### 3.4.4 Milestone 4.3 Novel molecular markers for identifying resistant phenotypes in red deer

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| Description | To develop molecular assays for gene expression transcripts demonstrated to be differentially expressed during ruminant immune response to mycobacterial challenge. The polarised presentation of phenotypes for disease resistance and susceptibility to MAP infection in deer provides an accessible model to identify candidate genes as a signature for the resistant or susceptible phenotype. The identification of such genes could be used in future studies to select animals with a resistant phenotype or to cull animals with susceptibility traits. It is likely that common features of host resistance to MPA infection among ruminants will allow finding in deer to be applied to cattle or sheep. |
| Alignment with JDRC Strategy | Tool: Diagnostic test based on gene markers for identification of Johne's disease resistant animals |
| Status | Ongoing program. On target, achieved contracted milestones 2008-2011 |

**2011 Science Report**

**Milestone 4.3: Report for Science Review**
**Frank Griffin and Rory O'Brien**

**Introduction**

Our ongoing studies to identify novel molecular (immune) markers have been designed to deliver a composite phenotype to discriminate between uninfected animals that are resistant or susceptible to Johne's disease. Deer present polarised phenotypes for resistance and susceptibility to Johne's disease caused by *Map* infection where juveniles may succumb to clinical disease with 8 months of birth. There is unique genetic homogeneity within individual deer breeds (*Cervus elaphus*) that have evolved in geographic isolation throughout Europe. This appears fortuitously to have facilitated selection within some breeds that are raised in proximity with domestic livestock, that express polarised resistance to pathogenic mycobacteriae (*M.bovis/M. paratuberculosis).* By contrast, deer raised in forest habitats seem to have evolved towards a more susceptible phenotype in the absence of continuous pathogenic bacterial challenge.

We have solid genetic evidence (available confidentially to the SAG), based on death and disease rates within NZ deer herds, exposed to high levels of *M. paratuberculosis* infection, that deer populations which originally colonised habitats no more than 500 kms apart in Europe, express polarised phenotypes for Resistance and Susceptibility to *Map* infection and clinical Johne's disease. It seems plausible to suggest that deer that have co-evolved in domesticated farming environments where infectious exposure to tuberculosis caused by *M. bovis* appear intrinsically more resistant to other pathogenic mycobacteria (*M. paratuberculosis*). The translocation of diverse international deer breeds into the NZ farming environment has exposed them to constant challenges from *M. bovis* (TB) and more latterly (*M. paratuberculosis*). While assisted reproductive technologies, developed and now used routinely by NZ deer farmers (AI/Embryo Transfer), have accelerated selective breeding traits, they have largely focussed on temperament, production (venison) traits and trophy heads. While NZ deer farmers have focussed on selection for superior production traits in their herds they have not selected for disease resistance. Similar issues could arise in the dairy industry where a small number of highly productive bulls are used extensively in the AI programme.

Our hypothesis for disease is that resistance to infectious disease is a positively selected trait, that is sustained in the presence of challenge by pathogens, it is a cost that may become redundant in an environment that lack pathogens; disease resistance may have an associated physiological 'cost' which might constrain other production/reproduction capacities. We should reflect on the farming philosophy that 'bigger-is-always-better' in the world of domestic livestock, in the face of increased intensification, stocking rates and exposure to microbial pathogens**.**

While our customised Paralisa™ antibody test can identify susceptible animals in a diseased herd, there is no test yet available to diagnose susceptible animals in non-infected herds. Susceptibility/Resistance to pathogenic mycobacterial infections in humans provides clear evidence of heritability, mediated by cellular immune responses (Macrophages/T cells). This is to be expected in a disease where immune reactivity is involved (infection; allergy; autoimmunity), as the host genotype plays a major role in defining quality of effector immune reactivity. A major resource available to our laboratory is the diagnostic facility that provides an immunodiagnostic service for the NZ deer industry - we currently process >27,000 blood samples annually. These diagnostic activities give us access to many of NZ's leading deer farms and the majority of its stud farms and constantly challenge us to advise farmers as to what are expected outcomes and why they may not be achieved. Our diagnostic platform is now supported by internationally accredited platforms (USA/Europe) using molecular techniques (qPCR) that monitor *M.ptb* levels in faeces and gut tissues and that have been extended to *Map* strain typing (VNTR). Our diagnostics have actively supported Milestone 4.1 (Crawford); Milestone 4.2 (Mackintosh) and Milestone 5.1 (Heuer) of the current JDRC programme.

While our original diagnostic platform (Griffin *et al* , 2005) (1) concentrated on the ability to detect every animal that harboured *Map* infection, we have recently become more preoccupied with diagnosing animals with the minimal level of infection, that might affect the productive/reproductive capacity of the host, or progress to clinical Johne's disease. In this regard we have focussed on parallel diagnostic testing (*Paralisa*™ / qPCR) because low level seroreactive animals do not necessarily progress to develop Johne's disease. Recent experimental infection studies by Colin Mackintosh show that deer with a resistant genotype, when experimentally challenged with *Map,* may produce low-titre, transient antibody levels before they resolve infection and express their resistance phenotype (Milestone 4.2). By contrast, susceptible animals produce persistently high levels of antibody and progress to develop clinical disease within one year of experimental infection with virulent *Map.*

In recent years DRL has identified a herd of deer comprising diverse European breeds that express polarised resistance and susceptibility to Johne's disease. Retrospective genetic analysis has established that resistance or susceptible heritability to Jd is 0.30 +/-0.05. The deer in the herd had been exposed to very high levels of *Map* for ten years, which disclosed polarised groups of animals that expressed the Resistant (Non-infected) or Susceptible (Diseased) phenotype. Extensive use of embryo transfer in an assisted breeding programme provided multiple progeny from individual dams/sires that were ideal for detailed genetic analyses. We now have Estimated Breeding Values (EBVs) for 3,982 animals that allow us to identify individual animals that express extremes of resistance or susceptibility. A selected number of these animals were used assist Colin Mackintosh's experimental challenge study (Reported under Milestone 3.2). The comparison between progeny from R & S sires, used for AI in unselected donor females, affirmed that either resistance or susceptibility to *Map* infection is highly heritable.

In the past two years, under the JDRC project, DRL has developed technology to isolate high quality mRNA from bulk cultures of *in vitro* matured cervine macrophages. At the inception of this project we anticipated that we should be able to isolate sufficient RNA from antigen stimulated Peripheral Blood Mononuclear Cells (PBMCs) to quantify relative expression levels of small number (~20) of candidate genes expressed by immune cells. Recognising the limitations of a candidate gene approach, the methodology has been refined over the past two years so we can now isolate RNA of sufficient quantity/quality to undertake whole transcriptome analysis of cultured macrophages. We can use peripheral blood macrophages, matured by *in vitro* culture,

stimulated either with specific ligands (CD40L), non-specific microbial stimulants (LPS) or specifically following laboratory exposure to *M.ptb* organisms.

**Methodology**

The road towards the *in vitro* maturation, *Map* challenge and gene expression interrogation of cervine macrophages is as described in the SAG science review for this milestone in 2010. With a robust culture system in place, the candidate gene panel has been expanded to include key molecules including -

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| B2M | STAT6 | SOCS4 | IFNg | MyD88 | GM-CSF | INOS | TNF β |
| STAT1 | RANK | IFNb | GATA3 | TRAF6 | NOD1 | NOD2 | CTLA 4 |
| STAT2 | PIAS1 | MAPK8 | RORC | P53 | IL-1b | SOCS1 | GITR |
| STAT3 | PIAS2 | MAPK11 | TLR1 | ATF2 | IL-18 | IL-19 | IL 25 |
| STAT4 | PIAS4 | HLA-DMB | TLR2 | CREB | NLRP3 | MAPK14 | |
| STAT5A | SOCS2 | RANTES | TLR4 | FOS | IRF3 | IL17 F | |
| STAT5B | SOCS3 | Tbet | TLR6 | JUN | NF-kB | RORα | |

In addition to the candidate gene approach, a parallel goal of Milestone 4.3 has been to apply transcriptomics technology to *Map*-infected macrophage cultures in order to assay the entire transcriptomic output of the cells for those gene markers displaying the most polarised expression responses to Map infection. A transcriptomics approach circumvents the limitations of 'cherry picking' particular gene markers because they are *hypothesised* to be differentially expressed and allows targets to be selected because they demonstrably *are*, even though their actual biological function may be entirely unknown. We also propose to investigate interesting targets arising from Colin Mackintosh's transcriptome experiment in lymph node biopsy samples recovered from experimentally challenged R and S animals, to complement the array of genes to be included in the final analytical platform.

Deer are undeniably a 'non-model' organism, without a well established reference genome against which to align and identify the outputs from high throughput sequencing experiments, a fact which must be taken into account in selecting an appropriate transcriptomics sequencing platform. This is a significant shortcoming of the shorter read length technologies as a necessary requirement of a transcriptomics experiment for this application is that transcripts be identifiable. If outputs from shorter read technologies such as SOLiD (30bp) and Illumina (70-100bp) cannot be unambiguously identified then, no matter how promising a transcript as a phenotypic marker, it cannot be utilised downstream in an *in vitro* expression assay and its value is greatly diminished. This makes the longer transcript read lengths returned by Roche 454 chemistry (500bp) an attractive option; even though the 'depth' of sequence returned may not be as great, there is a very much greater chance that differentially expressed genes identified via 454 will be readily identifiable by alignment against the fully annotated bovine genome.

It has been a turbulent year for Next Generation Sequencing (NGS) in NZ with the recent cessation of custom sequencing activities at both the Otago High Throughput Sequencing Facility (454) and the Massey Genome Service (Illumina). While the establishment of NZ Genomics Ltd promises to make NGS technology available to researchers in NZ in the coming year, at present there are no NGS service providers in the country. Currently, our intention is to work closely with Dr Jo Stanton (previously of the Otago based High Throughput Sequencing Facility) to elevate our mRNA recoveries from macrophages, with a view to using 454 technology for our transcriptomics work either using the 454 FX GLX platform operated by the Australian Genome Research Facility in Brisbane, the FX GLX at NZGL in Otago or the GS Junior platform operated at Otago. Having headed up the Otago Facility prior to its transfer to NZGL, Dr Stanton has extensive experience of NGS sample and library preparation for 454 sequencing projects, as well as analysis of same, and has expressed confidence that our macrophage RNA yields can be sufficient for a 454 transcriptomics experiment.

**Results**

A sample of the results obtained by Brooke Dobson (DRL) in her studies on the expression of candidate genes in *Map* infected macrophages from animals with an R or S genotype is given in Figure 1, below. There results were obtained using blood samples from uninfected deer with known breeding values for either Susceptibility or Resistance based on their herd EBVs. Note a high EBV (> +0.5) is indicative of susceptibility whereas a value of > -0.5 is indicative of resistance. A small group of animals with 0.0 values were included for comparison. These results are striking for three reasons:

- Pro-inflammatory markers (IL-1α, TNFα, iNOS and IL23p19) are significantly elevated in the S animals, relative to R.
- Low levels of pro-inflammatory markers are seen in the R animals while anti-inflammatory markers (IL-10 and IL12p35) are similar in S and R animals
- The data from the intermediate animals is no different from the R animals and clusters very tightly. This is particularly noteworthy as all three of these animals were bred from a single sire.

The conclusions that can be drawn from this preliminary dataset are:

- Naïve animals with a susceptible genotype appear to exhibit a dysfunctional, hyper-responsive inflammatory response to challenge by *Map in vitro*
- Resistant animals produce a controlled inflammatory response to infection.
- Gene expression levels in susceptible and resistant animals are unlikely to provide a mirror image of each other and may involve completely different sets of genes. This further suggests that we are unlikely to understand mechanisms of resistance by studying susceptible animals alone. It also highlights the future challenge of distinguishing between animals with Intermediate or Resistance genotypes.

Preliminary studies carried out on pairs of S and R animals, by Blake Gibson (DRL), using the expanded geneset listed above also show clear evidence of disregulation of pro-inflammatory markers in S animals compared to R. These studies will be extended to include larger groups of animals to select the most promising target genes. The results obtained by Robinson *et al*. (2) (Figure 2) on gene expression in lymph node biopsies from R and S animals, challenged experimentally with virulent *Map* in Colin Mackintosh's study (Milestone 4.2) show a very similar trend. While the lymph node RNA from these animals included the transcriptional output of lymphocytes, macrophages within the generalised cellular milieu of the lymph node tissue matrix, the impact on the inflammatory readout is compatible with the data obtained from Dobson's infected macrophage cells from naïve S and R animals. These data show clear differences in the gene readout from lymph nodes obtained from R (infected) and S (diseased) at necropsy. The most striking findings are:

- There is a significant elevation of pro-inflammatory cytokines (IFNγ, IL-1α and IL-17) in susceptible (diseased) animals.
- There is a significant elevation of regulatory molecules (IL-4, TGF-β and FoxP3) in resistant (infected) animals.

Taken together these data infer that the emergence of disease in susceptible animals is linked with an apparently disregulated inflammatory response to infectious challenge. By contrast, resistant animals produce a highly regulated and controlled response to infection that may be associated with containment of infection. The challenge that remains is to identify the set of genes that are expressed in resistant animals that facilitate the emergence of appropriate controlled, protective responses to infectious challenge and may allow them to be identified by predictive assay.

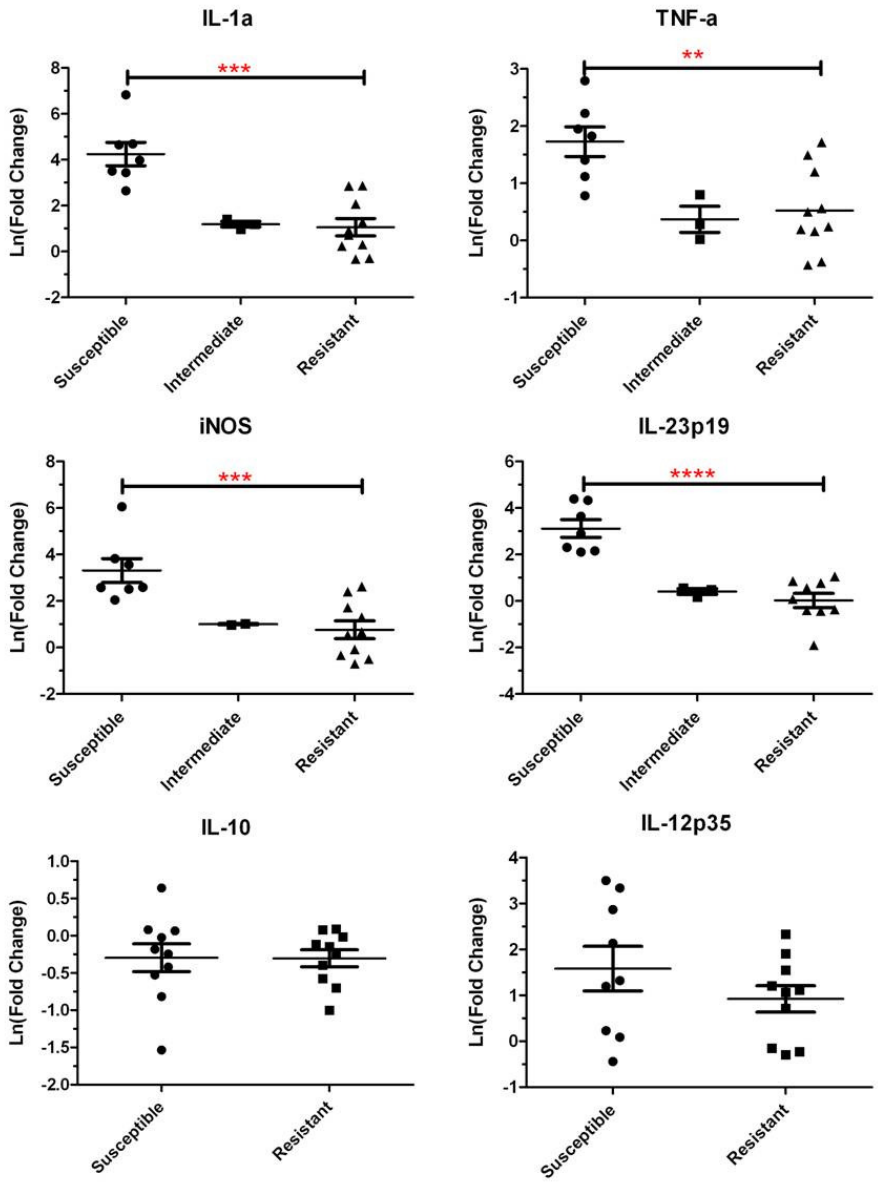


Figure 1. Differential expression of proinflammatory genes in response to macrophage challenge with *Map* in Resistant and Susceptible lines of deer.

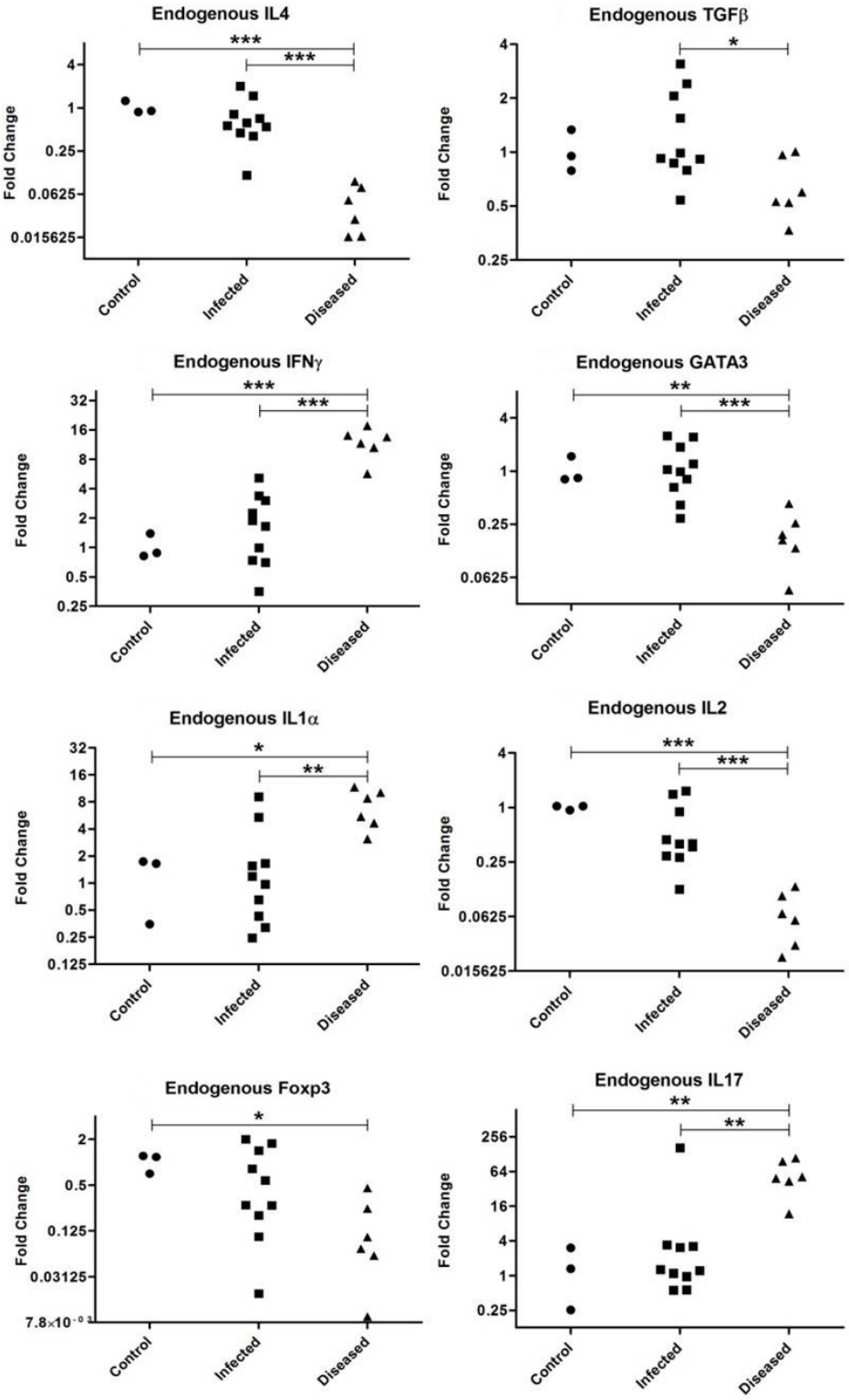


Figure 2. Differential expression of proinflammatory and regulatory cytokines in infected vs. severely diseased animals (2).

**Discussion**

We now have the necessary technology to predictably isolate, culture and invoke gene expression in peripheral blood derived macrophages. This has been demanding as macrophages are among the more difficult mammalian cells to isolate and manipulate *in vitro*, confounded by the fact that cervines are far from model organisms and available reagents tailored for their use are non-existent. This was an essential prelude for the accurate measurement of gene expression to minimise experimental variables and establish meaningful differences that may be correlated with either resistance or susceptibility. In the past two years, we have systematically identified breeds and subgroups of deer in a single stud herd, that express extreme levels of resistance or susceptibility to *M.ptb* infection. We are currently examining 3 other stud herds where our diagnostic programme suggests there will be a similar polarisation of phenotypes between different breeds. Access to multiple breeds of deer with extreme phenotypes provides an ideal experimental system to study the biology of heritable resistance. The fact that assisted breeding (AI and ET) has been used widely in the deer studs that we are studying, provides a unique aggregation of genotypes appropriate for genetic analyses.

**Conclusion**

We have established a technology platform to monitor gene expression predictably in deer macrophages. This technology will now be applied to selected animals that display extreme phenotypes for resistance or susceptibility to *M.ptb* infection. While initial studies look at the expression of a small number of candidate genes, we anticipate that a transcriptomic study will identify other genes that may be appropriate to develop a 'diagnostome' for resistance or susceptibility traits in outbred animals. The particular technology platform for the proposed transcriptome work remains to be confirmed pending investigations into cost and depth of coverage necessary and access to appropriate bioinformatics support for interpretation of the data. A 'minimal' approach is favoured over a 'maximum depth' rationale as it is anticipated that to be truly useful in a diagnostic sense the fundamental differences between R and S transcriptomes would need to be readily apparent. A lower resolution experiment will also keep costs down and allow multiple biological replicates to be performed.

**Recommendations for future directions**

Having demonstrated proof of principle that resistant and susceptible phenotypes may be distinguished by the nature of their gene expression response to *Map* challenge *in vitro*, our next task is to further develop and refine our suite of immunological markers to try and maximise observable demarcation between groups. The markers chosen thus far have been selected by virtue of their already well established roles in particular immunological pathways (inflammatory, TH1, TH2, TH17, TLR signalling, etc...); as all transcripts are constrained by some degree of transcriptional regulation, some genes will be more or less suitable as markers of differential expression than others, depending on the extent to which their expression is under strict regulatory control. Within a biological signalling pathway, there are a great many different points at which transcriptional activity may be assayed to maximise the strength of the discriminatory signal; some genes will be more useful than others for this purpose, depending on their capacity to be differentially expressed in response to the treatment, the amplitude of the expression signal and the extent to which their expression may be co-influenced by unrelated pathways. A scan of a panel of representative targets from pathways of interest will attempt to identify those individual targets most appropriate for use as expression markers to display polarised susceptibility phenotypes.

Ultimately, it is envisioned that a single blood sample collected in a standard vacutainer tube could be routinely processed for macrophage maturation in a small scale format such as a 75cm$^2$ culture flask. The expression readout of *in vitro* matured macrophages supplemented with autologous serum and infected with *Map* in a standardised fashion could be assayed against a suite of 50-100 gene expression markers (which have been empirically demonstrated to best

disclose R and S phenotypes in response to *Map* infection) simultaneously by qPCR within a single, 384 well microtitre plate. The cumulative data from these targets could be compared against a sliding scale of *Map* reactivity and used to identify phenotypes reflecting the hyper-responsive immune state seen to be associated with the susceptible phenotype. This would embody a test for immunological fitness/response to *Map* challenge that could be applied to the live animal with no more interference than that required for a Paralisa. Such a boutique test would likely be of particular interest to stud breeders and providers of foundation genetics. In addition to the selection of *Map* resilient founders, this could also have application in the breeding of animals that not only have resistance to *Map* but to other bacterial pathogens.

**References**

1. Griffin, J. F., E. Spittle, C. R. Rodgers, S. Liggett, M. Cooper, D. Bakker, and J. P. Bannantine. 2005. Immunoglobulin G1 enzyme-linked immunosorbent assay for diagnosis of Johne's Disease in red deer (*Cervus elaphus*). Clin. Diagn. Lab. Immunol. 12:1401-1409.
2. Robinson, M. W., R. O'Brien, C. G. Mackintosh, R. G. Clark, and J. F. Griffin. Immunoregulatory cytokines are associated with protection from immunopathology following *Mycobacterium avium* subspecies *paratuberculosis* infection in red deer. Infect. Immun. In Press.