Gross anatomy of the muscle systems and associated innervation of *Apatemon cobitidis proterorhini* metacercaria (Trematoda: Strigeidea), as visualized by confocal microscopy

M. T. STEWART[1], A. MOUSLEY[1], B. KOUBKOVÁ[2], Š. ŠEBELOVÁ[1], N. J. MARKS[1] *and* D. W. HALTON[1]*

[1] *Parasitology Research Group, School of Biology and Biochemistry, Medical Biology Centre, Queen's University Belfast, Belfast BT9 7BL, UK*
[2] *Department of Zoology and Ecology, Faculty of Science, Masaryk University, 611 37 Brno, Czech Republic*

(*Received 14 September 2002; revised 8 October 2002; accepted 8 October 2002*)

SUMMARY

The major muscle systems of the metacercaria of the strigeid trematode, *Apatemon cobitidis proterorhini* have been examined using phalloidin as a site-specific probe for filamentous actin. Regional differences were evident in the organization of the body wall musculature of the forebody and hindbody, the former comprising outer circular, intermediate longitudinal and inner diagonal fibres, the latter having the inner diagonal fibres replaced with an extra layer of more widely spaced circular muscle. Three orientations of muscle fibres (equatorial, meridional, radial) were discernible in the oral sucker, acetabulum and paired lappets. Large longitudinal extensor and flexor muscles project into the hindbody where they connect to the body wall or end blindly. Innervation to the muscle systems of *Apatemon* was examined by immunocytochemistry, using antibodies to known myoactive substances: the flatworm FMRFamide-related neuropeptide (FaRP), GYIRFamide, and the biogenic amine, 5-hydroxytryptamine (5-HT). Strong immunostaining for both peptidergic and serotoninergic components was found in the central nervous system and confocal microscopic mapping of the distribution of these neuroactive substances revealed they occupied separate neuronal pathways. In the peripheral nervous system, GYIRFamide-immunoreactivity was extensive and, in particular, associated with the innervation of all attachment structures; serotoninergic fibres, on the other hand, were localized to the oral sucker and pharynx and to regions along the anterior margins of the forebody.

Key words: Trematoda, Strigeidea, *Apatemon cobitidis proterorhini*, muscle, phalloidin, nerve, FaRP neuropeptide, 5-HT, confocal microscopy.

INTRODUCTION

Natural infections of strigeid metacercariae occur in amphibia, reptiles and mammals but are perhaps most common in fish where they can be responsible for a number of pathologies. For example, ocular infections of *Diplostomum spathaceum* metacercariae have been reported from 105 species of freshwater fish in Europe and North America and 23 species in the UK, resulting in eye lesions and cases of blindness (Chubb, 1979; Smyth, 1994). Heavy infections of strigeids can cause serious problems in fish stocks reared commercially. Thus, rainbow trout infected with *Apatemon gracilis* metacercarie in the pericardial cavity have been shown to exhibit cardiac dysfunction, as well as poorness of condition and stunted growth (Tort *et al*. 1987; Watson *et al*. 1992).

* Corresponding author: Parasitology Research Group, School of Biology and Biochemistry, Medical Biology Centre, Queen's University Belfast, Belfast BT9 7BL, UK. Tel: +44 (0)28 90 335792. Fax: +44 (0)28 90 236505. E-mail: d.halton@qub.ac.uk

Although several studies have examined the nature of the immune response of fish to invading strigeid metacercariae (Bortz *et al*. 1984; Stables & Chappell, 1986; Whyte *et al*. 1986, 1988, 1989), none has succeeded in exploiting it as basis for control. Numerous chemicals are used in the aquaculture industry, many of which accumulate harmful residues in the flesh of the fish or are themselves toxic to non-target organisms. Their misuse helps promote drug resistance, necessitating the search for novel drug targets. One such target with potential for chemotherapeutic exploitation is the FaRPergic component of the flatworm nervous system. FaRPs (FMRFamide-related peptides) are short-chain neuropeptides and, as far as can be determined, occur exclusively in invertebrates. They have been shown to potently alter muscle baseline tension and contraction frequency in all parasitic flatworms examined, including trematodes (Pax *et al*. 1996; Marks *et al*. 1996). However, understanding muscle physiology requires both an awareness of the innervation involved and also an understanding of the functional anatomy of the muscles themselves.

Parasitology (2003), **126**, 273–282.
DOI: 10.1017/S0031182002002780 Printed in the United Kingdom

The aim of this study was to examine the muscle systems and associated innervation of a strigeid metacercaria, using phalloidin fluorescence microscopy and indirect immunocytochemistry, respectively, interfaced with confocal scanning laser microscopy. The strigeid metacercaria chosen for study was *Apatemon cobitidis proterorhini* which occurs encysted in large numbers in the body cavity of the tubenose goby, *Proterorhinus marmoratus*.

MATERIALS AND METHODS

Encysted metacercariae (='tetracotyle' larvae) of *Apatemon* were dissected from the body cavity of the tubenose goby, *Proterorhinus marmoratus* caught from the Musov reservoir on the river Dyje in the Czech Republic and transferred to Hanks' saline and air mailed to Belfast. Metacercariae were excysted (after Kearn, Clevland & Wilkins, 1989) by first placing cysts for 30 min in 0·1% pepsin pH 2 at 41 °C, followed by brief washing in Hanks' saline and exposure to 0·1% trypsin and 0·1% bile salt solution, resulting in excystment within 1 min. Staining of muscle was by the fluorescence-phallodin method after Mair *et al*. (1998), and for the nervous system the indirect immunofluorescence technique of Coons, Leduc & Connolly (1955) was employed, using antisera to the turbellarian (*Bdelloura candida*, *Girardia* [=*Dugesia*] *tigrina*) FMRFamide-related neuropeptide (FaRP), GYIRFamide and to serotonin (5-hydroxytryptamine, 5-HT). Excysted metacercariae ($n>50$) were washed briefly in Hanks' saline and flat-fixed for 1 h in 4% paraformaldehyde (w/v) in 0·1 M phosphate-buffered saline (PBS; 0·145 M NaCl; 0·025 M $NaH_2PO_4 . 2H_2O$; 0·075 M Na_2HPO_4; pH 7·4) and then transferred to fresh fixative for a further 3 h.

Following an overnight wash in antibody diluent (AbD) (PBS containing 0·35% (v/v) Triton X-100; 0·1% (w/v) sodium azide; 0·1% (w/v) bovine serum albumin), specimens were incubated in primary antisera for 5 days, washed in AbD for 24 h and subsequently immersed in secondary rabbit anti-guinea pig immunoglobulin (IgG; for GYIRFamide) or swine anti-rabbit IgG immunosera (for 5-HT), in both cases with the fluorophore, tetramethylrhodamine isothiocyanate (TRITC) for 3 days. Specimens were washed again in AbD and incubated for 24 h with AbD containing 200 ng/ml fluorescein isothiocyanate (FITC) conjugated with phalloidin (Sigma Chemical Company), as a means of staining the F-actin of muscle. After a final overnight wash in AbD, metacercariae were mounted in PBS/glycerol (1:9, containing 2·5% (w/v) 1,4 diazabicyclo[2.2.2]-octane) and viewed using a Leica TCS-NT confocal scanning laser microscope (Leica Microsystems, Milton Keynes, UK). Immunocytochemical controls included (a) omission of primary antiserum; (b) substitution of primary antiserum with non-immune serum; and (c) liquid-phase pre-adsorption of primary antisera by addition of appropriate antigen (50–200 ng antigen/ml of diluted antiserum; GYIRFamide supplied by Sigma-Genosys Biotechnologies (Europe) Ltd).

For visualization of the gross anatomical structure, some metacercariae ($n=10$) were fixed for 3 h at room temperature within 15 min of excystment, using an aqueous mixture of 4% glutaraldehyde and 0·5% osmium tetroxide (3:1), after which they were critical-point dried, sputter-coated with gold and examined in a JSM Jeol 35C scanning electron microscope operated at 15 kV.

RESULTS

Muscle

Typical of strigeid trematodes, the metacercarial body of *Apatemon* consists largely of a flattened forebody bearing a full complement of attachment organs and the alimentary system, separated by a constriction from a small, rounded prominence or hindbody containing the genital rudiment. Within 15 min of excystment, the forebody transforms into a cup-shaped structure with all elements of the attachment apparatus on the inner surface of the cup, as evidenced by scanning electron microscopy (Fig. 1A). Both flattened and cup-shaped forebodies were examined.

Body wall. There is a clear demarcation in muscle organization between the body wall of the forebody and that of the hindbody (Fig. 1B). In the forebody, the wall comprises an outer circular, intermediate longitudinal and inner diagonal arrangement of fibres, the latter running in pairs at a 60° angle to the longitudinal fibres (Fig. 2A). The hindbody wall includes outer circular and intermediate longitudinal fibres, but instead of diagonal fibres there is an inner layer of more widely spaced (3 μm on average) circular fibres (Fig. 2E, inset).

Attachment apparatus. Strigeid trematodes are distinguished by a complex arrangement of attachment structures, comprising oral sucker, paired lappets, ventral sucker and holdfast organ. The bulk of the oral sucker is made up of well-developed radial muscle fibres; meridional fibres are also present and in apparent continuity with longitudinal fibres of the body wall (Fig. 1C, D). Dense bands of equatorial muscle encircle the mouth opening and form a sphincter (Fig. 1C). On either side of the oral sucker is an ear-like projection or lappet, connected to the oral sucker by 4 bands of muscle each comprising on average 5 fibres (Fig. 1B, D). Structurally, the lappets themselves are localized muscular infolds of the body wall composed of transverse, longitudinal and diagonal muscle fibres; a dense array of circular

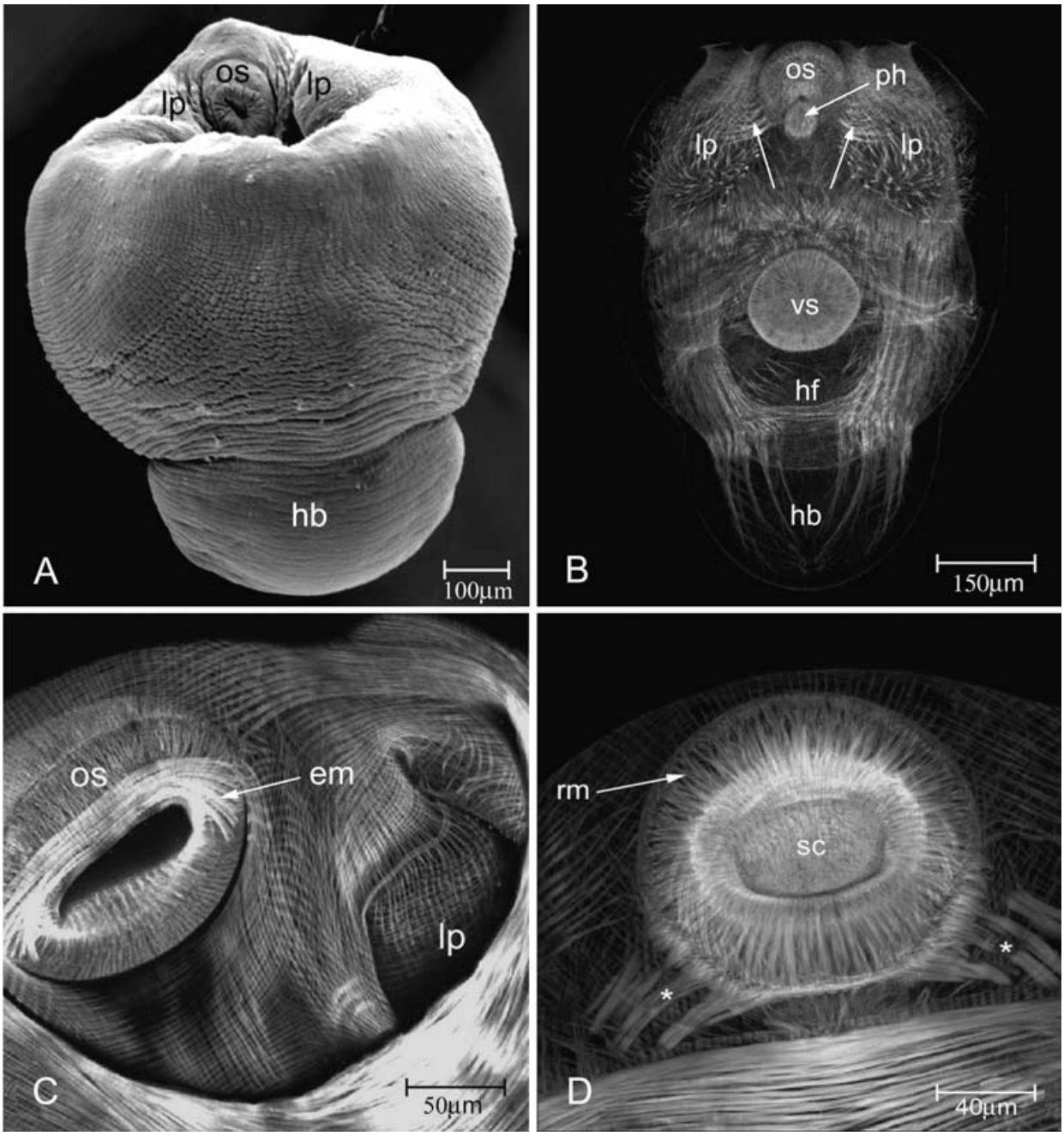

Fig. 1. (A) Scanning electron micrograph of an excysted *Apatemon* metacercaria fixed within 15 min of excystment, showing the cup-shaped forebody with oral sucker (os), lappets (lp) and undeveloped hindbody (hb). (B–D) Whole-mounts of *Apatemon* metacercaria stained with FITC-labelled phalloidin and viewed by confocal microscopy. (B) General view of the forebody and undeveloped hindbody, (hb); oral sucker (os), pharynx (ph), paired lappets (lp), ventral sucker (vs), holdfast (hf). Note the paired bands of muscle fibres that connect the lappets to the oral sucker (unlabelled arrows). (C) The oral sucker (os) opens on the inner surface of the cupped forebody. Bands of equatorial muscle (em) run around the rim of the oral sucker. Infoldings of the body wall form the lappets (lp). (D) Radially orientated fibres (rm) dominate the musculature of the oral sucker which is linked to the lappets by 4 bands of muscle (*); sucker concavity (sc).

muscle surrounds the opening of these so-called pseudosuckers (Fig. 2B). In common with the oral sucker, the ventral sucker or acetabulum consists largely of radial muscles extending between the inner and outer surfaces of the sucker, together with meridional fibres that follow its cup-shaped concavity and equatorial fibres that run around the rim (Fig. 2C). The holdfast is a musculo-glandular organ located just posterior to the ventral sucker (Fig. 1B). It is unique to strigeid trematodes and is of variable form. In *Apatemon* it is a bilobular structure with well-differentiated longitudinal and diagonal fibres and, in places, is connected to the body wall by dense, regularly-spaced bands of muscle (Fig. 2D).

Alimentary tract. The pharynx or feeding organ is composed mainly of radial muscles (Fig. 3C) and leads to 2 gut caeca that end blindly near the hindbody

https://doi.org/10.1017/S0031182002002780 Published online by Cambridge University Press

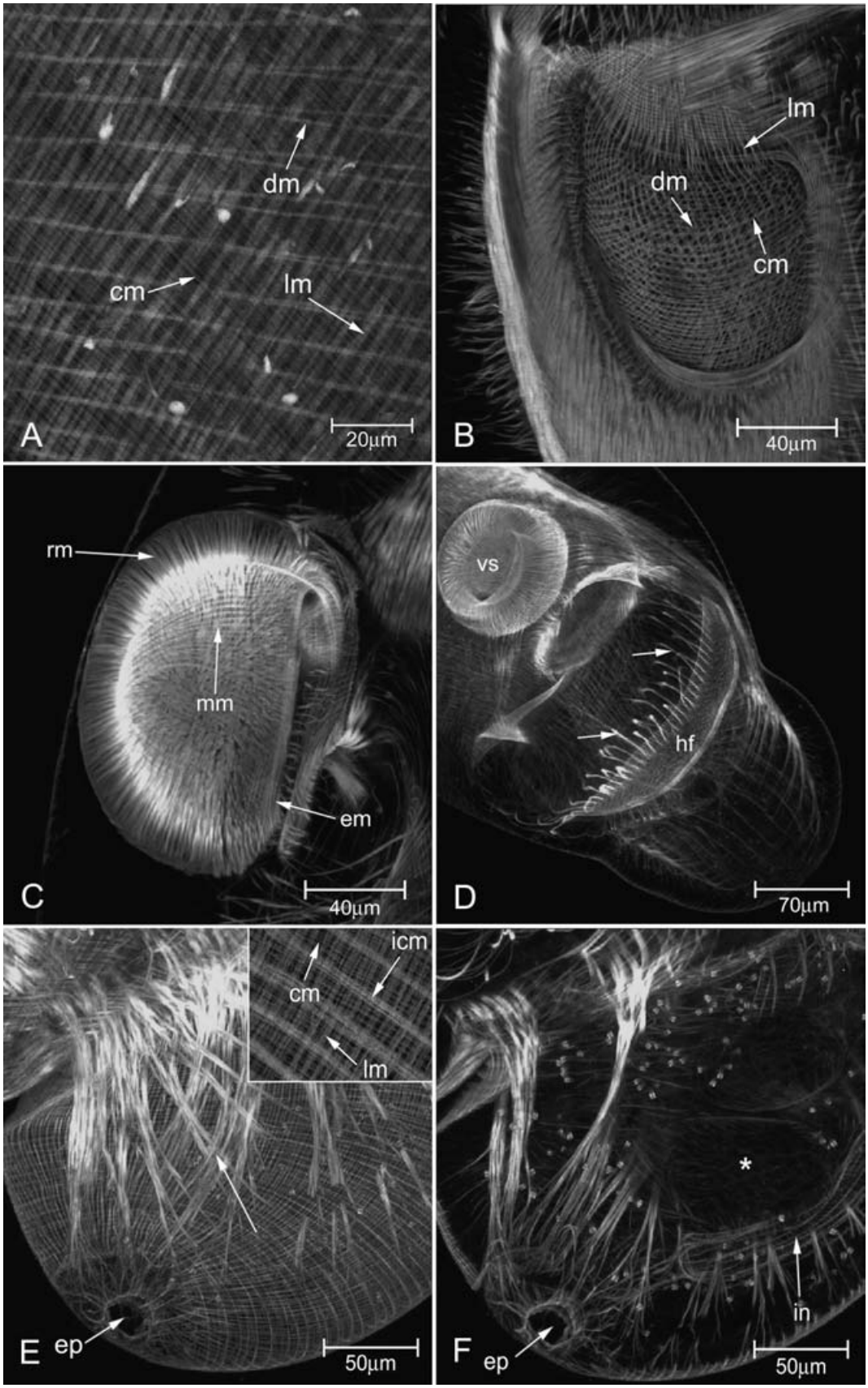

Fig. 2. Whole-mounts of *Apatemon* metacercaria stained with FITC-labelled phalloidin and viewed by confocal microscopy. (A) The forebody wall musculature comprises outer circular (cm), intermediate longitudinal (lm) and inner diagonal fibres (dm). (B) Circular (cm) and diagonal muscle (dm) of the lappet. Longitudinal muscle fibres (lm) of the body wall can be seen entering the lappet. (C) Muscle fibre arrangement in the ventral sucker comprising radial (rm), meridional (mm) and equatorial muscle (em). (D) Posterior to the ventral sucker (vs) lies the retracted holdfast (hf),

https://doi.org/10.1017/S0031182002002780 Published online by Cambridge University Press

of the worm (Fig. 2F). A layer of loosely-spaced (2 μm) inner circular and outer longitudinal fibres support the caecal epithelium and its associated basal lamina (Fig. 3D).

Hindbody. Large extensor and flexor muscles, derived from the longitudinal muscles of the forebody, project into the hindbody where they branch and insert at different levels down the body wall of the hindbody or end blindly in its interior (Fig. 2E, F). The reproductive system is undeveloped at this stage but undifferentiated cells, presumed to be the genital anlage, are discernable within the hindbody (Fig. 2F). A distinct band of circular fibres, derived from the body wall, encircles the terminal excretory pore as a sphincter (Fig. 2E, F).

Nervous system

The gross anatomical arrangement of the nervous system of *Apatemon* metacercaria follows the basic flatworm plan of central (CNS) and peripheral (PNS) nerve elements (Fig. 3A). Typically, the CNS in *Apatemon* consists of a pair of cerebral ganglia, representing a brain, interconnected by a dorsal commissure, from which emanate 3 pairs (ventral, dorsal and lateral) of longitudinal nerve cords (LNCs) that run posteriorly for much of the length of the forebody (Fig. 3B, C); other nerves project anteriorly from the brain and provide innervation to the oral region (Fig. 3B, C). The PNS comprises fibres and associated cell bodies originating in the nerve cords of the CNS and extending to innervate the pharynx, oral sucker and adjacent lappets (Fig. 3B, C, D); similarly, other peripheral fibres supply the ventral sucker and holdfast organ (Fig. 4B) and, in places down the body, there are connections to a subsurface plexus of fine nerves as well as to the excretory sphincter (Fig. 4D).

Peptidergic system. FMRFamide-related peptide (FaRP)-immunoreactivity (IR) was strong and extensive, revealing well-differentiated major central nerve elements. Thus, the butterfly-shaped brain was seen to comprise a pair of cerebral ganglia connected by a dorsal commissure just posterior to the pharynx (Fig. 3B). Neuronal elements projected in a posterior direction from the central neuropile of the brain, forming 2 pairs of longitudinal nerve cords (LNCs), the ventral longitudinal ones being best developed. These extended either side of the pharynx and ran the length of the worm before joining at the posterior of the worm in the vicinity of the excretory pore (Fig. 4D). Transverse cross-connectives (not shown) intersected the ventral nerve cords at regular intervals and accumulations of nerve fibres and associated cell bodies connected at these junctions. A pair of FaRPergic fibres ran anteriorly from the brain to anastomose with the different muscle types of the oral sucker (Fig. 3B). Nerve fibres and their cell bodies were also in close association with the muscle fibres of the lappets (Fig. 4A). Strong FaRPergic innervation was evident to both the ventral sucker and the holdfast, but was more extensive and varicose in appearance in the latter without exhibiting defined nerve tracts (Fig. 4B). Optical sectioning of the body wall revealed an extensive submuscular plexus of fine, FaRPergic nerves arranged in a distinct hexagonal array and in continuity with the ventral LNCs (Fig. 4C).

Serotoninergic system. Relatively weak IR was observed for 5-HT, localized mainly in the anterior portion of the worm but always in nerves separate from those immunoreactive for peptides. Staining in the CNS was in large cell bodies ($<15\,\mu$m) and associated fibres in the cerebral ganglia and in the ventral longitudinal nerve cords (Fig. 3C). In the PNS, 5-HT-IR occurred in ganglia lying lateral to the pharynx and in close association with the oral sucker. Fine fibres, derived from the brain, provided innervation to the anterior margin of the worm, and numerous immunoreactive cell bodies were seen in close association with each lappet.

A schematic of the gross anatomical arrangement of central nerve elements (red) and major muscle systems (black) in *Apatemon* metacercaria, based on the staining described, is presented in Fig. 5.

DISCUSSION

Whole-mount preparations of *Apatemon* metacercaria stained with phalloidin-FITC and examined by CSLM have provided novel insight into the anatomical arrangement of the major muscle systems of a strigeid trematode. Essentially, the subtegumental or body wall musculature comprises an outer sheath of circular fibres within which there is an array of closely packed longitudinal fibres and an inner lattice-like arrangement of diagonal fibres. All 3 fibre types are organized spatially and sequentially in a manner comparable to that described for the marine turbellarian, *Macrostomum hystricinum marinum* (Rieger *et al*. 1994) and the trematodes, *Fasciola*

linked to the body wall by extrinsic bundles of muscle fibres (arrows). (E) Extensor and flexor muscles (unlabelled arrow) derived from the forebody wall muscle run posteriorly into the hindbody. The excretory pore (ep) is surrounded by a sphincter-like arrangement of muscle. Insert: bodywall musculature in the hindbody comprises outer circular (cm), intermediate longitudinal (lm) and an inner layer of more widely spaced circular fibres (icm). (F) Optical section below the body wall showing the terminal region of 1 of the 2 intestinal caeca (in) supported by a thin layer of circular and longitudinal fibres. Undifferentiated cells (*) are present in the hindbody. Extensor and flexor muscles connect to the musculature surrounding the excretory pore (ep).

https://doi.org/10.1017/S0031182002002780 Published online by Cambridge University Press

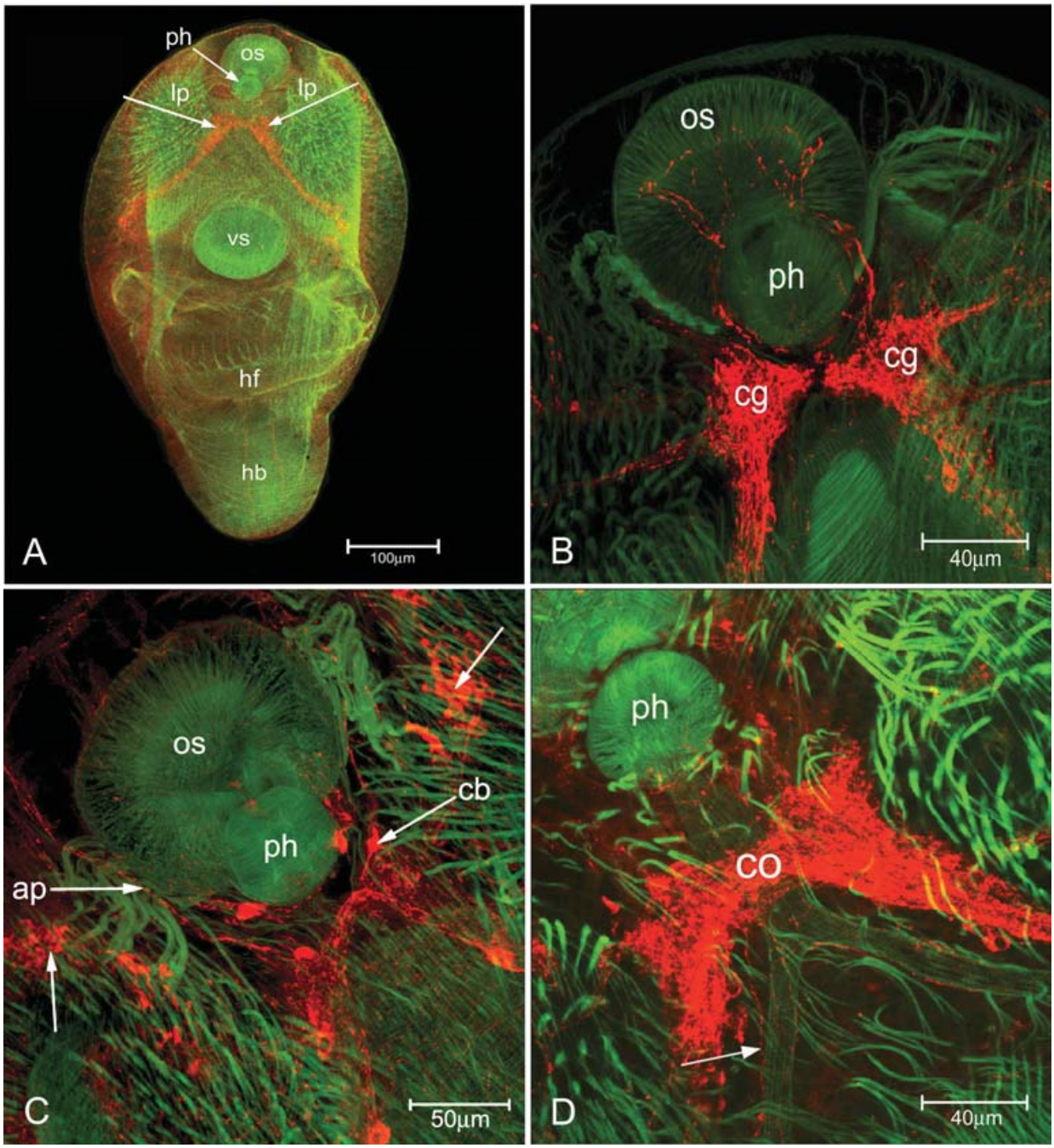

Fig. 3. Confocal scanning laser micrographs of *Apatemon* metacercaria demonstrating immunoreactivity to the FMRFamide-related peptide GYIRFamide and 5-hydroxytryptamine (5-HT). (A) Gross morphology of the GYIRFamide-immunoreactive nerve (red) and muscle (green). The cerebral ganglia (unlabelled arrows) give rise to the longitudinal nerve cords which run posteriorly into the undeveloped hindbody (hb). os, oral sucker; ph, pharynx; lp, lappet; vs, ventral sucker; hf, holdfast. (B) The oral sucker (os) and pharynx (ph) are innervated by projections derived from the cerebral ganglia (cg) (GYIRFamide). (C) Serotonin immunoreactivity in nerves closely associated with the musculature of the oral sucker (os) and pharynx (ph). Large 5-HT-immunoreactive cell bodies (cb) occur in the cerebral ganglia and in the anterior region of the lappets (unlabelled arrows). Note the nerve fibres (ap) that extend anteriorly to innervate muscle of the anterior body wall. (D) The paired cerebral ganglia are connected by a broad commissure (co). Note the 2 gut caeca are innervated by fine GYIRFa-immunoreactive fibres (arrow). ph, pharynx.

hepatica and *Schistosoma mansoni* (Mair *et al*. 1998, 2000), reflecting a high degree of conservation in platyhelminth muscle anatomy. The complement of muscle fibres described and their perceived innervation are consistent with the migratory/exploratory behaviours of freshly-excysted worms when observed moving along substrate: thus, contraction of the longitudinal and circular muscles, respectively, shorten and elongate the worm, while contraction of the diagonal muscles enable sweeping side-to-side movements and a degree of torsion. The regional differences in the layers of body wall muscle between

https://doi.org/10.1017/S0031182002002780 Published online by Cambridge University Press

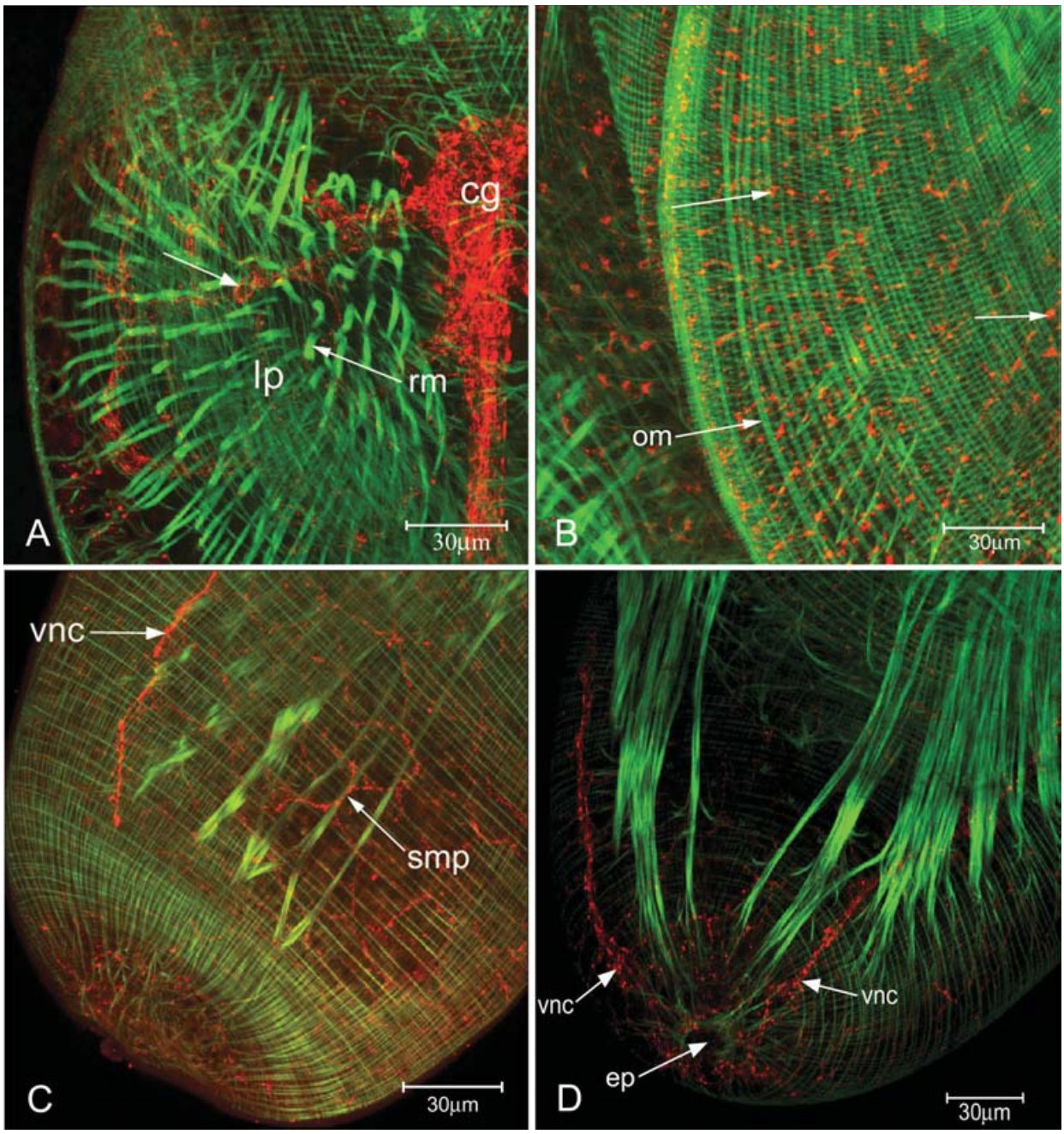

Fig. 4. Confocal scanning laser micrographs of *Apatemon* metacercariae demonstrating immunoreactivity to GYIRFamide. (A) Innervation to the musculature of the lappets (lp) derived from the cerebral ganglia (cg). The unlabelled arrow indicates a FaRP-IR cell body in close association with the radial muscle fibres (rm) of the lappet. (B) Varicose FaRPergic innervation (unlabelled arrows) of the outer musculature (om) at the leading edge of the holdfast. (C) The ventral nerve cord (vnc) runs into the hindbody giving rise to a netlike submuscular plexus (smp). (D) The paired ventral nerve cords (vnc) fuse posteriorly through a plexus of nerve fibres that surround the terminal excretory pore (ep).

forebody and hindbody reflect the relative roles of these 2 regions in strigeid biology. Forebody muscle is in general structurally adapted for attachment and alimentation by way of suckers, lappets and holdfast organ, while the hindbody largely serves a reproductive function for purposes of insemination (the worms mate tail to tail) and oviposition. In this regard, the second layer of circular muscle in the hindbody wall may be responsible for the vigorous peristaltic contractions of this region that occur during cross-insemination and egg release in the adult worm (personal observations).

As expected, the cup-shaped oral and ventral suckers are highly muscularized, with 3 groups of well-differentiated muscle fibres operating in sequence for purposes of attachment. These are the meridional muscles, whose contractions open the sucker and so enable it to flatten and flare against host mucosa; radial muscles, whose contractions return the sucker to a cup-shape, thereby drawing up a plug

https://doi.org/10.1017/S0031182002002780 Published online by Cambridge University Press

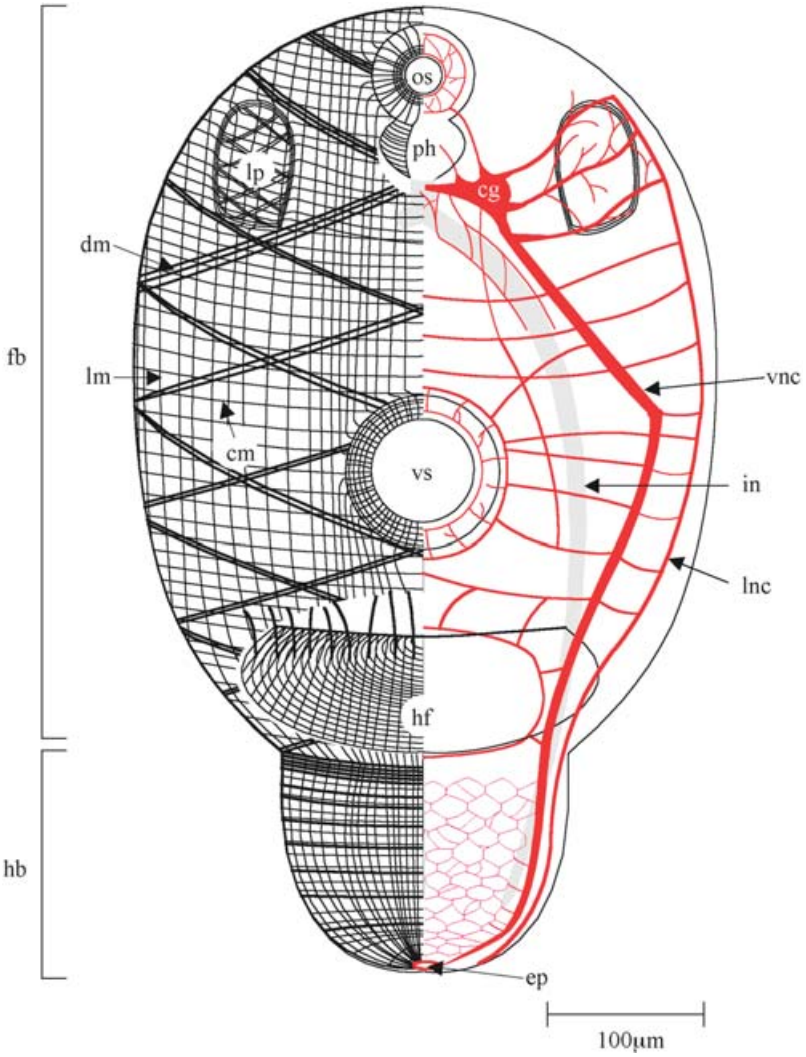

Fig. 5. Schematic showing the gross anatomical arrangement of the major neuronal pathways (red) and muscle systems (black) in excysted *Apatemon* metacercaria, based on immunostaining of FaRPergic neurons and phalloidin staining of F-actin, respectively. Key to abbreviations: cg, cerebral ganglion; cm, circular muscle; dm, diagonal muscle; ep, excretory pore; fb, forebody; hb, hindbody; hf, holdfast; in, intestine; lp, lappet; lm, longitudinal muscle; lnc, longitudinal nerve cord; os, oral sucker; ph, pharynx; vnc, ventral nerve cord; vs, ventral sucker.

of host tissue, which is then secured by contraction of the sphincter-like equatorial muscles at the rim.

The paired lappets are ear-like musculo-glandular structures arising at the anterior margins of the worm. They are capable of rapid inversion in the excysted metacercaria so extraneous tissue can be quickly drawn into them to secure attachment to host mucosa. Ducts from clusters of unicellular glands located in the forebody run among the lappet muscle fibres and convey secretory bodies to the exterior through apertures in their tegumental linings, suggesting the lappets are involved in both attachment and secretion (Erasmus, 1969).

The pharynx is non-glandular but composed largely of well-developed radially orientated fibres. Trematodes are largely suctoral feeders and draw food into their gut through the combined muscular action of the oral sucker and pharynx (Smyth & Halton, 1983). There are no documented accounts of pharynx mechanics in trematodes but it is assumed the structure serves as a muscular pump. It is possible that during feeding the lumen of the anterior portion of the pharynx is dilated by contraction of its radial muscles, such that fluid/semi-fluid food is sucked in, followed by dilation of the posterior pharynx and closure of the anterior pharynx to force food into the oseophagus and caeca. Observations on the ingestion of fluid food in *Philophthalmus* metacercariae by Howell (1970) indicate that feeding is accompanied by rhythmical extensions and contractions of the forebody musculature and that this probably aids pharyngeal pumping.

The intestinal caeca bifurcate behind the pharynx and short oesophagus and their epithelial lining is invested with a diffuse arrangement of predominately circular, but also some longitudinal fibres. The circular fibres can be assumed to provide peristaltic movement of food through the intestine, while the longitudinal fibres, which were more noticeable in the hindbody, may be required in adult worms to shorten

https://doi.org/10.1017/S0031182002002780 Published online by Cambridge University Press

the gut during the extensive hindbody contractions that have been observed to accompany oviposition.

Large longitudinal extensor and flexor muscles derived from the longitudinal body wall muscle in the forebody extend into the hindbody. Branches from these muscle bands run both towards the subtegumental layer and into the interior of the hindbody. They presumably serve in the support and posture of the rapidly developing hindbody, and may also be involved in the organization and function of the various reproductive structures that become established in this region of the adult worm.

In common with the metacercariae of other strigeid trematodes previously examined, namely, *Diplostomum spathaceum* and *Cotylurus erraticus* (Barton *et al*. 1993), the nervous system of *Apatemon* is well-differentiated and reactive for both neuropeptides and 5-HT. Analysis of the patterns of staining in the worm showed serotoninergic pathways were distinct from those immunoreactive for neuropeptide, although in places, such as in the suckers, there was often a close and intricate association between the two. Moreover, serotoninergic cell bodies were somewhat larger and more symmetrical in arrangement along the body of the worm, than the smaller and more randomly disposed FaRPergic somata.

Immunostaining for 5-HT revealed fibres emanating from the brain to innervate anterior regions in a manner comparable to that observed in the monogenean, *Entobdella soleae* by Marks *et al*. (1994), who suggested a role for 5-HT in contact perception. This hypothesis is corroborated by the presence of 5-HT-IR cell bodies along the inner surface of the anterior margins of each lappet. In the PNS, 5-HT-IR occurred in the ganglia in close association with the musculature of the oral sucker and pharynx. Serotonin has been shown to have an excitatory effect on muscle of both free living (Blair & Anderson, 1994) and parasitic helminths (Hillman, 1983; Maule *et al*. 1989*a*; Humphries *et al*. 2000), and as such may serve as a neuromodulator in *Apatemon*.

FaRP antisera produced extensive staining comparable to peptidergic patterns obtained in other flatworms examined: the turbellarian, *Bdelloura candida*, (Johnston *et al*. 1996), monogenean, *Diclidophora merlangi* (Maule *et al*. 1989*b*), and trematodes, *S. mansoni* (Skuce *et al*. 1990), *F. hepatica* (Magee *et al*. 1989) and *C. erraticus* (Barton *et al*. 1993). FaRP was also widely expressed within the PNS of *Apatemon*. FaRPergic fibres were found surrounding and anastomosing throughout the musculature of the oral sucker and acetabulum, suggesting FaRP involvement in neuronal control of sucker function. As with the other trematodes investigated, the paired ventral nerve cords showed the strongest immunoreactivity for neuropeptide and provide the major source of innervation in the undeveloped hindbody.

The presence of FaRP-IR fibres in nerves which innervate the musculature is not surprising. In addition to their extensive distribution within the CNS and PNS of representative species from all major classes of flatworms (Shaw *et al*. 1996; Halton *et al*. 1994), physiological studies have shown synthetic RFamide peptides induce dose-dependent contractions in species such as *B. candida* (Johnston *et al*. 1996), *S. mansoni* (Day *et al*. 1994), *F. hepatica* (Marks *et al*. 1996) and *Echinostoma caproni* (Humphries *et al*. 2000). In *B. candida* and *S. mansoni*, FaRPs had an excitatory effect on isolated muscle fibres, suggesting direct interaction with a muscle receptor. As such, the FaRPergic nervous system represents a promising target for chemotherapy.

This cytochemical investigation has given insight into the complexity of the neuromusculature of a 'resting' stage of a trematode. The potential to culture strigeid parasites to ovigerous adults *in vitro* within a few days, as demonstrated by Kannangara & Smyth (1974) and Mitchell, Halton & Smyth (1978), presents the strigeid metacercaria as an attractive model for future work examining structural and cytochemical changes accompanying development of the neuromusculature of the trematode reproductive complex. In particular, it offers an opportunity to experimentally evaluate the parasite's neuronal messenger system as a potential drug target, particularly in view of the finding that in the innervation of the trematode ootype FaRP neuropeptides are first expressed at the onset of egg production, implicating their involvement in regulating reproductive function (Armstrong *et al*. 1997).

This work was funded in part by a studentship (M.T.S.) from the European Social Fund, a postdoctoral fellowship (Š.Š.) from Queen's University Belfast, and the Grant Agency of the Czech Republic #524/00/0844 (D.W.H., B.K.).

REFERENCES

ARMSTRONG, E. P., HALTON, D. W., TINSLEY, R. C., CABLE, J., JOHNSTON, R. N., JOHNSTON, C. F. & SHAW, C. (1997). Immunocytochemical evidence for the involvement of a FMRFamide-related peptide (FaRP) in egg production in the flatworm parasite, *Polystoma nearcticum*. *Journal of Comparative Neurology* **377**, 41–48.

BARTON, C. L., HALTON, D. W., SHAW, C., MAULE, A. G. & JOHNSTON, C. F. (1993). An immunocytochemical study of putative neurotransmitters in the metacercaria of two strigeoid trematodes from rainbow trout (*Oncorynchus mykiss*). *Parasitology Research* **79**, 389–386.

BLAIR, K. L. & ANDERSON, P. A. V. (1994). Physiological and pharmacological properties of muscle cells isolated from the flatworm *Bdelloura candida* (Tricladia). *Parasitology* **109**, 325–335.

BORTZ, B. M., KENNY, G. E., PAULEY, G. B., GARCIA-ORTIGOZA, E. & ANDERSON, D. P. (1984). The immune response in immunised and naturally infected rainbow trout (*Salmo gairdneri*) to *Diplostomum spathaceum* as detected by ELISA. *Developmental and Comparative Immunology* **8**, 813–822.

COONS, A. H., LEDUC, E. H. & CONNOLLY, J. M. (1955). Studies on antibody production: 1. A method for the

histochemical demonstration of specific antibodies and its application to a study of the hyper-immune rabbit. *Journal of Experimental Medicine* **102**, 49–60.

CHUBB, J. C. (1979). Seasonal occurrence of helminths in freshwater fishes. *Advances in Parasitology* **17**, 141–313.

DAY, T. A., MAULE, A. G., SHAW, C., HALTON, D. W., MOORE, S., BENNETT, J. L. & PAX, R. A. (1994). Platyhelminth FMRFamide-related peptides (FaRPs) contract *Schistosoma mansoni* (Trematoda: Digenea) muscle fibres *in vitro*. *Parasitology* **109**, 455–459.

ERASMUS, D. A. (1969). Studies on the host-parasite interface of strigeoid trematodes. IV. The ultrastructure of the lappets of *Apatemon gracilis minor*, Yamaguti, 1933. *Parasitology* **59**, 193–201.

HALTON, D. W., SHAW, C., MAULE, A. G. & SMART, D. (1994). Regulatory peptides in helminth parasites. *Advances in Parasitology* **34**, 163–227.

HILLMAN, G. R. (1983). The neuropharmacology of schistosomes. *Pharmacology and Therapeutics* **22**, 103–115.

HOWELL, M. J. (1970). Ingestion by two species of digenetic trematodes. *Journal of Parasitology* **56**, 391–392.

HUMPHRIES, J. E., MOUSLEY, A., MAULE, A. G. & HALTON, D. W. (2000). Neuromusculature: structure and functional correlates. In *Echinostomes as Experimental Models for Biological Research* (ed. Fried, B. & Grazyk, T. K.), pp. 213–227. Kluwer Academic Publishers, Amsterdam.

JOHNSTON, R. N., HALTON, D. W., ANDERSON, P. A. V., JOHNSTON, C. F. & SHAW, C. (1996). The peptidergic nervous system of the triclad turbellarian *Bdelloura candida* (Maricola, Bdellouridae): an immunocytochemical study using an antiserum raised to an endogenous neuropeptide, GYIRFamide. *Journal of Comparative Neurology* **376**, 214–222.

KANNANGARA, D. W. W. & SMYTH, J. D. (1974). *In vitro* cultivation of *Diplostomum spathaceum* and *Diplostomum phoxini* metacercaria. *International Journal for Parasitology* **4**, 667–673.

KEARN, G. C., CLEVLAND, G. & WILKINS, S. (1989). An *in vitro* study of the forcible ejection of the metacercaria of the strigeid digenean *Apatemon* (*Australapatemon*) *minor* Yamaguti, 1933 by rapid inward expansion of the cyst wall. *Journal of Helminthology* **63**, 63–71.

MAGEE, R. M., FAIRWEATHER, I., JOHNSTON, C. F., HALTON, D. W. & SHAW, C. (1989). Immunocytochemical demonstration of neuropeptides in the nervous system of the liver fluke *Fasciola hepatica* (Trematoda, Digenea). *Parasitology* **98**, 227–238.

MAIR, G. R., MAULE, A. G., SHAW, C., JOHNSTON, C. F. & HALTON, D. W. (1998). Gross anatomy of the muscle systems of *Fasciola hepatica* as visualised by phalloidin-fluorescence and confocal microscopy. *Parasitology* **117**, 75–82.

MAIR, G. R., MAULE, A. G., DAY, T. & HALTON, D. W. (2000). A confocal microscopical study of the musculature of adult *Schistosoma mansoni*. *Parasitology* **121**, 163–170.

MARKS, N. J., HALTON, D. W., KEARN, G. C., SHAW, C. & JOHNSTON, C. F. (1994). 5-hydroxytryptamine-immunoreactivity in the monogenean parasite *Entobdella soleae*. *International Journal for Parasitology* **24**, 1011–1018.

MARKS, N. J., JOHNSTON, S., MAULE, A. G., HALTON, D. W., SHAW, C., GEARY, T. G., MOORE, S. & THOMPSON, D. P. (1996). Physiological effects of platyhelminth RFamide peptides on muscle-strip preparations of *Fasciola hepatica* (Trematoda: Digenea). *Parasitology* **113**, 393–401.

MAULE, A. G., HALTON, D. W., ALLEN, J. M. & FAIRWEATHER, I. (1989*a*). Studies on the motility *in vitro* of the ectoparasitic monogenean *Diclidophora merlangi*. *Parasitology* **98**, 85–93.

MAULE, A. G., HALTON, D. W., JOHNSTON, C. F., FAIRWEATHER, I. & SHAW, C. (1989*b*). Immunocytochemical demonstration of neuropeptides in the fish-gill parasite, *Diclidophora merlangi* (Monogenoidea). *International Journal for Parasitology* **19**, 307–316.

MITCHELL, J. S., HALTON, D. W. & SMYTH, J. D. (1978). Observations on the *in vitro* culture of *Cotylurus erraticus* (Trematoda: Strigeidae). *International Journal for Parasitology* **8**, 389–397.

PAX, R. A., DAY, T. A., MILLER, C. L. & BENNET, J. L. (1996). Neuromuscular physiology and pharmacology of parasitic flatworms. *Parasitology* **113**, S83–S86.

RIEGER, R. M., SALVENMOSER, W., LEGNITI, A. & TYLER, S. (1994). Phalloidin-rhodamine preparations of *Macrostomum hystricinum marinum* (Platyhelminthes): morphology and postembryonic development of the musculature. *Zoomorphology* **114**, 133–147.

SHAW, C., MAULE, A. G. & HALTON, D. W. (1996). Platyhelminth FMRFamide-related-peptides. *International Journal for Parasitology* **26**, 335–345.

SKUCE, P. J., JOHNSTON, C. F., FAIRWEATHER, I., HALTON, D. W. & SHAW, C. (1990). A confocal scanning laser microscope study of the peptidergic and serotoninergic components of the nervous system in larval *Schistosoma mansoni*. *Parasitology* **101**, 227–234.

SMYTH, J. D. (1994). *Introduction to Animal Parasitology*. 3rd Edn. Cambridge University Press, Cambridge.

SMYTH, J. D. & HALTON, D. W. (1983). *The Physiology of Trematodes*. 2nd Edn. Cambridge University Press, Cambridge.

STABLES, J. N. & CHAPPELL, L. H. (1986). Putative immune response of the rainbow trout, *Salmo gairdneri*, to *Diplostomum spathaceum* infection. *Journal of Fish Biology* **29**, 115–222.

TORT, L., WATSON, J. J. & PRIEDE, I. G. (1987). Changes in *in vitro* heart performance in rainbow trout, *Salmo gairdneri* Richardson, infected with *Apatemon gracilis* (Digenea). *Journal of Fish Biology* **30**, 341–347.

WATSON, J. J., PIKE, A. W. & PRIEDE, I. G. (1992). Cardiac pathology associated with the infection of *Oncorynchus mykiss* Walbaum with *Apatemon gracilis* Rud. 1819. *Journal of Fish Biology* **41**, 163–167.

WHYTE, S. K., ALLAN, J. C., SECOMBES, C. J. & CHAPPELL, L. H. (1986). Cercariae and diplostomules of *Diplostomum spathaceum* (Digenea) elicit an immune response in rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology* **31** (Suppl A), 185–190.

WHYTE, S. K., CHAPPELL, L. H. & SECOMBES, C. J. (1988). *In vitro* transformation of *Diplostomum spathaceum* (Digenean) cercariae and short term maintenance of post penetration larvae *in vitro*. *Journal of Helminthology* **62**, 293–302.

WHYTE, S. K., CHAPPELL, L. H. & SECOMBES, C. J. (1989). Cytotoxic reactions of rainbow trout, *Salmo gairdneri* Richardson, macrophages for larvae of the eye fluke *Diplostomum spathaceum* (Digenea). *Journal of Fish Biology* **35**, 333–345.