4. General Discussion

4.1 Background:

Project 03-16 is a key project in Program 2A. In the first 3 years of the project, the CRC and CSIRO established the National Facility for Immunogenomics at AAHL, which has allowed the development of a world-class microarray capability that has fed into a range of new projects within the CRC. The research has been successful in that several potential novel health products and productivity enhancers were identified as discussed in more detail below.

During the first 3 years of this project commercially valuable IP was developed in two areas both of which have the potential to significantly improve the competitiveness of the poultry industry. Three International patents have been filed.

One technology involves the development sophisticated RNAi constructs that can alter the phenotypic traits of poultry. This development may lead to the ability to control the sex of chickens (by silencing particular sex-determination genes) or to enhance muscle growth in chickens (by modulation of developmental genes). Both of these uses have major commercial potential.

The second technology involves the discovery of a new immuno-modulator, interferon lambda. This technology is expected to have applications as a vaccine adjuvant and therapeutic agent for viral diseases.

During years 4-6, the project focused on developing further data required to support claims in complete patent applications in both areas and thereby added value to the CRC's IP assets. New Milestones for 2006-2009 was recently submitted and approved by the CRC.

Major Outcomes:

- The establishment of a National Facility for Immuno-genomics has provided a state-of-the-art resource to a range of projects and collaborators within the CRC
- Microarray facility fully functional and used across several CRC projects
- Models of CAV (day old and 3-week old) infection established
- Assessment of cytokine profiles of the spleen during CAV and IBV infection
- Recombinant cytokines produced for trials and impact of their administration assessed

- Adjuvant effect of cytokines in vaccinated, CAV and IBV challenged birds tested
- Candidate therapeutics (IL-6 and IFNλ) have been identified and biological activity characterised
- International patents for IFNλ have been filed and are pending.
- Additional data to support the patent application (enhancement of disease resistance and vaccine efficacy in commercial birds) has been developed.
- The nature of the immune response during a mucosal CAV infection has been identified and candidate therapeutics were assessed.
- RNAi has been developed and validated for in vitro and in ovo use as a gene knock-down tool to study the function of genes identified by genomic studies
- RNAi was assessed as an anti-viral agent for CAV (provisional patent filed)
- Developed proof of concept for the use of RNAi gene silencing to control production traits.
- Data to support the patent application covering RNAi gene silencing approaches to combating viral diseases.
- Development of a bacterial delivery vector for the administration of new therapeutics
- Major input and resource for new gut health project in Program 1 (05-09)
- Major input into formation of a new project for the development of a novel RNAi based MDV vaccine (04-14)
- Successful training of three Post Docs (Tamsyn Crowley, Peng Guo and Anthony Keyburn)
- Successful training of 3 PhD students:
    - Anthony Keyburn – breakthrough with regards to the role of β toxin in NE
    - Jesse Thomas – role of cytokines in immune responses
    - Adam Karpala – RNAi applications

Commercial potential:

At this stage we envisage future commercial applications in two areas that the CRC and CSIRO has a strong competitive position in:

1. RNAi Technology: This technology potentially provides a mechanism to significantly improve the efficiency of poultry production. Chickens normally hatch at a ratio of 50:50 males:females. For the egg laying industry the ability to hatch all chicks as females would result in a more efficient and welfare-friendly system as it would eliminate the need for the wasteful mass disposal of male chicks. On the other hand, the ability to hatch all broiler chicks as males (rather than the current 50:50 mix) would result in around a 10% increase in feed efficiency and increased productivity as males grow faster and convert feed to meat more efficiently than females. A major outcome of project 03-16 is the filing of a provisional patent covering this capability.

2. Interferon Technology: Interferon lambda is a newly identified natural immune system stimulant in poultry and has health benefits such as anti-viral and immune-enhancing activity. The latter effect is known to produce significant growth enhancement of 10-20% using other interferons.

5. Implications and Recommendations

5.1 Genomics

We have developed a strong genomics capability which has contributed to a number of other projects in the CRC and has ongoing applications in a wide range of projects of relevance to the poultry industry. For example, the genomics work has informed our ongoing studies of CAV infection in chickens and has assisted in identifying and appropriately using and studying IFN-λ as a therapeutic agent. The genomics capacity is a core capability that needs to maintained to service the needs of multiple projects that are anticipated to be supported in the new CRC. This capability is not, in its own right, directed at producing specific commercial outcomes for the industry but is rather an enabling technology used by other more specifically focused projects. We see major opportunities in continuing host-pathogen studies, more focused work on monitoring the changes induced by therapeutic treatments such as IFN-λ, nutrigenomic and productivity studies, and in monitoring and understanding the effects of more advanced genomic manipulations such as those envisioned in the chicken sex selection work and our ongoing work to produce avian influenza resistant chickens.

Recommendation 1: The CRCII and CSIRO continue to co-invest in the National Facility for Poultry Immuno-genomics:

- **Provide resources to maintain the facility**
- **Support the provision of critical Bioinformatics input into data analysis**

5.2 RNA interference

In this project we have developed significant intellectual property in the area of using RNAi technology to modulate sex determination in chickens by targeting the sex determining gene (*DMRT1*). We now propose to develop further proof-of-concept data followed by the development of commercial applications that will have significant welfare and productivity benefits for the poultry industry. Through colleagues at Melbourne University, we recently confirmed that *DMRT1* is the major regulator of male sex determination in chickens (Smith *et al*., NATURE 2009, 461:267). By using RNAi to reduce expression of *DMRT1* in embryos, development was strongly biased towards females as measured by the development of ovaries rather than testis. Through this proposed project, we will develop novel, non-transgenic approaches to deliver RNAi to the developing embryo.

Specifically, modulation of *DMRT1* gene expression in male embryos provides an innovative approach to generate single sex female populations for the layer industry. In addition, we will collaborate with the Smith lab to also look at the potential to generate all-male populations and furthermore, to identify additional key genes involved in sex development of chickens. We will also functionally analyse two key candidate female-determining genes, R-Spondin1 and FOXL2 and test whether knock-down of these in embryos will result in sex bias toward to males. This important basic research will be fundamental to our understanding and manipulation sex ratios in chickens. Delivery of these molecules to embryos is a major challenge. There are two strategies for delivery of RNAi to chicken embryos: (1) viral vectors that express short hairpin RNA (shRNA) targeting *DMRT1* and; (2) synthetic "drug-like" delivery of small interfering RNA (siRNA) targeting *DMRT1*.

Recommendation 2: That the CRCII supports ongoing work in this important area to:

- **Develop proof of concept for modulation of males to females (layers)**
- **Develop proof of concept for modulation of females to males (broilers)**
- **Develop commercially acceptable delivery methods for this process**
- **Assess the biological and phenotypic characteristics of modulated chickens**
- **Work closely with a commercial partner from the onset of the work**

5.3 New therapeutics:

The increasing demand for "clean and green" poultry products has increased the pressure to develop natural, non-chemical alternative strategies to manage infectious diseases in poultry. Compounding this is the observation that for many diseases the current vaccines offer less than complete protection and, in the case of newly emerging pathogens, there may no vaccination strategies available. This, coupled with changes in production approaches, including reduced reliance on antibiotics and a trend towards free-range systems, has put further strain on existing protection strategies. Cytokines are proteins that control immune responses following infection or vaccination and represent excellent, naturally occurring therapeutics. In this project we have developed significant intellectual property in the area of cytokines as natural therapeutics and anti-virals. We propose that this proof-of-concept work now be extended to develop commercial applications that will have significant health benefits for the poultry industry.

The use of the natural cytokines as therapeutics or vaccine adjuvants has the potential to enhance Australia's "clean and green" image when it comes to sustainable food production. In addition, the availability of these novel immunomodulatory molecules provides the poultry health area with products will increase Australia's preparedness against disease outbreak risks. This then assists in

solving the major challenge of achieving sustainable, ethical poultry production and maintaining a supply of healthy and welfare conscious poultry products.

There is a great emphasis on the development of new approaches to enhance existing vaccine approaches to provide long-term protection and to meet the industry demands of enhanced quality, health and food safety. However, existing adjuvants can have deleterious side-effects, such as inflammation, which may result in the down-grading of meat quality and a subsequent reduction in profits. Therefore, to enhance the use and effectiveness of vaccination, alternative adjuvants must be developed. Natural immunomodulators provide an attractive alternative, and as such the use of recombinant cytokines is drawing considerable attention. Infact, chicken interferon gamma (a molecule discovered and developed by this CSIRO group) is currently undergoing commercial development by Merial as a growth and health enhancer in poultry. The recent identification of a number of chicken cytokine genes has provided the possibility to study their effectiveness in enhancing the immune response during infection and vaccination. In both human and veterinary health, major vaccine companies have indicated a need for such approaches and see this as a viable step in the development of improved vaccine strategies. Of particular relevance is Pfizer's interest in developing new vaccines, adjuvants and therapeutics for *in ovo* delivery.

We propose that these technologies be explored and developed by undertaking studies assessing the biological function of cytokines and assess their anti-viral potential and, similarly, to assess their ability to enhance vaccine efficacy. Furthermore, identification of the regulatory molecules involved in generating effective immune responses will provide mechanisms to manipulate the immune response to direct it towards an appropriate and controlled protective response.

We suggest the following areas of study will be important in developing this area:

Interferon-λ and its receptor as therapeutics for poultry: The first line of defence against viral infections is mediated by IFNs that are produced rapidly by the infected host. As described in this report, we have identified a novel class of cytokine, IFN-λ, in the chicken. Furthermore, we have also identified its receptor and observed the likely existence of a soluble form of this receptor. Based on the observed anti-viral activity of this cytokine and its potential to induce typical IFN-inducible genes, these molecules have the potential to be used as an anti-viral therapeutics.

New IFNs, the next wave of anti-viral therapeutics: We have recently identified several novel IFN and cytokine candidate molecules that represent excellent therapeutic candidates.

Type I IFNs on the Z chromosome and the anti-viral battle of the sexes: The identification of genes that control immune pathways could provide new ways to combat viral infection providing valuable insights into immune regulation. Intriguingly, the male chicken carries a double dose of the principal antiviral genes, the type 1 IFNs, this eventuates as these genes reside on the Z chromosome (of which males have 2 and females just 1). The regulatory mechanisms that compensate for male Z dosage of the type 1 IFNs are currently unknown and provide a unique opportunity to understand the processes that control these immune pathways. Moreover, by understanding how females double their production of type 1 IFNs or how males halve this production, important information as to how to manipulate the immune response will be gained and may provide potential novel immunostimulatory approaches or new anti-inflammatory strategies. We suggest that by taking functional genomics approaches such as those described in Chapter 1, we can compare and contrast male and female immune control genes. This will not only provide insights into immune related sex determined genes, but may also provide breeders with genetic information to support breeding strategies

Immune response analysis of 18 day old embryos for enhanced *in ovo* delivery of vaccines: Very little information exists as to the level of immune responsiveness in day 18 embryos – which are the targets of in ovo vaccine strategies. This information is required to assist in the rational choice of adjuvants to enhance *in ovo* vaccination. Using genomic approaches an ontogenic assessment of the immune receptor expression and the related immune responsiveness of the developing embryo can made. With this knowledge we can evaluate the administration of a variety of new adjuvants to determine which induces the highest level of response.

Chicken NALP3: Aluminium salt based adjuvants, commonly referred to as 'alum', are the most commonly used adjuvants in human and animal vaccines worldwide, yet there is a paucity of information around the mechanism underlying the action of these molecules. In mammals it has been recently postulated that alum adjuvants activate an innate immune response receptor known as NALP3, which then triggers the production of the inflammasome related pro-inflammatory cytokines interleukin-1 and interleukin-18. We have recently identified the chicken orthologue of the NALP3 receptor. We propose to investigate the Nalp3 inflammasome as a crucial element in the adjuvant effect of alum. Additionally, we investigate the role of the innate inflammasome pathway in humoral adaptive immune response and investigate the potential for other non-Alum based molecules to trigger NALP3 in a search for new adjuvants. The impact of this research has potential to develop new strategies to modify the design of effective and safe adjuvants for the future.

IFN-λ a potent adjuvant for poultry: We have shown in this report that ChIFNλ protein increased antibody responses five-fold in chickens when administered with a vaccine antigen, compared to

vaccination with antigen alone. This remarkable observation supports the concept that ChIFNλ may have the potential to augment an immune response when used as an adjuvant with a variety of vaccines. Similarly, this boosting effect may have the potential to allow a lower dose of antigen to be used, which may result in more cost effective use of vaccines.

Mesenchymal stromal cells (MSC) as RNAi and therapeutic delivery vehicles: MSC are adherent stromal cells capable of self-renewal and differentiation. These MSCs can be recovered from distinct tissue sources and appear are easily manipulated in cell culture. RNAi has the potential to be a unique therapeutic agent, however, as yet there are a number of barriers to simple delivery of these molecules. Furthermore, since the immune system has many receptors directed at detecting dsRNA molecules and then initiating a response against this dsRNA, the delivery of RNAi has been further been fraught with difficulties in delivery. Since MSC provide a possible delivery system and are non immunogenic, this provides the potential to use chicken MSC as a feasible cellular delivery system for RNAi.

Recommendation 3: That the CRCII supports ongoing work in this important area to:

- **Extend proof of concept for IFNλ to develop it towards a commercial product**
- **Assess potential candidates as commercial adjuvants for in ovo delivery**
    - **Eg. NALP3, IFNλ, plus new discoveries**
- **Further development of novel anti-viral therapeutics**
- **Development of MSCs as delivery vehicles for RNAi**
- **Assessment of immune system status in E18 embryos**
- **Work closely with a commercial partner from the onset of the work to develop further commercial development**

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Appendix 1

Journal Publications for 03-16

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Conference Presentations for 03-16 involvement in other Projects:

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Appendix 2

list of abbreviations

aa	amino acid(s)
Amp^R	ampicillin resistance
APP	acute phase protein(s)
bp	base pair(s)
BSA	bovine serum albumin
cDNA	DNA complementary to mRNA
Ch	chicken
cMGF	chicken myelomonocytic growth factor
ConA	concanavalin A
CPM	counts per minute
CRP	C reactive protein
CS	culture supernatant
CSF	colony stimulating factors(s)
C-terminus	carboxy terminus
CTL	cytotoxic T lymphocyte(s)
DEAE-Dextran	diethylaminoethyl-Dextran
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dpi	days post infection
EID	egg infectious dose
E. coli	Escherichia coli
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
FAV	fowl adenovirus
FCA	Freund's complete adjuvant
FCS	feotal calf serum
FIA	Freund's incomplete adjuvant
FPV	fowlpox virus
GAPDH.	glyceraldehyde-3-phosphatase dehydrogenase
gp130	glycoprotein 130
GST	glutathione S-transferase

HA	hemagglutinating antibody
HAT	hypoxanthine aminopterin thymidine
HRP	horse radish peroxidase
HVT	herpes virus of turkeys
IFN-	IFN-
IBV	infectious bronchitis virus
Ig	immunoglobulin(s)
IL	IL
IL-6R	IL-6 receptor
IPTG	isopropyl-β-D-thiogalactopyranoside
i.v.	intravenous
i.m.	intramuscular
i.p.	intra-peritoneal
LPS	lipopolysaccharide
mAb	monoclonal antibody(ies)
MDV	Marek's disease virus
MHC	major histocompatability complex
MW	broad range molecular weight markers
NDV	Newcastle disease virus
Ni-NTA	nickel-nitrilotriacetic acid
N-terminus	amino terminus
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PHA	phytohemagglutinin
PMA	phorbyl 12-myristate 13-acetate
po	porcine
PWM	pokeweed mitogen
qRT-PCR	quantitative real time PCR
R	receptor(s)
r	recombinant
RT	room temperature
RT-PCR	reverse transcription PCR
SAC	*staphylococcus* aureus cowen I
s.c. .	subcutaneous

SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM	standard error of the mean
SF	serum free
sIL-6R	soluble IL-6 receptor
SPF	specific pathogen free
SRBC	sheep red blood cell(s)
TCR	T cell receptor
TGF	transforming growth factor
Th1	T helper 1
Th2	T helper 2
TNF	tumor necrosis factor
TSB	trypticase soy broth
^{3}HT	methyl ^{3}H-thymidine
6x His	6x Histidine tag
α-MM	methyl α-D-mannopyranoside
α_1AG	alpha-1-acid glycoprotein

Plain English Compendium Summary

Project Title:	
Project No.:	03-16
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Objectives	
Background	A major problem faced by Australian poultry industries is reduced productivity due to disease. Over the past several decades the two main mechanisms used to control disease have been the use of vaccines and antibiotics. Emergence of new virulent strains of viruses and anti-biotic resistant bacteria has driven the search for new types of health products. The availability of detailed genomic information for chickens has allowed us to develop genomic based methods for identifying new vaccines and therapeutic approaches to control disease in poultry.
Research	We have established a state-of-the-art national facility for poultry immunology and genomic research that will service the Australian poultry industry. We have employed new technologies such as microarrays, RNA interference and sophisticated immunological analysis to understand how chickens respond to pathogens such as CAV and MDV. This provides crucial insights into the identification and assessment of new health products that can be used more widely by the poultry industry.
Outcomes	• Establishment of the National Facility for Immuno-genomics provides a state-of-the-art resource to a range of projects and collaborators within the CRC. • A new candidate therapeutic (interferon-λ) identified and assessed in trials. • RNA interference technology has been developed and validated for use to control production and sex traits. • 3 International patents have been filed and are pending. • Training and development of young scientists to meet the future needs of the Australian Poultry Industry. • Strong support from commercial partners in converting proof-of-concept research into commercial applications.
Implications	This project has identified proof-of-concept for several new approaches that can be used to develop and commercialise novel health products for the Australian poultry industry. The new facility infrastructure and technologies will be used in the Poultry CRC-II to further develop these candidates and provides further support for other CRC participants. This approach is aimed at enhancing the health and productivity of the poultry industry and ensure that it operates in a sustainable manner to meet growing consumer demands. The outcomes of this project provides a strong springboard to providing innovative solutions to address significant poultry health and welfare issues.
Publications	During this six year project, the research team has been highly successful in generating world class research. Success indicators include the publication of 22 papers in peer-reviewed scientific journals, presentations at 18 international and 18 national scientific and industry conferences and the filing of three International patent applications covering potential commercial opportunities for the use of RNAi to control sex ratios and the discovery of novel therapeutics and vaccine adjuvants. In addition the project has graduated three PhD students and trained three Post-Doctoral scientists which will be important future resources for the Australian poultry industry.