

# Adult triclabendazole-resistant *Fasciola hepatica*: morphological changes in the tegument and gut following *in vivo* treatment with artemether in the rat model

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## Abstract

A study has been carried out to determine the morphological changes to the adult liver fluke, *Fasciola hepatica* after treatment *in vivo* with artemether. Rats were infected with the triclabendazole-resistant Sligo isolate of *F. hepatica*, dosed orally with artemether at a concentration of 200 mg/kg and flukes recovered at 24, 48 and 72 h post-treatment (p.t.). Surface changes were monitored by scanning electron microscopy and fine structural changes to the tegument and gut by transmission electron microscopy. Twenty-four hours p.t., the external surface showed minor disruption, in the form of mild swelling of the tegument. The tegumental syncytium and sub-tegumental tissues appeared relatively normal. Forty-eight and seventy-two hours p.t., disruption to the tegumental system increased, with isolated patches of surface blebbing and reduced production of secretory bodies by the tegumental cells being the main changes seen. The gastrodermal cells showed a relatively normal morphology 24 h p.t. By 48 h, large numbers of autophagic vacuoles and lipid droplets were present. Autophagy increased in magnitude by 72 h p.t. and substantial disruption to the granular endoplasmic reticulum was observed. Results from this study show that flukes treated *in vivo* with artemether display progressive and time-dependent alterations to the tegument and gut. Disruption to the gut was consistently and substantially more severe than that to the tegument, suggesting that an oral route of uptake for this compound predominates. This is the first study providing ultrastructural information on the effect of an artemisinin compound against liver fluke.

## Introduction

*Fasciola hepatica* is a parasite of ruminant animals prevalent in temperate regions across the world. It is a species of considerable agricultural and economic

importance, with losses resulting from infection estimated at US\$2000–3000 million annually (Boray, 1994). In addition, fascioliasis has emerged as a major zoonotic disease in many countries (Mas-Coma *et al.*, 2005), with an estimated 17 million people currently infected and up to 180 million at risk (WHO, 2007). During recent years, an upsurge in cases of fascioliasis in livestock has been noted (Mitchell, 2002), which is

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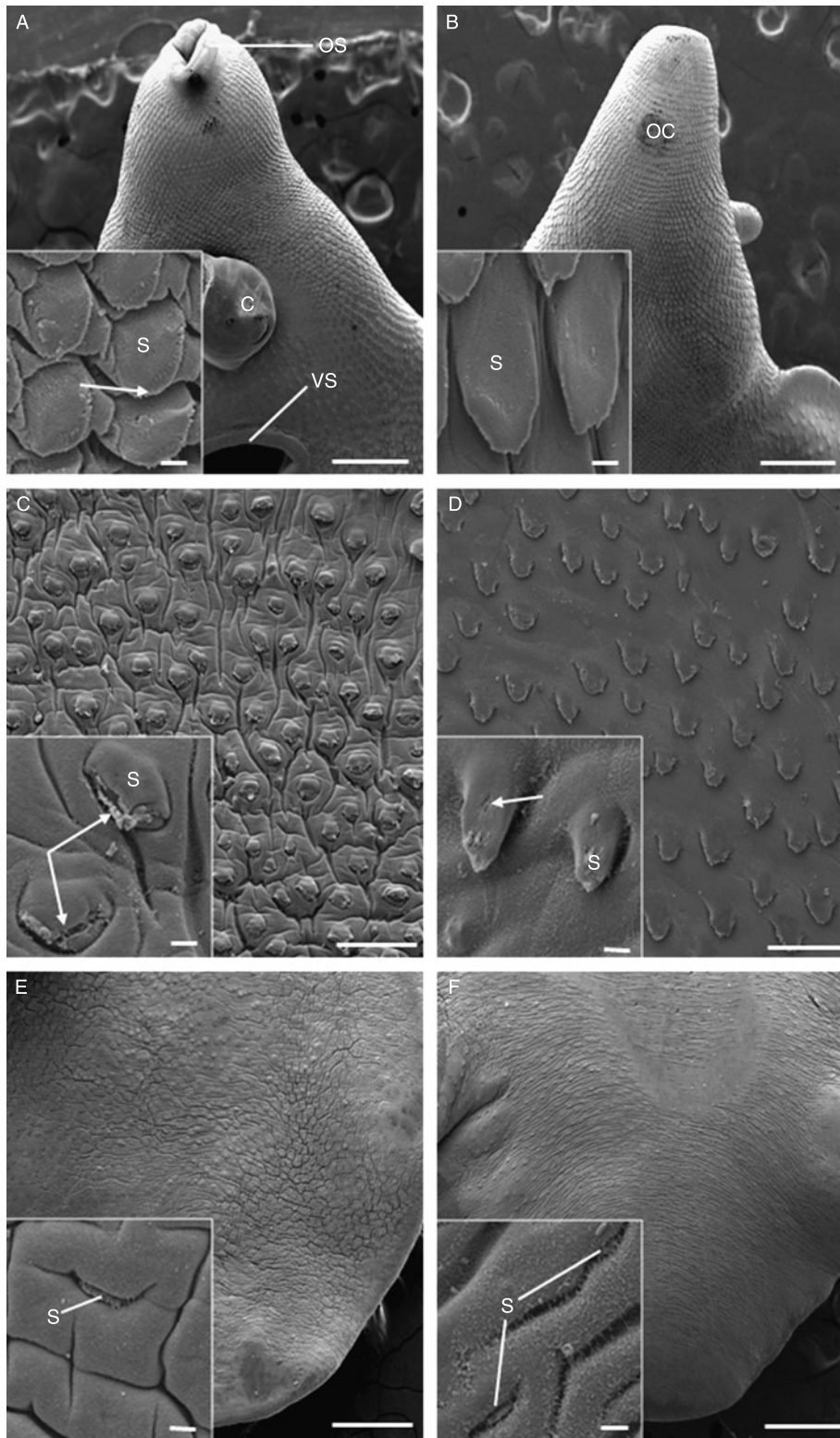


Fig. 1. A–F, Scanning electron micrographs of the tegumental surface of adult triclabendazole-resistant *Fasciola hepatica*, 24 h p.t. *in vivo* with 200 mg/kg artemether. (A) Ventral surface of the oral cone region, showing the oral sucker (OS), cirrus (C) and ventral sucker (VS). Scale bar = 500  $\mu$ m. Inset shows prominent spines (S) with apical spinelets (arrow). Scale bar = 20  $\mu$ m. (B) Dorsal surface of the oral cone

believed to be the result of climate-related changes in distribution of the intermediate snail host, *Galba truncatula* (Mitchell, 2002; Mas-Coma *et al.*, 2005). Current control of fascioliasis relies heavily on the drug of choice, triclabendazole (TCBZ) (Fairweather, 2005, 2009; Keiser *et al.*, 2005), due to its almost unique efficacy against both juvenile and mature flukes (Boray *et al.*, 1983). In the continued absence of an effective vaccine, chemotherapy will remain the mainstay of treatment for the foreseeable future.

Fluke populations demonstrating resistance to TCBZ have been well documented, with the first record on Australian farms in the mid-1990s (Overend & Bowen, 1995). Since then, resistant populations have been reported in Ireland, the Netherlands (references in Fairweather, 2005), Spain (Álvarez-Sánchez *et al.*, 2006) and much of the UK, with mounting evidence suggesting that resistance is underestimated and spreading (Wolstenholme *et al.*, 2004). The spread of resistance to the drug of choice creates a significant need for new strategies for fluke control. Such strategies should aim both at prolonging the life of currently available drugs (Wolstenholme *et al.*, 2004) as well as addressing the need for effective novel compounds to be introduced. One family of potential candidates to be considered in the search for novel flukicides is the artemisinins, derived from the wormwood plant, *Artemisia annua*. The original parent compound, *qinghaosu*, extracted in the 1960s in China as part of a co-ordinated governmental effort to uncover novel antimalarial compounds, was later named artemisinin and, since this discovery, several semi- and fully-synthetic derivatives have been developed (Woodrow *et al.*, 2005; Keiser & Utzinger, 2007). Today artemisinin derivatives and artemisinin combination treatments are the first-line drugs for uncomplicated falciparum malaria. In addition, artemisinin derivatives are known to be effective against juvenile stages of all major blood fluke species infecting humans (Utzinger *et al.*, 2007). Artemisinin-type compounds have also been shown to be effective against different food-borne trematodes such as *Opisthorchis viverrini* (Keiser *et al.*, 2006a) and *Clonorchis sinensis* in rodents (Keiser *et al.*, 2006a, 2007b). A number of artemisinin compounds have been tested against *F. hepatica*: the semi-synthetic artemether and artesunate and the fully-synthetic trioxolane derivative, OZ78. They show high efficacy against adult and juvenile flukes in the rat model (Keiser *et al.*, 2006b, c, 2007a). Recently, preliminary studies have reported promising fasciolicidal properties in sheep and even in humans (Hien *et al.*, 2008; Keiser *et al.*, 2008). Significantly, both artemether and OZ78 have shown substantial activity against the TCBZ-resistant Oberon isolate in the rat host (Keiser *et al.*, 2007a).

The aim of the current study was to evaluate morphological changes to flukes belonging to another TCBZ-resistant isolate of *F. hepatica*, the Sligo isolate, following treatment *in vivo* in the rat model with 200 mg/kg artemether. Flukes were recovered at three time-points post-treatment (p.t.) (24, 48 and 72 h), in an attempt to determine a time-scale for drug action. Morphological observations were made using scanning and transmission electron microscopy and concentrated on the tegument and gut. These tissues provide the two main surfaces across which drugs can enter the fluke and were selected in the hope of gathering information on the route of uptake of artemether by the fluke. This paper is the first to present ultrastructural information on the effect of an artemisinin compound against the liver fluke.

## Materials and methods

### Fluke material

The Sligo isolate of *Fasciola hepatica* used for the current study was first reported in 1995 in county Sligo, Ireland (Fairweather, 2005). It has been characterized as showing resistance to the action of triclabendazole *in vivo* at both adult and juvenile stages (Coles *et al.*, 2000; Coles & Stafford, 2001; McCoy *et al.*, 2005; McConville *et al.*, 2009a), as well as resistance to the TCBZ metabolite, triclabendazole sulphoxide *in vitro* (Robinson *et al.*, 2002; Mottier *et al.*, 2006a). Six male Sprague–Dawley rats under isoflurane anaesthesia were each orally infected, by means of a stomach tube, with 20 metacercarial cysts of the Sligo isolate of *F. hepatica*.

### In vivo drug treatment

Five months post-infection, rats were weighed individually and dosed orally with 200 mg/kg body weight artemether. The stock solution used comprised 1.0 g of artemether dissolved in 7% (v/v) Tween 80 and 3% (v/v) ethanol. The dose used has been shown to effect a 100% adult worm burden reduction in the TCBZ-susceptible Cullompton isolate (Keiser *et al.*, 2006b). Following dosing, flukes were recovered from the bile ducts at necropsy 24 h, 48 h and 72 h p.t. Eighteen flukes were recovered in total, each of which was washed briefly in NCTC 135 culture medium at 37°C. Zero-hour untreated specimens were prepared as controls. Specimens were prepared for scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as follows.

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region (OC). Scale bar = 100 µm. Inset shows the regular and uniform arrangement of the spines (S). Scale bar = 20 µm. (C) Ventral surface, midbody region showing the slight swelling of the interspinal tegument. Scale bar = 100 µm. Inset shows splitting of the tegument (arrows) at the tips of the spines (S). Scale bar = 20 µm. (D) Dorsal surface of the midbody region. Scale bar = 100 µm. Inset shows slight disruption (arrow) to the tegumental covering of the spines (S). Scale bar = 20 µm. (E) Ventral surface of the tail region, showing swelling of the interspinal tegument. Scale bar = 500 µm. Inset shows a spine (S) which appears to be submerged by the swollen tegument surrounding it. Scale bar = 20 µm. (F) Dorsal surface of the tail region, showing swelling of the interspinal tegument. Scale bar = 500 µm. Inset shows the roughened surface of the tegument. The spines (S) are barely visible, due to the swelling of the tegument surrounding them. Scale bar = 20 µm.



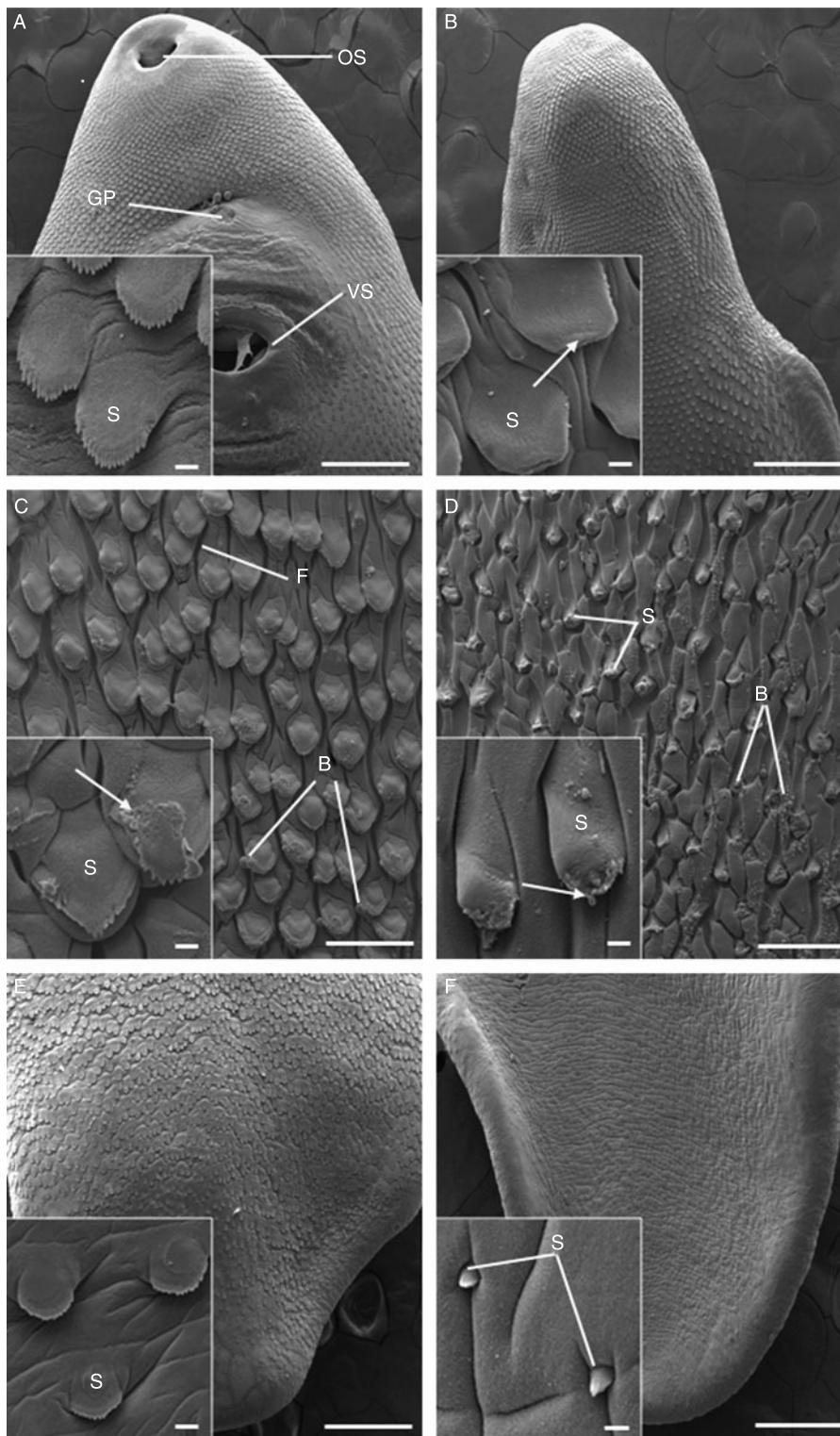


Fig. 2. A–F, Scanning electron micrographs of the tegumental surface of adult triclabendazole-resistant *Fasciola hepatica*, 72 h p.t. *in vivo* with 200 mg/kg artemether. (A) Ventral surface of the oral cone region, showing the oral sucker (OS), gonopore (GP) and ventral sucker (VS). Scale bar = 500  $\mu$ m. Inset shows well-defined spines (S) and smooth tegumental surface. Scale bar = 20  $\mu$ m. (B) Dorsal surface of the oral cone region. Scale bar = 100  $\mu$ m. Inset shows slight disruption to spines (S), as the spinelets are poorly defined (arrow).

### Tissue preparation for SEM

Flukes were lightly flat-fixed for 1 h at room temperature in 4% (w/v) glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4). They were then free-fixed for a further 3 h using fresh fixative before being post-fixed in 1% osmium tetroxide for 1 h. Specimens were then washed several times with cacodylate buffer before being dehydrated in an ascending series of ethanol. Following dehydration, flukes were dried using hexamethyldisilazane and mounted on aluminium stubs which were sputter-coated with gold-palladium. Samples were viewed using a FEI Quanta 200 scanning electron microscope operating at an accelerating voltage of 10 keV.

### Tissue preparation for TEM

Flukes were lightly flat-fixed for 1 h at room temperature in 4% (w/v) glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4). Specimens were then dissected into oral cone, midbody and tail regions, which were further split into transverse slices approximately 3 mm in thickness. These slices were free-fixed in fresh fixative for 3 h, before being washed several times in sodium cacodylate buffer. The samples were stored in buffer overnight.

Following washing, fluke samples were post-fixed for 1 h in 1% osmium tetroxide, washed in buffer and dehydrated through an ascending series of ethanol. Fluke material was then embedded using Agar 100 resin. Ultrathin sections (~60–75 nm in thickness) were cut on a Reichert Ultracut E ultramicrotome and mounted on uncoated 200-mesh copper grids. The sections were double-stained with alcoholic uranyl acetate (9 min) and aqueous lead citrate (6 min) and viewed in a FEI CM100 transmission electron microscope at 100 keV.

## Results

### Visual observations

The flukes recovered from the untreated control rats were very active and possessed full gut contents. Twenty-four hours p.t. with artemether, recovered flukes showed normal activity and gut contents were present. No difference was observed between treated and control specimens. Forty-eight hours p.t. with artemether, flukes were highly active and gut contents were retained. Seventy-two hours p.t. although still motile, the flukes appeared sluggish in their movement. Gut contents were again retained in all specimens.

### Scanning electron microscopy

The surface architecture of the control flukes appeared normal, matching the images presented by Bennett (1975) and Fairweather *et al.* (1999).

#### Twenty-four hours post-treatment

Little disruption was evident to the surface of the flukes in the oral cone region (fig. 1A and B). Tegumental spines were prominent, regularly arranged and separated by a smooth and uniform interspinal tegument (insets, fig. 1A and B). Individual spinelets were clearly visible on the spines (fig. 1A inset). The midbody region showed slight swelling of the interspinal tegument on the ventral surface (fig. 1C). At high magnification, disruption of the spinelets was observed and, in some cases, there was localized splitting of the tegument covering the spines (fig. 1C inset). In contrast, there was minimal swelling on the dorsal surface in this region, with a small number of spines showing minor disruption of their tegumental covering (fig. 1D and inset). In the tail region, the interspinal tegument was swollen both ventrally and dorsally, so that the spines appeared submerged (fig. 1E and F). On the dorsal surface, the interspinal tegument had a roughened and fibrous appearance (insets, fig. 1E and F).

#### Forty-eight hours post-treatment

For economy of space, micrographs for the 48 h treatment will not be included, but the results will be described. On examination, the surface morphology of the flukes was mostly normal, particularly in the oral cone region. The midbody region of these flukes showed isolated areas of tegumental swelling on the ventral surface, with slight tegumental furrowing. On the dorsal midbody surface there was mild swelling of the interspinal tegument. In the tail region, flukes exhibited substantial swelling of the interspinal tegument, causing the spines to appear submerged by the tegument surrounding them. This occurred to a similar extent on both surfaces and was greater than that observed in the 24 h-treated specimens.

#### Seventy-two hours post-treatment

Specimens recovered 72 h after dosing displayed no disruption to the ventral surface in the oral cone region (fig. 2A and inset). In contrast, the dorsal surface displayed a lack of spinelet definition due to minor swelling of the tegument covering the spines (fig. 2B and inset). In the midbody region, on both surfaces, swelling of the interspinal tegument was observed. On the ventral

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Scale bar = 20  $\mu$ m. (C) Ventral surface of the midbody region, showing substantial disruption to the interspinal tegument which is furrowed (F) and bears blebs (B). Scale bar = 100  $\mu$ m. Inset shows the swelling of the interspinal tegument, as well as the loss of tegument (arrows) on one of the spines (S). Scale bar = 20  $\mu$ m. (D) Dorsal surface of the midbody region, demonstrating evidence of more widespread blebbing (B) and swelling to the tegument. S, spines. Scale bar = 100  $\mu$ m. Inset shows blebs (arrowed) forming at the tip of a spine (S). Scale bar = 20  $\mu$ m. (E) Ventral surface of the tail region. Scale bar = 500  $\mu$ m. Inset shows normal spine morphology (S), and only a slight swelling of the interspinal tegument is evident. Scale bar = 20  $\mu$ m. (F) Dorsal surface of the tail region. Scale bar = 500  $\mu$ m. Inset shows swelling of the interspinal tegument with the spines (S) appearing to be submerged by the surrounding tegument. Scale bar = 20  $\mu$ m.

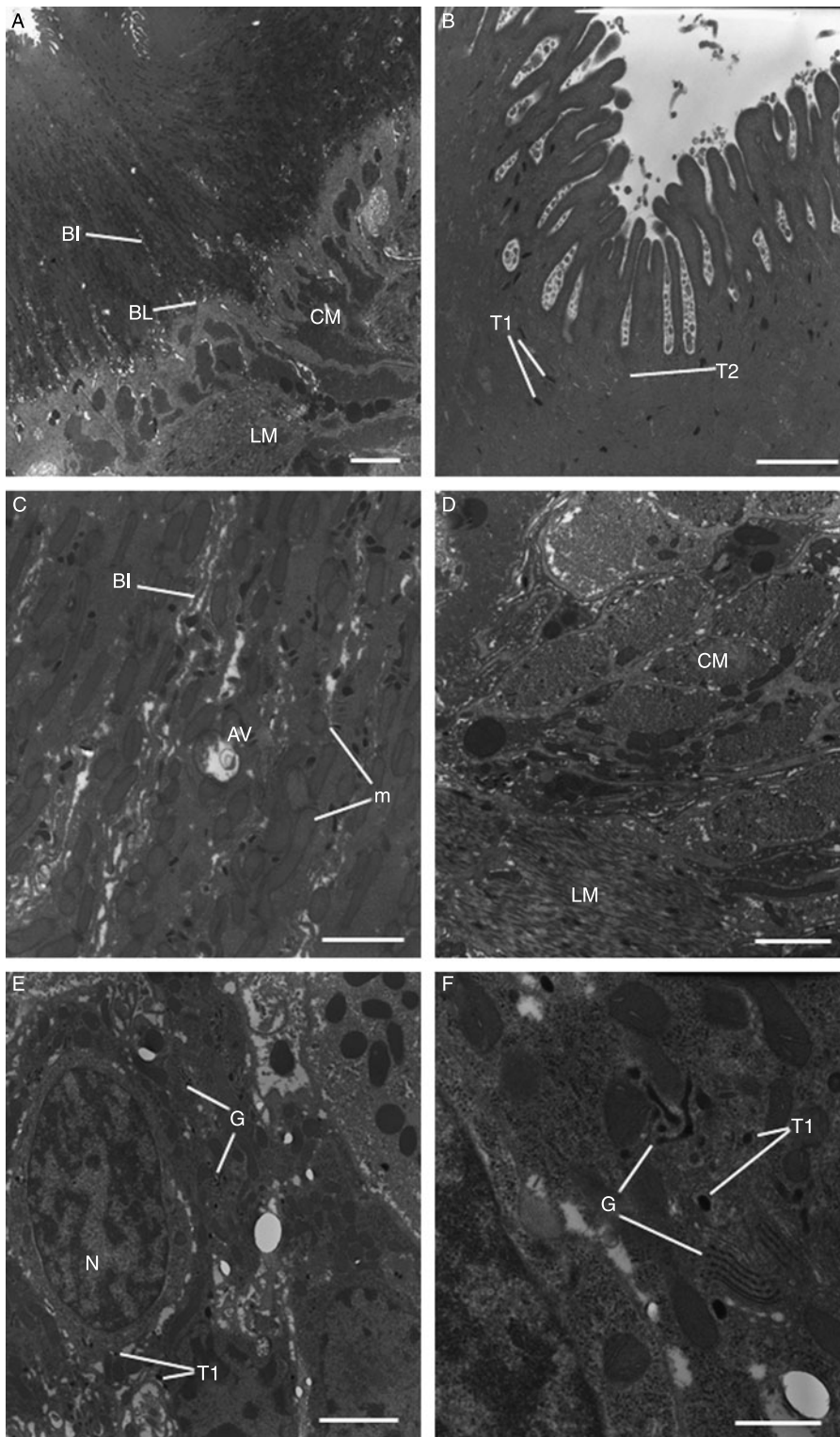


Fig. 3. A–F, Transmission electron micrographs of the tegument and sub-tegumental tissues of adult triclobandazole-resistant *Fasciola hepatica*, 24 h p.t. *in vivo* with 200 mg/kg artemether. (A) Tegument and sub-tegumental region, showing the full depth of the syncytium. The basal infolds (BI) and basal lamina (BL) retain a normal morphology. The circular (CM) and longitudinal (LM) muscle bundles also appear normal. Scale bar = 3  $\mu$ m. (B) A high magnification micrograph of the apical region of the tegument. Type 1 (T1) and type 2 (T2)



surface, this resulted in furrowing of the tegument and some isolated spines showed a partial loss of their tegumental covering (fig. 2C and inset). Swelling of the interspinal tegument on the dorsal midbody surface (fig. 2D) was coupled with disruption to the spine tips, as evidenced by the loss of spinelets (fig. 2D inset). On both dorsal and ventral surfaces there was a scattering of small blebs (fig. 2C and D and inset), although this was more evident on the dorsal surface (fig. 2D). The tail region of 72 h-treated specimens appeared reasonably unaffected, with only the dorsal surface displaying significant swelling of the interspinal tegument, so that the spines appeared to be partially submerged (fig. 2E and F).

#### *Transmission electron microscopy: tegument and sub-tegument*

The ultrastructure of the tegumental system in the control specimens remained normal, as described by Threadgold (1963, 1967) and Fairweather *et al.* (1999).

#### *Twenty-four hours post-treatment*

The tegumental syncytium of flukes appeared normal, with concentrations of tegumental secretory bodies at the apex of the syncytium (fig. 3A and B). Slight swelling of the mitochondria was visible and a few autophagic vacuoles were present (fig. 3C). The basal infolds appeared normal (fig. 3A and C) and the sub-tegumental musculature (both circular and longitudinal) retained a normal morphology (fig. 3D). Type 1 and type 2 tegumental cell bodies contained normal numbers of their secretory bodies (fig. 3E). At higher magnification, a number of Golgi complexes were observed (fig. 3F), many of which possessed dilated and irregularly stacked cisternae.

#### *Forty-eight hours post-treatment*

Again, no micrographs of this time period will be included. The tegumental syncytium of flukes recovered 48 h p.t. with artemether appeared normal, with the basal infolds and sub-syncytial basal lamina retaining a normal morphology. The mitochondria appeared swollen in a number of specimens, although no evidence of autophagy was observed. Tegumental secretory bodies were present in normal numbers and distribution within the syncytium. The sub-tegumental musculature appeared normal throughout, although the cytoplasmic connections from the tegumental cells that run between the muscle blocks appeared swollen and distended. In the tegumental cell bodies, a large proportion of the Golgi complexes contained dilated and irregularly stacked cisternae, although there were normal numbers of secretory bodies in the cells.

#### *Seventy-two hours post-treatment*

Flukes recovered from rats 72 h p.t. with artemether displayed changes to the tegumental syncytium and underlying tissues. In isolated areas of the syncytium, for example, there was substantial swelling of the mucopolysaccharide masses surrounding the basal infolds, although the basal infolds themselves appeared normal (fig. 4A). Both types of tegumental secretory body were present in normal numbers and distribution throughout the syncytium with a concentration at the apex (fig. 4B). Gross swelling and distension of the cytoplasmic connections was observed (fig. 4C and D), although the muscle bundles appeared normal. Tegumental cell bodies contained numerous Golgi complexes with irregularly stacked and dilated cisternae, together with swollen mitochondria (fig. 4F). The number of type 2 tegumental secretory bodies present in the cells appeared normal (fig. 4E), whereas there were few type 1 secretory bodies.

#### *Transmission electron microscopy: gastrodermal cells*

The gastrodermal cells of the control specimens retained a normal morphology similar to that described by Robinson & Threadgold (1975).

#### *Twenty-four hours post-treatment*

Twenty-four hours p.t. with artemether, the gastrodermal cells appeared relatively normal (fig. 5A). The number of secretory vesicles in the cells was normal, but they appeared somewhat elongated and irregularly shaped. The cisternae of the granular endoplasmic reticulum were swollen (fig. 5B(I) and B(II)).

#### *Forty-eight hours post-treatment*

Forty-eight hours p.t. large numbers of autophagic vacuoles and lipid droplets were observed in the cells (fig. 5C and D). The number of secretory vesicles present appeared normal, although they were small and oddly shaped. The cisternae of the granular endoplasmic reticulum were dilated (fig. 5D).

#### *Seventy-two hours post-treatment*

Specimens viewed 72 h p.t. with artemether showed a substantial increase in the level of autophagy and large lipid droplets were present throughout the tissue (fig. 5E). Dilation of the cisternae of the granular endoplasmic reticulum was visible, even at low magnification (fig. 5E). There were few secretory vesicles in the cells and they appeared small and irregularly shaped (fig. 5F).

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tegumental secretory bodies are present in normal numbers. Scale bar = 1  $\mu\text{m}$ . (C) High-power image of the syncytium showing the presence of autophagic vacuoles (AV), as well as slight distension of the mitochondria (m). The basal infolds (BI) appear normal. Scale bar = 1  $\mu\text{m}$ . (D) The circular (CM) and longitudinal (LM) muscle bundles appear normal. Scale bar = 2  $\mu\text{m}$ . (E) Type 1 tegumental cell body, containing a Golgi complex (G) and the nucleus (N). T1, type 1 secretory body. Scale bar = 2  $\mu\text{m}$ . (F) High magnification view of the Golgi complex (G) within a type 1 tegumental cell body. The complex appears abnormal, with swelling and distension of its cisternae. T1, type 1 secretory body. Scale bar = 1  $\mu\text{m}$ .

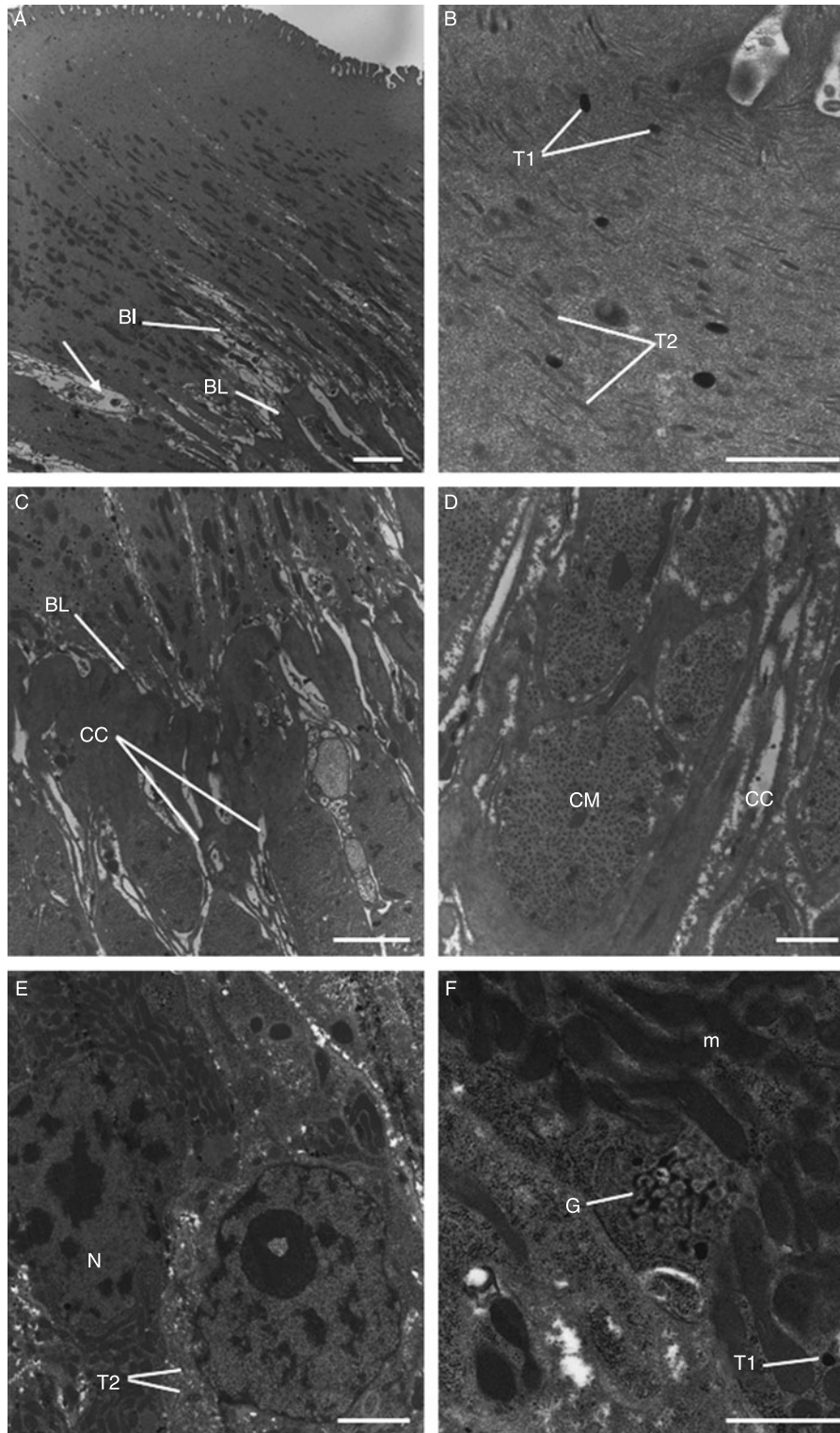


Fig. 4. A–F, Transmission electron micrographs of the tegument and sub-tegumental tissues of adult triclabendazole-resistant *Fasciola hepatica*, 72 h p.t. *in vivo* with 200 mg/kg artemether. (A) Tegument and sub-tegumental region, showing the basal infolds (BI) and basal lamina (BL), which are normal. The mucopolysaccharide masses surrounding the basal infolds appear swollen and distended (arrow). Scale bar = 3  $\mu$ m. (B) High-power micrograph of the apical region of the tegument, showing the presence of normal numbers of type 1



## Discussion

In the present study, changes to the tegumental system, the underlying musculature and gastrodermal cells were examined over a 72-h period, to monitor the response of the TCBZ-resistant fluke to treatment *in vivo* with artemether. While disruption to the tegument was relatively mild, the disruption to the gut became increasingly severe with time, with accumulations of lipid and extensive autophagic breakdown of the gastrodermal cells. The results will be discussed in terms of the time-course of drug action and the route of uptake of artemether.

The changes to the tegumental surface were relatively mild, not progressing beyond swelling and areas of scattered blebbing. There was no spine loss or tegumental sloughing, although there was some disruption and splitting of the tegumental covering of the spines. In previous SEM studies on *F. hepatica* involving artemisinins, instances of more severe blebbing, also spine loss and even sloughing have been described (Keiser *et al.*, 2006b, c; Keiser & Morson, 2008a, b). Whether this is due to a different response of this particular isolate of *F. hepatica* to artemether is not known, although other studies on the fluke have shown that different isolates respond in different ways to the same drug (Walker *et al.*, 2004; McKinstry *et al.*, 2007, 2009). That is, while the changes seen are similar, the extent of those changes or degree of disruption differs, so that the sensitivity of an isolate to a particular drug varies. The present result may be related to the TCBZ-resistant status of the Sligo isolate, but this point needs to be examined further, with more detailed analysis of the efficacy of artemisinin compounds against different isolates of *F. hepatica*.

More dramatic changes to the fluke surface stemming from *in vivo* treatment have also been observed in studies with other flukicides: e.g. closantel (Skuce & Fairweather, 1990), clorsulon (Meaney *et al.*, 2003, 2006), nitroxylin (McKinstry *et al.*, 2003), TCBZ (Halferty *et al.*, 2008) and the TCBZ derivative, compound alpha (McConville *et al.*, 2008). The lack of serious disruption to the surface morphology of the fluke was reflected in the limited internal changes to the tegumental system observed by transmission electron microscopy. Transport of tegumental secretory bodies continued, as evidenced by the numbers of secretory bodies in the syncytium although, by 72 h p.t., production of type 1 secretory bodies had declined and there were few secretory bodies in the cells. This decline in production coincided with increasing disruption of the Golgi complexes responsible for their packaging. In contrast, production of type 2 secretory bodies remained normal and numbers present in the cells remained high.

The tegumental secretory bodies are known to be responsible for the maintenance of the apical tegumental membrane (Fairweather *et al.*, 1999), so ultimately their decline would lead to severe disruption of the syncytium: that point was not reached at 72 h p.t.

The results provide some idea of the time-scale of drug action. For example, the reduction in motility that is evident at 72 h p.t. This will compound the autophagic disruption of the gut cells observed from 48 h p.t. onwards, as the fluke is unable to feed when it becomes inactive and essentially enters a state of starvation. The internal changes triggered by the degeneration of the gut are not manifested directly in any marked changes to the tegumental system because, although progressive changes to this tissue and to the external surface of the fluke were observed, they were not very severe. So, drug action is relatively slow, despite the relatively rapid attainment of peak plasma levels and the short half-life of artemether (Lefèvre & Thomsen, 1999). Established pharmacokinetic data for artemether, detailing a low bioavailability when administered orally (19% in rats: Li *et al.*, 1998), suggests that artemether undergoes extensive first-pass metabolism (Navaratnam *et al.*, 2000). The delayed drug effect observed in this study is consistent with observations made in previous studies on *F. hepatica* following treatment with artemether, artesunate and OZ78. In those studies, the death and removal of flukes was seen to take 72–96 h to become apparent (Keiser *et al.*, 2006b; Keiser & Morson, 2008a, b). The apparent contradiction between drug peaks, drug half-life and drug action has been seen in other drug studies on *F. hepatica* (e.g. Meaney *et al.*, 2003; McConville *et al.*, 2008, 2009b). For these drugs, which are relatively slow-acting, it may take some time before their action (and the morphological changes they induce) to become apparent; however, the effects are seemingly irreversible, as the drug continues to act following its elimination from the host. In contrast, the morphological changes induced by TCBZ more closely match its pharmacokinetics in the host (Halferty *et al.*, 2008) and the data on nitroxylin fit this pattern, too (McKinstry *et al.*, 2003, 2007).

The gut was more severely affected than the tegument at all time-points p.t. and the disruption became increasingly severe with longer treatment times. There was a progressive build-up of lipid droplets, disruption of the granular endoplasmic reticulum cisternae and extensive autophagy of the gastrodermal cells. The secretory activity of the cells was progressively affected over time, so that by 72 h p.t. few secretory vesicles were present. This would compromise the ability of the fluke to feed, compounding the effect of starvation referred to previously. The presence of lipid droplets is an indication of metabolic disruption in the cells, and the cells themselves appeared to be undergoing breakdown, as

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(T1) and type 2 (T2) tegumental secretory bodies. Scale bar = 2  $\mu\text{m}$ . (C) The sub-syncytial cytoplasmic connections (cc) appear swollen and distended. BL, basal lamina. Scale bar = 2  $\mu\text{m}$ . (D) The circular muscle (CM) bundles appear normal, although the surrounding cytoplasmic connections (cc) are swollen. Scale bar = 1  $\mu\text{m}$ . (E) A type 2 tegumental cell body, containing type 2 secretory bodies (T2) and a nucleus (N). Scale bar = 2  $\mu\text{m}$ . (F) High magnification image of swollen mitochondria (m) and a disrupted Golgi complex (G) within a type 1 tegumental cell body. The Golgi complex possesses dilated and irregular cisternae. T1, type 1 secretory body. Scale bar = 1  $\mu\text{m}$ .

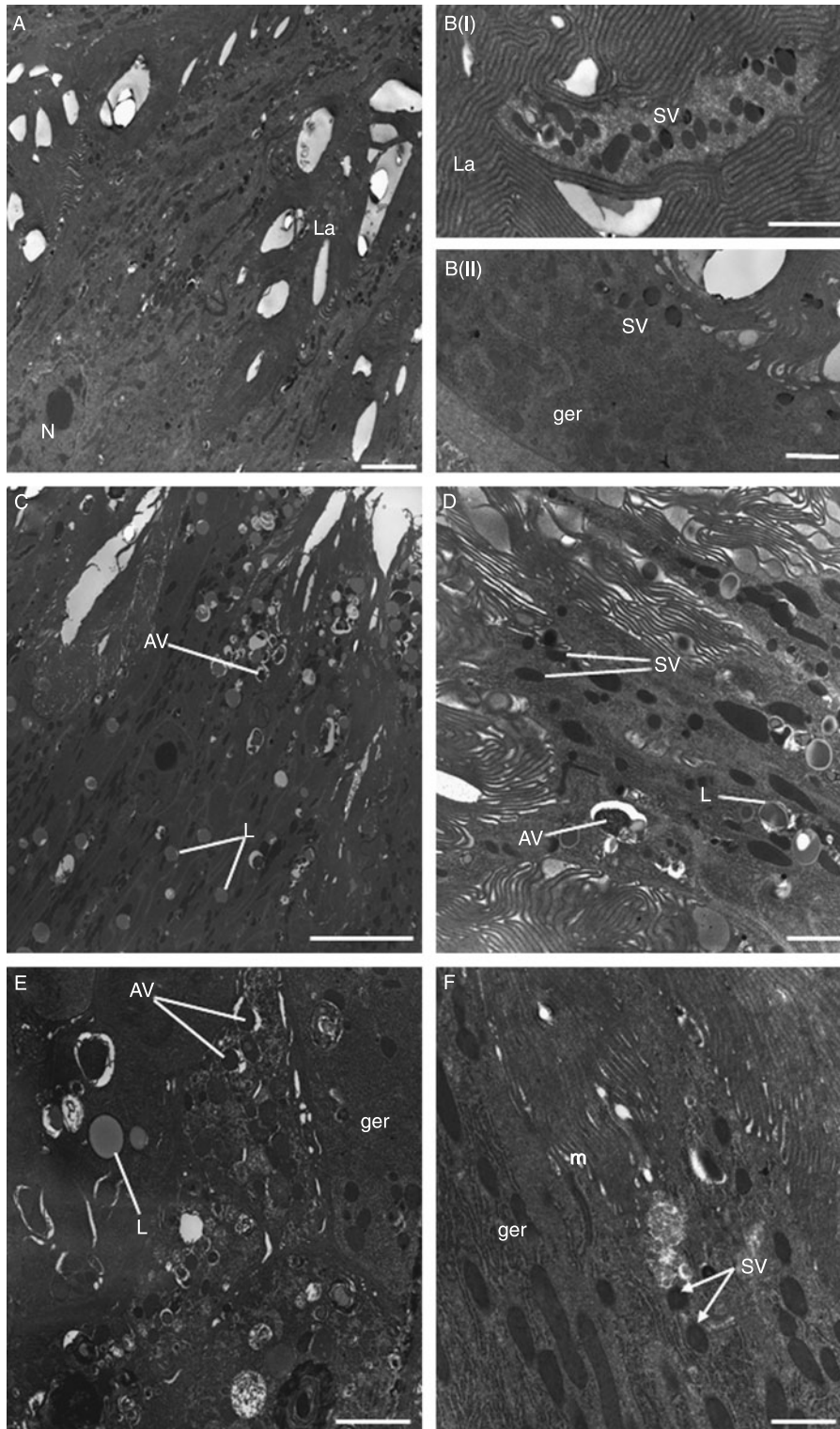


Fig. 5. Transmission electron micrographs of gastrodermal cells from adult tricloabendazole-resistant *Fasciola hepatica* 24 h (A and B), 48 h (C and D) and 72 h (E and F) p.t. *in vivo* with 200 mg/kg artemether. (A) Gastrodermis showing an epithelial cell containing a nucleus (N). Lamellae (La) project from the cell surface into the lumen. Scale bar = 3  $\mu$ m. (B(I)) High magnification image of secretory vesicles (SV) in the apical region of a gastrodermal cell. Lamellae (La) project from the surface of the cell. Scale bar = 1  $\mu$ m. (B(II)) High magnification

evidenced by the presence of autophagic vacuoles. Comparing the results for the tegument and gut, it seems most likely that oral uptake is the predominant route of entry for artemether, rather than trans-tegumental diffusion. This ties in with what is known about drug entry and activation in schistosomes. For these parasites, it has been suggested that the artemisinins need to be ingested by the flukes in order for drug activation to occur (Xiao *et al.*, 2003). Drug activation depends on the presence of an iron-containing compound, as would be derived from haemoglobin in the blood, and this leads to cleavage of the peroxide bridge in the drug molecule and the generation of free radicals. It is these free radicals which are damaging to the parasite (Xiao *et al.*, 2003). A similar mechanism may operate in *F. hepatica*, because of its haematophagous feeding behaviour. In support of this idea, greater disruption to the tegument of the liver fluke was observed *in vitro* when haemin was incorporated into the culture medium (Keiser & Morson, 2008a, b). Entry of an artemisinin compound across the tegument would not allow the interaction between the drug and an iron compound, and this would lead to a reduction in its activity. Oral uptake of drug is also the predominant mechanism for the fasciolicides, clorsulon and nitroxylin (Meaney *et al.*, 2005a, b, c; McKinstry *et al.*, 2007). It is not a universal phenomenon among fasciolicides, however, because entry of TCBZ has been shown to take place principally across the tegument (Mottier *et al.*, 2006b; Toner *et al.*, 2009). So, for artemether, its activation and efficacy are dependent on oral intake from the blood by the fluke, rather than relying on trans-tegumental diffusion as the drug is being excreted in the bile.

Some correlation can be made between the changes seen and the known action of artemisinins, via the production of free radicals. For example, treatment with artemisinins and free radicals causes collapse of the membrane potential of mitochondria, leading to their swelling and inhibition of electron transfer and oxidative phosphorylation (Dubin & Stoppani, 2000; Wakabayashi & Karbowski, 2001; Li *et al.*, 2005). This action could explain the mitochondrial changes seen and would have a significant impact on energy production by the fluke, contributing to the state of general ill-health. Such changes are also known to be part of the process leading to cell death, or apoptosis (Dubin & Stoppani, 2000; Wakabayashi & Karbowski, 2001; Li *et al.*, 2005). Furthermore, artemisinins and free radicals are known to cause the oxidation of membrane proteins, such as ion pumps (Rohn *et al.*, 1996; Berman & Adams, 1997; Mason *et al.*, 1997; Sumegi *et al.*, 2005). One target of artemisinin action in malarial parasites is the sarco(endo)plasmic reticulum  $\text{Ca}^{2+}$ -ATPase, which regulates calcium levels within the parasite (Eckstein-Ludwig *et al.*, 2003; Jung

*et al.*, 2005; Uhlemann *et al.*, 2007). The swelling of the granular endoplasmic reticulum cisternae seen in the present study could be due to inhibition of pumps associated with their membranes. It would severely affect the synthetic activity of the cells.

This study has shown that artemether treatment of a TCBZ-resistant isolate of *F. hepatica* induces progressive changes to the morphology of the fluke and that the changes are more severe in the gastrodermal cells than the tegument. This observation suggests that the drug enters the fluke via the oral route, but this idea could be tested further in experiments involving ligatures (to block the oral route) and the incorporation of haemin in the incubation medium (to activate the drug); similar experiments have been carried out previously on fluke (Meaney *et al.*, 2005a, b, c; Mottier *et al.*, 2006b; Keiser & Morson, 2008a, b; Toner *et al.*, 2009). In addition to the severe disruption of the gut, artemether causes considerable disruption to the reproductive tissues (vitelline cells and testes) and this will be published in a separate communication.

### Acknowledgements

This study was supported by a postgraduate studentship awarded to James O'Neill by the Department of Agriculture and Rural Development, Northern Ireland (DARDNI). J. Keiser (project no. PPOOA-114941) is grateful to the Swiss National Science Foundation for financial support.

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image of a gastrodermal cell containing secretory bodies (SV), as well as dilated cisternae of the granular endoplasmic reticulum (ger). Scale bar = 1  $\mu\text{m}$ . (C) Gastrodermis showing the presence of lipid droplets (L) throughout the epithelium. Numerous autophagic vacuoles (AV) are present. Scale bar = 10  $\mu\text{m}$ . (D) High-power micrograph showing the irregular shape of the secretory vesicles (SV), as well as the presence of numerous autophagic vacuoles (AV) and lipid droplets (L). Scale bar = 2  $\mu\text{m}$ . (E) Large lipid droplets (L) are present in the gastrodermis, as well as numerous autophagic vacuoles (AV). The cisternae of the granular endoplasmic reticulum (ger) appear dilated. Scale bar = 2  $\mu\text{m}$ . (F) High magnification image showing the irregular shape of secretory vesicles (SV), as well as distension of the cisternae of the granular endoplasmic reticulum (ger). Scale bar = 2  $\mu\text{m}$ .



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(Accepted 16 March 2009)

First Published Online 16 April 2009

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