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Review of the CSIRO Biological Control of Cane Toad Program to April 2008

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Executive Summary

This document is the second review of the federally funded project "The Development of a Cane Toad Biological Control" Commonwealth ID 56832. Terms of reference for the review are to assess: (1) progress of research by CSIRO on the development of a self-disseminating viral vector that disrupts the development of cane toads; (2) the technical feasibility of this research being developed to the point of offering a new and effective control method for cane toad populations in Australia (including consideration of the regulatory situation for release of such an organism); and (3) the opportunities provided by other current avenues of research into cane toad control, and the relative potential for these to provide a broad scale cane toad control.

The research performed by the CSIRO team has been of high scientific quality and a number of aspects of the research program have been successful and published in the peer-reviewed literature. These include the development of a cane toad breeding colony, the identification of genes differentially expressed in tadpoles and toadlets, and the construction of BIV recombinant viruses. However, there are still major technical hurdles to be overcome in the development of a self-disseminating genetically modified cane toad control agent. The long term feasibility of the approach is also questionable on several counts including the availability of an acceptable viral vector, the difficulty of generating an appropriate immune response from virally expressed proteins, and the major hurdle of obtaining approval for release. The lack of a national and international risk assessment and management plan for the release of a virally vectored GMO regardless of exact product specification is also a major deficit and should be an essential part of any further program in this area.

The first review (Hazell et al. 2003) acknowledged that the development of a fully tested and approved biocontrol for cane toads was a long-term solution and hence recommended that the then Environment Australia consider other avenues to address the problem in the short to medium term. However, key constraints in assessing the relative merits of alternative control approaches (including integrated control), and in comparison to the CSIRO biocontrol approach, are: (1) incomplete knowledge of ecological impacts; (2) lack of benefit-cost analyses of each approach; (3) lack of environmental impact assessments for each approach; and (4) uncertainty associated with regulatory approvals, especially for genetic approaches. We recommend that tight selection and performance criteria be used for future investment decisions in alternative approaches to cane toad control, and that this process include more comprehensive population modelling of their practicality and efficacy. No practical alternative approach to broadscale cane toad control has yet been demonstrated and hence continued investment in multiple alternative approaches is still required. Of all alternative approaches reviewed, the following are assessed as having potential opportunity for more effective local and possibly broader-scale control, either singularly or in combination, and could be considered for future investment by a technical panel facilitated by the Invasive Animals CRC: (1) the cane toad-specific lungworm parasite and the use of an alarm pheromone; (2) the bioprospecting approach to search for new pathogens overseas; (3) development of a cane toad-specific poison; and (4) the "daughterless male" approach for the special condition of a closed population.

Recommendations

1. Funding for the CSIRO Cane Toad Biocontrol Program should be discontinued in its current form.

2. The CSIRO cane toad biocontrol team and the Invasive Animals CRC should form a collaborative multidisciplinary partnership with clear mutual benefits identified at the outset.

3. The expertise developed by the CSIRO Biocontrol Team should be maintained if at all possible and channelled into related control efforts, such as the use of GMO control agents in non-disseminating systems.

4. A national and international risk assessment for the release of a virally vectored GMO should be undertaken prior to any further resourcing of the project. The opinion of the Office of the Gene Technology Regulator with respect to the likelihood of obtaining an approval for such a release should be sought.

5. Studies should be resourced to: (i) clarify the severity of cane toad impacts on native predators; and (ii) ascertain ecosystem level impacts of cane toads.

6. The following alternative approaches to cane toad control are assessed as having potential opportunity for more effective local and/or possibly broader-scale control, either singularly or in combination, and could be considered for future investment subject to more comprehensive technical assessments by a scientific panel facilitated by the Invasive Animals CRC:

a) The cane toad-specific lungworm parasite and alarm pheromone.
b) The bioprospecting approach to search for additional pathogens.
c) Designing a cane toad-specific poison based on knowledge of the cane toad-specific ion channel.
d) The “daughterless male” approach for closed island populations.

7. More comprehensive population modelling of the feasibility and efficacy of different control approaches is required, including integrated control, and encompassing more realistic spatial and stochastic processes.

8. Benefit-cost analysis of alternative control approaches should be undertaken, including combinations of different approaches (integrated control).

9. Tight selection and performance criteria should be used for future investment decisions in alternative cane toad control approaches, based on current levels of knowledge and/or knowledge gained from Recommendations 5, 7 and 8 above.

Background

Brief history cane toad research & management

Cane toad control was first placed on the national agenda in 1983 when federal funds were allocated to undertake initial studies in the Northern Territory and Queensland on ecology and diseases (Tyler 2006a). It was assumed that an *in situ* native pathogen could be identified and used in biological control without the need to import another exotic species. These initial studies ended in 1989 and further funds were allocated for the period 1990-93, which included research teams based in South America (both Venezuela and Brazil) searching for potential pathogens. The focus of this second phase was on finding a natural biological control agent, and studies on ecological impacts were considered minor. Several ranaviruses were isolated in South America and additional funds were allocated for the 1994-1996 period to import them into Australia for testing. Although highly lethal, they were not species-specific (i.e. they were ubiquitous with a broad host range) and hence the research was discontinued. Concomitantly, research focus began to shift to the ecological impacts of cane toads as recommended by the Kikkawa Committee (Tyler 2006a). Hence, it was recognised that knowledge of impacts was required to make rational long-term investment decisions for the control of cane toads. However since 1986, no suitable biocontrol agent had yet been identified. In 2000 CSIRO was successful in obtaining government funds to renew the search for a biocontrol agent. Given the previous failures in finding naturally-occurring viruses, the idea of using a virus that despite considerable research effort could be modified to contain a gene that would interfere with some essential life history process in the cane toad, was proposed (Robinson et al. 2006). Since 2002, CSIRO has received significant funding from the Department of Environment, Water, Heritage and the Arts (DEWHA) to continue this line of research.

Since 2002, there has been substantial activity on cane toad research and management (see summary in Table 1), including a number of successful workshops. A National Cane Toad Task Force (NCTTF) was formed in 2004 as a sub-committee of the Vertebrate Pests Committee (VPC) and replaced all previous cane toad committees. The NCTTF was recently replaced by the Cane Toad Advisory Group (CTAG), which was endorsed by the VPC and facilitated by the Invasive Animals CRC. While much information on cane toad biology and their impacts has accumulated in just over two decades, the development of cost-effective effective local and broadscale control methods remains elusive. Nevertheless, communication between all researchers in the field has been high in recent years as exemplified by the very high quality of workshops since 2002.

Ecological and Socio-cultural Impact.

<u>Ecological</u>

Two broad ecological impacts are considered here:

- impacts on native predators of cane toads; and
- ecosystem impacts (e.g. competition for shelter & food, food web cascades).

The National Cane Toad Taskforce (NCTTF) facilitated a workshop in June 2005 to review the impact and control of cane toads in Australia and to recommend future

research and management approaches to the VPC (Taylor & Edwards 2005, Robinson 2006a). The review was comprehensive and workshop participants concluded that:

- While it was acknowledged that some species such as the northern quoll might be seriously impacted, much research to date remained inconclusive at a species and population level. It was uncertain to what degree species that initially declined would recover in the long-term.
- There were insufficient data to quantify impacts from cane toad predation on aquatic and terrestrial invertebrates, and the impacts that might have occured on native species due to competition with cane toads for food and shelter. While it was suspected that predation and competition played a significant role in reducing populations of native species and altering ecosystems (see Doody et al. 2006a), data investigating such impacts were neither complete nor comprehensive.

Similar conclusions were made by Freeland (2005) when assessing potential cane toad impacts in the Eastern Kimberley Region for the WA government. It is interesting to note here that the only other species besides the northern quoll to suffer a demonstrated long-term decline is a proteocephalid tapeworm of the python, *Antaresia maculosus* (Freeland 1994). The decline appears to be associated with destabilisation of frog communities, although no frog species have been lost (Freeland 2005).

The most up-to-date reviews of the ecological impacts of cane toads in the Northern Territory are those by Shine et al. (2006) for the Fogg Dam area (near Darwin) and Doody et al. (2006b) for the Daly River area (south of Darwin). Shine et al. (2006) suggested that, although early days, the impacts of toad arrival on native fauna have been relatively minor except for widespread mortality of varanid lizards. Additionally, they argued for innovative ecologically-based approaches for reducing toad impacts. Doody et al. (2006b) confirmed the results of Shine et al. (2006) for three species of varanid, as significant declines in numbers occurred with the synchronous arrival of cane toads in the 2004-05 wet season (*Varanus panoptes* 77-90% population reduction; *V. mertensi* 86-92%; *V. mitchelli* 28-40%). They speculated that delayed impact might occur with the more aquatic varanid species (*V. mitchelli*) and freshwater crocodiles, and recommended continuous monitoring to determine short and long-term impacts.

During an interview, Dr Doody – whose self-funded study was part-funded by CSIRO in latter years – provided updated survey data to the 2006-07 wet season. His recent results show that, in contrast to current conventional wisdom that predators will rapidly recover from the initial toxic impact of the cane toad dispersal front, goanna populations in the Daly River area have not yet started to recover after three years (i.e. the low post-toad numbers have "flat-lined"). The assumption that native predators rapidly recover from the initial effects of cane toad invasion is used as an argument by many people interviewed not to invest funds in biocontrol and take the risk of releasing a GMO virus. Most quoted Freeland's (1990) early study where he concluded that, over the long-term, goanna populations recovered fully from cane toad impacts. However, his initial goanna study sites in the Gulf of Carpentaria (GoC) were not re-surveyed, and his conclusion appeared to be based on anecdotal information on goanna abundance in the Townsville region where toads had been present since 1935. He may be right, but it is important to note that in this often quoted study the recovery of goannas has never been quantified. Additionally, much

anecdotal information exists also that show cane toads cause significant population declines in native predators (Burnett 1997). Hence, the assumption that ecosystem impacts are temporary because genetic resistance and/or predator learning will eventually kick in needs to be re-assessed in a systematic manner because equivocal observational and anecdotal information are now emerging (i.e. rapid recovery goannas vs. little/no recovery, or Freeland/Shine vs. Green/Doody results). The re-assessment should review whether or not recovery rate is less in habitats that are optimal for cane toads as found in the Daly River, in comparison to that for suboptimal habitats as found in the Gulf of Carpentaria and at Fogg Dam (i.e. the Freeland & Shine studies, respectively). Sub-optimal habitats would have lower cane densities and hence may have fewer ecological impacts.

Socio-cultural (e.g. bush-tucker)

McRae *et al*. (2005) outline potential socio-economic impacts that cane toads may have on Northern Territory communities, in particular the impact on hunting practices of Indigenous people. Declines in bush tucker species such as varanid lizards, snakes and turtles have had significant cultural and economic impacts upon Aboriginal communities, although this has never been systematically quantified (van Dam et al. 2002).

Key recommendations not followed through since 2002

Despite the many cane toad workshops since 2002 and the formation of new cane toad committees and national taskforces, some key recommendations have not been implemented and yet are necessary pre-requisites for making rational future investment decisions with respect to long and short-term cane toad control, either locally or broadscale, and these are listed below:

- Collate, document and assess all current knowledge on the short and long-term impacts of cane toads (Hyatt and Robinson 2004).
- Undertake a risk assessment of short and long-term cane toad impacts to guide future control investments (Hyatt and Robinson 2004).
- Undertake a cost-benefit analysis of different control approaches (McCallum 2005), which will eventually require development of more realistic spatial population models to simulate different control scenarios including integrated control (this report & McCallum and Bashford 2008).
- Initiate research into ecosystem-level impacts of cane toads, with particular reference to invertebrate communities (Taylor and Edwards 2005, various workshop papers since 2002, and most people interviewed for this review).

Table 1. Summary of cane toad research and management events (workshops, committees, reports) since 2002.

	Date	Event	Scope
1	2002	Desktop risk assessment of cane toads on Kakadu National Park values (van Dam et al. 2002)	For DEH/PAN
2	2003	Progress report (July 2002-June 2003) for CSIRO Project "The Development of a Cane Toad Biological Control" (Robinson 2003)	For DEH
3	Feb 2003	Review of project "The Development of a Cane Toad Biological Control" (Hazell et al. 2003).	First review for DEH funds
4	Feb 2004	Workshop on Biological Control of Cane Toads (Hyatt & Robinson 2004).	For DEH
5	April 2004	Natural Resources Ministerial Council Communiqué. VPC directed to investigate options for a national approach to eradicate cane toads, and to review the threat posed by toads, assess the research in place to address the threat, and assess the costs and benefits of national action.	VPC, State & federal environment departs.
6	2004	Progress report (July 2003-June 2004) for CSIRO Project "The Development of a Cane Toad Biological Control" (Robinson 2004)	For DEH
7	Sept 2004	National Cane Toad Task Force (NCTTF) formed	Sub-committee of VPC
8	2005	Cane toads listed as a Key Threatening Process under the EPBC 1999	For DEH
9	April 2005	Desktop assessment of cane toad impacts on native wildlife, with special reference to East Kimberley Region (Freeland 2005)	For WA Dept. Industry & Res
10	2005	Progress report (July 2004-June 2005) for CSIRO Project "The Development of a Cane Toad Biological Control" (Robinson 2005)	For DEH
11	June 2005	Workshop on "A review of the impact and control of cane toads in Australia with recommendations for future research & management approaches (Taylor & Edwards 2005)".	For NCTTF/VPC
12	2006	Progress report (July 2005-June 2006) for CSIRO Project "The Development of a Cane Toad Biological Control" (Robinson 2006)	For DEH
13	June 2006	Workshop on "Science of Cane Toad Invasion & Control" (Molloy & Hendersen 2006)	For CSIRO/IA CRC /Qld NRM&W
14	2007	NCTTF replaced by Cane Toad Advisory Group (CTAG). Endorsed by VPC & facilitated by IA CRC.	VPC/IA CRC
15	2007	Progress report (July 2006-June 2007) for CSIRO Project "The Development of a Cane Toad Biological Control" (Robinson 2007)	For DEH
16	Nov 2007	Final report on project: "Potential short-term control measures for cane toads – focussing on spawning site preferences and trapping of tadpole and juvenile cane toads (Shine & Hagman 2007)".	For DEH & ARC
17	April 2008	Report on "Cane Toad Chemical Ecology: Developing Natural Control Strategies (Capon 2008)	For IA CRC
18	2008	Progress report (July 2007-June 2008) for CSIRO Project "The Development of a Cane Toad Biological Control" (Hyatt 2008)	For DEWHA
19	May 2008	Discovery of a cane toad-specific lungworm by Prof. Rick Shine (Australian Academy of Science)	Public announcement
20	June 2008	Review of project "The Development of a Cane Toad Biological Control" (Shannon & Bayliss 2008).	This report: 2nd review/DEWHA
21	June 2008	Cane toad session & one-day Cane Toad Control Research Forum will be held in Darwin at the 14th AVPCC.	For CTAG

ToR 1. Progress of research by CSIRO on the development of a self-disseminating viral vector that disrupts the development of cane toads

The **overall aim** of the project is to develop a strategy that will contribute to the trans-continental reduction of the cane toad population using a recombinant self-disseminating virus. The **concept** is to insert a specific gene(s) into a virus such that when the tadpoles are infected with the virus, an antibody response ensues and this response then targets that endogenous protein at a specific time during metamorphosis or adult development preventing development and/or survival.

The project has eight main objectives and progress on each aim will be reviewed in turn.

1. **Establish a cane toad colony.**

   The establishment of cane toad colony has been successful and Mr Daryl Venables and his colleagues should be congratulated on this achievement (Hamilton et al 2005). This knowledge has been disseminated to other users around the country. The group is also in the process of establishing the first known line of inbred cane toads.

2. **Identify an Australian virus that could be engineered as a model to test the hypothesis.**

   The virus identified for these purposes was Bohle iridovirus (BIV). It is the only amphibian virus isolated in Australia and while it is known to infect cane toads, it should be noted that it is not specific to cane toads. It is able to survive in the environment. It is a large double stranded DNA virus which makes it relatively easy to manipulate. It was chosen as a virus to model viral delivery to cane toads.

3. **Identify genes expressed in toadlets but not in tadpoles.**

   Microarray technology was successfully used to identify genes differentially expressed in toadlets and not in tadpoles. Nine genes were chosen from this analysis, gene fragments isolated, sequenced and searched against databases. Differential expression was confirmed by PCR. Novel sequences with altered expression throughout development were also identified in this screen. These data have recently been accepted for publication. (Damien C.T. Halliday, Gavin C. Kennedy, Nicholas H.R. Hamilton, Suze Tarmo, James Alderman, Nicole A. Siddon, Anthony J. Robinson Genes induced during the early developmental stages of the Cane Toad, *Bufo (Chaunus) marinus*. *In Press, Gene Expression Patterns, Accepted Manuscript, Available online 26 April 2008.)*

   Three genes have been chosen for follow up at this stage: adult globin (because of previous experiments in a published paper) (Maniatis et al. 1969), trefoil factor, and gastrokine. Cane toad trefoil factor 1 is a homologue of trefoil factor 1 (TFF1) in mammals (previously known as pS2) and xP1 in *X. laevis.* TFFs are small (7-12 kDa) protease-resistant proteins abundantly secreted onto mucosal surfaces of the gastrointestinal tract, where they are thought to play key roles in gut protection and the process of restitution (Taupin and Podolsky, 2003). TFF1 has an

important role in regulating the balance between gastrointestinal cell proliferation, death and differentiation. Trefoil factor family is comprised of the: (i) gastric peptides pS2/TFF1; (ii) spasmolytic peptide (SP)/TFF2; and (iii) the intestinal trefoil factor (ITF)/TFF3. Larval forms are not noted in the literature. Gastrokine represents a recently characterised protein of humans, mice, rats, cows and pigs that has strong, specific expression in the gastric epithelium of the stomach (Oien et al., 2004; Yoshikawa et al., 2000). As a secreted protein, gastrokine appears important for normal gastric function such as gastric mucosal protection, and is also found to be down-regulated in gastric cancer (Oien et al., 2004). A recent study (Baus-Loncar et al., 2007) concludes that the regulation of GKN2 parallels that of TFF genes, indicating that together they may play an important role in maintaining the homeostasis of the gastrointestinal tract.

Both gastrokine and trefoil factor expression dramatically increase late in tadpole to toadlet development. The gut of anurans alters abruptly during metamorphosis, developing from a long basic tube to a shorter more complex organ. Possibly these genes are induced in late metamorphosis to regulate the balance between cell differentiation, proliferation and death occurring within the gastrointestinal tract during this period. Because these proteins might play a role in gut development and because they are secreted proteins and maybe available for antibody recognition, they were chosen as possible targets.

Although the identification of differentially expressed genes has been successful, whether the genes identified to date will serve as good targets for the immune system and the details of their role in metamorphosis and/or survival needs much more in-depth investigation before conclusions can be drawn.

4. Assess species specificity of target genes.

To date only GenBank Blast searches have been undertaken in the project. These searches show that (a) cane toad Gastrokine reveals no matches to any amphibian, and (b) cane toad TFF shows *Xenopus laevis* and *X. tropicalis* are the only amphibians to have TFF homologues. However, it should be noted that beyond Xenopus genome sequences, there will be only fragmented information on other species. Amino acid sequence alignments have also been carried out (see Halliday et al).

In addition, the specificity of the target genes will be assessed by screening cDNA libraries made from native frog species. Advice was obtained from experts at the Amphibian Research Centre in order to ensure coverage of all 27 genera of Australian native frogs. So far cDNA libraries have been prepared from 11 native frog species across six genera with variable habitat distributions around Australia and are in long-term storage. These libraries will not be screened until chosen target genes have been verified as useful. Given limited resources this is a reasonable decision.

5. Search for alternative (Australian) viral vectors.

So far 30 toads obtained from the Townsville have been screened for Ranaviruses, Adenovirsuses and Herpervirus (in progress). So far no viruses have been detected. Around 100 cane toad liver samples (collected from 10 sites; each

contained 10) showed negative results for adenovirus screening. Sites were chosen across Australia to cover almost all potential areas where cane toads have spread. Although this screening has so far wielded negative results, the PCR technology developed for adenovirus and herpervirus has been transferred into another DEWHA-funded project examining amphibian diseases in Australia. This is a positive outcome from this section of the project.

6. Manipulate the virus to incorporate at least one adult toad gene.

The BIV virus was initially passaged in VERO cells in culture to attempt to attenuate the virus. Two recombinant viruses have been constructed, one in DNA from early passage virus and one from late passage virus. Both these viruses have a deletion in the translation control protein, eIF-2e, due to the insertion of the transgene and/or reporter gene The new viruses contained the adult globin gene. It should be noted that the viruses also contain the neo^r gene as a selectable marker. The construction of these viruses has been published (Pallister et al 2007) and was an excellent technical accomplishment by Dr Jackie Pallister.
The questions then asked of these viruses were:

a) Are the recombinant viruses still capable of infecting cane toads? The answer here is a clear yes.

b) Are they significantly attenuated? Three viruses were titrated along with wt virus to assess infectivity and attenuation by measuring mortality. The viruses were clearly still capable of infecting the animals but showed some evidence of attenuation.

c) Does the attenuated virus stimulate an immune response? The results here are equivocal. It would appear that in some of the trials the virus is being cleared by the animals but more extensive trials are required. One key experiment showed that when the initial inoculate was the recombinant virus and the challenge was the wt virus, then neither the initial inoculate nor the challenge virus could be detected in the screen. The challenge virus was detected in negative control tests.

A recombinant virus containing the trefoil factor gene has been constructed and another containing the gastrokine gene is underway.

7. Conduct animal trials to (i) replicate the Maniatis experiment with globin protein and (ii) evaluate effectiveness of recombinant viruses.

(i) Replication of the globin experiment.

These experiments were conducted in two ways. Firstly, recombinant adult globin was used for injection into tadpoles at a time when adult globin was not yet expressed. Following injection there was no indication of effects at the physical level as measured by body weight during metamorphosis or the speed of metamorphosis. There was no change in expression of adult globin gene nor in the level of haemoglobin protein detected. No antibody (IgG or IgM) was detected against the injected globin protein. Similar results were obtained following virus infection (see (ii) below).

This experiment has not shown any evidence of success of this approach but there are many unknowns that could be investigated if time and funding permitted. It is not clear if this approach to raising an immune response is likely to be successful with only one previous (1969) report of success. There is insufficient knowledge of the cane toad/tadpole immune system.

Antibodies are being raised against trefoil protein and gastrokine and these antibodies will be injected into tadpoles as an alternative experimental design to determine if blocking these proteins with an antibody will have an effect on metamorphosis. The direct antibody approach may have a higher chance of success if these proteins play a role in development, an unknown at this stage. It will also be a faster way of demonstrating whether these proteins are good targets.

(ii) **Evaluate effectiveness of recombinant viruses.**

The recombinant virus containing the adult globin gene has been shown to make the globin protein at least in vitro and tests are underway to examine expression after infection of animals.
Animals were infected with different doses of the recombinant virus containing only the neo^r gene and the virus containing both the neo^r gene and the adult globin gene. Virus detection was carried out using PCR and virus as detected in a considerable number of the animals, especially at higher doses of virus infection. There was no effect on the level of adult globin protein detected in the infected animals with either virus. This implies, at least in these trials, that either the recombinant globin is not being made or that an immune reaction, sufficient to effect endogenous globin expression, is not occurring.

Overall, the results of these trials did not indicate that this approach will be successful without considerable further trials or investigation of mechanism.

8. Assess the results in respect to (i) supporting the hypothesis and (ii) identify future directions for biocontrol of *Bufo marinus*.

(i) The starting hypothesis for this work was that toad-specific proteins expressed in tadpoles using a viral vector would elicit an immune response. This immune response would then target the endogenous protein at the time of its expression during development. If the target protein was important for development then the immune response to the protein would prevent development and/or survival. Experiments have been conducted on one target protein so far, i.e. adult globin. *There was no evidence either from injection experiments or from viral infection experiments that this protein elicited an immune response. There was also no evidence that these treatments had any impact on expression of the endogenous protein or on toad development. Thus, the basic concept on which the proposal was based has not proven successful.*
Since only one protein has been tested, it is impossible at this stage to assess the likelihood of success with other target proteins. Two other target proteins are currently being tested and these results should provide more conclusive

evidence as to the likely success of raising an immune response to adult proteins in tadpoles and the possible downstream consequences.

(ii) Will any of the achievements be useful for future approaches to cane toad control? While specific outcomes have not been positive, the most generally useful outcomes of this project so far are; a) the establishment of a cane toad colony, b) the ability to engineer a ranavirus to insert foreign genes, c) the establishment of PCR-based technology for screening for amphibian viruses, d) the identification of genes that *MAY* be useful targets for control, and e) the general technologies around cane toad development and manipulation.
If other efforts to control the cane toad based on molecular approaches are funded in the future then the availability of these enabling technologies should be considered/remembered.

ToR 2. Technical feasibility of this research being developed to the point of offering a new and effective control method for cane toad populations in Australia (including consideration of the regulatory situation for release of such an organism).

A. Feasibility at Proof of Principle stage.

1. ***Identification of suitable target genes.***

Genes that show differential expression between tadpoles and toadlets have clearly been identified. A number have been chosen for follow-up based on known information about these genes/proteins. The evidence that these genes will constitute good targets is not strong and would need considerably more research to prove. This may prove to be a major hurdle.

2. ***Identification of a suitable virus vector.***

The BIV virus was always intended to be used only as a "test" virus to determine the feasibility of insertion of target genes into such viruses and if such viruses could be delivered to cane toads. It appears that this would not be a suitable virus for release. There is no progress on the identification of other useful viruses despite reasonably extensive screening. This is a very serious technical hurdle.

3. ***Infection of tadpoles with the virus and expression of the recombinant proteins.***

Tadpoles can be infected with BIV but there is no evidence as yet that the engineered viruses express the proteins after infection. This is unlikely to be a major hurdle.

4. ***Immune response to expressed proteins.***

Evidence was presented that suggested an immune response against the virus but more precise experiments and analysis are required. There is no evidence so far that proteins either injected into toadlets or expressed from the virus (see 3 above) can illicit an immune response. This is an important result as the entire strategy relies on the generation of an immune response again these proteins. The strategy

also relies on the generation of immune memory to these proteins and a stronger response to the proteins when they are expressed later in development. So far the project has not progressed to testing the second part of these requirements.
This part of the project is likely to provide a major hurdle.

Overall there are still many major technical hurdles even in the proof of principle stages of this project. If these hurdles were overcome then the following considerations would become important.

B. Feasibility beyond Proof of Principle steps.

1. Dissemination of the virus – population modellin.

Most research funds for the control of cane toads have been invested in the search for a natural pathogen that is likely self-disseminating, or to genetically engineer one. The reason is obvious – self-disseminating biocontrol agents once released should entail little or no additional control costs (i.e. no *ad infinitum* maintenance costs). Population modelling has been used to examine disseminating (virally vectored) and non-disseminating (traps, sterile males, daughterless male) strategies to control cane toads (McCallum 2006, Thresher and Bax 2006, McCallum and Bashford 2008, Bashford and McCallum 2008). McCallum (2006) concluded that the ideal biocontrol agent would be a transmissible fertility-reducing agent, and that to propagate successfully through existing populations it would need to be either a pathogen novel to cane toads in Australia or one that has a higher reproductive rate than the wild type (and this may be a constraint when considering the use of existing pathogens to control density, see ToR 3 below). McCallum and Bashford (2008), in follow-up modelling for CSIRO, concluded also that most non-disseminating approaches to control toads over landscape-scales were either too impractical or too inefficient, and Thresher and Bax (2006) reached similar conclusions using different population models (see review in Appendix 1). Thus, on this basis, continuing the search for a self-disseminating control agent may be justified.

2. Genetic resistance – shelf life of biological control agent

Hinds et al. (1996) examined the consequences of genetic resistance associated with viral solutions for the control of rabbits, summarised here because it is highly relevant to assessing the potential of the virally-vectored genetically modified organism (GMO) approach to cane toad control. When myxomatosis was first released it spread rapidly and more than 95-99% of rabbits became infected and died over 18 months. However, there was a rapid change in the host-pathogen relationship as virus strains emerged that were less virulent (i.e. attenuated), and the rabbit developed a degree of genetic resistance. A dynamic balance has now established between the rabbit and the virus and, consequently, the number of susceptible rabbits that die during disease outbreaks ranges from less than 50% to 90% (Hinds et al. 1996). Nevertheless, myxomatosis still remains an effective mortality agent and, in combination with Rabbit Calicivirus Disease (RCD), has helped control rabbit density (Williams *et al*. 1995). Nevertheless, it is anticipated that genetic resistance to RCD will rapidly develop also. It is likely, therefore, that even if a GMO virus could be developed for cane toad control, genetic resistance and a reduced shelf-life would be additional

technological challenges to overcome and should be incorporated in future benefit-cost analyses of the approach.

3. Potential environmental impacts of a GMO virus to control cane toads

There are two key issues:

1. *Specificity*: the range of species that need to be tested to demonstrate "specificity" of the GMO virus before approval for release is granted; and the probability of a mutation whereby the virus crosses the species "barrier".
2. *Containment*: the inadvertent introduction of the GMO virus to cane toads in their native range in the USA-South America region; and/or if specificity is an issue the potential adverse impact on the world's bufonid species and/or all amphibian species (i.e. global diversity).

Once again the only analogue for discussion and limited assessment comes from specificity issues associated with the genetic viral approaches used to control rabbits in Australia. Hinds et al. (1996) examined specificity issues associated with release of the RCD virus, which is summarised here because of its relevance to the GMO virus approach adopted by CSIRO for the control of cane toads. They noted that the safety of the RCD virus is often raised despite the testing more than 40 animal species for susceptibility. A very comprehensive and robust testing design, therefore, would need to be applied to cane toads given that the virus may impact on a huge number of closely related native anuran species and fish.

The other specificity issue of the RCD virus raised by Hinds et al. (1996) was public concerns that it might mutate to affect other species. However, they argued that the ability of a virus to gain a new host was a very rare event and hence, the probability of the rabbit calicivirus doing so was extremely low. Nevertheless, they concluded that the only way to be sure how each virus will behave is to carry out extensive testing (as was apparently done with RCD).

In contrast, McCallum and Hocking (2005) suggested that almost all emerging diseases of humans are zoonotic (i.e. they occur in humans by cross-species transmission from an animal host). For example: the Ebola virus and HIV both appear to have transferred to humans from the great apes in East Africa; SARS appears to have a variety of wild mammalian hosts in southern China; and avian influenza occurs in wild waterfowl and is transferred to humans by domestic poultry. Nevertheless, Hinds et al. (1996) acknowledged that once a recombinant virus was released into the environment it would not be able to be retrieved, and that this possibility might cause widespread community concern. They recognised also that genetic engineering, upon which the GMO virus approach for cane toads depends on, might alarm people.

Containment to Australia of a GMO virus used for cane toad control is a key risk issue not dealt with during any of the CSIRO funding cycles that focused on "proof of concept". The only study that attempted to assess the risk of inadvertently exporting from Australia a genetically modified virus control agent is that by Williams (2007) for live mice (*Mus musculus domesticus*). The environmental risks associated with specificity and containment issues of a virally vectored control agent for cane toads, therefore, should be formally assessed and continually updated as a high priority if funding for the CSIRO biocontrol program continues.

4. Ethical and regulatory considerations for use of a virally vectored GMO

Ethical

One of the most difficult ethical issues in wildlife management concerns the use of infectious disease as a biological control agent (McCallum & Hocking 2005). For example, animal welfare issues associated with the use of myxomatosis and rabbit haemorrhagic (RHD/RCD) disease to control rabbits in Australia, synonymous with painful death, are generally "traded-off" against the enormous benefits to agricultural production and biodiversity conservation. McCallum and Hocking (2005) however, argued that there were likely additional and more significant ethical issues associated with the proposed use of genetically modified pathogens to control vertebrate pest populations, such as the GMO virus being developed for cane toads and past advocacy of a virally vectored immunocontraceptive (VVIC) to control rabbits, mice and foxes in Australia, and introduced possums in New Zealand. For example:

- the general principle of whether or not it is wise to release genetically modified infectious agents into the environment in the first place because there is no recall; and
- the possibility that such agents may affect species other than those targeted, either within the same region in which the release takes place or by transfer of the pathogens from the region in which it has been released to a region in which the target species is not a pest.

Both these ethical issues are treated as environmental risks above and placed within a regulatory and approvals framework below.

Regulatory

Assuming that the technical barriers highlighted above can be overcome to develop a virally vectored GMO for the control of cane toads, a number of complex legislative approvals are required before any strategic release can proceed. The legal framework within which release of GMOs for control of wildlife in Australia would be regulated is provided by a range of intersecting acts (McCallum & Hocking 2005). For example (and probably not exhaustive):

1. Under the federal Gene Technology Act 2000 (and Gene Technology Regulations 2001) and corresponding State and Territory laws, an approvals process is undertaken by the Gene Technology Regulator before a decision is made on whether or not to issue a licence to release. This act is the primary legislation regulating release of GMOs in Australia, and it is the Office of the Gene Technology Regulator established under this legislation that would ultimately be responsible for deciding whether or not such a release would be approved. The decision is based upon a Risk Assessment and Risk Management Plan prepared by the Regulator in accordance with the *Risk Analysis Framework* and in consultation with a wide range of experts, agencies, authorities and the public (see Recommendation 4).
2. The environmental impact of the proposed cane toad GMO virus must be assessed under the EPBC Act 1999, and under applicable State and Territory laws. Note that even though a specified product is not yet available the general concept of a virally vectored GMO can be assessed.

3. Cane toads must be declared a “target organism” and the virus and GMOs an “agent organism” under the Biological Control Act 1984. The capacity to sue to prevent release, or to recover damages from the consequences of release, are limited once an agent is declared a “biological control agent” under Federal and State Biological Control Acts (McCallum & Hocking 2005).
4. The Quarantine Act 1908 as amended by the Quarantine Amendment (Health) Act 2003. AQIS would need to be consulted in relation to its relevant legislation as the parent virus is not already present throughout all of Australia.
5. Any release of a GMO biocontrol pathogen would have international implications under the Cartegena Protocol on Biosafety (Cartegena Protocol 2000a, b). While Australia has not ratified the protocol, there would be moral obligation to accept responsibility for unintentional damage associated with the release of a GMO virus in Australia that escapes overseas (McCallum & Hocking 2005).

We note that the Office of the Gene Technology Regulator has not been asked for, and nor has it yet provided, an advisory opinion on whether or under what conditions release of a GMO pathogen for biocontrol of vertebrates might be approved. The opinion of the Regulator should therefore be sought before further funding is directed towards a GMO virus control program. Detailed information on the comprehensive assessment undertaken for licence applications to release a GMO into the environment is available from the Office of Gene Technology Regulator (OGTR) (http://www.ogtr.gov.au).

The legislative processes described above exist to ensure that the safety and effectiveness of the GMO virus has been properly assessed so that users will be protected from any future claims for compensation. The critical question then, is whether or not the public and hence the Australian Government, will accept the risks associated with the release of a GMO virus to control a vertebrate pest population. Technically it cannot be 100% guaranteed that loss and/or lack of specificity, and/or the accidental introduction of the GMO to the target species natural range, will not occur (i.e. there will always be a non-zero probability of occurrence). While the probability of occurrence of such an adverse event may arguably be very small (and this has yet to be formally assessed), the negative consequences to national and global biodiversity will always remain unacceptably large. Given that both the likelihood and consequences of exposure comprise the risk calculation ($P_{risk} = P_{exposure} \times P_{effects}$), there may be doubts that the Australian Government will underwrite (i.e. accept national and international liabilities) the release of a GMO virus to control cane toads whose real ecological impacts, while still uncertain, are generally not thought to be substantial or irreversible (see Introduction – Ecological impacts).

Additionally, while approvals have been granted for the release of a GMO food or agricultural product (e.g. cotton), approval has never been granted for the release of a GMO virus to kill and/or to inhibit reproduction of a target species. Given that the risk assessment process would involve a lengthy period of intense public engagement and debate, it appears highly unlikely given current public/political perceptions of GMO risk that a GMO virus would be released in the short to medium term (say up to 50 years). Even a release in the long-term (50+ years) seems unlikely based on long approval times for less novel feral animal control methods such as 1080 in baits (Glen Saunders pers. comm.).

An interesting analogue of public and political acceptance of a virally vectored GMO approach to vertebrate pest control is that provided over the past decade or so by the concept of virally vectored immunocontraception (VVIC) fertility control for rabbits, mice and foxes (Tyndale-Biscoe and Hinds 2007, Hinds 2007, McCleod et al. 2007). However, while the risk and public perception issues were examined and discussed, they were, on the whole, never formerly assessed. As mentioned, the only exception is the risk assessment by Williams (2007) of inadvertently exporting from Australia a genetically modified immunocontraceptive virus in live mice.

ToR 3. The opportunities provided by other current avenues of research into cane toad control, and the relative potential for these to provide a broad scale cane toad control.

A selection of alternative methods are described and discussed below, being those considered to have potential for more effective local and possibly broader-scale control (see Hyatt and Robinson 2004, Taylor and Edwards 2005). Table 2 is a preliminary and, because of a lack of detailed review of each possible approach, a subjective comparison of the different approaches. Included is scale (local vs. transcontinental), potential environmental risk, whether or not approval time may be prohibitive for medium-term control (say 10-50 years), issues that may need to be addressed and/or which may limit the effectiveness of the approach, and a subjective rating for chance of success for the chosen scale. We stress as in Recommendations 6 and 9 that all possible approaches should be subject to detailed scrutiny before any investment decisions are made.

1. Removals (traps, catch-dispose) and barriers (e.g. fences)

The capture and removal of cane toads by trapping has been extensively discussed by a number of people at a number of workshops (e.g. the 2005 and 2006 Cane Toad workshops), hence details are not reported here. Alford (2005) identified two types of traps, pitfall and cage, but gives no assessment of their relative merits in reducing toad density either locally or broadscale. Trapping success can be increased by attracting toads to traps using light, olfactory attractants, acoustic signalling or moisture (Alford 2005, Schwarzkopf and Alford 2006, Tyler 2006b for sex pheromones). Trapping may be an effective local control method if used in combination with other control approaches (integrated control), but only if trapping efficiency is high (Schwarzkopf and Alford 2006). McCallum (2006), McCallum and Bashford (2008) and Thresher and Bax (2006) used population modelling to assess the likelihood of success of different control approaches for cane toads, and concluded that removals would not be effective in reducing cane toad density. Hence, with respect to broadscale control of cane toads, trapping is likely impractical, cost-prohibitive and ineffective even at local levels unless sustained by tremendous effort. Nevertheless, trapping is well suited to community participation in local cane toad control (Sawyer 2006, Boulter et al. 2006, Sawyer and Taylor 2005), and Taylor (2005) examined factors that needed to be considered when identifying high priority sites for cane toad exclusion (via fences or local control).

Brook and Whitehead (2005) examined the strategy of using exclusion barriers to mitigate cane toad impacts and provided estimates of potential impact on native fauna from isolation, and potential capital and recurring costs of erecting and maintaining a barrier. They estimated the initial cost of a 6 km fence at $4-$6 million, with *ad*

infinitum annual maintenance costs of $0.5-$1.0 million, effectively demonstrating that such methods were cost-prohibitive at both small and large scales.

2. Biotechnology approaches

Virally-vectored GMO (attenuated BIV & cane toad specific gene)

This approach is addressed in Terms of Reference 1 and 2 above. Borrell (2008) provides a good plain English description of CSIRO's virally vectored GMO approach to cane toad control. Additionally, the 2004 workshop on Biological Control of Cane Toads facilitated by CSIRO provides a comprehensive technical overview (Hyatt & Robinson 2004), and an update was provided by Robinson et al. (2006) and Pallister et al. (2006) at the 2006 Cane Toad workshop.

Daughterless males

Koopman (2006) argued that a new genetic strategy for the control of cane toads is needed, one that is "safer" than the virally vectored approach to biocontrol, and proposed the "daughterless male" concept. The strategy is based on skewing sex ratios in favour of males and hence ultimately limiting the number of female breeders. Furthermore, he argued that major advantages of this method were: it was humane (non-toxic and non-lethal); because it was species-specific it posed no risk to other species in Australia; and it would not pose a risk to cane toads in their native range in the event of an accidental introduction because the success of the daughterless strategy relied on vigorous re-stocking. Nevertheless, the financial costs of initial stocking and continuous re-stocking in combination with the environmental costs in terms of greater local impact might be prohibitive. Additionally, although the method is non-disseminating and so with greater control on application, there would be penalties associated with an expected lengthy approval process prior to release because it is a GMO (Table 2).

Population modelling undertaken by McCallum and Bashford (2008) suggests that daughterless male genetic constructs will not prevent colonisation or reduce densities of open populations because of the need to constantly restock to high densities. Diffusion of daughterless male genes out of an area with concomitant diffusion of wild-type genes into an area will reduce effectiveness of control over time. However, they concluded that the approach may be useful in reducing the existing density of "closed" populations of cane toads such as on islands. Thresher and Bax (2006) used a different population model to investigate the potential of the daughterless male approach and reached similar conclusions, but with a reduced need for high volume restocking. Nevertheless, both modelling exercises did not incorporate spatial processes that could produce entirely different results, particularly when coupled to more appropriate spatially-based release designs.

RNAi approaches.

The use of RNAi to inhibit the expression of a gene in a very specific manner has gained increasing levels of interest in recent years. It may be possible to develop RNAi-based approached, such as targeting genes essential in development, for non-disseminating local control. The use of RNAi to target the cane-toad specific ATPase (see below) may also provide a useful avenue of investigation. However, the usefulness of RNAi in biological control scenarios has still to be proven.

3. Chemical control

General

Hayes et al. (2006) proposed using knowledge of cane toad chemicals to disrupt their survival. In 2006, the Institute of Molecular Bioscience at the University of Queensland received funds from the Queensland government and the Invasive Animals CRC to generate baseline knowledge of cane toad chemical ecology with a view to using this knowledge to develop natural control strategies (Capon 2008). Areas examined and reported on in detail encompassed: alarm pheromones, bufadienolides, alkaloids, microbiology, sex pheromones, peptides, and cane toad chemical defence mechanisms (Capon 2008). The project is linked to the alarm pheromone discovery by Professor Rick Shine's team at the University of Sydney, and the search for a sex pheromone by Associate Professor Michael Tyler at the University of Adelaide (see below).

Cane toad –specific poison

Toxins are still currently the main means of controlling vertebrate pests. Robinson and Alford (2005) first discussed the advantages and disadvantages of a cane toad specific toxin. The major advantage was that it was non-disseminating and, provided that there was no persistence, its use could be tightly controlled. If species-specificity could be obtained then it could be distributed widely in water bodies or in baits. The main disadvantages would be non-target kill if specificity could not be obtained, and the potential prohibitive costs of production and distribution. Robinson and Alford stated that there were few examples of using toxins to control amphibians, and those that were used were non-specific. However, Robinson (2006b) proposed the novel concept of using knowledge of cane toad poison to develop a cane toad-specific toxin. Cane toads are one of the most poisonous amphibians in the world and considerable research into the nature of the toxins in *Bufo* venom has been undertaken. Basically the toxins resemble digitalis, a mixture of compounds (cardiotonic steroids) found in a number of plant species, and act on the Na^+K^+ATPase or sodium pump of the cell (Capon 2008). Cane toads have modified their sodium pump so that it does not bind their own toxin, and it was proposed at the 2006 progress review workshop of the CSIRO Biocontrol Program that knowledge of these modifications could be used to design a cane toad-specific toxin (Robinson et al. 2006). The cane toad ATPase has a significantly different amino acid sequence in the toxin-binding loop of the protein compared to the *Xenopus* sequence, and any other species where the Na^+K^+ATPase sequence is known. The structure of the pig Na^+K^+ATPase was recently solved by X-ray crystallography (Capon 2008). Preliminary modelling by the CSIRO team has shown that the amino acid differences lead to an altered structure and may explain the reason why the cane toad is resistant to its own toxin. If further modelling and experimentation showed that this region of the protein formed a unique structure that could be targeted by a specific and novel toxin, then it may form the basis of a novel chemical control approach (Robinson et al. 2006). Approaches could include chemical library screening or computational modelling for a drug design.

Sex pheromones (attractants in traps)

Pheromones can act as either attractants or repellents (alarm signals), and both kinds have been reported for amphibians – a sex attractant (splendiferin) in *Litoria splendia* and an alarm reaction in the tadpoles of two toad species, one of which is the cane toad (Tyler 2006). Assoc. Prof. Tyler (University of Adelaide) has received departmental funds to construct an "Olfactorium" (basically a Y-maze tunnel, Tyler

2008) to determine whether or not male or female sex pheromones exist in cane toads, with a view to using this knowledge to enhance trapping efficiency at greater than local scales. The project was linked to the project on chemical ecology of cane toads by Prof. Capon (Institute of Molecular Bioscience, UQ), but this collaboration is no longer operating. Capon (2008) stated that "as yet no compelling experimental evidence exists for a cane toad sex pheromone". Tyler (pers. comm.), however, stated that he had conducted 100 preliminary trials to date but had yet to analyse the data and report results. This situation needs to be carefully monitored.

Tadpole alarm pheromones

The only reproducible pheromone behavioural response reported for cane toads to date is the "alarm" pheromone in tadpoles, whereby injured conspecifics are repelled by chemical cues (Shine & Hagman 2007). Tadpoles of native frogs do not appear to be repelled by the same cue, suggesting that the chemical is species-specific in its action (Shine & Hagman 2007). Additionally, chronic exposure to chemical cues from injured conspecifics increased mortality by 50% in laboratory-based tests and reduced size at metamorphosis by about 30% (Shine pers. comm.), suggesting that manipulation of pheromonal communication may play a role in toad control. Needless to say, these results need to be tested under field conditions.

A major impediment to assessing the utility of tadpole alarm pheromones under field conditions is that their active chemical constituents have yet to be identified by the Institute of Molecular Bioscience (Tony Peacock pers. comm.). If the chemicals can be identified and species-specificity guaranteed, then it could be produced commercially and distributed widely in water bodies or by baits, although the practicality and cost-effectiveness of this approach would need to be assessed. Additionally, the regulatory approvals process for broad-scale application of pheromones may be just as time consuming and complex (i.e. uncertain) as that for the release of a GMO virus to control cane toads.

Nevertheless, if laboratory results that demonstrate significantly increased tadpole mortality and size at metamorphosis in the presence of an alarm pheromone can be replicated under field conditions then, despite the potential limitations outlined above, this approach in comparison to all other alternative approaches appears to have more potential for more effective local control and, possibly, for larger areas depending of control conditions (Table 2). However, population modelling has yet to be undertaken to investigate whether or not an additional 50% mortality in tadpoles and reduced size at metamorphosis would translate to a significant reduction in the density of terrestrial post-metamorphic toads, and under different assumptions of density-dependency at all early life-history stages. This is critical assessment step as cane toads may compensate for reduced density in pre-metamorphlings through enhanced survival of post-metamorphlings, although the reduced size at metamorphosis is a good sign. Demographic compensation to a reduction in density is a common feature of the population ecology of many vertebrate species, and is a key assumption that underlies sustained-yield harvesting and pest control. The only way to unambiguously determine the existence of such compensation and hence whether or not the alarm pheromone has real potential to reduce cane toad density to acceptable levels, is to conduct carefully designed field experiments as highlighted above.

4. Conventional (or non-GMO) biocontrol methods

Sterile male release with inherited sterility

With respect to population control the sterile male release approach is conceptually similar to the daughterless male release approach discussed above. Sterile male release programs have been successful in controlling some insect pests over wide areas (the Sterile Insect Technique or SIT) and, hence, Mahony and Clulow (2006) suggested that such an approach may work for cane toads. The assumption is that released sterile males will compete for, and mate with, wild females. This would reduce reproductive output and, in the long-term, density, and needs to be tested. Mahony and Clulow (2006) argued that major advantages of the approach were that: it was species-specific, non-toxic, and entailed no risk to global amphibian biodiversity. However, a key disadvantage is the fact that male cane toads are small vertebrates rather than small insects. The sterile male release method relies on the mass rearing, sterilisation and release of a large number of individuals with fitness equivalent to wild types. Whist the SIT approach may be practical for some insect populations it may be impractical for application to small vertebrates. For example, for control of the New World screw worm, mass sterilisation is achieved via low dose radiation. However, Mahony and Clulow (2006) have developed a genetically modified stock of cane toads for making sterile males via triploid and tetraploidy. This approach is classed under "Conventional biocontrol" because the genetic modifications involve non-directed manipulation of the genome rather than genetic engineering.

Population modeling by McCallum and Bashford (2008), however, suggests that, as for the daughterless male approach, the sterile male release approach will not be practical in preventing colonisation nor reducing densities of open populations because of the need to constantly restock at high densities. Diffusion of sterile males (and in this case their genes) out of an area with concomitant diffusion of wild-type genes into an area will reduce effectiveness of control over time. Model results indicated also that at least two orders of magnitude more males would need to be released than those initially present. This would obviously increase ecological impacts perhaps by a similar order of magnitude, and represents a major disadvantage of the method (Table 2).

Parasites/pathogens

The search for *Bufo*-specific pathogens in Australia and South America has a long history of failure as highlighted by Robinson et al. (2005), and was one of the main reasons why CSIRO chose to investigate a long-term GMO strategy despite the investment risk. The discovery of a pathogen capable of acting as a control agent of cane toads would undoubtedly be a significant step in developing non-GMO methods for broadscale control of cane toads because it would likely be self-disseminating and so entail little or no additional control cost once released. In fact this has been the primary justification for CSIRO adopting the virally vectored GMO approach to cane control over costly alternative approaches more suited to local control than broadscale control. However, a major disadvantage is that genetic resistance of host to pathogen is likely to co-evolve as found for myxomatosis used to control rabbits (see Part B2 of this report above; Hinds et al. 1996), reducing the pathogen's effectiveness as a mortality agent over time. Nevertheless, Hinds et al. (1996) highlighted that, even in the face of rapidly evolving genetic resistance and concomitant reductions in

lethalities, the combined effects of myxomatosis and RCD still makes significant reductions in rabbit density across Australia. Hence, it is envisaged that more than one natural pathogen will need to be discovered and used, either sequentially or simultaneously, for long-term cane toad control (Andrew Peackock, pers. comm.).

Regardless of the above, Robinson et al. (2005) argued that the likelihood of finding such a pathogen for cane toad control was low based on the very low success rate of searches for pathogens as biocontrol agents for vertebrate pest species in general. They highlighted that the only examples of species-specific, or limited host-range, pathogens being found and used successfully for biocontrol agents for vertebrate pests on a continental scale were myxoma virus (restricted to a few species of lagomorphs) and rabbit haemorrhagic disease (RHD or rabbit calicivirus RCD restricted to the European rabbit).

Undiscovered natural pathogen

Robinson et al. (2005) suggested that if the search for a pathogen was to resume then the approach adopted in earlier projects would need to be modified to increase the likelihood of discovery. They suggested the following two approaches based on experience from the discovery of myxomatosis and RHD disease:

a) Search for a disease in cane toads in areas where they had just been introduced, either in the wild or in captivity, or the sudden appearance of a high mortality event where they have been established for sometime (this was the model used in Brazil between 1990-1995, with no success as significant declines in abundance were correlated to food shortages, not disease; Bayliss 1995).

b) Use a search strategy to screen other *Bufo* spp. for micro-organisms with the potential to control cane toads, particularly in areas where the cane toad is not present (and as suggested Andrew Peacock/SA government & Ross Alford/JCU, pers. comm.). By analogy with the myxomatosis experience, such pathogens would not necessarily produce large-scale mortality in their natural hosts.

The search strategy of seeking a biocontrol agent for cane toads where it was not present, but bioclimatically suited, was applied to a published CLIMEX modelling study (Sutherst et al. 1995) by Peacock (2006). Northern Argentina was identified as a region fitting the search success criteria and recommended by Peacock (2006) for investigation (i.e. importing cane toads and exposing them to local pathogen sources such as aquatic leeches and mosquitoes). Peacock (2006) recommended also that anuran trypanosomes be investigated as a potential pathogen biocontrol agent because: previous experimental studies showed that *T. cruzi* can cause significant mortality in both adult and juvenile cane toads; key invertebrate vectors may already exist in Australia; and the likelihood of host specificity is high because of the absence of anuran trypanosomes in Australia. Given that an effective approach to broadscale control of cane toads in Australia has yet to emerge, and irrespective of the discovery of a cane toad-specific lungworm in Australia (see immediately below), this approach is worthy of consideration and has been subjectively rated as having more opportunity than other alternative approaches (Table 2). The reasons are twofold: (i) as far as is known the lungworm only acts on metamorphlings (see below) and, to avoid possible compensatory mortality responses in metamorphlings and post-metamorphlings, self-disseminating mortality agents that independently act on terrestrial juvenile and adult life stages may have a greater overall chance of success in reducing adult toad density

(see below, and note that the potential issue of genetic resistance outlined above may also apply here); and (ii) all discovered pathogens may have the potential to be genetically modified to increase their effect (e.g. lethality, transmissibility). Peacock (pers. comm.) suggested further that the search strategy could be greatly enhanced using better range models, such as the one developed by Urban et al. (2007).

Cane toad-specific lungworm

Professor Shine and his research group (Team Bufo) from the University of Sydney announced on 7th May 2008 the discovery of a species-specific parasitic lungworm *Rhabdias* sp.) in Australian cane toad populations that, in laboratory tests, increased metamorphling mortality by 30% and significantly reduced the growth rate of survivors (Shine pers. comm.). The parasite had not been previously considered for biocontrol potential because it was thought to be an Australian species that had shifted from frogs to toads, and so might have the ability to shift back again. However, genetic studies show that the lungworm is species-specific to cane toads and originates from South America. This is a new and significant research finding that may have potential for cane toad control, particularly if integrated with other approaches as suggested by Shine (Table 2). However, as with all untested approaches, it requires robust examination (see below).

5. Adaptation and Impact abatement

Adaptation

Most people interviewed for the review assumed that the initial toxic impact of cane toads on native predators (e.g. goannas) at the invasion front was ameliorated over time due to behavioural (e.g. learned avoidance), environmental (e.g. micro-niche partitioning), biochemical (e.g. decreased toxicity in smaller toads), molecular (e.g. genetic resistance to toad toxins) factors, or a combination of these factors (Firestone and Robinson 2006). For example, while the Northern Territory northern quoll populations are facing severe localised extinctions, most likely due to the arrival of cane toads on top of other threats, some Queensland populations have persisted despite the long-term presence of toads (Firestone & Robinson 2006). If rapid adaptation of native predators to toad toxins is true, and impacts are minimal in the long-term, then the "do nothing" approach is a management option that needs to be seriously considered with respect to the risks and costs associated with developing pan continental control options, such as the virally vectored GMO approach and, even alternative approaches such as broadscale application of chemicals into waterways. However, as highlighted in our section on "Ecological impacts", this assumption needs urgent re-appraisal because both anecdotal and observational data are equivocal (see Table 2).

Relocate susceptible species to island refuges

Taylor and Keenan (2005) reviewed the mechanisms of toad dispersal to islands and the implications of choice of islands for translocation of impacted species. In 2006 the NT Department of Natural Resources, Environment and the Arts (NRETA) initiated the "Island Arks – Northern Quoll Translocation" program as a major response to the arrival of cane toads. Research indicated that quolls were virtually disappearing from areas colonised by cane toads. Breeding populations of northern quolls on two islands off the north-east Arnhem Land coast have been established, and Aboriginal ranger groups have been trained to monitor quoll populations and to

keep their islands free of cane toads. No assessment of the success or otherwise of this strategy has yet been provided by NRETA. This is a costly yet most likely cost-effective strategy for endangered species that are easily relocated, but is obviously unsuitable as a broadscale ecosystem solution to mitigating cane toad impact,s so is not considered in Table 2.

Modification of spawning habitat.

Shine and Hagman (2007) advocated modification of spawning site preference as a short-term control measure for cane toads (toads prefer open shallow water bodies with no edge vegetation). Whilst this would be a costly option even for localised control, it is obviously unsuitable for broadscale control, so is not considered in Table 2.

6. Integrated control

Most people interviewed suggested that a combination of approaches to cane toad control, or integrated control, would be the most effective approach given that all approaches had their own set of weaknesses and strengths, particularly with respect to control scale. However, only one integrated control design has been specifically proposed, being that advocated by Shine and his team, summarised below.

- Release a large number of small sterile males in front of the dispersal front so that native predators will learn to avoid the imminent arrival of larger, more lethal cane toads at the front (called "teacher toads"). Shine (pers. comm.) argues that producing large numbers of sterile males is practical due to research undertaken by Prof. Michael Mahony at Newcastle University, and that the additional impacts from releasing males into uncolonised areas would be more than compensated for by reduced mortality by "learning" (needless to say both assumptions need to be tested).
- I'm assuming here that Shine advocates also infecting these sterile males with lungworm parasites to disseminate them in establishing populations more rapidly than would otherwise occur.
- Concomitantly, use the alarm pheromone as a broad-scale chemical control method to increase the mortality of early life stages.

There are many issues to address with this proposal, even before approvals for field trials are granted. For example: basic epidemiological knowledge is lacking; comprehensive screening tests for species-specificity of both lungworm and chemicals duplicating alarm pheromones will need to be undertaken; associated risk assessments and environmental impact assessments, especially with the release of a large number of males into uncolonised areas, will need to be undertaken also; and the practicality and cost-effectiveness of distributing sufficiently large numbers of sterile small male toads infected with lungworm should be assessed, as it may be prohibitive over broad scales.

With respect to control potential, it should be noted also that this parasite has most likely been present in Australian cane toad populations since introduction more than 35 years ago, with apparently little effect on their rapid colonisation of new areas (H McCallum & A Peacock pers. comm.). However, it may be a major reason why long established populations generally collapse to lower densities than at the eruption front and this fact demands close scrutiny. Even so, and as outlined above (B1), McCallum

(2006) concluded from modelling that in order for a biocontrol agent to propagate successfully through existing populations, it would need to be either a pathogen novel to cane toads in Australia, or one that has a higher reproductive rate than the wild type. The first condition is not satisfied and, with respect to the second condition, the implications of genetic resistance need to be considered once again. Furthermore, and as outlined above for the ability of alarm pheromones to reduce post-metamorphic density, population modelling has yet to be undertaken to investigate whether or not additional mortality in metamorphlings due to the early introduction of lungworms would translate to significant reductions in the density of terrestrial post-metamorphic toads, and under different assumptions of density-dependency at different life-history stages. This is a critical assessment step as cane toads may compensate for reduced density in pre-metamorphlings through enhanced survival of post-metamorphlings, although the reduction in growth rates of surviving metamorphlings is a good sign. As for a population level response to alarm pheromones discussed above, the only way to unambiguously determine the existence of such compensation and hence the effectiveness of the early introduction of lungworms, is to conduct carefully designed field experiments.

Regulatory considerations for alternative methods

The major consideration here when comparing alternative control options is how long it would take to be granted approval to undertake field trials once a product was developed and, following this, local or broadscale control. Such detailed assessments are beyond the scope of this review, particularly with respect to genetic approaches, hence a subjective opinion only is given in Table 2 and is based on discussions with pest control experts that are familiar with approval times for non-GMO approaches.

Table 2. Comparison of selected alternative control method

Method & Proponents	Control scale	Requires GMO?	Potential environmental risk	Approval time may be prohibitive?	Issues that need to be considered	Chance success for scale
Removals – traps (intelligent traps, traps enhanced with acoustics/smells) Ross Alford (JCU)/community groups	Local	No	Low	No	Ineffective except at very local scales with substantial effort	Very low
Barriers Community groups	Local	No	Medium (impacts on native fauna)	No	Ineffective & cost-prohibitive except at very local scales	Zero-very low
GMO virus Alex Hyatt CSIRO Entomology & Sustainable Ecosystems	Trans-continental	Yes	High but not formally assessed	Yes	No technical breakthroughs with proof of concept. Long research & development time. High risk (specificity, to global biodiversity). Public & political acceptance unlikely, approval unlikely.	Very low at current development stage
Tadpole alarm pheromone Rick Shine (US) & Rob Capon (UQ)	Local	No	Low-medium (unknown for wider range native aquatic species)	Possibly	Chemicals in pheromone need to be identified & commercially produced. More comprehensive specificity screening tests likely required before application Long approval time may be prohibitive.	Low-medium
Sex pheromone Michael Tyler (Adelaide University)	Local	No	Unknown	Possibly	Existence of sex pheromone still needs to be confirmed & active chemicals identified. Long approval time may be prohibitive.	Very low
Toad-specific poison (NaK ATPase) Microbial disruption of life cycle (use of antibiotics or probiotics) Rob Capon (UQ)	Local Local	Yes Yes	Unknown Unknown	Yes Yes	No knowledge, research & development time unknown. Environmental & investment risks highly uncertain. Extensive screening to test species specificity required. Long approval time may be prohibitive.	Low Low

Daughterless male Peter Koopman (UQ)	Local	Yes	Low – medium (create additional impacts)	Yes	Need to continually re-stock may be costs-prohibitive except on local scale. Public/political acceptance unknown, unlikely to be high. Long approval time may be prohibitive.	Low-medium
Sterile male release (Michael Mahony & John Clulow)	Local	No	Low – medium (create additional impacts)	Possibly	Ineffective as high volume (~4 time density) release required (McCallum 2008). Long approval time may be prohibitive.	Low
Cane toad specific lungworm Rick Shine (US)	Local at invasion front, trans-continental post-invasion if self-disseminating	No	Low – medium (create additional impacts)	Possibly	No detailed knowledge of life history & epidemiology of CT lungworm. Extensive screening to test species specificity required. Long approval time may be prohibitive.	Low-medium
Undiscovered natural pathogen (e.g. trypanosomes) David Peacock (SA Gov)	Trans-continental	No	Low	Possibly	No natural bufo-specific pathogen identified, requires focused search (bioprospecting) in high probability areas overseas. Extensive screening to test species specificity required if search successful & local tests confirm pathenogicity. Long approval time may be prohibitive.	Low-medium

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Appendix

Appendix 1: Review of population models used to assess different control options.

Population models can be used to assess the efficacy of different control methods, such as conventional lethal control (traps and poisons), biocontrol (natural or genetically engineered solutions) or a combination of methods (integrated control). Models scenarios could incorporate also the consequences to control efficiency of the evolution of genetic resistance. Modelling is particularly useful in identifying critical gaps in knowledge needed for cost-effective control of any invasive species. Model parameters generally comprise: population and life history parameters of the target species and biocontrol agent if relevant (kill rate, infection rate, rate of population increase/colonisation); identified socially-acceptable control targets based on impacts (assuming that eradication is cost-prohibitive); and a formal analysis/assessment of the risks, costs and benefits of control options. Although there is no exact product specification for the biocontrol of cane toads, population models can be used to guide research direction at the molecular level given desired performance criteria.

CSIRO commissioned Prof. Hamish McCallum (University Tasmania) in the 2006-07 financial year to model the feasibility of biocontrol approaches to cane toad control (McCallum and Bashford 2008, Bashford and McCallum 2008). The goal was "to determine, via modelling, the feasibility of disseminating and non-disseminating approaches to biological control of the cane toad". Specific aims were to model the impacts of a disseminating and non-disseminating biocontrol agent on a population of cane toads across a range of infection rates, percentage lethalities, life states and cane toad densities. Given the level of funding and the basic scope of works specified in the sub-contract, the modeling work undertaken is more than adequate and of high quality. Virally-vectored and specific non-disseminating approaches (daughterless males, sterile males, traps/removals) to suppress cane toad density at the dispersal front were examined using simple epidemiological disease-compartment models (see Andersen 1979). While it's a necessary first step, the simulation results were predictable and clear-cut only because the deterministic population models that they used had simple and certain underlying assumptions. Nevertheless, some important insights were obtained, and methods and results are summarised below.

1. A deterministic modelling environment was used in preference to a more realistic stochastic modeling environment. However, only generic questions were asked about the behaviour of the system and, additionally, the true uncertainty level of demographic parameters of both disease and host were unknown. Hence, the deterministic approach is a suitable first step.
2. They differentiated between two control approaches: (i) reduction of density in areas where toads already exist; and (ii) prevention of colonisation into new areas.
3. Their modeling is in three parts:
    a) Strategies for controlling recent invasion of toads (McCallum 2006, McCallum and Bashford 2008), such as removals (baits, lures and traps), sterile male release and transmissible (self-disseminating) control agents. Hence, a key simplifying assumption is that no density-dependent population processes operate in recently colonised areas.

b) Simulation of a "daughterless male" release into a closed population in which density-dependent factors are operating (Bashford and McCallum 2008). Density-dependence was simulated using simple fisheries stock-recruitment models (Beverton and Holt for tadpoles, Ricker for adults).

c) In an addendum they model the potential efficacy of control agents acting on different life history stages of toads. Specifically they ask: (i) is an agent that affects juvenile survival more effective than one that targets adult survival?; (ii) is an agent that targets adult fecundity more effective than one that targets either juvenile or adult survival?; (iii) do model outcomes depend on the life history at which density-dependence primarily occurs; and (iv) are model outcomes influenced by whether or not transmission is density or frequency-dependent?

4. Key results suggest that:

a) Transmissible (self-disseminating) control agents are unlikely to prevent colonisation of new areas, but could reduce established densities, especially if fecundity is targeted instead of mortality (but see 4f below – both are valid).

b) Removal by trapping is unlikely to achieve much as the caveats are prohibitive (i.e. a very high and sustained catch rate over broad areas is required);

c) Sterile male release is unlikely to work for either control approach because at least two orders of magnitude more males would need to be released than those initially present.

d) "Daughterless" male genetic constructs are unlikely to be useful for preventing colonisation for similar reasons as for the sterile male approach; diffusion of daughterless male genes out of an area with concomitant diffusion of wild-type genes into an area will reduce effectiveness of control over time. However, they may be useful in reducing existing density of "closed populations".

e) Regardless of disease dynamics or the life stage at which regulation/density-dependence occurs, diseases transmitted directly between tadpoles fail to have significant lasting effects on adult toad densities. However, the impact of an adult-transmitted pathogen is much greater if density-dependence occurs in the tadpole stage.

f) The best prospects for biocontrol is when population regulation occurs during the tadpole stage, and a mortality-inducing or fecundity-reducing pathogen is transmitted between adults, particularly if transmission is frequency-dependent rather than density-dependent.

g) Spatial and stochastic processes were not included in the initial modeling.

In general, the modelling results of McCallum and Bashord suggest that: removals, even with efficient traps, will not be effective and nevertheless, cannot be sustained; the sterile male approach, normally applied to insect populations, should not be contemplated because it is costly, highly ineffective and produces greater impacts; similarly the daughterless male approach will not work because of the need to constantly restock as, except on islands, there are no "closed" populations of toads in Australia; and the lack of knowledge of basic population dynamics, such as the degree of density-dependence at different life stages, seriously constrains use of modelling to assess the efficacy of different control approaches including integrated control using a combination of approaches. Limitations of the above modelling that need to be

addressed in future studies are: spatial and stochastic processes were not included; the influence of model uncertainty on conclusions is not made explicit; and genetic resistance (i.e. shelf life of the GMO virus) was not considered in simulations of the transmissible control agent.

Additional to the CSIRO commissioned modelling studies there is the work undertaken by Thresher and Bax (2006) for the Invasive Animals CRC and presented at the Brisbane Cane Toad Workshop in 2006. Methodology and results are summarised below.

1. They used a genetic/population model to investigate the strengths, weaknesses and potential for physical removal in comparison to five genetic techniques (sterile male release, "daughterless", female-specific lethality, female-specific sterility) to eradicate cane toads.
2. An age-structured deterministic population model was used to simulate recruitment, mortality, sex ratios and gene frequencies in a freely interbreeding population (i.e. no immigration and emigration). Density-dependence in the juvenile stages was incorporated by using a discrete logistic (Ricker) model. Environmental stochasticity was incorporated by arbitrarily (albeit reasonably) linking recruitment to rainfall and rainfall to the SOI.
3. Results suggest that:
    a) Physical removal of post-metamorphlings can eradicate cane toads but that an extreme and most likely cost-prohibitive amount of effort is required.
    b) Similarly for sterile male release.
    c) Three genetic approaches ("daughterless", female-specific sterility and lethality) all lead to population extinction within a relatively short period of time (<20 years) and at modest levels of stocking effort.
    d) All model results and, hence, overall comparison of efficacies between control approaches, are highly sensitive to the assumed degree of density-dependence in each life stage (as are the models of McCallum & Bashford above).
    e) Spatial processes were not incorporated.

In general the modelling results of Thresher and Bax (2006) suggest that: removal and the sterile male approaches will not work because of the extreme amount of effort that would be required to sustain control (McCallum and Bashford 2007 conclude the same); the "daughterless" male approach will work (similar to McCallum and Bashford's 2007 conclusion for "closed" populations); the female-specific sterility and lethality approaches will also work (as McCallum and Bashford 2007 found); and, in complete agreement with McCallum and Bashford (2007), the lack of knowledge on basic population dynamics, such as the degree of density-dependence at each life stage, seriously constrains use of modelling to assess the efficacy of different control approaches. Limitations of the above modelling that need to be addressed in future modelling studies are: spatial processes were not included; the modeling objective of "eradication" was unrealistic and generally not used as a control objective in the real world except on islands; the influence of model uncertainty on conclusions were not made explicit; and genetic resistance (i.e. shelf life of the GMO virus) was not considered in simulations of the transmissible control agent although their genetic models were well suited to do so. Nevertheless, this was also a very useful first-pass modelling exercise. The modelling approach of both groups were essentially similar, the differences in conclusions simply reflecting differences in assumptions with

respect to the degree of density-dependence at each life history stage and, of course, estimates of other demographic parameters and their associated uncertainties.

It's surprising that the modelling work has only been done at the end of quite a few biocontrol funding cycles and not in parallel. The Marsupial CRC, for example, developed population control models for introduced possums in New Zealand to examine the efficacy of using a non-disseminating bait system to deliver an immunocontraceptive in combination with conventional 1080 lethal control. Although the biotechnology was never realised, the simulated integrated control scenarios showed that target density reductions could be achieved at reasonable cost and with a socially acceptable reduction in the broadscale use of 1080 poison (Bayliss and Choquenot 1999). Hence, the molecular work had the opportunity to focus away from virally-vectored delivery systems that entailed significant risk to native Australian possums.

Both Thresher and Bax (2006), and McCallum and Bashford (2008), highlighted their model limitations. In particular the consequences of spatial dynamics and/or stochasticity (both model and environmental uncertainty) on simulation results were not examined in any great detail, and which might produce counter-intuitive results and/or unexpected insights. Real populations of cane toads exhibit huge spatial and temporal variability, and their population dynamics can be characterised as open non-equilibrium complex systems (in contrast to the simple closed equilibrium models used by both sets of modellers). Additionally, cane toad populations are comprised of highly mobile individuals (i.e. an enormous amount of continual mixing) that are differentially structured because they have a complex life cycle (e.g. aquatic eggs and tadpoles; terrestrial metamorphlings vs. juveniles vs. adults, males vs. females). On top of this we have complex disease compartments (infecteds vs. sucsceptibles) with potentially different transmission modes and rates depending on life history stage and also what disease model you choose to use.

If funding for CSIRO biocontrol work continues then the next modeling phase should encompass simulations using more realistic although more complex spatial behavioural models (a tradeoff between model complexity and utility then needs to be made). That is, different population modeling approaches need to be used other than those underpinned by simple equilibrium rules or logistic population growth assumptions. For example:

- agent-based or individual-based models that use cellular automaton rules to simulate spatial and/or behavioural dynamics;
- risk-based or probabilistic models;
- models that assess the benefits and costs of single control options vs. integrated control options (maximising conservation benefit vs. minimising costs);
- models that incorporate genetic resistance to GMOs (i.e. deal with the "shelf life" of the product); or
- a combination of the above modeling approaches.

There are sufficient observational data on population dynamics, life history, disease/parasite incidence, demography, sexual/foraging and physiological behaviour, habitat use and movements, gathered over a couple of decades in a diversity of habitats, to develop such models. It would require an appropriate level of resourcing

but the initial investment would be highly beneficial in terms of guiding overall investments into future control work irrespective of the approach adopted.