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Research paper

Fasciola hepatica: a light and electron microscope study of the ovary and of the development of oocytes within eggs in the uterus provides an insight into reproductive strategy

R.E.B. Hanna[a,*], D. Moffett[a], F.I. Forster[a], A.G. Trudgett[b], G.P. Brennan[b], I. Fairweather[b]

[a] Veterinary Sciences Division, Agri-Food and Biosciences Institute (AFBI), Stormont, Belfast BT4 3SD, United Kingdom
[b] School of Biological Sciences, The Queen's University of Belfast, Belfast BT9 7BL, United Kingdom

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ABSTRACT

The ultrastructure of the ovary of *Fasciola hepatica* collected from field-infected sheep, was compared with that of flukes from laboratory-infected rats harbouring the Oberon or the Cullompton fluke isolate. At the periphery of the ovarian tubules, in all flukes, interstitial tissue was identified that appears to provide physical support and facilitate the metabolism of the germinal-line cells. Oogonia undergo mitotic division to maintain the cell population and to produce oocytes. Early oocytes feature conspicuous synaptonemal complexes in the nucleoplasm, and these become less evident as the oocytes grow in size, move towards the core of the ovarian tubule, and synthesise osmiophilic bodies. The latter may represent cortical granules, and serve to block polyspermy. The identity of the synaptonemal complexes was confirmed by immunocytochemical labelling of synaptonemal proteins. The occurrence of synaptonemal complexes in the oocytes of all fluke types examined indicates that pairing of bivalent chromosomes, with the potential for genetic recombination and chiasmata formation, is a feature of the triploid aspermic parthenogenetic Cullompton flukes, as well as of the wild-type out-breeding field-derived and Oberon isolate flukes. In oocytes within shelled eggs in the proximal uterus of all flukes, condensed chromosomes align at meiotic metaphase plates. Following the reduction division, two equal pronuclei appear in each oocyte in the distal uterus. On the basis of these observations, a mechanism of facultative parthenogenesis for *F. hepatica* is proposed that accommodates the survival and clonal expansion of triploid aspermic isolates.

1. Introduction

The liver fluke, *Fasciola hepatica*, is capable of an impressively high reproductive output, reaching 25,000 eggs per fluke per day in sheep (Happich and Boray, 1969). *Schistosoma mansoni* worm pairs, by contrast, produce approximately 350 eggs per day (Cheever et al., 1994). A large proportion of the body of each liver fluke is occupied by testis and vitelline tissue, and it is evident that production of spermatozoa and egg components represent the major energy-consuming activities of the adult (Hanna et al., 2008; Hanna, 2015).

The processes of spermatogenesis, spermiogenesis, vitellogenesis, egg formation and its neural control are well understood, and were reviewed by Fairweather et al. (1999). However, while early electron microscope studies of the ovary were carried out by Björkman and Thorsell (1964) and Gresson (1964), the resolution of the images was constrained by the technical limitations of the instruments and preparative methods in use at the time.

F. hepatica is hermaphrodite and self-fertile, because single flukes can produce eggs (Lagrange and Gutmann, 1961), albeit with a low level of auto-insemination (Hanna et al., 2011). Despite the extensive development of testis tissue and the massive production of spermatozoa in most isolates and individuals of *F. hepatica* that have been studied, some authors have discussed the possibility that parthenogenesis may also be an element in the reproductive repertoire of flukes (Sanderson, 1953, 1959; Terasaki et al., 2000; Fletcher et al., 2004; Hanna et al., 2008, 2011). Aspermic triploid isolates of *F. hepatica* have been described by various workers (Itagaki et al., 1998; Terasaki et al., 2000) and meiotically dysfunctional aspermic diploid isolates are also known (Agatsuma et al., 1994; Terasaki et al., 1982, 2001). Half of the triclabendazole-resistant Sligo isolate flukes studied by Hanna et al. (2008) were function-

* Corresponding author.
E-mail addresses: bob.hanna@afbini.gov.uk, robert.hanna160549@gmail.com (R.E.B. Hanna).

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ally aspermic, while the Cullompton isolate of *F. hepatica*, which has been widely used in molecular and chemotherapeutical studies (Fairweather, 2011), is triploid and aspermic, spermatogenesis not proceeding beyond the primary spermatocyte stage (Fletcher et al., 2004). It is likely that all these aspermic isolates propagate by a parthenogenetic mechanism (Fletcher et al., 2004). It would be expected that self-fertilisation and parthenogenesis, like asexual reproductive strategies, would restrict genetic recombination and the degree of genetic diversity generated by each fluke (Fletcher et al., 2004). Genetic diversity might be generated within trematode parasite populations by mitotic recombination during the asexual stage of the life cycle in snails, as appears to be the case for *S. mansoni* (Grevelding, 1999; Bayne and Grevelding, 2003), but currently there is no evidence that mitotic recombination occurs in the intermediate host of *F. hepatica.* Agatsuma et al. (1994) found a high level of heterozygosity in flukes from Japan, Korea, Australia and USA, indicating a predominantly out-breeding strategy, a finding supported by Walker et al. (2007), who showed that individual hosts can carry genetically diverse infra-populations of flukes. It is important to know the relative frequencies of cross-fertilisation, self-fertilisation and parthenogenesis in *F. hepatica* populations in order to inform epidemiological models relating to the spread of anthelmintic resistance (Fletcher et al., 2004; Hanna et al., 2008, 2011).

The main aim of the present study is to document and compare the morphology of the ovary in out-breeding wild-type and Oberon isolate *F. hepatica*, and in aspermic triploid Cullompton isolate flukes, which are assumed to reproduce parthenogenetically. Special attention is directed to features relating to meiosis and early development of the oocytes because it is possible that events up to and including the first meiotic division (reduction division, Meiosis 1) of the oocyte, and the second meiotic division (cleavage division, Meiosis 2) of the zygote, predispose to subsequent development either in an out-breeding or a parthenogenetic direction, depending on whether or not spermatozoa are available for fertilisation.

2. Materials and methods

2.1. Sources of material

The field-derived flukes used in this investigation were collected from the liver of two sheep taken from each of five lowland sheep farms in Northern Ireland (NI). The sheep were killed using a captive bolt, followed by exsanguination, at the Veterinary Research Laboratories, Stormont. The flocks of origin had taken part in a survey of triclabendazole resistance in fluke populations in NI, which was reported by Hanna et al. (2015), and are considered to be representative of the intensive sheep farming industry in NI. At least 30 flukes were collected from each liver. In total, approximately 200 field-derived flukes were examined histologically.

Flukes of the Oberon and Cullompton isolate were obtained from the main bile ducts of male Hooded Lister rats, each experimentally infected with up to 8 metacercariae, 16 weeks prior to euthanizing with carbon dioxide. The full details of these infections were recorded previously (Hanna et al., 2011). In total, 128 Cullompton flukes (from 63 rats) and 50 Oberon flukes (from 34 rats) were examined histologically. The flukes examined were collected from rats with worm burdens ranging from 1 to 5 flukes per host. While significant differences were found in testis size and development between flukes from rats with single as opposed to multiple fluke burdens, no differences were recorded in ovarian development or in number of eggs packing the uterus, irrespective of the fluke burden (Hanna et al., 2011). The provenance of the Oberon and Cullompton isolate flukes was described in detail by Fairweather (2011). These isolates were maintained by passage through both rats (Fairweather, 2011) and sheep (Flanagan et al., 2011) and showed no change in drug sensitivity over time. No data is available regarding the number of times each isolate was passaged.

2.2. Tissue preparation for histology

Flukes for histological examination were placed in 10 cm-square plastic Petri dishes and flat-fixed for 2–4 h underneath glass microscope slides using 10% (v/v) neutral buffered formalin (NBF). Thereafter, the flukes were free-fixed in NBF at 4 °C overnight. Following this, each fluke was sliced into equal right and left halves along the median plane. The two halves were dehydrated through an ascending series of ethanol, cleared in xylene and embedded in wax blocks following conventional procedures, with the cut surfaces presented at the block face. Sections 3 μm in thickness were cut from each block face and stained with haematoxylin and eosin (H&E) using standard histological protocols. Duplicate sections were stained by the acid Feulgen method to demonstrate DNA (Bancroft and Gamble, 2008) and counter stained with light green. Sections were examined and the reproductive structures were photographed using a Leica DM LBZ microscope with a Nikon Coolpix 5000 camera system.

2.3. Tissue preparation for transmission electron microscopy

Initially, each fluke destined for electron microscopy was flat-fixed under a microscope slide for 30 min at room temperature in 4% (w/v) glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2). A cube of tissue, each side 2–3 mm, was then cut from the anterior body region of the fluke, behind the acetabulum and left of the uterus, viewed from the ventral surface. The cubes were free-fixed for 4 h in 4% (w/v) glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.2) and subsequently transferred to and stored in 5% (w/v) sucrose in 0.1 M sodium cacodylate buffer (pH 7.2) at 4 °C until further processing. Fluke tissue was then post-fixed in 1% osmium tetroxide for 1 h prior to dehydration through an ascending series of ethanol to propylene oxide. The samples were infiltrated and embedded in Durcupan epoxy resin (Sigma-Aldrich Co. Ltd., UK) which was polymerised for 48 h at 60 °C. Using 1 μm thick sections stained with 1% (w/v) aqueous toluidine blue and examined by light microscopy to select appropriate areas of each block face, ultrathin sections (90–120 nm thick) were cut from the resin blocks using a Leica EM UC7 ultramicrotome, mounted on uncoated nickel grids, double-stained with uranyl acetate and lead citrate, and viewed in a JEOL JEM-1400 transmission electron microscope with an AMT Activue XR16 digital camera system, operating at an accelerating voltage of 80 kV.

2.4. Immunocytochemical demonstration of synaptonemal proteins

Three different commercial antisera were used. They were designed to label specific synaptonemal proteins in histological sections, and were as follows:

Anti-synaptonemal protein 1(Novus Biologicals, Abingdon, UK. Cat.no.NB300-229) (anti-SCP1), polyclonal, prepared in rabbit.

Anti-synaptonemal protein 3 (Biorbyt Ltd. Cambridge, UK. Cat.no.ORB6921) (anti-SCP3 [Biorbyt]), polyclonal, prepared in rabbit.

Anti-synaptonemal protein 3 (Novus Biologicals, Abingdon, UK. Cat.no.NB300-232) (anti-SCP3 [Novus]), polyclonal, prepared in rabbit.

Preliminary experiments were carried out using dilutions of each antiserum from 1/50 to 1/1000 with tris-buffered saline (pH