Taxonomy, morphology and host-parasite interactions of *Gyrodactylus* of poeciliid fish.

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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Contents

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Gareth Ronald Richards

A series of wide ranging taxonomic and experimental studies of the monogenean parasite species *Gyrodactylus bullatarudis* and *G. turnbulli* of the poeciliid fish host *Poecilia reticulata* were carried out.

G. bullatarudis Turnbull, 1956 *sensu* Harris (1986) from *Xiphophorus* sp. was reassigned to *G. rasini* Lucky,1973. Careful examination of *Gyrodactylus* cirrus spines, particularly the large spines, proved valuable for species differentiation.

A new technique applying modified Mallory stain during transfer of *Gyrodactylus* specimens from ammonium picrate-glycerin to a permanent mountant was developed. It was found excellent for study of general anatomy (muscles and tendons, cell nuclei, tegument and gland cells were all stained) and in the rendition of the taxonomically important dorsal and ventral bars. Improved descriptions of the ventral and dorsal bars of *G. turnbulli* resulted from its use.

G. bullatarudis had larger attachment sclerites when reared at 25°C than at 19°C. Sclerite sizes of *G. turnbulli* were not significantly different at these two temperatures. Samples of sclerites from *Gyrodactylus* infrapopulations originating from a single parasite were less variable than those from mixed infrapopulations, suggesting a correlation between genetic variation and sclerite morphometric variation.

Freeze fixation-dehydration was used in the preparation of *Gyrodactylus* for SEM viewing. Freeze fixation-dehydration caused little shrinkage and provided instant immobilisation of specimens. Others fixed using 10% neutral buffered formalin showed more shrinkage and signs of stress before death. Freeze fixation-dehydration was the best preparatory method for the study of *Gyrodactylus* external gross morphology.

G. turnbulli and *G. bullatarudis* were shown to have opisthaptor attachments which were similar overall to those of other *Gyrodactylus*, but detailed differences were described. An hypothesis relating opisthaptor attachment to marginal hook morphology of *Gyrodactylus* species was presented.

In the laboratory *G. bullatarudis* and *G. turnbulli* had similar rates of increase on naive fish at 26°C but *G. bullatarudis* was more pathogenic. *G. bullatarudis* induced host mortality was 0.004 per host per day per parasite and *G. turnbulli* induced host mortality was 0.002 per host per day per parasite. Differences in host-site specificity, attachment and feeding were identified as probable causes of this difference in pathogenicity.

The initial host response of *P. reticulata* to *Gyrodactylus* was shown to be non (*Gyrodactylus*) species-specific and was probably localised. However, the exact nature of the response remains unknown.

Investigation of *G. turnbulli* infections of adult *P. reticulata* demonstrated these parasites capable of maintaining low intensity, randomly distributed infections on just six hosts in a 50l aquarium for at least 3 months without regular addition of susceptible fish.

CHAPTER 1

1. Introduction: an overview of *Gyrodactylus* von Nordmann, 1832

The aims of this chapter are to:

1) Explain the nomenclature used throughout my investigations.

2) Describe the anatomy of *Gyrodactylus* species paying particular attention to *G. bullatarudis* Turnbull, 1956 and *G. turnbulli* Harris, 1986 from *Poecilia reticulata* Peters. *Gyrodactylus* locomotion, feeding and reproductive biology are discussed at some length as they are particularly relevant to this thesis.

3) Discuss host (species) specificity within the genus *Gyrodactylus*, with particular emphasis on those infecting poeciliid fish.

4) Give an overview of the important features of *Gyrodactylus* epidemiology which have relevance to this thesis.

5) Outline the objectives of my thesis.

Although this chapter is primarily a literature review, on discussing *Gyrodactylus* anatomy and host specificity, some results from my own work have been included. These results were from scanning electron microscope (= SEM) studies and exploratory studies in host specificity. Both of these studies were not comprehensive enough to warrant description in separate chapters. The SEM studies illustrated features of the anatomy of *Gyrodactylus* better than most previous works due to an improved preparatory (fixation and dehydration) technique which is described at length in Chapter 6. The studies in host specificity report some new findings.

In section 1.4 reference is made to the genus *Macrogyrodactylus*. This genus is also a viviparous gyrodactylid (Malmberg, 1956). It closely resembles *Gyrodactylus* and observations from studies of *Macrogyrodactylus* species are relevant to discussions of *Gyrodactylus* host-parasite relationships.

1.1. Notes on nomenclature and an introduction to the genus *Gyrodactylus*

The taxon Monogenea is also frequently referred to as the Monogenoidea Bychowsky, 1937. The group has been given ordinal [van Beneden (1858); Carus (1863) cited with others from Boeger and Kritsky (1993)], infra class (Brooks, 1989) and class (Bychowsky, 1957) status. There have been numerous systematics proposed for the Monogenea, three within the past ten years (Boeger and Kritsky, 1993; Malmberg, 1990; Lebedev, 1988). Nomenclature of the group is therefore controversial. Hereafter the taxon shall be referred to as the class Monogenea as accepted by the round table discussion held during the Fourth International Congress on Parasitology, Warszawa, 1978 (see Euzet and Prost, 1981). This thesis is not concerned with the investigation of the systematics of the Monogenea as a whole although some interrelations within the genus *Gyrodactylus* von Nordmann, 1832 are discussed. The systematics for *Gyrodactylus* described by Malmberg (1970) have been adopted for this purpose (see below).

Gyrodactylus are parasites of the external surfaces of marine and freshwater fish and amphibians (Malmberg, 1970; Wootton *et al.*, 1993). They are characterised by a posterior attachment organ (or opisthaptor) which consists of sixteen marginal hooks and two central large hooks (or hamuli) which are connected, with few exceptions, by a ventral and a dorsal bar (Malmberg, 1970); two cephalic lobes each bearing one spike

sensilla (see Fig. 1.1) and by a viviparous mode of reproduction.

The genus *Gyrodactylus* incorporates a diverse array of over 400 species which may vary in length from between about 0.3 to 1mm (Malmberg, 1970; Harris, 1985a; personal observations). Malmberg (1970) published the widely accepted systematics for the genus. The *Gyrodactylus* were divided into six subgenera on the basis of their excretory systems; *G.* (*Gyrodactylus*) Malmberg, 1964, *G.* (*Mesonephrotus*) Malmberg, 1964, *G.* (*Metanephrotus*) Malmberg, 1964, *G.* (*Paranephrotus*) Malmberg, 1964, *G.* (*Neonephrotus*) Malmberg, 1964 and *G.* (*Limnonephrotus*) Malmberg, 1964. Within these subgenera there were further divisions into species groups based on the morphologies of the hamuli, ventral and dorsal bars, marginal hooks and the cirrus.

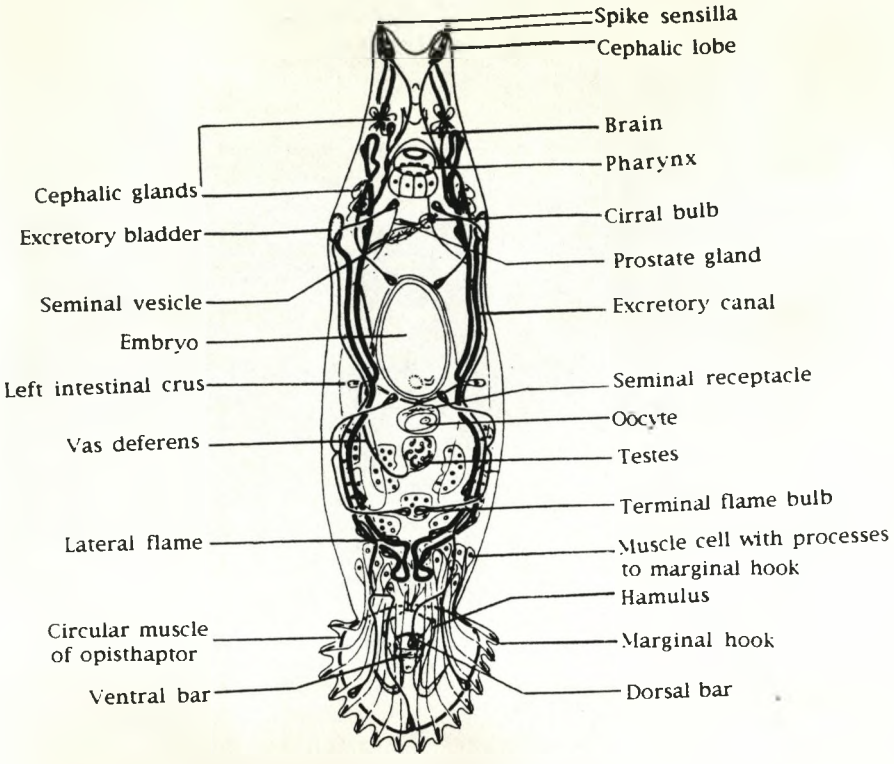

Fig. 1.1. *G. callariatus*-complex (Malmberg, 1957) [*G.* (*Metanephrotus*) (Malmberg, 1970)] as illustrated by Malmberg (1957), p. 35, figure 3B, except for the addition of the spike sensillae, and the labelling. This figure was chosen as a good model for reference in the discussion of *Gyrodactylus* anatomy (section 1.2).

1.2. *Gyrodactylus* anatomy and functional morphology

1.2.1. The opisthaptor, anterior attachment apparatus and locomotion

The posterior attachment organ, or opisthaptor

The *Gyrodactylus* opisthaptor consists of an anterior and posterior lobe. The sclerite components of the anterior lobe are the hamuli and their

supporting bars. The dorsal lobe covers the anterior lobe in attachment and supports the sixteen marginal hooks and their finger-like processes along its lateral and posterior margins (see Fig. 1.1). Fig. 1.2 shows a *G. rasini* (see Chapter 3) attached to a *Xiphophorus* hybrid. Fig. 1.3 shows the underside of the opisthaptor of a *G. rasini* following removal from the fish using double-sided Sellotape (see Chapter 6, section 6.2).

Each marginal hook consists of a shaft, a sickle and a sickle-filament loop (Fig. 1.4). The join between the marginal hook shaft and the marginal hook sickle is articulated to allow dorsoventral (up and down), but no lateral movement. These movements of the marginal hooks were described by Braun (1966). Each marginal hook finger-like process is capable of being extended outward from the main body of the dorsal lobe of the opisthaptor engaging the marginal hook sickle in a clawing motion. Each marginal hook can undertake this movement independently of the others.

There are muscle processes attached to each marginal hook which originate from cells situated in the posterior part of the preopisthaptoral part (= main body) of the parasite (Malmberg, 1957) (see Fig. 1.1).

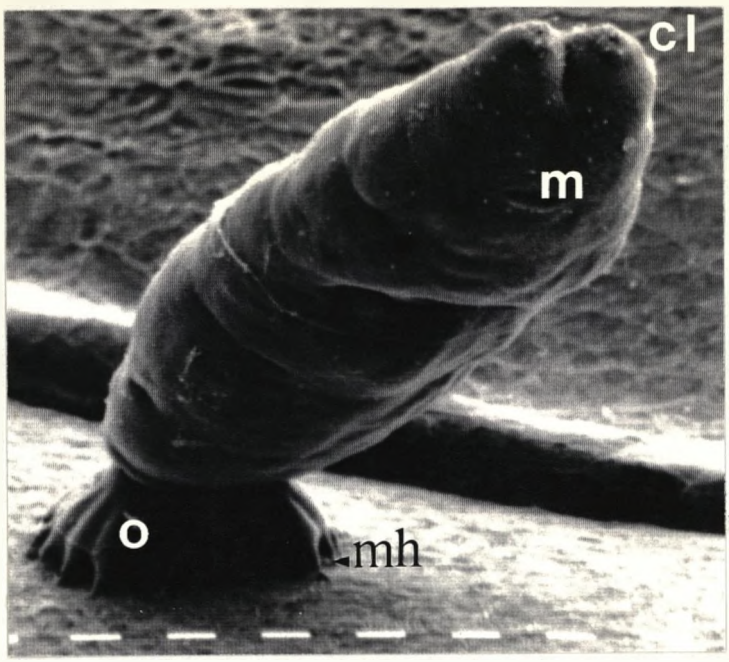

Fig. 1.2. *G. rasini* attached to a *Xiphophorus* hybrid. cl = cephalic lobe, mh = marginal hook, m = mouth, o = opisthaptor. (1 micromarker = 10μm)

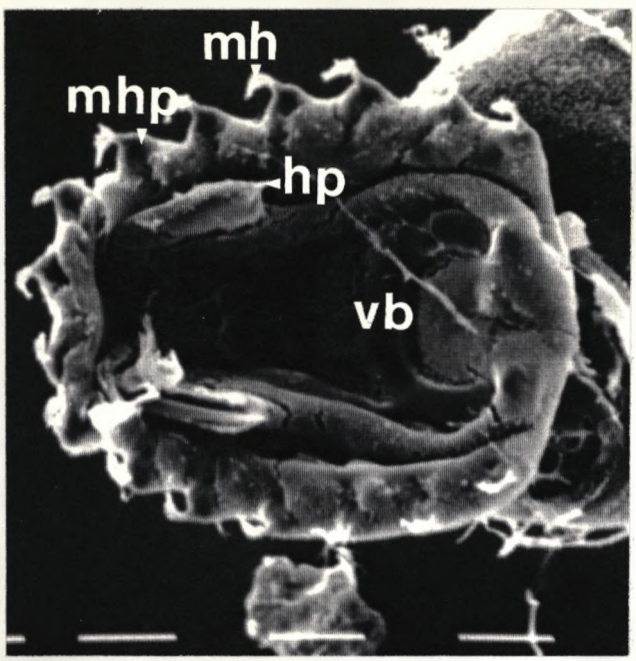

Fig. 1.3. Underside (= ventral surface) of the opisthaptor of a *G. rasini* following its removal from a *Xiphophorus* hybrid. hp = hamulus points, mh = marginal hook, mhp = marginal hook finger-like process, vb = ventral bar. (1 micromarker = 10μm)

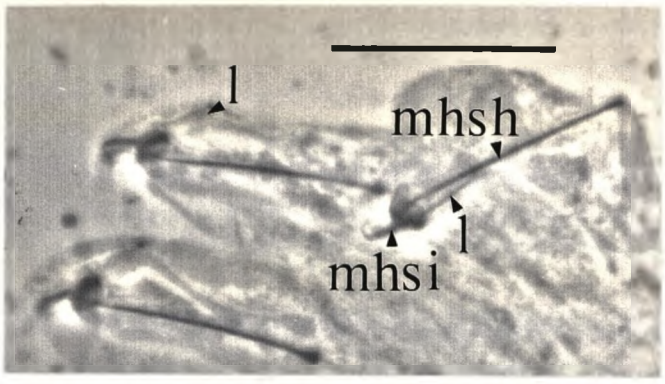

Fig. 1.4. Marginal hooks of a *G. bullatarudis* fixed and mounted in ammonium picrate-glycerin and viewed by phase contrast transmission microscopy. mhsh = marginal hook shaft, mhsi = marginal hook sickle, l = marginal hook sickle filament loop. Marker = 20μm.

Opisthaptoral attachment of *Gyrodactylus* was first described by Lester (1972) for *G. alexanderi* on *Gasterosteus aculeatus*. Opisthaptoral attachment of *G. bullatarudis* and *G. turnbulli* are described and investigated in detail in Chapter 7.

The anterior attachment apparatus

The anterior attachment apparatus (Smyth and Halton, 1983) in *Gyrodactylus* consists of the cephalic lobes and their associated glands and ducts.

Each cephalic lobe is provided with a cup-like cavity which opens anteroventrally (Kritsky, 1978). Each cavity is surrounded by numerous uniciliate sense organs (Lyons, 1969a) and, slightly dorsally, a single spike sensilla (Lyons, 1969b). The cephalic lobes are capable of extending and retracting from and into (though not involuting into) the main body of the fluke. The spike sensillae may be protruded and retracted out of and into the cephalic lobes and the cup-like cavities may be opened and closed (see Figs 1.5 to 1.8).

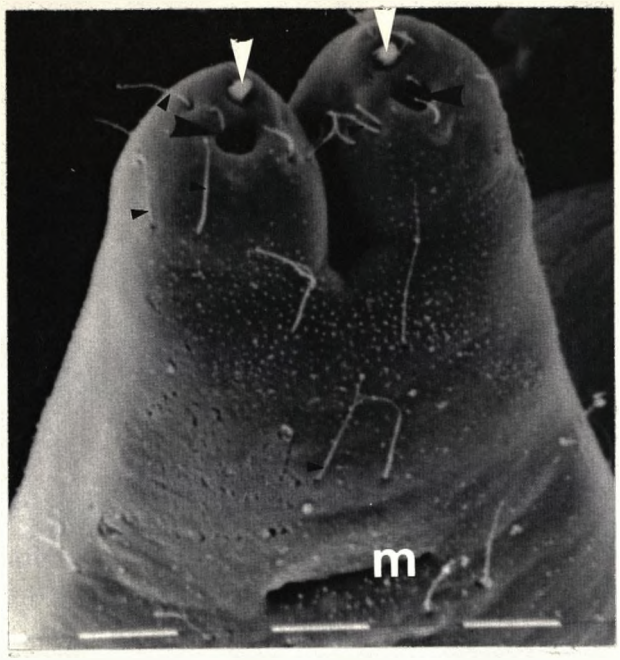

Fig. 1.5. Cephalic lobes of *G. bullatarudis*. The cup-like cavities (Kritsky, 1978) (large black arrows) are slightly open. The spike sensillae (Lyons, 1969b) (large white arrows) and uniciliate sensillae (Lyons, 1969a) (small black arrows) were clearly illustrated on this specimen. The spike sensillae are not fully protruded. m = mouth. (1 micromarker = 10μm)

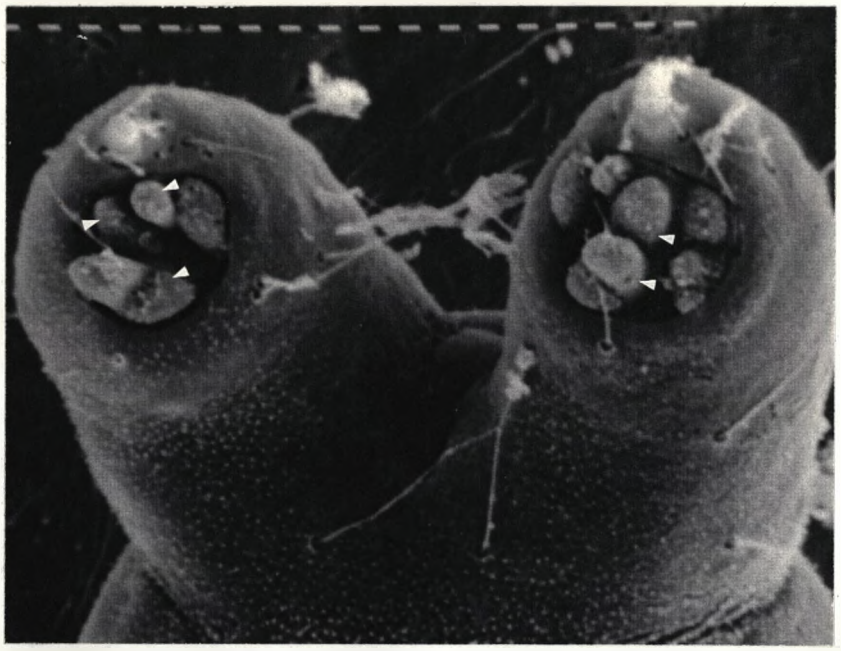

Fig. 1.6. Cephalic lobes of *G. bullatarudis*. The cup-like cavities are more open than those in Fig. 1.5, less open than those in Fig. 1.7 and the secretory papillae (Kritsky, 1978) are visible (small white arrows). (1 micromarker =1μm)

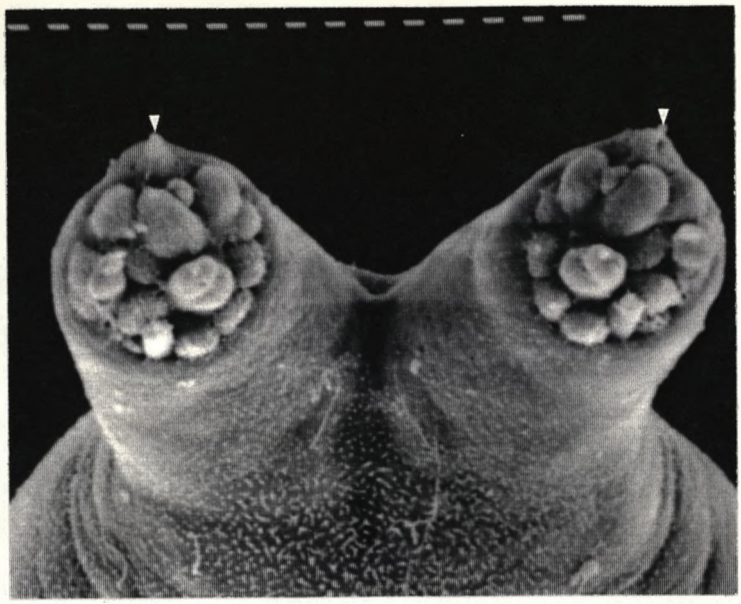

Fig. 1.7. Cephalic lobes of *G. bullatarudis*. The cup-like cavities are more open than those in Figs. 1.5 and 1.6. The two spike sensillae are arrowed. 1 (1 micromarker = 1μm)

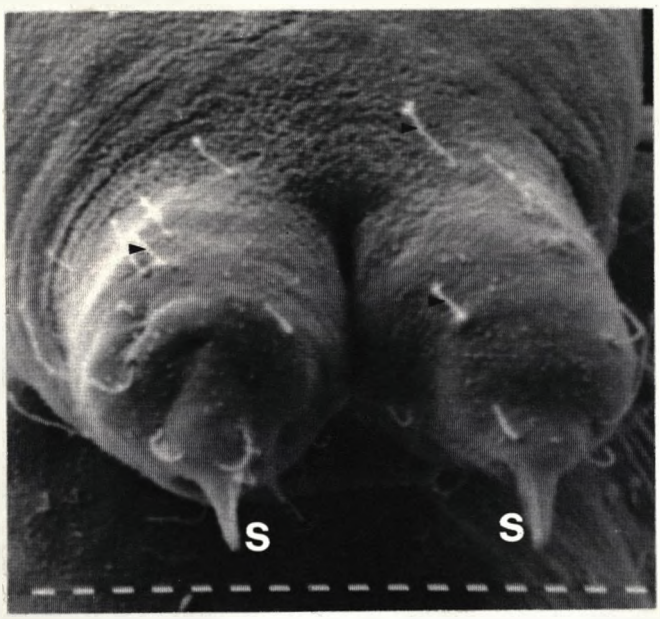

Fig. 1.8. Dorsal view of the cephalic lobes of *G. bullatarudis*. The two spike sensillae (s) are fully protruded. Uniciliate sensillae (three are arrowed) are also clearly depicted. (1 micromarker = 1μm)

Kritsky (1978) described the cephalic glands and associated structures of *G. eucaliae* Ikezaki and Hoffman, 1957. These structures were not studied in detail in my study and so Kritsky's (1978) findings were used for the following account.

Numerous ducts terminate in each cup-like cavity. Before they terminate they converge into several groups, though not combining. Each one of these groups of ducts opens into the cup-like cavity of the cephalic lobe through the tip of a small papilla (see Figs. 1.6 and 1.7). The ducts arise from three types of unicellular glands arranged in homogeneous groups (see Fig. 1.9):

> "Three bilaterally paired groups of dorsal glands produce elongate acidophilic secretion units; a single paired group of anteroventral glands lying anterior to the pharynx produce a basophilic secretion resembling that of mucoid glands of turbellarians; the posteroventral glands lie immediately posterior to the pharynx and secrete a granular, acidophilic unit." (Kritsky, 1978).

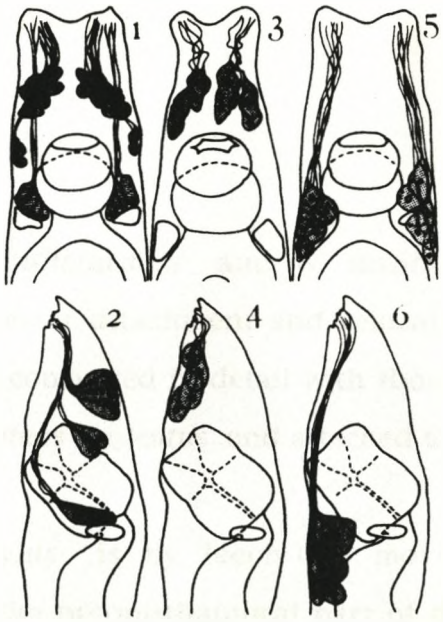

Fig. 1.9. Cephalic glands and associated ducts of *G. eucaliae* as illustrated by Kritsky (1978), p. 38, figures 1 to 6: "1, 2. Ventral and lateral views of the dorsal glands, respectively. 3, 4. Same of anteroventral glands. 5, 6. Same of posteroventral glands."

The main function of the cephalic glands is thought to be adhesion (Kritsky, 1978). Following adhesion, for example during a relocation cycle (see below), it is also important for the head organs to be quickly released from the fish surface. It is possible that the function of one or more of these types of glands is to secrete a substance which aids release of the cephalic lobes from the substrate. Lyons (1969b) commented on the close proximity of the *Gyrodactylus* spike sensillae (see section 1.2.3) to the secretory region of the cephalic lobes and suggested that a coating of secretory substance may be important to these sense organs for absorbing molecules during olfaction.

Kritsky (1978) found that the tegument covering the cephalic lobes of *G. eucaliae* differed from the rest of the tegument by containing numerous distinct secretory granules and "in uniting via ducts with syncytial subtegumental areas which apparently produce these secretion units", speculating that these granules, on release, may also have adhesive properties.

Locomotion

My observations of *G. bullatarudis* and *G. turnbulli*, outlined in the paragraph below, on anterior attachment and relocation of both live and SEM prepared specimens concurred in detail with those of Lester (1972) of *G. alexanderi* on *Gasterosteus aculeatus* and attached to glass slides.

Relocation of *Gyrodactylus* is by leech-like movements. Whilst the opisthaptor is attached, the preopisthaptoral part of the body is extended and directed anteriorly. At this stage the body may be stretched up to two and a half times its relaxed length. As the body extends forward, the cephalic lobes are also extended anteriorly and diverge from each other.

The cephalic lobes are placed on the surface and, as this is done, a sticky secretion is exuded from the openings of the gland canals situated at the anterior of the cephalic lobes (Kritsky, 1978, see above). This sticky secretion enables the attachment of the cephalic lobes to the surface. Fig. 1.10 shows the adhesion of a *G. bullatarudis* to the surface of a fish and Figs. 1.11A and 1.11B show the adhesion of a *G. turnbulli* to the surface of a cover slip. Fig. 1.11B shows the sticky substance covering an area of 25 to 30μm in diameter about each cephalic lobe. Once the anterior is attached the opisthaptor can be released and placed immediately behind the anterior attachment (see Fig. 1.12). The opisthaptor, anterior to marginal hooks VII or VIII[1], overlaps the coating of the sticky secretion on the substrate. Once this manoeuvre is completed and the opisthaptor has attached, the anterior is released and the relocation cycle completed. The partial overlapping of the opisthaptor over the sticky secretion laid down by the anterior attachment apparatus enables its adhesion to hard surfaces which cannot be pierced by the marginal hooks. Thus relocation, almost identical to relocation on fish, is also observed on hard surfaces.

Fig. 1.10. *G. bullatarudis* attached to the surface of a *P. reticulata* by its anterior attachment apparatus. c = cephalic lobe, o = opisthaptor. (1 micromarker = 1μm)

[1] Marginal hooks are numbered from the posterior toward the anterior as recommended by Llewellyn (1963) (see Euzet and Prost, 1981).

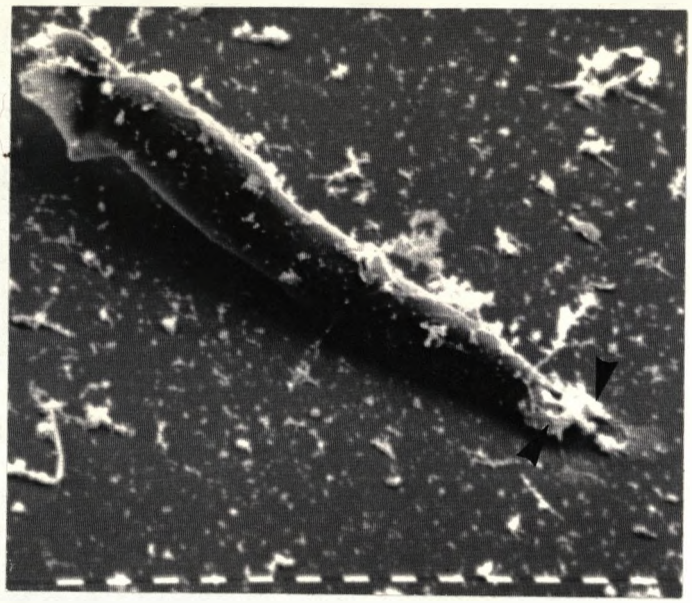

Fig. 1.11A. *G. turnbulli* attached to a cover slip by its anterior attachment apparatus (the cephalic lobes are arrowed). (1 micromarker = 10μm)

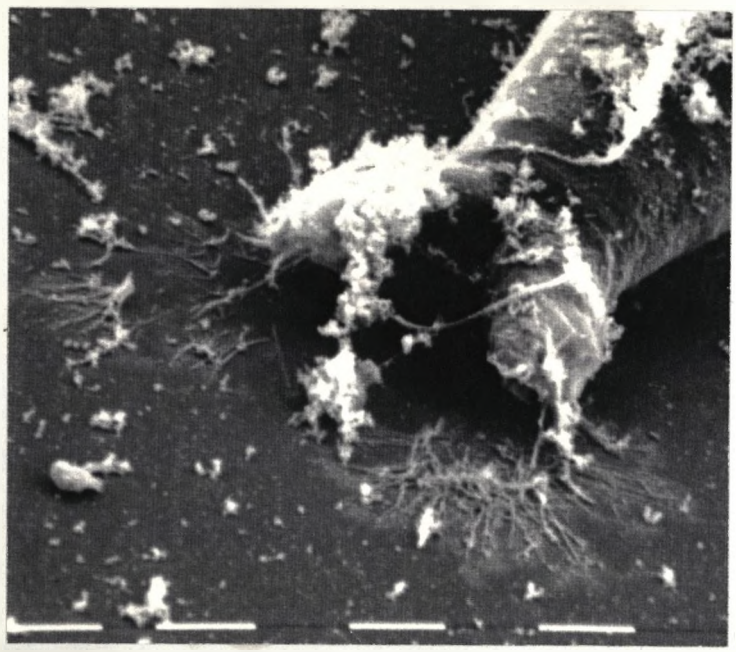

Fig. 1.11B. The same *G. turnbulli* attached to a cover slip as that in Fig. 1.11A. Note the sticky substance covering an area of 25 to 30μm in diameter about each cephalic lobe. (1 micromarker = 10μm)

Fig. 1.12. *G. bullatarudis* on *P. reticulata*. This fluke was nearing the end of a relocation cycle on the host surface. The opisthaptor has been placed slightly posterior to the attachment of the anterior attachment apparatus. In due course the anterior attachment apparatus would have detached and the fluke would then have completed the relocation cycle. The opisthaptor has attached slightly skewed (marginal hooks VIII left and right are arrowed). (1 micromarker = 10μm)

1.2.2. The digestive system and feeding

The pharynx and feeding

Fig. 1.13 shows the protruded pharynx of *G. bullatarudis*. The anterior of the pharynx of *G. bullatarudis* consists of eight cells which are fused to each other along their lateral borders. They are protruded as a barrel shaped unit. The interior of the anterior pharyngeal bulb of *G. bullatarudis* is lined with ridges running along its length (see Fig. 1.14).

The anterior part of the pharynx of *G. turnbulli* consists of eight protrusible, pyramid shaped cells. They are not fused with each other along their lateral borders (see Figs. 1.15 and 1.16). Fig. 1.17 shows a *G. turnbulli* on a fish which had been dead for 2h 20mins before being prepared for the SEM by freeze fixation - dehydration. This fluke appears to be retrieving from feeding, having caused the wound on the right of the figure.

Fig. 1.13. *G. bullatarudis* on *P. reticulata* with a partially protruded pharynx. (1 micromarker = 10μm)

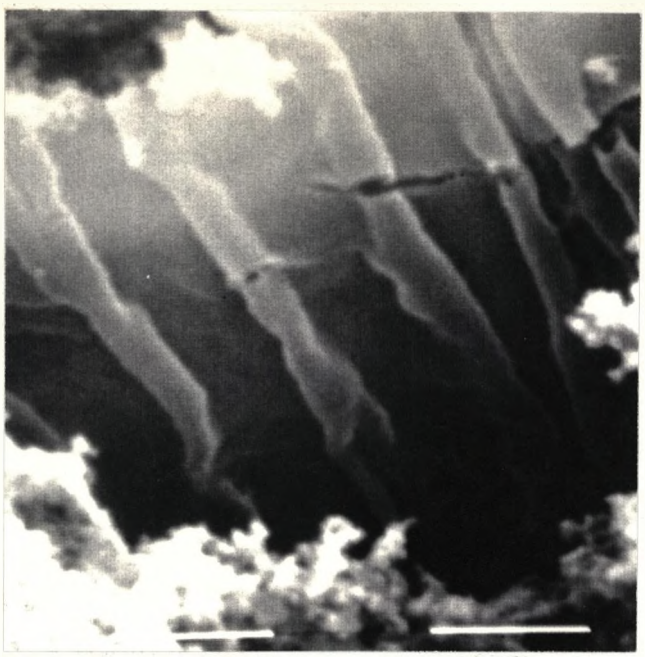

Fig. 1.14. The internal tegument of the pharynx of the *G. bullatarudis* in Fig. 1.13 made visible by removing the fluke from the fish and cutting it with a razor blade (procedure performed by Mr. C. J. Veltkamp). The ridges can also be seen using light microscopy running dorsoventrally within the anterior pharyngeal bulb of fixed and live specimens of *G. bullatarudis.* (1 micromarker = 1μm)

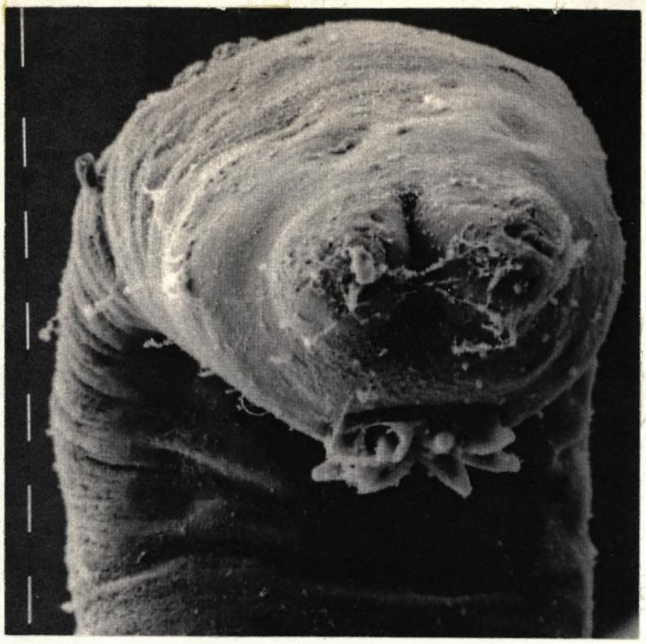

Fig. 1.15. *G. turnbulli* on *P. reticulata* with a protruded pharynx. (1 micromarker = 10μm)

Fig. 1.16. Ventral view of the protruded pharynx of the *G. turnbulli* illustrated in Fig. 1.15 following removal from the fish. Note the pyramidal shaped anterior portions of the eight pharyngeal cells (two are arrowed). (1 micromarker = 1μm)

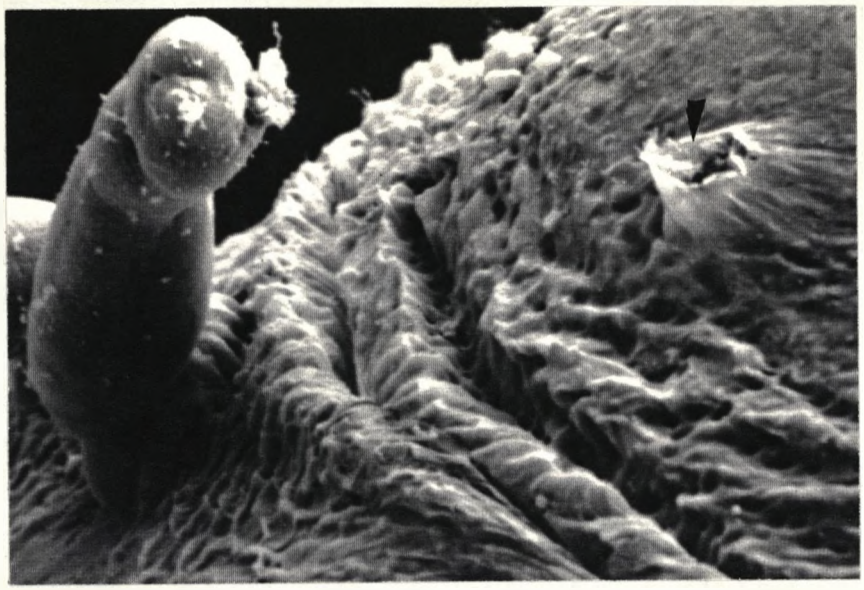

Fig. 1.17. *G. turnbulli* on a *P. reticulata* which had been dead for 2h 20mins before freeze fixation. The fluke appears to be retrieving from feeding, having caused the wound on the right (arrowed). (1 micromarker = 10μm)

Kritsky (1970) described the pharynx of *G. eucaliae* following evaluation by transmission electron microscopy. The gross anatomy of the pharynx of *G. eucaliae* as described by Kritsky (1970) corresponds to my observations of live specimens and SEM studies of *G. turnbulli*. These two species both belong to the *G. eucaliae* species group Malmberg, 1964 (Harris, 1986). The following description of the pharynx was principally made with reference to Kritsky (1970). The description appears fully applicable to *G. turnbulli* but only partially applicable to *G. bullatarudis* :

The pharynx consists of eight radially arranged cells, anterior to the oesophagus and posterior to the buccal tube (see Fig. 1.18). The eight cells are each divided into an anterior and a posterior potion by an incomplete

septum. The anterior and posterior portions make up the anterior and posterior pharyngeal bulbs. The anterior parts of these cells appear quite different in *G. bullatarudis* and *G. turnbulli* (see Figs. 1.13 and 1.15 and text above). The eight pharyngeal cells of *G. eucaliae* are described by Kritsky (1970) as unicellular glands. The posterior portions contain the cell nucleus but the cytoplasm in the two portions are identical. The two parts are connected by a fine communicating passage which passes through the pharyngeal septum. Single ovate secretion granules congregate near these communicating ducts. Although found throughout the cytoplasm, these granules also aggregate near the discharging ducts of the anterior portion of the pharyngeal cells. Each cell empties through an individual papillae which is 7 to 8μm long and arises near the rear of the anterior pharyngeal bulb. These papillae are filled with secretion granules.

The pharynx of *G. eucaliae* has longitudinal, circular and radial muscles (see Fig. 1.19). The anterior portions of these pharyngeal cells have significantly more myofibrils than the posterior (Kritsky, 1970).

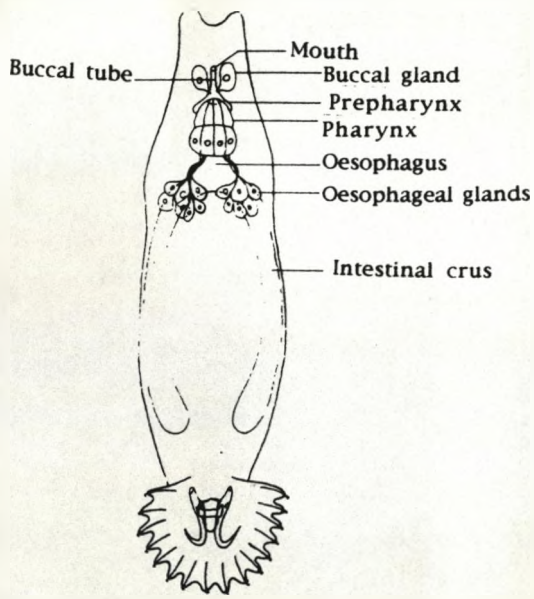

Fig. 1.18. The digestive system of *G. eucaliae* as illustrated by Kritsky (1970), p. 75, figure 4.

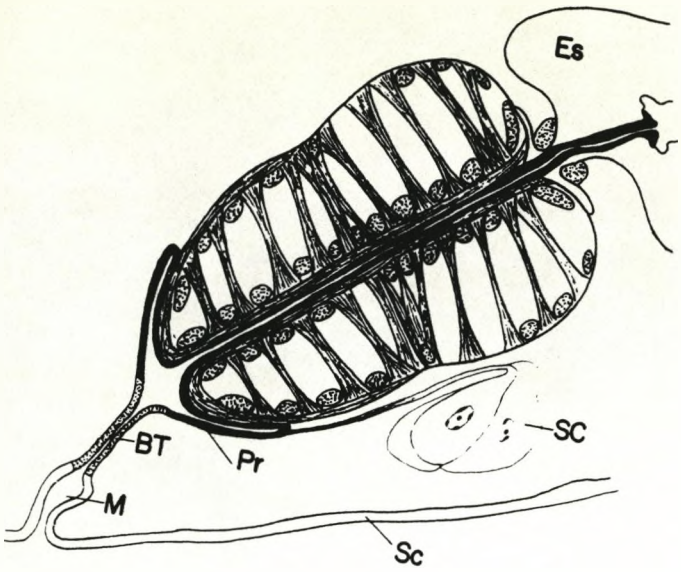

Fig. 1.19. Mid saggital section of the pharyngeal region of *G. eucaliae* as illustrated by Kritsky (1970), p. 78, figure 5. "Note the longitudinal, circular and radial muscles of the pharynx." Labelling; buccal tube (BT), oesophagus (Es), mouth (M), prepharynx (Pr), somatic cuticle (Sc) and subcuticular cells of the pharyngeal cuticle (SC).

Current opinion on feeding is that the anterior of the pharynx is everted and placed over the fish skin, enzymes are released onto the host epidermis and the resulting digest is ingested by sucking into the gut (Malmberg, 1993; Mo, 1994). Fig. 1.17 suggests that when the anterior of the pharynx is stellate, for example, the pharynx of *G. turnbulli*, the pyramidal anterior portions of the pharyngeal cells are capable of gripping host epidermis, perhaps loosened following extracorporeal digestion by secreted enzymes. Kritsky (1970) found the anterior pharyngeal bulb to be well distributed with myofibrils. When observed within wet mounts of living flukes, retracted within the buccal cavity, the tips of the anterior pharyngeal bulb can be seen to move independently of each other. These movements are, however, very small within the restricted space of the pharyngeal cavity.

Detailed observations on the feeding behaviour of *G. bullatarudis* and *G. turnbulli* are reported in Chapter 8.

The posterior parts of the pharyngeal cells are similar in appearance in both *G. bullatarudis* and *G. turnbulli* and it is assumed that the eight pharyngeal cells of *G. bullatarudis* are also glandular in character.

Apart from the pharyngeal cells there are two other sets of glands associated with the anterior digestive tract, the oesophageal and buccal glands (Kritsky, 1970). These glands discharge into the buccal tube and the oesophagus respectively (see Fig. 1.18).

Kritsky (1970) also describes an extrapharyngeal muscle around the oesophagus just posterior to the pharynx. He suggested that this muscle serves as an oesophageal sphincter.

The oesophagus and intestine

The oesophagus is short and the gut soon bifurcates into two crura (see Figs. 1.1 and 1.18). These two blind ending sacs have a gastrodermis consisting of syncytial epithelium with large widely spaced cell nuclei (Kritsky *et al.*, 1994). Kritsky *et al.* (1994) also found "few widely spaced lamellae projecting into the intestinal lumen".

There have been few studies of the contents of *Gyrodactylus* gut lumen. Harris (pers. comm.) has frequently observed epidermal cell nuclei within the gut crura. Ikezaki and Hoffman (1957) cultured gram positive motile rod-shaped bacteria and yeast-like fungi from the gut contents of *G. eucaliae*. I have observed what appear to be forming organic crystals within the gut lumen of both *G. bullatarudis* and *G. turnbulli* from *P. reticulata* (see Fig. 1.20). I am unsure whether they were ingested or

whether they started forming within the gut before or after fixation in ammonium picrate-glycerin (see Chapter 2, section 2.2.3). They had a maximum width and length of up to about 8μm and 25μm respectively.

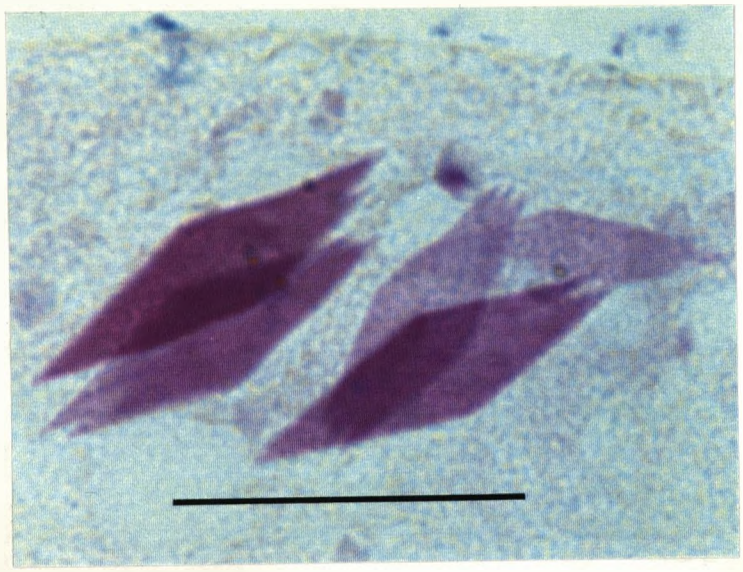

Fig. 1.20. Objects observed within the gut lumen of both *G. bullatarudis* and *G. turnbulli* from *P. reticulata* observed following fixation and mounting in ammonium picrate-glycerin. This preparation was trichrome stained with modified Mallory solution following fixation and mounting in ammonium picrate-glycerin using the procedure described in Chapter 5. The objects were stained pink by the acid fuchsin in the solution. Their regular shapes suggest that they are crystals, their jagged edges suggest that they are forming and their staining with acid fuchsin suggests that they are organic. Marker = 20μm.

1.2.3. The excretory system

The comparative anatomies of the excretory systems of the six subgenera of *Gyrodactylus* were described by (Malmberg, 1970). Small canals which are

terminated by flame bulbs drain into paired main anterior and posterior canals (Malmberg, 1970). The *Gyrodactylus* investigated within this study were from the *G.* (*Mesonephrotus*) and *G.* (*Metanephrotus*) groups (Harris, 1986; also see Chapters 2 and 3). Members of both these subgenera have excretory bladders into which the main excretory canals drain (see Fig. 1.1). These bladders, which can be observed to expand and contract on filling and emptying, dispel their contents to the exterior via two anterodorsolaterally placed, crescent shaped excretory pores (see Figs. 1.21 and 1.22).

Fig. 1.21. Dorsal view of a *G. turnbulli* on *P. reticulata*. The right excretory pore is arrowed. 1 micromarker = 10μm.

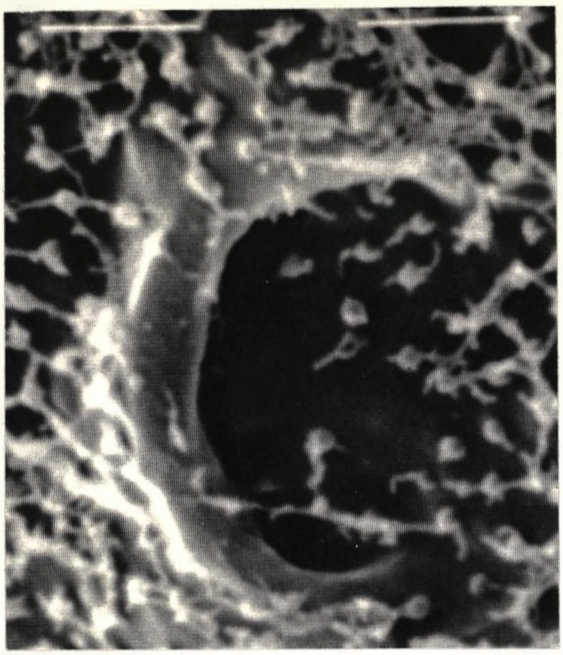

Fig. 1.22. Open excretory pore of a *G. turnbulli*. Excretory pores of *G. bullatarudis* were of similar size and shape. (1 micromarker = 1μm)

1.2.4. Surface and sub-surface sensory receptors

Four different types of sensory receptor have been described from *Gyrodactylus* species:

1) Sense organs (presumed tangoreceptors) which end in a single cilium are widely distributed over the body surface but are not found on the opisthaptor (Lyons, 1969a). These sense organs are most concentrated on and around the cephalic lobes (see Figs. 1.5 to 1.7), the pharyngeal region and the posteroventral surface of the preopisthaptoral part of the body. Their distribution is species specific and may be used for species identification (Shinn *et al.*, 1993).

2) The paired spike sensillae situated at the anterior of the cephalic lobes (see Figs. 1.5 to 1.8) are "compound organs consisting of a cluster of individual sensilla" capable of being retracted and protruded inward and

outward (Lyons, 1969b). Lyons (1969b) speculated that these are chemoreceptors.

3) and 4) Two further types of sensory receptors were described by Watson and Rhode (1994) from *Gyrodactylus* sp. parasitising *Xiphophorus helleri*. One pair of each receptor were found on the dorsal aspect of the cephalic lobes, one of each type of receptor on each lobe. Both types were sub-surface ciliary receptors. The anterior pair were situated close to the spike sensillae and the second pair posterior to these and slightly more lateral. Watson and Rhode (1994) speculated that the anterior receptors may have "pressure/contact or photoreceptive function" and the posterior type "closely resembles presumed photoreceptors found in several other parasitic and free living platyhelminths".

1.2.5. The reproductive system

The male reproductive system

The testes lie posterior to the seminal receptacle. From the testes the vas deferens runs anteriorly around the left gut crus (Kritsky, 1970) to a region just posterior to the pharynx and terminates into the seminal vesicle. I found the vas deferens difficult to observe in both live and fixed specimens, but the seminal vesicle was almost always visible in specimens with a fully developed cirrus. From the seminal vesicle there is a short pars prostatica (Kritsky, 1970) which leads to the cirral bulb (see Fig. 1.23). The ejaculatory duct runs through the cirral bulb and opens into the cirrus which connects to the exterior through the gonopore (unobserved in my investigation) which in *G. eucaliae* is situated on the mid ventral surface, anterior to the uterine pore (Kritsky, 1970). Surrounding the ejaculatory duct at the distal region of the cirral bulb are the cirrus spines.

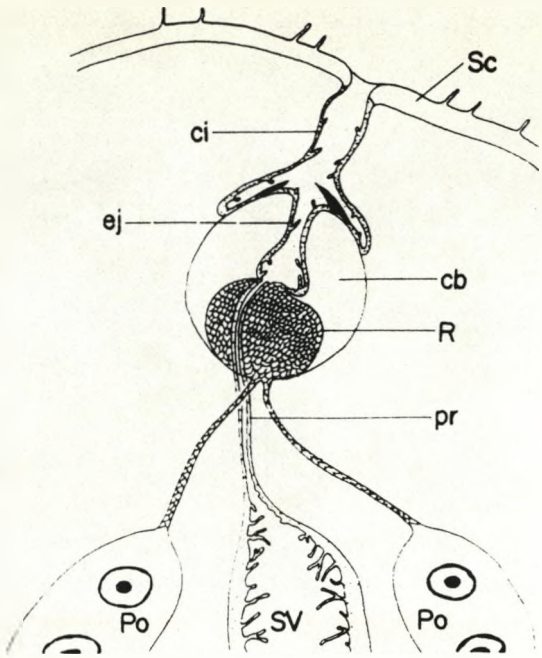

Fig. 1.23. "Diagram of the anterior portion of the male reproductive system [*G. eucaliae*]" as illustrated by Kritsky (1970), p. 125, figure 7. Labelling; cirral bulb (cb), cirrus (ci), ejaculatory duct (ej), prostates (po), pars prostatica (pr), somatic cuticle (Sc), seminal vesicle (SV) and prostatic reservoir (R).

The cirrus spines consist of one large spine and one or more rows of small spines (Malmberg, 1970). The exact morphology of the spines are difficult to determine but are valuable in species identification (see Chapters 2 and 3). The three species of *Gyrodactylus* investigated in this thesis appeared to have other sclerotised structures in the cirral bulb surrounding the ejaculatory duct (see Chapters 2 and 3).

Harris (1982) stated that the intromittent organ in monogeneans "is protruded rather than everted, and should therefore be referred to as a penis". However, although the cirral bulb of *Gyrodactylus* is protruded

during copulation (see Harris, 1989), it seems likely that the cirrus spines (see Fig. 1.23) are everted. Furthermore, Kritsky (1970) refers to the short lumen connecting the ejaculatory duct to the exterior as the "involuted cirrus". There is some justification for both terms. However, I have chosen to follow the nomenclature of Kritsky (1970) because of its anatomical precision. In addition, the term "cirrus" was used in the majority of previous works concerning *Gyrodactylus* (Turnbull, 1956; Malmberg, 1970; Mackenzie, 1970 and others).

The female reproductive system

Perhaps the most dominating anatomical feature of *Gyrodactylus* sp. is the large central uterus which is frequently seen containing a developing embryo. The uterus opens to the exterior via the uterine canal and uterine pore situated mid ventrally (Kritsky, 1970, not observed in my studies). Immediately posterior to the uterus is the sac-like seminal receptacle (Malmberg, 1957; Harris, 1982) usually made conspicuous by the presence of an oöcyte (see Fig. 1.1). The seminal receptacle also functions as an ovary to which it has previously been referred (Kritsky, 1970). Kritsky (1970) describes the division of the single layered seminal receptacle wall into germinal and epithelial regions. The germinal region consists of "relatively few germ cells ... which periodically produce an oöcyte which protrudes into and frequently fills the ovarian cavity". Following birth of a daughter (see below), the oöcyte within the seminal receptacle may move anteriorly into the uterus and commence embryonic development (Harris, 1993b).

1.2.6. *Gyrodactylus* reproduction

In a new-born *Gyrodactylus*, a well developed embryo can be seen within the uterus and a prominent oöcyte within the seminal receptacle (see

section 1.2.5). The weight of evidence suggests that the developing embryo within the uterus is derived from mitotic proliferation and is the product of a form of polyembryony (see Harris, 1993a). Following the birth of the first-born daughter (nomenclature from Harris, 1993a), the oöcyte moves from the seminal receptacle into the uterus. Harris (1993a) cites his own unpublished evidence in observing that the origin of the second-born daughter, into which the oöcyte develops, is "clearly parthenogenic". Evidence discussed below indicates that further daughters may be of sexual or asexual origin.

Copulation has been observed between *Gyrodactylus* on a number of occasions (Braun, 1966; Lester and Adams, 1974; Harris, 1989; Harris *et al.*, 1994; personal observations). Harris (1989) described copulation between *G. turnbulli* on *P. reticulata* :

> "Copulation was observed when reaching flukes made contact with their neighbours. The anterior of the reaching fluke was tilted back and the penis [= cirrus] ... used to grasp the partner. This individual thrashed violently, looping over and initiating a mutual copulation. The penis may be inserted into the co-copulant at any point on the body surface between the uterus and seminal receptacle, although it is normally placed close to the latter."

The male reproductive system develops after the female system in *Gyrodactylus* (Turnbull, 1956; Lester and Adams, 1974; Harris, 1985b; Harris, 1989; Harris *et al.*, 1994). Lester and Adams (1974) observed fully developed cirri at the age of four days in *G. alexanderi* at 15° C. This would appear to be near to the day on which this species gave birth for the first time. The interval before the birth of the first-born daughter was not given, however the interval between the births of the first- and second-born daughters averaged 5.3 days at 15° C. In *G. gasterostei*, *G. turnbulli* and *G. salaris*, a fully functional male system becomes fully mature with a complete cirrus and spermatozoa in the testes between the births of the first- and second-born daughters (Harris, 1985b; Harris, 1989; Harris *et al.*, 1994 respectively). Harris (1989) and Harris *et al.* (1994) found that, on

microscopical examination of live flukes, only those with fully developed male reproductive systems were inseminated, with whorls of sperm visible in their seminal receptacles. They concluded that copulation mainly occurred between flukes with functional male systems.

Although copulation does occur between older individuals, cross insemination is not necessary in *G. turnbulli* or *G. salaris* for the production of post second-birth daughters. Scott (1982)[2] recorded a maximum of three progeny from isolated *G. turnbulli* and Jansen and Bakke (1991), a maximum of four from isolated *G. salaris*.

Harris (1993a) concluded of gyrodactylids, that "the relative importance of sexual reproduction depends on population age structure and mortality". He regarded *G. turnbulli* as a cyclic parthenogen which reproduces primarily asexually but with an increasing sexual component at higher population densities.

1.3. *Gyrodactylus* host specificity - with special reference to those infecting poeciliid fish

Malmberg (1970) found that "with very few exceptions the examined *Gyrodactylus* species seem to be host specific (species specific)". Bakke *et al.* (1992) suggested that the degree of host gyrodactylid specificity had been over estimated and that "narrow specificity is an artifact based on numerous species descriptions of gyrodactylids collected from only a single host". They describe variation among the degrees of *Gyrodactylus* host specificity. Some *Gyrodactylus* can only utilise a single host whereas some

[2] Scott and co-workers (1982 to 1985), and probably Madhavi and Anderson (1985) misidentified *G. turnbulli* as *G. bullatarudis* (see Harris, 1986).

can "successfully attach and reproduce on several host genera".

Guppies (*Poecilia reticulata* Peters; order, Cyprinodontiformes; family, Poeciliidae) are oöviviparous and are native to moderately low elevation freshwater rivers and streams of Trinidad, Tobago, Barbados and Venezuela (Rosen and Bailey, 1963). They are common aquarium fish, easily maintained within the laboratory and have been used as experimental animals by a number of workers (see Scott, 1985a). Two species of *Gyrodactylus* have been found to infect *P. reticulata*, *G. bullatarudis* Turnbull, 1956 and *G. turnbulli* Harris, 1986. *G. bullatarudis* has been recorded from *P. reticulata* (Turnbull, 1956; Harris and Lyles, 1992), *P. sphenops* (Kritsky and Fritts, 1970) and *Xiphophorus* (*helleri* x *maculatus*) hybrids (Harris, 1986). This third host record is shown to be erroneous in Chapter 3. *G. turnbulli* has only been recorded from *P. reticulata* (Harris, 1986; An *et al.*, 1991). However, Lyles (1990) showed that *G. turnbulli* was able to survive and reproduce on one out of two *P. latipinna* and three out of four *Poeciliopsis lucida* under laboratory conditions.

The host specificity of these two species of *Gyrodactylus* deserve detailed study. However, as a preliminary investigation, I devised a simple protocol (described in the next three paragraphs) to test the ability of *G. turnbulli* and *G. bullatarudis* from *P. reticulata* to colonise and reproduce on two other cyprinodonts, another poeciliid (*Xiphophorus* hybrids) and *Ameca splendens* of the Goodidae.

A "donor" *P. reticulata* infected with at least 20 *Gyrodactylus* was placed with 4 uninfected "recipient" fish of a different species in 500ml dechlorinated tap water. All fish used were *Gyrodactylus*-naive, laboratory bred and under 20mm standard length.

After 24h the recipient fish were examined for flukes using the procedure

described in Chapter 8, section 8.2.3. If a recipient fish was found to be infected, the number of infecting flukes was recorded and it was placed in a separate container holding 200ml dechlorinated tap water. If it was not infected it was placed back with the original donor fish. Fish which remained uninfected after 48h with the donor fish were considered insusceptible to the parasite.

Once a recipient fish was infected and separated from the others it was examined for flukes every second day using the same procedure as above. This was continued until the fish died or until its parasite infrapopulation became extinct.

G. turnbulli did not readily transfer to *A. splendens* and could not survive for longer than 24h on this host. The results of the other experiments are shown in Figs. 1.24 to 1.26. Both *G. bullatarudis* and *G. turnbulli* would readily transfer to *Xiphophorus* hybrids and could survive and reproduce on these hosts. *G. bullatarudis* could also transfer, survive and reproduce on *A. splendens.*

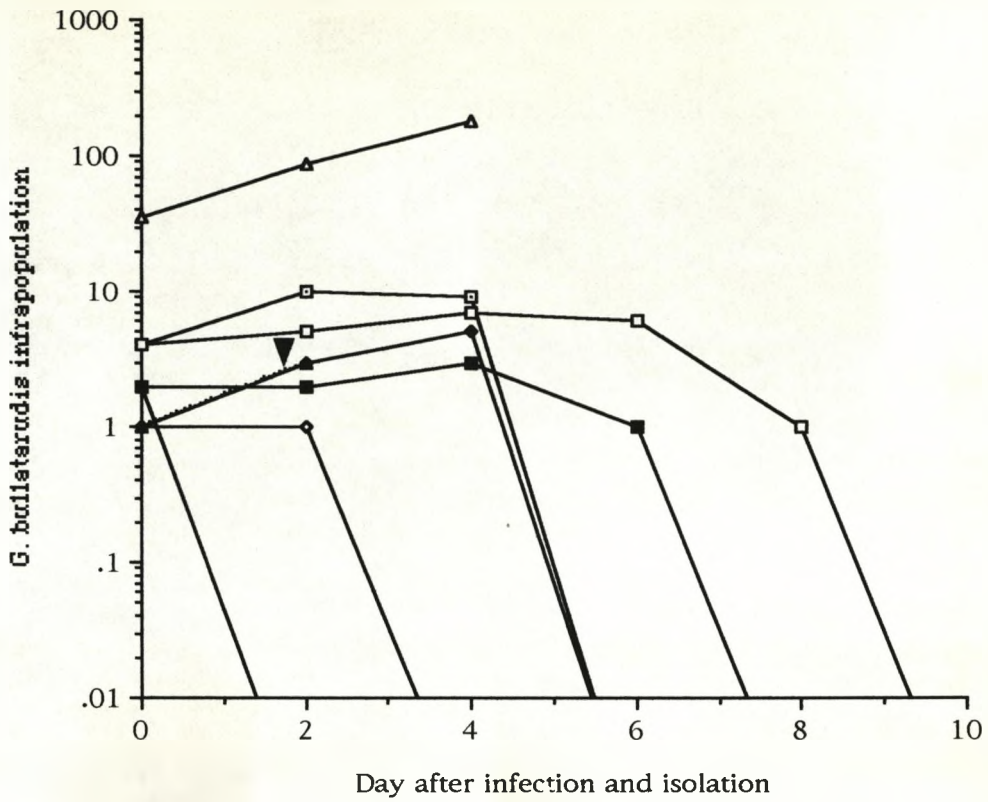

Fig. 1.24. Infrapopulations of *G. bullatarudis* (plotted on a log scale) on eight laboratory bred *Xiphophorus* hybrids counted every second day after infection from a donor *P. reticulata* (see protocol in section 1.3). The fish represented by the broken line (arrowed) was infected 48h after placement with the donor fish.

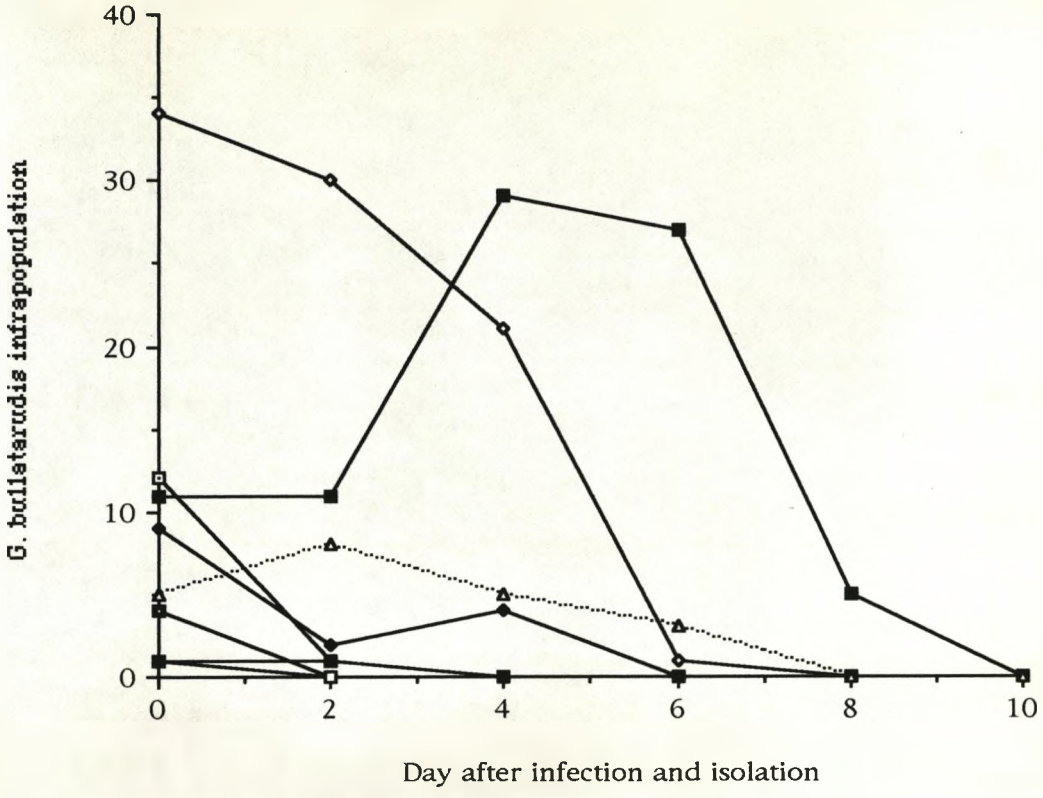

Fig. 1.25. Infrapopulations of *G. bullatarudis* on eight laboratory bred *Ameca splendens* counted every second day after infection from a donor *P. reticulata* (see protocol in section 1.3). The fish represented by the broken line was infected 48h after placement with the donor fish.

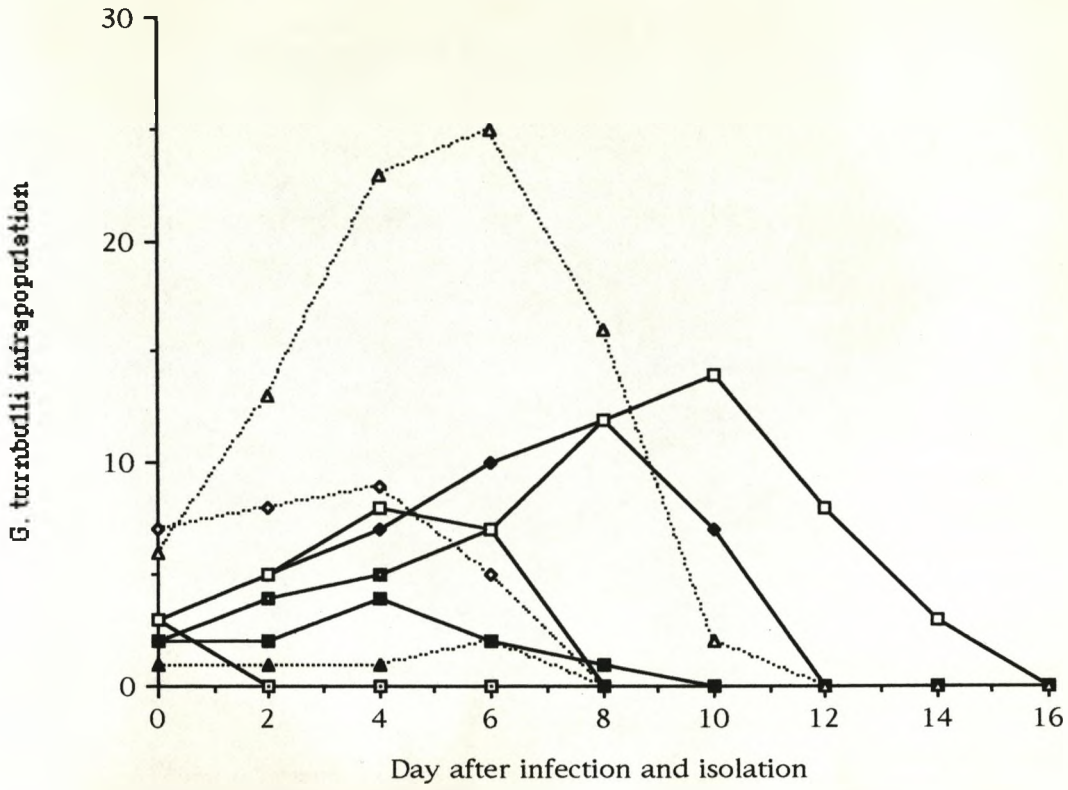

Fig. 1.26. Infrapopulations of *G. turnbulli* on eight laboratory bred *Xiphophorus* hybrids counted every second day after infection from a donor *P. reticulata* (see protocol in section 1.3). The fish represented by the broken lines were infected 48h after placement with the donor fish.

Bakke *et al.* (1992) recognised three types of mechanisms which might help maintain host specificity. 1) Behavioural mechanisms, for example, the reluctance of *G. turnbulli* to transfer to *A. splendens* in my experiments may have been due to the behaviour of the *Gyrodactylus* rather than a mechanism of host origin. 2) Physiological mechanisms such as the speculated prevention of feeding of *G. salaris* by the thick mucous layer found on eel skin (Bakke *et al.*, 1991). 3) Innate host resistance. Different strains of the same host species were shown to have different susceptibilities to the same *Gyrodactylus* (Madhavi and Anderson, 1985; Mackenzie and Mo, 1993) therefore host resistance has a genetic component. Consequently, different species may have innate resistance to certain *Gyrodactylus* species which serve to restrict *Gyrodactylus* host specificity.

The three types of mechanisms enforcing host specificity outlined above deserve further investigation as there is little evidence of their relative importance. The results of studies of host specificity of *G. salaris* (see Bakke, 1991) and my own observations on the host specificities of *G. bullatarudis* and *G. turnbulli* suggest *Gyrodactylus* species have a cline of potential hosts from those to which it will not attach through to host species which are fully susceptible. It is the breadth of this cline that determines the extent of *Gyrodactylus* host species specificity.

1.4. Epidemiology - with special reference to *Gyrodactylus* of poeciliid fish

1.4.1. General features of *Gyrodactylus* population biology

Host-parasite relationships of *Gyrodactylus* are similar to those defined by Anderson and May (1979) for microparasites. *Gyrodactylus* have a direct

life cycle whereby new born daughters infect the same host as their parent. They also have relatively short generation times. At 25°C *G. turnbulli* has an average life expectancy of 4.2 days (Scott, 1982). The time to birth of the first daughter is 1 day, there is then an average of 2.5 days before birth of the second daughter and the third daughter is born, on average, 2 days after that (Scott, 1982).

Fig. 1.27 illustrates the movement of host fish between the subgroups; susceptible hosts, infected hosts and resistant hosts. Scott and Anderson (1984) found that recurrent epidemics were a characteristic feature over long-term observations of *G. turnbulli*-infected immature *P. reticulata* in experimental arenas so long as there were regular influxes of *Gyrodactylus*-naive fish into the population. Their theoretical models which best fitted these observations were those which incorporated a refractory period of resistance to reinfection post recovery. Further studies (Scott and Robinson, 1984; Scott, 1985b) demonstrated that hosts that recovered from an initial infection remained refractory to reinfection for up to about six weeks post recovery at 25°C. There was, however. considerable variation in susceptibility to challenge infections and in the duration of the refractory period.

Parasite-induced host mortality was also an important observation from Scott and Anderson's (1984) long-term experimental arenas. *G. turnbulli* - induced host mortality was thought to be a significant regulatory force affecting the host population in these systems. Lyles (1990) found indirect evidence of parasite-induced host mortality of *P. reticulata* by *G. turnbulli* in the Paria River in Trinidad.

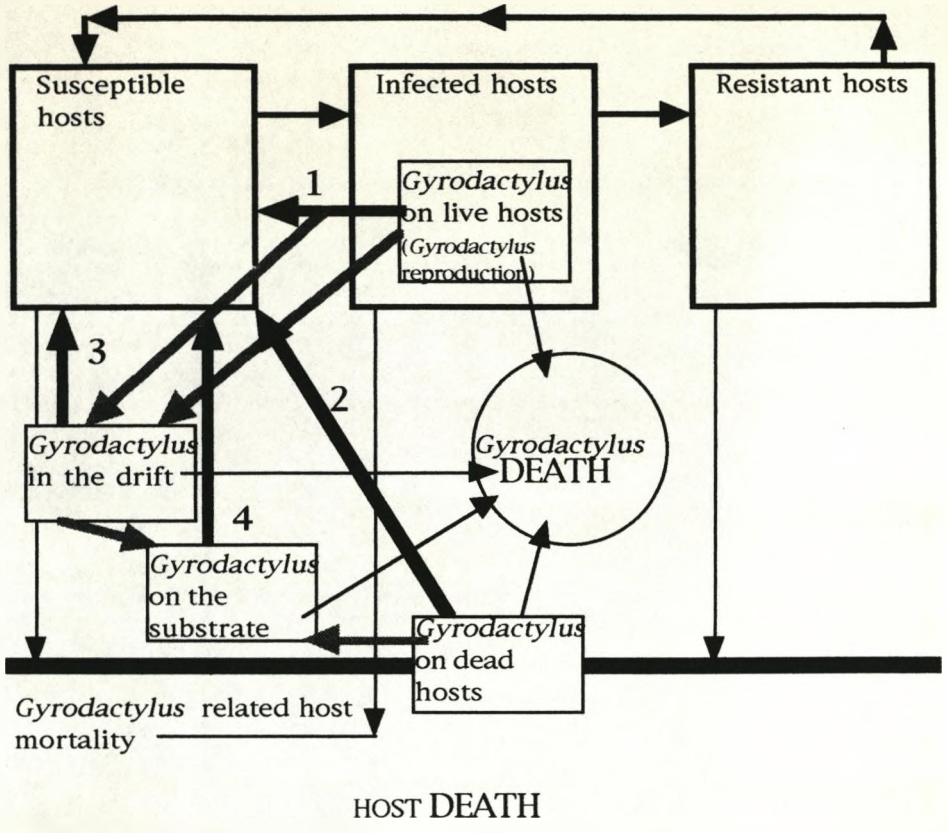

Fig. 1.27. Flow chart summarising the main features of *Gyrodactylus* infection within a host population. Host reproduction is not represented as it takes place at a much slower rate than that of *Gyrodactylus*. Medium thickness arrowed lines denote movement of fish from susceptible to infected, to resistant and back to the susceptible components of the total population. Thicker arrowed lines (black) show possible movement and transmission strategies of *Gyrodactylus* ; 1) from live host to recipient, 2) from dead host to recipient, 3) from the drift to recipient and 4) from the benthic substrate to a recipient. The thinnest arrowed lines represent parasite and host mortality and the grey lines represent other movements of flukes.

1.4.2. *Gyrodactylus* transmission

There are four routes of *Gyrodactylus* transmission (Bakke *et al.*, 1992) (see Fig. 1.27):

> 1) Transmission from live host to recipient, 2) from dead host to recipient, 3) from the drift to recipient and 4) from the benthic substrate to recipient.

The relative importance of the four routes of infection depends largely on the behaviour of the host species. Bakke *et al.* (1992) noted that "host-host contact must be most important for gyrodactylids of pelagic hosts as dead hosts and detached parasites would rapidly be swept out of contact with living hosts". The first two of these routes were considered most important for *G. turnbulli* on *P. reticulata* by Scott and Anderson (1984). Their results showed that transmission between living fish entailed some risk and only 35 to 39% of parasites that attempted to transfer succeeded. They also found that the rate of transmission between living fish (0.0052 / parasite / host / 5l water / unit time) took place at a much slower rate than from dead donor to living recipients (0.052 / parasite / host / 5l water / unit time). However, the average life expectancy of *G. turnbulli* on dead hosts (12h) was considerably shorter than on living hosts (4.2 days) and so transmission from dead hosts was more ephemeral.

Transmission by detached *G. turnbulli* was not studied by Scott and Anderson (1984). However, transmission of unattached *G. turnbulli* is reported in Chapter 10 of my study and has been reported for other *Gyrodactylus* species on a number of occasions (see Bakke et al., 1992). One of the consequences of the response of the lungfish, *Polypterus senegalus*, to the gyrodactylid, *Macrogyrodactylus polypteri*, is an increase in fluke detachment rate (Harris, 1993a, 1993b) . Detached flukes were observed to

survive for "a few days" attached to the substrate (Harris, 1993b). Consequently, transmission of flukes from the substrate may be important to the dispersal strategy of this *Macrogyrodactylus* which infects a predominantly benthic host. Bakke *et al.* (1992) comment that the importance of detached, drifting *Gyrodactylus* may have been underestimated, particularly in lotic conditions. They reported unpublished observations by P. A. Jansen and T. A. Bakke of the infection of 35 1+ salmon parr suspended in a cage in a *G. salaris* infected river over 20 days resulting in a mean intensity of 6.6 flukes per fish.

1.4.3. Gyrodactylosis and *Gyrodactylus* pathogenicity

Gyrodactylus infections of high intensity may cause the disease gyrodactylosis. Damage results to the host epidermis caused by opisthaptoral attachment and feeding. Many different symptoms have been described from different host species, for example, loosened scales, haemorrhages, peeling skin (Bykhovskaya-Pavlovskya *et al.*, 1962), frayed fins (Mackenzie, 1970), skin discolouration (Cone and Odense, 1984), emaciation and morbidity (Lester and Adams, 1974). The nature of the gyrodactylosis caused is *Gyrodactylus* species specific (Cone and Odense, 1984). In addition to direct damage caused to the host epidermis, wounds may lead to secondary infestation by bacteria and fungi (Malmberg, 1993; personal observations). Damage to the epidermis may also cause osmoregulatory problems for the host (Mo, 1994).

Gyrodactylosis has only been reported of fish supporting high intensities of *Gyrodactylus* infection. Some species of *Gyrodactylus* can infect their hosts in high numbers without causing obvious symptoms of disease (Cone and Odense, 1984). This may be due to the rapid turn over of epidermal cells in fish epidermis. Lester and Adams (1974) calculated that *Gasterosteus*

aculeatus replaced all epidermal cells every 11 days at 15°C.

1.5. Aims

Much work has already been devoted to the study of *Gyrodactylus* infecting poeciliid fish (Turnbull, 1956; Scott, 1982, 1985a, 1985b; Scott and Anderson, 1984; Madhavi and Anderson, 1985; Harris, 1986, 1988, 1989; Lyles, 1990). The aim of this project was to perform a wide ranging comparative study of *Gyrodactylus* species infecting *P. reticulata* with work of sufficient depth to add to the knowledge within a variety of subject areas. Three avenues of investigation were followed; taxonomy, functional morphology and host-parasite interactions.

1.5.1. Taxonomy

The aims of the taxonomic investigations were two-fold:

1) To identify *Gyrodactylus* species found infecting locally obtained *P. reticulata* recently imported from ornamental fish farms in Singapore and bound for retail distribution. A consequence of this investigation was a re-examination of a *Gyrodactylus* from *Xiphophorus* hybrids after Harris (1986).

2) To study changes in sclerite morphometrics of these *Gyrodactylus* at different temperatures and on different host species and to investigate whether sclerite variability could be indicative of parasite genetic variability.

1.5.2. Functional morphology

The study of functional morphology of attachment was compared between *G. bullatarudis* and *G. turnbulli*. Comparison of *Gyrodactylus* attachment has rarely been considered with respect to the variety of sclerites present in the genus (Malmberg, 1970). Functional morphology of attachment is new and interesting way of investigating *Gyrodactylus* interrelationships.

1.5.3. *P. reticulata* - *Gyrodactylus* interrelations

There were three broad aims of the investigations of guppy-*Gyrodactylus* host-parasite interactions:

1) To identify differences in the host-parasite relationships of *G. bullatarudis* and *G. turnbulli* on *P. reticulata*. For example, host-site specificity, parasite-induced host mortality and rates of parasite increase.

2) To investigate whether the host response of *P. reticulata* to *G. bullatarudis* or to *G. turnbulli*, would be effective against challenge infections regardless of which species was used in the initial infection.

3) Most previous investigations of *G. turnbulli* epidemiology (Scott, 1982, 1985a, 1985b; Scott and Anderson, 1984; Scott and Nokes, 1984; Scott and Robinson, 1994; Madhavi and Anderson, 1985) have used immature fish of 16mm or smaller standard length. The third aim of this part of the project was to investigate differences in rates of parasite induced mortality, host-site specificity and patterns of infection in small populations of adult fish infected with *G. bullatarudis* and *G. turnbulli* and to compare these results with those of the previous studies which used immature fish.

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CHAPTER 2

2. Identification of *Gyrodactylus* species from *Poecilia reticulata.*

2.1. Introduction

The first description of a *Gyrodactylus* species from poeciliid fish was that of *G. bullatarudis* from aquarium guppies (*Poecilia reticulata* Peters) in Canada (Turnbull, 1956). This species was later reported by Rogers and Wellborn (1965) from aquarium guppies in the USA and was first recorded in the wild by Kritsky and Fritts (1970) on *P. sphenops* in Costa Rica. *G. bullatarudis* has also been reported on *P. reticulata* native to Trinidad (Harris and Lyles, 1992).

Harris (1986) re-examined gyrodactylids from aquarium guppies previously identified as *G. bullatarudis* for Scott and co-workers' laboratory studies (see Scott, 1985). Live and fixed specimens were compared with a species he identified as *G. bullatarudis* from *Xiphophorus* (*X. maculatus* x *X. helleri*) hybrids. This was the first recording of *G. bullatarudis* from these hosts. The species used in Scott's experiments were found to be a new species which he described and named *G. turnbulli.*

G. turnbulli has since been found on introduced, feral guppies in Peru (An, Jara and Cone, 1991) and on native Trinidadian guppies (Harris and Lyles, 1992).

A summary of reports of *Gyrodactylus* species from poeciliid fish is given in Table 2.1.

Gyrodactylus species	Subgenus	Species-group	Host species	Origin
G. bullatarudis [12357]	*G.(Mesonephrotus)* [5]	*G. arcuatus* [5]	*Poecilia reticulata* [127]	Aquaria (Canada[1], USA[2]) Trinidad[7]
			Poecilia sphenops [3]	Costa Rica[3]
			Xiphophorus hybrids [5]	Aquaria (UK[5])
G. gambusiae [2]	*G.(Mesonephrotus)* [5]	*G. arcuatus* [5]	*Gambusia affinis* [2]	Fish hatchery, Florida[2]
G. costaricensis [3]	*G. (Mesonephrotus)* [5]	*G. arcuatus* [5]	*Poecilia sphenops* [3]	Costa Rica[3]
G. rasini [4]	*G. (Mesonephrotus)* [4]	*G. arcuatus**	*Xiphophorus helleri* [4]	Aquaria (Czechoslovakia[4])
G. turnbulli [567]	*G.(Metanephrotus)* [5]	*G. eucaliae* [5]	*Poecilia reticulata* [567]	Aquaria (UK[5]) Trinidad[6] Peru[7]

Table 2.1. Records of *Gyrodactylus* species from poeciliid fish. References: 1 Turnbull (1956), 2 Rogers and Wellborn (1965), 3 Kritsky and Fritts (1970), 4 Lucky (1973), 5 Harris (1986), 6 An *et al.* (1991), 7 Harris and Lyles (1992) and * Chapter 3, this thesis.

Of the two species of *Gyrodactylus* known to infect *P. reticulata*, until now, only *G. turnbulli* has been recorded from guppies in the UK. In this chapter the identification of two species of *Gyrodactylus* obtained from guppies purchased in Liverpool is described.

2.2. Materials and methods

2.2.1. Origin of fish and parasites

Two batches of about 30 guppies (*Poecilia reticulata* Peters) were obtained from a wholesaler in Liverpool no later than two weeks after import from ornamental fish farms in Singapore. In both batches, fish were found to be infected with *Gyrodactylus*. Differences in the site specificity of the parasites infecting the two batches were noted; in one group flukes were commonly seen on the head and opercular regions and in the other the posterior surfaces of the fish were favoured by the parasite. Gills from autopsied infected fish showed no evidence of infection.

2.2.2. Maintenance of *Gyrodactylus*

Populations of the parasites were ensured by keeping groups of three to five infected fish of standard length up to 30mm in jars containing 500ml of tap water which had been left standing for at least 48h in the environmentally controlled aquarium room regulated to maintain a water temperature of about 25°C (referred to hereafter as standing tap water). When one infected fish was removed for autopsy or when one died, a replacement fish was added which had been treated with a 1:4000 dilution of formalin for 1h to remove all gyrodactylid ectoparasites (Lester, 1972) at least 6 weeks before (see Scott and Robinson, 1984) or by a *Gyrodactylus* -

naive laboratory bred fish. Parasites and fish were kept in a 12: 12, light: dark regime at water temperatures of between 24 and 26° C.

2.2.3. Taxonomic investigation of sclerites

Each infected guppy to be autopsied for parasite collection was anaesthetised in a petri dish containing a solution of 0.02% MS222 until motionless, killed by inserting a needle behind an eye and into the brain (Malmberg, 1970) and then placed into another dish containing filtered standing tap water.

This petri dish was placed in a fridge for 10 to 15mins. *Gyrodactylus* which had been chilled to about 4°C were almost motionless and could be more easily detached from fish skin or glass. Flukes were dislodged using a size 0 entomological pin and were pipetted onto clean slides. An improved method of detaching *Gyrodactylus* from fish is described in Chapter 3.

Before placing cover slips (size No. 0, 22mm diameter, circular), slides were viewed under a binocular dissecting microscope and dirt was removed from the drops of water using pins and forceps.

Specimens to be used in the investigation of hard parts were prepared using the following method based on that of Malmberg (1970): water was drawn out from under the cover slip using filter paper in order to flatten the parasites. This operation was begun using a binocular microscope until the flukes became still. Then the slide was transferred to a phase contrast compound light microscope. Once found using the x10 objective, the parasite opisthaptor was observed under the x40 objective - a x10 ocular lens was used throughout this work. Water was drawn out until the hamuli became splayed apart and flattened as far as possible without damaging

them. At this stage the fluke tegument would start to rupture.

A small drop of ammonium picrate-glycerin (1:1 ammonium picrate: glycerol) was then placed on the slide near to the edge of the cover slip. The ammonium picrate-glycerin was mixed in a watch glass when needed. This was considered to be more economic than using a stock solution and it also allowed minor adjustments to be made to the viscosity of the mixture for the reasons given below. The mixture could be made more viscous by further addition of glycerol and more runny by further addition of ammonium picrate solution. The drop of fixative was spread, increasing the area of slide which it covered and reducing its height. A fine line of fixative would then be directed towards the cover slip using a pin until contact with water under the edge allowed the mixture to be drawn under, replacing evaporating water. This manipulation of the drop of fixative on the slide was necessary to avoid excessive lifting of the cover slip caused by too rapid a flow of fixative under the cover slip. Too rapid a flow of fixative under the cover slip also occurred if the fixative mixture was too runny. Alternatively, the replacement of water could be too slow if the fixative mixture was too viscous, resulting in the drying of specimens during preparation.

The diffusion of ammonium picrate-glycerin under the cover slip would take half an hour or more and so slides were removed from the microscope and placed on a clean, flat surface to allow completion of this process.

Measurements of the hard parts were made using an ocular micrometer and an oil immersion x100 objective lens. Sclerites of ten specimens from each group were drawn using a Wild M11 microscope and a Wild camera lucida. The system used for taxonomic analysis was that outlined by Harris (1985) with the omission of the examination of the excretory system, which was not necessary for species identification, and with the addition of a more

detailed study of the cirrus sclerites which was lacking from previous species descriptions. The measurements taken were those listed in Harris (1986) so that a complete comparison could be made with that work (see Fig. 2.1). The cirrus sclerites of both species were also drawn using the camera lucida. An attempt was made to elucidate their finer structure, particularly the morphology of the large spine.

Key to measurements

1 Hamulus total length
2 Hamulus shaft length
3 Hamulus root length
4 Hamulus point length

5 Ventral bar total length
6 Ventral bar total width
7 Ventral bar process length
8 Ventral bar membrane length

9 Marginal hook total length
10 Marginal hook shaft length
11 Marginal hook sickle length
12 Marginal hook sickle distal width
13 Marginal hook sickle proximal width

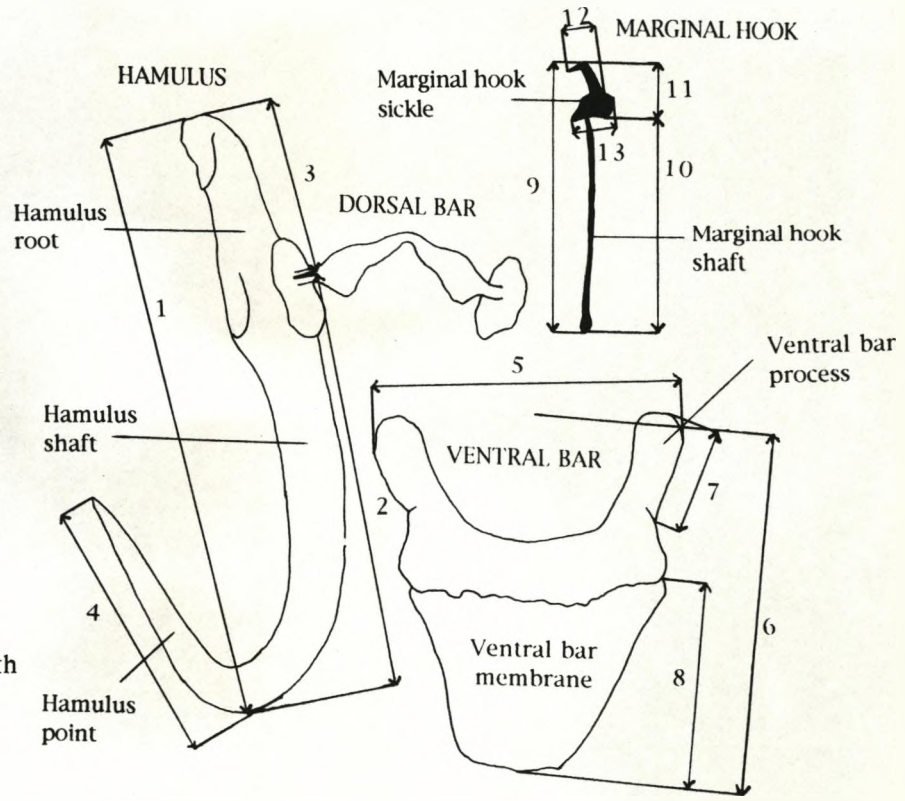

Fig. 2.1. Nomenclature of opisthaptoral sclerites and the measurements used in this thesis for taxonomic investigation of *Gyrodactylus* species (after Harris, 1985).

2.2.4. Preparation of archival specimens

Opinions differ on the longevity of ammonium picrate-glycerin preparations. Ergens (1969) argued that even after use of a suitable sealant, archival permanence of ammonium picrate-glycerin preparations is doubtful. Because of this, some specimens initially prepared in ammonium picrate-glycerin were transferred to a permanent mount of Canada balsam using the following method based upon that of Ergens (1969). Later, some specimens were prepared in the modified version of Ergens' method described in Chapter 5.

First small rings were drawn in Indian ink on the bottom of the slide encircling each specimen. The alignment of the cover slip was recorded by making four marks at its edge, each corresponding with a mark on the upper surface of the slide.

This preparation was placed in a petri dish containing absolute ethanol and was left for 5 mins. The cover slip was then lifted off. Care was taken to ensure that the cover slip was lifted straight up and that there was no sliding between cover slip and slide. This operation was best performed using a pair of blunt and a pair of fine forceps - one point of the fine forceps could be forced between cover slip and slide to initiate the lifting process while the two points of the blunt forceps were held firm against the opposite edge to stop sliding of the cover slip during the lifting process. Flattened specimens stuck to either the cover slip or to the slide.

The cover slip was then placed up-side down next to the slide and both were left for a further 5mins. Both cover slip and slide were then transferred to a petri dish containing a 1: 1 mixture of ethanol: xylene and were left there for 5 mins before transfer to a final petri dish which contained pure xylene. Here they were left for 2 mins before mounting in Canada balsam.

The best method of mounting was found to be as follows; first both cover slip and slide were placed on a clean sheet of paper with their specimen sides uppermost. Once excess xylene had evaporated, a small amount of Canada balsam was placed on the cover slip. The slide could then be lowered, specimen side downward, onto the cover slip, making sure that the markings on the cover slip were aligned with those on the slide.

The best specimens prepared in this way were deposited in the Natural History Museum, London (reference numbers, BM(NH)1994.11.24.1-6 for *G. bullatarudis* and BM(NH)1994.11.24.7-12 for *G. turnbulli*).

2.3. Results

Two species of *Gyrodactylus* were identified; *G. bullatarudis* and *G. turnbulli*.

2.3.1. General species descriptions

G. turnbulli Harris, 1986

Host: *Poecilia reticulata* (Harris, 1986; An *et al.*, 1991; Harris and Lyles, 1992).

Host-site specificity: External surface. Posterior of the host, particularly the caudal peduncle and caudal fin.

Synonym: *G. bullatarudis sensu* Scott (1985) (see Harris, 1986).

Description: Body fusiform. A "very small" species according to the scheme

of Harris (1985) (average length under 400μm).

Careful study of live specimens reveals that the anterior part of the pharynx consists of eight protrusible, pyramid shaped cells (see Chapter 1, Figs. 1.15 and 1.16). The tips of each of these cells is capable of a small amount of independent movement. They are not fused with each other along their lateral borders. Although this type of pharynx is not unique to *G. turnbulli* among *Gyrodactylus* species, it does allow quick differentiation of living *G. turnbulli* from *G. bullatarudis* at total magnifications of x200 or greater.

The cirrus consists of 1 large spine and a single row of 4 - 7 small spines (section 2.3.2).

The attachment sclerites were found to be the most useful organs for species diagnosis. Hamuli, 53 - 59μm long, with straight shafts, 38 - 41μm long, and roots of about half that length, 15 - 19μm. The ventral bar has two anterior processes of moderate length, 5 - 8μm and a membrane roughly rectangular in shape. The ventral bar membrane has a median notch along its posterior edge, see Chapter 5, section 5.4, fourth paragraph, and Fig. 5.5. The most distinctive elements of the attachment sclerites are the dorsal bar and the marginal hooks (see Fig 2.2.). The flexible dorsal bar bears "small lugs on either side of the mid point" (Harris, 1986). When stained, dorsal bar posteriad supporting attachments (Malmberg, 1970) are visible, see Chapter 5, section 5.4, third paragraph, and Figs. 5.3 and 5.4. Each marginal hook has a point which extends beyond the level of the toe. The blade of the marginal hook sickle is gently curved towards the point.

Table 2.2 compares sclerite measurements taken from *G. turnbulli* in this work with those of previous studies.

G. bullatarudis Turnbull, 1956

Hosts: *Poecilia reticulata* (Turnbull, 1956; Rogers and Wellborn, 1965; Harris and Lyles, 1992), *P. sphenops* (Kritsky and Fritts, 1970)

Host-site specificity: External surface. Anterior of the host particularly the head and opercular regions.

Description: Body fusiform. A "very small" species according to the scheme of Harris (1985) (average length under 400μm).

The anterior of the pharynx consists of eight cells which are fused to each other along their lateral borders. They can be protruded as a barrel shaped unit (see Fig. 1.13, Chapter 1). Lines can be seen delineating ridges running postero-anteriorly on the interior surfaces of these cells (see Fig. 1.14, Chapter 1). Although this type of pharynx is not unique to *G. bullatarudis* among *Gyrodactylus* species, it does allow quick differentiation of living *G. bullatarudis* from *G. turnbulli* at total magnifications of x200 or greater.

The cirrus consists of one large spine and a single row of 4 - 7 small spines (section 2.3.2).

The attachment sclerites were again found to be the most useful organs for species diagnosis. Hamuli 50 - 59μm long, with shafts 33 - 41μm long, and roots of length 14 - 18μm. The ventral bar has relatively long antero-lateral processes of 7 - 11μm and a membrane which tapers slightly posteriorly. The most distinctive elements of the attachment sclerites are the hamuli

connecting bars and the marginal hooks (see Fig 2.3). The dorsal bar has an obvious notch at the mid point with two swellings on either side. The ventral bar has pronounced, straight processes. Each marginal hook has a point which does not extend beyond the level of the toe. The blade of each marginal hook sickle has an abrupt bend near the point turning an angle of about 90°.

Table 2.3 compares sclerite measurements taken from *G. bullatarudis* in this work with those of previous studies.

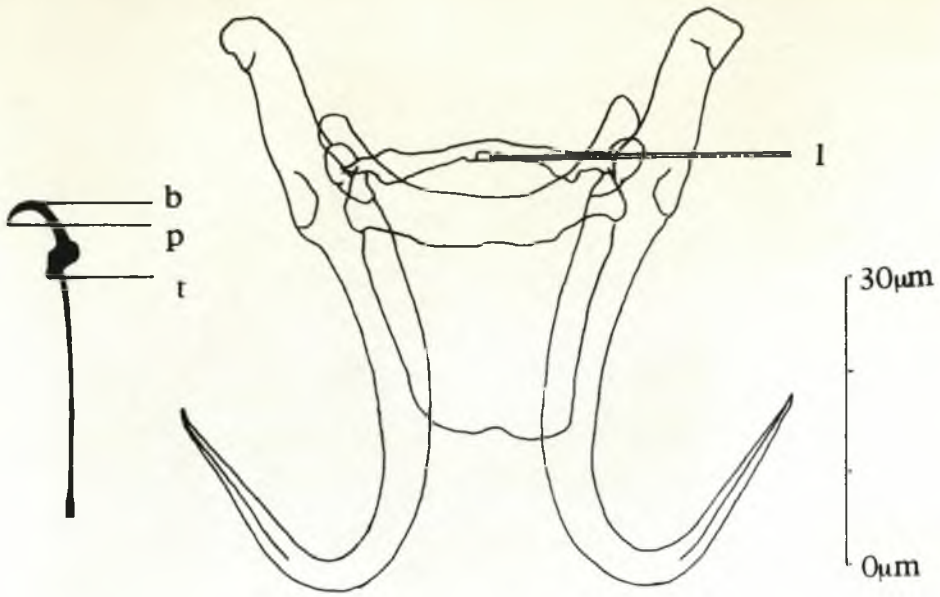

Fig. 2.2. Haptoral sclerites of *G. turnbulli*. Note the distinctive lugs on each side of the mid point of the dorsal bar (l) and the gently curved blade (b) of the marginal hook with a point (p) which extends beyond the level of the toe (t).

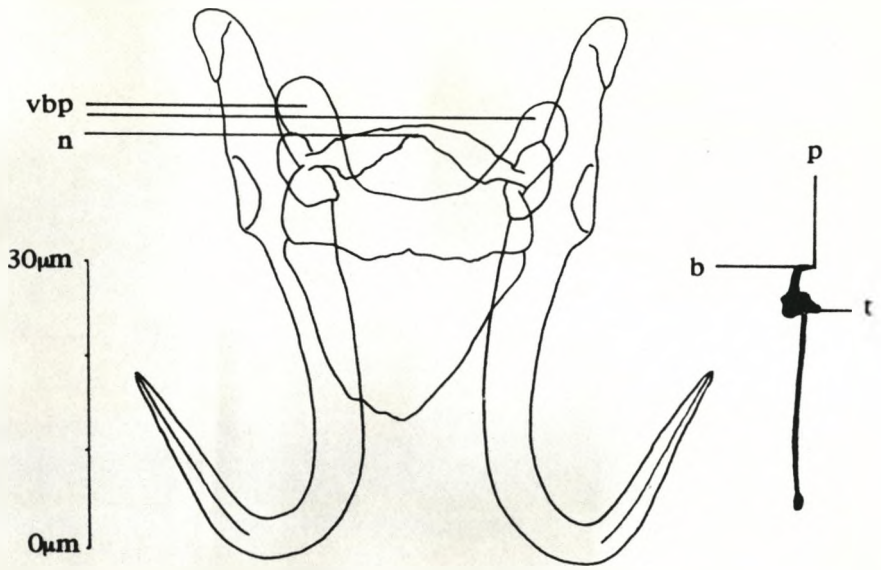

Fig. 2.3. Haptoral sclerites of *G. bullatarudis*. Note the distinctive notch at the mid point of the dorsal bar (n), the pronounced ventral bar process (vbp) and the abruptly curved blade (b) of the marginal hook with a point (p) which does not extend beyond the level of the toe (t).

Investigation	Present study			Harris (1986) (Own material)			Harris (1986) (Material from Scott, 1982)			An, Jara and Cone (1991)	
Parasite species	*G. turnbulli*			*G. turnbulli*			*G. turnbulli*			*G. turnbulli*	
Host species	*Poecilia reticulata*			*Poecilia reticulata*			*Poecilia reticulata*			*Poecilia reticulata*	
Location	External surface			External surface			External surface			External surface	
Collection site/origin	UK, imported from Singapore			UK, imported from Singapore			UK, imported from Singapore			Moche River, Peru	
Method of preparation	Ammonium picrate-glycerin			Ammonium picrate-glycerin			Fixed in formalin, mounted in Canada balsam			Formalin fixed specimens mounted in glycerin jelly	
	Mean	Std. Dev.	Range	Mean	Std. Dev.	Range	Mean	Std. Dev.	Range	Mean	Range
Hamulus total length.	56	1.9	53-59	52.9	1.5	50-55	55	0.4	54-55	57	55-58
Hamulus shaft length.	39	0.8	38-41	39.5	1.4	37-41	41.3	2	40-45	44	40-44
Hamulus root length.	17	1.0	15-19	16	1.1	14-17	15	0.7	14-16	16	16-19
Hamulus point length.	26	0.6	24-27	22.5	1.1	21-24	23	1.2	21-24	25	23-25
Ventral bar total length.	33	2.2	28-38	29.3	2	25-31	-	-	-	21	20-24
Ventral bar total width.	32	2.8	25-36	29	1.1	27-31	-	-	-	-	-
Ventral bar process length.	6	0.8	5-8	5.5	0.6	4-7	-	-	-	3	2-5
Ventral bar membrane length.	21	1.8	18-25	16.8	1.8	15-21	-	-	-	14	8-17
Dorsal bar length.	28	2.2	24-31	-	-	-	-	-	-	20	19-21
Marginal hooks total length.	33	1.0	31-35	30.9	1.2	29-33	31.5	1.5	30-35	32	30-34
Marginal hooks shaft length.	26	1.0	24-28	24	0.6	23-25	23	1	22-24	25	22-27
Marginal hooks sickle length.	8	0.4	7-9	7	0.6	6-8	8	0.6	7-9	8.5	8-9
Marginal hooks sickle distal width.	5	0.4	3-5	5.8	0.6	5-8	4.3	0.4	not given	4	3.5-5
Marginal hooks sickle proximal width.	5	0.6	3-5	3.8	0.6	3-5	4	not given	not given	3.5	3-4
Cirrus spines.	1 (large) 4-7 (small)			1 (large) 5-7 (small)			Not mentioned in publication			1 (large) 6 (small)	
No. Specimens examined.	14			Not mentioned in publication			Not mentioned in publication			17	

Table 2.2. Measurements of *G. turnbulli* Harris, 1986 from Harris (1986), An *et al.* (1991) and the present study. All measurements are in μm.

Investigation	Present study			Harris (1986)			Turnbull (1956)		Rogers and Wellborn (1965)		Kritsky and Fritts (1970)	
Parasite species	*G. bullatarudis*			*G. bullatarudis* *			*G. bullatarudis*		*G. bullatarudis*		*G. bullatarudis*	
Host	*Poeclila reticulata*			*Xiphophorus spp.*			*Poecilia reticulata*		*Poecilia reticulata*		*Poecilia sphenops*	
Location	External surface			External surface			External surface		External surface		External surface	
Collection site/origin	UK, imported from Singapore			UK, imported from Singapore			Aquarium fish, Canada		Aquarium fish, Alabama, USA		Near Rincón, Costa Rica	
Method of preparation	Ammonium picrate-glycerin			Ammonium picrate-glycerin			Live in methyl cellulose		Formalin-hardenen in glycerin-jelly		Formalin fixed in glycerin-jelly	
	Mean	Std. Dev.	Range	Mean	Std. Dev.	Range	Mean	Range	Mean	Range	Mean	Range
Hamulus total length.	55	1.8	50-59	47.4	1.2	46-50	51	48-54	46	43-48	54	51-59
Hamulus shaft length.	38	1.4	33-41	38.2	1.5	36-39	-	-	-	-	-	-
Hamulus root length.	17	0.9	14-18	11.1	1.5	9-13	-	-	-	-	-	-
Hamulus point length.	25	1.0	24-30	21.1	1.6	18-24	24	23-25	22	21-23	-	-
Ventral bar total length.	29	1.7	26-35	21.9	1.1	20-25	23	22-25	23	22-24	27	24-29
Ventral bar total width.	32	2.1	27-39	29.2	1.8	26-32	-	-	-	-	-	
Ventral bar process length.	9	1.0	7-11	9.4	1.2	7-12	-	-	-	10-11	-	
Ventral bar membrane length.	17	1.3	14-22	13.8	0.7	12-15	14	not given	not given	-	-	
Dorsal bar length.	26	2.3	23-32	19.1	1.3	not given	23	20-25	22	21-23	20	18-23
Marginal hooks total length.	25	0.5	24-27	23.2	0.9	21-25	-	-	-	-	28	27-29
Marginal hooks shaft length.	22	0.6	20-23	18.6	0.9	17-20	20	19-21	19	18-21	-	-
Marginal hooks sickle length.	6	0.3	5-7	4.7	0.4	4-5	5	5-6	not given	5-6	6	not given
Marginal hooks sickle distal width.	2	0.0	2	3.7	0.3	-	-	-	-	-	-	-
Marginal hooks sickle proximal width.	5	0.9	3-6	3.8	0	-	-	-	-	-	-	-
Cirrus spines.	1 (large) 4-7 (small)			1 (large) 4-5 (small)			1 (large) 4-7 (small)		1 (large) 4-6 (small)		1 (large) 4-5 (small)	
No. Specimens examined.	37			13			20		15		20	

Table 2.3. Measurements of *G. bullatarudis* Turnbull, 1956 from Harris (1986)*, Turnbull (1956), Rogers and Wellborn (1965), Kritsky and Fritts (1970) and the present study. All measurements are in μm.

* *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) is a synonym of a parasite tentatively identified as *G. rasini* Lucky, 1973, see Chapter 3.

2.3.2. The cirrus sclerites

58% (25/43) of the specimens of *G. bullatarudis* and 57% (8/14) of the specimens of *G. turnbulli* examined had cirral bulbs with refractive sclerites.

A schematic diagram to show the interpretation of the morphology of the cirrus armature found for *G. turnbulli* and *G. bullatarudis* is given in Fig. 2.4.

Although both *G. turnbulli* and *G. bullatarudis* had one large spine and a similar number of small spines (4 - 7 for both *G. turnbulli* and *G. bullatarudis*) surrounding the opening of the ejaculatory duct (see Kritsky, 1970) in the cirral bulb, the morphology of both the these elements of armature, particularly of the large spine, was significant enough to allow species differentiation (see Fig. 2.5).

The surrounds of the opening of the ejaculatory ducts of both species were associated with a number of structures which appeared dark under phase contrast microscopy and these were all considered to be parts of the cirrus armature and are illustrated in Fig. 2.4. In describing the cirrus armature "anterior" is arbitrarily denoted by the direction of the large spine from base to point when viewed from directly above the armoured surface of the cirral bulb.

The small spines were of simple structure. Those of *G. turnbulli* were longer and thinner (more pin-like) than those of *G. bullatarudis* (see Fig. 2.5).

The large spine is a much more complicated structure which curves downwards (see Fig. 2.4B) and is composed of two bilaterally symmetrical

structural elements which appear dark under phase contrast microscopy (Fig. 2.4A). These elements converge on each other to form the point and diverge nearer the base of the spine. Each of these elements has a posterior root and a lateral root. The lateral and posterior roots of the large spines of both *G. turnbulli* and *G. bullatarudis* are joined to form a lateral ring (see Figs. 2.4 and 2.5). The structural elements appear to be thickened areas of the large spine. Whether they are completely separate or joined by a thinner element is unclear as the material between the two structural elements making up the blade of the large spine appears less dense and its borders are not well defined posteriorly.

The large cirrus spine of *G. turnbulli* is also longer and thinner than of *G. bullatarudis*, Fig. 2.5F. *G. bullatarudis* also exhibited a dark line running between the two lateral structural elements of the large spine in the region of the divergence of the lateral roots, *G. turnbulli* did not (compare Figs. 2.5A and 2.5C). This dark line was interpreted as a bar joining the two lateral structural elements of the large spine.

Posterior to the large spine is a cap-like structure consisting of a curved posterior supporting bar and a thinner, posterior supporting membrane. Both these structures appear dark under phase contrast microscopy (see Figs. 2.4 and 2.5). Lateral supporting bars (Fig. 2.4) were seen on many specimens. These presumably function as additional strengthening of the opening of the ejaculatory duct.

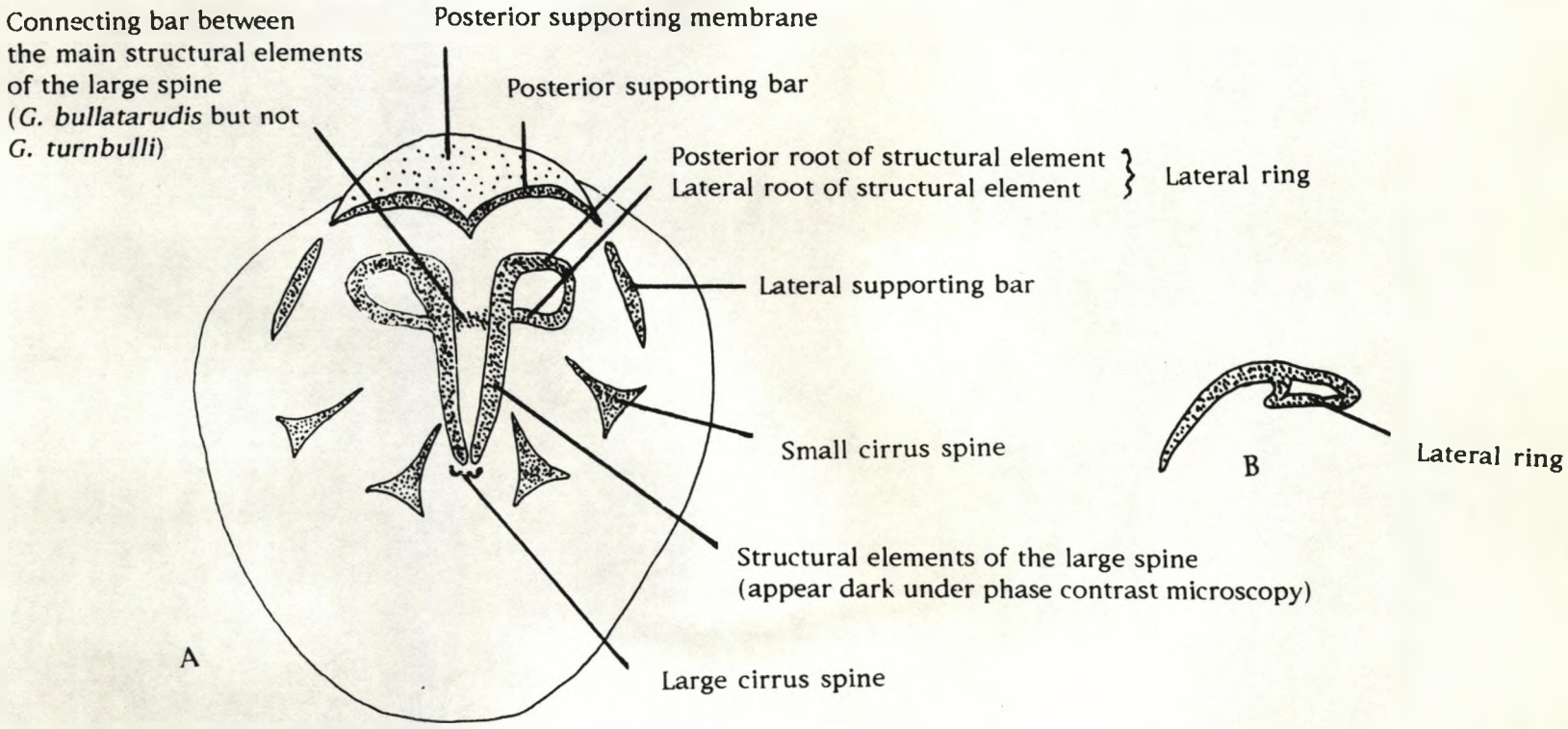

Fig. 2.4. Schematic diagrams of the *Gyrodactylus* cirrus armature as interpreted by phase contrast microscopy of ammonium picrate - glycerin prepared specimens of *G. turnbulli* and *G. bullatarudis*. The shaded areas appear darker or refractory on mature cirral bulbs. A, view of the cirrus armature from above ; B, side - on view of the large cirrus spine.

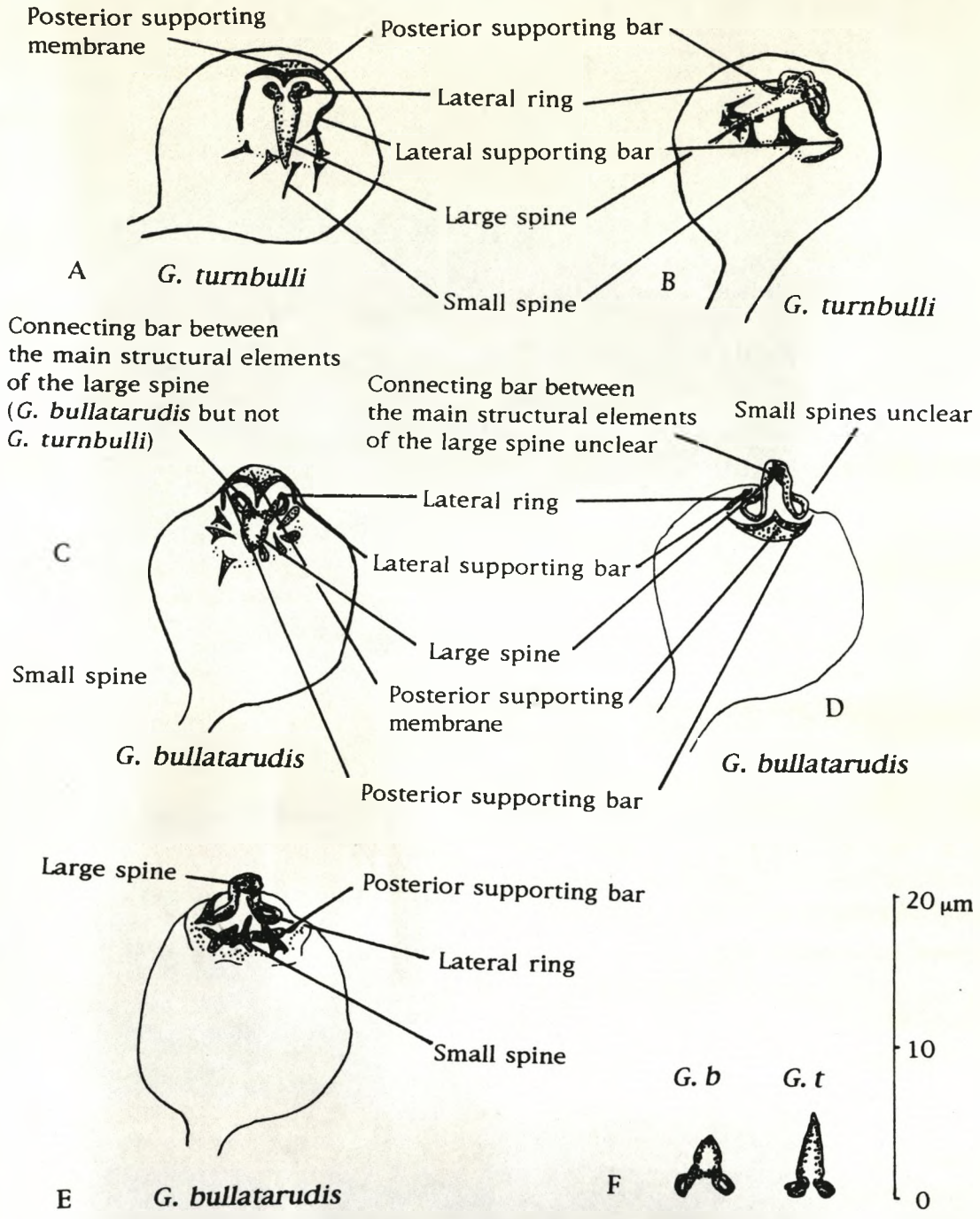

Fig. 2.5. Cirrus armature of *G. turnbulli* (A and B) and *G. bullatarudis* (C, D and E) fixed and mounted in ammonium picrate-glycerin, viewed with a phase contrast microscope and drawn with the aid of a camera lucida. F is a direct comparison of the large cirrus spine of *G. bullatarudis* and *G. turnbulli* against a scale bar applicable to all the drawings.

2.4. Discussion

Since the early 1980's *G. turnbulli* and *P. reticulata* have been obtained for use in a number of studies of host - parasite interactions in the UK (see Scott, 1985; Madhavi and Anderson, 1985; Harris, 1988 and 1989), their occurrence was therefore considered likely to be common on ornamental fish stocks in this country.

Identification of *G.turnbulli* was confirmed later by comparison with material deposited by Scott (BM(NH) 1981.3.15.2) and paratypes deposited by Harris (BM(NH) 1985.3.15.2) in the Natural History Museum, London.

G. bullatarudis had not previously been recorded from aquarium guppies in the UK, their presence is noteworthy as this implies that both species of native *Gyrodactylus* parasites of *P. reticulata* (Harris and Lyles, 1992 see Table 2.1) are being maintained in stocks of Singaporean and, possibly, British suppliers of ornamental fish.

Drawings of ventral bars of *G. bullatarudis* produced in this work were of greater similarity to those drawn by Turnbull (1956)[1] (Fig. 2.6A), also of *G. bullatarudis* from *P. reticulata*, than to those drawn by Harris (1986) of specimens he collected from *Xiphophorus* hybrids (Fig. 2.6D) and of specimens he drew collected by Kritsky and Fritts (1970) from *P. sphenops* (Fig. 2.6C). In particular, the ventral bar processes drawn by Harris (1986) appear longer and thinner and curve markedly in on each other in comparison to those drawn by Turnbull (1956) and to those drawn in the present study. The ventral bar processes drawn by Kritsky and Fritts (1970) (Fig. 2.6B) were more similar to those observed in the present study and to those drawn by Turnbull (1956) than those drawn by Harris (1986).

[1] The sclerites of *G. bullatarudis* are not illustrated in Rogers and Wellborn (1965).

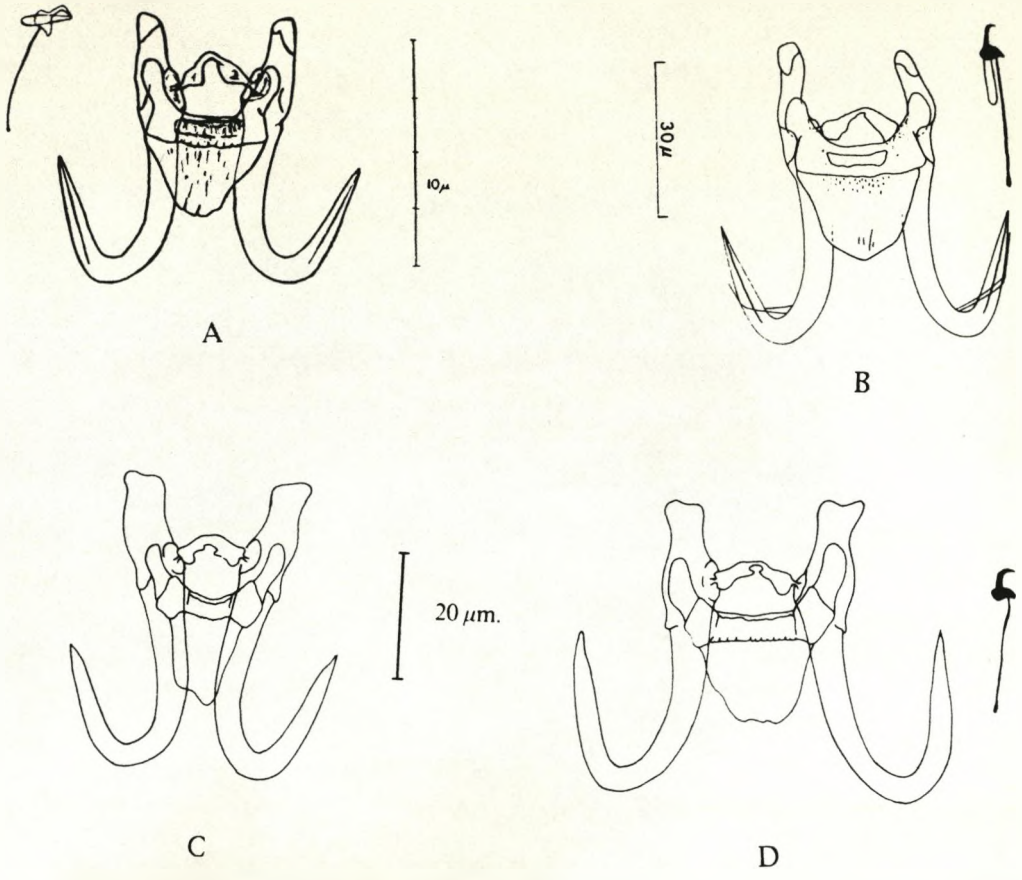

Fig. 2.6. Drawings of the haptoral sclerites of *G. bullatarudis* from, A, *P. reticulata* (Turnbull, 1956), B, *P. sphenops* (Kritsky and Fritts, 1970), C, *P. sphenops* (Harris, 1986 from a specimen collected by Kritsky and Fritts, 1970) and, D, *Xiphophorus* hybrids (Harris, 1986)

The average marginal hook sickle distal width of specimens obtained in this study (2μm) was less than half the average marginal hook proximal width (5μm) but only just over half of the average marginal hook sickle distal width found by Harris (1986) for his specimens from *Xiphophorus* hybrids (3.7μm - almost equal to the corresponding average proximal width of 3.8μm). Harris's (1986) own average measurement of marginal hook sickle distal width from two specimens deposited by Turnbull (1956) (1.8μm) was less than half of the corresponding average measurement for specimens he collected from *Xiphophorus* hybrids and exactly half the value of average marginal hook sickle proximal width from the same two

specimens (3.6μm). The marginal hook illustrated in Fig. 2.6B drawn by Kritsky and Fritts (1970) is more similar to those collected in the present study and by Turnbull (1956) from *P. reticulata* than to those collected from *Xiphophorus* hybrids by Harris (1986) (Fig. 2.6D). One specimen collected by Kritsky and Fritts (1970) and measured by Harris (1986) had a distal width of 2.4μm and a proximal width of 3.6μm

Given the above discrepancies in ventral bar morphology and marginal hook dimensions in comparisons of drawings and measurements of specimens of *G. bullatarudis* collected from *P. reticulata* (present study and Turnbull, 1956), from *P. sphenops* (Kritsky and Fritts, 1970) and from *Xiphophorus* hybrids (Harris, 1986), Harris's (1986) statement, "it is possible that *G. bullatarudis* exists as a series of indistinguishable strains on different poeciliids", was cast in doubt. A further study investigating the morphological differences between *G. bullatarudis* infecting *P. reticulata* and those infecting *Xiphophorus* hybrids is the subject of Chapter 3.

Kritsky and Fritts (1970) illustrated the cirral bulb of *G. bullatarudis* (Fig 2.7A). Lateral strengthening elements of the large spine are in evidence and also the lateral rings of these elements as defined in section 2.3.2. A structure similar to the connecting bar illustrated in Fig. 2.5C was also shown but the posterior supporting membrane and the posterior and lateral supporting bars were not. The length of the large spine (approximately 7μm by comparison with the scale bar) was larger than the large spines drawn from *G. bullatarudis* in this investigation (approximately 4μm). Drawings of the cirrus of *G. bullatarudis* by Turnbull (1956) "sketched from fresh material" also shows features compatible with the lateral rings of the lateral supporting elements of the large spine and the connecting bar between them (Fig. 2.7B). The small spines drawn by Turnbull (1956) are more similar to those drawn in the present investigation than those drawn by Kritsky and Fritts (1970) which are

more pin - like. However, the original drawing published by Kritsky and Fritts (1970) was small and it is difficult to interpret such fine detail from it.

Even though the exact fine structure of the cirrus armament could not be seen, camera lucida drawings of these sclerites and the patterns of their dark or refractory elements as visualised by phase contrast microscopy were species specific. There is further evidence of this in Chapter 3 in which *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) is separated from *G. bullatarudis sensu stricto* by various morphological characteristics of the attachment sclerites but was also separable by the considerably different large spines of the cirrus armature which have lateral and posterior roots of their lateral strengthening elements which are not joined to form a lateral ring as shown in Figs. 2.4 and 2.5.

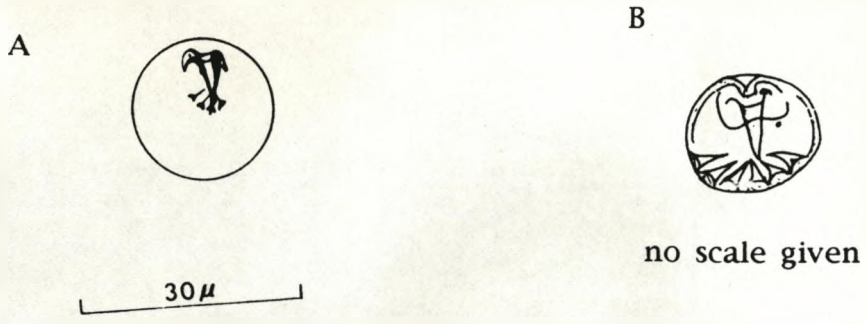

Fig. 2.7. Cirrus sclerites of *G. bullatarudis* from, A, *P. sphenops* (Kritsky and Fritts, 1970) and, B, *P. reticulata.* (Turnbull, 1956).

Although the number of small or large spines are invariably recorded (unless no specimens with a cirrus were observed) in species descriptions of *Gyrodactylus*, drawings of the cirrus sclerites are often omitted, for

example Harris (1986) and An *et al.* (1991). This omission is regrettable as the morphology of the cirrus spines in particular may be of considerable value to species identification and are worth recording in as much detail as possible. However, one factor which limits the usefulness of the cirrus sclerites for *Gyrodactylus* species identification is the protogynous nature of the genus. Population age structure and mortality of *Gyrodactylus* species differ along with their reproductive strategies and the proportion of functional males with a mature cirrus within a population (Harris, 1993). This proportion varies considerably, from, for example, 44% (Harris, 1989 - *G. turnbulli* on *P. reticulata*) to around 70% (Harris *et al.*, 1994 - *G. salaris* on *Salmo salar*).

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CHAPTER 3

3. Identification of a *Gyrodactylus* species from *Xiphophorus* hybrids.

3.1. Introduction

This chapter investigates the hypothesis of Harris (1986) that *G. bullatarudis* consists of morphologically indistinguishable strains, each infecting different species of fish. As a consequence, a re-description of *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) is given.

3.2 Materials and Methods

The haptoral sclerites of the groups of parasites listed in section 3.2.1 were compared using phase contrast microscopy, multivariate statistical analysis and, for Groups II, III and IV, scanning electron microscopy.

3.2.1 Origin and maintenance of *Gyrodactylus*

Group I; *G. bullatarudis* from *P. reticulata* , same population as those discussed in Chapter 2. The notation "Group I (*G. b* (*P. r*) 1)" shall hereafter be used in the text as a reminder that Group I were *G. bullatarudis* (*G. b*) from *P. reticulata* (*P. r*) and were from the first population of these parasites obtained (1):

The origin of these parasites is described in Chapter 2, section 2.2.1, and their maintenance in Chapter 2, section 2.2.2.

Group II; *G. bullatarudis* from *P. reticulata* , acquired after those discussed in Chapter 2. The notation "Group II (*G. b* (*P. r*) 2)" shall hereafter be used in the text as a reminder that Group II were *G. bullatarudis* (*G. b*) from *P. reticulata*: (*P. r*) and were from the second population of these parasites obtained (2):

About 20 adult *P. reticulata* infected with *G. bullatarudis* were obtained from the same wholesaler in Liverpool who supplied the hosts and parasites described in Chapter 2 (referred to as Group I (*G. b* (*P. r*) 1) in this chapter). However, the new host fish were obtained from a different importer, also within two weeks after arrival from Singapore.

By the time these parasites were obtained, a larger reservoir of naive, laboratory bred guppies had been established for use in parasite propagation and so, contrary to Group I, no larger, treated fish (see Chapter 2, section 2.2.2) were used in their propagation. They were maintained by keeping groups of four to six infected fish of standard length up to 15mm in jars containing 500ml of standing tap water (see Chapter 2, section 2.2.2). When one infected fish was removed for autopsy or when one died it was replaced by a naive laboratory bred fish. Parasites and fish were kept in a 12: 12, light: dark regime at water temperatures ranging between 24 and 26°C.

Group III; *G. bullatarudis* originally from *P. reticulata* (same population as Group II) but maintained on *Xiphophorus* hybrids for a minimum of 11 days (264 hours). The notation "Group III (*G. b* (*P. r*) 2 on *X*)" shall hereafter be used in the text as a reminder that Group III were *G. bullatarudis* (*G. b*) from *P. reticulata*: (*P. r*), were from the second population of these parasites obtained (2) and were maintained on experimentally infected

Xiphophorus hybrids (*X*):

One *P. reticulata*, heavily infected with *G. bullatarudis* from Group II, was killed using the method described in Chapter 2, section 2.2.3. and was placed overnight in 500ml of standing tap water with 5 laboratory bred *Xiphophorus* hybrids of standard length 10 - 20mm. All these *Xiphophorus* became infected with at least one *G. bullatarudis*. It was possible to maintain this population of *G. bullatarudis* on laboratory bred *Xiphophorus* hybrids by checking the cultures every four days and adding naive fish following removal of dead fish or recovered fish which had become refractory to reinfection (see Scott, 1985). This population of parasites was sampled 11 days after their establishment on the "atypical" host. Given that *G. bullatarudis* completes its life cycle on the guppy in about 60 hours at 25-27°C, development of an individual from ovum to birth takes about 42 hours (Turnbull, 1956) and that *G. turnbulli* has an average life span of 4.2 days at 25°C (Scott, 1985) and that both *G. turnbulli* and *G. bullatarudis* have a similar intrinsic rates of increase on individual fish (see Chapter 8), 11 days was considered to be enough time for most of the population to be made up of parasites which had developed and had been born on *Xiphophorus*.

Group IV; *G. bullatarudis* from *Xiphophorus* hybrids. The notation "Group IV (*G. b* (*X*))" shall hereafter be used in the text as a reminder that Group IV were *G. bullatarudis* (*G. b*) from *Xiphophorus* hybrids (*X*):

Four *Xiphophorus* hybrids imported from Singapore were obtained through a local wholesaler in Liverpool. Three of these fish were found to be infected with *Gyrodactylus*, tentatively identified as *G. bullatarudis*.

This population of flukes was propagated and maintained for three to four

weeks by keeping groups of three to five infected fish of standard length 20 to 40mm in jars containing 500ml standing tap water, replacing sacrificed fish, dead fish and fish which had lost their infection with uninfected *Xiphophorus* hybrids obtained from the same local wholesaler.

Group V; museum specimens of *G. bullatarudis* from *Xiphophorus* hybrids (BM(NH) 1985. 3. 15) deposited by Harris (1986). The notation "Group V (*G. b* (*X*) BM(NH))" shall hereafter be used in the text as a reminder that Group V were *G. bullatarudis* (*G. b*) from *Xiphophorus* hybrids (*X*) deposited in the Natural History Museum, London (BM(NH)).

Comparison of Groups I (*G. b* (*P. r*) 1) and II (*G. b* (*P. r*) 2) was to investigate the amount of variation that might occur between two different populations of *G. bullatarudis* which both infect *P. reticulata*.

Comparison of Groups II (*G. b* (*P. r*) 2) and III (*G. b* (*P. r*) 2 on *X*) was to establish whether morphological variation in *G. bullatarudis* may arise in parasites, originally from the same population, due to differences in microenvironments on different host species.

It was proposed that if any consistent differences could be found between the *Gyrodactylus* infecting different species of fish, the hypothesis of Harris (1986) should be rejected and a description of the form infecting *Xiphophorus* should be given as distinct from the description of *G. bullatarudis* Turnbull, 1956 given in Chapter 2.

3.2.2 Scanning electron microscopy

One heavily infected fish was taken from each of Groups II (*G. b* (*P. r*) 2), III (*G. b* (*P. r*) 2 on *X*) and IV (*G. b* (*X*)) and was prepared for scanning electron microscopy by freeze fixation-dehydration (see Chapter 6, section

6.2).

A sample of parasites was removed from each fish after first viewing using the SEM. The method of removal is described in Chapter 6, section 6.2. These preparations were used to allow SEM comparison of the attachment sclerites, particularly the marginal hooks.

3.2.3 Phase contrast microscopy

Taxonomic investigation of sclerites of Groups I (*G. b* (*P. r*) 1) and IV (*G. b* (*X*)) by phase contrast microscopy was carried out using the same protocol as in Chapter 2, section 2.2.3. Specimens from Groups II (*G. b* (*P. r*) 2) and III (*G. b* (*P. r*) 2 on *X*) were prepared later, using the improved method of removing *Gyrodactylus* from small fish described below.

After the infected fish was killed by the method described in Chapter 2, section 2.2.3, it was placed in a small Eppendorff tube, covered with standing tap water and the plastic lid snapped closed. This Eppendorff tube was left in the fridge for 1h and 30mins, by which time many flukes had translocated off the fish. The tube was then taken from the fridge and shaken briskly to detach as many flukes as possible. The dead fish was then removed and the water, carrying detached flukes, pipetted into a watch glass. It was then easy to pipette cold relaxed flukes from the watch glass onto a clean slide for mounting in ammonium picrate-glycerin as described in Chapter 2, section 2.2.3. Whilst individual slides were being prepared, the watch glass containing the other *Gyrodactylus* was placed back in the fridge to prolong the lives of the remaining living flukes and the integrity of dead flukes. Some preparations of sclerites were made from dead flukes as the sclerites remained intact for some time after death.

Measurements and drawings of attachment sclerites of specimens from

Group I (*G. b* (*P. r*) 1) used in Chapter 2 were also used in this investigation. Attachment sclerites of 30 specimens from each of Groups II (*G. b* (*P. r*) 2) and III (*G. b* (*P. r*) 2 on *X*) and 39 specimens from Group IV (*G. b* (*X*)) were measured as described in Chapter 2, section 2.2.3, (measurements taken are shown in Table 2.1) and sclerites from 5 specimens from each group were drawn using a Wild M11 microscope and a Wild camera lucida.

Five museum specimens of *G. bullatarudis* from *Xiphophorus* hybrids mounted on a single slide (BM(NH) 1985. 3. 15) deposited by Harris (1986) were measured (Group V), however it was only possible to obtain all 13 sclerite measurements (see Table 2.1) from one specimen.

3.2.4 Multivariate statistical analysis

Consider a multidimensional graph in which each axis represents a single variable - for example, one of the 13 measurements illustrated in Fig 2.1. On such a graph each specimen may be represented by a single point. Mahalanobis distances (d^2 distances) are representative of the distances from a point corresponding to the multivariate sample mean and the points representing individuals plotted on the same multidimensional graph (Manly, 1986).

To illustrate the differences between the multivariate means of Groups I to IV [(*G. b* (*P. r*) 1), (*G. b* (*P. r*) 2), (*G. b* (*P. r*) 2 on *X*) and (*G. b* (*X*))] , the Minitab procedure, DISCRIMINANT (Minitab Inc., 1991) was used to find the d^2 distance from each specimen, as represented by the values of the thirteen measurements shown in Fig. 2.1, to the multivariate mean of each of Groups I to IV. Where two values were obtained for a single measurement of a single specimen, for example for each of the measurements of the paired hamuli, only one of these values, chosen arbitrarily, was used.

Using Cricket Graph (Cricket Software, 1986 - 1989), graphs were made plotting the d^2 distances from each specimen to the multivariate mean of each of two chosen groups, each axis representing the d^2 distance to the multivariate mean of one of those groups.

The procedure, DISCRIMINANT, then carried out a discriminant function analysis of these samples in which each individual specimen was allotted to the group whose multivariate mean it most resembled. By comparing the number of specimens from one group allotted by the discriminant function analysis to a second group and *vice versa*, it was possible to estimate how similar the two groups were.

3.3 Results

3.3.1 Scanning electron microscopy

Morphological differences were observed between the marginal hooks of specimens of *G. bullatarudis* from *P. reticulata* (Group II) and those of *G. bullatarudis* from *Xiphophorus* hybrids (Group IV). Marginal hooks of *G. bullatarudis* from *Xiphophorus* hybrids had a pronounced curvature near the point of the sickle whereas those from *P. reticulata* did not (see Figs. 3.1. and 3.2.).

No difference in the marginal hook morphology of specimens from Groups II and III (*G. b* (*P. r*) 2 on *X*) were observed. Differences between Groups II and IV were not therefore variations caused by the microenvironments of different host species.

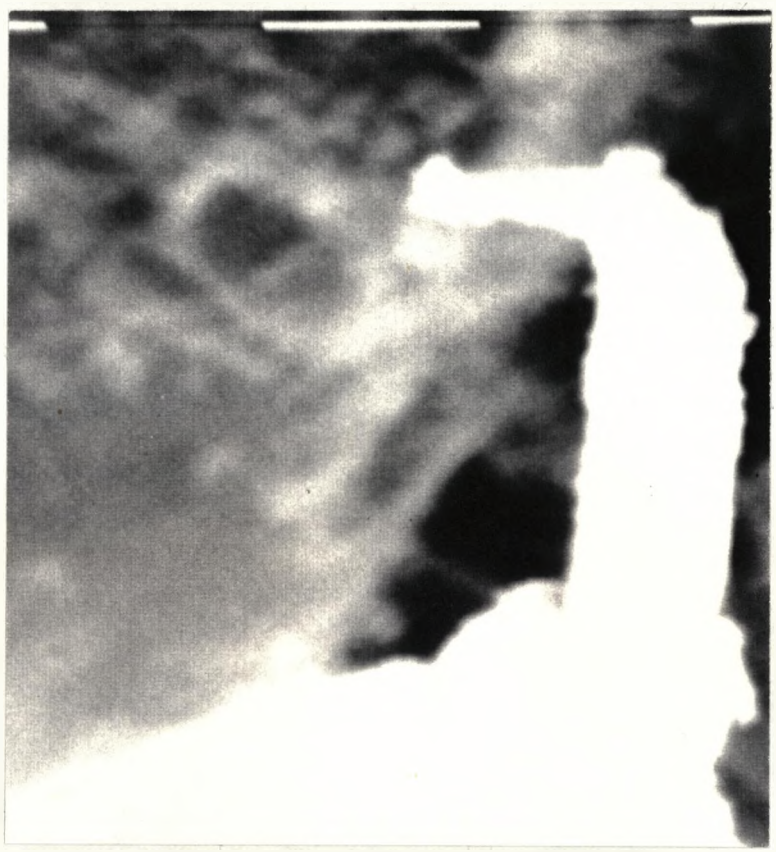


Fig. 3.1. Marginal hook of a *G. bullatarudis* (Group II) detached from a *P. reticulata.* . Note the absence of curvature near the point. (1 marker = 1μm)

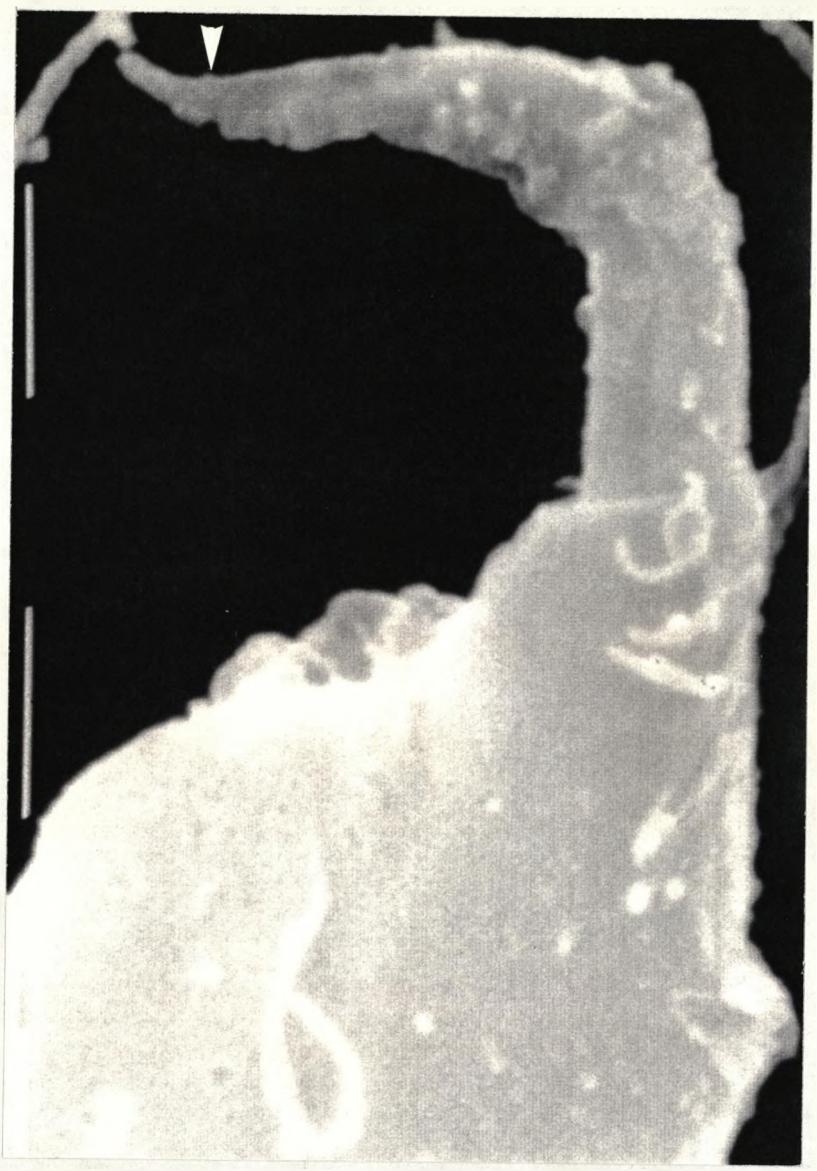


Fig. 3.2. Marginal hook of a *G. bullatarudis* (Group IV) detached from a *Xiphophorus* hybrid. Note the pronounced curvature near the point of the sickle (arrowed). (1 marker = 1μm)

3.3.2 Phase contrast microscopy

Measurements (Table 3.1) and observations (see Figs. 3.3, 3.4 and 3.5) showed the sclerites of *G. bullatarudis* from *Xiphophorus* hybrids (Groups IV and V) to be generally smaller than those of *G. bullatarudis* from *P. reticulata* (Groups I to III). They, however, had longer ventral bar processes, each pair, slightly curved and converging on each other (Figs. 3.4 and 3.5). The ventral bar processes of specimens from Groups I to III were shorter and had less curvature. The distal and proximal widths of marginal hooks from Groups IV and V were similar to each other whereas the marginal hook sickle distal widths from Groups I to III were distinctly shorter than their corresponding proximal widths.

No distinction could be made between specimens from Groups II (*G. b* (*P. r*) 2) and III (*G. b* (*P. r*) 2 on *X*).

Group I (*G. b* (*P. r*) 1) was morphologically indistinguishable from Groups II (*G. b* (*P. r*) 2) and III (*G. b* (*P. r*) 2 on *X*) and no average measurement differed by more than 1μm between any of these three groups - evidence that the differences between Groups I, II and III and Groups IV and V were more than could be accountable for by intra specific variation.

Measurements made	Group I *G. b (P. r) 1*			Group II *G. b (P. r) 2*			Group III *G. b (P. r) 2 on X*			Group IV *G. b (X)*			Group V *G. b (X) BM(NH)*		
	Average	Range	N	Average	Range	N	Average	Range	N	Average	Range	N	Average	Range	N
Hamulus total length.	55	50 - 59	74	55	49 - 58	60	55	50 - 57	60	50	47 - 52	78	48	47 - 49	5
Hamulus shaft length.	38	33 - 41	74	38	34 - 40	60	38	34 - 40	60	36	33 - 37	78	34	33 - 36	5
Hamulus root length.	17	14 - 18	74	17	11 - 18	60	17	16 - 18	60	15	13 - 16	78	14	13 - 15	5
Hamulus point length.	25	24 - 30	74	26	23 - 27	60	26	25 - 27	60	24	22 - 25	78	24	23 - 24	5
Ventral bar total length.	29	26 - 35	36	28	24 - 30	30	28	26 - 31	30	27	26 - 29	39	26	25 - 26	4
Ventral bar total width.	32	27 - 39	37	31	27 - 35	30	31	26 - 34	30	31	29 - 34	39	31	28 - 38	4
Ventral bar process length.	9	7 - 11	73	8	6 - 11	60	8	7 - 9	60	10	9 - 15	78	10	10 - 11	8
Ventral bar membrane length.	17	12 - 19	37	16	10 - 20	30	16	11 - 21	30	16	15 - 17	39	15	14 - 16	4
Marginal hooks total length.	25	24 - 27	68	26	25 - 28	60	26	25 - 28	60	24	21 - 25	76	23	23	1
Marginal hooks shaft length.	22	20 - 23	68	21	20 - 22	60	21	20 - 22	60	19	17 - 20	76	18	18	1
Marginal hooks sickle length.	6	5 - 7	68	6	6	60	6	5 - 6	60	5	5	76	5	5	1
Marginal hooks sickle distal width.	2	2	50	3	2 - 3	60	2	2 - 3	60	4	3 - 5	76	4	4	1
Marginal hooks sickle proximal width.	5	3 - 6	50	5	4 - 5	60	5	4 - 5	60	4	4 - 5	76	4	4	1
No. specimens examined	37			30			30			39			4		

Table 3.1. Measurements (in μm) of sclerites taken from samples of specimens from Groups I to V.

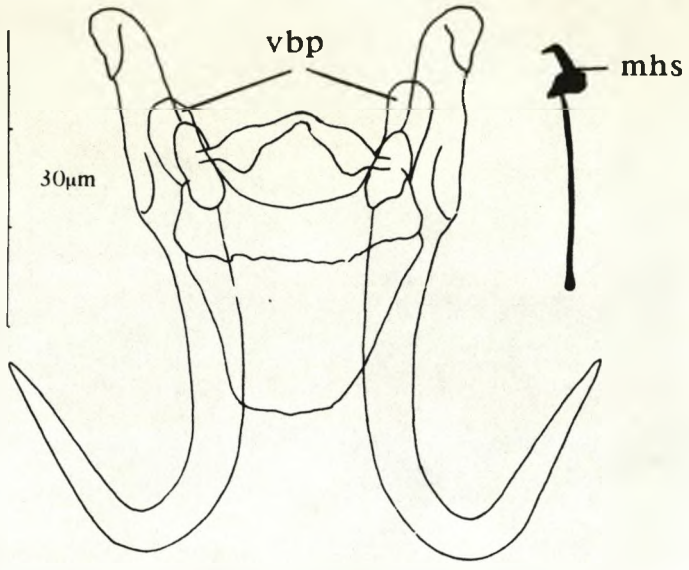


Fig. 3.3. Attachment sclerites of *G. bullatarudis* from a *Poecilia reticulata* (Group II). Note the shorter ventral bar processes (vbp) and different marginal hook sickle (mhs) morphology in comparison to sclerites in Figs. 3.4 and 3.5.

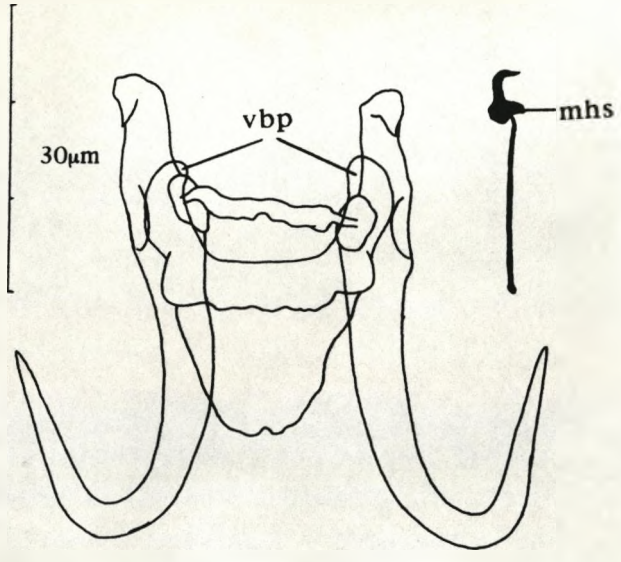


Fig. 3.4. Attachment sclerites of *G. bullatarudis* from a *Xiphophorus* hybrid (Group IV). Note the longer ventral bar processes (vbp) and different marginal hook sickle (mhs) morphology in comparison to sclerites in Fig. 3.3. Also note the similarity of these sclerites to those in Fig. 3.5.

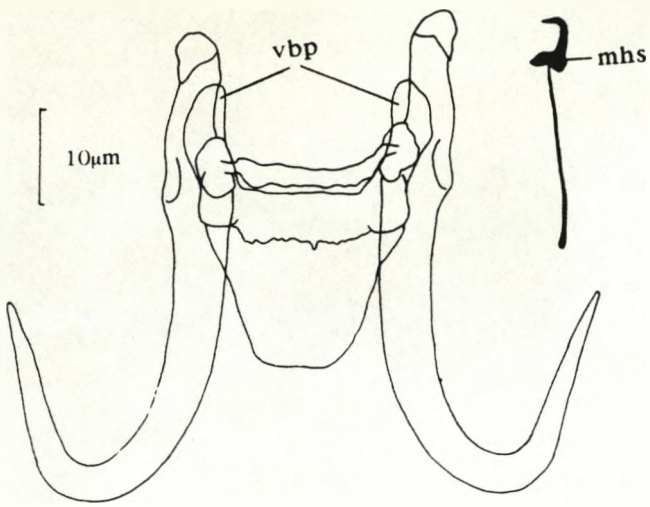


Fig. 3.5. Attachment sclerites of *G. bullatarudis* from a *Xiphophorus* hybrid (Group V, museum specimen deposited by Harris (1986), ref., BM(NH) 1985.3.15). Note the longer ventral bar processes (vbp) and different marginal hook sickle (mhs) morphology in comparison to sclerites in Fig. 3.3. Also note the similarity of these sclerites to those in Fig. 3.4.

3.3.3 Multivariate statistical analysis

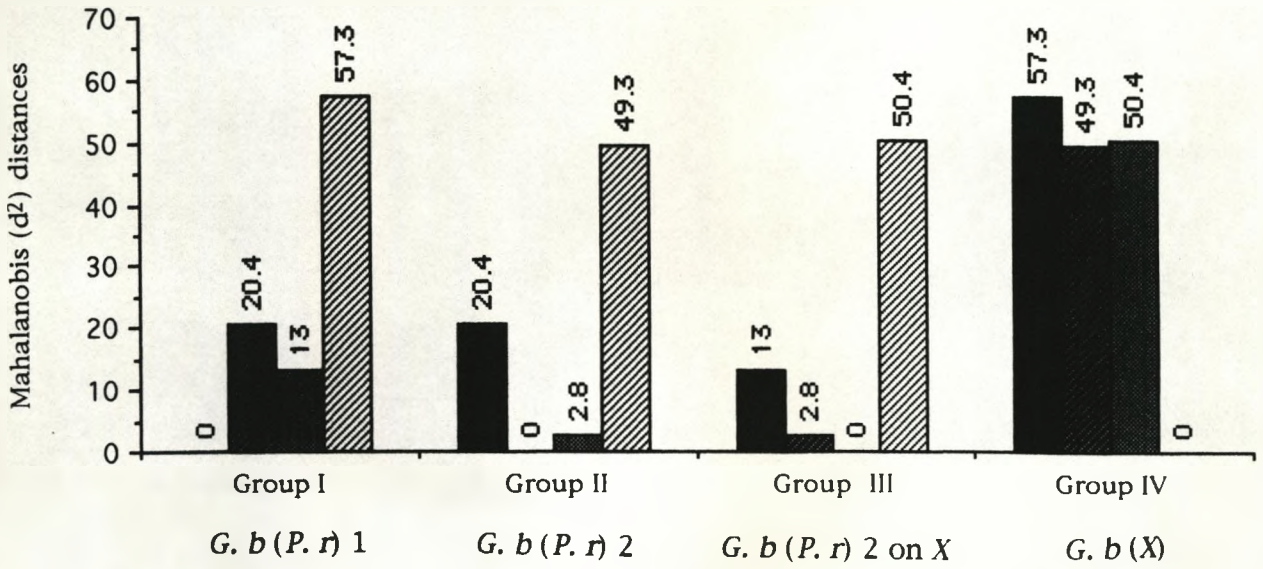
d^2 distances from observations to their group centres "should be approximately χ^2 variates with" degrees of freedom equal to the number of variables used in the analysis (Manly, 1986). As d^2 distances approximate to χ^2 variates with degrees of freedom equal to the number of variables (= measurements in the present context) used in the analysis (= 13), it can be said that any specimen which has a d^2 distance less than 22.36 (= the χ^2

variate where P = 0.05 with 13 degrees of freedom) from one of the group's multivariate means has a chance greater than 5% of actually belonging to a sample with the same multivariate mean and variance of that group.

The d^2 distances from the multivariate means of each of Groups I to IV from each other are shown in Fig 3.6. The multivariate means of Groups I to III [(*G. b* (*P. r*) 1), (*G. b* (*P. r*) 2) and (*G. b* (*P. r*) 2 on *X*)] are all very similar, in particular groups II and III. All of Groups I to III have multivariate means which differ markedly from the multivariate mean of Group IV (*G. b* (*X*)).

The lines a and b on Fig 3.7 represent the d^2 distances from the multivariate means of Groups II (*G. b* (*P. r*) 2) and IV (*G. b* (*X*)) respectively which correspond with the χ^2 variate where P = 0.05 with 13 degrees of freedom. Irrespective of their actual group, specimens whose points lie between line a and the y axis have a 5% or better probability of belonging to a sample with the same variance and multivariate mean as Group II and specimens whose points lie between line b and the x axis have a 5% or better probability of belonging to a sample with the same variance and multivariate mean as Group IV.

Fig 3.7 shows that specimens from Groups I to III [(*G. b* (*P. r*) 1), (*G. b* (*P. r*) 2) and (*G. b* (*P. r*) 2 on *X*)] were more similar to each other than to specimens from Group IV (*G. b* (*X*)). Considerable variation was observed within all samples.



Groups from whose multivariate means the corresponding Mahalanobis distances are given (see legend)

Fig. 3.6. Mahalanobis (d^2) distances from the multivariate means of each of Groups I to IV to each other. ■, d^2 distance from the multivariate mean of Group I (*G. b* (*P. r*) 1), ■ d^2 distance from the multivariate mean of Group II (*G. b* (*P. r*) 2), ■ d^2 distance from the multivariate mean of Group III (*G. b* (*P. r*) 2 on *X*) and ▨ d^2 distance from the multivariate mean of Group IV (*G. b* (*X*)).

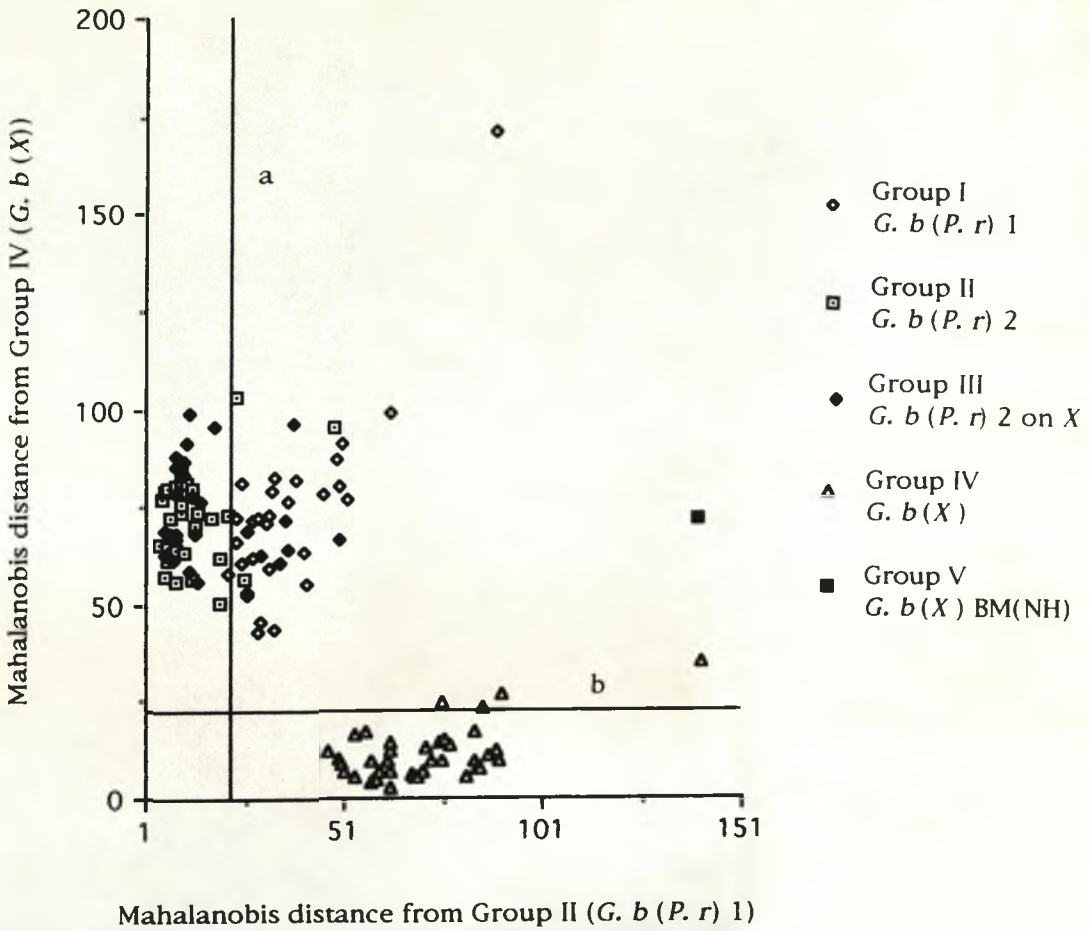


Fig. 3.7. Mahalanobis distances of specimens' sclerite dimensions (Groups I to V) from the multivariate means of Group II (*G. b* (*P. r*) 2), x axis, and Group IV (*G. b* (*X*)), y axis. Specimens plotted between line a and the y axis have a greater than approximately 5% probability of actually belonging to Group II and specimens plotted between line b and the x axis have a greater than approximately 5% probability of actually belonging to Group IV (see section 3.3.3).

Groups as predicted by discriminant function analysis	Actual groups from specimens came			
	Group I *G. b (P. r) 1*	Group II *G. b (P. r) 2*	Group III *G. b (P. r) 2 on .*	Group IV *G. b (X)*
Group I	26	0	1	0
Group II	0	25	8	0
Group III	1	5	21	0
Group IV	0	0	0	39
Total	27	30	30	39
No. correct	26	25	21	39
Prop. correct	0.963	0.833	0.7	1

Table 3.2. Results of the discriminant function analysis. The numbers of specimens in true groups (columns) are shown corresponding to the numbers of specimens in each group as predicted by the discriminant function analysis (rows). For example, Group III consisted of 30 specimens. However, the discriminant function analysis of sclerite measurements predicted that 8 of those specimens were more likely to belong to Group II and that 1 of those specimens was more similar likely to belong to Group I than to Group III.

Only one of the five museum specimens was included in this analysis - it was the only specimen from which all 13 attachment sclerite measurements could be accurately taken. It was the only specimen which was flattened sufficiently to present one marginal hook in absolute profile. It was shown morphometrically to be much more similar to Group IV (*G. b (X)*) than to any of the other groups, although its similarity to the multivariate mean of this group was not significant.

3.3.4. A re-description of *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986)

Host: *Xiphophorus* sp.

Host-site: External surface.

Description: Body length of moderately flattened, moderately contracted specimens, 200 - 400μm (a very small *Gyrodactylus* species according to the scheme of Harris (1985)).

Flattened specimens mounted in ammonium picrate-glycerin for sclerite examination had a pharynx of average length of 41μm (SD 5.2μm) and average width of 37μm (SD 4.3μm). The anterior and posterior pharyngeal bulbs (Kritsky, 1970) are of approximately equal width, although the width of the posterior part was greater for a larger number of specimens. The anterior of the pharynx consists of eight cells which are fused to each other along their lateral borders. They can be protruded as a barrel shaped unit. Lines can be seen delineating ridges running postero-anteriorly on the interior surfaces of these cells.

The cirrus consists of one large spine and a single row of 4 - 7 small spines (Fig. 3.8). The large spine has lateral strengthening elements with lateral and posterior roots which do not join to from a lateral ring (see section

2.3.2 for explanation of nomenclature).

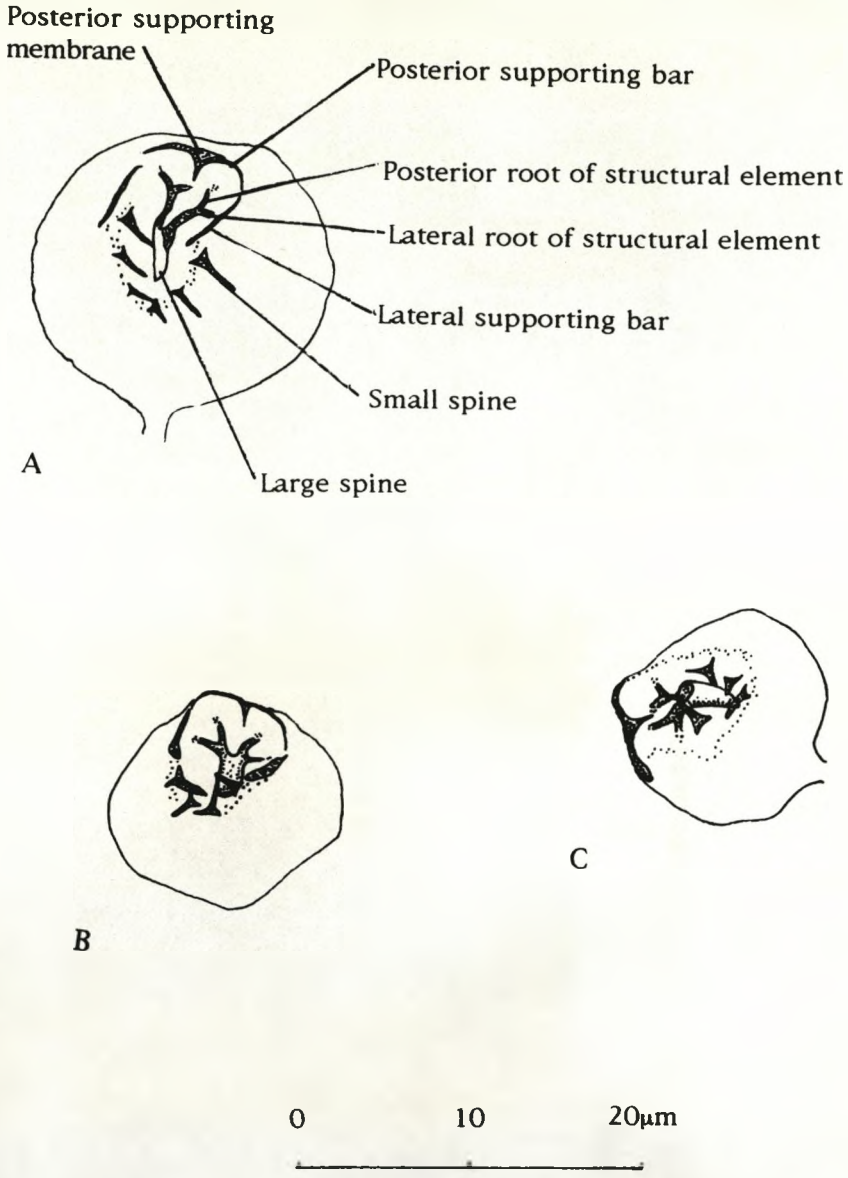


Fig. 3.8A, B and C. Cirrus armature of *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) fixed and mounted in ammonium picrate-glycerin, viewed with a phase contrast microscope and drawn with the aid of a camera lucida (see section 2.3.2 for an explanation of the nomenclature).

The hamuli are 47 - 52 μm in length with shafts and roots 33 - 37 μm and 13 - 16 μm long, respectively. The dorsal bar has a median notch, though this was not obvious on all specimens. Of the attachment sclerites (see Figs. 3.4 and 3.5), the ventral bars and marginal hooks are the most distinctive. The ventral bar processes are relatively long (9 - 15 μm), slightly curved and converge on each other distally. The marginal hooks have distal and proximal widths which are approximately equal. The marginal hooks also have a curve near the point of the sickle blade which is only just visible using the phase contrast microscope and causes the point to be directed slightly distally, away from the main body of the haptor (Figs. 3.1, 3.4 and 3.5).

3.4 Discussion

3.4.1 Scanning electron microscopy

After examining "more than 85" *Gyrodactylus* species Malmberg (1970) found the marginal hook "to be the most decisive body part in the determining of *Gyrodactylus* species".

The difference in the blade morphology of the marginal hooks of specimens of *G. bullatarudis* from *P. reticulata* as compared with *G. bullatarudis* from *Xiphophorus* hybrids is striking (Figs. 3.1. and 3.2.).

As this pronounced difference in marginal hook morphology was exhibited by both Groups II (*G. b* (*P. r*) 2) and III (*G. b* (*P. r*) 2 on *X*) it was not caused by the different microenvironments provided by different host species. Harris's (1986) hypothesis that *G. bullatarudis* consists of morphologically indistinguishable strains, each infecting different species of fish was rejected and it was proposed that Groups II (*G. b* (*P. r*) 2) and III (*G. b* (*P. r*)

2 on *X*) were of a different *Gyrodactylus* species to Group IV (*G. b* (*X*)).

3.4.2 Phase contrast microscopy

The differences in marginal hook morphology illustrated by SEM could only be observed using phase contrast light microscopy when special attention was paid to particularly well prepared specimens mounted in ammonium picrate-glycerin and would have been overlooked without the results of the SEM investigation. This was not surprising given that the maximum resolution of a light microscope is 0.2 - 0.5μm and that of an SEM is about 2nm (Alberts *et al.*, 1983) and the width of a marginal hook is less than 0.5μm near its point. So this character difference was not useful in distinguishing specimens of Groups II (*G. b* (*P. r*) 2) or III (*G. b* (*P. r*) 2 on *X*) from specimens of Group IV (*G. b* (*X*)) by phase contrast microscopy. However, differences in marginal hook proximal and distal widths and differences in the lengths and morphology of the ventral bar processes were observed using the phase contrast light microscope and could be used to distinguish the *G. bullatarudis* from *P. reticulata* from the *G. bullatarudis* from *Xiphophorus* hybrids (see section 3.3.2).

Having established criteria for separating Groups II (*G. b* (*P. r*) 2) and III (*G. b* (*P. r*) 2 on *X*) from Group IV (*G. b* (*X*)) using phase contrast microscopy, it was then possible to confirm the identification of specimens from Group IV as of the same taxa as the museum specimens deposited by Harris (1986) (see Table 3.1 and Figs. 3.4 and 3.5).

On the strength of the above findings, it is proposed that *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) from *Xiphophorus* hybrids is not *G. bullatarudis* Turnbull, 1956 *sensu stricto*.

It was clear that these two taxa could be readily confused when using

standard taxonomic procedures (Malmberg, 1970) for identification. The differences in the ventral bar process morphology are difficult to establish without direct comparison of specimens from both taxa (but see Chapter 5 where a staining technique is described which improves the rendition of *Gyrodactylus* haptoral bars). Differences in measurements of marginal hooks which may also differentiate the two taxa are very small < 4μm and may be unnoticed or untrusted by taxonomists. Furthermore, marginal hooks are notoriously difficult to prepare adequately flattened to give a good profile for measurement (see Ergens, 1977; Harris, 1986).

The difficulties encountered in differentiating between the two taxa when using phase contrast microscopy is in contrast to the ease and certainty by which this was done using the SEM, even though, using the latter method, only the marginal hook blades were observed with clarity. There is a distinct advantage to the SEM, when its use is possible, as a tool for taxonomic comparisons of *Gyrodactylus* species. Since the present study was completed, a paper was published, Shinn *et al.* (1993), which describes a method for extraction of the attachment sclerites of gyrodactylids for taxonomic observation using the SEM. The power of this method as an aid to species diagnosis is recognised, and is advocated, when possible, for future use.

3.4.3 Multivariate statistical analysis

The calculation and plotting of d^2 distances was a useful method to show quantitatively how different the various groups were from each other using haptoral sclerite measurements.

The results from the multivariate analysis showed Groups II (*G. b* (*P. r*) 2) and III (*G. b* (*P. r*) 2 on *X*) to be not significantly different from each other.

The discriminant function analysis showed a high proportion of specimens from Group II (17%) being classified as more likely to belong to Group III and *vice versa* (27% of Group III) (see Table 3.2). Group I (*G. b* (*P. r*) 1) was very similar to Groups II and III, however, the analysis showed these specimens to be less similar to Groups II and III than Groups II and III were to each other. The dissimilarity between Groups II and III and Group I was considered no greater than may be expected due to intra specific variation between two isolated populations of the same species. This dissimilarity is not nearly as great as that exhibited between Groups I to III and Group IV (*G. b* (*X*)). Therefore, using morphometric criteria, it was shown that the dissimilarity of Group IV to Groups I to III was greater than may be expected from intraspecific variation alone.

The one museum specimen (Harris, 1986) included in the analysis was shown unlikely to belong to any of the other groups, however, it was a great deal more similar to specimens of Group IV (*G. b* (*X*)) (Fig 3.7.), as would be expected from inspection of Table 3.1. and Figs. 3.3 - 3.5. It is proposed that the dissimilarity between this museum specimen and Group IV is due to intraspecific variation.

3.4.4 Conclusions from the morphology and morphometric comparisons

Evidence from all three of the comparative studies leads to the following conclusions:

1. *G. bullatarudis* Turnbull, 1956 from *P. reticulata* and *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) from *Xiphophorus* are morphologically distinguishable from each other using the standard protocol for *Gyrodactylus* identification (Malmberg, 1970).

2. These morphological differences are not due to the different

microenvironments found on different species of fish host.

3. The morphological differences, in particular the size and shapes of the marginal hooks, are significant enough to establish that *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) from *Xiphophorus* hybrids is a separate species to *G. bullatarudis sensu stricto.* The identity of *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) is discussed in the next section.

3.4.5 Consequences and implications of the establishment of *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) as a separate species to *G. bullatarudis sensu stricto*

A description of the excretory system of *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) was given in Harris (1986). He showed that this species was of the *G.* (*Mesonephrotus*) subgenus and, using this description and the characteristics of the attachment sclerites, showed it to belong to the *G. arcuatus* species group (Malmberg, 1970). The excretory system of *G. bullatarudis sensu stricto* remains to be described. However, the close morphological resemblance of *G. bullatarudis sensu stricto* to *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) indicates that these two taxa are closely related and that they are both mesonephrotids of the *G. arcuatus* species group.

One of the species of *Gyrodactylus* most resembling *G. bullatarudis sensu stricto* and *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) is *G. gambusiae* Rogers and Wellborn, 1965 from *Gambusia affinis.* Indeed, Harris (1986) suggests that *G. gambusiae* may be a synonym for *G. bullatarudis.* This is, however, unlikely given that the marginal hook sickle length of *G. gambusiae* was 10 μm "from the tip of the base to the most distant point of curvature" (Rogers and Wellborn, 1965) - approximately double the measurement recorded in this study for both *G. bullatarudis*

sensu stricto and *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986). Marginal hook sickle length was found to be one of the most consistent and less variable sclerite measurements used in the present study. Furthermore, the ventral bar processes of the ventral bar drawn of *G. gambusiae* by Rogers and Wellborn (1965) are straight and do not curve inward on each other at all - this contrasts with the present study's observations of both *G. bullatarudis sensu stricto* and *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986).

Kritsky and Fritts (1970) recorded *G. bullatarudis* on *Poecilia sphenops* from Costa Rica. Owing to the results of the present study, the question arises as to whether this identification was of *G. bullatarudis sensu stricto* or *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986). Measurements from Kritsky and Fritts (1970) of 21 specimens and Harris (1986) of one specimen collected by Kritsky and Fritts (1970), particularly those of the hamulus total length, 51-59μm and 51.6μm respectively, and the marginal hook sickle distal and proximal widths taken by Harris (1986) of 2.4 and 3.6μm respectively (in the present study these measurements would have been rounded to 2 and 4μm) suggest that the specimens concerned were indeed *G. bullatarudis sensu stricto.* The investigation of the cirral bulb of *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) (see Fig. 3.8)showed that the large spine of the cirral bulb had a marked difference in appearance to that of *G. bullatarudis sensu stricto* (Figs. 2.5C, D and E) especially in the former having an absence of lateral rings and an absence of an apparent connecting bar associated with the main structural elements of the large spine (see section 2.3.2 for explanation of nomenclature). The cirral bulb illustrated by Kritsky and Fritts (1970) (see Fig. 2.7A of Chapter 2) shows structures apparently similar to the lateral rings and connecting bar of the main structural elements of the large cirrus spine of *G. bullatarudis* shown in Fig. 2.5C - further evidence that the *G. bullatarudis* from *P. sphenops* (Kritsky and Fritts, 1970) was indeed *G. bullatarudis sensu stricto.*

sensu stricto and *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986). Marginal hook sickle length was found to be one of the most consistent and less variable sclerite measurements used in the present study. Furthermore, the ventral bar processes of the ventral bar drawn of *G. gambusiae* by Rogers and Wellborn (1965) are straight and do not curve inward on each other at all - this contrasts with the present study's observations of both *G. bullatarudis sensu stricto* and *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986).

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G. bullatarudis Turnbull, 1956 *sensu* Harris (1986) may be a synonym for *G. rasini* Lucky, 1973, first described from *X. helleri* which had been bred in Czechoslovakia (Lucky, 1973). The measurements of Lucky (1973) of *G. rasini* from the gills of *X. helleri* are very similar to those of Harris (1986) of *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) and those of the present study (see Table 3.3). Their synonymity would be more likely were it not for the difference in site specificity recorded for the two taxa. However, host-site specificity may not be used to differentiate *Gyrodactylus* species. For example, Malmberg (1970) comments that *G. arcuatus* Bychowsky *sensu* Bychowsky and Poljansky parasitising *Gasterosteus aculeatus* prefers the skin and fins in the warmer parts of the year and the inside of the mouth in the cooler. Harris (1993) comments that "*G. arcuatus* [on *G. aculeatus*] is generally restricted to the gill chamber of its host in southern England, although it can infect the entire body of its host in Scandinavia (Malmberg, 1970)".

Lucky's drawings of the haptoral and cirrus sclerites of *G. rasini* are poor, making qualitative morphological comparisons with sclerites of specimens examined in this study impossible. So, in conclusion, *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) is tentatively identified as *G. rasini* Lucky, 1973[1] . There is, however, a need for close comparison of archival specimens of *G. rasini* with specimens from the present study in order to confirm this identification.

1
Voucher specimens of the *Gyrodactylus* I obtained from *Xiphophorus* hybrids have been deposited in the Natural History Museum, London, as *G. rasini*, reference number, BM(NH) 1994.11.24.13-18.

Investigation	Lucky (1973)	Harris (1986)		Present study	
Parasite species	*G. rasini*	*"G. bullatarudis"*		*"G. bullatarudis"*	
Host	*X. helleri*	*Xiphophorus* hybrids		*Xiphophorus* hybrids	
Host origin	Aquarium fish	Aquarium fish		Aquarium fish	
	Czechoslovakia	Singapore		Singapore	
	Range	Average	Range	Average	Range
Hamulus total length.	45 - 50	47.4	46-50	50	47 - 52
Hamulus shaft length.	35 - 38	38.2	36-39	36	33 - 37
Hamulus root length.	12 - 15	11.1	9-13	15	13 - 16
Hamulus point length.	20 - 23	21.1	18-24	24	22 - 25
Ventral bar total length.	21 - 25	21.9	20-25	27	26 - 29
Ventral bar total width.		29.2	26-32	31	29 - 34
Ventral bar process length.	11 - 13	9.4	7-12	10	9 - 15
Ventral bar membrane length.	12 - 17	13.8	12-15	16	15 - 17
Marginal hooks total length.	16 - 23	23.2	21-25	24	21 - 25
Marginal hooks shaft length.	13 - 18	18.6	17-20	19	17 - 20
Marginal hooks sickle length.	3 - 5	4.7	4-5	5	-
Marginal hooks sickle distal width.	-	3.7	-	4	3 - 5
Marginal hooks sickle proximal width.	-	3.8	-	4	4 - 5
No. specimens examined	Not mentioned	13		30	

Table 3.3. Haptoral sclerite measurements (in μm) of *G. rasini* Lucky, 1973 (Lucky, 1973), *G. bullatarudis* Turnbull, 1956 *sensu* Harris, 1986 (Harris, 1986) and parasites from this study identified as *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986).

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CHAPTER 4

4. The effects of temperature and host species on the opisthaptor sclerite morphometrics of *G. bullatarudis* and *G. turnbulli.*

4.1 Introduction

Many studies have shown a relationship between temperature and opisthaptor sclerite size of species of *Gyrodactylus* and other Monogenea. In the overwhelming majority of experimental (Ergens and Gelnar, 1985; Mo, 1991b and Tomnatik, 1990) and field observations (Ergens, 1976; Ergens, 1981; Ergens, 1983; Ergens, 1991; Kulemina, 1976; Malmberg, 1970; Mo, 1991a, b and c, 1993) sclerites are smaller at higher and larger at lower temperatures. In only one instance has a contrary observation been observed, reported of *G. osoblahensis* on *Leuciscus cephalus* and *L. leuciscus* (Ergens, 1988).

All studies so far have been of species parasitic on fish native to a temperate climate. The first aim of this study was to test whether a similar change in sclerite size of *G. bullatarudis* and *G. turnbulli* occurs in relation to temperature.

There have been reports of differences in sclerite morphometrics of single *Gyrodactylus* species on different species of hosts (Mo, 1991a; Mo, 1993; Prost, 1991). The second aim of this chapter was to examine whether changes in opisthaptor sclerite dimensions could be observed in *G.*

bullatarudis which had been maintained on *Xiphophorus* hybrids under experimental conditions.

Although previously it has been alluded that variation in opisthaptor sclerite morphometrics is proportionate to the genetic variation within a population of *Gyrodactylus*, there is no direct evidence for this. For example, Harris (1993) relates variation in hamulus dimensions to the frequency of sexual reproduction observed in a variety of different gyrodactylids. The third aim of this study was to test this assumption by comparing infrapopulations of *G. bullatarudis* and *G. turnbulli* originating from more than one *Gyrodactylus* with infrapopulations originating from a single *Gyrodactylus*.

4.2. Materials and methods

4.2.1. Investigation of the effect of temperature on sclerite morphometrics

Sclerites from both species kept at two temperatures were measured. The first temperature was 25±1°C - the temperature at which all stocks of fish and *Gyrodactylus* were maintained in a controlled environment room (referred to hereafter as the c. e. room). The other temperature (19±1°C) was maintained in an incubator with the same 12: 12 light: dark regime as the c. e. room but at a temperature at which the *Gyrodactylus* could be expected to produce a large difference in sclerite size corresponding to the findings of most of the works quoted in the opening paragraph of the introduction (section 4.1). A detailed explanation for this choice of the second temperature is given in the Discussion, section 4.4.6.

4.2.2. Origin and maintenance of the groups of *G. bullatarudis*

The *G. bullatarudis* used in this work were from the same population as Groups II (*G. b* (*P. r*) 2) and III (*G. b* (*P. r*) 2 on *X*) in Chapter 3, see section 3.2.1.

Four infected fish of standard length of 10 to 15mm were removed from a container of 500ml of standing tap water (see Chapter 2, section 2.2.2). Both pairs of infected fish were placed in two similar containers of the same size. Three uninfected fish of similar length were added to each container. One of these containers was placed in an incubator set to keep water at 18 to 20°C and the other remained in the c. e. room where water was kept between 24 and 26°C.

After 6 days, all the fish remaining in the container in the c. e. room (two fish had died) were heavily infected with *G. bullatarudis*. One of these fish was killed as described in Chapter 2, section 2.2.3 and was placed in a separate container with 5 naive laboratory bred *Xiphophorus* hybrids. This dead *P. reticulata* was removed after 24h by which time all the *Xiphophorus* were infected with at least one *G. bullatarudis*. Another *P. reticulata* from the container which remained in the c. e. room was killed. Individual *Gyrodactylus* from this fish were placed, one on each of four, naive laboratory bred *P. reticulata* which were placed in separate containers in the c. e. room. A detailed description of the method of the experimental infection of *P. reticulata* with individual *Gyrodactylus* is given in Chapter 8, section 8.2.2. The remaining *G. bullatarudis* from this second dead fish were prepared for sclerite investigation as described in Chapter 3, section 3.2.3. These *Gyrodactylus* specimens are referred to hereafter as group Gb h25 (= *G. bullatarudis*, heterogeneous-culture kept at an approximate average water temperature of 25°C), they have been referred to previously as Group II (*G. b* (*P. r*) 2) in Chapter 3.

The maintenance of the infections of *Xiphophorus* hybrids has previously been described - see entry for Group III (*G. b* (*P. r*) 2 on *X*) in Chapter 3, section 3.2.1. After 11 days of maintenance, one fish was removed from this culture and killed. Specimens of *G. bullatarudis* from this fish were prepared as described in Chapter 3, section 3.2.3. The resulting *Gyrodactylus* specimens are referred to hereafter as group Gb X h25 (= *G. bullatarudis* on *Xiphophorus* hybrids, heterogeneous-culture kept at an approximate average water temperature of 25°C), they have been referred to previously as Group III (*G. b* (*P. r*) 2 on *X*) in Chapter 3.

13 days after its initial infection, one of the fish which had been infected with a single *G. bullatarudis* had accrued a large infrapopulation. This fish was killed. Specimens of *G. bullatarudis* from this fish were prepared as described in Chapter 3, section 3.2.3. The resulting *Gyrodactylus* specimens are referred to hereafter as group Gb mono25 (= *G. bullatarudis*, mono-culture kept at an approximate average water temperature of 25°C).

Meanwhile, the infections of fish which had been placed in the incubator were maintained by checking the cultures every six to eight days. Naive fish were added following removal of dead fish or fish which had recovered and become refractory to reinfection (see Scott, 1985). These naive laboratory bred *P. reticulata* had been placed in the incubator for at least 12h to avoid overly stressing them with a sudden temperature change on addition to the *G. bullatarudis* culture. After 14 days in the incubator, one heavily infected fish was removed from the container, killed and *G. bullatarudis* specimens were prepared from it using the method described in Chapter 3, section 3.2.3. The resulting *Gyrodactylus* specimens are referred to hereafter as group Gb h19 (= *G. bullatarudis*, heterogeneous-

culture kept at an approximate average water temperature of 19°C).

A summary flow diagram of the preparation of the groups of *G. bullatarudis* is given in Fig. 4.1.

4.2.3. Origin of the groups of *G. turnbulli*

The *G. turnbulli* used in this investigation were those originating from the population used in the initial taxonomic investigation described in Chapter 2. Their method of maintenance was, however, changed. They were now maintained on approximately 30 naive laboratory bred guppies of about 15mm standard length, kept in a 12l aerated aquarium. To maintain the infestation, dead or autopsied fish were replaced or at least 5 uninfected fish were added per week if no fish needed replacing.

Two infected fish of standard length up to 15mm were removed from the *G. turnbulli* maintenance aquarium. They were placed in two different containers, each with four uninfected fish in 500 ml of standing tap water. One of these containers was placed in an incubator at 18 to 20°C and the other remained in the c. e. room where water was kept between 24 and 26°C.

After 6 days, all the fish in the container remaining in the c. e. room were heavily infected with *G. turnbulli.* A *P. reticulata* from this container was killed. Individual *Gyrodactylus* from this fish were placed, one on each of five naive laboratory bred *P. reticulata* of standard length 20 to 30mm. These fish were isolated in separate containers in the c. e. room (a detailed description of the method of the experimental infection of *P. reticulata* with *Gyrodactylus* is given in Chapter 8, section 8.2.2). The remaining *G. turnbulli* from the dead fish were prepared for sclerite investigation as

described in Chapter 3, section 3.2.3. The resulting *Gyrodactylus* specimens are referred to hereafter as group Gt h25 (= *G. turnbulli*, heterogeneous-culture kept at an approximate average water temperature of 25°C).

14 days after its initial infection, one of the fish which had been infected with a single *G. turnbulli* had accrued a large infrapopulation. This fish was killed and *G. turnbulli* specimens were removed from it and prepared for sclerite examination using the method described in Chapter 3, section 3.2.3. The resulting *Gyrodactylus* specimens are referred to hereafter as group Gt mono25 (= *G. turnbulli*, mono-culture kept at an approximate average water temperature of 25°C).

Infections of fish which had been placed in the incubator were maintained as described earlier for the maintenance of the *G. bullatarudis* infections which were used to provide specimens for group Gb h19. After 9 days following placement of the culture in the incubator, one heavily infected fish was removed from the container and killed. *G. turnbulli* specimens were prepared from this fish using the method described in Chapter 3, section 3.2.3. The resulting *Gyrodactylus* specimens shall be referred to hereafter as group Gt h19 (= *G. turnbulli*, heterogeneous-culture kept at an approximate average water temperature of 19°C).

A summary flow diagram of the preparation of the groups of *G. turnbulli* is given in Fig. 4.2.

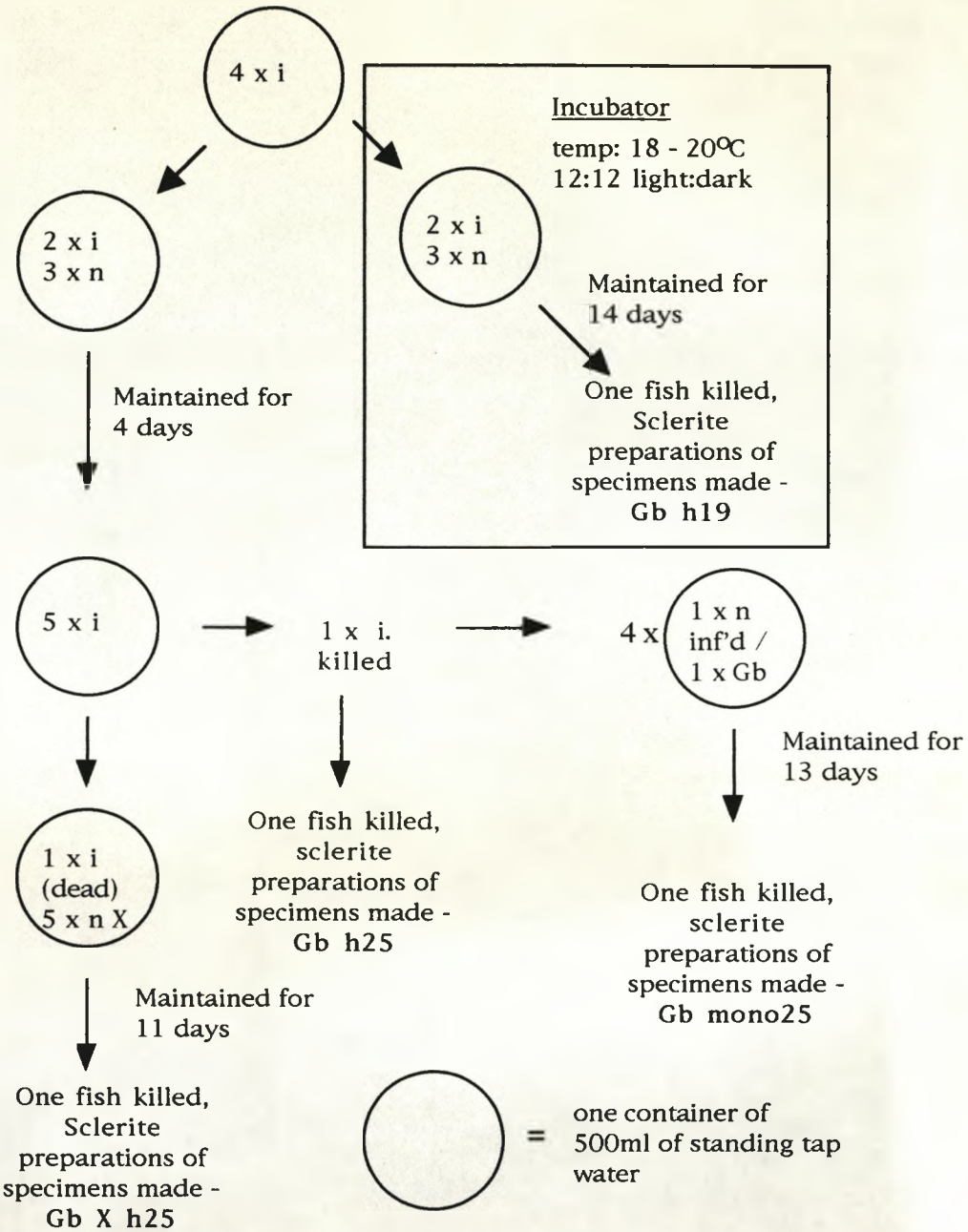


Fig. 4.1. Flow diagram summarising the maintenance of the *G. bullatarudis* groups. All groups except for Gb h19 were maintained in a controlled environment room in which the water temperature was kept between 24 and 26°C with a 12:12 light: dark regime. i = infected fish, n = naive laboratory bred fish. n X = naive laboratory bred *Xiphophorus* hybrids of standard length between 10 and 20mm (all other fish were *Poecilia reticulata* of standard length 10 to 15mm except for those infected with monocultures which were between 20 and 30mm standard length).

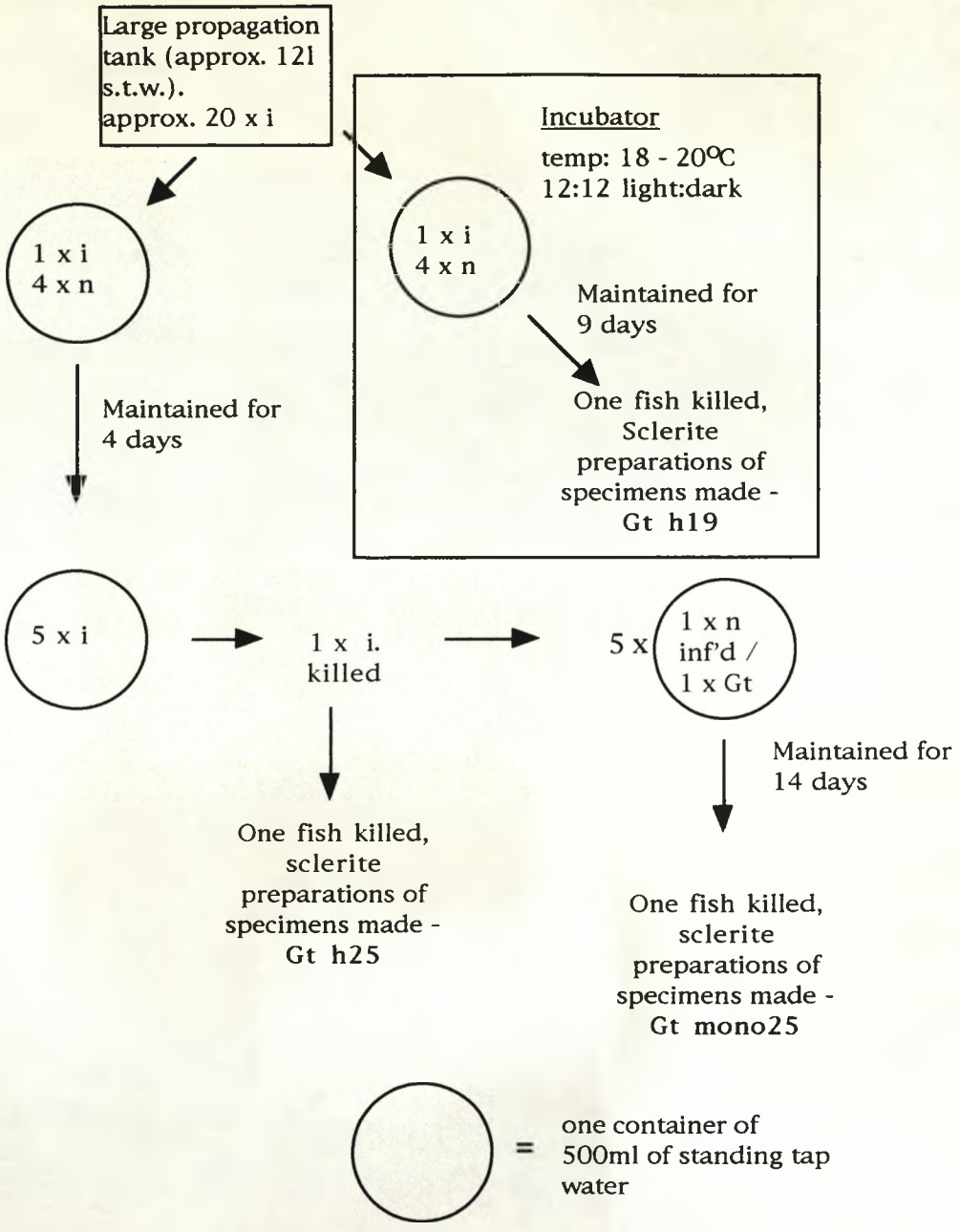


Fig. 4.2. Flow diagram summarising the maintenance of the *G. turnbulli* groups. All groups except for Gt h19 were maintained in a controlled environment room in which the water temperature was kept between 24 and 26°C with a 12:12 light: dark regime. i = infected fish, n = naive laboratory bred fish. All fish were *Poecilia reticulata* of standard length up to 15mm except for those infected with monocultures which were between 20 and 30mm standard length.

4.2.4 Measurement of sclerites and analysis of sclerite measurements

Sclerites of 30 specimens from each group were measured as described in Chapter 2, section 2.2.3. The measurements shown in Fig. 2.1 and the abbreviations for those measurements used in this chapter are shown in Table 4.1.

Measurement	Abbreviation
Hamulus total length	htl
Hamulus shaft length	hshl
Hamulus root length	hrl
Hamulus point length	hpl
Ventral bar total length	vbtl
Ventral bar total width	vbtw
Ventral bar process length	vbpl
Ventral bar membrane length	vbml
Marginal hook total length	mhtl
Marginal hook shaft length	mhshl
Marginal hook sickle length	mhsil
Marginal hook distal width	mhsidw
Marginal hook proximal width	mhsipw

Table 4.1. Table showing the abbreviations for the sclerite measurements illustrated in Fig. 2.1 used in this investigation. These abbreviations have been used in this chapter in the principal component formulae (section 4.3.2) and to annotate diagrams and tables.

Univariate comparative analyses were carried out using InStat 2.01 (Graph Pad Software, 1993) and multivariate analyses using Mac Minitab (Minitab inc., 1991). Graphs were produced using Cricket Graph 1.3.2 (1986 - 1989).

As the conversion factors for the ocular graticule values were the same for the groups of measurements taken from the samples compared with each other (for all *G. bullatarudis* groups 90μm = 9.80 ocular graticule units and for *G. turnbulli* 90μm = 9.85 ocular graticule units) it was decided to use them in the analysis of comparisons between samples. This was done to avoid the accumulation of errors by the numerical rounding of conversions from ocular graticule units to μm. For example, a graticule measurement of 0.6 or 0.7 would both be converted to 6μm when rounding to the nearest μm when 90μm = 9.80 ocular graticule units.

Where two values were obtained from each specimen for measurements from paired organs, for example from measurement of the hamuli, only one value per specimen was used in the comparative analysis. Each one of these values was selected arbitrarily. This was so that all the values for each variable would be independent of each other.

4.2.5. Test for normal distribution and transformation of not-normal data sets

Each measurement from each sample was tested for a normal distribution using Mac Minitab in the following way.

The command NSCORES was used to produce normal probability values for each value in a sample. This command first considers the size of the sample involved. It then ranks the numbers and computes corresponding values (normal scores) such that the normal score for the lowest number in the sample is the lowest number expected in a normally distributed sample of

the same size. The normal score for the next highest number is the second highest expected number and so on (Minitab inc., 1991). A plot of sample values against their theoretical values yields straight line if the distribution of the sample values is normal. The correlation of sample values against their corresponding normal score values is a "powerful test for normality, essentially equivalent to the Shapiro-Wilk test" (Mac Minitab Manual, Minitab inc., 1991). Thus the command CORRELATION was used to test this association. The resultant correlation coefficients were compared with values given in Exhibit 5.1 "Correlation Test for Normality" (Mac Minitab Manual, Minitab inc., 1991). If the correlation coefficients fell below the value given for N=30, P=0.05, it was assumed that there was a less than 5% chance of the sample being normally distributed and the hypothesis of normality was rejected. All samples of raw measurements were examined in this way. The results are shown in Tables 4.2 and 4.3.

		htl	hshl	hrl	hpl	vbtl	vbtw	vbpl	vbml	mhtl	mhshl	mhsil	mhsidw	mhsipw
Gb h25	Mean	55	38	17	26	28	30	8	16	26	21	6	2	4
	Standard deviation	1.9	1.2	1.4	0.6	1.4	2.2	1.0	1.9	0.6	0.7	0.0	0.5	0.3
	Range	49 - 58	35 - 40	11 - 18	24 - 27	24 - 30	23 - 35	6 - 11	10 - 20	25 - 28	20 - 22	6	2 - 3	4 - 5
		*		*			*							
Gb h19	Mean	53	37	16	25	28	30	8	16	26	21	6	2	5
	Standard deviation	1.8	1.3	1.3	0.8	1.5	1.4	0.8	1.1	0.5	0.4	0.2	0.4	0.2
	Range	50 - 56	34 - 40	13 - 18	24 - 27	24 - 30	26 - 32	6 - 9	14 - 18	25	20 - 22	6	2 - 3	4 - 5
							*							
Gb mono25	Mean	54	37	17	25	28	31	9	16	26	21	6	2	5
	Standard deviation	1.6	1.1	0.9	0.7	0.9	1.2	0.7	1.0	0.5	0.6	0.0	0.5	0.2
	Range	51 - 57	35 - 40	15 - 18	25 - 27	27 - 30	28 - 32	6 - 10	13 - 17	25 - 27	20 - 22	6.0	2 - 3	5 - 6
Gb X h25	Mean	55	38	17	26	29	31	9	16	26	21	6	2	4
	Standard deviation	1.4	1.4	0.7	0.6	1.2	1.9	0.5	1.7	0.5	0.4	0.3	0.4	0.4
	Range	51 - 57	34 - 40	16 - 18	25 - 27	26 - 31	27 - 34	7 - 9	11 - 21	25 - 27	20 - 22	5 - 6	2 - 3	4 - 5
		*					*		*					

Table 4.2. Descriptive statistics of the *G. bullatarudis* groups. N = 30 for all variables. All values are in μm. * = hypothesis of normality dropped following application of the Minitab approximation to the Shapiro-Wilk test for normality. Abbreviations for measurements are shown in Table 4.1.

		htl	hshl	hrl	hpl	vbtl	vbtw	vbpl	vbml	mhtl	mhshl	mhsil	mhsidw	mhsipw
Gt h25	Mean	56	38	18	26	32	32	6	19	34	26	8	5	4
	Standard deviation	2.1	2.0	0.9	0.8	2.0	2.0	0.7	1.3	0.8	0.7	0.2	0.5	0.4
	Range	50 - 59	32 - 41	15 - 19	24 - 28	26 - 34	26 - 35	5 - 7	16 - 21	31 - 35	25 - 28	7 - 8	4 - 6	4 - 5
		*		*		*								
Gt h19	Mean	55	38	17	26	32	32	6	20	35	27	8	5	4
	Standard deviation	2.3	1.7	1.3	0.9	1.3	2.1	0.8	1.5	1.0	1.0	0.0	0.4	0.4
	Range	49 - 60	34 - 40	13 - 19	25 - 29	29 - 35	25 - 34	5 - 8	16 - 22	33 - 37	25 - 29	8	5 - 6	4 - 5
							*							
Gt mono25	Mean	58	40	18	26	32	32	7	19	34	26	8	5	4
	Standard deviation	1.3	1.3	0.9	0.5	1.2	1.6	0.7	1.1	1.0	1.1	0.3	0.3	0.5
	Range	55 - 60	37 - 42	16 - 19	25 - 27	29 - 34	29 - 36	6 - 8	17 - 22	32 - 36	25 - 29	7 - 8	5 - 6	4 - 5

Table 4.3. Descriptive statistics of the *G. turnbulli* groups. N = 30 for all variables and all values are in μm. * = hypothesis of normality dropped following application of the Minitab approximation to the Shapiro-Wilk test for normality. Abbreviations for measurements are shown in Table 4.1.

In order to satisfy the prerequisites of the univariate and multivariate tests to be performed later, the data which were not normally distributed were transformed in order to "normalise" (Fowler and Cohen, 1990) it. Frequency distribution charts of the measurement samples not considered to be of normal distribution were consistently truncated at the maximal end of the distribution (Figs. 4.3 to 4.13). In order to resolve this, the data were transformed such that x became e^x. The effect of this on one of these distributions is shown in Figs. 4.14a and 4.14b. Only sets of particular measurements which were to be compared with each other with at least one of their samples shown to be significantly not-normal were transformed in this way. From the samples of *G. bullatarudis*, the measurements of hamulus total length, hamulus root length, ventral bar total length and ventral bar membrane length were transformed, and from the samples of *G. turnbulli*, hamulus total length, hamulus root length, ventral bar total length and ventral bar total width were transformed. All sets of transformed data were retested for a normal distribution. All *G. turnbulli* samples of measurements were found to have been "normalised" as were all *G. bullatarudis* samples of measurements except for Gb h25 hamulus total length and hamulus root length which were closer to being normally distributed but not significantly so. These two sets of measurements were then transformed such that the original values of x became 10^x in the transformed data. These new sets of transformed data were again tested for normality. This time the hypothesis of normality was retained.

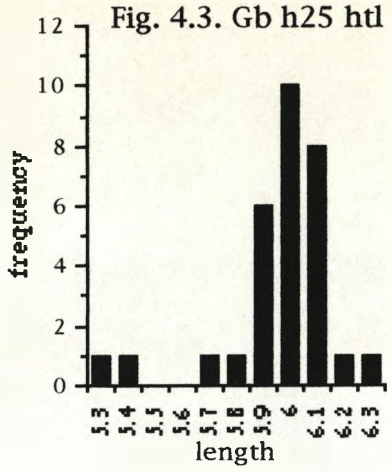


Fig. 4.3. Gb h25 htl

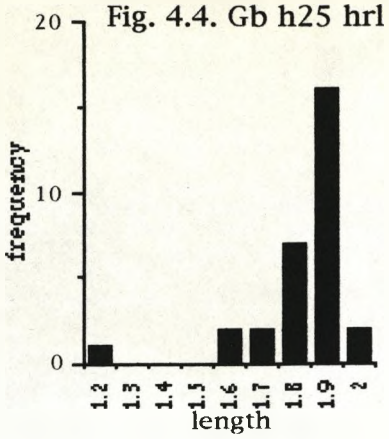


Fig. 4.4. Gb h25 hrl

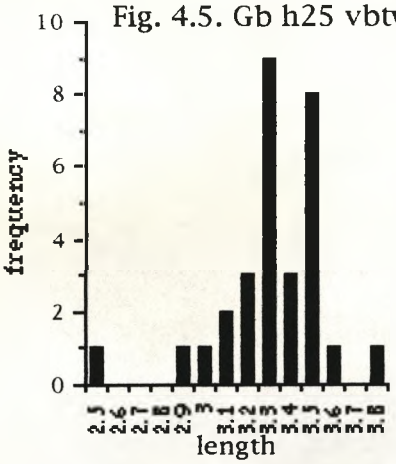


Fig. 4.5. Gb h25 vbtw

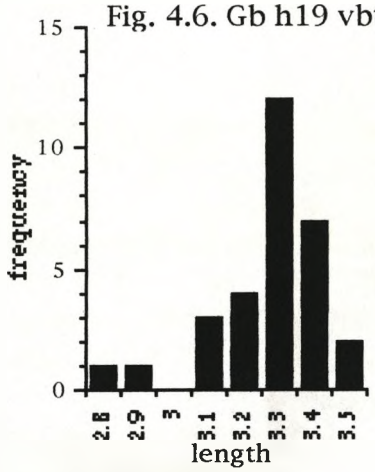


Fig. 4.6. Gb h19 vbtw

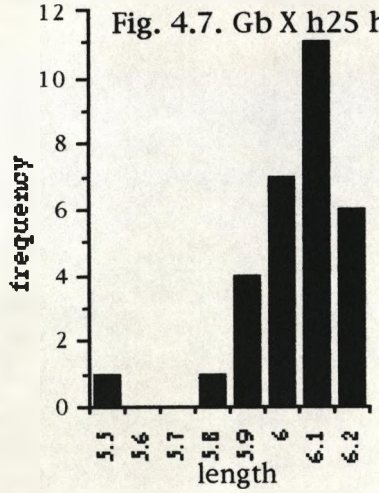


Fig. 4.7. Gb X h25 htl

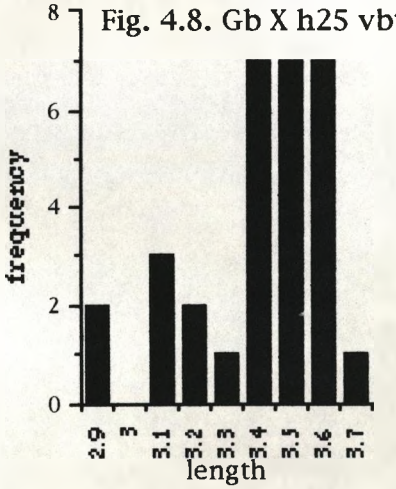


Fig. 4.8. Gb X h25 vbtw

Fig. 4.9. Gb X h25 vbml

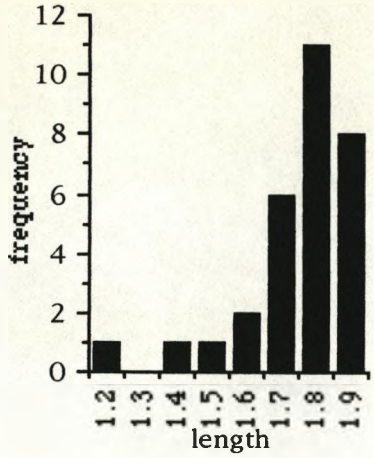


Fig. 4.10. Gt h25 htl

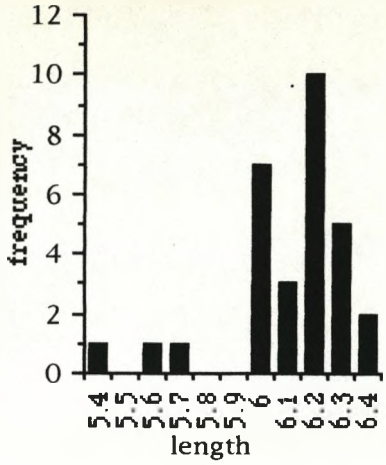


Fig. 4.11. Gt h25 hrl

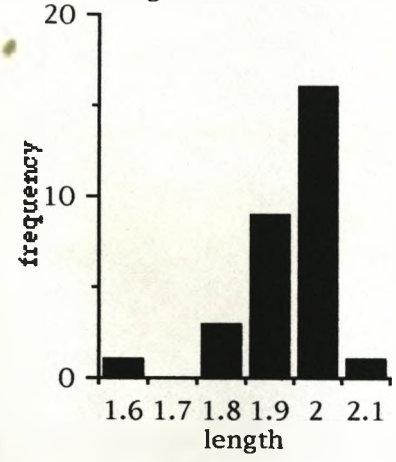


Fig. 4.12. Gt h25 vbtl

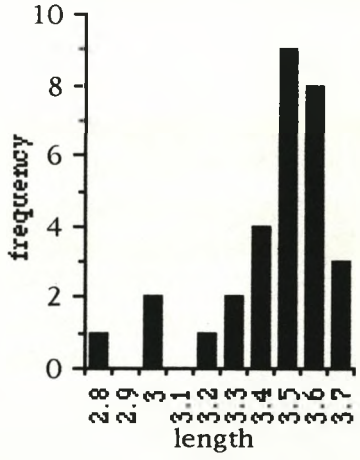
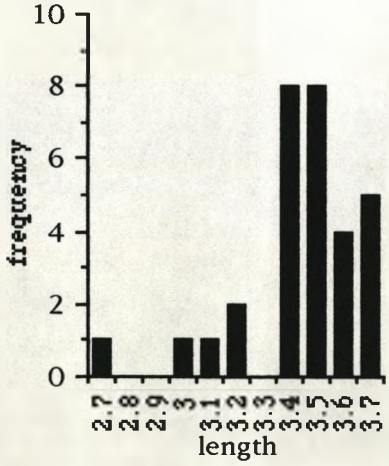


Fig. 4.13. Gt h19 vbtw



Figs. 4.3-13. Length-frequency graphs of all the measurements which were not normally distributed according to the Minitab approximation to the Shapiro-Wilk test for normality. The abbreviations for each group are explained in sections 4.2.2 and 4.2.1. The abbreviations for the different sclerite measurements are given in Table 4.1. All measurements are given in ocular graticule units.

Fig. 4.14a Gt h25 ventral bar total length. Length-frequency plot of untransformed measurements. Measurements are in ocular graticule units. Note the pronounced skew to the left. See section 4.2.5.

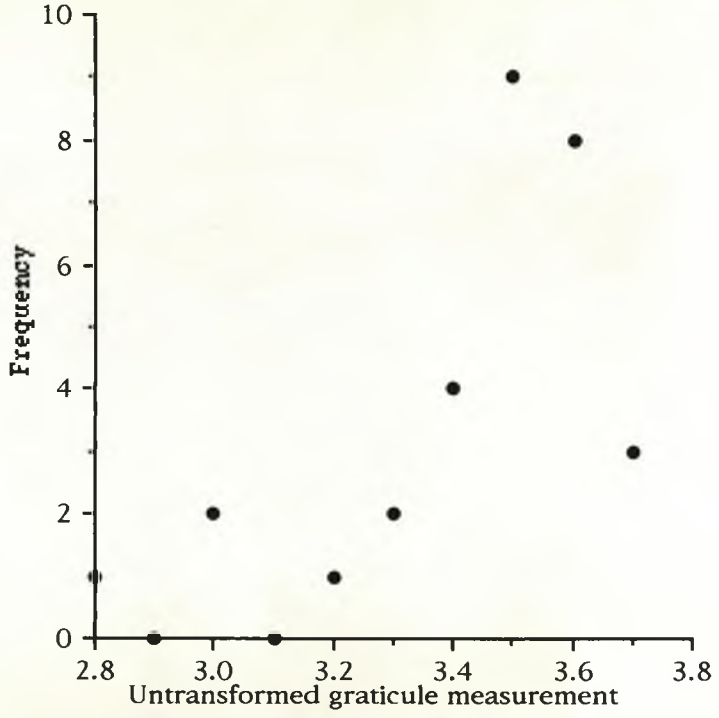
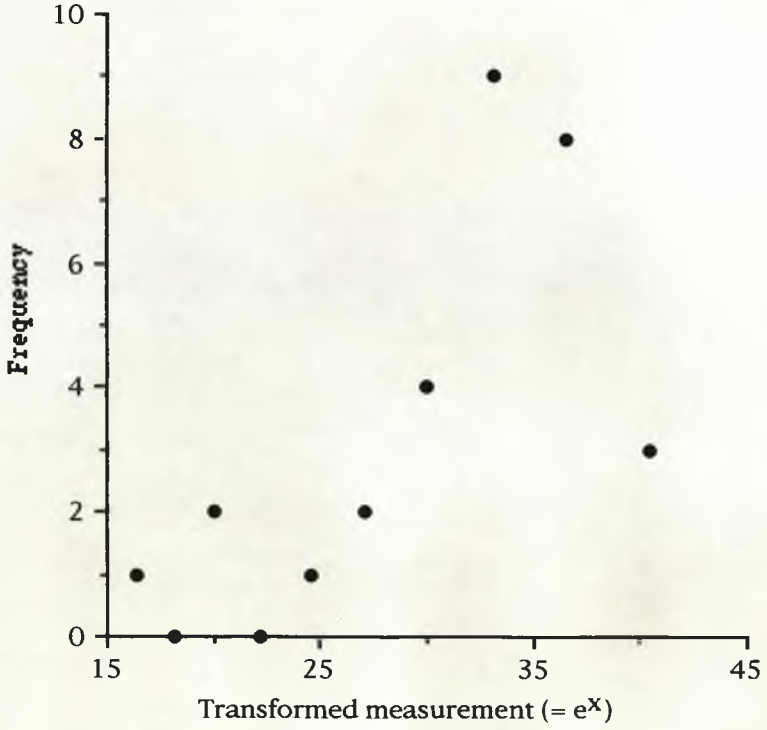


Fig. 4.14b Gt h25 ventral bar total length. Length-frequency plot of transformed measurements such that x(transformed) = $e^{x(original)}$. Note the reduced skew to the left. See section 4.2.5.



4.2.6. Univariate statistical analysis

Since the aim of the experiment was to examine the morphometric differences between the "h25" groups and their corresponding related groups kept under different conditions, it was not primarily concerned with the differences between the test groups themselves. Therefore, each variable from each test group was tested against each corresponding variable within the related "h25" group. First the two-tailed F-test was applied to see if the standard deviations of the two corresponding samples were significantly different and then either the Student t test, if the standard deviations were not found to be significantly different, or Welch's alternate t test were used to test for a significant difference between the means. Welch's alternate t test assumes that both samples to be tested have normal frequency distributions but does not assume that they have equal standard deviations.

4.2.7. Multivariate statistical analysis

Principal Components Analysis (PCA)

The following account of PCA was written with reference to Manly (1986):

PCA is a method by which variation within a set of multivariate data can be accounted for as concisely as possible as a set of uncorrelated indices or "Principal Components" (referred to hereafter as PC's) which are calculated from combinations of the original variables. As the PC's are uncorrelated, they are, in effect, different dimensions that can be used to describe the data instead of the original variables. Their calculation is complex and best undertaken by a computer. However, their usefulness in the analysis of morphometric data can be appreciated by considering that they are a) uncorrelated, separate dimensions and b) calculated to account

for as much variation within the data set as possible within two constraints. One of the constraints is that they are uncorrelated, the other is described below. PC's can be written in the form:

[1] $$z_i = a_{ij}x_j + a_{ij}x_j + ... + a_{ip}x_p$$

where z_i is the i^{th} PC and a_{ij} is the constant by which the j^{th} variable (x_j) is multiplied by in the i^{th} PC. There are always the same number of PC's as there are variables, for example, there are p principal components in the above data set. The a_{ij} constants are constrained such that:

[2] $$a_{ij}^2 + a_{ij}^2 + ... + a_{ip}^2 = 1$$

"Thus the variance of z_1 ,var (z_1), is as large as possible given this constraint on the constants a_{ij}" (Manly, 1986). The variance of z_2 is as large as possible given the same constraint of the a_{ij} constants as in z_1, with the added constraint that z_1 and z_2 are uncorrelated.

Therefore following PCA analysis of this theoretical data set, p principal components were formulated. z_1 accounted for more of the variance within the data set than z_2 which accounted for more of the variance within the data set than z_3 ... z_p accounted for the least variance within the data set than any other of the PCs. All these PCs were uncorrelated. If there was considerable correlation between the variables of the raw data from this theoretical set, a very large proportion of the variance within the data would be accounted for by, for example, the first three PCs. Thus the data set could be comprehensively analysed using the values from just three PCs

instead of p variables.

Furthermore, it is possible to examine which of the p variables contribute the most variation to each of the PCs. This can be done by comparing the a_{ij} constants shown in equation 1. The j^{th} variable with the highest a_{ij} value is the variable which contributes most variance to the i^{th} PC. Therefore the variable with the highest a_{ij} value, where i = 1, is probably the variable which accounts for most variance within the whole data set.

Having obtained PC formulae, it was possible to calculate the value of each PC for each specimen. The first three PC values obtained for each group were plotted. The means and the standard deviations of PCs 1 to 3 for Groups Gb h19, Gb mono25 and Gb X h25 and those for Groups Gt h19 and Gt mono25 were compared with those of Gb h25 and Gt h25 respectively, using the F test and the Student t test or Welch's alternate t test (see section 4.2.6).

Discriminate Function Analysis

The Minitab procedure DISCRIMINANT (Minitab Inc., 1991) was used as described in section 3.2.4 to investigate the similarities between the groups of *G. bullatarudis* and *G. turnbulli*, respectively. In both instances only the seven variables identified for each species to be of most significance in PC1 of the PCAs were used in the analyses.

4.3. Results

4.3.1 Results from the univariate statistical analysis

The results of these tests are given in Tables 4.4 - 4.8.

Measurement	transformation	SD comparison (F test)	SD difference	Mean comparison (t test)	M. compari. (Welch)	Difference
ht1	**10^x**	ns	-	**P=0.0003**	-	**h25 > h19**
hshl	untransformed	ns	-	ns	-	-
hr1	**10^x**	ns	-	ns	-	-
hp1	untransformed	ns	-	ns	-	-
vbt1	untransformed	ns	-	ns	-	-
vbtw	**e^x**	**P=0.0058**	**h25 > h19**	-	ns	-
vbp1	untransformed	ns	-	**P=0.0258**	-	**h19 > h25**
vbm1	**e^x**	**P=0.0041**	**h25 > h19**	-	ns	-
mht1	untransformed	ns	-	ns	-	-
mhshl	untransformed	**P=0.0112**	**h25> h19**	-	ns	-
mhsidw	untransformed	ns	-	ns	-	-
mhsipw	untransformed	ns	-	ns	-	-

Table. 4.4. Results of the F test, t test / Welch alternate t test comparisons of the dimensions of the Gb h25 group with the Gb h19 group. Abbreviations for measurements are given in Table 4.1.

Measurement	transformation	SD comparison (F test)	SD difference	Mean comparison (t)	M. compara. (Welch)	Difference
ht1	**10^x**	*ns*	-	*ns*	-	-
hshl	untransformed	*ns*	-	*ns*	-	-
hr1	**10^x**	*ns*	-	*ns*	-	-
hp1	untransformed	*ns*	-	*ns*	-	-
vbt1	untransformed	**P=0.0055**	**h25 > mono25**	-	*ns*	-
vbtw	**e^x**	**P=0.0029**	**h25 > mono25**	-	*ns*	-
vbp1	untransformed	**P=0.0192**	**h25 > mono25**	-	*ns*	-
vbm1	**e^x**	**P=0.0192**	**h25 > mono25**	-	*ns*	-
mht1	untransformed	**P=0.0004**	**h25 > mono25**	-	*ns*	-
mhshl	untransformed	*ns*	-	**P= 0.021**	-	**h25 > mono25**
mhsidw	untransformed	*ns*	-	-	-	-
mhsipw	untransformed	*ns*	-	*ns*	-	-

Table 4.5. Results of the F test, t test / Welch alternate t test comparisons of the dimensions of the Gb h25 group and the Gb mono25 group. Abbreviations for measurements are given in Table 4.1.

Measurement	transformation	SD comparison (F test)	SD difference	Mean comparison (t)	M. compara. (Welch)	Difference
ht1	10^x	ns	-	ns	-	-
hshl	untransformed	ns	-	ns	-	-
hr1	10^x	P=0.0312	h25 > X h25	-	ns	-
hp1	untransformed	ns	-	ns	-	-
vbt1	untransformed	ns	-	ns	-	-
vbtw	e^x	ns	-	ns	-	-
vbp1	untransformed	P=0.0015	h25 > X h25	-	ns	-
vbm1	e^x	ns	-	ns	-	-
mht1	untransformed	ns	-	ns	-	-
mhshl	untransformed	P=0.021	h25 > X h25	-	ns	-
mhsidw	untransformed	ns	-	ns	-	-
mhsipw	untransformed	P=0.0347	X h25 > h25	-	ns	-

Table. 4.6. Results of the F test, t test / Welch alternate t test comparisons of the dimensions of the Gb h25 group with the Gb X h25 group. Abbreviations for measurements are given in Table 4.1.

Measurement	transformation	SD comparison (F test)	Difference in SDs	Mean comparison (t)	M. compara. (Welch)	Difference in Means
ht1	e^x	ns	-	ns	-	-
hsh1	untransformed	ns	-	ns	-	-
hr1	e^x	ns	-	**P=0.0061**	-	**h25 > h19**
hp1	untransformed	ns	-	**P=0.0397**	-	**h19 > h25**
vbt1	e^x	ns	-	ns	-	-
vbtw	e^x	ns	-	ns	-	-
vbp1	untransformed	ns	-	ns	-	-
vbm1	untransformed	ns	-	ns	-	-
mht1	untransformed	ns	-	**P<0.0001**	-	**h19 > h25**
mhsh1	untransformed	ns	-	**P=0.0016**	-	**h19 > h25**
mhsidw	untransformed	ns	-	ns	-	-
mhsipw	untransformed	ns	-	ns	-	-

Table. 4.7. Results of the F test, t test / Welch alternate t test comparisons of the dimensions of the Gt h25 group with the Gt h19 group. Abbreviations for measurements are given in Table 4.1.

Measurement	transformation	SD comparison (F test)	Difference in SD's	Mean comparison (t)	M. compara. (Welch)	Difference in Means
ht1	**e^x**	*ns*	-	**P<0.0001**	-	**mono25 > h25**
hshl	untransformed	**P=0.0142**	**h25 > mono25**	-	**P=0.0006**	**mono25 > h25**
hr1	**e^x**	*ns*	-	**P=0.0141**	-	**mono25 > h25**
hp1	untransformed	**P=0.0027**	**h25 > mono25**	-	*ns*	-
vbt1	**e^x**	**P=0.015**	**h25 > mono25**	-	*ns*	-
vbtw	**e^x**	*ns*	-	*ns*	-	-
vbp1	untransformed	*ns*	-	**P=0.0474**	-	**mono25 > h25**
vbm1	untransformed	*ns*	-	*ns*	-	-
mht1	untransformed	*ns*	-	*ns*	-	-
mhshl	untransformed	**p=0.0177**	**mono25 > h25**	-	*ns*	-
mhsidw	untransformed	*ns*	-	*ns*	-	-
mhsipw	untransformed	*ns*	-	**P=0.0165**	-	**h25 > mono25**

Table. 4.8. Results of the F test, t test / Welch alternate t test comparisons of the dimensions of the Gt h25 group with the Gt mono 25 group. Abbreviations for measurements are given in Table 4.1.

4.3.2. Results from the multivariate statistical analysis

Principal Components Analysis (PCA)

The combined data for all the *G. bullatarudis* groups and all the *G. turnbulli* groups were run through two separate PCA's incorporating all the measured variables except, in both instances, marginal hook sickle length, for which, in most samples consisted of just one repeated value. The first three PC indices and the proportions of the total variance they accounted for are shown in Tables 4.9 and 4.10.These initial PCAs showed that neither sets of data were suitable for effective PCA - the first three PC's accounting for just 52.6% and 59.4% of the variance in the *G. bullatarudis* and *G. turnbulli* data sets respectively.

Variable	PC1 ("a" constants)	PC2 ("a" constants)	PC3 ("a" constants
htl	0.46	-0.009	0.185
hshl	0.389	0.038	0.235
hrl	0.341	-0.088	0.195
hpl	0.333	0.15	0.034
vbtl	0.301	-0.031	0.196
vbtw	0.322	-0.32	-0.396
vbpl	0.344	-0.121	0.083
vbml	0.196	-0.326	-0.516
mhtl	0.194	0.575	-0.336
mhshl	0.128	0.635	-0.15
mhsidw	0.059	-0.038	-0.515
mhsipw	-0.04	0.097	-0.046
Proportion of var. accounted for	0.29	0.139	0.097
Cumulative	0.29	0.429	0.526

Table 4.9. "a" constants of PCs 1,2 and 3 (see equation 1, section 4.2.7) of the initial PCA of the *G. bullatarudis* data. Note that these three indices accounted for just 52.6% of the variance of the whole data set. Abbreviations for measurements are given in Table 4.1.

Variable	PC1 ("a" constants)	PC2 ("a" constants)	PC3 ("a" constants)
htl	0.408	-0.235	-0.184
hshl	0.407	-0.164	-0.174
hrl	0.207	-0.312	-0.023
hpl	0.176	0.385	0.191
vbtl	0.382	0.05	0.192
vbtw	0.403	-0.011	0.17
vbpl	0.271	-0.192	-0.249
vbml	0.405	0.075	0.201
mhtl	0.15	0.509	-0.312
mhshl	0.132	0.48	-0.397
mhsidw	0.025	0.305	-0.067
mhsipw	0.091	0.213	0.687
Proportion of var. accounted for	0.31	0.18	0.104
Cumulative	0.31	0.49	0.594

Table 4.10. "a" constants of PCs 1,2 and 3 (see equation 1, section 4.2.7) of the initial PCA of the *G. turnbulli* data. Note that these three indices accounted for just 59.4% of the variance of the whole data set. Abbreviations for measurements are given in Table 4.1.

Secondary PCA's of both sets of samples, this time after reduction of the number of variables used in each data set from 12 to 7 by disregarding all variables whose a_{ij} constant were below 0.200 in the first PC of the first PCA, were more satisfactory. The first three PC's accounted for 73.3% and 79.9% of the total variance the *G. bullatarudis* and *G. turnbulli* data sets respectively. The first three PC's of both sets of data are given below. The abbreviations used for the various measurements are explained in Table 4.1.

First three PC's resulting from PCA of the collective data set, Gb h25, Gb h19, Gb mono25 and Gb X h25:

PC1 = - 0.487htl - 0.416hshl - 0.370hrl - 0.335hpl - 0.320vbtl - 0.323vbtw - 0.366vbpl

PC2 = 0.120htl - 0.311hshl + 0.529hrl + 0.563hpl - 0.333vbtl - 0.250vbtw - 0.345vbpl

PC3 = - 0.071htl - 0.047hshl - 0.204hrl + 0.318hpl + 0.652vbtl - 0.649vbtw + 0.065vbpl

PC's 1, 2 and 3 accounted for 46.4%, 14.5% and 12.3% of the total variance of the data, respectively - a total of 73.2% of the variance.

First three PC's resulting from PCA of the collective data set, Gt h25, Gt h19 and Gt mono25:

PC1 = - 0.443htl - 0.434hshl - 0.250hrl - 0.373vbtl - 0.409vbtw - 0.302vbpl - 0.395vbml

PC2 = - 0.381htl + 0.024hshl - 0.762hrl + 0.037vbtl + 0.309vbtw + 0.268vbpl + 0.323vbml

PC3 = 0.190htl + 0.290hshl - 0.105hrl - 0.429vbtl - 0.245vbtw + 0.709vbpl - 0.349vbml

PC's 1, 2 and 3 accounted for 51.1%, 16.4% and 12.3% of the total variance of the data, respectively - a total of 79.9% of the variance.

Initially, for both data sets, PC1 was plotted against PC2. However, no separations of groups were clearly visible. Plotting each PC individually was found to be a better way of illustrating the results to display the separation of specimens on account of their PC values. The plots of PC's 1 to 3 are shown in Figs. 4.15 to 4.20. The results of the comparisons of the means and standard deviations of PCs 1 to 3 for Groups Gb h19, Gb mono25 and Gb X h25 and those for Groups Gt h19 and Gt mono25 with those of Gb h25 and Gt h25 respectively, are shown in Tables 4.11 and 4.12.

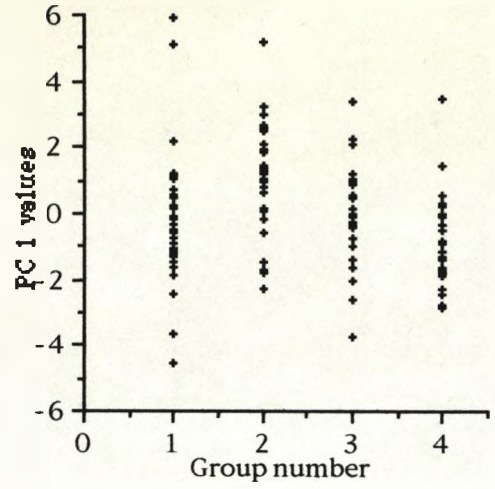


Fig. 4.15. PC1 values from the second PCA of the *G. bullatarudis* data using all variables except the marginal hook dimensions and vbml. Group 1 = Gb h25, 2 = Gb h19, 3 = Gb mono25 and 4 = Gb X h25. PC1 accounted for 46.4% of the variance.

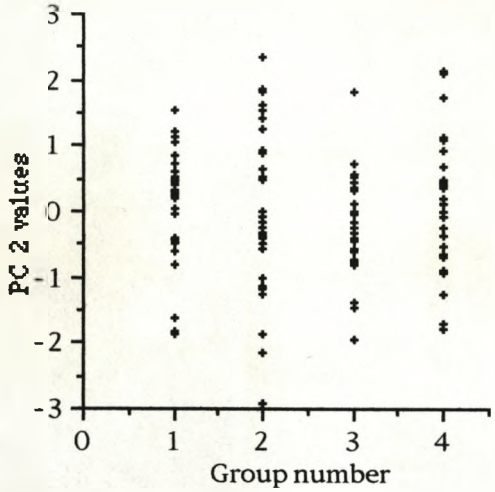


Fig. 4.16. PC2 values from the second PCA of the *G. bullatarudis* data using all variables except the marginal hook dimensions and vbml. Group 1 = Gb h25, 2 = Gb h19, 3 = Gb mono25 and 4 = Gb X h25. PC2 accounted for 14.5% of the variance.

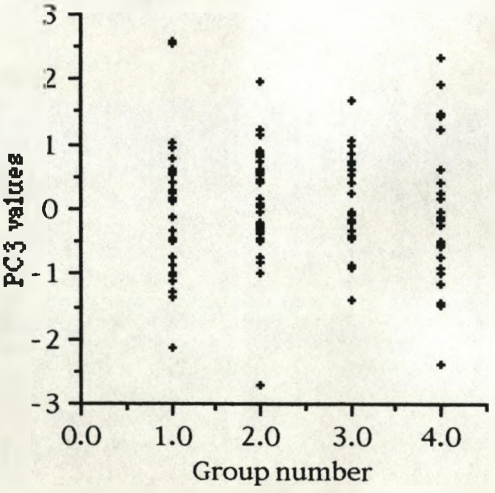


Fig. 4.17. PC3 values from the second PCA of the *G. bullatarudis* data using all variables except the marginal hook dimensions and vbml. Group 1 = Gb h25, 2 = Gb h19, 3 = Gb mono25 and 4 = Gb X h25. PC3 accounted for 12.3% of the variance.

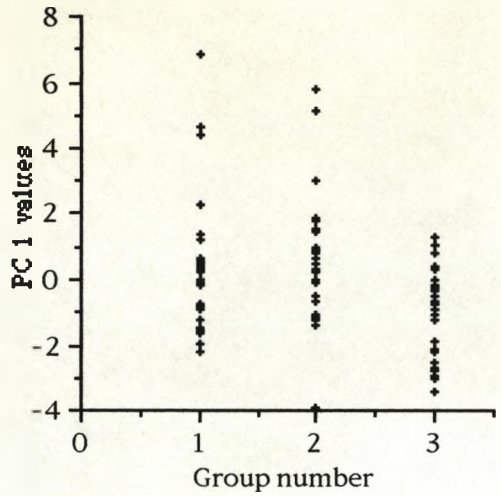


Fig. 4.18. PC1 values from the second PCA of the *G. turnbulli* data using all variables except the marginal hook dimensions and hpl. Group 1 = Gt h25, 2 = Gt h19 and 3 = Gt mono25. PC2 accounted for 51.1% of the variance.

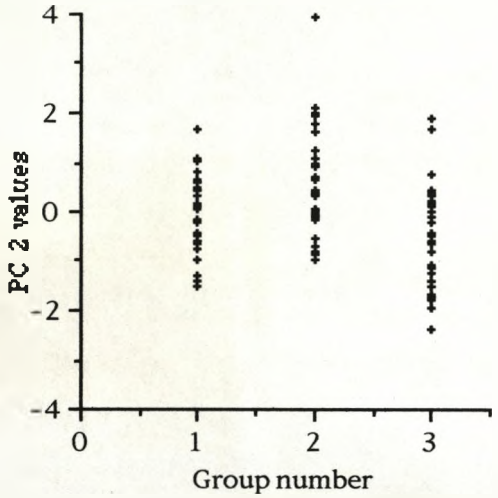


Fig. 4.19. PC2 values from the second PCA of the *G. turnbulli* data using all variables except the marginal hook dimensions and hpl. Group 1 = Gt h25, 2 = Gt h19 and 3 = Gt mono25. PC1 accounted for 16.4% of the variance.

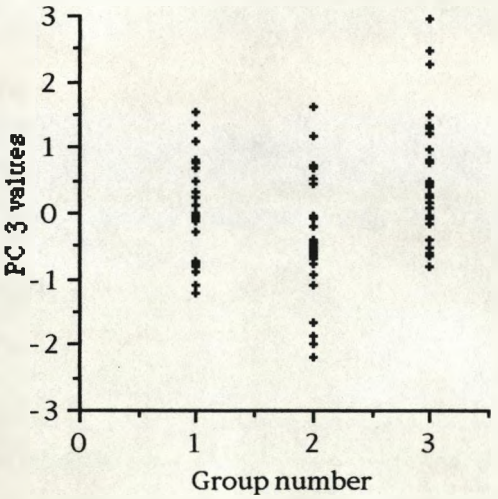


Fig. 4.20. PC3 values from the second PCA of the *G. turnbulli* data using all variables except the marginal hook dimensions and hpl. Group 1 = Gt h25, 2 = Gt h19 and 3 = Gt mono25. PC3 accounted for 12.3% of the variance.

Group	PC	SD comparison (F test)	SD difference	Mean comparison (t)	M. compari. (Welch)	Difference
Gb h19	PC1	*ns*	-	**0.0138**	-	**h19 > h25**
Gb h19	PC2	**0.024**	**h19 > h25**	-	*ns*	-
Gb h19	PC3	*ns*	-	*ns*	-	-
Gb mono25	PC1	**0.046**	**h25 > mono25**	-	*ns*	-
Gb mono25	PC2	*ns*		**0.0493**	-	**h25 > mono25**
Gb mono25	PC3	**0.005**	**h25 > mono25**	-	*ns*	-
Gb X h25	PC1	**0.0098**	**h25 > X h25**	-	*ns*	-
Gb X h25	PC2	**0.016**	**X h25 > h25**	-	**0.0004**	**h25 > X h25**
Gb X h25	PC3	*ns*	-	*ns*	-	-

Table. 4.11. Results of the F test, Student t / Welch alternate t test comparisons of the PCs calculated from the second PCA for the *G. bullatarudis* groups. Means and variances of the first three PCs of Gb h19, Gb mono 25 and Gb X h25 are compared with the respective values for the Gb h25 group.

Group	PC	SD comparison (F test)	SD difference	Mean comparison (t)	M. compari. (Welch)	Difference
Gt h19	PC1	*ns*	-	*ns*	-	-
Gt h19	PC2	**0.0308**	**h19 > h25**	-	**0.009**	**h25 > h19**
Gt h19	PC3	*ns*	-	*ns*	-	-
Gt mono25	PC1	**0.0178**	**h25 > mono25**	-	**0.005**	**h25 > mono25**
Gt mono25	PC2	**0.045**	**mono25 > h25**	-	*ns*	-
Gt mono25	PC3	*ns*	-	**0.02**	-	**mono25 > h25**

Table. 4.12. Results of the F test, Student t / Welch alternate t test comparisons of the PCs calculated from the second PCA for the *G. turnbulli* groups. Means and variances of the first three PCs of Gt h19 and Gt mono 25 are compared with the respective values for the Gt h25 group.

The results of the DFAs for *G. bullatarudis* and *G. turnbulli* are summarised in Figs. 4.21 to 4.25 and 4.26 to 4.29 respectively.

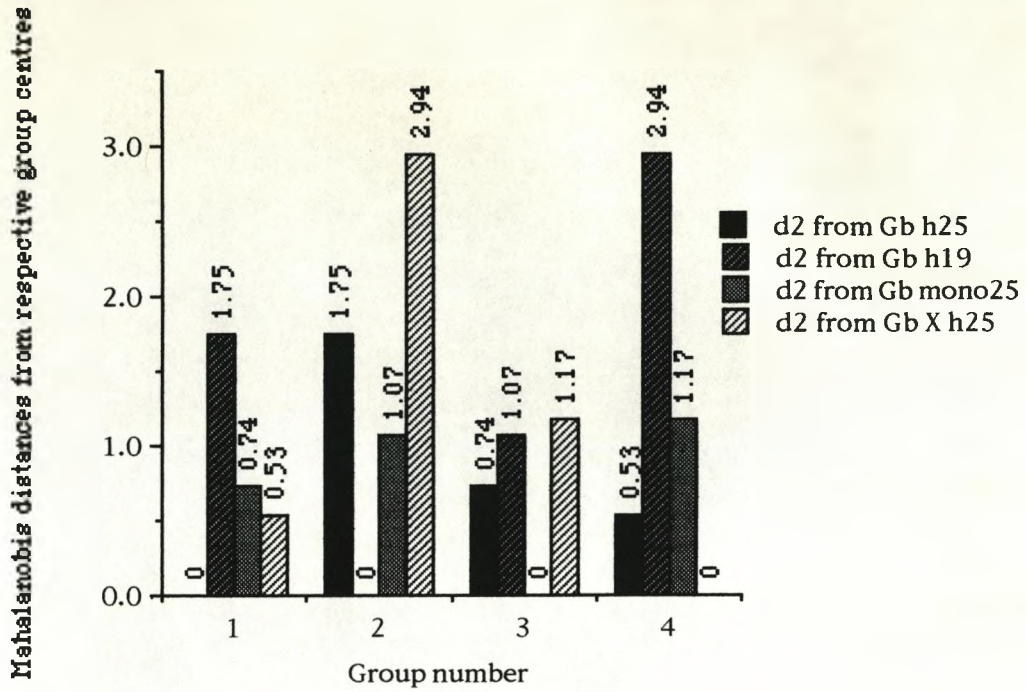


Fig. 4.21. Mahalanobis (d2) distances from the multivariate means (=group centres) of each of the *G. bullatarudis* groups to each other. Group 1 = Gb h25, 2 = Gb h19, 3 = Gb mono25 and 4 = Gb X h25.

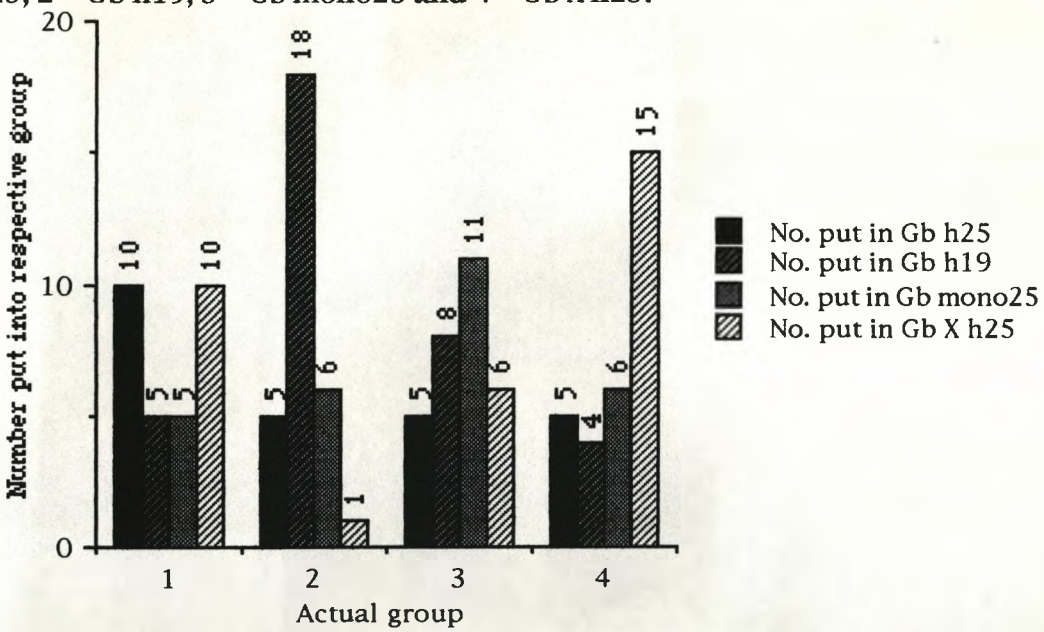
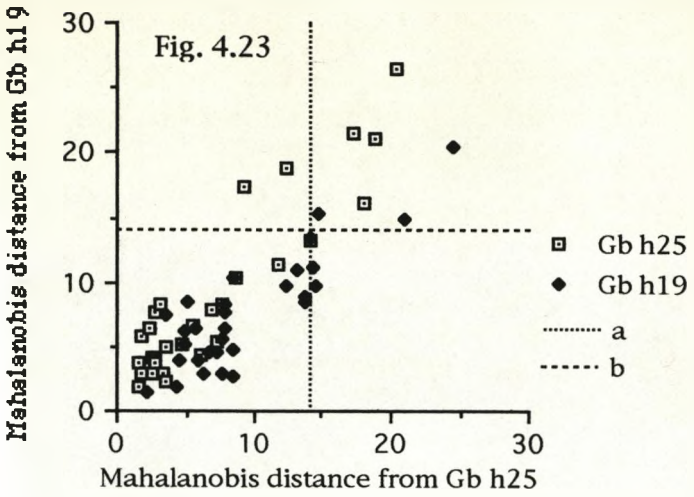
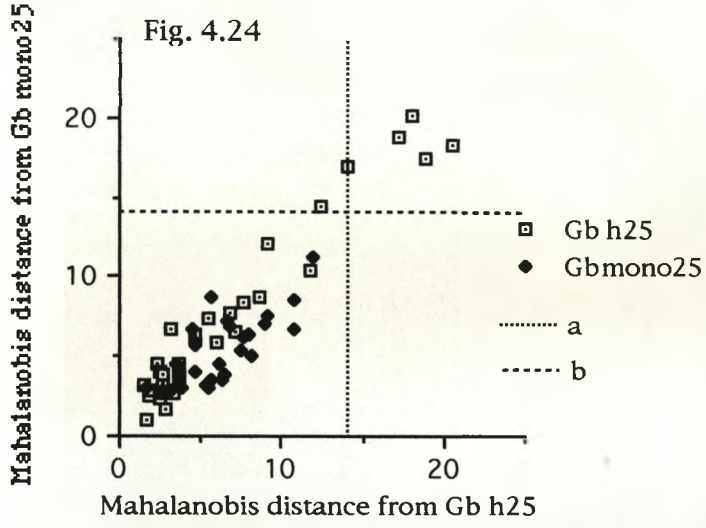
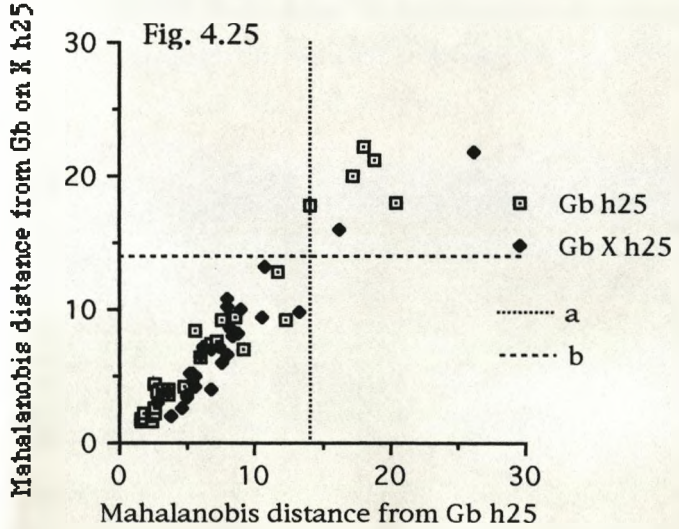


Fig. 4.22. Results of the DFA of the *G. bullatarudis* groups. The distribution of specimens from each actual group (see labelling of horizontal axis and the key below) among all the groups as predicted by the DFA (vertical bars). Group 1 = Gb h25, 2 = Gb h19, 3 = Gb mono25 and 4 = Gb X h25.







Figs. 4.23 - 4.25. Mahalanobis distances of specimens' sclerite measurements from Gb h25 and Gb h19, Gb mono25 and Gb X h25, respectively, from the multivariate means of those same groups.

Specimens plotted between line a and the y axis have a greater than 5% probability of actually belonging to Gb h25 and specimens plotted between line b and the x axis have a greater than 5% probability of actually belonging to the group whose multivariate mean is represented by $y = 0$.

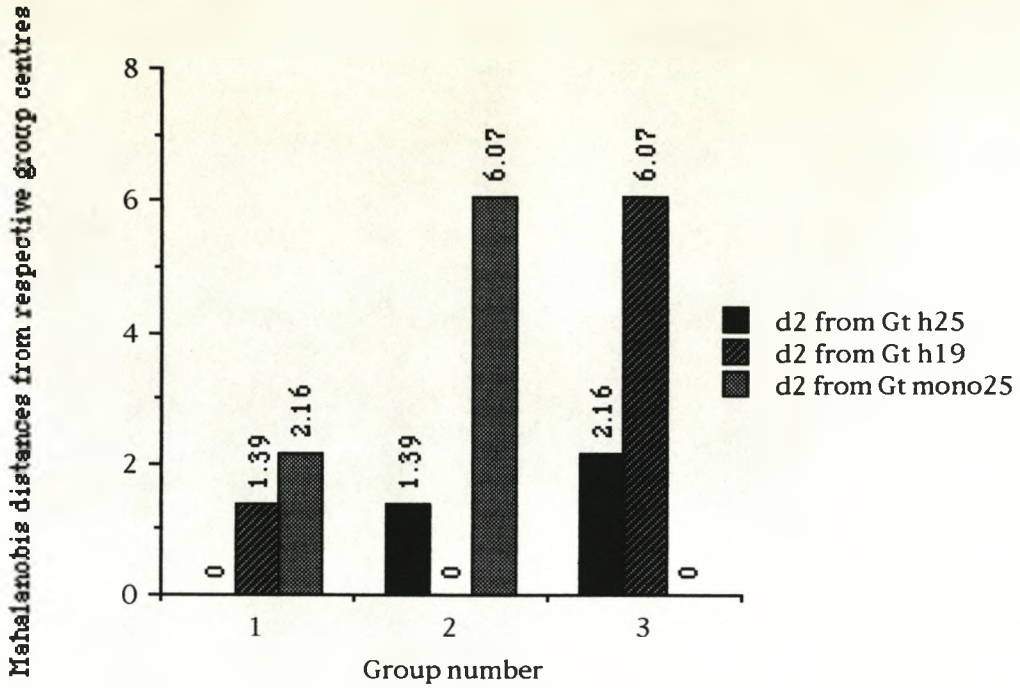


Fig. 4.26. Mahalanobis (d2) distances from the multivariate means (=group centres) of each of the *G. turnbulli* groups to each other. Group 1 = Gt h25, 2 = Gt h19 and 3 = Gt mono25.

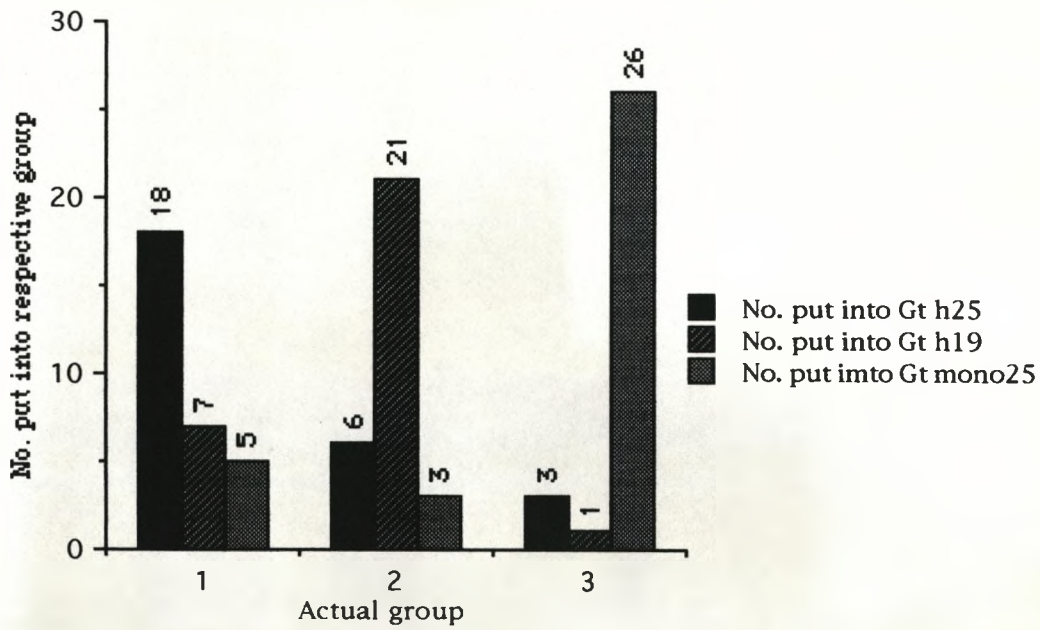
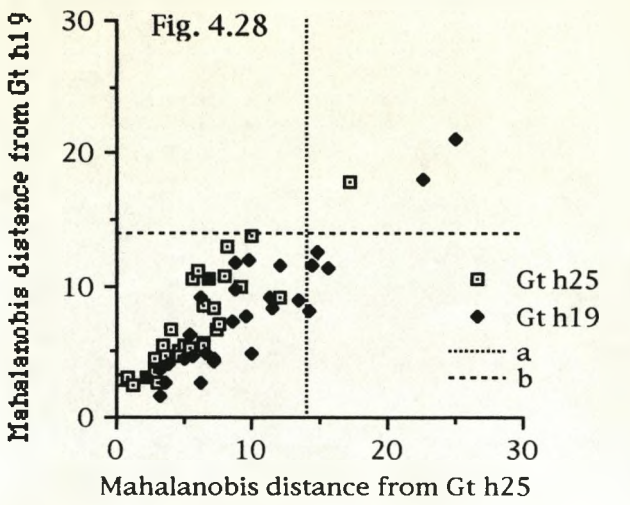
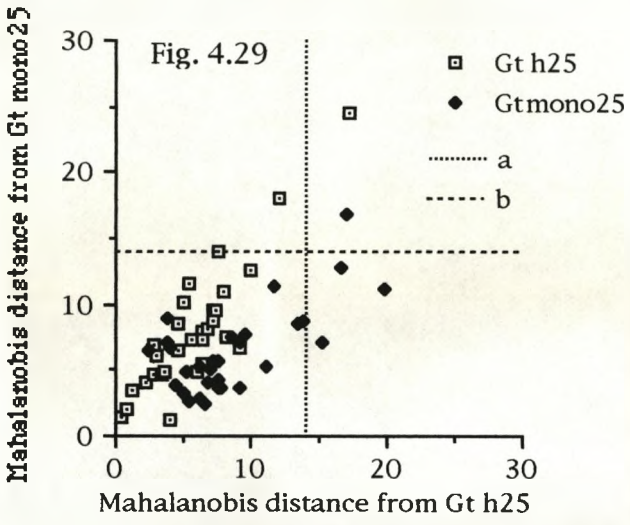


Fig. 4.27. Results of the DFA of the *G. turnbulli* groups. The distribution of specimens from each actual group (see labelling of horizontal axis and the key below) among all the groups as predicted by the DFA (vertical bars). Group 1 = Gt h25, 2 = Gt h19 and 3 = Gt mono25.





Figs. 4.28 - 4.29. Mahalanobis distances of specimens' sclerite measurements from Gt h25 and Gt h19 and Gt mono25, respectively, from the multivariate means of those same groups.

Specimens plotted between line a and the y axis have a greater than 5% probability of actually belonging to Gt h25 and specimens plotted between line b and the x axis have a greater than 5% probability of actually belonging to the group whose multivariate mean is represented by y = 0.

4.4. Discussion

4.4.1. Length-frequency distribution of the measurements

Figs. 4.3 to 4.13 show the measurement frequency distributions which deviated significantly from a normal distribution. They all skew to the left. A skew to the right is more common in biological data (Fowler and Cohen, 1990). The following hypothesis is proposed.

It is assumed that the greatest contributor to error was the measurement of sclerites which were not adequately flattened. Measurement of insufficiently flattened *Gyrodactylus* would, due to parallax, invariably lead to an underestimation of flat length.

In order to examine the effect that this might have on the frequency distribution of measurement data, I have produced a model based on probability plots of binomial expansions with differing "p" and "q" values where $p + q = 1$ (see Fig. 4.30). An arbitrary assumption is made that distances measured erroneously are 4 times more likely to be shorter than longer than their actual measurements because inadequate flattening is the most likely cause of error. Therefore, in the model, the probability of an incorrect measurement being shorter than its actual measurement = $p = 0.8$ and the probability of an incorrect measurement being longer than its actual measurement = $q = 0.2$. The line through the open triangles represents the distribution of points measured erroneously. The line running through the open circles represents the distribution of correct measurements from the sample. This distribution was calculated using the probabilities $p = 0.5$ and $q = 0.5$. Only about 3/30 measures are usually outlying the rest in the not-normal frequency distributions (see Figs. 4.3 to 4.13). It is therefore assumed that the frequency distribution of

erroneously measured points accounts for about 10% of the variance of the total frequency distribution and the actual frequency distribution about 90%. The total frequency distribution represented by the line running through the closed circles is the cumulative of both correctly and erroneously measured distances. The shape of the latter curve conforms quite well to the shapes of the frequency distributions in Figs. 4.3 to 4.13.

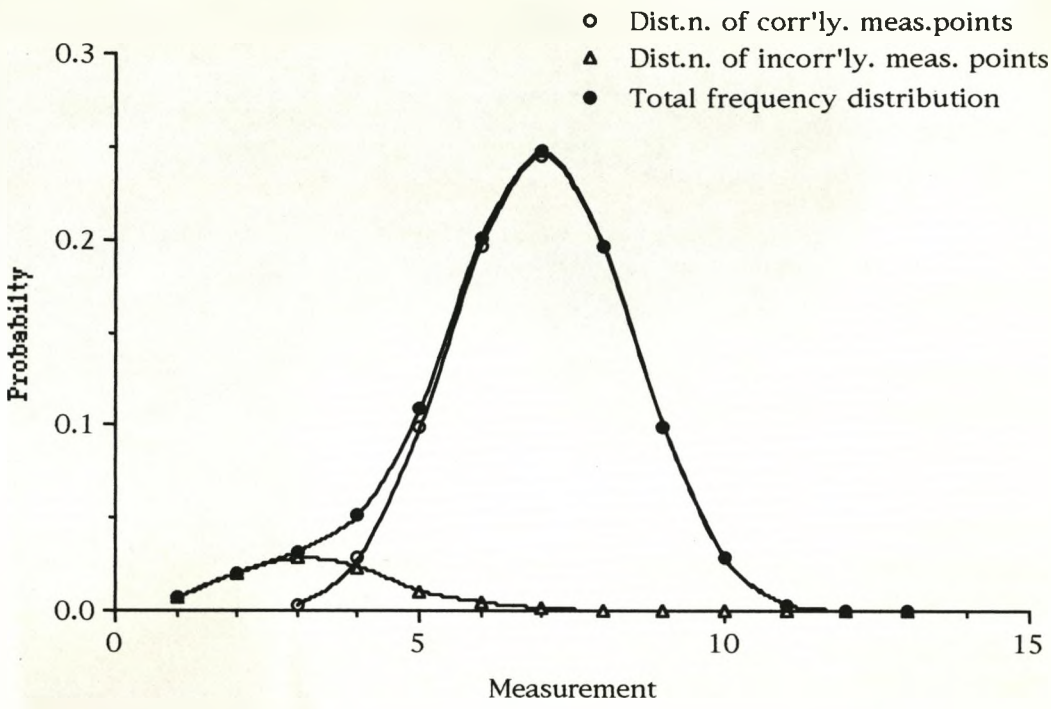


Fig 4.30 This model assumes that the measurements of greatest error were from inadequately flattened specimens. An inaccurate measurement was taken to be four times more likely to be less than the actual length than greater. 10% of measurements are assumed to be inaccurate and to err enough to conform to the frequency distribution designated by the open triangles. See section 4.4.1.

4.4.2. Univariate statistical analysis

Comparison of the groups of G. bullatarudis

None of the groups showed consistent significant differences in size when compared to the Gb h25 group, although Gb h19 had a significantly smaller mean value for hamulus total length, the largest of the sclerite measurements.

The dimensions of the ventral bar were less variable in the mono-culture than in the hetero-culture. This conforms to the assumption [for example in Harris (1993), see section 4.1] that the variation inherent in at least some sclerite measurements is related to genetic variation.

There was also evidence to suggest that the Gb h25 group was also more variable than the Gb h19 and Gb X h25 groups. This may be indicative of a selection process within the two latter groups. Some genotypes less suited to the different temperature or host species may have become reduced in number, thereby causing a reduction in the overall genetic variation within those groups.

Comparison of the groups of G. turnbulli

Again there was no consistent evidence to suggest a significant general increase or decrease in size of sclerites at the lower temperature.

There was evidence to suggest that the mono-culture sample had sclerites which were generally larger than the hetero-culture.

Again there was evidence that variation in sclerite measurements is related

to the amount of genetic variation in the population. This was not as convincing as the comparison of Gb h25 with Gb mono25. There was little evidence in the comparison of Gt h25 and Gt mono25 consistent with the comparison of the corresponding *G. bullatarudis* groups to suggest that variation in the ventral bar dimensions were more affected by genetic variation than the other sclerite components.

4.4.3. Principal Components Analysis

Choice of variables used in the second PCAs

The initial PCAs, which included all the variables except marginal hook sickle length, were ineffective because too many variables were relatively uncorrelated or exhibited little variance within and between the different samples.

Figs. 4.31 and 4.32 show a standardised measure of variance (standard deviation divided by the mean) plotted against the mean for all the sclerite measurement variables of *G. bullatarudis* and *G. turnbulli* respectively, excepting measurements of marginal hook sickle length.

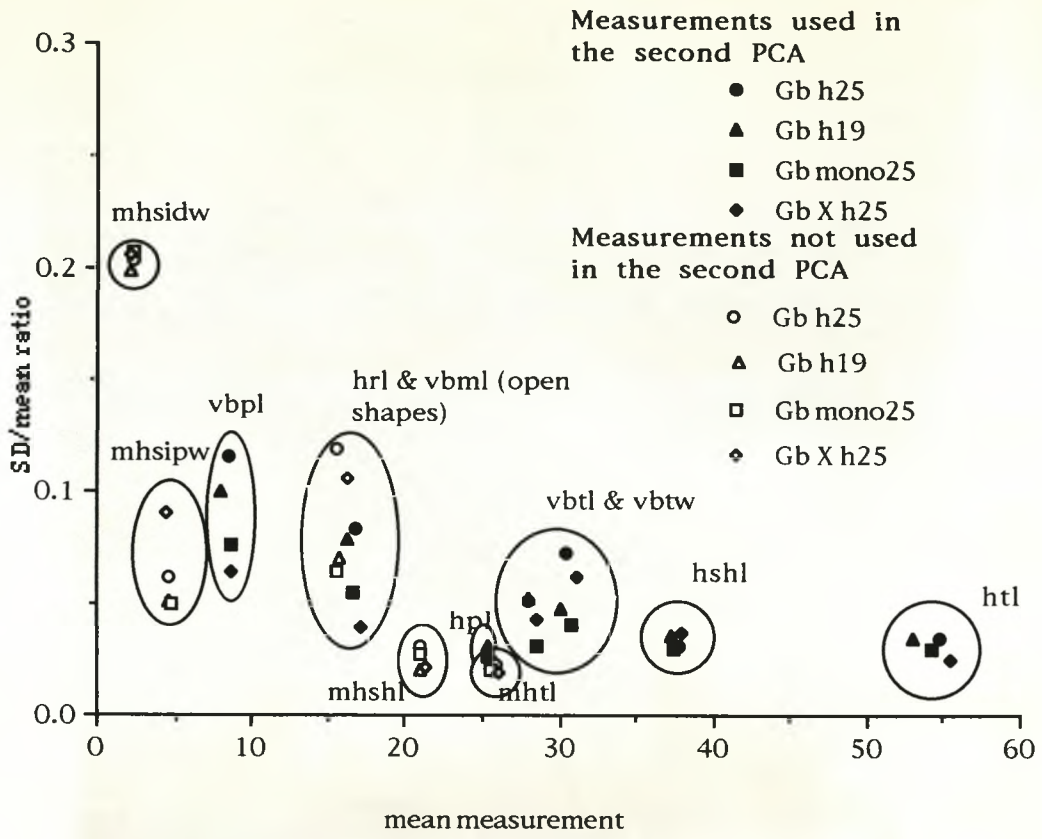


Fig 4.31. Graph plotting standard deviation to mean ratio of the measurements from the samples of *G. bullatarudis*. The graph compares those measurements used in the first PCA which were not used in the second. Note that the measurement of mhsil was not used in either PCA because in at least one sample it consisted of a single value. All values are in μm. See Table 4.1 for an explanation of the abbreviations for the different sclerite measurements.

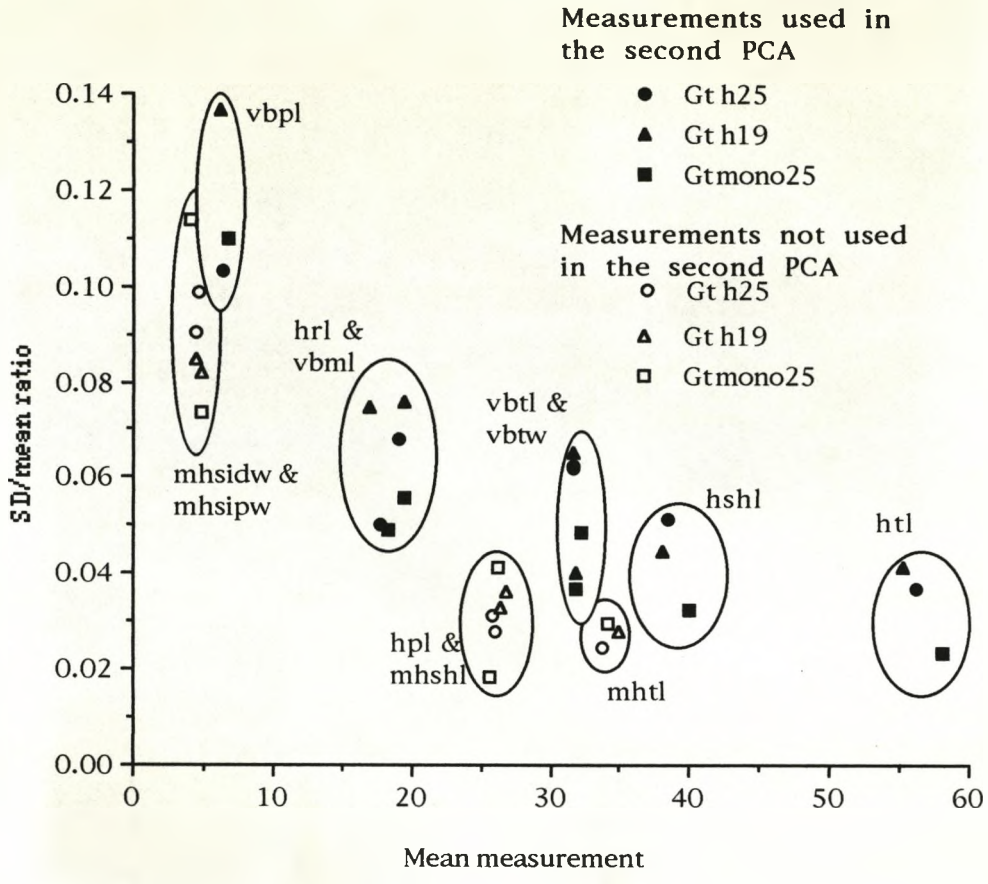


Fig 4.32. Graph plotting standard deviation to mean ratio of the measurements from the samples of *G. turnbulli.* The graph compares those measurements used in the first PCA which were not used in the second. Note that the measurement of mhsil was not used in either PCA because in at least one sample it consisted of a single value. All values are in μm. See Table 4.1 for an explanation of the abbreviations for the different sclerite measurements.

In Fig. 4.31 (*G. bullatarudis*) all the variables which were discarded for the second PCA, with the exception of measurements of ventral bar membrane length, show less standardised variance with respect to their means in comparison to the variables which were retained for the second analysis. There insignificance in the first PCA, as shown by their low "a" multiples (see equation 1, section 4.2.7 and Table 4.9), can be explained by their very small contribution to overall variance within the samples. The low value of the "a" coefficient for ventral bar membrane length in PC1 of the initial PCA, however, was more likely due to lack of correlation with other variables.

The coefficients of the PC1s of the initial PCAs for both *G. bullatarudis* and *G. turnbulli* were all of the same sign, with the exception of marginal hook sickle proximal width in the *G. bullatarudis* analysis which was of little significance (-0.04). In morphometrics it is generally accepted that the first PC of a PCA, when all coefficients are of the same sign, is an index of size (Blackith and Reyment, 1971; Manly, 1986; Sundberg, 1989). The evidence therefore suggests that ventral bar membrane length of *G. bullatarudis* specimens have less correlation with the overall size of the sclerites than the other ventral bar and hamulus dimensions.

Fig. 4.32 (*G. turnbulli*) also shows a clear correlation between the comparatively low standardised variance and the relatively insignificant "a" constants (see Table 4.10) of the measurements discarded for the second PCA.

For both species, hamulus point length and the marginal hook measurements show less standardised variance in relation to their mean values than the other hamulus and ventral bar measurements.

Of the variables incorporated into PC1 of the initial PCAs, those of the

marginal hooks were the least influential (see Tables 4.9 and 4.10). This indicates that they were the less variable elements of the attachment sclerites under different conditions. The marginal hook measurements are therefore the best indicators for species identification being more constant and less variable under different treatments. Malmberg (1970) also found the marginal hooks were most consistent characters for species identification.

Hamulus total length and hamulus shaft length were the most influential measurements on the value of the first PCs and were therefore the best indicators of overall sclerite size.

Comparisons of PC1 values from the second PCA (Figs. 4.15 and 4.18 and Tables 4.11 and 4.12)

Both PC1 indices of the second PCAs for the two species were indices of size (see above) and for both species hamulus total length and hamulus shaft length were the major contributors.

The comparisons of PC1 values of the second PCAs for the *G. bullatarudis* groups indicated that the sclerites of Gb h19 were generally smaller than those of the Gb h25 group and the sclerites of both the Gb mono25 and Gb X h25 were less variable. Similar comparisons of the *G. turnbulli* groups indicated that the sclerites of the Gt h19 sample were neither significantly different in size nor variability to those of the Gt h25 group whereas the sclerites of the Gb mono25 group were significantly larger and less variable.

As both sets of PC1 values are considered to be indices of size (see previous page), it is worth comparing the results of their comparisons to the results of the univariate comparisons.

Consistent findings were obtained from both methods in comparison of the *G. bullatarudis* groups. However, an overall difference in size between the Gb h25 and Gb h19 groups was more clearly demonstrated in the comparison of PC1 values than in the univariate analyses and no difference in variance was found between Gb h19 and Gb h25 in the comparisons of PC1 standard deviations.

Similarly, results of comparison of *G. turnbulli* groups were consistent for both methods. However, the difference in variance between the mono- and hetero-cultures was more convincingly shown by comparison of PC1 values than in the univariate analyses and no significant difference in size was indicated between Gt h19 and Gt h25 in the PCA.

Comparisons of PCs 2 and 3 values from the second PCA (Figs. 4.16, 4.17, 4.19 and 4.20; Tables 4.11 and 4.12)

PCs 2 and 3 of the second PCAs of the *G. bullatarudis* and *G. turnbulli* groups were indices of shape (Manly, 1986), or size and shape (see Sundberg, 1989), rather than just size. In total, they accounted for just over one quarter of the variance in each set of samples and were therefore of less consequence than the first PCs which, alone, accounted for roughly half the variances in each set.

PC2 of the *G. bullatarudis* groups contrasted measurements of hamulus root length, hamulus point length and, to a lesser extent, hamulus total length with the other measurements. Although Gb mono25 and Gb X h25 were not significantly different to Gb h25 along the size parameter, PC1, they were along this shape parameter. The PC2 values for Gb h19 and Gb X h25 were significantly more variable than those for Gb h25.

PC3 of the same PCA contrasts hamulus point length, ventral bar total length and ventral bar process length with the other measurements. None of the Gb h19, mono25 and X h25 groups were significantly different to the Gb h25 group along this parameter although the Gb mono25 group was significantly less variable.

PC2 of the second *G. turnbulli* PCA contrasted hamulus total length and hamulus root length with the other parameters. As for Gb h19, the Gt h19 group was significantly different to its corresponding "h25" group along this first shape parameter. The Gt mono25 was significantly more variable than the Gt h25 group along this parameter, contradicting the assumption that sclerite variability is correlated to genetic variability.

PC3 of the second *G. turnbulli* PCA contrasts hamulus total length, hamulus shaft length and ventral bar process length with the other variables. This parameter, was significantly different in mean between Gt mono25 and Gt h25.

It was interesting that groups similar to the "h25" groups along the first PC, indicating similar overall size, differed along the second PC, indicating a difference in shape. In the of comparisons of variance, Gb X h25 and Gt mono25 were significantly less variable than their corresponding "h25" group along the former, and more variable along the latter indices.

Summary of the PCA investigations

Marginal hook measurements were shown to be the least variable of characters in the intraspecific comparisons between groups. Hamulus total length and hamulus shaft length were the most influential characters in distinguishing between groups of the same species along the first PCs. They were therefore the best indicators of general sclerite size.

The results of the PCAs showed that size rather than shape accounted for most of the variance within and between groups.

Comparisons of PC1 confirmed the implications of the univariate statistical analyses - sclerites of Gb h19 being generally smaller than those of Gb h25, and those of Gb mono25 and Gb X h25, slightly less variable; sclerites of Gt mono25 were shown to be generally larger and less variable than those of Gt h25. No distinction could be made using this size parameter between Gt h25 and Gt h19.

4.4.4. Discriminant Function Analysis

The results of the DFAs using the same variables as the second PCAs showed that the centroids of the various groups were not significantly different. Thus it was impossible to accurately assign specimens to their correct groups using the measured variables.

4.4.5. A summary comparison of the univariate, PCA and DFA analyses

The results of the univariate analyses were not conclusive and were therefore of little value by themselves. However, patterns observed in the univariate analysis of measurements were also shown in the comparisons of first PCs of the second PCAs. This was not surprising given the assumption that the first PC of a morphometric analysis is a size, rather than a shape parameter (Manly, 1986) or a size and shape component (see Sundberg, 1989). Sundberg (1989) stated that "biometric analysis should be based on several characters simultaneously, since selection presumably acts upon whole phenotypes and not on single characters". The values of the first PCs from the second PCAs were therefore very useful variables which accounted for 46.1 and 51.1% of the variance in the *G. bullatarudis*

and *G. turnbulli* groups respectively and confirmed that, as indicated by some of the results of the univariate comparisons, there were significant mean size and variation in size differences between groups.

The DFAs showed that the multivariate means of each group of the same *Gyrodactylus* species were not significantly different. The DFAs were of little use in separating the groups, but were good demonstrators of overall similarities, not withstanding the differences illustrated by the separate PCs of the PCA analyses. The results of the DFAs were not a contradiction of the significant differences found between certain groups along certain PCs because the DFAs considered all variables together whereas the PCAs identified differences along uncorrelated dimensions calculated from combinations of the original variables, *ie.*, along different PCs.

4.4.6. Discussion of results in relation to previous works investigating the effects of temperature on sclerite size

The results from this study, assuming that PC1 of the second PCAs were both indices of size, are contrary to the overwhelming majority of previous findings (see Ergens, 1976; Ergens, 1981; Ergens, 1983; Ergens, 1991; Ergens and Gelnar, 1985; Kulemina, 1976; Malmberg, 1970; Mo, 1991a, b and c, 1993), indicating that sclerite size is positively correlated with temperature for *G. bullatarudis* and not affected by temperature for *G. turnbulli.*

My choice of temperatures was carefully considered. The studies of Mo (1991b) and Jansen and Bakke (1991) were consulted as outlined in the next three paragraphs.

Mo (1991b) found a significant negative correlation between sclerite measurements of *G. salaris* on *Salmo salar* and temperatures ranging from

1.5°C to 20°C but did not find sclerites larger than those obtained from "the River Batnfjordselva, where the temperature was 0.0°C for about four months of the year (Mo, 1991[1])".

Jansen and Bakke (1991) recorded a significant negative correlation between average life span of *G. salaris* on *Salmo salar* at temperatures between 2.6°C and 18.0°C. However, although there was an appreciable increase in life span from 5.8 days at 16.5°C through 12.5 days at 13°C, to 31.4 days at 6.6°C, there was only a small increase to 33.7 days at 2.6°C. Indeed, these two means are not significantly different when compared in isolation using the Student t test. The results suggested that at temperatures below about 6°C, *G. salaris* begins to show signs of stress, although its intrinsic rate of increase remains above 0.

Taking the results of Mo (1991b) and Jansen and Bakke (1991) into account, the largest difference in sclerite size could be anticipated where *Gyrodactylus* are kept at a temperature which was not low enough to reduce the rate of reproduction below 0, but where indications of stress become apparent.

Scott and Nokes (1984) showed that the average expected life span of *G. turnbulli* under experimental conditions increased from 30°C, where the intrinsic rate of increase was well below 0, to 21°C, where the life span was 5.5 days. Previously Scott (1982) had shown that the average life span of *G. turnbulli* at 25°C was 4.2 days. From 21°C to 17°C the average life span decreased from 5.5 days to approximately 4.8 days but the intrinsic rate of increase was still above 0. The parasites and fish used in those experiments

[1] Mo (1991a) in references, section 4.5.

were of ornamental stock - the parasites were actually obtained from Harrods Department Store in London (Scott, pers. comm.) - and so were assumed to have had similar recent evolutionary histories to the stocks used in this work.

The results from Scott and Nokes (1984) suggested that *G. turnbulli* started to become stressed at temperatures below 21°C and above 17°C and, at the top of their temperature range, above 27.5°C. The incubator was set to maintain a water temperature of 19°C in accordance with the work of Scott and Nokes (1984) as a temperature slightly below where stress became apparent.

Atkinson (1994) found that out of 109 studies showing a significant effect of rearing temperature on ectotherm organism size, 83.5 % showed a decrease, 11.9%, an increase, and 4.6% a mixture of increases and decreases in organism size at a particular stage in development. The temperatures at which all these studies operated were restricted to a range which were not considered to stress the organisms involved.

Gyrodactylus sclerite size is fixed at birth except for negligible growth of the hamulus roots (Ergens, 1965a and b). If the opisthaptor sclerites are correlated with overall body size, one would suspect that the differences in sclerite size at different temperatures (Ergens, 1976; Ergens, 1981; Ergens, 1983; Ergens, 1991; Ergens and Gelnar, 1985; Kulemina, 1976; Malmberg, 1970; Mo, 1991a, b and c, 1993) are homologous with the general pattern shown in other ectotherms (Atkinson, 1994) and may be caused by an adaptive response to changes in other population parameters at different temperatures similar to those outlined in Sibly and Atkinson (1994).

Much of the interpretation of these findings depends on whether *G.*

turnbulli and *G. bullatarudis* were overly stressed at the lower temperature and whether they would show the same mortality and reproductive changes in response to temperature as Scott and Nokes (1984) found for *G. turnbulli*.

If the two species were overly stressed at the lower temperature, the general reduction in sclerite size may simply have been due to a lack of the ability to maintain sufficient embryonic growth under stress.

If the two species were not overly stressed at the lower temperature, the results either demonstrate genuine exceptions to the general findings for *Gyrodactylus* and other ectotherms, or they demonstrate a loss of an adaptive response to different temperatures due to the constant, narrow temperature range at which they have been kept for a considerable number of generations in artificial conditions.

Both these possible interpretations are of considerable interest and should be considered in future work.

4.4.7. Effects of different host species on sclerite size

The finding that no morphometric difference was observed between the sclerites of *G. bullatarudis* on *P. reticulata* and on *Xiphophorus* hybrids suggests, as previously discussed in Chapter 3, that changes in sclerite morphology are not caused by different micro environments provided by different host species.

The significant reduction in variation along the respective PC1 index between the *G. bullatarudis* on *P. reticulata* and those on *Xiphophorus* hybrids may be an indication of selection occurring among the latter group for *G. bullatarudis* lines more suited for survival on the atypical

host. These results provide support for a hypothesis that variation in sclerite morphology found between the same species of *Gyrodactylus* on different species of fish (Prost, 1991 and Mo, 1991a) are due to genetic drift between populations separated by barriers caused by differences in the behaviour or geographical distribution of their hosts rather than phenotypic differences caused by different microenvironments.

4.4.8. Sclerite variability among mono- and hetero-cultures

Comparisons of the PC1 indices for the secondary PCAs for the mono- and hetero-culture groups of both species showed significantly less variation in each respective mono-culture group, thus confirming the assumption that sclerite variability is, at least in part, influenced by genetic variation.

The significant difference in size exhibited between the sclerites of the *G. turnbulli* hetero- and mono-culture groups may have been caused by an initial selection of a *Gyrodactylus* from the hetero-culture with sclerites considerably larger than the mean as the origin of the mono-culture. Future studies could compare a number of different mono-cultures derived from the same hetero-culture to see whether any exhibit a trend towards smaller or larger sclerites. Furthermore, the development of a mono-culture could be followed, continued by propagation, to see whether there was any change in the mean size of the sclerites and to measure the rate of increase of their variation that would presumably occur following phases of sexual reproduction within the population (Harris, 1989 and 1993).

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CHAPTER 5

5. Trichrome staining of *Gyrodactylus* sclerites and soft tissues following fixation in ammonium picrate-glycerin, including an improved rendition of the haptoral bars of *G. turnbulli*.[1]

5.1. Introduction

Ergens (1969) recommended the ammonium picrate-glycerin method (Malmberg, 1957) for preparation of specimens of the lower Monogenea for study of their haptoral and genital sclerites. Ergens noted that the properties of ammonium picrate-glycerin "as a mounting media expire after about six months" and described a method for transferring specimens from ammonium picrate-glycerin into Canada balsam. The latter preparations retain their qualities for sclerite observation using phase contrast microscopy and have the advantage of archival permanence.

The disadvantages of the combined method of Malmberg (1957) and Ergens (1969) are that the soft tissues are not differentially stained and sometimes the detailed features of the ventral and dorsal bars are difficult to discern, particularly the attachment of the dorsal bar to the hamuli and the definition of the thin ventral bar membrane.

Kritsky *et al.* (1978) described a method of staining the hamuli connecting bars of formalin fixed *Gyrodactylus* specimens with Gomori's trichrome

[1] On 25. 1. 95 a manuscript based upon this chapter was accepted for publication in the *Journal of Helminthology* (1995) 69, 1 - 6.

solution. This method clearly differentiated the hamuli connecting bars and the ventral bar membrane, however, the hamuli and marginal hooks were unstained, the soft tissues were not clearly differentiated and the technique required individual specimens to be oriented with their ventral surfaces uppermost before mounting.

This chapter describes the incorporation of a simple trichrome staining technique during the transfer of *Gyrodactylus* specimens fixed and mounted in ammonium picrate-glycerin to a permanent mount which clearly demonstrates the hamuli, dorsal and ventral bars, cirrus spines and differentially stains the soft tissues.

5.2. Materials and Methods

Specimens of *G. bullatarudis* and *G. turnbulli* and the species tentatively identified as *G. rasini* from *Xiphophorus* hybrids which had been mounted in ammonium picrate-glycerin and used in the investigations described in the previous three chapters were transferred into Canada balsam using the following adaptation of the transferal method of Ergens (1969):

Each slide was placed in a petri dish containing pure ethanol for 5 min. The cover slip was then lifted off using a pair of blunt and a pair of fine forceps - one point of the fine forceps could be forced between cover slip and slide to initiate the lifting process while the two points of the blunt forceps were held firm against the opposite edge of the cover slip to stop it sliding. Flattened specimens stuck to either the cover slip and/or to the slide. The cover slip was placed up-side down next to the slide and both were left for a further 5 min. Both cover slip and slide were taken through the following procedures to avoid loss of any part of a specimen. They were transferred to a petri dish containing a 70: 30 mixture of pure ethanol:

recently filtered modified Mallory solution (phosphotungstic acid, 1g; orange G, 2g; aniline blue, 1g; acid fuchsin, 3g - dissolved in 200ml distilled water) for 1h to 1h 30mins. Both were then washed in two changes of pure ethanol and left in the second change for 5mins. They were then transferred to a petri dish containing a 1: 1 mixture of ethanol: xylene for 5mins before being placed in pure xylene for 2mins. The cover slip was placed, specimen downward, onto a drop of Canada balsam on a clean slide. A drop of Canada balsam was also placed over the specimen(s) on the slide and was covered with a clean cover slip. So, when there were more than one specimen on an original slide, each ammonium picrate-glycerin preparation usually resulted in two modified Mallory stained preparations.

Some specimens were split during separation of cover slip and slide. However, this was not serious because when single specimens split owing to one part of the body sticking to the slide and the other part to the cover slip, the pieces remained intact, allowing taxonomic investigation of the sclerites and anatomical examination of other parts of the body.

When only one specimen was mounted in ammonium picrate-glycerin on the original slide, the procedures were followed as above until the cover slip and slide were put in the first change of pure ethanol after their 1h to 1h 30mins in modified Mallory solution. The stained specimens could be seen with the naked eye or under a dissecting microscope enabling determination of whether it had remained intact, sticking to either the cover slip or to the slide, or whether it had split and stuck to both. If the specimen had remained intact, the procedures thereafter were applied to either the cover slip or to the slide depending on to which the specimen had stuck. If the specimen had split, both cover slip and slide were processed and two separate preparations resulted, each displaying one part of the body.

Stained specimens were observed and photographed using a Leitz Wetzlar photomicroscope.

5.3. Results

The hamuli and marginal hook sickles stained yellow except for the tendon attachment caps of the hamulus roots which stained red. Marginal hook shafts (distal halves) stained yellow but the marginal hook shaft (proximal halves) stained red. The dorsal and ventral bars stained red. The large cirrus spines stained both yellow and red but the small cirrus spines only stained red.

Of the soft tissues, muscles and tendons stained dark purple; cell nuclei, dark purple or pink; tegument, blue and gland cells, different shades of purple.

See Figs. 5.1 to 5.9.

Figs. 5.1 and 5.2. (over leaf) Hamuli and connecting bars of *G. rasini* (Fig. 5.1) and *G. bullatarudis* (Fig. 5.2) fixed and mounted in ammonium picrate-glycerin and then transferred to Canada Balsam incorporating staining with Modified Mallory solution. Note the clear definition of the ventral bars (vb), the ventral bar processes (vbp) and ventral bar membranes (vbm) - essential in differentiation of these two species. Also note the staining of the tendons associated with the hamuli (h); anterior hamulus tendons (aht), ring tendon through which the anterior tendons pass (rt) and the hamulus filaments (hf). Additional labelling; hamulus tendon attachment cap (htac), hamulus membrane (hm), dorsal bar (db) and the aperture of the hamulus membrane (ahm). Scale bar = 10μm.

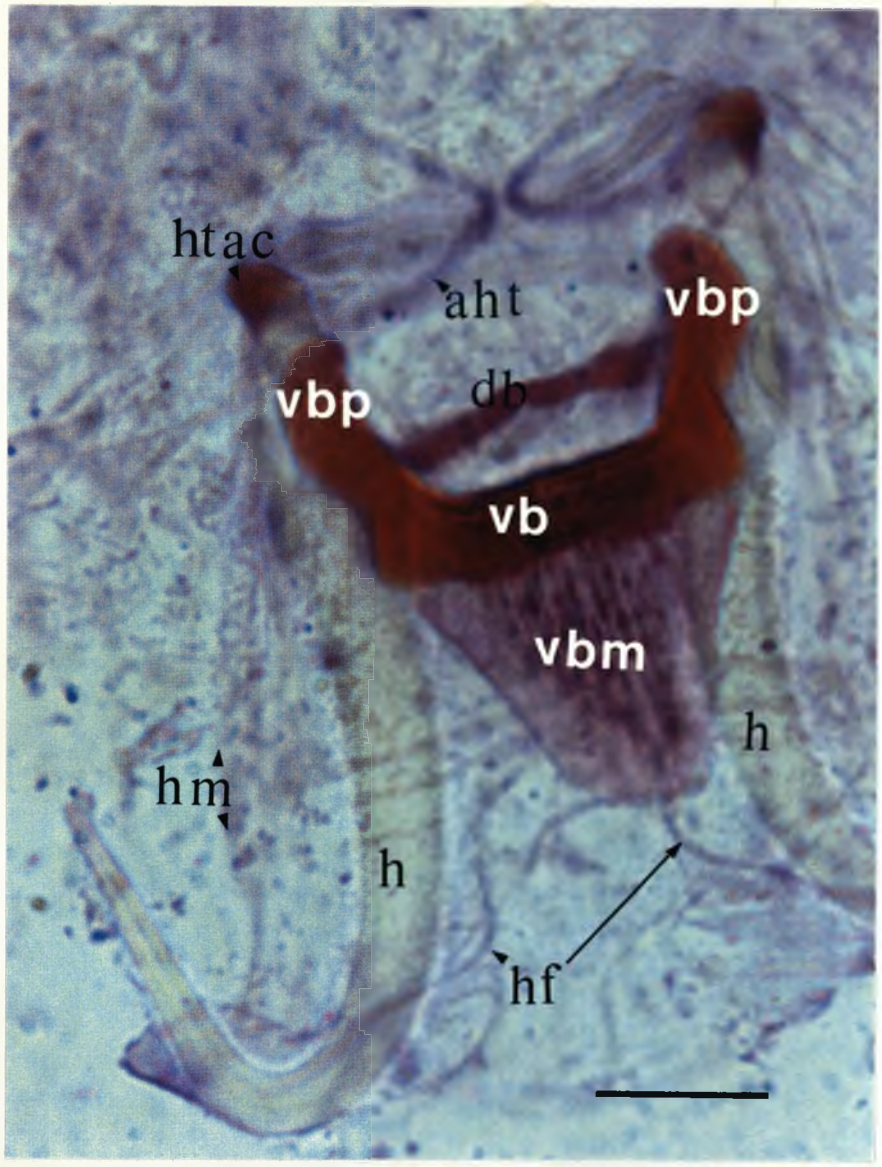

Fig. 5.1. (see page 6 - 4)

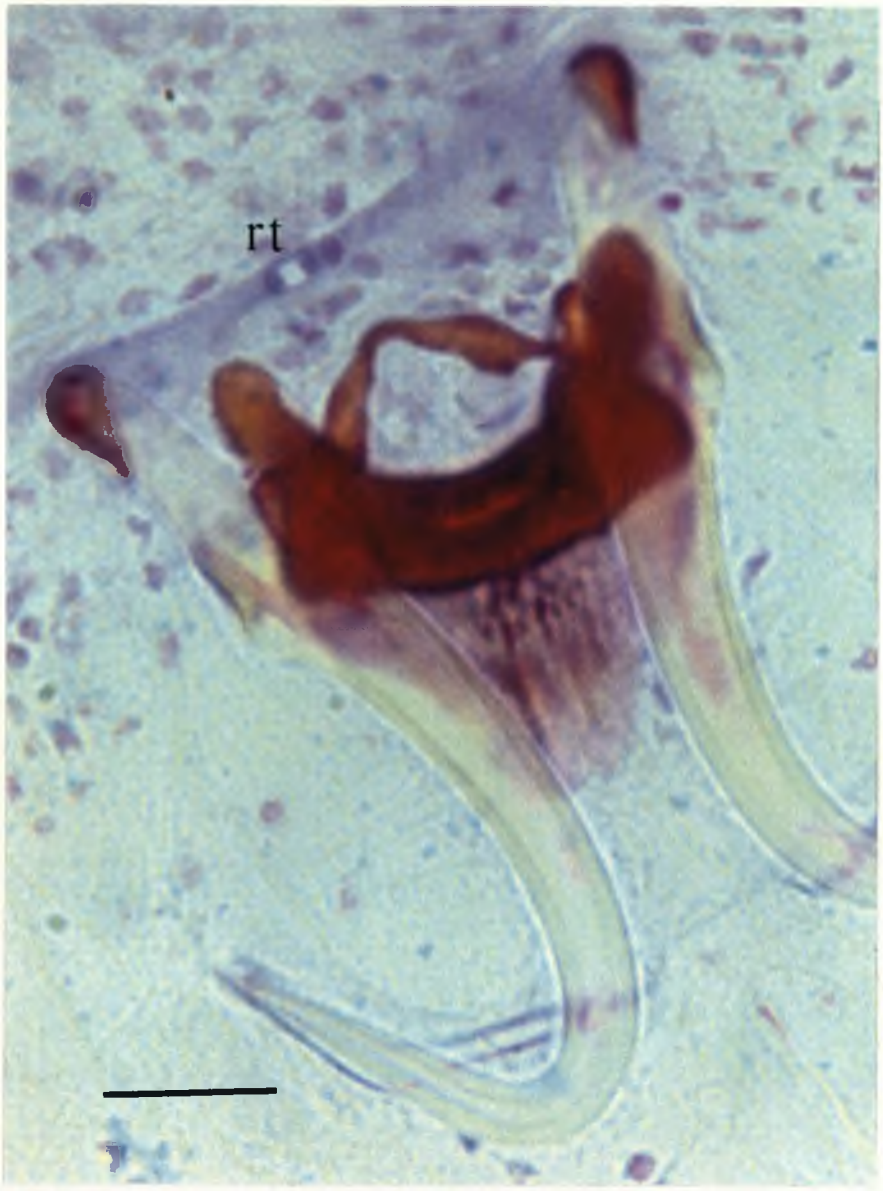

Fig. 5.2. (see page 5 - 4)

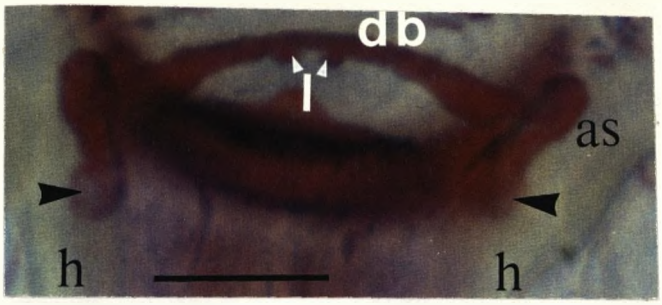

Fig. 5.3. Dorsal bar of *G. turnbulli* (ammonium picrate-glycerin, Modified Mallory, Canada balsam). Note the large attachment swellings (as) on either side of the dorsal bar and the median lugs (l) characteristic of this species. Also note the faintly stained part of the attachment swellings of the dorsal bar (arrowed), the dorsal bar posteriad supporting attachments (Malmberg, 1970). Additional labelling; dorsal bar (db) and hamuli (h). Scale bar = 10μm.

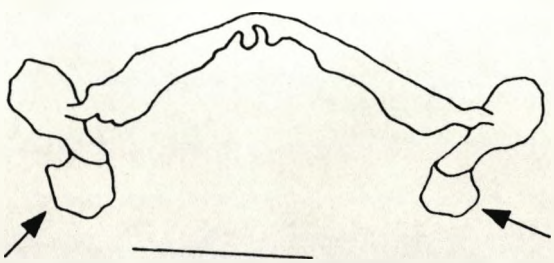

Fig. 5.4. Dorsal bar of *G. turnbulli* (ammonium picrate-glycerin, Modified Mallory, Canada balsam) drawn with the aid of a camera lucida. Note the faintly stained part of the attachment swellings (arrowed), the dorsal bar posteriad supporting attachments (Malmberg, 1970). Scale bar = 10μm.

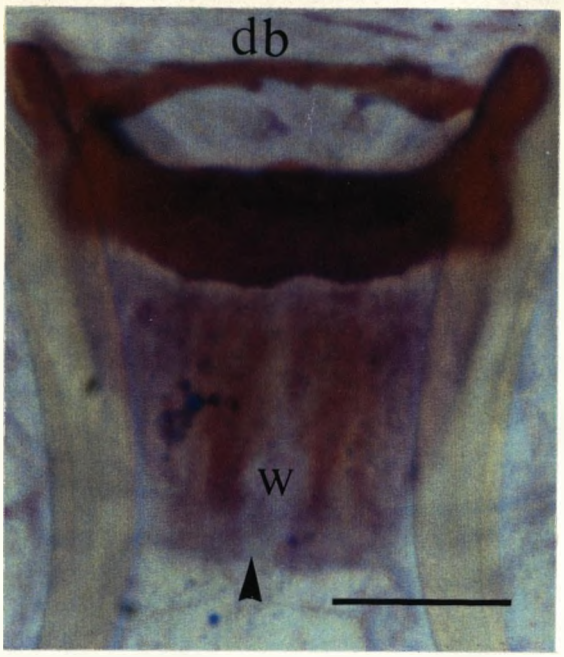

Fig. 5.5. Ventral bar of *G. turnbulli* (ammonium picrate-glycerin, Modified Mallory, Canada balsam). Note the median notch (arrowed) in the distal edge of the ventral bar membrane and the weakly stained area (w) with which it is associated. Additional labelling; dorsal bar (db). Scale bar = 10μm.

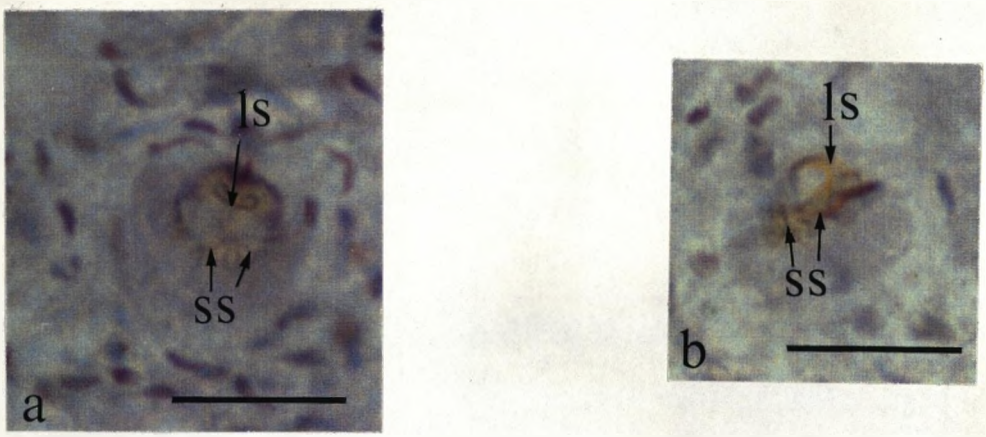

Fig. 5.6 a and b. Cirral bulb of *G. turnbulli* (ammonium picrate-glycerin, Modified Mallory, Canada balsam) viewing the spines, a., from above the opening of the ejaculatory duct and, b., from the side. Note the small spines (ss), which were stained red, and the large spine (ls), which was stained yellow and red. Scale bars = 10μm.

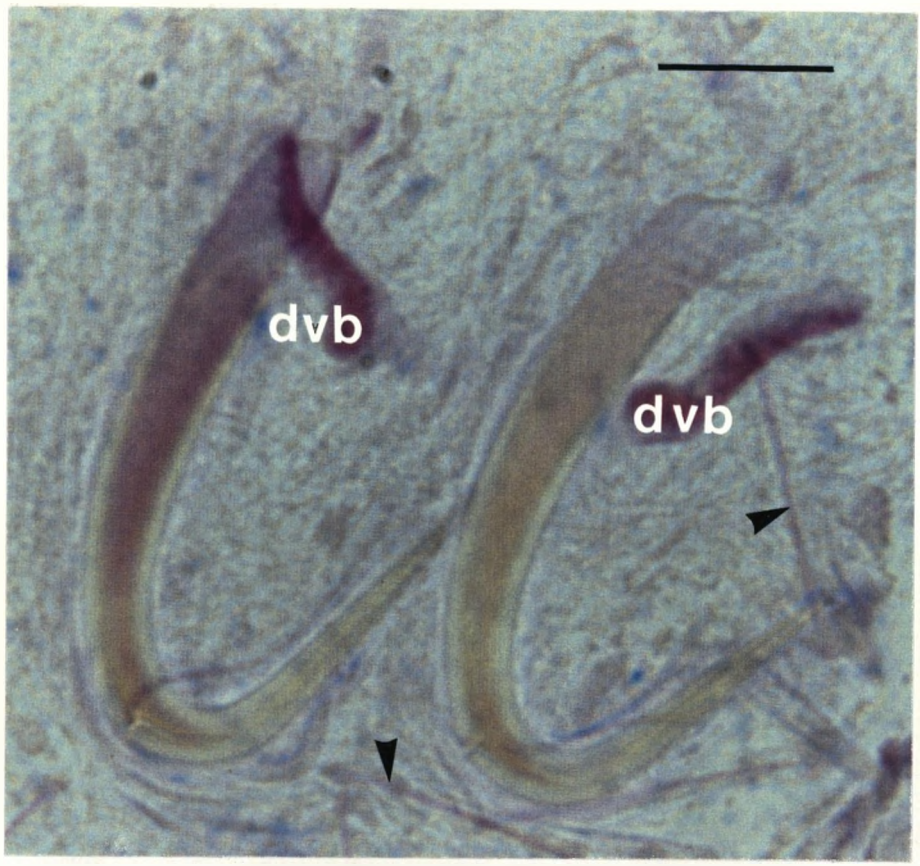

Fig. 5.7. Developing hamuli and ventral bar of an embryonic *G. rasini* (ammonium picrate-glycerin, Modified Mallory, Canada balsam) photographed through the wall of the uterus. Note the two lateral, heavily stained loci, red (dvb) of the developing ventral bar, the yellow-stained hamulus points (light grey) and the red-stained forming end of the hamuli (dark grey). Also note the embryonic marginal hook shafts, stained red (arrowed). Scale bar = 10μm.

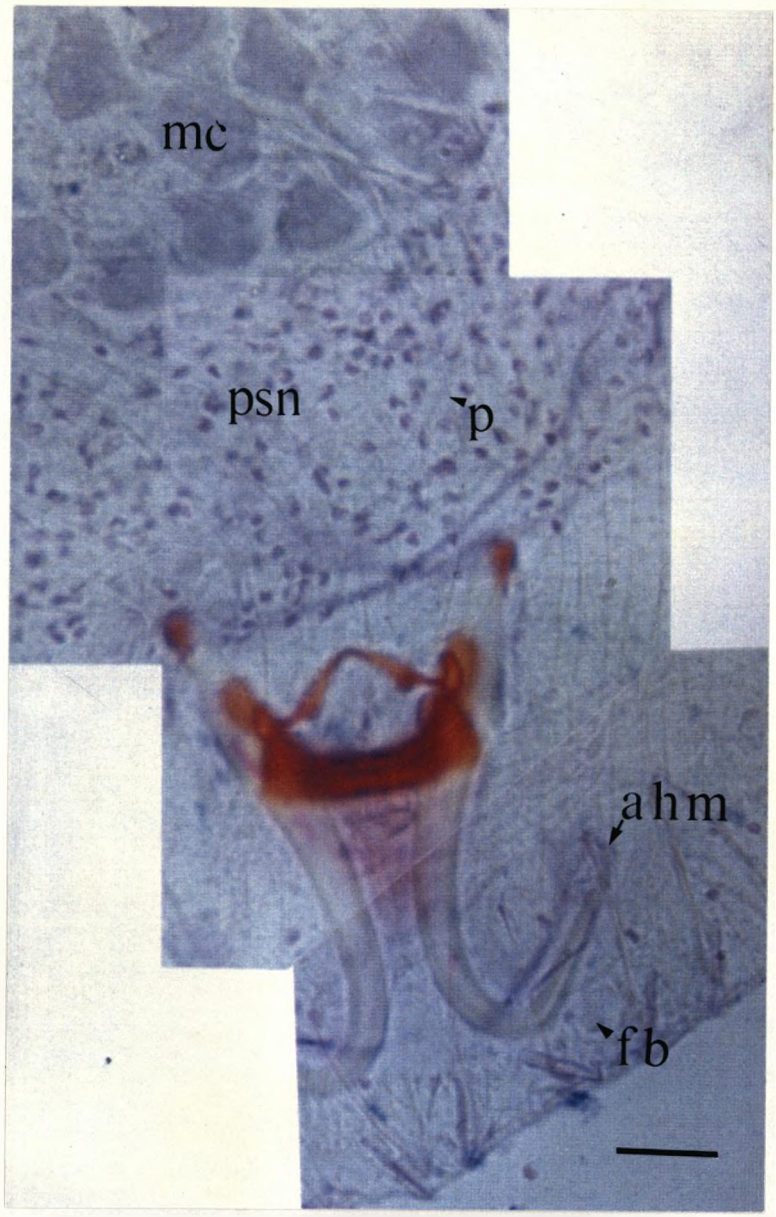

Fig. 5.8. Photomicrograph of the haptoral region of *G. bullatarudis* (ammonium picrate-glycerin, Modified Mallory, Canada balsam). Note the muscle cells (mc) with processes (p) attaching to marginal hooks, the hamuli, ventral and dorsal bars, the hamulus filaments, hamulus tendon attachment cap, flame bulb (fb) and the aperture of the hamulus membrane (ahm). Also note the pink-stained cell nuclei presumed to be associated with the nervous system (psn). Scale bar = 10μm.

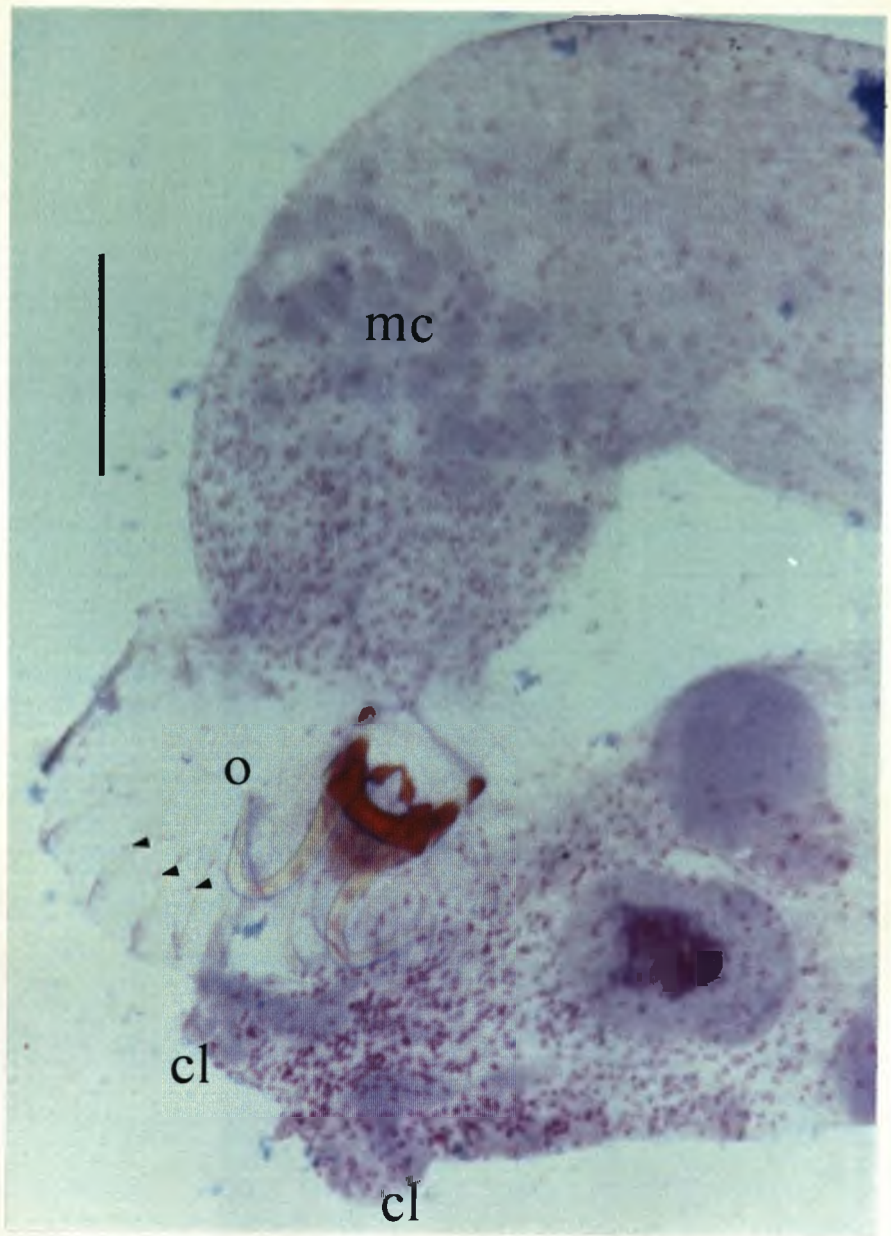

Fig 5.9. Anterior and posterior regions of a single *G. bullatarudis* (ammonium picrate-glycerin, Modified Mallory, Canada balsam). Note the pink-stained cell nuclei (three arrows) presumed to be associated with the nervous system. The greatest concentrations of these cells are between the band of muscle cells with processes leading to the marginal hooks (mc) and the opisthaptor (o) and, anteriorly, between the two cephalic lobes (cl) and around the pharynx (p). Also note that one of these cells (arrowed) is associated with each marginal hook. Scale bar = 50μm.

5.4. Discussion

The acid fuchsin in the trichrome stain coloured the hamuli connecting bars red. This was the same as the result obtained by Kritsky *et al.* (1978). In the present study, however, the hamuli were more clearly seen due to the yellow staining of ammonium picrate.

The staining of the ventral bars and the ventral bar membranes enhanced the differences in morphology of this structure in two easily confused species, *G. bullatarudis* and *G. rasini* (Figs. 5.1 and 5.2), see Chapter 3, 3.4.

Figs. 5.3 and 5.4 show the definition of the dorsal bar of *G. turnbulli* following staining with ammonium picrate and Modified Mallory solution. The swellings which articulate with the hamulus - a feature not illustrated in detail by Harris (1986) or by An *et al.* (1991) - are shown to consist of an anterior heavily stained part and a posterior faintly stained part. Only the heavily stained part of these swellings can be clearly differentiated by phase contrast microscopy following conventional fixation and mounting in ammonium picrate-glycerin. This poses the question as to whether the lightly stained part is thinner than the rest of the structure, is different in substance to the rest, or both. Malmberg (1970, figure 8, p. 30; figure 12, p. 49) described similar appendages to the dorsal bars of *G. kutikovana* Malmberg, 1964, *G. elegini* Bychowsky, 1948 and *G. macrochiri* Hoffman and Putz, 1964 naming them "posteriad supporting attachments". It is likely that dorsal bar posteriad supporting attachments are more common among *Gyrodactylus* species than presently recognised owing to their poor differentiation in unstained specimens.

Specimens stained with Modified Mallory showed a median notch in the ventral bar membrane which was made conspicuous by an association with

a weaker-stained, possibly thinner, area (Fig. 5.5). Re-examination of drawings made with the aid of a camera lucida of the haptoral sclerites of *G. turnbulli* under phase contrast microscopy confirmed the consistent appearance of a median notch in the distal edge of the ventral bar membrane (see Chapter 2, Fig. 2.2). This observation had gone unnoticed as a species characteristic. Paratype specimens from the Natural History Museum, London (BM(NH) 1985.3.15.2-7) (Harris, 1986) also showed the median notch in the distal edge of the ventral bar membrane, confirming that this is a species characteristic which had not been illustrated by Harris (1986) or An *et al.* (1991).

In addition to the attachment sclerites, the hard parts associated with the cirrus were also differentially stained by the new technique (Fig. 5.6a and 5.6b). This phenomenon was not reported by the two previous works describing stains for attachment sclerites of *Gyrodactylus* (Zdárska, 1976; Kritsky *et al.*, 1978). The cirrus spines are often of value to systematic evaluation of *Gyrodactylus* species (Malmberg, 1970) and so their staining is desirable. In many stained specimens the prostate gland and vesicular seminalis (Malmberg, 1957) were also visible.

The only sclerites not clearly stained by this method were the marginal hooks. Although these are stained yellow by ammonium picrate, they can be seen more clearly using phase contrast microscopy. However, the marginal hooks and hamuli of well flattened specimens prepared in ammonium picrate-glycerin showed up well when observed by phase contrast microscopy and so further staining was not required for their study.

Embryonic, developing sclerites stained differently to those of mature specimens. In particular, hamuli, which develop from the tip of the point back (Ergens, 1965a, 1965b, 1983), when mature, stained completely yellow,

except for their tendon attachment caps (see Figs. 5.1 and 5.2), and when developing, stained red near their forming ends and yellow nearer the points (Fig. 5.7). The marginal hook shafts of mature sclerites stained yellow near the articulation with corresponding marginal hook sickles and red near the region of their muscle attachment caps (Shinn *et al.*, 1993). However, when developing within the embryo, the marginal hook shafts appeared completely red (Fig. 5.7). Mo and Appleby (1990) showed a developing hamulus, isolated from the soft tissues by artificial digestion, with its shaft apparently surrounded by a sheath. They suggest that a sheath is present during the formation of the hamulus and is homologous with the structure they called the "hood-shaped structure" (after Malmberg, 1970), which I have termed the hamulus tendon attachment cap. It is possible that these sheaths surrounding developing hamuli stain red in modified Mallory solution and that similar sheaths surround marginal hook shafts during their development and have this same property. Alternatively, the different stained appearance of embryonic sclerites may be caused by changes in the composition of the sclerite protein(s) (Lyons, 1966; Kayton, 1983) during their development. "The general rule of trichrome staining is that the less porous tissues are coloured by the smallest dye molecule; wherever a dye of large molecular size is able to penetrate, it will always do so at the expense of the smaller molecule" (Bradbury and Gordon, 1982). As the molecular weight of acid fuchsin is greater than that of ammonium picrate and molecular weight can be used as an indication of molecule size (Bradbury and Gordon, 1982), it is possible that the protein(s) which make up the hamuli and marginal hook shafts become less porous as they mature.

One of the major advantages of this technique over the two previous methods described for staining the haptoral sclerites of *Gyrodactylus* (Zdárska, 1976; Kritsky *et al.*, 1978) was that the soft tissues stained differentially. The hamulus filaments and their association with the

openings of the hamulus membrane were clearly visible as were the sickle-filament loops of the marginal hooks and their association with the apertures of the finger-like processes of the dorsal lobe of the opisthaptor. Muscles and tendons associated with the opisthaptor and the attachment sclerites were well defined (see Figs. 5.1, 5.2 and 5.8).

Heavy concentrations of pink-stained nuclei were seen in the anterior and posterior of the preopisthaptoral part of the *Gyrodactylus*. Similar nuclei were associated with each marginal hook (Figs. 5.8 and 5.9), one nucleus per hook. Closer examination of these nuclei revealed that they were associated with a filament, possibly a muscle, attached to the muscle cap of the marginal hook shaft and the marginal hook sickle. Heavy concentrations of these nuclei correspond in position to the posterior nerve plexus associated with the opisthaptor, the region of the brain, nerve supply to the anterior and the nerve ring encircling the mouth described by Reuter (1987) for *G. salaris*. This evidence suggested that these nuclei were associated with the neuromuscular system.

In conclusion, the method described is a time efficient staining technique applicable to *Gyrodactylus* specimens on routine transferal from temporary to permanent mounting media. The technique should be used as an aid to taxonomic investigation of sclerites, in particular, when the morphology of the dorsal bar and ventral bar need clarification. However, for rendition of the taxonomically important marginal hooks (Malmberg, 1970), the use of ammonium picrate-glycerin prepared specimens and phase contrast microscopy (Malmberg, 1957; Ergens, 1969) is preferred. The new method has the following advantages over the method of Kritsky *et al.* (1978): it is applicable to specimens fixed and mounted in ammonium picrate-glycerin [a technique "which takes little time and a minimum of technical equipment" (Ergens, 1969)], in addition, the hamuli are stained yellow and the cirrus sclerites and soft tissues are differentially stained

thereby facilitating general anatomical study.

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CHAPTER 6

6. Freeze fixation-dehydration (Veltkamp *et al.*, 1994) as a method of preparation of *Gyrodactylus* for the SEM.

6.1 Introduction

Veltkamp *et al.* (1994) described "freeze dehydration" for preparing epizoonts and epiphytes on aquatic invertebrates and plants for study under the SEM. This method provided "instant immobilisation" of living materials. "Freeze dehydration" involves instantly freezing specimens by plunging living material into absolute ethanol at -18°C. Following this initial freeze, the temperature is slowly raised. As the ice melts slowly within the material, each water molecule is sequentially replaced by an ethanol molecule. Thus the specimens are fixed[1] and dehydrated by the technique.

Materials which have been fixed by methods other than freezing can be washed in distilled water and dehydrated using "freeze dehydration" (Dr. L. Gibbons, pers. comm.). This avoids the time and cost of taking specimens through a graded series of ethanol.

The term "freeze fixation-dehydration" is used here to discriminate the use

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[1] Abercrombie *et al.* (1992):

"FIXATION. (1) In microscopy, the first step in making permanent preparations of organisms, tissues etc., for study. Aims at killing the material with the least distortion. Solutions of formaldehyde and osmium tetroxide often used. Some artifacts of structure usually produced."

of the procedure described by Veltkamp *et al.* (1994) ("freeze dehydration") as a method of fixation and dehydration from its use in dehydrating specimens which have already been fixed.

This study compares fixation by freezing in ethanol with fixation using a chemical fixative, 10% neutral buffered formalin, in the preparation of *Gyrodactylus* for the SEM.

6.2 Materials and methods

G. bullatarudis on *P. reticulata* from the same source as those used in Chapter 3, section 3.2.1, were used. Infected fish were taken straight from the stock maintenance containers for preparation by one of the two methods outlined below.

Anaesthesia

In both of the protocols outlined below, infected fish were anaesthetised in 0.02% MS222 by the method described in Chapter 8, section 8.2.2. They were not, however, pithed prior to fixation. Anaesthetised fish could be handled easily with fine forceps resulting in very little disturbance to their infected external surfaces before fixation. The anaesthetic did not notably alter the mobility nor did it produce a reaction from the *Gyrodactylus.*

Fixation using 10% neutral, buffered formalin followed by freeze dehydration (formalin fixation - freeze dehydration)

One heavily infected fish (15mm, standard length) was anaesthetised using MS222 and then plunged into a vial containing 10% formalin (100ml of 40% formaldehyde in 900ml distilled water), neutral buffered with sodium

dihydrogen phosphate monohydrate (4g) and disodium hydrogen phosphate anhydrous (6.5g) (Bancroft and Stevens, 1982)(referred to hereafter as 10% NBF) and was left overnight in a refrigerator at 4°C. It was then washed in two changes of distilled water, dehydrated by plunging in -18°C ethanol and following the same procedure as used in "freeze dehydration" (described below), critical point dried using carbon dioxide, mounted on a stub, sputter-coated with 60% gold/palladium and observed in a Phillips 501B SEM using accelerating voltages of between 7.2 - 15kV.

Fixation by freezing followed by freeze dehydration (freeze fixation - dehydration)

Whole *G. bullatarudis* infected fish (10 - 15mm, standard length) were anaesthetised in 0.02% MS222 and plunged in a vial containing 100% ethanol which had been cooled to about -18°C in a freezer. Specimens were left in the same freezer for 3h, put into a refrigerator overnight at 4°C and then placed into a change vial of -18°C, 100% ethanol and stored at 4°C. Finally, specimens were prepared and viewed under the SEM as described above.

A sample of parasites was removed from each fish viewed using the SEM. This was accomplished by holding the working surface of a second stub covered with double-sided Sellotape close enough to the fish on the original stub to touch the exposed surfaces of *Gyrodactylus* attached to this fish so that they stuck to the Sellotape. The flukes were gently detached by withdrawing the second stub. This procedure was performed with the aid of a binocular microscope. These preparations were sputter-coated to allow investigation of the attachment surface of opisthaptors in functional positions.

Comparisons were made between the two techniques and with observations of wet mounts of live specimens attached to pieces of fish fin and to fish scale, photographed using a Leitz Wetzlar research photomicroscope. In order to compare the relative amount of shrinkage in specimens prepared by formalin fixation and freeze fixation, two measurements were taken from each of 40 *Gyrodactylus*, 20 prepared by formalin fixation and 20 by freeze fixation. The measurements, illustrated in Fig 6.1, were taken from the attached opisthaptors of specimens of which all the marginal hooks were piercing the surface of the fish epidermis. 20 specimens prepared using 10% NBF and 20 specimens prepared by freeze-dehydration were measured. There were two possible values of "measurement A" (see Fig. 6.1) from each specimen but only one was chosen arbitrarily.

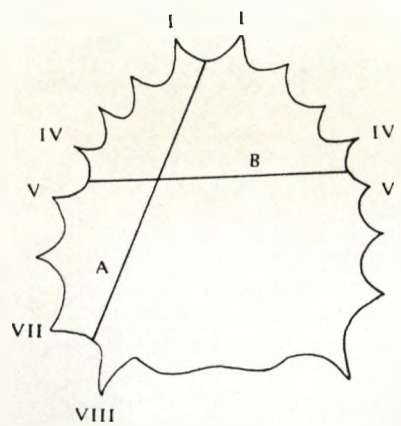

Fig. 6.1. Schematic diagram to show the two measurements of attached opisthaptors of *G. bullatarudis* used to compare specimens prepared by freeze fixation and 10% NBF fixation. Measurement A was taken from a point on the edge of the dorsal lobe of the opisthaptor mid-way between marginal hooks VII and VIII to a point on the edge of the dorsal lobe mid-way between marginal hooks I (left) and I (right). Measurement B was taken from a point on the edge of the dorsal lobe of the opisthaptor mid-way between marginal hooks IV and V (left) to a point on the edge of the dorsal lobe mid-way between marginal hooks IV and V (right).

6.3. Results

Material fixed using 10% NBF showed conspicuous differences to both the material prepared by freeze fixation and the live specimens. The tegument of the opisthaptor and preopisthaptoral parts of the body was conspicuously more wrinkled than the material prepared by freeze dehydration (compare Figs. 6.2 and 6.3 with Fig. 6.4). The morphology of the opisthaptors of flukes attached to the anterior of the fish were conspicuously different to those attached posterior to the gill opercula. The two flukes in Fig. 6.3 each exhibit one of these two types of appearances in attachment. Flukes attached anteriorly generally showed pronounced extension of the finger-like processes of the marginal hooks with comparatively little distortion of the fish epidermis, whereas the typical appearance of the opisthaptors of flukes attached posteriorly was of less extended finger-like processes but with greater distortion of the fish epidermis.

Flukes prepared by freeze fixation were less wrinkled than those fixed in 10% NBF (see Figs. 6.4 and 6.5). There were some instances of the finger-like processes of the marginal hooks being more extended than others but consistent differences in appearance of flukes attached to the anterior and posterior of the fish were not observed.

Fig. 6.2. *G. bullatarudis* fixed using 10% NBF on the head of the host fish. Note the deeply wrinkled tegument and the long and thin finger-like processes of the marginal hooks (two are arrowed). (1 micromarker = 10μm)

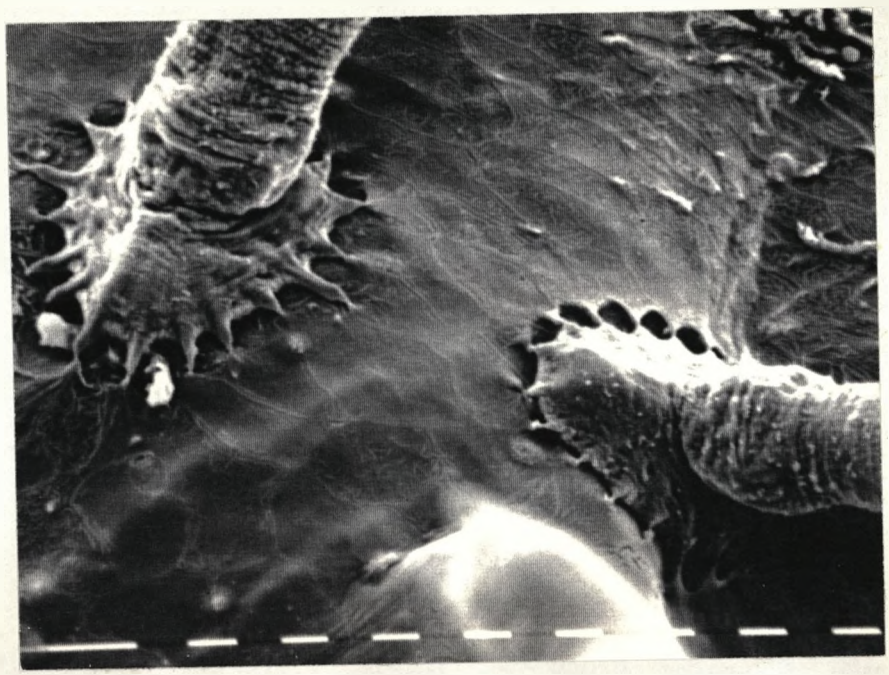

Fig. 6.3. Opisthaptoral attachments of two *G. bullatarudis* fixed using 10% NBF. The attachment on the left is characteristic of those observed in the head region of the fish. Note the long, thin appearance of the finger-like processes of the marginal hooks and that the pull of the marginal hooks does not markedly stretch the fish epidermis. The one on the right is characteristic of those observed in more posterior regions. The opisthaptor is more compact, the finger-like processes of the marginal hooks are less spread but they cause distinct distortions of the fish epidermis. (1 micromarker = 10μm)

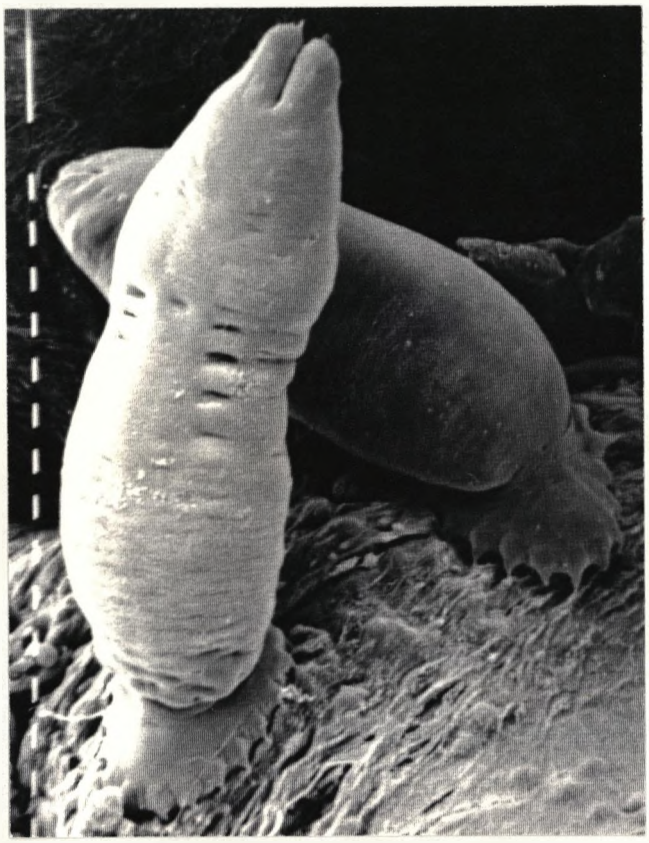

Fig. 6.4. Two *G. bullatarudis* prepared for the SEM by freeze fixation. Note the less wrinkled appearance in comparison to the parasites in Figs. 6.2 and 6.3 and the appearance of the opisthaptors are intermediate between the two extremes shown in Fig. 6.3. (1 micromarker = 7μm)

Fig. 6.5. *G. bullatarudis* on a fish prepared for SEM by freeze dehydration. Note the very extended nature of the central parasite. This was one of the most extended of all the flukes observed. The photograph is illustrative of the variety of postures in which flukes were frozen on preparation by this technique. (1 micromarker [arrowed]= 6μm)

Measurement A averaged 51.36μm (standard deviation [=SD], 4.52μm) and B, 37.75μm (SD, 3.90μm) for the 20 10% NBF fixed specimens and 61.81μm (SD, 4.00μm) and 48.67μm (SD, 4.79μm), respectively, for the 20 freeze dehydrated specimens. Both average measurements for the 10% NBF fixed specimens were about 20% shorter than those of the freeze fixed specimens and were significantly different according to the Student t test $(P < 0.05)$.

The appearance of the opisthaptors of live flukes was more similar to those of flukes prepared by freeze fixation (see Fig. 6.6). Their marginal hook finger-like processes were not as spread out as those specimens attached to the anterior of the fish fixed with 10% NBF and the tension from the marginal hooks did not distort the fish epidermis as much as those specimens attached to the posterior of the fish fixed with 10% NBF.

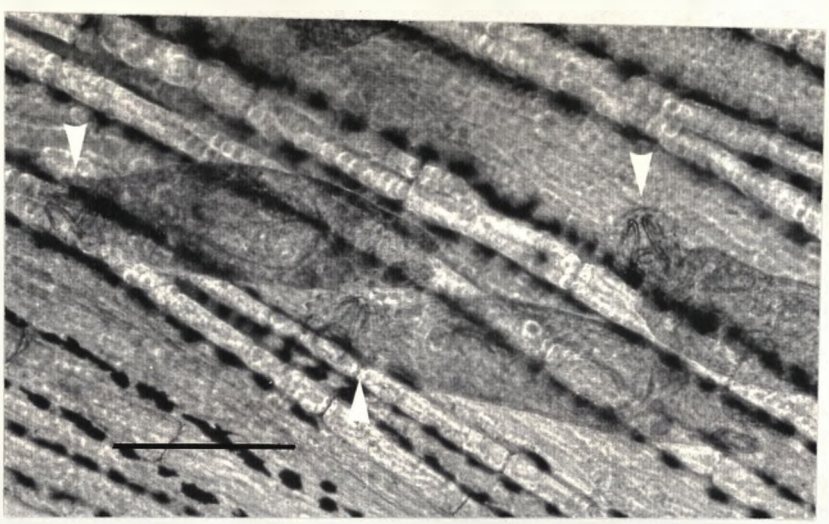

Fig. 6.6. Photomicrograph of living, attached *G. bullatarudis* viewed through the pectoral fin of a *P. reticulata* (three attached opisthaptors are arrowed). Note the spread of the marginal hooks and their finger-like processes are closer to those shown in Fig. 6.4 than those shown in Figs. 6.2 and 6.3. (Scale bar = 100μm)

The hamulus membranes of specimens removed from the infected fish which had been fixed using 10% NBF were consistently better preserved than those of specimens removed from the fish prepared using freeze fixation (compare Figs. 6.7 and 6.8). Notice the characteristic cracking hamulus membrane of Fig. 6.8 (arrowed).

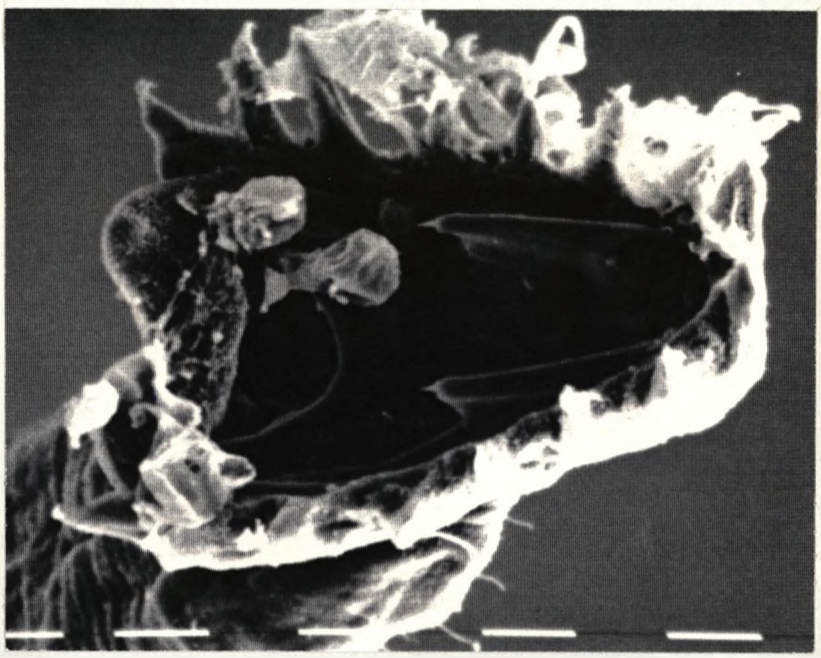

Fig. 6.7. Underside of the opisthaptor of a *G. bullatarudis* in a functional position following fixation with 10% NBF. (1 micromarker = 8μm)

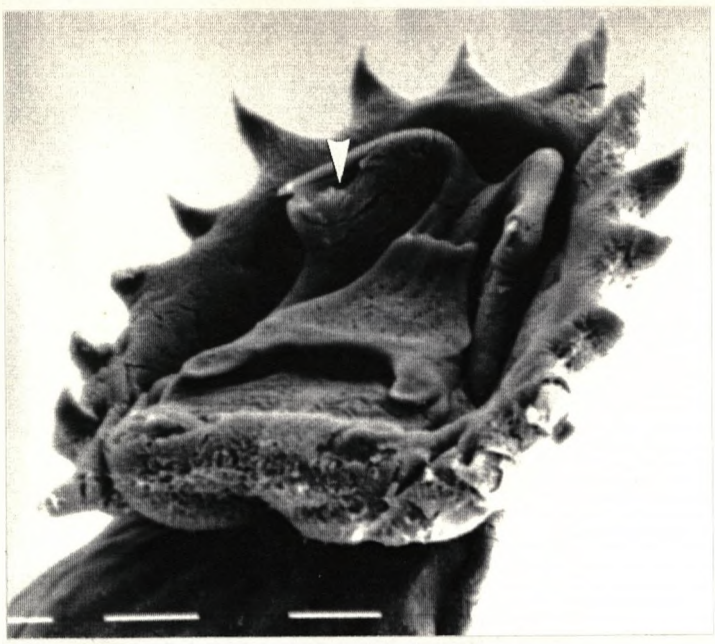

Fig. 6.8. Underside of the opisthaptor of a *G. bullatarudis* prepared by freeze dehydration. Note the characteristic cracking of the hamulus membrane (arrowed). (1 micromarker = 10μm)

Surface features such as ciliary sensory receptors of the parasites and ridges on the fish epidermal cells were preserved using both methods. However, wrinkling of the tegument of *G. bullatarudis* specimens fixed with 10% NBF obscured details of some ciliary sensory receptors.

6.4. Discussion

The wrinkled appearance of the tegument and the shrunken appearance of the opisthaptor in attachment of specimens fixed using 10% NBF were

similar to illustrations from previous works: *G. avalonia* on *Gasterosteus aculeatus* and *G. salmonis* on *Salmo gairdneri* fixed using 10% formalin (Cone and Odense, 1984); *Ooegyrodactylus farlowellae* on *Farlowella amazonum* fixed in 1% osmium tetroxide and post fixed in 2% gluteraldehyde (Harris, 1983); *G. colemanensis* and *G. salmonis* on *Salvelinus fontinalis* fixed in 10% formalin (Cone and Cusack, 1988); *G. aculeati* on *Gasterosteus aculeatus* fixed in 5% gluteraldehyde and post fixed in 2% osmium tetroxide (Schmahl and Taraschewski, 1987).

The differences in appearance of the opisthaptors of *G. bullatarudis* on different parts of the fish following fixation suggested that either a different method of attachment was used by the parasites on these different regions and/or the mechanical properties of the epidermis were different in these regions before fixation and/or after fixation. Attached opisthaptors of specimens prepared by freeze fixation and of live flukes were of relatively constant form irrespective of their situation on the host. The differences in appearance of the 10% NBF fixed specimens were therefore considered to be artificial and caused by fixation as explained below.

Shrinkage of the opisthaptor and preopisthaptoral parts occurred due to 10% NBF fixation and this caused wrinkling of the tegument all over the body of the fluke. It also resulted in at least an approximate 20% reduction in diameter of the main body of the dorsal lobe of the opisthaptor in attachment which caused the two characteristically different appearances of the opisthaptor on different regions of the body of the fish. In the anterior regions of the fish, where the epidermis was able to resist the extra tension exerted by the attachment of each marginal hook, there was comparatively little movement of the marginal hooks themselves and the shrinkage caused the finger-like processes of the marginal hooks to stretch and appear more spread out and thinner than in life. Where the

fish epidermis was less able to resist this extra tension caused by shrinkage, stretching of the epidermis occurred, resulting in the characteristic distortions shown in Fig. 6.3.

The use of an anaesthetic to immobilise infected fish prior to immersion in the respective fixing medium was an important part of both protocols. The external, infected surfaces of the fish suffered far less disturbance than if the fish had had to be caught, pithed and plunged into fixing solutions whilst still active. Although the anaesthetic did not noticably affect movement of the infecting *Gyrodactylus*, it was appreciated that gyrodactylids do respond to changes in water currents (Harris and Tinsley, 1987) and so observations by SEM and of live flukes could not be considered as demonstrative of the behaviour of flukes on a swimming fish in a natural situation.

Plunging infected fish into cold ethanol at -18°C instantly froze the monogeneans on the external surface of the fish. Attached flukes were found to be in a variety of positions and states of contraction and extension (see Fig. 6.5) so the method appeared to provide instant immobilisation of the parasites under study. The size and shape of flukes prepared using 10% NBF were found to be more uniform in shape and it was doubtful that they had been instantly immobilised and had probably reacted to the fixative before death.

An outline of the theory of freeze dehydration was given by Veltkamp *et al.* (1994) and a comparison was made between freeze dehydration and freeze drying, for which "the quenching liquid is much colder (below -60°C), to form smaller ice crystals". The point was made that "although larger ice crystals are formed in the freeze dehydration technique, they affect only the contents of the cell, and not its outward appearance". However, the

disruption of the structural contents of cells or multinucleate syncitia (in helminth tegument, for example) on dehydration would result in the material becoming more brittle than if it had been prepared by fixation or freeze drying and therefore more prone to damage during subsequent manipulation. This may be the explanation why, on observation of the undersides of opisthaptors following removal of flukes from their host fish, the hamulus membranes of *G. bullatarudis* prepared by freeze dehydration were more often found to be damaged than those of specimens prepared following 10% NBF fixation.

Results from this experiment have shown that for the study of gross external functional morphology, freeze fixation is a better preparatory procedure for the investigation of gyrodactylids than conventional tried fixation techniques. It is also less time consuming as it does not require a chemical fixation or post fixation scheme or the use of a conventional alcohol series for dehydration. However, the use of 10% NBF is a better method of preserving the integrity of fine, membranous tissue like the hamulus membrane.

6.5. References

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CHAPTER 7

7. A comparison of the opisthaptor attachment of *G. turnbulli* and *G. bullatarudis.*

7.1 Introduction

The method of opisthaptor attachment of *G. alexanderi* Mizelle and Kritsky, 1967 on *Gasterosteus aculeatus* was described in detail by Lester (1972). Since then, attachment of only nine species of *Gyrodactylus* have been studied in detail (Harris 1982, Cone and Odense 1984, Cusack and Cone 1986, Cone and Cusack 1988, Cone and Wiles 1989). Considering the wide range of sclerite morphologies present within the genus (Malmberg 1970) there is a deficit of studies of *Gyrodactylus* relating details of morphology to the basic method of attachment described by Lester (1972).

This chapter is a comparative study of opisthaptor morphology and attachment of *G. bullatarudis* and *G. turnbulli.* Although the attachment of *G. bullatarudis* has been studied previously (Harris 1982, Cone and Odense 1984), a re-examination is justified by a recent preparatory technique [freeze fixation - dehydration (see Chapter 6)] for the SEM which worked particularly well and by the need for direct comparison with *G. turnbulli.*

7.2. Materials and Methods

The origins of the parasites used in this work are described in Chapter 2, section 2.2.1 and their maintenance is described in section 2.2.2.

Live *G. bullatarudis* and *G. turnbulli* attached to pieces of fish skin on scale and fin mounted in water with a cover slip in place were observed and photographed using a Leitz Wetzlar research photomicroscope.

Observations of other live specimens attached to fish fin and scale were recorded on video tape using a Sony video camera on the research microscope. The video recordings of attached flukes were studied repeatedly.

As this was a study of functional morphology, instantaneous fixation by freezing (freeze fixation - dehydration) was used in preference to a chemical fixative for preparation of specimens for the SEM (see Chapter 6, section 6.4, last paragraph). Heavily infected fish were prepared by freeze fixation - dehydration (Veltkamp *et al.*, 1994) as described in Chapter 6, section 6.2. Investigation of the impressions left by opisthaptors on the skin of fish was done following their removal using the method described in Chapter 6, section 6.2.

Comparisons of the attachment sclerite morphology of *G. bullatarudis* and *G. turnbulli* made in Chapters 2 to 4 were also used in this work.

7.3. Observations

The basic method of attachment of both these *Gyrodactylus* species was as described by (Lester 1972) for *G. alexanderi.* The opisthaptor can be likened to a tent. The marginal hooks act as pegs to which tension is applied, part of the reaction to the tension resulting in the hamuli being directed downward, pressing, but not piercing the host skin (see Cone and Wiles 1989) (Figs. 7.1, 7.2, 7.3, 7.4 and 7.5). There are, however, differences between the two species which mainly concern attachment of the marginal

hooks.

The finger-like processes which the marginal hooks subtend are generally longer for *G. turnbulli* (Figs. 7.1 and 7.6) than for *G. bullatarudis* (Fig. 7.2 and 7.7) and are spread further apart.

In addition, the angle of insertion (see Fig. 7.8A and 7.8B) is generally smaller for *G. turnbulli* (α_{Gt}) than it is for *G. bullatarudis* (α_{Gb}). This can be seen on the freeze - fixed flukes shown in Figs. 7.1 and 7.2 and also on the living flukes shown in Figs. 7.9 and 7.10.

G. bullatarudis tends to pull the fish epidermis upward more than *G. turnbulli* - the marginal hooks and their supporting processes are retracted upward and into the dorsal lobe of the haptor so that the epidermis is stretched upward and inward in cone shapes, the marginal hooks supporting the cone apices (Fig. 7.11). This particular kind of contortion of epidermal cells was very rarely observed in attachments of *G. turnbulli* where pierced cells were mainly stretched horizontally parallel to the surface of the fish (Fig. 7.12). Conical contortions of the host epidermis were also seen during attachment of live flukes, particularly *G. bullatarudis*, although shrinkage of host epidermis slightly exaggerates this effect in material prepared for the the SEM.

Impressions left by the hamuli of *G. bullatarudis* were found generally to be deeper into the host epidermis than those of *G. turnbulli* (see Figs. 7.4 and 7.5).

A degree of versatility in attachment was observed, see for example Fig. 7.13 showing a *G. bullatarudis* attached to the edge of a pectoral fin.

The opisthaptor is constantly moving in attachment. Each marginal hook, whilst remaining embedded in the host epidermis, is capable of independent movement. This allows alteration in the muscular tensions applied to each marginal hook to compensate for the changes in direction of detachment forces acting on the haptor from both external (*eg.* arising from water currents) and self (*eg.* caused by movement of the upper, preopisthaptoral body of the parasite) origin.

Fig. 7.1. *G. turnbulli* attached to *Poecilia reticulata*. (1 marker = 10μm)

Fig. 7.2. *G. bullatarudis* attached to *P. reticulata*. (1 marker = 7μm)

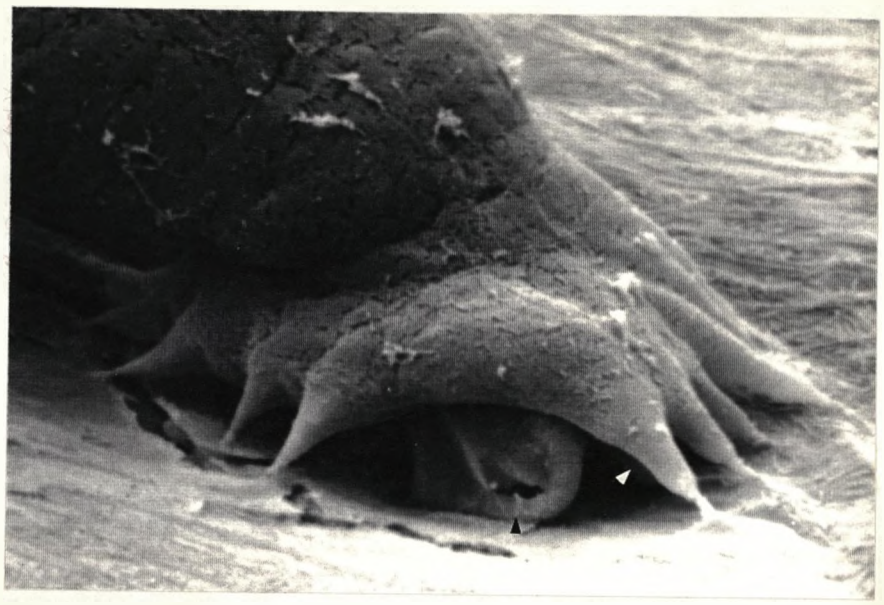


Fig. 7.3. Attached opisthaptor of *G. turnbulli*. While the marginal hooks pierce the fish epidermis, the hamuli (arrowed) do not. (1 marker = 10μm)

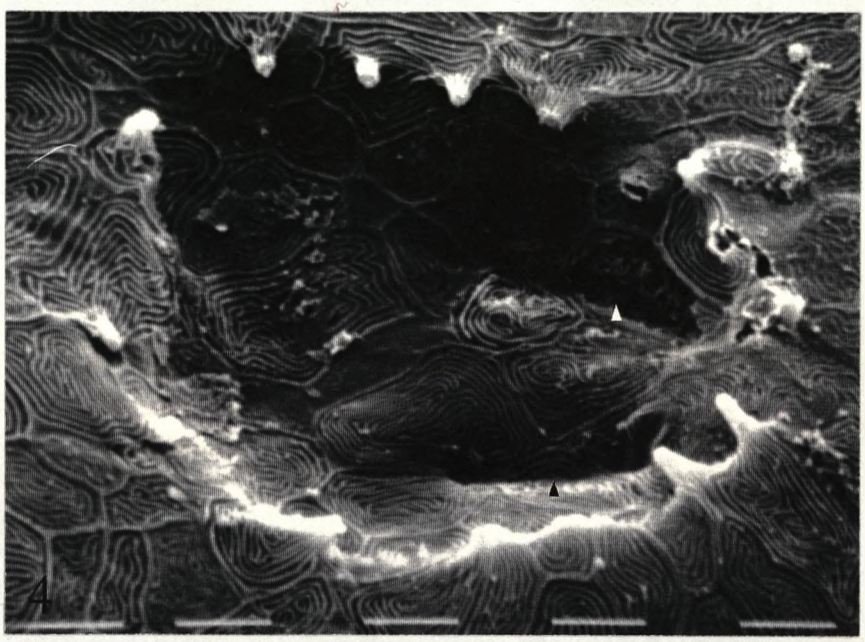


Fig. 7.4. Scar from the attachment of a *G. bullatarudis* left after its removal using double-sided Sellotape. Tear marks have only been left by the attachment of the marginal hooks whilst the hamuli have left impressions (arrowed) indicating that they did not pierce the host epidermis. (1 marker =10μm)

Fig. 7.5. Scar from the attachment of a *G. turnbulli* left after its removal using double-sided Sellotape. The hamuli impressions do not appear as deep as those left by the *G. bullatarudis* removed to leave the scar in Fig. 7.4. (1 marker = 10μm)

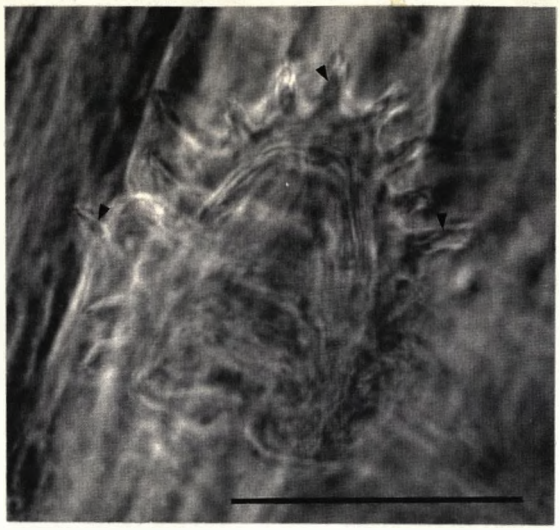


Fig. 7.6. Photomicrograph of the underside of an attached opisthaptor of *G. turnbulli* viewed through the caudal fin. The marginal hook processes (arrowed) are relatively more extended in comparison to those of *G. bullatarudis* (Fig. 7.7). (marker bar = 50μm)

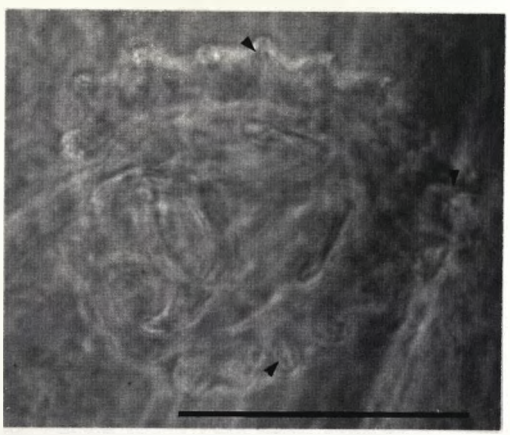


Fig. 7.7. Photomicrograph of the underside of an attached opisthaptor of *G. bullatarudis* viewed through the pectoral fin. The marginal hook processes (arrowed) are relatively less extended in comparison to those of *G. turnbulli* (Fig. 7.6). (marker bar = 50μm)

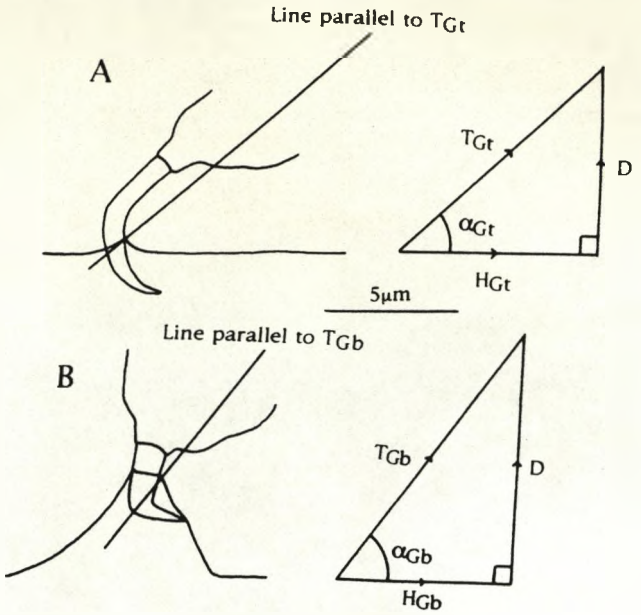
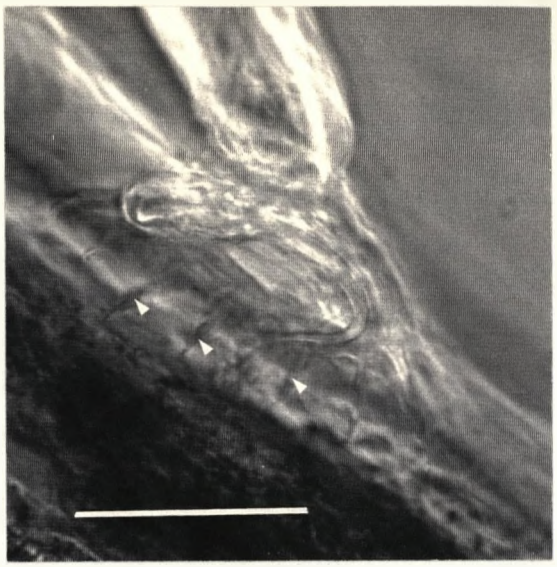


Fig. 7.8. Diagram illustrating marginal hook attachment of, A, *G. hullatarudis* and, B, *G. turnbulli*, showing α, the angle of insertion, T, the tension acting through the marginal hook when a detachment force, D, acting perpendicular to the surface of the fish, is resisted, and, H, the horizontal component of that force.



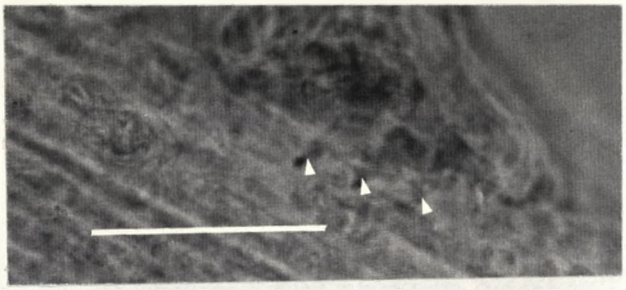


Fig. 7.9. (top) Photomicrograph of the lateral view of an attached opisthaptor of *G. turnbulli* attached to a pectoral fin. The marginal hook processes (arrowed) are relatively more extended in comparison to those of *G. bullatarudis* (Fig. 7.10). (marker bar = 50μm)

Fig. 7.10. (bottom) Photomicrograph of the lateral view of an attached opisthaptor of *G. bullatarudis* attached to a pectoral fin. The marginal hook processes (arrowed) are relatively less extended in comparison to those of *G. turnbulli* (Fig. 7.9). (marker bar = 50μm)

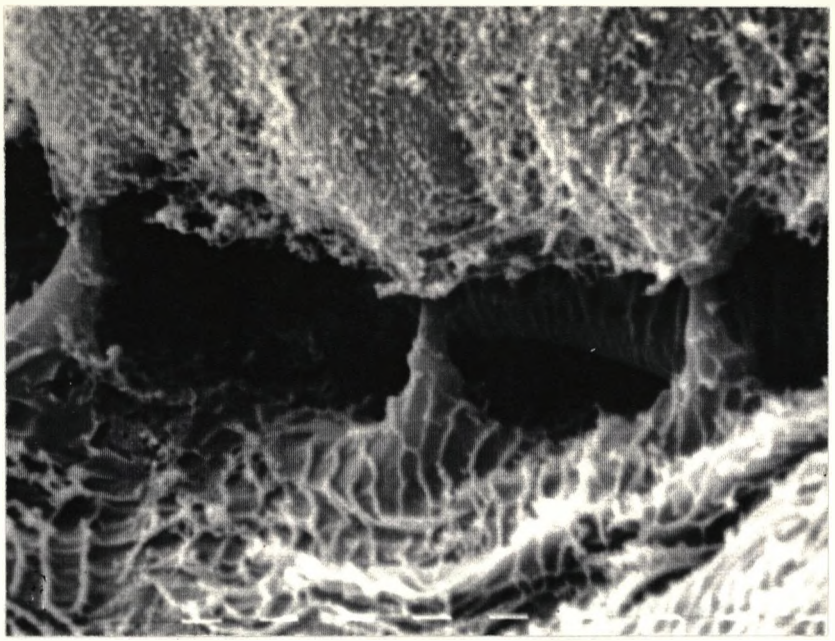


Fig. 7.11. Marginal hooks of *G. bullatarudis* in attachment. (1 marker = 1μm)

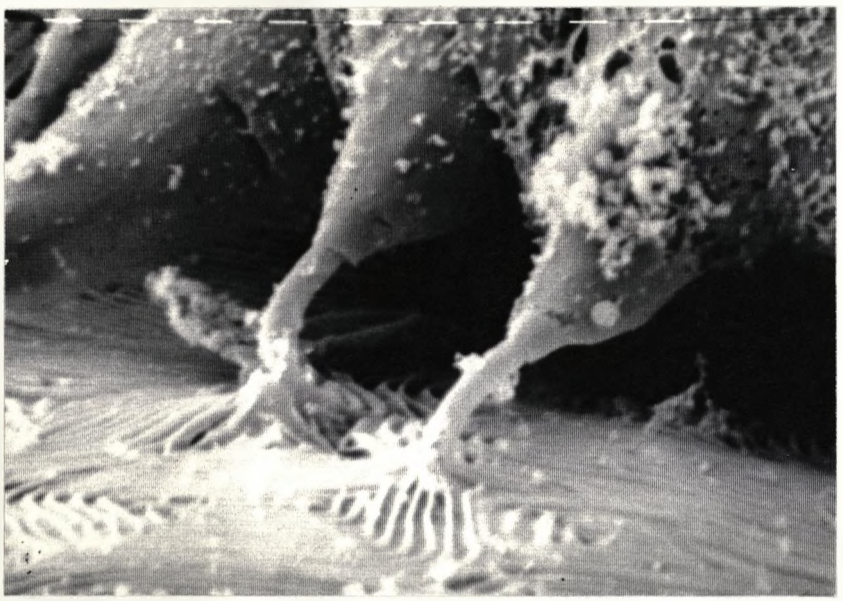


Fig. 7.12. Marginal hooks of *G. turnbulli* in attachment. (1 marker = 1μm)

Fig. 7.13. Opisthaptor of *G. bullatarudis* attached to the edge of a pectoral fin showing the ability of the attachment apparatus to adjust on uneven surfaces. (1 marker = 10μm)

7.4. Discussion

7.4.1. Differences between the opisthaptor attachments of *G. bullatarudis* and *G. turnbulli*

The function of the hamuli / dorsal bar / ventral bar complex was the same for both species and indeed the dimensions and appearance of the hamuli are very similar (see the drawings of sclerites and measurement tables in Chapter 2). Despite their appearance as attachment hooks, the hamuli are predominantly used as support structures (the central "tent pole" in Cone and Wiles' (1989) tent analogy). However, Lester (1972) noted (with reference to *G. alexanderi*) their ability to passively pierce the skin and

protect against dislodgement when strong shearing forces, directed parallel to the fish surface, acted on flukes. Mo (1994), in studies on *G. salaris* and *G. derjavini* on salmonids, reported finding punctures in the host epidermis caused by the hamuli. He said that the hamulus points were protruded from the hamulus membrane and used to assist attachment when flukes were giving birth or feeding. No evidence was provided during my study to confirm or contradict this. However, one *G. bullatarudis* was fixed whilst giving birth and was found to have one hamulus point piercing the fish epidermis. This fluke and its daughter, protruding from the birth pore, had been disturbed when their host fish was mounted on a stub and it was not clear whether the hamulus pierced the host skin during the SEM preparatory procedures or whether this had occurred during life.

Although both species of *Gyrodactylus* used the same basic method, small differences were observed in the general appearance of their opisthaptors in attachment. These differences concerned the lengths and widths of the dorsal lobe finger-like processes, the degree to which they were extended and the angles which they subtended the fish surface.

G. bullatarudis : The dorsal lobe processes are shorter and less spread out than those of *G. turnbulli* but the angle of incidence between the processes and the host surface is greater (compare angles α_{Gt} and α_{Gb} in Figs. 7.8A and 7.8B). The points of the marginal hooks act more like the barbs on a gaff than as grappling irons and are pulled upwards and into the haptor dorsal lobe causing characteristic upward stretching of the epidermis. Hereafter, I refer to this type of attachment as *G. bullatarudis*-like.

G. turnbulli : The dorsal lobe processes are longer and thinner and more spread out than those of *G. bullatarudis*. The angle of incidence of

the dorsal lobe finger-like processes and the host surface is relatively small (see Figs. 7.8A and 7.8B). The marginal hooks act similarly to grappling irons, the tips of the hooks piercing the epidermal cells and then, when they are drawn back in towards the haptor, because the angle of incidence of the dorsal lobe process and the host surface is small (see Fig. 7.8A), characteristic horizontal stretching of the epidermis along the surface of the fish results. Hereafter, this type of attachment is called *G. turnbulli*-like.

7.4.2. Relationships between structure and function

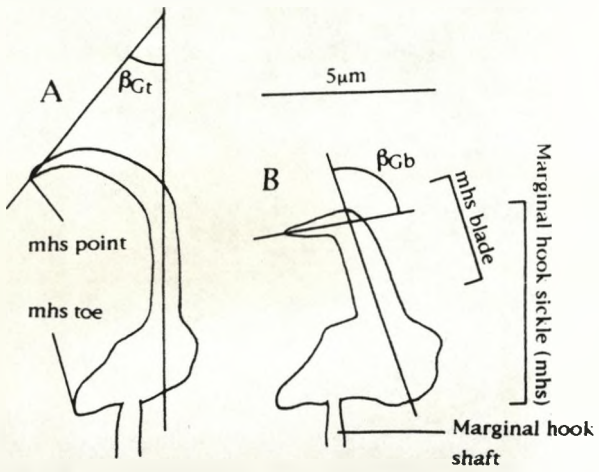


Fig. 7.14. A comparison of the marginal hook morphology of, A, *G. turnbulli* and, B, *G. bullatarudis*. Angle β is the angle of incidence between a line which bisects the marginal hook sickle point and a line which runs parallel to the main axis of the marginal hook sickle blade.

When the opisthaptor of a *Gyrodactylus* puts down on a new site, first the ventral lobe, including the hamulus complex, is placed in contact with the fish surface. Then the marginal hooks are extended outward, in unison, on their finger-like processes and each are brought down in a clawing action to pierce the host epidermis and thus form the tent-like attachment

described above.

The clawing action of each marginal hook is made possible by the flexibility between the marginal hook shaft and sickle (see Braun, 1966). The angles β_{Gt} and β_{Gb} between the lines shown in Figs. 7.14A and 7.14B, one which bisects the point and the other which runs parallel to the shaft of the marginal hook sickle blade is important. When a marginal hook is talon shaped, and this angle is small, puncture of the host surface is more easily accomplished when the marginal hook is operated in a position where the angle shown in Figs. 7.8A and &.8B as α_{Gb} or α_{Gt} is small. In order to appreciate this, consider an extreme situation. If the angle α_{Gt} in Fig. 7.8A was particularly large (just less than 90°, for example), a clawing *G. turnbulli* marginal hook would present the curved part of the sickle blade to the fish skin instead of the stabbing point. But, a marginal hook with less curvature in the blade would be more likely to be able to present its point to penetrate the fish surface. I consider that the morphology of the marginal hooks of *G. turnbulli* and *G. bullatarudis* can be related to their slightly different methods of attachment as follows.

Marginal hooks of *G. turnbulli* have sickle points which extend ventrally beyond the marginal hook sickle toe. The curvature of the marginal hook sickle blade causes the point to be directed inward in relation to the outward directed marginal hook process. These marginal hooks are shaped like talons. Thus, angle β_{Gt} is appreciably more acute than β_{Gb} (see Figs. 7.14A and 7.14B). It is therefore more difficult for these marginal hooks to pierce the fish epidermis close to the edge of the haptor dorsal lobe, *ie.* with a large angle α_{Gt} (Fig. 7.8A). The angle of attack of the point in relation to the surface of the epidermis would be too acute to allow enough purchase for penetration. Consequently the marginal hooks are extended on

relatively long dorsal lobe finger-like processes which, in attachment, subtend a relatively acute angle (α_{Gt}, Fig.7.8A) with the fish surface.

Conversely, the marginal hooks of *G. bullatarudis* have a sickle point which does not extend beyond the sickle toe and has a much more abrupt curvature within the blade causing the point to be directed such that β_{Gb} (Fig. 7.14A) is slightly greater than 90°. The marginal hooks of *G. bullatarudis* are shaped like small pins with their points bent through an angle of 90°. These marginal hooks do not need to be extended as far out from the dorsal lobe of the opisthaptor as those of *G. turnbulli* before they are capable of piercing the host epidermis by a clawing action. Consequently they are extended on relatively short dorsal lobe finger-like processes which, in attachment, subtend a relatively obtuse angle (α_{Gb}, Fig.7.8B) with the fish surface.

7.4.3. Investigation of the marginal hook structure and function hypothesis by referring to other works

In order to test the relationships between morphology and modes of action of the marginal hooks of *G. bullatarudis* and *G. turnbulli* proposed in the last section, previous SEM studies of *Gyrodactylus* attachment have been investigated with reference to descriptions of marginal hook morphology.

G. avalonia Hanek and Threlfall, 1969 illustrated in figure 1 of Cone and Odense (1984) has an attachment to *Gasterosteus aculeatus* which is similar to that of *G. bullatarudis*. Its dorsal lobe finger-like processes are short and similar in shape to those of *G. bullatarudis* but they appear to be pulling the host epidermis laterally, rather that upward and into the opisthaptor as observed for *G. bullatarudis*. However, the specimen illustrated in Cone and

Odense (1984) was fixed in 10% formalin and shows the typical appearance, caused by shrinkage, associated with the use of this chemical fixative (see Chapter 6, Fig. 6.3) and has a greater similarity to specimens of *G. bullatarudis* fixed in this way. Illustrations of marginal hooks of *G. avalonia* in Hanek and Fernando (1971) (their figures 9 and 10) show profiles which exhibit the important criteria detailed above as adaptations for this particular type of attachment. Figures 11 and 12 of the same publication, however, illustrate marginal hook profiles from *G. avalonia* with less similarity to those of *G. bullatarudis*. Figure 1 in Hanek and Threlfall (1969) shows two *G. avalonia* marginal hook profiles with characteristics consistent with those we have defined as important as adaptations for a *G. bullatarudis*-like attachment; the angle defined as β in Figs. 7.14A and 7.14B greater than 90° and a marginal hook sickle point which does not extend beyond the marginal hook sickle toe.

G. colemanensis Mizelle and Kritsky, 1967 illustrated in figures 5 to 7 of Cone and Cusack (1988) attached to *Salvelinus fontinalis* also shows an attachment with dorsal lobe finger-like processes acting and appearing more similar to those of *G. bullatarudis* than to those of *G.turnbulli*, causing conical contortions of the host epidermis. There is a marked similarity of marginal hook profiles of *G. colemanensis* [figure 3C, Cone *et al.* (1983) and figure 4, Mizelle and Kritsky (1967)] to that of *G. bullatarudis*.

Fig. 3.2 shows the marginal hook of *G. rasini* in profile and Fig. 1.2 shows *G. rasini* attached to a *Xiphophorus* hybrid. The marginal hook profile of this species is more similar to that of *G. bullatarudis* than *G. turnbulli*. For *G. rasini*, the angle defined as β in Figs. 7.14A and 7.14B is greater than 90° but its marginal hook sickle point extends as far laterally as the marginal hook sickle toe. Its opisthaptor (see Fig. 1.2) conforms more to a *G. bullatarudis*-like than *G. turnbulli*-like shape in attachment.

G. salmonis Yin and Sproston, 1948 has an attachment illustrated in Cone and Odense (1984) (their figures 3 to 5) to *Salmo gairdneri* and in Cone and Cusack (1988) (their figures 2 to 4) to *Salvelinus fontinalis* which is more similar to that of *G. turnbulli* than *G. bullatarudis*. In the latter publication, the word "pedunculate"[1] was used to describe its opisthaptor in attachment - a term which could also be applied to the attached opisthaptor of *G. turnbulli*. Figure 1C of Cone *et al.* (1983) shows a marginal hook profile of *G. salmonis*, its marginal hook sickle point extends beyond the toe and its angle β (see Figs. 7.14A and 7.14B) is less than 90°, although greater than that of *G. turnbulli*.

G. salaris Malmberg, 1957 as seen on Atlantic salmon parr, figures 3 and 6 of Mo (1994) also has a "pedunculate" appearance in attachment and marginal hooks which are talon shaped (Mo, 1994, his figure 4; Malmberg, 1993, his figure 6a).

So my comments made in section 7.4.2 relating the morphology of the marginal hooks of *G. turnbulli* and *G. bullatarudis* and to the differences observed in the morphologies of their opisthaptors in attachment may be applied equally reliably to five other *Gyrodactylus* species for which illustrations of attachment and marginal hook morphology are available.

7.4.4. Evolutionary significance

G. bullatarudis, *G. avalonia* and probably *G. colemanensis* are all of the subgenus *G.* (*Mesonephrotus*) Malmberg, 1964 (Malmberg 1970). *G. bullatarudis* and *G. avalonia* are both of the *G. arcuatus* species-group

[1] I interpret the term pedunculate to mean stalked, *ie.* the opisthaptor appears to bear stalks. Abercrombie, *et al.* (1992):

"PEDUNCLE. Stalk of an inflorescence."

Malmberg, 1964 (Malmberg 1970).

G. turnbulli is of the subgenus *G.* (*Metanephrotus*) Malmberg, 1964 (Harris 1986), *G. salmonis* and *G. salaris* are of the subgenus *G.* (*Limnonephrotus*) Malmberg, 1964 (Malmberg 1970).

The evidence presented suggests that the mode of action of *Gyrodactylus* marginal hooks in attachment relates to marginal hook morphology. Given the importance of marginal hook sickle morphology in *Gyrodactylus* systematics (Malmberg 1970), further studies relating marginal hook morphology to attachment would be of considerable interest.

Following inspection of the marginal hooks illustrated in Malmberg (1970) I speculate that the *G. bullatarudis*-like attachment is a more primitive form, less common in the more advanced *Gyrodactylus* subgenera [*G.* (*Metanephrotus*), *G.* (*Paranephrotus*) Malmberg, 1964, *G.* (*Neonephrotus*) Malmberg, 1964 and *G.* (*Limnonephrotus*)], but more common in *G.* (*Gyrodactylus*) Malmberg, 1964 and *G.* (*Mesonephrotus*).

7.4.5. Functional significance of the different methods of attachment of *G. bullatarudis* and *G. turnbulli*

Although differences have been shown between the attachments of the two species which are related to marginal hook morphology, the general strategies of both systems are the same. It is difficult, therefore, to attach any particular significance to these differences in relation to the two species' preferred sites of attachment (see Chapters 8, 9 and 10). My observations have shown that both species attach and relocate anywhere on the host external surface without difficulty. This view concurs with that of Malmberg (1970) who found it difficult to categorise sclerite morphologies with site specificity and who stated emphatically that he

observed no correlation between marginal hook sickle morphology and site specificity. However, the methods of attachment of *G. turnbulli* and *G. bullatarudis* stress the host epidermis in slightly different ways.

Consider a detachment force applied to both species acting at right angles to the fish epidermis and running through the main body of the parasites (see force "D" in Figs. 7.8A and 7.8B). In both instances this force would be spread and resisted equally by the 16 marginal hooks. However, comparing the resolution of forces about one marginal hook from each species, a greater vertical component would have to be resisted by the attachment of marginal hooks of the *G. bullatarudis* system than those of the *G. turnbulli* system where the force would be divided with a comparatively greater component resisted in the horizontal plane (see Figs. 7.8A and 7.8B and inequalities 1 and 2 below).

[1] $$\alpha_{Gb} > \alpha_{Gt}$$

[2] $$T_{Gb} / H_{Gb} > T_{Gt} / H_{Gt}$$

By extension, for a stable attachment, *G. turnbulli* relies more on the host epidermis maintaining integrity in a horizontal plane than *G. bullatarudis* which relies more on the host epidermis maintaining integrity in a vertical plane.

Another consequence of the different modes of attachment is that, when forces of attachment are in equilibrium, the method used by *G. bullatarudis* is likely to result in a greater downward pressure being exerted by the

hamuli into the fish surface. Consider tensions applied, in attachment, to the muscle processes which are attached to the muscle attachment caps (Shinn *et al.*, 1993) of the marginal hook shafts. This is the tension which maintains the stability of the opisthaptor in attachment - the tension of the guy ropes in the tent analogy. The muscle cells and their processes which attach to the muscle attachment caps of the marginal hook shafts are shown in Chapter 1, Fig. 1.1 and in Chapter 5, Fig. 5.8. The tensions, as they would be predicted to act through a marginal hook of *G. turnbulli* and a marginal hook of *G. bullatarudis* would be directed similarly to T_{Gt} and T_{Gb} in Figs. 7.8A and 7.8B respectively. Because $\alpha_{Gb} > \alpha_{Gt}$ and assuming T_{Gb} is approximately equal to T_{Gt}, a greater downward vertical reaction component, transmitted through the hamuli, would be expected in the attachment of *G. bullatarudis* than in the attachment of *G. turnbulli* where the tension would again be divided with a comparatively greater component acting through the horizontal plane. The general observation that impressions left by the hamuli of *G. bullatarudis* were deeper into the host epidermis than those of *G. turnbulli* (see Figs. 7.4 and 7.5) lends support to this argument. Cone and Wiles (1989) demonstrated that the cells compressed by the hamuli become necrotic and vacuolated at attachment sites of *G. colemanensis* on *Salmo gairdneri*. This is discussed further in Chapter 8, section 8.4.4 in a comparison of the damage caused to the fish by the two different modes of attachment of *G. turnbulli* and *G. bullatarudis*.

7.5. References

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CHAPTER 8

8. Comparison of the infrapopulation dynamics of *G. bullatarudis* and *G. turnbulli.*

8.1. Introduction

The epidemiology of *G. turnbulli* [misidentidied as *G. bullatarudis* - see Harris (1986)] was studied by Scott (1982) and Scott and Anderson (1984); by Scott and Nokes (1984) with particular emphasis on relationships with temperature; by Scott and Robinson (1984) and Scott (1985a) with particular reference to the population dynamics of challenge infections, and by Madhavi and Anderson (1985) in a study of the relationships between host genetics and susceptibility. The epidemiology of *G. bullatarudis*, however, has not been investigated in detail since the observations on its life cycle recorded with its original description (Turnbull, 1956).

The first aim of this chapter was to compare the infrapopulation dynamics of *G. bullatarudis* and *G. turnbulli* on isolated fish with similar genetic histories, kept at a constant temperature.

Detailed studies (Cusack, 1986; Cone and Cusack, 1989; Harris, 1988; Jensen and Johnsen, 1992) have shown that dispersal patterns of *Gyrodactylus* that infect the external surfaces of fish are species specific. A further aim of this chapter was to study the host-site specificities of the two species of parasite and how they change over the time-course of infection. The host-site specificity of *G. turnbulli* had previously been investigated by Harris (1988) who found that these flukes were mainly restricted to the caudal

peduncle whilst infrapopulations were growing, but increasingly spread to the fins, particularly the caudal fin, during the decay phase (Scott, 1985b) of the infection. A note in Turnbull (1956) stated that *G. bullatarudis* mainly infected the head but there have been no detailed studies to improve upon this observation.

An additional aim of this chapter is to compare the pathology caused by *G. bullatarudis* and *G. turnbulli*, by simple observations of fluke behaviour and histological sectioning of infected fish.

8.2. Materials and methods

The origins of the parasites used in this work are described in Chapter 2, section 2.2.1 and their maintenance is described in Chapter 2, section 2.2.2.

8.2.1. Experimental infections

The experiments were carried out in the controlled environment room with air temperature between 27 and 28°C, water temperature between 25°C and 27°C and a 12:12 light:dark regime. Guppy and parasite stocks were also kept in this room.

25 *Gyrodactylus*-naive laboratory bred guppies of standard length 10 to 15mm were infected under anaesthesia with three *G. bullatarudis* each, and 45 with three *G. turnbulli* using the procedure described in section 8.2.2. Each infected fish was isolated in 200ml standing tap water (see section 2.2.2) contained in the bottom part of a thoroughly washed 1.5l plastic fizzy drinks bottle which had been cut into two pieces about 15cm from the base. In addition, 20 control fish were handled, anaesthetised and kept isolated similarly to those fish which were infected and had their *Gyrodactylus*

infrapopulations regularly observed; however, the control fish were not infected.

Every second day each fish was anaesthetised and the number of parasites on the external surface were counted using the procedure described in section 8.2.3. Fish were examined in this way until they either died or their parasite infrapopulation became extinct. Detailed accounts of the infection and observation procedures are given in sections 8.2.2 and 8.2.3. Control fish were anaesthetised for 3.5 to 4 mins (see section 8.2.3.) every second day for 30 days.

In order to get an idea of the site specificity of both species of parasite, the number of flukes found on each of the fins, head, flanks and caudal peduncle (see section 8.2.3 for a more detailed definition of these zones) were recorded.

8.2.2. Infection procedure

A heavily infected guppy from one of the infected stocks was anaesthetised in a 0.02% solution of 3-aminobenzoic acid ethyl ester (MS222) (Scott and Robinson, 1984), mixed on the day of use (0.04g MS222 in 200ml standing tap water), and then killed by insertion of an entomological pin just behind one of the eyes (Malmberg, 1970). This dead donor fish was placed in standing tap water.

Recipient fish were infected one at a time in the following way. They were first caught in a 5cm diameter petri dish, excess water was pipetted out so that there was just enough for it to remain upright and immersed if the dish was tilted. Anaesthetic solution was then added to fill the dish and a stopwatch was started. Some of the anaesthetic was then pipetted out so that there was just enough for the fish to remain upright and immersed if the

dish was tilted. A scale or piece of fin to which one or more flukes were attached was taken from the sacrificed donor fish and held near the anaesthetised recipient with fine forceps. Having already removed some of the anaesthetic solution, the recipient's petri dish could be held in the left hand, at a tilt, whilst presenting the infected fish tissue with the right. The operation was observed using a binocular microscope with fibre optic illumination so that the number of flukes passing onto the recipient fish could be counted. Each initial inoculation consisted of three flukes and the placement of the initial attachment of each fluke was recorded. The recipient fish remained in the anaesthetic for 4 to 4.5mins at which time, after removal of all excess anaesthetic, the fish was poured into another petri dish containing standing tap water. If the operation of fluke transference had not been completed at this stage, it was continued until three flukes had attached. Fish usually took about 2 mins to become fully mobile following removal from the anaesthetic solution.

The recipient fish was then placed in 200ml standing tap water in an isolated fish container (described in section 8.2.1).

8.2.3. Examination procedure

On examination, each fish was removed from its container using a 5cm diameter petri dish and anaesthetised as described for the initial inoculation (see section 8.2.2).

The numbers of flukes on the caudal, dorsal, anal, pelvic and pectoral fins; the caudal peduncle (the surface of the body posterior to an imaginary line from the most anterior point of the base of the dorsal fin to the most anterior point of the base of the anal fin), the flanks (the surface of the body between the fore said imaginary line and the posterior edge of the operculum) and the head and opercula were counted and recorded. These

zones were first used for recording host site specificity of *G. turnbulli* on *P. reticulata* by Harris (1988). Blunt forceps were used to push the fish under the dissecting microscope, manoeuvring it to allow complete examination of the external surfaces.

Fish were removed from the anaesthetic after 3.5 to 4mins following the same procedure as detailed in section 8.2.2. If necessary, examination would continue following removal from the anaesthetic so long as the fish remained still. The standing tap water in the isolated fish container was changed before replacing the examined fish. Water was therefore changed every second day. When high infestations were encountered, over 150 flukes on one side of one fish, the total infrapopulation and their distribution were estimated by doubling the numbers recorded in each zone on the first side to be examined.

All fish were fed once daily with tropical fish flakes.

8.2.4. Observations of live *Gyrodactylus* on fish

Infected fish from the infected stock containers were used in these investigations.

Individual infected fish were caught in a 5cm diameter petri dish and lifted out of the infected stock container. Enough water was removed so that the fish were still covered with water but restricted of movement enough to allow detailed observations of individual flukes at x200 total magnification using a Kyowa binocular dissecting microscope.

One fluke from each fish was observed for 15mins. The number of translocations made by each *Gyrodactylus* and the number of times each applied its mouth to the surface of the fish for feeding was recorded. In

addition, the standard length of, and *Gyrodactylus* infrapopulation carried by each fish used were recorded.

15 *G. bullatarudis* and 15 *G. turnbulli* were observed from 30 different fish for 15 mins each.

8.2.5. Sectioning of infected fish

Three fish were sectioned for examination using the light microscope. All fish were initially *Gyrodactylus*-naive and of 8mm standard length. One fish had been infected with four *G. bullatarudis* following the procedure outlined in section 8.2.2. On the fourth day after inoculation it was anaesthetised, flukes were counted and it was placed in a vial containing 3% gluteraldehyde, buffered with 0.1M sodium cacodylate at a pH of 7.2. A second fish was infected with four *G. turnbulli* and also fixed on the fourth day post infection. The third fish was a control and was anaesthetised and fixed as above but remained uninfected.

On fixation, the fish infected with *G. bullatarudis* had 19 flukes on its head and opercula (as defined in section 8.2.3) and the fish infected with *G. turnbulli* had 24 flukes on its caudal peduncle, 3 on its caudal fin and 1 on its flanks.

The following preparatory procedures were carried out by Mr. J. Smith.

The fish were left for 48h in the fixative and then washed in two changes of 0.1M sodium cacodylate buffer, 15 mins in each change.

The fish were post fixed for 2 hours in 1% osmium tetroxide, also buffered with 0.1M sodium cacodylate, and then washed as before.

The materials were dehydrated by taking them through a series of ethanol solutions and were embedded in Spurr's resin (Spurr, 1969) (standard formula for medium hardness). 2μm thick sections were cut from the head region and the caudal peduncle of each fish using a Reichert Ultracut E ultramicrotome with 45° cutting knives. The sections were then transferred to slides and flattened on a hot plate at 60 to 70°C. Sections were stained for 2 mins in Toludine blue, washed in tap water, washed in distilled water, dried on a hot plate and mounted in "Eukitt" quick-hardening mounting medium.

8.3. Results

8.3.1. Results from experimental infections

Out of the 25 fish infected with *G. bullatarudis*, only one survived. None of the fish infected with *G. turnbulli* survived. The majority of infections showed an exponential increase in *Gyrodactylus* which eventually led to the death of the host (see Fig. 8.1). Five fish infected with *G. bullatarudis* and two fish infected with *G. turnbulli* did not show a continuous exponential increase in *Gyrodactylus* until death, although all but one of these fish died. These fish exhibited evidence of a response to their infections which led to at least a temporary decrease or plateau in parasite infrapopulation growth. The population dynamics of *Gyrodactylus* on these fish are shown in Figs. 8.2 and 8.3.

None of the 20 fish from the control group died during 30 days of procedures.

The host-site specificities of the *Gyrodactylus* infecting fish that showed no sign of a response are illustrated in Figs. 8.4 and 8.5.

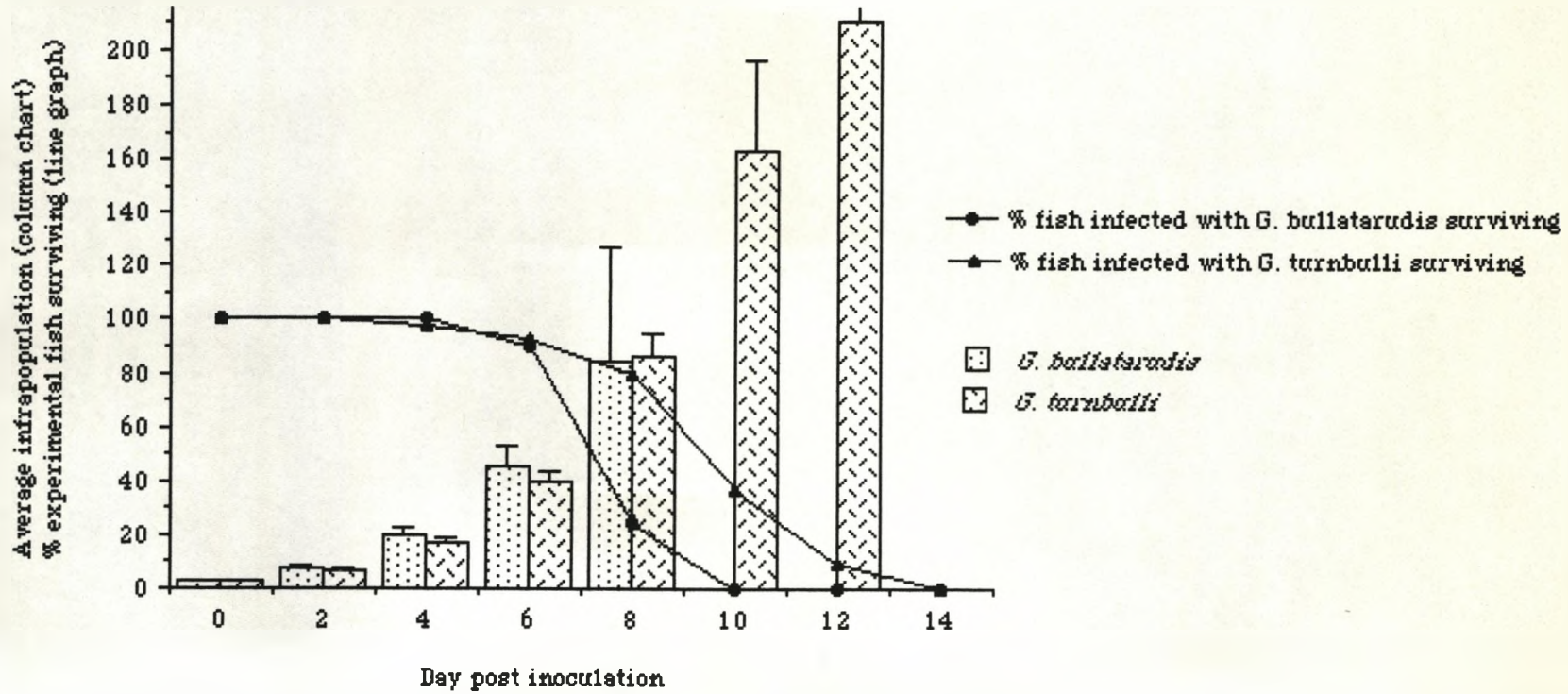

Fig. 8.1. Column chart of the mean infrapopulations of *Gyrodactylus* on the experimentally infected fish which showed no signs of recovery showing 95% confidence limits for each mean. This is contrasted against line graphs of the percentage of fish surviving on corresponding days post infection. Although the mean longevity of fish infected with *G. bullatarudis*, 7.53 days (SD 1.28 days) post inoculation, and the mean longevity of fish infected with *G. turnbulli*, 9.56 days (SD 2.15 days) post inoculation, were significantly different according to the Student t test, on days 2, 4, 6 and 8, the mean infrapopulations of those fish infected with *G. bullatarudis* and those infected with *G. turnbulli* were not.

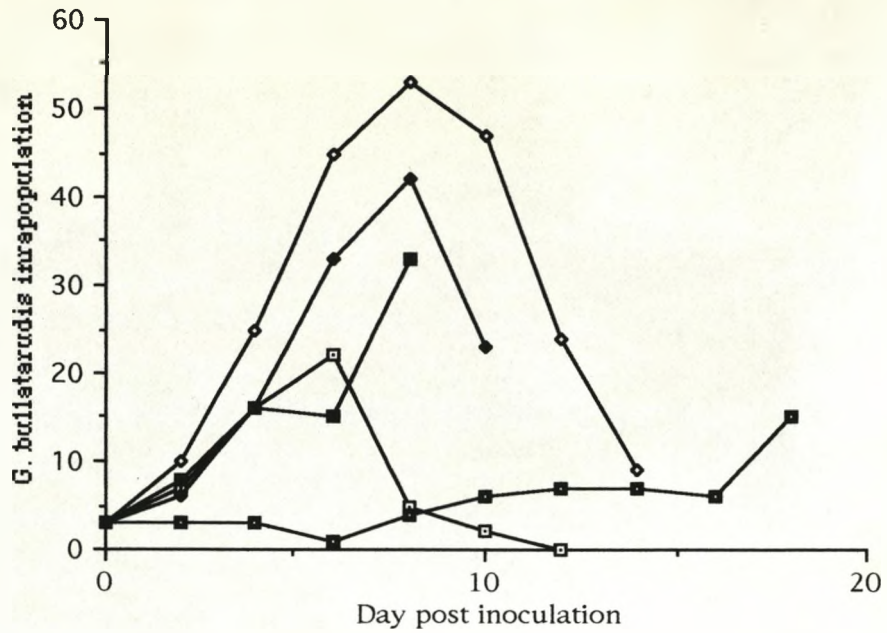

Fig. 8.2. Infrapopulation dynamics of *G. bullatarudis* on the five fish which showed some evidence of a host response.

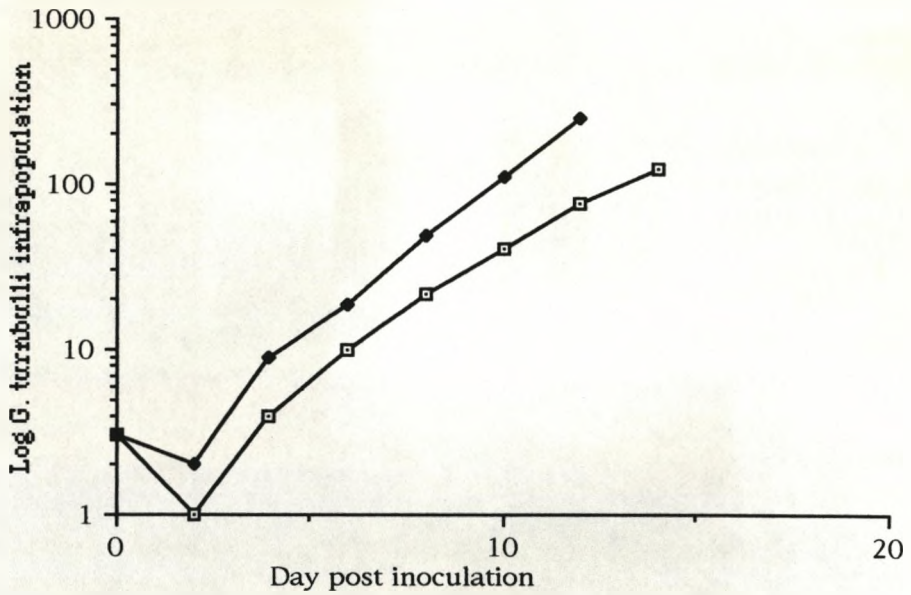

Fig. 8.3. Infrapopulation dynamics of *G. turnbulli* on the two fish which showed evidence of a host response. The evidence of a host response in these fish was more dubious than that of the five fish illustrated in Fig. 8.2 because, following the establishment of an increasing infrapopulation on day two, there was no decrease or plateau in the growth of the infrapopulations.

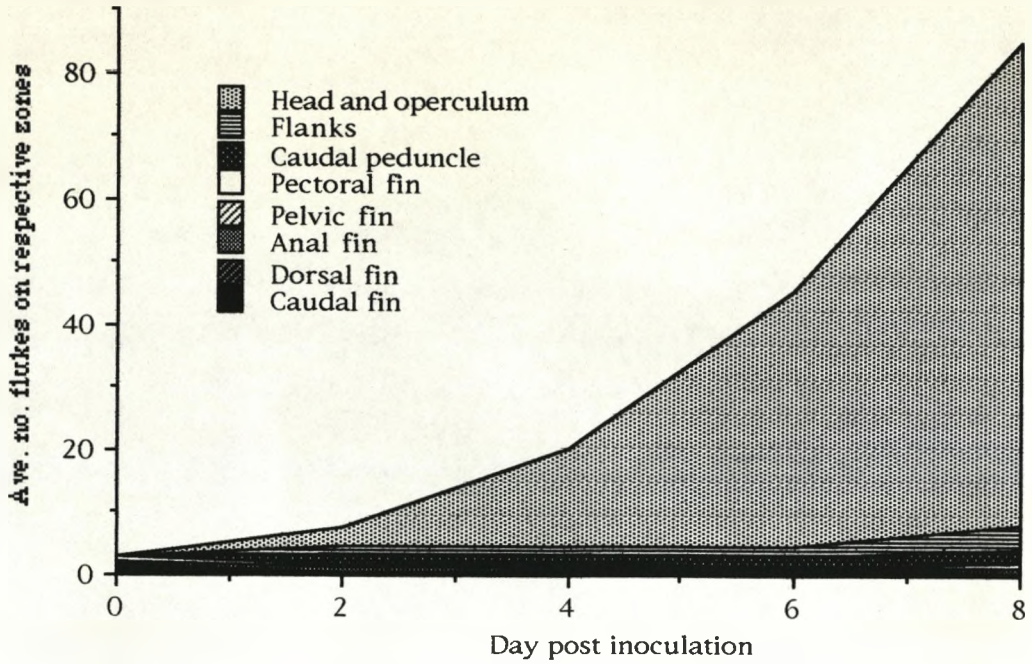

Fig. 8.4. Area graph showing the average number of *G. bullatarudis* in each of the eight host-site zones defined in section 8.2.1 on every second day post experimental infection with three flukes. Only results from fish showing no signs of recovery from infection have been used.

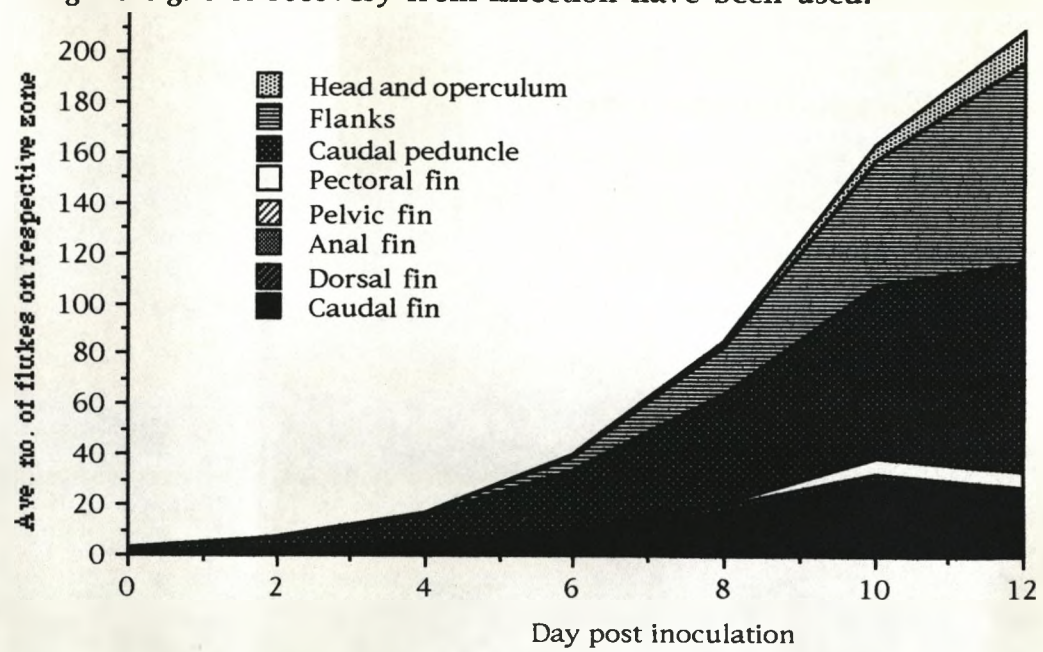

Fig. 8.5. Area graph showing the average number of *G. turnbulli* in each of the eight host-site zones defined in section 8.2.1 on every second day post experimental infection with three flukes. Only results from fish showing no signs of recovery from infection have been used.

8.3.2. Results from observations of live *Gyrodactylus* on fish

Relocation

Observations from fish infected with an established infection of either parasite species showed that flukes rarely relocated more than once in quick succession. However, flukes would frequently relocate 2 or more times in quick succession following placement on a naive recipient fish by the method described in section 8.2.2. An account of *Gyrodactylus* locomotion is given in Chapter 1, section 1.2.1.

The results of the studies of frequencies of relocation of *G. bullatarudis* and *G. turnbulli* are given in Tables 8.1 and 8.2.

Feeding

Feeding was accomplished by both species in a similar way. First the preopisthaptoral part of the body was stretched forward and the anterior placed in contact with the fish surface. The prohaptor may have been used in the initial attachment of the anterior of the fluke to the fish surface when the pharynx was placed into the position for its attachment, but once the pharynx had attached to the fish and feeding was underway, the cephalic lobes were lifted off the fish surface but remained protruded and diverging from each other.

Whilst feeding was taking place, the pharyngeal region of the flukes was expanded. Both species usually fed with their preopisthaptoral bodies stretched longer than they were when relaxed and unmoving. However *G. turnbulli* occasionally stretched exceptionally far in order to feed, their preopisthaptoral body stretched two to two and a half times its relaxed length.

Following feeding, the pharynx was released from the fish and the preopisthaptoral body of the parasite was relaxed and a more normal attitude was adopted. For a few seconds following release from feeding, the anterior of the flukes sporadically contracted, possibly pushing the recently acquired digest from the oesophagus into the gut [Harris (1982) observed a similar action following feeding of *G. gasterostei* and attributed the movement toward the same function].

The length of time each species took for a feeding bout differed, *G. turnbulli* usually had its pharynx in contact with the fish surface for between 30 and 70 seconds and *G. bullatarudis* for between 10 and 40 seconds.

The results of the studies of frequencies of feeding of *G. bullatarudis* and *G. turnbulli* are given in Tables 8.1 and 8.2.

Fish standard length (mm)	*G. bullatarudis* infrapopulation	No. of relocations in 15mins	No. of feeding attitudes in 15mins
9	15	0	1
9	1	0	4
10	22	3	2
10	12	1	7
10	12	0	5
10	29	0	5
10	5	0	2
11	74	0	2
11	14	1	2
13	12	2	5
13	29	0	1
14	36	1	10
14	10	0	7
15	71	1	3
19	1	1	2

Table 8.1. Observations of relocation and frequency of feeding of individual *G. bullatarudis* on 15 different *P. reticulata.* of different lengths and carrying different infrapopulations.

Fish standard length (mm)	*G. turnbulli* infrapopulation	No. of relocations in 15mins	No. of feeding attitudes in 15mins
9	7	0	0
9	14	1	0
10	1	1	1
11	9	0	1
12	35	0	1
12	4	0	1
13	8	0	0
13	16	2	0
14	268	3	1
14	17	0	0
14	5	0	0
15	20	1	1
16	60	0	1
17	8	2	0
17	6	1	0

Table 8.2. Observations of relocation and frequency of feeding of individual *G. turnbulli* on 15 different *P. reticulata.* of different lengths and carrying different infrapopulations.

8.3.3. Sections of infected fish

On sectioning infected fish no great differences were found between the extent of damage caused by *G. bullatarudis* and *G. turnbulli*. Both species had marginal hooks which, in opisthaptoral attachment pierced the host epidermis by 2 to 4μm (see Figs. 8.6 and 8.7). Damage through feeding was not assessed as it was impossible to distinguish injury through feeding from incidental damage to the host epidermis from other sources.

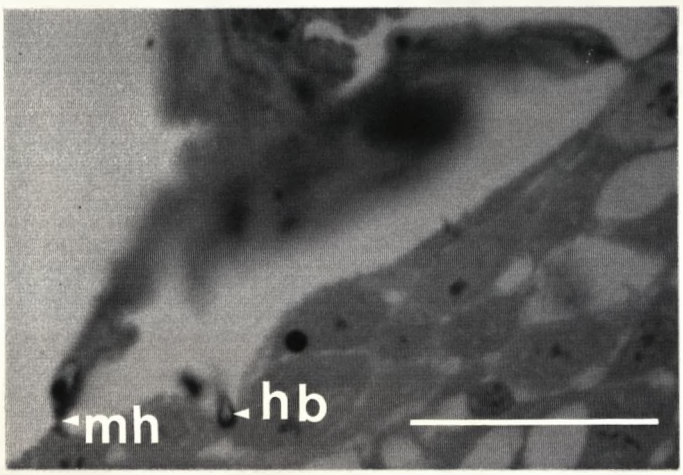

Fig. 8.6. Marginal hook (mh) and hamulus blade (hb) of a *G. bullatarudis* attached to the dorsal surface of the head of a *P. reticulata*. Note the compression caused by the hamulus. Scale bar = 20μm.

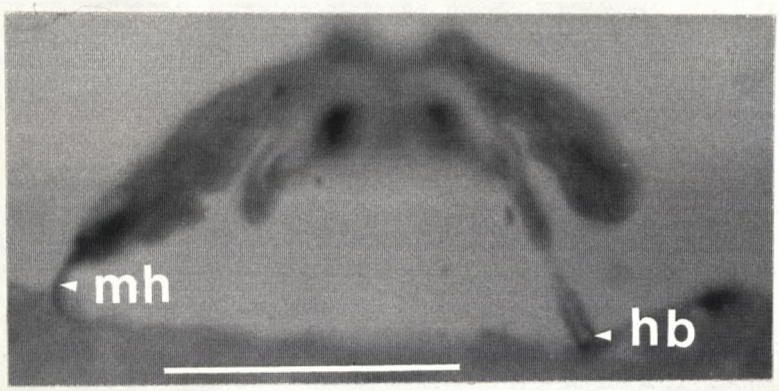

Fig. 8.7. Attached opisthaptor of a *G. turnbulli* in section [marginal hook (mh) and hamulus blade (hb)] attached to the surface of the caudal peduncle of a *P. reticulata*. Note that less compression was caused by the hamulus than shown in Fig. 8.6. Scale bar = 20μm.

8.4. Discussion

Scott (1985b) said of guppies infected with *G. turnbulli*:

"[they] can be divided into three groups on the basis of the parasite population dynamics on isolated hosts: on some, the parasite never establishes, some fish recover from the infection, and some fish die during the exponential growth of the parasite population."

The overwhelming majority of fish used in this investigation were of the third of these categories.

The aim of my experiment was to compare the population dynamics of *G. bullatarudis* and *G. turnbulli* on individual fish. There were not enough fish exhibiting growth and decay in their parasite infrapopulations to allow a comparison of the dynamics of infections of that kind. So, only the infrapopulation dynamics on fish which showed no sign of a response to infection were compared in detail.

8.4.1. *Gyrodactylus* infrapopulation intrinsic rates of increase

The infrapopulations of *G. bullatarudis* and *G. turnbulli* on fish which showed no sign of a response to infection were not significantly different in standard deviation (F test) or mean (Student t test) on coinciding days 2, 4, 6 and 8 after their initial inoculation with three flukes. Average intrinsic rates of increase were worked out per parasite per day for each day on which parasite infrapopulations were counted using the equations below:

[1] $N_{t+1} = rN_t$ therefore $N_{t+2} = r^2N_t$ therefore $r = \surd(N_{t+2} / N_t)$

Where: N_t = the infrapopulation on day t.

N_{t+1} = the infrapopulation one day after day t.

N_{t+2} = the infrapopulation two days after day t.

r = the intrinsic rate of increase on day N_{t+2}.

The average intrinsic rates of increase for both *G. bullatarudis* and *G. turnbulli* infections are given in Fig. 8.8 . The differences between the means and standard deviations of these values for *G. bullatarudis* and *G. turnbulli* on coinciding days 2, 4, 6 and 8 post infection were not significant according to comparisons using the F and Student t tests.

The estimates of intrinsic rates of increase were considerably greater than the values obtained by Scott and Nokes (1984), who calculated the intrinsic rates of increase of *G. turnbulli* at the temperatures of 17, 21, 25.5, 27.5 and 30°C and found the maximum of about 0.21 per parasite per day to be at a temperature of 27.5°C, and Scott (1982), who calculated the intrinsic rate of increase of *G. turnbulli* at 25°C to be 0.19 per parasite per day. However, the calculations of these two previous works were from data collected from experiments investigating the birth and death processes of individual flukes on individual fish. The rates of increase may have been reduced by the use of intrusive experimental methods - daily examination of fish under anaesthetic was required. Also, the calculations from my work were from infected fish that showed no obvious sign of a response, thus reducing the chance of a reduction in parasite rate of reproduction caused by host resistance.

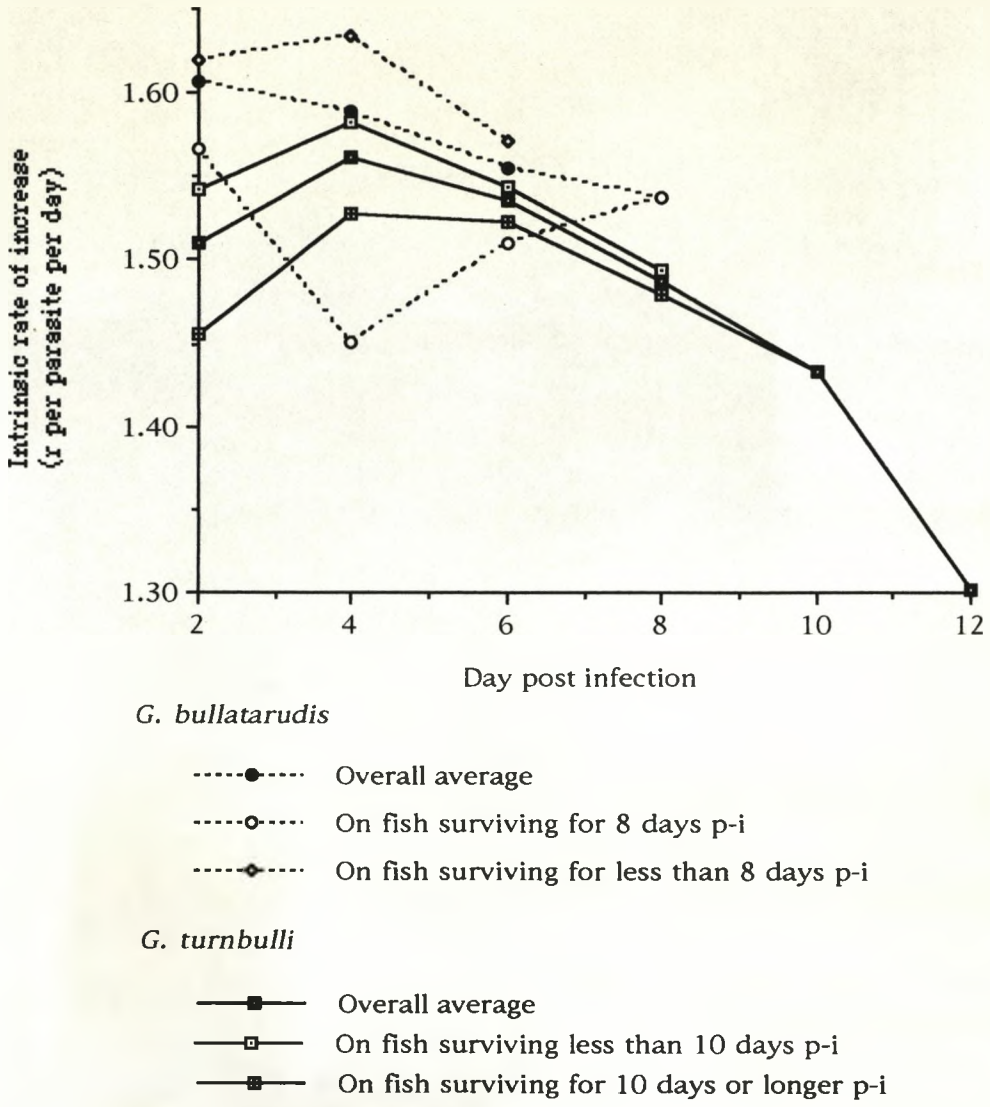

Fig. 8.8. Average intrinsic rates of increase for *G. bullatarudis* and *G. turnbulli* on fish showing no signs of a response to infection. For both parasite species average intrinsic rates of increase of two sub-groups are also shown. *G. bullatarudis* sub-groups are; those flukes on fish surviving for eight days post infection (= p-i) and those flukes on fish surviving for less than eight days post infection. *G. turnbulli* sub-groups are; those flukes on fish surviving for ten days or longer post infection (= p-i) and those flukes on fish surviving for less than ten days post infection. The method of calculation of the intrinsic rate of parasite increase is given in equation [1], section 8.4.1.

A feature of the mean intrinsic rates of increase of both *G. bullatarudis* and *G. turnbulli* shown in Fig. 8.8 was that they were (with the exception of the infrapopulations on fish infected with *G. bullatarudis* which survived for 8 days), at least after day 4, decreasing with time post inoculation and, therefore, with increasing infrapopulation size.

Two likely explanations for the decreases in the mean rates of parasite infrapopulation increase over time are given below. They are not mutually exclusive.

1. Increased infrapopulations led to over-crowding and intraspecific competition which led to decreased rates of infrapopulation growth. Evidence supporting this explanation was obtained from observations of the development of *Gyrodactylus* dispersal patterns over their hosts. This is discussed in section 8.4.3.

2. Some fish provided micro-environments which were more suited to the parasites than others and the average intrinsic rate of increase decreased as those fish supporting higher rates of increase were among the first to die.

8.4.2. Per capita rate of parasite induced host mortality

The following method was adopted from Scott and Anderson (1984) to estimate the parasite induced host mortality per host per day per parasite. Average per capita instantaneous death rates of the hosts were calculated for every second day post infection for each fish infected with *G. bullatarudis* and *G. turnbulli* and showing no signs of recovery. This was done by dividing the the number of fish which had died over the previous two days by the number of fish alive at the start of those two days, by the number of days (two). These values were plotted against average intensities

of infection on the corresponding days (Fig. 8.9) to obtain the linear model:

[2] $A = b + \alpha M$

Where: A = instantaneous rate of host mortality

b = natural instantaneous rate of host mortality

M = average parasite burden (intensity) per host

α = per capita parasite induced host mortality per host per unit time per parasite

In this instance b was assumed to be 0, seeing as none of the control fish had died within 30 days, and values for α were only required for a comparison of *G. bullatarudis* and *G. turnbulli* under these experimental conditions. In fact, the graphs obtained for *G. bullatarudis* and *G. turnbulli* were not linear (see Fig. 8.9) but both followed a similar pattern. Parasite induced host mortality rose slowly in relation to the mean parasite burden until a threshold was reached, approximately 50 flukes/host for *G. bullatarudis* and approximately 100 flukes/host for *G. turnbulli*, after which a steeper gradient was realised. In order to obtain comparative measures of per capita rates of parasite induced host mortality a straight line was fitted to all the points plotted for each species and their gradients taken as values of parasite induced host mortality per host per day per parasite. The actual values of α were estimated as 0.004/host/day/parasite for *G. bullatarudis* and 0.002/host/day/parasite for *G. turnbulli*. Thus *G. bullatarudis* was estimated to be twice as lethal per host per day per parasite than *G. turnbulli* in infections where the host showed no apparent response to infection.

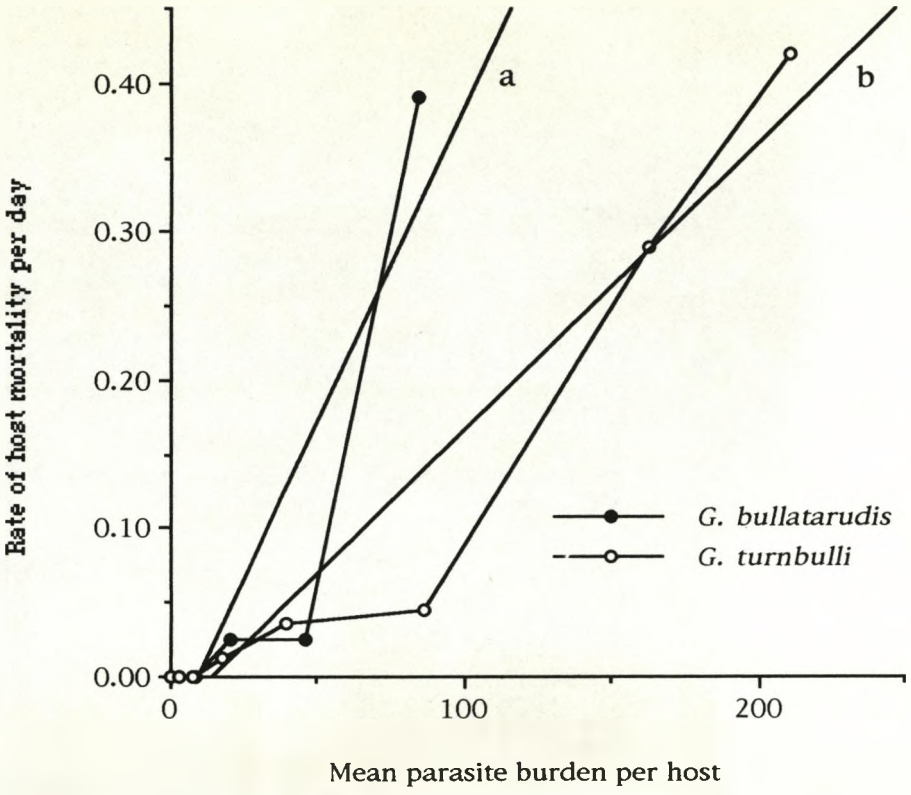

Fig. 8.9. Graph used to estimate and compare the rate of host mortality caused per host per day per parasite by *G. bullatarudis* and *G. turnbulli*. The lines "a" and "b" were fitted by the line-fit facility of Cricket Graph.

Line "a" has the equation: $y = -0.04 + 0.004x$

Line "b" has the equation: $y = -0.03 + 0.002x$

These equations are equivalent to equation [2] in section 8.4.2. As explained in the text, the constants "-0.04" and "-0.03" were assumed to be close enough to to be disregarded but the gradients 0.004 for line "a" and 0.002 for line "b" were used as approximations to the rates of parasite induced host /host/day/parasite of fish infected with *G. bullatarudis* and *G. turnbulli* showing no signs of a response to infection.

Although *G. bullatarudis* and *G. turnbulli* had indistinguishable rates of increase on individual fish at a water temperature of 26°C, *G. bullatarudis* caused higher mortality per host per day per parasite. This suggested that other differences in life histories are likely to exist. For example, *G. bullatarudis* might rely proportionately more on transmission from dead donor to live recipient fish than from living donor to live recipient fish than *G. turnbulli*. A comprehensive study of the epidemiology of *G. bullatarudis* is lacking but would make interesting comparison with the studies of Scott (1982) and Scott and Anderson (1984).

These findings were based upon values calculated under experimental conditions where the effects of fluke infections on mortality caused by factors such as predation and reduction in foraging efficiency due to infection were excluded.

8.4.3. Host-site specificity

Recordings of host-site specificity (see Figs. 8.4 and 8.5) showed that the dispersal patterns of *G. bullatarudis* and *G. turnbulli* on their hosts were different, not only in their preferred sites of attachment - the head and opercula for *G. bullatarudis* and the caudal peduncle for *G. turnbulli* - but also because *G. bullatarudis* tended not to disperse over other areas of the fish when infrapopulations became very large. These differences in host-site dispersion caused different signs of gross pathology associated with *G. bullatarudis* and *G. turnbulli* infections.

When large infrapopulations occurred, those of *G. bullatarudis* were usually much more crowded and confined to a smaller area than *G. turnbulli* infections of a similar number, which tended to be spread relatively evenly over the posterior of the fish. In severe infections the

epidermis of the anterior of the head, including the cornea and the surrounds of the mouth and nasal cavities became visibly roughened. This is likely to have resulted in decreased quality of vision and stress due to the irritation of important sense organs.

There was a greater tendency for *G. turnbulli* to infect the fins of their hosts, particularly the caudal, dorsal and anal fins, as infrapopulation size increased. This caused the fin rays to converge on each other, resulting in the symptom commonly referred to by aquarists as "clamped fins". The host subsequently lost swimming efficiency and manoeuvrability. Such an effect was not observed as a result of *G. bullatarudis* infections. The order of fins to become "clamped" - usually as the infrapopulation grew above about 30 flukes - was dorsal fin, anal fin, caudal fin, pelvic fin and pectoral fin. However, infrapopulations on these small fish seldom grew large enough to cause clamping of the pectoral fins before the host had either died or recovered.

The results of the calculations of the intrinsic rates of increase for *G. bullatarudis* and *G. turnbulli* (Fig. 8.8) are interesting when considered together with recordings of host-site specificity (Figs. 8.4 and 8.5). There is evidence that flukes of both species increased at greater rates on their preferred host-sites; the head and opercula for *G. bullatarudis* and the caudal peduncle for *G. turnbulli.*

The overall average intrinsic rates of increase for *G. bullatarudis* declined steadily from day 2 to day 8 post infection. However, the intrinsic rates of increase on fish surviving to day 8 and on those that did not, are quite different (Fig. 8.8).

The average intrinsic rates of increase of the *G. bullatarudis* on fish that did not survive for 8 days post infection followed a similar pattern to all the

fish infected with *G. turnbulli*, increasing from day 2 to day 4 and then decreasing. The average intrinsic rates of increase of *G. bullatarudis* on fish that survived for 8 days or longer showed a decrease in average intrinsic rate of increase from days 2 to 4 post infection, and then an increase to day 8 post infection. The recorded host-site locations of flukes infecting both these groups of fish are shown for day 2 post infection in Table 8.3. There is a significant ($P < 0.01$) difference between the distribution of flukes from these two groups according to the χ^2 test.

On day 2, the fish which survived to day 8 post infection had an unusual distribution for *G. bullatarudis*, with less occurring on the head and opercula than on the caudal peduncle, flanks and fins. On those fish which did not survive to 8 days post infection *G. bullatarudis* had a more typical distribution, the number of flukes on the head making up the larger of the three groups. On day 2 post infection the average infrapopulations of *G. bullatarudis* on these two groups of fish were not significantly different and so there was not likely to be more over-crowding on one group of fish than the other.

Areas of fish surface (see section 8.2.4)	No. of flukes on fish not surviving for 8 days p-i	No. of flukes on fish surviving for 8 days p-i
Fins	22 (18.3%)	9 (25.0%)
Caudal peduncle & Flanks	43 (35.8%)	21 (58.3%)
Head & operculum	55 (45.8%)	6 (16.7%)
Total	120	36

Table 8.3. A comparison of the host-site locations of *G. bullatarudis* on day 2 post infection on fish that survived for 8 days with those on fish that did not survive for 8 days post infection (= p-i). A χ^2 test showed a significant association between the rows and columns of this table, $P < 0.01$.

Areas of fish surface (see section 8.2.4)	No. of flukes on fish not surviving for 8 days p-i	No. of flukes on fish surviving for 8 days p-i
Caudal fin	23 (54.8%)	10 (66.7%)
Caudal peduncle, Anal fin & Dorsal fin	7 (16.7%)	5 (33.3%)
Flanks, Pelvic fin, Pectoral fin & Head & operculum	12 (28.6%)	0 (0.0%)
Total	42	15

Table 8.4. A comparison of the original placement of *G. bullatarudis* on the day of infection on fish that survived for 8 days with those on fish that did not survive for 8 days post infection (= p-i). A χ^2 test showed no significant association between the rows and columns of this table.

Two possible causes of a decrease in the rate of increase from day 2 to day 4 were, firstly, a response to infection by the host which, although slowing the rate of increase of the infrapopulations, did not result in a decrease or plateau in the overall parasite burden or, secondly, a reduced rate of increase due to random events leading to fewer parasites being located on their preferred area for attachment. Table 8.4 shows a comparison of the original random placement of flukes on day 0 of the experiment. The lower two categories of this table were summed to allow χ^2 analysis. There were no significant correlations between the data in the resultant rows and columns. Even so, 29% of flukes on fish that died before day 8 were originally placed on the anterior regions, but none were placed on these regions on fish that did survive 8 or more days post infection and which had a lower rate of increase on day 2.

So, where greater proportions of *G. bullatarudis* were located on preferred areas, the head and opercula, there was evidence that infections were likely to yield higher intrinsic rate of increase. There was also evidence indicating that original placement sites of infecting flukes may influence the rate of growth of the infrapopulation.

Fig. 8.5 illustrates the tendency for *G. turnbulli* to spread from the preferred site of infection, the caudal peduncle, onto the flanks, caudal fin, pectoral fins and head and opercula , respectively, as the infrapopulation increases.

The intrinsic rates of increase of *G. turnbulli* on all groups of fish in Fig. 8.8 followed the same trend, an increase from days 2 to 4 post infection was followed by a steady decrease which was steepest between days 10 and 12. The decrease in the rate of increase from day 4 to day 6 in both groups coincided with the spread of flukes onto the flanks, probably due to the

infections on the caudal peduncle reaching a carrying capacity after which over-crowding occurred. Even though there was little crowding of flukes on the flanks at this time, the rates of increase of *G. turnbulli* began to decline, and continued to decline as a larger proportion of flukes were to be found on areas other than the caudal peduncle. Assuming the decrease in rates of increase was caused by the effects of intraspecific competition due to over-crowding, the question arises as to why the infections did not spread to other parts of the host's external surface before these effects became apparent.

Possible explanations for this are given in the paragraphs below. They are not mutually exclusive.

The first explanation is based upon the assumption that *G. turnbulli* are specifically adapted for reproduction and survival on the caudal peduncle. As the infrapopulation grows, the caudal peduncle becomes more crowded. The effects of intraspecific competition increases until the benefits to flukes remaining in the most suitable microenvironment on the fish become overridden by the effects of crowding and so the infection starts to spread to other regions. As a greater proportion of flukes have to survive on regions of the host's external surface to which they are not optimally adapted, the infrapopulation's rate of increase decreases - in addition, crowding and, therefore, the effects of competition on the caudal peduncle continue to increase.

The second explanation makes no assumption that *G. turnbulli* are specifically adapted for reproduction and survival on the caudal peduncle. The spread of the infrapopulation onto other parts of the fish is delayed because of benefits from aggregation, for example, to increase the probability of copulation between sexually mature flukes, consistent with the hypothesis put forward by Rhode (1991) of habitat restriction to

enhance the probability of intraspecific contact, thereby facilitating mating. Harris (1993) classifies *G. turnbulli* as a fluke which increases its proportion of sexually produced offspring at higher population densities. There may therefore be a trade-off within the infrapopulation between the disadvantages of over-crowding due to intraspecific competition and the advantages of an increased rate of sexual reproduction.

8.4.4. *Gyrodactylus* attachment

Attachment has already been discussed in detail in Chapter 7, however, it is referred to in this chapter with particular reference to the difference in pathogenicity observed in the experimental work.

Observations of the sections of attached *G. bullatarudis* and *G. turnbulli* showed the marginal hooks of both species only penetrated the host epidermis by 2 to 4μm. However, of the four attached *G. bullatarudis* and five attached *G. turnbulli* observed in section, the hamuli of the *G. bullatarudis* were estimated to compress the host epidermis more than those of the *G. turnbulli*. Similar results were obtained in Chapter 7 (see Figs. 7.4 and 7.5). Cone and Wiles (1989) reported damage to the epidermis of *Salmo gairdneri* by the "pressure exerted by the hamulus blades" in addition to the damage caused by the piercing of the marginal hooks of *G. colemanensis*. The evidence suggests that greater damage may be caused to the host epidermis by *G. bullatarudis* due to greater compression caused under the hamuli blades in attachment. This is consistent with the differences in the mode of attachment of *G. bullatarudis* and *G. turnbulli*, described in Chapter 7, where it was postulated that a tension applied to the marginal hooks of *G. bullatarudis* acting through the muscles attached to the marginal hook shafts would have a reaction with a proportionately greater vertical component, equivalent to a compressive force acting through the hamuli, than a similar tension applied to the attached

marginal hooks of *G. turnbulli*, which, in turn, would have a reaction with a proportionately greater horizontal component (see section 7.4.5).

Many host epidermal cells which were pierced by marginal hooks were more deeply stained with toludine blue than surrounding cells. This may have been indicative of a build up of microfibrils in these cells similar to that observed by Cone and Wiles (1989) on examination of the attachment sites of *G. colemanensis* on *S. gairdneri*. Unfortunately this was not investigated further by transmission electron microscopy.

8.4.5. *Gyrodactylus* relocation rates

Frequency of relocation on the host may have important implications for the pathology caused by *Gyrodactylus* infections. Cone and Wiles (1989) wrote that the frequent relocation of *G. colemanensis* on *S. gairdneri* (= *Oncorhynchus mykiss*) "explains the absence of a gross pathological response by the fish", "distinct wounds" and of inflammation. Both *G. bullatarudis* and *G. turnbulli* relocated on their hosts at similar intervals (usually once or twice every 15mins) and so differences in pathogenicity were not caused by differences in relocation behaviour.

Cone and Wiles (1989) speculated that frequency of movement may be determined by the rate at which individual flukes deplete food sources in their immediate range or by the rate of deterioration of their opisthaptoral attachment sites over use. An alternative explanation may be to reduce the pathology caused to the fish by chronic inflamation and autoimmune reactions thus leading to an increased infrapopulation carrying capacity per host which would be beneficial to the survival of the fluke population as a whole.

The observations of locomotion showed no correlation between intensity

(equivalent to density) of infection and the rate of relocation of individual flukes. A more convincing study of such a relationship would have been achieved by observing several samples of flukes, each from a different fish carrying a different infrapopulation, and testing for a correlation between the sample means of rates of relocation and infrapopulation size. A relationship between fluke density and rate of relocation would have been expected if rate of relocation was determined by the rate of depletion of food resources in the vicinity of individual flukes, hence, the evidence from these observations indicates that the latter two explanations for the determination of rates of relocation given in the previous paragraph are the more likely.

8.4.6. Observations of feeding behaviour

The feeding behaviours of the two species were quite different. This was not surprising given the very different morphologies of the pharynx of *G. bullatarudis* and *G. turnbulli* (see Chapter 1, section 1.2.2). *G. bullatarudis*, on average, fed about 4 times every 15mins whereas *G. turnbulli* fed about once every 30mins. Although *G. bullatarudis* fed more often than *G. turnbulli*, the latter species usually had their pharynx in contact with the fish for longer than the former during feeding times. *G. turnbulli* were usually in contact with the fish epidermis between 30 and 70 seconds whilst timings for *G. bullatarudis* ranged between 10 and 40 seconds. Although it was shown that, on average, the pharynx of a *G. bullatarudis* would be in contact with the host epidermis for longer over a given period than a pharynx of a *G. turnbulli*, actual quantification of the damage caused by individual flukes was not achieved. Harris (1982) describes feeding wounds from *G. gasterostei* from which he estimated that "a parasite would remove 25 - 40 cells in each feeding bout". It is only through such observations of actual feeding wounds that quantification of damage can be achieved. A more extensive comparison of feeding of *G. bullatarudis* and *G. turnbulli*

would entail observation of flukes until feeding had occurred, followed by careful excision of the fin or scale in order to study the damage by direct observation and sectioning followed by histological staining.

The observations of feeding of both *G. bullatarudis* and *G. turnbulli* differed from those of *G. gasterostei* (Harris, 1982) and of *G. salaris* Mo (1994) in that both the latter species were observed to attach to the host using their cephalic lobes whilst the pharynx was placed on the host skin and to remain attached during feeding. My observations were that the cephalic lobes may have attached to the fish skin initially, but once feeding was taking place they were released and were actually lifted off the host skin whilst feeding contractions of the pharynx were occurring. Another difference was the length of time the pharynx was in contact with the fish skin, Harris (1982) recorded times of between 2 and 3 minutes for *G. gasterostei* on *Gasterosteus aculeatus.* Lester (1972), however, recorded times comparable with my study of between 20 and 30 seconds for *G. alexanderi* on *G. aculeatus*. However, Lester (1972) also stated that feeding occurred less than once per hour at 15°C.

In discussing the pathology of *G. salaris* on Norwegian Atlantic salmon (*Salmo salar*) Malmberg (1993) and Mo (1994) attribute greater damage to the host epithelium by feeding than to the damage caused by attachment. Feeding behaviour of *Gyrodactylus* differs markedly between species, albeit at different temperatures, however, this has been insufficiently studied, leaving a dearth of knowledge of particular interest given the apparent importance of feeding in the determination of pathogenicity.

8.4.7. Summary

1. *G. bullatarudis* and *G. turnbulli* have similar intrinsic rates of increase

on naive fish at 26°C. Intrinsic rates of increase - after a threshold density - became negatively correlated with infrapopulation size. This was presumably due to the affects of over-crowding and intraspecific competition.

2. Under experimental conditions *G. bullatarudis* was approximately twice as lethal as *G. turnbulli* per parasite per host per day on fish showing no signs of an immune response at 26°C.

3. *G. bullatarudis* and *G. turnbulli* had different host-site specificities which caused different signs of gross pathology. Heavy infections of *G. bullatarudis* caused visible damage to the anterior of the head, including the cornea, whilst heavy infections of *G. turnbulli* caused their host's fins to become "clamped", leading to a loss of swimming efficiency and manoeuvrability.

4. There was evidence that *G. bullatarudis* infecting the preferred region (the head and operculum) performed better than flukes of the same species infecting other regions of the host. Because of this, the site of initial inoculation was shown, on some fishes, to affect the future rate of development of the resulting infrapopulation.

5. The feeding behaviour of the two *Gyrodactylus* species were different. Over a given time, the pharynx of a *G. bullatarudis* was likely to be in contact with the host epidermis for longer than a pharynx of *G. turnbulli*. A more detailed study is required for an exact quantification of damage caused by each species per fluke per feeding bout.

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CHAPTER 9

9. Infrapopulation dynamics of initial and challenge infections of two *Gyrodactylus* species on *P. reticulata.*

9.1. Introduction

Scott and Anderson (1984) found that oscillating prevalence and intensity of *Gyrodactylus* was a feature of *G. turnbulli* [misidentified and named as *G. bullatarudis* - see Harris (1986)] epidemiology on guppies in experimental arenas with a regular in-flow of susceptible hosts. They found that the model which best fitted these observations was one which incorporated a temporary refractory response of the host post-recovery from infection.

Studies by Scott and Robinson (1984) demonstrated that *P. reticulata* were refractory to challenge infections of *G. turnbulli* initiated one day after treatment, one or two weeks following an initial infection.

The first aim of this chapter was to test the protection acquired by *P. reticulata* from initial infections, treated three days post inoculation, against challenge infections of either *G. bullatarudis* or *G. turnbulli*, regardless of which species was used in the initial infection.

By comparing the host-site specificities of initial and challenge infections, the second aim was to investigate whether the host response, if initiated, was localised or spread over the whole of the external surface.

9.2. Materials and methods

The origins of the parasites used in this work are described in Chapter 2, section 2.2.1 (*G. turnbulli*) and Chapter 3, section 3.2.1, entrance for "Group II" (*G. bullatarudis*) and their maintenance in Chapter 4, sections 4.2.2 and 4.2.3. The fish used were *Gyrodactylus*-naive immature guppies of standard length 11mm which had been out bred in the laboratory from a varied ornamental stock.

The experiments were carried out in a room with air temperature kept between 27 and 28°C, water temperature between 24.5 and 26.0°C and a 12:12 light:dark regime. Experiments were carried out in the same room in which guppy and parasite stocks were kept.

9.2.1. Experimental groups

Treatments were performed on groups of 19 or 20 fish as follows:

Group c/c (Host mortality control group): 20 fish anaesthetised for 4 to 4.5mins on day 0, 3.5 to 4mins followed by a treatment procedure on day 3, anaesthetised for 4 to 4.5mins on day 4 and and for 3.5 to 4mins every following third day for 30 days. The results from this group were used to control for mortality caused to the experimental fish by factors other than *Gyrodactylus* infection.

Group c/Gb (*G. bullatarudis* challenge control group): 19 fish anaesthetised for 4 to 4.5mins on day 0, 3.5 to 4mins followed by a treatment procedure on day 3, infected with 3 *G. bullatarudis* on day 4 and examined every following third day until there were two counts of 0 on consecutive

examinations or the fish died. The results from this group were used to provide a control for comparison with the two groups which used *G. bullatarudis* as the challenge infection.

Group Gb/Gb (*G. bullatarudis* initial and challenge infections group): 20 fish infected with 3 *G. bullatarudis* on day 0, examined and treated on day 3, reinfected with 3 *G. bullatarudis* on day 4 and examined every following third day until there were two counts of 0 on consecutive examinations or the fish died. The results from this group were used to investigate the dynamics of a challenge infection of *G. bullatarudis* following an initial infection of *G. bullatarudis.*

Group Gt/Gb (*G. turnbulli* initial infection followed by *G. bullatarudis* challenge infection group): 20 fish infected with 3 *G. turnbulli* on day 0, examined and treated on day 3, reinfected with 3 *G. bullatarudis* on day 4 and examined every following third day until there were two counts of 0 on consecutive examinations or the fish died. The results from this group were used to investigate the dynamics of challenge infections of *G. bullatarudis* following an initial infection of *G. turnbulli.*

Group c/Gt (*G. turnbulli* challenge control group): 20 fish anaesthetised for 4 to 4.5mins on day 0, 3.5 to 4mins followed by a treatment procedure on day 3, infected with 3 *G. turnbulli* on day 4 and examined every following third day until there were two counts of 0 on consecutive examinations or the fish died. The results from this group were used to provide a control for comparison with the two groups which used *G. turnbulli* as the challenge infection.

Group Gt/Gt (*G. turnbulli* initial and challenge infections group): 20 fish infected with 3 *G. turnbulli* on day 0, examined and treated on day 3, reinfected with 3 *G. turnbulli* on day 4 and examined every following third

day until there were two counts of 0 on consecutive examinations or the fish died. The results from this group were used to investigate the dynamics of a challenge infection of *G. turnbulli* following an initial infection of *G. turnbulli.*

Group Gb/Gt (*G. bullatarudis* initial infection followed by *G. turnbulli* challenge infection group): 20 fish infected with 3 *G. bullatarudis* on day 0, examined and treated on day 3, reinfected with 3 *G. turnbulli* on day 4 and examined every following third day until there were two counts of 0 on consecutive examinations or the fish died. The results from this group were used to investigate the dynamics of challenge infections of *G. turnbulli* following an initial infection of *G. bullatarudis.*

Experimental isolated fish were kept in 200ml water obtained from a 55l aquarium used to hold two male and seven female *P. reticulata*, uninfected with *Gyrodactylus* (referred to hereafter as aquarium water[1]). The tank made use of an under-gravel filter and also contained two broad-leafed plants and some algae. The experimental fish were isolated, each in the bottom part of a thoroughly washed plastic fizzy drinks bottle which had been cut in two about 15cm from the base.

Infection and examination procedures were as described in Chapter 8, sections 8.2.2 and 8.2.3 respectively, except water from the aquarium (mentioned above) was used instead of standing tap water.

All fish were fed daily with tropical fish flakes. Water was changed every three days following infection, examination or treatment procedures.

9.2.2. Treatment procedure

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[1] See 9.4. Discussion for an explanation for the change in the source of water for use in experiments.

The treatment solution used was 1ml formalin solution added to 4l aquarium water (Lester, 1972; Scott and Robinson, 1984). Each fish to be treated was removed from its container using a 5cm diameter petri dish, excess water was pipetted out and it was then placed in 200ml treatment solution in a similar container. After 1h the fish was removed from the solution in the same way as above and placed in a clean container of 200ml aquarium water.

9.2.3. Analysis

The following parameters based upon those used by Scott and Robinson (1984) were used to compare the dynamics of challenge infections with those of initial and control infections:

Establishment of infrapopulations following challenge or control infections.

Comparisons of mean parasite burden on corresponding days post challenge / control infection.

Comparison of mean peak parasite burdens (the maximum infrapopulations recorded on fish which recovered from infection).

Comparisons of mean duration of infection.

Comparisons of host-site specificity.

All comparative statistics were carried out using InStat 2.01 for Macintosh (GraphPad Software, 1993).

9.3. Results

None of the fish in the c/c group died during the experiment.

The other fish were divided into three categories depending on the course of their control / challenge infection; 1, fish that recovered from infection, 2, fish that died whilst infected and, 3, fish that died whilst infected but showed a reduction in infrapopulation on consecutive checking days at least once. Note that divisions 2 and 3 are not mutually exclusive. The number of fish in each of these categories from the various experimental groups are shown in Table 9.1.

Group	No. of fish that recovered from infections	No. of fish that died whilst infected	No. of fish that died but showed signs of a response to the infection	Total no. of fish in each group
c/Gb	19	0	-	19
Gb/Gb	20	0	-	20
Gt/Gb	19	1	0	20
c/Gt	12	8	0	20
Gt/Gt	15	5	1	20
Gb/Gt	16	4	2	20

Table 9.1. The number of fish from the six different groups which either recovered from infection, died whilst infected, or died whilst infected but showed signs of a response by a reduction in infrapopulation at least once on consecutive checking days.

Group	No. of fish on which infrapopulations established	No. of fish on which infrapopulations did not establish
c/Gb	8	11
Gb/Gb	7	13
Gt/Gb	3	17
c/Gt	17	3
Gt/Gt	16	4
Gb/Gt	19	1

Table 9.2. A comparison of the numbers of fish from each group on which *Gyrodactylus* infrapopulations established according to the criteria of Scott and Robinson (1984). The numbers only apply to challenge or control infections.

9.3.1. Establishment of infrapopulations following challenge or control infections

The criteria used to determine when a parasite infrapopulation was successfully established were taken from Scott and Robinson (1984) - "(1) the parasite population increased above three parasites at some time during the infection, (2) the fish remained infected at least until day six post-infection". The second criteria meant that only control or challenge infections could be assessed for establishment - initial infections were treated before "day six post-infection".

Table 9.2 shows a comparison of the numbers of fish on which infrapopulations successfully established in the challenge and control groups.

Sections 9.3.2 to 9.3.4 involve only those fish which recovered from their challenge / control infections.

9.3.2. Comparisons of mean parasite burden

Figs. 9.1 and 9.2 show the mean parasite burdens on fish challenged with *G. bullatarudis* or *G. turnbulli* in comparison with their respective control groups and initial infections.

The Wilcoxon matched pairs test was used to compare mean parasite burden of the various challenge groups on day three of their initial infections compared with the mean parasite burden on day three of their challenge infections. The results of these tests are shown in Table 9.3.

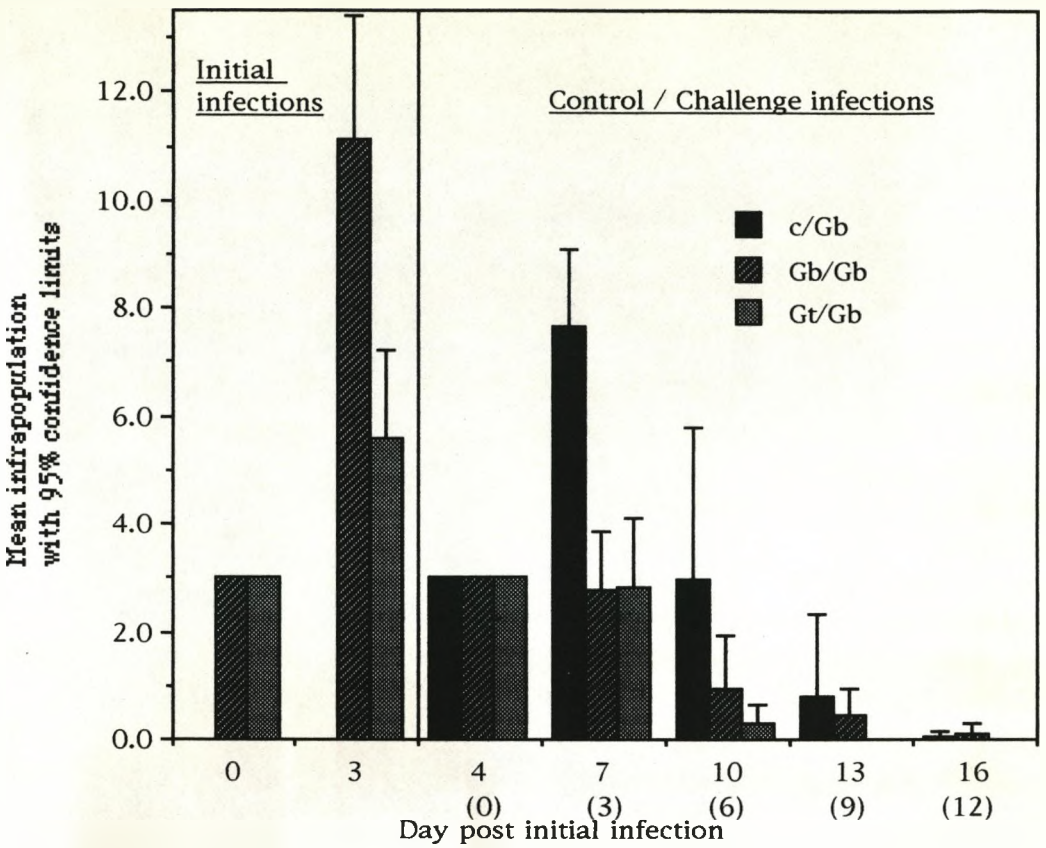

Fig. 9.1. Mean parasite burdens of initial and challenge / control infections of groups c/Gb, Gb/Gb and Gt/Gb with 95% confidence limits. Numbers in parentheses denote days post - challenge / control infections.

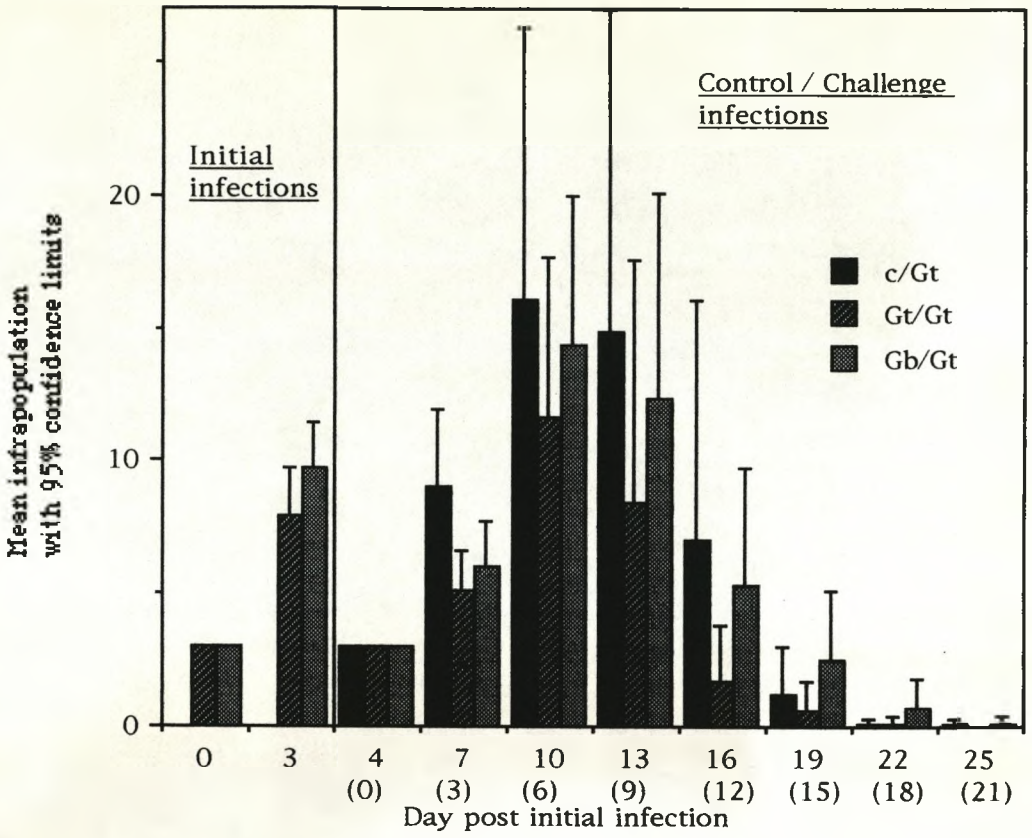

Fig. 9.2. Mean parasite burdens of initial and challenge / control infections of groups c/Gt, Gb/Gt and Gt/Gt with 95% confidence limits. Numbers in parentheses denote days post - challenge / control infections.

Group	No. of pairs (N)	T	P
Gb/Gb	20	202	<0.0002
Gt/Gb	16	85	0.03
Gt/Gt	12	65	0.01
Gb/Gt	14	82	0.01

3 pairs were excluded from groups Gt/Gb and Gt/Gt and 2 pairs from group Gb/Gt because the paired numbers were of equal value

Table 9.3. Results of the Wilcoxon matched pairs test used to compare mean parasite burden of the various challenge groups on day three of their initial infections compared with the mean parasite burden on day three of their challenge infections.

In addition, mean parasite burdens of respective challenge and control infections were compared on day 3 post challenge / control infection using the Kruskal-Wallis nonparametric ANOVA test with Dunn's multiple comparison post-test. The mean parasite burdens of the *G. bullatarudis* challenge and control groups were found to be significantly different ($P < 0.0001$) by the Kruskal-Wallis test and the post-test found the mean parasite burdens of the control infections to be significantly different to both challenge groups ($P < 0.001$) but groups Gb/Gb and Gt/Gb were not found to be significantly different to each other. Similarly, the *G. turnbulli* challenge and control groups were found to have significantly different mean parasite burdens on day three post-inoculation ($P < 0.01$), however, Dunn's post-test found only the means from c/Gt and Gt/Gt to be significantly different ($P < 0.05$).

The means of these respective groups were not significantly different on subsequent days post inoculation according to the same tests.

9.3.3. Comparisons of mean peak parasite burdens

Using Kruskal-Wallis nonparametric ANOVA test and Dunn's multiple comparison post-test, the mean peak parasite burdens of respective control and challenge infections were compared.

Mean peak burdens of the *G. bullatarudis* challenge and control infections (8.74 for c/Gb, 2.95 for Gb/Gb challenge infection and 2.84 for Gt/Gb challenge infection) were found to be significantly different by the nonparametric ANOVA test ($P < 0.0001$). The post-test showed the average peak parasite burdens of c/Gb to be significantly different from those of both challenge infections. However, mean peak burdens of Gb/Gb and Gt/Gb were not found to be significantly different.

Mean peak burdens of the *G. turnbulli* challenge and control infections (23.9 for c/Gb, 15.6 for Gt/Gt challenge infection and 17.2 for Gb/Gt challenge infection) were not found to be significantly different.

9.3.4. Comparisons of mean duration of infection

The mean durations of infections - recorded as the number of days from the inoculation to the last day on which fish observed to be infected - were recorded for all challenge and control groups. They were 4.7, 3.9 and 2.84 days for groups c/Gb, Gb/Gb and Gt/Gb and 8.5, 8.4 and 11.25 days for groups c/Gt, Gt/Gt and Gb/Gt respectively.

Bartlett's test for homogeneity of variances showed that within the sets of *G. bullatarudis* and *G. turnbulli* challenge and control groups, the variance of the durations of infection were not significantly different and so it was decided to use parametric ANOVA to test for significant differences between mean durations of infection within the two sets of groups.

However, for both *G. bullatarudis* and *G. turnbulli* challenge and control groups, no significant differences between mean durations of infection were found.

9.3.4. Comparisons of dynamics of infrapopulations of fish that died

Comparisons of the numbers of mortalities caused by respective control and challenge infections showed no significant differences by χ^2 analysis.

The dynamics of infrapopulations of the fish from group Gt/Gb and those that died following challenge / control infections of *G. turnbulli* are shown in Figs. 9.3 and 9.4 respectively.

Table 9.4 shows a comparison of the mean maximum parasite burden and mean longevity post-challenge / control infection of fish from groups c/Gt, Gt/Gt and Gb/Gt that died. The samples of fish from these groups that died were too small to allow further statistical analysis.

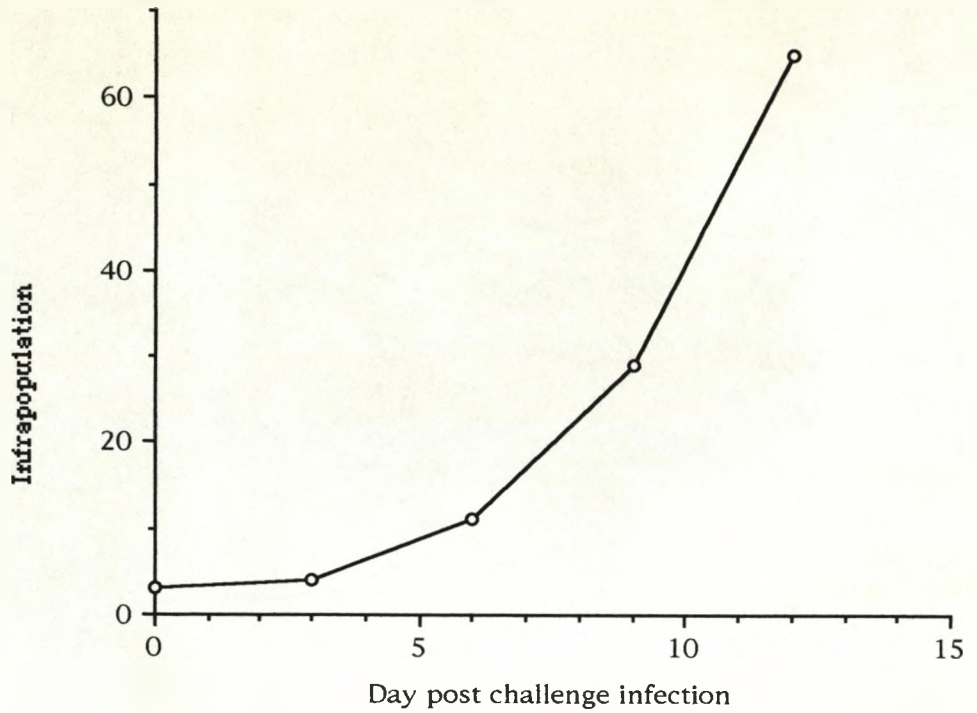

Fig. 9.3. Infrapopulation dynamics of *G. bullatarudis* on the fish of the group Gt/Gb that died.

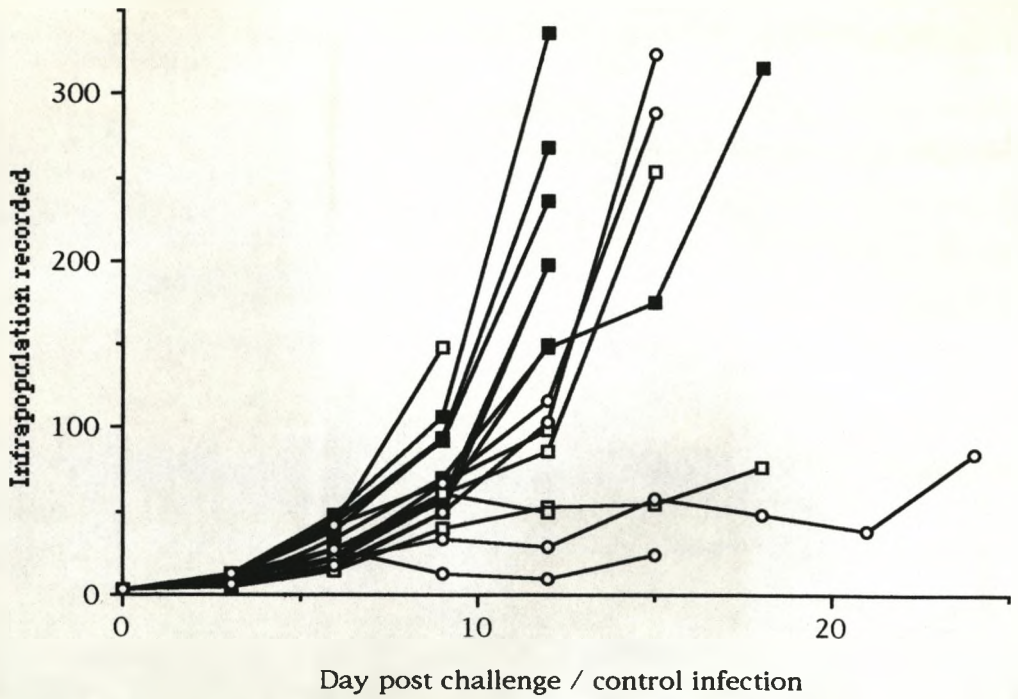

Fig. 9.4. Infrapopulation dynamics of *G. turnbulli* on the fish of the groups c/Gt (solid squares), Gt/Gt (open squares) and Gb/Gt (open circles) that died.

	Mean longevity post challenge / control inoculation (days)			Mean maximum infrapopulation recorded		
	c/Gt	Gt/Gt	Gb/Gt	c/Gt	Gt/Gt	Gb/Gt
Mean	15	15.2	18.25	225	127.8	181.5
Std. Dev.	2.27	3.56	5.19	81.81	77.79	147.49
N	8	5	4	8	5	4
SEM	0.801784	1.59	2.59	28.93	34.79	73.75
Range	12 - 20	10 - 20	15 - 26	95 - 338	61 - 254	27 - 324

Table 9.4. A comparison of the mean longevity post-challenge / control infection and mean maximum parasite burden of fish from groups c/Gt, Gt/Gt and Gb/Gt that died. The samples of fish from these groups that died were too small to allow further statistical analysis.

9.3.5. Comparisons of host-site specificity

In order to examine whether the distribution of parasites of challenge infections were different to that of initial or control infections, the following comparisons of host-site specificities were made using χ^2 analysis of the following 3 X 2 contingency tables.

i, Comparison of host-site specificity on day 3 of c/Gb* with that of Gt/Gb* (asterisk denotes the infections being compared, for example, above, the *G. bullatarudis* control infection from group c/Gb is being compared with the *G. bullatarudis* challenge infection from group Gt/Gb).

Group set	No. flukes on the fins.	No. flukes on the caudal peduncle and flanks.	No. of flukes on the head and operculum.
c/Gb*	24 (16.4%)	54 (37.0%)	68 (46.6%)
Gt/Gb*	5 (9.1%)	4 (7.2%)	46 (83.7%)

Results: $\chi^2 = 23.393$, $P < 0.0001$, host-site specificities of *G. bullatarudis* infections on day three of the control infection and of the challenge infection following an initial infection of *G. turnbulli* were significantly different.

ii, Comparison of host-site specificity on day 3 of Gb*/Gb with that of Gb/Gb*.

Group set	No. flukes on the fins.	No. flukes on the caudal peduncle and flanks.	No. of flukes on the head and operculum.
Gb*/Gb	78 (35.0%)	37 (16.6%)	108 (48.4%)
Gb/Gb*	12 (21.8%)	11 (20.0%)	32 (58.2%)

Results: $\chi^2 = 3.49$, $P = 0.17$, host-site specificities of *G. bullatarudis* infections on day three of the initial infection and of the following challenge infection were not significantly different.

iii, Comparison of host-site specificity on day 3 of c/Gt with that of Gb/Gt*.

Group set	No. flukes on the caudal fin.	No. flukes on the caudal peduncle.	No. flukes on the other fins, flanks, head and operculum.
c/Gt*	53 (29.6%)	113 (63.1%)	13 (7.3%)
Gb/Gt*	27 (20.6%)	97 (74.0%)	7 (5.3%)

Results: $\chi^2 = 4.14$, $P = 0.1264$, host-site specificities of *G. turnbulli* infections on day three of the control infection and of the challenge infection following an initial infection of *G. bullatarudis* were not significantly different.

vi, Comparison of host-site specificity on day 3 of Gt*/Gt with that of Gt/Gt*.

Group set	No. flukes on the caudal fin.	No. flukes on the caudal peduncle.	No. flukes on the other fins, flanks, head and operculum.
Gt*/Gt	49 (30.6%)	100 (62.5%)	11 (6.9%)
Gt/Gt*	20 (16.9%)	87 (73.7%)	11 (9.3%)

Results: $\chi^2 = 6.904$, $P = 0.03$, host-site specificities of *G. turnbulli* infections on day three of the initial infection and of the following challenge infection were significantly different.

The following comparisons showed significantly different host-site specificities on day three post infection - c/Gb* vs Gt/Gb*, Gt*/Gt vs Gt/Gt*. In order to test whether these differences resulted from significantly different placements of flukes on their artificial infection (day 0), the following comparisons by χ^2 analysis were made.

v, Comparison of host-site specificity on day 0 of c/Gb* with that of Gt/Gb*.

Group set	No. flukes on the caudal fin,	No. flukes on the caudal peduncle, anal and dorsal	No. of flukes on the pectoral and pelvic fins, flanks and head and operculum.
c/Gb*	13 (22.8%)	10 (17.5%)	34 (59.6%)
Gt/Gb*	16 (26.7%)	13 (21.7%)	31 (51.7%)

Results: χ^2 = 0.764, P = 0.683, host-site specificities of *G. bullatarudis* infections on day 0 of the control infection and of the challenge infection following an initial infection of *G. turnbulli* were not significantly different.

vi, Comparison of host-site specificity on day 0 of Gt*/Gt with that of Gt/Gt*.

Group set	No. flukes on the caudal fin,	No. flukes on the caudal peduncle, anal and dorsal	No. of flukes on the pectoral and pelvic fins, flanks and head and operculum.
Gt*/Gt	16 (26.7%)	16 (26.7%)	28 (46.7%)
Gt/Gt*	18 (30.0%)	15 (25.0%)	27 (45%)

Results: χ^2 = o,168, P = 0.919, host-site specificities of *G. turnbulli* infections on day 0 of the initial infection and of the following challenge infection were not significantly different.

9.4. Discussion

Comparisons of mean parasite burdens on day 3 between initial and challenge infections and control and challenge infections showed that an initial infection of either parasite species caused a significantly reduced infrapopulation on day three post challenge infection. The response from *P. reticulata* to an initial *Gyrodactylus* infection provided some protection against a challenge infection three days after its termination regardless of

whether the same or a different species of parasite was used in the initial and challenge infection.

The results showed that, under the experimental conditions, *G. bullatarudis* were more susceptible to the response of the host than *G. turnbulli.* Mean durations of infections were markedly lower for challenge / control groups of *G. bullatarudis* in comparison to those of *G. turnbulli* and far fewer of the challenge / control infections of *G. bullatarudis* established than those of *G. turnbulli.* In addition, comparisons of mean peak parasite burdens were found to be significantly different for the control and challenge infections of *G. bullatarudis* but not for the challenge and control infections of *G. turnbulli.*

Contrary to the results of Scott and Robinson (1984), there were no significant differences between the numbers of naive and challenged fish on which parasite infrapopulations established. Scott and Robinson (1984) used a paired comparison between initial infections and challenge infections of *G. turnbulli*. Such a comparison was not possible in this study because the initial infection was not allowed to run for six days to assess establishment (see section 9.3.1). Consequently, proportions of fish samples on which *Gyrodactylus* established were compared between respective control and challenge infections.

Also contrary to the results of Scott and Robinson (1984), no significant differences were found between the duration of challenge and control infections. This was true for both *G. bullatarudis* and *G. turnbulli.*

The evidence above suggests that the host response to *Gyrodactylus* infection takes time to develop and becomes more effective as the duration of infection increases. Comparable trends are evident in Scott and Robinson (1984) who found that parasite burdens on fish challenged after

2 weeks were lower than those challenged after 1 week although "statistically significant differences were not detected". Similarly, in other comparisons of the results from that work between the dynamics of infrapopulations on fish challenged after two weeks and those of fish challenged after one week; the peak parasite burdens of the former and latter were 10.89 and 8.52 flukes respectively, the mean times to peak burdens were 5.25 and 3.68 days and the mean times to recovery 12.25 and 10.95 days.

The refractory period post-recovery from infection is temporary. Scott (1985) compared the dynamics of initial infections with those of challenge infections initiated immediately, and one, two, four and six weeks post natural recovery from *G. turnbulli* infection. She found that fish partially susceptible to reinfection immediately after recovery from an initial infection usually regained full susceptibility four to six weeks post-recovery. Fish completely refractory to reinfection immediately post-recovery became partially refractory to reinfection four to six weeks post-recovery. These results suggested a cline of susceptibility probably due to host genetic variation (see Madhavi and Anderson, 1985).

Infections of *G. bullatarudis* on fish which had had an initial infection of *G. turnbulli* were found to be significantly more concentrated on the head and opercula regions than the other infections of *G. bullatarudis* on day 3 post inoculation. The site specificity of the challenge infection of the group Gt/Gt was significantly different to its initial infection on day three post inoculation. The main difference was that proportionately fewer flukes were found on the caudal fin in the challenge than in the initial infection. Both these significantly different results showed proportionately fewer flukes from challenge infections on areas which were more heavily infected in the initial infections (see Figs. 9.5 and 9.6). These comparisons of host-site specificity provided further evidence that

the response to *Gyrodactylus* was non-specific and also that it was most effective over the main sites of infection.

A relatively localised response could conceivably explain changing host-site specificity observed by Harris (1988) during the course of *G. turnbulli* infections, where greater proportions of flukes were found on the caudal peduncle during the growth of the infrapopulations, but proportionately more on the fins during the decay phase of the infrapopulation dynamics. However, no similar results were seen in my observations of *G. turnbulli.*

The evidence suggests that the host response to *Gyrodactylus* is not species specific but may be relatively localised on areas of the fish surface which are most heavily infected. Therefore, where two species of fluke co-exist on the same host species in the same geographical location, when a fluke infects a new host, it is less likely to be subjected to an immediate localised host response caused by a previous infection if it relocates to a region not favoured by the other co-existing parasite species. Therefore, by occupying a site on the host unlikely to have been occupied by a sympatric species it increases its chances of survival to reproduce. This may provide a selective pressure making it advantageous for different sympatric species of *Gyrodactylus* to occupy different regions of the same host.

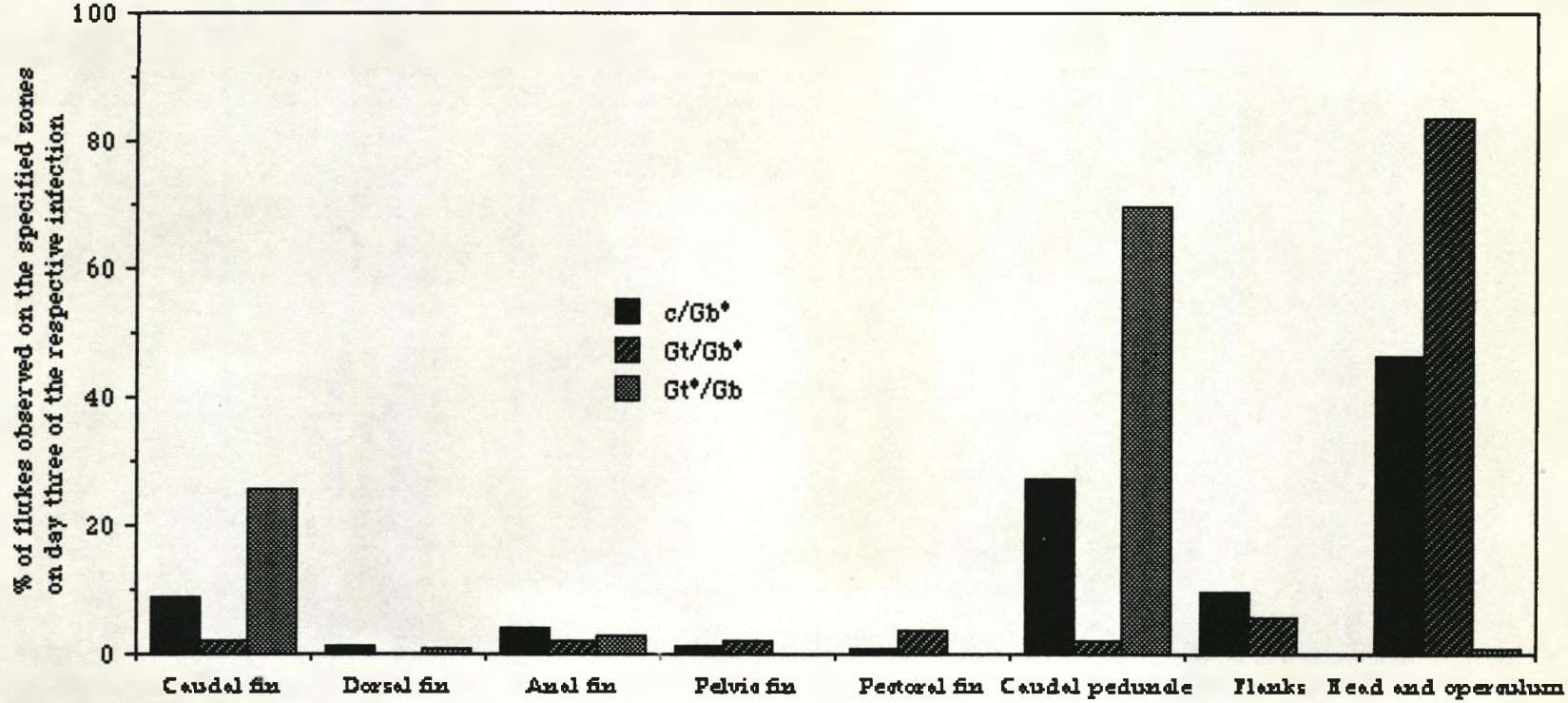

Fig. 9.5. Percentage occurrence of flukes on day three of the c/Gb*, Gt/Gb* and Gt*/Gb infections (asterisk denotes the respective infection). Note the relative absence of flukes on the caudal fin and caudal peduncle from the Gt/Gb* infection in comparison to the c/Gb* infection. It is postulated in the text that this difference was due to avoidance by flukes of the challenge (Gt/Gb*) infection of those areas of the external surface most heavily infected by the initial infection (Gt*/Gb) due to the initiated host response being most effective in these areas.

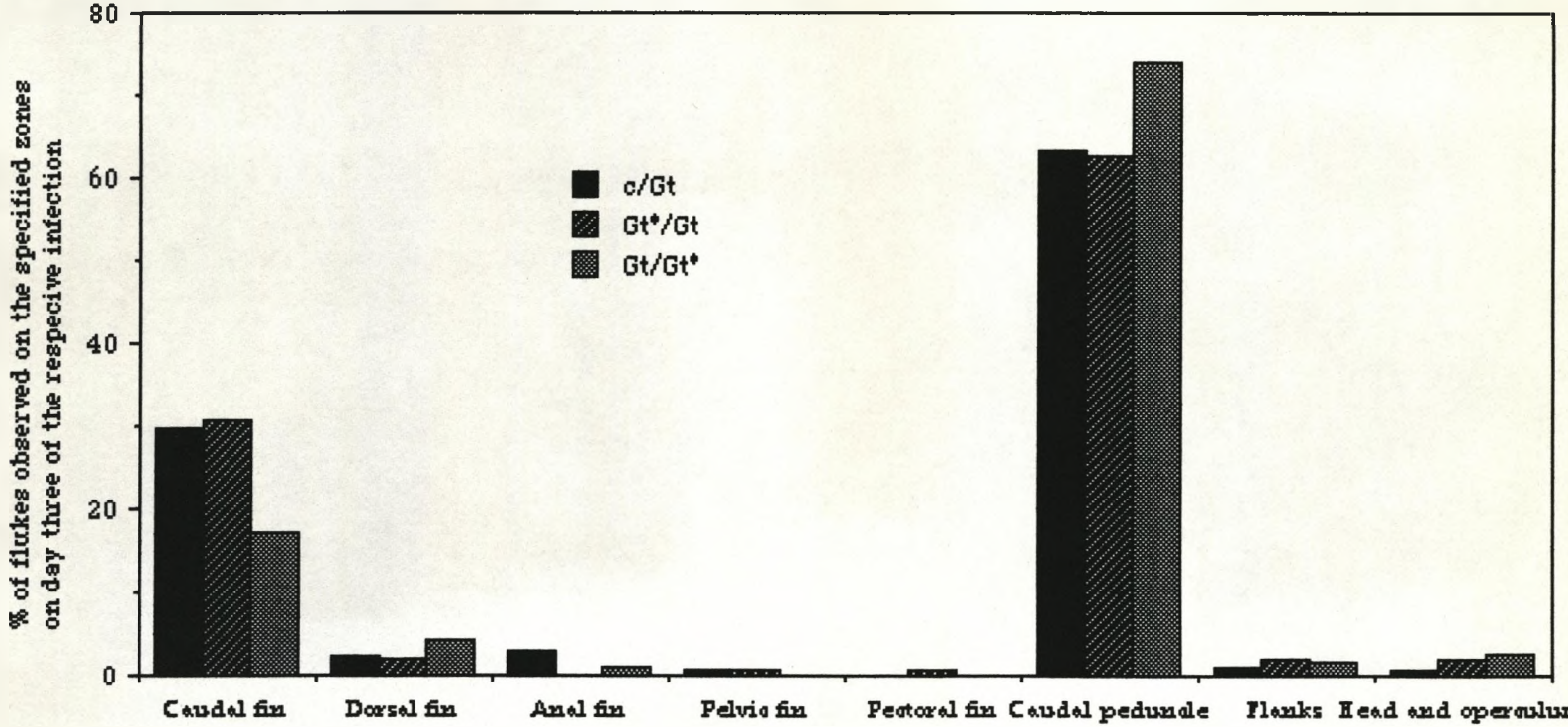

Fig. 9.5. Percentage occurrence of flukes on day three of the c/Gt*, Gt/Gt* and Gt*/Gt infections (asterisk denotes the respective infection). Note the relative absence of flukes on the caudal fin from the Gt/Gt* infection in comparison to the c/Gt* and Gt*/Gt infections. It is postulated in the text that this difference was due to avoidance by flukes of the challenge (Gt/Gt*) infection of those areas of the external surface most heavily infected by the initial infection (Gt*/Gt) due to the initiated host response being most effective in areas.

Although adding to the knowledge of the host response of the guppy to *Gyrodactylus* - it has been shown that the response is not species specific and is most effective over the main sites of infection - the exact nature of the response remains unknown. Lester (1972) described "cuticular shedding" of *Gasterosteus aculeatus* in response to *G. alexanderi* infections and noted that though "not produced in reaction to *Gyrodactylus*, its increase in density is associated with the flukes". No similar observations were made of the fish used in this experiment, however, I sometimes observed guppies with *G. turnbulli* develop a thickening of the epidermis of the caudal peduncle which appeared white and translucent. This thickening disappeared following recovery from infection, although sloughing was not observed.

There was a marked difference in the mortality resulting from the control infections compared to those fish infected in Chapter 8 (0/19 of c/Gb and 8/20 of c/Gt in comparison to 20/25 of those fish infected with *G. bullatarudis* and 43/45 of those fish infected with *G. turnbulli* in Chapter 8). The differences between the treatment of fish used in the present experiments and those described in Chapter 8 were:
(1) Control fish (present work) were pretreated with anaesthetic and a 1: 4000ml formalin bath whereas fish used in Chapter 8 were not manipulated until their infection. (2) Control fish (present work) were subjected to anaesthetic for counting of fluke infrapopulations every third day, not every second day, post infection. Consequently, water was changed every third day and not every second day post-infection. (3) Water used in the present work was taken from an aquarium used for breeding *Gyrodactylus*-naive guppies and water used for the experimental isolated fish used in Chapter 8 was taken straight from the reservoir tank after it had been left for 48 hours or more (standing tap water, see Chapter 2, section 2.2.2).

The protocol was changed to make use of water from the breeding tank in

preference to standing tap water because of a change in tap water quality which was noticed during a run of experiments undertaken after those described in Chapter 8 but before those described in this chapter. These intermediate experiments ran well until isolated fish started to die prematurely - even those with very low parasite burdens, and fish from an uninfected control group. By using water from an aquarium used as a guppy breeding tank instead of water from the standing reservoir, premature mortality of experimental fish was eliminated. However, because of this change in protocol, the experiments had to be entirely repeated. Control infections of *G. turnbulli* from this intermediate run of experiments (before the adverse mortalities caused by the change in water quality), whose treatment differed from those presently described (Group c/Gt) only in the source of water used, resulted in a similar mortality (30/30) to those fish whose infections of *G. turnbulli* are described in Chapter 8.

These differences in mortalities caused by infection experiments show that water composition is important in the determination of the recovery rate of fish to *Gyrodactylus* infections, although the mechanism of the relationship in this instance is unknown. Unfortunately, no records of the water chemistry were taken from the standing tap reservoir water before the problems started to arise in the experimental work and so the differences in water composition which resulted in these changes in host-parasite interaction remain unidentified.

9.5. References

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CHAPTER 10

10. Long-term *Gyrodactylus* infections of adult *P. reticulata* in experimental arenas.

10.1. Introduction

The epidemiology of *G. turnbulli* [misidentified as *G. bullatarudis* - see Harris (1986)] on *P. reticulata* was studied in a variety of experiments which are summarised by Scott (1985). However, all these experiments used host fish which were immature and under 16mm standard length.

Cusack (1986) found a negative correlation between the size of *G. colemanensis* infections of *Salmo gairdneri* and host size. Scott (1985) reports that in Scott and Anderson's (1984) long-term infections of *P. reticulata* with *G. turnbulli*, infections were more aggregated amongst the smaller fish. Mughal and Manning (1986) found evidence that the humoral defences of the immune system of juvenile thick-lipped grey mullet (*Chelon labrosus* Risso) were not as well developed as adults of the same species. There are therefore differences in the host - parasite interactions of parasitic infections of juvenile and adult fish. The first aim of this chapter was to use simple experimental designs to investigate the dynamics of *G. bullatarudis* and *G. turnbulli* infections on small numbers of adult fish contained within 50l aquaria.

I have found high prevalences of *G. turnbulli* on *P. reticulata* imported from ornamental fish farms in Singapore and from unknown origin purchased from a local retailer in Liverpool. It is of interest to aquarists to

know how long infestations of *Gyrodactylus* might persist on adult *P. reticulata* under ordinary aquarium conditions.

The second aim of these experiments was to examine whether there are any differences in the host-site specificities exhibited by *Gyrodactylus* infections of adult as opposed to immature *P. reticulata.*

10.2. Materials and methods

10.2.1. Sources of fish and parasites

The origins of the parasites used in this work are described in Chapter 2, section 2.2.1 (*G. turnbulli*) and Chapter 3, section 3.2.1, entrance for "Group II" (*G. bullatarudis*) and their maintenance in Chapter 4, sections 4.2.2 and 4.2.3. The fish used were *Gyrodactylus*-naive immature guppies of standard length 11mm which had been out bred in the laboratory from a varied ornamental stock.

The fish were sexually mature laboratory bred *Gyrodactylus*-naive fish with standard lengths between 22 and 30mm.

10.2.2. Procedures for the long-term *G. turnbulli* and *G. bullatarudis* experiments

Three 50l aquaria were set up, each with an under gravel filter covered with 2.5cm of gravel and left for 1 month to allow maturation of water. To encourage the growth of nitrogen fixing bacteria within the gravel, small amounts of fish food flakes were placed in the tank approximately every second day for two weeks before fish were introduced.

10 fish were put into each tank, 5 males and 5 females, and were left for two weeks. Coloured drawings were made of each fish's caudal fin to allow them to be individually identified. Fish in the long-term *G. bullatarudis* infection experiment were named Gbm1, Gbm2, Gbm3, Gbm4 , Gbm5, Gbf1, Gbf2, Gbf3, Gbf4 and Gbf5 ("Gb" = *G. bullatarudis* experiment, "m" = male, "f" = female and the numbers arbitrarily distinguish the five fish of each sex). Fish in the long-term *G. turnbulli* infection experiment were named Gtm1...5 and Gtf1...5. Fish in the mortality control experiment were not named individually except for the female (named Cf1) which was anaesthetised first to act as a control for the fish used to initiate the *G. bullatarudis* and *G. turnbulli* experiments (see below).

Long-term G. turnbulli *infection experiment*

A female fish was taken from the tank which was to be infected with *G. turnbulli*. It was placed in a 12l aerated tank with around 20 smaller fish which were infected with *G. turnbulli*. This was, in fact, the *G. turnbulli* stock tank (see Chapter 4, section 4.2.3). After 19 hours it was removed from the *G. turnbulli* stock tank, anaesthetised using 0.02% MS222 and the numbers of flukes infecting it were counted and their positions recorded (see Chapter 8, section 8.2.3). This female fish was then placed in the tank with the 9 other adult *P. reticulata* which were to take part in the long-term *G. turnbulli* infection experiment. The day on which the fish was added to the tank was called day 0 of this experiment. Once approximately every 10 days from day 0 until termination of the experiment on day 210, see section 10.2.4, each fish was removed from the tank and checked for parasites using the procedures described in section 10.2.5.

Long-term G. bullatarudis *infection experiment*

To initiate the infections of *G. bullatarudis* in the second tank, a female

fish was infected by placing with two fish of approximately 16mm standard length with heavy infections of *G. bullatarudis* in a 500ml converted fizzy drinks container (see Chapter 8, section 8.2.1). The fish was left there for 24 hours. Again, the day of addition of this infected fish back into the second tank with the nine other fish, following a count of parasites whilst under anaesthetic, was called day 0 of the experiment. Each fish was checked for parasites every 10 days thereafter using the procedures outlined in section 10.2.5. Infections within the tank were followed in this way until no parasites were recorded on any fish for two consecutive checks. Later, the surviving fish were used, in this aquarium, in the investigation of the importance of transmission of detached *G. turnbulli* (see section 10.2.4).

Long-term adult mortality control experiment

On the initiation of the long-term adult mortality control experiment, a female fish was removed from the respective tank. This fish (named Cf1) was treated with anaesthetic to correspond to the two fish used to initiate the long-term *G. bullatarudis* and *G. turnbulli* infection experiments when they were initially checked for parasites. Cf1 was then placed back into the mortality control tank. Following that, approximately every 10 days for 164 days, each fish in the tank was treated with anaesthetic as outlined in section 10.2.5. Deaths of fish from this tank were recorded throughout the experiment.

Throughout these experiments all fish were fed daily with Tetra tropical fish food flakes. Dead fish were removed from the tanks approximately twelve hours after they were first noticed. This delay in removing the dead fish was to allow all possible transmission of *Gyrodactylus* from the dead to the surviving fish.

New-born fish were removed from the three aquaria as soon as they were

observed. All tanks were observed carefully at least once every day throughout these experiments in order to carry this process out. Three broods were removed from the long-term *G. turnbulli* experiment aquarium and three from the mortality control aquarium.

10.2.3. Challenge infection of fish infected with *G. turnbulli.*

Low intensities of *G. turnbulli* persisted in the long-term infection experiment for 13 weeks following the deaths of three heavily infected fish before day 50. To test the response of the remaining six fish to a challenge infection, a heavily infected fish was added to the aquarium as follows.

A female fish (27mm standard length) was placed in the main *G. turnbulli* 12l stock tank for three days. It was then checked for parasites using the standard procedures described in section 10.2.5 and found to be infected with 282 *G. turnbulli.* This fish was placed in the aquarium with the six remaining fish in the long-term *G. turnbulli* infection experiment on day 146. All fish were checked, as normal, on day 152 and the "challenge fish" was removed on day 154.

The long-term *G. turnbulli* infection experiment was terminated on day 210 but the aquarium, following removal of the fish (three of which were infected with *G. turnbulli*), was used in the experiment to investigate the importance of detached flukes in the transmission of *G. turnbulli* between hosts at low prevalence and intensities (see section 10.2.4, second paragraph).

10.2.4. Investigation of the importance of transmission of detached *G. turnbulli*

The "challenge fish" after being removed from the long-term *G. turnbulli*

experiment (see section, 10.2.3, second paragraph) was placed with the eight fish that had recovered from *G. bullatarudis* infections (see section. 10.2.2, fourth paragraph). This was eight weeks after the recovery of the latter fish from their infections. The day of introduction of *G. turnbulli* to this aquarium was called day 0 of this experiment. On day 20, all fish were removed after being checked for parasites using the protocol outlined in section 10.2.5. In total 1562 *G. turnbulli* were counted on the nine fish. Immediately after the fish were removed, nine *Gyrodactylus*-naive fish, two males and seven females, of 20 to 30mm standard length were placed in the tank. Four days afterwards, on day 24, these nine recipient fish, were checked for *G. turnbulli* infections.

The second investigation of detached *G. turnbulli* transmission was carried out using the six remaining fish from the long-term *G. turnbulli* infection experiment. On day 210 of that experiment, the six remaining fish were removed and checked for parasites. Instead of replacing them, six laboratory bred *Gyrodactylus*-naive fish of similar size were added in their place. That day was called day 0 of the small parasite population detached *G. turnbulli* transmission experiment. These six potential recipient fish were checked for parasite infections on days 4 and 13 using the standard procedures outlined in section 10.2.5.

10.2.5. Procedures for checking adult fish for *Gyrodactylus* infections

When all the fish in an aquarium were to be checked for infections, first the anaesthetic was mixed on the day using water from the same aquarium. A number of 250ml crystallising dishes, one for each fish, were placed in front of the aquarium and approximately 200ml of water from the respective aquarium was placed in each dish. In quick succession each fish was removed from the aquarium using a small aquarium net and was placed in a separate crystallising dish. After all the fish were removed from the

tank and isolated, each fish was taken in turn, anaesthetised and checked for parasites as described in section 8.2.4 - the only differences being that 250ml crystallising dishes were used instead of 5cm diameter petri dishes and all the water used during the protocol was from the tank which contained the fish being checked.

Before replacing the fish in the respective aquarium, an equivalent volume of water from the standing tap water reservoirs was used to top up the tank to compensate for that used in the checking procedures. Once all the fish from the particular tank had been checked, their infrapopulations recorded and were fully active and swimming following anaesthesia, they were replaced in the aquarium.

10.3. Results

10.3.1. Infections of *G. bullatarudis* on adult fish

Fig. 10.1 shows the mean intensity of *G. bullatarudis* infections on the 10 adult fish and the number of fish surviving plotted against time. Fig. 10.2 shows the prevalence (number of infected divided by total number of fish) of the *G. bullatarudis* on these experimental fish throughout the experiment.

Two fish died during the course of the experiment, one on day 2, before the first check on day 10, and the other on day 27, following a count of 1 fluke on day 20. Neither fish were suspected of dying as a result of *Gyrodactylus* infection.

One fish remained uninfected throughout the experiment. The fish used to initiate the infection with 68 flukes had recovered by day 10 and then

remained uninfected. All the other fish became infected. All but one (excluding the one that died on day 27) lost their infection before day 30. The remaining fish had lost its infection by day 40.

Of those fish that sustained and recovered from an infection, the maximum peak parasite burden recorded was 23 (excluding the infrapopulation on the fish introduced to begin the experiment) and the minimum was 1. No fish was found to be reinfected following recovery.

An area graph was prepared (Fig. 10.3) plotting the average percentage of the total infrapopulations of *G. bullatarudis* found on the defined areas of the host plotted against the total infrapopulation.

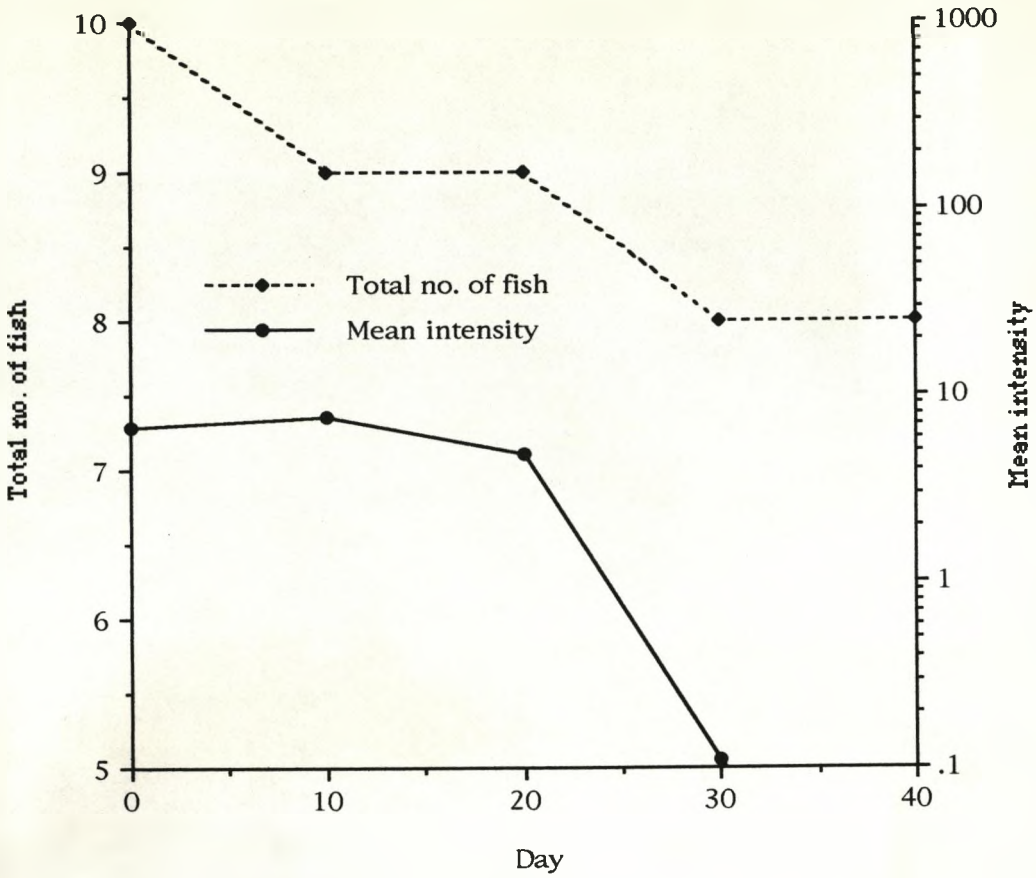

Fig. 10.1. Mean intensity (right axis, log scale) of *G. bullatarudis* infections and the total number of fish (left axis) remaining throughout the long-term infection experiment using ten adult *P. reticulata*.

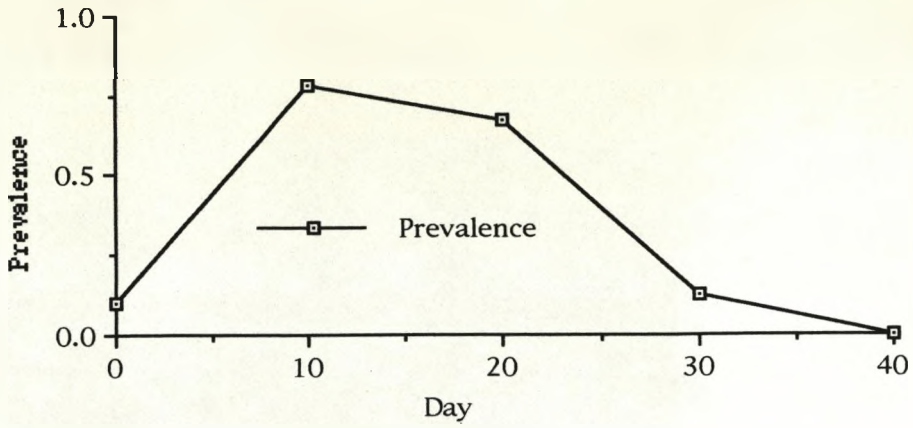

Fig. 10.2. Prevalence (number of infected fish / total number of fish) of *G. bullatarudis* throughout the long-term infection experiment using adult *P. reticulata*.

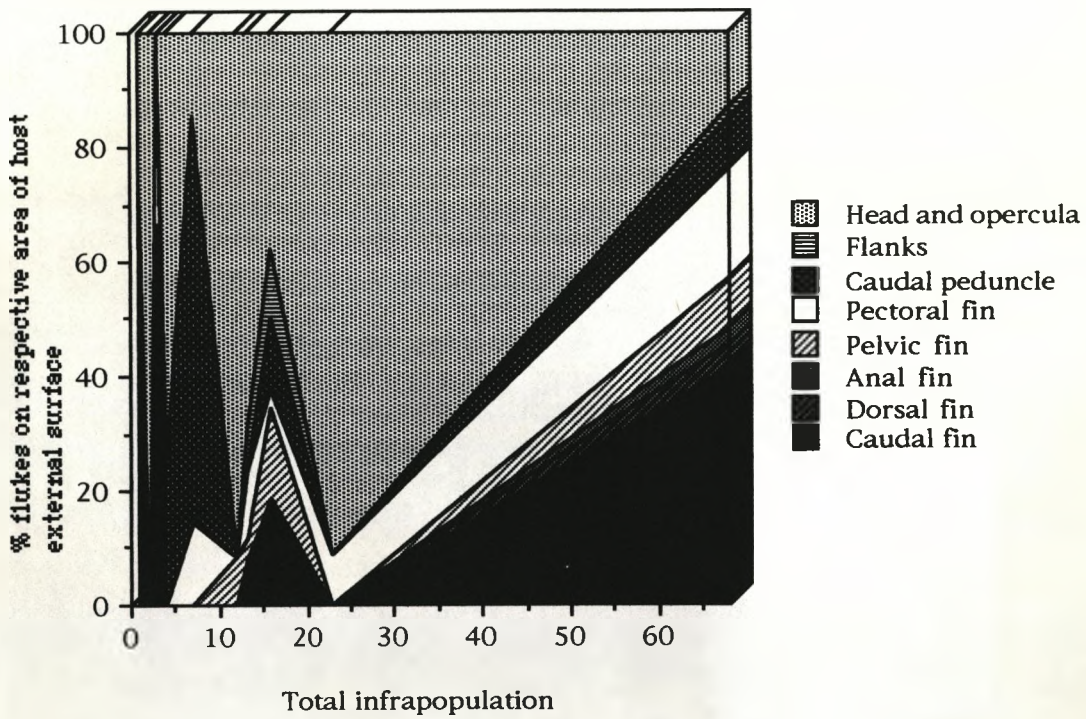

Fig. 10.3. *G. bullatarudis* host-site specificity during the long-term *G. bullatarudis* infection experiment on ten adult *P. reticulata*. Percentage of infrapopulations recorded on the eight defined areas of the host external surface plotted against actual total infrapopulation.

10.3.2. Infections of *G. turnbulli* on adult fish

Fig. 10.4 shows the mean intensity of *G. turnbulli* infections on the fish in the experiment and the number of fish surviving plotted against time. Fig. 10.5 shows the prevalence of *G. turnbulli* amongst the surviving experimental fish plotted against time. Note that on day 144 an additional fish (the "challenge fish" - see section 10.2.3) was placed in the tank carrying an infection of 282 *G. turnbulli*. The "challenge fish" was removed from the tank on day 154.

Four fish died during the course of the experiment. Three of those died following *G. turnbulli* infections which increased more or less exponentially. They died on days 33, 40 and 46 - their infrapopulations plotted against time are shown in Fig. 10.6. Another fish died on day 19 following a count of 9 flukes on day 10. It was unlikely that the *Gyrodactylus* infection was the cause of the death of this fish.

All fish were infected with *G. turnbulli* at some stage during the experiment. The *G. turnbulli* population was maintained for a long period at a low intensity. From days 50 to 144, when the challenge infection was introduced, the average intensity ranged from 0.33 to 3.3. During this time the maximum infrapopulation on any one fish was 6.

All of these six surviving fish, during days 50 to 144, were observed to have lost their infections at least once and then regain an infection, see Fig. 10.7. On day 122 all fish were observed to have lost their infections but on day 132 fish Gtf1 and Gtf4 were observed to have gained infections of 1 and 2 flukes respectively.

On addition of the challenge infection on day 144 all the six remaining fish became infected but by day 162 had again returned to a mean intensity of

infection of less than 3. These low levels of intensity of infection were maintained until the termination of the experiment on day 210.

As observed between days 50 and 142, even though the six fish were supporting a very small total population of parasites ranging between 2 and 10, some fish regained infections after previously becoming uninfected.

Fig. 10.8 shows the relationship between dispersion, given as variance/mean "standardised by multiplying by the number of observations in the sample minus one (N - 1), that is, the degrees of freedom," to give the product, chi-square (= χ^2) (Fowler and Cohen, 1990) and the total fluke population. There is a significant correlation ($P < 0.05$ according to the Spearman Rank Correlation test) between the standardised value of dispersion and mean intensity of infection.

Fig. 10.9 shows calculated χ^2 values plotted against respective degrees of freedom superimposed upon zones of 95% confidence for clumped, random and regular dispersal obtained from Figure 8.2 in Fowler and Cohen (1990). The χ^2 values between days 50 to 144 and days 154 to 210, when six fish were present, was nearly within or within the 95% confidence zone indicating random dispersal.

Area graphs are shown (Figs. 10.10 and 10.11) plotting the average percentages of the total infrapopulations of *G. turnbulli* found on the eight predefined areas of the host external surface plotted against respective total infrapopulations. Fig. 10.10 summarises the host-site specificity of flukes parasitising the fish that died as a result of infection and Fig. 10.11 that of flukes parasitising the six surviving fish.

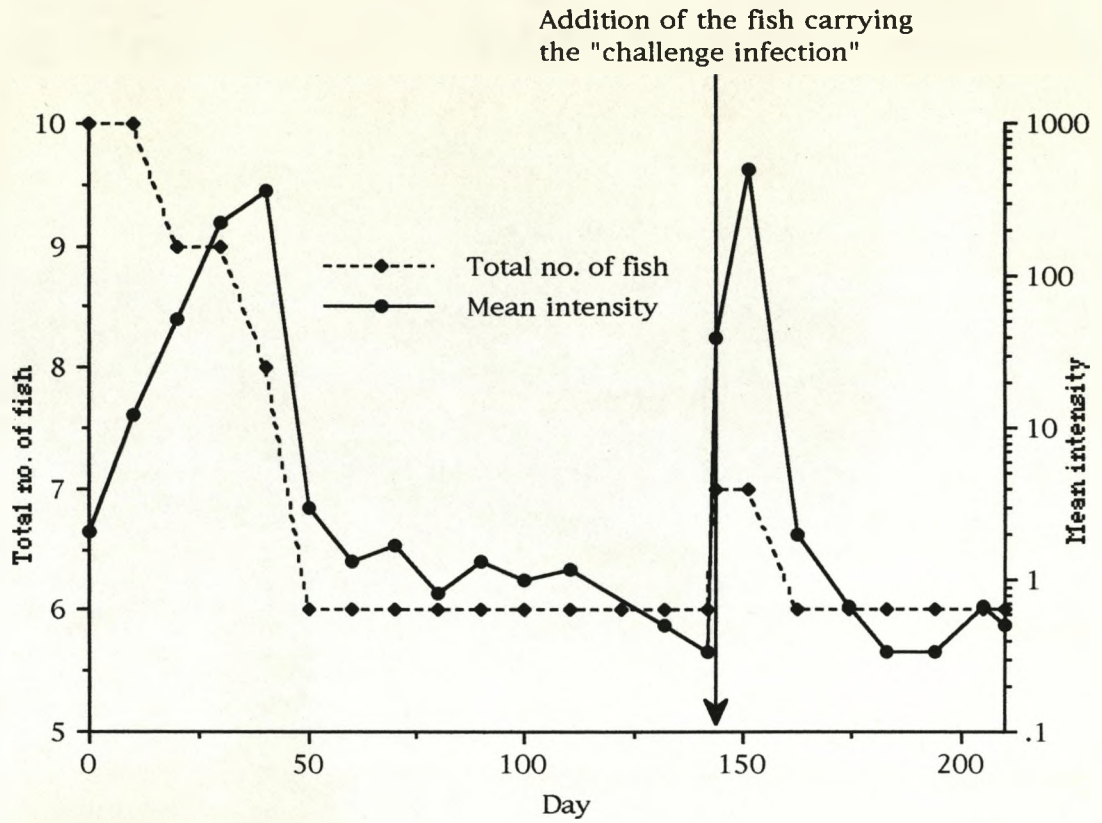

Fig. 10.4. Mean intensity (right axis, log scale) of *G. turnbulli* infections and the total number of fish (left axis) remaining throughout the long-term infection experiment using adult *P. reticulata*. Note that the fish placed in the aquarium to provide a high intensity challenge infection was added on day 144 and removed on day 151. Calculations of mean intensity during that time include the parasites counted on the "challenge fish".

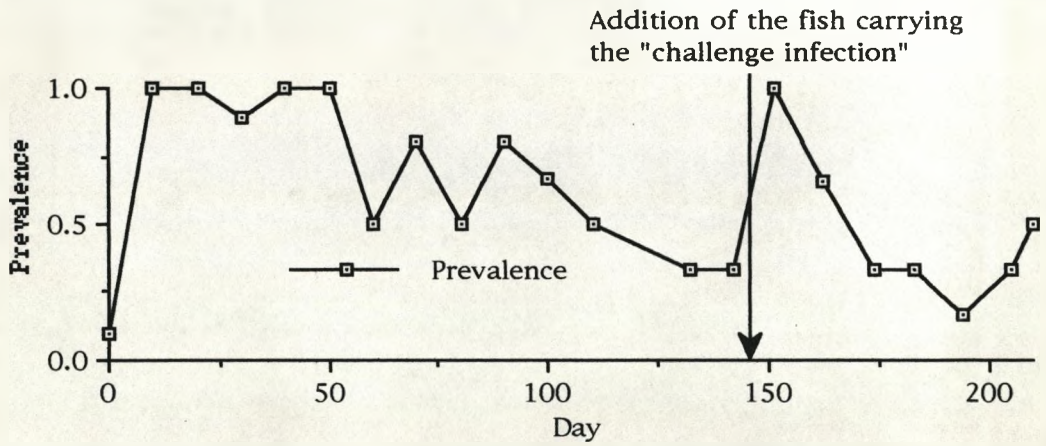

Fig. 10.5. Prevalence of *G. turnbulli* thoughout the long-term infection experiment using adult *P. reticulata*. As in Fig. 10.4, the "challenge fish" has been included in the calculations.

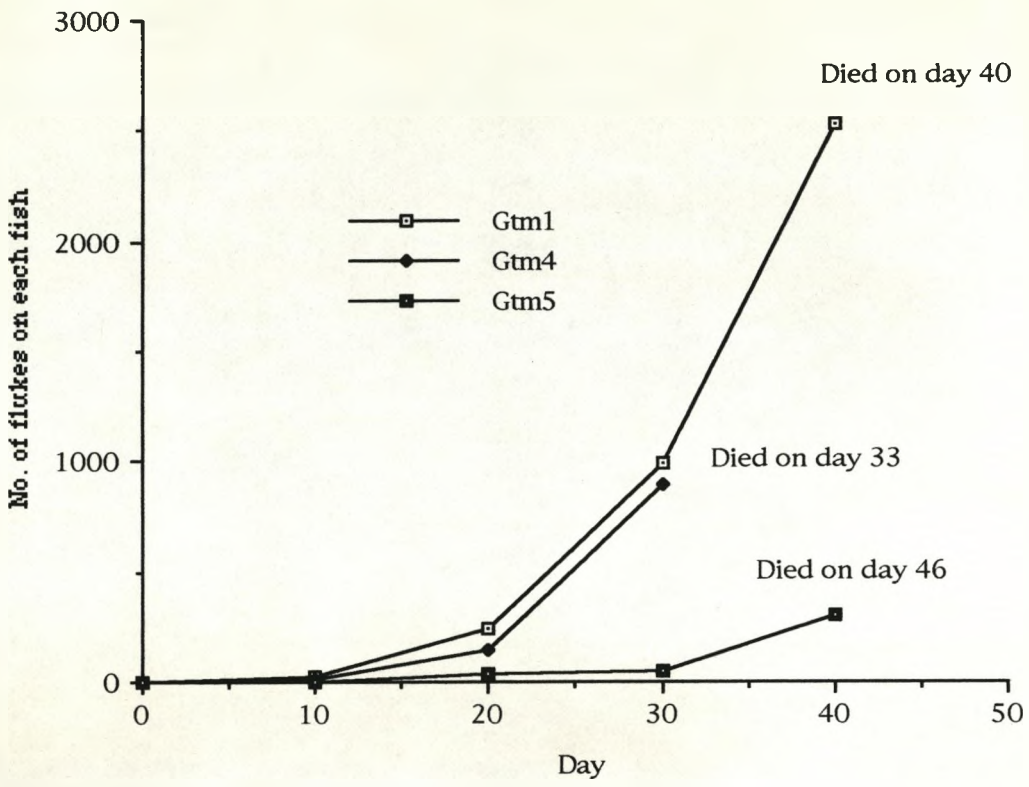

Fig. 10.6. Infrapopulations plotted against time for adult *P. reticulata* infected with *G. turnbulli* which died during the course of the long-term *G. turnbulli* infection experiment (fish Gtm1, Gtm2 and Gtm5) presumably due to heavy *Gyrodactylus* infection.

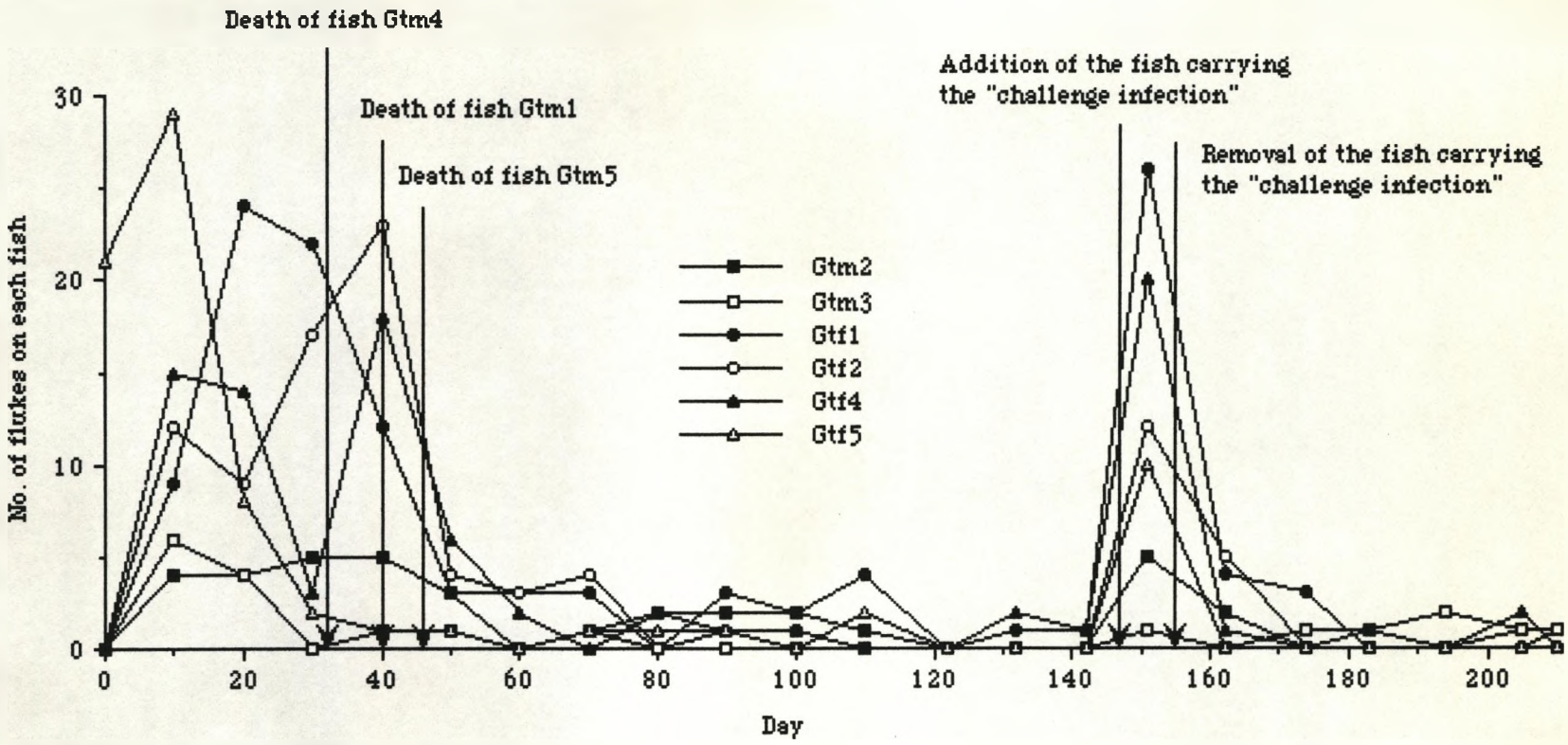

Fig. 10.7. Adult *P. reticulata* infected with *G. turnbulli* which survived the course of the long-term experiment (fish Gtm2, Gtm3, Gtf1, Gtf2, Gtf4 and Gt5). Note the falls in infrapopulations following the deaths of the three heavily infected fish whose infrapopulations are shown in Fig. 10.6 and the rises and falls in infrapopulations as the "challenge fish" was added and removed to and from the aquarium.

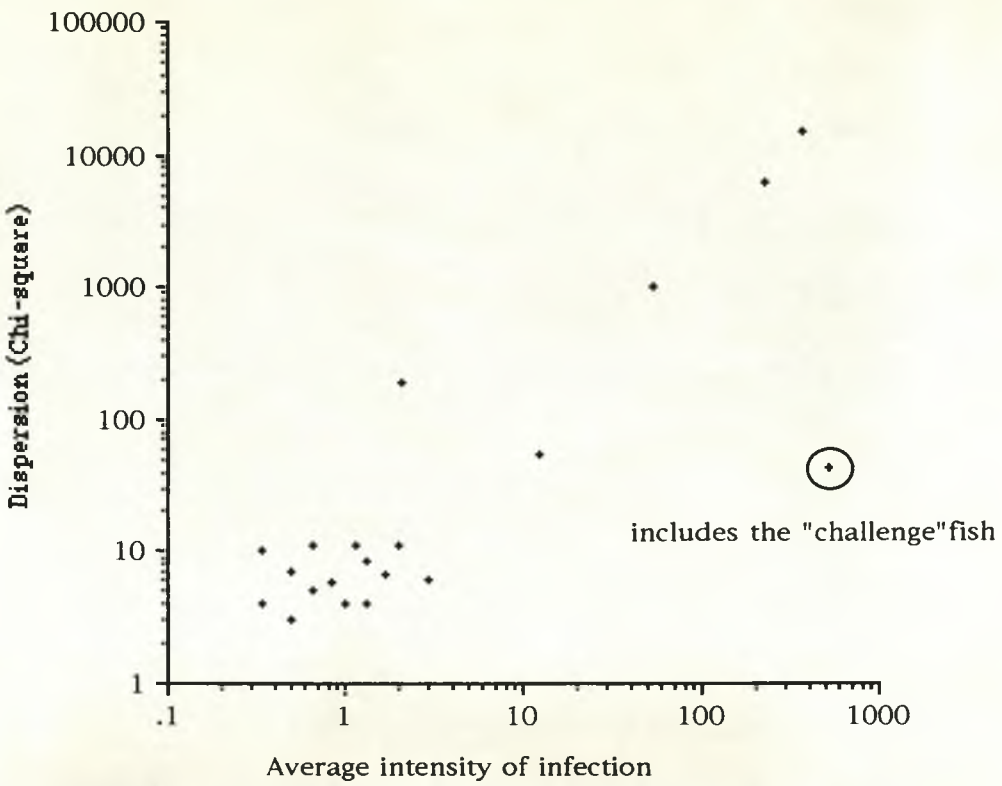

Fig. 10.8. Parasite dispersion in the long-term *G. turnbulli* infection experiment, as represented by variance: mean ratio multiplied by the number of observations in the sample minus one (= χ^2), plotted against mean intensity of *G. turnbulli* infection (log scales used for both axes).

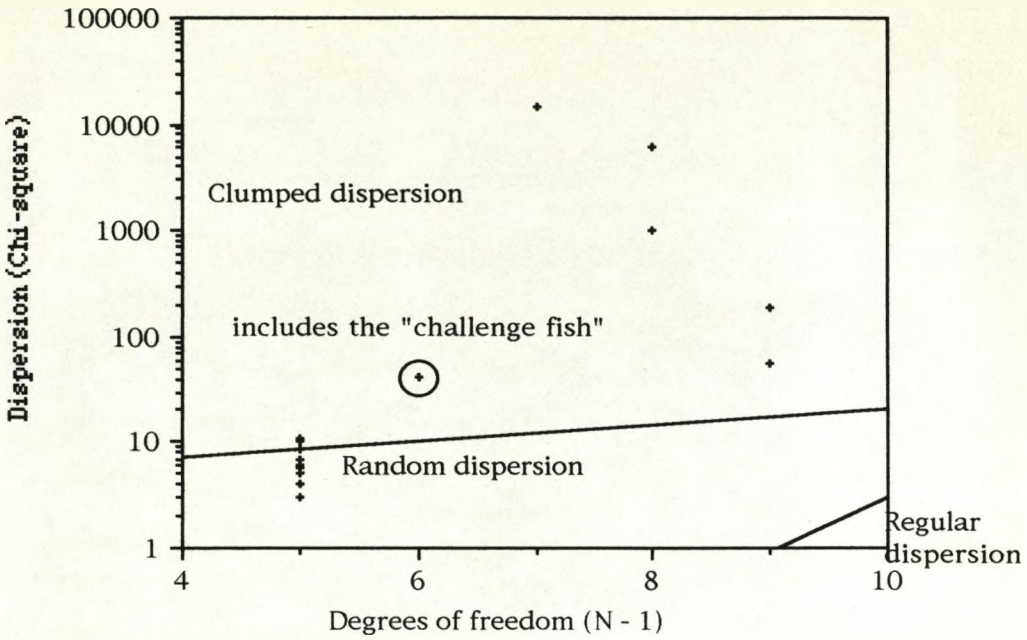

Fig. 10.9. Parasite dispersion in the long-term *G. turnbulli* infection experiment, as represented by variance: mean ratio multiplied by the number of observations in the sample minus one (= χ^2), plotted on a log scale against the degrees of freedom (= N - 1). The 95% confidence zones of clumped, random and regular dispersion are given, as in Fowler and Cohen (1990).

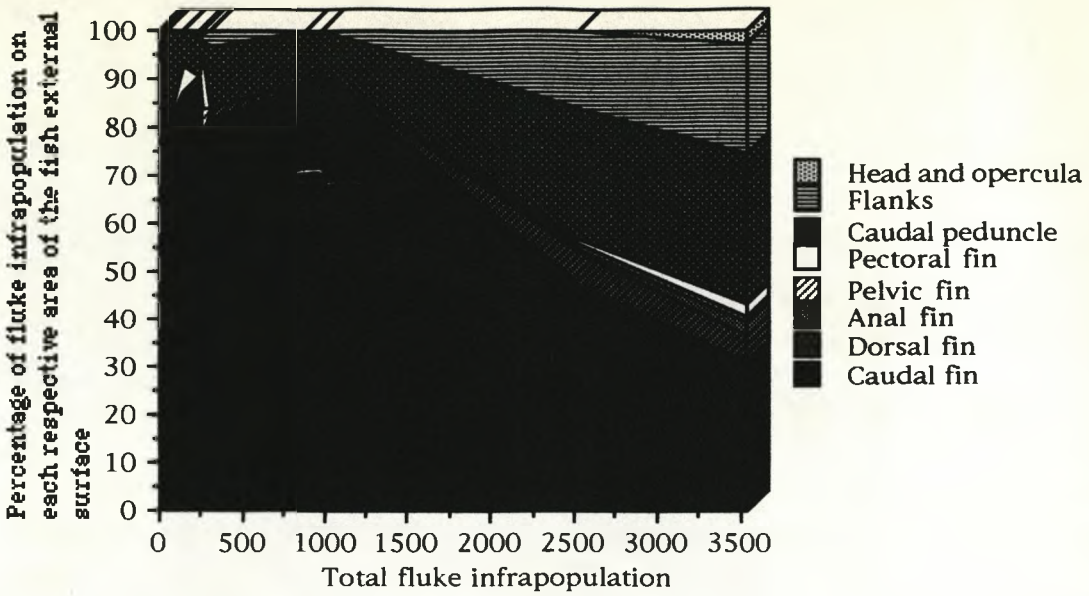

Fig. 10.10. Host-site specificity of *G. turnbulli* on adult *P. reticulata* which died due to excessive parasite burdens during the course of the long-term experiment. Percentage of infrapopulations recorded on the eight defined areas of the host external surface plotted against actual infrapopulation.

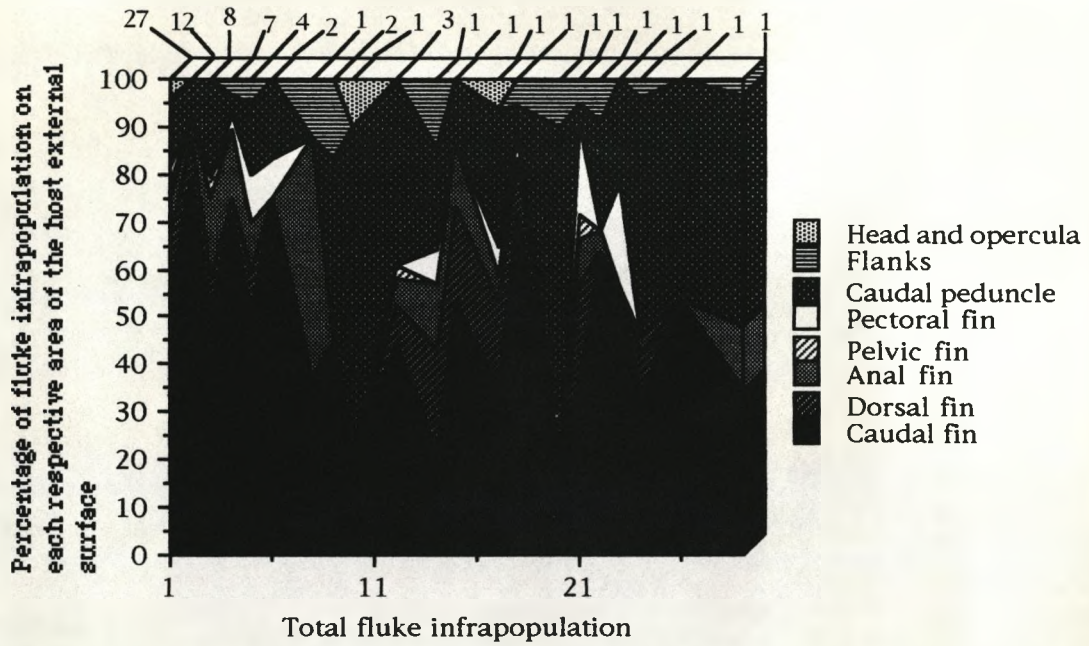

Fig. 10.11. Host-site specificity of *G. turnbulli* on adult *P. reticulata* which survived the long-term experiment. Percentage of infrapopulations recorded on the eight defined areas of the host external surface plotted against actual infrapopulation. Where a size of infrapopulation has been recorded more than once (the frequency of each infrapopulation value is given along the top of the graph), an average percentage value was taken for each host-site zone.

10.3.3. Investigations of transmission of detached *G. turnbulli*

Tables 10.1 and 10.2 show the results of the two experiments investigating the transmission of detached *G. turnbulli* in aquaria of the same size as those used in the above long-term experiments.

The results showed that the transmission of detached *Gyrodactylus* is important when parasite infrapopulations are high, but is less likely to occur in aquaria where they are low, such as those maintained on the adult experimental fish on day 210.

10.3.4. Results from the adult host mortality control tank

Fig. 10.12 shows the steady decline of the numbers of fish surviving in the mortality control aquarium.

		Number of flukes on each fish on respective days			
		Day 0	Day 20		Day 24 (4)
	Gbm1	0	4	Rim1	4
	Gbm2	0	8	Rim2	9
	Gbm4	0	438	Rif1	0
Fish i. d.	Gbm5	0	1100	Rif2	11
	Gbf2	0	7	Rif3	5
	Gbf3	0	3	Rif4	23
	Gbf4	0	2	Rif5	2
	Gtf6	3500	0	Rif6	1
			REMOVED -	Rif7	4
			RECIPIENTS ADDED	Rif8	6

Table 10.1. Results of the first experiment investigating the transmission of detached *G. turnbulli*. The donor fish, which, except for the initiator (Gtf6) were those that survived the long-term *G. bullatarudis* experiment, have their original names. Recipient fish were given the prefix "Ri", a letter denoting their sex and an individual number. Recipient fish were checked four days after placement in the tank from which the donors had been removed.

		Number of flukes on each fish on respective days			
	Day	0 (210)		4	13
	Gtm2	1	Riim1	0	-
	Gtm3	1	Riim2	0	0
Fish i. d.	Gtf1	0	Riim3	0	0
	Gtf2	1	Riif1	0	0
	Gtf4	0	Riif2	0	0
	Gtf5	0	Riif3	0	0
		REMOVED -			
		RECIPIENTS ADDED			

Table 10.2. Results of the second experiment investigating the transmission of detached *G. turnbulli*. The donor fish were those that survived the long-term *G. turnbulli* experiment and have their original names. Recipient fish were given names as described above except the prefix "Rii" was used instead of "Ri". Recipient fish were checked four and thirteen days after placement in the tank from which the donors had been removed.

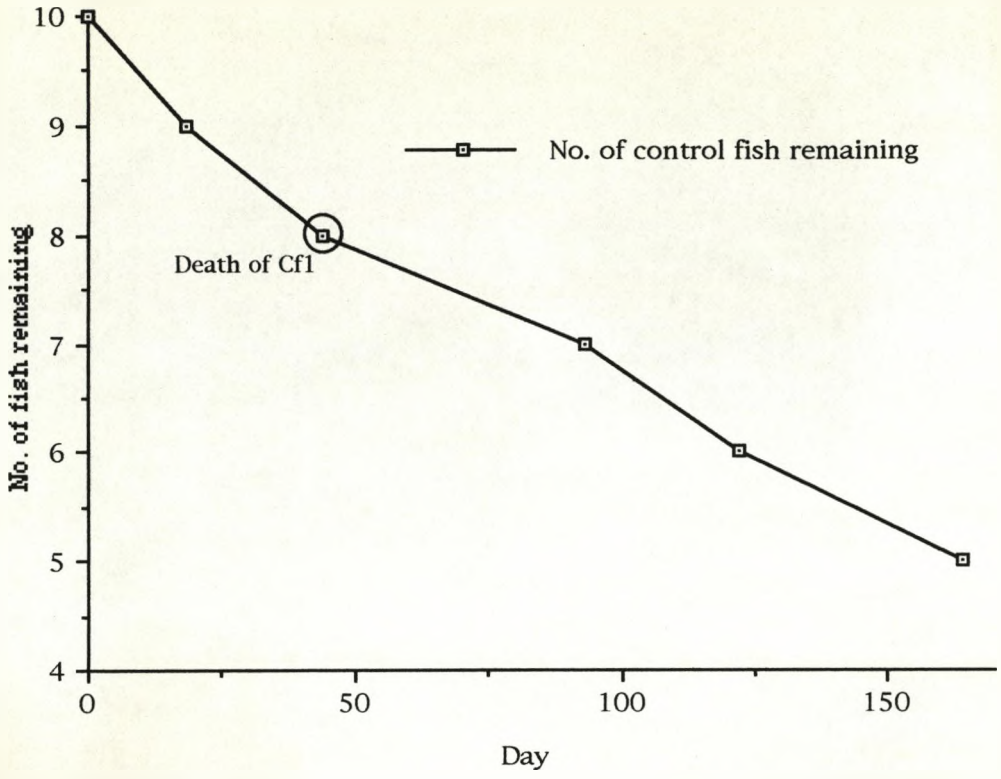

Fig. 10.12. The number of fish surviving throughout the control experiment. The rate of mortality is approximately 0.03 per fish per day.

10.4. Discussion

10.4.1. Discussion of the results from the long-term *G. bullatarudis* infection experiment

None of the fish in the aquarium into which *G. bullatarudis* was introduced showed a high susceptibility to this parasite leading to host mortality. The total parasite population was seen to increase from day 0 to day 10 and then decrease to extinction between day 30 and day 40. This pattern of parasite population growth and decline in a very small population of hosts where there is no regular in-flow of susceptible fish, excepting for the absence of parasite-induced host mortality, follows the pattern demonstrated by Scott and Anderson (1984) using immature *P. reticulata* and *G. turnbulli*. They found that in two replicates of experiments where a fish of 10mm standard length infected with 10 *G. turnbulli* was placed with 49 naive fish of standard length 10mm in 5l of standing tap water, host-parasite dynamics were characterised by "rapid exponential growth of the parasite population followed by a dramatic decline in abundance and prevalence concomitant with heavy host mortalities". The decline resulted in extinction of the parasite population by 100 days post initiation. They found that parasite populations could only be maintained longer than 100 days in similar experiments when naive fish were added to the experimental arenas at regular intervals.

The failure of the maintenance of the *G. bullatarudis* infections of adult *P. reticulata* for longer than 40 days in the present work would seem to be a decline in the number of suitable hosts. After fish had recovered from their initial infections they presumably became refractory to reinfection. The present findings were consistent with the results presented in Chapter 9 where host responses of immature fish were found to be more effective

against *G. bullatarudis* than against *G. turnbulli*. Madhavi and Anderson (1985) showed that host genetics was a factor which influenced susceptibility of the host towards *Gyrodactylus*. It may have been that host genetics was influential in the observed differences in the susceptibility of the laboratory fish used in these experiments to *G. bullatarudis* and *G. turnbulli*.

Alternatively, Chapter 9 (last two paragraphs of the discussion) describes the evidence found during this work that showed that water composition may influence the dynamics of guppy-*Gyrodactylus* host-parasite interactions under experimental conditions. Harris and Lyles (1992) sampled guppies from nine sites on a variety of streams on the south slope of the northern mountains in Trinidad and found that *G. bullatarudis* and *G. turnbulli* coexisted at 3 sites, *G. bullatarudis* was exclusive at four sites and *G. turnbulli* was exclusive at two sites. I have found the prevalence of *G. bullatarudis* on ornamental fish obtained for this study to be much less than that of *G. turnbulli*. Koskivaara *et al.* (1991) found that out of four naturally occurring *Gyrodactylus* parasites of roach, one favoured oligotrophic water bodies and the others performed better in eutrophic conditions. It is possible that in the maintenance of ornamental fish the aquatic conditions are less suitable for *G. bullatarudis* than for *G. turnbulli*.

10.4.2. Discussion of the results from the long-term *G. turnbulli* infection experiment

The results of the long-term investigation of *G. turnbulli* infections were markedly different to the results of the equivalent *G. bullatarudis* experiment. Initially, the pattern of infection was as might have been expected, consistent with the results of Scott and Anderson (1984), see above. Three fish, susceptible to infection, sustained increasing *G.*

turnbulli infrapopulations until they died, whilst the remaining six fish started to recover from their initial infections and the total parasite population declined until about day 50 when the mean intensity of infection fell to around 3.0 on the six surviving fish. From then on, the course of infections differed from what was expected according to the findings of Scott and Anderson (1984). Instead of becoming extinct, low intensities of infection, though not at 100% prevalence, persisted at least until day 144, when an additional heavily infected fish was introduced to provide the challenge infections.

Although there were three mortalities of adult fish, owing to increasing populations of *G. turnbulli*, over 164 days just as many fish died in the mortality control aquarium as in the *G. turnbulli* experiment. The relative importance of *Gyrodactylus*-induced host mortality to total mortality under these conditions was therefore not important and there were other unidentified causes of death.

There was a significant correlation between mean intensity of infection and the degree of over-dispersion as represented by the χ^2 product (Fowler and Cohen, 1990). During the prolonged periods of low parasite intensities on just six fish (days 50 to 144 and 154 to 210), the dispersion was, or was nearly, randomly dispersed (Fig. 10.9). This was also different to the results from the two "zero immigration" experiments of Scott and Anderson (1984), where aggregated dispersion of the parasites was maintained throughout. A prolonged period of random dispersion of the parasites was an unexpected result, especially as there is known heterogeneity of genetic susceptibility of *P. reticulata* to *Gyrodactylus* infection (Madhavi and Anderson, 1985) and the parasites undergo direct reproduction on the host - two generalities which, it is claimed, tend to lead to over-dispersion of parasites amongst their hosts (Anderson and Gordon, 1982).

Persistence of low intensity infections of adult *P. reticulata* may be possible where the response of the fish to infection is not enough to cause outright extinction of its infrapopulation, but is enough to reduce it to relatively few parasites (approximately < 10). Evidence was presented in Chapter 9 to suggest that the host response to *Gyrodactylus* is localised. Movement of *G. turnbulli* about the external surfaces of their hosts may allow a low, persistent infection of adult fish by avoiding areas made inhospitable to the parasites by localised immune response. Such a persistence of infection may not be possible on smaller fish, where the external surface area is much less and there is therefore less, chance of persistently avoiding a host response in this manner. Where there are more than one adult fish confined together, transmission between hosts would also serve to avoid areas of fish surface under the influence of an effective response to *Gyrodactylus* infection.

Lyles (1990) found that, in the Pariar River, Trinidad, where *G. turnbulli* were the only monogenean parasites of *P. reticulata*: "The smallest classes of new-born guppies harbour no flukes. As the fish mature, they acquire infections. Prevalences appear to peak in the size classes which are sexually differentiating (gonopodium develops at 12-13mm)." She notes in discussion of guppy natural life histories that there is spatial separation of natural populations according to size: "Larger, more powerful adults may forage in deeper, swifter waters; juveniles tend to be restricted to calm pools and shallow margins." She gives two possible explanations for the changes in the prevalence of *G. turnbulli* amongst different age classes: The first assumes that new-born fish are uninfected with flukes and that *G. turnbulli* infections do not occur until the fish "physically join the adult population" and the other, that juveniles are more susceptible "but death does not necessarily follow" and higher prevalences of infection are found as sexual maturation compromises fluke resistance, or is caused by

movement into the adult population. Both these explanations for her findings suggest that that there is a persistent low intensity of infection of adult fish such as has been demonstrated by the results of the *G. turnbulli* infections my work. Furthermore, the results of my work suggest that persistent *G. turnbulli* infections could be maintained in relatively small *P. reticulata* populations.

The result should be noted by aquarists and ornamental fish breeders and importers. It has been shown that small numbers of adult *P. reticulata*, when kept together, may persistently carry, for at least three to four months, low intensity *Gyrodactylus* infections, undetectable by observation of common gross pathological signs (see Chapter 8, section 8.4.3). These low level infections may cause mortalities of *Gyrodactylus*-susceptible fish added to the tank or bred from the infected adults.

The only comparable study of long-term experimental gyrodactylid infections is that by Harris (1993a) of *Macrogyrodactylus polypteri* on *Polypterus senegalus*. He recorded infections of this parasite on individual fish kept in pairs in 54 or 90l aquaria at 25°C lasting up to at least one year, with fluctuations between about 5 and 40 individuals. He also noticed that in the decline phases of infections, the number of flukes on the gravel lining the bottom of the aquaria increased, suggesting an increase in detachment rate concomitant with an effective host response. Detached flukes could survive for a few days and could therefore facilitate reinfection of the same or the other cohabiting fish (Harris, 1993b).

Scott and Anderson (1984) showed that, at 25°C, detached *G. turnbulli* had a life expectancy of about 12h. In a study of the age structure of *Gyrodactylus* infections of *P. reticulata*, Harris (1989) collected detached *G. turnbulli* in petri dishes placed in the bottom of 300ml aquaria containing groups of 4

infected fish ("10-30mm in length, both sexes"). He collected relatively few detached flukes ("only 51 detached flukes were recovered" in comparison to a sample of 308 *Gyrodactylus* from autopsied guppies) and found that they were made up of a significantly larger proportion of older individuals than those attached to fish. These two results suggested that detached flukes were relatively insignificant in the transmission of *G. turnbulli* from one host to another. In the light of those previous results and the results of my experiments examining the rates of transmission of detached *G. turnbulli* under experimental conditions, it would seem that it, although significant in high parasite populations, was likely to be of little significance when the fluke population was as low as was observed between days 50 and 144 and days 162 and 210. Transmission when intensities of infection were low, however, was observed, this is discussed below.

Scott and Anderson (1984) found that transfer of *G. turnbulli* between living hosts was quite risky for the parasite, in fact, they calculated that on average only about 35% of flukes that attempted to move from one host to another did so successfully, the remaining 65% became detached. One of the interesting results from my study was the observations of transmission of *G. turnbulli* from hosts with extremely low infrapopulations to other hosts, as indicated by the observations of flukes on fish which were uninfected at the previous checking between days 50 and 144 and days 162 and 210 (see Fig. 10.7). In the present experiment chance contact between infected parts of fish with other fish would probably have been relatively infrequent at those times with a host density of 0.15 fish/l between days 50 and 144 and days 162 and 210. It is possible that transmission may have been augmented between male and female fish during copulation, although overt sexual behaviour was not seen. Another possibility was that new-born fish, which were not observed before being cannibalised by adults, augmented transmission. It was considered unlikely that new-born fish would have gone unnoticed, but the possibility cannot by ruled out.

It was possible that flukes were unobserved on some fish during the checking procedures and so actual transmission rates of *G. turnbulli* were lower than those perceived. Adult fish had a large surface area which had to be checked for flukes and their darkly pigmented skin often made the *Gyrodactylus* difficult to see, particularly at low intensities of infection.

Observations of the six surviving *G. turnbulli* infected *P. reticulata* following the addition of the host carrying the challenge infection on day 154 of the long-term *G. turnbulli* infection experiment showed that they had remained at least as resistant to a heavier infection as they were at the start of the experiment. The result was consistent with the findings of Kahlil (1964) who found that *Polypterus senegalus* remained resistant to heavy *Macrogyrodactylus polypteri* infections when heavily infected fish were added to the same aquaria if they had retained a small infrapopulation of the parasite from their previous infection.

10.4.3. Host-site specificity

The host-site specificity of *G. turnbulli* differed on the adult fish used in this investigation in comparison to infections of immature fish described in the preceding two chapters. Compare the area graph denoting the changes in proportionate host-site specificity of flukes infecting fish that did not recover from infections in the experiment using adult fish (Fig. 10.10) with Fig. 8.5 which illustrates the host-site specificity of immature fish (less than 15mm standard length) which showed no sign of recovery from infection. The main difference is that a far greater proportion of the flukes infecting the adult fish were observed attached to the caudal fin. This was a consistent observation at low and high intensities of infection. The caudal fins of adult fish usually have proportionately larger areas than those of immature fish, particularly males. In addition, adult caudal fins do

not become completely "clamped" (see Chapter 8, section 8.4.3.) as those of immature fish do at high *G. turnbulli* infestations. Of those fish used in the experiments in Chapters 8 and 9 a very considerable reduction in caudal fin surface area was observed for immature fish of 10-15mm standard length with 30 or more *G. turnbulli*. The caudal fins of such fish invariably became clamped to such an extent that they appeared to end in a sharp point and did not flex along their lengths while the fish were swimming.

There is a similar difference in the comparison between the host site specificities of the *G. turnbulli* infecting the surviving fish of group c/Gt in Chapter 9 (standard length 11mm) with those infecting fish which did not die during the course of the present experiment. A comparison of the proportions of flukes from a total of 436 individual observations of flukes from the survivors of the present work with 612 observations of flukes from the survivors of the c/Gt group of Chapter 9 is shown in Fig. 10.13. The observations of Harris (1988), also shown in Fig. 10.13, of flukes infecting fish between 10 and 30mm in length (presumably encompassing both adult and immature fish) are intermediate in the comparisons of the proportions of flukes from these three groups infecting the caudal fin and caudal peduncle.

A comparison of the total proportions of *G. bullatarudis* observed on the eight specified host-site zones during the experiment using adult fish (181 observations of individual flukes) with those observed during the infections of the c/Gb group in Chapter 9 (see Fig. 10.14) also showed a significant difference (5 x 2 χ^2 analysis, $P < 0.0001$ combining all the fin categories except for the caudal fin). Again, more than twice the proportion of the total number of flukes were observed on the caudal fins of the immature fish (11mm, standard length) than were observed on the caudal fins of the adult fish and a greater proportion of the flukes were

observed on the caudal peduncle of the smaller fish than were observed on the caudal peduncle of the adult fish.

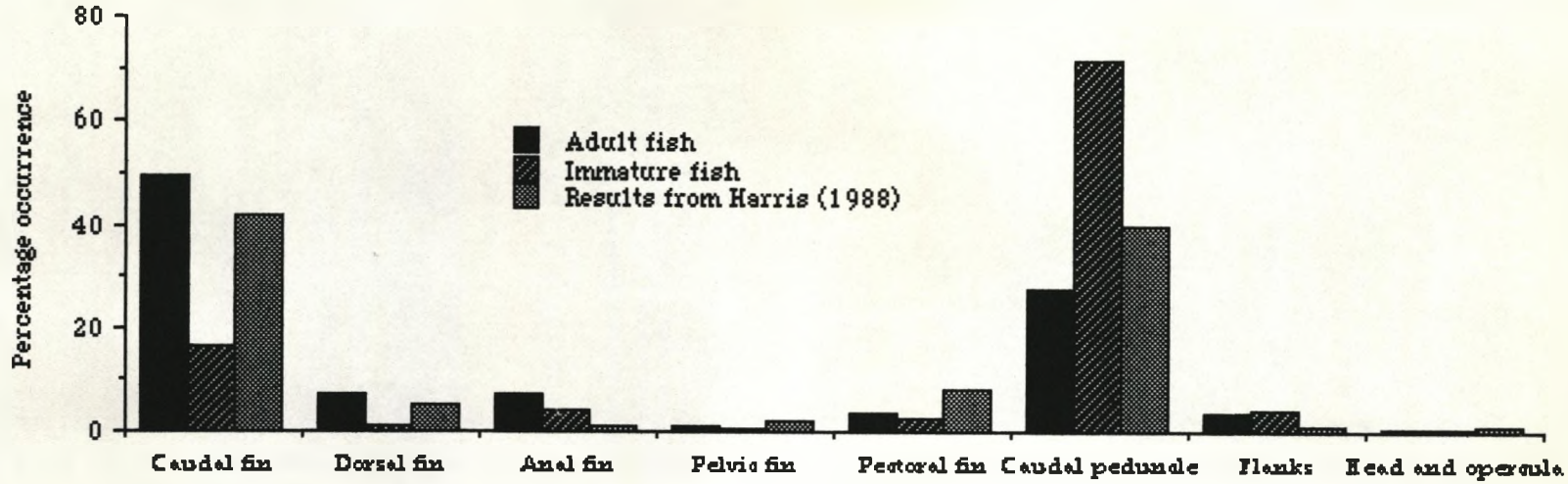

Fig. 10.13. A comparison of the host-site specificities of *G. turnbulli* as shown by the accumulated observations of individual flukes from group c/Gt (Chapter 9, immature fish, 11mm standard length) the adult fish used in the long-term experiments in this chapter and those of Harris (1988) from fish of 10 to 30mm in length. All the fish from which these observations were taken survived the course of their infections.

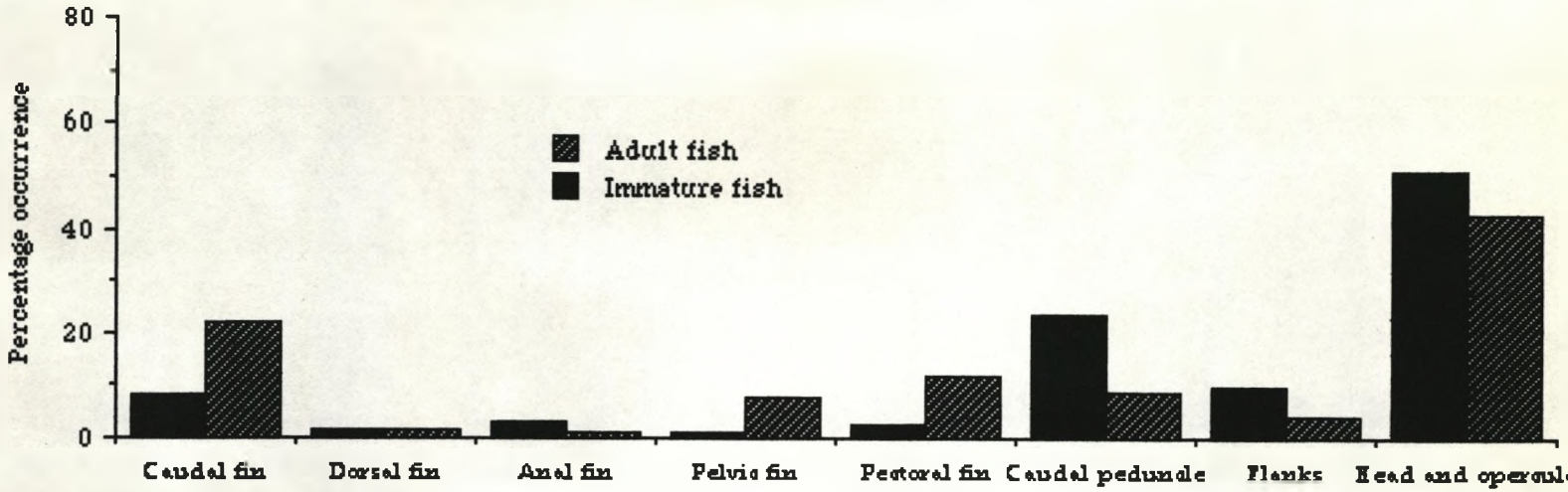

Fig. 10.14. A comparison of the host-site specificities of *G. bullalarudis* as shown by the accumulated observations of individual flukes from group c/Gb (Chapter 9, immature fish, 11mm standard length) and the adult fish used in the long-term experiments in this chapter. All the fish from which these observations were taken survived the course of their infections.

10.5. References

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CHAPTER 11

11. Conclusions and direction for further studies

The aim of this chapter is to summarise the conclusions from this thesis and to give direction to future work.

G. bullatarudis Turnbull, 1956 *sensu* Harris (1986) from *Xiphophorus* sp. was found to be different from *G. bullatarudis sensu stricto* and was reassigned to *G. rasini* Lucky,1973, although this requires confirmation (Chapters 2 and 3). Attempts to acquire type specimens of *G. rasini* are in progress. Thus the perceived "natural" host range of *G. bullatarudis* Turnbull, 1956 now only includes fish of the genus *Poecilia* [*P. reticulata* (Turnbull, 1956;, Rogers and Wellborn, 1965; Harris and Lyles,1992) and *P. sphenops* (Kritsky and Fritts, 1970)].

Careful examination of cirrus spines, particularly the large spines proved valuable in species differentiation (Chapters 2 and 3). The large cirrus spines of the previously confused *G. bullatarudis* Turnbull, 1956 *sensu* Harris (1986) (= *G. rasini*) and *G. bullatarudis* Turnbull, 1956 were markedly different. Better illustration of the morphology of these characters would greatly improve species descriptions.

A simple technique using modified Mallory stain in the transfer of *Gyrodactylus* specimens from ammonium picrate-glycerin to a permanent mountant was described (Chapter 5). As well as being useful in the study of general anatomy, the technique enhanced the rendition of the taxonomically important dorsal and ventral bars. The dorsal bar of *G. turnbulli* was shown to have posteriad supporting attachments (Malmberg,

1970). It is likely that dorsal bar posteriad supporting attachments are more common among *Gyrodactylus* species than presently recognised owing to their poor differentiation in unstained specimens. The modified Mallory staining technique is a useful addition to the armoury of the *Gyrodactylus* taxonomist. Existing species descriptions should be improved by re-examination of specimens following staining.

Assuming that PC1 of the second PCAs in Chapter 4 were both indices of size, sclerites of *G. bullatarudis* were smaller when reared at 19 than at 25°C and sclerite sizes of *G. turnbulli* were not significantly different at the two test temperatures. The majority of previous works (see Ergens, 1976; Ergens, 1983; Ergens, 1991; Ergens and Gelnar, 1985; Kulemina, 1976; Malmberg, 1970; Mo, 1991a, b and c, 1993), all of which used *Gyrodactylus* from temperate fish species, found a negative correlation between sclerite size and temperature. My results were contrary to previous findings and should be followed by a more comprehensive series of experiments. Sclerites from *G. bullatarudis*, *G. turnbulli* and other neotropical *Gyrodactylus* should be measured following rearing in a series of temperatures within their natural range to see if my observations of the effect of temperature on the size of *G. bullatarudis* sclerites are repeatable and consistent with other neotropical *Gyrodactylus*. In addition, a population of *G. turnbulli*, with a similar history to those I have used, could be exposed to a fluctuating range of temperatures between 17 and 28°C (see Scott and Nokes, 1984) for over a year or more. A series of sclerite measurements at different temperatures could then determine whether the evolution of differences in sclerite size at different temperatures could be induced in this way.

The comparisons between samples from mono-cultures and hetero-cultures (Chapter 4) of *G. bullatarudis* and *G. turnbulli* confirmed the (see for

example Harris, 1993) link between genetic variation and sclerite morphometric variation. Harris (1993) and Malmberg (1993) stressed dangers of perturbations of natural fish stocks and the maintenance of *Gyrodactylus* in fish culture facilities which may cause genetic drift altering host specificity and pathogenicity of their parasites. Monitoring of sclerite variability in both laboratory maintained *Gyrodactylus* and of *Gyrodactylus* from wild, introduced and cultivated fish stocks may provide important information on the rates of such processes.

Two linear measurements of the opisthaptor showed 20% more shrinkage of *Gyrodactylus* fixed using 10% neutral buffered formalin than using freeze fixation for preparation of specimens for SEM viewing (see Chapter 6). Freeze fixation-dehydration provided instant immobilisation of specimens whereas those treated with formalin showed signs of stress before death. Freeze fixation-dehydration was shown the best preparatory method for the study of *Gyrodactylus* external gross morphology. Formalin, however, better preserved the delicate hamulus membrane. This method for SEM preparation based upon that originally described by Veltkamp *et al.* (1994) was therefore shown to have great potential for the study of fish ectoparasites and can be applied in a variety of different contexts.

G. turnbulli and *G. bullatarudis* were shown to have methods of opisthaptor attachment which were similar overall to those of other *Gyrodactylus* species. However, differences in detail of the mode of action of their opisthaptors were described. An hypothesis relating opisthaptor attachment to differences in the marginal hook morphology of *Gyrodactylus* species was presented and discussed in Chapter 7. It was argued that the type of attachment used by *G. bullatarudis* is more primitive than that of *G. turnbulli* and more common in the subgenera *G. (Gyrodactylus)* and *G. (Mesonephrotus)*. Given the importance of marginal hook morphology to *Gyrodactylus* systematics, similar studies of other

species to test this hypothesis may provide information contributing to the understanding of the evolution of the genus.

Chapter 8 showed that *G. bullatarudis* and *G. turnbulli* have the same intrinsic rates of increase on naive fish at 26°C. Under experimental conditions *G. bullatarudis* was approximately twice as lethal as *G. turnbulli* per host per day per parasite on fish showing no signs of a host response at 26°C. Probable causes of this difference in pathogenicity were identified as:

1)Different host-site specificities which caused different gross pathology.

2) Differences in opisthaptoral attachment which were discussed in Chapter 7. Evidence suggested that the hamuli of *G. bullatarudis* exert greater pressure on the host epidermis than those of *G. turnbulli* in static attachment.

3) Differences in feeding. Over a given time, the pharynx of a *G. bullatarudis* was likely to be in contact with the host epidermis for longer than a pharynx of a *G. turnbulli*. But more detailed study is required for an exact quantification of damage caused by each species per fluke per feeding bout.

The guppy-*Gyrodactylus* host-parasite system could be used to investigate other aspects of the *Gyrodactylus* infection. Studies on the effects of infection on foraging efficiency and predator avoidance could be easily undertaken in aquarium arenas. These factors probably contribute significantly to the deleterious effects of these parasites on *P. reticulata* in natural situations and should be considered when interpreting experimental analyses of pathogenicity.

There is great scope for further comparative studies on the epidemiology of *G. bullatarudis* and *G. turnbulli*. My studies (Chapters 8, 9 and 10) have shown that both these parasites have different host-parasite relationships

with *P. reticulata*. *G. turnbulli* are less pathogenic and less vulnerable to host response, whereas *G. bullatarudis* are more pathogenic but more likely to succumb to the host response. Populations of *G. bullatarudis* were found to be less stable than those of *G. turnbulli*, both whilst culturing them in maintenance of the stock populations and also in the long-term adult host infection experiments (Chapter 10). Once an understanding of the differences between the various components of *G. bullatarudis* and *G. turnbulli* epidemiology has been achieved, future work could study concurrent infections of these two parasites on single hosts and, in larger experimental arenas, within host populations.

The initial host response of *P. reticulata* to *Gyrodactylus* was non-specific and localised on heavily infected areas of the fish surface (Chapter 9). The exact nature of the response remains unknown. Future challenge infections using *G. bullatarudis* and *G. turnbulli* should be undertaken using a similar protocol to Scott (1985) to test the duration of the refractory period of *P. reticulata* against both parasite species regardless of which species was used in the initial infection.

The long-term investigation of *G. turnbulli* infections of adult *P. reticulata* produced most interesting results (Chapter 10). The parasites maintained low intensity, randomly distributed infections on just six hosts over a period of about 3 months. This result is in marked contrast to those of the long-term experiments of Scott and Anderson (1984) in which *G. turnbulli* could not be maintained on a population (50, declining to about 13 largely due to parasite induced host mortality) of immature *P. reticulata* (16 mm maximum standard length) for over 100 days unless susceptible fish were regularly added. Comparisons of these results emphasise the need for understanding host-parasite interactions in hosts of all age groups before attempting to model patterns of infection in natural populations.

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