**	9.26	***
BULB WIDTH	**67.2**	11.4	***	**51.6**	7.79	***
PROBOSCIS LENGTH	25.1	4.7	***	32.2	5.25	***
PROBOSCIS WIDTH	**41.5**	6.73	***	29.1	4.75	***
BODY SHAPE	12.2	3.06	**	1.4	0.90	NS
NECK SHAPE	26.3	-4.92	***	19.9	-3.83	***
BULB SHAPE	22.7	4.3	***	0.0	-0.03	NS
PROBOSCIS SHAPE	0.1	0.26	NS	1.5	-0.92	NS

Table 3.3.6: Regression of non-hook characters with total length for male and female *P. laevis* of English origin.

	FEMALE			MALE		
	R^2%	t-ratio	p	R^2 %	t-ratio	p
TRUNK LENGTH	**95.0**	28.0	***	**94.5**	25.2	***
TRUNK WIDTH	**63.1**	7.95	***	**60.0**	6.92	***
NECK LENGTH	**54.2**	6.79	***	**59.8**	7.32	***
NECK WIDTH	0.0	0.002	NS	11.2	-2.13	*
BULB LENGTH	1.1	0.69	NS	9.5	1.97	NS
BULB WIDTH	3.0	1.14	NS	4.2	1.24	NS
PROBOSCIS LENGTH	0.2	0.26	NS	3.8	-1.21	NS
PROBOSCIS WIDTH	0.1	0.15	NS	0.0	0.01	NS
BODY SHAPE	4.1	-1.26	NS	16.0	-2.47	*
NECK SHAPE	17.4	-2.87	**	**47.6**	-5.72	***
BULB SHAPE	0.0	0.10	NS	0.5	-0.42	NS
PROBOSCIS SHAPE	0.2	-0.32	NS	2.7	1.02	NS

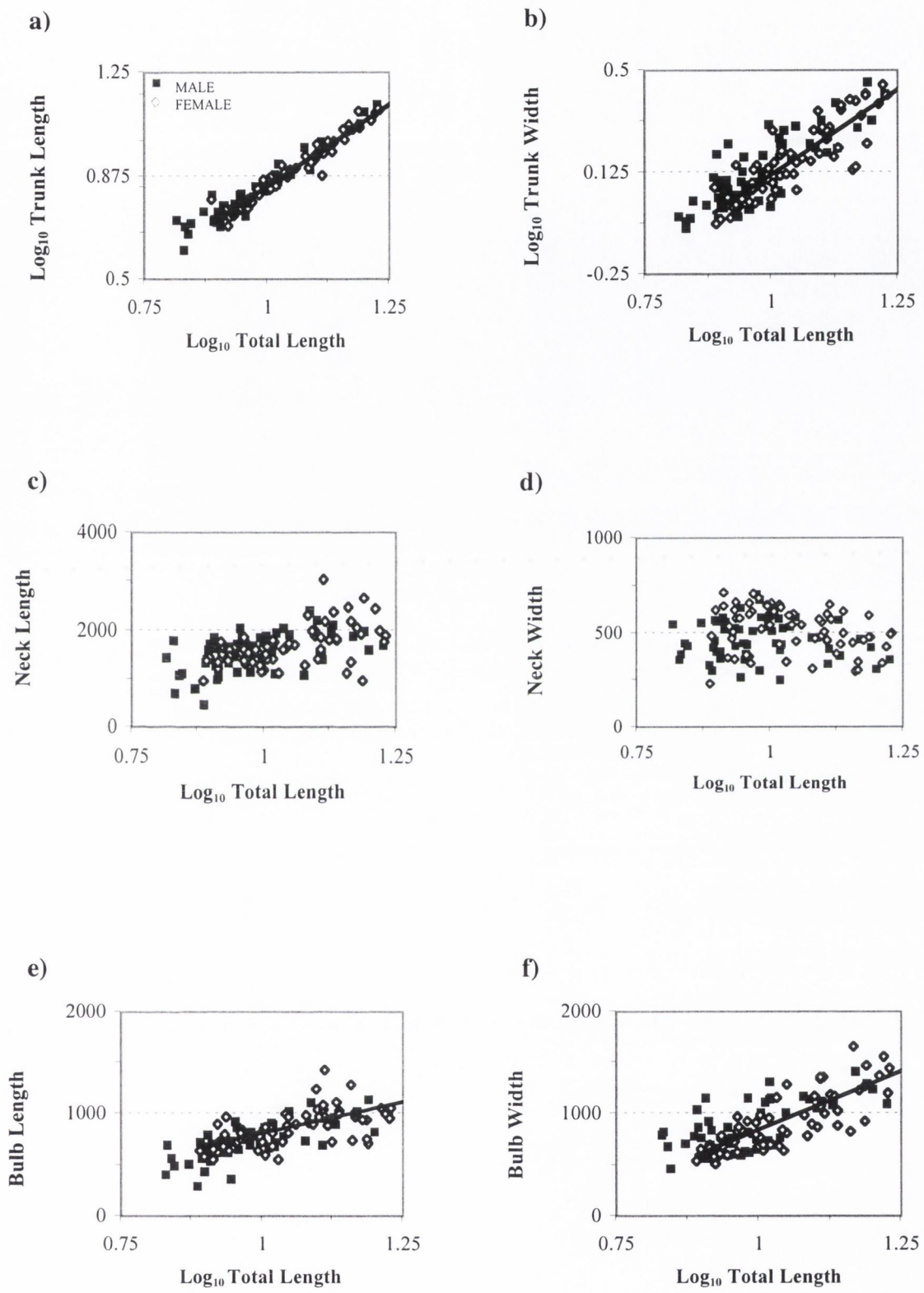


Fig. 3.3.9(a - f) Scatterplot of $\log_{10}$ trunk length, $\log_{10}$ trunk width, neck length, neck width, bulb length and bulb width against $\log_{10}$ total length for male and female *P. laevis* of Irish origin.

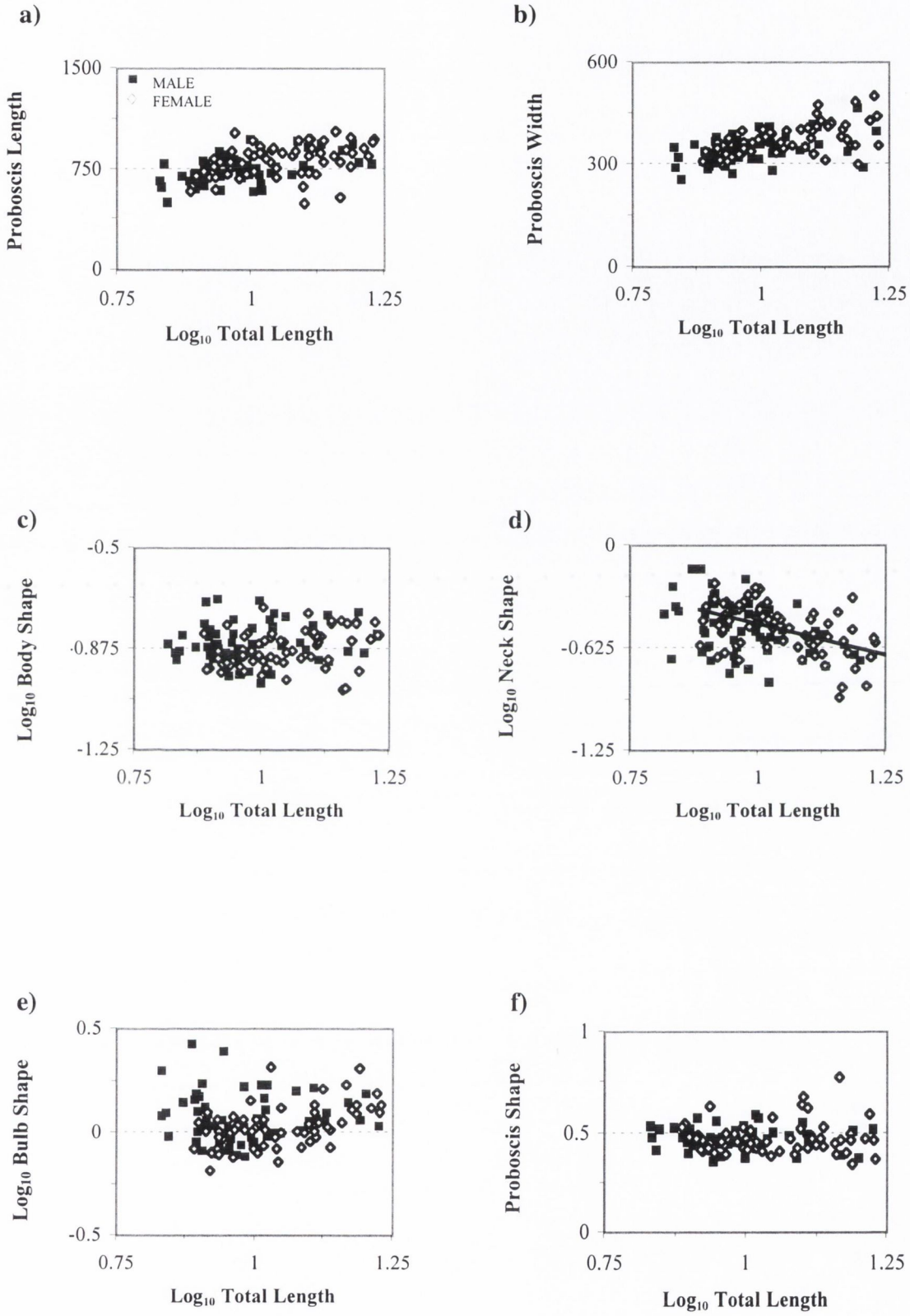


Fig. 3.3.10(a - f) Scatterplot of proboscis length, proboscis width, $\log_{10}$ body shape, $\log_{10}$ neck shape, $\log_{10}$ bulb shape and proboscis shape against $\log_{10}$ total length for male and female *P. laevis* of Irish origin.

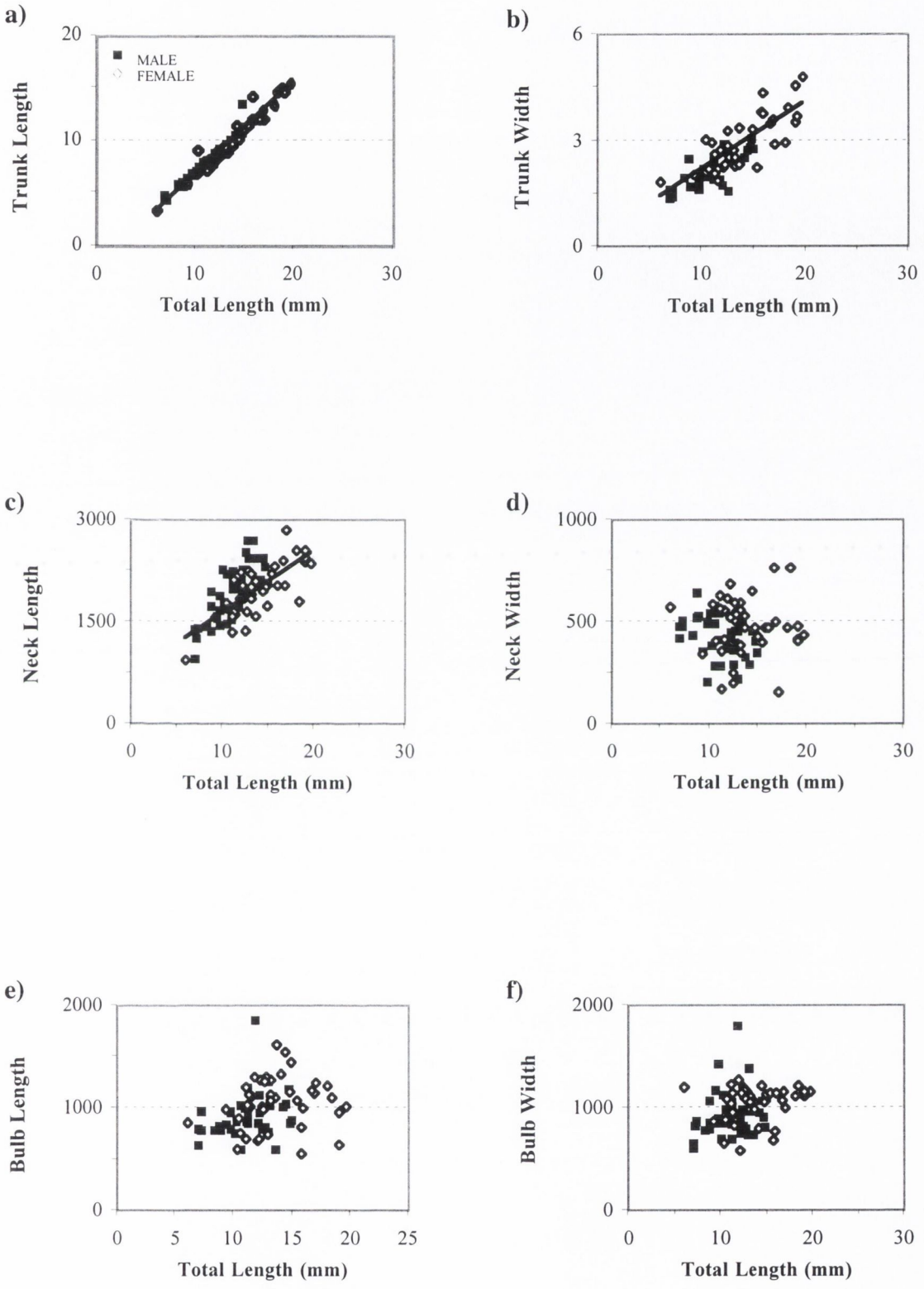


Fig. 3.3.11(a - f) Scatterplot of trunk length, trunk width, neck length, neck width, bulb length and bulb width against total length for male and female *P. laevis* of English origin.

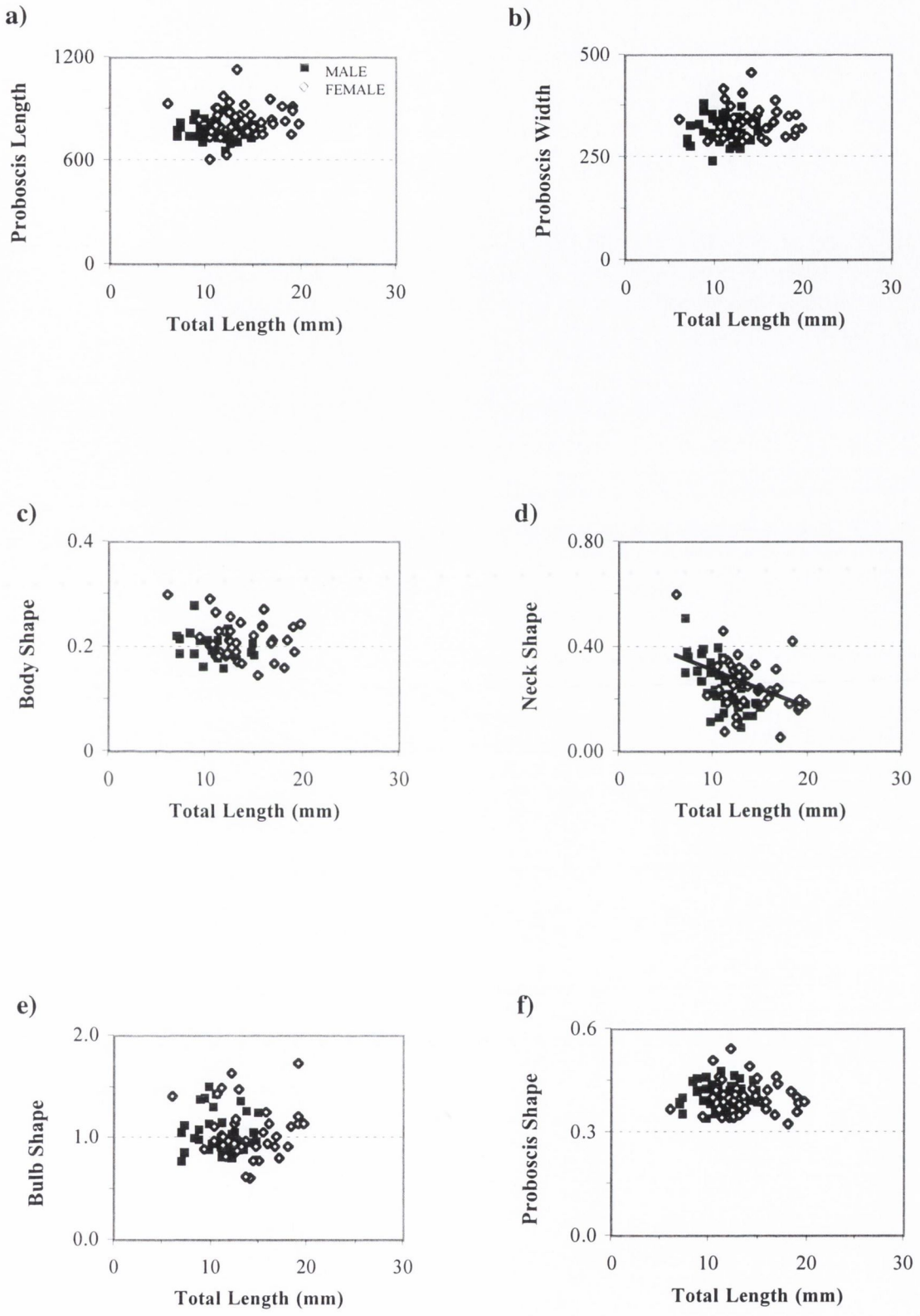


Fig. 3.3.12(a - f) Scatterplot of proboscis length, proboscis width, body shape, neck shape, bulb shape and proboscis shape against total length for male and female *P. laevis* of English origin.

Linear Regression of non-hook characters with total length

A linear regression analysis was carried out between all non-hook characters and total length for Irish *P. laevis* (table 3.3.5, figures 3.3.9 and 3.3.10). Trunk length and width of both males and females were found to be strongly related to total length, which is an expected result. Variation in bulb length and width for both males and females and the variation in proboscis width for females were also found to be strongly related to total length. The relationship between most of the non-hook characters and length was positive except for neck width and neck shape. No relationship was found between the remaining shape characters and length.

A similar investigation was conducted for the English sample (table 3.3.6, figures 3.3.11 and 3.3.12). Again a strong relationship was shown to exist between both trunk length and width and total length. Unlike the Irish situation neck length related strongly to total length though not bulb length and width, nor proboscis width. A mild negative regression was found between neck shape and total length for English males.

Hook Characters

Distribution and Variability

Number of hooks per row and number of longitudinal rows are important characters when describing different species of acanthocephalans. The distributions of these characters and those of the positions of the stoutest and longest hooks and the length of the longest hook of Irish *P. laevis* can be seen in figure 3.3.13. Apart from the position of the stoutest hook, the characters show normal distributions. It is clear from figure 3.3.13 a and b and table 3.3.7 that important diagnostic characters such as the number of hooks per row and the number of rows vary to quite a degree in Irish *P. laevis*. The stoutest hook seems to be found equally at the 4^{th} and 5^{th} position. The position of the longest hook seems to be more fixed at the 2^{nd} position. In Irish *P. laevis* the longest hook has a mean length of 66.4 ± 6.17 μm and 61.9 ± 5.14 μm in females and males respectively (table 3.3.9).

Figure 3.3.14 shows the distributions of the aforementioned hook characters for English *P. laevis*. Distributions are normal except in the case of the position of the stoutest hook (figure 3.3.14c). The number of hooks per row is not as variable as that

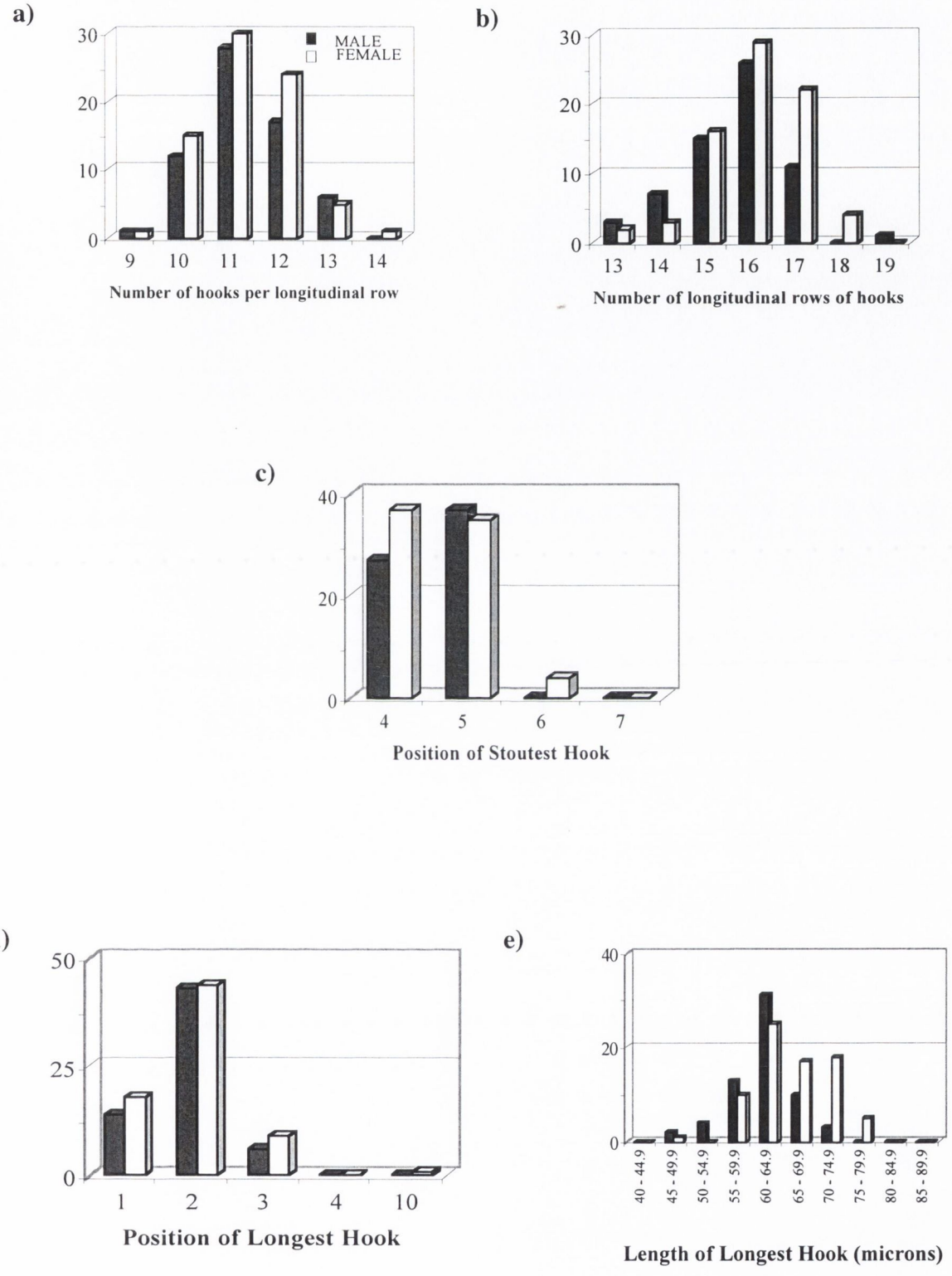


Fig. 3.3.13(a - e) Frequency distribution of the number of hooks per longitudinal row, number of longitudinal rows, the position of the stoutest hook, the position of the longest hook and the length of the longest hook for male and female *P. laevis* of Irish origin.

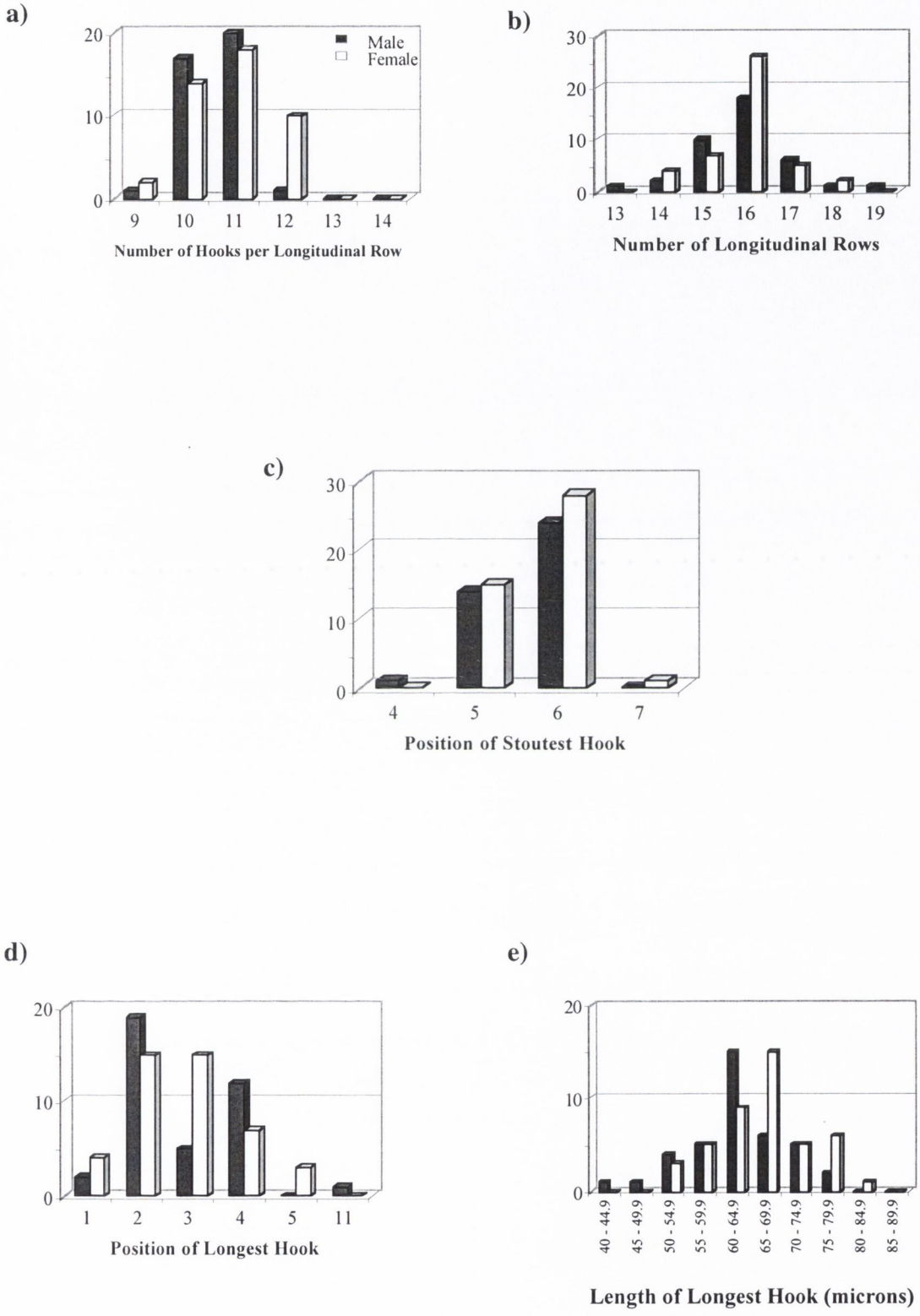


Fig. 3.3.14(a - e) Frequency distribution of the number of hooks per longitudinal row, number of longitudinal rows, position of the stoutest hook, position of the longest hook and the length of the longest hook for male and female *P. laevis* of English origin.

seen in Irish *P. laevis*, ranging from 9 to 12 though with the same median value of 11 hooks per row (tables 3.3.7 and 3.3.8). The number of rows does seem to vary to the same extent as in Irish *P. laevis* and also has the same median value of 16 (tables 3.3.7 and 3.3.8). The stoutest hook is found more at the 6^{th} position, which contrasts with the Irish situation and the longest hook is found at more positions including 2^{nd}, 3^{rd} and 4^{th}. In English *P. laevis* the longest hook has a mean of 66.7 ± 6.86 μm and 62.8 ± 7.1 μm in females and males respectively (table 3.3.10).

The lengths and widths of the first three anterior hooks and the stoutest hook of Irish *P. laevis* are shown in figure 3.3.15. The distributions of all these characters are normal. Hook 2 is the longest of the anterior hooks shown and supports the fact that the longest hook is found most often at this position. The width of the first three anterior hooks seems to increase slightly with position though the width of the stoutest hook is clearly the largest of the four hooks. Figure 3.3.16 shows the lengths and widths of three of the posterior hooks and the basal hook. Again all characters possess a normal distribution. The lengths and widths of the three posterior hooks are quite similar with the seventh hook being the longest of the three. The length of the basal hook is more on the scale of an anterior hook though its width is more like that of a posterior hook but slightly larger.

It seems clear that the length and width of a hook varies with its position in the row. Hook shape was investigated in order to determine if this character was conserved among hooks at different positions. Hook area was also studied. Figure 3.3.17 shows the shape and area of the first three anterior hooks and the stoutest hook of Irish *P. laevis*. The characters are normally distributed. The shape of the first and second anterior hooks is similar. Hook shape increases in the third anterior hook to its highest level in the stoutest hook. That the stoutest hook has the largest value for shape is not surprising as it is a shorter and wider hook than the previous three and hook shape is really a gauge of 'stoutness'. Area also changes between the anterior hooks, increasing from the first to the second hook and remaining similar between the second, third and stoutest hooks. Hook shape and area of the posterior hooks and the basal hooks are shown in figure 3.3.18. The characters are again normally distributed with the exception of the area of hook 9 and the basal hook which show a slight skew to the right. The shape of these four hooks is similar and is also comparable to the shape of the first three anterior hooks. The area of the three posterior hooks is also similar. The area of the basal hook is larger and has more extreme values than the other hooks. It

Table 3.3.7: Summary statistics of four hook characters for male and female *P. laevis* of Irish origin including results of a chi-squared test for sexual differences.

	FEMALE				MALE					
	N	Median	Min	Max	N	Median	Min	Max	χ^2 (df)	p
NO. HOOKS/ROW	76	11	9	14	64	11	9	13	0.71 (3)	NS
NO. LONGITUDINAL ROWS	76	16	13	18	63	16	13	19	4.56 (3)	NS
POSITION OF LONGEST HOOK	72	2	1	10	63	2	1	3	0.64 (2)	NS
POSITION OF STOUTEST HOOK	76	5	4	6	64	5	4	5	1.15 (1)	NS

Table 3.3.8: Summary statistics of four hook characters for male and female *P. laevis* of English origin including results of a chi-squared test for sexual differences.

	FEMALE				MALE					
	N	Median	Min	Max	N	Median	Min	Max	χ^2 (df)	p
NO. HOOKS/ROW	44	11	9	12	39	11	9	12	7.55 (2)	*
NO. LONGITUDINAL ROWS	44	16	14	18	39	16	13	19	1.86 (2)	NS
POSITION OF LONGEST HOOK	44	3	1	5	39	2	1	11	6.77 (2)	*
POSITION OF STOUTEST HOOK	44	6	5	7	39	6	4	6	0.03 (1)	NS

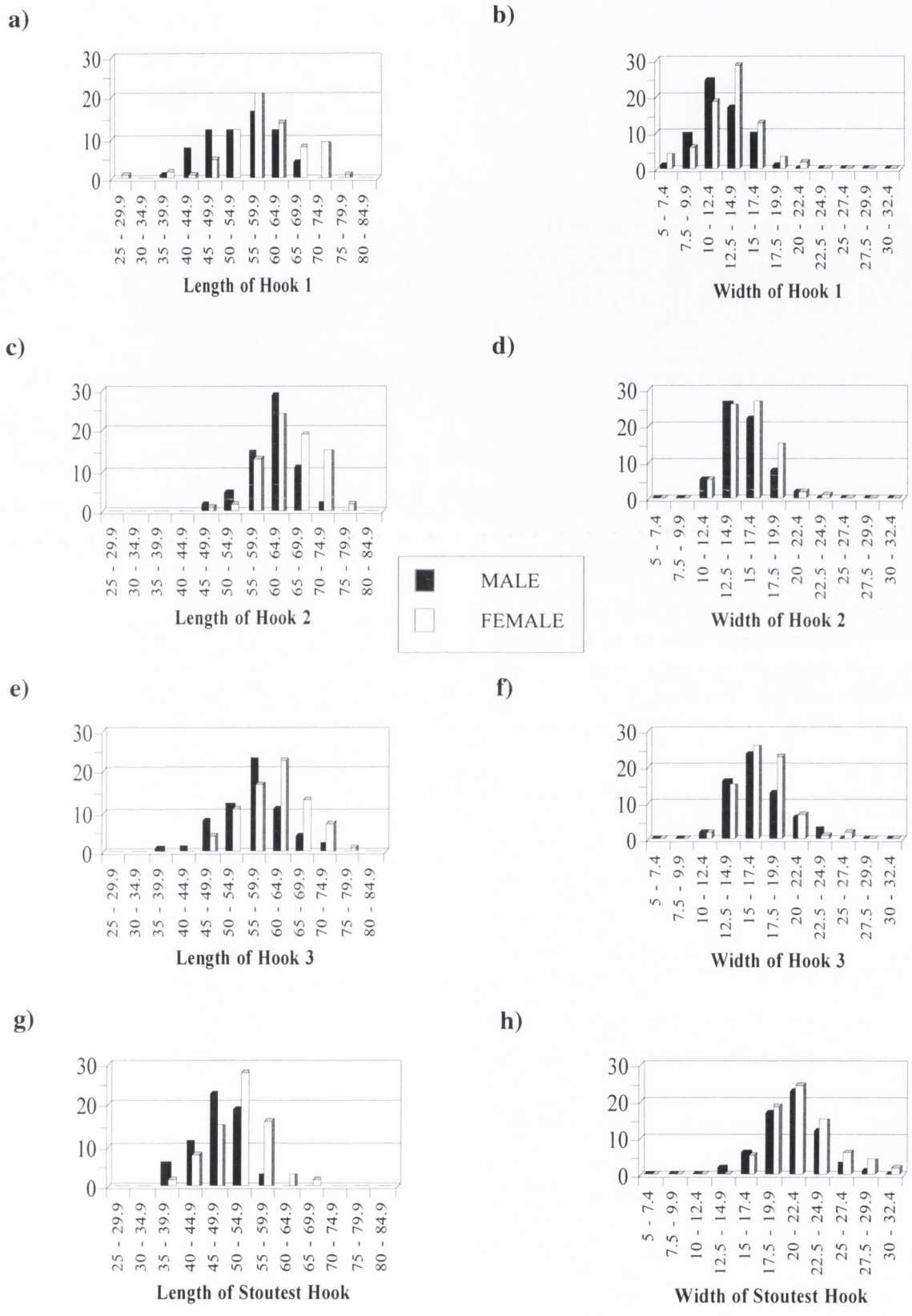


Fig. 3.3.15 Frequency distribution of anterior hook length and width **(a - f)** and stoutest hook length and width **(g - h)** for male and female *P. laevis* of Irish origin.

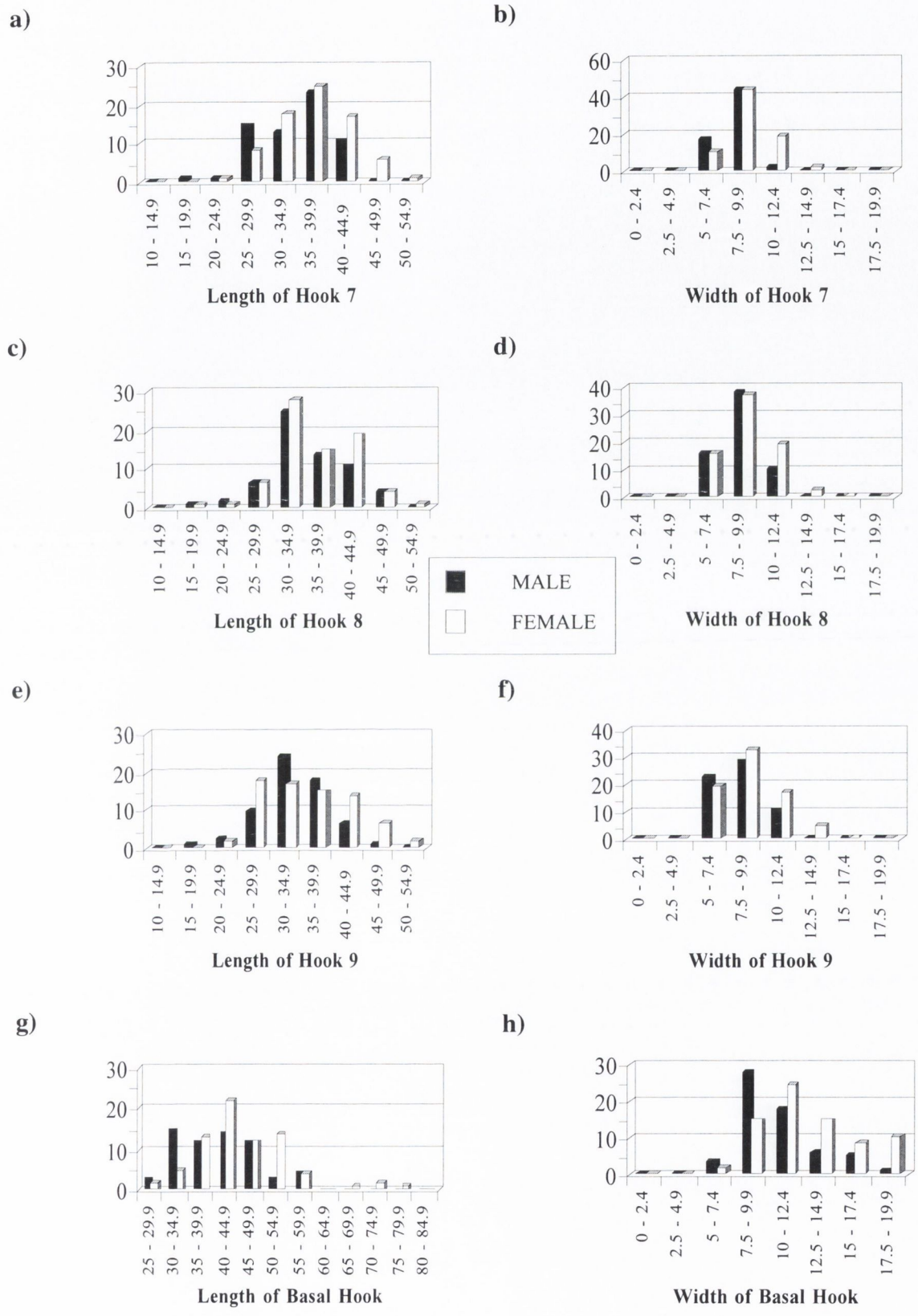


Fig. 3.3.16 Frequency distribution of posterior hook length and width **(a - f)** and basal hook length and width **(g - h)** for male and female *P. laevis* of Irish origin.

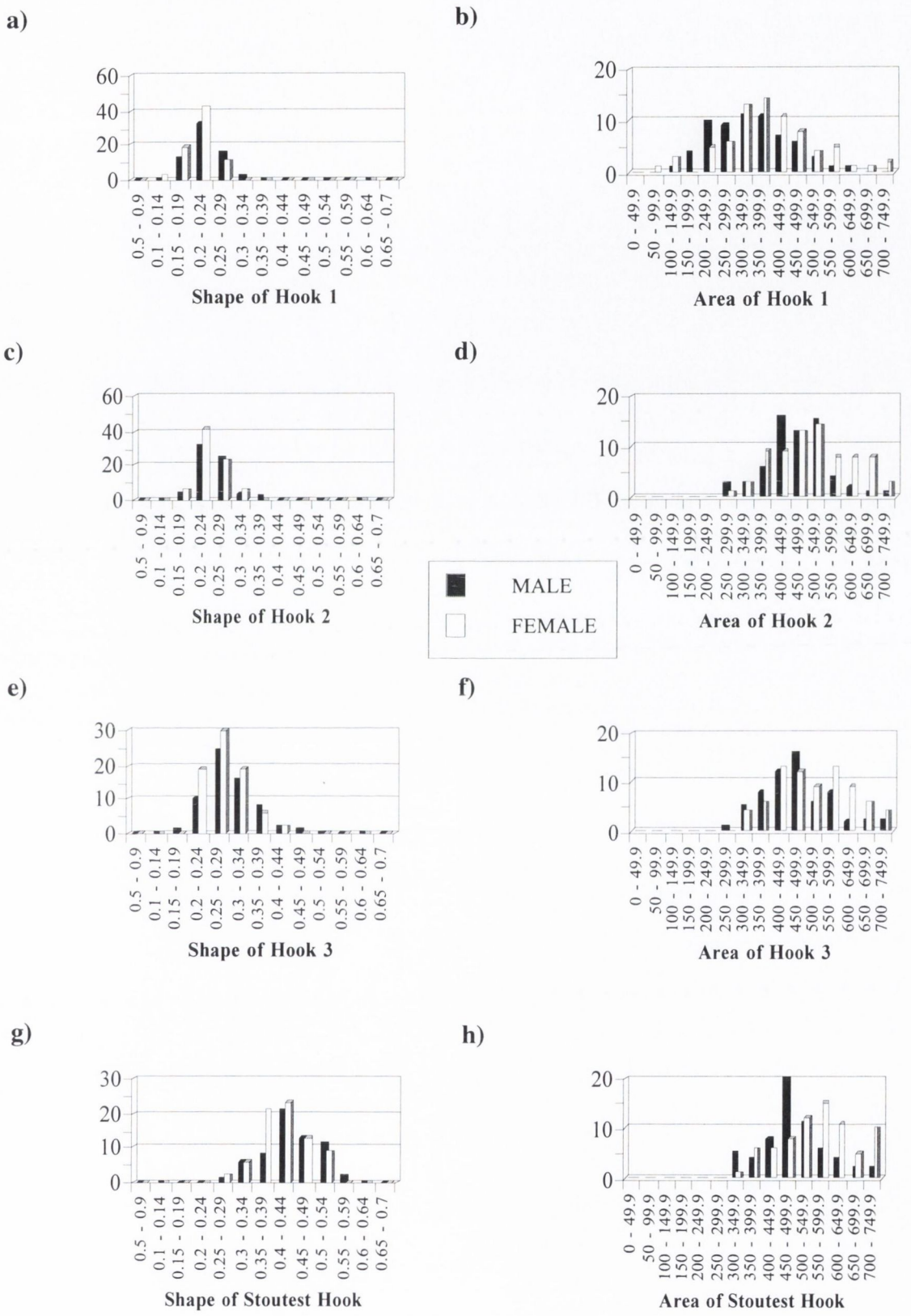


Fig. 3.3.17 Frequency distribution of anterior hook shape and area **(a - f)** and stoutest hook shape and area **(g - h)** for male and female *P. laevis* of Irish origin.

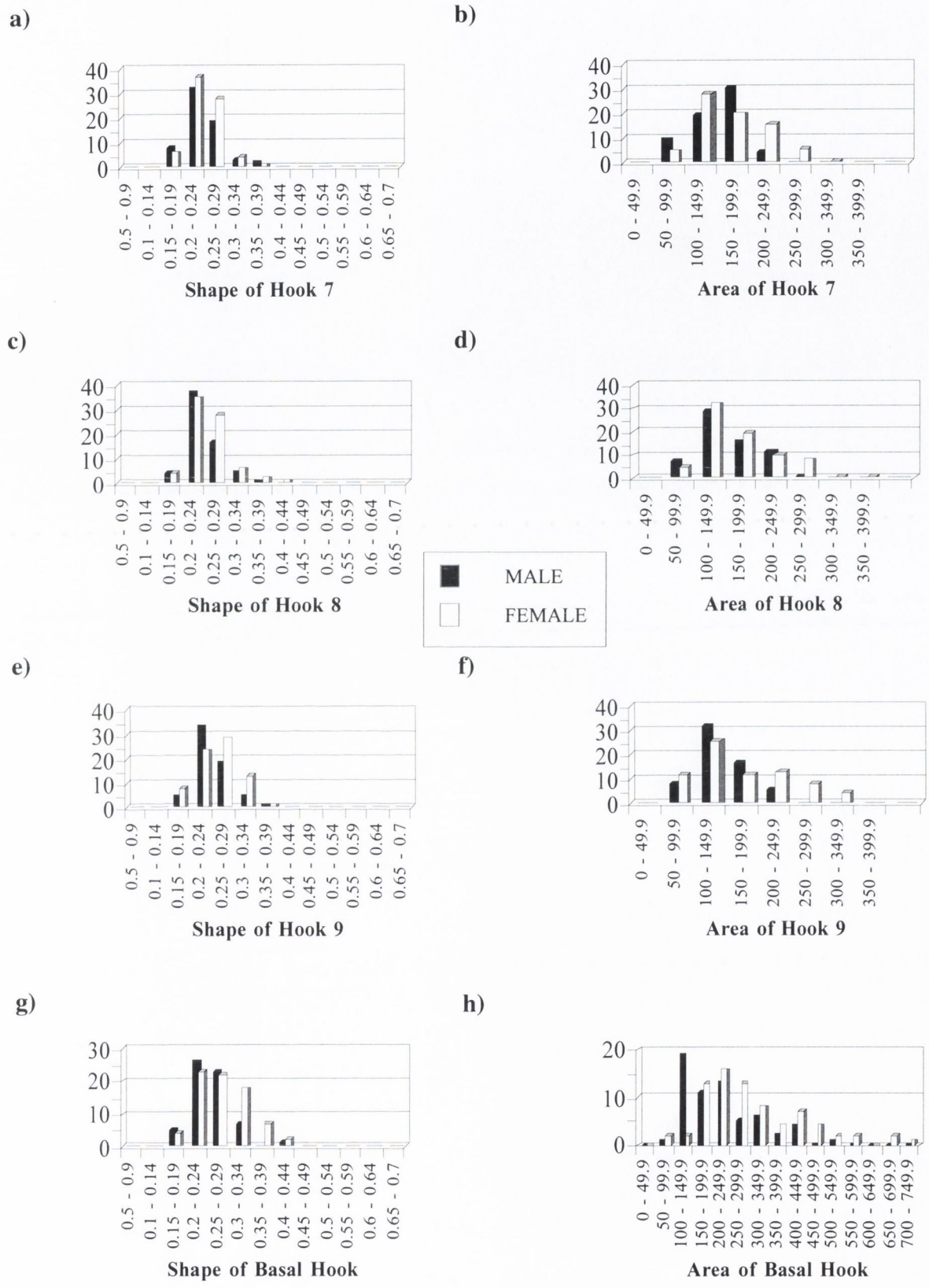


Fig. 3.3.18 Frequency distribution of posterior hook shape and area **(a - f)** and basal hook shape and area **(g - h)** for male and female *P. laevis* of Irish origin.

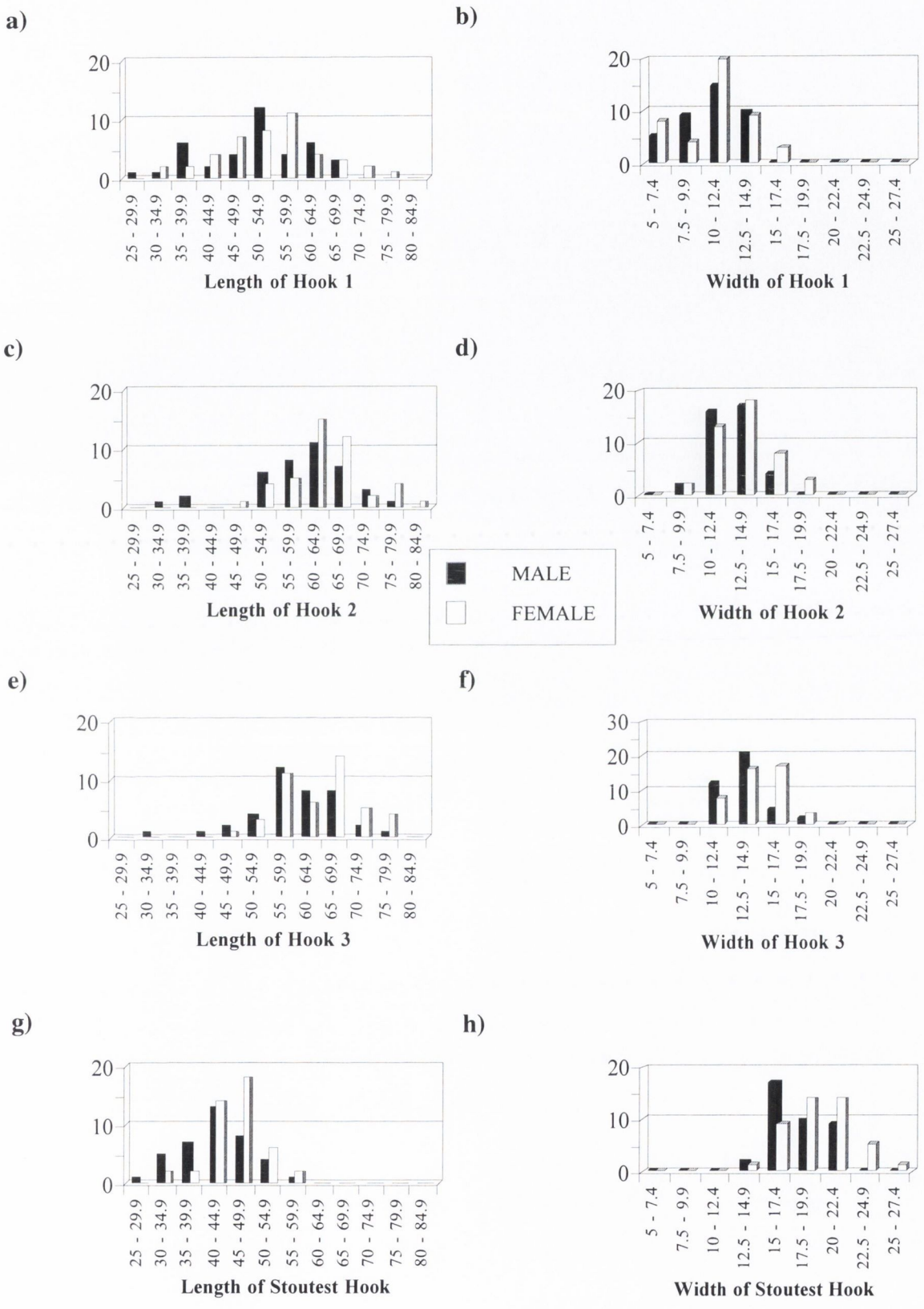


Fig. 3.3.19 Frequency distribution of anterior hook length and width **(a - f)** and stoutest hook length and width **(g - h)** for male and female *P. laevis* of English origin.

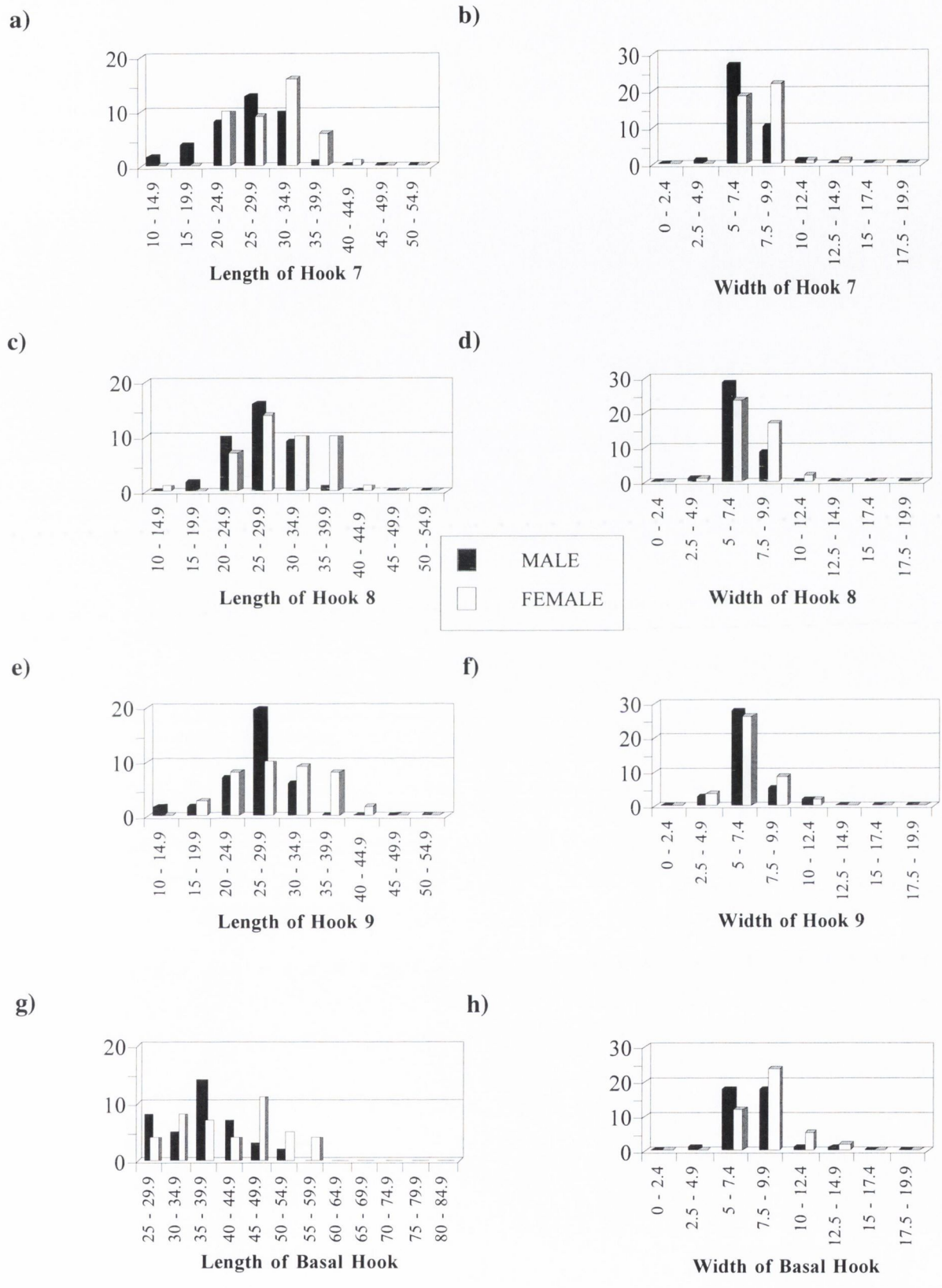


Fig. 3.3.20 Frequency distribution of posterior hook length and width **(a - f)** and basal hook length and width **(g - h)** for male and female *P. laevis* of English origin.

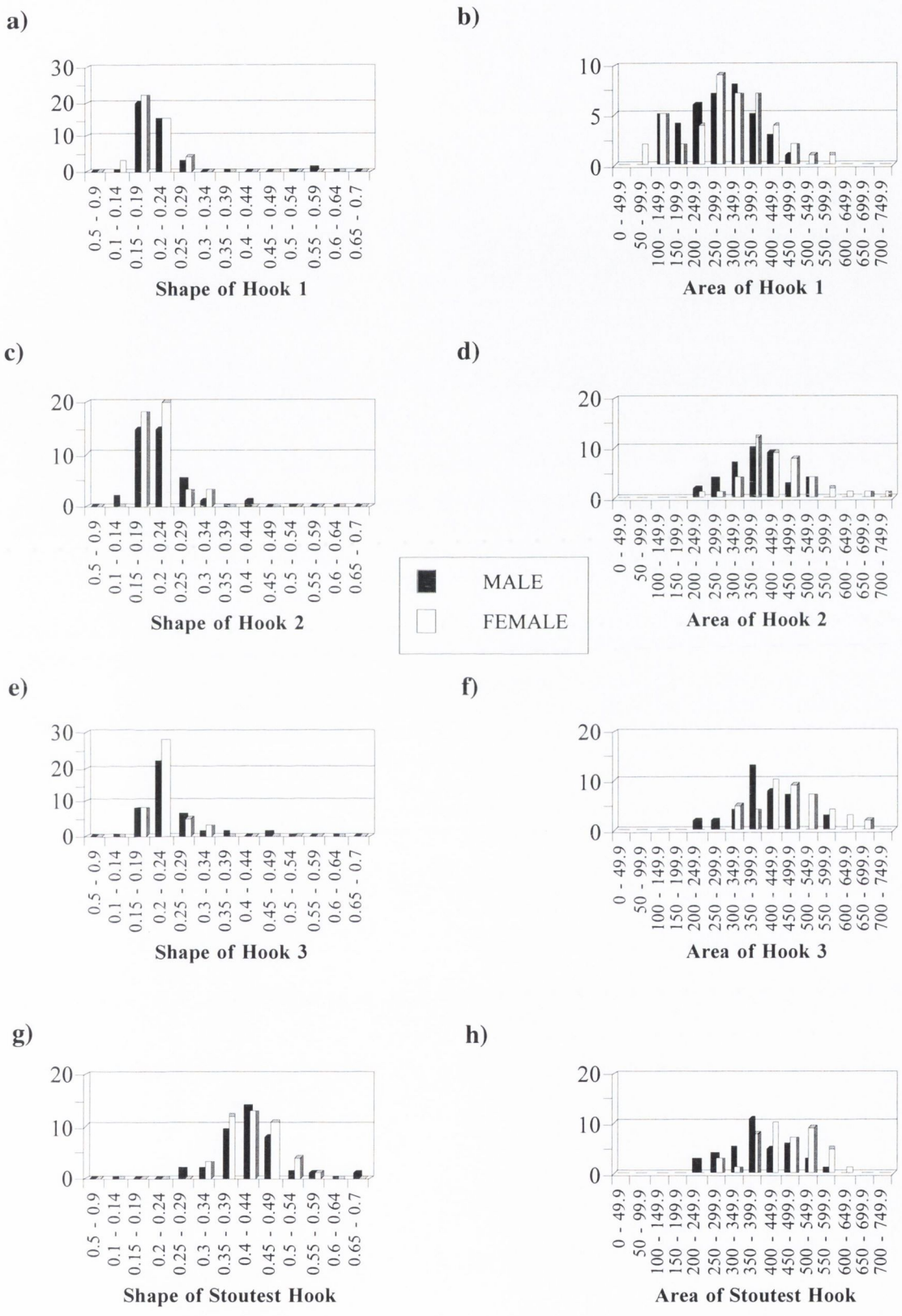


Fig. 3.3.21 Frequency distribution of anterior hook shape and area **(a - f)** and stoutest hook shape and area **(g - h)** for male and female *P. laevis* of English origin.

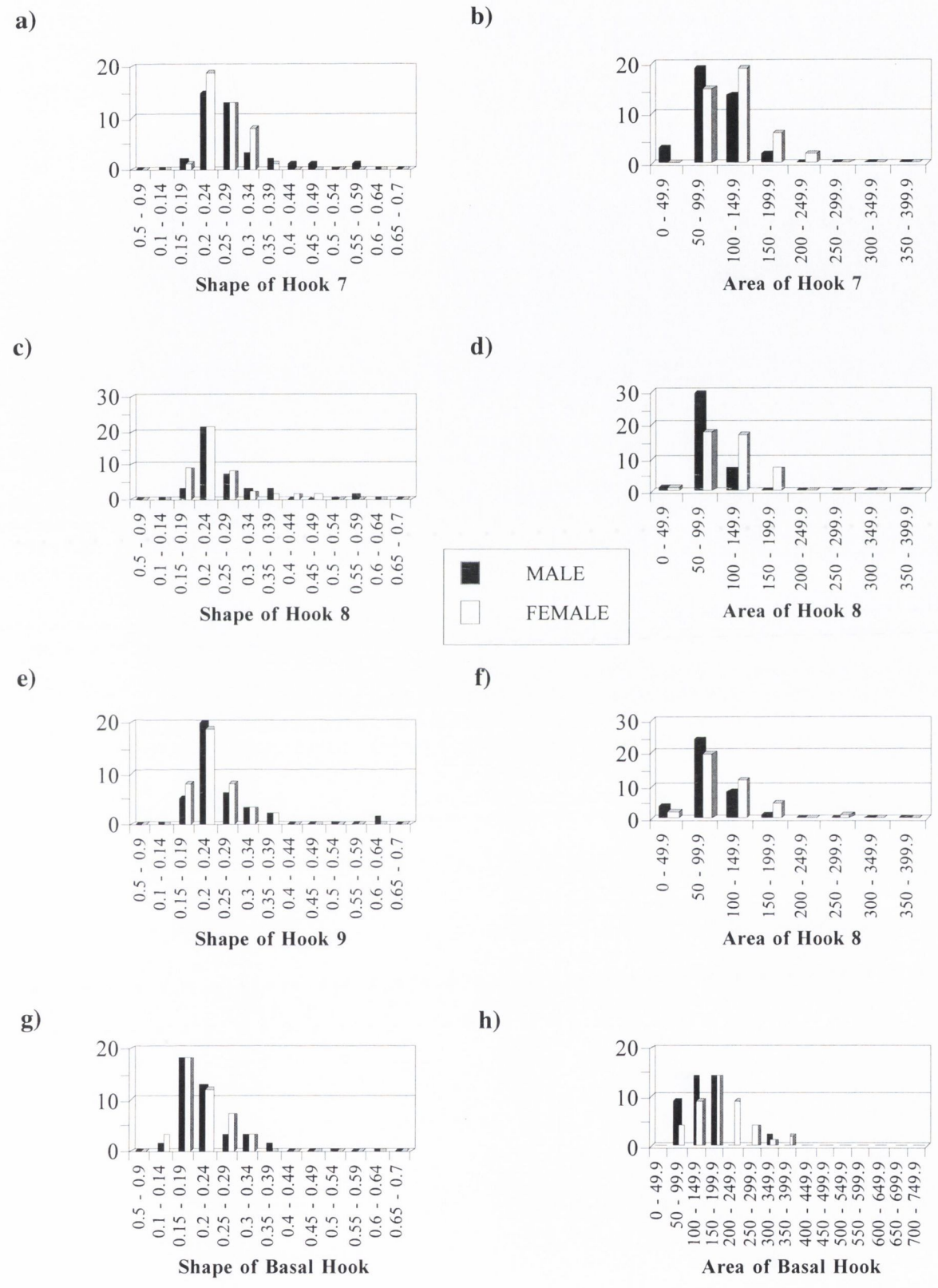


Fig. 3.3.22 Frequency distribution of posterior hook shape and area **(a - f)** and basal hook shape and area **(g - h)** for male and female *P. laevis* of English origin.

does seem from these observations that hook shape is conserved amongst the majority of the hooks except in the case of the stoutest hook. The lengths and widths of the first three anterior hooks and the stoutest hook of English *P. laevis* are shown in figure 3.3.19. The characters are normally distributed. The changes in hook length and width between positions mirrors those as described for Irish *P. laevis*. An exception being hook 3, which seems longer than hook 2. Figure 3.3.20 shows the length and width of three of the posterior hooks and the basal hook. Again a similar pattern of change between hooks at different positions as that seen in the Irish is shown. Hook shape and area of the first three anterior hooks and the stoutest hook are shown in figure 3.3.21. The distribution of these characters approximates the normal distribution. The shape of the first three hooks is similar though that of the first hook has a slightly smaller value. Again the stoutest hook has the largest value for shape. Change in hook area also follows the Irish pattern with the area of hook 1 being most widely distributed. Figure 3.3.22 shows the distributions of hook shape and area of the posterior and basal hooks. Shape would again seem to be conserved among hooks at different positions with the exception of the stoutest hook. Hook area is also similar among the three posterior hooks. Basal hook area is not as widely distributed in this sample as in the Irish sample.

Sex

The number of hooks per row and the number of rows did not differ between the sexes of Irish *P. laevis* (table 3.3.7) as predicted by the null hypothesis. The null hypothesis states that the number of hooks, rows and the positions of the stoutest and longest hooks should be similar for males and females. Neither was any sexual dimorphism found in the positions of the stoutest and longest hooks. The number of hooks per row did differ between the sexes of English *P. laevis* (table 3.3.8). There were more females with 12 hooks per row than expected by the analysis and fewer males. English males and females had similar numbers of longitudinal rows of hooks. The position of the longest hook also differed between the sexes. The longest hooks of females were found at the 3rd position more often than expected.

There was a large degree of sexual dimorphism found among hook length, hook width and hook area in both samples. As for the non-hook characters, females were larger. This was not the case for hook shape. All hook lengths differed

Table 3.3.9: Summary statistics of hook lengths for male and female *P. laevis* of Irish origin.

		FEMALE			>	MALE			
		N	Mean	Std. Dev.	N	Mean	Std. Dev.	t	p
ANTERIOR HOOKS:	LONGEST HOOK	76	66.4	6.17	64	61.9	5.14	4.73	***
	HOOK 1	74	59.2	9.18	64	54.3	7.63	3.45	***
	HOOK 2	76	64.8	5.95	64	61.2	5.31	3.81	***
	HOOK 3	76	60.8	6.58	62	56.7	6.27	3.71	***
	STOUTEST HOOK	74	51.8	5.94	62	47.4	5.07	4.67	***
POSTERIOR HOOKS:	HOOK 7	76	37.0	5.93	64	34.6	5.69	2.47	*
	HOOK 8	76	35.9	6.08	64	35.1	5.98	0.78	NS
	HOOK 9	75	36.0	7.41	64	34.0	5.62	1.83	NS
	BASASL HOOK	76	45.70	9.68	63	40.8	7.85	3.32	**

Table 3.3.10: Summary statistics of hook lengths for male and female *P. laevis* of English origin.

		FEMALE			>	MALE			
		N	Mean	Std. Dev.	N	Mean	Std. Dev.	t	p
ANTERIOR HOOKS:	LONGEST HOOK	44	66.7	6.86	39	62.8	7.1	2.54	*
	HOOK 1	44	53.8	10.11	39	50.9	10.41	1.3	NS
	HOOK 2	44	63.9	7.14	39	59.9	9.45	2.12	*
	HOOK 3	44	64.3	7.15	39	59.3	8.38	2.89	**
	STOUTEST HOOK	44	46.0	4.87	39	42.7	6.50	2.64	**
POSTERIOR HOOKS:	HOOK 7	44	29.4	7.12	39	25.6	7.28	2.37	*
	HOOK 8	43	29.4	7.43	39	26.3	6.24	2.04	*
	HOOK 9	41	28.9	7.65	38	25.6	6.55	2.01	*
	BASASL HOOK	43	42.4	9.08	39	36.8	7.76	3.05	**

Table 3.3.11: Summary statistics of hook widths for male and female *P. laevis* of Irish origin.

		FEMALE			>	MALE			
		N	Mean	Std. Dev.	N	Mean	Std. Dev.	t	p
ANTERIOR HOOKS:	HOOK 1	76	13.0	2.89	64	12.4	2.56	1.42	NS
	HOOK 2	76	15.9	2.54	64	15.2	2.21	1.67	NS
	HOOK 3	76	17.2	2.84	64	16.8	2.77	0.78	NS
	STOUTEST HOOK	76	21.8	3.35	64	20.7	2.86	2.06	*
POSTERIOR HOOKS:	HOOK 7	76	9.1	1.66	64	8.4	1.27	3.02	**
	HOOK 8	76	9.1	2.0	64	8.5	1.46	2.11	*
	HOOK 9	75	9.2	2.10	63	8.3	1.52	3.05	**
	BASASL HOOK	76	12.8	3.5	63	11.0	5.01	2.44	*

Table 3.3.12: Summary statistics of hook widths for male and female *P. laevis* of English origin.

		FEMALE			>	MALE			
		N	Mean	Std. Dev.	N	Mean	Std. Dev.	t	p
ANTERIOR HOOKS:	HOOK 1	44	10.8	2.87	39	10.6	2.32	0.24	NS
	HOOK 2	44	13.6	2.13	39	12.7	1.78	2.03	*
	HOOK 3	44	14.6	2.09	39	13.6	1.86	2.35	*
	STOUTEST HOOK	44	19.6	2.56	38	17.9	2.22	3.29	**
POSTERIOR HOOKS:	HOOK 7	43	7.8	1.43	39	7.0	1.18	2.77	**
	HOOK 8	44	7.2	1.52	39	6.7	1.03	1.8	NS
	HOOK 9	41	6.9	1.52	38	6.5	1.44	1.16	NS
	BASASL HOOK	43	8.9	1.81	39	7.7	1.83	2.92	**

Table 3.3.13: Summary statistics of hook area for male and female *P. laevis* of Irish origin.

		FEMALE			>	MALE			
		N	Mean	Std. Dev.	N	Mean	Std. Dev.	t	p
ANTERIOR HOOKS:	HOOK 1	74	391.6	129.50	64	341.9	106.64	2.47	*
	HOOK 2	76	517.1	111.24	64	466.5	88.97	2.99	**
	HOOK 3	76	525.5	114.82	62	477.3	103.54	2.59	**
	STOUTEST HOOK	74	565.8	125.27	62	492.1	97.77	3.85	***
POSTERIOR HOOKS:	HOOK 7	76	171.5	52.97	64	146.5	39.62	3.19	**
	HOOK 8	76	167.2	58.78	64	151.7	45.77	1.76	NS
	HOOK 9	75	170.7	67.73	63	141.7	43.11	3.04	**
	BASASL HOOK	76	303.4	138.70	63	228.1	110.62	3.56	***

Table 3.3.14: Summary statistics of hook area for male and female *P. laevis* of English origin.

		FEMALE			>	MALE			
		N	Mean	Std. Dev.	N	Mean	Std. Dev.	t	p
ANTERIOR HOOKS:	HOOK 1	44	300.7	119.90	39	277.0	98.80	0.99	NS
	HOOK 2	44	435.6	94.68	39	282.2	87.04	2.68	**
	HOOK 3	44	470.9	94.50	39	403.5	83.11	3.46	***
	STOUTEST HOOK	44	453.1	85.41	38	383.7	86.52	3.64	***
POSTERIOR HOOKS:	HOOK 7	43	116.0	43.03	39	89.7	33.85	3.09	**
	HOOK 8	44	107.9	40.51	39	87.8	24.59	2.76	**
	HOOK 9	41	103.0	44.17	38	83.28	30.11	2.33	*
	BASASL HOOK	43	191.8	69.95	39	144.3	56.65	3.39	**

Table 3.3.15: Summary statistics of hook ratio for male and female *P. laevis* of Irish origin.

		FEMALE			>	MALE			
		N	Mean	Std. Dev.	N	Mean	Std. Dev.	t	p
ANTERIOR HOOKS:	HOOK 1	74	0.22	0.03	64	0.23	0.034	1.76	NS
	HOOK 2	76	0.25	0.04	64	0.25	0.034	0.64	NS
	HOOK 3	76	0.29	0.05	62	0.30	0.06	1.52	NS
	STOUTEST HOOK	74	0.42	0.06	62	0.44	0.07	1.70	NS
POSTERIOR HOOKS:	HOOK 7	76	0.25	0.04	64	0.25	0.04	0.45	NS
	HOOK 8	76	0.26	0.04	64	0.24	0.04	1.53	NS
	HOOK 9	75	0.26	0.05	63	0.25	0.04	1.62	NS
	BASASL HOOK	76	0.28	0.06	63	0.27	0.16	0.33	NS

Table 3.3.16: Summary statistics of hook ratio for male and female *P. laevis* of English origin.

		FEMALE			>	MALE			
		N	Mean	Std. Dev.	N	Mean	Std. Dev.	t	p
ANTERIOR HOOKS:	HOOK 1	44	0.20	0.03	39	0.21	0.06	1.35	NS
	HOOK 2	44	0.22	0.04	39	0.22	0.05	0.37	NS
	HOOK 3	44	0.23	0.04	39	0.24	0.06	0.50	NS
	STOUTEST HOOK	44	0.43	0.06	38	0.43	0.07	0.18	NS
POSTERIOR HOOKS:	HOOK 7	42	0.26	0.04	38	0.28	0.08	1.04	NS
	HOOK 8	43	0.25	0.06	38	0.26	0.07	0.67	NS
	HOOK 9	40	0.24	0.05	37	0.25	0.08	0.89	NS
	BASASL HOOK	43	0.21	0.05	39	0.21	0.06	0.04	NS

between the sexes of Irish *P. laevis* except for those of hooks 8 and 9 (table 3.3.9). Except for hook 1, English females had significantly longer hooks than males (table 3.3.10). The widths of the first three anterior hooks did not differ between Irish males and females though they did differ for the remaining hooks (table 3.3.11). The hook widths of English females were significantly larger than those of males except in the case of hooks 1, 8 and 9 (table 3.3.12). Irish females had larger hook areas than males though no significant difference was found for the area of hook 8 (table 3.3.13). Again the hook areas of English females were larger than those of males though not in the case of hook 1 (table 3.3.14). Hook shape is the only character which showed no significant sexual dimorphism in both Irish and English *P. laevis* (tables 3.3.15 and 3.3.16).

Effect of number of hooks per row on hook size

As already explained in materials and methods, not all hooks have been compared due to the fact that individual worms have different numbers of hooks per row. Therefore hooks which were common to all worms, whether those worms had 9 or 13 hooks per row were chosen for the analyses. Whether or not the number of hooks per row had a general effect on hook size was investigated with a two-way ANOVA. In most cases the number of hooks per row had no effect on hook length or width.

Number of hooks per row did have an effect on the lengths of hooks 1, 3 and 9 of Irish *P. laevis* (table 3.3.17). The length of hook 3 increased with an increase in hook

Table 3.3.17: Results showing the effects of number of hooks per row and parasite sex on the hook lengths of Irish *P. laevis*.

	SEX			HOOKS/ROW			SEX*HOOKS/ROW		
	F-stat	df	p	F-stat	df	p	F-stat	df	p
LONGEST HOOK	13.0	1, 136*	***	0.9	3, 136	NS	0.2	3, 136	NS
HOOK 1	7.3	1, 134	**	3.1	3, 134	*	0.5	3, 134	NS
HOOK 2	9.5	1, 136	**	1.0	3, 136	NS	0.1	3, 136	NS
HOOK 3	10.8	1, 134	**	8.6	3, 134	***	0.2	3, 134	NS
STOUTEST HOOK	11.5	1, 132	***	1.8	3,132	NS	0.5	3,132	NS
HOOK 7	4.0	1, 136	*	1.9	3, 136	NS	0.1	3, 136	NS
HOOK 8	0.4	1, 136	NS	2.1	3, 136	NS	0.0	3, 136	NS
HOOK 9	1.9	1, 136	NS	3.2	3, 136	*	0.6	3, 136	NS
BASAL HOOK	6.4	1, 135	*	1.3	3, 135	NS	0.2	3, 135	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

Table 3.3.18: Results showing the effects of number of hooks per row and parasite sex on the hook widths of Irish *P. laevis*.

	SEX			HOOKS/ROW			SEX*HOOKS/ROW		
	F-stat	df	p	F-stat	df	p	F-stat	df	p
HOOK 1	2.8	1, 136*	NS	4.8	3, 136	**	0.5	3, 136	NS
HOOK 2	2.2	1, 136	NS	0.7	3, 136	NS	0.6	3, 136	NS
HOOK 3	0.6	1, 136	NS	2.3	3, 136	NS	0.3	3, 136	NS
STOUTEST HOOK	4.5	1, 136	*	0.7	3, 136	NS	0.2	3, 136	NS
HOOK 7	12.2	1, 136	***	2.6	3, 136	*	1.2	3, 136	NS
HOOK 8	3.6	1, 136	NS	0.9	3, 136	NS	0.2	3, 136	NS
HOOK 9	8.6	1, 136	**	1.2	3, 136	NS	0.7	3, 136	NS
BASAL HOOK	16.4	1, 134	***	3.4	3, 136	*	0.2	3, 136	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

number. The third hooks from individuals with 11($p < 0.05$, Scheffe Post-Hoc test), 12 ($p < 0.0001$, SPH test) and 13 hooks ($p < 0.01$, SPH test) were significantly longer than those hooks from worms with 10 hooks per row. For hook 9, hooks increased in size with number of hooks per row but no significant differences were elucidated by a Scheffe Post-Hoc test. The opposite situation was true for hook 1. The length of this hook seemed to decrease with an increase in number of hooks per row though significant differences were not found with a Scheffe Post-Hoc test.

Table 3.3.19: Results showing the effects of number of hooks per row and parasite sex on the hook lengths of English *P. laevis*.

	SEX			HOOKS/ROW			SEX*HOOKS/ROW		
	F-stat	df	p	F-stat	df	p	F-stat	df	p
LONGEST HOOK	3.0	1, 79*	NS	0.2	2, 79	NS	1.8	2, 79	NS
HOOK 1	0.0	1, 79*	NS	1.0	2, 79	NS	1.5	2, 79	NS
HOOK 2	1.2	1, 79*	NS	0.4	2, 79	NS	1.3	2, 79	NS
HOOK 3	2.6	1, 79*	NS	0.1	2, 79	NS	1.3	2, 79	NS
STOUTEST HOOK	4.8	1, 79*	*	0.3	2, 79	NS	1.0	2, 79	NS
HOOK 7	1.7	1, 76	NS	0.9	2, 79	NS	0.0	2, 79	NS
HOOK 8	2.4	1, 77	NS	0.2	2, 77	NS	0.1	2, 77	NS
HOOK 9	4.3	1, 76	*	0.1	2, 76	NS	1.2	2, 76	NS
BASAL HOOK	9.3	1, 78	**	1.6	2, 78	NS	1.1	2, 78	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

Table 3.3.20: Results showing the effects of number of hooks per row and parasite sex on the hook widths of English *P. laevis*.

	SEX			HOOKS/ROW			SEX*HOOKS/ROW		
	F-stat	df	p	F-stat	df	p	F-stat	df	p
HOOK 1	0.0	1, 79*	NS	0.5	2, 79	NS	0.3	2, 79	NS
HOOK 2	0.0	1, 79	NS	0.5	2, 79	NS	0.3	2, 79	NS
HOOK 3	3.5	1, 79	NS	1.0	2, 79	NS	0.6	2, 79	NS
STOUTEST HOOK	7.3	1, 78	**	1.2	2, 78	NS	1.0	2, 78	NS
HOOK 7	2.2	1, 78	NS	1.4	2, 78	NS	0.3	2, 78	NS
HOOK 8	0.7	1, 79	NS	2.2	2, 79	NS	0.3	2, 79	NS
HOOK 9	0.7	1, 78	NS	0.1	2, 78	NS	0.0	2, 78	NS
BASAL HOOK	9.1	1, 78	**	0.7	2, 78	NS	1.9	2, 78	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

The width of hooks 1 and 7 and of the basal hook also seemed to be affected by the number of hooks per row for Irish *P. laevis* (table 3.3.18). The width of hook 1 reflected the pattern shown by the length of this hook. The width of hook 1 from worms with 10 ($p < 0.05$, SPH test) and 11 hooks ($p < 0.01$, SPH test) per row were significantly bigger than that from worms with 12 hooks per row. The width of hook 7 and the basal hook seemed to increase with the hook number. None of the lengths and widths of English hooks were affected by hook number (tables 3.3.19 and 3.3.20). There were also no interactions between number of hooks per row and parasite sex in either sample.

Seasonal effects within the Irish sample

The number of hooks per row, the number of longitudinal rows and the position

Table 3.3.21: Results showing the effects of season on four hook characters of male and female Irish *P. laevis*. Missing values (-) indicate insufficient cell size.

	FEMALE		MALE	
	χ^2 (df)	p	χ^2 (df)	p
NO. HOOKS/ROW	8.83 (6)	NS	3.29 (4)	NS
NO. LONGITUDINAL ROWS	5.40 (4)	NS	0.91 (4)	NS
POSITION OF LONGEST HOOK	4.91 (4)	NS	-	-
POSITION OF STOUTEST HOOK	1.79 (2)	NS	7.92 (2)	*

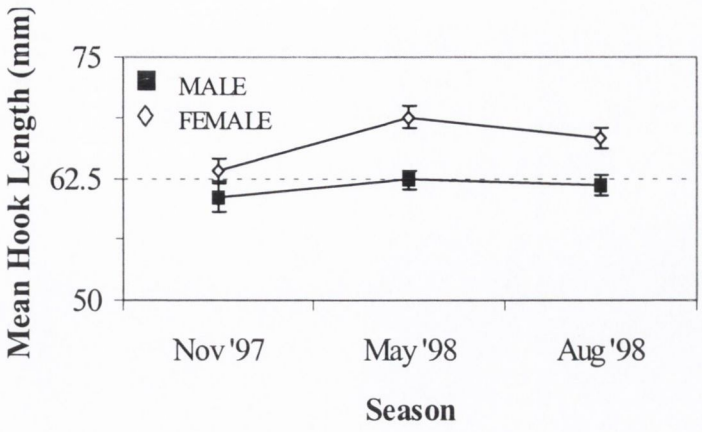


Fig. 3.3.23: Change in mean length of the longest hook with season for male and female *P. laevis* of Irish origin.

of the longest hook did not change with season for Irish *P. laevis* (table 3.3.21) as predicted by the null hypothesis, which states that these characters should not change with season. The position of the stoutest hook of males did change with season. The stoutest hook was found at the 5th position more often than expected in August and less often at the 4th position. In November the opposite is true, with more of the stoutest hooks found at the 4th position.

Statistically significant differences were found in the length, width, area and shape of Irish hooks, with a few exceptions (tables 3.3.22 –3.3.25). The growth pattern of the hooks is slightly different to that of the non-hook characters. Instead of

Table 3.3.22: Results showing the effects of season and parasite sex on the hook lengths of Irish *P. laevis*.

	SEX			SEASON			SEX*SEASON		
	F-stat	df	p	F-stat	df	p	F-stat	df	p
LONGEST HOOK	20.4	1, 139*	***	4.2	2, 139	*	1.2	2, 139	NS
HOOK 1	10.9	1, 137	**	3.4	2, 137	*	1.1	2, 137	NS
HOOK 2	13.4	1, 139	***	4.1	2, 139	*	1.3	2, 139	NS
HOOK 3	12.5	1, 137	***	3.2	2, 137	*	0.9	2, 137	NS
STOUTEST HOOK	14.8	1, 135	***	0.3	2, 135	NS	1.1	2, 135	NS
HOOK 7	4.4	1, 139	*	3.1	2, 139	*	2.3	2, 139	NS
HOOK 8	0.4	1, 139	NS	2.6	2, 139	NS	3.1	2, 139	*
HOOK 9	1.8	1, 138	NS	3.1	2, 138	*	4.2	2, 138	*
BASAL HOOK	8.3	1, 138	**	0.4	2, 138	NS	1.6	2, 138	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

Table 3.3.23: Results showing the effects of season and parasite sex on the hook widths of Irish *P. laevis*.

	SEX			SEASON			SEX*SEASON		
	F-stat	df	p	F-stat	df	p	F-stat	df	p
HOOK 1	2.2	1, 139*	NS	3.6	2, 139	*	0.9	2, 139	NS
HOOK 2	3.1	1, 139	NS	6.5	2, 139	**	1.9	2, 139	NS
HOOK 3	0.9	1, 139	NS	4.5	2, 139	*	0.8	2, 139	NS
STOUTEST HOOK	3.7	1, 139	NS	5.9	2, 139	**	1.3	2, 139	NS
HOOK 7	8.6	1, 139	**	10.6	2, 139	***	2.1	2, 139	NS
HOOK 8	4.0	1, 139	*	7.7	2, 139	***	3.0	2, 139	NS
HOOK 9	8.6	1, 137	**	7.4	2, 137	***	2.0	2, 137	NS
BASAL HOOK	6.6	1, 138	*	2.2	2, 138	NS	1.5	2, 138	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

increasing from November through to August, the hooks increased in size from November to May and peaked in May. In August the size of the hook either reached a plateau or slightly decreased.

There were no significant changes in the lengths of the stoutest hook, hook 8 and the basal hook. The lengths of hooks 1, 2 and the longest hook and the widths of hooks 1, 2 and 3 increased from November to May, where they peaked and decreased slightly in August (figures 3.3.23 and 3.3.24). The length of hook 3 stabilised in August as did the length and width of the stoutest hook. The length of hook 7 continued to increase

Table 3.3.24: Results showing the effects of season and parasite sex on the hook areas of Irish *P. laevis*.

	SEX			SEASON			SEX*SEASON		
	F-stat	df	p	F-stat	df	p	F-stat	df	p
HOOK 1	6.2	1, 137*	*	4.0	2, 137	*	1.0	2, 137	NS
HOOK 2	9.2	1, 139	**	8.3	2, 139	***	2.7	2, 139	NS
HOOK 3	6.7	1, 137	**	6.0	2, 137	**	1.6	2, 137	NS
STOUTEST HOOK	11.2	1, 135	**	3.6	2, 135	*	1.9	2, 135	NS
HOOK 7	8.7	1, 139	**	8.0	2, 139	***	2.9	2, 139	NS
HOOK 8	2.7	1, 139	NS	6.4	2, 139	**	3.6	2, 139	*
HOOK 9	8.0	1, 137	**	7.6	2, 137	***	3.0	2, 137	*
BASAL HOOK	10.8	1, 138	**	1.3	2, 138	NS	1.9	2, 138	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

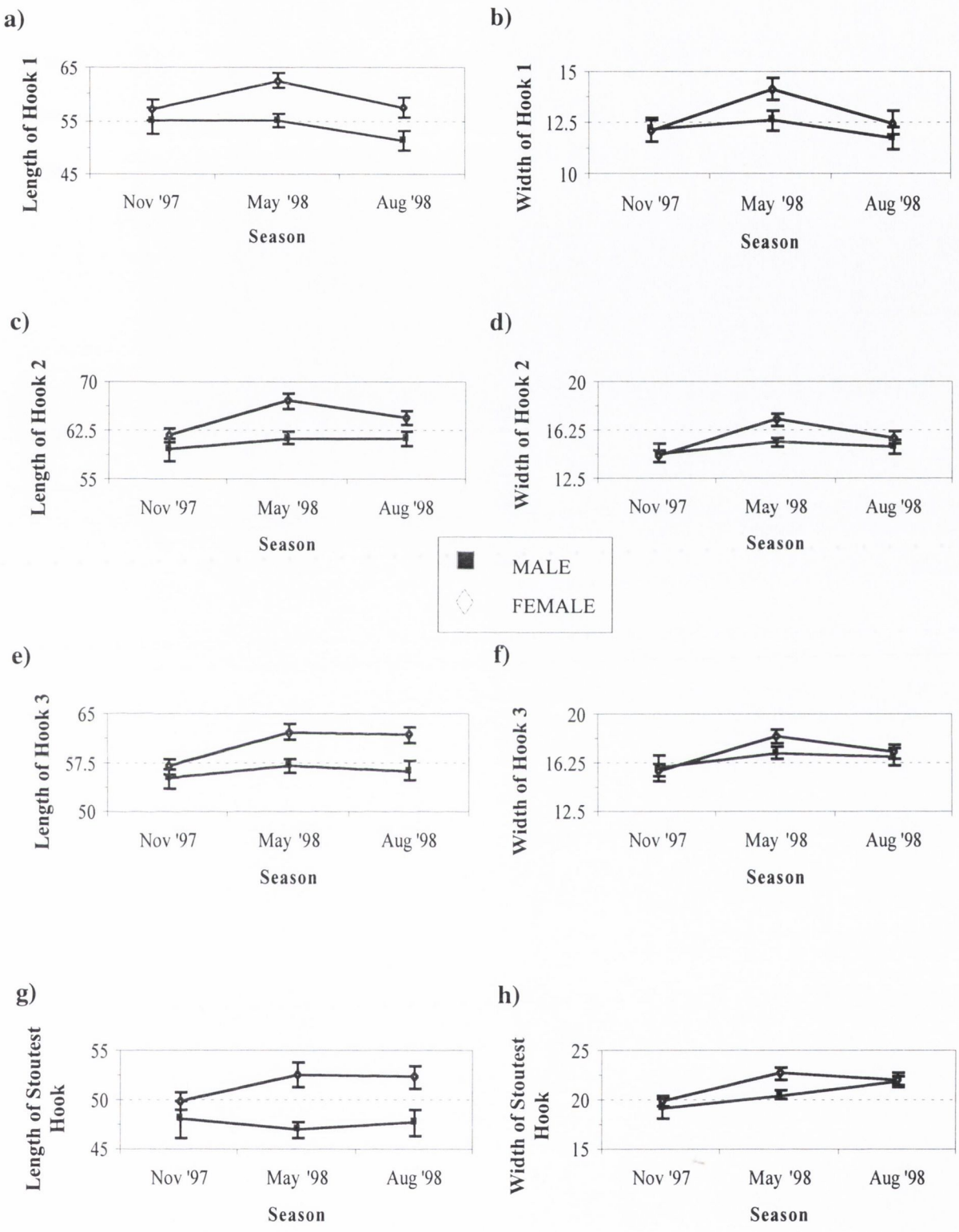


Fig. 3.3.24 Change in mean anterior hook length and width **(a -f)** and mean stoutest hook length and width **(g - h)** with season for male and female *P. laevis* of Irish origin. Error bars represent standard error.

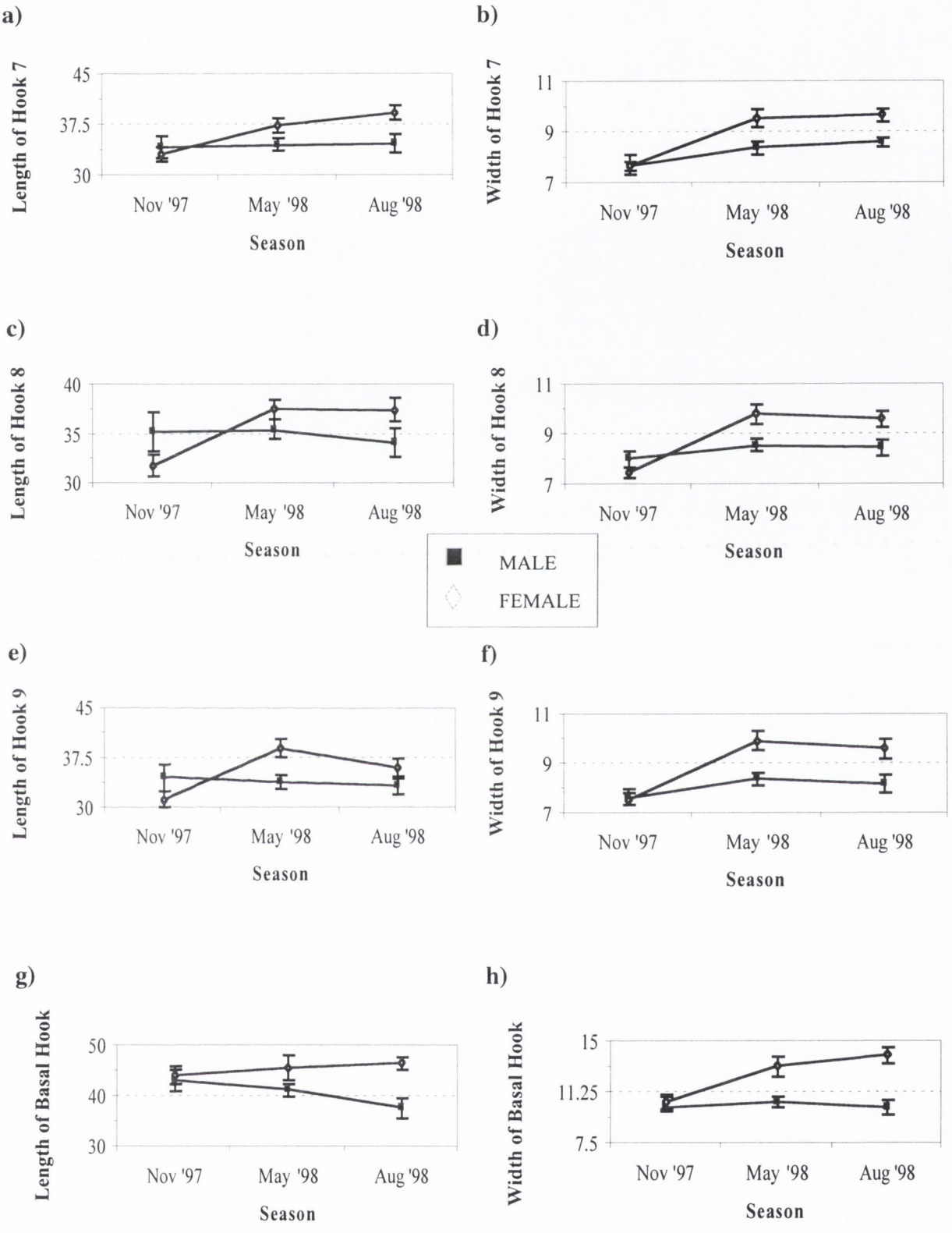


Fig. 3.3.25 Change in mean posterior hook length and width **(a -f)** and mean basal hook length and width **(g - h)** with season for male and female *P. laevis* of Irish origin. Error bars represent standard error.

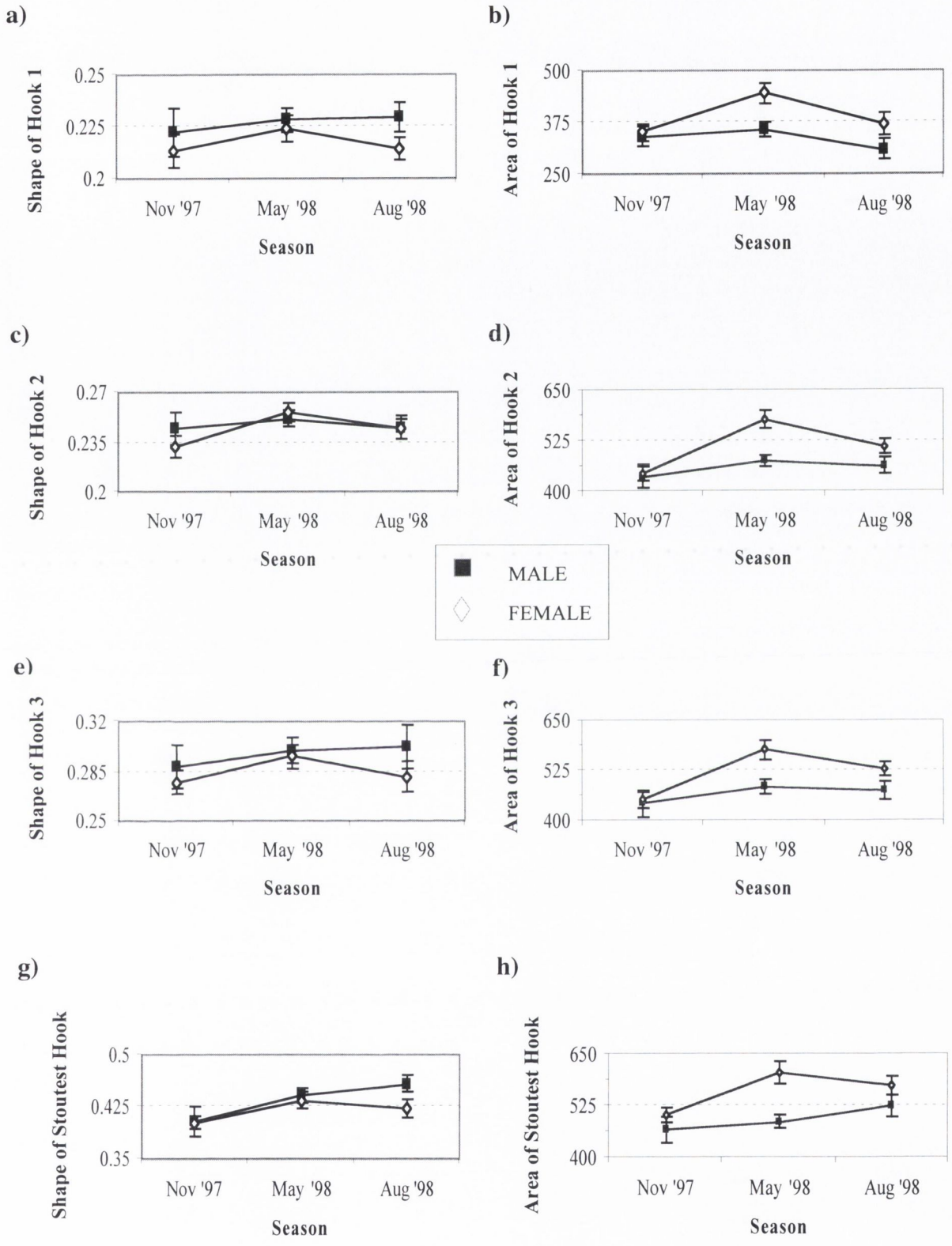


Fig. 3.3.26 Change in mean anterior hook shape and area **(a -f)** and mean stoutest hook shape and area **(g - h)** with season for male and female *P. laevis* of Irish origin. Error bars represent standard error.

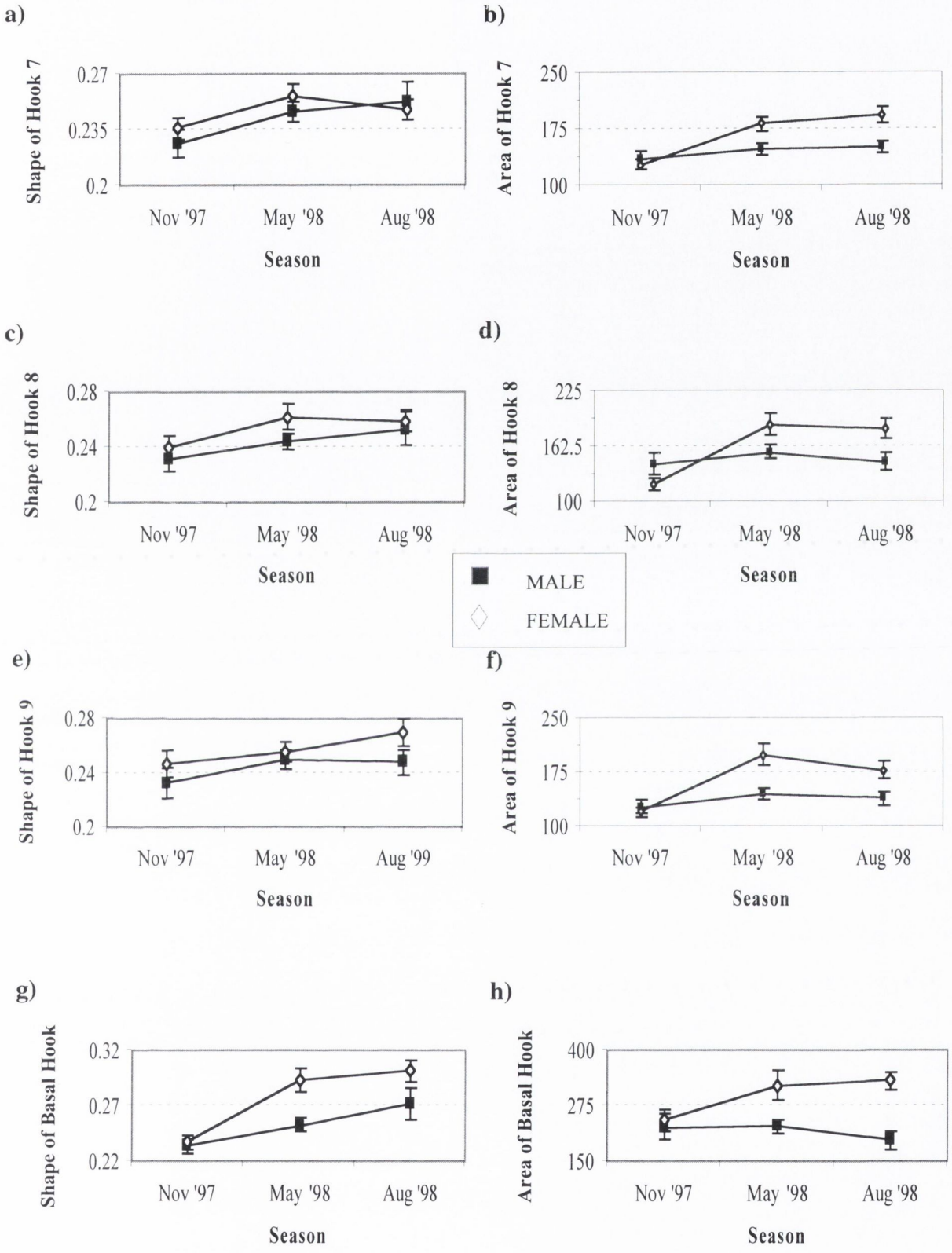


Fig. 3.3.27 Change in mean posterior hook shape and area **(a -f)** and mean basal hook shape and area **(g - h)** with season for male and female *P. laevis* of Irish origin. Error bars represent standard error.

from May to August and the length of hook 9 slightly decreased (figure 3.3.25). The widths of the posterior hooks seemed to stabilise in August. Basal hook width did not differ significantly between seasons. Neither were there any differences in anterior hook shape (figure 3.3.26). The shape of the stoutest hook did change significantly, increasing in value from November to May and stabilising in August. The area of the anterior hooks increased from November to May where it peaked and then dropped slightly in August. Hook 7 is the only posterior hook to change in shape significantly with season (figure 3.3.27). The shape of this hook increased from November to May and stabilised in August. The area of the posterior hooks followed the growth trend of the shape of hook 7. Neither the shape nor area of the basal hook showed any significant change. For more information on the statistical significance of the changes in hook dimensions with time, refer to Appendix A.

Table 3.3.25: Results showing the effects of season and parasite sex on the hook ratios of Irish *P. laevis*.

	SEX			SEASON			SEX*SEASON		
	F-stat	df	p	F-stat	df	p	F-stat	df	p
HOOK 1	2.3	1, 137*	NS	0.6	2, 137	NS	0.4	2, 137	NS
HOOK 2	0.2	1, 139	NS	2.0	2, 139	NS	0.5	2, 139	NS
HOOK 3	1.6	1, 137	NS	0.8	2, 137	NS	0.3	2, 137	NS
STOUTEST HOOK	1.7	1, 135	NS	3.7	2, 135	*	0.9	2, 135	NS
HOOK 7	0.5	1, 139	NS	3.3	2, 139	*	0.6	2, 139	NS
HOOK 8	2.0	1, 139	NS	2.2	2, 139	NS	0.3	2, 139	NS
HOOK 9	3.0	1, 137	NS	1.6	2, 137	NS	0.5	2, 137	NS
BASAL HOOK	0.4	1, 138	NS	2.3	2, 138	NS	0.2	2, 138	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

Linear Regression of hook characters with proboscis length

A linear regression analysis was carried out between hook length, width, area and shape and proboscis length for both Irish and English *P. laevis* (tables 3.3.26 – 3.3.33). Generally there is little relationship between the dimensions of the hooks and the length of the proboscis (figure 3.3.28). In a few cases there are weak coefficients of determination, highlighted in bold in the tables. For Irish *P. laevis* these include the length of hooks 3 and 7, the width of hook 7 and the basal hook and the area of hook 7. The coefficients of determination for these hooks fall in the range of 20 – 27%, which

does not indicate a particularly strong relationship. For English *P. laevis* the width and area of hook 3 and the stoutest hook also show a weak relationship. For the males of both populations in particular, hook size seems to be completely unrelated to proboscis length. Refer to Appendix B for more graphical representations of hook size against proboscis length.

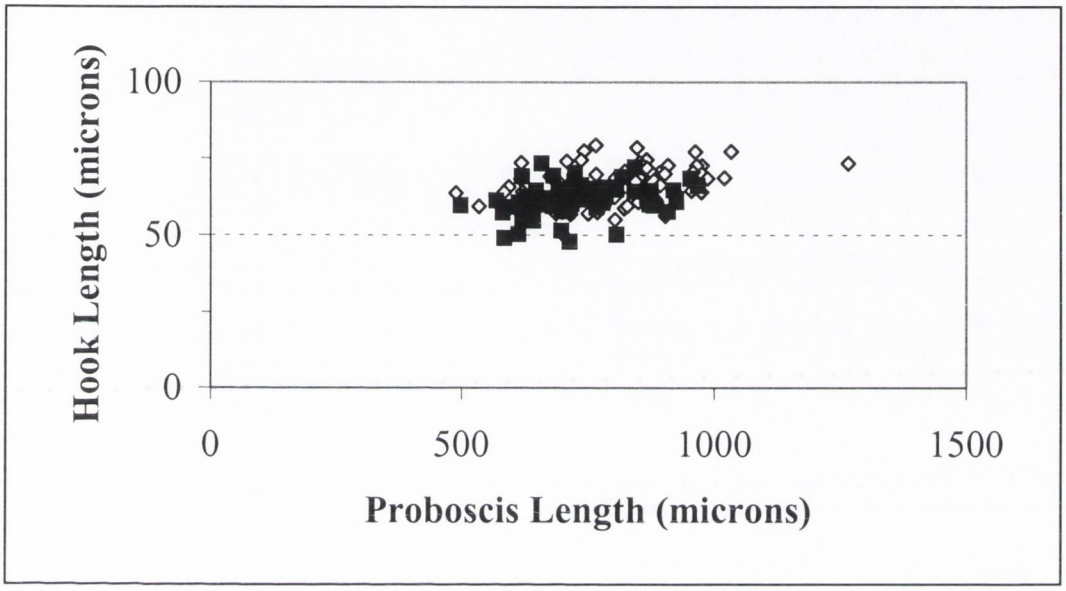


Fig. 3.3.28: Scatterplot of the length of the longest hook against proboscis length for male and female *P. laevis* of Irish origin.

Table 3.3.26: Regression of hook length with proboscis length for male and female *P. laevis* of Irish origin.

		FEMALE			MALE		
		R^2 (%)	t-ratio	p	R^2 (%)	t-ratio	p
ANTERIOR HOOKS	LONGEST HOOK	12.0	12.20	***	7.4	2.21	*
	HOOK 1	0.2	-0.34	NS	0.1	0.28	NS
	HOOK 2	12.0	3.17	***	7.0	2.14	*
	HOOK 3	**26.9**	5.21	***	**20.4**	3.89	***
POSTERIOR HOOKS	STOUTEST HOOK	10.8	2.95	**	12.5	2.91	**
	HOOK 7	**23.1**	4.71	***	5.7	1.91	NS
	HOOK 8	18.6	4.11	***	7.9	2.28	*
	HOOK 9	13.2	3.33	**	7.7	2.26	*
	BASAL HOOK	4.1	1.79	NS	0.7	0.66	NS

Table 3.3.27: Regression of hook length with proboscis length for male and female *P. laevis* of English origin.

		FEMALE			MALE		
		R^2 (%)	t-ratio	p	R^2 (%)	t-ratio	p
ANTERIOR HOOKS	LONGEST HOOK	0.8	0.57	NS	5.9	1.52	NS
	HOOK 1	1.0	0.64	NS	0.1	-0.17	NS
	HOOK 2	1.0	-0.64	NS	3.8	1.21	NS
	HOOK 3	4.9	1.47	NS	9.2	1.9	NS
POSTERIOR HOOKS	STOUTEST HOOK	6.9	1.76	NS	5.9	1.53	NS
	HOOK 7	0.1	-0.18	NS	7.9	1.78	NS
	HOOK 8	0.5	0.46	NS	0.5	0.44	NS
	HOOK 9	0.6	0.49	NS	1.0	0.60	NS
	BASAL HOOK	0.1	0.18	NS	1.5	0.75	NS

Table 3.3.28: Regression of hook width with proboscis length for male and female *P. laevis* of Irish origin.

		FEMALE			MALE		
		R^2 (%)	t-ratio	p	R^2 (%)	t-ratio	p
ANTERIOR HOOKS	HOOK 1	0.5	-0.60	NS	0	0.08	NS
	HOOK 2	0.1	0.31	NS	1.8	1.06	NS
	HOOK 3	0.3	0.45	NS	0.8	0.71	NS
	STOUTEST HOOK	5.6	2.09	*	2.0	1.11	NS
POSTERIOR HOOKS	HOOK 7	**20.2**	4.33	***	1.1	0.82	NS
	HOOK 8	10.6	2.96	**	4.2	1.63	NS
	HOOK 9	12.5	3.23	**	1.7	1.01	NS
	BASAL HOOK	**24.9**	4.95	***	0.1	0.24	NS

Table 3.3.29: Regression of hook width with proboscis length for male and female *P. laevis* of English origin.

		FEMALE			MALE		
		R^2 (%)	t-ratio	p	R^2 (%)	t-ratio	p
ANTERIOR HOOKS	HOOK 1	3.9	1.3	NS	2.6	-1.0	NS
	HOOK 2	0.6	0.51	NS	0.1	0.23	NS
	HOOK 3	**27.1**	3.95	***	3.4	1.14	NS
	STOUTEST HOOK	**29.3**	4.18	***	6.4	1.57	NS
POSTERIOR HOOKS	HOOK 7	4.4	1.37	NS	15.4	2.59	*
	HOOK 8	19.3	3.17	**	9.7	2.0	*
	HOOK 9	7.8	1.81	NS	3.8	1.19	NS
	BASAL HOOK	12.6	2.43	*	0	-0.003	NS

Table 3.3.30: Regression of hook area with proboscis length for male and female *P. laevis* of Irish origin.

		FEMALE			MALE		
		R^2 (%)	t-ratio	p	R^2 (%)	t-ratio	p
ANTERIOR HOOKS	HOOK 1	0.3	-0.45	NS	0.0	0.03	NS
	HOOK 2	3.0	1.51	NS	4.6	1.72	NS
	HOOK 3	8.3	2.59	*	8.3	2.31	*
	STOUTEST HOOK	8.1	2.52	*	7.4	2.17	*
POSTERIOR HOOKS	HOOK 7	**25.6**	5.05	***	3.2	1.42	NS
	HOOK 8	16.5	3.83	***	6.5	2.05	*
	HOOK 9	14.7	3.55	***	4.9	1.77	NS
	BASAL HOOK	12.3	3.22	**	0.3	0.39	NS

Table 3.3.31: Regression of hook area with proboscis length for male and female *P. laevis* of English origin.

		FEMALE			MALE		
		R^2 (%)	t-ratio	p	R^2 (%)	t-ratio	p
ANTERIOR HOOKS	HOOK 1	2.3	1.0	NS	1.3	-0.7	NS
	HOOK 2	0.0	-0.07	NS	2.2	0.9	NS
	HOOK 3	**23.2**	3.56	***	9.9	2.01	*
	STOUTEST HOOK	**26.9**	3.94	***	7.2	1.67	NS
POSTERIOR HOOKS	HOOK 7	0.7	0.55	NS	12.6	2.31	*
	HOOK 8	6.7	1.74	NS	4.5	1.31	NS
	HOOK 9	4.8	1.4	NS	1.8	0.80	NS
	BASAL HOOK	5.3	1.51	NS	0.3	0.32	NS

Table 3.3.32: Regression of hook ratio with proboscis length for male and female *P. laevis* of Irish origin.

		FEMALE			MALE		
		R^2 (%)	t-ratio	p	R^2 (%)	t-ratio	p
ANTERIOR HOOKS	HOOK 1	3.3	-1.58	NS	0.0	0.17	NS
	HOOK 2	1.0	-0.87	NS	0.0	-0.15	NS
	HOOK 3	7.4	-2.43	*	4.6	-1.68	NS
	STOUTEST HOOK	0.3	-0.44	NS	2.1	-1.13	NS
POSTERIOR HOOKS	HOOK 7	0.3	0.44	NS	1.6	-0.99	NS
	HOOK 8	0	0.07	NS	0.7	-0.66	NS
	HOOK 9	0.1	0.19	NS	4.0	-1.59	NS
	BASAL HOOK	**20.0**	4.3	***	0.0	0.02	NS

Table 3.3.33: Regression of hook ratio with proboscis length for male and female *P. laevis* of English origin.

		FEMALE			MALE		
		R^2 (%)	t-ratio	p	R^2 (%)	t-ratio	p
ANTERIOR HOOKS	HOOK 1	5.2	1.52	NS	2.2	-0.91	NS
	HOOK 2	2.2	0.98	NS	2.2	-0.91	NS
	HOOK 3	10.3	2.2	*	1.4	-0.72	NS
	STOUTEST HOOK	7.4	1.83	NS	0.1	-0.21	NS
POSTERIOR HOOKS	HOOK 7	0.7	0.52	NS	6.1	-1.53	NS
	HOOK 8	3.1	1.15	NS	0.7	-0.52	NS
	HOOK 9	0.1	0.20	NS	1.5	-0.72	NS
	BASAL HOOK	7.6	1.84	NS	3.6	-1.18	NS

3.4 DISCUSSION

Non-Hook Characters

Overall variability

The variability shown by non-hook characters was similar in both populations of *P. laevis*. Body and trunk length, trunk width and bulb width were the most variable characters. This is not surprising if one has sampled a heterogeneous population consisting of established and newly acquired individuals. *P. laevis* can grow to lengths of over 20mm though its infective stage measures less than 5mm. Of the four external features measured, the proboscis proved the least variable and varied less in the English population. Though this finding is satisfying from a taxonomic point of view, the proboscis did vary in size and also overlapped in both populations to a large degree.

These findings agree with those of the two previous investigations into the morphology of this parasite. The measurements presented here overlap those of Brown (1987), the only difference being that these measurements are slightly more wide ranging. Brown sampled individuals from only one host, so the greater amount of variation encountered in this study is not unexpected. It is interesting though that Brown detected most of the variability of these characters with just one infrapopulation. These findings also agree with those of Edwards (Unpublished). More differences are seen between the wild Irish population and Edwards' laboratory grown Irish population. The proboscis of wild English *P. laevis* overlaps in length and width that of the laboratory population. Wild Irish *P. laevis* have probosces with more wide ranging dimensions than those grown in the laboratory. Bulb width was also similar between the wild and experimental populations of *P. laevis* from both countries.

Sexual dimorphism

Non-hook characters overlapped to a large extent between males and females of both populations, though in almost all cases females were significantly bigger. Neck length did not differ in the English population though the reasons for this are not clear. There were no differences in bulb width between Irish males and females. Bulb width was a very wide ranging character in both sexes and this may have masked any slight

differences between them. *P. laevis* is far less sexually dimorphic than other species *e.g. A. jacksoni*, the females of which are twice the size of the males (Bullock, 1962). Farrell (2001) also found the trunk length of female *Acanthocephalus clavula* to be almost twice as long as that of males. Both Brown (1987) and Edwards (Unpublished) found similar levels of sexual dimorphism in other populations of *P. laevis*.

Seasonal effects within Irish P. laevis

The seasons over which characters were compared represent a complete adult phase in the life cycle of *P. laevis*, from infection of the definitive host in November to maturation and reproduction in August. Because there is no overlap of generations in Irish *P. laevis*, the situation is much clearer. Parasites measured at the same time of year should be of an approximately similar age and developmental stage and therefore act as replicates. All characters increased between November 1997 and August 1998 with one exception. Female characters increased more than male characters. The pattern of growth was similar between all characters except for neck width. Most characters increased steadily from November to August or from May to August. The neck on the other hand increased in width between November and May and decreased in August by a similar amount. A possible explanation for this might be that the parasites are much heavier in August and this extra weight might cause the neck to stretch and therefore change the shape of the neck.

The danger of comparing samples collected at different times of the year is two fold. Parasites collected at different times of the year might differ in age and parasites, as most organisms, grow with age (Amin, 1975b). Also care should be taken when comparing parasites with seasonal cycles of growth. As has been shown clearly in the preceeding chapter, generations of English *P. laevis* overlap througout the year (Hine and Kennedy, 1974b; Brown, 1989), whereas Irish *P. laevis* have a distinctly seasonal growth cycle (Molloy *et al*, 1995). It seems more acceptable to compare this Irish sample, which contains three different monthly groups with an English sample taken from just one month as both should contain young and old parasites.

What may be contentious about these results is the extent to which the proboscis increased in size. Similar changes in character size between older and younger *E. salmonis* were recorded by Amin and Redlin (1980). Edwards (Unpublished) found no change in the size of the proboscis over the course of a 200 day experiment for males

and females of both populations. It is possible that the results presented here do not represent growth of the proboscis over time but rather the differential loss of individuals with smaller probosces. This is not wholly convincing though as proboscis size is still quite wide ranging in August and shows the continued survival of individuals with smaller probosces (to a minimum of 488.74μm). Edwards' experimental conditions may have been sufficiently different from those of the natural environment of *P. laevis* in Ireland as to cause a different pattern to emerge. It seems there is more evidence in favour of proboscis growth than against it, though due to the contradictory nature of the evidence the issue remains unresolved.

Relationship between non-hook characters and total length

The strong relationship between trunk length and trunk width and total length seen in both populations is not unexpected and is due to parasite growth. The size of the bulb is highly correlated with total length in Irish *P. laevis* though not in the English. This difference may be due to host suitability. Chub is considered a preferred definitive host for *P. laevis* and is known to mount a strong immunuological reaction against this parasite (Hine and Kennedy, 1974a). English *P. laevis* seem to have a fast growing bulb which does not grow at the same pace as the rest of the body. The fast growing bulb of English *P. laevis* is perhaps an adaptation to the immune response of chub. Proboscis characters in Irish *P. laevis* show only a weak relationship with body length. This may seem to contradict the previous finding of growth over time. Though it seems the proboscis of Irish *P. laevis* does grow to some degree, it does not do so in tandem with the rest of the parasite. The neck of English *P. laevis* has a strong relationship with body length which is not the case for Irish *P. laevis*. The neck of Irish *P. laevis* does increase in length between November and August though it does not seem to do so at the same rate as the entire body. Brown (1987) found a strong relationship between body length and width, body length and bulb size and body length and neck size. He found a very weak relationship between proboscis size and body length, which compares well with the findings presented here.

Hook Characters

Overall variability

Both English and Irish populations had numbers of longitudinal rows of hooks ranging between 13 and 19. This incorporates the range of 14 to 18 rows observed by Brown (1987) and is more wide ranging than the number of rows observed by Edwards (Unpublished) which was 15 to 18. The individuals with extreme numbers of rows *e.g.* those with 13, 14, 18 and 19 rows represent a very small proportion of the two samples. Irish *P. laevis* had higher ranging numbers of hooks per row, 9 to 14 than the English, which had 9 to 12 hooks. These values are again comparable to those observed by Brown (1987), 8 to 13 and Edwards, 9 to 13. The range of positions at which the stoutest hook is found overlaps between the two populations but is often found at a more posterior position, position 6 on the English proboscis. The range of positions at which the longest hook is found in English *P. laevis* is far greater than that of Irish *P. laevis*. Edwards found the longest hook of Irish *P. laevis* to be positioned most frequently at the second position, which corresponds to these findings. Edwards found the longest hook of English *P. laevis* most often at position 3, which differs from the wide range presented here.

Variability of the four hook characters, length, width, shape and area was very similar between the two populations. It is obvious from the barcharts of the length and area of hook 1, that this was the most variable hook. Hook shape was the least variable character both along the hook row and between populations. The stoutest hook had the most distinctive shape of all the hooks. The change in hook length and width between anterior and posterior hooks was also very similar between English and Irish *P. laevis*.

Sexual dimorphism

The number of longitudinal rows of hooks did not vary between the sexes. English females had slightly more hooks per row than males. Females had longer and wider hooks in almost all cases. The preceeding examples of sexual dimorphism have already been documented for the Acanthocephala (Bullock, 1962; Amin, 1974; Edwards, unpublished observations). Hook shape did not differ between the sexes of either population, which shows it to be the most conservative hook character.

Effect of number of hooks per row on hook size

Hooks did not differ in size between English worms with different numbers of hooks per row. Some hooks were found to differ in size in the Irish situation. Hook 1 was again the most variable hook. Irish individuals were still pooled together for comparisons of hook size with English *P. laevis*, as only 3 of the 8 hooks differed with hook number and these differences were not consistent with hook number.

Seasonal effects within Irish P. laevis

The anterior hooks excluding the stoutest hook and posterior hooks 7 and 9 were statistically longer in May than November. Hooks were also wider in May than November. Hook area reflected the same pattern but no change in hook shape was found. The seasonal pattern of change in hook size differs from the continued change of the non-hook characters. Hook size increased between November and May but not after May. The difference in patterns between males and females is striking. Male hooks do not seem to change as much as female hooks. Edwards (Unpublished) found no change in the size of the longest hook of either English and Irish *P. laevis* grown experimentally in rainbow trout, which contradicts these findings. Now that it is known that at the centre of the acanthocephalan hook lies a core of living material (Hutton and Oetinger, 1980) it is easier to accept the possibility of hook growth after the larval developmental phase.

Relationship between hook characters and proboscis length

Very little correlation was found between hook length, width, area and shape and proboscis length. Hooks and probosces of *P. laevis* from both populations seem to grow independently of one another.

Conclusions

This study represents the first comprehensive investigation of morphological variation in wild Irish *P. laevis* and only the second for wild English *P. laevis*. Modern techniques such as digital photography and analysis applied to this investigation has

increased reliability and reduced human error. Morphological variability due to parasite sex, season, parasite age and host species was detected. Seasonal variation in proboscis size and hook characters of Irish *P. laevis* was an unexpected result. These findings cast doubt over the genetic predetermination of proboscis and hook size though the results are not conclusive. English and Irish populations of *P. laevis* exhibited very similar levels of variability in all characters measured. In general the lengths and widths of hooks and non-hook characters overlapped between English and Irish worms. Edwards (Unpublished) concluded that there were sufficient differences between the means of such characters as to identify the two strains based on a survey of large samples. The variation uncovered in this study casts considerable doubt over this approach. Better, more clear cut characters are required in order to separate the strains with confidence.

CHAPTER 4

A MORPHOLOGICAL COMPARISON OF AN IRISH AND ENGLISH POPULATION OF *POMPHORHYNCHUS LAEVIS*

4.1 INTRODUCTION

This chapter is a logical continuation of chapter 3. In chapter 3 the degree of morphological variability exhibited by an Irish and English population of *P. laevis* was studied. Using the information gathered in chapter 3, these two populations will now be compared. The aim of this chapter is to identify any character(s) with which these two populations can be identified, if indeed they do differ at all. Each character, all non-hook and hook characters will be systematically compared using statistical tests, between the two groups. Finally, a multivariate analysis, Principal Components Analysis will be applied to the data in an attempt to split the two populations.

4.2 MATERIALS AND METHODS

The worms studied here are those which were used in chapter 3 to study the variability within an Irish and English population of P. *laevis*. Therefore the methods followed in their preparation are exactly those described in section 3.2 and will not be redescribed here. The characters compared between the two populations are those listed in table 3.2.1 and consist of hook and non-hook characters and some derived characters. The non-hook characters are the length and width of the trunk, neck, bulb and proboscis. The hook characters include the length and width of anterior hooks 1 to 3, posterior hooks 7 to 9 and the basal hook and also the the number of longitudinal rows and the number of hooks per longitudinal row.

Statistical Analysis

Non-hook characters were compared between English and Irish *P. laevis* using a two-way analysis of variance. The number of hooks per row, the number of longitudinal rows and the positions of the stoutest and longest hooks were compared between English and Irish samples using chi-squared analysis, as these data consist of frequencies. Hook lengths and widths were also compared using a two-way analysis of variance. A derived variable, anterior/posterior ratio was also studied. The ratio of number of anterior hooks to total hook number and that of number of posterior hooks to

total hook number was calculated for each worm. The posterior hook ratio was then divided into anterior hook ratio and 1 was subtracted from the result to give the anterior/posterior ratio for each worm.

A multivariate analysis, Principle Components Analysis was applied to the data. This is a data reduction technique, which allows one to examine all the variables describing the population(s) simultaneously. The method uses matrices to determine which of the variables are responsible for the majority of the variation in the data.

Table 4.2.1: Characters chosen for the Principal Components Analysis.

Total Length
Trunk Length and Width
Neck Length and Width
Bulb Length and Width
Proboscis Length and Width
Area of anterior hooks 1 – 3, posterior hooks 7 – 9, stoutest and basal hooks
Anterior /Posterior Ratio
Position of the Stoutest Hook

It produces new variables, the principle components, which are combinations of existing ones. This process makes the data more manageable as generally one only needs to look at two or three of the principle components in order to understand the data. Fifty characters describe each worm. It was decided to reduce this number in order to make the analysis more clear and easy to interpret. The characters chosen are listed in table 4.2.1. These characters were chosen as they highlighted the differences between the two populations while still maintaining the variability within the samples. It is advised to use only variables that have a multivariate normal distribution. Two non-normal variables are included in the analysis, the position of the stoutest hook and anterior/posterior hook ratio.

4.3 RESULTS

Non-Hook Characters

The results of the comparisons of all thirteen non-hook characters between English and Irish samples can be seen in table 4.3.1 (figures 4.3.1 and 4.3.2). In seven out of the thirteen cases, English *P. laevis* was statistically significantly larger than Irish *P. laevis*. Irish *P. laevis* were significantly bigger than the English in neck width, proboscis width and neck, bulb and proboscis shape (figures 4.3.1d, 4.3.2b, d –f).

Table 4.3.1: Comparison of non-hook characters between male and female *P. laevis* of English and Irish origin.

	SEX			COUNTRY			SEX*COUNTRY		
	F – Stat	df	p	F – Stat	df	p	F – Stat	df	p
TOTAL LENGTH	28.6	1, 213*	***	24.0	1, 213	***	0.5	1, 213	NS
TRUNK LENGTH	27.2	1, 214	***	16.7	1, 214	***	1.4	1, 214	NS
TRUNK WIDTH	24.9	1, 103	***	156.7	1, 103	***	4.9	1, 103	*
NECK LENGTH	4.5	1, 211	*	34.0	1, 211	***	0.9	1, 211	NS
NECK WIDTH	10.7	1, 211	**	4.8	1, 211	*	0.0	1, 211	NS
BULB LENGTH	25.1	1, 219	***	48.6	1, 219	***	0.0	1, 219	NS
BULB WIDTH	8.4	1, 212	**	10.2	1, 212	**	0.4	1, 212	NS
PROBOSCIS LENGTH	25.3	1, 221	***	4.8	1, 221	*	0.02	1, 221	NS
PROBOSCIS WIDTH	20.1	1, 214	***	27.3	1, 214	***	1.4	1, 214	NS
BODY SHAPE	2.5	1, 202	NS	263.3	1, 202	***	8.8	1, 202	**
NECK SHAPE	0.2	1, 211	NS	27.5	1, 211	***	0.3	1, 211	NS
BULB SHAPE	2.2	1, 212	NS	5.8	1, 212	*	1.2	1, 212	NS
PROBOSCIS SHAPE	0.0	1, 214	NS	56.4	1, 214	***	0.8	1, 214	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

As described in the previous chapter the size of the Irish characters varies significantly over time. Since Irish worms were sampled in three different months and the English in just one, August, the characters from worms sampled in August only were compared. No significant differences in total length, trunk length, neck width and neck shape were found between the two populations in August (table 4.3.2). English *P. laevis* were significantly wider than the Irish. English *P. laevis* also had longer bulbs than the Irish. Body shape had a significantly higher value in English *P. laevis*. Irish *P.*

laevis had significantly longer and wider probosces than the English worms. This was also reflected in proboscis shape. Irish worms from August had wider bulbs than the English.

From the comparison of worms from August, it seems that the Irish proboscis is larger than that of the English though the rest of the organs are at least of similar size if not bigger in the English population.

Table 4.3.2: Comparison of non-hook characters between male and female *P. laevis* of English and Irish origin during August.

	SEX			COUNTRY			SEX*COUNTRY		
	F – Stat	df	p	F – Stat	df	p	F – Stat	df	p
TOTAL LENGTH	15.5	1, 126*	***	0.6	1, 126	NS	0.5	1, 126	NS
TRUNK LENGTH	14.0	1, 136	***	2.0	1, 136	NS	1.2	1, 136	NS
TRUNK WIDTH	17.6	1, 116	***	28.3	1, 116	***	4.8	1, 116	*
NECK LENGTH	1.7	1, 122	NS	5.4	1, 122	*	0.3	1, 122	NS
NECK WIDTH	8.2	1, 122	**	0.0	1, 122	NS	0.3	1, 122	NS
BULB LENGTH	12.1	1, 126	***	6.9	1, 126	**	0.1	1, 126	NS
BULB WIDTH	6.1	1, 123	*	5.8	1, 123	*	0.1	1, 123	NS
PROBOSCIS LENGTH	19.1	1, 126	***	4.5	1, 126	*	0.3	1, 126	NS
PROBOSCIS WIDTH	12.9	1, 123	***	43.1	1, 123	***	1.5	1, 123	NS
BODY SHAPE	1.1	1, 116	NS	90.3	1, 116	***	5.7	1, 116	*
NECK SHAPE	0.5	1, 122	NS	3.3	1, 122	NS	0.0	1, 122	NS
BULB SHAPE	2.8	1, 123	NS	27.6	1, 123	***	2.0	1, 123	NS
PROBOSCIS SHAPE	0.1	1, 123	NS	23.6	1, 123	***	0.2	1, 123	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

Hook Characters

Table 4.3.3: Comparison of four hook characters between male and female *P. laevis* of English and Irish origin.

	MALES		FEMALES		ALL	
	χ^2 (df)	p	χ^2 (df)	p	χ^2 (df)	p
NO. HOOKS/ROW	12.0 (2)	**	2.3 (2)	NS	10.9 (2)	**
NO. LONGITUDINAL ROWS	0.2 (2)	NS	6.9 (2)	*	5.1 (2)	NS
POSITION OF LONGEST HK	25.8 (3)	***	25.5 (3)	***	49.8 (3)	***
POSITION OF STOUTEST HK	55.7 (2)	***	58.3 (2)	***	113.1 (2)	***

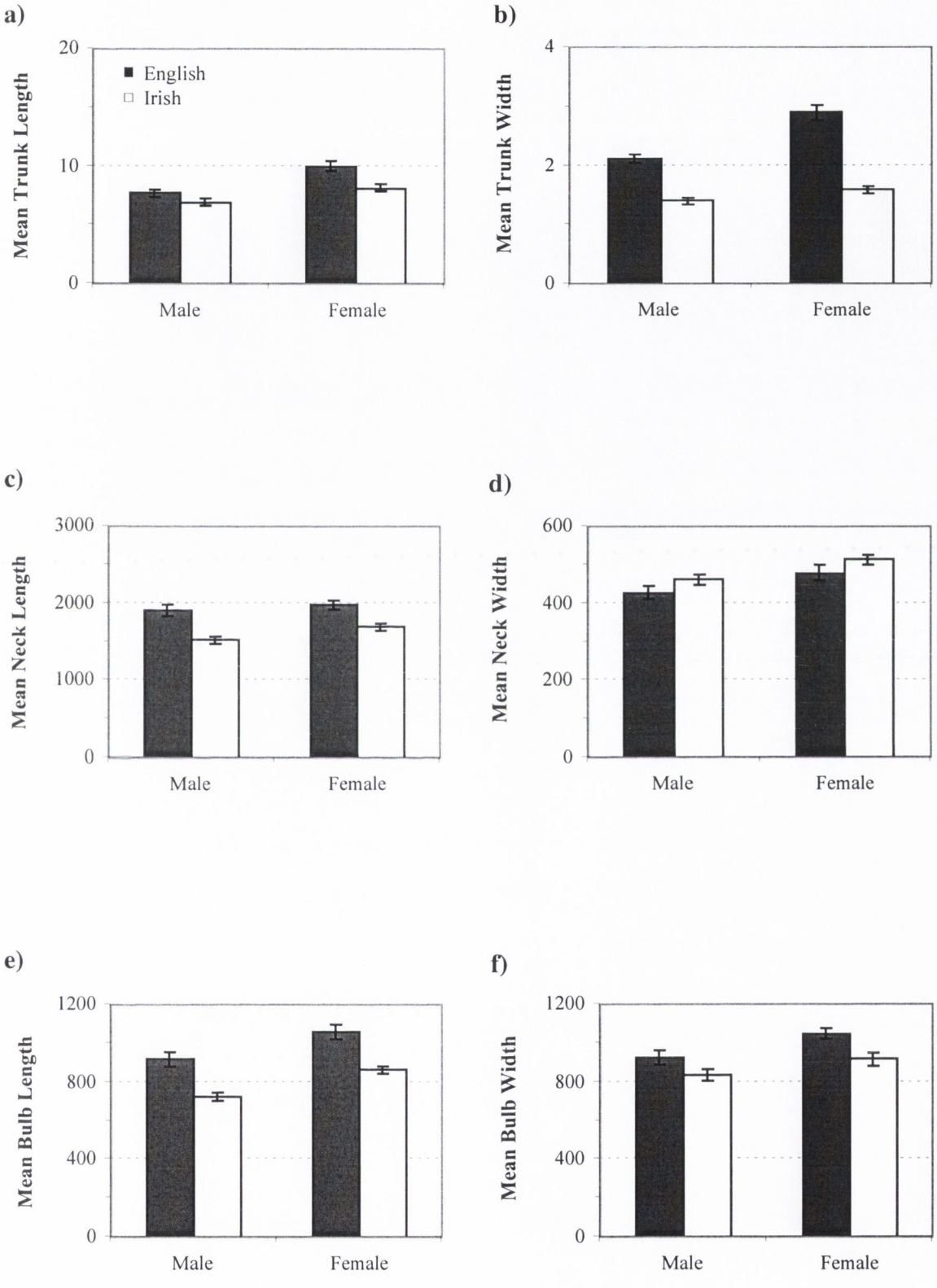


Fig. 4.3.1(a - f) Comparison of the mean trunk length, mean trunk width, mean neck length, mean neck width, mean bulb length and mean bulb width between male and female *P. laevis* of Irish and English origin. Error bars represent standard errors.

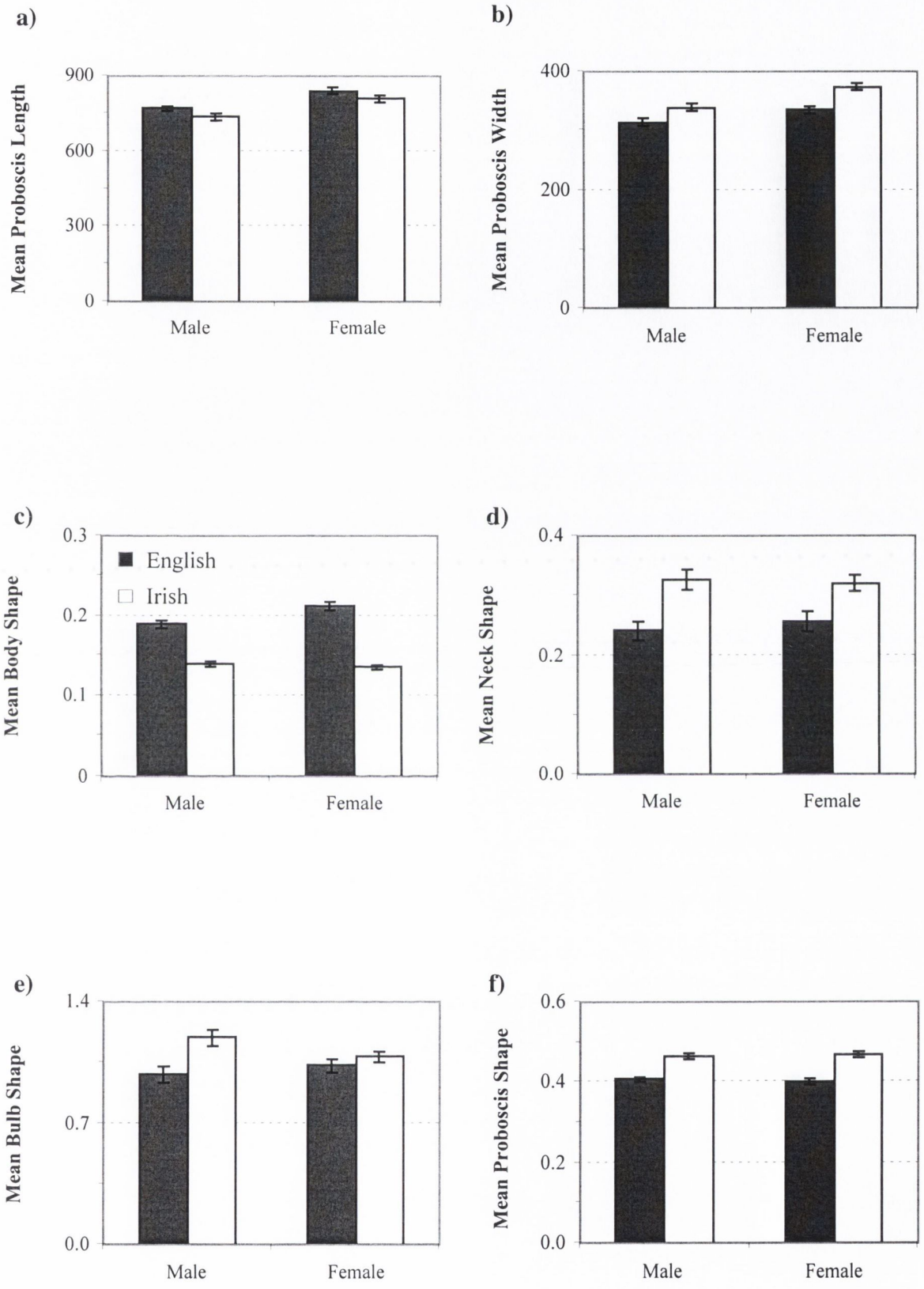


Fig. 4.3.2(a - f) Comparison of the mean proboscis length, mean proboscis width, mean body shape, mean neck shape, mean bulb shape and mean proboscis shape between male and female *P. laevis* of Irish and English origin. Error bars represent standard errors.

The number of hooks per row differed significantly between the males of the two populations though not the females (table 4.3.3). The null hypothesis does not expect any differences in characters between worms from different populations. Fewer Irish males had 10 hooks per row than expected and more had 12 hooks per row. More English males had 10 hooks per row and fewer 12 hooks per row than expected. No difference was found in the number of longitudinal rows between Irish and English males. Irish females had 17 rows more often than expected and 16 rows was more common among the English females. The longest hook was found more often at the 1st and 2nd position of Irish males and females than expected. This hook was found more often at the 3rd and 4th positions in English worms. The stoutest hook occurred at positions 4 and 5 more often in Irish *P. laevis* and more at position 6 in English *P. laevis*.

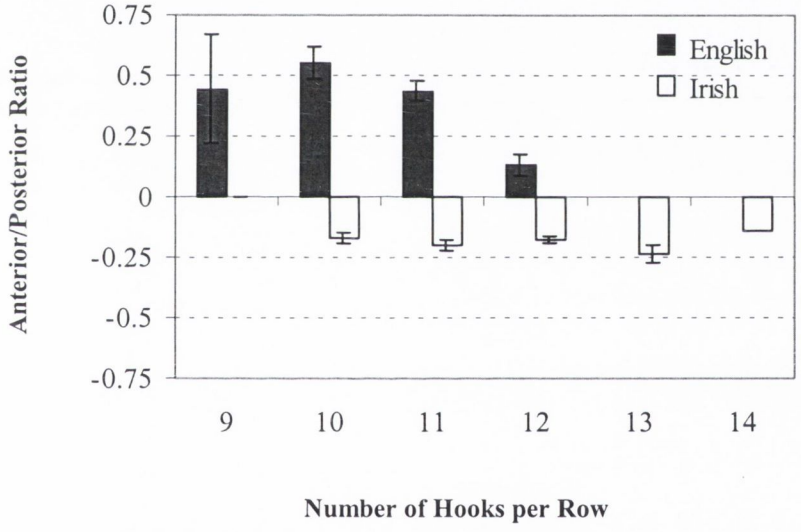


Fig. 4.3.3: Comparison of the mean proportion of anterior and posterior hooks (±standard error) between Irish and English *P. laevis*.

As has been described in materials and methods the hooks can be categorised into anterior and posterior hooks. Due to the striking differences in the location of the stoutest hook between the two populations it was questioned whether this reflected differences in numbers of anterior and posterior hooks or the proportions of them. Figure 4.3.3 shows means of the ratio of anterior to posterior hooks for each number of hooks per row for both populations. A negative number signifies a worm having more posterior hooks than anterior and a positive number signifies more anterior than posterior hooks. As can be seen from the figure, Irish worms tend to have more

Table 4.3.4: Comparison of hook lengths between male and female *P. laevis* of English and Irish origin.

	SEX			COUNTRY			SEX*COUNTRY		
	F-stat	df	p	F-stat	df	p	F-stat	df	p
LONGEST HOOK	23.8	1, 222*	***	0.5	1, 222	NS	0.1	1, 222	NS
HOOK 1	9.5	1, 222	**	11.9	1, 222	***	0.6	1, 222	NS
HOOK 2	16.2	1, 222	***	1.4	1, 222	NS	0.0	1, 222	NS
HOOK 3	21.8	1, 220	***	9.8	1, 220	**	0.2	1, 220	NS
STOUTEST HOOK	24.6	1, 218	***	45.7	1, 218	***	0.4	1, 218	NS
HOOK 7	12.3	1, 221	***	88.1	1, 221	***	0.6	1, 221	NS
HOOK 8	4.8	1, 222	*	75.2	1, 222	***	1.6	1, 222	NS
HOOK 9	7.4	1, 217	**	64.6	1, 217	***	0.4	1, 217	NS
BASAL HOOK	18.9	1, 220	***	8.8	1, 220	**	0.1	1, 220	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

Table 4.3.5: Comparison of hook widths between male and female *P. laevis* of English and Irish origin.

	SEX			COUNTRY			SEX*COUNTRY		
	F-stat	df	p	F-stat	df	p	F-stat	df	p
HOOK 1	1.1	1, 222*	NS	28.4	1, 222	***	0.5	1, 222	NS
HOOK 2	6.1	1, 222	*	57.4	1, 222	***	0.1	1, 222	NS
HOOK 3	3.9	1, 222	*	69.3	1, 222	***	0.9	1, 222	NS
STOUTEST HOOK	12.2	1, 221	***	39.2	1, 221	***	0.7	1, 221	NS
HOOK 7	15.0	1, 221	***	46.3	1, 221	***	0.0	1, 221	NS
HOOK 8	6.3	1, 222	*	64.7	1, 222	***	0.1	1, 222	NS
HOOK 9	7.4	1, 216	**	67.0	1, 216	***	1.3	1, 216	NS
BASAL HOOK	9.1	1, 220	**	52.7	1, 220	***	0.5	1, 220	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

posterior hooks than anterior ones and the English tend to have more anterior ones regardless of hook number itself.

The length, width, area and shape of the hooks were compared between Irish and English *P. laevis*. The general trend was that Irish hooks tended to be larger than those of the English. Six of the nine hook lengths were significantly longer in Irish *P. laevis* (table 4.3.4, figures 4.3.4 and 4.3.5). The longest hook and hook 2 did not differ significantly between the samples. Hook 3 was the only hook that was significantly longer in English *P. laevis*. All Irish hooks were significantly wider than those of the English (table 4.3.5, figures 4.3.4 and 4.3.5). The trend was also true of hook area (table 4.3.6, figures 4.3.6 and 4.3.7). Irish hooks had significantly higher values for

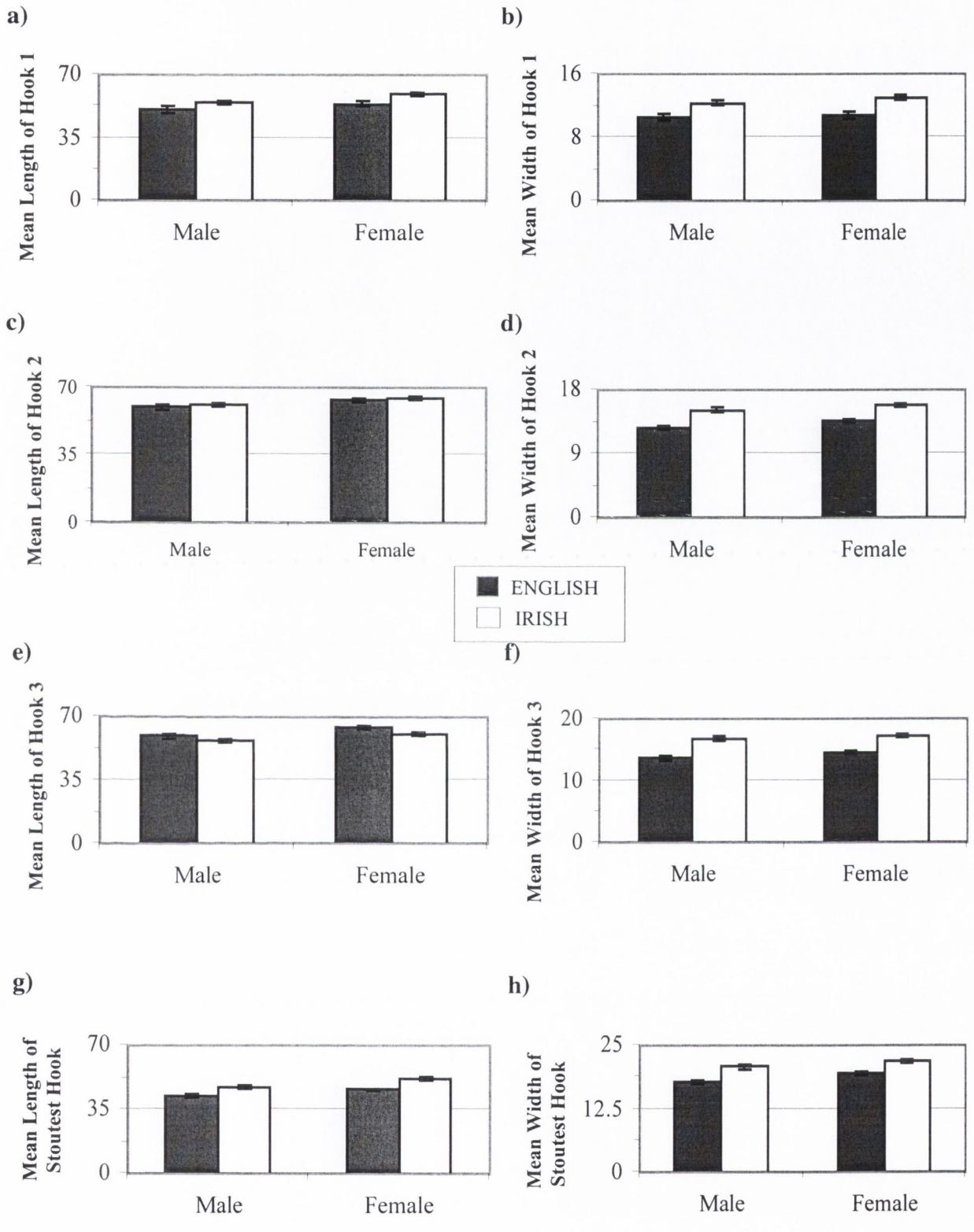


Fig. 4.3.4 Comparison of the mean anterior hook length and width **(a - f)** and mean stoutest hook length and width **(g - h)** between male and female *P. laevis* of Irish and English origin. Error bars represent standard error.

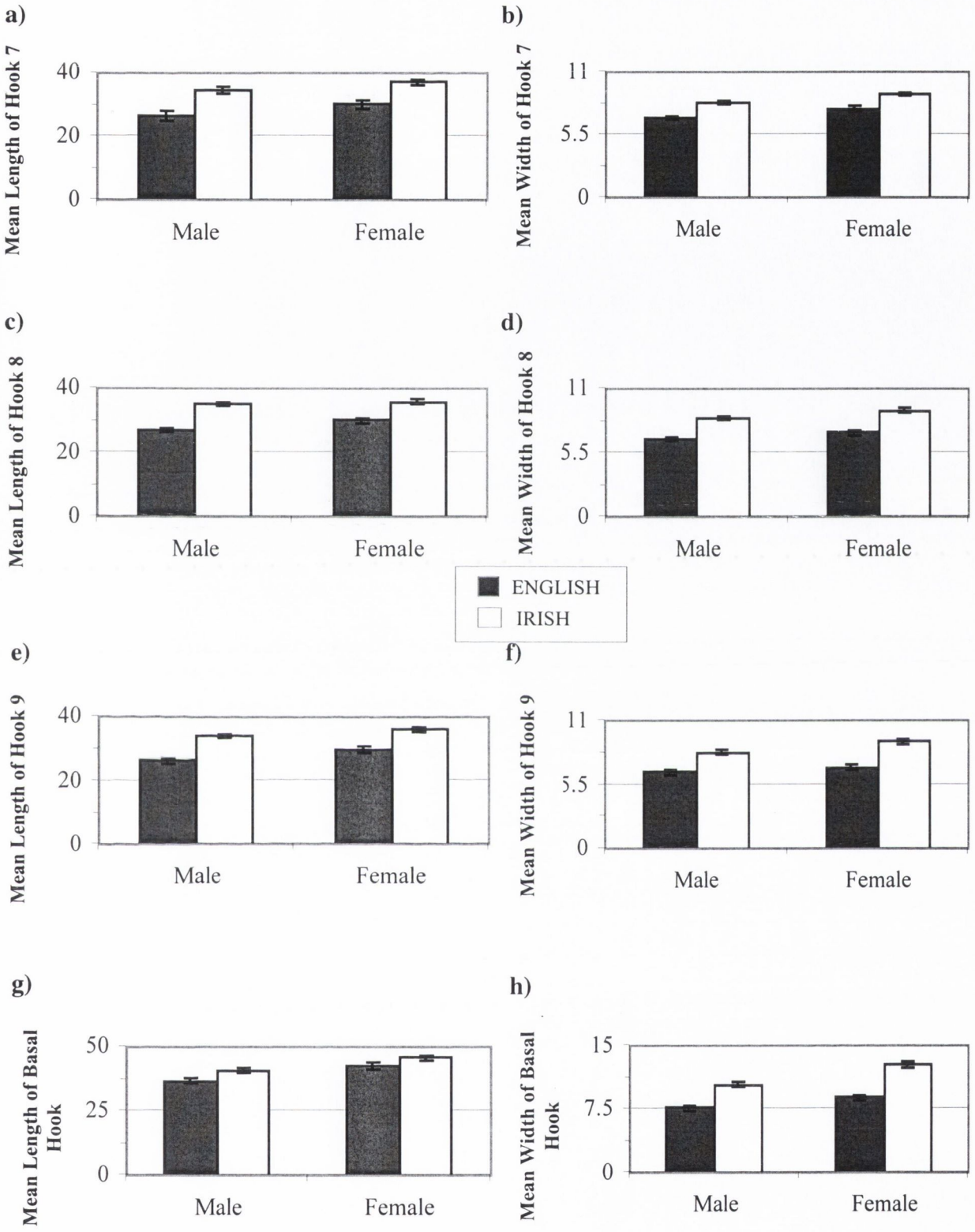


Fig. 4.3.5 Comparison of the mean posterior hook length and width **(a - f)** and mean basal hook length and width **(g - h)** between male and female *P. laevis* of Irish and English origin. Error bars represent standard error.

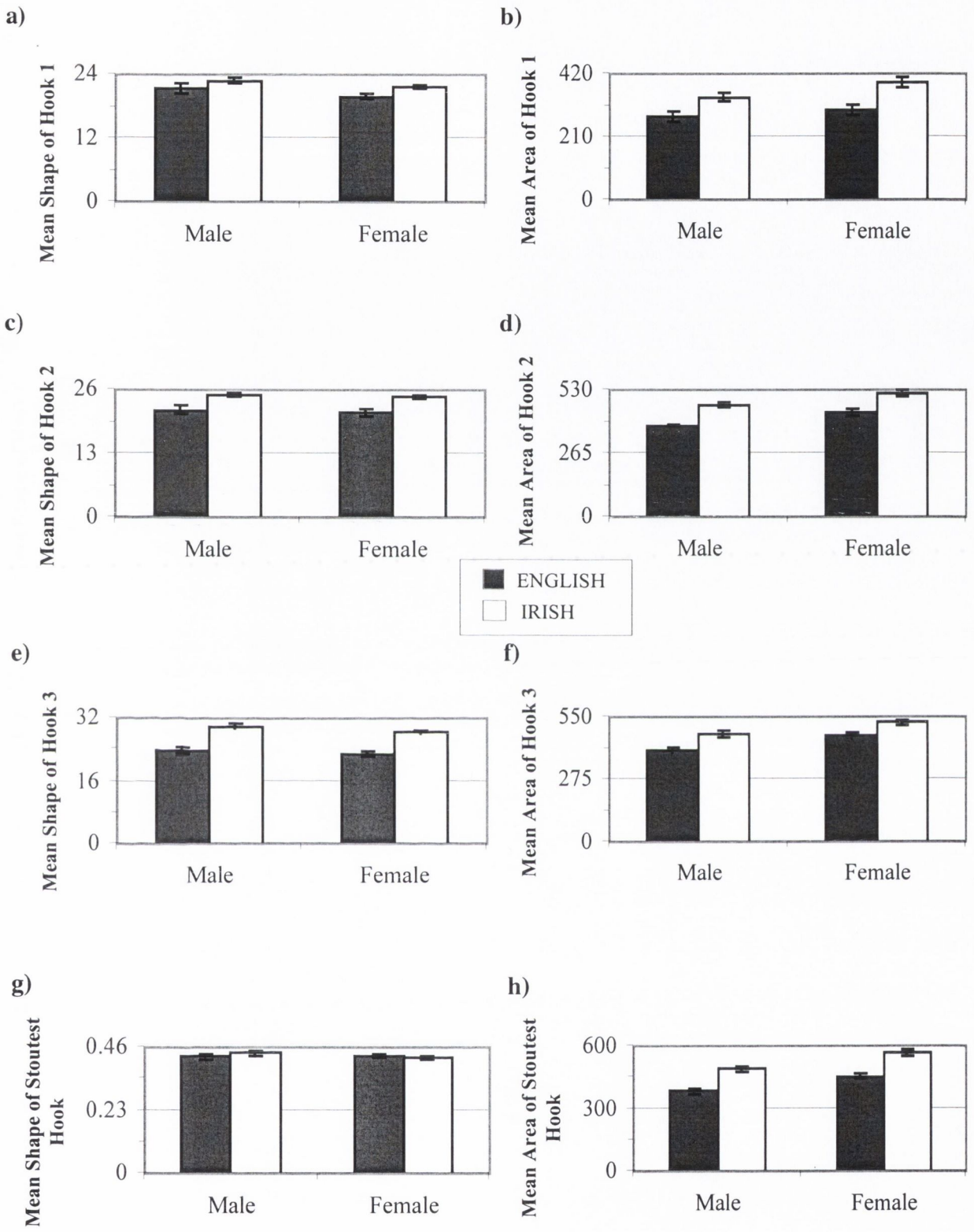


Fig. 4.3.6 Comparison of the mean anterior hook shape and area **(a - f)** and mean stoutest hook shape and area **(g - h)** between male and female *P. laevis* of Irish and English origin. Error bars represent standard error.

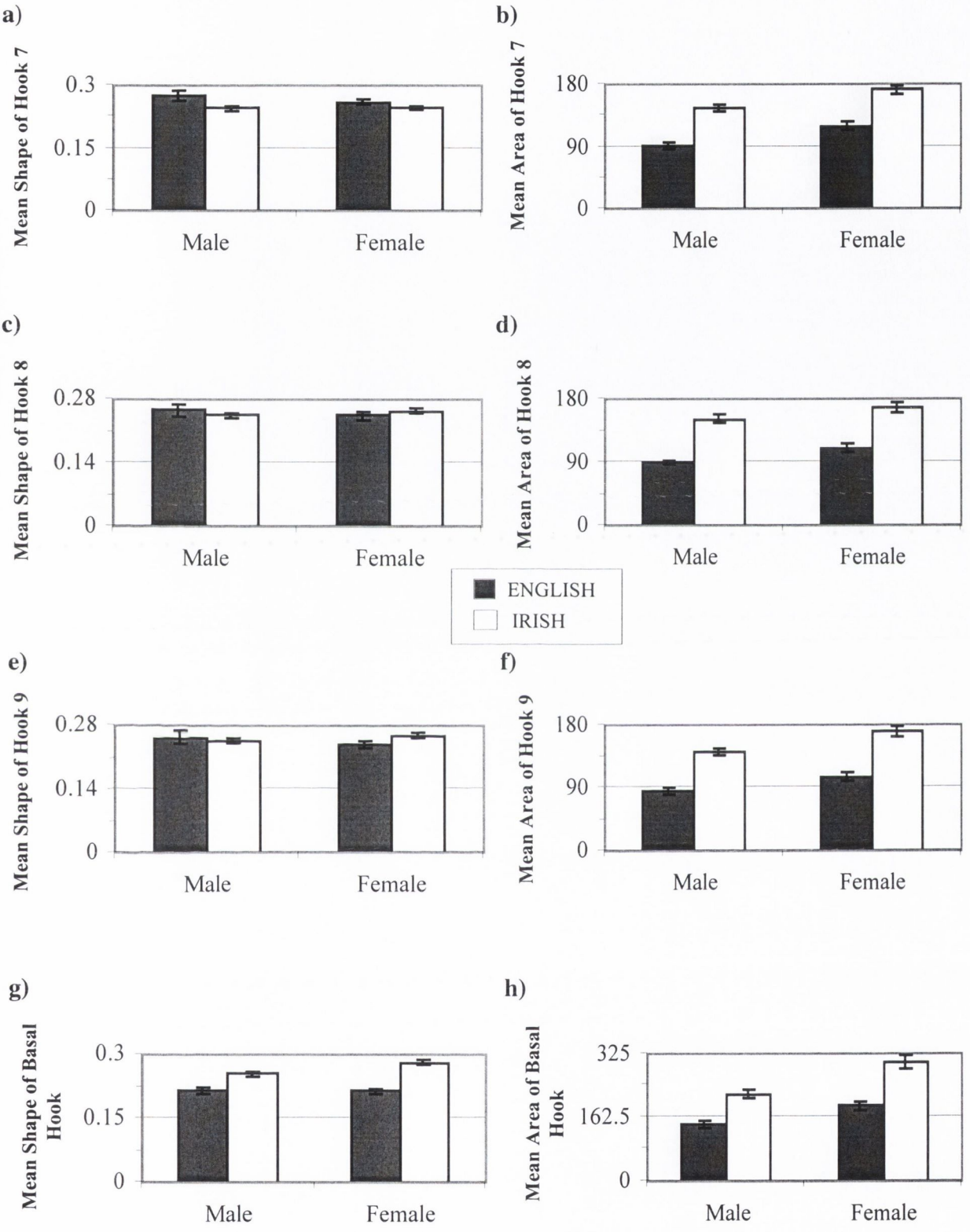


Fig. 4.3.7 Comparison of the mean posterior hook shape and area **(a - f)** and mean basal hook shape and area **(g - h)** between male and female *P. laevis* of Irish and English origin. Error bars represent standard error.

shape in four cases (table 4.3.7, figures 4.3.6 and 4.3.7). There were no significant differences in the shape of the stoutest hook and hooks 8 and 9. The shape of hook 7 had a significantly higher value in English worms.

Table 4.3.6: Comparison of hook areas between male and female *P. laevis* of English and Irish origin.

	SEX			COUNTRY			SEX*COUNTRY		
	F-stat	df	p	F-stat	df	p	F-stat	df	p
HOOK 1	5.1	1, 220*	*	23.2	1, 220	***	0.7	1, 220	NS
HOOK 2	14.6	1, 222	***	37.2	1, 222	***	0.0	1, 222	NS
HOOK 3	16.3	1, 220	***	20.1	1, 220	***	0.5	1, 220	NS
STOUTEST HOOK	24.0	1, 217	***	57.3	1, 217	***	0.0	1, 217	NS
HOOK 7	17.2	1, 221	***	82.1	1, 221	***	0.0	1, 221	NS
HOOK 8	7.4	1, 222	**	89.0	1, 222	***	0.1	1, 222	NS
HOOK 9	11.2	1, 216	***	74.3	1, 216	***	0.4	1, 216	NS
BASAL HOOK	16.6	1, 220	***	42.0	1, 220	***	0.9	1, 220	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

Table 4.3.7: Comparison of hook ratios between male and female *P. laevis* of English and Irish origin.

	SEX			COUNTRY			SEX*COUNTRY		
	F-stat	df	p	F-stat	df	p	F-stat	df	p
HOOK 1	5.1	1, 220*	*	8.8	1, 220	**	0.2	1, 220	NS
HOOK 2	0.5	1, 222	NS	29.8	1, 222	***	0.0	1, 222	NS
HOOK 3	1.8	1, 220	NS	73.8	1, 220	***	0.4	1, 220	NS
STOUTEST HOOK	0.8	1, 217	NS	0.1	1, 217	NS	1.4	1, 217	NS
HOOK 7	0.8	1, 219	NS	11.3	1, 219	***	1.8	1, 219	NS
HOOK 8	0.0	1, 220	NS	0.0	1, 220	NS	2.0	1, 220	NS
HOOK 9	0.0	1, 214	NS	0.7	1, 214	NS	2.9	1, 214	NS
BASAL HOOK	0.1	1, 220	NS	22.3	1, 220	***	0.1	1, 220	NS

*The first number refers to the degrees of freedom (df) associated with the factor and the second number refers to the total degrees of freedom.

Principal Components Analysis

Multivariate analyses are the best tools with which to analyze a multivariate dataset. Principle components analysis is a very powerful tool as it can reduce a myriad of variables to just one or two that best describe the variation in the dataset. Not all the variables measured were entered into the analysis, as this would have made the interpretation of the results more difficult and also confounded any differences between

the two samples. Therefore variables were chosen carefully so as to highlight the differences between the two groups without losing too much information. The variables chosen for the analysis are presented in the materials and methods section of this chapter.

Figure 4.3.8 represents the first two principle components, which together incorporate 58.8% of the variation in the data-set. The x-axis labelled U1 basically describes the range of the position of the stoutest hook, the anterior/posterior ratio and hook area. As one moves from the negative side of the axis to the positive, the values of the first two variables mentioned increase, while hook area decreases. The most negative U1 values describe worms with the stoutest hook at position 4, negative anterior/posterior ratios (more posterior than anterior hooks) and large hook areas (ranging from 200 to 950 μm^2). The most positive U1 values represent worms with the stoutest hook at position 6, a positive anterior/posterior ratio (more anterior than posterior hooks) and smaller hook areas (ranging from 50 – 400 μm^2). The y-axis labelled U2 describes the size of the worms and non-hook characters. As one moves from the negative to the positive side of the axis the size of the non-hook characters

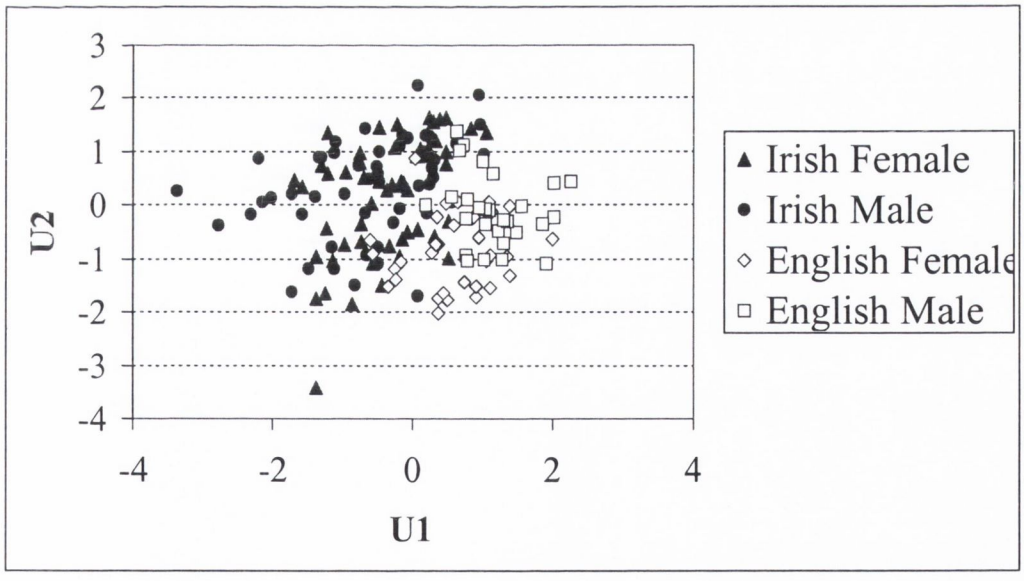


Fig. 4.3.8: Scatterplot showing the first (U1) and second (U2) principle components for English and Irish *P. laevis*.

diminishes. Therefore at the negative end are large worms with long probosces, large bulbs and bodies (*e.g.* 970 μm, 1045 μm and 17mm in length respectively), while at the positive end are smaller worms (*e.g.* 500μm, 818μm and 7mm being the length of the

proboscis, bulb and body respectively). Though a clearly defined separation of the two populations is not apparent from figure 4.3.8, the populations are divided very well along the x-axis. Despite the large overlap in sizes, the Irish sample being the more variable, the position of the stoutest hook and the ratio of anterior to posterior hooks could be important taxonomic characters when identifying these populations.

4.4 DISCUSSION

Non-Hook Characters

Despite finding a great overlap in size between Irish and English *P. laevis*, statistical differences were found. One population was not found to be larger than the other for all characters. English *P. laevis* had longer and wider trunks, longer necks and probosces and longer and wider bulbs than Irish *P. laevis*. Their bodies also had a significantly different shape. Conversely the Irish population had wider necks and probosces and the shape of their necks, bulbs and probosces also meant that they were wider in general than the English.

Due to the large seasonal variation in size within the Irish population, the two populations were compared for the month of August only. This revealed fewer statistical differences between the two populations. Results which differed from those already listed were that in August Irish *P. laevis* now had longer probocides and wider bulbs than the English. Edwards (Unpublished) found the English proboscis to be longer and wider than that of the Irish. He also found the Irish bulb the wider of the two.

A striking difference in the appearance of the neck, apart from its length and width, was observed between the two populations, though it did not enable a statistical comparison to be made. The neck of Irish *P. laevis* has the same appearance along its entire length. The appearance of the neck of English *P. laevis* changes distinctly approximately half way along its length. The lower half of the neck attached to the trunk, is quite stout and normal in appearance. The upper half of the neck attached to the proboscis and before the bulb, is much narrower and seems to be little more than a single walled tube. The reason for this seems to be the host encapsulation process. In chub, the capsule made of host tissue which envelopes the proboscis and bulb of English *P. laevis* constricts the neck so severely as to alter its appearance. Though the encapsulation process also occurs in brown trout, it does not have the same effect in altering the shape and appearance of the neck. It was impossible to measure this difference and so it remains an observed difference without being borne out by statistics.

It seems obvious that none of the non-hook characters could be used reliably to separate the two populations. Firstly the overlap in measurements is too large and

secondly the results change depending on the season in which Irish *P. laevis* are sampled. These findings disagree with those of Edwards (Unpublished) who stated that both the length and width of the proboscis could distinguish between the Irish and English strain of *P. laevis*.

Hook Characters

The positions of the longest and stoutest hooks differed significantly between the two populations. Due to the greater degree of variability in the position of the longest hook, the position of the stoutest hook was a more useful character for distinguishing the two populations. Though there seemed to be a good deal of overlap in this character statistically, in practical terms this character does separate the populations. The stoutest hook occurs at positions 4 and 5 in Irish *P. laevis* and positions 5 and 6 in English *P. laevis*. If one just examined this character on one row as was presented here for simplicity, then this character would not separate these populations very effectively. It is well known that hooks alternate their position between rows in the Acanthocephala and that neighbouring rows sometimes differ in numbers of hooks by one hook per row. Therefore if one examined two rows on each individual worm, on English *P. laevis* the stoutest hook would occur on one row at either position 5 or 6 and on the neighbouring row at the other position. Due to the fact that the stoutest hook only rarely occurs at position 6 on Irish *P. laevis* and at position 4 on English *P. laevis*, this character would separate these populations. For the few worms that have the same number of hooks in all rows, it may prove difficult to assign those worms to a particular strain with 100% confidence. Edwards (Unpublished) observed this difference in experimental populations of English and Irish *P. laevis*, "the English hook 6 is similar to the anterior hooks whereas the Irish hook 6 is similar in size and shape to the posterior hook". Edwards was confident that the two populations could be distinguished based on the proboscis and so failed to see the significance of his observation.

The numbers of hooks in a longitudinal row which were anterior and posterior were noted. The proportion of the hooks which were anterior and posterior proved to be another distinguishing character of Irish and English *P. laevis*. English *P. laevis* always had either similar numbers of anterior and posterior hooks per row or more anterior hooks. Irish *P. laevis* either had similar numbers of anterior and posterior hooks per

row or more posterior hooks. Despite the overlap, if one observed the two neighbouring rows as explained for the position of the stoutest hook then this character would also separate the populations to a high degree of certainty.

Two species of *Pomphorhynchus* in North America, *P. bulbocolli* and *P. rocci* have been identified based on similar types of differences (Huffman and Nickol, 1978; Gleason and Huffman, 1981). These two species were found to differ in the length of a hook at a certain position on the proboscis. If this hook is greater than 37μm, then *P. rocci* is identified and if the hook is less, then the worm is probably *P. bulbocolli*. This information coupled with the difference in hook shape along the length of the proboscis successfully identifies these two species morphologically.

Of the anterior hooks, neither the longest hook nor the first hook differed in length between the populations. Hook 2 was longer in Irish *P. laevis* and hook 3 longer in English *P. laevis*. The remaining hooks were significantly longer in Irish *P. laevis*. Irish *P. laevis* also had wider hooks than English *P. laevis*. As a result Irish *P. laevis* also had statistically greater hook areas. Hook shape did not differ between the populations. These results differ dramatically from those of Edwards (Unpublished). Edwards found the hooks of English *P. laevis* to be consistently longer than those of Irish *P. laevis* and their hook widths the same. The difference between results is certainly due to differences in data analysis. Edwards only included worms with 11 hooks per row in his analysis. Also he pooled data from all 5 anterior hooks and all 5 posterior hooks to get an average and compared this between the two populations. In this study of wild *P. laevis* populations, individual hook sizes were compared. Due to differences in hook numbers between individuals, only the first three anterior hooks were compared and the stoutest hook was analysed separately. Hook 3 is longer in English *P. laevis*, which might also be true for hooks 4 and 5, though this was not tested. This might explain why, overall, Edwards found longer anterior hooks in the English sample. In this latest study, digital image analysis was used and individual measurements were compared, which lends a higher degree of reliability to this data.

Despite all of the morphological variation documented for wild Irish and English *P. laevis*, a principal components analysis succeeded in separating the populations. This analysis confirmed the usefulness of the position of the stoutest hook and the ratio of numbers of anterior to posterior hooks as distinguishing characters of the two populations. Multivariate analyses have been used in other studies to confirm or deny the usefulness of morphological criteria in the identification of closely related species.

A principle components analysis was used to analyse rostellar hook characters, such as length and number of hooks in two studies of *Echinococcus granulosus*. Morphological criteria did not differentiate between Tasmanian and mainland Australian strains despite epidemiological differences between the two (Hobbs, Lymbery and Thompson, 1990). An attempt to distinguish sylvatic from domestic strains on the mainland was more successful. The relationship between total length of large hooks and the number of hooks separated the two cycles to some degree though there was an overlap similar to that seen in this study (Hobbs *et al*, 1990). In Spain, the same morphological characters clearly identified three strains of *E. granulosus*, a sheep and cattle strain, a pig strain and a horse strain (Ponce Gordo and Cuesta Bandera, 1997). The same characters, hook length and number of hooks identified these strains. Ponce Gordo and Cuesta Bandera (1997) extended their analysis by applying a discriminant function analysis to the data. This produced two linear functions which allowed correct classification in 100% of samples. Jacquiet *et al* (1995) found the differences in occurrence of females with different vulvar morphology i.e. whether they were smooth, knobbed or linguiform helped to distinguish between three closely related species, *Haemonchus placei*, *H. contortus* and *H. longistipes*. Principal components analysis of morphological criteria of four closely related nematode species revealed seven assemblages (Duncan *et al*, 1999). It should be noted that in all of the preceeding studies, morphological criteria alone was not accepted for the discrimination of species or strains. Genetic analysis such as RAPD's (Jacquiet *et al*, 1995; Ponce Gordo and Cuesta Bandera, 1997) and 28S rDNA sequence analysis (Duncan *et al*, 1999) was carried out in tandem with the morphological studies.

Conclusions

Two characters have proved successful in distinguishing a wild English and wild Irish population of *P. laevis*. It is unclear whether these differences are purely phenotypic and related only to the host or whether there is some genotypic basis for them. Other researchers have found the morphology of acanthocephalans to vary between host species (Bullock, 1962; Amin, 1975b; Buckner and Nickol, 1975). This may be in part due to the differential ability of hosts to provide the best environment in which *P. laevis* can grow. Lymbery (1998) found rostellar traits of E. granulosus such as blade length (hook length), to differ phenotypically from 55 to 68% between isolates

in the same host species. This variation was completely attributed to environmental influence as opposed to having a genetic basis. It is also unknown if these populations are representative of all Irish and all English *P. laevis*. Further work needs to be done to validate these results. Whether these differences would be enough to identify two different species is also unclear. Species identification within the Acanthocephala based on slight morphological differences due to the degree of phenotypic plasticity exhibited by them is not satisfactory (Brown, 1987). Within the two populations presented here, the position of the stoutest hook and the ratio of the number of anterior to posterior hooks overlapped a little, though in general were very consistent characters.

CHAPTER 5

A STUDY OF THE POPULATION GENETICS OF *POMPHORHYNCHUS LAEVIS* USING A MITOCHONDRIAL GENE.

5.1 INTRODUCTION

New data presented in the previous chapter have revealed morphological characters which can separate the English and Irish strains. Two characters, the position of the stoutest hook and the proportion of anterior to posterior hooks, when used together separate the two strains in most cases. Previous attempts to separate the marine strain from other strains morphologically were never completely satisfying, due to the conservation of characters and phenotypic plasticity. The lack of distinguishing morphological criteria in the past was probably the strongest evidence against strain differentiation in *P. laevis* (Brown, 1987, Kennedy *et al*, 1989).

Only four studies have been published which have employed molecular techniques such as enzyme electrophoresis and RAPD's (random amplified polymorphic DNA) to study species differences in the Acanthocephala. Three of them successfully resulted in the differentiation of congeneric species, which were morphologically very similar (de Buron *et al*, 1986; Aho *et al*, 1992; Dezfuli and Tinti, 1998). The other study carried out RFLP analysis (restriction fragment length polymorphism) of the marine and English strains of *P. laevis* and found putative differences between the two (Munro *et al*, 1990). The work presented in this chapter is the first known attempt to use direct sequencing of mitochondrial DNA to study the population genetics of an acanthocephalan species.

Direct sequencing of subunit I of cytochrome c oxidase, a mitochondrial gene, was carried out for *P. laevis* from nine different freshwater populations in Ireland, the U.K. and Italy. The aim of the work is to compare these sequences and identify strains within this species and to correlate these with the ecologically specialised strains described by Kennedy *et al* (1989).

5.2 MATERIALS AND METHODS

Sample Collection

Nine different populations of *Pomphorhynchus laevis* in Ireland, the United Kingdom and Italy were sampled. Table 5.2.1 lists the number of worms sequenced from each population, their location and the host from which they were removed. Worms taken from brown trout from L. Feeagh in August and November, 1999 were placed in liquid nitrogen after their removal from the fish. The worms were then stored at –80 °C. *P. laevis* sampled from the river Lee and the river Liffey in September 2001 were dissected out of deep frozen fish (-20 °C) and then stored at –80 °C. English *P. laevis* from the river Culm and the river Avon, sampled in 1999 and the river Otter, sampled in May 2001 were transported in dry ice while still attached to the gut of the fish. After their dissection from the gut the worms were stored at –80 °C. *P. laevis* from Scotland, sampled in August 2001 and Italy, sampled in May 1999 were transported and stored in 100% ethanol.

Table 5.2.1: The location, number and host of samples collected for sequencing.

LOCATION	N	HOST
L. Feeagh, Co. Mayo, Ireland	17	Brown Trout
R. Lee, Co. Cork, Ireland	8	Brown Trout
R. Liffey, Co. Dublin, Ireland	4	Brown Trout
R. Culm, Dorset, England	12	Chub
R. Avon, Hampshire, England	10	Bullhead
R. Otter, Devon, England	5	Brown Trout
R. Otter, Devon, England	5	Flounder
R. Snizort, Isle of Skye, Scotland	5	Sea Trout
R. Brenta, Italy	9	Chub

Extraction of Total Genomic DNA

Whole worms (some with heads removed) were vortexed in sterile water in order to remove any debris and placed in 1.5 ml eppendorf tubes. 250 µl of digestion

buffer (Appendix C.I) was added to each worm. The eppendorfs were left in a water bath at 55 °C for 2 hours and overnight at 37 °C.

DNA was extracted according to the protocol described in appendix D.I. All extractions took place in the fume cupboard due to the toxic nature of phenol. The resultant pellet of DNA was left overnight at –20 °C. It was then re-centrifuged at 13,000 rpm for 3 minutes. The solution in which the pellet was sitting was removed and discarded. The pellet was then washed with 200 µl of 75% ethanol to remove any residues from the extraction process (which might inhibit PCR) and left to dry in air for approximately 30 minutes. After drying, the pellet was resuspended in 100 µl of TE buffer (Appendix C.II).

Amplification of Cytochrome c oxidase, subunit I

A mitochondrial gene cytochrome c oxidase, subunit I (COI) was amplified using the polymerase chain reaction (PCR). The purpose of the technique is to yield enough material comprised of millions of copies of the target sequence in order to facilitate subsequent analyses or tests on the sequence. The technique is based on essential cellular functions such as transcription and DNA replication. Short oligonucleotide sequences (primers), 15 – 35 bp in length, which match regions flanking both ends of the target sequence are needed to prime the reaction (Hoelzel and Green, 1998).

Essential reagents needed to carry out the technique include template DNA, an excess of the two primers, a polymerase (an enzyme which catalyses the addition of new nucleotides to a growing chain of nucleotides *i.e.* a new DNA molecule) and excess free nucleotides. The first step in the process is to heat the template DNA to a very high temperature *e.g.* 94 °C in order to denature the DNA (break the bonds between the two strands in the double helix resulting in single strands). The mixture is then cooled to allow the primers to anneal to the template strands. Polymerisation takes place once the mixture is heated to 72 °C. At the end of this procedure there are now two new copies of the sequence. The procedure is then repeated between 30 to 40 times, greatly increasing the copy number of the target sequence.

The primers used to amplify COI in *P. laevis* were based on studies carried out on cestodes and nematodes (Zarlenga, Hoberg, Stringfellow and Licthenfels, 1998;

Bowles, Blair and McManus, 1992; and Garey and Wolstenhome, 1989). The following forward and reverse primers were used initially:

COI p1 (for): TTT TTT GGG CAT CCT GAG GTT TAT

COI p2 (rev): TAA AGA AAG AAC ATA ATG AAA ATG

New primers were designed as some samples did not amplify successfully using the above pair of primers:

COI p4 (for): GGG CAT CCT GAG GTT TAT ATT CTC

COI p3 (rev): TGT CAT GAA ATA CCG TAT CCA AGC

Primers were synthesised by Oswel, UK. The optimal composition of the 50 µl PCR mixture for the nine samples was similar and is described in appendix D.II. COI sequences from Irish *P. laevis* from L. Feeagh and the R. Lee were amplified using the primers COI p1 and COI p2. COI sequences from the remaining samples were amplified with the same forward primer and COI p3 as the reverse primer. The only other difference between PCR mixtures was the $MgCl_2$ concentration. *P. laevis* from L. Feeagh required $MgCl_2$ with a working concentration of 15 mM whereas the remaining samples required $MgCl_2$ with a working concentration of 20 mM. Oil was added to the mixture to prevent evaporation during the high temperature stages of the PCR process.

The conditions to which the PCR mixture was subjected differed slightly between samples (table 5.2.2). Conditions were altered for different samples only to optimise the yield. The samples from the R. Liffey were kept at the annealing and extension temperatures for a longer time. More cycles were also carried out. The optimal annealing temperature for the primer pair COI p1 and COI p3 is 55 °C and this was reduced to 53 °C in the case of the Italian samples. The reaction was repeated 35 times for the R. Lee samples and some of the Italian samples in order to increase the yield of the PCR product.

An amount of the PCR product (5 µl) was visualised in order to verify successful amplification and also to check the size and quality of the product (product size is an aid to verify whether the correct sequence has been amplified). 5 µl of the product was mixed with 2 µl of tracking dye (0.25 % Bromophenol blue, 0.25 % Xylene cyanol FF and 30 % glycerol). This was then electrophoresed on a 1 % agarose gel which was stained with Ethidium bromide (plate 5.2.1). DNA glows orange in the presence of Ethidium Bromide and ultraviolet light. All agarose gels were run on a

Horizon 58, a Gibco BRL Horizontal Gel Electrophoresis Apparatus maufactured by Life Technologies in the presence of 1X TBE buffer (Appendix C.III).

Table 5.2.2: PCR conditions

CONDITIONS	L. Feeagh/ R. Lee*	R. Liffey	UK	Italy
	Temp/Time	Temp/Time	Temp/Time	Temp/Time
Initial Denaturation	94 °C/ 4 min	94 °C/ 4 min	94 °C/ 4 min	94 °C/ 4 min
Denaturation	94 °C/ 40 s	94 °C/ 40 s	94 °C/ 40 s	94 °C/ 40 s
Annealing	**50 °C/ 40 s**	**50 °C / 1 min**	**55 °C/ 40 s**	**53 °C/ 40 s**
Extension	72 °C/ 1 min	**72 °C / 1.5 min**	72 °C/ 1 min	72 °C/ 1 min
Final Extension	72 °C/ 10 min	72 °C/ 10 min	72 °C/ 10 min	72 °C/ 10 min
No. of Cycles	**30/35***	**35**	30	**30/35**

After successful amplification, the PCR products were cleaned to remove primer dimer (when the primers bond together instead of to the target sequence), unused nucleotides and other residuals of the PCR process. Cleaning was carried out using a Gibco BRL Concert Rapid PCR Purification System, manufactured by Life Technologies (Appendix D.III).

Direct Sequencing of COI

The sequence that was isolated and copied in the PCR process now needs to be broken down into its constituent bases so that it can be read and analysed. The sequencing method used is known as Liquid Cool sequencing. This method is a slight variation on the dideoxy termination method incorporating ^{35}S-labelled nucleotides developed by Sanger, Nicklen and Coulson (1977). The PCR product is mixed with primer (forward or reverse), excess dNTP's (NTP stands for nucleotide tri phosphate), a sequenase enzyme, ^{35}S-labelled dATP and ddNTP's (dd stands for dideoxy) as well other reagents used to stabilise and improve the reaction. The most important ingredient is ddNTP's. These differ from normal dNTP's by the addition of dideoxy to the nucleotide. Four reactions are carried out simultaneously but separately, corresponding to each of the four ddNTP's. The sequencing reaction is similar to a PCR as double stranded DNA is denatured at a high temperature and cooled to allow the primer to anneal to the single strands. Then a copy is made of the template sequence to which the primer has annealed, catalysed by the enzyme and using the excess dNTP's.

ddNTP's are present in smaller proportions than the dNTP's and every so often these are added to the new copy instead of a normal nucleotide. When this happens, the copy ceases to be produced from that base forward and only a partial sequence remains. Because there are thousands of copies of target sequence present due to the intial PCR, at the end of the sequencing reaction there will be thousands of partial sequences of different sizes terminated at every possible base by the addition of a ddNTP at that base.

When the products of the sequencing reaction are electrophoresed on a polyacrylamide gel of appropriate concentration, because the partial sequences are a different size due to the different number of bases, they separate out from each other and form bands. Also the reaction products from the four reactions are electrophoresed in separate lanes. An autoradiograph (X-ray film) is then exposed to the gel and a banding pattern forms on the film due to the presence of the radioactive isotope (figure 5.2.1). Each band corresponds to a partial sequence of a certain size and therefore terminated at a particular base. Because the sequences terminated by different ddNTP's are run out on different lanes one can read off the bases which make up the sequence in question.

The sequencing method used is known as a Liquid Cool sequencing method because after the double stranded DNA has been denatured, it is plunged into liquid nitrogen. This prevents the now single strands from re-annealing, which can occur due to the relatively small size of the strands (Hoelzel and Green, 1998). The exact protocol followed is described in appendix D.IV.

After the sequencing reaction has been stopped by the addition of formamide, the sequencing products are denatured by placing the terisaki plate onto a layer of water, which has been heated to boiling point on a heating block. The samples are allowed to denature for approximately 2 to 3 minutes. Then 1.5 µl of each sample (four per individual worm) was loaded on a 6 % vertical polyacrylamide gel, set up on a Model S2, Gibco BRL Sequencing System maufactured by Life Technologies (Appendix C.IV). 1x TBE buffer was added to the tanks at the top and bottom of the gel. 6M ammonium acetate was also added to the bottom tank to aid resolution of the bands. Each sample was run twice, once at 70W for six hours and once at 70W for two and a quarter hours. DNA polymerisation starts at the primer, which is the 5' end of the sequence (if the forward primer is used) and therefore the lighter partial sequences occur at this end. Light molecules do not need as long to separate out as heavier

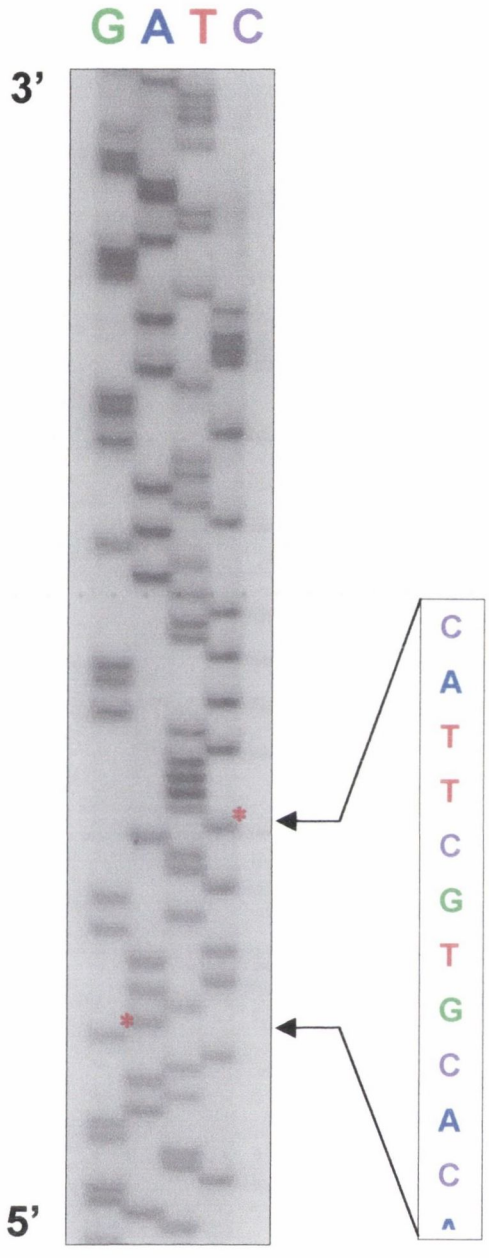


Fig. 5.2.1 A photograph of a section of an autoradiograph exposed to ^{35}S labelled DNA. The sequence shown comes from an individual worm from brown trout from the River Otter. The bands at the bottom are nearest the primer and therefore the sequence is read from the bottom up. The bases highlighted are those numbered 167 to 180 in the segment of the COI gene sequenced.

molecules, which is the basis of electrophoresis. The long run allowed the bases near the 3' end of the sequence to be visualised. Because the sequence is over 400 bp in length, the entire sequence could not be visualised in one run and this necessitated two runs.

After the samples had been electrophoresed for a sufficient length of time, the gel was adhered to filter paper and dried at 80 °C for 50 minutes. An autoradiograph was then exposed to the gel for a period of between one day and three weeks depending on the strength of the isotope. All work, which involved the radioactive isotope was performed in a designated radioactive area and all necessary precautions followed.

The sequence of both the forward and reverse strands was detected, to ensure reliability of the data. Not all sequences were manually sequenced in the laboratory. 23 sequences were automatically sequenced using dye terminator technology by MWG Biotech, Germany and LARK Technologies, UK.

Data Analysis

Multiple alignments were carried out in ClustalX. Median networks were constructed as described by Bandelt *et al* (1995). A neighbor joining tree was created using the PHYLIP package (Felsenstein, 1993). Values of genetic distance were first calculated using the DNADIST programme. Distances were calculated according to the formula based on the Kimura 2-parameter method. Once this distance matrix was calculated, the matrix was fed into the tree construting programme NEIGHBOR.

Analysis of Molecular Variance (AMOVA) was calculated using ARLEQUIN ver 2.000 (Schneider, Roessli and Excoffier, 2000). This analysis takes into account both sequences of and the number of mutations between haplotypes. The total variance is partitioned into covariance components due to intra-individual differences, inter-individual differences and inter-population differences. The covariance components are recorded in the final output under the heading variance components. Three components Va, Vb and Vc are associated with among group variance, among populations within groups variance and between populations within groups variance respectively. Fixation indices associated with each covariance component are also calculated. These are:

$F_{CT} = Va/ Vt$

$F_{SC} = Vb/(Vb + Vc)$

$F_{ST} = (Va + Vb)/ Vt$

F_{CT} is associated with among group variance and represents the proportion of the total variance attributed to among group variance. The significance of this index only was tested.

5.3 RESULTS

General Observations

Figures 5.3.1 and 5.3.2 show partial sequences of the cytochrome c oxidase gene (subunit I) for *P. laevis* from Ireland and Italy respectively. The primers chosen were expected to amplify 460 base pairs of the middle of the COI gene as described from *F. hepatica* (Bowles *et al*, 1992; Garey and Wolstenholme, 1989). The total length of this gene in *F. hepatica* is 1,533 base pairs. As can be seen from figures 5.3.1 and 5.3.2, it was not possible to read the sequence to the end of the reverse primer as less than 420 bases were readable.

The average GC content for this gene from worms from Ireland and Britain was 43.4 % (calculated from just the aligned part of the sequence) and that for worms from Italy was 42.1%. The GC content was not as low as that described for the entire mitochondrial genome of nematodes which was 26.2 % ± 2.15 (Saccone *et al*, 1999).

Three types of mutations were found in the COI gene of *P. laevis*. These were transitions, transversions and indels (insertion/ deletion). The bases guanine (**G**) and adenosine (**A**) are known chemically as purines. These have a different chemical structure to cytosine (**C**) and thiamine (**T**), which are known as pyrimidines. In the double helix structure of DNA, **A** always pairs with **T** and vice versa and **G** with **C** and vice versa. Through mutation, a base can be transcribed or translated erroneously during the replication process. If a base is exchanged for another base of the same chemical make up, then this is known as a transition. If a base is exchanged for a base with a different chemical composition, this is known as a transversion. For example if **A** was substituted by either **T** or **C**, this is a transversion as a purine is being substituted by a pyrimidine. Transitions are much more common, particularly in mtDNA.

Out of 25 variable sites in total among sequences from worms from Ireland and Britain, only 5 displayed transversions, which gave a transition/ transversion ratio of 5 : 1 (table 5.3.1). Out of 60 variable sites among the Italian sequences, only 6 were as a result of a transversion (table 5.3.1). This gives a transition/ transversion ratio of 10 : 1.

One insertion/deletion was found in five sequences from two populations. These included the four sequences from the R. Liffey and one sequence from chub,

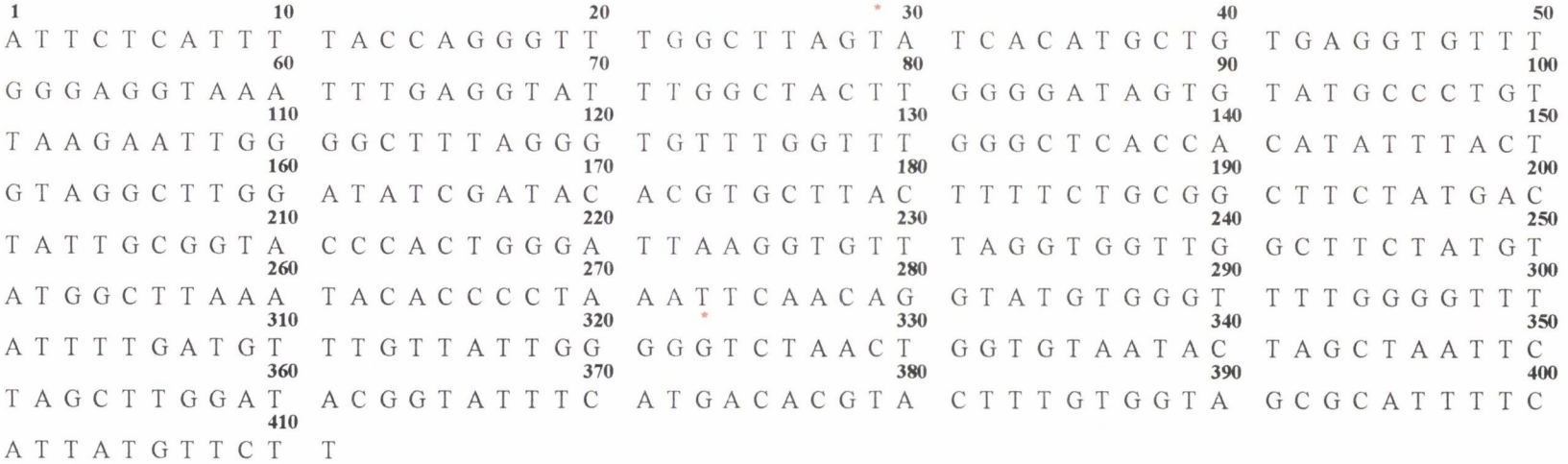


Figure 5.3.1: The entire sequence of one individual *P. laevis* from L. Feeagh, Co. Mayo, I11BT. The sequence delineated by two red asterisks corresponds to the 295 bases aligned for analyses.

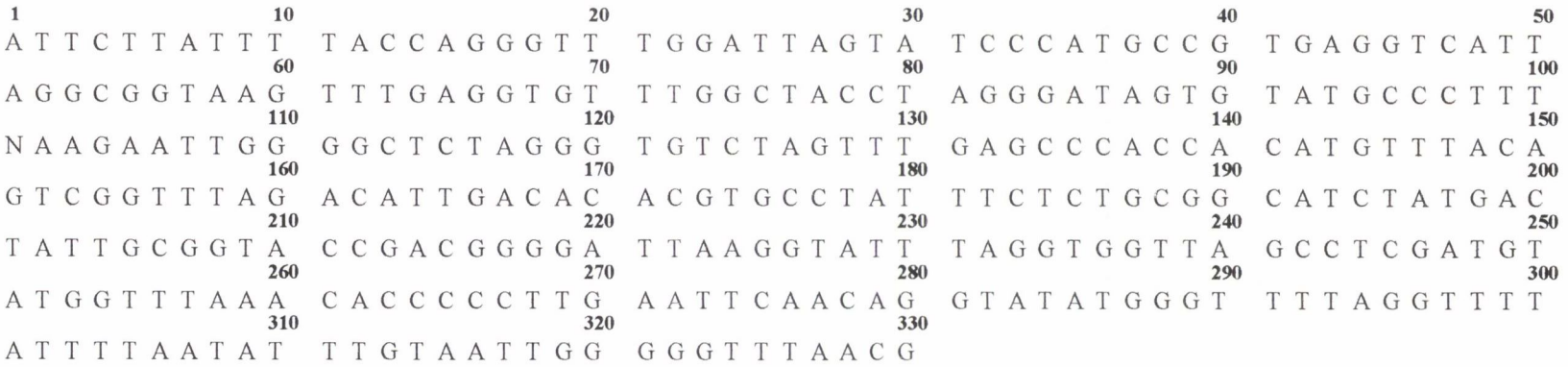


Figure 5.3.2: The entire sequence of one individual *P. laevis* from R. Brenta, Italy, IT81C.

Table 5.3.1: Table showing the polymorphic sites found in partial sequences of the COI gene of *P. laevis* in Ireland, the UK and Italy.

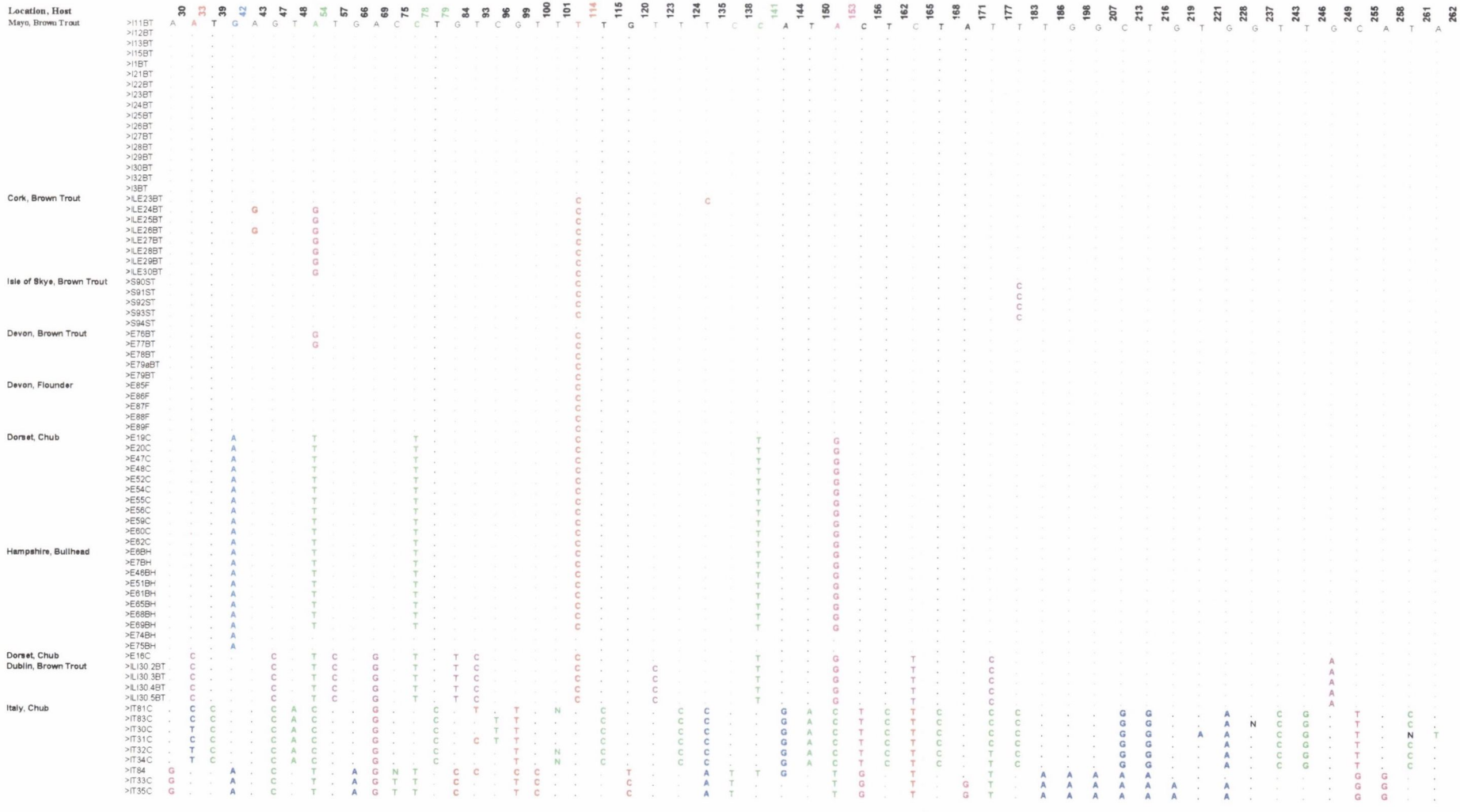
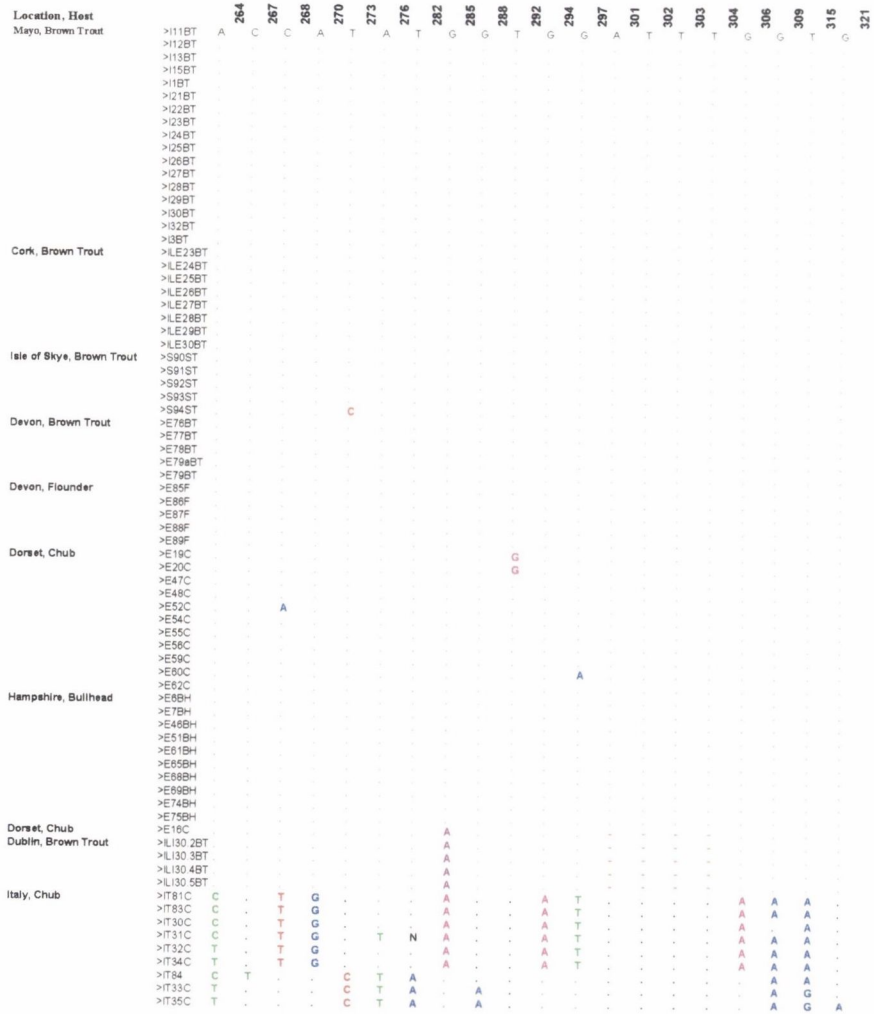


Table 5.3.1 contd.: Table showing the polymorphic sites found in partial sequences of the COI gene of *P. laevis* in Ireland, the UK and Italy.



which only differed from the former by 1 base. These sequences are grouped together at the end of table 5.3.1. An indel refers to a section of the sequence, which has either been inserted or deleted. As can be seen from table 5.3.1, bases 301 to 304 (**ATTT**) are missing from the R. Liffey sequences and E16C, the unique sequence from the River Culm.

Of the 25 mutations recorded for sequences from Ireland and Britain, four of these were at non-third base positions. Three bases form a codon and each codon codes for a particular amino acid. Because COI is a coding gene, how mutations in the sequence affect the amino acid structure of the protein can be determined. Generally mutations at the third base of a codon do not change the amino acid coded for. These mutations are known as silent changes or synonymous changes. Therefore four of the mutations in the Irish and British sequences occurred at either the first or second position in the codon.

Table 5.3.2: A summary of the non third base mutations found in partial sequences of the COI gene in *P. laevis* from various locations.

LOCATION	MUTATION	POSITION IN SEQUENCE	AMINO ACID CHANGE
Cork	A – G*	43	Serine - Glycine
Dublin & Dorset	T – C	47	Cysteine - Leucine
Dorset	C – A	268	Leucine - Methionine
Dorset	T – G	292	Leucine - Valine
Italy	T – A	221	Isoleucine - Asparagine
Italy	A – T	261	Threonine - Serine

*The first base is that in the reference sequence and the second base is the result of a mutation.

Two sequences from the river Lee in Cork have a mutation at base 43, which is at the first position in the codon (table 5.3.2). The original codon reads **AGG** and with the mutation it reads **GGG**. **AGG** in invertebrates codes for serine (Garey and Wolstenholme, 1989), which bears a hydroxyl side chain and **GGG** codes for glycine, which is an aliphatic amino acid. This is quite a substantial change as serine is a hydrophilic molecule, whereas glycine is a hydrophobic molecule and is found in the interior of the protein (Matthews, Holde and Ahern, 2000). However glycine is the least hydrophobic of all the aliphatic amino acids.

The five most different sequences found in Ireland and Britain, those of the four worms found in the river Liffey and one from chub in the river Culm possess a mutation at base 47, which is at the second position in the codon (table 5.3.2). The original codon reads **TGT** and with the mutation it reads **CTT**. **TGT** codes for cysteine and **CTT** codes for leucine. Cysteine bears a sulphur containing side chain and is hydrophilic, whereas leucine is hydrophobic due to its relatively extensive side chain (Matthews *et al*, 2000).

The final two non-synonymous changes occurred in three of the sequences from chub in the river Culm (table 5.3.2). The mutation at base 268 changes the amino acid from leucine to methionine, which is another amino acid with a sulphur containing side chain. Since methionine is the most hydrophobic of this class of amino acids the biochemical change is not substantial. The final mutation at position 292 changes the amino acid from leucine to valine, which is another of the aliphatic amino acids. Therefore this change is probably of little consequence to protein functions.

Only two of the base substitutions in the Italian sequences occurred at non-synonymous positions (table 5.3.2). The mutation at position 221 is quite a significant change as isoleucine is a hydrophobic aliphatic amino acids and asparagine is a hydrophilic molecule. At position 262, the changed amino acid is in the same hydroxyl side chain bearing class as the original.

Median Network of Irish and British Sequences

Figure 5.3.3 represents all of the 14 unique haplotypes found in *P. laevis* from the 8 populations sampled in Ireland and the UK. These networks were produced by first aligning the sequences in ClustalX and highlighting positions where polymorphisms occurred as shown in table 5.3.1. Each haplotype is represented by a circle, the area of which is proportional to the number of individuals sharing that haplotype. The number of individuals with a particular haplotype is also written beside the circle within parentheses. Each haplotype is shaded according to the population it originates from as described by the legend. The degree of separation between haplotypes is proportional to the number of mutations between them. One mutation is designated by a single line, which is delineated by a black dot at both ends. Smaller numbers in blue refer to the position of the mutation in the sequence, the first base after the forward primer is numbered 1. Beside some of the position numbers are small black

letters written in parentheses. These indicate a transversion substitution. The first letter is the base at that position in the reference sequence and the second letter is the resulting base.

The haplotypes cluster into three groups, which are separated by a number of mutations. The first group at the top left of the network is composed mainly of *P. laevis* from brown trout. All of the populations from brown trout, except the Dublin population are represented. This group includes two Irish brown trout populations, one from Mayo (the most frequent haplotype) and the other from Cork, the Scottish brown trout population and the English brown trout population from the river Otter. Two non-salmonid populations are also present. These are the flounder population also from the river Otter and two individuals from bullhead. The presence of the flounder population here may be explained in terms of the preferred definitive host. In freshwater, *P. laevis* does not mature in flounder, although it can survive in this host (Kennedy, 1984). The worms found in flounder are most likely members of the brown trout population, which cycle through flounder due to its feeding on the intermediate host. The most numerous haplotype present, that encoutered in *P. laevis* in the Mayo sample, displays no intra-population diversity. *P. laevis* from Scottish brown trout are represented by two haplotypes, separated by four bases. *P. laevis* from English brown trout also exhibit two haplotypes though these are only separated by one base change. *P. laevis* from brown trout from Cork are the most heterogeneous of the group, consisting of three haplotypes, one of which is also found in English brown trout. The mean percentage divergence within this group is 0.70% ± 0.30 (± SD). Percentage divergences between all haplotypes are shown in table 5.3.3.

The next group is that in the lower left-hand corner of the network. This consists of two populations of cyprinid fish, chub and bullhead. The dominant haplotype in this group is present in both populations, which are also from different river systems. *P. laevis* from chub from the river Culm, represent the most diverse population as five haplotypes were recovered. Three haplotypes differ from the main one by just one unique mutation. Though the sample size is small, the orientation of haplotypes in this group is reminiscent of a starburst pattern. Tentatively, a starburst pattern can be identified with one frequent haplotype in the centre with numerous rare haplotypes emanating from it and differing from it by only one mutation (Bandelt *et al*, 1995). This pattern describes a founding event by the central haplotype and subsequent

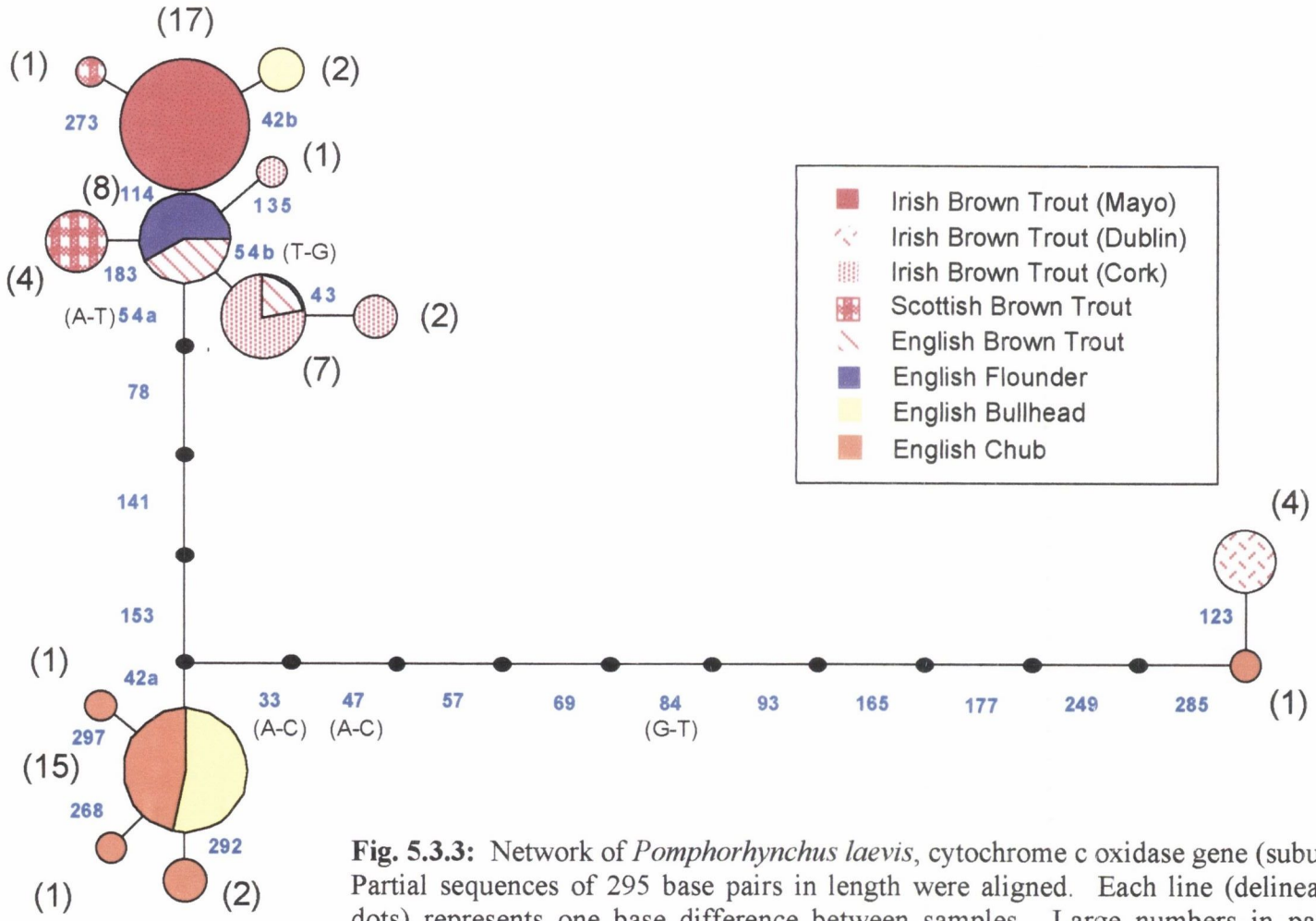


Fig. 5.3.3: Network of *Pomphorhynchus laevis*, cytochrome c oxidase gene (subunit I). Partial sequences of 295 base pairs in length were aligned. Each line (delineated by small black dots) represents one base difference between samples. Large numbers in parentheses represent sample sizes and small blue numbers show the position of each mutation within the gene sequence.

Table 5.3.3: Percentage differences between haplotypes of *P. laevis* from Ireland, the UK and Italy. Above the diagonal, base differences are shown (excluding indels) and below the diagonal percentage differences are shown.

LOCATION, HOST		MST1	EST1	CST1	CST2	CST3	SST1	SST2	ELC1	ELC2	ELC3	ELC4	ELC5	ECG1	DST1	ItLC1	ItLC2	ItLC3	ItLC4	ItLC5	ItLC6	ItLC7	ItLC8
Mayo, Brown Trout	**MST1**	*	1	2	3	2	2	1	6	7	7	7	15	1	16	45	46	48	47	45	44	43	43
Devon, Brown Trout	**EST1**	0.35	*	1	2	1	1	2	5	6	6	6	14	2	16	46	47	49	48	46	45	44	44
Cork & Devon, Brown Trout	**CST1**	0.68	0.35	*	1	2	2	3	5	6	6	6	14	3	15	46	47	49	48	46	45	44	44
	CST2	1.02	0.68	0.35	*	3	3	4	6	7	7	7	15	4	16	47	48	50	49	47	46	45	45
	CST3	0.68	0.35	0.68	1.02	*	2	3	6	7	7	7	15	3	16	45	46	48	47	45	45	44	44
Isle of Skye, Brown Trout	**SST1**	0.68	0.35	0.68	1.02	0.68	*	3	6	7	7	7	15	3	16	45	46	48	47	45	46	45	45
	SST2	0.35	0.68	1.02	1.36	1.02	1.02	*	7	8	8	8	16	2	17	46	47	48	47	45	43	42	42
Dorset, Chub & Hampshire, Bullhead	**ELC1**	2.03	1.69	1.69	2.03	2.03	2.03	2.37	*	1	1	1	11	5	12	49	50	52	51	49	43	42	40
	ELC2	2.37	2.03	2.03	2.37	2.37	2.37	2.71	0.35	*	2	2	12	6	13	50	51	53	52	50	44	43	41
	ELC3	2.37	2.03	2.03	2.37	2.37	2.37	2.71	0.35	0.68	*	2	12	6	13	49	50	52	51	49	44	43	41
	ELC4	2.37	2.03	2.03	2.37	2.37	2.37	2.71	0.35	0.68	0.68	*	12	6	13	49	50	52	51	49	44	43	41
	ELC5	5.08	4.75	4.75	5.08	5.08	5.08	5.42	3.73	4.07	4.07	4.07	*	16	1	46	47	47	50	49	51	50	47
Hampshire, Bullhead	**ECG1**	0.35	0.68	1.02	1.36	1.02	1.02	0.68	1.69	2.03	2.03	2.03	5.42	*	17	46	47	48	47	45	43	42	42
Dublin, Brown Trout	**DST1**	5.42	5.42	5.08	5.42	5.42	5.42	5.76	4.07	4.41	4.41	4.41	0.35	5.76	*	47	48	49	48	46	44	43	44
Italy, Chub	**ItLC1**	15.25	15.59	15.59	15.93	15.25	15.25	15.59	16.61	16.95	16.61	16.61	15.59	15.59	15.93	*	2	4	8	3	52	51	51
	ItLC2	15.59	15.93	15.93	16.27	15.59	15.59	15.93	16.95	17.29	16.95	16.95	15.93	15.93	16.27	0.68	*	2	6	5	54	53	53
	ItLC3	16.27	16.61	16.61	16.95	16.27	16.27	16.27	17.63	17.97	17.63	17.63	15.93	16.27	16.61	1.36	0.68	*	4	7	56	55	55
	ItLC4	15.93	16.27	16.27	16.61	15.93	15.93	15.93	17.29	17.63	17.29	17.29	16.95	15.93	16.27	2.71	2.03	1.36	*	9	60	59	59
	ItLC5	15.25	15.59	15.59	15.93	15.25	15.25	15.25	16.61	16.95	16.61	16.61	16.61	15.25	15.59	1.02	1.69	2.37	3.05	*	54	53	54
	ItLC6	14.92	15.25	15.25	15.59	15.25	15.59	14.58	14.58	14.92	14.92	14.92	17.29	14.58	14.92	17.63	18.31	18.98	20.34	18.31	*	1	12
	ItLC7	14.58	14.92	14.92	15.25	14.92	15.25	14.24	14.24	14.58	14.58	14.58	16.95	14.24	14.58	17.29	17.97	18.64	20.00	17.97	0.34	*	11
	ItLC8	14.58	14.92	14.92	15.25	14.92	15.25	14.24	13.56	13.90	13.90	13.90	15.93	14.24	14.92	17.29	17.97	18.64	20.00	18.31	4.07	3.73	*

expansion resulting in mutational events. The mean percentage divergence within this group is 0.35% ± 0.00 (± SD).

The final group in the lower right-hand corner consists of only two haplotypes from two populations. The haplotype, which represents all four *P. laevis* from the river Liffey in Dublin, differs from that of one worm from chub from the river Culm by one base. The mean percentage divergence within this group is 0.35% ± 0.00 (± SD). The presence of such a divergent haplotype in Ireland is interesting especially as chub are absent there. It is possible that this haplotype appears in Irish *P. laevis* due to the introduction of *G. pulex* from England into Wiclow. *G. duebeni* is the most widespread gammarid in Irish freshwaters. In 1988, *G. pulex*, the common British gammarid was introduced into the river Brittas, Co. Wicklow as a result of an accidental escape from a local fish farm (Keatinge, 1989). It has since spread to other areas within the local catchment, which includes the river Liffey and has outcompeted the native gammarid (McLoughlin and Reynolds, in press). If some of the founding members of *G. pulex* were infected with *P. laevis* cystacanths then it is possible that the haplotype found is a result of this introduction.

The most frequent haplotype in the mainly salmonid group differs from that in the cyprinid group by 6 base changes. Overall the mean percentage divergence between these two groups is 2.20% ± 0.27 (± SD). The third group is very different from the salmonid group as it differs from it by a mean percentage of 5.30% ± 0.30 (± SD). The third group differs from the cyprinid group by a mean percentage of 4.20% ± 0.24 (± SD).

Median Network of Italian Sequences

All Italian worms sequenced are from the same host, chub and the same river, the Brenta. As can be seen from figure 5.3.4, the amount of variation exhibited by the COI gene from these 9 worms is far greater than seen in Irish and British worms. 8 unique haplotypes were found, only one of which occurred in more than one worm. The haplotypes form 2 distinct groups. The left-hand cluster consists of 6 worms exhibiting 5 haplotypes. This group has a mean percentage divergence of 1.7% ± 0.83 (±SD). The right-hand group consists of 3 worms each showing a unique haplotype. This group has a mean percentage divergence of 2.7% ± 2.1 (± SD). The two Italian

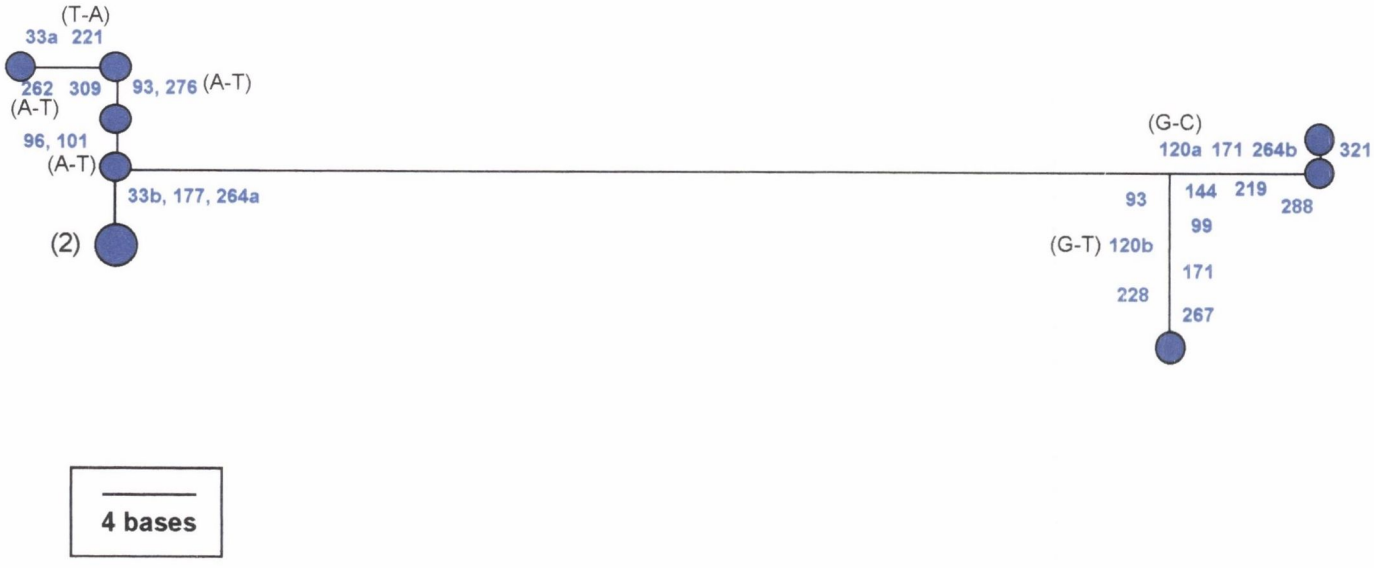
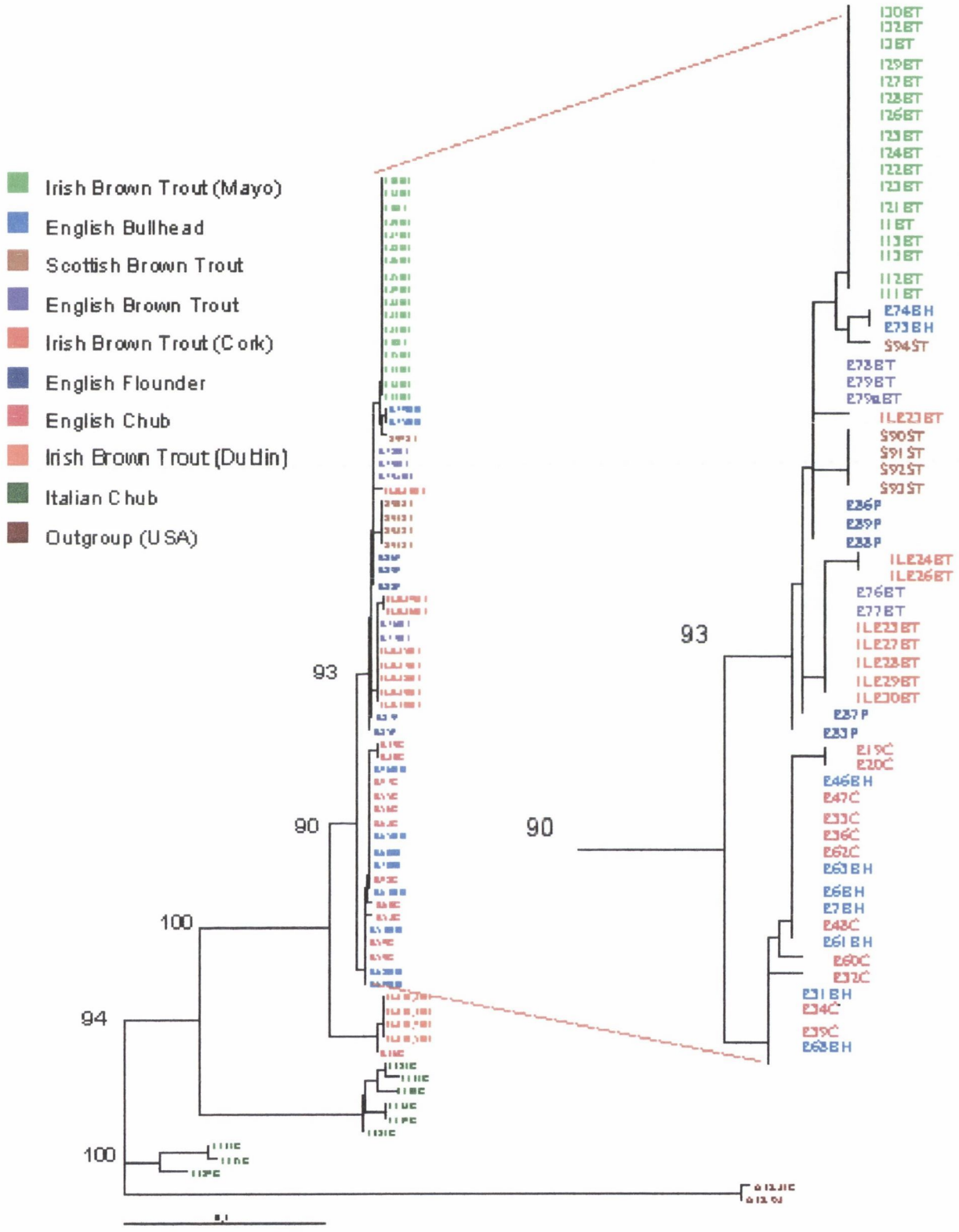


Fig. 5.3.4: Network of *Pomphorhynchus laevis* from the river Brenta, Italy, cytochrome c oxidase gene (subunit I). Partial sequences of 295 base pairs in length were aligned.


Irish Brown Trout (Mayo)
English Bullhead
Scottish Brown Trout
English Brown Trout
Irish Brown Trout (Cork)
English Flounder
English Chub
Irish Brown Trout (Dublin)
Italian Chub
Outgroup (USA)
93
90
100
94
100
93
90

groups differ considerably from each other by a mean percentage divergence of 18.5% ± 0.96 (± SD).

Neighbor Joining Tree

This tree (figure 5.3.5) was constructed as a way to visualise the Irish, British and Italian sequences together. The outgroup consists of two sequences from *Leptorhynchoides thecatus* (ATK23C and ATK7M), a parasite of freshwater fish in the United States. The tree validates the relationships borne out by the median networks. The sequences from the salmonid and cyprinid groups form a distinct clade, reflecting their close relationship relative to the other sequences. As in the network, sequences from brown trout from Mayo and Cork in Ireland, Scotland and England form one branch of the clade along with the sequences from flounder and two from bullhead. Most of the remaining sequences from the English cyprinids form the other branch. The heterogeneous group comprising a sequence from one English chub and those from the Liffey brown trout is outside of this clade. The two groups found among the Italian haplotypes do not cluster together on the tree and that they are as divergent from each other as they are from the British/ Irish clade is clearly shown. The Italian group containing sequences IT33C, IT35C and IT84C differ from the British/ Irish clade by an average percentage of 14.9% ± 0.7 (± S.D.). The remaining Italian sequences differ from the British/ Irish clade by 16.3% ± 0.7 (±S.D.). The Italian group containing only three sequences seems to be more closely related to the outgroup than the other.

AMOVA Analysis

Table 5.3.4: Results of an AMOVA analysis where populations have been grouped by country.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	VARIANCE COMPONENTS	PERCENTAGE OF VARIATION
AMONG GROUPS	2	32.724	0.448	23.5
AMONG POPULATIONS WITHIN GROUPS	4	32.945	0.912	47.8
WITHIN POPULATIONS	55	30.267	0.550	28.8
TOTAL	61	95.935	1.911	

The hypothesis of strain formation in *P. laevis* states that *P. laevis* exists as two distinct strains in Ireland and England (Kennedy *et al*, 1989). By adhering to this hypothesis, it follows then that genetic variation among all of the haplotypes should be greatest between country. AMOVA allows one to test the existence of population genetic structure according to user defined hypotheses. Three hypotheses were tested. A population genetic structure was imposed on the data using country, host species and host tolerance to saline (*e.g.* euryhaline versus stenohaline) to partition the populations into particular groups.

The Italian population was not included in the following tests, as European populations are not dealt with by the strain hypothesis. The Irish population from brown trout in the Liffey was also excluded as it probably represents a stocked population rather than a wild population. The first population genetic structure tested for was that of country. The populations described in table 5.2.1 were divided into three groups according to the country in which they were found *i.e.* Scotland, Ireland or England. Only 23.5% of the genetic variation was assigned to groups, whereas 47.8% of the variation was found among populations within groups (table 5.3.4). Neither the

Table 5.3.5: Results of an AMOVA analysis where populations have been grouped by host species.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	VARIANCE COMPONENTS	PERCENTAGE OF VARIATION
AMONG GROUPS	3	49.712	0.824	41.54
AMONG POPULATIONS WITHIN GROUPS	3	15.95	0.609	30.71
WITHIN POPULATIONS	55	30.27	0.550	27.75
TOTAL	61	95.94	1.983	

Table 5.3.6: Results of an AMOVA analysis where populations have been grouped by host species (minus flounder).

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	VARIANCE COMPONENTS	PERCENTAGE OF VARIATION
AMONG GROUPS	2	47.40	1.052	46.8
AMONG POPULATIONS WITHIN GROUPS	3	15.95	0.603	26.8
WITHIN POPULATIONS	51	30.27	0.594	26.4
TOTAL	56	93.61	2.248	

variation among groups nor the fixation index, F_{CT} was found to be statistically significant, $p = 0.216$.

The next hypothesis tested was that population genetic structure in *P. laevis* was partitioned by host species. The seven populations were grouped according to which fish species hosted that population, *i.e.* brown trout, chub, bullhead or flounder. Variation was found to occur almost equally among groups, among populations within groups and within populations (table 5.3.5). The variation found among groups was slightly higher at 41.5% than in the other situations. Again neither group variation nor the fixation index, F_{CT} were significant, $p = 0.148$.

As explained earlier it is more than likely that *P. laevis* found in flounder is not separate from the population occurring in brown trout in the river Otter, due to the fact that brown trout is the definitive host and *P. laevis* in freshwater rarely attains maturityin flounder. For this reason another test of groups differentiated by host species was conducted, this time leaving out the flounder group. In this test, a greater proportion of the genetic variation was assigned to group, 46.8% (table 5.3.6). Both the group variation component and the fixation index, F_{CT} were found to be almost significant, $p = 0.075$.

Table 5.3.7: Results of an AMOVA analysis where populations have been grouped by host tolerance to saline.

SOURCE OF VARIATION	D.F.	SUM OF SQUARES	VARIANCE COMPONENTS	PERCENTAGE OF VARIATION
AMONG GROUPS	1	46.833	1.47609	60.83
AMONG POPULATIONS WITHIN GROUPS	5	18.836	0.40001	16.49
WITHIN POPULATIONS	55	30.267	0.5503	22.68
TOTAL	61	95.935	2.4264	

The final hypothesis tested was that the level of tolerance of the host to saline could explain the population genetic structure of *P. laevis*. Brown trout and flounder are euryhaline fish and able to tolerate both fresh and sea water and ranges in between, while chub and bullhead are strictly stenohaline and do not tolerate saline conditions. The populations were assigned to one of two groups, based on the salinity tolerance exhibited by the host, euryhaline or stenohaline. 60.8% of the genetic variation was

explained by this genetic structure and this was found to be statistically significant, p = 0.051 (table 5.3.7).

5.4 DISCUSSION

The cytochrome c oxidase gene was chosen for this study as it has proved invaluable in other studies of intraspecific variation within different helminth species (Bowles *et al*, 1992; Bowles and McManus, 1994; Blair *et al*, 1997; Itagaki *et al*, 1997; Morgan and Blair, 1998). Mitochondrial DNA mutates at a faster rate than nuclear DNA, at least at synonymous sites (Saccone *et al*, 1999). This makes it more suitable for studying relationships between closely related organisms. Its mode of maternal inheritance and lack of recombination also allow for easier determination of relationships. The synonymous rate of mutation is similar between all of the mitochondrial genes but the non-synonymous mutation rate in the cytochrome c oxidase subunits is the slowest (Saccone *et al*, 1999). Therefore, the chance that the amino acid changes found among the different *P. laevis* populations represent actual adaptations to local conditions is increased because of the functional constraints on this gene.

Irish and British Sequences

Based on clear differences between *P. laevis* in Ireland and England, it was proposed that this species exists as three ecologically specialised strains in these countries (Kennedy *et al*, 1989). The purpose of this research was to find some support for this hypothesis on a molecular level. As reported in the literature review, the term 'strain' should imply a partitioning of genetic variation between populations from different hosts or areas rather than within populations (Thompson and Lymbery, 1990). Analyses such as the construction of a median network and AMOVA have uncovered just that. 12 of the 14 haplotypes formed two distinct clusters separated from each other by an average percentage of 2.2%. This level of difference at first might seem small but in light of other studies, it is significant. Low intra-strain variability was also found which adds to the significance of the percentage divergence.

In comparable work, an extensive study of variation in the COI gene of isolates of *E. granulosus* from a range of hosts and geographical locations identified 9 distinct genotypes (Bowles *et al*, 1992; Zhang *et al*, 1998). Though the level of difference between some of the genotypes was almost equal to that between known subspecies of *E. granulosus*, small differences were found between others. Those strains that differed from the reference sequence by only one to three bases maintained their status due to

concomitant important epidemiological differences. Some studies *e.g. S. japonicum* and *Fasciola* sp in Asia have revealed very little genetic differentiation between proposed strains despite epidemiological and morphological differences (Bowles *et al*, 1993; Itagaki *et al*, 1998).

The composition of these distinct clusters is unexpected. Based on the strain hypothesis it was anticipated that if differences were found, they would occur between Ireland and Britain (Kennedy *et al*, 1989). The results did not correspond to this theory. In one of the groups, sequences from all over Ireland, Scotland and England cluster together while the other group is formed by sequences of English origin only. Therefore a clear geographical split was not found. Instead it was the definitive host that seemed to account for the 2.2% divergence between groups. What grouped the Irish, English and Scottish sequences together was that for the most part they came from worms that were found in brown trout. The other group was composed of sequences from English cyprinid fish, chub and bullhead. That the host rather than geographical location accounted for the sequence divergence was corroborated by the AMOVA analysis. Very little correlation between genetic variation and geography was found. Both host species and the host's tolerance to salinity explained a much larger percentage of the variation. Though host salinity tolerance explained 61% of the variation, which was greater than that explained by host species, 47%, a possible separation of the strains by host species should not be dismissed.

The sequences from worms found in English chub and bullhead grouped together. This supports the hypothesis that the salinity tolerance of the host rather than the host species might be responsible for strain differentiation in *P. laevis*. Bullhead are not definitive hosts of *P. laevis* as the parasite rarely matures in this fish (Hine and Kennedy, 1974a). The bullhead came from the river Avon and it is well documented that chub are the definitive host of *P. laevis* in this river (Hine and Kennedy, 1974a, b). If the sequences from bullhead actually represent a population from chub, then the divergence between the strains may represent a chub/ brown trout split, rather than a stenohaline/euryhaline split.

The presence of the 'brown trout' strain in flounder is explicable as probably being due to *P. laevis* from brown trout cycling through flounder. A similar explanation can also be put forward for the presence of the 'brown trout' strain in a single bullhead. Brown trout are also found in the river Avon and are responsible for the maintenance of *P. laevis* in the upper reaches, though at much lower levels (Hine and Kennedy, 1974a;

Kennedy *et al*, 1978). It is therefore possible that bullhead are host to the 'brown trout' strain due to its natural presence in *G. pulex* or due to stocking of fish infected with this strain and subsequent transmission to bullhead.

The third cluster shown in the median network of the Irish and British sequences is the most mysterious. The presence of such a diverse sequence in *P. laevis* of Irish brown trout might be explicable by the introduction of infected *G. pulex* from England into Wicklow in 1988 (Keatinge, 1989). How this sequence arose in England is unknown. It is possible that it represents ancestral polymorphism, which existed at a low frequency in the colonising population. It may also represent a stocking event.

One of the assumptions of the strain hypothesis is that the Irish strain is the most recently evolved (Kennedy *et al*, 1989). This is based on the fact that neither of the cyprinid hosts of *P. laevis*, chub and barbel occur in Ireland. Therefore it was assumed that *P. laevis* could only have colonised Ireland due to the stocking of Irish rivers with infected cyprinids. Because of the depauperate nature of Ireland's ichthyofauna, it became popular to stock rivers with non-native species for recreational purposes. The stocking of infected cyprinids is thought to have occurred within the last 500 years, approximately. A record for the introduction of carp and tench goes back to 1634 (Went, 1950).

Based on work carried out on the mitochondrial DNA of higher animals, it is thought that a sequence divergence of 2% represents a divergence date of one million years ago (Hewitt, 1996). Similar estimates of between 1.7 and 2.3% exist for arthropods (Hewitt, 1996). The rate of evolution of acanthocephalan mtDNA is unknown but using these estimates as a rough guideline it is feasible to state that it seems highly unlikely that a mean divergence of 2.2% arose in P. *laevis* over the last 500 years. Britain and Ireland were re-colonised after the last ice age, more than 7,500 years ago, (Wheeler, 1977) and it is likely that the level of divergence predates even this. It is more plausible that the divergence occurred as a result of the last ice age, due to the division of populations into separate refugia. Alternatively because substitution rates in the Acanthocephala are unknown, mutations may accumulate very quickly in mitochondrial DNA. Therefore the possibility of an introduction of *P. laevis* into Ireland within the last 500 years and its subsequent evolution into the Irish brown trout strain cannot be dismissed (Kennedy *et al*, 1989). It is not possible to ascertain from these data if *P. laevis* is native to Ireland and colonised with the salmonids or if it was introduced via human intervention.

A divergence of between 2 and 4 million years ago has been estimated between three sibling species within *Contracaecum osculatum* (Nascetti *et al*, 1993). This estimate was based on enzyme electrophoresis though and is difficult to compare with the mitochondrial data presented here. 7 of 17 loci discriminated at least one of the sibling species from the other two if not all three. The three species, though sometimes found sympatrically, use different definitive hosts and are found in different geographical areas. Also despite some niche overlap, no individuals were found with heterozygous loci, implying reproductive isolation. The divergence date is thought to coincide with glaciations during the Pliocene and the Pleistocene periods and it is thought that allopatric speciation could have occurred in the Baltic Sea. The pattern of speciation in *C. osculatum* compares well with that in another nematode of seals, *Pseudoterranova decipiens* (Paggi *et al,* 1991).

Italian Sequences

The neighbor joining tree highlighted the enormous diversity within the Italian sequences and within *P. laevis* as a whole. The two groups revealed in the river Brenta differed by a mean percentage of 18% and they both differed from the British and Irish groups by a similar amount. Inter-specific percentage difference in the COI gene of *Echinococcus* sp was found to peak at 11.5% (Bowles *et al,* 1992). Due to the ability of cestodes to self-fertilise it is possible that speciation can occur at lower levels of genetic difference than in purely sexually breeding species such as *P. laevis*. An average difference of 15.6 % was found in the ND4 gene between *Haemonchus placei* and *H. contortus* (Blouin *et al*, 1997).

The level of intra-specific variation seen within *P. laevis* among the geographically isolated populations studied here can be explained by the effect of ice ages on species. Ice ages can have a dramatic effect on species' gene pools and can also result in extinctions. During the last ice age which peaked 18,000 years ago (Hewitt, 1996), ice sheets covered most of Ireland, Britain and northern Europe (Wheeler, 1977). Therefore any species which are extant today survived the last ice age and many preceeding ones, in refugia, which were located in southern parts of Europe (Hewitt, 2000). Species populations found in southern parts today have much higher levels of genetic heterogeneity than their northern counterparts. Mountain ranges in the south provide a much more heterogeneous environment, which allows speciation to proceed at

higher rates (Hewitt, 1996). This coupled with constant range restrictions and hence shuffling of genomes over many ice ages has resulted in the increased genetic diversity (Hewitt, 1996). Organisms in northern Europe exhibit extensive genetic homogeneity over much of their range. When the ice sheets retreated, new uninhabited territory became available and individuals on the periphery of the refugia were able to colonise. Such colonisation is known as leading edge expansion and tends to occur rapidly (Hewitt, 1996, 2000). As species moved further north, a continued loss of alleles would result in increased homozygosity.

P. laevis from Italy represent the diversity of a southern refuge and *P. laevis* from Britain and Ireland represent the homogeneity of rapid colonisation. Another reason for the degree of difference between *P. laevis* from Britain and Ireland and *P. laevis* from Italy is that it is doubtful that Italian individuals colonised northern Europe due to the barrier of the Alps (Durand *et al*, 1999). Based on studies of the fish hosts chub and brown trout, it is more likely that *P. laevis* in Britain and Ireland were colonised by individuals from the Balkans (Bernatchez *et al*, 1992; Durand *et al*, 1999).

European chub were found to comprise of four distinct lineages when a 600bp section of their cytochrome b gene was analysed (Durand *et al*, 1999). Two lineages were found in the north of Europe and two in the south, one Adriatic and one Aegean. The Atlantic group, which included fish from the UK, Germany, France and the Netherlands had a mean sequence divergence of 0.27 ± 0.05% compared to a value of 2.9 ± 1.38% found for the Adriatic group (Durand *et al*, 1999). In fact the 11 populations which make up the former group were dominated by one haplotype. Divergence between the four lineages was estimated to have happened in the Pliocene based on a sequence divergence rate of 2.8% per million years. A study of 640bp of the mitochondrial control region in brown trout revealed similar findings to those of Durand *et al* (1999). Five phylogeographic groupings were revealed and again Atlantic populations were dominated by one haplotype (Bernatchez *et al*, 1992). Atlantic groups were found to be more similar to Danubian groups, although unique haplotypes were also found in the Danube. Application of a molecular clock of 2% sequence divergence per million years allowed an estimate of 450,000 to 700,000 years ago for the divergence of brown trout. It is possible that *P. laevis* in brown trout and chub differ genetically due to Ireland and Britain having been colonised by brown trout and chub from different refugia.

Conclusions

This study has revealed *P. laevis* to be composed of multiple strains on a genetic level, but only two of these are also supported by different ecological and morphological characteristics. Two major British Isles *P. laevis* strains are distinguished by a 2.2% difference in subunit I of the mitochondrial COI gene. These strains most closely resemble the Irish and English strains described by Kennedy *et al* (1989). The strains revealed by this study do not seem to be separated by geography and there is evidence to suggest that each strain is restricted to a particular type of host. A more appropriate name for the Irish strain might be the euryhaline or salmonid strain as it is found in Ireland, Scotland and England but is mostly found in brown trout with a few exceptions. A more appropriate name for the English strain might be the stenohaline or cyprinid strain as this strain was only found in the chub and bullhead of two English rivers.

The 2.2% difference found seems to contradict the divergence time of 500 years ago suggested by Kennedy *et al* (1989) when compared to divergence times in other species. This level of difference suggests a possible divergence time of up to one million years ago. However, mutation rates in the Acanthocephala are unknown at present so this estimate is unsubstantiated. It is not possible from these data to substantiate the method of introduction of *P. laevis* into Ireland. Two routes are possible. Firstly, *P. laevis* may have colonised Ireland via stockings of infected cyprinids (Kennedy *et al*, 1989). Before this time Ireland would have remained free of this parasite due to the fact that the stenohaline hosts of *P. laevis* were unable to colonise after the last ice age, as the Irish Sea constituted a barrier. Secondly, Ireland may have been colonised naturally due to the ability of its host there, brown trout to tolerate saline conditions. It is also possible that *P. laevis* in Ireland is more closely related to the marine strain as this strain is also able to tolerate both freshwater and marine conditions (Kennedy, 1984; Munro *et al*, 1989). This hypothesis requires testing. A more complete phylogeographic study, including more populations in Britain and northern Europe, is needed to enable a more accurate dating of strain divergence in *P. laevis*.

CHAPTER 6

GENERAL DISCUSSION

GENERAL DISCUSSION

The overall aim of this work was to investigate strain differentiation in *P. laevis* found in Ireland and the U.K. The investigation was conducted by examining three separate areas in detail, namely the biology, morphology and the population genetics of *P. laevis*. Ecological, morphological and molecular data were collected from several populations. Previously, much evidence existed to support the ecological differences between the proposed strains. In this thesis, the ecological evidence has been corroborated. For the first time, strong morphological and molecular data have been revealed in support of the strain hypothesis.

The results of the ecological study of Irish *P. laevis* supports current knowledge. *Irish P. laevis* is located in the posterior region of the gut of brown trout, which confirms work by Fitzgerald and Mulcahy (1983) and Molloy *et al* (1995). Two years of data were collected and in both years, the processes of growth and maturation followed a distinctly seasonal cycle, a feature first observed in Irish *P. laevis* by Molloy *et al* (1995). *P. laevis* in L. Feeagh only produce one generation per year, which differs from the overlap of generations seen in *P. laevis* in the rivers Avon, Severn and on the continent (Hine and Kennedy, 1974b; Brown, 1989, Moravec *et al*, 1997). Irish *P. laevis* do not reach as large a size nor mature as frequently as their British and European counterparts and as a result brown trout are merely suitable hosts as defined by Lyndon and Kennedy (2001), rather than preferred definitive hosts. It now seems likely that temperature mediated effects on the intermediate and definitive hosts are affecting the cycles of growth and maturity in Irish *P. laevis* and in a much more pronounced way in the Irish situation.

For example, Fitzgerald (1987) studied the population dynamics of *G. duebeni* in the river Shournagh and found that it produced only one generation per year, which differed from reports of *G. duebeni* and *G. pulex* in Britain having two generations per year (Hynes, 1954; Sheader, 1983). Fitzgerald (1987) concluded that this affected the availability of susceptible gammarids to infection by the parasite, which would have a knock-on effect on the parasite's own life cycle. In the bivoltine situation the first generation appeared in spring as expected. High summer temperatures resulted in a rapid maturation process whereby the new generation bred and released a second generation by the end of the summer (Sheader, 1983). The impact of a univoltine strategy in *G. duebeni* on the population in L. Feeagh is that *P. laevis* may only infect

the intermediate host in the spring and summer. This is the only time when young, susceptible gammarids are available (Fitzgerald, 1987). Also, L. Feeagh is an oligotrophic lake and as a result its energy production is relatively low (Byrne, 2000). This coupled with winter temperatures could be responsible for the lack of winter growth seen in *P. laevis* between November and April, which might also prevent it from reaching the larger weights of other populations due to a shorter growing season. A more detailed study of the intermediate host in L. Feeagh could probe some of these possibilities.

The morphological study of a wild Irish and English population of *P. laevis* has revealed characters capable of separating them. The stoutest hook is found in the fifth or sixth position in the English population and in the fourth or fifth position in the Irish population. Also English *P. laevis* had either the same numbers of anterior and posterior hooks or more anterior hooks while the opposite was true for Irish *P. laevis*. Though these characters overlap to some degree between the two populations, they should prove successful in separating them in most situations. In ambiguous cases observations of more rows on the proboscis and consideration of other factors such as hook area, which was generally greater in Irish *P. laevis* should result in identification. These results provide evidence favourable to the idea of separate strains in *P. laevis,* which did not seem possible considering the overwhelming morphological variation exhibited by acanthocephalans (Bullock, 1962; Amin, 1975a and b; Brown, 1987; Amin and Redlin, 1998). The impediment of morphological variability to clear taxonomic identification was superseded in North America for two similar species of *Pomphorhynchus*. Characters similar to those described here distinguished *P. bulbocolli* and *P. rocci*, which were initially thought to be one and the same species (Huffman and Nickol, 1978; Gleeson and Huffman, 1981).

It was vital to the strain hypothesis to conduct a study at the molecular level, due to the lack of distinguishing morphological markers. At the beginning of this project it was not known if morphological characters could be found and even though characters capable of differentiating the two freshwater strains were revealed, more supporting evidence was required. Parasites in general do not possess many taxonomically useful organs. In species where differentiation was suspected though not supported by morphology, molecular techniques were the only methods capable of providing supportive evidence, for example, studies conducted on *Contracaecum osculatum* (Nacetti *et al*, 1993) and *Macvicaria crassigula* and *Monorchis parvis* (Jousson *et al*,

2000). Even in studies where morphological characters were found to distinguish between strains or species, genetic analysis was also conducted (Ponce Gordo and Cuesta Bandera, 1997; Jacquiet *et al*, 1995).

The population genetics study revealed a heterogeneity in one mitochondrial gene in *P. laevis*. Differences of 2.2% were found between two haplotype clusters in particular. These clusters fitted best to the description of the two freshwater strains by Kennedy *et al* (1989). A difference of 2.2% is certainly acceptable for defining strains when compared to other studies (Bowles *et al*, 1992) especially in light of definite epidemiological and morphological differences. Schistosomes show interspecific differences of 13 to 21% in the COI gene (Bowles *et al*, 1993) and Echinostoma species show interspecific differences of 12.3 to 30.5% (Morgan and Blair, 1998). Therefore the inter-strain differences shown for *P. laevis* are much lower than those between known species.

What was unexpected about the molecular findings was that the genetic variation between strains was more closely related to the host or host related factors than to geographical separation. It was hypothesised that a process of allopatric speciation may have been instigated in *P. laevis* due to an introduction into Ireland and selective adaptation to a new environment (Kennedy *et al*, 1989). The results do not concur with this particular hypothesis as strains similar to those found in Ireland were also found in two rivers in England and one in Scotland. The pattern of genetic variation suggests a process of sympatric or parapatric speciation, though exactly when and where this process began is unclear.

The level of variation uncovered in the Italian population was unexpected. These parasites were removed from the same host species, chub and from the same river, the Brenta though whether they came from exactly the same location is unknown (Dezfuli, pers. comm.). It is possible that some of this variation can be explained by events such as the Quarternary ice ages (Hewitt, 1996) though whether some of this variation belies another speciation process requires further investigation. A recent and ongoing study into variation within the COI gene of three palaeacanthocephalans has revealed up to 30% sequence difference (France *et al*, 2002). The species compared were from three different families. More work needs to be conducted on *P. laevis* in Italy to confirm whether the sequence difference of 18.5% is representative of separate species.

When considering all three aspects of the biology of *P. laevis* together, the results support a theory of divergent evolution. Ecologically, the Irish and English freshwater populations use different intermediate and definitive hosts. Morphological differences are emerging between the two strains. On a mitochondrial level, high inter-strain variation has been found. The new results uncovered here coupled with the recent discovery of possible reproductive isolation between the marine and freshwater strains in the river Thames (Guillen-Hernandez and Whitfield, 2001) provide strong evidence for strain formation in *P. laevis*. Also the populations for which ecological and morphological differences were found display distinct haplotypes which are 2.2% different. Whether evolutionary processes will continue to separate these strains or whether continuing transfers of fish between Ireland and the U.K. will serve to keep them in a single evolutionary unit is unknown. Though identifying biological and evolutionary species is an arduous task it is an important one to continue. The accurate identification of species and diverging populations is vital for the correct management of biodiversity and for identifying the correct epidemiology of disease causing agents such as parasites.

Further Work

Ecology:

A year long study needs to be conducted into the lifecycle of *G. duebeni* in L. Feeagh and into the occurrence of the various larval stages of the parasite in this host. This is needed in order to confirm at what times of the year gammarids are available for infection by acanthors and when gammarids are infected with cystacanths and hence exactly at what times of year transmission to the final host occurs.

It might be interesting to carry out an experiment with the Irish strain, by maintaining the adult phase of the life cycle in the laboratory. The focus of the experiment would be to investigate the growth and maturation cycle and whether under altered conditions the parasite would still produce just one generation per year.

Morphology:

It is important to validate the effectiveness of the two morphological characters, position of the stoutest hook and the proportions of anterior and posterior hooks. This is necessary as their phenotypic plasticity needs to be assessed. Now that various populations with a similar genetic makeup to both the L. Feeagh population and the R. Avon population have been identified, their morphological characteristics should be compared to the results presented here, which would confirm the usefulness of the two characters as identifying features.

Population Genetics:

The COI gene of *P. laevis* from more brown trout, chub and other populations across Ireland and the U.K. needs to be sequenced in order to determine the frequency of the identified haplotypes in these populations. It is also necessary to clarify the division of haplotypes between brown trout and chub to be certain that these haplotypes are specific to particular strains or whether this is an artefact of sampling.

It may be possible to develop an assay to conduct the aforementioned work instead of sequencing every individual, which is more time consuming. The assay might consist of a restriction enzyme pattern specific to each haplotype. If new patterns were identified then these individuals could be sequenced and new polymorphisms identified.

Another molecular marker needs to be sequenced in order to determine if the variation in the COI gene is just a feature of mitochondrial DNA. Gene trees are not necessarily equivalent to species trees especially when closely related organisms are considered (Anderson, 2001). Alleles have been known to segregate even in the same population giving a false picture of speciation which is not actually occurring (Anderson, 2001). To safeguard against this it is wise to use a multi-locus technique. A nuclear gene such as one of the ITS sequences in the ribosomal DNA would provide a good comparison to the mitochondrial gene.

More populations from large catchments on the continent such as the rivers Rhine, Danube *etc.* could be analysed in order to determine which populations are most closely related to the Irish and English strains. This would help determine which

populations are ancestral to those in Ireland and England and it might enable a more accurate dating of their separation.

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APPENDIX A

Table A.1: Results of comparisons (Scheffe Post-Hoc test) of the hook lengths of Irish *P. laevis* between the three sampling periods

	AUG > MAY p	AUG > NOV p	MAY > AUG p	MAY > NOV p	NOV > MAY p	NOV> AUG p
LONGEST HOOK		NS	NS	*		
HOOK 1			*	NS		NS
HOOK 2		NS	NS	*		
HOOK 3		NS	NS	*		
STOUTEST HOOK						
HOOK 7	NS	*		NS		
HOOK 8						
HOOK 9		NS	NS	*		
BASAL HOOK						

Table A.2: Results of comparisons (Scheffe Post-Hoc test) of the hook widths of Irish *P. laevis* between the three sampling periods.

	AUG > MAY p	AUG > NOV p	MAY > AUG p	MAY > NOV p	NOV > MAY p	NOV> AUG p
HOOK 1			NS	NS		NS
HOOK 2		NS	NS	**		
HOOK 3		NS	NS	*		
STOUTEST HOOK	NS	**		*		
HOOK 7	NS	***		***		
HOOK 8		**	NS	***		
HOOK 9		*	NS	**		
BASAL HOOK						

Table A.3: Results of comparisons (Scheffe Post-Hoc test) of the hook areas of Irish *P. laevis* between the three sampling periods.

	AUG > MAY p	AUG > NOV p	MAY > AUG p	MAY > NOV p	NOV > MAY p	NOV> AUG p
HOOK 1			*	NS		NS
HOOK 2		NS	NS	***		
HOOK 3		NS	NS	**		
STOUTEST HOOK		NS	NS	*		
HOOK 7	NS	**		**		
HOOK 8		*	NS	**		
HOOK 9		*	NS	***		
BASAL HOOK						

Table A.4: Results of comparisons (Scheffe Post-Hoc test) of the hook shape of Irish *P. laevis* between the three sampling periods.

	AUG > MAY p	AUG > NOV p	MAY > AUG p	MAY > NOV p	NOV > MAY p	NOV> AUG p
HOOK 1						
HOOK 2						
HOOK 3						
STOUTEST HOOK	NS	*		*		
HOOK 7		NS	NS	*		
HOOK 8						
HOOK 9						
BASAL HOOK						

APPENDIX B

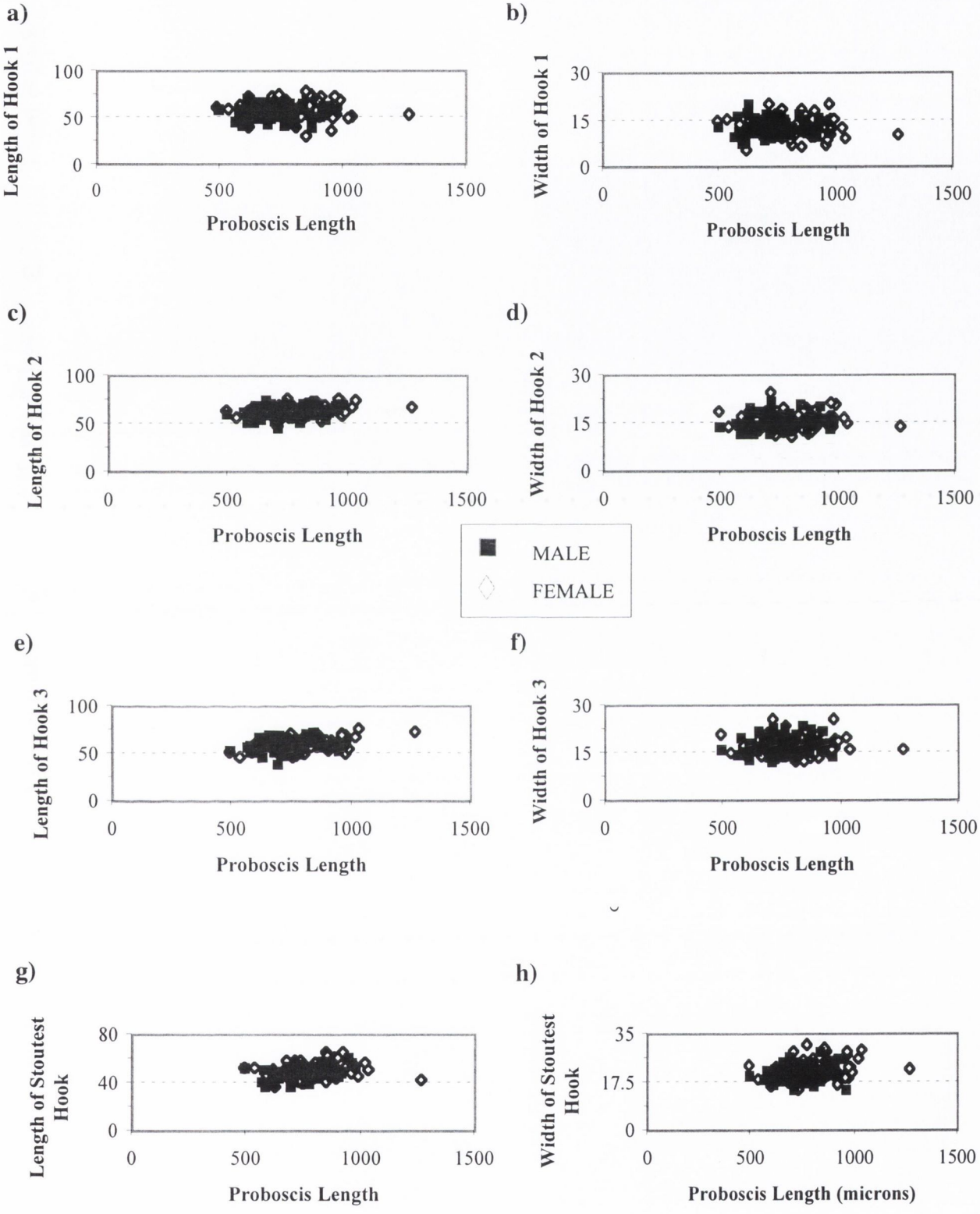

Fig. B.1: Scatterplot of anterior hook length and width **(a - f)** and stoutest hook length and width **(g - h)** against proboscis length for male and female *P. laevis* of Irish origin.

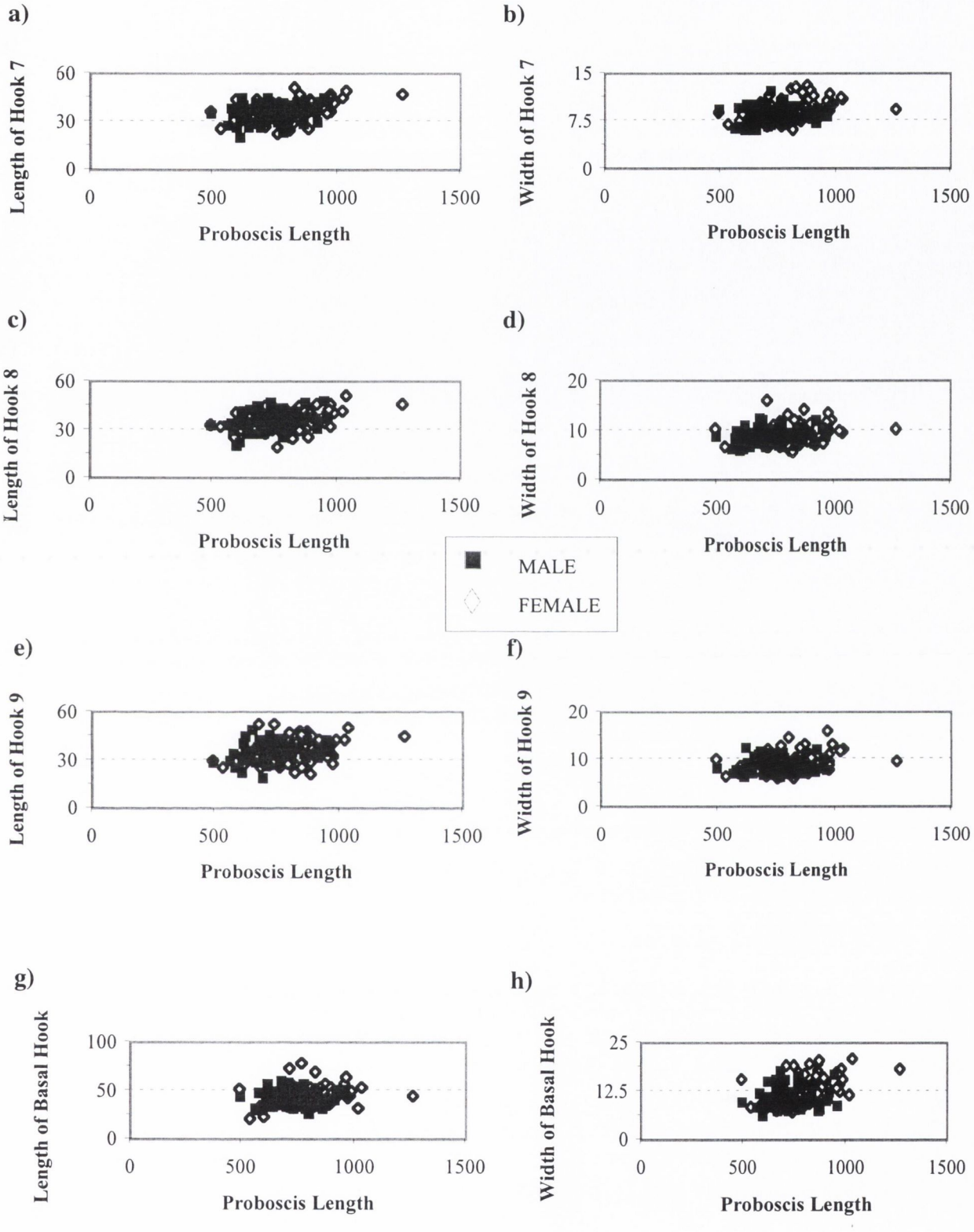

Fig. B.2: Scatterplot of posterior hook length and width **(a - f)** and basal hook length and width **(g - h)** against proboscis length for male and female *P. laevis* of Irish origin.

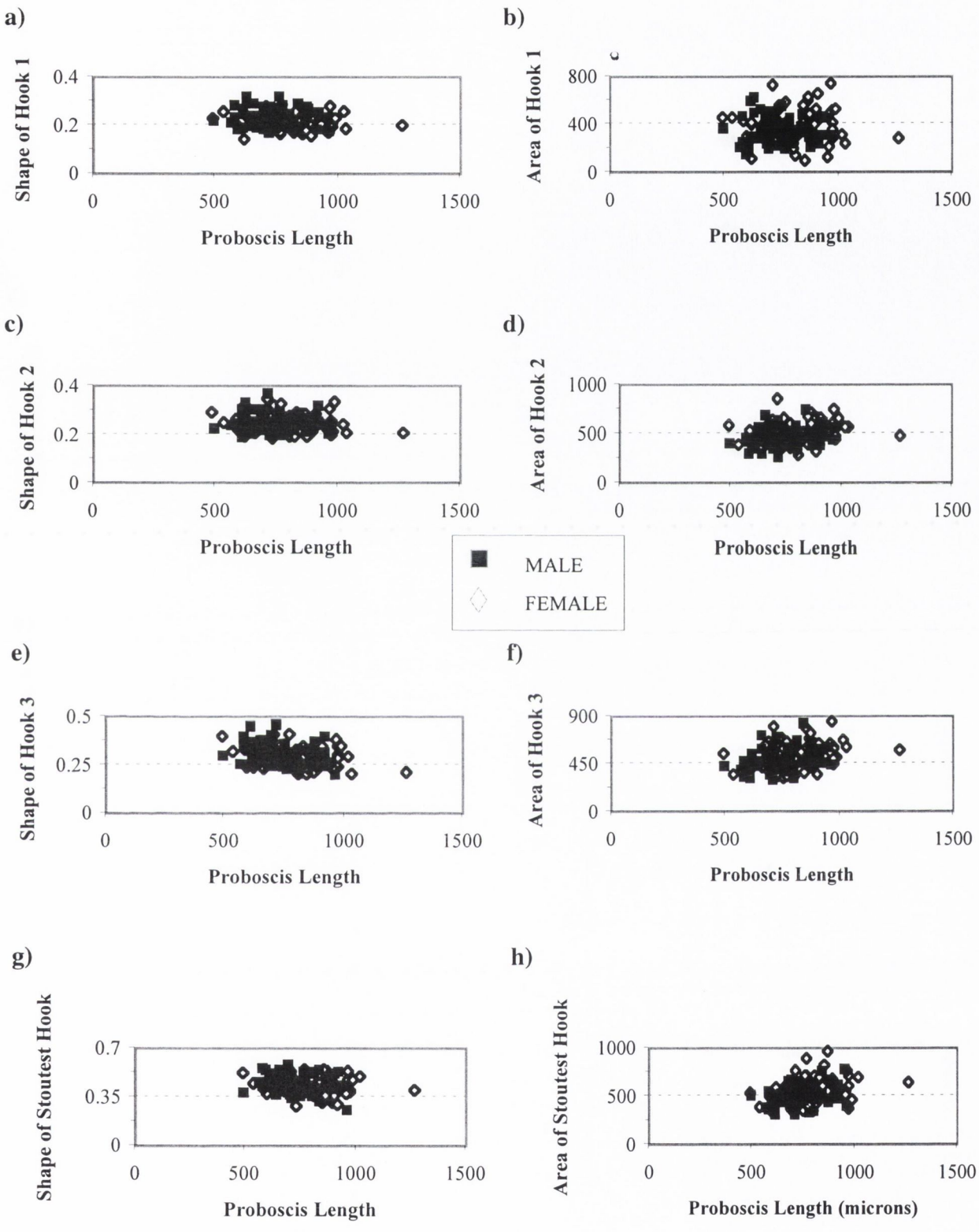

Fig. B.3: Scatterplot of anterior hook shape and area **(a - f)** and stoutest hook shape and area **(g - h)** against proboscis length for male and female *P. laevis* of Irish origin.

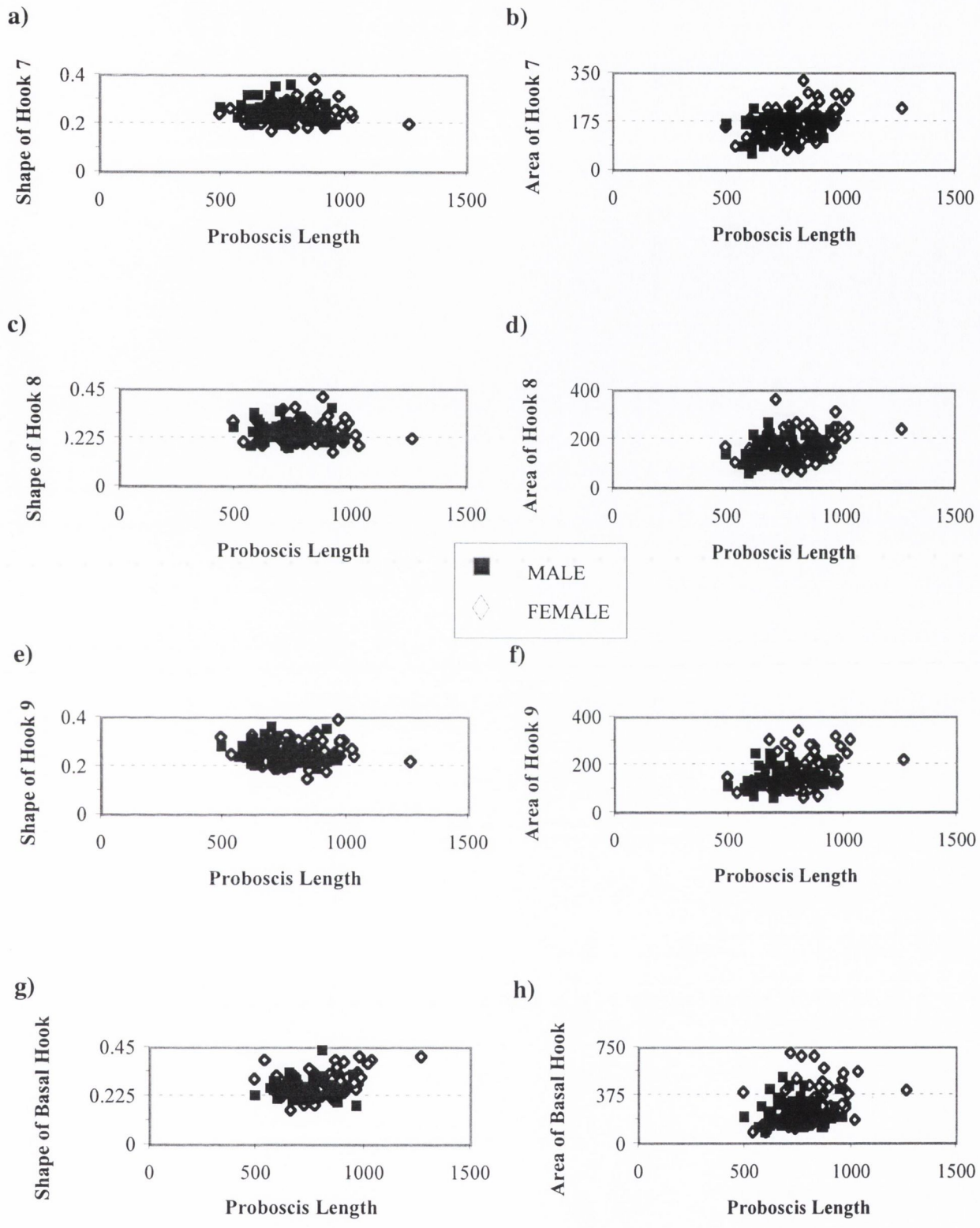

Fig. B.4: Scatterplot of posterior hook shape and area **(a - f)** and basal hook shape and area **(g - h)** against proboscis length for male and female *P. laevis* of Irish origin.

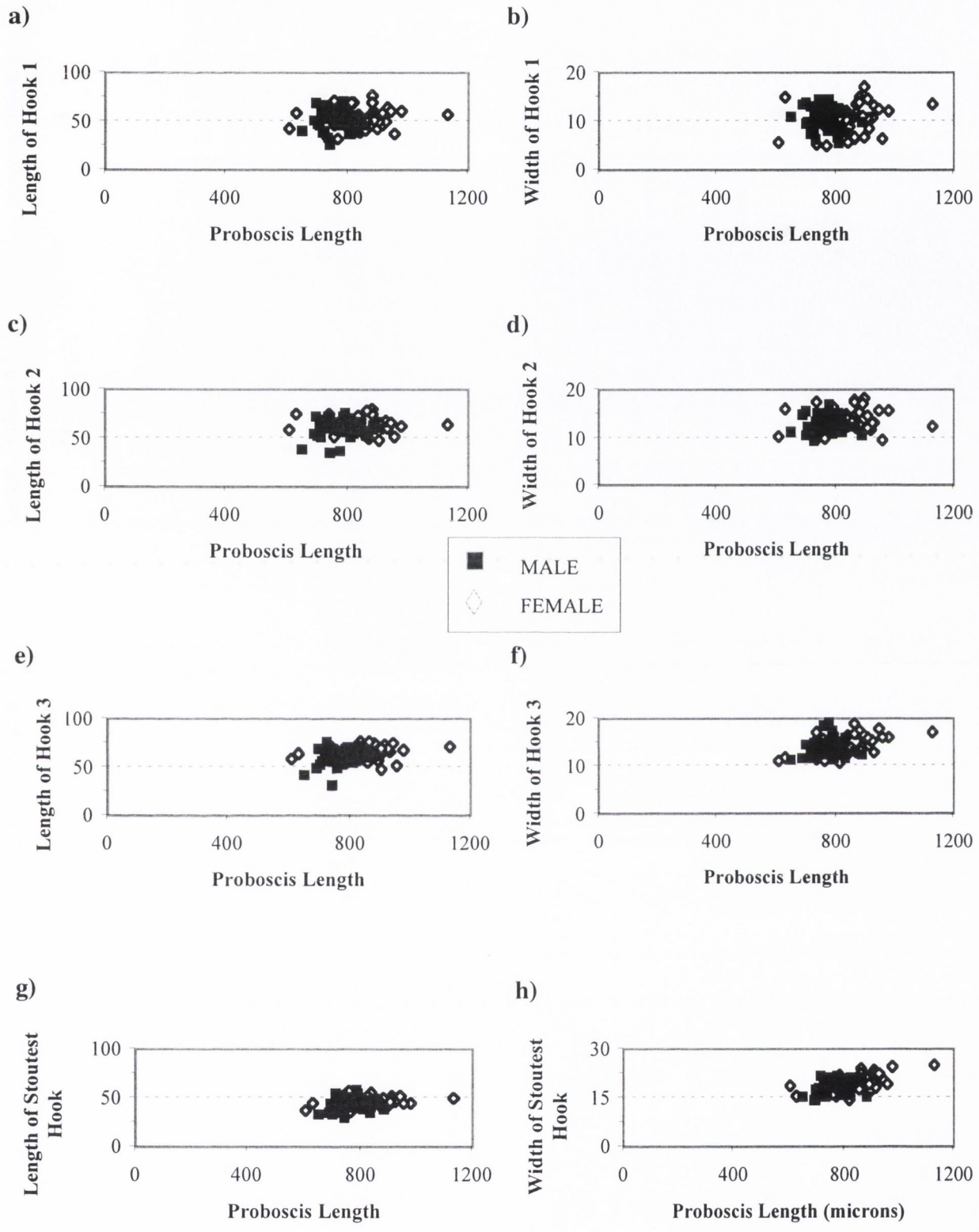

Fig. B.5: Scatterplot of anterior hook length and width **(a - f)** and stoutest hook length and width **(g - h)** for male and female *P. laevis* of English origin.

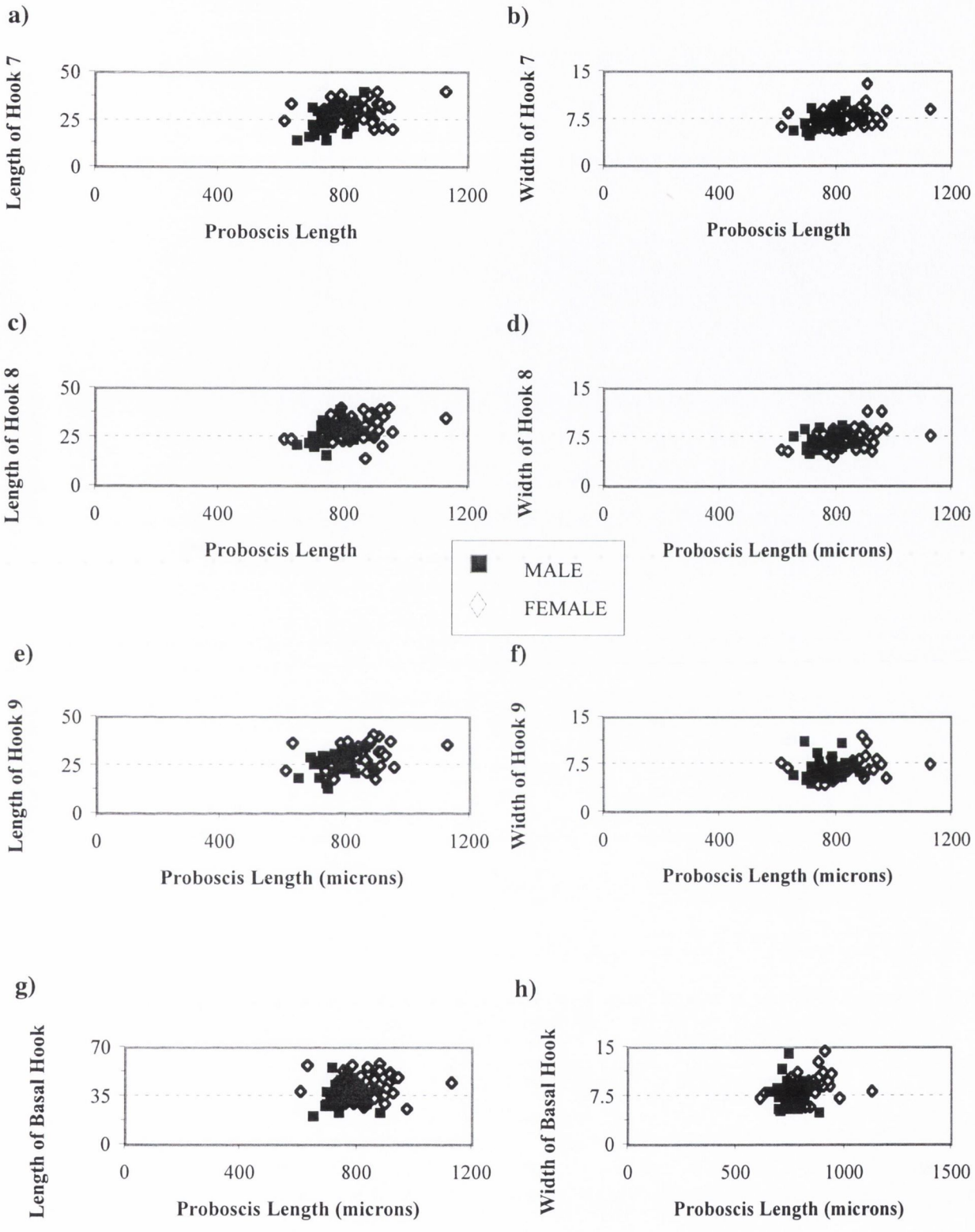

Fig. B.6: Scatterplot of posterior hook length and width **(a - f)** and basal hook length and width **(g - h)** for male and female *P. laevis* of English origin

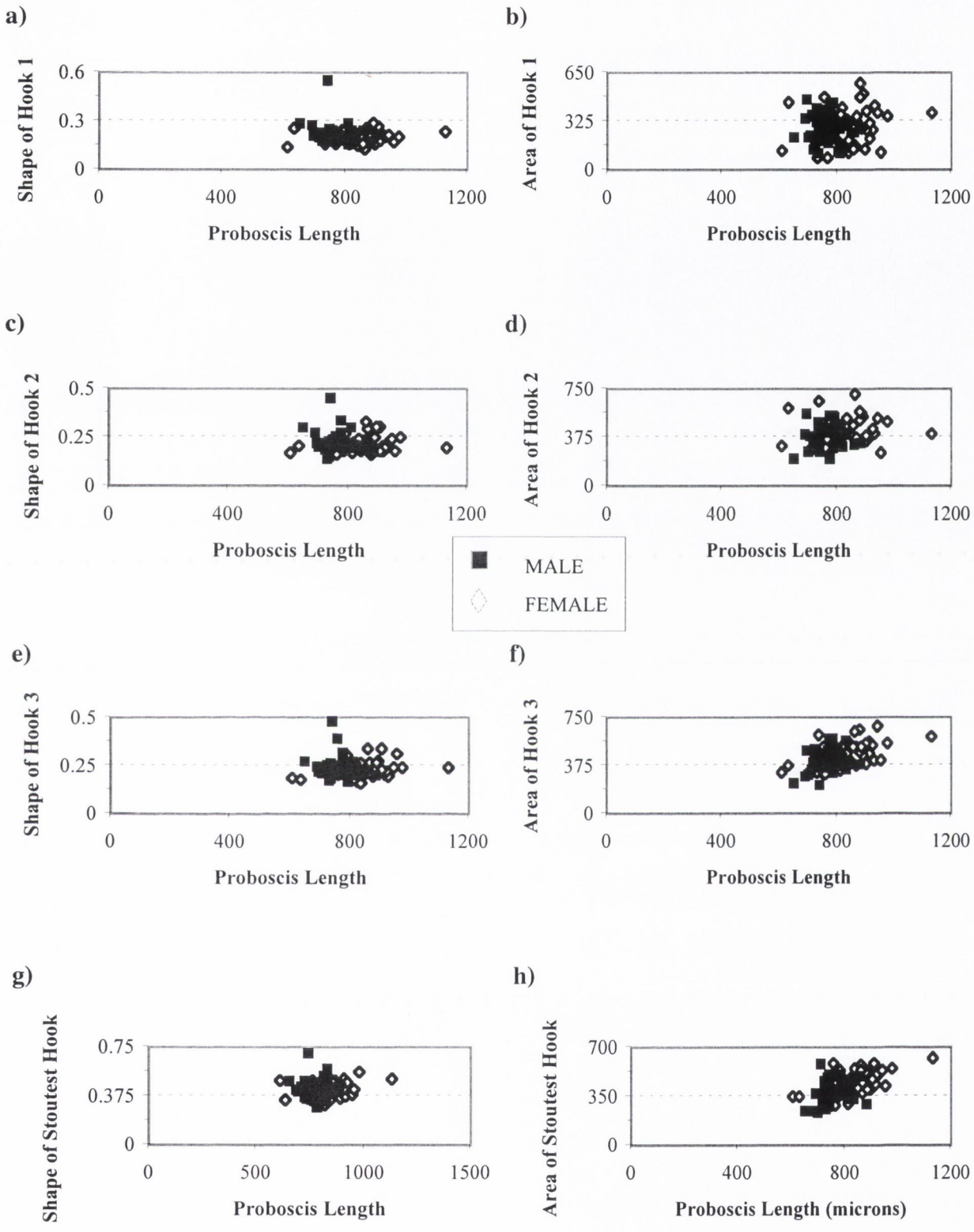

Fig. B.7: Scatterplot of anterior hook shape and area **(a - f)** and stoutest hook shape and area **(g - h)** for male and female *P. laevis* of English origin.

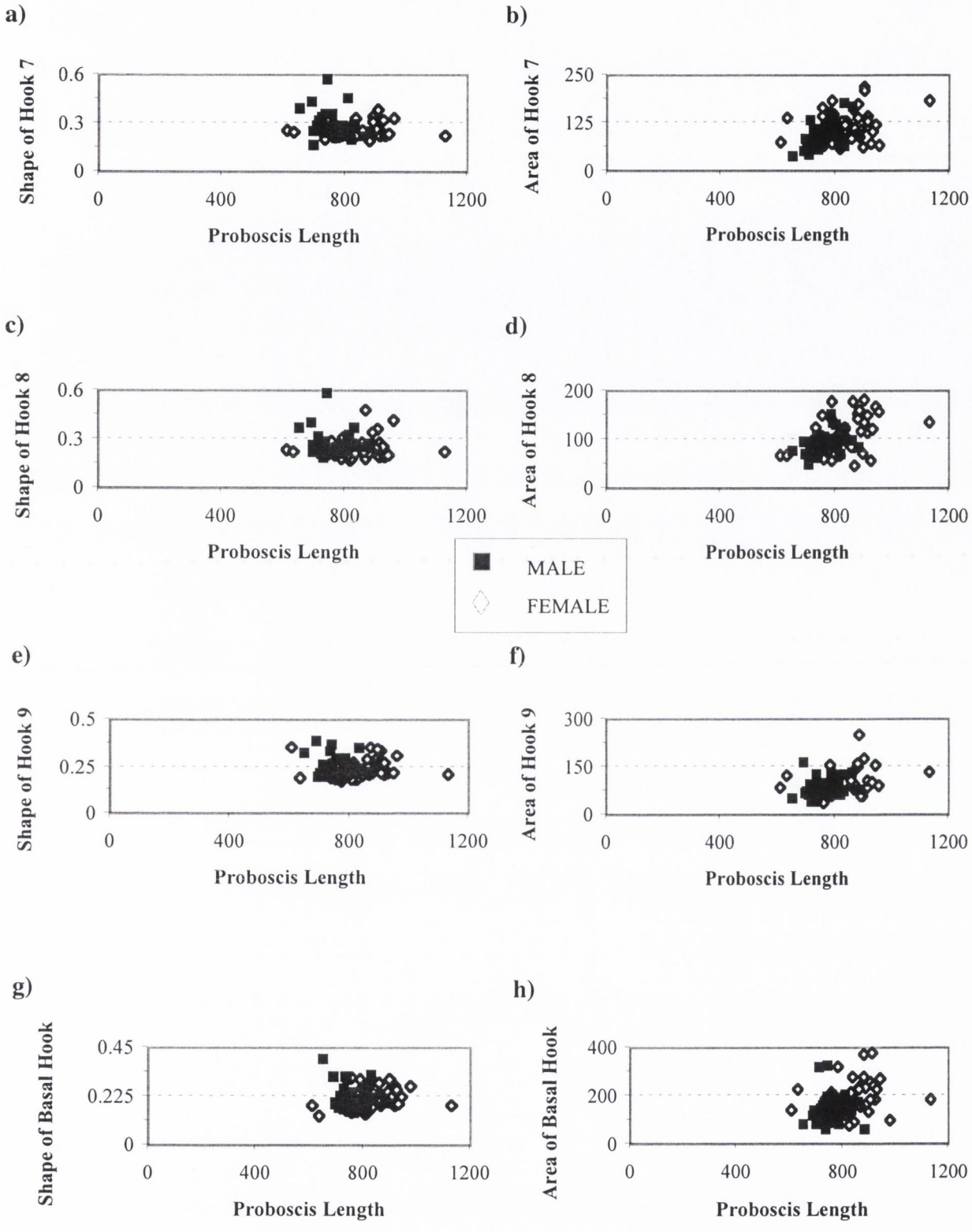

Fig. B.8: Scatterplot of posterior hook shape and area **(a - f)** and basal hook shape and area **(g - h)** for male and female *P. laevis* of English origin.

APPENDIX C: Recipes

I) Digestion Buffer

	Working Concentration	Volume
NaCl	1 M	5 ml
Tris – HCl (pH 8.0)	1 M	500 µl
EDTA (pH 8.0)	0.5 M	2 ml
Sarkosyl / SDS 1 %	30 %	1.65 ml
Proteinase K	19 mg / ml	-
ddH_2O	-	40.35 ml

100 µl of proteinase K was added to 9.9 ml of the above solution. The resulting solution was then diluted 2 fold by the addition of 10 ml of ddH_2O.

II) 100X TE Buffer

1.0 M	Tris HCl (pH 8.0)
.01 M	EDTA

III) 10X TBE Buffer

1.0 M	Tris
0.9 M	Boric Acid
0.01 M	EDTA

IV) 6 % Polyacrylamide Gel

Polyacrylamide was made up using reagents from the SequaGel Sequencing System, maufactured by BioSciences. 1L of gel composed of-

100 ml	Buffer
250 ml	Concentrate
650 ml	Diluent

480 µl of APS and 48 µl of TEMED were added to 60 ml of the above solution to cause the gel to polymerise.

APPENDIX D: Laboratory Procedures

I) DNA Extraction

1. Add 250µl of phenol (pH 8) to degraded tissue.
2. Vortex briefly
3. Spin for 3 minutes at 13,000 rpm.
4. Remove aqueous layer into a clean eppendorf.
5. Add 250 µl of phenol:chloroform:isoamyl alcohol (in these proportions 25:24:1).
6. Repeat stages 2 to 4.
7. Repeat stages 5 to 6.
8. Add 500 µl of cold (-20 °C) absolute alcohol to the final aqueous layer in a clean eppendorf tube.
9. Add 70 to 75 µl of 3M sodium acetate in order to have a final concentration of 0.3M.
10. Invert tubes gently to precipitate the DNA.
11. Spin for 3 minutes at 13, 000 rpm.
12. If pellets are not seen or are very small, leave in freezer overnight and re-centrifuge.

II) 50 µl PCR Mixture

REAGENTS	FINAL CONC.	VOLUME
PCR Buffer	1X	5.0 µl
dNTP's	200 µM	5.0 µl
$MgCl_2$	1.5 / 2.0 mM	5.0 µl
Primers (A\B)	0.5 µM	5.0 µl
Platinum Taq Polymerase	2.5 units	0.5 µl
Template DNA	-	12.5 µl
ddH_2O	-	17.0 µl
TOTAL VOLUME	-	50.0 µl
Oil Overlay	-	20.0 µl

A: COI p1 + COI p2 B: COI p1 + COI p3

10X PCR Buffer: 200 mM Tris HCl (pH 8.4) and 500 mM KCl

III) Cleaning of amplification product

1. **Sample Preparation:** Add 400 µl of Binding Solution to the amplification reaction and mix thoroughly. Removal of oil is not necessary.
2. **Cartridge Loading:** Place a spin cartridge into a 2 ml wash tube. Load the sample from step 1 into the spin cartridge. Centrifuge the mixture in a microcentrifuge at 12,000 rpm for 1 minute. Discard the flow-through.
3. **Cartridge Wash:** Place the spin cartridge back into the 2 ml wash tube. Add 700 µl of Wash Buffer to the spin cartridge. Centrifuge at 12,000 rpm for 1 minute. Discard the flow-through. Centrifuge again at 12,000 rpm for 1 minute to remove all residual wash buffer.
4. **DNA Elution:** Place the spin cartridge into a 1.5 ml recovery tube. Add 50 µl of TE Buffer directly to the centre of the spin cartridge. Incubate at room temperature for 1 minute, then centrifuge at 12,000 rpm for 2 minute.

IV) Liquid Cool Sequencing Procedure

1. Label 0.5 ml PCR tubes: one for each sample; 4 for the terminators (G A T C); one for the Labelling Reaction; and one for the Annealing Reaction.
2. Make up (sample # +1) times the Annealing Reaction mastermix (Appendix E.I) and add 7 µl to 4 µl of each DNA sample.
3. Place tubes into a rack and heat to 93° C for 2 to 3 minutes in a pre-heated PCR machine. Drop immediately into liquid nitrogen. The annealed template/primer mix can be kept at this stage for up to 30 minutes.
4. Set up (sample # +1) times the Labelling Reaction mastermix (Appendix E.II), excluding ^{35}S, enzyme (and Mn if required).
5. Set up (sample # +1) times the Termination Reaction (Appendix E.III) for each base (G A T C) and add 2.5 µl to each respective well on a Terisaki plate labelled ([G A T C] x sample #). Place on ice.
6. Add radioactive label, enzyme (and Mn if required) to labelling reaction mastermix.
7. Quickly remove annealed template/primer from liquid nitrogen, uncap and place in MicroCentaur centrifuge.
8. Quickly add 5 µl of labelling mix to each annealed template/primer. Pulse spin to mix and thaw, and incubate in the centrifuge at room temperature for 2 minutes.

9. Place terisaki plate on heat block at 37 °C. Transfer 3.5 µl of labelled template to each termination well (G A T C) over the course of 5 minutes – the timing of this step is crucial.
10. Stop the reaction by adding 4 µl of formamide to each well over 5 minutes.

APPENDIX E: Sequencing reaction mixtures

I) Annealing Reaction Mastermix

2 µl	Primer
2 µl	5X Sequenase Buffer
1 µl	50 % DMSO
2 µl	ddH_2O

II) Labelling Reaction Mastermix

1.5 µl	100 mM DTT
0.5 µl	50 % DMSO
0.4 µl	Labelling Mix
2.1 µl	ddH_2O

0.5 µl	^{35}S-dATP	These three components are added to the labelling reaction mastermix at a later stage in the process.
0.25 µl	T7 Sequenase enzyme	
(0.2 µl	Mn Buffer)	

III) Termination Reacion Mastermix

2.5 µl	Terminator Mix (ddNTP's)
0.25 µl	50 % DMSO