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Refugia and anthelmintic resistance: Concepts and challenges

Jane E. Hodgkinson[a], Ray M. Kaplan[b], Fiona Kenyon[c], Eric R. Morgan[d], Andrew W. Park[b,e], Steve Paterson[f], Simon A. Babayan[g], Nicola J. Beesley[a], Collette Britton[g], Umer Chaudhry[h], Stephen R. Doyle[i], Vanessa O. Ezenwa[b,e], Andy Fenton[f], Sue B. Howell[b], Roz Laing[g], Barbara K. Mable[g], Louise Matthews[g], Jennifer McIntyre[g], Catherine E. Milne[j], Thomas A. Morrison[g], Jamie C. Prentice[g], Neil D. Sargison[h], Diana J.L. Williams[a], Adrian J. Wolstenholme[b], Eileen Devaney[g,*]

[a] Institute of Infection and Global Health, University of Liverpool, Liverpool, L69 7ZJ, UK
[b] Department of Infectious Diseases, College of Veterinary Medicine, University of Georgia, Athens, GA, 30602, USA
[c] Moredun Research Institute, Pentlands Science Park, Edinburgh, EH26 0PZ, UK
[d] School of Biological Sciences, Queen's University Belfast, Chlorine Gardens, Belfast, BT9 5BL, UK
[e] Odum School of Ecology, University of Georgia, Athens, GA, 30602, USA
[f] Institute of Integrative Biology, University of Liverpool, L69 7ZB, UK
[g] Institute of Biodiversity, Animal Health and Comparative Medicine, University of Glasgow, Glasgow, G61 1QH, UK
[h] Royal (Dick) School of Veterinary Studies, Easter Bush Veterinary Centre, Roslin, EH25 9RG, UK
[i] Wellcome Sanger Institute, Hinxton, Cambridgeshire, CB10 1SA, UK
[j] SRUC, Peter Wilson Building, West Mains Road, Edinburgh, EH9 3JG, UK

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ABSTRACT

Anthelmintic resistance is a threat to global food security. In order to alleviate the selection pressure for resistance and maintain drug efficacy, management strategies increasingly aim to preserve a proportion of the parasite population in 'refugia', unexposed to treatment. While persuasive in its logic, and widely advocated as best practice, evidence for the ability of refugia-based approaches to slow the development of drug resistance in parasitic helminths is currently limited. Moreover, the conditions needed for refugia to work, or how transferable those are between parasite-host systems, are not known. This review, born of an international workshop, seeks to deconstruct the concept of refugia and examine its assumptions and applicability in different situations. We conclude that factors potentially important to refugia, such as the fitness cost of drug resistance, the degree of mixing between parasite sub-populations selected through treatment or not, and the impact of parasite life-history, genetics and environment on the population dynamics of resistance, vary widely between systems. The success of attempts to generate refugia to limit anthelmintic drug resistance are therefore likely to be highly dependent on the system in hand. Additional research is needed on the concept of refugia and the underlying principles for its application across systems, as well as empirical studies within systems that prove and optimise its usefulness.

1. Introduction

The term 'refugium' is classically defined as an area in which a population of organisms can survive through a period of unfavourable conditions. In the context of drug resistance in animal parasites, a refugium refers to untreated hosts or environments that allow the maintenance of drug sensitive parasites in the face of drug exposure. Refugia-based control for livestock parasites has gained increasing traction in the last 20 years, starting with the 'call to arms' by van Wyk (2001), who proposed that refugia should be incorporated more widely into rational anthelmintic use as a means of slowing the spread of resistance. In practice, this frequently relies upon treatment of only a proportion of animals, rather than the whole group, leaving some part of the parasite population untreated and thus free from the selection pressure applied by exposure to drug. At a recent BBSRC-funded meeting in Scotland, an international group of scientists met to discuss the theoretical principles under-pinning refugia-based control strategies and their practical application in the management of drug resistance in

* Corresponding author.
E-mail address: eileen.devaney@glasgow.ac.uk (E. Devaney).

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parasitic helminths. This short paper summarises the discussion and presents a critical appraisal of the concept of refugia in principle and practice in three host-parasite systems.

2. Refugia in helminth parasites - a theoretical perspective

Exploiting refugia as a means of delaying the evolution of resistance has been explored in other biological systems, in particular for the management of agricultural pests (Tabashnik et al., 2008). Here, the planting of insecticidal GM crops was accompanied by planting of non-GM standard crops to provide refugia for insects. This widespread practice has allowed analysis of the multiple factors determining the success of refugia-based approaches (Jin et al., 2015). These systems have benefitted from adopting a theoretical approach and emphasise that knowledge of both epidemiological and evolutionary dynamics (and their interaction) is essential for the development of optimal control strategies (see for example (Brown and Stankova, 2017). While the use of refugia emerges as a potential control strategy against both *Bacillus thuringiensis* (Bt) insect resistance and anthelmintic resistance, there are important differences. Genetically modified crops producing Bt toxins are ingested by juvenile insect stages (Bravo and Soberon, 2008), whereas advanced larval stages and adult parasites encounter drug product within a host. This alters the time frames, and sequence, over which benefits and costs of resistance play out. In Bt resistance, the benefits of resistance primarily manifest in juvenile survivorship and hence recruitment (Raymond et al., 2011), whereas in helminths a range of effects on larval establishment, adult survival and fecundity may occur.

For gastro-intestinal (GI) nematodes of livestock, control has largely depended upon treatment of all animals in a group with broad-spectrum anthelmintics, which kills susceptible worms, leaving only resistant genotypes to seed the pasture. By minimising exposure to drug, refugia-based control strategies aim to conserve susceptible alleles within the parasite population. The resulting mixture of resistant and susceptible genotypes on pasture should then allow the potential for cross-breeding and/or dilute the frequency of resistant genotypes within a population. Nowadays, best-practice recommendations promote targeted selective treatment (TST), in which only a proportion of a flock is treated (see section 3 for discussion) or strategic timing of drug application to preserve refugia. The success of refugia-based control depends upon many factors; these include the potential fitness costs of harbouring resistant alleles in the absence of drug exposure, the existing level of resistance in the population, the genetic diversity of the parasite population, mechanisms and modes of inheritance of resistance alleles (dominant or recessive), the proportion of the worm population exposed to the drug, which may vary with the particular parasite species (e.g. not all life cycle stages may be exposed or susceptible), and the efficacy and frequency of treatment with a particular drug. In addition, an understanding of environmental factors such as the climate and its effect on the survival of parasite populations in refugia on pasture, and host and pasture management practices, are all relevant for optimising the worm population in refugia. Many of the factors influencing the success of refugia-based approaches in control of GI nematodes were covered in a comprehensive review (Kenyon et al., 2009); in this section we limit our discussion to the impact of the fitness costs of resistance, the influence of parasite life-history traits on the success of refugia-based strategies and the use of modelling approaches.

2.1. Fitness costs of resistance

Evolutionary theory predicts that resistance may come at a cost but there are few examples in the literature that have explored fitness costs in metazoan parasites in relation to refugia-based control. The potential fitness costs of drug resistance are central to the discussion of refugia. In a refugium, which is not subject to drug treatment, a fitness cost to a resistance allele will favour and maintain susceptible alleles in a population and so slow the spread of resistance. While theoretically, without a fitness cost, refugia would still slow resistance by reducing selection for resistant alleles, the effect would be less marked than with a fitness cost. *A priori*, one expects such fitness costs. After all, if a protein has been functioning well in a worm for millions of years, ablating or changing it to confer drug resistance is likely to have a cost to the worm in the absence of a drug. Compensatory mutations elsewhere in the genome, however, can ameliorate these costs. In the free-living nematode, *Caenorhabditis elegans*, compensatory mutations can restore wild-type fitness to lines carrying deleterious mutations (Estes et al., 2011). The mechanistic basis of a compensatory mutation has been defined for mercury resistance in *Pseudomonas fluorenscens* (Harrison et al., 2015). In the presence of heavy metals, the plasmid conferring mercury resistance results in significant fitness benefits, while in the absence of heavy metals, the plasmid causes a major disruption to transcription and a large fitness cost. In such populations, secondary, compensatory mutations rapidly emerge that restore the regulation of the transcriptome and, consequently, restore fitness and allow carriage of the plasmid, even in the absence of heavy metals. There is a clear lesson for drug resistance in helminths. Evolution may rapidly find ways to ameliorate any cost of resistance, equalising the fitness of drug resistant and susceptible alleles and so reducing the benefits of refugia in allowing drug susceptible alleles to be maintained. There are few publications that have analysed the fitness costs of anthelmintic resistance; however, a recent review covers the relevant issues in the context of insecticide resistance (Ffrench-Constant and Bass, 2017) and highlights many issues that also apply to anthelmintic resistance, e.g. the need to fully understand fitness costs in field populations of pests. For the insect growth regulator cyromazine, for instance, resistance can be selected rapidly but is highly unstable and efficacy is restored in the absence of selection, implying a high fitness cost (Khan and Akram, 2017). While there are parallels between these respective systems, much remains to be learned about the interplay between the fitness costs of anthelmintic resistance and their possible impact on refugia populations with additional empirical data needed to reach a full understanding.

2.2. Parasite life-history traits

The evolution of parasite life history traits (such as establishment, growth and reproduction) will also impact on the development of resistance and refugia. Drug resistance is often considered a question of molecular biology: the variants in drug targets or detoxification systems that are selected for following a drug regime. However, evolution will act on any trait that maximises fitness, and life-history traits of parasitic helminths, unrelated to drug action, have also been proposed to respond to, and ameliorate, the effects of drug (or other) treatment. Although this is not well studied empirically in parasitic helminths, some good examples come from other areas of parasitology. In the rodent malaria parasite *Plasmodium chabaudi,* there is evidence of selection for greater virulence in response to vaccination with the AMA-1 antigen, but no evidence of selection at the *ama-1* locus (Barclay et al., 2012). Increased virulence of *Plasmodium* results from greater reproduction of blood-stage parasites, which can compensate for the increased level of immunity provided by a vaccination that provides only partial protection. In helminths, Skorping and Read (1998) suggested that migrating stages of helminth species may be less susceptible to drug, with treatment selecting for parasites that migrate for longer and grow larger. Empirical support for this hypothesis came from experimental evolution in *Strongyloides ratti*, where ‘fast’ and ‘slow’ lines of worms were generated by passaging eggs obtained from either early or late in an infection, respectively (Paterson and Barber, 2007); drug treatment would be expected to have a similar effect to the ‘fast’ lines by abbreviating infections and selecting for early reproduction. Consistent with this, ‘fast’ lines were found to have higher base-line levels of reproduction, but suffered more from the effects of immunity and

density-dependence (crowding effects) on reproduction than 'slow' lines. These factors will also play important roles in the success of refugia-based strategies for GI nematodes of livestock, where fecundity levels vary markedly between parasite species, and where the age of the host, and therefore immune status, will influence the numbers of L3 on pasture.

2.3. Modelling refugia

In other biological systems where the concept of refugia has been adopted to slow the spread of resistance, mathematical modelling has contributed to understanding the complex interactions between refugia and the prevalence and frequency of resistance (see for example, (Cerda and Wright, 2004; Crowder and Carriere, 2009). In the parasitic helminth field, several approaches to understanding the flow of resistance alleles in nematodes have been published (Leathwick, 2013; Learmount et al., 2016), with recent work explicitly encompassing the impact of refugia on resistance (Park et al., 2015; Cornelius et al., 2016). To address the evolutionary epidemiology of resistance, Park et al. (2015) used S–I (susceptible or infected) models to study two main parameters: the degree of mixing between treated and untreated hosts, and the extent of drug coverage. The expectation was that more mixing would increase the force of infection but also enhance the effect of refugia by increasing dilution by the drug-susceptible strain, limiting the frequency of resistance in treated hosts at a potentially acceptable cost in terms of disease burden. However, the model generated somewhat counter-intuitive results, predicting that the extent of drug coverage and mixing would affect resistance in complex ways, so that increased mixing can either decrease or increase the frequency of resistance depending upon model variables (Park et al., 2015). In particular, for a fixed level of drug coverage, the long-term prediction for the frequency of resistance in treated hosts is minimized at intermediate levels of mixing. Further, for low coverage, increased mixing tends to increase the prevalence of infection in the treated group whereas the opposite is true for high levels of coverage, findings that will be driven by the relative balance of susceptible versus resistant strains at equilibrium. This model suggested that refugia-based strategies could work, but in a highly conditional way; the apparently counter-intuitive results suggest further investigation is merited into the role density-dependence plays in these findings and what the implications of drug coverage and mixing are for resistance and the prevalence of infection for the whole population (refugia and treated), not just the treated group. Nevertheless, this model highlights the importance of the interaction between mixing and coverage. Variation in mixing may occur by design, such as in a controlled co-grazing strategy (see (Leathwick and Besier, 2014). It may also occur naturally, for example in mass drug administration programmes, such as those in use for soil-transmitted helminths and schistosomiasis, which focus on school-aged children, and thus have the potential to result in age-structured refugia (Webster et al., 2008). These models underscore the complexity of the numerous interactions that affect resistance and thus refugia. Future models could be adapted to include the consequences of recessive or dominant genotypes and polygenic mechanisms of resistance; the role of standing genetic variation versus *de novo* mutation; the parasite life history traits affected by drug treatment (e.g. longevity or fecundity of adult worms) and the potential costs (or benefits) of resistance. Additionally, models may be extended to other ecological transmission settings, particularly those that generate an unequal distribution of parasites in infected hosts such as spatial clumping of free-living parasite stages (Cornell et al., 2003) or vector-borne transmission (Churcher and Basáñez, 2008). Thus, knowledge of both epidemiological and evolutionary dynamics (and their interaction) will be essential to develop the most effective refugia-based strategies.

3. Refugia in practice - gastro-intestinal nematodes as a case study

The application of refugia-based strategies to slow the spread of drug resistance in helminths has been best studied in GI nematode infection in small ruminants. While this strategy has gained increasing acceptance in recent years (e.g. see SCOPS, http://www.scops.org.uk), significant knowledge gaps remain. Anthelmintic resistance has a genetic basis and may be either pre-adaptive or spontaneous, such that alleles conferring resistance may either be present in populations prior to drug exposure, or may occur *de novo* during the period of drug exposure (Redman et al., 2015). The frequency of resistance genes should increase as the population is exposed to drugs, but whether the converse is true (i.e. a reversion to susceptibility over time once selection pressure is removed) remains unclear. Multiple studies performed on different continents have repeatedly demonstrated that resistance to the benzimidazole (BZ) class of drugs was not reversible: phenotypic resistance remained high many years after cessation of BZ treatment (Hall et al., 1982; Herd et al., 1984; Borgsteede and Duyn, 1989). In contrast, a 5-year study in New Zealand, where a range of best practices were applied, including refugia-based control and combination anthelmintics, demonstrated partial reversion toward susceptibility for levamisole and ivermectin, but not BZ, in multi-drug resistant *Teladorsagia circumcincta* populations (Leathwick et al., 2015). The lack of reversion to susceptibility to BZ compounds may be an indication of longer-term or more frequent exposure of parasites to this drug class and the consequent genetic fixation of resistance in the worm population. It could also indicate differences in the dominance/recessive nature of the alleles conferring resistance to each anthelmintic, or differences in fitness costs.

The best characterised of current refugia-based control strategies for GI nematodes is targeted selective treatment (TST) of small ruminants. By treating only a proportion of the flock, most animals and their worms remain unexposed to drug, thereby conserving susceptible alleles. A major challenge with TST, and other refugia-based methods, is deciding which animals to treat. GI nematodes are classically overdispersed in their host; i.e. a few hosts contain most of the worms (Anderson and May 1978) and potential indicators to treat grazing ruminants include faecal egg counts, milk yield or various production scores (reviewed in Kenyon et al. (2009); Charlier et al. (2014). However, adoption of a specific TST approach depends on the practicalities of the situation on the farm in question, as any modification of standard practice has to be easy to adopt and cost effective. For example, in *H. contortus* endemic zones, a relatively simple measure of anaemia such as FAMACHA©, in which the colour of the mucus membrane is assessed, has been widely used (van Wyk and Bath, 2002). The feasibility of using the FAMACHA© score to monitor the health of infected goats and maintain refugia in the field was exemplified by studies on smallholder farms in Botswana, where *H. contortus* is endemic. Here a TST approach achieved the same health outcome as treating the whole herd, but for 24% of the drug input (Walker et al., 2015). Interestingly, four years later most of the farmers were still applying the same general approach, demonstrating the potential for sustained impact of a relatively limited intervention in a resource–poor setting. Studies from other regions, such as Brazil, confirm that TST based on FAMACHA© scores can be applied practically by farmers to control parasite burdens while generating refugia (Maia et al., 2015).

In regions where *T. circumcincta* predominates, lower than expected weight gain has been shown to be a reliable indicator of sheep requiring treatment. For example, in a 5-year study Kenyon et al. (2013) demonstrated that this TST approach could reduce the number of anthelmintic treatments, while maintaining live weight gain and drug efficacy. Furthermore, the TST strategy appeared to conserve the diversity of nematode species present within the host over time, implying a refugium of multiple susceptible species on the farm. In contrast, monthly treatment of the whole flock resulted in the dominance of a single, pathogenic and presumably drug-resistant species, *T.*

circumcincta (Melville et al., 2016), a scenario likely to result in clinical disease. Although TST based on weight gain as an index of performance can be semi-automated using electronic identification and weigh crates, further research is needed to develop selection tools for TST that are easy to apply and are economically attractive in different contexts. Further work is also required to ensure that TST strategies use suitable treatment thresholds to preserve production whilst minimising anthelmintic use; if treatment thresholds are too low then more treatments may be administered than necessary, reducing available refugia, whereas thresholds too high could result in production loss. In the future, these could draw on new technologies, e.g. automated monitoring of performance or behaviour (Vercruysse et al., 2018). It is important to acknowledge that TST could result in increased pasture contamination relative to more conservative whole flock treatment, as more resilient hosts could shed large numbers of eggs while still maintaining productivity (Bisset and Morris, 1996). The higher pasture larval burden, which might be expected from this scenario, although assisting in the maintenance of refugia, might impact production gains in parasite naïve animals (Coop et al., 1982); however, studies to date have shown no negative effect of well-managed refugia on pasture contamination or lamb production (Kenyon et al., 2013).

While TST appears to offer a practical solution to the spread of anthelmintic resistance in some systems, we still have no methods to directly determine the extent to which refugia-based strategies influence the rate of spread of resistance alleles within a worm population. This situation will be remedied as molecular methods are developed for tracking anthelmintic resistance (Laing et al., 2016; Doyle and Cotton, 2019); evidence for the slowing of resistance through refugia will be important if livestock producers are to be persuaded to alter existing management practices in the interests of sustained drug efficacy.

3.1. Environmental considerations in GI nematode studies

In addition to factors that affect the worm population in the host, refugia-based strategies are influenced by environmental factors. Certainly, in temperate climates, the majority of the GI nematode population is free-living on pasture for much of the year. As refugia–based strategies rely on dilution of resistant genotypes with susceptible genotypes, or breeding between resistant and susceptible worms, the dynamics of L3 on pasture and the optimisation of mixing between genotypes are key considerations. The net consequence of multiple interacting factors on larval availability is hard to predict. Elevated temperatures increase the rate of both development and mortality of GI nematode larvae, leading to non-linear relationships between temperature and transmission potential (Kao et al., 2000). Moreover, migration of L3 from the faecal pat onto the herbage is both strongly moisture-dependent (Wang et al., 2014) and dynamic over short time spans (Rose et al., 2015) and thus pasture-level processes will significantly affect the mixing of susceptible and resistant genotypes and their availability to grazing livestock. Although untreated hosts within a group may generate refugia of predictable size in terms of parasite eggs produced, the size of the refugia will subsequently vary according to climatic and other factors, and may dwindle to insignificant levels or, conversely, become large enough to negatively impact production.

A variety of mathematical models have been used to predict the effect of climate and climate change on the epidemiology of GI nematodes, building upon established frameworks (Smith and Grenfell, 1994). For example, the dynamics of *H. contortus* infection in the UK was studied using simulation models under current and projected climates (Rose et al., 2016). Current studies are elaborating upon these to include genetic mixing between selected populations and refugia. Spatial clumping of L3 is predicted to promote inbreeding (Cornell et al., 2003), so factors determining the fine-scale distribution of L3 in space and time are potentially important considerations for the selection of resistance in GI nematodes, as is the potential of over-lapping generations. The way in which susceptible and resistant genotypes mix is likely to be highly system-specific, suggesting that the generation and effectiveness of refugia will be similarly so.

While parasite life cycle stages in the environment are typically thought of as a refugium, much may depend upon the drug in use. For example, the macrocyclic lactone (ML), ivermectin, is excreted largely intact in the faeces (Canga et al., 2009) and parasite stages present in the faecal pat may still be exposed to drug in the immediate post-treatment period. Moreover, where parasites actively migrate into the soil they may be exposed to a range of microbial products, e.g. ivermectin itself derives from a soil bacterium *Streptomyces avermitilis* (Campbell, 1985; Him et al., 2009). These data suggest that there may be degrees of refugium in the environment; selective pressure could be applied to free-living nematode stages at genetic loci relevant to drug resistance and thus the assumption that the external environment represents a 'neutral' refugium may be naïve.

Other environmental factors that can impact refugia include the presence of alternative hosts, which could remove infective larvae and/or deposit new eggs. Wild ungulates, for instance, have been shown both to remove GI nematode L3 from livestock pasture (Walker et al., 2018), and to carry and propagate resistant genotypes (Chintoan-Uta et al., 2014), and presumably could also import drug-sensitive parasites onto a farm. Different livestock species and age classes on a farm could also be considered alternative hosts, differing from each other in grazing patterns and host competence. Planned rotational grazing could therefore be used to reduce highly resistant L3 populations through removal by refractory hosts, and/or supply susceptible genotypes from young or resilient untreated animals. Practical strategies to manage refugia on grazing land shared by multiple host classes might be feasible, if supported by new research in this area.

4. Refugia in a vector borne helminth - the enigma of dog heartworm

While there is a growing body of work on the use of refugia-based control for GI nematodes, much less is known about the potential of refugia-based control in other host-parasite systems. A case in point is the vector-borne nematode *Dirofilaria immitis* (the dog heartworm). Interestingly, the *D. immitis* scenario appears to contradict much of the dogma surrounding the concepts of refugia, as developed for GI nematodes, in that resistance has developed in a high-refugia situation. A sound grasp of the life cycle and transmission of *D. immitis* is required to explain this finding, along with knowledge of the stages targeted by the MLs used in prophylaxis. MLs were introduced for heartworm control in 1987, and the first proven case of resistance was reported in 2013 (with details subsequently published in 2015, Bourguinat et al. (2015), although the possibility that resistance had emerged was discussed almost ten years previously (Hampshire, 2005). A major cause of the apparent contradiction referred to above relates to the definition of 'resistance' in heartworm versus GI nematodes. For *D. immitis*, resistance is defined at the individual worm and individual animal level while for GI nematodes, resistance is typically defined at the level of the parasite population. Since the MLs are expected to be 100% effective when used prophylactically against *D. immitis* (Seward et al., 1986), any deviation from full activity could constitute resistance. Furthermore, distinguishing resistance from issues of owner compliance is complex (Atkins et al., 2014). MLs target the L3 and L4 stages for the first 4–6 weeks post-infection, during which time the parasite migrates through the tissues of the dog. Once the juveniles arrive in the pulmonary vasculature, around day 90 post-infection, the drug loses its ability to kill the worms (McCall et al., 2001). For *D. immitis*, the population in refugia therefore comprises worms in untreated animals, the adult worm (largely unaffected by the MLs) and the stages in the mosquito (unexposed to MLs). The extent of refugia in *D. immitis* infection is thought to be quite large, since most pet dogs do not receive chemoprophylaxis, and there are large numbers of feral and wild canids (such as coyotes) that are susceptible to infection and that tend to have high

infection rates (Prichard, 2005; Brown et al., 2012). In addition, the mosquito populations responsible for transmission can be enormous. According to the accepted dogma, high refugia and low treatment coverage should prevent the development of resistance and consequently many experts thought that resistance would not occur in *D. immitis* (Prichard, 2005). Furthermore, if resistance did appear it was assumed that it would spread rapidly as there is no practical means to prevent it; high societal mobility means that dogs move throughout the country freely and in large numbers, and there is only a single drug class available to prevent the infection.

However, almost everything we know about resistance in *D. immitis* contradicts such expectations. Resistance is largely confined to the Mississippi Delta region (Pulaski et al., 2014), yet this area has the highest predicted level of refugia for a variety of socio-economic, climatic, physiographic and agricultural reasons. In addition, there appears to be little spread of ML resistant heartworms outside this region. How then to explain such contradictions? It seems that various factors are at play, perhaps the most important being the transmission bottleneck caused by the mosquito intermediate host. The processes involved with infection of mosquitoes, development of the larvae to the infective stage inside the mosquito and transmission to another dog, are complex and can fail at multiple points; consequently, the probability of transmission of resistant worms between dogs via the mosquito appears to be very low. Additionally, there may be a fitness cost associated with resistance, and the impact of any fitness deficit will be exponentially amplified through the multi-step process of the lifecycle. This latter possibility is of particular relevance, as a fitness cost could render the resistant genotype unstable. This would decrease the likelihood of transmission and make it most probable where levels of innate transmission are greatest, such as the Mississippi Delta region. Much remains to be learned about resistance in this particular host-parasite system and factors such as the possible genetic sub-structuring of the population, the level of standing genetic variation, and the mechanism/s of resistance are all important unanswered questions. This case illustrates that risk factors for anthelmintic resistance inferred in one system might not hold in others, and that general principles of how refugia impact on the development of drug resistance are elusive in practice.

5. Refugia in a helminth that undergoes clonal expansion

Another host-parasite system for which refugia-based strategies may be applicable is the liver fluke, *Fasciola hepatica*. The *F. hepatica* life cycle involves both a mammalian definitive host, primarily sheep and cattle, and a snail intermediate host. There are some similarities between liver flukes and GI nematodes: e.g. the role of climate on pre-parasitic stages (eggs, miracidia, cercariae and metacercariae), the presence of the parasite in wildlife, the ability of the metacercariae to survive on pasture and their tendency towards spatial aggregation. However, there are several factors specific to fluke infection, such as the ability of adult worms to both self- and cross-fertilize, the clonal expansion within the snail and the availability of snail vectors, all of which will impact potential refugia. Adult parasites have a huge reproductive potential with up to 20,000 eggs produced per fluke per day (Boray, 1969; Gonzalez-Lanza et al., 1989) and a single miracidium infecting a snail can give rise to ~3000 metacercariae. Thus a large population exists outside the mammalian host (and therefore in refugia), including a large clonal population in the snail vector. However, clonal expansion also provides the potential for amplification of resistance genes (Beesley et al., 2017) so the effect of the asexual reproducing stages on refugia is unclear.

Currently several flukicide drugs are licensed to control liver fluke, including triclabendazole (TCBZ), clorsulon, nitroxynil, closantel, albendazole, and oxyclozanide (Coles and Stafford, 2001). Unique amongst these drugs, TCBZ, a benzimidazole derivative, demonstrates high efficacy against both adult and immature fluke (Boray et al., 1983) and is the drug of choice to treat livestock and human infection. In livestock, frequent treatment of whole groups, especially in high-risk years, has been a major driver of drug resistant parasite populations, despite the fact that at the time of treatment there is considered to be multiple refugia: eggs on pasture, viable, un-ingested metacercariae on herbage, parasite stages within infected snails and other farm livestock and wildlife reservoirs. Despite these extensive refugia, resistance to TCBZ was first reported in Australia in 1995, is now found in many locations worldwide and is considered a substantial threat (Kelley et al., 2016; Kamaludeen et al., 2019). As control regimes move away from reliance on TCBZ, resistance to other flukicides is emerging (Novobilsky and Hoglund, 2015). It is clear that liver fluke poses challenges distinct to those relevant for GI nematodes, such as the impact of asexual versus sexual reproduction and direct versus indirect life cycles. Given the high pathogenicity of *F. hepatica*, a strong evidence base is required if refugia-based control strategies, such as leaving a proportion of animals untreated, are to be encouraged.

Refugia-based strategies to slow the development of drug resistance in *F. hepatica* have yet to be evaluated, either theoretically or empirically. With this in mind we have the potential to build on an existing mathematical model that describes development of liver fluke within a population of cattle (Turner et al., 2016). This model has been used successfully to investigate the potential value of a prototype vaccine and has since been modified to include the impact of drug treatment. Functions incorporated in the model include host susceptibility (based on parasite aggregation), death rate of immature and mature fluke, fluke fecundity and density dependent effects, and fluke maturation time. The model also includes seasonal addition of metacercariae to the pasture, temperature-dependent mortality of metacercariae on the pasture and consumption of metacercariae by definitive hosts. Currently the model allows investigation of the effect of multiple treatments with anthelmintics of different efficacies in the same year. The timing of each treatment can be specified, as can the proportion of animals treated i.e. determining the size of refugia from infected, untreated hosts.

As for GI nematodes, in order to derive genetically explicit models that can predict the success of refugia-based strategies, an understanding of the mechanism of resistance, whether resistance is a monogenic or polygenic trait and the mode of inheritance is needed. Ongoing studies are using genetic crosses of drug-resistant and drug-susceptible isolates to map resistance loci (Hodgkinson et al., 2013). To date it is known that a single locus, dominant trait can confer resistance to TCBZ (Hodgkinson et al., unpublished data). However, given the difference between *F. hepatica* and nematodes in terms of their biology and transmission, the focus remains on providing empirical data to parameterise appropriate models to determine whether maintenance of refugia could impact the spread of resistance in this system.

6. Conclusions

The discussion of three separate host-parasite systems demonstrates that while using refugia to slow the spread of anthelmintic resistance is intuitive, it will likely operate differently under each scenario. To fully understand the specific nuances of refugia-based control, additional studies will be required. Many issues of fundamental significance (see Table 1 for examples) have received little experimental attention and, thus their likely impact on refugia is poorly understood. In a recent article, which canvassed key questions in the livestock helminthology field, 10 of 100 proposed concerned refugia (Morgan et al., 2019). The significant progress in parasitic helminth genomics and a clearer understanding of the mechanisms and evolution of resistance will be important to further our understanding of the applicability of refugia-based strategies to metazoan parasites, and providing appropriate genetic markers with which to measure and best generate and utilize refugia. However, while identifying molecular mechanisms of anthelmintic resistance will give us the tools to properly assess whether refugia can slow the spread of resistance, increased interaction between

Table 1
Outstanding questions relating to refugia in practice.

Do parasites of different genotypes inter-breed freely?
Is there competition between parasites of different genotype in the host?
Are there always trade-offs in fitness (e.g. growth rate versus fecundity)?
Are fitness costs more or less important than the dilution factor in refugia?
To what extent can refugia work to slow the development of resistance even where fitness costs are absent or minimal?
Are fitness costs of resistance negated by compensatory mutations, and how quickly?
Do experiments with laboratory isolates faithfully mimic what happens in the field?
How much refugia is enough?
Can parasite community replacement be effective as a way of restoring drug susceptibility?

evolutionary biologists, geneticists, mathematical modellers and parasitologists will be key to maximising the impact of such developments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijpddr.2019.05.001.

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