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## Proceedings of the Vertebrate Pest Conference

### Title

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### Permalink

<https://escholarship.org/uc/item/75344338>

### Journal

Proceedings of the Vertebrate Pest Conference, 22(22)

### ISSN

0507-6773

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### Publication Date

2006

### DOI

10.5070/V422110092

# Fertility Control for Wildlife Management – The Brushtail Possum in New Zealand

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**ABSTRACT:** Fertility control vaccines are under development for a number of pest wildlife species, mostly based on whole zona pellucida (ZP) or individual ZP proteins. Such vaccines must be effective, long lasting, cheap and readily deployed. One approach to deployment is oral delivery in baits. This is one strategy being taken in New Zealand for control of brushtail possums, 2- to 4-kg marsupials introduced from Australia in the 1850s, and now major pests of both conservation and agricultural production. New Zealand has highly effective aerial and ground systems for delivery of toxic baits to possums that could be adapted readily to deliver vaccine baits. Recent trials in captivity where female possums were immunised with recombinant possum ZP3 and ZP2 proteins demonstrated 70-75% reductions in fertility in natural and assisted breeding trials. Immunisation with possum-specific epitopes of the ZP2 and ZP3 proteins has also proved effective at reducing the numbers of fertilised eggs recovered from immunised females. For field delivery of an oral vaccine, we are investigating the use of bacterial “ghosts”. These are the empty cell walls of bacteria that have been modified to express possum ZP proteins in their cell walls. The possum’s immune system recognises the bacterial ghost as foreign, and produces antibodies against them. At the same time, it is tricked into developing antibodies against the possum egg proteins, causing a contraceptive effect. In a recent proof of concept trial, female possums immunised with a possum ZP2-bacterial ghost vaccine by nasal spray showed a significant reduction in fertility. Oral delivery may require protection of vaccine ghosts from degradation. We have developed a protective system and are currently repeating this trial using oral delivery of the ZP2 ghost vaccine. Our future priority is increasing the vaccine efficacy and longevity ahead of limited field trials in 2009.

**KEY WORDS:** antifertility agents, biological control, immunocontraception, Marsupialia, New Zealand, possums, safety, *Trichosurus vulpecula*, vertebrate pest control

Proc. 22<sup>nd</sup> Vertebr. Pest Conf. (R. M. Timm and J. M. O’Brien, Eds.)  
Published at Univ. of Calif., Davis. 2006. Pp. 102-105.

## INTRODUCTION

There is a widening range of species for which fertility control vaccines are under development as alternatives to lethal methods of management. Most of these vaccines are based on porcine zona pellucida or species-specific zona pellucida proteins (e.g., Kirkpatrick *et al.* 1996, Hardy *et al.* 2002, Kitchener *et al.* 2002). While immunisation against zona pellucida proteins is known to induce infertility in a wide range of eutherian mammals, little is known of the effects of such vaccines on marsupial species despite an urgent management need for such non-lethal control technologies for pest marsupials such as the common brushtail possum (*Trichosurus vulpecula*) (Cowan 2000). Possums were introduced to New Zealand from their native Australia in the 1850s and are now major pests of both conservation and agricultural production (Cowan 2005). They damage native forests by browsing, prey on native birds, and are the principal wildlife host of bovine tuberculosis. About US\$45 million is spent annually on lethal control of this pest, mostly trapping and poisoning (Morgan and Hickling 2000, Cowan 2005).

Since its inception in the 1990s, the research programme on biological control of possums has been guided by a National Science Strategy overseen by a committee of key end-user management agencies ([www.frst.govt.nz/About/possum.cfm](http://www.frst.govt.nz/About/possum.cfm)). In the strategy, biocontrol is just one approach, but seen as a key one for a long-term solution to New Zealand’s possum problem.

Part of the strategy is focussed on fertility control, and specifically on development of an oral, bait-delivered immunocontraceptive vaccine (Cowan 2000, Duckworth *et al.* 2006). This paper reviews progress to date in development of such a vaccine for possums based on zona pellucida antigens, including recent research on the potential of bacterial ghost technology (Eko *et al.* 1999, Walcher *et al.* 2004) as a platform for oral vaccine delivery.

## METHODS

Following on from recent characterisation of the structure of the possum ZP proteins (Mate and McCartney 1998, McCartney and Mate 1999, Mate *et al.* 2003, Cui and Duckworth 2005), various trials were conducted to examine the effects of alloimmunisation with purified recombinant possum ZP antigens or epitopes on the immune responses and fertility of treated female possums.

The general testing protocol used wild adult possums housed indoors in individual cages for a physical and immunological acclimatisation period of six to twelve weeks (Buddle *et al.* 1992). Ten days before the experimental period, female possums were transferred, in pairs matched by body weight, into 4 m × 4 m outdoor pens with shelter and sacks for nesting. Possums were fed *ad libitum* fresh fruit and cereal-based possum pellets, with water freely available.

Female possums were assigned at random to experi-

mental groups and immunised with Freund's-based vaccine containing either ZP antigen or PBS with adjuvant only (Control Group) in weeks 0, 3, and 6. Vaccines were prepared by mixing each recombinant protein with phosphate buffered saline (PBS) and then emulsifying the mixture with an equal volume of complete Freund's adjuvant for the primary immunisation and with Incomplete Freund's Adjuvant (IFA) for booster immunisations. Control animals received the same adjuvants emulsified with plain PBS.

For trials that involved assessments of bacterial ghosts expressing possum ZP proteins, individually housed female possums were immunised by various routes (subcutaneous, nasal, and/or oral) at various doses 3 times at 2 weekly intervals, with control females given plain *Escherichia coli* ghosts only. The nasal/conjunctival route of immunisation evoked the strongest immune response, and so it was used in a trial to assess the effects on fertility of immunisation with bacterial ghosts expressing ZP2 proteins in the periplasmic space. Various gastric acid inhibitors were also tested for their ability to improve immune response to bacterial ghosts given orally.

Immune responses in all trials were assessed by standard techniques such as indirect Enzyme-Linked Immunosorbent Assay (ELISA) and Western blotting (Duckworth *et al.* 1999, Mate *et al.* 2003). The secondary antibody for ELISA (supplied by David Kay, Newcastle University) was raised in sheep against possum serum immunoglobulin (Ig) and reacted with possum IgG, IgA, and IgM.

Fertility was assessed in two ways. In natural breeding trials, a mature male was introduced to the pens and left with females for the duration of the breeding season, with pouches of females checked regularly and any pouch young removed so that females would come back into oestrus repeatedly (Duckworth *et al.* 1999). In SO/AI trials, females were treated with hormones to induce superovulation and then artificially inseminated as previously described (Molinia *et al.* 1998) except a vaginal rather than uterine route was used. Possums were killed, and the reproductive tract was removed and ovaries separated for assessment of numbers of ovulations sites and remaining unovulated follicles (>2 mm). Oviducts, uteri, and the left lateral vagina were flushed to recover eggs for assessment of fertilisation. Fluid from 2-4 unovulated ovarian follicles in the left ovary was collected by puncturing each follicle with a fine glass capillary tube.

Initial assessments of non-target safety were carried out using laboratory mice and domestic chickens. These were immunised by injection with Freund's-based vaccine containing either possum ZP antigen/epitope or PBS with adjuvant only (Control Group), with boosters in IFA at weeks 2, 4, and 6. Mice and chickens were then bred to assess any effects on reproductive performance.

All projects were approved by the Animal Ethics Committee of Landcare Research, Lincoln and were performed in accordance with the 1987 Animals Protection (Codes of Ethical Conduct) Regulations of New Zealand.

## RESULTS

Various trials were conducted using antigens based on possum ZP3, the N- or C-terminal regions of possum ZP-2, and possum ZP1. An epitope of possum ZP3, homologous to one shown previously to produce infertility in mice (Millar *et al.* 1989), was also tested. With the exception of ZP1, all antigens tested produced significant reductions of 60-87% ( $P < 0.05$ ) in the number of pouch young or embryos produced by treated females compared with control females (Table 1). Immunisation with ZP antigens appeared to have two effects in SO/AI trials— a reduction in numbers of ovulations and a reduction in the percentage of ovulated eggs that were fertilised (Table 1).

Bacterial ghosts expressing N- or C- terminal halves of possum ZP2 evoked immune responses in most possums. The percentage of fertilised eggs declined by 37% in possum immunised with ZP2-C bacterial ghosts (Table 2;  $P < 0.05$ ). This is the first demonstration of a mucosally-delivered vaccine for possums that produced a significant reduction in fertility. Dose response and route of immunisation trials aiming to improve immune responses to orally delivered ghosts identified an optimal dose of ghosts, which when given orally with a gastric acid inhibitor, produces as strong an immune response as a similar dose given nasally/conjunctivally. An experiment is currently in progress to assess the effects on fertility of oral immunisation with bacterial ghosts expressing ZP2 and ZP3 using this optimal protocol.

To test for non-target effects, mice and chickens have been immunised by injection with ZP3, ZP3-epitope, ZP2-C, or ZP2-epitope. No significant effects were detected on the percentage of animals breeding or laying, numbers and size of litters/eggs laid, or birth weights/hatching rate of treated animals compared with controls.

**Table 1. Summary of results of immunisation trials with various possum ZP antigens. Fertility assessment methods are described in the text. Imm = ZP-treated; Cont = controls; PY = pouch young.**

Antigen	Fertility Assessment	Matings or Ovulations		% Fertilized		No. PY or Embryos
		Imm	Cont	Imm	Cont	
ZP3	Natural	6/15	11/15	17	75	87% ↓
ZP3	SO-AI*	1.0	3.2	25	73	80% ↓
ZP2-C	SO-AI	1.7	3.7	25	43	72% ↓
ZP2-N	SO-AI	1.4	3.7	33	43	75% ↓
ZP3-peptide	SO-AI	2.4	3.6	42	70	60% ↓
ZP1	SO-AI	No effect		No effect		No effect

\*superovulation / artificial insemination

**Table 2. Numbers of possums showing humoral immune responses to bacterial ghosts and percentage of fertilised eggs recovered after superovulation and artificial insemination (\*P < 0.05; \*\*P < 0.001 different from control).**

	Control (Plain Ghosts)	ZP2-N Bacterial Ghosts	ZP2-C Bacterial Ghosts
Humoral immune response	ZP2-N 0/20 ZP2-C 1/20	ZP2-N 17/20**	ZP2-C 17/20**
% eggs fertilised	90%	76%	57%*

## DISCUSSION

Progress to date with development of an oral ZP-based fertility control vaccine for possums is encouraging. Proteins and epitopes have been identified that have the capacity to produce the levels of infertility that computer modelling and field trials using surgical sterilisation (Ramsey 2005) have suggested are necessary for the vaccine to be a useful addition to the possum management toolbox. Our future priorities are to increase vaccine efficacy and longevity, so that by 2013 we will have a bait-delivered vaccine that will decrease possum fertility by at least 60% for at least 2 years.

The long separation of the lineages of marsupial and eutherian mammals is associated with low amino acid homology between possum ZP proteins and the equivalent proteins in eutherian species. For example, possum ZP3 has only 46% homology with the equivalent protein in mice, and 45-46% with equivalent proteins in a range of other eutherian species (Harris *et al.* 1994; McCartney and Mate 1999). If we consider only the ZP3 epitope used in our trials, there is even less homology between possums and mice (26%) or other eutherians (15-28%). This difference provides a potential basis for vaccine specificity, which appears to be borne out by the lack of effects on fertility in our trials to date with mice and chickens immunised with possum ZP proteins and epitopes. Our next step will be to test bacterial ghost constructs known to affect fertility in possums for possible effects in mice and chickens. Ultimately, a wide range of non-target species will have to be tested to satisfy regulatory requirements before a bait-delivery fertility vaccine could be deployed. As an example of the likely scale of such requirements, the evaluation of rabbit haemorrhagic disease virus for release in Australia and New Zealand involved testing in 33 different animal species (Bureau of Rural Sciences 1996).

## ACKNOWLEDGEMENTS

This research was funded under contract to the New Zealand Foundation for Research, Science and Technology, and the Animal Health Board. Thanks are due to Susie Scobie, Denise Jones, Jane Arrow, Vaughan Myers, Julie Turner, and animal facility staff for assistance with all testing trials. The draft manuscript was edited by Anne Austin.

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