Counterintuitive increase in observed Mycobacterium avium subspecies paratuberculosis prevalence in sympatric rabbits following the introduction of paratuberculosis control measures in cattle

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Counterintuitive increase in observed *Mycobacterium avium subspecies paratuberculosis* prevalence in sympatric rabbits following the introduction of paratuberculosis control measures in cattle.

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Abstract

Paratuberculosis (Johne's disease) is caused by the bacterium *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). Achieving herd-level control of mycobacterial infection is notoriously difficult, despite widespread adoption of test-and-cull based control strategies. The presence of infection in wildlife populations could be contributing to this difficulty. Rabbits are naturally infected with the same *Map* strain as cattle, and can excrete high levels in their faeces. The aim of this study is to determine if implementation of paratuberculosis control in cattle leads to a decline in *Map* infection levels in rabbits.

An island wide, test-and-cull based paratuberculosis control programme was initiated on a Scottish island in 2008. In this study annual tests were obtained from 15 cattle farms, from 2008-2011, totaling 2,609 tests. Rabbits (1,564) were sampled from the 15 participating farms, from 2008-2011, and *Map* detected by faecal culture.

Map seroprevalence in cattle decreased from 16% to 7.2%, whilst *Map* prevalence in rabbits increased from 10.3% to 20.3%. Results indicate that efforts to control paratuberculosis in cattle do not reduce *Map* levels in sympatric rabbits. This adds to mounting evidence that if *Map* becomes established in wild rabbit populations, rabbits represent a persistent and widespread source of infection, potentially impeding livestock control strategies.

Introduction

Paratuberculosis (also known as Johne's disease) is a chronic enteritis caused by *Mycobacterium avium* subspecies *paratuberculosis* (*Map*). At the herd level the disease impacts on animal welfare and is responsible for substantial financial losses from decreased productivity, infertility, and direct costs of control (Carta and others 2013). Annual test and removal of seropositive animals supported by changes in herd management is commonly employed to control the disease in livestock but despite the widespread adoption of such control strategies, paratuberculosis remains notoriously difficult to eradicate from livestock populations (Juste, 2012). The existence of wildlife reservoirs has been considered to be a barrier to eradication of *Mycobacterium bovis* from cattle herds (Delahay and others 2002) and it has been postulated that infected rabbit populations may have an impact on the control of paratuberculosis (Shaughnessy and others 2013).

Map can infect a broad range of wildlife hosts including rabbits, foxes, stoats, weasels, rodents and carrion eating birds (Beard and others 2001a). However, evidence suggests that of all susceptible non-ruminant species, rabbits (*Oryctolagus cuniculus*) pose the greatest transmission risk to livestock (Daniels and others 2003a; Judge and others 2005). When rabbits from wild colonies have been examined they have been found to have high levels of infection in their tissues relative to other non-ruminant wildlife hosts (Greig and others 1997). Infected rabbits excrete an average of 7.6×10^5 colony forming units (cfu) of bacteria per gram of faeces (Daniels and others 2003b), and there is potential for rabbits to contribute 170 million cfu of *Map* bacteria per hectare per day (Daniels and others 2003b). Furthermore the prevalence of infection within rabbit populations in Scotland has been found to be as high as 67% (Greig and others, 1997). As paratuberculosis is transmitted by the faecal oral route this represents an infection risk to grazing cattle, which may be exacerbated by their grazing behaviour. Grazing herbivores avoid grazing swards contaminated with faeces of their conspecifics and sympatric wildlife, however they do not exhibit avoidance of rabbit faeces (Daniels and others 2001; Judge, and others 2005; Smith and others 2008). The ingestion of a small number of rabbit pellets could constitute an infective dose to cattle, as each infected pellet carries a mean of 1.3×10^5 cfu

(Daniels and others 2003b). Evidence for interspecies transmission has been provided by molecular typing of cattle and rabbit *Map* isolates, which has shown that the same strains infect both species (Greig and others, 1999; Stevenson and others, 2009). Furthermore, the ability of rabbit isolates of *Map* to infect calves has been demonstrated experimentally (Beard, and others 2001b). Likewise, rabbits have been experimentally infected with cattle *Map* strains (Mokresh and Butler 1990).

There is a relationship between the current and past levels of paratuberculosis in cattle and the presence and prevalence of *Map* in rabbits (Greig and others, 1999), and there is an association between paratuberculosis persistence in cattle herds enrolled on disease control programs and *Map* infection levels in sympatric rabbits (Shaughnessy and others 2013). However, previous studies do not show if rabbits are a competent reservoir, capable of maintaining the infection in their population in the absence of re-infection from cattle, or merely a spill-over host. Within rabbit populations, transmission can occur via horizontal, vertical, and pseudo-vertical routes (Judge and others, 2006), and simulation modelling suggests that the rates of transmission are sufficient for *Map* to persist in rabbit populations (Judge and others, 2007). However, there is no empirical evidence of *Map* persistence in rabbits following control strategies in cattle. The aim of this study is to determine if implementation of paratuberculosis control in cattle leads to a decline in *Map* infection levels in rabbits

Materials and methods

Study population

Cattle sampling

An island wide paratuberculosis control scheme was initiated on a Scottish Island, in the UK, in 2008, with all cattle farms on the island participating in the Premium Cattle Health Scheme (PCHS). The

PCHS (http://www.sruc.ac.uk/info/120112/premium_cattle_health_scheme), is a Cattle Health Certification Standards (CHeCS) licensed scheme (http://www.checs.co.uk/). All animals over 24 months in the herds are tested annually using a commercially available ELISA test on blood (ID Screen Paratuberculosis, IDvet) and all positive animals, and offspring of positive females, are culled. Additionally, exposure management is employed, to avoid exposure of susceptible calves to faecally contaminated food and water sources, which includes increased hygiene measures around calving to decrease faecal contamination of the cows' teats, skin, and the environment. The cattle population in this study comprised 15 cattle farms, with a mixture of both beef and dairy herds. As all cattle farms on the island joined the PCHS in 2008, this enabled an initial screen and coordinated starting point in this longitudinal study, with annual test results from all 15 farms from 2008-2011, giving a total of 2609 tests.

Rabbit sampling

Using a standardized sampling regime, a total of 1,564 rabbits were sampled from the 15 participating farms (see Judge and others (2006) for details). Each farm was visited once per year, in spring, from 2008-2011, and an average random sample of 27 adult rabbits selected and euthanized (range 22- 48) per farm each year. Samples were examined using procedures outlined in Shaughnessy and others, (2013), and summarised here**.**

Rabbit carcasses were sexed, weighed, and subjected to superficial gross examination prior to post-mortem procedures being carried out as described by Judge and others, (2006). Pooled samples were taken from mesenteric lymph nodes and sections of intestine (sacculus, appendix, ileum, and caecum). Each rabbit was tagged with a unique identification number, and to reduce risk of cross-contamination each rabbit was dissected upon a fresh sheet of Benchkote (Whatman), using sterile scissors, forceps and disposable scalpels.

Culture and PCR examinations

Bacteriological culture on Middlebrook 7H11 slopes was used to determine the *Map* infection status of each rabbit (Greig and others, 1997). The tissue pool was homogenised in 10mL of sterile distilled water with a Colworth Stomacher 80 (Seward Medical). Homogenates were decontaminated by adding 10mL of 1.5% hexadecyl pyridinium chloride and stood overnight at room temperature for settlement of particulate material. Supernatants were centrifuged at 3080g for 20 minutes and each pellet was re-suspended in 10mL of sterile distilled water. Centrifugation was repeated and the pellet re-suspended in 1mL of sterile distilled water. The suspension was centrifuged at 3080g for 5 minutes. The pellets were finally re-suspended in 250µL of sterile distilled water. Two slopes of Middlebrook 7H11 agar supplemented with Selectatabs (amphotericin B, polymyxin B, ticarcillin and trimethoprim; code MS24, MAST Laboratories), 10% Middlebrook oleic acid–albumin–dextrose–catalase enrichment medium (Becton–Dickinson), and 2µg/mL of mycobactin J (Allied Monitor) were each inoculated with 100µL of the prepared suspension. The cultures were incubated for up to 52 weeks at 37^{o}C and examined regularly for bacterial growth, enabling growth and identification of both fast (Type C (cattle-type)) and slow (Type S (sheep-type)) growing *Map* strains. Identification of cultured *Map* was confirmed by PCR detecting the IS*900* insertion sequence (Greig and others, 1999). Briefly, 250µL of sterile distilled water were inoculated with a single suspect colony from each positive culture. DNA was extracted from the bacterial suspension by heating to 100^{o}C for 15 minutes and then centrifuged at 16,060g for 2 minutes; 2µL of the supernatant was analysed by PCR for the presence of the IS*900* gene (Whittington and others 1998). Isolates found weakly positive or negative for IS*900* were analysed for the presence of IS*901* according to the PCR described by Kunze and others (1992).

Molecular typing of isolates

To confirm the strain types present and further characterise the isolates, 32 were typed by Mycobacterial Interspersed Repetitive Unit-Variable Number Tandem Repeat (MIRU-VNTR) analyses as described by Thibault and others (2007). INMV profiles were assigned according to the Mac INMV database (http://mac-inmv.tours.inra.fr). Five microlitres of the heat killed cell bacterial suspension was used for each locus PCR.

Statistical analysis

The relationship between changes in paratuberculosis prevalence in cattle, and the changes in *Map* prevalence in rabbits over time was inferred using a generalised linear mixed model (GLMM), with a logit link function and binomial error distribution. The Wald test from the GLMM model was used to determine significant differences, with the Wald statistic (*W*) presented with the probability value for the fixed effects. In the model, *Map* status was the response variable, farm identification (and farm by species interaction) was used as a random effect, and species, year, and species by year interaction were included as the fixed effects. Statistical analysis was carried out using GenStat (16th Edition)

Results

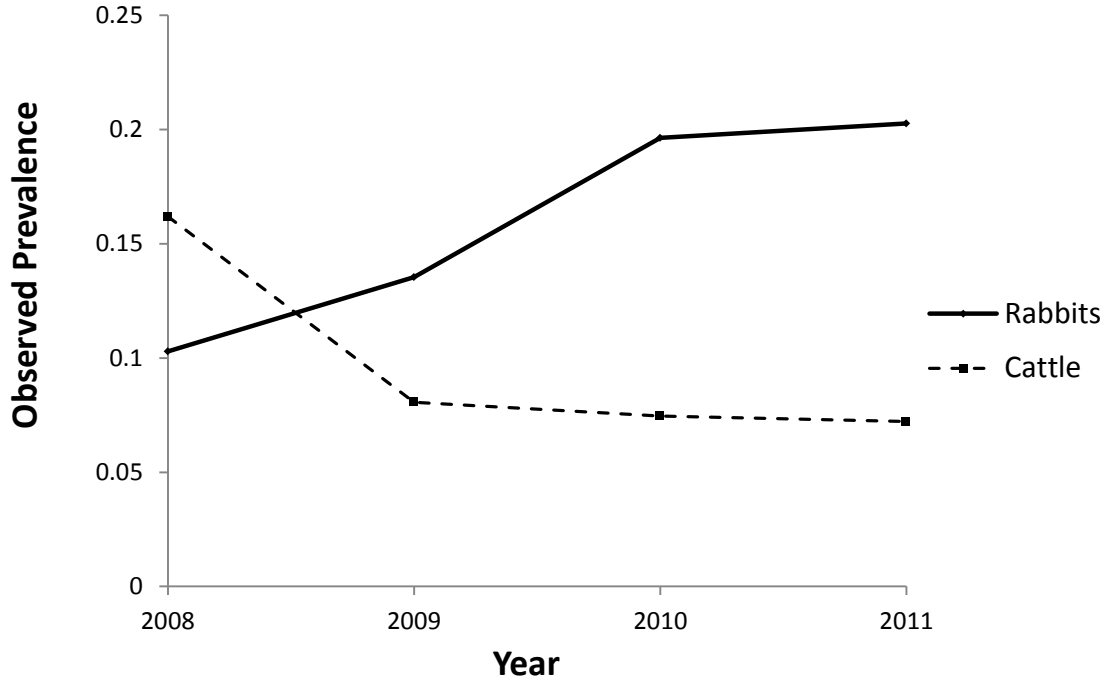

Fig 1. Observed prevalence of paratuberculosis ELISA positive cattle and *Map* culture positive rabbits from 2008 to 2011, from cattle study farms.

A total of 2,609 ELISA tests were carried out on cattle from the 15 farms over the study period. As farms were spatially contiguous, transmission could have transferred between them due to the ranging behaviour of rabbits (Daniels and others, 2003c), hence in figure 1 cattle and rabbit data across all farms were combined. On joining the scheme, cattle included in the study had an overall paratuberculosis prevalence of 16%. This prevalence decreased monotonically over the study period, and by 2011 had dropped to 7.2%. A total of 1,564 rabbits were sampled from the 15 farms, of which 256 were identified as *Map* positive in culture and 1,308 samples were classed as negative. *Map* prevalence in rabbits monotonically increased over the study period, from a prevalence of 10.3% in 2008 to 20.3% in 2011. There was no evidence that a decrease in paratuberculosis prevalence in cattle is associated with decrease in *Map* prevalence in rabbits in this study (figure 1). The observed trend in rabbits over time was significantly different from the observed trend in cattle over time, at the 95% confidence level (species.year interaction, $W = 22.3$, $P<0.001$). There was no significant effect

of the other fixed terms in the model (Species: $W = 0.36$, $P = 0.557$, year: $W = 3.61$, $P = 0.307$, species.year: $W = 22.34$, $P < 0.001$)

All 15 farms had cattle test positive for antibodies to paratuberculosis in at least one annual ELISA test during the study period, and nine farms had all cattle test negative for paratuberculosis antibodies on at least one annual test. Thirteen of the 15 farms had rabbits test positive for *Map* for at least one year of the study, and eight farms had rabbits test positive each year. However, there were not enough farms in the study to conclude whether the presence of *Map* in sympatric rabbits affected the likelihood of paratuberculosis control in cattle at the farm scale.

The rabbit cultures were checked for *Map* growth after 16 weeks, and again after 52 weeks to determine if slow growing Type S strains were present. Type S (subtype Type I) strains have been isolated from sheep on Shetland previously (Stevenson and others 2002). Over the study period, 218 rabbit samples tested positive after 16 weeks (13% of submissions). After 52 weeks of culture, a further 38 samples tested positive (2.3% of submissions). Hence 85% of samples that tested positive for *Map* over the course of the study did so after 16 weeks. This value ranged from 74-98% for individual years of the study, with no linear temporal trend.

Thirty two rabbit isolates were typed by MIRU-VNTR. Of these 16 grew within 16 weeks and 16 grew between 16 and 52 weeks. The results are shown in Table 1. This typing confirms that the strains isolated from the rabbit samples are Type C strains and that the slow growth of some cultures was probably due to a low initial bacterial load in the sample.

Table 1: Growth, PCR and MIRU-VNTR results for 32 rabbit isolates obtained during the study.

Number of isolates	Time for growth	IS*900*	IS*901*	INMV profile
15	Up to 16 wks	Positive		1
1	Up to 16 wks	Positive		3
15	16 - 52 wks	Positive		1
1	16 - 52 wks	Negative	Positive	88

One of the isolates was IS*901* positive *Mycobacterium avium* with an INMV profile consistent with this identification. All other isolates were confirmed as *Map* and with the exception of one, were INMV profile 1. This is a common profile among Type C (Type II) strains (Stevenson et al 2009). One *Map* isolate was INMV3, also indicative of a Type C strain..

Discussion

All farms in the study joined the PCHS in 2008, and by 2009 there had been a substantial drop in seroprevalence (Fig. 1). However, when control is targeted at livestock, prevalence in rabbits does not echo the trends observed in the cattle. The finding that cattle prevalence trends are not echoed in rabbit infection levels is consistent with previous studies that indicate *Map* can be maintained within a rabbit population (Judge and others, 2007; Maio and others, 2011). This is the first study to show that efforts to control paratuberculosis in cattle do not reduce *Map* levels in sympatric rabbits.

The increase in *Map* prevalence in rabbits following implementation of control in cattle was unexpected. There are a number of possible drivers of observed patterns of *Map* prevalence in rabbits. The mild winters of 2008 and 2009 were above the long term average (LTA) winter temperature for the island, whilst the colder winters of 2010 and 2011 were below the winter LTA

temperatures (1932-2013 LTA Met Office climate data). Lower temperatures increase disease susceptibility and facilitate outbreaks, as the increased costs of thermoregulation and thermal stress combined with lower food availability leave fewer energetic resources to combat infections (Rödel and others 2004). The increase in *Map* prevalence in rabbits could also be due to a 'perturbation effect'. First reported in badger:tuberculosis disease systems, the perturbation effect describes increased infection levels resulting from social perturbations, increased ranging, mixing between social groups, and territory disruption following the non-selective removal of individuals (Carter and others 2007). Modelling studies predict this effect is widespread in disease systems (Prentice et al, 2014), and the rabbit *Map* system has many attributes associated with a perturbation effect, including spatially structured host populations and heterogeneous infection patterns (Prentice et al, 2014).

Our results add to mounting evidence indicating that if *Map* becomes established in rabbit populations they will represent a persistent and widespread source of infection, potentially impeding control strategies in livestock. Despite a decrease in prevalence in cattle, eradication was not achieved within the study period. This is consistent with known difficulties in control, previously blamed on poor test sensitivities. However, Shaughnessy and others, (2013) demonstrated that farms that responded well to the PCHS *Map* control and had low levels of *Map* in sympatric rabbits could achieve effective paratuberculosis control in their herds, suggesting that lack of control may not be a sole consequence of low test sensitivities. Shaughnessy and others, (2013) also demonstrated that PCHS enrolled farms with high *Map* levels in rabbits were unable to control paratuberculosis in cattle despite implementing PCHS control measures. Combined with our finding that *Map* levels in rabbits are independent of levels in cattle, this suggests rabbits are maintenance hosts, rather than merely incidental hosts.

For rabbits to be considered a reservoir for paratuberculosis in cattle they must be able to transmit the pathogen to cattle, and the pathogen must be capable of being permanently maintained within the rabbit population (Haydon and others, 2002). The ability of rabbits to transmit *Map* to cattle has been demonstrated (Beard and others 2001b), and it has been demonstrated here that implementation of control programmes in cattle herds does not necessarily lead to a decline in *Map* in rabbits (Fig. 1).

This empirical finding is consistent with simulation studies modelling *Map* dynamics in rabbit populations (Judge and others 2007). The findings of this study indicate that rabbits may be a reservoir, but it is not clear what role they play in disease maintenance in cattle. In the absence of control strategies paratuberculosis can be maintained in cattle populations without reinfection from external sources (e.g. rabbits), as cattle are the main transmission source for other cattle. The disease can be maintained in cattle if the herd stays above the critical community size (the minimum size of a closed population required to maintain a pathogen indefinitely (Haydon and others, 2002)). However disease control strategies can increase the size of population required to maintain infection, and can effectively push the cattle population below the critical community size whilst keeping the herd size constant. If the critical community size becomes larger than the cattle population through implementing control measures (e.g. test and cull, vaccination, exposure management), the cattle can become a non-maintenance population**.** If this cattle population were then isolated from external sources of infection, then the pathogen would die out. However, if cattle-centric control does not concurrently decrease *Map* levels in rabbits (as is shown here), rabbits could facilitate infection persistence in cattle herds brought below the critical community size – in such a scenario a rabbit reservoir would be required for paratuberculosis maintenance in the multi-host system.

Although test and cull schemes like the PCHS can control infection levels in cattle, eradication success is likely to be hindered if *Map* is persistent in the local rabbit population. As active paratuberculosis control in cattle does not lead to contemporaneous declines in rabbit *Map* prevalence, control should be focused in concert across both livestock and wildlife domains, with disease management planned at the system level. Control through culling is often employed to reduce infection in wildlife populations, however transmission reduction through culling sympatric rabbits seems unfeasible as sustained annual rabbit population reductions of 40-50% would be required to eradicate *Map* in rabbits (Davidson and others, 2009). Additionally, culling of rabbits may inadvertently lead to an increase in transmission through the perturbation effect. Contact between livestock and rabbit pellets could be reduced through modifying husbandry practices (Miller and others 2013); it is possible to restrict rabbit access to cattle grazing areas, lowering transmission risk

through reducing pellet numbers and increasing sward height (Daniels and others 2003b). Likewise, areas of pasture can be fenced off to prevent cattle grazing areas where rabbit pellet concentrations are highest (Daniels and others 2003c). However preventing rabbit to cattle transmission is tricky for a pathogen of a grazing animal transmitted via the faecal oral route and shed in high numbers from a highly ubiquitous wildlife reservoir. Access restriction to high risk areas should be prioritised for young livestock, as they are more susceptible to infection (Windsor and Whittington 2010).

Conclusions

Test and cull in the cattle population can decrease paratuberculosis seroprevalence even in the presence of infected rabbits. However, our results indicate that control in cattle does not concomitantly lead to *Map* reduction in rabbits, this suggests that if test and cull implementation drives cattle herds below the critical community size rabbits could thwart eradication efforts. Ideally, paratuberculosis outbreaks in cattle should be avoided in the first place to prevent spill-over into the local rabbit population – this can be aided by thorough testing, hygiene management, and avoiding introduction of infected cattle into clean herds. Our findings suggest that if paratuberculosis is established in both populations, eradication will be dependent on the implementation of a unified control strategy that incorporates control within cattle and rabbit hosts. This could include direct control in the cattle population (e.g. test and cull), blocking tactics to prevent transmission between the cattle and reservoir populations (e.g. fencing), and direct reservoir control (e.g. culling). For successful disease eradication, an understanding of transmission dynamics within and between livestock and wildlife reservoir populations is required.

Conflict of interest statement

G. Caldow is the manager of PCHS. None of the other authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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