Genome engineering in poultry

opportunities and impacts

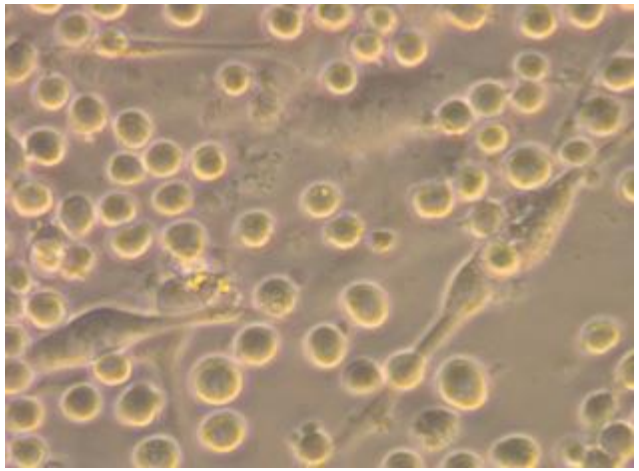
Dr Tim Doran | Australian Centre for Disease Preparedness

Why genetically engineer birds?

- Biology
  - chicken and quail are excellent model organisms for developmental biology research
- Agriculture - improve poultry production (meat and eggs)
  - generating chickens that are resilient to disease, have improved production traits, improved welfare and safer food products
- Biotechnology - generate valuable biologicals for medicine
  - chicken eggs as bioreactors for pharmaceutical proteins
- Conservation
  - genetic rescue of endangered bird species

Primordial Germ Cells (PGCs)

- Avian embryonic PGCs migrate through the vasculature on their path to the gonad where they become the sperm or ova producing cells



- This unique feature of avian PGC migration through blood has led to a transformational advance in the generation of genetically engineered chickens

- PGCs “OUTSIDE” – highly skilled culture (van de Lavoir *et al*, 2006)
  - establish PGC cultures
  - introduce genetic modifications into cultured cells
  - expand the modified cells into clonal populations
  - inject selected cells into recipient embryos to create germline chimeras

- PGCs “INSIDE” – *In vivo* transfection of PGCs (Tyack *et al*, 2014)
  - direct Injection - no selection step available which can lower success rate
  - Avoid imported biologicals required for PGC culture system

Disease Resilience – avian influenza (ANP32A)

- Avian influenza is a major problem
  - Consistent and extensive outbreaks in recent years
  - Vaccination not fully effective
  - Significant threat of human flu pandemic
- Avian influenza virus polymerase does not function well in mammalian cells (host restriction)
- Species specific difference in ANP32A protein - avian ANP32A proteins have a 33 amino acid insertion

- Long et al 2019 used CRISPR to remove the 33 amino acid insertion from chANP32A or to knockout the entire protein in chicken cells
  - Edited cells that expressed the short chANP32A supported mammalian-adapted but not avian polymerase activity
  - Cells completely lacking chANP32A did not support either mammalian or avian influenza polymerase activity and were refractory to infection

eLIFE elifesciences.org RESEARCH ARTICLE

Species specific differences in use of ANP32 proteins by influenza A virus

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Abstract Influenza A viruses (IAV) are subject to species barriers that prevent frequent zoonotic transmission and pandemics. One of these barriers is the poor activity of avian IAV polymerases in human cells. Differences between avian and mammalian ANP32 proteins underlie this host range barrier. Human ANP32A and ANP32B homologues both support function of human-adapted influenza polymerase but do not support efficient activity of avian IAV polymerase which requires avian ANP32A. We show here that the gene currently designated as avian ANP32B is evolutionarily distinct from mammalian ANP32B, and that chicken ANP32B does not support IAV polymerase activity even of human-adapted viruses. Consequently, IAV relies solely on chicken ANP32A to support its replication in chicken cells. Amino acids 129I and 130N, accounted for the inactivity of chicken ANP32B. Transfer of these residues to chicken ANP32A abolished support of IAV polymerase. Understanding ANP32 function will help develop antiviral strategies and aid the design of influenza virus resilient genome edited chickens.

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Introduction

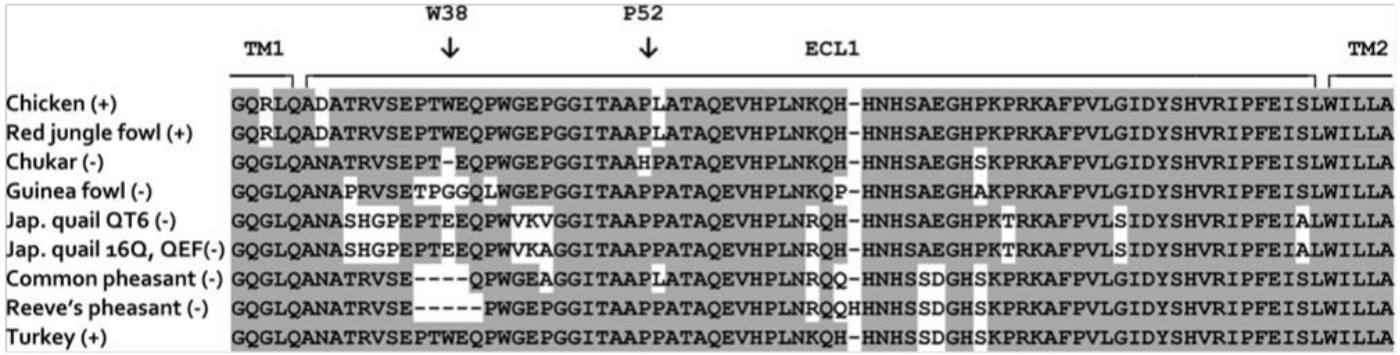
Influenza A viruses (IAV) infect a wide range of host species but originate from wild birds. Zoonotic transmission from the avian reservoir is initially restricted by host specific species barriers. Infection of new host species requires the virus to bind to cell surface receptors, utilise foreign host cellular proteins whilst evading host restriction factors in order to replicate its genome, and finally transmit between individuals of the new host.

The negative sense RNA genome of influenza A virus (IAV) is replicated in the cell nucleus using a virally encoded RNA-dependent RNA polymerase, a heterotrimer composed of the polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acidic (PA) proteins together with nucleoprotein (NP) that surrounds the viral RNA, forming the viral ribonucleoprotein complex (vRNP) (***Te Velthuis and Fodor, 2016***).

Crucially, the viral polymerase must co-opt host factors to carry out transcription and replication (***Te Velthuis and Fodor, 2016***). The PB2 subunit is a major determinant of the host restriction of the viral polymerase (***Almond, 1977***). Avian IAV polymerases typically contain a glutamic acid at position 627 of PB2, and mutation to a lysine, the typical residue at this position in mammalian-adapted PB2 (***Subbarao et al., 1993***), can adapt the avian polymerase to function efficiently in mammalian cells. We have suggested that the restriction of avian IAV polymerase is due to a species specific difference in host protein ANP32A (***Long et al., 2016***). Avian ANP32A proteins have a 33 amino acid insertion, lacking in mammals, and overexpression of chicken ANP32A (chANP32A) in human cells

Disease Resilience – avian leucosis virus subgroup J (ALV-J)

- ALV-J causes significant economic losses in the poultry industry
- Virus receptor is chNHE1
- Deletion or substitution of a single amino acid (tryptophan 38) confers resistance to infection



Precise CRISPR/Cas9 editing of the NHE1 gene renders chickens resistant to the J subgroup of avian leukosis virus

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Avian leukosis virus subgroup J (ALV-J) is an important concern for the poultry industry. Replication of ALV-J depends on a functional cellular receptor, the chicken Na^+/H^+ exchanger type 1 (chNHE1). Tryptophan residue number 38 of chNHE1 (W38) in the extracellular portion of this molecule is a critical amino acid for virus entry. We describe a CRISPR/Cas9-mediated deletion of W38 in chicken primordial germ cells and the successful production of the gene-edited birds. The resistance to ALV-J was examined both in vitro and in vivo, and the ΔW38 homozygous chickens tested ALV-J–resistant, in contrast to ΔW38 heterozygotes and wild-type birds, which were ALV-J–susceptible. Deletion of W38 did not manifest any visible side effect. Our data clearly demonstrate the antiviral resistance conferred by precise CRISPR/Cas9 gene editing in the chicken. Furthermore, our highly efficient CRISPR/Cas9 gene editing in primordial germ cells represents a substantial addition to genotechnology in the chicken, an important food source and research model.

avian leukosis virus subgroup J | Na^+/H^+ exchanger type 1 | CRISPR/Cas9 genome editing in chicken | primordial germ cells | disease resilience in poultry

Background

Since its identification during the first outbreak in the United Kingdom (1), avian leukosis virus subgroup J (ALV-J) has been an important concern for the poultry industry. European and American ALV-J strains, such as the prototypic HPRS103, mostly induced myelocytomatosis in broiler chickens. In China and Southeast Asia, however, ALV-J evolved into various strains with diversified pathologies in both meat- and egg-type chickens (2). The last big ALV-J outbreak in China, with surprisingly high mortality of infected chickens, occurred in 2018, despite an eradication program geared toward the virus (3).

Chicken Na^+/H^+ exchanger type 1 (chNHE1) has been recognized as a receptor for ALV-J, and its prominent and glycosylated extracellular loop 1 is necessary for virus entry (4). Comparison of its amino acid sequence in ALV-J–susceptible species (domestic chicken, jungle fowls, turkey) and resistant species (most galliform birds) pointed to W38 being a residue critical for the receptor function (5). The single amino acid deletion of W38 introduced by CRISPR/Cas9 in cultured chicken cells rendered the cells resistant to ALV-J and confirmed the importance of W38 for virus entry (6). Unfortunately, chNHE1 is well conserved in a wide range of chicken breeds, including indigenous breeds of Asian origin (7). As a result, there is no known source of genetic variability to be used to selectively breed an ALV-J–resistant chicken. This obstacle might be overcome by precise gene editing using CRISPR/Cas9.

The current approach to chicken genome manipulation relies on primordial germ cell (PGC) technology (8). Male PGCs are derived from chicken embryos infected with a retroviral vector or DNA-transfected during in vitro culture and finally returned to the embryos, where they differentiate and mature into functional sperms in the cockerels after hatching. This is now a well-established technology providing transgenic chickens (9) and even genetic knockouts (10). However, the knock-in technology and CRISPR/Cas9 editing of endogenous loci in the chicken remained to be expanded, with the low efficiency of embryonal PGC application being the main obstacle. This has now changed with our method of orthotopic PGC transplantation into sterilized adult cockerels (11). This technique improves the efficiency of transgenesis in the chicken and makes such gene editing feasible. In this report, we describe the preparation of a chicken line with CRISPR/Cas9-introduced ΔW38 into chNHE1. This chicken line is fully resistant to ALV-J infection.

Results

Generation of chNHE1-Edited Chickens. We derived PGCs from blood aspirations of 14- to 17-stage (H & H) male embryos of inbred line CB (*SI Appendix*, Fig. S1), which is homozygous for the wt chNHE1 allele and susceptible to ALV-J infection. For

Significance

The current progress of genome editing techniques accelerates the knock-out and knock-in studies in animal models and production of genetic modifications in livestock. Increased resistance to viral pathogens is a particular goal because many monogenic host cell factors are necessary for productive infection and pathogenesis. For example, virus receptors with their specific virus binding sites are direct targets for the CRISPR/Cas9 gene editing. We introduced a single amino acid deletion into the gene encoding the receptor that is required for avian leukosis virus subgroup J to infect chicken cells. Here, we demonstrate that this mutation confers the resistance of chickens to avian leukosis virus subgroup J, an important pathogen in poultry. In addition, we present highly efficient genome-editing technology in chicken.

Author contributions: A.K., P.T., and J.H. designed research; A.K., P.T., J.M., M.R., J.P., J.K., D.K., J.G., V.K., B.L., and J.H. performed research; A.K., P.T., J.M., M.R., J.P., J.K., D.K., J.G., V.K., and J.H. analyzed data; and A.K., P.T., J.M., J.K., and J.H. wrote the paper.

Competing interest statement: A.K., P.T., J.M., M.R., J.P., J.K., D.K., and J.H. are inventors in a patent application related to this work (CZ patent application no. PV 2019-392). The other authors declare no competing interests.

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Increased food safety – egg allergy

- Egg allergy is widespread and impacts more than 40 million children worldwide
    - Cost of childhood food allergies in the USA alone is $25 billion per year
- Major food safety issue and implications for vaccines grown in eggs
- Egg allergy is caused by 4 proteins within the egg white
- Ovomucoid (Ovm) is the most allergenic egg white protein
    - makes up a small amount of the total egg white protein
    - Resistant to cooking and digestion
- We are using CRISPR to knockout Ovm using our well-established poultry genome engineering capability
    - OVM gene knockout – safe in cooked egg products
    - Our goal is to have 1st gene edited animal food product in Australia
    - We can achieve something truly novel and disruptive in the free-from-food industry

Production and characterization of eggs from hens with ovomucoid gene mutation

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ABSTRACT Ovomucoid is a major egg white protein which is considered as the most dominant allergen in chicken eggs. Owing to the difficulty of separating ovomucoid from egg whites, researchers have adopted genetic deletion for development of hypoallergenic eggs. Previously, we used CRISPR/Cas9 to establish chickens with ovomucoid gene (*OVM*) mutations, but it remained unknown whether such hens could produce eggs at maturity. Here, we have reported on eggs laid by *OVM*-targeted hens. Except for watery egg whites, the eggs had no evident abnormalities. Real-time PCR revealed alternative splicing of *OVM* mRNA in hens, but their expression was limited. Immunoblotting detected neither mature ovomucoid nor ovomucoid-truncated splicing variants in egg whites. Sixteen chicks hatched from 28 fertilized eggs laid by *OVM*-targeted hens, and fourteen of the sixteen chicks demonstrated healthy growth. Taken together, our results demonstrated that *OVM* knockout could almost completely eliminate ovomucoid from eggs, without abolishing fertility. Thus, the eggs developed in this study have potential as a hypoallergenic food source for most patients with egg allergies.

Key words: ovomucoid (*OVM*)-knockout egg, egg allergen depletion, gene targeting, genome editing, CRISPR/Cas9

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INTRODUCTION

Chicken eggs are one of the most common allergenic foods, especially in children, in whom allergy prevalence ranges between 0.5 and 2.5% (Rona et al., 2007; Caubet and Wang, 2011). The glycoprotein ovomucoid (**OVM**) is a major allergenic protein that constitutes 11% of the egg white protein (Kovacs-Nolan et al., 2005). OVM is believed to be the most dominant allergenic protein (Bernhisel-Broadbent et al., 1994; Cooke and Sampson, 1997; Mine and Yang, 2008). Therefore, removal of OVM or elimination of its allergenicity may result in hypoallergenic egg products. Food-processing methods such as proteinase and heat treatments are frequently used to reduce allergenicity of various foods (Verhoeckx et al., 2015). Such methods have been used to reduce egg allergenicity, but their use in egg products is limited because OVM is highly resistant to such treatments (Matsuda et al., 1983; Kovacs-Nolan et al., 2000; Hirose et al., 2004). Physical removal of OVM from egg whites has also been explored, with methods that include solvent extraction and rinsing of boiled egg whites (Urisu et al., 1997; Tanabe et al., 2000). However, physical removal is impractical and difficult to use in food manufacturing because of insufficient OVM elimination, poor cost-efficiency, and destruction of egg white properties such as gelling and foaming (Mine and Zhang, 2001; Chang et al., 2018). In contrast, genetic deletion of OVM from hens implies that OVM protein will not be expressed, thereby potentially generating hypoallergenic OVM-free eggs that reduce immune response in patients with egg allergies (Park et al., 2014; Chojnacka-Puchta and Sawicka, 2020).

We previously used the CRISPR/Cas9 system to produce biallelic *OVM* knockout ($OVM^{-/-}$) chickens (Oishi et al., 2016), but we did not examine their egg production capacity. Here, we aimed to determine the egg-laying ability of $OVM^{-/-}$ hens and studied properties of eggs from such hens. To that end, we raised $OVM^{-/-}$ hens to sexual maturity and then evaluated

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Selectively hatching female chicks – a high priority for the global egg industry

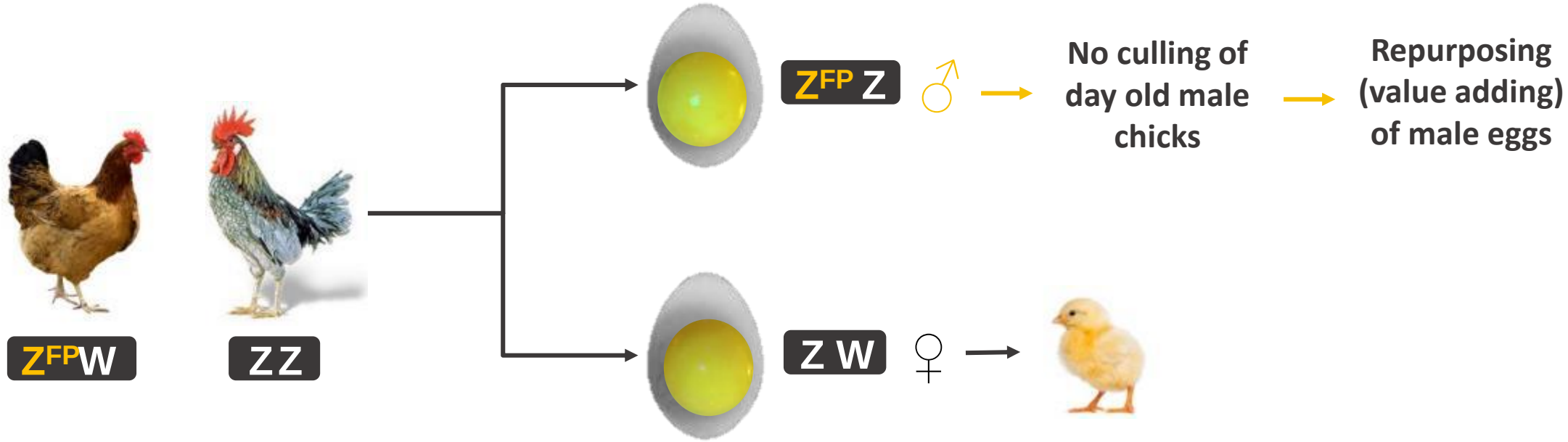
- Challenge: develop a technology to detect and remove male embryos prior to hatch
  - At point of lay
  - Without physically penetrating the shell
  - High through-put, high accuracy
  - Low cost

- Global industry demand for a solution

“Null segregation” : offspring are **not GMOs**

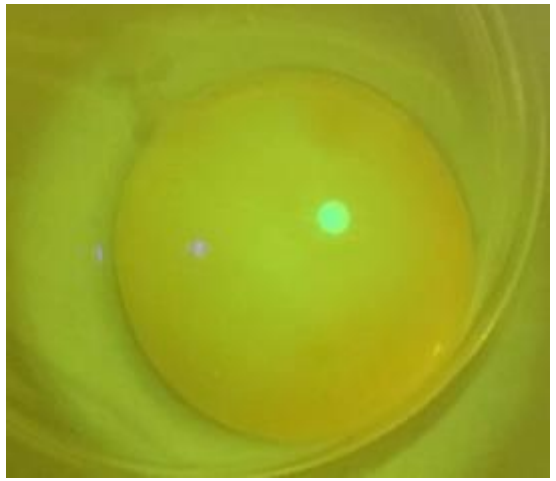
Gene Technology Regulations - Amendments (for clarification of definitions)

- Null-segregant offspring of GMO parents will **not** be classified as GMOs

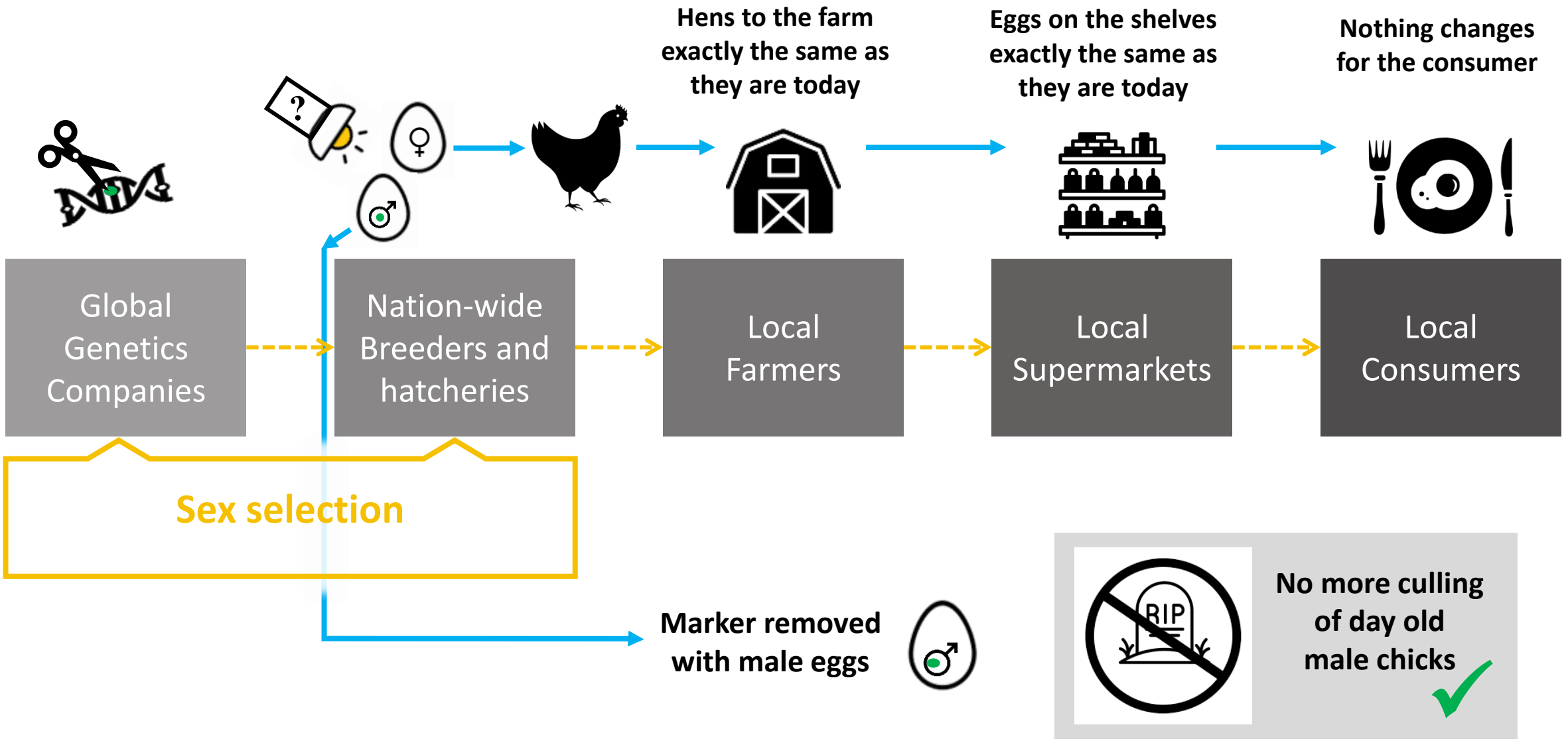


Null-segregant process also being recognised (and excluded from GMO definition) by Food Standards Australia New Zealand (FSANZ)

Production of chromosome marked chickens through direct injection



Sex selection - benefiting the entire supply chain



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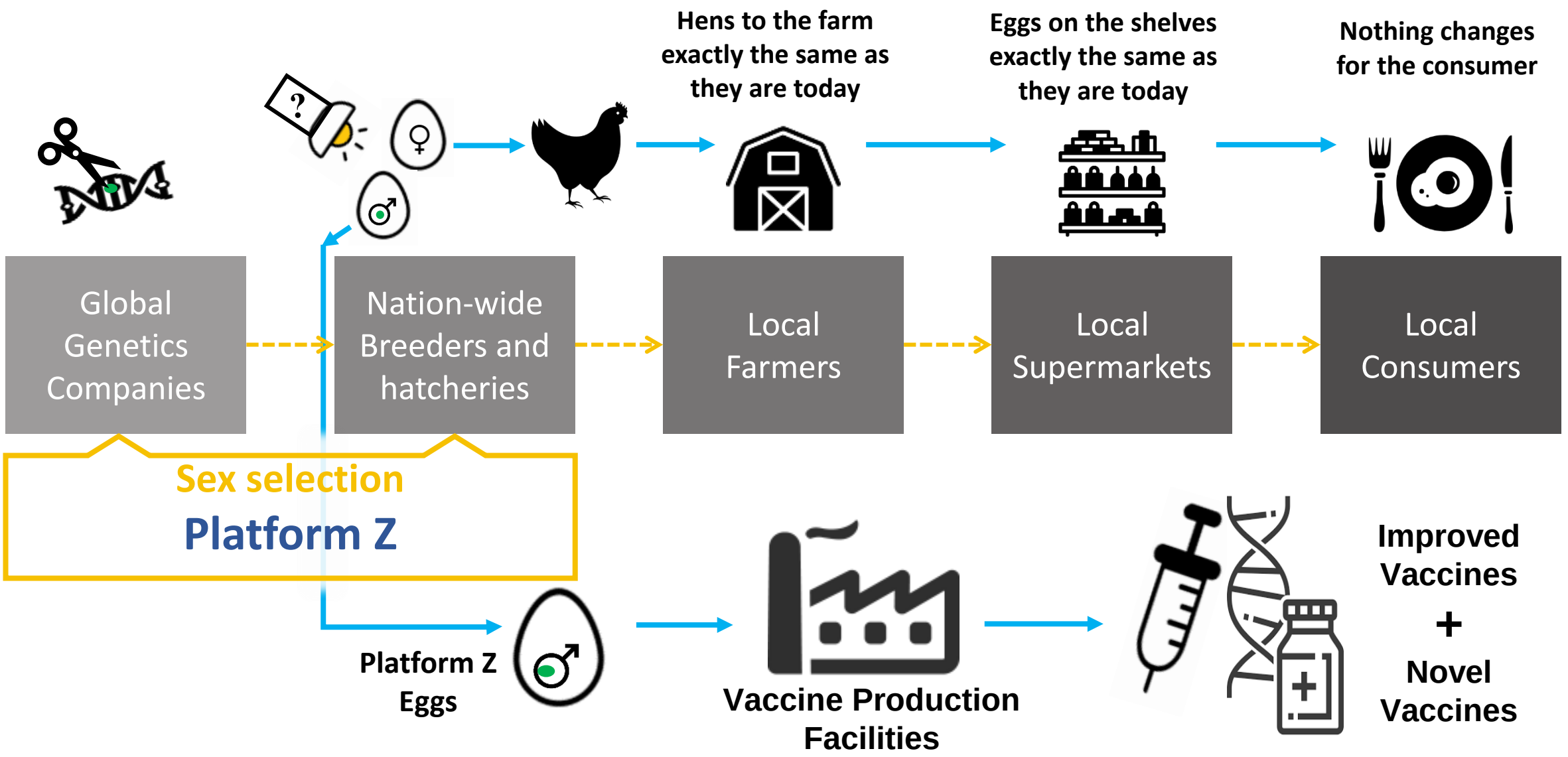

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