The effect of a parenteral ivermectin/closantel injection on the growth and reproductive development of early immature *Fasciola hepatica* in cattle

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**Abstract**

Sixteen calves approximately 6 months old were each infected with 500 metacercariae of *Fasciola hepatica*. Thirty-two days later they were weighed and divided into two groups, and on day 35 all calves in one of the groups were injected subcutaneously with an ivermectin/closantel combination. Both groups were sacrificed between days 70 and 72 to enable counting and examination of the flukes recovered from the bile ducts. Eggs released by the flukes were collected for incubation, hatching and estimation of egg viability. Flukes were counted, flat-fixed in 70% ethanol, stained with catechol and carmine and measured. The reproductive organs, namely testis, vitelline glands, ovary and uterus, were examined and scored on a 0–3 scale according to their state of development. This was visually assessed on the basis of size, distribution and staining density of their constituent tissues and the abundance of eggs in the uterus. A representative sample of flukes from each animal was fixed in formalin for histological sectioning to enable more detailed examination of the reproductive structures.

Treatment of the immature flukes reduced the population in cattle by 42.6% as compared with the controls and as a result of the stunting effect due to the presence of closantel during early development the size of treated flukes was reduced by 43.9%. A bimodal pattern of size and reproductive score was also observed in flukes from treated cattle, suggesting that the stunting effect on individual flukes differed depending on whether or not they had gained access to the bile ducts or were still migrating in the hepatic parenchymal tissue at the time of drug exposure with the effect being greater once the fluke had gained access to the bile ducts. The mean reproductive score for untreated flukes was 8.76 and for treated flukes 5.64, a 35.6% reduction. This difference was highly significant (\( p < 0.001 \)). Egg shedding from treated flukes was significantly less than that from controls (\( p < 0.05 \)), but there were no differences in hatchability, suggesting that whilst drug treatment reduced the energy supply available for gametogenesis/oogenesis, it did not induce functional defects in the gonads or accessory reproductive organs.

Histological examination confirmed that there was a reduction in development of testes, ovaries and vitellaria in treated flukes, with a consequent reduction in egg production. In the treated flukes, early spermatogonia and oogonia were the predominant cell types in the testes and ovary, whilst undifferentiated stem cells were abundant in the vitelline follicles. In untreated flukes, cells representing more advanced stages in gametogenesis and vitellogenesis predominated in the respective organs. It is likely that this inhibition of gametogenesis and vitellogenesis was caused by the effects of closantel treatment on intermediary metabolism in the flukes. Clearly these effects were evident even at a relatively early stage of
fluke growth, and because of the impact on egg output may have epidemiological importance in addition to the reduction in fluke numbers.

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1. Introduction

Closantel is a salicylanilate anthelmintic that binds extensively to plasma albumin (Michiels et al., 1987). As a result its activity is mainly directed against blood-feeding internal parasites such as Fasciola hepatica, Haemonchus contortus, Oestrus ovis and Oesophagostomum larvae. It has been combined with the macrocyclic lactone anthelmintic, ivermectin, in a novel combination in an injectable formulation. The resultant pharmacokinetic and pharmacodynamic properties result in efficacy against a wide range of internal and external parasites in cattle (Cromie et al., 2006; Borgsteede et al., 2006). Previous studies on the effects of closantel on the ultrastructure of Fasciola hepatica in sheep have been reported by Verhayen et al. (1980). They observed changes in the morphology of absorptive tissues in the fluke intestine. Later damage to reproductive organs was also noted. Maes et al. (1988) in experiments with both sheep and rats indicated that as well as removing a high percentage of adult flukes and to a lesser extent immature flukes, the size of any flukes surviving in treated animals was stunted and was much less than in the control animals or in those treated with triclabendazole. Closantel is much less effective on young immature flukes than on adults and late immature flukes that have reached the bile ducts (Boray, 1997; Fairweather et al., 1998). These effects have also been examined by Pax et al. (1989) who postulated that they were due to closantel’s ability to interfere with the proton gradient in parasite mitochondria, giving rise to subsequent effects on intrategumental pH and mobility. Skuce and Fairweather (1990) continued such studies and observed the effects on in vitro mobility and fluke ultrastructure especially those affecting the cells associated with the structure of the tegument. The study described in this paper is a more detailed report on the effect of a parenteral closantel/ivermectin combination on the size, reproductive organ development, egg production and egg hatching of experimental F. hepatica infections in cattle.

2. Materials and methods

All experiments were approved by the Animal Use Committee of Norbrook Laboratories Limited.

Two groups of eight cattle were used. All were Friesian x male and approximately 6 months of age. Although they had never grazed on pasture, faeces samples were taken to ensure that they were not excreting any helminth eggs and therefore unlikely to be infected. On day −35 all were weighed and infected with 500 F. hepatica metacercariae (Compton Paddock Laboratories, Newbury, Berkshire, UK) in gelatin capsules. The metacercariae were of a strain not exposed to anthelmintics and known to be susceptible to all flukicides. On day −3 all cattle were weighed and the dose of ivermectin/closantel to be administered was calculated for those selected for treatment. After weighing they were randomly assigned to treatment groups A and B according to descending weight. Those of identical weight were further allocated by descending ear tag number. On day 0 Group A were injected subcutaneously in the neck with the ivermectin/closantel injection at a dose rate of 200 \( \mu g \) kg\(^{-1}\) of ivermectin and 5 mg kg\(^{-1}\) of closantel. Group B remained as untreated controls. Blood samples were taken from Group A on days +7 and +14 to monitor closantel plasma concentrations. All animals were humanely slaughtered by exsanguination following stunning with a captive bolt on days 70–72 of the study.

2.1. Preparation of flukes for staining and histology

Immediately after slaughter the liver of each animal was removed and all flukes present in bile ducts were removed manually and placed in normal saline at 37 °C until collection from the specific liver was complete. Flukes were then counted and arranged on the bottom of a 20 cm square Perspex tray. When all the flukes from a particular liver had been satisfactorily spaced on the tray, a square of 6 mm thick plate glass, cut to fit loosely into the Perspex tray, was applied over the flukes. The weight of this plate was sufficient to flatten the flukes to uniform thickness. The tray was flooded with 70% ethanol that flowed around the flukes from the sides of the glass plate and from a 1 cm diameter hole that had been drilled in the center of the plate.

The flukes were left in the tray to flat-fix overnight. The glass plate was then lifted and the flukes were gently dislodged from the base of the tray and the
underside of the plate. They were stored in 70% ethanol to await further processing.

Ten flukes were selected randomly from each population, placed in a 10 cm square plastic petri dish and flat-fixed under a 2 mm thick glass plate using 10% neutral buffered formalin. After flat-fixing overnight, these flukes were stored in 10% neutral buffered formalin to await preparation for histological sectioning.

2.2. Assessment of egg viability

After removal of the flukes for fixation, the container in which each batch had been held prior to fixation was allowed to stand for 10 min to enable the eggs that had been shed to settle to the bottom. The saline was then gently decanted, leaving the fluke eggs as sediment at the bottom. The eggs were washed by sedimentation from several changes of dechlorinated tap water, and a comparative estimate of the abundance of eggs shed by each batch of flukes was recorded as a score of 1 (fewest eggs) to 6 (most eggs).

Approximately 1500–2000 eggs from each batch of flukes were then resuspended in 50 ml of tap water in 300 ml plastic honey jars with loosely applied metal lids, and stored in darkness at 22 °C for 15 days. After this period the jars were brought into full daylight and 50 ml of chilled tap water (10 °C) was added to each.

Two hours after exposure of the eggs to daylight, 100 ml of 10% neutral buffered formalin was added to each jar and the hatched and unhatched eggs were allowed to sediment. Sub-samples of this sediment were examined using a dissecting microscope, and approximately 500 eggs from each batch were scored as ‘hatched’ or ‘unhatched’. The proportion of hatched eggs was recorded in an Excel spreadsheet as a ‘hatched’ or ‘unhatched’. The proportion of hatched eggs was recorded in an Excel spreadsheet as a 'hatched' or 'unhatched'. The proportion of hatched eggs was recorded in an Excel spreadsheet as a 'hatched' or 'unhatched'.

2.3. Preparation and examination of stained whole-mounts

Flukes that had been fixed with 70% ethanol were incubated overnight at 22 °C in 0.1% catechol dissolved in 35% ethanol. Following a rinse in 35% ethanol they were transferred to borax carmine solution (2% sodium tetraborate with 1.5% carmine in 35% ethanol) and left to stain for 24 h.

The flukes were then rinsed with 35% ethanol and transferred to 70% acidified ethanol (1 ml of concentrated HCl in 500 ml of 70% ethanol) to destain. The destaining process was carefully observed, with frequent changes of acidified ethanol, until the flukes just retained an overall pink colour. They were then rinsed in non-acidified 70% ethanol and transferred through two 3-h changes of absolute ethanol to xylene.

The stained flukes were placed in xylene in a glass petri dish, and examined using a low-power dissecting microscope with sub-stage illumination. The length and maximum width of each fluke was measured against a transparent 2 mm grid attached to the microscope stage. Since the true size of any fluke is better represented by its surface area (bearing in mind that the flukes were flattened to uniform thickness) than by either the length or the width alone, the product of length and width (proportional to the surface area) was used in subsequent comparisons and calculations.

The testes, vitelline tissue, uterus and ovary of each were examined and a score 0–3 was allocated to each reproductive structure using the following criteria:

2.3.1. Testis

Representation and thickness of the dendritic tubules comprising the testicular tissue in the posterio-medial region of the body. In flukes given a score of 3 for testis development, the dendritic tubules were well defined and turgid throughout the posterio-medial field (Fig. 1), whilst in those with a testis score of 2 or 1 the tubules were less defined and at least in some areas appeared shrunken or collapsed (Figs. 2 and 3). Those flukes in which the testis tubules could not be distinguished from the ramifying gut caeca were given a score of 0 (Fig. 4).

2.3.2. Vitellaria

Distribution and density of catechol-induced tanning in the posterior and posterio-lateral regions of the body. Catechol acts as a substrate for phenol oxidase, an enzyme associated with the shell precursor proteins in mature vitelline cells and involved in the quinone-tanning process during maturation of the eggs in the uterus. The intensity and distribution of dark brown staining in the posterior and posterio-lateral regions of flukes incubated in catechol was taken to reflect the abundance and localisation of mature cells in the vitelline tissue. Those flukes given a score of 3 showed dense staining throughout the vitelline field, ootype and uterus (Fig. 1). In some cases the intensity of staining was reduced (Fig. 2) or patchy in distribution (Fig. 3). These flukes were scored 1 or 2 for vitelline development. The smallest flukes often showed no evidence of catechol tanning, and these were given a score of 0 (Fig. 4).

2.3.3. Uterus

Presence and abundance of tanned eggs immediately posterior to the acetabulum. In those individuals given a...
score of 3 for the uterus, the antero-medial area of the body was occupied by a thick, extensively coiled uterine tube packed with tanned eggs (Fig. 1). Flukes in which the uterine tube was narrower containing fewer eggs, were given a score of 2 (Fig. 2). If only a few tanned eggs were evident a score of 1 was considered appropriate (Fig. 3), whilst flukes with no eggs in the uterine area were given a score of 0 (Fig. 4).

2.3.4. Ovary

Representation and thickness of the dendritic tubules comprising the ovarian tissue that lies in a pre-testicular position to the observer’s left of the uterus (with the acetabulum facing the observer). The ovary in those flukes showing maximum development (score 3) was a clearly defined dendritic structure, in which the individual tubules were relatively thick and densely stained with carmine (Fig. 1). Flukes in which the ovarian tubules were thinner and less well defined were scored 2 or 1 (Figs. 2 and 4). If the ovarian tubules could not be distinguished clearly from local ramifications of the gut caeca, a score of 0 was considered appropriate (Fig. 3).

The size measurements and the individual scores for the reproductive structures for each fluke were recorded in an Excel spreadsheet to facilitate subsequent statistical analysis. The ‘reproductive score’ for each fluke was the sum of the individual scores for testis, vitellaria, uterus and ovary (maximum score possible = 12). The ‘size index’ was the product of the length and width measurements.

2.4. Preparation and examination of histological sections

The purpose of histological examination of the individual reproductive structures was to characterise in greater detail the variations previously identified and quantified in the whole-mounts.
Each formalin-fixed fluke was transected at the level of the Mehlis’ gland (at the posterior margin of the uterus, where the width of the body is greatest) and the posterior and anterior ends were processed separately through conventional procedures of alcohol-dehydration and wax-embedding for histological sectioning. The ten posterior ends in each batch were aligned together in a single wax block so that the plane of section was parallel to the planes of transection of the flukes. The ten anterior ends were aligned similarly in a separate wax block. The dimensions of each fluke section reflected the width and thickness of the fluke.

Histological sections 3 μm thick were cut from each wax block and stained with haematoxylin and eosin following conventional procedures. In general, sections of the posterior ends of the flukes were used to study histological variations in testis and vitelline structure in different individuals, whilst sections of the anterior ends revealed details relating to the ovary and uterus.

3. Results

3.1. Plasma closantel concentrations

At 7 and 14 days post treatment the mean plasma concentrations of closantel in Group A were 36.2 (S.D. 5.4) and 26.7 (S.D. 6.7) μg ml⁻¹.

3.2. F. hepatica counts

The geometric mean fluke count of the untreated Group B was 184.6 (range 93–281). That of Group A was 105.8 (range 51–226). The reduction in fluke count for Group A was 42.6%.

3.3. F. hepatica sizes

Within each batch of flukes (i.e. the population of flukes that developed within a single host animal) variation was found in size. The length of the flukes
varied from 5 to 32 mm, whilst the width varied from 2 to 11 mm.

The mean size index for control Group B was 186.3 mm$^2$ (n = 514), for Group A 107.4 mm$^2$ (n = 596). The reduction in size for Group A when compared to Group B was 43.9%. After conversion to logarithms for analysis flukes in Group A were significantly smaller than those in Group B (p < 0.001). Log conversion was used because the range of sizes was wide and differences in S.D.s were too large for normal analysis. The size distribution of all flukes measured in both of the experimental groups is illustrated by Fig. 5. Whilst in the control Group B there was a normal distribution of fluke sizes with a single peak at 175–225 mm$^2$, Group A shows a much broader pattern of size distribution with a peak in the smaller size range of 25–77 mm$^2$.

3.4. *F. hepatica* reproductive score

Within each batch of flukes variation was found in reproductive score.

The reproductive score for the control Group B was 8.76 (S.D. 2.57, n = 514). For Group A it was 5.64 (S.D. 3.81, n = 596). The difference between the Groups was significant (p < 0.001). The percentage reduction was 35.6 for Group A.

There was strong correlation between the size index and reproductive score for both groups ($r^2$ for the untreated Group B was 0.6356, and for Group A 0.8354).

3.5. *F. hepatica* egg shedding

The egg shedding score for Group B was 4.62 (S.D. 1.2). The corresponding score for Group A was 2.25 (S.D. 1.3). After non-parametric analysis the score for Group B was significantly greater (p < 0.05) than that for Group A.

3.6. *F. hepatica* egg hatching

Although there were differences in the number of eggs produced by Group A compared to Group B, no differences were observed in the percentage that hatched after incubation. The percentage hatch for Group A was 77.6, and for control Group B 71.3.

3.7. Histological sections

3.7.1. Testis

Low magnification examination of profiles of testis tubules in transverse sections of the posterior ends of flukes revealed variation in the abundance and variety of cell types present. In general, sections of wider flukes (i.e. those with a higher size score) predominantly displayed tubules of relatively wide diameter that were densely packed with cells in various stages of spermatogenesis (Figs. 7 and 8). At the periphery of the tubules were numerous primary and secondary...
spermatogonia that had basophilic cytoplasm and a high nucleo-cytoplasmic ratio. Many tertiary spermatogonia (the most abundant cell type) with larger intensely basophilic were located towards the core of the tubules. Amongst these were rosettes of smaller cells with eosinophilic cytoplasm and dense peripheral nuclei. These represent the primary and secondary spermatoocytes, which undergo two divisions of meiosis.

Sections of narrower flukes (i.e. those with a lower size score) generally displayed testis tubules of smaller diameter than those described above. These tubules often contained fewer cells and considerable ‘empty’ (non-cellular) space (Figs. 9 and 10). The cells present were predominantly primary and secondary spermatogonia. Fewer tertiary spermatogonia and spermatoocytes were present than in the densely packed tubules described above.

3.7.2. Vitellaria

Vitelline follicles were present towards the lateral margins of all the sections examined, and were clustered in a broad zone of the parenchyma immediately beneath the peripheral musculature and tegumental perikarya (Figs. 11 and 12).

Each follicle comprised a small group of 10–20 cells, amongst which three types were distinguishable at light microscope level (Fig. 13). The S (stem) cells were located peripherally in the follicles and each featured a highly basophilic nucleus with a high nucleo-cytoplasmic ratio. Intermediate (I) cells, within which significant amounts of shell precursor protein had been synthesised, had a larger cytoplasmic volume than the S cells and contained numerous eosinophilic granules. Mature (M) vitelline cells were the largest of the three types, tending to bulge from the surface of the follicle. The large cytoplasmic area of these cells, which contained glycogen as well as clusters of shell protein, was largely unstained by H&E.

Whilst the vitelline tissue in most of the sections of flukes examined was found to contain all three types of cell, the diameter of the follicles was larger and the proportion of M cells was greater in the widest flukes (i.e. those with the largest size index) (Figs. 12 and 13). Smaller flukes tended to have smaller vitelline follicles.

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with fewer M type cells (Fig. 11). This was particularly evident in those flukes less than 5 mm wide, in which the vitelline follicles contained mainly stem cells (Fig. 14).

3.7.3. Uterus

Profiles of the coiled uterine tube were a conspicuous feature in sections of the anterior portions of flukes. In general, these profiles encompassed sections of many eggs, each of which was enveloped with a thin shell (often broken or displaced during processing) and contained mature vitelline cells together with an ovum. The eggs usually appeared distorted due to dehydration (Fig. 15). The wall of the uterus itself was a thin cytoplasmic layer enclosing elongated nuclei at intervals and exhibiting fine microvilli or microlamellae on the luminal aspects. In smaller flukes (width 6 mm or less), the uterine profiles often appeared as empty spaces, with no eggs evident (Fig. 16). This corresponded with the appearance of the uterine field in whole mount preparations of small flukes, which often received a score of 0 for presence of eggs (Fig. 4).

3.7.4. Ovary

Profiles of the dendritic tubules of the ovary were located dorso-laterally to the uterus in sections of the anterior portions of flukes. The diameter of these profiles varied from fluke to fluke, and to some extent within each individual fluke. In general, the wider flukes (corresponding to those with a larger size index and higher reproductive score) had thicker ovarian tubules (Fig. 15) than small flukes with a low reproductive score (Fig. 16). The wall of each ovarian tubule was relatively thick, containing muscle tissue lined with ‘nurse cells’. Oogonia, which are small cells with densely basophilic nuclei and a high nucleo-cytoplasmic ratio, were distributed peripherally in each tubule. Primary oocytes, derived from the oogonia by mitotic division and subsequent differentiation, were located towards the centre of each tubule. The primary oocytes were relatively large, rounded cells with abundant pale-staining vesiculated cytoplasm. Each contained a large nucleus that was less basophilic than those in the oogonia, and which bore one or two conspicuous nucleoli. The primary oocytes arrest at the preprophase of the first meiotic

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division and do not continue meiosis until after they leave the ovary.

Whilst primary oocytes appeared to be the most abundant cells and occupied most space in the wider ovarian tubules (Fig. 17), they were fewer in number in profiles of narrow ovarian tubules where oogonia predominated (Fig. 18).

4. Discussion

Whilst ivermectin is not an effective fasciolicide, closantel uncouples oxidative phosphorylation in flukes. It is also believed to have a significant neurotoxic effect that causes spastic paralysis resulting in the detachment from the food source in vivo and starvation (Fairweather et al., 1998). Closantel retards the development of immature flukes in sheep and extends the prepatent period of the surviving fluke populations. The level of efficacy depends on the age of the infection, ranging from 50% at 6 weeks to over 90% at 12 weeks. In cattle it has previously been reported that the minimum age of flukes in which closantel is >90% effective is 12 weeks (Boray, 1997; Fairweather et al., 1998). In an unpublished study the injectable formulation used in this study was >90% effective when used on late immature flukes at 9 weeks of age (Borgsteede et al., 2006). In the current study, the anthelmintic combination containing closantel was administered 35 days after experimental infection with metacercariae. The efficacy of the treatment, in terms of reduction of final worm burden was expected to be less than 50%. This was found to be the case in that the fluke burdens of calves in Group A, which had received the drug combination showed a mean reduction of 42.6% compared to the untreated calves. It is likely that the early peak plasma concentrations of closantel achieved by the subcutaneous injectable formulation were high enough to cause metabolic and neurotoxic effects experienced by the immature flukes. The effect of the drug regime on the flukes was reflected by other parameters examined, namely reduction of fluke size and reproductive score.

Egg shedding in the groups of flukes was stimulated by incubation in saline at 37 °C, an artificial situation that bears little resemblance to the normal process of egg shedding in vivo. The flukes in the untreated control
group shed significantly more eggs than those from calves that had been injected with closantel/ivermectin. There was no difference between the groups in the viability of the eggs that were shed. This was determined by their ability to embryonate and hatch, yielding motile miracidia. Thus closantel, which by its inhibitory effects on feeding and intermediary metabolism in flukes reduces the availability of ATP for the energy-demanding processes of gametogenesis and oogenesis, causes a decrease in egg production but does not appear to induce functional defects in the gonads or accessory reproductive organs.

Examination of the distribution of size and reproductive score amongst all the flukes within both of the groups revealed the emergence of a bimodal pattern in the drug treated group, when compared to the normal distribution patterns seen in the untreated control group. This may indicate that the drug acted differently on two distinct populations of flukes that co-existed in each infected animal, causing stunting and retardation in one of the populations, while allowing the other population of flukes to proceed in growth and development towards the fully functional adult stage. It is likely that these two populations reflect the tissue location of the immature flukes at the time of administration of closantel. Boray et al. (1967) observed that in experimental infections a proportion of flukes may enter the liver tissues 1–3 weeks later than the majority, presumably taking longer after intestinal penetration to travel to the liver. They also proposed that this behaviour may be an explanation for the number of flukes that survived anthelmintic treatment. Flukes that are migrating through the hepatic parenchymal tissue are in a distinctly different physical and physicochemical environment from those that have gained access to the biliary network, and undoubtedly differ from them in physiological attributes and in the partitioning of energy resources. There is evidence that once flukes gain access to the bile ducts a spurt in growth followed by reproductive maturation occurs. This is facilitated by a reduction in the need to constantly renew the surface glycocalyx of the tegument in the face of humoral and cell mediated attack by the host (Hughes et al., 1981). Newly excysted juvenile flukes rely on
aerobic metabolism and are dependant on Kreb’s cycle activity. During the period of migration in the liver parenchyma, the activity of Kreb’s cycle decreases with acetate becoming the major end-product. Oxygen is still required to re-oxidise NADH. Adult flukes in the bile ducts are anaerobic and utilise malate dismutation to re-oxidise NADH (Tielens, 1998). Apparently these switches in metabolism are triggered by a decrease in oxygen availability and driven by the changing ratio of the key enzymes pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK). The fundamental metabolic differences between flukes in the liver parenchyma and those in the bile ducts may underlie the differences in susceptibility to closantel. If the primary effect of the drug is to induce spastic paralysis and detachment from the food source as suggested by Fairweather et al. (1984), then flukes in the hepatic parenchyma might have an advantage over those in the bile ducts because even if paralyzed they would still lie in a nutritive medium and energy substrates such as glucose could still enter by tegumental diffusion.

Comparison of whole-mount preparations with equivalent histological sections from flukes of various sizes in the experimental groups supported the interpretation that a reduction in reproductive score was associated with a decline in the activity of cell division and maturation in the reproductive organs rather than the development of drug-induced lesions. In the drug-treated populations there were more small flukes with a low reproductive score than was the case in the untreated control populations. This probably reflects restrictions in energy metabolism caused by closantel during critical phases in the development of flukes.

In the testis follicles of small drug-treated flukes, primary spermatogonia were usually the commonest type of cells present. These are stem cells, and their predominance indicates relative inactivity in the follicle. The small size of the testis follicles together with the presence of ‘empty’ space within them gave poor resolution in the whole-mount preparations. Presumably the energy-demanding process of spermatogenesis and spermiogenesis, which involve mitotic and meiotic divisions as well as cellular differentiation, proceed at rates dictated by the availability of ATP. By contrast, the testis follicles of larger flukes, which predominated in the control population, had several cell types. Here...
spermatogonia and spermatocytes were more abundant than primary spermatogonia. A full account of spermatogenesis and spermiogenesis in *F. hepatica* has been given by Stitt and Fairweather (1990).

Similarly histological sections of the ovary in small flukes were narrow in comparison to those in larger flukes. They contained numerous peripheral oogonia (the equivalent of stem cells) but few primary oocytes, which packed the core of ovarian tubules in flukes with a high reproductive score. The narrow diameter of the ovarian tubules in small flukes was also evident in the whole-mount preparations. The predominance of oogonia in the ovarian tubules of flukes with a low reproductive score reflects a reduced level of functional activity including cellular division and differentiation. During oogenesis the oogonia undergo a number of mitotic divisions to form primary oocytes which are much larger than the oogonia (25 μm in diameter compared to 10 μm of the oogonia) and show cytoplasmic differentiation. As with spermatogenesis, the production of oocytes, and ultimately mature ova, undoubtedly demands high energy expenditure, and is likely to be retarded by uncouplers of energy metabolism. Descriptions of oogenesis in *F. hepatica* have been given by Bjorkman and Thorsell (1964), Gresson (1964) and Fairweather et al. (1998).

Bearing in mind that each egg assembled in the ootype of *F. hepatica* incorporates about 30 vitelline cells, and that each fluke produces some 25,000 eggs each day, cell division and differentiation in the vitelline follicles consumes the majority, possibly the largest, proportion of the energy generated by intermediary metabolism. Therefore it is not surprising that drug-induced restriction of available ATP had a marked effect on the functional activity in the vitelline tissue.

In histological sections, reduced cell division was manifested by the preponderance of stem cells in the follicles and the relative reduction in the glycogen-filled mature type of cells. The processes of cell division and maturation in the vitelline follicles have been described in detail by Irwin and Threadgold (1970) and Threadgold (1982). In the present study, catechol was used to give an indication of the amount and distribution...
of the enzyme phenol oxidase in the vitelline tissue of treated and untreated flukes. This enzyme is associated with egg-shell precursor protein in the vitelline cells of *F. hepatica* (Johri and Smyth, 1956) and is manufactured in the intermediate type cells. Its presence was taken to indicate active cytoplasmic differentiation in the vitelline follicles, and the reduction in staining found in whole mounts of small flukes from the drug-treated populations can be interpreted as retardation in differentiation of vitelline cells due to lack of available energy.

Not surprisingly, the presence of eggs in the uterus of flukes was closely related to the size and to the state of development of the other reproductive structures. For the successful formation of egg-shells in the ootype, the correct number of mature vitelline cells, each with an appropriate complement of precursor proteins and tanning enzymes, must be present. It is likely that defects in the quality of the vitelline cells would prevent successful shell formation and therefore render the eggs non-viable. The fact that eggs shed by the drug-treated flukes hatched as successfully as did those from the control flukes provides evidence that each of the individual components incorporated in the eggs (ovum, vitelline cells) retained functional integrity.

5. Conclusion

In conclusion the study gives additional information on the stunting effects that closantel has on the growth and reproductive capacity of immature *F. hepatica*. This effect could be of epidemiological significance if egg availability is considered (Maes et al., 1988) when treatment is applied at critical periods and used as a tool in strategic control programmes (Taylor et al., 1994).

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