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Submission on AgResearch Application GMD01194

Submitter: New Zealand Institute of Gene Ecology

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1. Introduction

1.1. This submission concerns AgResearch Application GMD01194, with particular reference to Appendix 6, and the application of the analysis in Appendix 6 to assessments of the impact of modified gene transfers and the associated technologies accompanying the modifications. Appendix 6 purports to advance reasons why the risk of gene escape from the proposed GMO developments is very low. Not all pertinent scientific considerations are brought to light by this analysis, however. Thus the statement (Summary of Effects 1.1.1) "Because Tg cattle subject of this application will never leave containment or enter any food chain, issues of direct horizontal gene transfer are not applicable…" may not be valid.

1.2. AgResearch rightly, in our view, recognises that gene transfer is "an area requiring much more research". This submission is intended to highlight where that research might best be directed and applied by the Authority in its deliberations on GMD01194 and/or on subsequent controls and conditions that might be imposed on GMD01194 should it be approved.

1.3. Submitter. The New Zealand Institute of Gene Ecology (NZIGE) is a research organisation (www.nzige.canterbury.ac.nz). The NZIGE has no commercial interest in the technology being developed by the applicant. Our submission is, however, informed by our extensive experience in the research areas discussed below.

1.4. Disclaimer. As a research organisation it is partly in our own interest to identify issues worthy of public investment in research. We submit that, before the risks of gene escape or associated technologies proposed in GMD01194 can properly be assessed, better research is needed. The Authority should be aware that the NZIGE is one research organisation that could help to do the needed research and could compete for funding to conduct the needed research. Our interest in the research, however, is not a bias on how we view AgResearch's research or in how we would conduct subsequent research.

2. Summary. The key points we wish to raise in this submission are: (1) horizontal gene transfer (HGT) is a broader worry than AgResearch has allowed, and (2) some risks from

the proposed research are indirect, concerning not Tg animals themselves but the possible impact of co-technologies employed in their construction, maintenance or use. As will become clear, these two key points are related. We will discuss them in turn.

2.1. HGT and its potential impacts are not limited to events wherein the transferred genes become a part of a recipient organism's genome (either the initial Tg animal or through unplanned transfers), as implied by the AgResearch analysis.

2.1.1. AgResearch has limited its analysis to the assumed effects on the descendants of an organism "transformed" (ie, made recombinant) by a horizontally transferred gene and the effects such populations of recombinants might have on the environment.

2.1.2. We will argue that many, perhaps the vast majority, of HGT events do not transform organisms in this way but nevertheless are important for considerations of risk. We will detail some of those considerations and draw the Authority's attention to the literature on these matters.

2.1.3. The evolution of viruses, which are horizontally transferred genes that can, but rarely do, transform organisms in the sense used by AgResearch, is a familiar example. More often, their evolution is strictly by HGT. To ignore the impact of viruses and other horizontally transferred genes because they do not routinely transform recipient organisms is to invoke a definition of HGT that is arbitrary, and restrictive, without scientific justification.

2.2. **In discussing the potential impact of the Tg animals being constructed, AgResearch has neglected to consider the impact of the co-technologies inherent in their construction and maintenance (and eventual use).**

2.2.1. The Tg animals are to be transformed by genes other than those expected to be developed for their therapeutic value, such as puromycin and neomycin resistance genes, and the tetracycline-on control system. The former genes

might, or migh not (at AgResearch's discretion), be removed by cre/lox, but use of the tetracycline control system implies the ongoing use of tetracycline as a co-technology.

2.2.2. These genes are examples of two co-technologies that deserve two additional assessments. First, AgResearch should assess the potential impact of unplanned transfers of these genes to other organisms. Second, AgResearch should assess the environmental impact of the antibiotics.

3. Issue 2.1, the difference between gene transfer and gene transmission and how that difference should be used in risk analysis.

3.1. Preamble. That HGT is real and an important mechanism by which some genes reproduce is by now widely acknowledged. Yet that acknowledgment is only recent. Had this application been made even five years earlier, the debate on its acceptability would have been at the level of arguing whether HGT happened at all. This is to say that the science of HGT is young even though the effects of HGT have been described since the mid-20th Century (Ferguson and Heinemann, 2002). HGT's role in evolution is just starting to be studied outside of specialist biological examples (eg, Agrobacterium and plants). Technologies purpose designed for its study are only just appearing. So it is understandable, perhaps, that despite the realisation by the larger scientific community that HGT is real and frequent, HGT is not universally incorporated into the daily working analyses of molecular biologists, botanists and zoologists. Moreover, it will take time for this new specialist branch of genetics to become widely incorporated in curricula through the publication of new textbooks. Still, the incorporation of HGT in risk analysis must transcend a cursory knowledge of HGT and cultural barriers to these ideas within some branches of biology.

3.2. Horizontal gene transfer is any occurrence of heritable material passing between organisms asynchronously with reproduction of the organisms, that is, reproduction of heritable material outside the context of parent to offspring (ie, vertical) reproduction.

3.3. Horizontal gene transfer is not only the passage of DNA between species.

3.4. Usually, however, the evidence of HGT is the demonstration of DNA of mixed species origin in a particular genome. The evidence derives from sequencing DNA. The number of genes known to have been inherited following gene transfer is still relatively small in part because of the difficulty in determining whether a particular DNA sequence (composed of some combination of only four nucleotides) is significantly different from what would be expected to occur in that genome (Heinemann, 1991; Heinemann, 2000; Lawrence and Ochman, 2002).

3.5. The statement that gene transfers are rare, or only occur over evolutionary time, is an inappropriate conclusion to draw from available evidence for the following reasons.

3.5.1. Many mechanisms for HGT are known and all are operative (or potentially operative in nature) at least to some degree in all organisms (de la Cruz and Davies, 2000).

3.5.2. Transferred DNA may be of the form of short but potent sequences (for phenotypes) and not large enough to detect beyond the statistical noise of comparing nucleotide sequences (Heinemann, 2000; Lawrence and Ochman, 2002).

3.5.3. Transferred DNA may be long sequences of similar but not identical composition to indigenous sequences and again not distinguishable from statistical noise.

3.5.4. The statement is based solely on genomic data which is inherently biased toward detecting genes that transit from reproduction by HGT to ones that reproduce vertically (ie, synchronously with the reproduction of the organism). Many genes transfer without making this transition, such as viruses.

3.6. The arbitrary treatment of risk as only arising from HGT events that transform a recipient organism, and thus may then be passed vertically, does not acknowledge the literature in a balanced way. Therefore, we feel it is useful to reiterate the definition of transfer and transmission.

3.6.1. Transfer is defined as the movement of heritable material, as in HGT (3.2), but does not require that the heritable material subsequently pass to descendants of that organism (Clark and Warren, 1979; Heinemann, 1992; Heinemann, 1997).

3.6.2. Transmission is defined as the movement of heritable material, as in HGT, and its subsequent inheritance by descendant organisms (Clark and Warren, 1979; Heinemann, 1991).

3.6.3. Transmission is an appropriate criterion for demonstrating transfer; the absence of evidence for transmission is inappropriate evidence to conclude transfer did not occur or occurs infrequently (Ferguson and Heinemann, 2002; Heinemann and Sprague Jr., 1989). This is because many other important variables could affect the heritable material on its conversion to becoming part of the genetic constitution of the organism, but not be relevant to its impact. For example:

3.6.3.1. In the case of viruses, their retention in the host organism may be irrelevant to their impact and their continued evolution.

3.6.3.2. In the case of mosaic genes (those that arise by recombination between similar but not necessarily homologous genes), the impact of incorporating only a small number of nucleotides (eg, 1) is invisible to most types of DNA analysis but not necessarily to phenotype.

3.6.4. Failure to distinguish between the concepts of transfer and transmission have led to significant problems. One clear example is the mid-20th Century predictions about the evolution of antibiotic resistance in bacteria of medical

importance and current claims about how quickly antibiotic resistance will disappear should antibiotics be withdrawn (Heinemann et al., 2000).

3.7. Recent significant advances in empirical studies of evolution offer hope of predicting when genes have the potential to cause harm by HGT independently of transmission. Until that hope can be realised as fact, these studies are essential reading for those who assess the risk of HGT.

3.7.1. Currently, evolution science cannot predict when an entity will evolve to cause harm. In the biomedical literature, this harm is usually equated with the term virulence, such as when a virus or bacterium will evolve to be virulent to humans (Lipsitch et al., 1996).

3.7.2. The characteristic of some genes, when they occur in combination, can cause them to be evolutionarily more successful reproducing by HGT than vertically. This demonstration has been made with what would have been considered the least likely type of genes, those that kill their own hosts, and are therefore virulence factors (Cooper and Heinemann, 2000; Heinemann and Roughan, 2000; Naito et al., 1995).

3.7.3. Some of these same genes in isolation would not demonstrate those qualities and, indeed, have been mistaken as having evolved for different reasons (Naito et al., 1995).

3.7.4. This branch of empirical evolution is referred to as “within-host” evolution and draws upon the realisation that genes react to selection at the intracellular level, not just selection from some hypothetical external environment.

3.7.5. It is often incorrectly assumed that selection is only a factor of the environment external to the organism (Bateson, 1992; Lewontin, 2000). AgResearch acknowledges this oversimplification when it recognises that some parts of the genome can experience higher recombination and mutation frequencies than other parts. Thus, selective pressures exist even at the

molecular level, and these selective pressures can shape the evolution of the organism (Heinemann, 1993; Heinemann, 2000). The genes themselves can generate selective pressures that govern the evolution of the organism, rather than act as passive factors upon which external selective pressures determine the fate of the organism (Cooper and Heinemann, 2000). The power of selection at the molecular level is revealed through the organisation of genes in some genomes (Lawrence and Roth, 1996) and other ways. The internally generated selective pressures favour combinations of genes that evolve best by HGT (Cooper and Heinemann, 2000). Within-host selection is a powerful demonstration of the legitimacy of such views.

3.7.6. Thus, it should be taken as standard practice, in our view, to comment on the possibility that transgenes could acquire properties that make them more or less likely to reproduce by HGT. Following such analysis, the Authority should erect monitoring controls that could detect such events.

4. Issue 2.2, the impact of co-technologies on risk analysis.

4.1. AgResearch states: "The wide transfer of genes that might have occurred naturally weakens the argument that human intervention through genetic engineering is placing genes where they have never been before."

4.1.1. That statement is acceptable as a comment on the impression of genomes as unchanging and divorced from the effects of the environment.

4.1.2. However, it is misleading in that it could be interpreted to mean that there are no differences between the potential impacts of gene transfers conducted by humans and that conducted by nature. To be accurate, the statement should be followed by an accounting of:

4.1.3. any unique or associated selective pressures accompanying the gene transfers, such as artificial selection for, or maintenance of, Tg organisms, or

unanticipated contributions to phenotype that could be made by the transgene in the transgenic genome.

4.2. Sometimes genes are used to initially select for the Tg organism or to maintain the Tg phenotype. In this case, AgResearch includes certain antibiotic resistance genes and visual marker genes. Resistance genes can adapt organisms to environmental selection and both the resistance and marker genes can create selective pressures. Even GFP, for instance, can cause free-radical damage as a side effect of its fluorescence (Greenbaum et al., 2000; van Thor et al., 2002). Thus, it can select for changes in genes that repair such damage in DNA or proteins or possibly increase the mutation rate of transgenic cells.

4.3. When humans introduce a selective pressure in conjunction with the gene transfer, they couple two events that occur randomly in nature, making the effect potentially quite different in a number of ways. For example:

4.3.1. The additional selective pressure (eg, as from antibiotics) can increase mutation rates in populations of the Tg or other organisms (Funchain et al., 2001; Negri et al., 2002).

4.3.2. The additional selective pressure can change receptivity of non-target organisms in the affected environment to gene transfer (Funchain et al., 2001).

4.3.3. The additional selective pressure maintains Tg organisms at potentially higher population levels than would occur in nature without the selective pressure or human protection, and thereby increase the probability of subsequent gene transfers.

4.4. An illustrative example with sequela of extreme interest to the NZIGE is the AgResearch proposal to control gene expression using the antibiotic tetracycline. *We are extremely concerned to see no analysis of risk in the use of tetracycline as a co-technology.*

4.4.1. It is now routinely acknowledged that the most important factor behind the spread of antibiotic resistant bacteria is the use of antibiotics that select for bacteria that acquire a resistance gene.

4.4.2. The transgenes to be used by AgResearch do not confer tetracycline resistance.

4.4.3. However, their use of a tetracycline responsive gene expression element implies that AgResearch will increase the use of tetracycline, the very practice routinely acknowledged to be the cause of widespread resistance to this antibiotic.

4.4.4. It can be argued that tetracycline resistance is not a unique risk factor, has lost some of its clinical relevance, and is used in other agricultural settings in equivalent or greater amounts.

4.4.5. In our view, however, such arguments are naïve for the following reasons.

4.4.6. The use of one antibiotic has implications for resistance to other antibiotics (Heinemann, 1999; Heinemann et al., 2000; Salyers and Amábile-Cuevas, 1997).

4.4.6.1. Vectors that convey antibiotic resistance genes in nature tend to carry more than one resistance gene, meaning that selection for any of those genes maintains all linked resistance genes (eg, Holmberg et al., 1984).

4.4.6.2. Tetracycline resistance can lead to an increased likelihood of cross-resistance to other drugs (Heinemann, 1999). For example, one study found that tetracycline can select *Escherichia coli* with a "multiple antibiotic resistance" (mar) phenotype and those strains were 1000 times more likely to acquire resistance to structurally unrelated fluoroquinolone antibiotics, a class which is of extreme clinical importance (reviewed in Heinemann, 1999).

4.4.6.3. Tetracycline responsive elements are also found in conjugative transposons of both gram-negative and gram-positive bacteria; tetracycline stimulates HGT rates by controlling expression of the genes that cause these elements to transfer (Salyers, 1995).

4.4.6.4. Tetracycline has immuno-modulating activities (Heinemann et al., 2000). This should be considered in relation to the long-term exposure of the animals and consequent potential for increased infection rates and increased use of other antibiotics.

4.4.6.5. Tetracycline's immuno-modulating activities make it relevant to medical applications other than treating specific bacterial infections. The risk of over-use of this antibiotic should be explored beyond considering its clinical uses as an anti-infective chemotherapeutic.

4.4.7. The use of these drugs can have clinical implications for relevant pathogens.

4.4.7.1. Recent work on *E. coli* O157 (STEC and non-STEC), a human pathogen of interest to New Zealand because it is carried by food animals, found that "selection pressure imposed by the use of tetracycline derivatives…whether therapeutically in human or veterinary medicine or as prophylaxis in the animal production environment, is a *key driving force* (emphasis ours) in the selection of antimicrobial resistance in STEC and non-STEC O157" (Schroeder et al., 2002).

4.4.7.2. *E. coli* O157:H7 has the additional ability to be taken up by the roots of plants (eg, lettuce) that are eaten raw, and migrate to edible tissues. Hence, this pathogen, which appears to be acquiring clinically relevant antibiotic resistance traits in part because of the use of tetracycline, also can evade surface decontamination and heat sterilization through foods commonly ingested raw by humans (Solomon et al., 2002). The authors of this study concluded: "Under natural conditions, even a low level of

contamination could present a significant human health risk, since the infective dose of *E. coli* O157:H7 is less than 1,000 cells" (Solomon et al., 2002).

4.4.8. We note that in these many ways the use of the co-technology is very different when applied to the development of modified bacteria grown in monoculture in a laboratory. This is because in the laboratory the co-technology has no impact on the larger microbial community and the laboratory bacteria have no opportunity to acquire genes from, or transfer them to, other bacteria. So whereas the Tg animals may be considered in a state of containment, there does not exist adequate evidence for the conclusion that the facilities will contain the impacts of the co-technologies.

5. Conclusions

5.1. Food chain and HGT. It may be incorrect to consider that the animals will be secure from entry into "any" food chain, since they are not being maintained in adiabatic chambers, gnotobiotic or pest free.

5.1.1. AgResearch considers the HGT scenario: Tg GM animal → Tg GM bacteria → Tg GM plants → Tg GM animal. It does not seriously consider other relevant possibilities such as Tg GM animal → Tg GM virus → Tg GM animals/plants/bacteria or Tg GM animal → Tg GM parasite → Tg GM insect/bacteria. This strikes us as odd, given the literature on virus transfer ranges (eg, Gibbs and Weiller, 1999) and animal-to-animal gene transmission (Kidwell, 1993). In fact, the scenario AgResearch highlights may be the least likely in view of the literature.

5.1.2. AgResearch has not discussed in what ways these genes or associated selection/marker genes might adapt other organisms, such as blood-sucking parasites and soil microorganisms, to known or unknown selective pressures.

5.1.3. The maintenance of the animals and their ability to be increased in number through subsequent breeding or re-creation by humans does differ from the "natural" condition, contrary to what is claimed in the application, wherein these same genes might have transferred to the bovine genome without human intervention.

5.1.3.1. In nature, the probability of an HGT event being maintained in the bovine genome and affecting the phenotype of the animal's descendants is the product of the [probability of the HGT event · probability of expression · probability of selection ("positive") · the magnitude of the selective advantage].

5.1.3.2. In the development case, the HGT event, expression and selection (at least by humans) variables are (near) certainties. Whether these changes could affect estimates of subsequent HGT events to other organisms or contribute to other long-term changes in the Tg animal's lineage are unknown, but that uncertainty should be acknowledged.

5.1.3.3. An example from the biomedical experience with antibiotics and resistance evolution is illustrative here. Many newly emerging antibiotic resistant strains of bacteria are less competitive than their antibiotic-susceptible parents in environments free of the antibiotic. This early disadvantage, however, is soon lost. Many have assumed that resistance to current antibiotics would fade when new antibiotics were developed partly because resistant strains were less fit in antibiotic-free environments. However, it is clear now that resistant strains can acquire competition-compensatory mutations while growing in antibiotics (Bjorkman et al., 1998; Bjorkman et al., 2000; Schrag and Perrot, 1996; Schrag et al., 1997). By the time the antibiotic is removed from the environment, the strains are as fit or more fit than their parents even in antibiotic-free environments. Antibiotics, in this case, serve as an umbrella supporting the evolution of initially uncompetitive phenotypes.

5.1.3.4. Human maintenance of Tg animals or derivative organisms could have a similar "umbrella effect" on the ecology of recombinants, impacting on the evolution of ecologically undesirable organisms even if the initial Tg organism appears less fit. This point also applies to the discussion on co-technologies, below.

5.1.4. AgResearch plans "to set in place a quality assurance program to track any cattle Tg (GM) DNA that may find its way into the soil bacteria of the burial disposal site."

5.1.4.1. This is a laudable goal. However, many such monitoring attempts have produced negative results. We believe that some assessment of the detection limits of these techniques in the relevant soil should be required *a priori*. Those detection limits should not preclude AgResearch from detecting reasonably low concentrations of Tg DNA and should be verified by disinterested experts.

5.1.4.2. Moreover, it should be acknowledged that the transgenes will only be detected in microorganisms whose own genomes are different enough to the transgene sequences to be identified by comparing DNA sequences. Can AgResearch provide empirical or modelling evidence of limits of detection of transgenes, or parts of transgenes, in different genomes?

5.1.4.3. Far more likely than transfer of the transgene as a whole is its recombination with other nucleotide sequences in soil microorganisms or other organisms. Such recombinations result in retention of 'short patches' of nucleotides transmitting to the recipient genome (Coic et al., 2000; Mezard et al., 1992). There is compelling laboratory evidence that such recombination events are stimulated considerably when recipient organisms are under stress, as they would be in the presence of antibiotics or other changes in the environment, eg, the introduction of fresh carcasses into the soil. How does AgResearch plan to monitor for short patch recombination or assess the impact of its operations on indigenous

organisms affected by the work? Controls should be in place that are sufficiently sensitive to detect these changes.

5.2. Co-technologies and ecological impact.

5.2.1. Controls should be in place to monitor expected and unknown perturbations to the ecosystem from the proposed development. Such controls are technically feasible but may require new research to test and develop.

5.2.2. AgResearch should address concerns about the use of co-technologies, particularly the effects of antibiotics.

Respectfully submitted by

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