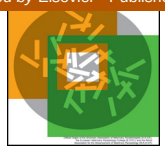
Veterinary Parasitology 208 (2015) 101–111

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar



Fasciola hepatica vaccine: We may not be there yet but we're on the right road

Verónica Molina-Hernández[a], Grace Mulcahy[b], Jose Pérez[c], Álvaro Martínez-Moreno[c], Sheila Donnelly[d], Sandra M. O'Neill[e], John P. Dalton[a], Krystyna Cwiklinski[a,*]

[a] *School of Biological Sciences, Queen's University Belfast, Belfast, UK*
[b] *Veterinary Science Centre, University College Dublin, Belfield, Dublin, Ireland*
[c] *School of Veterinary Medicine, University of Cordoba, Córdoba, Spain*
[d] *The i3 Institute & School of Medical and Molecular Biosciences, University of Technology, Sydney, Australia*
[e] *School of Biotechnology, Dublin City University, Ireland*

ARTICLE INFO

Keywords:
Fasciola hepatica
helminth
vaccines
hepatic pathology
immune response

ABSTRACT

Major advances have been made in identifying potential vaccine molecules for the control of fasciolosis in livestock but we have yet to reach the level of efficacy required for commercialisation. The pathogenesis of fasciolosis is associated with liver damage that is inflicted by migrating and feeding immature flukes as well as host inflammatory immune responses to parasite-secreted molecules and tissue damage alarm signals. Immune suppression/modulation by the parasites prevents the development of protective immune responses as evidenced by the lack of immunity observed in naturally and experimentally infected animals. In our opinion, future efforts need to focus on understanding how parasites invade and penetrate the tissues of their hosts and how they potentiate and control the ensuing immune responses, particularly in the first days of infection. Emerging 'omics' data employed in an unbiased approach are helping us understand liver fluke biology and, in parallel with new immunological data, to identify molecules that are essential to parasite development and accessible to vaccine-induced immune responses.

1. Introduction

According to the United Nations, the world population will increase to 9 billion by 2050. The demand for animal-derived food, which has been growing at a rate of >7% over the last two decades, will correspondingly increase, both in developed and developing countries (FABRE Technology Platform working group). Therefore, going forward, to support this population growth, we need to secure a sustainable means of producing the quantity of food required, and we need to do this in a safe and ethical manner to protect the consumer, the health and welfare of animals and our environment (Foresight, 2011).

This global population growth will open up attractive market prospects for countries with the capacity and technology to produce large quantities of high-quality animal products (particularly beef, dairy, pig and sheep). The value of farm-level animal production to the EU is over €130 billion; farmers represent >8% of the workforce, and farms employ >15 million people (FABRE Technology Platform working group, 2025). As farming intensifies, however, issues surrounding disease control, the prevention of disease transmission from animals to humans (zoonosis), the health and welfare of animals and the quality/safety of

* Corresponding author.
E-mail address: k.cwiklinski@qub.ac.uk (K. Cwiklinski).

http://dx.doi.org/10.1016/j.vetpar.2015.01.004

the food we consume will come to the forefront (FABRE Technology Platform working group; Meat & Livestock Australia: http://www.mla.com.au).

Ireland is a good example of a small European country whose economy depends heavily on agriculture and at the same time has the ability to take advantage of these future trends. Irish agriculture is primarily a grass-based industry; of the total land area of 6.9 million hectares, 4.2 million (64%) is devoted to agriculture, 90% of which is pasture. There are about 6.5 million cattle in Ireland and the Irish sheep flock exceeds 4.8 million head, with a breeding flock of 2.51 million head. During 2011, 548,000 tonnes of beef and 37,000 tonnes of sheep meat were produced, most of which was exported. Each year, 17,000 dairy farms produce 5.4 billion litres of milk (Teagasc). These figures provide a snapshot of how important the livestock industry, altogether valued at €5–6 billion per annum, can be to a small economy such as Ireland's with a population of under 5 million (Bloemhoff et al., 2014).

2. Parasitic diseases impact hugely on the animal production industry

Helminth parasites cause >55% of all farm animal diseases in Europe and effective control strategies would have a major impact on the sustainability of the livestock industry (Nieuwhof and Bishop, 2005; Murphy et al., 2006; Morgan et al., 2013). In Europe, parasitic diseases of farm animals are caused principally by infections with the liver fluke Fasciola hepatica, the lungworm *Dictyocaulus viviparus*, and the gastrointestinal nematode parasites of sheep (*Haemonchus contortus*, *Teladorsagia circumcincta*) and cattle (*Ostertagia ostertagi*, *Cooperia oncophora*). These parasites impact on livestock productivity by affecting growth rates, fertility and the quality of wool and milk production. In particular, during seasons of major fluke outbreaks, infection can result in many animal fatalities (Piedrafita et al., 2010). In general, about 17% of animal production is estimated to be lost through infection of livestock with pathogens including these parasites (FABRE Technology Platform working group).

The losses to the livestock industry as a result of parasitic helminths are compounded by the significant effort and cost of trying to control these infections with regular chemical treatments. Within Europe, the annual spend on anthelmintic drugs for parasitic helminths of ruminants has been estimated to be €400 million (Selzer, 2009; Morgan et al., 2013). These costs contribute to the overall economic losses related to disease, which for *F. hepatica*, in particular, are estimated to be US $3 billion globally (Spithill et al., 1999a; Mas-Coma et al., 2005; Piedrafita et al., 2010). However, anthelmintic resistance, especially in gastrointestinal nematode parasites (Kotze et al., 2014), has spread to the point that it is now a global concern and is already impacting significantly on livestock productivity in many regions. Resistance is also becoming a serious issue in liver fluke (Fairweather, 2011; Dalton et al., 2013). The use of chemical products to control parasitic infections is, therefore, not sustainable in the long term because of the likelihood of continual emergence of drug-resistant parasite populations. Additionally, there are growing consumer concerns about chemical residues in food, and also the detrimental impact they have on the environment (Dalton and Mulcahy, 2001; Cooper et al., 2012). In particular, an assessment report adopted by the Committee for Medicinal Products for Veterinary Use (European Commission) has recently established maximum residue limits for triclabendazole (TCBZ) in bovine and ovine milk, in addition to those already set for ruminant muscle, fat, liver and kidney (EMA, 2012). Recent flukicide licensing restrictions for lactating dairy cows only allow the use of albendazole (ABZ) and oxyclosanide (OCZ) for fluke control (HRPA, 2013). Unlike TCBZ which is active against both immature and adult parasites, ABZ and OCZ have restrictive activity against the adult parasites and use of these anthelmintics often requires repeated treatments, which are not always carried out in the field (Fairweather and Boray, 1999; Power et al., 2013).

Vaccines, on the other hand, are considered safe and are acceptable to both users and consumers. New vaccine technologies and adjuvant development are further improving food safety by ensuring that these leave no residues in food (Parker et al., 2009; Adams et al., 2009). As no chemical residues are passed onto pasture, vaccines are also considered environmentally friendly. However, despite the huge successes we have made in vaccine development against viruses and bacteria over the past 200 years (and in recombinant vaccines in the last 30 years), with the exception of the live attenuated Huskvac vaccine for lungworm, there are no commercially viable vaccines for animal (or human) helminth parasitic pathogens. More recently, a new vaccine for *H. contortus*, Barbervax, consisting of native gut-derived antigen complex has been launched in Australia (www.barbervax.com.au).

3. Liver fluke disease: a food-borne zoonotic disease

Liver fluke disease, or fasciolosis, in sheep and cattle results from infection with the trematode, *F. hepatica* (Andrews, 1999). Due to the emergence of parasites resistant to the frontline drug triclabendazole (TCBZ) and also to climate changes favourable to the survival of the intermediate host (*Galba truncatula*) and the infective stage (metacercariae) of the parasite (Piedrafita et al., 2010), consistently high prevalence levels of liver fluke have been recorded across Europe (recently reviewed by Charlier et al., 2014). Within the UK, the prevalence of fasciolosis has shown a marked increase, specifically in dairy herds from 48 to 72% from 2003 to 2006 (McCann et al., 2010) and, more markedly, the distribution of liver fluke has spread to include areas of Scotland and East Anglia (Pritchard et al., 2005; Kenyon et al., 2009). The increased prevalence of *F. hepatica* further adds to the economic impact of this parasite and indicates that the potential losses are greatly under-represented.

F. hepatica is also an important pathogen of humans in certain regions of the world where farm management practices allow infected animals to roam amongst vegetation consumed by humans. Global human infections are estimated to be between 2 and 17 million with 180 million people at risk; prevalence is particularly high in the Andean Altiplano (Bolivia, Peru and Ecuador) with lower

infection numbers recorded in Egypt, Iran and South East Asia (Mas-Coma, 2005; Mas-Coma et al., 2009; Gonzalez et al., 2011). The widening geographical distribution of liver fluke also has an impact on human infections, with fasciolosis recently being reported in areas of Pakistan (Afshan et al., 2014). Besides better farm management procedures, control of human infections would greatly benefit from a vaccine targeting animal infections.

The discovery of novel means of control and intervention for fasciolosis requires us to be cogniscent of each stage of the parasite's life cycle and interpret this as a complex interplay between parasite and host. Parasite stages termed cercariae are released by snails that encyst on grass and other vegetation as metacercariae, which can survive for up to a year (Andrews, 1999). Following ingestion by grazing animals, the parasites emerge from their cysts in the intestine (we term these newly excysted juveniles, NEJ), traverse the intestinal wall, which takes just a few hours, before migrating through the liver capsule and into the parenchyma. Here their feeding and migratory activities cause tissue perforation and haemorrhage, leading to extensive tissue damage. After about 7–8 weeks, the parasites migrate into the bile ducts, mature and produce 20,000–24,000 eggs per fluke per day which are released onto the pasture with the faeces (Boray, 1969). Following embryonation on pasture, the eggs hatch and release motile miracidia that infect mud snails and initiate another cycle of infection.

The liver damage caused by these migrating parasites reduces animal performance, fertility and wool and milk production (reviewed by Sangster, 2001; Mezo et al., 2008; Knubben-Schweizer et al., 2010; Animal Health Ireland, 2011; Charlier et al., 2014). These injurious pathogenic effects are compounded by the negative impact the parasite has on the immune system; potent immunosuppressive molecules secreted by the parasite compromise the natural resistance of the host and promote co-infections resulting in microbial diseases such as salmonellosis, clostridiosis and tuberculosis (Aitken et al., 1979, 1981; Hall et al., 1981; Brady et al., 1999; Flynn et al., 2007, 2009; Dalton et al., 2013). The parasites can live for very long periods (longest recorded 11 years in sheep; Durbin, 1952) inflicting long-term damage, causing low performance (Piedrafita et al., 2010) and possibly reduced ability to resist other infections.

4. *F. hepatica*-induced tissue damage and the immune response: partners in hepatic pathology

The early pathogenesis of fasciolosis involves two stages. A pre-hepatic stage is caused by the penetration of NEJ through the host intestine wall and migration within the peritoneal cavity, which lasts for approximately 3 days after the primary infection (Kendall and Parfitt, 1962). Animals display no clinical signs at this time and pathological findings are rare, except for small haemorrhagic foci in the peritoneum layer. No changes are observed in the intestine wall (Kendall and Parfitt, 1962; Boray, 1969; Kelly, 1993; Zafra et al., 2013a, 2013b), which suggests that the parasite moves swiftly through this tissue and/or possesses a mechanism that suppresses immune activation within the tissue. However, the parasites clearly induce an 'immunological presence' as the cellular component of the peritoneal fluid does change, with the infiltration of a large number of immune cells such as lymphocytes, macrophages and, especially, eosinophils (Zafra et al., 2013a, 2013b). Control of the host immune system by *F. hepatica* likely begins here as experiments in laboratory animals have demonstrated that recruitment and activation of M2 macrophages is evident within 24 h after infection (Donnelly et al., 2005, 2008; Adams et al., 2014) and that flukes induce an apoptotic effect on peritoneal immune cells (Serradell et al., 2007; Guasconi et al., 2012). This early immunomodulatory effect likely plays a critical role in determining the ultimate outcome of *F. hepatica* infection and is triggered directly by molecules (such as proteases, protease inhibitors and antioxidants) secreted from the parasite and by host molecules (alarmins) that signal tissue damage to stimulate immune repair mechanisms (Dalton et al., 2013).

Between 4 and 6 days post-infection, the majority of NEJ have penetrated the liver capsule and established themselves firmly within the parenchymal tissue, which initiates the hepatic stage of pathogenesis. Depending on the definitive host, the time of migration from intestine to liver can extend up to 10 days (Dawes and Hughes, 1964; Sukhdeo and Sukhdeo, 2002). Here, the parasites move randomly, forming characteristic transects/tunnels and feed on the hepatic cellular components and blood for 5–6 weeks (parenchymal phase), before reaching the bile duct where they develop into their adult form (biliary phase) at 7–8 weeks post-infection (Sinclair, 1967; Dow et al., 1967; Rushton and Murray, 1977). As a result of their parenchymal feeding activity, NEJ grow and develop rapidly, causing increased hepatic necrotic tracts and haemorrhaging resulting in acute and subacute fasciolosis.

The hepatic-stage histopathology is characterised by acute necrotic foci and tracts, some of which are associated with haemorrhages. In the early stages of infection, migrating NEJ are surrounded by relatively healthy hepatic parenchyma, whereas necrotic tracts and haemorrhages are found several millimetres behind the larvae, suggesting that products shed or released by NEJ are responsible for hepatic necrosis (Zafra et al., 2013a, 2013b). Migrating larvae in sheep (Meeusen et al., 1995) and goats (Zafra et al., 2013a, 2013b) are not surrounded by the inflammatory infiltrate, a feature which has been attributed to the rapid migration of NEJ (Meeusen et al., 1995). By contrast, in recent vaccine trials in goats, we found that animals showing little hepatic damage exhibited severe infiltration of eosinophils surrounding migratory flukes at early stages of infection (Zafra et al., 2013a, 2013b). Necrotic hepatic tissue induces chemotaxis of inflammatory cells, particularly eosinophils, macrophages and lymphocytes, which are found at the periphery of necrotic areas. Macrophages become activated, increasing in size and accumulate around the necrotic areas in a palisade pattern, surrounded with lymphocytes to form necrotising granulomas (Marcos et al., 2007; Zafra et al., 2013a, 2013b). Small necrotic foci or tracts are phagocytosed and removed by activated macrophages, but larger necrotic areas are encapsulated by fibrous connective tissue, leading to tortuous fibrous scars (chronic tracts) after necrosis or haemorrhages are removed. Even in a

single dose infection, it is common to find acute, subacute and chronic tracts in the liver due to the same larvae migrating for weeks, and thus the simultaneous migration of many immature forms can cause severe damage to the liver parenchyma. The clinical signs characterising this stage of infection include jaundice, ill-thrift and anaemia and can even result in death, particularly of young animals.

The presence of adult flukes within the bile ducts induces a severe chronic cholangitis with erosion and hyperplasia of the biliary epithelium, infiltrate of eosinophils, lymphocytes and plasma cells in portal areas, intraepithelial infiltration of globule leukocytes and portal fibrosis (Behm and Sangster, 1999; Perez et al., 1999, 2002; Tliba et al., 2000; Zafra et al., 2008; Golbar et al., 2013). These lesions, together with chronic tracts induced during the migratory stage, are typical of chronic fasciolosis (Perez et al., 2005; Zafra et al., 2008, 2013a, 2013b). Chronic fasciolosis is the most common form in ruminants and occurs when the adults reach the bile ducts. Clinically, it is characterised by similar signs as the acute/subacute phase, together with persistent diarrhoea, pallor of mucous membranes, weight loss, weakness, with some animals developing oedema under the jaw ('bottle jaw') (Behm and Sangster, 1999).

Adult flukes feed on blood and hepatic parenchyma adjacent to bile ducts, causing damage and focal rupture of bile ducts, allowing some parasite eggs to reach the liver parenchyma causing severe eosinophilic and granulomatous inflammatory responses (Perez et al., 2002, 2005; Zafra et al., 2008, 2013a, 2013b). At this stage, it has been shown that in sheep, parasite excretory-secretory (ES) products continue to stimulate a favourable immune environment for parasite survival and induce apoptosis of inflammatory cells in the pericholangiolar inflammatory infiltrates (Escamilla et al., unpublished). The migratory behaviour of the parasite may be an immune evasion tactic as parasites are rarely found in the tracts themselves but have moved on leaving behind a trail of destruction. Lesions can also be found in the liver where parasites are absent, suggesting that antigens released by the parasite can become trapped in the tissue and attract immune cell infiltration.

A major factor that contributes to hepatic damage is the level of the infective dose. Previous studies revealed that, after high infective doses, the lesions found were more severe, even causing death of the animals during early infection, whereas under low infective doses the disease develops chronically (Behm and Sangster, 1999). Moreover, studies carried out in sheep (Perez et al., 2002) and goats (Martinez-Moreno et al., 1999; Perez et al., 1999) have shown that small repeat infections, similar to those which occur in natural infections (Clery et al., 1996), produce more severe hepatic lesions than a single infective dose of equal metacercariae number. These findings suggest that, while the mechanical tissue-penetrating action of the parasite may be the cause of hepatic damage, it is the ensuing immune, or wound-healing, response that plays the major role in the pathology of fasciolosis. The immunopathological cells comprise T lymphocytes (CD4+, CD8+ and gamma-delta), B cells and macrophages, eosinophils and mast cells which lead to the formation of large granulomas (Chauvin and Boulard, 1996; Martinez-Moreno et al., 1999; Perez et al., 1999, 2002, 2005; Zafra et al., 2009).

Understanding how the immune system of various ruminant hosts leads to tissue damage following primary and subsequent infection with *F. hepatica* is still a huge challenge to researchers but is essential to lay the basis for effective vaccine development. Dissecting the myriad of cytokines, chemokines and innate and adaptive cellular pathways that are elicited to infection is critical to identify those that are responsible for protecting against infection whilst preventing excessive tissue damage. To date, we know that *F. hepatica* infection drives the host immune system towards an immunomodulatory response leading to the activation of T helper (Th) 2 immune response within 7 days of infection, with the concurrent production of high titres of IgG1 antibodies without IgG2 (sheep and cattle: Mulcahy et al., 1998, 1999; Walsh et al., 2009 and murine models: Donnelly et al., 2005, 2008), and a down-regulation of Th1 immune response in murine models (Brady et al., 1999; O'Neill et al., 2000) and cattle (Flynn et al., 2009). Thus, mouse studies indicate that while the parasites induce host hepatic damage, it has the ability to limit the extent of pro-inflammatory Th1-driven protective immune responses and promote anti-inflammatory healing mechanisms, involving a Th2-mediated immune response, M2 macrophages (Donnelly et al., 2005, 2008) and mast cells (Vukman et al., 2013). Collectively, this prevents the development of a protective immune response against the parasite allowing the development of a chronic infection. It is possible that, similar to infections with schistosome parasites (Colley and Secor, 2014), the elaboration of an anti-inflammatory/regulatory immune response is important to protect the host from excessive immune-mediated liver damage (McSorley and Maizels, 2012). These anti-inflammatory immune responses may be effective in preventing host mortalities in low-level infections, but in high-level infections severe liver damage caused by the migrating flukes may overwhelm this protective response and cause organ failure. This proposal is important to consider for future vaccine development as their efficacy may be highly dependent on infection dose, or in a field/herd situation a vaccine may only be effective in animals that acquire low-level infections. More studies that investigate the effect of infection dose and repeated infection on host immune responses are needed to understand the impact of these schedules on liver tissue damage, immune-related pathology and the overall pathogenesis of liver fluke infection.

5. There is no strong evidence of protective immunity in ruminants to *F. hepatica* infection

Compounding the difficulty of *F. hepatica* vaccine development is the lack of evidence for natural innate and cellular immune-mediated resistance in ruminants to these parasites (also reviewed by Piedrafita et al., 2004). Few studies have directly addressed this question, though Clery et al. (1996) have shown that cattle chronically infected in the field do not develop resistance to an experimental challenge. Studies by Doy and Hughes (1984) attempted to identify the tissue where resistance to

infection in cattle is mediated. While they found that cattle infected 18 or 26 weeks previously exhibited high levels of resistance to a secondary challenge infection (56 and 94%, respectively, assessed by fluke number 15/16 weeks after challenge infection), no protection was observed if they sacrificed animals earlier, at 4–14 days after the challenge infection. From this data, they suggested that resistance mechanisms were effective against the challenge infection after the parasites penetrated the liver capsule. However, whether these 'protective mechanisms' were immunologically mediated or result from tissue damage and fibrosis caused by the primary infection is open to debate. Indeed, early studies by Ross (1967), Boray (1967) and Doyle (1971) suggested that protection in cattle to re-infection was related to tissue fibrosis caused by the primary infection which retards the migration of the parasites in the challenge infection; therefore, a high primary challenge leads to greater liver fibrosis and, consequently, seemingly greater protection compared to low primary infections. A more recent study by Hoyle and colleagues (2003) has indicated that infections with TCBZ-drug abbreviated *F. hepatica* could induce a partial protective response as judged by measuring the serum levels of liver enzymes. However, this study was not supported by estimations of parasite burden and would need to be repeated to validate these suggestions.

The only commercially available vaccine for any ruminant helminth is Huskvac. This consists of radiation-attenuated 3rd-stage larvae and is highly effective against lungworm (husk) caused by the nematode, *D. viviparus* (McKeand, 2000). Likewise, radiation-attenuated larvae (cercariae) of *Schistosoma mansoni* induce high levels of protection (which is transferable with antibodies and immune cells) against a heterologous parasite challenge infection in mice and non-human primates and is the basis of many approaches to vaccine development (Anderson et al., 1999; Eberl et al., 2001; Bickle, 2009). Irradiation of *F. hepatica* metacercariae can attenuate the parasites; however, while parasites successfully excyst and the NEJ are capable of crossing the intestine and penetrating the liver tissue, they do not develop beyond the equivalent of 10-day-old normal parasites and, hence, they cause little pathology (Boray, 1969; Rickard and Howell, 1982; Hughes, 1987). Early studies by Dargie et al. (1974) in cattle have shown that high dose infections with radiation-attenuated parasites did not confer any resistance to a challenge infection. In experiments in which some protection was observed following immune priming with radiation-attenuated metacarceriae, protection was attributed to flukes that escaped attenuation, developed normally and induced liver pathology and fibrosis. Results of these experiments would argue that the early migratory stages of the parasite do not induce potent immunologically driven protective responses and that liver pathology/fibrosis is predominantly responsible for protection against challenge.

As recently discussed by Toet et al. (2014), sheep appear to offer no immunological resistance to infection with *F. hepatica*, and even Indonesian Thin Tailed (ITT) sheep that elicit a protective innate and adaptive response to *Fasciola gigantica* are susceptible to infection with *F. hepatica*. Furthermore, while peritoneal cells removed from *F. gigantica*-infected ITT sheep (predominantly eosinophils and macrophages) mediate antibody-dependent cytotoxicity against *F. gigantica* NEJ *in vitro* by the production of superoxide radicals, this mechanism was ineffective against *F. hepatica* NEJ (Piedrafita et al., 2007). These studies suggest that *F. hepatica* NEJ possess a unique ability to resist killing by reactive oxygen species released by sheep innate immune effector cells, which may involve the high expression of antioxidant enzymes such as superoxide dismutase, glutathione *S* transferase (GST) or peroxiredoxin (Piedrafita et al., 2000, 2007; Donnelly et al., 2005, 2008). Sheep do not develop such a marked fibrotic reaction in the liver parenchyma or calcification of the bile ducts compared to cattle infected with *F. hepatica* (reviewed by Behm and Sangster, 1999) leaving them highly susceptible to disease which can be fatal at high doses. From a vaccine perspective, the differences between cattle and sheep immune responses are important to delineate. In particular, while the targets at which the vaccine may be directed could be the same for cattle and sheep, the formulation, delivery regime and administration methods may need to be tailored to elicit efficacious immune responses in each species.

We propose that infection of ruminants with *F. hepatica* induces potent immune suppression or modulation to an extent that animals are susceptible to further infection with liver flukes (and other pathogens). The developing Th2-driven immune response, and suppressed Th1 response, that lead ultimately to an immune regulatory environment, may be necessary to secure parasite survival and at the same time prevent excessive immune-mediated damage to the liver (particularly by containing the damage within the parasite tracts, and laying down fibrotic tissue). However, this immune response may not be sufficient to prevent death resulting from organ failure caused by high-level or repeated infections. It follows that future vaccine programmes need to target parasite molecules that are involved in early stage migration, tissue penetration and immune evasion/suppression.

6. Vaccine candidates

The recent review by Toet et al. (2014) has summarised our progress in vaccine development against *F. hepatica*, and the related parasite *F. gigantica*, over the last three decades. Interestingly, most of the vaccine candidates were first isolated as native proteins, usually from adult worm ES products, because this antigen preparation was not very complex and was easy to obtain. Several of these early antigens, including cathepsin L proteases, GST and fatty acid binding protein (FABP) induced significant protection in cattle and sheep (reviewed by Toet et al., 2014), reducing not only worm burden (and egg output), but also liver pathology. Recent reports of vaccine trials in sheep with several recombinant but functional forms of these antigens such as cathepsin L1 (CL1), GST and peroxiredoxin (FhPrx) from *F. hepatica*, or Sm14 peptide from *S. mansoni*, have not reported significant reduction of worm burdens but have shown reduced liver pathology (Piacenza et al., 1999; Zafra et al., 2009, 2013a, 2013b; Mendes et al., 2010; Perez-Ecija

et al., 2010; LaCourse et al., 2012; Toet et al., 2014). While enthusiasm for bringing these antigens forward as future vaccines has waned largely because of inconsistency of vaccine results (such as with GST and cathepsin L, see Spithill et al., 1999b; Toet et al., 2014), in our opinion, there has not be an exhaustive assessment of these antigens in terms of formulation and delivery (as well as combination vaccines) and, hence, we would disagree with Toet et al. (2014) in dismissing molecules such as GST and FABP as potential future vaccines because of the discrepancy between available data. Furthermore, recent genomics/transcriptomic data reveal these proteins belong to large families with complex developmental expression patterns (Cwiklinski et al., unpublished) that need to be explored further to ensure that the appropriate member(s) are those that are brought into vaccines trials. The observation that adult worm-derived antigens can induce protection at the level of the liver (and possibly earlier) would suggest that these antigens are either also expressed by the juvenile/immature parasite stages or that the immune response cross-reacts between the various members of the same family. If the latter scenario is the case, then perhaps focusing on family members that are expressed by the early stage parasites would prove more promising.

Encouraging results from schistosome vaccine trials has shown that utilising combinations of antigens can increase the efficacy of vaccines (Nascimento et al., 2002). Nascimento et al. (2002) showed that using a triple antigen vaccine with a combination of Sm14, IrV5 and ECL antigens, showed the highest level of protection when compared with single antigens alone. Similar studies have also recently been carried out using a multi-antigen approach for the nematode parasites, *H. contortus* (Smith et al., 2001; LeJambre et al., 2008) and *T. circumcincta* (Nisbet et al., 2013) with promising results suggesting that a vaccine combination approach may be the only way to combat these complex parasites, including *F. hepatica*.

7. Cathepsins L and B proteases: important to the parasite and prime vaccine targets

F. hepatica relies on proteolytic activity for many of its pivotal functional activities in the host, including tissue penetration, migration, feeding and immune evasion and, hence, it is not surprising that these have been the most encouraging candidates for vaccine development for some time (Dalton et al., 2013). Adult parasites secrete an abundance of cathepsin L cysteine proteases, representing about 80% of the total protein from adult ES, that they use for digesting the protein contents of the blood, including haemoglobin, albumin and immunoglobulin, consistent with ES protein analysis (Wilson et al., 2011). This provides free amino acids required for synthesising egg proteins (Robinson et al., 2008a). Native cathepsin L proteases are readily isolated from adult ES products by standard gel permeation and ion exchange chromatography into two 'homogeneous' fractions (termed FhCL1 and FhCL2) (Smith et al., 1993; Dowd et al., 1994). The highly significant protection (regularly 50–55% reduction in worm burden and egg production) observed using these native preparations when delivered in Freund's complete adjuvant was further enhanced (72.4%) when combinations were made with proteins contained in a high molecular weight haem-containing (Hb) fraction. The Hb fraction itself induced low but significant protection (43.8%) in cattle against a challenge infection and recent proteomic analysis has revealed that this mixture includes peroxiredoxin (FhPrx), the helminth defense molecule (FhHDM) and fatty acid binding protein (FABP), all of which can induce modulation of host immune responses including alterations in macrophage function (Robinson et al., 2011b, 2012; Thivierge et al., 2013; Dalton et al., 2013; Figueroa-Santiago and Espino, 2014). In sheep, a combination of the FhCL1 and FhCL2 induced 60% protection but were less efficacious when used individually (34 and 33%, respectively). Surprisingly, neither FhCL1 nor FhCL2 enhanced the efficacy of *F. hepatica* leucine aminopeptidase, which alone was highly efficacious (89%) in sheep (Piacenza et al., 1999).

Our understanding of *F. hepatica* proteases, and other secreted proteins, has been greatly improved by the availability of proteome data (Jefferies et al., 2001; Morphew et al., 2007; Robinson et al., 2008b, 2009) and by integrating this with an analysis of the transcriptome (Robinson et al., 2009). We now know that FhCL1 and FhCL2 are not expressed and secreted by the NEJ as they penetrate the liver and intestine; these early-stage parasites produce different members of the cathepsin L family, FhCL3 and FhCL4. FhCL3 is particularly abundant and secreted by NEJ while FhCL4 may play a more housekeeping role (Cancela et al., 2008; Robinson et al., 2008b, 2009). The expression of FhCL3 is down-regulated soon after the parasite enters the liver parenchyma while FhCL1, FhCL2 and another family member, FhCL5, become more predominant as the parasite migrates and prepares to enter the bile ducts. These changes in protease expression reflect the parasite's adaptation to its changing environment as it migrates through different tissues and encounters new macromolecules. Molecular and biochemical analysis of FhCL3 (Corvo et al., 2009, 2013; Robinson et al., 2011a) have shown that this enzyme possesses a constellation of residues in its active site that confer it with a unique ability to digest collagen, suggesting that this protease is critical to parasite penetration of the intestine and liver capsule (Dalton et al., 2006). Once within the liver parenchyma FhCL2, which also exhibits collagenolytic activity (albeit, not are effective as FhCL3), together with FhCL1 and FhCL5, which both lack collagenolytic activity (Robinson et al., 2011a), combine to generate an efficient tissue-degrading and feeding mechanism. Once in the bile ducts, the parasites rely most heavily on FhCL1 which has an active site arrangement that gives this protease a marked preference for cleaving peptide bonds involving residues (Leu, Ala and Phe) that are common in haemoglobin and, hence, appears to be specifically adapted to blood feeding (Lowther et al., 2009).

Interestingly, a similar highly regulated pattern of expression occurs with the cathepsin B proteases. Several cathepsin B proteases (FhCB1, FhCB2 and FhCB3) show parallel expression with the FhCL3, i.e. are secreted by the early NEJ but are down-regulated as the parasite migrates in the liver tissue. This would suggest that the concerted action of FhCL3/FhCBs is essential for successful intestine and

liver penetration. In support of this suggestion, McGonigle et al. (2008) demonstrated that RNAi-mediated knockdown of either the NEJ FhCLs or FhCBs blocked their ability to traverse the intestine wall. Once in the liver, the parasite cathepsin B proteases (FhCB1, FhCB2 and FhCB3) are switched off but, in contrast to the cathepsin Ls, are not replaced by other family members (other members exist but present studies indicate that these are not secreted and may play a role in the internal tissues/cells of the parasite). It appears that the role of liver migration, tissue feeding and then blood digestion while residing in the bile ducts is the sole responsibility of the FhCL1, FhCL2 and FhCL5.

The exquisite control of the expression of the cysteine proteases as the parasites migrates and establishes a long-lived infection in the host is now guiding our strategy for vaccine development. Given that partial protection against infection can be achieved in ruminants by vaccination with a single recombinant protease such as FhCL1, which is predominantly secreted in the bile duct, it may be judicious for sheep and cattle vaccines to combine this with other family members, most particularly FhCL3, or with members of the cathepsin B family (FhCB1, 2 or 3), that are more specifically expressed in the early juvenile stages. Experiments in rats achieved a level of 83% protection by combining FhCB2 with FhCL5 (Jayaraj et al., 2009).

8. Future direction using next-generation technologies

Following the development and advancement of next-generation sequencing technologies, large sequence data sets are now being generated for several parasites (Huang et al., 2013; Gobert et al., 2014; Young et al., 2014; Forrester and Hall, 2014). Genome data sets can inform functional genomics analysis, including transcriptomics, proteomics, secretomics and epigenomics. Utilising all available 'omics' data for a particular parasite provides an unbiased approach to understanding parasite biology, rather than focusing on a particular gene or protein. Furthermore, we can now perform in-depth analysis of the structure and expression of complex gene families. This will provide a comprehensive understanding of which genes/proteins are important at each stage of development, especially those acting at the host–parasite interface, which are of potential importance for invasion and infection. Furthermore, we are now beginning to probe the interfaces between the host and parasite and monitor their dynamics. Accordingly, those molecules released by the parasite into the host tissues either as soluble mixtures (Robinson et al., 2008b, 2009) or part of the cargo of vesicles/microparticles (Marcilla et al., 2012) have been reported. A detailed characterisation of the fluke surface tegument has also been carried out (Wilson et al., 2011; Toet et al., 2014) which may also be exploited as vaccine and diagnostic targets (Prasanphanich et al., 2013). Reverse genetics tools such as RNAi can be used for *F. hepatica* and currently represents a most direct means to investigate the functionality of NEJ proteins (McGonigle et al., 2008; Rinaldi et al., 2008; McVeigh et al., 2014).

Recently, a draft *F. hepatica* genome has been completed together with extensive transcriptome datasets for several lifecycle stages, NEJ (1-, 3- and 24-h post-excystment), 21–day-old juveniles and mature adult flukes (Cwiklinski et al., unpublished). Using clustering analysis, genes exhibiting similar temporal expression patterns have been grouped to provide a more complete picture of which genes are important at each stage of development, which will allow us to investigate how the functions are inter-connected. For example, when this analysis was applied to cathepsin L and cathepsin B genes, it supported our previous findings regarding their strict developmental regulation as described above, as well as providing a more detailed and reliable clarification of the gene family members. Exploiting this cluster analysis will allow the development of a framework from which a multi-antigen vaccine programme targeted towards a specific lifecycle stage or several stages can be implemented.

The extensive sequencing carried out for several *F. hepatica* isolates has also revealed that these helminths are highly polymorphic and, therefore, likely to be highly adaptable (Cwiklinski et al., unpublished). In particular, the high level of genetic diversity could explain the emergence of anthelmintic resistance in diverse *F. hepatica* populations across the world and should influence future control strategies, using drugs and/or vaccines. Anthelmintic resistance is likely to be multi-genic, and possibly involving different genes each time resistance evolves in different fluke populations across the world, which poses several questions regarding how data from different fluke populations can be interpreted. Will different parasite populations, even within similar geographical locations, respond differently to new drug and vaccine control strategies? Is the inconsistency observed in vaccine trials (inter- and intra-laboratory) due to the use of different fluke populations or genetic variation within populations? Potential variations between fluke populations need to be considered for any control strategy and, more relevantly, suggest that a multi-antigenic approach to vaccine development may have a better chance of succeeding.

9. Conclusion

Over the last few decades, much progress has been made in the isolation, characterisation and testing of a number of native and recombinant molecules as vaccines against liver fluke disease in ruminant hosts (Toet et al., 2014). Despite significant success, however, data between trials, even within the same laboratories, have shown a large degree of inconsistency. Much of this could be attributed to variations in a number of elements within the protocols employed, including vaccine schedules and formulations, age of animals, breed of animals, number of animals within groups, infection doses and infections regimes. Indeed, it is important that as we move forward within the research field, a standardised vaccine protocol is established to allow comparison of results between trials. For the same reason, as new immunological reagents become available to improve our analysis of ruminant immune responses, it is critical that we develop standard operation procedures (SOPs) for both sheep and cattle, and other species.

Fasciolosis is a hepatobiliary disease and, therefore, dysfunction of liver is the predominant physio-pathological

complication that affects the total health status of the animal. This damage is caused directly by the parasite's migratory activity and indirectly by the host's immune response to parasite-secreted molecules and tissue damage. Therefore, reduction of the magnitude of the pathological damage by vaccinations may be as significant as reducing worm burden. While fluke burden and liver damage are likely to be interrelated, judging vaccine efficacy solely by enumerating fluke burden may not provide an overall accurate assessment of the benefit of a vaccine. Many vaccine trial protocols involve sacrificing animals at the chronic stage of infection when the challenge parasites are in the bile ducts and easily recovered. However, at this time point, liver damage cannot be easily graded as it contains both acute- and chronic-associated damage. Perhaps, we should consider placing a greater importance on diagnostic methods of hepatic pathology, so that protection against liver disease at early stages of infection can be quantified: such as estimating liver enzymes in serum (aspartate transaminase: AST, alkaline phosphatase: ALP, gamma-glutamyl transferase: GGT and glutamate dehydrogenase: GLDH) or finding novel serum biomarkers, such as the study by Rioux et al. (2008) that indicates several serological markers could be used for assessing liver damage.

The pursuit of a *F. hepatica* vaccine needs to focus on understanding fluke biology, specifically the proteins involved in the tissue invasion and migration within the definitive host. It is our opinion that the most effective vaccine would be one that is directed against the early migratory stages of the parasite, including surface tegumental proteins/glycoproteins and secreted molecules, with the primary aim of preventing the penetration of the liver capsule by the parasites. However, other vaccine strategies may include targeting those molecules that suppress the development of Th1-mediated immune responses, those antigens that induce liver damage and/or attract the infiltration of immune cells or, indeed, the sensory molecules that facilitate the migration of the fluke from intestine to liver to bile duct. This presents us with the challenge of learning more about the early migratory stages of *F. hepatica*, a stage that has traditionally been neglected, particularly in ruminants, due to the difficulty of obtaining workable levels of parasite material. However, genomic, transcriptomic and proteomic methods have made the molecular dissection of this parasite stage possible and will facilitate the rational design of single and multiple antigen vaccine cocktails. It is imperative that we bolster this molecular progress with new methodologies and by combining robust immunological analysis of innate and adaptive responses with pathological analysis of the early stages of infection to understanding how and when the parasite initiates control of host immune responses.

Finally, translating experimental findings to the field will throw up many challenges including issues related to parasite polymorphism as described above. Also, developing effective vaccine formulations that will induce potent protection in the field with low antigen dose and the minimum number of injections (preferably two or less) will be required to make these commercially viable. Nevertheless, field trials will provide valuable information of vaccine performance (e.g., potency and longevity) at a 'herd' level where infection rates can vary significantly between animals and will help answer the critical question of whether vaccinating is more beneficial or financially worthwhile than not vaccinating to the end-user, i.e. farmers. Clearly, we are not there yet but the road ahead looks very promising.

Acknowledgements

V.M.H., K.C. and J.P.D. were funded by grants from the Ministère Économie, Innovation et Exportation (MEIE), Québec, award, a Marie Curie Intra European Fellowship within the 7th European Community Framework Programme (VMH) and a European Research Council Advanced Grant (HELIVAC, 322725) awarded to J.P.D. G.M., J.P., A.M.M. and J.P.D. are members of the FP7-funded Consortium PARAVAC.

References

Adams, L.G., Babiuk, L., McGavin, D., Nordgren, R., 2009. Special issues around veterinary vaccines. In: Barrett, A.D.T., Stanberry, L.R. (Eds.), Vaccines for Biodefense and Emerging and Neglected Diseases. Elsevier, pp. 225–254.

Adams, P.N., Aldridge, A., Vukman, K.V., Donnelly, S., O'Neill, S.M., 2014. Fasciola hepatica tegumental antigens indirectly induce an M2 macrophage-like phenotype in vivo. Parasite Immunol. 36 (10), 531–539, http://dx.doi.org/10.1111/pim.12127.

Afshan, K., Fortes-Lima, C.A., Artigas, P., Valero, A.M., Qayyum, M., Mas-Coma, S., 2014. Impact of climate change and man-made irrigation systems on the transmission risk, long-term trend and seasonality of human and animal fascioliasis in Pakistan. Geospat Health 8 (2), 317–334.

Aitken, M.M., Hughes, D.L., Jones, P.W., Hall, G.A., Smith, G.S., 1979. Immunological responses of fluke-infected and fluke-free cattle to *Salmonella dublin* and other antigens. Res. Vet. Sci. 27 (3), 306–312.

Aitken, M.M., Jones, P.W., Hall, G.A., Hughes, D.L., Brown, G.T., 1981. Responses of fluke-infected and fluke-free cattle to experimental reinfection with *Salmonella dublin*. Res. Vet. Sci. 31 (1), 120–126.

Anderson, S., Coulson, P.S., Ljubojevic, S., Mountford, A.P., Wilson, R.A., 1999. The radiation-attenuated schistosome vaccine induces high levels of protective immunity in the absence of B cells. Immunology 96 (1), 22–28, pii:imm661.

Andrews, S., 1999. The life cycle of *Fasciola hepatica*. In: Dalton, J.P. (Ed.), Fasciolosis. CABI Publishing, pp. 1–29.

Animal Health Ireland, 2011. Liver Fluke—The Facts, Available from http://www.animalhealthireland.ie/ckfinder/userfiles/files/Animal%20Health%20Liver%20Fluke%206pp-web%281%29.pdf

Behm, C., Sangster, N., 1999. Pathology, pathophysiology and clinical aspects. In: Dalton, J.P. (Ed.), Fasciolosis. CABI Publishing, pp. 185–224.

Bickle, Q.D., 2009. Radiation-attenuated schistosome vaccination—a brief historical perspective. Parasitology 136 (12), 1621–1632, http://dx.doi.org/10.1017/S0031182009005848.

Bloemhoff, Y., Danaher, M., Andrew, F., Morgan, E., Mulcahy, G., Power, C., Sayers, R., 2014. Parasite control practices on pasture-based dairy farms in the Republic of Ireland. Vet. Parasitol. 204 (3–4), 352–363, http://dx.doi.org/10.1016/j.vetpar.2014.05.029.

Boray, J., 1967. The effect of host reaction to experimental *Fasciola hepatica* infections to sheep and cattle. In: Soulsby, E. (Ed.), The Reaction of the Host to Parasitism. Proceedings of the Third International Conference of the World Association for the Advancement of Veterinary Parasitology. Elwertm Marburg, Germany, pp. 185–224.

Boray, J.C., 1969. Experimental fascioliasis in Australia. Adv. Parasitol. 7, 95–210.

Brady, M.T., O'Neill, S.M., Dalton, J.P., Mills, K.H., 1999. *Fasciola hepatica* suppresses a protective Th1 response against *Bordetella pertussis*. Infect. Immun. 67 (10), 5372–5378.

Cancela, M., Acosta, D., Rinaldi, G., Silva, E., Duran, R., Roche, L., Zaha, A., Carmona, C., Tort, J.F., 2008. A distinctive repertoire of cathepsins is expressed by juvenile invasive *Fasciola hepatica*. Biochimie 90 (10), 1461–1475, http://dx.doi.org/10.1016/j.biochi.2008.04.020.

Charlier, J., Vercruysse, J., Morgan, E., van Dijk, J., Williams, D.J., 2014. Recent advances in the diagnosis, impact on production and

prediction of *Fasciola hepatica* in cattle. Parasitology 141 (3), 326–335, http://dx.doi.org/10.1017/S0031182013001662.
Chauvin, A., Boulard, C., 1996. Local immune response to experimental Fasciola hepatica infection in sheep. Parasite 3 (3), 209–215.
Clery, D., Torgerson, P., Mulcahy, G., 1996. Immune responses of chronically infected adult cattle to *Fasciola hepatica*. Vet. Parasitol. 62 (1–2), 71–82, http://dx.doi.org/10.1016/0304-4017(95)00858-6.
Colley, D.G., Secor, W.E., 2014. Immunology of human schistosomiasis. Parasite Immunol. 36 (8), 347–357, http://dx.doi.org/10.1111/pim.12087.
Cooper, K.M., Kennedy, D.G., Danaher, M., 2012. Pro SafeBeef and anthelmintic drug residues—a case study in collaborative application of multi-analyte mass spectrometry to enhance consumer safety. Anal. Bioanal. Chem. 404 (6–7), 1623–1630, http://dx.doi.org/10.1007/s00216-012-6310-2.
Corvo, I., Cancela, M., Cappetta, M., Pi-Denis, N., Tort, J.F., Roche, L., 2009. The major cathepsin L secreted by the invasive juvenile *Fasciola hepatica* prefers proline in the S2 subsite and can cleave collagen. Mol. Biochem. Parasitol. 167 (1), 41–47, http://dx.doi.org/10.1016/j.molbiopara.2009.04.005.
Corvo, I., O'Donoghue, A.J., Pastro, L., Pi-Denis, N., Eroy-Reveles, A., Roche, L., McKerrow, J.H., Dalton, J.P., Craik, C.S., Caffrey, C.R., Tort, J.F., 2013. Dissecting the active site of the collagenolytic cathepsin L3 protease of the invasive stage of *Fasciola hepatica*. PLoS Negl. Trop. Dis. 7 (7), e2269, http://dx.doi.org/10.1371/journal.pntd.0002269.
Dalton, J.P., Mulcahy, G., 2001. Parasite vaccines—a reality? Vet. Parasitol. 98 (1–3), 149–167, http://dx.doi.org/10.1016/S0304-4017(01)00430-437.
Dalton, J.P., Robinson, M.W., Mulcahy, G., O'Neill, S.M., Donnelly, S., 2013. Immunomodulatory molecules of *Fasciola hepatica*: candidates for both vaccine and immunotherapeutic development. Vet. Parasitol. 195 (3–4), 272–285, http://dx.doi.org/10.1016/j.vetpar.2013.04.008.
Dalton, J.P., Caffrey, C., Sajid, M., Stack, C., Donnelly, S., Loukas, A., Don, T., McKerrow, J., Halton, D.W., Brindley, P.J., 2006. Proteases in trematode biology. In: Maule, A.G., Marks, N.J. (Eds.), Parasitic Flatworms: Molecular Biology, Biochemistry, Immunology and Physiology. CABI International, pp. 348–369.
Dargie, J.D., Armour, J., Rushton, B., Murray, M., 1974. Immune mechanisms and hepatic fibrosis in fascioliasis. In: Soulsby, E.J.L., Marks, N.J. (Eds.), Proceedings of the Sixth International Conference of the World Association for the Advancement of Veterinary Parasitology. Academic Press, New York, pp. 119–122.
Dawes, B., Hughes, D.L., 1964. Fascioliasis: the invasive stages of *Fasciola hepatica* in mammalian hosts. Adv. Parasitol. 2, 97–168.
Donnelly, S., O'Neill, S.M., Sekiya, M., Mulcahy, G., Dalton, J.P., 2005. Thioredoxin peroxidase secreted by *Fasciola hepatica* induces the alternative activation of macrophages. Infect. Immun. 73 (1), 166–173, http://dx.doi.org/10.1128/IAI.73.1.166-173.2005.
Donnelly, S., Stack, C.M., O'Neill, S.M., Sayed, A.A., Williams, D.L., Dalton, J.P., 2008. Helminth 2-Cys peroxiredoxin drives Th2 responses through a mechanism involving alternatively activated macrophages. FASEB J. 22 (11), 4022–4032, http://dx.doi.org/10.1096/fj.08-106278.
Dow, C., Ross, J.G., Todd, J.R., 1967. The pathology of experimental fascioliasis in calves. J. Comp. Pathol. 77 (4), 377–385.
Dowd, A.J., Smith, A.M., McGonigle, S., Dalton, J.P., 1994. Purification and characterisation of a second cathepsin L proteinase secreted by the parasitic trematode *Fasciola hepatica*. Eur. J. Biochem. 223 (1), 91–98.
Doy, T.G., Hughes, D.L., 1984. *Fasciola hepatica*: site of resistance to reinfection in cattle. Exp. Parasitol. 57 (3), 274–278.
Doyle, J.J., 1971. Acquired immunity to experimental infection with *Fasciola hepatica* in cattle. Res. Vet. Sci. 12 (6), 527–534.
Durbin, C.G., 1952. Longevity of the liver fluke, *Fasciola* sp. in sheep. In: Otto, G.F. (Ed.), Proceedings of the Helminthological Society of Washington. The Helminthological Society of Washington, p. 120.
Eberl, M., Langermans, J.A., Frost, P.A., Vervenne, R.A., van Dam, G.J., Deelder, A.M., Thomas, A.W., Coulson, P.S., Wilson, R.A., 2001. Cellular and humoral immune responses and protection against schistosomes induced by a radiation-attenuated vaccine in chimpanzees. Infect. Immun. 69 (9), 5352–5362.
EMA, 2012. European public MRL assessment report (EPMAR): Triclabendazole (extrapolation to bovine and ovine milk). Available from http://www.ema.europa.eu/docs/en_GB/document_library/Maximum_Residue_Limits_-_Report/2012/03/WC500124511.pdf
FABRE Technology Platform Working Group. Sustainable Farm Animal Breeding and Reproduction. A Vision for 2025. Available from www.fabretp.org
Fairweather, I., 2011. Reducing the future threat from (liver) fluke: realistic prospect or quixotic fantasy? Vet. Parasitol. 180 (1–2), 133–143, http://dx.doi.org/10.1016/j.vetpar.2011.05.034.
Fairweather, I., Boray, J.C., 1999. Fasciolicides: efficacy, actions, resistance and its management. Vet. J. 158 (2), 81–112, http://dx.doi.org/10.1053/tvjl.1999.0377.
Figueroa-Santiago, O., Espino, A.M., 2014. *Fasciola hepatica* fatty acid binding protein induces the alternative activation of human macrophages. Infect. Immun., http://dx.doi.org/10.1128/IAI.02541-14.
Flynn, R.J., Mannion, C., Golden, O., Hacariz, O., Mulcahy, G., 2007. Experimental *Fasciola hepatica* infection alters responses to tests used for diagnosis of bovine tuberculosis. Infect. Immun. 75 (3), 1373–1381, http://dx.doi.org/10.1128/IAI.01445-06.
Flynn, R.J., Mulcahy, G., Welsh, M., Cassidy, J.P., Corbett, D., Milligan, C., Andersen, P., Strain, S., McNair, J., 2009. Co-infection of cattle with *Fasciola hepatica* and *Mycobacterium bovis*—immunological consequences. Transbound Emerg. Dis. 56 (6–7), 269–274, http://dx.doi.org/10.1111/j.1865-1682.2009.01075.x.
Foresight, 2011. The Future of Food and Farming. Final Project Report. The Government Office for Science, London, pp. 1–211.
Forrester, S.J., Hall, N., 2014. The revolution of whole genome sequencing to study parasites. Mol. Biochem. Parasitol. 195 (2), 77–81, http://dx.doi.org/10.1016/j.molbiopara.2014.07.008.
Gobert, G.N., You, H., McManus, D.P., 2014. Gaining biological perspectives from schistosome genomes. Mol. Biochem. Parasitol. 196 (1), 21–28, http://dx.doi.org/10.1016/j.molbiopara.2014.07.007.
Golbar, H.M., Izawa, T., Juniantito, V., Ichikawa, C., Tanaka, M., Kuwamura, M., Yamate, J., 2013. Immunohistochemical characterization of macrophages and myofibroblasts in fibrotic liver lesions due to *Fasciola infection* in cattle. J. Vet. Med. Sci. 75 (7), 857–865, http://dx.doi.org/10.1292/jvms.12-0536.
Gonzalez, L.C., Esteban, J.G., Bargues, M.D., Valero, M.A., Ortiz, P., Naquira, C., Mas-Coma, S., 2011. Hyperendemic human fascioliasis in Andean valleys: an altitudinal transect analysis in children of Cajamarca province. Peru. Acta Trop. 120 (1–2), 119–129, http://dx.doi.org/10.1016/j.actatropica.2011.07.002.
Guasconi, L., Serradell, M.C., Masih, D.T., 2012. *Fasciola hepatica* products induce apoptosis of peritoneal macrophages. Vet. Immunol. Immunopathol. 148 (3–4), 359–363, http://dx.doi.org/10.1016/j.vetimm.2012.06.022.
Hall, G.A., Hughes, D.L., Jones, P.W., Aitken, M.M., Parsons, K.R., Brown, G.T., 1981. Experimental oral *Salmonella dublin* infection in cattle: effects of concurrent infection with *Fasciola hepatica*. J. Comp. Pathol. 91 (2), 227–233, http://dx.doi.org/10.1016/0021-9975(81)90027-X.
Hoyle, D.V., Dalton, J.P., Chase-Topping, M., Taylor, D.W., 2003. Pre-exposure of cattle to drug-abbreviated *Fasciola hepatica* infections: the effect upon subsequent challenge infection and the early immune response. Vet. Parasitol. 111 (1), 65–82, http://dx.doi.org/10.1016/S0304-4017(02)00326-6.
HRPA, 2013. Use of Flukicides in Dairy Cows, Available from http://www.hpra.ie/homepage/veterinary/safety-information/safety-notices/item?t=/use-of-flukicides-in-dairy-cows&id=b1d4f825-9782-6eee-9b55-ff00008c97d0
Huang, Y., Chen, W., Wang, X., Liu, H., Chen, Y., Guo, L., Luo, F., Sun, J., Mao, Q., Liang, P., Xie, Z., Zhou, C., Tian, Y., Lv, X., Huang, L., Zhou, J., Hu, Y., Li, R., Zhang, F., Lei, H., Li, W., Hu, X., Liang, C., Xu, J., Li, X., Yu, X., 2013. The carcinogenic liver fluke, *Clonorchis sinensis*: new assembly, reannotation and analysis of the genome and characterization of tissue transcriptomes. PLoS ONE 8 (1), e54732, http://dx.doi.org/10.1371/journal.pone.0054732.
Hughes, D.L., 1987. Fasciola and Fascioloides. In: Soulsby, E. (Ed.), Immune Responses in Parasitic Infections: Immunology, Immunopathology and Immunoprophylaxis. Volume II. Trematodes and Cestodes. CRC Press, Boca Raton, pp. 91–114.
Jayaraj, R., Piedrafita, D., Dynon, K., Grams, R., Spithill, T.W., Smooker, P.M., 2009. Vaccination against fasciolosis by a multivalent vaccine of stage-specific antigens. Vet. Parasitol. 160 (3–4), 230–236, http://dx.doi.org/10.1016/j.vetpar.2008.10.099.
Jefferies, J.R., Campbell, A.M., van Rossum, A.J., Barrett, J., Brophy, P.M., 2001. Proteomic analysis of *Fasciola hepatica* excretory-secretory products. Proteomics 1 (9), 1128–1132, http://dx.doi.org/10.1002/1615-9861(200109)1:9<1128::AID-PROT1128>3.0.CO;2-0.
Kelly, W., 1993. The liver and biliary system. In: Jubb, K., Kennedy, P., Palmer, N. (Eds.), Pathology of Domestic Animals. Academic Press, San Diego, CA, pp. 319–406.
Kendall, S., Parfitt, J., 1962. The chemotherapy of fascioliasis. British Vet. J. 118, 1–10.
Kenyon, F., Sargison, N.D., Skuce, P.J., Jackson, F., 2009. Sheep helminth parasitic disease in south eastern Scotland arising as a possible consequence of climate change. Vet. Parasitol. 163 (4), 293–297, http://dx.doi.org/10.1016/j.vetpar.2009.03.027.
Knubben-Schweizer, G., Ruegg, S., Torgerson, P.R., Rapsch, C., Grimm, F., Hassig, M., Deplazes, P., Braun, U., 2010. Control of bovine fasciolosis

in dairy cattle in Switzerland with emphasis on pasture management. Vet. J. 186 (2), 188–191, http://dx.doi.org/10.1016/j.tvjl.2009.08.003.
Kotze, C., Hunt, P.W., Skuce, P., von Samson-Himmelstjerna, G., Martin, R.J., Sager, H., Krücken, J., Hodgkinson, J., Lespine, A., Jex, A.R., Gilleard, J.S., Beech, R.N., Wolstenholme, A.J., Demeler, J., Robertson, A.P., Charvet, C.L., Neveu, C., Kaminsky, R., Rufener, L., Alberich, M., Menez, C., Prichard, R.K., 2014. Recent advances in candidate-gene and whole-genome approaches to the discovery of anthelmintic resistance markers and the description of drug/receptor interactions. Int. J. Parasitol: Drugs Drug Resist. 4, 164–184, http://dx.doi.org/10.1016/j.ijpddr.2014.07.007.
LaCourse, E.J., Perally, S., Morphew, R.M., Moxon, J.V., Prescott, M., Dowling, D.J., O'Neill, S.M., Kipar, A., Hetzel, U., Hoey, E., Zafra, R., Buffoni, L., Perez Arevalo, J., Brophy, P.M., 2012. The sigma class glutathione transferase from the liver fluke *Fasciola hepatica*. PLoS Negl. Trop. Dis. 6 (5), e1666, http://dx.doi.org/10.1371/journal.pntd.0001666.
LeJambre, L.F., Windon, R.G., Smith, W.D., 2008. Vaccination against *Haemonchus contortus*: performance of native parasite gut membrane glycoproteins in Merino lambs grazing contaminated pasture. Vet. Parasitol. 153 (3–4), 302–312, http://dx.doi.org/10.1016/j.vetpar.2008.01.032.
Lowther, J., Robinson, M.W., Donnelly, S.M., Xu, W., Stack, C.M., Matthews, J.M., Dalton, J.P., 2009. The importance of pH in regulating the function of the *Fasciola hepatica* cathepsin L1 cysteine protease. PLoS Negl. Trop. Dis. 3 (1), e369, http://dx.doi.org/10.1371/journal.pntd.0000369.
Marcilla, A., Trelis, M., Cortes, A., Sotillo, J., Cantalapiedra, F., Minguez, M.T., Valero, M.L., Sanchez del Pino, M.M., Munoz-Antoli, C., Toledo, R., Bernal, D., 2012. Extracellular vesicles from parasitic helminths contain specific excretory/secretory proteins and are internalized in intestinal host cells. PLoS ONE 7 (9), e45974, http://dx.doi.org/10.1371/journal.pone.0045974.
Marcos, L.A., Yi, P., Machicado, A., Andrade, R., Samalvides, F., Sanchez, J., Terashima, A., 2007. Hepatic fibrosis and *Fasciola hepatica* infection in cattle. J. Helminthol. 81 (4), 381–386, http://dx.doi.org/10.1017/S0022149X07850231.
Martinez-Moreno, A., Jimenez-Luque, V., Moreno, T., Redondo, E.S., de las Mulas, J.M., Perez, J., 1999. Liver pathology and immune response in experimental *Fasciola hepatica* infections of goats. Vet. Parasitol. 82 (1), 19–33, http://dx.doi.org/10.1016/S0304-4017(98)00262-3.
Mas-Coma, S., 2005. Epidemiology of fascioliasis in human endemic areas. J. Helminthol. 79 (3), 207–216.
Mas-Coma, S., Bargues, M.D., Valero, M.A., 2005. Fascioliasis and other plant-borne trematode zoonoses. Int. J. Parasitol. 35 (11–12), 1255–1278, http://dx.doi.org/10.1016/j.ijpara.2005.07.010.
Mas-Coma, S., Valero, M.A., Bargues, M.D., 2009. Chapter 2. Fasciola, lymnaeids and human fascioliasis, with a global overview on disease transmission, epidemiology, evolutionary genetics, molecular epidemiology and control. Adv. Parasitol. 69, 41–146, http://dx.doi.org/10.1016/S0065-308X(09)69002-3.
McCann, C.M., Baylis, M., Williams, D.J., 2010. Seroprevalence and spatial distribution of *Fasciola hepatica*-infected dairy herds in England and Wales. Vet. Rec. 166 (20), 612–617, http://dx.doi.org/10.1136/vr.b4836.
McGonigle, L., Mousley, A., Marks, N.J., Brennan, G.P., Dalton, J.P., Spithill, T.W., Day, T.A., Maule, A.G., 2008. The silencing of cysteine proteases in *Fasciola hepatica* newly excysted juveniles using RNA interference reduces gut penetration. Int. J. Parasitol. 38 (2), 149–155, http://dx.doi.org/10.1016/j.ijpara.2007.10.007.
McKeand, J.B., 2000. Vaccine development and diagnostics of *Dictyocaulus viviparus*. Parasitology 120 (Suppl.), S17–S23.
McSorley, H.J., Maizels, R.M., 2012. Helminth infections and host immune regulation. Clin. Microbiol. Rev. 25 (4), 585–608, http://dx.doi.org/10.1128/CMR.05040-11.
McVeigh, P., McCammick, E.M., McCusker, P., Morphew, R.M., Mousley, A., Abidi, A., Saifullah, K.M., Muthusamy, R., Gopalakrishnan, R., Spithill, T.W., Dalton, J.P., Brophy, P.M., Marks, N.J., Maule, A.G., 2014. RNAi dynamics in juvenile *Fasciola* spp. liver flukes reveals the persistence of gene silencing in vitro. PLoS Negl. Trop Dis. 8 (9), e3185, http://dx.doi.org/10.1371/journal.pntd.0003185.
Meeusen, E., Lee, C.S., Rickard, M.D., Brandon, M.R., 1995. Cellular responses during liver fluke infection in sheep and its evasion by the parasite. Parasite Immunol. 17 (1), 37–45.
Mendes, R.E., Perez-Ecija, R.A., Zafra, R., Buffoni, L., Martinez-Moreno, A., Dalton, J.P., Mulcahy, G., Perez, J., 2010. Evaluation of hepatic changes and local and systemic immune responses in goats immunized with recombinant Peroxiredoxin (Prx) and challenged with *Fasciola hepatica*. Vaccine 28 (16), 2832–2840, http://dx.doi.org/10.1016/j.vaccine.2010.01.055.
Mezo, M., Gonzalez-Warleta, M., Castro-Hermida, J.A., Ubeira, F.M., 2008. Evaluation of the flukicide treatment policy for dairy cattle in Galicia (NW Spain). Vet. Parasitol. 157 (3–4), 235–243, http://dx.doi.org/10.1016/j.vetpar.2008.07.032.
Morgan, E.R., Charlier, J., Hendrickx, G., Biggeri, A., Catalan, D., von Samson-Himmelstjerna, G., Demeler, J., Müller, E., van Dijk, J., Kenyon, F., Skuce, P., Höglund, J., O'Kiely, P., van Ranst, B., de Waal, T., Rinaldi, L., Cringoli, G., Hertzberg, H., Torgerson, P., Wolstenholme, A., Vercruysse, J., 2013. Global change and helminth infections in grazing ruminants in Europe: impacts, trends and sustainable solutions. Agriculture 3, 484–502, http://dx.doi.org/10.3390/agriculture3030484.
Morphew, R.M., Wright, H.A., LaCourse, E.J., Woods, D.J., Brophy, P.M., 2007. Comparative proteomics of excretory-secretory proteins released by the liver fluke *Fasciola hepatica* in sheep host bile and during in vitro culture ex host. Mol. Cell. Proteom. 6 (6), 963–972, http://dx.doi.org/10.1074/mco.M600375-MCP200.
Mulcahy, G., O'Connor, F., Clery, D., Hogan, S.F., Dowd, A.J., Andrews, S.J., Dalton, J.P., 1999. Immune responses of cattle to experimental anti-*Fasciola hepatica* vaccines. Res. Vet. Sci. 67 (1), 27–33, http://dx.doi.org/10.1053/rvsc.1998.0270.
Mulcahy, G., O'Connor, F., McGonigle, S., Dowd, A., Clery, D.G., Andrews, S.J., Dalton, J.P., 1998. Correlation of specific antibody titre and avidity with protection in cattle immunized against *Fasciola hepatica*. Vaccine 16 (9–10), 932–939, http://dx.doi.org/10.1016/S0264-410X(97)00289-2.
Murphy, T.M., Fahy, K.N., McAuliffe, A., Forbes, A.B., Clegg, T.A., O'Brien, D.J., 2006. A study of helminth parasites in culled cows from Ireland. Prev. Vet. Med. 76 (1–2), 1–10, http://dx.doi.org/10.1016/j.prevetmed.2006.04.005.
Nascimento, E., Leao, I.C., Pereira, V.R., Gomes, Y.M., Chikhlikar, P., August, T., Marques, E., Lucena-Silva, N., 2002. Protective immunity of single and multi-antigen DNA vaccines against schistosomiasis. Mem. Inst. Oswaldo Cruz 97 (Suppl. 1), 105–109.
Nieuwhof, G.J., Bishop, S.C., 2005. Costs of the major endemic diseases of sheep in Great Britain and the potential benefits of reduction in disease impact. Anim. Sci. 81 (1), 23–29, http://dx.doi.org/10.1079/ASC41010023.
Nisbet, A.J., McNeilly, T.N., Wildblood, L.A., Morrison, A.A., Bartley, D.J., Bartley, Y., Longhi, C., McKendrick, I.J., Palarea-Albaladejo, J., Matthews, J.B., 2013. Successful immunization against a parasitic nematode by vaccination with recombinant proteins. Vaccine 31 (37), 4017–4023, http://dx.doi.org/10.1016/j.vaccine.2013.05.026.
O'Neill, S.M., Brady, M.T., Callanan, J.J., Mulcahy, G., Joyce, P., Mills, K.H., Dalton, J.P., 2000. Fasciola hepatica infection downregulates Th1 responses in mice. Parasite Immunol. 22 (3), 147–155, http://dx.doi.org/10.1046/j.1365-3024.2000.00290.x.
Parker, R., Deville, S., Dupuis, L., Bertrand, F., Aucouturier, J., 2009. Adjuvant formulation for veterinary vaccines: Montanide™ Gel safety profile. Proc. Vaccinol. 1 (1), 140–147, http://dx.doi.org/10.1016/j.provac.2009.07.026.
Perez, J., Martin de las Mulas, J., Carrasco, L., Gutierrez, P.N., Martinez-Cruz, M.S., Martinez-Moreno, A., 1999. Pathological and immunohistochemical study of the liver and hepatic lymph nodes in goats infected with one or more doses of *Fasciola hepatica*. J. Comp. Pathol. 120 (2), 199–210, S0021997598902717 [pii].
Perez, J., Ortega, J., Bravo, A., Diez-Banos, P., Morrondo, P., Moreno, T., Martinez-Moreno, A., 2005. Phenotype of hepatic infiltrates and hepatic lymph nodes of lambs primarily and challenge infected with *Fasciola hepatica*, with and without triclabendazole treatment. Vet. Res. 36 (1), 1–12, http://dx.doi.org/10.1051/vetres:2004047.
Perez, J., Ortega, J., Moreno, T., Morrondo, P., Lopez-Sandez, C., Martinez-Moreno, A., 2002. Pathological and immunohistochemical study of the liver and hepatic lymph nodes of sheep chronically reinfected with *Fasciola hepatica*, with or without triclabendazole treatment. J. Comp. Pathol. 127 (1), 30–36, http://dx.doi.org/10.1053/jcpa.2002.0561.
Perez-Ecija, R.A., Mendes, R.E., Zafra, R., Buffonni, L., Martinez-Moreno, A., Perez, J., 2010. Pathological and parasitological protection in goats immunised with recombinant cathepsin L1 and challenged with *Fasciola hepatica*. Vet. J. 185 (3), 351–353, http://dx.doi.org/10.1016/j.tvjl.2009.07.004.
Piacenza, L., Acosta, D., Basmadjian, I., Dalton, J.P., Carmona, C., 1999. Vaccination with cathepsin L proteinases and with leucine aminopeptidase induces high levels of protection against fascioliasis in sheep. Infect. Immun. 67 (4), 1954–1961.
Piedrafita, D., Estuningsih, E., Pleasance, J., Prowse, R., Raadsma, H.W., Meeusen, E.N., Spithill, T.W., 2007. Peritoneal lavage cells of Indonesian thin-tail sheep mediate antibody-dependent superoxide radical cytotoxicity in vitro against newly excysted juvenile *Fasciola gigantica* but not juvenile *Fasciola hepatica*. Infect. Immun. 75 (4), 1954–1963.
Piedrafita, D., Raadsma, H.W., Prowse, R., Spithill, T.W., 2004. Immunology of the host-parasite relationship in fasciolosis

(*Fasciola hepatica* and *Fasciola gigantica*). Can. J. Zool. 82, 233–250, http://dx.doi.org/10.1139/Z03-216.
Piedrafita, D., Spithill, T.W., Dalton, J.P., Brindley, P.J., Sandeman, M.R., Wood, P.R., Parsons, J.C., 2000. Juvenile *Fasciola hepatica* are resistant to killing in vitro by free radicals compared with larvae of *Schistosoma mansoni*. Parasite Immunol. 22 (6), 287–295, http://dx.doi.org/10.1046/j.1365-3024.2000.00300.x.
Piedrafita, D., Spithill, T.W., Smith, R.E., Raadsma, H.W., 2010. Improving animal and human health through understanding liver fluke immunology. Parasite Immunol. 32 (8), 572–581, http://dx.doi.org/10.1111/j.1365-3024.2010.01223.x.
Power, C., Danaher, M., Sayers, R., O'Brien, B., Clancy, C., Furey, A., Jordan, K., 2013. Investigation of the migration of triclabendazole residues to milk products manufactured from bovine milk, and stability therein, following lactating cow treatment. J. Dairy Sci. 96 (10), 6223–6232, http://dx.doi.org/10.3168/jds.2013-7016.
Prasanphanich, N.S., Mickum, M.L., Heimburg-Molinaro, J., Cummings, R.D., 2013. Glycoconjugates in host-helminth interactions. Front. Immunol. 4, 240, http://dx.doi.org/10.3389/fimmu.2013.00240.
Pritchard, G.C., Forbes, A.B., Williams, D.J., Salimi-Bejestani, M.R., Daniel, R.G., 2005. Emergence of fasciolosis in cattle in East Anglia. Vet. Rec. 157 (19), 578–582.
Rickard, M.D., Howell, M.J., 1982. Comparative aspects of immunity in fascioliasis and cysticercosis in domesticated animals. In: Symons, L.E.A., Donald, A.D., Dineen, J.K. (Eds.), Biology and Control of Ectoparasites. Academic Press, Sydney, pp. 343–374.
Rinaldi, G., Morales, M.E., Cancela, M., Castillo, E., Brindley, P.J., Tort, J.F., 2008. Development of functional genomic tools in trematodes: RNA interference and luciferase reporter gene activity in *Fasciola hepatica*. PLoS Negl. Trop. Dis. 2 (7), e260, http://dx.doi.org/10.1371/journal.pntd.0000260.
Rioux, M.C., Carmona, C., Acosta, D., Ward, B., Ndao, M., Gibbs, B.F., Bennett, H.P., Spithill, T.W., 2008. Discovery and validation of serum biomarkers expressed over the first twelve weeks of *Fasciola hepatica* infection in sheep. Int. J. Parasitol. 38 (1), 123–136, http://dx.doi.org/10.1016/j.ijpara.2007.07.017.
Robinson, M.W., Alvarado, R., To, J., Hutchinson, A.T., Dowdell, S.N., Lund, M., Turnbull, L., Whitchurch, C.B., O'Brien, B.A., Dalton, J.P., Donnelly, S., 2012. A helminth cathelicidin-like protein suppresses antigen processing and presentation in macrophages via inhibition of lysosomal vATPase. FASEB J. 26 (11), 4614–4627, http://dx.doi.org/10.1096/fj.12-213876.
Robinson, M.W., Corvo, I., Jones, P.M., George, A.M., Padula, M.P., To, J., Cancela, M., Rinaldi, G., Tort, J.F., Roche, L., Dalton, J.P., 2011a. Collagenolytic activities of the major secreted cathepsin L peptidases involved in the virulence of the helminth pathogen *Fasciola hepatica*. PLoS Negl. Trop. Dis. 5 (4), e1012, http://dx.doi.org/10.1371/journal.pntd.0001012.
Robinson, M.W., Dalton, J.P., Donnelly, S., 2008a. Helminth pathogen cathepsin proteases: it's a family affair. Trends Biochem. Sci. 33 (12), 601–608, http://dx.doi.org/10.1016/j.tibs.2008.09.001.
Robinson, M.W., Donnelly, S., Hutchinson, A.T., To, J., Taylor, N.L., Norton, R.S., Perugini, M.A., Dalton, J.P., 2011b. A family of helminth molecules that modulate innate cell responses via molecular mimicry of host antimicrobial peptides. PLoS Pathog. 7 (5), e1002042, http://dx.doi.org/10.1371/journal.ppat.1002042.
Robinson, M.W., Menon, R., Donnelly, S.M., Dalton, J.P., Ranganathan, S., 2009. An integrated transcriptomics and proteomics analysis of the secretome of the helminth pathogen *Fasciola hepatica*: proteins associated with invasion and infection of the mammalian host. Mol. Cell. Proteomics 8 (8), 1891–1907, http://dx.doi.org/10.1074/mcp.M900045-MCP200.
Robinson, M.W., Tort, J.F., Lowther, J., Donnelly, S.M., Wong, E., Xu, W., Stack, C.M., Padula, M., Herbert, B., Dalton, J.P., 2008b. Proteomics and phylogenetic analysis of the cathepsin L protease family of the helminth pathogen *Fasciola hepatica*: expansion of a repertoire of virulence-associated factors. Mol. Cell. Proteomics 7 (6), 1111–1123, http://dx.doi.org/10.1074/mcp.M700560-MCP200.
Ross, J.G., 1967. Studies of immunity to *Fasciola hepatica*: acquired immunity in cattle, sheep and rabbits following natural infection and vaccine procedures. J. Helminthol. 41 (4), 393–399.
Rushton, B., Murray, M., 1977. Hepatic pathology of a primary experimental infection of Fasciola hepatica in sheep. J. Comp. Pathol. 87 (3), 459–470, http://dx.doi.org/10.1016/0021-9975(77)90035-4.
Sangster, N.C., 2001. Managing parasiticide resistance. Vet. Parasitol. 98 (1–3), 89–109, http://dx.doi.org/10.1016/S0304-4017(01)00425-3.
Selzer, P.M., 2009. Preface. In: Antiparasitic and Antibacterial Drug Discovery from Molecular Targets to Drug Candidates. Wiley-Blackwell, Hoboken, USA, pp. 11–12.
Serradell, M.C., Guasconi, L., Cervi, L., Chiapello, L.S., Masih, D.T., 2007. Excretory-secretory products from *Fasciola hepatica* induce eosinophil apoptosis by a caspase-dependent mechanism. Vet. Immunol. Immunopathol. 117 (3–4), 197–208, http://dx.doi.org/10.1016/j.vetimm.2007.03.007.
Sinclair, K., 1967. Pathogenesis of Fasciola and other liver flukes. Helminth. Abstr. 3, 115–134.
Smith, A.M., Dowd, A.J., McGonigle, S., Keegan, P.S., Brennan, G., Trudgett, A., Dalton, J.P., 1993. Purification of a cathepsin L-like proteinase secreted by adult *Fasciola hepatica*. Mol. Biochem. Parasitol. 62 (1), 1–8, http://dx.doi.org/10.1016/0166-6851(93)90171-S.
Smith, W.D., van Wyk, J.A., van Strijp, M.F., 2001. Preliminary observations on the potential of gut membrane proteins of *Haemonchus contortus* as candidate vaccine antigens in sheep on naturally infected pasture. Vet. Parasitol. 98 (4), 285–297, http://dx.doi.org/10.1016/S0304-4017(01)00400-9.
Spithill, T.W., Smooker, P.M., Copeman, D.B., 1999a. *Fasciola gigantica*: epidemiology, control, immunology and molecular biology. In: Dalton, J.P. (Ed.), Fasciolosis. CABI Publishing, pp. 465–525.
Spithill, T.W., Smooker, P.M., Sexton, J.L., Bozas, E., Morrison, C.A., Creaney, J., Parsons, J.C., 1999b. Development of vaccines against *Fasciola hepatica*. In: Dalton, J.P. (Ed.), Fasciolosis. CABI Publishing, pp. 377–410.
Sukhdeo, M.V., Sukhdeo, S.C., 2002. Fixed behaviours and migration in parasitic flatworms. Int. J. Parasitol. 32 (3), 329–342, http://dx.doi.org/10.1016/S0020-7519(01)00334-4.
Thivierge, K., Cotton, S., Schaefer, D.A., Riggs, M.W., To, J., Lund, M.E., Robinson, M.W., Dalton, J.P., Donnelly, S.M., 2013. Cathelicidin-like helminth defence molecules (HDMs): absence of cytotoxic, anti-microbial and anti-protozoan activities imply a specific adaptation to immune modulation. PLoS Negl. Trop Dis. 7 (7), e2307, http://dx.doi.org/10.1371/journal.pntd.0002307.
Tliba, O., Sibille, P., Boulard, C., Chauvin, A., 2000. Local hepatic immune response in rats during primary infection with *Fasciola hepatica*. Parasite 7 (1), 9–18, http://dx.doi.org/10.1051/parasite/2000071009.
Toet, H., Piedrafita, D.M., Spithill, T.W., 2014. Liver fluke vaccines in ruminants: strategies, progress and future opportunities. Int. J. Parasitol. 44 (12), 915–927, http://dx.doi.org/10.1016/j.ijpara.2014.07.011, pii:S0020-7519(14)00197-0.
Vukman, K.V., Adams, P.N., Metz, M., Maurer, M., O'Neill, S.M., 2013. *Fasciola hepatica* tegumental coat impairs mast cells' ability to drive Th1 immune responses. J. Immunol. 190 (6), 2873–2879, http://dx.doi.org/10.4049/jimmunol.1203011.
Walsh, K.P., Brady, M.T., Finlay, C.M., Boon, L., Mills, K.H., 2009. Infection with a helminth parasite attenuates autoimmunity through TGF-beta-mediated suppression of Th17 and Th1 responses. J. Immunol. 183 (3), 1577–1586, http://dx.doi.org/10.4049/jimmunol.0803803.
Wilson, R.A., Wright, J.M., de Castro-Borges, W., Parker-Manuel, S.J., Dowle, A.A., Ashton, P.D., Young, N.D., Gasser, R.B., Spithill, T.W., 2011. Exploring the *Fasciola hepatica* tegument proteome. Int. J. Parasitol. 41 (13–14), 1347–1359, http://dx.doi.org/10.1016/j.ijpara.2011.08.003.
Young, N.D., Nagarajan, N., Lin, S.J., Korhonen, P.K., Jex, A.R., Hall, R.S., Safavi-Hemami, H., Kaewkong, W., Bertrand, D., Gao, S., Seet, Q., Wongkham, S., Teh, B.T., Wongkham, C., Intapan, P.M., Maleewong, W., Yang, X., Hu, M., Wang, Z., Hofmann, A., Sternberg, P.W., Tan, P., Wang, J., Gasser, R.B., 2014. The *Opisthorchis viverrini* genome provides insights into life in the bile duct. Nat. Commun. 5, 4378, http://dx.doi.org/10.1038/ncomms5378.
Zafra, R., Buffoni, L., Martinez-Moreno, A., Perez-Ecija, A., Martinez-Moreno, F.J., Perez, J., 2008. A study of the liver of goats immunized with a synthetic peptide of the Sm14 antigen and challenged with *Fasciola hepatica*. J. Comp. Pathol. 139 (4), 169–176, http://dx.doi.org/10.1016/j.jcpa.2008.06.004.
Zafra, R., Buffoni, L., Perez-Ecija, R.A., Mendes, R.E., Martinez-Moreno, A., Martinez-Moreno, F.J., Perez, J., 2009. Study of the local immune response to *Fasciola hepatica* in the liver and hepatic lymph nodes of goats immunised with a peptide of the Sm14 antigen. Res. Vet. Sci. 87 (2), 226–232, http://dx.doi.org/10.1016/j.rvsc.2009.02.013.
Zafra, R., Perez-Ecija, R.A., Buffoni, L., Moreno, P., Bautista, M.J., Martinez-Moreno, A., Mulcahy, G., Dalton, J.P., Perez, J., 2013a. Early and late peritoneal and hepatic changes in goats immunized with recombinant cathepsin L1 and infected with *Fasciola hepatica*. J. Comp. Pathol. 148 (4), 373–384, http://dx.doi.org/10.1016/j.jcpa.2012.08.007.
Zafra, R., Perez-Ecija, R.A., Buffoni, L., Pacheco, I.L., Martinez-Moreno, A., LaCourse, E.J., Perally, S., Brophy, P.M., Perez, J., 2013b. Early hepatic and peritoneal changes and immune response in goats vaccinated with a recombinant glutathione transferase sigma class and challenged with *Fasciola hepatica*. Res. Vet. Sci. 94 (3), 602–609, http://dx.doi.org/10.1016/j.rvsc.2012.10.026.