Gross anatomy of the muscle systems of *Fasciola hepatica* as visualized by phalloidin-fluorescence and confocal microscopy

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(*Received 26 January 1998; revised 18 February 1998; accepted 18 February 1998*)

SUMMARY

Neuropeptides, biogenic amines and acetylcholine are expressed abundantly within the nervous systems of parasitic flatworms, and are particularly evident in the innervation of the musculature. Such associations have implicated the nervous system in locomotion, host attachment and reproductive co-ordination. Information on the muscle systems of parasitic flatworms is generally sparse, in particular those muscles associated with the reproductive system, intestinal tract and attachment apparatus. Also, the use of sectioned material has left description of the 3-dimensional organization of the musculature largely unrecorded. Using fluorescein isothiocyanate (FITC)-labelled phalloidin as a site-specific probe for filamentous actin, applied to whole-mount preparations of adult *Fasciola hepatica* and examined by confocal scanning laser microscopy, the present work reports on the organization of the major muscle systems in this trematode parasite. A highly regular array of outer circular, intermediate longitudinal and inner diagonal fibres distinguishes the body wall musculature, which is also involved in the development of both ventral and oral suckers. Circular fibres dominate the duct walls of the male and female reproductive systems, whereas the muscles of the intestinal tract have a somewhat diffuse arrangement of fibres. An understanding of the structural complexity of the muscle systems of parasitic flatworms is considered as fundamental to the interpretation of results from physiological experiments.

Key words: Platyhelminthes, Trematoda, *Fasciola hepatica*, muscle, phalloidin, confocal microscopy.

INTRODUCTION

Native flatworm oligopeptides possessing carboxy-terminal RFamide motifs (e.g. YIRFamide and GNFFRFamide), generally known as FMRFamide-related peptides or FaRPs, elicit potent excitatory responses in both muscle-strip preparations and isolated muscle fibres from a number of free-living and parasitic flatworms (see Blair & Anderson, 1996; Pax *et al*. 1996; Marks *et al*. 1996; Moneypenny *et al*. 1997). Although serotonin (5-hydroxytryptamine, 5-HT) also stimulates the contractions of muscle-strip preparations that include neuronal tissue, and is a necessary medium component for isolated muscle fibre contractions, it does not, itself, induce contractions of isolated muscle fibres of *Schistosoma mansoni* and *Bdelloura candida* (Day, Bennett & Pax, 1994; Blair & Anderson, 1994). On the other hand, acetylcholine (ACh) evokes inhibitory effects on whole-mount or muscle-strip preparations of a number of parasitic flatworms (Chance & Mansour, 1953), and represses muscle contractions induced by FaRPs applied to isolated schistosome muscle fibres (Day *et al*. 1996). Collectively, these findings indicate a neurotransmitter or neuromodulatory role for FaRPs, 5-HT and ACh, and infer the presence of specific receptors on the smooth muscle of flatworms. However, none of the receptors which mediate the observed physiological effects has been characterized and the muscle fibres responding to these neurotransmitters are as yet unknown.

FaRPs, 5-HT and ACh represent putative neurotransmitters within flatworm nervous systems and, through peripheral plexuses, have been identified in the innervation of muscle fibres associated with locomotion (body wall musculature), host attachment (suckers, bothridia and haptors), alimentation (pharynx and intestine) and reproduction (ducts within the male and female reproductive systems) (see Halton & Gustafsson, 1996). Except for body wall or subtegumental musculature, detailed studies of muscle systems in large, parasitic flatworms such as *Fasciola hepatica* are rare (see review by Mair *et al*. 1998). There is an early description of the musculature of *F. hepatica* by Bettendorf (1897), using methylene blue staining on whole-mount preparations, and a more recent investigation using actin-antibodies and fluorescently-labelled phalloidin on sectioned material of the worm (Stitt *et al*. 1992). Phalloidin staining has also been employed

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https://doi.org/10.1017/S0031182098002807 Published online by Cambridge University Press

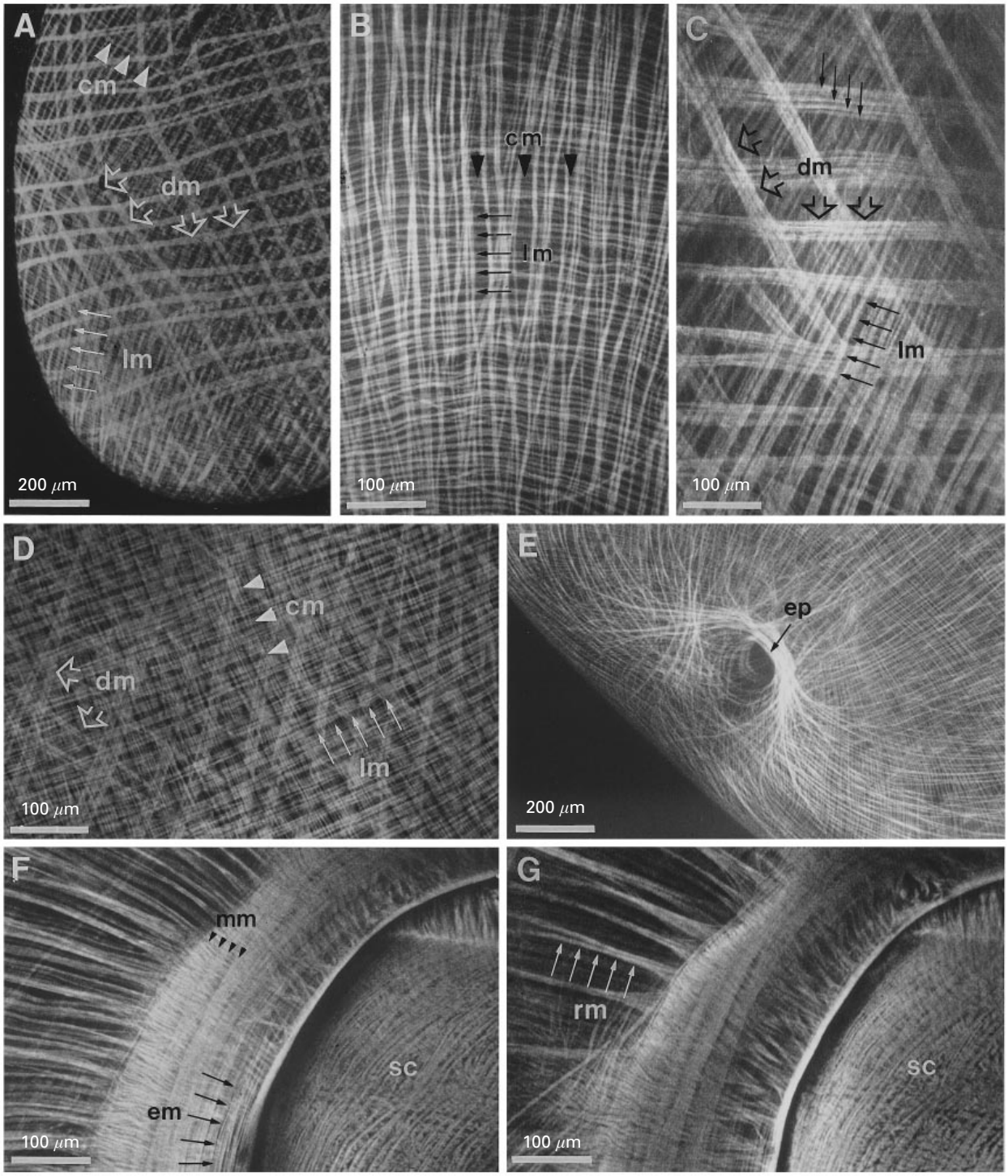

Fig. 1. Muscle fibre arrangement within the body wall (or subtegumental) musculature and the ventral sucker of *Fasciola hepatica* as visualized by FITC–phalloidin staining and CSLM. (A) Posterior of the worm showing circular (cm), longitudinal (lm) and diagonal (dm) muscle fibres arranged into distinct muscle bands (after Mair *et al*. 1998). (B) Circular (cm) and longitudinal (lm) muscle fibres within the subtegumental musculature. (C) Deeper plane of focus showing longitudinal muscle fibres (lm) and bands of diagonal muscle fibres (dm). Note individual muscle fibres within the diagonal muscle bands, indicated by arrows. (D) Muscle fibre arrangement with circular (cm), longitudinal (lm) and diagonal (dm) muscle fibres. (E) Sphincter-like muscle fibre arrangement at the excretory pore (ep). (F) Meridional (mm) and equatorial (em) muscle fibres of the ventral sucker are located on the sucker surface (sc, sucker cavity). (G) Radial muscle fibres (rm) of the ventral sucker (sc, sucker cavity).

to localize F-actin in developmental stages of the cestode, *Diphyllobothrium dendriticum* (Wahlberg 1997) and in the microturbellarian, *Macrostomum hystricinum marinum* (Rieger *et al*. 1994); the same technique was also used to study muscle differentiation in the turbellarians, *Hoploplana inquilina* and *M. hystricinum marinum* (Reiter *et al*. 1996).

In this paper, the phalloidin-fluorescence tech-

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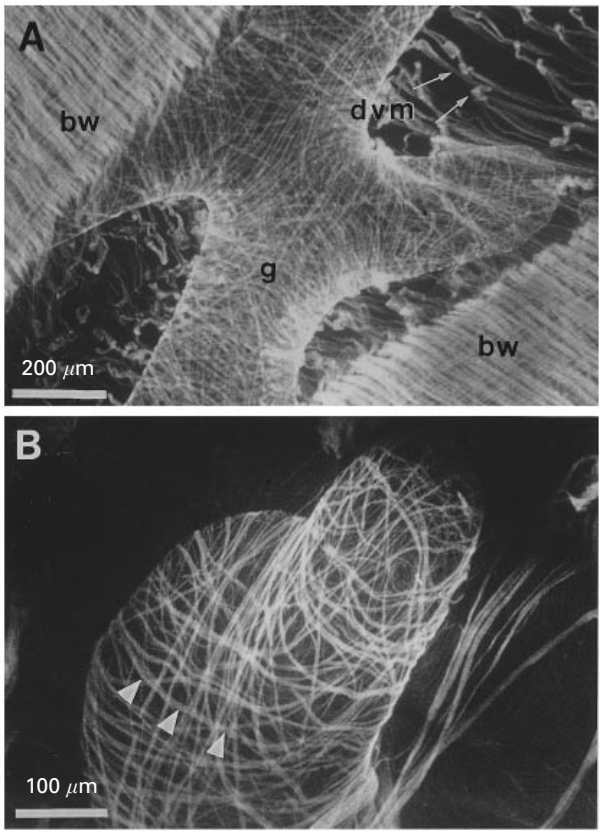

Fig. 2. Muscle fibre arrangement within the alimentary tract of *Fasciola hepatica* as visualized by FITC-phalloidin staining and CSLM. (A) Intestinal diverticula of the gut (g) and numerous dorsoventral muscle fibres (dvm), as seen through a split in the dorsal body wall (bw) musculature. (B) Terminal region of a gut diverticulum showing the diffuse organization of mostly circular muscle fibres (arrowheads) (after Mair *et al.* 1998).

nique has been used, in conjunction with confocal scanning laser microscopy (CSLM), to explore muscle organization in *F. hepatica*. Details of the musculature of the body wall, attachment apparatus, digestive and reproductive systems of the worm are described and, where appropriate, some of the functional correlations are discussed.

MATERIALS AND METHODS

Animals

Adult *Fasciola hepatica* (1·5–2 cm in length) were recovered from the bile ducts of bovine livers made available in a local abattoir (W. D. Meats, Coleraine) and kept briefly in 0·9% NaCl (w/v) at 37 °C prior to treatment.

FITC–phalloidin staining

Worms were flat-fixed between microscope slides in 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 1 h and then transferred to fresh fixative for another 3 h. After fixation, animals were washed overnight in antibody diluent (AbD, 0·1 M PBS, pH 7·4, containing 0·1% (w/v) Triton X–100, 1% (v/v) bovine serum albumin and 0·1% (w/v) NaN_3). Specimens were then incubated for 24 h at 4 °C with AbD containing 200 ng/ml fluorescein isothiocyanate (FITC)-conjugated phalloidin (Sigma Chemical Company). Finally, worms were washed for 2 h in AbD with several buffer changes and mounted in PBS/glycerol (1:9, containing 2·5% (w/v) 1,4-diazabicyclo[2.2.2]octane, Sigma Chemical Co.), and viewed and photographed using either an MRC 500 confocal scanning laser microscope (Bio-Rad, Lasersharp, Abingdon, UK) or a Leica TCS-NT confocal scanning laser microscope (Leica, Milton Keynes, UK). Phalloidin, a phallotoxin from *Amanita phalloides*, binds F-actin with a 0·9:1 stoichiometry. Bound FITC-labelled phalloidin exhibits significantly higher fluorescence than the unbound moiety; the fluorescence enhancement of FITC-phalloidin by actin reaches a factor of 1·5 (Huang *et al.* 1992).

RESULTS

Body wall musculature

The body wall or subtegumental musculature of *Fasciola hepatica* comprises circular, longitudinal and diagonal muscle fibres, which form a well-developed and highly organized lattice-like arrangement over the entire body (Fig. 1A). Each of the 3 fibre types forms distinct muscle layers whose interrelationships are derived from z-series scans using confocal microscopy, revealing an outer circular, intermediate longitudinal and inner diagonal arrangement of fibres (Fig. 1B–D). Individual circular and longitudinal muscle fibres appear evenly spaced, whereas diagonal fibres are arranged into muscle bands of 2 or more parallel fibres (Fig. 1C). Circular muscle fibres are generally orientated at right angles to the longitudinal body axis, but are skewed mid-ventrally and mid-dorsally so as to converge towards the posterior tip of the body; longitudinal fibres run parallel to the main body axis. The two sets of diagonal fibres run at angles of approximately 60° and 120°, respectively, in relation to the longitudinal muscle fibres, and their arrangement is particularly dense in the head-cone region. The subtegumental musculature is also involved in the development of the gonopore (situated anterior to the ventral sucker) and the excretory pore (situated at the posterior ventral tip; Fig. 1E). In both instances, circular and longitudinal muscle fibres enter the respective openings and form sphincter-like fibre arrangements. In addition, dorso–ventral muscle fibres extend between the dorsal and ventral body walls throughout the worm (Fig. 2A).

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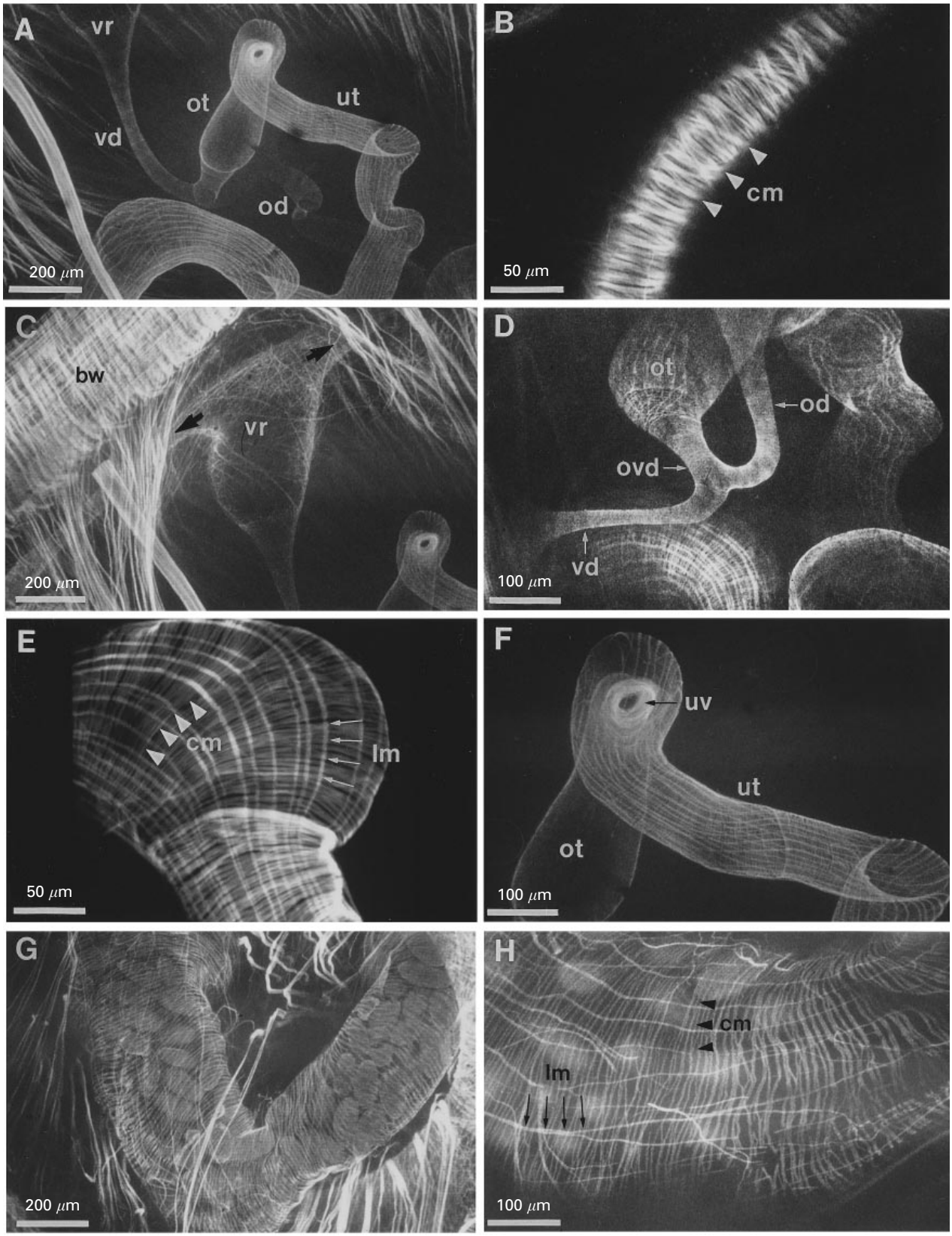

Fig. 3. Muscle fibre organization within the ducts associated with the female reproductive system of *Fasciola hepatica* as visualized by FITC-phalloidin staining and CSLM. (A) Overview of the egg-laying apparatus and associated ducts: vitelline reservoir (vr), vitelline duct (vd), oviduct (od), ootype (ot) and proximal uterus (ut). (B) Oviduct displays a dense organization of circular muscle fibres (cm). (C) Vitelline reservoir (vr) and the associated vitelline ducts (arrows) as seen through a gap in the body wall (bw). (D) Junction of oviduct (od) and vitelline duct (vd) with the short ovovitelline duct (ovd) that opens into the proximal ootype (ot). (E) Proximal ootype showing the dense arrangement of circular (cm) and fewer longitudinal (lm) muscle fibres. (F) The circular muscles of the ootype (ot)

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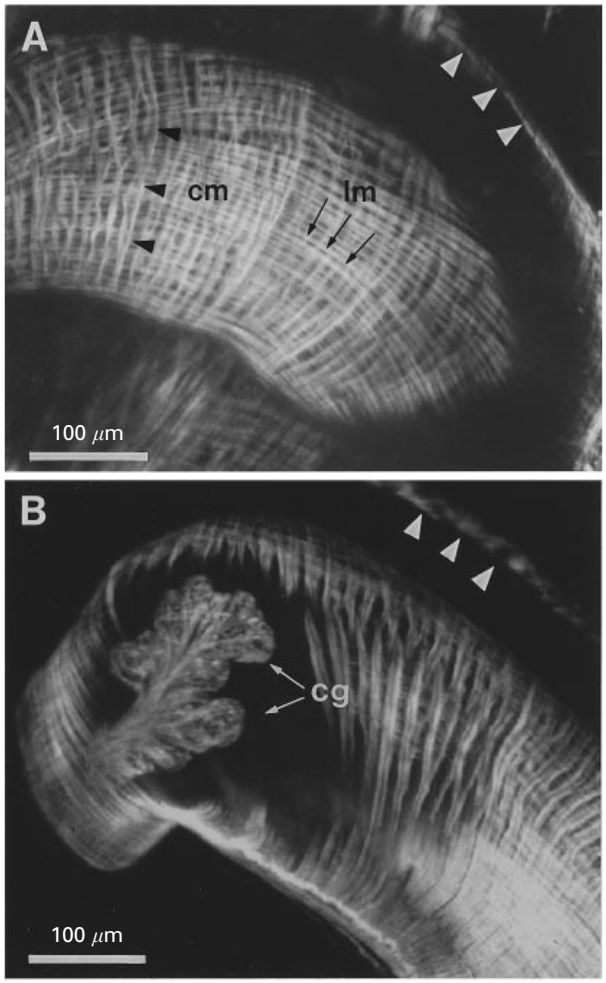

Fig. 4. Muscle fibre arrangement within the male copulatory system of *Fasciola hepatica* as visualized by FITC-phalloidin staining and CSLM. (A) The cirrus displays densely arranged circular (cm) and longitudinal (lm) muscle fibres, and lies within the cirrus sac, the lining of which is indicated by the white arrowheads. (B) At the proximal end of the cirrus is a gland-like structure (cg). Arrow heads indicate lining of the cirrus sac.

Attachment apparatus

Oral and ventral suckers display 3 types of densely organized muscle fibres. The musculature of both the ventral and oral suckers is partly derived from the subtegumental musculature and includes: longitudinal muscle fibres of the body wall which give rise to meridional fibres within the suckers, and circular fibres which become equatorially arranged fibres; a third set of fibres, which do not seem to be derived from the subtegumental musculature, run between the inner and outer faces of the sucker as radial muscles (Fig. 1 F, G).

Intestinal caeca

The alimentary tract of *F. hepatica* bifurcates into 2 major intestinal caeca immediately behind the pharynx and extends to the posterior of the worm. The caeca give rise to numerous lateral diverticula, each displaying a diffusely organized array of largely circular muscle fibres (Fig. 2 A, B).

Reproductive system

Within the female reproductive system, numerous muscle fibres were identified in all of the ducts associated with the egg-laying apparatus (Fig. 3 A–H). The walls of the vitelline ducts and the vitelline reservoir contain muscle fibres which have a diffuse arrangement similar to that of the gut musculature (Fig. 3 C). The remaining ducts of the female reproductive system (i.e. oviduct, ovovitelline duct, Laurer's canal, ootype and uterus) are distinguished by a dense and much more ordered arrangement of circular fibres, together with a few inner longitudinal fibres; diagonal fibres are absent (Fig. 3 B, E, F). Circular muscle fibres are particularly dense in the wall of the uterus (Fig. 3 G, H). At the transition of ootype and uterus, a sphincter-like arrangement of circular muscle fibres marks the junction and corresponds to the uterine valve (Fig. 3 F). The ootype itself also displays a compact arrangement of circular and longitudinal fibres in its walls (Fig. 3 E).

Numerous highly organized circular and longitudinal muscle fibres are also evident in the cirrus musculature and in the wall of the surrounding cirrus sac (Fig. 4 A, B). In common with the female reproductive system, diagonal fibres were not found in the ducts of the male system. Staining was also evident in a gland-like structure situated at the proximal end of the cirrus (Fig. 4 B). Both the cirrus and the distal uterus open into a common gonopore situated anteriorly to the ventral sucker.

DISCUSSION

Flat-fixed, whole-mount preparations of *F. hepatica*, stained with phalloidin–FITC and imaged by confocal microscopy, have allowed a detailed examination of the worm musculature and, in comparison with sectioned material, have revealed novel information on its 3-dimensional arrangement (see Fig. 5 for a schematic representation of the major muscle systems present in the worm).

Beneath the tegument, there is a complex network of outer circular, intermediate longitudinal and inner

are organized into a sphincter at the uterine valve (uv), which serves to regulate egg-release into the uterus (ut). (G) Distal portion of the (egg-filled) uterus displaying densely arranged circular fibres. (H) Higher magnification of the uterus reveals the numerous circular muscle fibres (cm) and few longitudinal muscle fibres (lm) of its walls.

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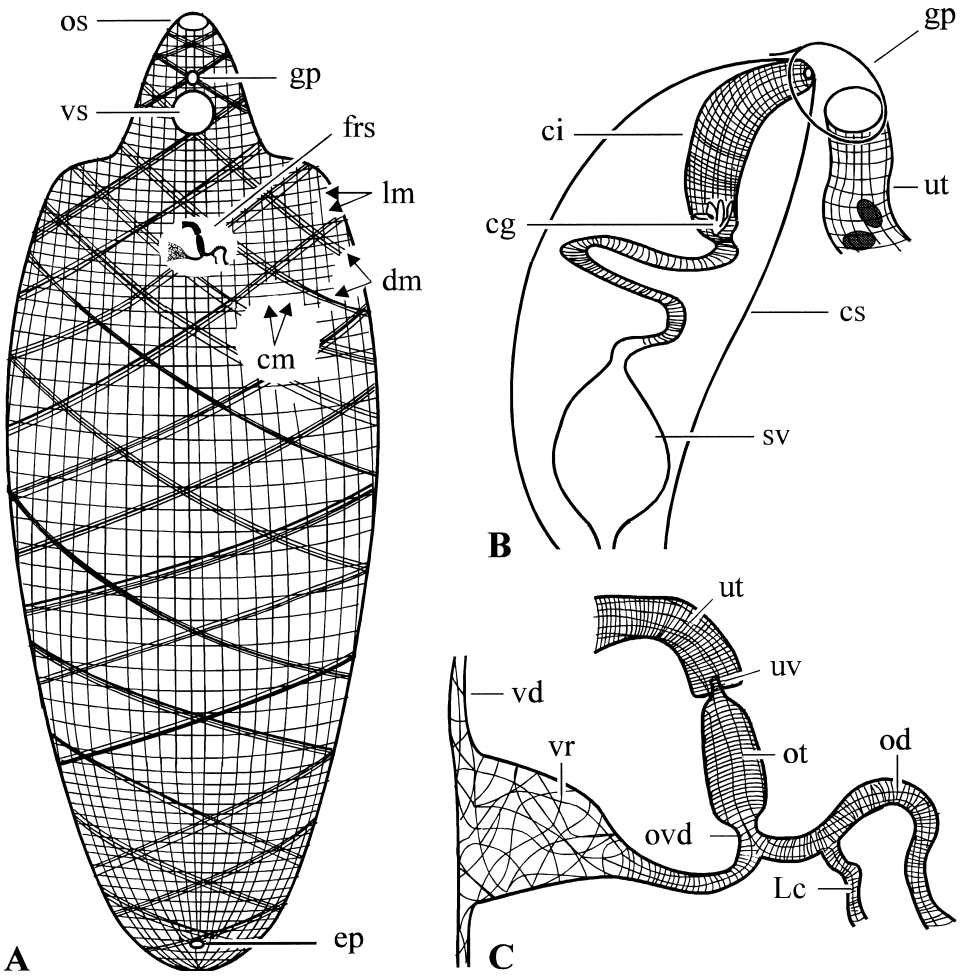

Fig. 5. Schematic representation of the major muscle systems in *Fasciola hepatica*. (A) Lattice-like arrangement of circular, longitudinal and diagonal muscle fibres is present within the body wall. (B) Male copulatory apparatus within the cirrus sac opens through the common gonopore. (C) Muscle fibre organization of the ducts associated with the egg-laying apparatus. cg, Gland-like structure; ci, cirrus; cm, circular muscle fibres; cs, cirrus sac; dm, diagonal muscle fibres; ep, excretory pore; frs, female reproductive system; gp, gonopore; Lc, Laurer's canal; lm, longitudinal muscle fibres; od, oviduct; os, oral sucker; ot, ootype; ovd, ovo-vitelline duct; sv, seminal vesicle; ut, uterus; uv, uterine valve; vd, vitelline duct; vr, vitelline reservoir; vs, ventral sucker.

diagonal muscle fibres, with diagonal fibres arranged into distinct muscle bands, and all 3 types of fibres organized into distinct muscle layers. A similar arrangement of somatic muscles was observed by Bettendorf (1897) in *F. hepatica*, and also has been demonstrated in the marine turbellarian, *M. hystricinum marinum* (Rieger *et al*. 1994). Although diagonal fibres represent a substantial portion of the subtegumental musculature, previous studies on sectioned material of *F. hepatica*, using FITC–phalloidin and actin antisera, failed to detect them within the subtegumental muscle sheath (Stitt *et al*. 1992).

From studies on small vermiform bilaterians, such as *M. hystricinum marinum*, it is assumed that the body wall musculature, together with the extracellular matrix (in particular the basal matrix), provides skeletal support to maintain body form and is used to facilitate ciliary locomotion (Rieger *et al*. 1994). A comparable arrangement of somatic muscle fibres within the body walls of *F. hepatica* and the monogenean, *Diclidophora merlangi* (Halton *et al*. 1998) indicates that they serve in both motility and in providing the overall body shape in larger platyhelminths. Also, the dorso–ventral fibres that extend between the dorsal and ventral sides of the worm may be involved in maintaining structural integrity by linking fibres of the body wall or, alternatively, the extracellular matrix. These fibres were not confined to any particular part of the body and may help maintain the flattened form that distinguishes most flatworms. In this respect, dorso–ventral fibres in *M. hystricinum marinum* are restricted to the head and tail region, which are the only parts of the animal flattened in form, the rest of the body being barrel-shaped.

Somatic musculature provides the principal means of body movements, and muscle contractions may be evoked by 5-HT and FaRPs in whole-mount (*D. merlangi*) or muscle-strip preparations (*F. hepatica*) of parasitic flatworms (Holden-Dye & Walker, 1993; Tembe *et al*. 1993; Marks *et al*. 1996; Graham,

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McGeown & Fairweather, 1997; Moneypenny *et al*. 1997). In addition, FaRPs have also been shown to affect individual (i.e. isolated) muscle fibres of *Schistosoma mansoni* and the turbellarian, *Bdelloura candida* (Day *et al*. 1994; Johnston *et al*. 1996). Pax *et al*. (1996) have raised the possibility that the 3 muscle layers present within the body wall may exhibit different functional properties. If this is the case, then the current approach to studying the physiological and pharmacological properties of somatic musculature on whole-mount or muscle-strip preparations may not be appropriate. In view of the lack of structural information for specific receptors of parasitic flatworms and, concomitantly, no information on the localization of putative FaRP, NPF and classical transmitter receptors, the interpretation of the responses recorded for tension, contraction frequency and amplitude may not reflect the actual effect of the studied compounds on single muscle fibres. For example, if all of the body wall muscles (circular, longitudinal and diagonal) responded in a similar manner to a test compound, then their overall effect on body tension would be difficult to predict as the muscles would respond antagonistically to alter worm length. Also, the differently orientated muscle fibres within the body wall may express different receptors, such that the observed effects could be a mixture of muscle responses. Moreover, the possibility of the numerous muscle fibres of the intestinal and reproductive tracts responding to FaRPs and classical transmitter molecules, and thereby contributing to observed isometric or isotonic contraction recordings, has not been taken into consideration. Observations on *F. hepatica in vitro* reveal co-ordinated peristaltic contractions of the intestinal caeca during movement of foodstuffs and in the regurgitation of undigested material. However, evidence of neuronal innervation of the caecal musculature such as by immunocytochemical staining for either neuropeptides or classical transmitters has not been reported (Magee *et al*. 1989; Marks *et al*. 1995).

Muscle fibres within the suckers were orientated in 3 directions and appeared to be derived, at least in part, from the body wall musculature. Both suckers have been shown to be extensively innervated by nerve fibres, expressing both classical and neuropeptide transmitters (Fairweather *et al*. 1987; Marks *et al*. 1995), indicating regulation of sucker function. Contractions of longitudinal (meridional) fibres serve to open and flatten the sucker against host substratum, whereas contractions of the radial fibres close the sucker into a cup-shape and, with the action of the circular (equatorial) fibres, create a suction force that draws up and secures host tissue within the sucker concavity.

The ducting of the female reproductive system (oviduct, ootype and uterus) displayed densely arranged circular fibres and only few longitudinal fibres, with the exception of the vitelline reservoir, where fibres were more diffusely arranged. Circular fibres most likely undergo peristaltic contractions and serve to transport egg material (oocytes and vitelline cells) into the ootype, and following egg formation and shaping, are responsible for the output of eggs to the uterus. The sphincter-like arrangement of circular fibres at the junction of the ootype and uterus likely provides a means of regulating the release of formed eggs into the uterus as no similar arrangements of fibres were detected elsewhere in the reproductive tract. FaRP as well as 5-HT immunoreactivities have been observed repeatedly in neuronal cells surrounding the ootype in both trematode and cestode species (Magee *et al*. 1989; Maule *et al*. 1993; Marks *et al*. 1995; Mair *et al*. 1997; Halton *et al*. 1998), suggesting a neuronal involvement in egg formation and output by regulating the motility of the musculature of the ootype and the uterus walls. Support for this comes from the immunocytochemical finding that in the monogenean, *Polystoma nearcticum* the ootype innervation displays temporal expression of FaRPs only during reproductively active periods, which are in synchrony with those of its host frog (Armstrong *et al*. 1997).

Within the male copulatory system, circular and longitudinal muscle fibres could be detected within the cirrus and the cirrus sac. The cirrus appeared highly muscularized with equally dense arrangements of circular and longitudinal muscle fibres.

Specific muscle systems have been identified within the intestine, reproductive system and adhesive organs of *F. hepatica*, and the results demonstrate the abundance and highly organized arrangement of the musculature within the worm. The data further implicate muscle in the coordination of locomotory, attachment, reproductive and digestive activities, and warrant further detailed studies of helminth musculature as a primary drug target (Thompson, Klein & Geary, 1996; Mair *et al*. 1998). Only through a better understanding of the complexity of these systems will it be possible to elucidate the basic mechanisms that regulate muscle function in parasitic flatworms.

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